CHAPTER

3

Plus-Strand RNA Viruses

INTRODUCTION

The plus-strand RNA [(+)RNA] viruses comprise a very large group of viruses belonging to many families. Among these are viruses that cause epidemic disease in humans, including encephalitis, hepatitis, polyarthritis, yellow fever, dengue fever, poliomyelitis, and the common cold. The number of cases of human disease caused by these viruses each year is enormous. As examples, dengue viruses infect an estimated 50 to 100 million people each year; most humans suffer at least one rhinovirus-induced cold each year, with the cases therefore numbering in the billions; and most humans during their lifetime will suffer several episodes of gastroenteritis caused by astroviruses or caliciviruses. In terms of frequency and severity of illness, the (+)RNA viruses contain many serious human pathogens, and we will begin our description of viruses with this group.

The human (+)RNA viruses belong to seven families (Table 3.1). These seven families also contain numerous viruses that infect other vertebrates, of which many are important pathogens of domestic animals. Large numbers of (+)RNA viruses that infect plants are also known; in fact, most plant viruses contain (+)RNA genomes. The plant viruses, however, belong to different families and are currently classified by the International Committee on Taxonomy of Viruses (ICTV) into nine families plus many unassigned genera. Because of their importance as disease agents of domestic crops, much is known about these viruses. Other families of (+)RNA viruses include two families of bacterial viruses, one of fungal viruses, and four families of insect viruses (the nodaviruses, in particular, have been intensively studied). Thus, the (+)RNA viruses have evolved into many distinctly different families and must have arisen long ago. In this chapter, the seven families of viruses that include human viruses as members are considered, followed by a brief discussion of the relationships of these viruses to the plant viruses and what this means in terms of virus evolution.

FAMILY *PICORNAVIRIDAE*

The picornaviruses are so named because they are small (*pico* = small), *RNA*-containing viruses. Nine genera of picornaviruses, five of which contain human pathogens, are currently recognized (Table 3.2), and more will probably be recognized as further studies of the known viruses occur and as new viruses are described. A dendrogram that illustrates the relationship of the nine genera to one another, as well as the relationships of a number of viruses within the various genera, is shown in Fig. 3.1. This dendrogram makes clear that all picornaviruses are closely related. They share significant nucleotide and amino acid sequence identity and form a well-defined taxon. The dendrogram also illustrates the rationale for grouping these viruses into nine genera.

As described in Chapter 2, the structures of several picornaviruses have been solved to atomic resolution by X-ray crystallography. The picornavirus virion is composed of 60 copies of each of four different proteins (called VP1–4) that form an icosahedral shell having T=3 symmetry (or pseudo-T=3) and a diameter of approximately 30 nm (see Figs. 2.1, 2.5, 2.7, and 2.8).

Organization and Expression of the Genome

The structure of the genome of poliovirus and comparison of it with the genomes of the other genera are shown in Fig. 3.2. The picornaviral genome is a single RNA molecule of about 7.5 kb. It contains one open reading frame (ORF) and is translated into one long polyprotein. This polyprotein is

TABLE 3.1 Families of Plus-Strand RNA Viruses That Contain Human Pathogens

Family	Size of genome (nucleotides)	Other vertebrate hosts	Representative human pathogens
Picornaviridae	~7500	Cattle, primates, mice	Poliovirus Human rhinovirus Hepatitis A
Caliciviridae	~7500	Rabbits, swine, cats	Norwalk
Hepeviridae	7200	Primates, swine	Hepatitis E
Astroviridae	6800–7900	Cattle, ducks, sheep, swine	Human astrovirus
Togaviridae	~11,600	Mammals, birds, horses	Semliki Forest, Ross River, WEE, VEE, EEE, Mayaro, rubella
Flaviviridae	9500–12,500	Swine, cattle, primates, birds	Dengue, yellow fever, JE, MVE, TBE, WNV, hepatitis C
Coronaviridae	20,000–30,000	Mice, birds, swine, cattle, bats	SARS coronavirus

Virus name abbreviations: WEE, VEE, EEE, Western, Venezuelan, Eastern equine encephalitis viruses; JE, Japanese encephalitis virus; MVE, Murray Valley encephalitis virus; TBE, tick-borne encephalitis virus; WNV, West Nile virus; SARS, severe acute respiratory syndrome.

TABLE 3.2 *Picornaviridae*

Genus/members	Virus name abbreviation[a]	Usual host(s)	Transmission	Disease	World distribution
Enterovirus					
Human enterovirus A	HEV-A	Humans	Oral–fecal, contact	See Table 3.5	Worldwide
Human enterovirus B	HEV-B	Humans	Oral–fecal, contact	See Table 3.5	Worldwide
Human enterovirus C	HEV-C	Humans	Oral–fecal, contact	See Table 3.5	Worldwide
Human enterovirus D	HEV-D	Humans	Oral–fecal, contact	See Table 3.5	Worldwide
Poliovirus (Types 1, 2, and 3)	PV	Humans	Oral–fecal, contact	See Table 3.5	Originally worldwide, extirpated in Americas
Bovine enterovirus, porcine enteroviruses A and B; Unassigned enteroviruses of humans and monkeys					
Rhinovirus					
Human rhinoviruses (>100 serotypes)	HRV-A, HRV-B	Humans	Aerosols, contact	Common cold	Worldwide
Cardiovirus					
Encephalomyocarditis Theilovirus	EMCV	Mice	Oral–fecal, contact	Encephalitis, myocarditis	Worldwide
Aphthovirus					
Foot and mouth disease	FMDV	Cattle, swine	Oral–fecal, contact	Lesions on mouth and feet	Worldwide (except United States, Australia)
Equine rhinitis A	ERAV	Horses			Worldwide
Hepatovirus					
Hepatitis A	HAV	Humans	Oral–fecal	Hepatitis	Endemic worldwide
Parechovirus					
Human parechovirus	HPeV	Humans	Oral–fecal	Gastroenteritis	Worldwide
Erbovirus					
Equine rhinitis B	ERBV	Horses	?	?	?
Kobuvirus					
Aichi	AiV	Humans	Oral–fecal	Gastroenteritis	Isolated in Japan (oysters)
Teschovirus					
Porcine teschoviruses (10 species recognized)	PTV-1	Swine	Oral–fecal	Paralysis, porcine encephalomyelitis	Britain, central and Eastern Europe

[a] Standard abbreviations are given for either the virus listed (such as poliovirus) or for the type member of the genus.

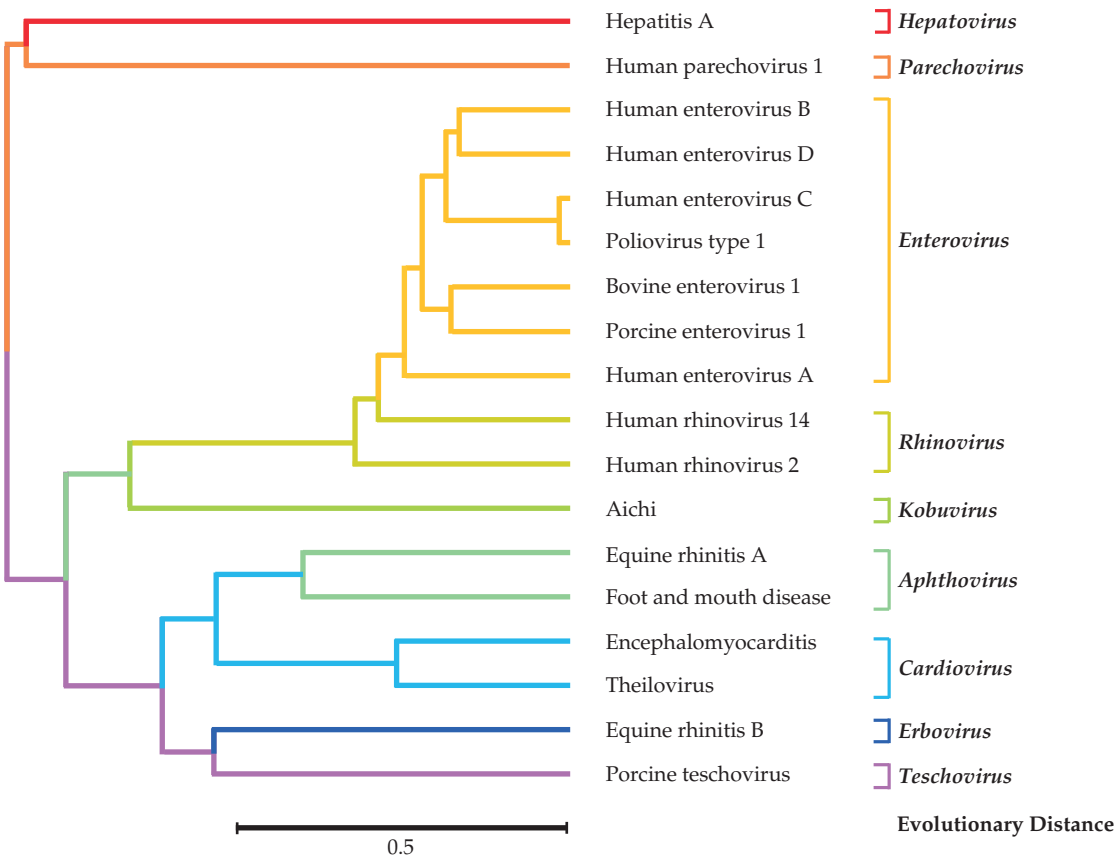

FIGURE 3.1 Relationships between 18 representative picornaviruses. The viruses shown have been classified into the 9 recognized genera. This tree was generated from the amino acid sequences of the $3D^{pol}$ proteins. Adapted from Yamashita et al. (2003), and updated with the taxonomy found in Fauquet *et al*. (2005). Evolutionary distance, calculated by the UPGMA (unweighted pair group method with averages) method, is the number of residue substitutions that have occurred between two sequences since their divergence from a common ancestor and is defined as D = number of base mismatches/total alignment length in nucleotides.

cleaved by one or more virus-encoded proteinases to form more than 25 different polypeptides, including processing intermediates (not all of which are shown in the figure) as well as final cleavage products. The ORF in the genome contains three regions, called P1 (the 5′ region), P2 (the middle region), and P3 (the 3′ region). Region 1 encodes the structural proteins and regions 2 and 3 encode proteins required for RNA replication. The genome organization of all picornaviruses is similar, but each genus differs in important details. For example, the aphthoviruses and the cardioviruses have a poly(C) tract near the 5′ end of the RNA that is important for virus replication. These two genera also have a leader polypeptide that precedes the structural protein region. The aphthovirus leader peptide is a papain-like protease that cleaves itself from the polyprotein and has a role in the shutoff of cellular protein synthesis. The function of the cardiovirus leader is not known. Hepatitis A virus, Aichi virus, and echovirus 22, representatives of three other genera, also have leaders.

The picornaviral genome has a small protein, VPg, covalently bound to the 5′ end, which is the primer for initiation of RNA synthesis. VPg is normally removed from RNA that serves as mRNA by a cellular enzyme, but its removal is not required for its translation. The 3′ end of the RNA is polyadenylated. As described in Chapter 1, the 5′ nontranslated region of a picornaviral RNA possesses an IRES (internal ribosome entry site) and the RNA is translated by a cap-independent mechanism. The translation of picornaviral RNA is greatly favored in the infected cell because picornaviruses interfere with host cell macromolecular synthesis and, in particular, interfere with host protein synthesis. Infection with entero-, rhino-, and aphthoviruses leads to proteolytic cleavage of a cellular protein called eIF4G that is a component of the cap-binding complex. Cleavage of this protein by $2A^{pro}$ of entero- and rhinoviruses or by the leader protease of aphthoviruses results in inhibition of the translation of RNAs that require the cap-binding protein complex, that is, capped host cell mRNAs. The cardioviruses, which are also cap independent, interfere with translation of host mRNAs in a different way, by interfering with phosphorylation of cap-binding protein. Poliovirus also interferes with host

protein synthesis by cleavage of poly(A)-binding protein by the viral $3C^{pro}$, but the mechanism by which this interference operates on cellular protein synthesis and not viral protein synthesis, since poliovirus RNA is also polyadenylated, is not yet clear. In addition, $3C^{pro}$ of apthoviruses cleaves initiation factors eIF4A and eIF4GI, and it is thought that these cleavages lead to a decrease in the level of viral protein synthesis later in infection, which facilitates packaging of the viral RNA.

The viral $3C^{pro}$ and its precursor $3CD^{pro}$ make multiple cleavages in the polyprotein translated from the genome, as illustrated for poliovirus in Fig. 3.2. Some cleavages occur *in cis* and some *in trans*. The crystal structures of $3C^{pro}$ of poliovirus and of a rhinovirus have been solved to atomic resolution, and their core structure resembles that of chymotrypsin (Fig. 1.19A). The catalytic center has the same geometry as that of chymotrypsin, but in $3C^{pro}$ the catalytic serine has been replaced by cysteine. Moreover, in many, but not all, picornaviruses the aspartic acid in the catalytic triad has been replaced by glutamic acid. Thus, $3C^{pro}$ is related to cellular serine proteases and may have originated by the capture of a cellular serine protease during the evolution of the viruses.

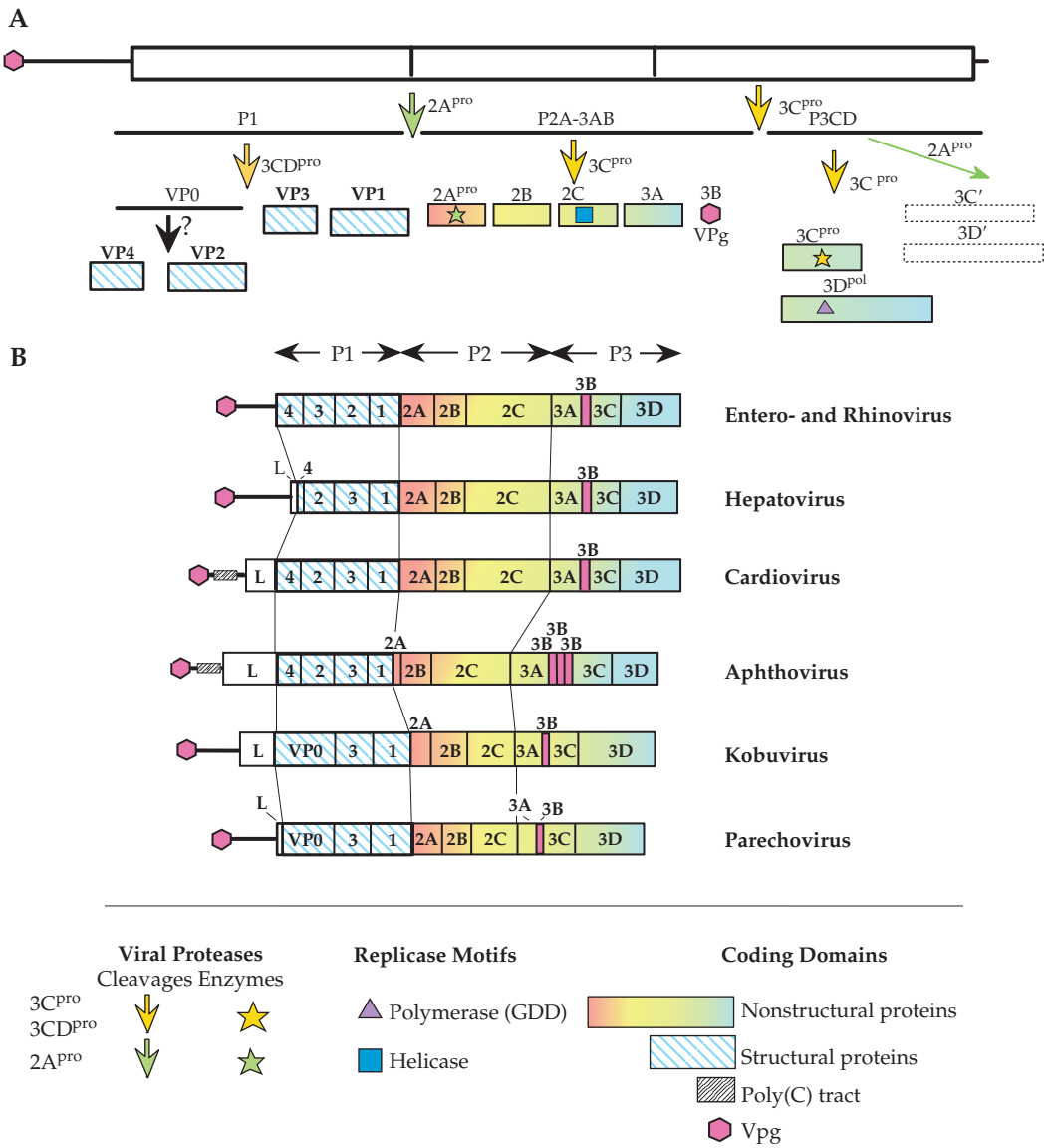

FIGURE 3.2 Genome organization of the *Picornaviridae*. (A) Genome organization of poliovirus showing the proteolytic processing steps. Both the $3C^{pro}$ and $2A^{pro}$ proteases are "serine-type proteases" with cysteine in the catalytic site. (B) Comparative genome organizations of representatives of seven of the nine genera of *Picornaviridae*. The key to the different shadings of coding domains and the symbols for various enzymatic motifs used in both (A) and (B) is given below. Adapted from Murphy *et al*. (1995) p. 300 and Yamashita *et al*. (1998).

Protease $3C^{pro}$ is present in all picornaviruses whereas protease $2A^{pro}$ is present in only a subset of picornaviruses. In poliovirus, $2A^{pro}$, like $3C^{pro}$, is a serine protease in which the active site serine has been replaced by cysteine. $2A^{pro}$ catalyzes one essential cleavage in the polyprotein of poliovirus, that between P1 and P2. This cleavage occurs *in cis*. The proteolytic activity of $2A^{pro}$ is also required for other functions during poliovirus replication, the nature of which have not been established. An interesting experiment is illustrated in Fig. 3.3 because it illustrates the power of molecular genetics and the tricks that modern virologists can play with viruses. This experiment will serve as a prelude to the discussion of the uses of viruses as vectors in Chapter 11. A poliovirus was constructed in which a stop codon was placed after the structural protein domain (region 1), so that $2A^{pro}$ was not needed to remove P1 from the polyprotein precursor. The stop codon was followed by an IRES and a new AUG start codon, so that P2A and the rest of the genome could be translated from the polycistronic RNA. This virus was viable. However, when the 2A proteinase was inactivated by changing the active site cysteine, the resulting virus was dead, showing that the proteolytic activity of P2A is required not only to separate regions 1 and 2 of the polyprotein but also for other function(s).

Protein 2A of rhinoviruses is also a protease. The crystal structure of protein 2A of human rhinovirus type 2 reveals that this protease is unrelated to $2A^{pro}$ of polioviruses, however. Thus, these two proteases in closely related viruses have different origins, and the viruses have solved the problem of how to separate regions 1 and 2 in the polyprotein in different ways. Furthermore, this finding illustrates that recombination to introduce new functions into viral genomes has been important in the evolution of these viruses, a theme to which we will refer many times in this book. Still another solution to the problem of separating regions 1 and 2 has been adopted by the cardio- and aphthoviruses. Protein 2A is not a protease in these viruses. Indeed, 2A is only 18 residues long, and cleavage between P1 and P2 is catalyzed by $3C^{pro}$. Another interesting feature of these viruses is that the cleavage at the 2A–2B junction occurs spontaneously during translation, catalyzed by the specific amino acid sequence at the scissile bond. This cotranslational cleavage occurs only during translation on eukaryotic ribosomes, and it has been proposed that no cleavage actually occurs, but that the 2A sequence prevents synthesis of the specific peptide bond at the 2A–2B junction.

In addition to these cleavages catalyzed by $2A^{pro}$, $3C^{pro}$, and the leader protease of aphthoviruses, VP0 is cleaved during virion maturation to VP2 and VP4 in most, but not all, picornaviruses. Available evidence suggests that this cleavage is not catalyzed by a protease.

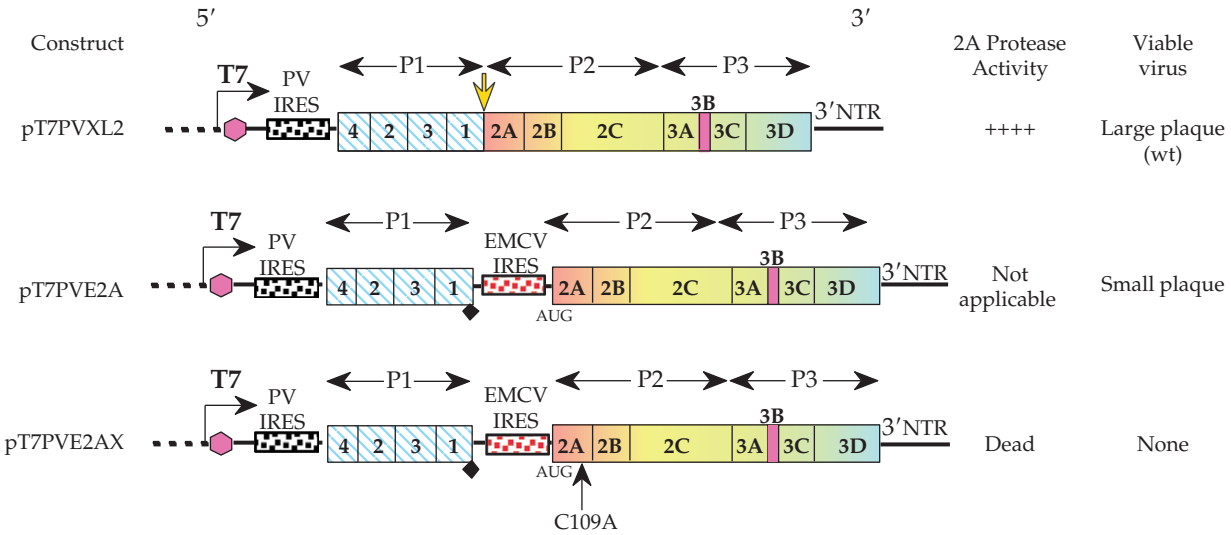

FIGURE 3.3 Diagramatic illustration of constructs used to unravel the functions of protein 2A in poliovirus replication. cDNA copies of the virus RNA can be manipulated by genetic engineering to insert IRES elements or make specific mutations. RNA can be transcribed from the clones *in vitro* and used to infect cells, which is possible for plus-strand RNA viruses because the first event after infection is translation of the genomic RNA. The wild-type construct pT7PVXL2 is shown in the top line. The 2A proteolytic activity normally cleaves the bond between domains P1 and P2 of the translated polyprotein. If this function is rendered nonessential, as in construct pT7PVE2A, by the insertion of a stop codon at the C terminus of P1 (solid diamond), followed by a second IRES and an initiation AUG at the beginning of 2A, virus is still produced, but forms small plaques. Thus separation of the structural region and the nonstructural region in this way results in viable virus. However, if the proteolytic activity of 2A is inactivated by mutation of the catalytic cysteine to alanine as in pT7PVE2AX, no virus is produced, demonstrating that the proteolytic activity of $2A^{pro}$ is necessary for other functions in addition to the P1/P2 cleavage. The pink hexagon is the VPg encoded in 3B and linked to the 5′ end of the RNA. Adapted from Lu *et al*. (1995) and Molla *et al*. (1993).

Functions of the Picornavirus Proteins

The cleavage product P1 consists of a polyprotein precursor for the four structural proteins of the virus, VP1–4. P1 is first cleaved *in trans* to VP0, VP1, and VP3 by $3CD^{pro}$ (Fig. 3.2A). VP0 is later cleaved to VP2 and VP4 during assembly of most picornaviruses.

The cleavage products of P2 and P3 are required for RNA replication. $2A^{pro}$ has been described. Protein 2B from a Coxsackie virus has been shown to induce the influx of extracellular Ca^{2+} and the release of Ca^{2+} from stores in the endoplasmic reticulum, and it is proposed that this protein induces lesions in cellular membranes that allow release of progeny virions. $2C^{ATPase}$ has been shown to be an ATPase, not a GTPase, and contains sequence motifs characteristics of helicases. Many, but not all, RNA viruses encode helicases to unwind duplex RNA during replication, and it is assumed that $2C^{ATPase}$ performs such a function. The precursor to 2B and to $2C^{ATPase}$, a protein called $2BC^{ATPase}$, has a different role in RNA replication. It is required for proliferation of membranous structures in poliovirus-infected cells that serve as sites for RNA replication.

Region 3 encodes VPg, $3CD^{pro}$/$3C^{pro}$, and the viral RNA polymerase $3D^{pol}$. Cleavages effected by $3C^{pro}$ are illustrated in Fig. 3.2. $3C^{pro}$ may also have a regulatory role in the virus life cycle, because the cleavage intermediate $3CD^{pro}$, which is fairly long lived, has properties that differ from $3C^{pro}$. One function of $3CD^{pro}$ is to bind the viral RNA in conjunction with 3AB, the precursor for VPg, or with a cellular protein, poly(C)-binding protein. Formation of a complex with the viral RNA is essential for its replication, and differential cleavage of the 3C–3D bond during the infection cycle may regulate replication. A strategy in which precursor proteins perform different functions than those performed by the final cleavage products, such as those illustrated by $2BC^{ATPase}$ and $3CD^{pro}$, allows the virus to optimize the coding capacity of its small genome, because a given sequence is used for more than one function.

Replication of Picornaviruses

The replication of poliovirus has been particularly well studied and the virus has served as a model for the replication of eukaryotic RNA viruses. All nonstructural poliovirus proteins, including cleavage intermediates, have been purified and studied for their possible function as enzymes or RNA-binding proteins. These studies have been complemented by studies of replication complexes isolated from infected cells, studies using replicons in which the luciferase gene replaces the P1 coding region (see, for example, Fig. 3.3 and Chapter 11), and studies of processes that occur in infected cells.

Replication of poliovirus RNA is associated with cellular membranes, as appears to be true of all eukaryotic plus-strand RNA viruses. The 3A protein has hydrophobic sequences that may be involved in this association. During replication, a full-length complementary copy of the genomic RNA is produced that serves as a template for the synthesis of genomic RNA (illustrated schematically in Fig. 1.11A). This complementary RNA template has been variously called minus-strand RNA [abbreviated (−)RNA], antigenomic RNA, or virion-complementary (vc) RNA. Much more (+)RNA than (−)RNA is produced, since (+)RNA is needed for translation and encapsidation into progeny as well as for replication, whereas (−)RNA is needed only as a template for making (+)RNA. It is probable that disproportionate amounts of (+) and (−) strands are synthesized because the promoters in the viral RNA recognized by the viral replication machinery for (+) and (−)RNA synthesis (which might also be called origins of replication) differ in their strength, but other mechanisms are known to be used in at least some RNA viruses.

The RNA-dependent RNA polymerase $3D^{pol}$ is strictly primer dependent. In the presence of template, $3D^{pol}$ can uridylate VPg on a specific tyrosine residue. This nucleotidyl peptide, VPgpU or VPgpUpU, then functions as a primer for the initiation of RNA synthesis. It is of interest that several viruses belonging to other families, such as hepatitis B virus (a virus that uses reverse transcription during the replication of its genome) and adenovirus (a DNA virus), have also adopted the strategy of using a protein primer for initiation of nucleic acid synthesis.

The nature and function of the promoters in the poliovirus genome that are involved in the initiation of RNA replication are incompletely understood. One essential element has been called a ***c****is*-acting ***r***eplication ***e***lement, abbreviated *cre*, or 3B-uridylation site, abbreviated *bus*. *cre* is a stem-loop structure that contains a motif in the loop, AAACA, that is conserved in all picornaviruses. This motif serves as a template for the uridylation of VPg described before, which is required for the initiation of RNA synthesis. The description of this element as *cis* acting is a misnomer because the element can act *in trans*, and there is a pool of VPgpUpU within poliovirus-infected cells that can be used to initiate RNA synthesis. The *cre* element is found in different locations in different picornaviruses. In poliovirus it is found in the coding sequence for 2C, in rhinovirus in the coding region for VP1, and in FMDV it is in the 5′ NTR. Furthermore, the element can be moved to other regions within a viral genome and still function normally.

A second sequence element required for RNA replication, present in polioviruses and rhinoviruses if not in all picornaviruses, is located within the 5′-terminal NTR. This element forms a cloverleaf that binds protein complexes containing $3CD^{pro}$.

In addition to the various viral proteins just described, a number of cellular proteins are also required for viral RNA replication. In fact, cellular proteins appear to be required for replication of all (+)RNA virus RNAs, but the identity

of these proteins and their function in viral RNA replication is only poorly understood. One such protein in the case of poliovirus is a cellular protein called heterogeneous nuclear ribonucleoprotein C1 (hnRNP C1), which interacts with RNA synthesis initiation complexes and appears to be required for the initiation of positive-strand RNA. A second protein is the poly(A)-binding protein. Efficient replication of polio RNA requires a poly(A) tract at the 3′ end of the RNA that is at least 20 residues in length, and it is believed that the poly(A)-binding protein binds this poly(A) tract and participates in the initiation of minus-strand RNA synthesis.

It has been possible to achieve a complete replication cycle of poliovirus in an extract of uninfected HeLa cells. RNA from poliovirus virions added to such an extract will direct the synthesis of all the poliovirus proteins, and these in turn will replicate the input RNA and encapsidate the progeny genomes. This cell-free, *de novo* synthesizing system for poliovirus, is as yet unique in virology.

In cell culture, most picornaviruses complete their replication cycle in about 6 hours. The infection is cytolytic, and large quantities of virus are produced. An exception is hepatitis A virus, which establishes chronic infections in cell culture and grows to very low titers.

Genus *Enterovirus*

Enteroviruses replicate primarily in the enteric tract where they usually cause only mild disease. More serious enteroviral disease may develop after spread to other organs, such as the central nervous system or the heart. Enteroviruses are normally contracted though ingestion of the virus, either in contaminated food or water or by exposure to the virus through contacts with individuals that are excreting the virus. The epidemiology of poliovirus has been the most intensively studied among the enteroviruses. Poliovirus is present in oropharyngeal secretions early after infection and is excreted in feces over a period of weeks following infection. The virus spreads readily and rapidly through households, which demonstrates the importance of close contacts in virus spread. The virus also has the ability to persist in the external environment for weeks under favorable conditions, and this may represent another source of infection during epidemics. Sewage surveys, for example, have been used to follow poliovirus epidemics, and poliovirus has been found in lakes and swimming pools.

In general, enteroviruses have a fairly narrow host range. Most of the well-studied viruses are human viruses, because humans take a particular interest in the viruses that cause them the most trouble, but enteroviruses of nonhuman primates, pigs, cattle, and insects are known. The more than 65 known human enteroviruses, many of which are important pathogens, normally infect only humans, but poliovirus will infect Old World monkeys. It has been suggested that the virus may be a natural pathogen of these monkeys but it is unlikely that nonhuman primates constitute a reservoir for it, which is important in relation to efforts spearheaded by the World Health Organization to eradicate poliovirus globally.

The classification of human enteroviruses has recently undergone extensive revision, based upon the wealth of sequence information that is increasingly available. Previously, classification was based upon the symptomology of disease caused or upon the characteristics of the growth of a virus in experimental animals or in cultured cells. Poliovirus has been known for more than a century as the causative agent of epidemic poliomyelitis. It was first shown to be a filterable virus in 1908. However, early experiments could only be conducted in monkeys, because the virus will only infect primates. Thus, the amount of information that could be obtained was limited, but such studies eventually showed that more than one poliovirus serotype existed. The development of methods for the cultivation of viruses in cell culture in the 1940s made it possible to screen human stool samples in an effort to type poliovirus isolates, which was necessary if a vaccine was to be produced. Such screening resulted not only in the identification of three serotypes of poliovirus, but also in the discovery of many other enteroviruses as well. The study of virology in the United States owes much to the campaign to develop a vaccine against poliomyelitis. This campaign generated a great deal of public support, which led to funding through private as well as governmental agencies, and the successful development of a vaccine reinforced this support.

The first of these other enteroviruses to be found were two Coxsackie viruses, found by screening patients in Coxsackie, New York, who were suffering from paralysis during a polio epidemic. Coxsackie viruses will infect mice and are classified into two subgroups, called A and B, which differ in their biological properties in mice. They were simply given serial numbers in the order of their isolation—23 Coxsackie A viruses and 6 Coxsackie B viruses are now recognized. Another series of enteroviruses that were first identified in these early studies were called echoviruses (***e***nteric ***c***ytopathic ***h***uman ***o***rphan virus), because these viruses infected the enteric tract of humans, caused cytopathology in cultured cells, and were orphans, not known to cause disease. Echoviruses were distinguished from Coxsackie viruses by their inability to infect suckling mice. Currently 29 echoviruses are recognized in the genus *Enterovirus*. The latest human viruses to be isolated are now simply called enteroviruses and given serial numbers. The first four such viruses to be recognized were thus called human enterovirus 68, 69, 70, and 71. Numbering started with 68 because at the time there were thought to be 67 polio, Coxsackie, and echoviruses. However, 5 of these (one Coxsackie A virus and 4 echoviruses) were subsequently found to be misidentified, and one (echovirus 22) is sufficiently distinct that it has been

renamed human parechovirus and classified into the genus *Parechovirus* (Table 3.2).

Thus, from such studies, a total of 65 human enteroviruses was isolated and, as indicated, classified according to their biological properties. As the genomes of these various viruses were sequenced, it became apparent that these viruses fell into five lineages or clades whose members are closely related to one another. As described in Chapter 1, the definition of a virus species is somewhat arbitrary, but the purpose of classification is to recognize evolutionary relationships, and 63 of these 65 human enteroviruses have now been reclassified into five species, called *poliovirus* and *human enterovirus A, B, C, D*. These assignments are shown in Table 3.3, and the various members of a species are now considered serotypes. This table contains information on serotypes accepted as of the 2005 ICTV report. More than 80 serotypes are now known and as new serotypes continue to be identified and characterized, it is to be expected that this number will continue to grow. The extensive sequence data have also uncovered examples of recombination that have occurred during the evolution of these viruses, both within species and between species.

Sequence information has also been used to identify one species of bovine enterovirus and two species of pig enteroviruses (*Porcine Enterovirus A and B*) in the genus *Enterovirus* (Table 3.3). There are 2 serotypes assigned to *Simian enterovirus A* and 17 other known monkey enteroviruses have as yet to be classified into species. The monkey viruses form a distinct clade related to porcine enterovirus 8 (*Porcine Enterovirus A*) and will probably be classified into one or two species.

Polioviruses

The best known of the enteroviruses are the three serotypes of poliovirus. These viruses are the causative agents of poliomyelitis, a disease characterized by the death of motor neurons in the spinal cord. Most poliovirus infections of susceptible humans are inapparent or result in a mild febrile illness in which cells of the pharynx and the gut are infected and recovery is uncomplicated. However, a transient viremia is established following infection (viremia = virus present in the blood), and in a small percentage (<2%) of infections the virus invades the central nervous system (CNS), where it infects motor neurons in the spinal cord and, in severe cases, other regions of the CNS. The mechanism by which the virus enters the CNS is still controversial. Current information supports the hypothesis that viremia allows the virus to enter by penetrating through the blood–brain barrier, but entry via retrograde transport in axons that serve the periphery may also be involved. In any event, infection of the CNS can result in paralysis, which can be severe enough to be fatal because of paralysis of respiratory muscles. The name *poliomyelitis* comes from the Greek words *polio* = gray and *myelo* = spinal cord, from the pathology caused by damage to the motor neurons in the spinal cord, which are located in the gray matter.

Polioviruses readily undergo recombination with other polioviruses and with at least some other enteroviruses. The distinguishing feature of a poliovirus, what makes it a poliovirus, is the structural protein module and not the nonstructural protein module associated with the structural proteins. This has importance implications for vaccines that protect against poliomyelitis, as described later.

TABLE 3.3 Current Taxonomy of the Genus *Enterovirus*

Species	Strains, subtypes, and serotypes
Human enterovirus A	Human Coxsackie viruses A2–8, 10, 12, 14, 16, human enterovirus 71, 76
Human enterovirus B	Human Coxsackie virus A9
	Human Coxsackie viruses B1–6 (including swine vesicular disease virus)
	Human echoviruses 1–7
	21 other human echoviruses
	Human enterovirus 69, 73–78
Human enterovirus C	Human Coxsackie viruses A1, 11, 13, 15, 17, 19–22, 24
Human enterovirus D	Human enteroviruses 68, 70
Poliovirus	Human poliovirus types 1, 2, and 3
Bovine enterovirus	Bovine enteroviruses 1, 2
Porcine enterovirus A	Porcine enterovirus 8
Porcine enterovirus B	Porcine enterovirus 9, 10
Simian enterovirus A	Simian enterovirus A1, A2-plaque
Unassigned viruses	17 simian enteroviruses

Epidemic Poliomyelitis

Polioviruses appear to have been important pathogens of humans for a very long time. The depiction of a lame priest on an Egyptian stele that dates from 3500 years ago suggests that poliovirus was present in ancient Egypt, and references to clubfoot in ancient Greek and Roman writings probably signifies that polio was present at these early times. However, although it is very likely that poliovirus has been widespread in humans for thousands of years, there is no firm evidence for poliomyelitis in human populations until about 200 years ago, when the virus appears to have been (or to have become) widespread. Serosurveys in the United States in the 1930s and 1940s, before the introduction of the Salk and Sabin vaccines, indicated that 80–100% of adults had been infected by poliovirus at some time in their lives. Studies in other areas of the world, including studies of lameness in populations, also suggest that, at least in the 1900s, the majority of the world's population had been infected with poliovirus.

Paradoxically, even though poliovirus was surely widespread earlier, poliomyelitis epidemics of large proportions evolved only during the twentieth century and they were concentrated at first in countries practicing the highest standards of hygiene. This startling phenomenon has been explained as resulting from changes in human behavior. Originally, the highly infectious virus was contracted by infants shortly after birth when they were still protected by maternal antibodies (see Chapter 10 for a discussion of maternal antibodies). This natural infection served to immunize the infant, protecting it from poliomyelitis for life. However, when the chain of immunization was interrupted upon removal of the virus from the environment by the development of hygienic conditions, unprotected children grew up, giving rise to susceptible populations. If the virus invades such populations, epidemics rapidly evolve.

Notice that this scenario requires that infants be infected very early, while still protected by maternal antibodies. After these antibodies wane, the infant is susceptible to poliomyelitis, although it has been thought that infection of susceptible but very young children is less likely to cause poliomyelitis. Statistics of the fraction of young children who contract poliomyelitis in societies in which the virus is endemic, rather than epidemic, are not well defined, in part because of the high death rate of children in such societies due to many infectious diseases. However, surveys conducted in the twentieth century of lameness in populations, most of which is probably due to paralytic polio, found similar extents of lameness whether the virus was endemic or epidemic.

In any event, it is clear that changes in human behavior can bring about serious complications relating to infectious disease, and such scenarios have recurred many times during the last century. However, it is important to note that although higher standards of hygiene led eventually to epidemics of poliomyelitis, these standards also led to a reduction in diseases caused by numerous other infectious agents, both viral and bacterial (see Fig. 1.1).

Control of Epidemic Poliomyelitis

Before it was controlled with vaccines, epidemic poliomyelitis was greatly feared, and it is hard now for people to realize the extent of fear that the disease induced. It was not simply that the disease could be fatal, but the specter of the iron lung and the wheelchair hanging over teenagers or young adults who were the most likely to contract the disease. Furthermore, the epidemics struck during the summer, during the summer breaks of schools or universities. Many human pathogenic viruses are known to prefer a season for attack on humans: influenza during the winter, measles in early spring, enteroviruses during the summer. It is thought that this phenomenon relates to air temperature and humidity. For example, poliovirus infections are correlated with humidity in the Americas and in Europe.

In the United States, there were huge poliovirus epidemics every summer in the 1950s in which more than 50,000 people, mostly children or adolescents, became ill. Of these cases, about 20,000 were paralytic and 2000–3000 people died (Fig. 3.4). Death was often the result of the paralysis of the muscles required for breathing, and iron lungs were introduced for mechanical ventilation of poliomyelitis patients until their muscles recovered sufficiently that they could breathe on their own. Wards containing dozens of patients in iron lungs became a common sight in the large epidemics of the 1950s (Fig. 3.5), and there were fears that larger wards containing still more iron lungs would be required as the epidemics became more virulent. Of the survivors of poliomyelitis, many were permanently paralyzed and confined to wheelchairs or required the use of crutches for walking. One of the best known poliomyelitis cases is that of Franklin D. Roosevelt, who contracted poliovirus in 1921 at the age of 39 and was in a wheelchair for the rest of his life, although he continued to lead an active political life.

Introduction of the Salk and Sabin vaccines in the 1950s and 1960s led to the elimination of poliovirus in the United States over a period of about 2 decades (Fig. 3.4) and more recently has led to the elimination of poliovirus throughout the Americas. The Salk vaccine, which was the first to be developed, is an inactivated virus vaccine that is given as a series of injections. Introduction of this vaccine resulted in a rapid decrease in the number of poliovirus cases. However, because the vaccine induces circulating antibodies but little in the way of mucosal immunity (see Chapter 10), it prevents poliomyelitis, the disease, by preventing spread of the virus from the gastrointestinal (GI) tract to the CNS, but not infection of the GI tract by the virus. The virus thus remained in circulation. The Sabin vaccine, introduced shortly thereafter,

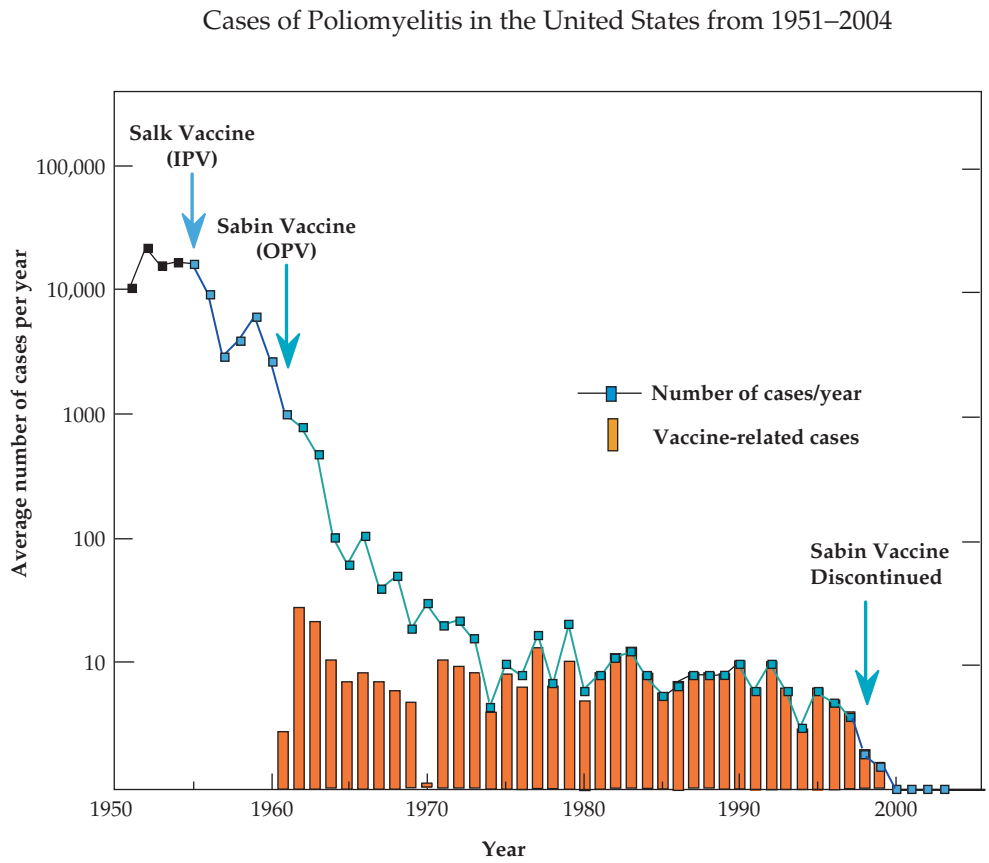

FIGURE 3.4 Total number of cases of poliomyelitis in the United States from 1951 to 2004 and the number of vaccine-related cases after the introduction of the live virus Sabin vaccine. IPV is ***i***nactivated ***p***olio ***v***accine; OPV is ***o***ral ***p***olio ***v***accine. Data from N. Nathanson *et al*. (1996) p. 556 and from *Morbidity and Mortality Weekly Report (MMWR)*. Note that the scale is logarithmic, with each division portraying 10 times as many cases as the one below.

is a live attenuated vaccine that is given orally. Attenuation was achieved by blind passage of the virus followed by testing of the resulting virus in monkeys. The changes resulting from passage are now known and are shown in Table 3.4; two changes are sufficient to make the virus avirulent in the case of types 2 and 3. The introduction of the Sabin vaccine led to a further rapid decline in paralytic poliomyelitis. This vaccine has the drawback that it induces a very small number of cases of paralytic disease, termed vaccine associated paralytic poliomyelitis (VAPP), that result from reversion of the attenuated virus to virulence. The incidence rate is about 1 per million persons inoculated, and there were about 10 such cases per year in the United States until use of this vaccine was discontinued in the year 2000 (Fig. 3.4). The efficacy of the Sabin vaccine is very high, however, because it induces mucosal immunity as well as other forms of immunity. It prevents subsequent infection by the wild-type virus, thus allowing eradication of the wild-type virus if coverage is sufficiently broad. In addition, it is much cheaper and simpler to manufacture and administer than the Salk vaccine (oral administration of relatively small doses of live virus versus injection of large amounts of inactivated virulent virus), making it suitable for widespread use in developing countries. Worldwide use of this vaccine has resulted in the eradication of wild-type poliovirus in the United States and throughout the Americas (the last case of indigenous poliovirus infection in the Americas occurred in Peru in 1991). Poliovirus is in the process of being eradicated in other parts of the world, although it is still endemic in areas of Africa and Asia. With the extirpation of polio in the United States, the use of Sabin vaccine was discontinued in this country and it has been replaced with the Salk vaccine, in order to eliminate VAPP.

As described before, polioviruses undergo recombination with other enteroviruses. It is important, therefore, to note that the mutations in the Sabin vaccines that render the virus attenuated are all found in the structural region of the genome (Fig. 3.6). Thus, recombination with other enteroviruses cannot restore the virulence of the virus. Reversion to virulence requires the back mutation of the attenuating

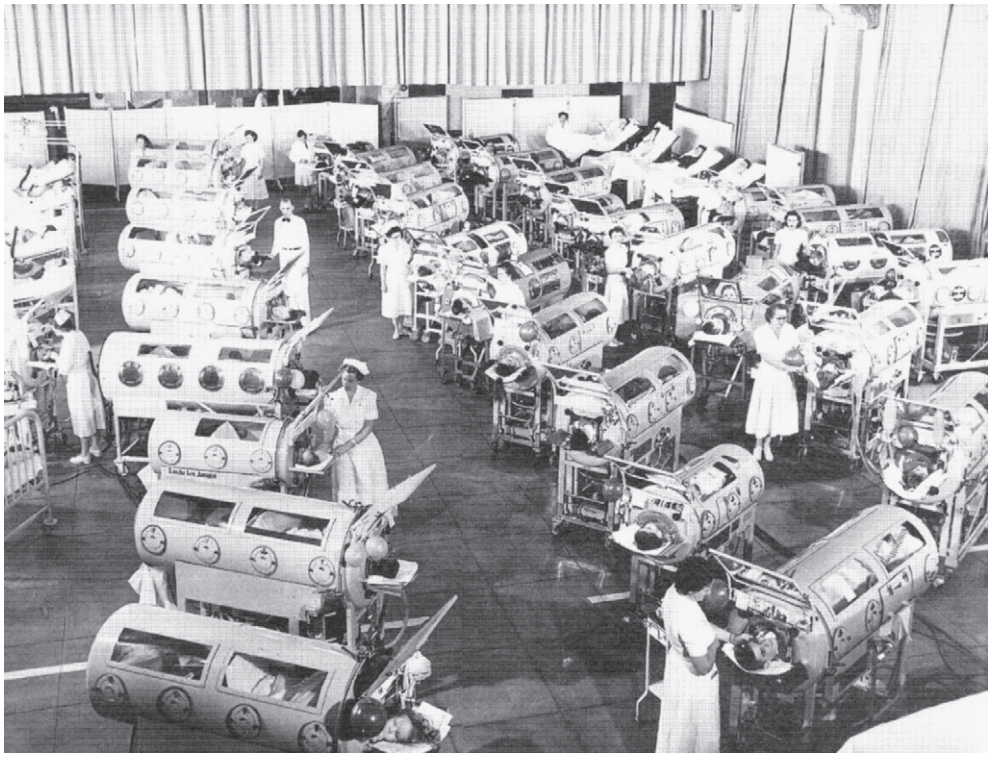

FIGURE 3.5 Ward of iron lungs and rocking beds at the poliomyelitis rehabilitation center in Rancho Los Amigos, California. From Halstead (1998) with the permission of the author and the publisher.

mutations in the vaccine viruses or, conceivably, recombination between two attenuated poliovirus strains that eliminates the attenuating mutations. In the latter case, however, it is unlikely that most recombinants would be virulent because of the incompatibility of the various nonstructural proteins with one another.

Development of the Polio Vaccine

The polio vaccine has been enormously successful in controlling this virus scourge, but the history of its development and the current difficulties in complete eradication of polio have important lessons for us. The original Salk vaccine was incompletely inactivated because the science of virology was insufficiently developed to assay for minute amounts of residual live virus in solutions containing very high concentrations of virus. The result was that this vaccine caused a small number of cases of poliomyelitis, but in retrospect the risk–reward ratio was favorable because of the significant decline in natural infections (Fig. 3.4). As soon as the problem was recognized, more stringent methods of inactivation

TABLE 3.4 Characteristics of Poliovirus Vaccines

Salk vaccine	**Inactivated wild-type poliovirus (three types)**					
Sabin vaccine	**Live poliovirus, attenuated by mutations in:**					
	Type 1		**Type 2**		**Type 3**	
	nt	aa	nt	aa	nt	aa
5′NTR	A480G	—	G481A	—	C472U	—
VP1	G2795A	A106T	C2909U	T143I	U2 493C	I6T
	C2879U	L134F				
VP3	U2438A	L225M			C2034U	S91F
VP4	G935U	A65S				

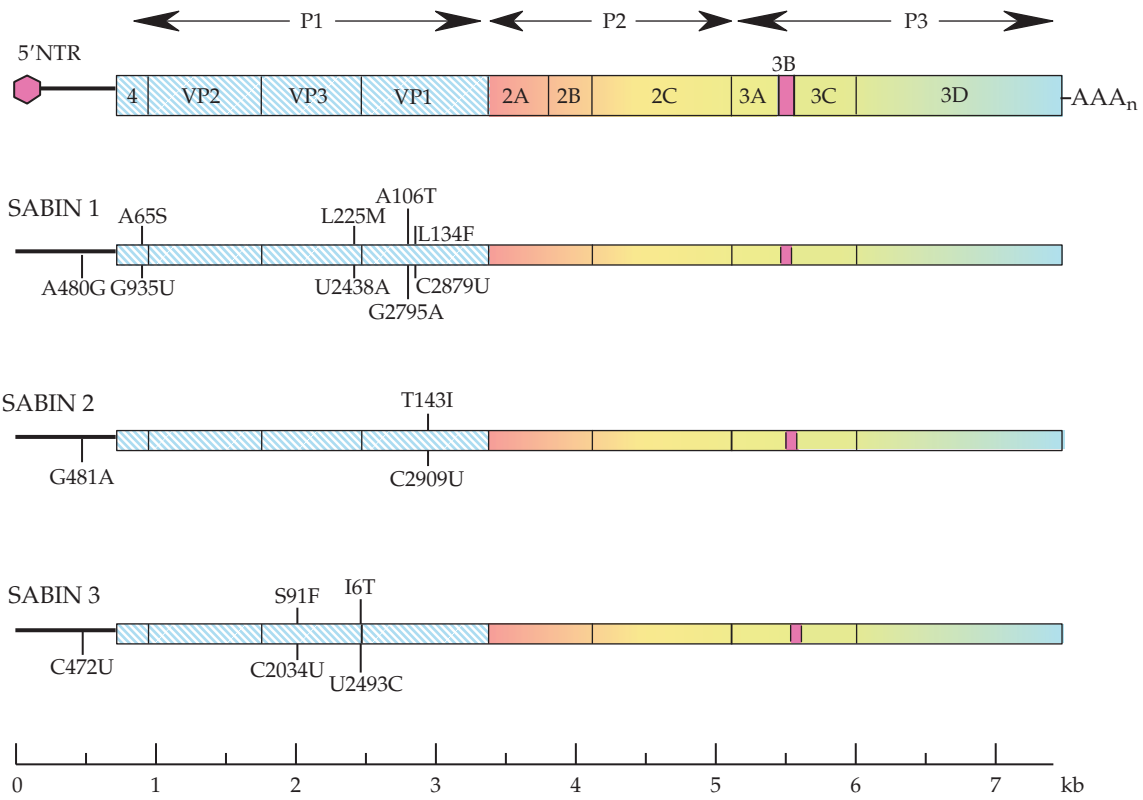

FIGURE 3.6 Diagrams of the genomes of the Sabin vaccine strains of poliovirus types 1, 2, and 3 showing the locations of the attenuating mutations.

were quickly developed that resulted in complete inactivation of the infectivity of the virus, solving this problem and serving as an example for development of other vaccines. The introduction of the Salk vaccine, although enormously successful in controlling polio, also suffered from early problems when it was found that early lots were contaminated with the monkey virus SV40 (described in Chapter 7). Infection of humans by this virus appears to be benign, although there is some evidence that, very rarely, brain tumors may be associated with infection. In any event, this episode brought to light the issue of adventitious contamination of cell cultures with viruses that infect the host supplying tissues for culture. A third problem that arose during development of polio vaccines was the infection of a number of laboratory workers in Germany with Marburg virus. These workers were employed in the isolation of cells from the kidneys of wild-caught monkeys that were to be used in propagating polioviruses, and some of the monkeys were infected with Marburg virus, at that time an unknown virus. Several people died in the ensuing epidemic (described in Chapter 4).

Eradication of Polioviruses

Introduction of the Sabin vaccine led to the eradication of poliovirus from the Americas, and in 1988 the World Health Organization (WHO) initiated a campaign, the Global Polio Eradication Initiative, to eradicate poliovirus worldwide by the year 2000. Although falling short of this goal, significant progress has been made. The number of polio cases worldwide fell from an estimated 300,000+ cases in the mid 1980s to fewer than 3000 by 2000 and subsequently to still lower levels (Fig. 3.7). In 2000 the Centers for Disease Control and Prevention (CDC) said that 2971 cases were reported of which 719 were confirmed by laboratory analysis. In 2001, 537 cases were reported of which 473 were confirmed by laboratory analysis, and these cases occurred in just 10 countries. It appeared that eradication would be achieved soon. The eradication campaign hit a snag recently, however, when Muslim clerics in Nigeria claimed that the vaccine could cause AIDS or infertility. In 2003, officials in some parts of Nigeria suspended local vaccination programs, and an epidemic of poliomyelitis in Nigeria resulted that then spread to neighboring countries that had been free of polio (Fig. 3.8). By 2005 polio had spread to a total of 16 countries that had previously been polio free. Further setbacks in the polio vaccination initiative have resulted from civil unrest in Sudan and other countries that resulted in interference with vaccine campaigns and the reestablishment of poliovirus transmission. Health ministers from Africa are stepping up vaccination

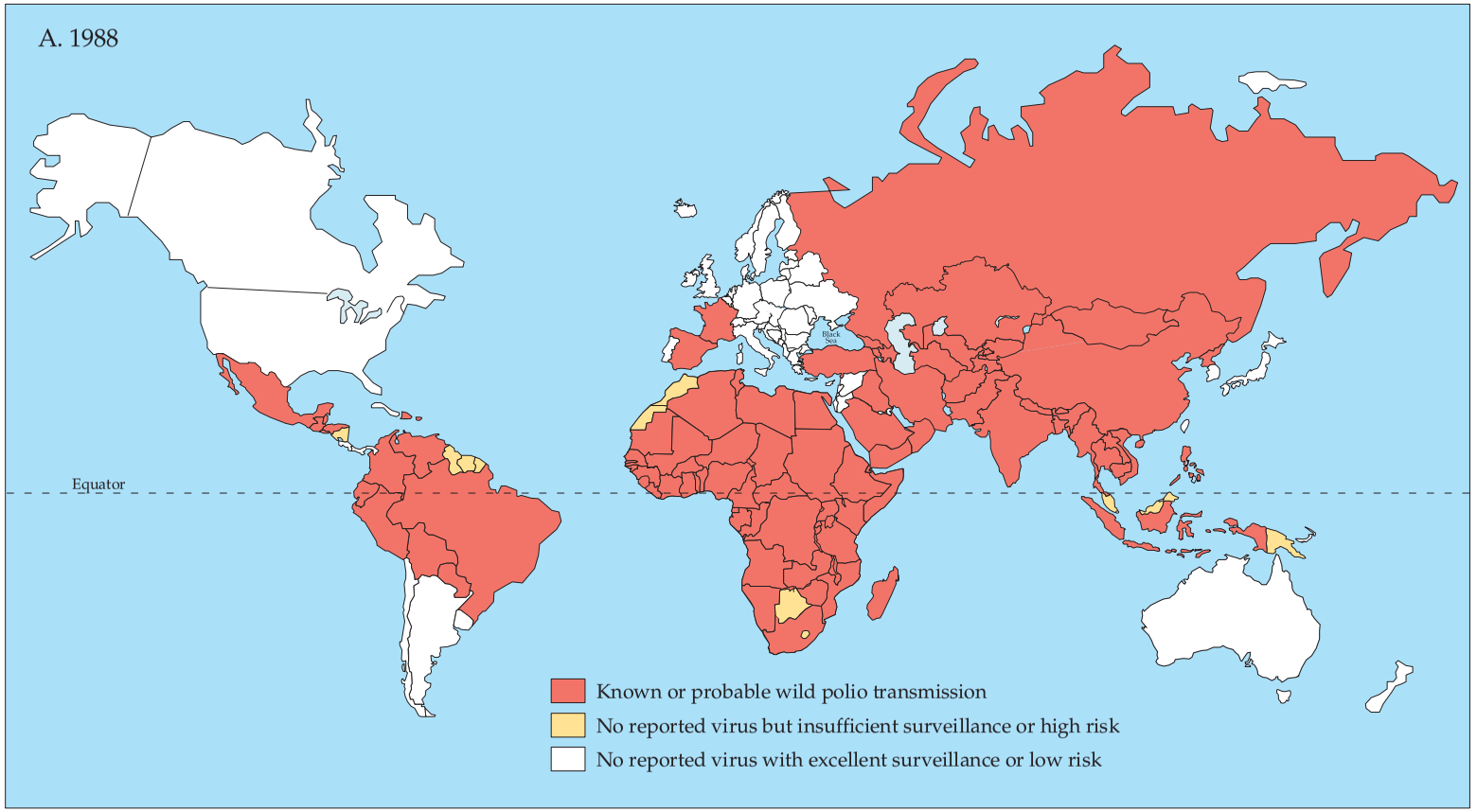

FIGURE 3.7 Maps showing the worldwide distribution of wild poliovirus and the effects of the global eradication efforts. (A) Wild poliovirus transmission in 1988. This is from the Web site www.who.int/gpv-surv/graphics/NY_graphics/global_polio_98.htm.

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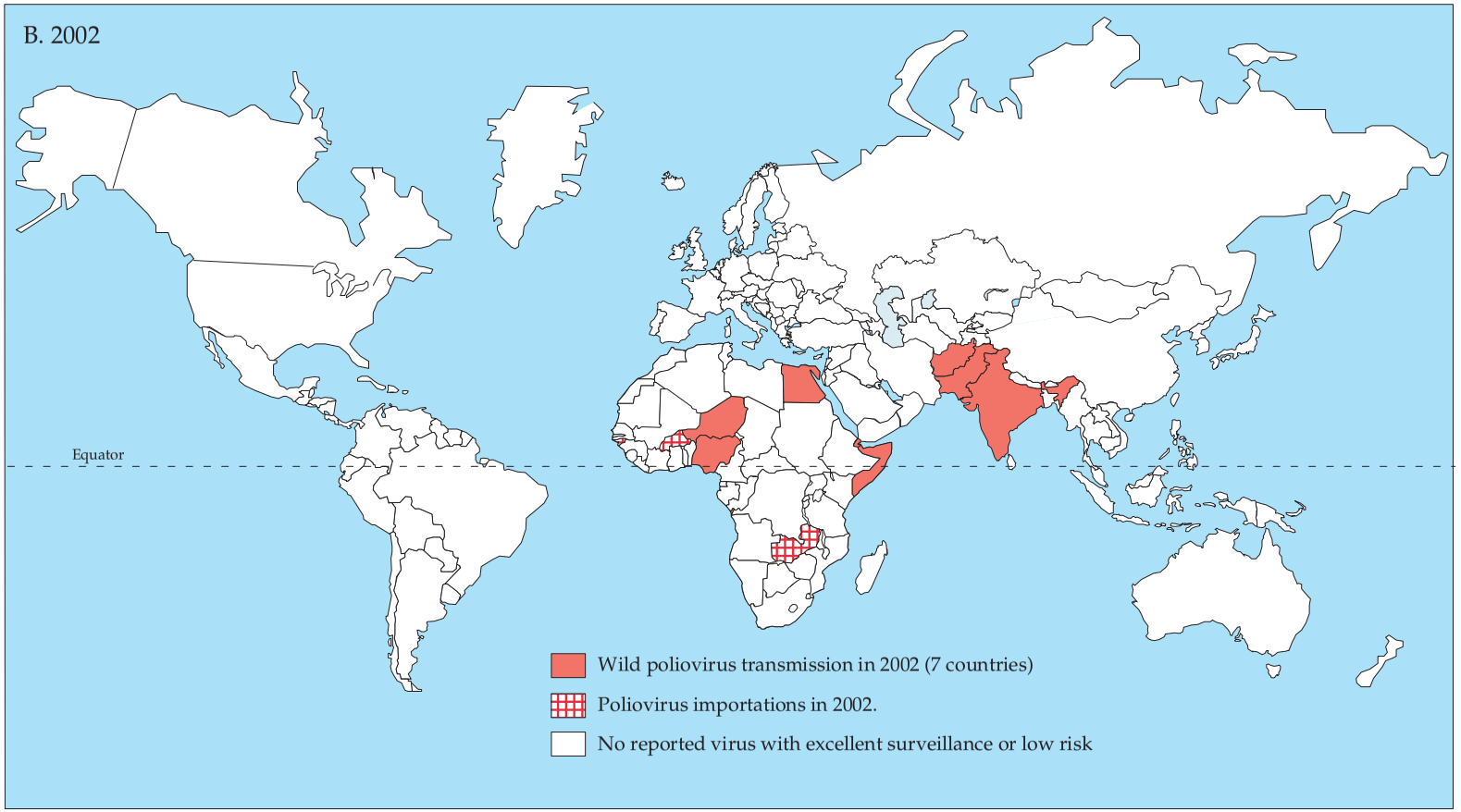


FIGURE 3.7 (Cont'd) (B) Wild poliovirus transmission as of 13 March 2002 (from *MMWR* Vol. 49, #16, p. 352).

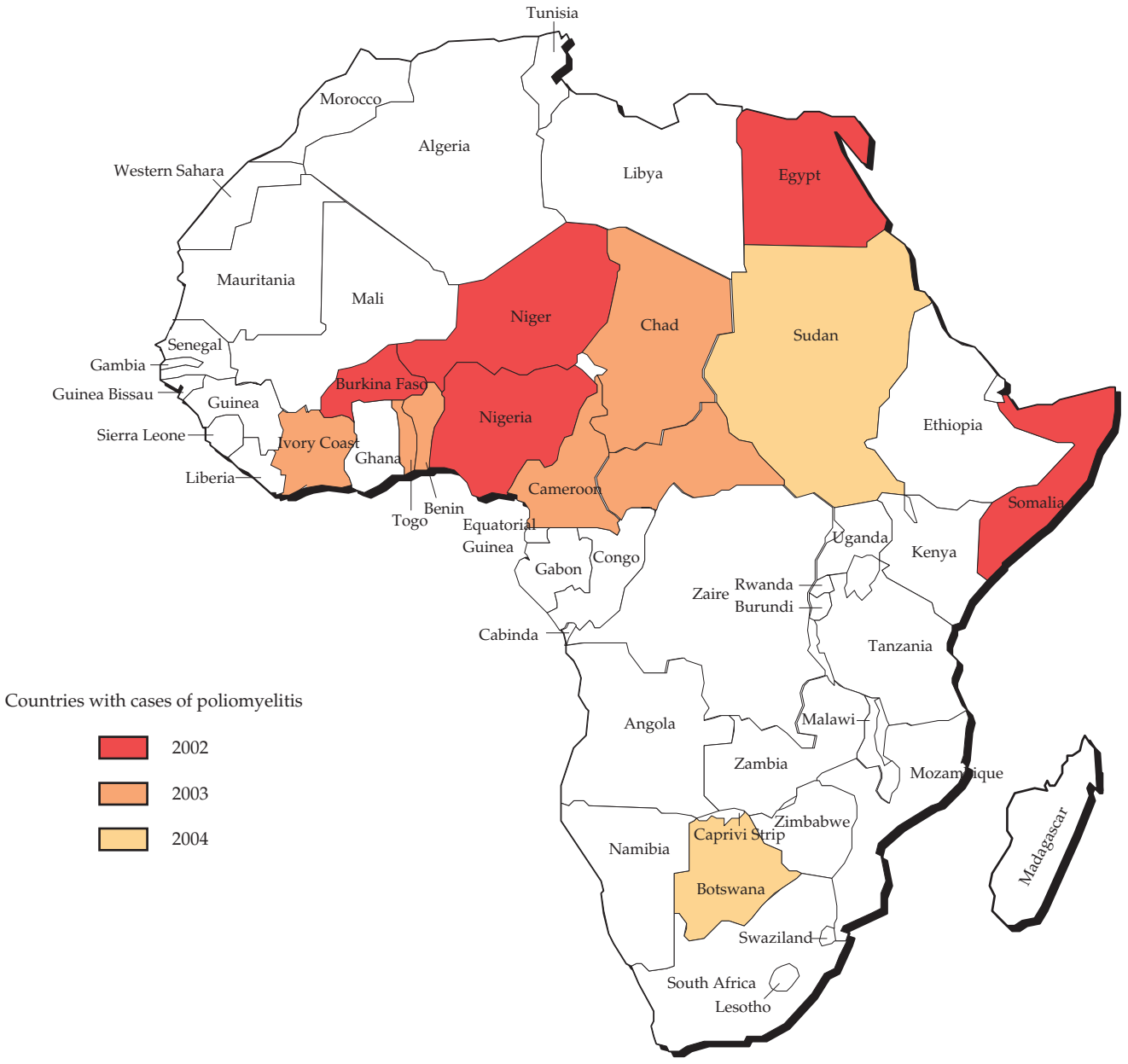


FIGURE 3.8 Reemergence of poliomyelitis in Africa 2002–2004. Data from *MMWR*.

programs to reestablish control of poliovirus transmission, but these situations illustrate problems that result from the continuing conflicts among societies.

Should Routine Poliovirus Immunization Be Eliminated?

If poliovirus is finally eradicated worldwide, should vaccination against the virus be scaled back? Limiting vaccination would be important because of the residual virulence of the vaccine virus. Virulent revertants of vaccine virus not only cause a small number of individual cases (Fig. 3.4), but have led to small epidemics of poliomyelitis when the virulent virus, derived by reversion, circulates in nonimmunized or incompletely immunized contacts (e.g., 21 cases in Hispaniola in 2000–2001), illustrating the potential for continued outbreaks arising from vaccination. The Salk vaccine could still be used in developed countries, but it seems unlikely that routine administration of Salk vaccine would be used in developing countries. It should be possible to design new attenuated viruses for vaccine purposes that would be safer than the Sabin vaccine, but in the absence of poliovirus epidemics no drug company would want to undertake the very expensive development of a new vaccine, especially in view of legal problems that would be sure to arise.

Associated problems are the difficulties in being sure that poliovirus is truly eradicated and the possibility that virulent poliovirus could reemerge. It is known that immunodeficient children who have received the vaccine virus can continue to secrete virus for long periods of time, during which the virus may revert to virulence. Even wild-type poliovirus can circulate silently because most infections are inapparent. In regions where poliomyelitis has reemerged following interruption of vaccination campaigns, it has been found in some cases that the virus had been circulating for at least 2 years before reemergence. In addition, poliomyelitis can be caused by enteroviruses other than poliovirus (see later), a possible source of confusion in diagnosis.

If vaccination were terminated and poliovirus were to reemerge in a naïve population, it could lead to a widespread epidemic. Possible sources of reemerging virus include new strains that might arise from other enteroviruses, circulating strains of wild poliovirus or vaccine-derived virus that have escaped detection, or the inadvertent or deliberate release of wild poliovirus by escape from a laboratory or introduction by terrorists. It would be necessary to maintain stockpiles of polio vaccine to counter such threats, and in the case of the Salk vaccine this stockpiling itself could serve as a possible source of accidental escape of the wild virus. Since the events of 9/11, it is also obvious that terrorists would have no compunctions about releasing a virulent virus into the U.S. population if such a virus could be obtained. Thus, even if (when?) poliovirus is eradicated, the policies with respect to vaccination will require careful consideration.

Post-Polio Syndrome

Although poliovirus has been eradicated from developed countries, there is a large cohort of people infected in the 1950s who are or were paralyzed. Many paralyzed poliomyelitis patients were ultimately able to resume almost normal activities. Through a process of axonal sprouting and reenervation of muscles by the motor neurons that survived the infection, many learned to walk and use their previously paralyzed limbs. In many, recovery was effectively complete. However, a syndrome called post-polio syndrome has emerged to plague a significant fraction, perhaps 40%, of the survivors of paralytic poliomyelitis. This syndrome appears 30–40 years after polio infection and is characterized by fatigue, pain, and weakness. The weakness may be severe enough to require the use of a wheelchair. The syndrome results from the degeneration of motor neurons, but the reasons for the degeneration are not clear. The favored hypothesis is that it is the result of overuse of the surviving motor neurons, which are forced to do the work of many. A second possibility is that the surviving neurons were damaged by the original poliovirus infection and fail prematurely. A third, albeit unlikely, possibility is that poliovirus persists in neurons and is somehow reactivated, even in the presence of anti-polio antibody. In model studies using Sindbis virus infection of mice, it has been found that the virus can persist in neurons in a latent state for at least 1–2 years. There is no evidence that poliovirus might similarly persist in humans for 40 years, however, and such persistence seems unlikely. Other possible explanations for the failure of motor neurons in post-polio syndrome have also been suggested. Fortunately, paralytic poliomyelitis and its sequelae may soon be a thing of the past.

Other Enteroviral Diseases of Humans

As described, 62 human enteroviruses other than polioviruses are currently recognized in the ICTV catalog and classified as serotypes of 4 different species. Although most of these have been known for 50 years, it is only recently that the association of many of these viruses with significant human illness has been shown. In fact, it has now been established that most enteroviruses do cause disease, and many of them cause significant episodes of serious disease (Table 3.5). Study of disease caused by these viruses has been complicated by the fact that there are so many enteroviruses, of which at least some have multiple strains that may differ in disease-causing potential, and by the fact that serious disease is an uncommon complication of infection by most enteroviruses (even for poliovirus most infections do not result in significant disease). This has made it difficult to ascribe any particular disease to infection by any particular virus. However, even though serious disease is an uncommon complication, enteroviral infections are very common, and the total number of cases of disease caused by these viruses is large. These illnesses include very infrequent paralytic disease essentially indistinguishable clinically from that caused by poliovirus; myocarditis and pericarditis (caused especially by the Coxsackie B viruses) that is usually subclinical but can be acute and result in significant cardiac compromise; aseptic meningitis; encephalitis; hepatitis; the common cold (perhaps a quarter of summer colds are due to enteroviruses); diarrheal disease; febrile illnesses; rash; hand-foot-and-mouth disease (a common childhood illness caused by several serotypes in human enterovirus A); and epidemic acute hemorrhagic conjunctivitis (an epidemic disease caused by enterovirus 70 that appeared recently and spread around the world). The Coxsackie B viruses are also associated epidemiologically with juvenile onset diabetes in humans but how (or even whether) they cause diabetes is still unresolved. There are no vaccines for any of these viruses.

Genus *Rhinovirus*

The human rhinoviruses are the causative agents of about half of human colds, the most characteristic symptom of which is rhinitis (inflammation of the nasal mucous membrane and characterized by a runny nose). Other viruses that

TABLE 3.5 Clinical Syndromes Associated with Human Enteroviruses

Clinical syndrome	Poliovirus	Enterovirus A	Enterovirus B	Enterovirus C	Enterovirus D
Paralysis	Types 1, 2, 3	Coxsackie A7, A9 Enterovirus 71	Coxsackie B2–B5 Echoviruses 4, 6, 9, 11, 30	—	Enterovirus 70
Aseptic meningitis	—	Coxsackie A2, A4, A7, A9, A10	Coxsackie B1–B6 All echoviruses except 12, 24, 26, 29, 32, 33	—	—
Pericarditis, myocarditis	—	—	Coxsackie B1–B5 Echoviruses 1, 6, 9, 19	—	—
Encephalitis	—	Enterovirus 71	Coxsackie B1–B5 Echoviruses 2, 6, 9, 19	—	Enterovirus 70
Hepatitis	—	Coxsackie A4	Coxsackie A9, B5 Echovirus 4, 9	—	—
Upper respiratory disease, pneumonia	—	—	Coxsackie B4, B5	Coxsackie A21, A24	Enterovirus 68
Hand, foot, and mouth disease	—	Enterovirus 71 Coxsackie A5, A10, A16	—	—	—
Acute hemorrhagic conjunctivitis	—	—	—	Coxsackie A24	Enterovirus 70
Undifferentiated febrile illness	Types 1, 2, 3	—	Coxsackie B1–B6	—	—

serve as major causes of the common cold include some of the enteroviruses, just described, and the coronaviruses, described later. One hundred serotypes of human rhinoviruses are currently recognized. Eighteen of these have been assigned to the species *human rhinovirus A* and three of them to *human rhinovirus B*. The remaining 79 serotypes have not yet been assigned to a species. There are also three serotypes of bovine rhinovirus known to exist, and there are rhinoviruses for other animals that have as yet to be well characterized. In general, rhinoviruses are specific for a particular species or for a limited range of species, and this restriction appears to work at the level of receptors required for virus entry (see Chapter 1).

The 100 serotypes of human rhinoviruses are not cross protective and the result is that we are subject to many rhinovirus colds during our lifetimes. Young children, not having been exposed to rhinoviruses and other viruses that cause colds, contract many colds a year. Adults, having become immune to many of these viruses through hard experience, have fewer colds per year, usually only about one. However, the extent and duration of immunity to a particular rhinovirus induced by infection are not well established. There are so many rhinoviruses (and although rhinoviral disease may be miserable it is not life threatening) that detailed studies on cohorts of people over many years have not been done to establish whether immunity to a particular rhinovirus following infection is long lived. For the same reasons, there are no vaccines for any of these viruses.

Rhinoviruses replicate in the upper respiratory tract and are transmitted by direct person-to-person contact. Coughing and sneezing, common syndromes of rhinovirus infection, help spread the virus to nearby contacts. It is not clear how much of the spread is due to aerosolization of the virus on coughing or sneezing followed by inhalation of the aerosolized virus by a susceptible contact, and how much is due to contact with mucus that contains virus, such as by handshake or contact with contaminated doorknobs, followed by transmission of the virus to mucosal membranes in the nose or the mouth.

It is an interesting and informative historical fact that early attempts to isolate rhinoviruses using standard cell culture techniques were unsuccessful. Most cells in the body are maintained at 37°C at a pH of 7.4, and cells in culture are normally maintained under these conditions. However, cells in the upper respiratory tract are maintained at a lower temperature, about 33°C, because the inhalation of outside air through the upper respiratory tract keeps this area cool, and at a pH significantly less than 7.4 because of the high concentration of CO_2 in expired air. Rhinoviruses replicate well in cultured cells under these altered conditions and appear

to require the lower temperature and lower pH for efficient growth. In part because of this, rhinovirus infection is limited to the upper respiratory tract, and rhinoviruses almost never cause lower respiratory tract infections.

It is also of interest that rhinoviruses are sensitive to very low pH, and infectivity is destroyed by exposure to pH 3. The related polioviruses, however, survive exposure to pH 2, which is necessary because, being enteroviruses, they must survive passage through the stomach in order to infect an animal.

Genus *Cardiovirus*

The *Cardiovirus* genus consists of several viruses of mice of which encephalomyocarditis virus (EMC) has been extensively studied as a model picornavirus. It is closely related to other picornaviruses (Fig. 3.1) although differing in certain important characteristics. The EMC IRES has proved more useful than the poliovirus IRES in experiments that require polycistronic mRNAs or that express proteins in a cap-independent fashion in vertebrate expression systems. Theiler's virus, another member of this genus, causes demyelinating disease in mice and has been extensively studied as a model for multiple sclerosis in humans.

Genus *Hepatovirus*

Hepatitis in Humans

Many different viruses, belonging to several virus families, are known to cause hepatitis (inflammation of the liver) in humans. These different viruses have different modes of transmission and cause illness of different degrees of severity (although all hepatitis is serious) that results from destruction of liver cells caused by growth of these viruses in the liver as a target organ. Hepatitis is characterized by fatigue and other symptoms that result from inadequate liver function, and it may be fatal if sufficient destruction of the liver takes place. A characteristic feature of acute hepatitis is the presence of elevated levels of liver enzymes circulating in the blood that results from the destruction of liver cells. Many cases of hepatitis are accompanied by jaundice (turning yellow) because of the destruction of the liver, which is responsible for clearing bilirubin from the blood.

The viruses whose primary disease syndrome in humans is hepatitis, and which therefore target the liver as the principal or only organ infected, or viruses that are closely related to viruses that cause such hepatitis, have historically been named hepatitis virus followed by a letter, in the order of isolation. Thus we have hepatitis A virus, the first to be isolated, hepatitis B virus, the second, and so forth. Because these viruses belong to a number of different families, confusion can arise because of the similar names even though the viruses are unrelated. For reference, Table 3.6 presents a description of the currently known viruses whose name includes hepatitis. Figure 3.9 shows the incidence of hepatitis in the United States in 1997 caused by hepatitis viruses A, B, and C, which are the most important causes of viral hepatitis in the United States. Identification of which hepatitis virus is responsible for any specific case of hepatitis requires immunologic tests or virus isolation, because symptoms are similar.

Hepatitis A virus is a picornavirus and will be considered here. The other viruses will be considered when their respective families are introduced.

Hepatitis A Virus

Hepatitis A virus (HAV) is a causative agent of infectious hepatitis in humans. The virus is worldwide in distribution. Only one serotype is known, but isolates from different areas or different times can be grouped into different genotypes or strains. The most distantly related HAV isolates share about 75% nucleotide sequence identity, but most isolates are much more closely related. HAV is a typical picornavirus but is an outlier in the family (Fig. 3.1). It shares only 28% amino acid identity in its structural proteins with any other picornavirus, whereas most picornaviruses are more closely related to one another.

The number of cases of hepatitis A in the world has been estimated to be more than 1.4 million each year. In 1998 in the United States, for example, ~37,000 cases of hepatitis were reported, of which two-thirds were diagnosed as caused by HAV. HAV is spread through contaminated food and water. Filter-feeding shellfish like oysters are known to concentrate the virus, and consumption of raw shellfish has been the cause of many epidemics of hepatitis A. A 2003 epidemic of more that 500 cases in Pennsylvania was caused by consumption of green onions that are believed to have been contaminated during harvest. Infection by HAV usually results in a self-limited illness in which the patient recovers with relatively few sequelae. The illness can be quite serious, even fatal, however, because 90% of the liver tissue can be destroyed by virus infection, and liver function is severely impaired until the liver recovers. The seriousness of disease is age dependent. Very young children suffer little disease but with advancing age infection by the virus becomes more serious. The mortality rate in children younger than 14 is only 0.1%, but HAV infection in people older than 40 results in a fatality rate of 2.1%.

Before the introduction of a vaccine, the only prophylaxis for HAV was injection of immune gamma globulin, which provided protection from the virus for a few weeks. Two inactivated virus vaccines against HAV were licensed in the mid 1990s that have been found to give long-lived protection. Results from a clinical trial in Thailand that showed the

TABLE 3.6 Causative Agents of Viral Hepatitis in Humans

Virus	*Family/ genus*	Genome type/ *size in kb*	Transmission	Chronicity?	Long-term effects	Annual[a] U.S. acute cases/*deaths* in 2004	Chronic hepatitis (millions of cases) U.S./*World*
Hepatitis A	***Picornaviridae***/ *Hepatovirus*	**ss (+) RNA**/ *7.5 kb*	Fecal–oral	Very little	Few if any	**5683**/*76*	**0**/0
Hepatitis B	***Hepadnaviridae***/ *Orthohepadnovirus*	**ds DNA (RT)**/[b] *3.2 kb*	Parenteral, sexual, vertical	10% of adults, 90% of neonates	HCC[c], cirrhosis	**6212**/*659*	**1.2**/*300–400*
Hepatitis C	***Flaviviridae***/ *Hepacivirus*	**ss (+) RNA**/ *9.4 kb*	Parenteral, sexual, vertical	>50%	HCC[c], cirrhosis	**720**/*4321*	**3**/*50–100*
Hepatitis D	*Deltavirus*	**ss, circular RNA**/*1.7 kb*	Parenteral, (sexual, vertical?)	Yes	Exacerbates symptoms of Hep B	**7500**/ *1000*	**0.07**/?
Hepatitis E	***Hepeviridae***/ *Hepevirus*	**ss (+) RNA**/ *7.5 kb*	Fecal–oral	No	Few if any	**Very rare**	**0**/0
Hepatitis F[d]	?						
Hepatitis G	***Flaviviridae***/ *Hepacivirus*	**ss (+) RNA**/ *9.4 kb*	Parenteral, other?	Yes	??	**??**/*none*	??

[a] Data from MMWR Summary of Notifiable Diseases—2004. It is noteworthy that acute cases of both hepatitis A and hepatitis B have declined significantly since 1990 with the introduction of vaccines that are now in widespread use; see Figure 3.9 below.
[b] RT is reverse transcriptase. Nucleic acid in virion is partially ds DNA, consisting of a full-length minus-strand DNA of 3.2 kb, and an incomplete plus-strand DNA that is variable in length.
[c] HCC, Hepatocellular carcinoma.
[d] Isolate from a fulminant case of hepatitis, not further characterized.

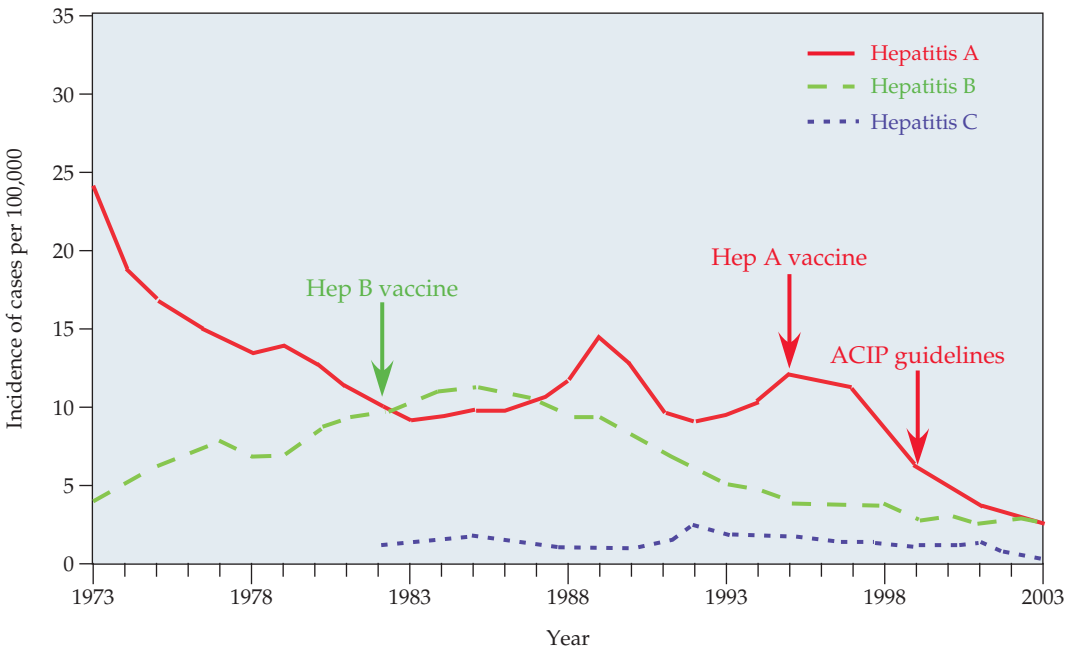


FIGURE 3.9 Incidence (cases per 100,000 population) of viral hepatitis in the United States between 1973 and 2003. Hepatitis A incidence was the lowest ever in 2004, but there has been a trend for cyclic increases every decade, and future increases could occur. However, with the expansion of recommended vaccination to include children in all communities where the incidence was consistently above the national average (1999 ACIP guidelines), the incidence of HAV has continued to plummet. No vaccine for hepatitis C exists, but an antibody test for hepatitis C was first introduced in May 1990. This graph is adapted from *MMWR* Vol. 52, #54, *Summary of Notifiable Diseases United States 2003*.

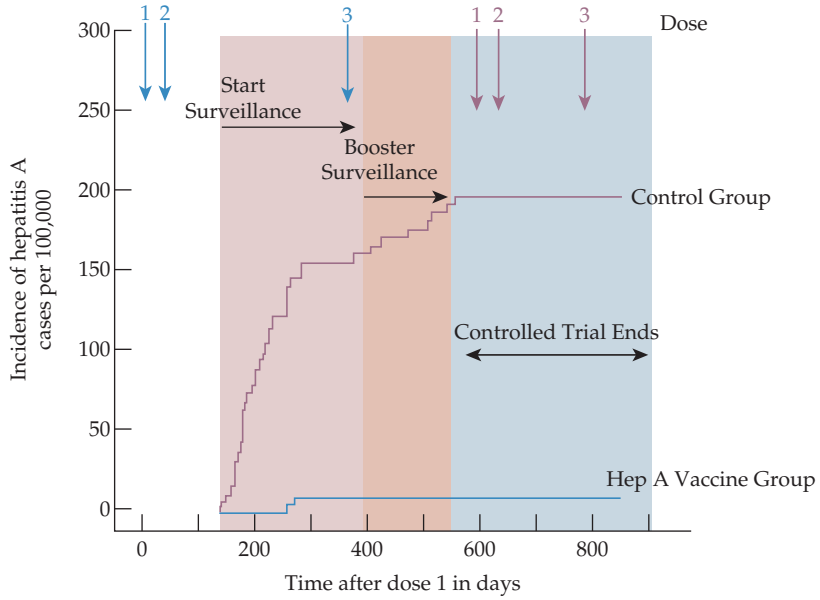


FIGURE 3.10 Controlled trial of a hepatitis A vaccine in Thailand. Children were divided into two groups, and at the times indicated by the blue arrows the vaccine group was given hepatitis A vaccine and the control group was given hepatitis B vaccine. There were two periods of surveillance for cases of hepatitis A, indicated by the pink overlays. The controlled trial ended at 540 days when the control group was given hepatitis A vaccine at the times indicated by the magenta arrows and the vaccine group was given hepatitis B vaccine. Adapted from Figure 3 in Innis *et al*. (1994).

efficacy of this vaccine are shown in Fig. 3.10, as an example of the type of data that can be obtained in clinical trials. The introduction of these vaccines and the recommendation in 1996 of routine childhood vaccination has resulted in a steady decline in the rate of hepatitis A in the United States. In 2003 there were 7653 cases reported (2.7 per 100,000 population), and childhood vaccination appears to have been important in the control of this disease. Of interest is the finding that hepatitis A rates were much higher in the western United States before the introduction of the vaccines, but the rates are now similar across the United States, and an increasing proportion of cases occur in adults.

Genus *Kobuvirus*

In Aichi, Japan in 1989, a stool specimen from a patient suffering from gastroenteritis associated with consumption of raw oysters was found to contain a new picornavirus, subsequently called Aichi virus. Since then the virus has been found not only in Japan but also in Pakistani children with gastroenteritis and in Japanese travelers returning from Southeast Asia with gastroenteritis, and the virus thus appears to be widespread and to be one of the many viruses that are causative agents of human gastroenteritis. The virus is sufficiently distinct in its sequence from other picornaviruses to be classified in a separate genus, *Kobuvirus* (Fig. 3.1). The name is derived from the Japanese word for bump, because the virion appears bumpy in the electron microscope. There are interesting differences in the replication of this virus from those of other picornaviruses, such as the presence of VP0 in virus particles rather than the cleaved products VP2 and VP4 (see Fig. 3.2). The worldwide burden of human gastroenteritis caused by Aichi virus or closely related viruses is unknown at present. As described later, many viruses belonging to a number of virus families cause epidemic gastroenteritis in humans, and sorting out the causative agents is difficult and requires time.

A virus related to Aichi virus appears to be widespread in cattle in the Aichi area of Japan. This virus, classified for now as a member of the *Kobuvirus* genus, apparently causes inapparent infection in cattle and has been called bovine kobuvirus. Thus, it is possible that kobuviruses are widely distributed in the world and infect a number of species.

Genus *Aphthovirus*

Foot-and-mouth disease viruses (FMDV) belong to seven currently recognized serotypes. They cause a debilitating disease, foot-and-mouth disease, in cattle and other animals, and are economically important pathogens. FMDV was eliminated from the United States many years ago by the simple expedient of killing all infected animals until such time as the virus was extirpated. The last epidemic in the United States occurred in 1929. The virus still circulates in Europe, South America, and other parts of the world, and the U.S. Department of Agriculture maintains

strict quarantines in order to prevent the virus from reappearing in this country. In the United States, work with the virus is allowed only on Plum Island in Long Island Sound, in order to prevent its accidental release. Because of its agricultural importance, the molecular biology of the virus has been intensively studied.

In 2001, a large epidemic of FMDV occurred in Western Europe. The epidemic began in February in British sheep and spread to cattle and pigs in Britain and on the continent. By June more than 2000 infected animals had been detected. The epidemic was controlled by restricting the movement of sheep, cattle, and swine, and culling of herds in which FMDV was found. Almost 4 million animals were destroyed in this process. The damage to the British cattle industry was particularly distressful because this epidemic occurred only a few years after widespread culling of cattle to control an epidemic of a prion disease called "mad cow disease" (Chapter 9). Beginning in March, many rural areas often visited by tourists were closed to prevent the spread of the virus, and the U.S. Department of Agriculture was especially vigilant in examining travelers returning from affected countries. British authorities considered vaccinating cattle with commercial vaccines to control the epidemic, but vaccines have not been used by the British to date. Vaccination for FMDV is used in some parts of the world, but is controversial because it is then difficult to distinguish between vaccinated animals and infected animals. Thus, for example, the United States does not allow the importation of beef from areas where vaccination is practiced because it is not possible to rule out the presence of FMDV infection. Although FMDV was thought to have been eradicated by June 2001, and restrictions were about to be lifted, 16 new cases of FMDV were then found in Britain that delayed lifting of the restrictions in that country. The last new case was found on September 30, 2001, and Britain was declared free of FMDV on January 15, 2002.

FAMILY *CALICIVIRIDAE*

The caliciviruses are nonenveloped viruses possessing icosahedral symmetry and having a diameter of about 30 nm. The name comes from the Latin word for cup or goblet (source of the English word chalice) because there are cuplike depressions in the surface of the virion when viewed in the electron microscope. The characteristics of a number of caliciviruses are shown in Table 3.7. Four genera of caliciviruses are currently recognized, two of which, genus *Norovirus* (whose members were formerly called the Norwalk-like viruses) and genus *Sapovirus* (formerly called the Sapporo-like viruses), contain agents that cause human gastroenteritis. The genera *Vesivirus* and *Lagovirus* contain viruses of cats, rabbits, pigs, and sea lions. Additional caliciviruses of cattle, dogs, mink, walrus, and chickens remain to be classified as to genus. Most or all caliciviruses are host specific, infecting only a single animal species.

TABLE 3.7 *Caliciviridae*

Genus/members	Virus name abbreviation	Usual host(s)	Transmission	Disease	World distribution
Vesivirus					
Vesicular exanthema of swine (includes San Miguel sea lion virus)	VESV	Swine	Oral, contact	Fever, lesions on snout and feet	California
Feline calicivirus	FCV	Cats	Contact	Rhinitis, pneumonia, fever	Worldwide
Rabbit vesivirus		Rabbits			
Lagovirus					
Rabbit hemorrhagic disease	RHDV	Rabbits	Water-borne, oral–fecal	Hemorrhages	China, Europe, Australia[a]
European brown hare syndrome	EBHSV	Hares			Europe
Norovirus					
Norwalk	NV	Humans	Water-borne, oral–fecal	Epidemic gastroenteritis	Worldwide
Sapovirus					
Sapporo	SV	Humans	Water-borne, oral–fecal	Epidemic gastroenteritis	Worldwide

Unassigned members of the family include caliciviruses of cattle, dogs, fowl, mink, and walrus

[a] RHDV was inadvertently introduced into Australia.

The human caliciviruses have been difficult to study because it has not been possible to grow them in cell culture or to infect experimental animals. As a consequence, we know less about them than we do about such well-studied viruses as the picornaviruses. The first calicivirus described was a virus of sea lions (San Miguel sea lion virus, genus *Vesivirus*), and most of what we know of the molecular biology of caliciviruses comes from studies of this virus and of other nonhuman viruses such as feline calicivirus, which will replicate in cultured cells. The complete sequences of a number of the human viruses have now been obtained, which has greatly expanded our knowledge of these viruses, but details of the molecular biology of their replication are still lacking.

The caliciviruses are distant relatives of the picornaviruses. Where known, the (+)RNA genome of about 8 kb has a 5′-terminal VPg and a 3′-terminal poly(A), as do the picornaviruses, and calicivirus proteins share sequence identity with the picornavirus $2C^{ATPase}$, $3D^{pol}$, and $3C^{pro}$. Unlike the picornaviruses, however, the nonstructural proteins are 5′ terminal and the structural protein(s) 3′ terminal (Fig. 3.11). Translation of the genomic RNA produces a polyprotein that contains the sequences of the nonstructural proteins. Cleavage of the polyproteins is presumably effected by $3C^{pro}$ (also called $3C^{pro}$-like or $3CL^{pro}$). In those caliciviruses that have been studied in cell culture, a subgenomic RNA is produced that is assumed to be an mRNA for the major capsid protein and, at least in one case, a minor capsid protein as well, and it is assumed that all caliciviruses produce a subgenomic RNA in order to produce the structural protein(s). There is evidence that this subgenomic RNA, once produced, can replicate independently of the viral genomic RNA. The organization of the calicivirus genome differs slightly among the genera as shown in Fig. 3.11. In the lagoviruses and sapoviruses, the major capsid protein is in frame with the nonstructural proteins and contiguous with them. Thus,

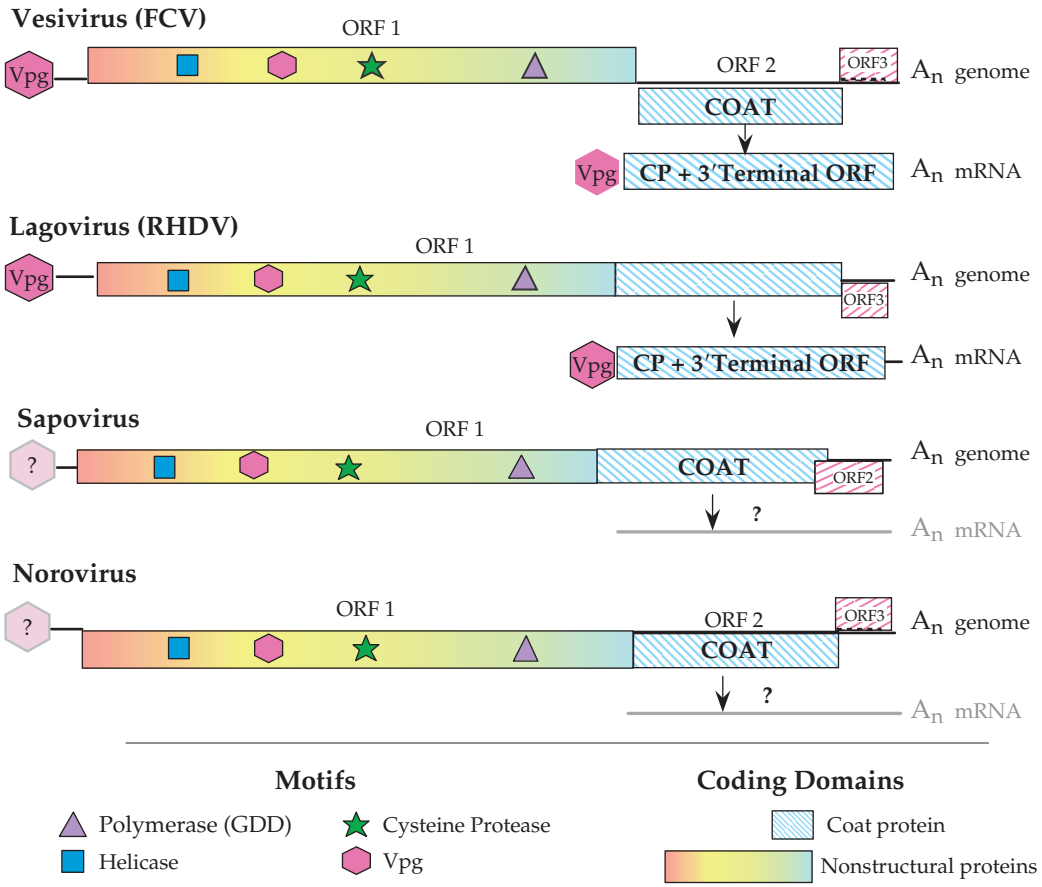


FIGURE 3.11 Diagrammatic representation of the genome organization of the four genera of *Caliciviridae*. Notice that the coat protein is encoded in ORF2 of vesiviruses and noroviruses but as the C-terminal portion of ORF1 in lagoviruses and sapoviruses. Subgenomic mRNAs have been identified for vesiviruses and lagoviruses. FCV, feline calicivirus; RHDV, rabbit hemorrhagic disease virus. Adapted from Fauquet *et al*. (2005), p. 847.

it is possible that the nonstructural polyprotein contains the sequences of the structural proteins as well, but is assumed that the major source of the structural proteins is translation of a subgenomic mRNA, although details of the viral expression strategy are still lacking. In the noroviruses and vesiviruses, the major capsid protein is in a different reading frame.

Another, fairly short, ORF is found in the 3′-terminal region of each calicivirus genome. The translation product of this ORF is a minor structural protein.

Caliciviruses have been shown to interfere with host protein synthesis. The mechanism used is the same as one of the mechanisms used by poliovirus, cleavage of poly(A)-binding protein by $3C^{pro}$. The norovirus protease cleaves this host protein at the same site as does the poliovirus $3C^{pro}$, whereas the feline calicivirus protease cleaves it at a site 24 residues downstream of the poliovirus site.

Noroviruses

Norwalk virus is a human virus that causes gastroenteritis. As described, there is no cultured cell line in which to propagate the viruses, nor even an animal model in which to grow it, and studies have relied on human volunteers for virus propagation. This has severely limited the amount of information (and material) that can be obtained. However, because of the power of modern gene cloning technology, the entire genome of Norwalk has been cloned and sequenced, starting from stools of experimentally infected human volunteers. These genome sequences can be aligned with the genomes of other caliciviruses (Fig. 3.11), and the mechanisms of the expression of the genome and the functions of the encoded proteins can be predicted from studies of the animal caliciviruses. Thus, for example, it is assumed that the Norwalk viruses possess a 5′-terminal VPg as does San Miguel sea lion virus, but to date it has not been possible to prove this. However, the genomic sequence of Norwalk virus contains a sequence related to the VPg of other caliciviruses, providing evidence that Norwalk virus RNA does in fact carry a 5′-terminal VPg. Clones of various regions of the viral RNA can be expressed in cultured cells to study the expression and function of various domains. For example, the capsid protein of Norwalk virus has been expressed in insect cells and these proteins spontaneously assemble into T=3 virus-like particles of 38-nm diameter whose structure has been determined to atomic resolution by X-ray diffraction techniques. Of interest is the recent discovery of a mouse norovirus. The virus causes only silent infections in immunocompetent mice but, importantly, it will replicate in cultured cells, and thus has the potential to serve as a surrogate virus to work out details of the molecular biology of replication of noroviruses. Another recent development is the finding that human noroviruses will replicate in a cell system in which the viral RNA is introduced by transcription from cDNA clones.

Several isolates of the Norwalk virus have been studied, all of which share more than 50% sequence identity and which are named after the location where they were first isolated. These include Norwalk virus, Hawaii virus, Snow Mountain virus, Southampton virus, Lordsdale virus, and Desert Shield virus. These viruses are extraordinarily infectious. In one epidemic investigated by the Centers for Disease Control and Prevention and local health authorities, a baker preparing food for a wedding was ill with gastroenteritis. After using the toilet, he washed his hands thoroughly before handling food, but his hands were still contaminated with virus, perhaps under the fingernails. He used his hands to stir a very large pot of icing used to glaze cakes and doughnuts that were distributed at the wedding reception, and managed to contaminate the icing with virus. Every guest at the reception who ate as much as a single doughnut contracted gastroenteritis.

The Norwalk viruses regularly cause epidemics of gastroenteritis. The incubation period is short (24 hours on average) and the course of disease is also short (1–2 days). Following recovery, immunity is established to the virus, but the duration of immunity appears to be fairly short (perhaps one or a few years). Studies of immunity are made difficult by the finding that some fraction of the human population seems to be resistant to any particular Norwalk virus studied, perhaps because of a lack of receptors for the virus, and by the fact that there are so many viruses that cause gastroenteritis.

The Norwalk group of viruses is worldwide in its distribution but epidemiological studies of these viruses have been difficult in the absence of a cell culture system or an experimental animal that can be infected by the virus. Foodborne infections are estimated to cause 76 million cases of human illness in the United States each year, of which 300,000 lead to hospitalization and 5000 are fatal. A significant fraction of these illnesses, perhaps one-third, are caused by bacteria, for which ready tests are available for use by health authorities and these epidemics have received wide attention. Until recently, however, little testing for Norwalk virus was performed. With the determination of the sequence of Norwalk RNA followed by the development of RT–PCR techniques to rapidly screen for Norwalk virus, the CDC has now attempted to assess the importance of Norwalk virus in food-borne outbreaks of gastroenteritis. Analysis of available data suggests that perhaps 50% of food-borne outbreaks in the United States are attributable to Norwalk virus, and these viruses are therefore responsible for the majority of outbreaks of nonbacterial gastroenteritis in the United States. Further, the Norwalk outbreaks are larger than those associated with bacteria, a median of 25 cases per outbreak versus 15 cases in bacterial outbreaks and 7 cases in outbreaks of unknown etiology. More than half of the Norwalk

outbreaks were associated with eating salads, sandwiches, or fresh produce, and in 40% of Norwalk outbreaks restaurants or caterers were associated. Some outbreaks have been associated with the consumption of raw oysters for reasons described earlier. Given the total number of cases of gastroenteritis in humans, it is clear that Norwalk virus is a very important disease pathogen.

Norwalk virus has also established itself as a bane of the cruise ship industry. In 2004, for example, there were 38 outbreaks of gastrointestinal illness on cruise ships, defined as at least 3% of the passengers and crew aboard the ship developing diarrhea or vomiting not due to seasickness and accompanied by other symptoms such as fever or aching muscles. Most of such outbreaks are due to noroviruses. In one series of episodes studied by the CDC, a cruise ship suffered outbreaks during six consecutive cruises, despite extensive efforts to cleanse the ship. After the second outbreak, the ship was taken out of service for a week and scrubbed exhaustively, but the outbreaks continued. The CDC investigation showed that one particular genotype of virus was present in all of the outbreaks, but that new genotypes were also introduced into the ship from time to time.

Sapoviruses

Many isolates of Sapporo virus have been made in association with outbreaks of gastroenteritis. Sapporo virus also appears to be a widespread virus that is an important cause of human gastroenteritis, but much less is known about the epidemiology of Sapporo virus than about Norwalk virus.

Rabbit Hemorrhagic Disease Virus

Rabbit hemorrhagic disease virus (RHDV), a member of the genus *Lagovirus*, causes an often fatal illness in European rabbits. It has been used in Australia and New Zealand in an attempt to control large and destructive populations of introduced rabbits, a topic that will be covered in more detail in Chapter 7 when we discuss rabbit myxoma virus. RHDV was being studied on an island off the south coast of Australia as a possible rabbit control agent when it was "inadvertently" introduced onto the mainland. Once introduced it spread rapidly, probably aided by local farmers. The virus was later introduced, at first illegally and then legally, into New Zealand. The virus has been only moderately successful in controlling the rabbit populations. When first introduced, it was highly pathogenic, causing high fatality rates. After some time, however, an increasing proportion of rabbits survived infection. In New Zealand, the virus appears to have developed the ability to establish persistent or latent infections, perhaps allowing the rabbit host to survive challenge with virulent virus. There may also have been a background level of immunity to the virus caused by the circulation of a similar virus in the rabbit population of New Zealand for many years before introduction of the virulent virus.

FAMILY *HEPEVIRIDAE*

Hepatitis E virus is similar in size and genome organization (Fig. 3.12) to the caliciviruses and until recently was considered to be a member of the *Caliciviridae*. It differs in important details, however, and classification as a distinct family was deemed appropriate. The viral RNA is capped rather than possessing a VPg, and the virus-encoded protease, whose sequence has been deduced from the sequence of the viral genome, appears to be a papain-like protease rather than a $3C^{pro}$-like enzyme, another difference from the caliciviruses. Like the caliciviruses, the capsid protein is 3′ of the nonstructural proteins and is translated from a subgenomic mRNA.

HEV is one of several viruses that cause human hepatitis (Table 3.6), in this case epidemic water-borne hepatitis. Infection is by the oral–fecal route and contaminated water is often the source of the infection. Unlike picornaviruses

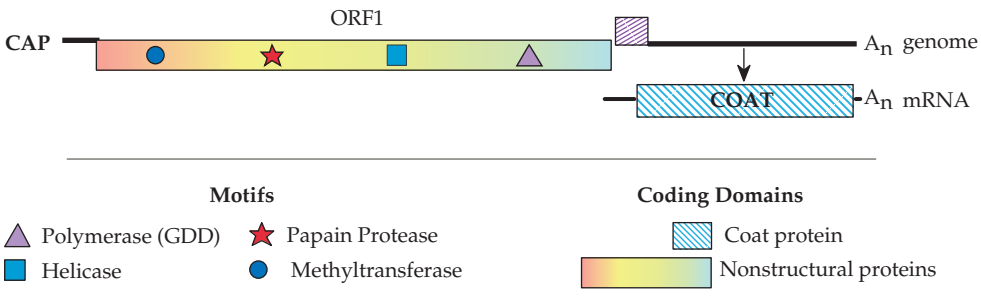


FIGURE 3.12 Genome organization of hepatitis E virus, adapted from Fauquet *et al*. (2005) p. 854.

and caliciviruses, the virus has a wide host range, infecting a variety of mammals. The virus is widespread in pigs, both domestic and wild, and pigs appear to be the major reservoir host for the virus. Thus, transmission of the virus is zoonotic, from animals to humans. In one particularly interesting study in Japan, four humans were infected by the virus upon eating raw deer meat from a wild deer. The nucleotide sequence of the virus isolated from the humans differed from the sequence of virus isolated from the deer by fewer than 8 nucleotides, clearly showing that the deer served as the source of the infection. From a wild boar captured in the same area a year later, virus was isolated that differed by up to 23 nucleotides from the deer–human viruses. It has been found that in Japan only about 1% of deer are positive for HEV antibodies, whereas 3 of 7 wild boars were found to be positive and almost 100% of Japanese pigs are positive by 6 months of age. Thus, pigs are the major reservoir and spread the virus to other mammals as well as to humans.

HEV is found in Asia, Africa, Southern Europe, and Mexico, where it causes thousands of cases of hepatitis each year (Fig. 3.13). A dendrogram of various geographical isolates of HEV illustrates that the New World strain has diverged significantly from the Asian isolates (Fig. 3.14). Thus, there is little or no circulation of virus between different geographic regions. The disease is severe but the fatality rate is low (<1%), with the prominent exception that the fatality rate in pregnant women can be 20%. There is no vaccine or treatment for the virus at present.

An avian HEV is also known. This virus shares about 50% nucleotide sequence identity with the mammalian virus. It is an important pathogen of commercial broiler chickens, but does not appear to infect mammals.

FAMILY *ASTROVIRIDAE*

Astroviruses constitute a recently described family of animal viruses. Some of these are human viruses that cause gastroenteritis, but astroviruses for cattle, pigs, sheep, and ducks are also known (Table 3.8). The name comes from the Greek word for star, from starlike structures on the surface of the virion. Unlike the human caliciviruses, the human astroviruses will grow in cultured cells, and progress on understanding their molecular biology has been more rapid. They are small viruses, 30 nm, with icosahedral symmetry, and a genome of 7 kb.

The replicase proteins of astroviruses are translated from the genomic RNA as two polyproteins (Fig. 3.15). The smaller translation product (1a) terminates at a stop codon; ribosomal frameshifting (Chapter 1) at a retrovirus-like slippery

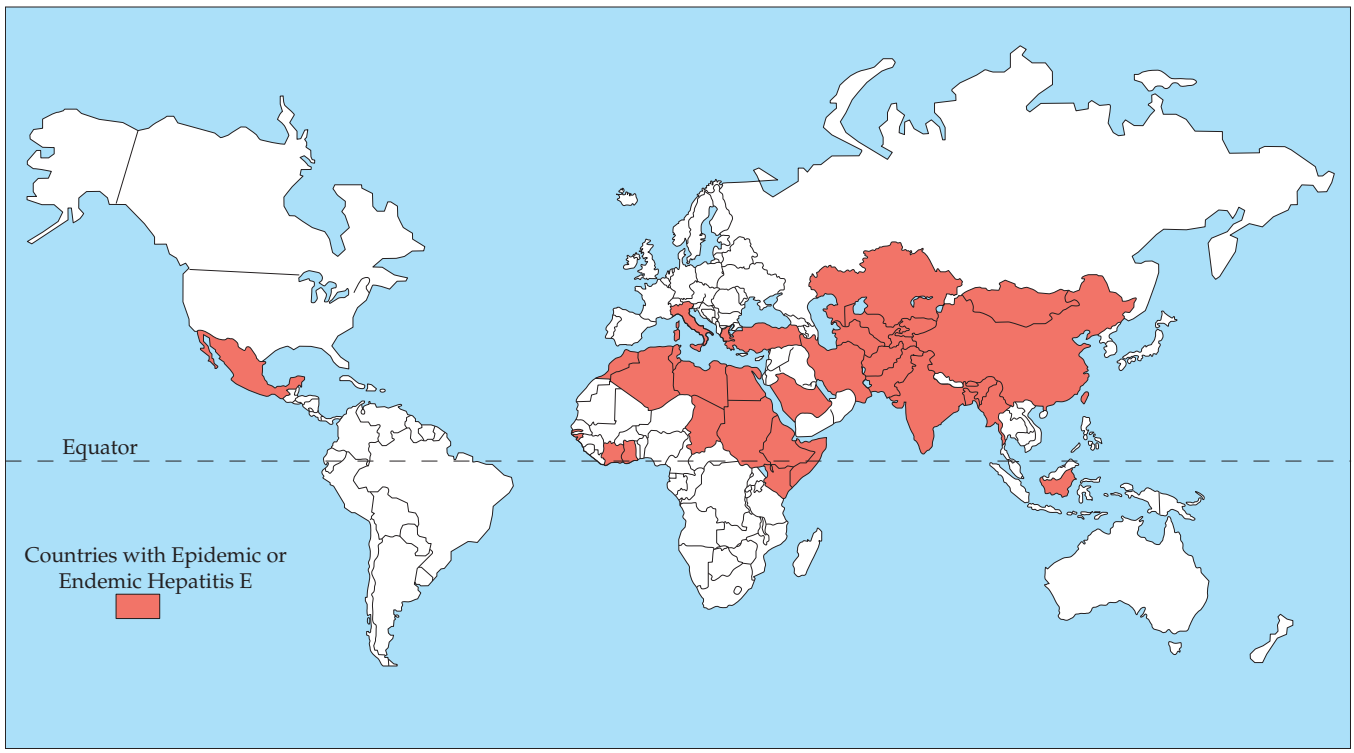


FIGURE 3.13 Worldwide incidence of epidemic and endemic hepatitis E. In addition, serosurveys indicate that 1–2% of blood donors in United States and Western Europe have detectable IgG antibodies to hepatitis E. Adapted from Fields *et al*. (1996) p. 2838.

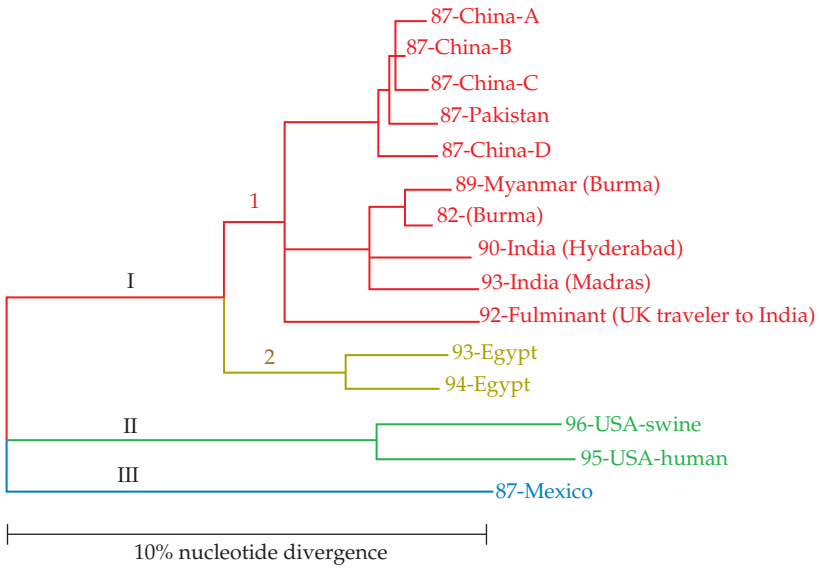


FIGURE 3.14 Phylogenetic tree of HEV isolates based on the complete sequences of ORF2 (1983 nt), encoding the coat protein. Isolates are listed by year and location of isolation. Branch lengths are proportional to the evolutionary distance between sequences. Roman numerals are used to denote genotypes (<85% nucleotide sequence identity), and arabic numbers denote subgenotypes (<92.5% nucleotide sequence identity). The genetic groupings of the HEV strains reflect the geographic relationships of the places from which they were isolated, showing that the viruses are geographically isolated and do not circulate over wide areas. Adapted from Figure 1 in Tsarev *et al*. (1999).

TABLE 3.8 *Astroviridae*

Genus/members[a]	Virus name abbreviation	Usual host(s)	Transmission	Disease
Mamastrovirus				
Human astrovirus (5)	HAstV	Humans	Water-borne, oral–fecal	Gastroenteritis
Bovine astrovirus (2)	BAstV	Cattle	Water-borne, oral–fecal	Gastroenteritis
Feline astrovirus	FAstV	Cats	Water-borne, oral–fecal	Gastroenteritis
Ovine astrovirus 1	OAstV	Sheep	Water-borne, oral–fecal	Gastroenteritis
Porcine astrovirus 1	PAstV	Swine	Water-borne, oral–fecal	Gastroenteritis
Avastrovirus				
Duck astrovirus	DAstV	Ducks	Water-borne, oral–fecal	Fatal hepatitis in ducklings
Turkey astrovirus	TAstV	Turkeys	Water-borne, oral–fecal	Gastroenteritis

[a] All astroviruses identified are worldwide in distribution.

sequence allows readthrough into a second reading frame (1b) to produce a longer polyprotein (1ab). A subgenomic mRNA is translated into the capsid protein of the virus.

Astroviruses encode a 3C-like serine protease (with serine in the active site) and an RNA polymerase related to other viral RNA polymerases, but there is no evidence for a helicase or a capping enzyme. It has been suggested that astroviruses contain a VPg, consistent with the lack of a capping enzyme, but no domain encoding a VPg has been identified in the genome.

Epidemiological studies suggest that astroviruses are an important cause of acute human gastroenteritis worldwide. A summary of virus families known to contain viruses that cause gastroenteritis and acute diarrhea in humans as well as other vertebrates is shown in Table 3.9. In addition to the picornaviruses, caliciviruses, and astroviruses described earlier, some adenoviruses and reoviruses also cause human gastroenteritis, and members of additional families may also cause human gastroenteritis, as shown in the table. In addition to the viruses listed, there may yet

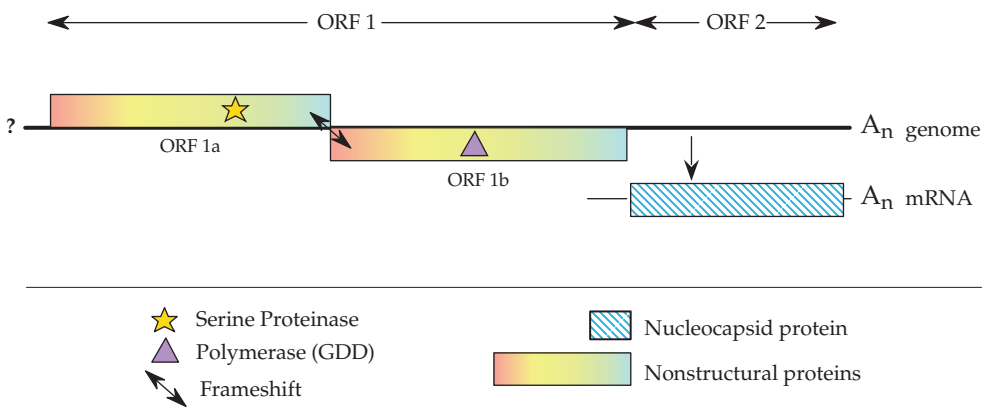


FIGURE 3.15 Genome organization of a human astrovirus. ORF 1a is in a different reading frame than ORF 1b. Ribosomal frameshifting (arrows) during translation is required to produce the ORF 1b protein. Adapted from Fauquet *et al*. (2005) p. 860.

TABLE 3.9 Viruses Causing Acute Diarrhea

Nucleic acid	Family	Virus[a]	Host range
Single-stranded, plus-sense RNA	*Caliciviridae*	Sapporo, Norwalk feline calicivirus	Humans, cattle, swine, chickens, dogs, cats
	Astroviridae	Numerous astroviruses	Humans, cattle, swine, cats, dogs, avian species
	Coronaviridae	PEDV, TGEV and others	Swine, cattle, foals, mice, rabbits, dogs, cats turkeys, (humans?)[b]
		Numerous toroviruses	Cattle, horses (goats, sheep, swine, rabbits, mice, humans?)
	Flaviviridae	Pestivirus BVDV	Cattle
	Picornaviridae	Aichi, human parechovirus 1	Humans
Single-stranded, minus-sense RNA	*Paramyxoviridae*	Canine distemper	Dogs
		Newcastle disease	Chickens, fowl
Double-stranded RNA	*Reoviridae*	Rotavirus A	Mammalian and avian species, humans
		Rotavirus B	Swine, cattle, sheep, rodents, humans
		Rotavirus C	Swine, ferrets, humans
		Rotaviruses D, F, G	Avian species
		Rotavirus E	Swine
Single-stranded DNA	*Parvoviridae*	Numerous parvoviruses	Cattle, cats, dogs, mink, (humans?)
Double-stranded DNA	*Adenoviridae*	Human Ad40,41	Humans

[a] Abbreviations: PEDV, porcine epidemic diarrhea virus; TGEV, transmissible gastroentieritis virus; BVDV, bovine viral diarrhea virus.
[b] The role of the listed viruses in causing diarrhea has not been proven for species listed in parentheses.
Source: Adapted from Granoff and Webster (1999), p. 442.

be other small viruses that cause gastroenteritis in humans. Virus particles have been seen in stools of humans suffering from gastroenteritis that have not as yet been characterized and are referred to simply as SRVs (small, round viruses). Some of these may be members of families not yet characterized.

FAMILY *TOGAVIRIDAE*

The family *Togaviridae* contains two genera, genus *Alphavirus* and genus *Rubivirus*. The family name comes from the Latin word for cloak, and the name was given to them because they are enveloped. The 30 alphaviruses have

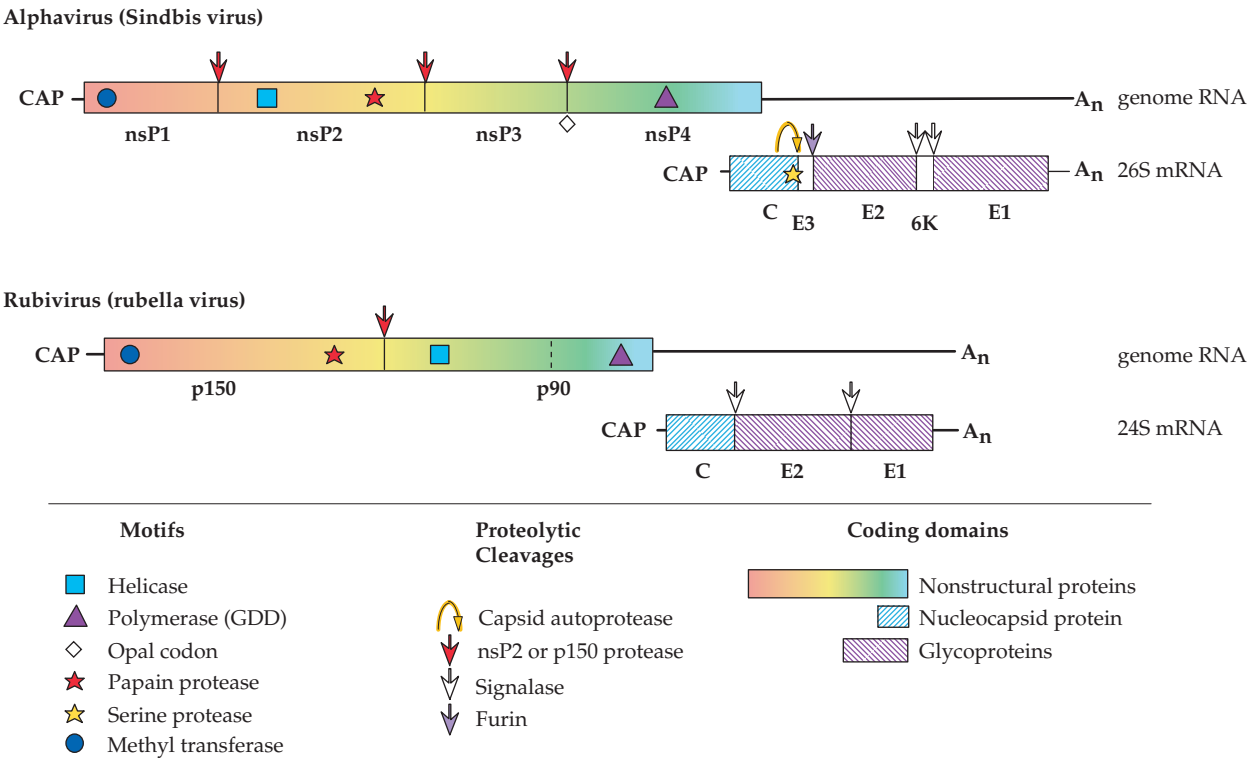


FIGURE 3.16 Genome organizations of the *Togaviridae*. A number of protein motifs are indicated, as well as the enzymes responsible for the proteolytic cleavages. The opal codon shown between nsP3 and nsP4 is leaky and is not present in all alphavirus isolates; readthrough produces small amounts of nsP4. Adapted from Strauss and Strauss (1994) Figure 34.

a (+)RNA genome of about 12 kb, whereas rubella virus, the only member of the *Rubivirus* genus, has a genome of 10 kb. The genomes of alphaviruses and of rubella virus are organized in a similar fashion, as illustrated in Fig. 3.16. The virions of the two groups are also roughly similar in size (70 nm for alphaviruses, 50 nm for rubiviruses) and structure (icosahedral nucleocapsids surrounded by a lipoprotein envelope). Structures of alphaviruses are illustrated in Figs. 2.5, 2.14, and 2.18. However, although the two genera exhibit similarities, they are only distantly related. As an historical footnote, the flaviviruses, described after the togaviruses, were once classified as a genus within the *Togaviridae*. When sequences of alphaviruses and flaviviruses were determined, however, they were found to be unrelated and the flaviviruses were placed into a new family.

Genus *Alphavirus*

The alphaviruses have a worldwide distribution. They get their name from the Greek letter alpha because they were once known as the Group A arboviruses. Many cause important illnesses in humans, and information for a representative selection of these viruses is presented in Table 3.10. Because of their importance as disease agents and aided by the fact that alphaviruses grow well in cultured cells and in animal models, this group of viruses has been well studied. The genomes of many of them have been sequenced in their entirety. All members of the genus are closely related and share extensive amino acid sequence identity, with the most distantly related alphaviruses sharing about 40% amino acid sequence identity on average and viruses belonging to the same lineage sharing higher sequence identity. A dendrogram that illustrates their relationships is shown in Fig. 3.17. A number of lineages or clades are present, including a clade of aquatic viruses, a clade of encephalitic viruses (EEE, VEE), the Sindbis clade (which includes Aura virus and many strains of Sindbis virus), the SFV clade (which includes many viruses which cause arthritis including polyarthritis), and a clade of recombinant viruses (the WEE lineage). The dendrogram illustrates the interesting fact that during evolution of alphaviruses, there was a singular recombination event between Eastern equine encephalitis virus and a Sindbis-like virus to produce Western equine encephalitis virus, which subsequently evolved into a number of different viruses (the WEE lineage). Recombination events

TABLE 3.10 *Togaviridae*

Genus/members	Virus name abbreviation	Usual host(s)	Transmission	Disease	World distribution
Alphavirus					
Sindbis	SINV	Mammals[a], Birds	Mosquito-borne	Arthralgia, rash, fever	Old World
Semliki Forest	SFV	Mammals[a]	Mosquito-borne	Arthralgia, fever	Africa
Ross River, Barmah Forest	RRV, BFV	Mammals[a]	Mosquito-borne	Polyarthritis, fever, rash	Australasia
Ft. Morgan	FMV	Birds	Vectored by swallow bug	?	North America
Chikungunya, O'Nyong-nyong	CHIKV, ONNV	Humans	Mosquito-borne	Arthralgia, fever	Africa
Mayaro	MAYV	Mammals[a]	Mosquito-borne	Arthralgia, fever	South America
Eastern, Western, Venezuelan equine encephalitis	EEEV, WEEV, VEEV	Mammals[a], Birds	Mosquito-borne	Encephalitis	Americas
Salmon pancreas disease virus	SPDV	Fish	No arthropod vector		
Rubivirus					
Rubella	RUBV	Humans	No arthropod vector	Rash, congenital abnormalities	Americas, Europe

[a] Humans can be infected by these viruses, but humans are not the primary mammalian reservoir.

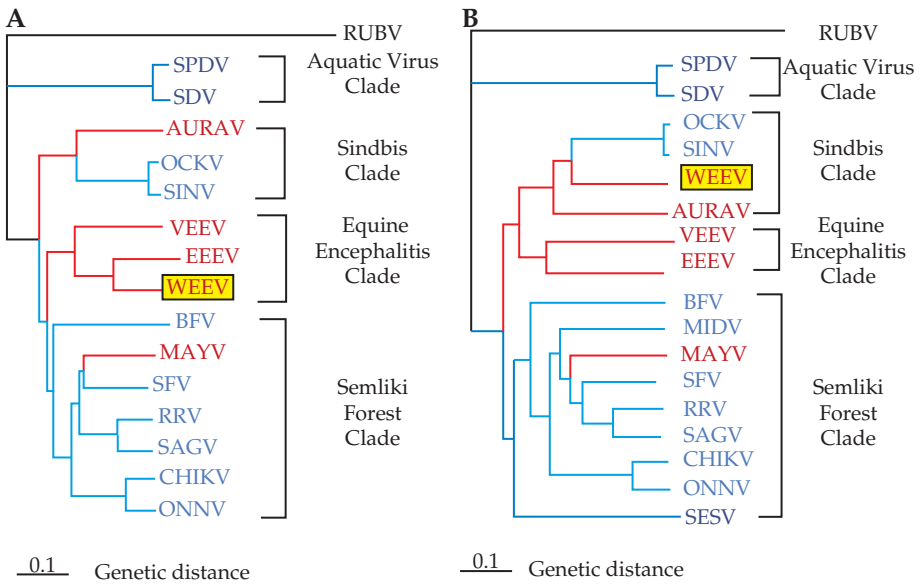


FIGURE 3.17 Phylogenetic trees of the alphaviruses, constructed using the neighbor-joining method from the distances computed using Clustal X v 1.81 software, with the sequences from rubella virus as outgroup. The Old World viruses are shown in blue, the New World viruses in red. The vertical distances are arbitrary, but the lengths of the horizontal branches indicate the number of amino acid substitutions along the branch. (A) A tree derived from the amino acid sequences of nonstructural proteins nsP1 through nsP4, comprising roughly 2475 amino acids (B) A tree derived from the sequences of the virion structural proteins or roughly 1245 amino acids. The trees are very similar in architecture with the exception of the position of WEE (boxed), whose nonstructural proteins are most closely related to EEE, but whose structural proteins resemble SIN, indicating that a recombinational event has taken place to produce WEE. Note that Fort Morgan, Buggy Creek, and Highlands J (not shown) are three recombinant New World viruses closely related to WEE. Most virus abbreviations are found in Table 3.10; AURAV, Aura; MIDV, Middelburg; SDV, sleeping disease; OCKV, Ockelbo; SAGV, Sagiyama; SESV, Southern elephant seal virus. These trees were adapted from Luers *et al*. (2005) with permission.

in which the recombinant virus persists and prospers as a distinct virus appear to be uncommon to rare, but there is much evidence that recombination has played an important role in the evolution of viruses. The dendrogram also illustrates the fact that there have only been a limited number of transfers of alphaviruses between the Americas and the Old World. In fact, three transfers between the Americas and the Old World are sufficient to explain the dendrogram, and the majority of members of the three major lineages are restricted to either the Americas or to the Old World. This contrasts with many virus families, where individual viruses are often worldwide in distribution and evidence is abundant for the mixing of lineages between the two hemispheres.

Expression of the Genome

Alphaviruses enter a cell via endosomal uptake and fuse with the endosomal membrane upon exposure to a suitably low pH that differs somewhat from virus to virus. The capsid protein has an affinity for ribosomes and there is evidence that ribosomes help disassemble the nucleocapsid upon its entry into the cytoplasm by binding the capsid protein. Release of the genomic RNA, which is capped and polyadenylated, is followed by its translation into a nonstructural polyprotein that is cleaved into four polypeptides by a viral protease (Fig. 3.16). Activities present in these proteins include a capping activity in the N-terminal protein (nsP1); helicase, papain-like protease, and RNA triphosphatase activities in nsP2; and RNA polymerase in nsP4. A viral encoded capping activity is required to cap the viral mRNAs (the genomic RNA and a subgenomic RNA) because replication occurs in the cytoplasm and the virus does not have access to cellular capping enzymes. The RNA triphosphatase in nsP2 is required to remove the terminal phosphate in the 5′ triphosphate on the RNA in order to prepare the RNA for capping by the viral enzyme, the RNA helicase is needed to unwind the RNAs during replication, and the protease is required to process the precursor polyprotein. The RNA polymerase is needed to synthesize viral RNA. All four nonstructural proteins are required to synthesize the viral RNA. Replication of the RNA and synthesis of a subgenomic RNA follow the pattern illustrated schematically in Figure 1.9B.

The function of the phosphoprotein nsP3 in RNA replication is unknown. It is phosphorylated on several threonines and serines in a nonconserved domain in the C-terminal region of the protein. The N-terminal domain of nsP3 is a conserved domain (often referred to as the X domain) that is also present in a number of other viruses (rubella virus, hepatitis E virus, coronaviruses) as well as being widely distributed in bacteria, archae, and eukaryotes. The virus domain shares up to 35–40% amino acid sequence identity with the cellular homologues, whose function is unknown.

Replication of the viral RNA takes place in association with cellular membranes. Small spherical invaginations form in the membranes, induced by viral proteins, in which replication occurs. Protein nsP1 has been shown to interact with membranes by means of a specific domain within the protein, and it is assumed that this association is important for the membrane association of the replication machinery.

Studies of the viral nonstructural protease have shown that the cleavages that process the polyprotein control viral RNA replication. During or shortly after translation, the full-length polyprotein precursor (called P1234) cleaves itself *in cis* to produce P123 and nsP4. These form an RNA synthetase, probably together with cellular proteins, that can make complementary (−)RNA from the genomic RNA template, but which cannot make (+)RNA efficiently. Subsequent cleavage of P123 *in trans,* between nsP1 and nsP2, gives rise to a synthetase that can make both (+)RNA and (−)RNA. A second cleavage between nsP2 and nsP3 gives rise to a synthetase that can make only (+)RNA. Thus, (−)RNA templates are made early, but as infection proceeds and the concentration of protease builds up, *trans* cleavage occurs and (−)RNA synthesis is shut down (Fig. 3.18). After this time, plus-strand RNA synthesis continues from the preformed minus-strand templates but no further increase in the rate of RNA synthesis takes place. This control mechanism may have evolved not only to make the infection process more efficient, since genomic RNA for progeny virions and subgenomic RNAs for translation of structural proteins required

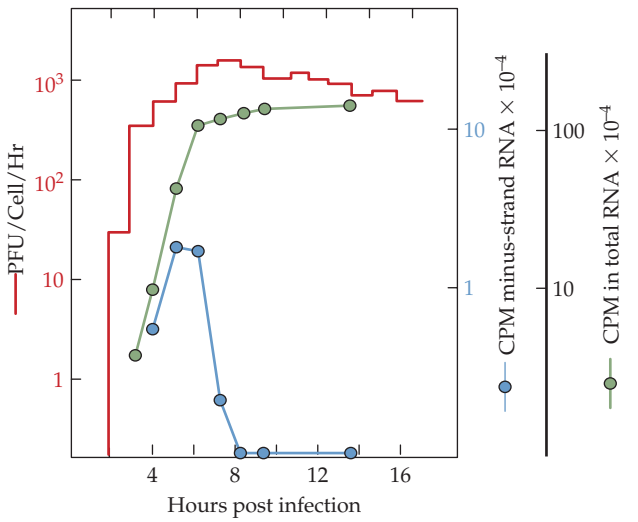


FIGURE 3.18 Growth curve of Sindbis virus infection in chicken cells at 30°C. At the times shown, the medium was harvested and replaced with fresh medium, and the titer was determined. The red line shows release of infectious virus into the culture fluid. For determining the rate of RNA synthesis, cells infected as for virus assay were pulsed with radioactive uridine for 1 hour at the times shown. Monolayers were harvested and incorporation into total RNA (green line) and minus-strand RNA (blue line) were determined. Adapted from Strauss and Strauss (1994) Figure 5.

for assembly of progeny virions are needed in much larger quantities than minus-strand templates, but also to control the virulence of the virus. In the case of alphaviruses, it is particularly important to control the virulence of the virus in the mosquito vector. As discussed in more detail later, it is important that the infection process in the mosquito be tightly controlled, and shutting down minus-strand RNA synthesis not only prevents further exponential increase in virus replication but also allows downregulation of virus replication as minus-strand templates decay. It also has the effect that the infected cell becomes resistant to superinfection by the same or a related virus because no (−)RNA templates can be made, which could be especially important in the mosquito vector. The resistance to superinfection by the same or related viruses is called superinfection exclusion or homologous interference.

The rate of cleavage of the early synthetase that makes (−)RNA to convert it into one that can make (+)RNA is thought to be controlled in part by a leaky stop codon between nsP3 and nsP4 that is present in most, but not all, alphaviruses (Fig. 3.16). Termination at this codon produces P123, which can act *in trans* as a protease but cannot act as a synthetase because it lacks the nsP4 RNA polymerase. In addition to a more rapid buildup of protease that accelerates the rate of conversion to (+)RNA synthesis, this additional P123 and its cleavage products may serve to accelerate the rate of RNA synthesis. There is evidence from genetic studies that domains in nsP1 and nsP2, among others, are required for the recognition of viral promoters and the initiation of RNA synthesis, and additional helicase activity could also speed up the rate of RNA synthesis.

During infection by alphaviruses, a subgenomic mRNA is produced that serves as the message for the production of the structural proteins of the virus, which consist of a capsid protein and two glycoproteins. The 4.1-kb subgenomic RNA is synthesized by the viral replicase from the (−)RNA template using an internal promoter of 24 nucleotides. The activity of this core 24-nucleotide promoter is increased by enhancer sequences present in the 100 nucleotides upstream of the core promoter. The structural proteins are translated as a polyprotein and cleaved by a combination of viral and cellular enzymes. The capsid protein is itself a serine autoprotease that cleaves itself from the N terminus of the nascent polyprotein. It has a fold that is similar to that of chymotrypsin (Fig. 2.14B), suggesting that it was derived from a cellular serine protease during evolution of the virus. After release of the capsid protein, N-terminal signal sequences and internal signal sequences in the glycoprotein polyprotein precursor lead to its insertion into the endoplasmic reticulum (Figure 3.19). This precursor is cleaved

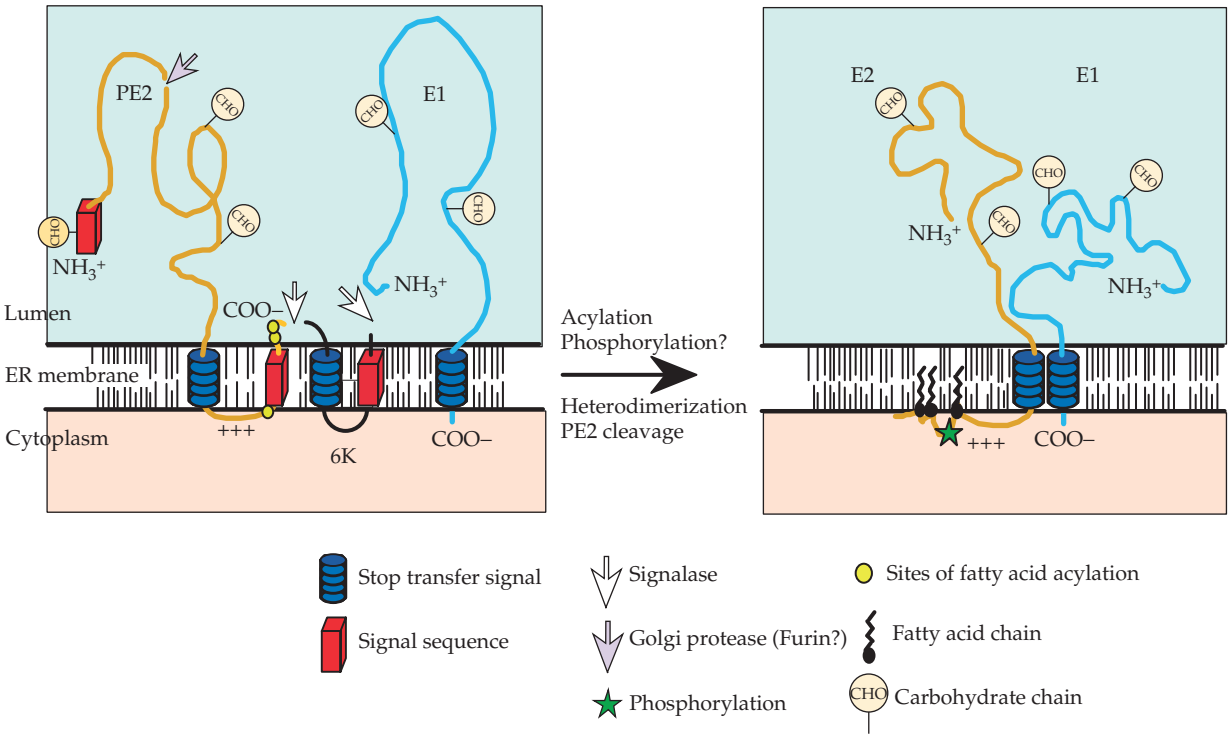


FIGURE 3.19 Schematic diagram of the configurations of glycoproteins PE2 and E1 in membranes. The left panel shows the configuration of PE2 immediately after signalase cleavage from 6K, with the C terminus in the lumen of the ER. The right panel shows the configuration of the E2-E1 heterodimer after phosphorylation–dephosphorylation with the C terminus in the cytoplasm. Adapted from Strauss and Strauss (1994), Figures 7 and 9.

by signalase, a cellular enzyme that resides in the lumen of the endoplasmic reticulum, to produce glycoprotein PE2 (a precursor to glycoprotein E2), 6K (a small hydrophobic peptide located between E2 and E1), and glycoprotein E1. PE2 and E1 quickly form a heterodimer. At some time, the C terminus of PE2/E2 is withdrawn from the lipid bilayer into the cytoplasm, where it can interact with the capsid during virus assembly. During transport of the heterodimer to the cell plasma membrane, PE2 is cleaved by another cellular enzyme, furin or a furin-like enzyme, to form E2 and E3. E3 is a small glycoprotein that in most alphaviruses is lost into the culture fluid.

Viral Promoters

The replication of alphaviral RNA requires the recognition of specific promoters in the viral RNA by the viral RNA synthetase. These promoters act *in cis*, that is, they must be present in the RNA to be used as a template, and both viral and cellular proteins may be involved in the recognition of these promoters. Four alphavirus promoters, or components of promoters, are illustrated in Figure 3.20. The best understood of these promoters is that for the production of the subgenomic mRNA for the structural proteins. The basal promoter consists of 24 nucleotides, of which 19 are upstream of the transcription start site and 5 are copied into the subgenomic RNA. This subgenomic promoter can be placed in front of any RNA sequence and the viral synthetase will use it to synthesize a subgenomic mRNA. This property of the promoter has made alphaviruses useful as expression vectors (Chapter 11).

The promoters for synthesis of full-length genomic RNA and the antigenomic (–)RNA template are less well understood. A linear sequence element at the 3′ end of the (+)RNA and two elements at or near the 5′ end of the genomic RNA that can form stem-loop structures (Fig. 3.20) are required for the efficient synthesis of both the antigenomic RNA from the genomic RNA and for genomic RNA synthesis from the antigenomic template. The genomic RNA (and presumably the antigenomic RNA as well) is known to cyclize in the absence of protein (Fig. 3.21), thus bringing the sequence elements at the two ends of the molecule into close proximity, allowing the viral replicase to interact with both ends of the RNA at once when initiating synthesis of new RNA. It seems likely that this mechanism evolved so that only full-length RNA molecules can be replicated. This eliminates replication not only of the subgenomic RNA but also of any broken RNA molecules.

It is noteworthy that the RNAs of many, perhaps most, RNA viruses, both plus stranded and minus stranded, cyclize at some stage of RNA replication or translation. In some cases, as in the alphaviruses, cyclization occurs by means of complementary sequences near the ends of the RNA and requires no protein to stabilize the interaction. In other cases, the interactions of the complementary sequences near the ends of the RNA are stabilized by the binding of viral or cellular proteins. And in still other cases, cyclization is effected by cellular proteins. In one scenario, a cellular protein such as the poly(A)-binding protein binds to the 3′ poly(A) tract and another cellular protein, such as one that binds to translation initiation signals, binds near the 5′ end of the molecule. These two cellular proteins then interact with each other to hold the two ends of the RNA near one another.

Assembly of Progeny Virions

The structure of alphaviruses has been described in Chapter 2. PE2-E1 heterodimers form in the endoplasmic reticulum and are transported to the cell surface. During transport, they are cleaved to form E2-E1 heterodimers. The PE2-E1 heterodimer is more stable to acidic pH than is the E2-E1 heterodimer and survives the mildly acidic pH of transport vesicles. Cleavage is necessary to prime the E2-E1 heterodimer for disassembly upon entry into a susceptible

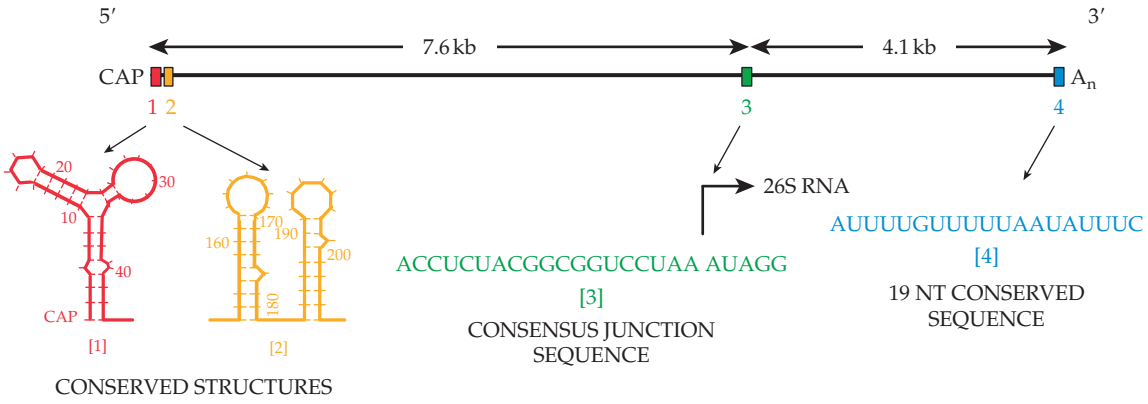


FIGURE 3.20 Promoters in the alphavirus genome. Adapted from Strauss and Strauss (1994), Figure 18.

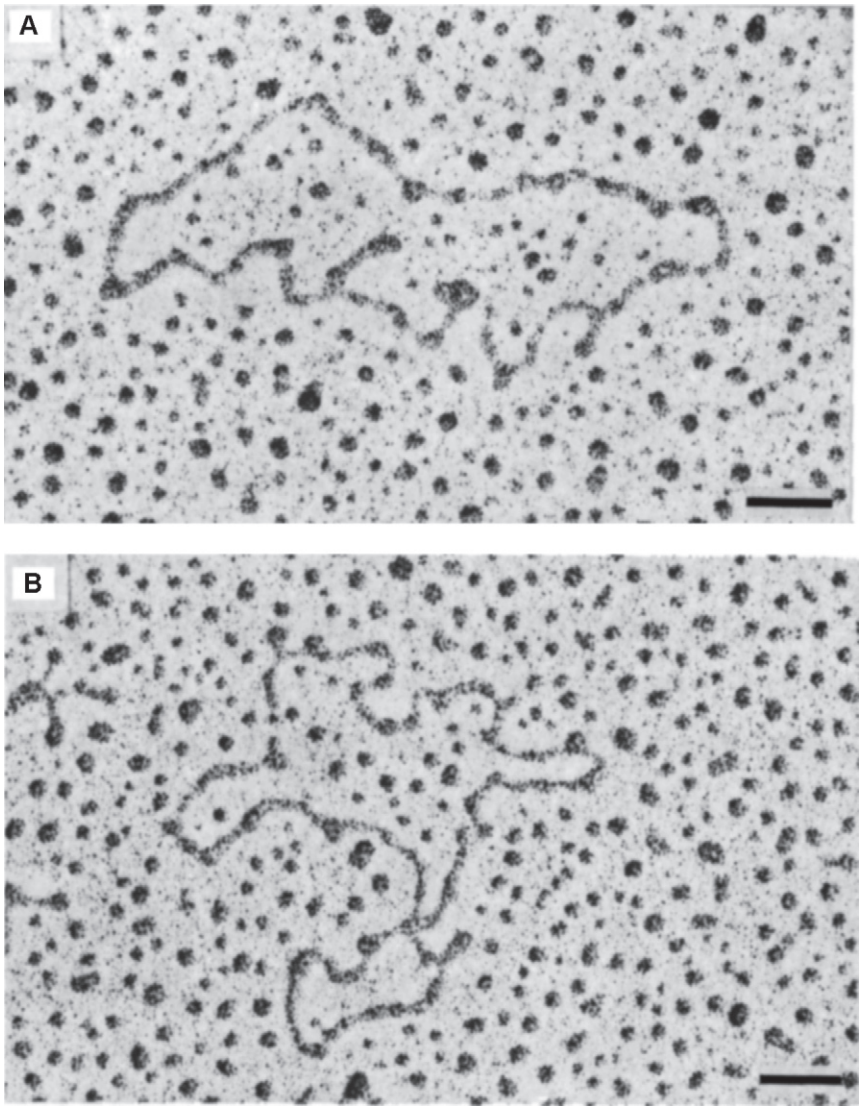


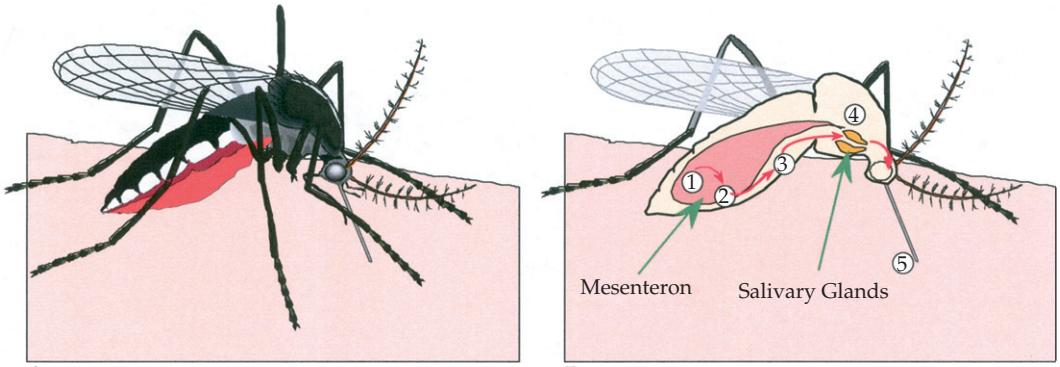
FIGURE 3.21 Electron micrographs of Sindbis virus 49S genomic RNA. The RNA was treated with 0.5 M glyoxal in 0.1 M phosphate buffer for either 30 min (A) or 40 min (B) at 35°C before spreading. Scale bar is 100 nm. Adapted from Frey *et al*. (1979) Figure 3.

cell, which is required for formation of E1 homotrimers that are responsible for fusion. During transport or virus assembly, three E2-E1 heterodimers trimerize to form the spikes found in the virion. Virions normally mature when a preassembled nucleocapsid consisting of the genomic RNA and 240 copies of capsid protein buds through the cell plasma membrane to acquire a lipoprotein envelope containing 240 copies of the E1-E2 heterodimer (Fig. 2.25C). The free energy for budding is provided by lateral interactions between the viral glycoproteins as they assemble around the nucleocapsid and by the interaction of the C-terminal domain of glycoprotein E2 with a docking site on each nucleocapsid protein molecule. The assembled virion has icosahedral symmetry in which the symmetry axes of the nucleocapsid are coordinated with those of the glycoprotein shell by the E2–capsid interactions. The diameter of the assembled virion is 70 nm (Figs. 2.5 and 2.14A).

The 6K protein is required for efficient assembly of virions. Virions that appear to be normal in every way will assemble in the complete absence of 6K but only inefficiently and virus that lacks the 6K gene produces only low yields. It has been shown that 6K expressed alone will form ion channels, but whether this is important for virus assembly is not known.

Alphaviruses Are Arboviruses

All alphaviruses are arboviruses (***ar***thropod-***bo***rne animal viruses), with the probable exception of the newly described fish viruses, and were once referred to as



A. A female mosquito takes a blood meal

B. Cutaway view of the mosquito showing steps in the replication and transmission of an arbovirus

FIGURE 3.22 Sequential steps necessary for a mosquito to transmit an arbovirus. (1) A female mosquito ingests an infectious blood meal and virus enters the mesenteron. (2) Virus infects and multiplies in mesenteronal epithelial cells. (3) Virus is released across the basal membrane of the epithelial cells and replicates in other tissues. (4) Virus infects salivary glands. (5) Virus is released from the epithelial cells of the salivary glands and is transmitted in the saliva during feeding. Adapted from Monath (1988).

the Group A arboviruses. In nature, they alternate between replication in arthropod vectors, usually mosquitoes, and higher vertebrates. A mosquito may become infected on taking a blood meal from a viremic vertebrate, which can have 10^8 or more infectious particles per milliliter of blood. The infection in the mosquito, which is almost asymptomatic and lifelong, begins in the midgut and spreads to the salivary glands, as illustrated in Fig. 3.22. After infection of the salivary glands, the mosquito can transmit the virus to a new vertebrate host when it next takes a blood meal, injecting saliva in the process. Infection in the vertebrate begins in the tissues surrounding the bite or in regional lymph nodes, but then spreads to other organs. The infection is usually self-limited and the vertebrate is capable of infecting mosquitoes for only a brief time after viremia is established but before an immune response limits circulating virus. The necessity to alternate between two such different hosts has constrained the evolution of arboviruses—changes that adapt the virus to one host or that are neutral in one host are often deleterious in the alternate host. Thus, the evolutionary pressures on arboviruses are different from those on viruses such as poliovirus, which infects only primates.

Different alphaviruses infect different spectra of mosquitoes and vertebrates in nature. It is useful to distinguish between reservoir hosts in which the virus is maintained in nature and dead-end hosts in which infection normally does not lead to continuity of the infection cycle. We can also distinguish between enzootic cycles, in which the virus is continuously maintained in nature and which may or may not result in disease in the enzootic host, and epizootic cycles, in which the virus breaks out and causes epidemics of disease that may die out with time. Two types of natural transmission cycles are illustrated in Fig. 3.23. In Fig. 3.23A, a simple transmission cycle is illustrated, such as that of urban yellow fever infection of humans (see the section on flaviviruses later). In this cycle, humans are the only vertebrate hosts and the virus alternates between infection of a human and infection of the mosquito vector *Aedes aegypti*. Figure 3.23B illustrates a complex transmission pattern, using as an example the transmission of Eastern equine encephalitis virus in North America. This virus has a vertebrate reservoir consisting primarily of migratory songbirds and is transmitted by the mosquito, *Culiseta melanura*, a common inhabitant of freshwater swamps in eastern North America. However, the virus is capable of infecting other mosquitoes and has even been isolated from naturally infected chicken mites. It also infects mammals, including humans. On occasion, the virus breaks out of its enzootic cycle to cause epidemics of disease in pheasants, transmitted and maintained by an epizootic vector mosquito. Either enzootic or epizootic vectors are capable of infecting humans or domestic animals if they invade the areas in which these mosquitoes are present, but these hosts are usually dead-end hosts and do not further spread the virus.

Most alphaviruses are capable of infecting both mammals and birds, and the nature of the vertebrate reservoir depends on the virus or even the strain of virus, which may differ by geographic location. Thus, for example, Sindbis virus in nature is normally maintained in birds, which are its usual vertebrate reservoir. However, the virus is capable of infecting mammals, including humans, and has also been isolated from amphibians and reptiles. Numerous species of mosquitoes form its insect reservoir, but it has also been isolated from other hematophagous arthropods, including mites. In contrast, Ross River virus is maintained in small marsupial mammals in Australia and does not appear to use birds as hosts.

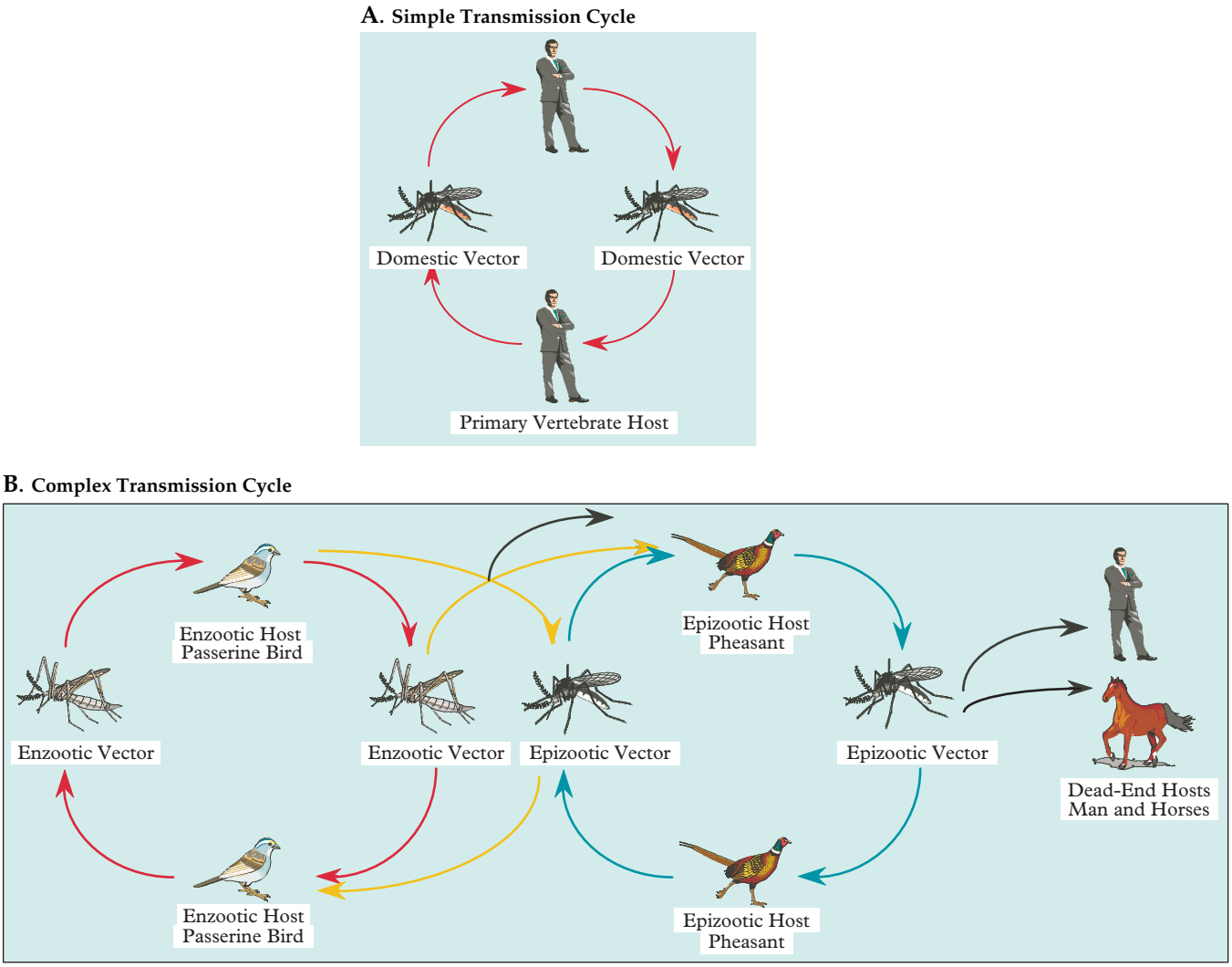


FIGURE 3.23 Two generalized transmission cycles of arboviruses. (A) Simple cycle, such as urban yellow fever, involving a single vector (*Aedes aegypti* mosquitoes) and a single vertebrate host (man). (B) Complex cycle, such as that for Eastern equine encephalitis, where the virus is maintained in an enzootic host (passerine birds) with an enzootic vector (*Culiseta melanura*), but can enter an epizootic vector (another insect) and be transmitted to epizootic hosts, and tangentially to dead-end hosts like man. An intermediate type of cycle is illustrated in Figure 5.12 for Colorado tick fever. Adapted from Monath (1988) p. 129.

Arboviruses that are transmitted by mosquitoes, ticks, sand flies, or other bloodsucking arthropods are known from several families of viruses. A selection of arboviruses from three virus families, most of which cause human disease, is listed in Table 3.11, together with the diseases they cause.

Pathology in the Mosquito Vector

The mosquito vector suffers little pathology upon infection by an arbovirus. This must be so if the virus is to persist, because transmission of the virus to a new vertebrate requires that the female mosquito survive long enough and be healthy enough to take another blood meal, which is required for egg development. The time between infection of the female and the time at which the mosquito is capable of transmitting the virus is called the extrinsic incubation period. This period varies widely with temperature, humidity, the virus, and the mosquito. It can be as short as 2 days or as long as 2 weeks, although in alphaviruses it is seldom longer than 1 week.

Although infection of the mosquito is relatively benign, recent studies have shown that there is some pathology associated with arboviral infection. There is limited pathology

TABLE 3.11 Representative arboviruses that cause human disease

Family/virus	Predominant disease manifestations[a]				
	Nonfatal systemic febrile illness	Encephalitis		Hemorrhagic Fever (HF)	
		Frequency[b]	%Mortality[c]	Frequency[b]	%Mortality[d]
Togaviridae					
Chikungunya	Most cases, Ep			Rare, Ep	rare
Mayaro	Most cases, Ep				
O'nyong-nyong	Most cases, Ep				
Ross River	Most cases, Ep				
Sindbis	Most cases, Ep				
EEE	Most cases	Rare	50–70		
VEE	Most cases, Ep	Rare	0.1–20[e]		
WEE	Most cases	Rare	5–10		
Flaviviridae					
Dengue (1–4)	Most cases, Ep			Rare, Ep	3–12
West Nile	Ep	En	~9%[f]		
Japanese encephalitis		<1%	30–40[g]		
Kyasanur Forest		En	5	En	5
Murray Valley		Ep	20–70		
Rocio		Ep	13		
St. Louis encephalitis		Ep	4–20		
Tick-borne encephalitis					
Eastern		Rare	30		
Central European		Rare	1–10		
Omsk hemorrhagic fever				En	1–2
Yellow fever				Most cases	5–20
Bunyaviridae					
Bunyamwera	En				
Germiston	En				
Sand fly fever	Ep				
Rift Valley fever	Ep			En	1–5
California encephalitis		En	1		
Crimean hemorrhagic fever				En	15–20

[a] In addition to the disease manifestations listed, most viruses in this table can cause mild febrile illness; some viruses are endemic (En) but others cause occasional outbreaks or epidemics (Ep).
[b] Frequency relates to the relative number of cases exhibiting encephalitis or HF relative to the total number of infections. This number can be difficult to estimate, since only the most seriously ill (e.g., hospital patients in an epidemic) may be reported as infections. Mortality ≥ 10% is highlighted in red.
[c] Percent mortality is the percent of those *with encephalitis* who succumb.
[d] Percent mortality is the percent of those *with HF* who succumb.
[e] Mortality in children is at the high end of the range given.
[f] Mortality from recent epidemics in the United States.
[g] Mortality generally lower in children.

in the gut, which might be important for virus spread from the gut into the hemolymph, from which it spreads to the salivary glands, in some cases after an increase in titer following replication in fat body cells. More importantly, however, are studies showing that arbovirus infection results in decreased survival and reproductive capacity. Thus, the persistence of an arbovirus requires a delicate balance. The virus must replicate to sufficiently high titer in the vertebrate to cause sufficient viremia to infect a mosquito, which usually results in symptomatic disease.

But in the mosquito the infection must be controlled so as to produce sufficiently high titers of virus in salivary fluid without damaging too extensively the ability of the mosquito to survive and reproduce.

Overwintering by Arboviruses

In humid tropical or subtropical areas in which mosquitoes are active throughout the year, an arbovirus can be maintained by continuous transmission between invertebrate and vertebrate hosts. Virus activity may vary during the year, for example it may be much greater during a rainy season when the number of mosquito vectors is higher, but the virus is active throughout the year and human infection can occur at any time. However, in temperate zones in which adult mosquitoes die off in the winter, or in very dry areas in which mosquitoes are only active after sporadic rains, the virus must have a mechanism by which it overwinters. Mosquitoes survive winters (or extended droughts) by suspending development of the young at some stage. In some mosquitoes, eggs are laid but do not develop until conditions are favorable. In others, developing young diapause, suspending development at some stage of embryonic development or larval development, until conditions are favorable, such as the return of spring. Thus, one mechanism for overwintering by some arboviruses is transovarial transmission, in which the virus infects oocytes in the infected female at an early stage of development; the replication cycle of the virus is suspended during diapause and the animal develops normally. When the newly hatched mosquito begins to fly, it is already infected. One way to search for transovarial transmission in the field is to look for virus in male mosquitoes. Since these do not feed on blood, the only way for them to become infected is by transovarial transmission. Some alphaviruses are known to use this mechanism whereas other alphaviruses do not. A second mechanism used by some arboviruses is persistent infection of a vertebrate host so that infected vertebrates are present when the mosquitoes emerge once again. Some alphaviruses are known to persist in humans or other vertebrates for extended periods, but such persistence is rare and it is not known whether this could be a means of overwintering. In some arboviruses, however, such as the coltiviruses (see Chapter 5), persistence is known to be essential to the maintenance of the virus in nature and the virus has evolved specific mechanisms to persist. A third mechanism is the reintroduction of the virus into an area from regions where it persists year-round, for example, by the return of migratory birds or by infected mosquitoes being blown over large distances by storms. The whole subject of overwintering is an interesting evolutionary and ecological study, and in the case of many arboviruses, including most alphaviruses, overwintering is only poorly understood.

Seasonality of Disease

It is obvious from the preceding discussion that mosquito-borne diseases are seasonal. In temperate areas, disease is absent in the winter. In spring when mosquitoes first arise, disease is also absent or rare. It is only with time that the intensity of virus transmission builds up to the point that humans become infected with some frequency. There have also been suggestions that the arboviruses that first appear may not be as virulent as viruses that appear later in the season, perhaps because they are adapted to the mosquito vector and must readapt to the vertebrate host. In any event, arboviral epidemics characteristically occur in mid to late summer and early fall in temperate regions. In other regions, epidemics may be associated with heavy rainfall that results in an increase in the mosquito population.

Encephalitic Alphaviruses

Most, perhaps all, alphaviruses are neurotropic. They readily infect neurons in culture or in experimental animals. Infection of neurons is dependent upon the age of the animal as well as dependent upon the strain of virus. Sindbis virus infection of the mouse has been used as an experimental model to study virus induced encephalitis (inflammation of the brain, from *enceph* = brain, *itis* = inflammation) and encephalomyelitis (inflammation of the brain and spinal cord, from the preceding plus *myel* = medulla or marrow). Some strains of the virus will invade the central nervous system after peripheral inoculation and cause encephalitis, whereas other strains of the virus do not invade the CNS following peripheral inoculation but will cause encephalitis upon direct inoculation of the virus into the brain, and still other strains do not cause overt encephalitis even though neurons may be infected. In all cases, the infection of neurons to cause encephalitis is age related. Very young mice are susceptible to most strains of the virus, whereas infection of older mice does not result in encephalitis for many strains of the virus. Manipulation of the viral genome in the laboratory has resulted in the identification of specific genes that are important for neurovirulence.

It is important to distinguish neurotropism, the ability to infect neurons or other cells of the CNS, from neuroinvasion, the ability of the virus to cross the blood–brain barrier and invade the CNS, from neurovirulence, the ability to cause brain disease once the CNS has been invaded. Although most alphaviruses studied are neurotropic, only three viruses regularly cause encephalitis in humans or domestic animals. These three viruses, Eastern (EEE), Western (WEE), and Venezuelan equine encephalitis (VEE) viruses, are New World alphaviruses that cause fatal encephalitis in horses. WEE and EEE regularly cause encephalitis in humans as

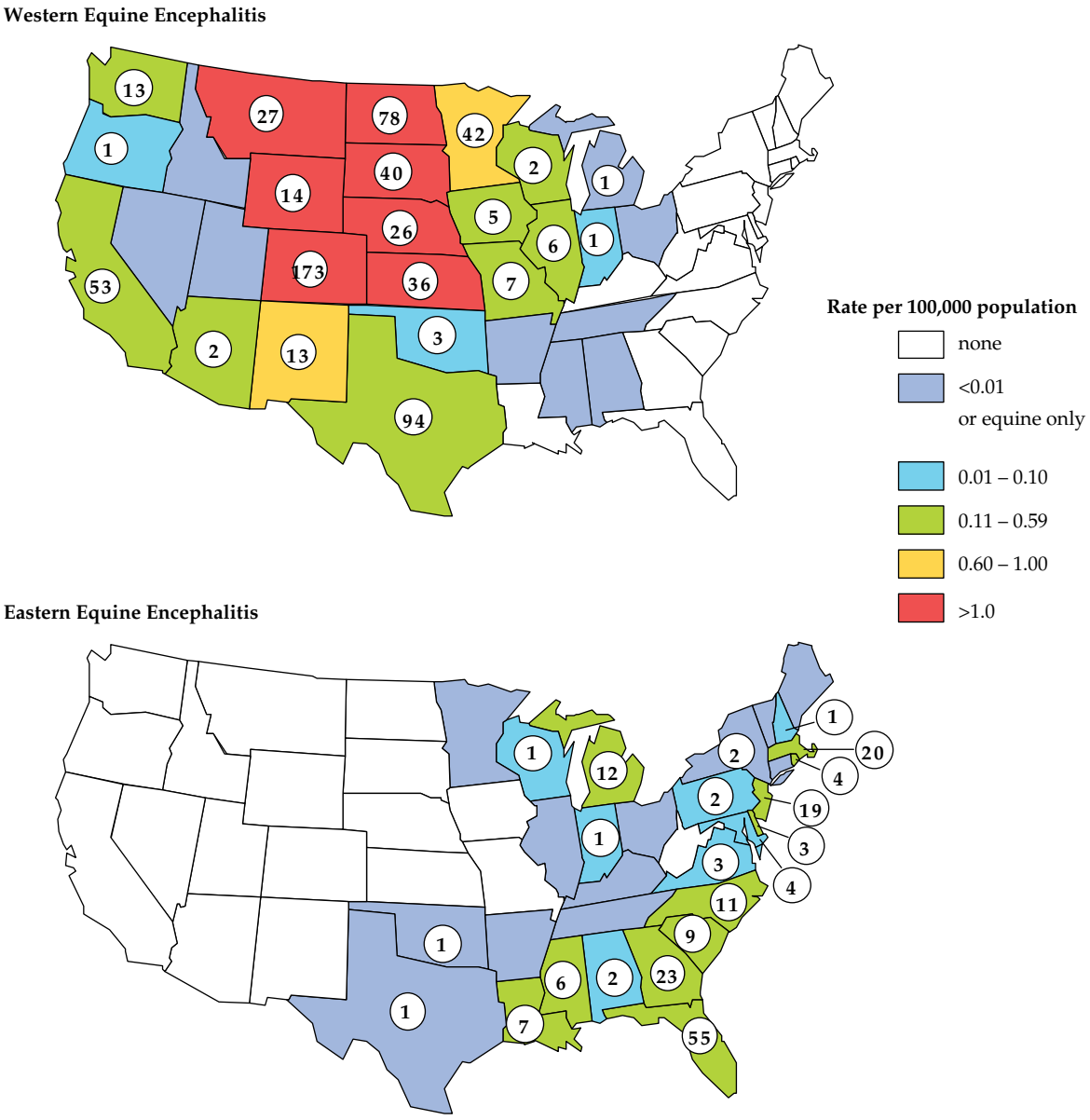


FIGURE 3.24 Geographic distribution of reported human cases of alphavirus encephalitis in the United States from 1964 to 2003. Colors indicate the rate in cases per 100,000 population by state and the actual numbers of cases are shown. Note that there were two human cases of WEE in 1994 and one in 1999, but none have been reported since then. Reported cases of EEE from 1994 through 1998, for which no state locations were given, were 1 in 1994, 4 in 1995, 5 in 1996, 14 in 1997, and 4 in 1998. There were 5 cases of EEE in 1999, 3 in 2000, 9 in 2001, 10 in 2002, and 14 in 2003. Since 1998 EEE cases are reported by state and the cases for 1999–2003 have been added to the cumulative state totals. Adapted from Fields *et al*. (1996) p. 875 and additional data from *MMWR, Summary of Notifiable Diseases, 1998, 1999, 2000, 2001, 2002, 2003*.

well, although the number of cases is small (Table 3.11 and Fig. 3.24). The mechanisms by which the CNS is invaded by alphaviruses or by viruses belonging to other families that cause encephalitis (poliovirus, certain flaviviruses, and others) are imperfectly known. Model studies in mice have indicated that Sindbis virus invades the CNS by retrograde axonal transport from the peripheral site of infection where primary replication occurs, one of the mechanisms that also may be important for the invasion of the CNS by poliovirus (see earlier). Infection of the nasal neuroepithelium, whose neurons project directly to the CNS, may also lead in infection of the CNS. Sindbis virus appears to use this mechanism as well as transport from the peripheral sites, and transport from the neural epithelium may be particularly important in VEE infection. A third possible mechanism is the establishment of viremia followed by infection of choroid plexus

epithelial cells such that the virus replicates across the blood–brain barrier. As described, this mechanism appears to be particularly important in poliovirus invasion of the CNS, but does not appear to be used during invasion by Sindbis virus. Thus, the primary mechanism used appears to differ among different viruses, and more than one mechanism may be important for any individual virus.

Alphaviruses have been used as a model system to study recovery of mice from virally induced encephalitis. The CNS is immunologically privileged (see Chapter 10) because neurons are not replaceable once destroyed, and the mechanisms used to cure the CNS of viruses differ from those used in other organs such as the liver or intestinal tract. It has been shown that humeral antibodies are required for clearance from the CNS but the mechanism is unknown. Cytotoxic T cells are also required for recovery from virally caused CNS disease, but by means of secretion of interferon-γ rather than by killing of infected cells.

Eastern Equine Encephalitis Virus

The majority of human infections by EEE are inapparent or result in febrile illness that is usually mild. Encephalitis results in only about 4% of infected adults but in more than 10% of children less than 10 years old, demonstrating the age-related aspects of the disease. EEE-caused encephalitis is fatal about half the time, with the highest fatality rates in the very young and the very old. Survivors usually have neurological deficits. There have been an average of seven cases of EEE encephalitis per year in the United States over the last 50 years. Horses are more sensitive to the virus and more likely to become infected, and the virus is of major concern to horse breeders in the eastern United States. The case fatality rate is 80–90%, and in years past there have been epidemics involving thousands of horses; the largest such epidemic on record killed more than 11,000 horses in Louisiana and Texas in 1947.

As described before, in North America EEE is maintained in an enzootic cycle in birds that inhabit freshwater swamps, vectored by *Culiseta melanura*. Perhaps because many of these birds are migratory and therefore capable of spreading the virus over large distances, the virus is fairly uniform throughout its North American range. Viruses isolated over a period of more than 60 years from many different areas of North America show less than 2% nucleotide sequence divergence. As illustrated in Fig. 3.23, epizootic cycles in other birds can occur. Mosquitoes vectoring either the enzootic cycle or an epizootic cycle are capable of transmitting the disease to horses or humans.

EEE in South America is a distinct virus. It is vectored primarily by *Culex* species and is not associated with severe disease in humans. In addition to birds, small mammals, especially rodents, which are sedentary, are an important reservoir for the virus. Perhaps because of this, the virus has evolved into a number of strains that differ by up to 25% in nucleotide sequence.

Western Equine Encephalitis Virus

WEE is less virulent for humans than is EEE. Encephalitis occurs in only 1 of 1000 adults infected by WEE, but in about 2% of children younger than 5, and the encephalitis produced by WEE is less severe, with an overall case fatality rate of about 3%, but about 8% in persons older than 50. The virus is also less virulent in horses, in which the case fatality rate is about 40%. The virus is a recombinant between EEE and a Sindbis-like virus, probably Aura virus present in South America (Fig. 3.17). Because the glycoproteins were derived from the Aura parent, the virus is serologically related to the Sindbis lineage. The RNA replication proteins are derived from EEE, as is the encephalitic potential of the virus. The fact that the virus is less virulent than EEE is consistent with laboratory studies that chimerization of a virus, a technique being used to produce live virus vaccines, usually results in lowered virulence (see, e.g., Chapter 11).

WEE is endemic from western Canada discontinuously to southern South America. In the western United States, the primary vector is *Culex tarsalis* and the vertebrate reservoir is again birds, although jackrabbits and possibly other mammals may be important in some areas. In the past, infection by the virus was common. An epizootic of WEE in 1912 killed an estimated 25,000 horses in the western United States. In 1960, 34% of humans tested in rural areas of California were found to be positive for antibodies to WEE, indicating past infection by the virus. Infection is now less common in the United States, and only about 2% of humans in similar areas of California were found to be seropositive for WEE in the mid 1990s. Confirming this trend, an average of 34 cases of human WEE encephalitis occurred per year in the United States from 1955 to 1984, but this number has declined thereafter and there has been only one case in the United States since 1994 (Fig. 3.24). This dramatic decline may have resulted from mosquito control measures, the widespread use of insect repellents, the adoption of air-conditioning and window screens that has resulted in fewer bites by mosquitoes, especially night flying mosquitoes, and because fewer horses, which are amplifying hosts for WEE, are used in farming. No vaccines for this virus are available for widespread use, although experimental vaccines exist that are given to laboratory personnel who work with the virus.

In Central and South America, WEE remains a widespread problem because the horse is still in widespread use as a farm animal. However, the virus is not associated with significant human disease in South America, for reasons unknown. In South America, small mammals appear to be an important vertebrate reservoir.

As described, WEE arose by recombination, an event that occurred at some unknown time but probably more that 1000 years ago. Since its origin, it has diverged into a number of different viruses, including Fort Morgan virus found in Colorado and Oklahoma and Highlands J virus present along the east coast of the United States. Fort Morgan virus is transmitted by swallow bugs present in the nests of swallows to the young birds of the year, an interesting adaptation. Highlands J virus is maintained in a cycle similar to that for EEE. Neither of these viruses has been associated with human disease.

Venezuelan Equine Encephalitis Virus Complex

The VEE complex consists of a number of closely related viruses that are endemic to tropical and subtropical areas of the Americas. These viruses were first classified by serology into six subtypes of VEE, numbered with Roman numerals from I to VI. Some of these subtypes were further subdivided; in particular, subtype I was subdivided into IAB, IC, ID, IE, and IF. Partial or complete sequences of most of these viruses are now available and these subtypes are now considered to be full species (Fig. 3.25). As a species, VEE virus consists of IAB, IC, and ID; IE, also considered a strain of VEE, will be considered in more detail later. Subtype II is now named Everglades virus and is endemic to Florida. Subtype IIIA is called Mucambo virus, IIIB is Tonate virus (of which Bijou Bridge virus is another isolate), IV is Pixuna virus, V is Cabassou virus, and VI is Rio Negro virus; most of these are South American viruses. Subtype IF (not shown in Fig. 3.25) is also a South American virus but is only distantly related to other subtype I viruses, and is now considered a separate species, named to the present by its isolate designation 78V353I virus. These eight virus species are maintained in an endemic cycle in which the principal mosquito vectors are species belonging to the genus *Culex*, subgenus *Melanoconin*, and the major vertebrate reservoirs are small mammals, primarily cotton rats and opossums. These endemic viruses are able to infect horses but replicate to only low titers in them, causing no disease and not establishing an epidemic cycle. Similarly, these endemic viruses are not associated with significant human disease.

The endemic VEE ID gives rise to epizootic viruses IAB and IC by a small number of mutations in envelope glycoprotein E2 that allow the virus to grow to high titers in horses. The horse serves as an important amplifying host that allows the virus to become epidemic and spread over wide areas. *Aedes taeniorhynchus* and *Psorophora confinnis* are important mosquito vectors in these outbreaks. In these epidemics, which occur at intervals of 10–20 years in Venezuela, Columbia, Peru, and Ecuador, large numbers of horses die of encephalitis and significant episodes of human illness occur. For example, an epidemic in Venezuela and Columbia in 1995 resulted in disease in an estimated 75,000 to 100,000 people, including 3000 cases of neurological disease and 300 deaths. As with the other encephalitic alphaviruses, children and older people are more at risk for serious illness.

Because of the importance of VEE epidemics, an inactivated virus vaccine was produced many years ago from a

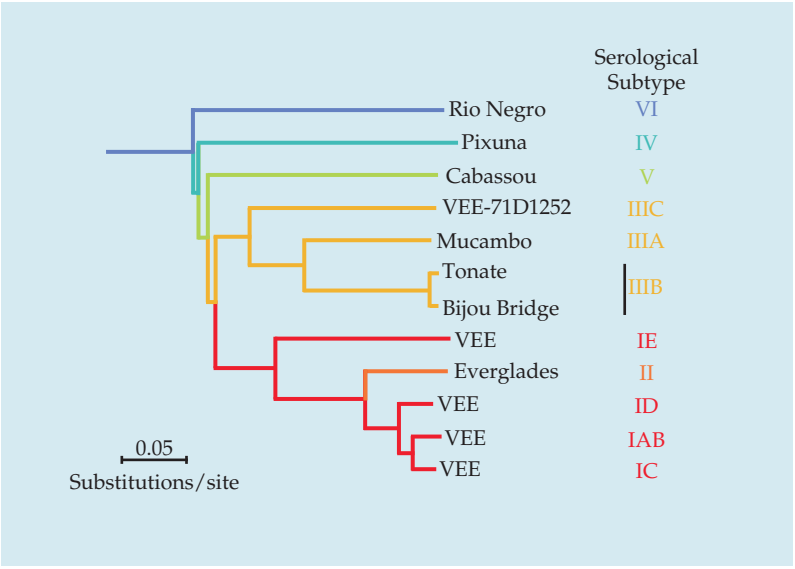


FIGURE 3.25 Phylogenetic tree of the various strains of Venezuelan equine encephalitis virus (VEE) and species of viruses formerly considered to be serological subtypes of VEE. This tree was generated from partial E1 envelope gene sequences using a neighbor-joining algorithm. Adapted from Fauquet *et al.* (2005), Figure 4 on p. 1007.

strain of IAB virus called Trinidad Donkey for use in horses and for laboratory personnel who work with the virus. This vaccine suffered from poor immunogenicity and insufficient inactivation. Use of the vaccine, for example, led to a wide-ranging epidemic of VEE in 1969–1973 that spread from South America up through Central America to southern Texas, causing many thousands of cases of disease in horses and humans. Because of these problems, use of this vaccine was abandoned and a new attenuated vaccine strain called TC-83, derived from the Trinidad Donkey virus by passage in culture, was developed. This vaccine is effective but is reactogenic and causes mild illness in many recipients. An inactivated version of this attenuated virus has also been produced as a vaccine but is not as effective as the live virus vaccine.

VEE virus can spread by aerosols as well as by mosquito transmission. Many cases of laboratory acquired infection have resulted from aerosols. Because of this property and the incapacitating disease caused by VEE, this virus was weaponized by the Russians in the past as a potential biowarfare agent.

Virtually all epidemics of epizootic VEE have been due to IAB and IC viruses. However, a recent epizootic of VEE in Mexico resulted from an IE strain that had a mutation in envelope glycoprotein E2. Unlike the IAB and IC strains, the epizootic IE virus grows to only low titers in horses. It is believed that the mutation in IE virus allowed a more efficient interaction with mosquito vectors that are widespread and numerous, allowing more efficient transmission of the virus. As shown in Fig. 3.25, IE virus is more closely related to Everglades virus than it is to ID virus, and the virus may be reclassified in the future.

Alphaviruses That Cause Arthritides

A number of alphaviruses cause disease in humans characterized by fever, rash, and joint involvement. The pain from arthritis (joint inflammation, from *arth* = joint and *itis* = inflammation) or arthralgia (joint pain, from *arth* and *algia* = pain) following infection by these viruses can be so severe as to be disabling and can last for a year or more with relapses of severe arthritis being common during this period. The names of some of these viruses come from the crippling pain caused by the disease resulting from viral infection.

Ross River Virus

Ross River virus is widespread in Australia, New Guinea, and the Solomon Islands. The disease induced by virus infection is known as epidemic polyarthritis and is characterized by pain, often accompanied by frank swelling, in the small joints of the hands and feet and in the knees. The principal vectors are *Aedes camptorhynchus* and *Aedes vigilax* in coastal regions, which breed in salt marshes, and *Culex annulirostris* and *Aedes notoscriptus* in other areas, which breed in freshwater. The vertebrate reservoir consists of various macropods. Because mammals constitute the vertebrate reservoir, different strains of the virus have evolved in different geographic regions for reasons discussed earlier. In some areas of Australia in which the virus is endemic, a majority of the population may be seropositive for RRV, indicating a high attack rate. It has been estimated that 2 to 30% of infected humans develop clinical symptoms following infection. As noted, recovery may be prolonged, with arthritic symptoms recurring over a period of a year or more.

Of interest was a wide-ranging epidemic of Ross River polyarthritis that swept through the South Pacific in 1979–1980. The epidemic began when a single viremic traveler from Australia landed in Fiji. The epidemic began near the airport and eventually spread throughout the island. From there it jumped to other islands having air contact with Fiji. During this epidemic, it is believed that humans were the primary or only vertebrate host, with the disease being transmitted from mosquito to human to mosquito without the intervention of another animal reservoir (the cycle illustrated in Fig. 3.23A). Sequencing studies have suggested that a mutation in glycoprotein E2 may have allowed this cycle to become established. The mosquito vector during this epidemic was *Aedes polynesiensis*. This epidemic was explosive with most of the people on the affected islands becoming infected by the virus and about 10% of them becoming ill. The epidemic eventually burned itself out because all the humans had become immune and the virus failed to establish an endemic cycle in other animals in the region. After 20 years without RRV in Fiji, three recent cases of RRV disease have been reported in travelers to Fiji. It is thought that RRV has been reintroduced into the island on these occasions and susceptible tourists infected, but that the native population is now largely immune to the virus and no new epidemic has occurred.

Sindbis Virus

The prototype alphavirus is Sindbis virus, named after the town of Sindbis, Egypt, where it was first isolated in 1953 from mosquitoes. It has the widest distribution of any alphavirus, endemic from northern Europe through the Middle East and India to South Africa, Southeast Asia, Indonesia, the Philippines, New Guinea, and Australia. As might be expected from this broad range, strains of virus isolated from different regions may differ by 20% or so in nucleotide sequence and differ in their epidemiology and disease potential. As far as known, birds are the vertebrate reservoir for the virus over its entire range with different mosquitoes serving as vectors in different areas. Over most of its range no human illness is associated with infection or illness is very rare, even though in regions such as the Nile Delta seroprevalence rates may be fairly high. However,

strains of the virus in northern Europe, South Africa, and Australia cause significant episodes of arthritic illness.

In northern Europe the strains of Sindbis virus that cause arthritic disease are called Ockelbo virus in Sweden (present between the 60th and 64th parallels), Pogosta virus, widespread in Finland, and Karelian Fever virus, present in far Western Russia. The virus is maintained in migratory birds or in game birds and the mosquito vectors are various species of *Culex* and *Culiseta*. *Aedes* species may spread the virus to humans. The disease in humans is typical of alphavirus arthritic disease with fever, rash, and joint inflammation and the number of cases can be large during epidemic years. Children are less likely to develop disease upon infection. In South Africa strains of Sindbis virus also cause human disease, but the number of cases appears to be small and the virus has been less well studied. Strains of Sindbis virus that are present in Australia also cause human disease, primarily in northeastern Australia. The virus appears to be fairly uniform throughout Australia but to be replaced every so often by new strains that presumably invade from the north.

Mayaro Virus

Mayaro virus is present in the northern half of South America (Trinidad, Surinam, Brazil, Columbia, Bolivia). It is maintained by *Haemagogus* mosquitoes and humans usually contract the virus while in humid tropical forests. Rubber workers are at risk of infection and the polyarthritis caused by the disease can be debilitating, preventing the workers from gainful employment. Mayaro belongs to the Semliki Forest virus clade (Fig. 3.17) and causes a disease that is similar to that caused by the related Ross River virus. It is the only known representative of this clade in the Americas and represents one of the very few transfers of alphaviruses between the Old and the New World.

Chikungunya Virus and Related Viruses

Chikungunya (CHIK) virus, a member of the Semliki Forest virus clade, is endemic or epidemic from sub-Saharan Africa through India and Southeast Asia to the Philippines. The name comes from Swahili meaning "that which bends up," from the intense arthralgia that causes patients to lie with joints flexed. In Africa the virus is maintained in an endemic cycle that is similar to that for yellow fever virus (see later). The mosquito vectors are *Aedes africanus* and *Aedes furcifer* and subhuman primates are the vertebrate reservoir. During explosive epidemics in urban areas of Africa and Asia, *Aedes aegypti* is the vector and human–mosquito–human cycles maintain the virus. During an epidemic, a large fraction of the susceptible human population may contract the disease. The epidemic then dies out, to return when reintroduced after a period of time that allows a susceptible population to build up. Epidemics often occur during the rainy season when the population of *Aedes aegypti* is highest.

In 2006, a hugh epidemic of CHIK, accompanied in many locations by infections of dengue virus as well (see later) whose disease symptoms can be similar to those caused by CHIK, occurred throughout the Indian Ocean region. The virus appears to have been imported from East Africa, and many thousands of cases occurred in the region, affecting the islands of Reunion, Mauritius, Madagascar, Mayotte, the Seychelles, and the Maldives as well as the Indian subcontinent. In Reunion there occurred more than 200,000 cases in a population of under 800,000. Many of these islands are popular tourist destinations, and many cases were imported into Europe. The primary mosquito vector was *Aedes albopictus* or *Aedes aegypti*, depending upon the location, both of which are also efficient vectors for dengue virus.

O'nyong-nyong (ONN) virus is a close relative of CHIK that has caused very large epidemics of disease in East Africa similar to that caused by CHIK. The name also comes from the very painful arthralgia accompanying the disease. Epidemics affecting up to two million people have occurred followed by the disappearance of the virus for long periods. In these epidemics the virus is transmitted by *Anopheles funestrus* and *Anopheles gambiae*, mosquitoes that are major vectors of malaria, and these epidemics represent the only known cases of epidemic transmission of an alphavirus by anopheline mosquitoes. An endemic cycle presumably maintains the virus during interepidemic periods but, if so, this cycle is unknown. A strain of ONN called Igbo-Ora virus, from the name of the Nigerian village in which the virus was first isolated, is present in West Africa.

Barmah Forest Virus

Barmah Forest virus is an Australian virus that is an outlier in the Semliki Forest clade (Fig. 3.17). It also causes polyarthritis in humans. It is probably maintained in a cycle similar to that for Ross River virus.

Other Alphaviruses

Other alphaviruses are known that infect higher vertebrates including humans, but most are not associated with disease in humans. Semliki Forest virus, named after the Semliki Forest in Uganda, has been extensively characterized as a model system to study the molecular biology of alphaviruses. Most strains cause no human illness, but strains from central Africa cause a disease characterized by exceptionally severe headache, fever, and rash. One case of fatal human encephalitis caused by this virus occurred in a laboratory worker, who is believed to have contracted the virus via aerosols. Getah virus, widespread in Asia, causes a mild febrile illness in humans.

Recent isolates of new alphaviruses include Southern elephant seal virus, currently unclassified. It is spread by a louse, *Lepidophthirus macrorhini*, that infests the seals.

Two fish alphaviruses have been recently isolated, salmon pancreas disease virus and sleeping disease virus, which are now considered to be strains of the same species, to be called salmonid alphavirus. A third strain of the virus, Norwegian salmonid alphavirus, has been found in western Norway. Salmonid alphavirus is an important pathogen of Atlantic salmon and rainbow trout in Norway, Britain, Ireland, and France. It causes significant economic losses in the farmed fish industry and is the leading cause of economic loss in Ireland. The viruses can be spread by direct fish-to-fish infection and are not known to have an arthropod vector, but since all other alphaviruses are vectored by arthropods there has been speculation that a sea louse *Lepeophtheirus salmonis* might be involved in the transmission of the virus. It is not known if the primary location of infection is saltwater or freshwater.

Rubella Virus

Rubella virus is less well understood than alphaviruses because it grows very poorly in cultured cells and its genome possesses an extraordinarily high GC content (70%), which retarded efforts to sequence and express the viral genome. The complete sequences of several strains are now known and detailed molecular studies are under way. The genome is approximately 10 kb in size and is expressed similarly to the alphavirus genome: The genomic RNA is translated into a polyprotein cleaved by a papain-like protease into two pieces, and a subgenomic mRNA is translated into structural proteins consisting of a capsid protein and two envelope glycoproteins (Fig. 3.16). Interestingly, minus-strand RNA synthesis is controlled in the same way as in alphaviruses. The uncleaved nonstructural polyprotein synthesizes minus-strand RNA, whereas the cleaved products synthesize only plus-strand RNA.

Rubella virus infects only humans and there is no other reservoir. Infection is by person-to-person contact, primarily through aerosols. It causes a relatively benign illness, sometimes called German measles, with a characteristic rash and is (or was) one of the typical childhood diseases. However, infection of a pregnant woman in the first trimester of pregnancy can have devastating effects on the developing fetus. The virus sets up a long-lived infection in the fetus that often causes developmental abnormalities resulting in children with severe handicaps (congenital rubella syndrome). An attenuated virus vaccine against rubella has been developed that is now routinely given to children as part of mumps–measles–rubella vaccination (MMR). Since the vaccine was introduced, there has been a drastic reduction in the number of cases of rubella in the United States (Fig. 3.26) and other developed countries.

Because the postnatal disease caused by rubella virus is trivial, the purpose of the rubella vaccine is to protect against future birth defects rather than to protect the individual vaccinated. This raises interesting ethical questions about its use. In some societies, only females were vaccinated, since they would want to protect their future children from the effects of rubella. However, because males remained susceptible to the virus, it continued to circulate in the population and rubella-caused birth defects continued to occur. To protect against this, the only solution is to vaccinate the entire population so as to eliminate the virus from the society.

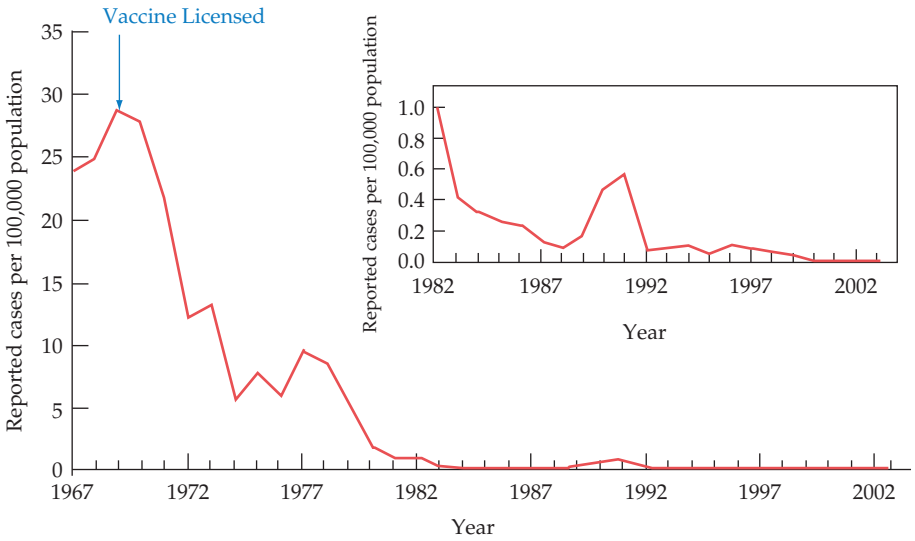


FIGURE 3.26 Incidence of rubella virus, by year, in the United States. From *MMWR Summary of Notifiable Diseases in the United States for 1997, 1998, 1999, 2000, 2001, 2002, 2003*.

The rubella vaccine has now been in use for many years and is generally safe and effective when given to children. The present vaccine has a high incidence of side effects in adults, however, especially arthralgia and arthritis. Nonetheless, vaccination is recommended for women of childbearing age, as well as for certain health care personnel, who have never been vaccinated and who are seronegative. The need exists to improve the vaccine, and current efforts to understand the molecular biology of the virus in more detail will hopefully lead to the development of a better vaccine.

FAMILY *FLAVIVIRIDAE*

The *Flaviviridae* are named after the prototype virus, yellow fever virus, *flavus* being the Latin word for yellow. The *Flaviviridae* are divided into three genera, the genus *Flavivirus*, the genus *Pestivirus*, and the genus *Hepacivirus*. A partial listing of viruses in the three genera is given in Table 3.12. In the following discussion, the term flavivirus refers only to members of the genus *Flavivirus* unless otherwise specified.

The genome organizations of members of the three genera are shown in Fig. 3.27. The genomes of the three genera are similar in size (11 kb for flaviviruses, 12.5 kb for pestiviruses, 9.4 kb for hepaciviruses) and organization. These viruses, like the picornaviruses, have a genome that contains only a single ORF. This ORF is translated into a long polyprotein that is processed by cleavage into 10 or more polypeptides. Processing of the precursor polyprotein is complicated. Cleavage is effected by a combination of one or two or three (depending on the virus) virus encoded proteases and two or more cellular proteases. The structural proteins are encoded in the 5′-terminal region of the genome (like picornaviruses). However, all members of the *Flaviviridae* are enveloped, unlike the picornaviruses, and the structural proteins consist of a nucleocapsid protein and two or three envelope glycoproteins. Cellular proteases make the cleavages that separate the glycoproteins, but the cleavages in the nonstructural region of the polyprotein, which is required for RNA replication, are made by one or two virus-encoded proteases. Even so, cellular signalase makes at least one of the cleavages in the nonstructural domain of flaviviruses. The cleavage pathways in this genus are described in detail next.

All members of the *Flaviviridae* encode a serine protease with a catalytic triad consisting of serine, histidine, and aspartic acid. The protease resides in the nonstructural

TABLE 3.12 *Flaviviridae*

Genus/species	Virus name abbreviation	Usual host(s)	Transmission	Disease	World distribution
Flavivirus					
Dengue (Types 1–4)	DENV	Humans	Mosquito-borne	Dengue fever, shock, hemorrhage,	Worldwide
Yellow fever	YFV	Primates[a]	Mosquito-borne	Hemorrhage, liver destruction	Africa, Americas
Japanese encephalitis	JEV	Mammals,[a] especially swine	Mosquito-borne	Encephalitis	Widespread in Asia
St. Louis encephalitis	SLEV	Mammals,[a] birds	Mosquito-borne	Encephalitis	North America
Murray Valley encephalitis	MVEV	Mammals,[a] birds	Mosquito-borne	Encephalitis	Australia
Tick-borne encephalitis	TBEV	Mammals[a]	Tick-borne	Encephalitis	Europe, Asia
West Nile	WNV	Mammals,[a] birds	Mosquito-borne	Encephalitis	Europe, Africa, North America
Hepacivirus					
Hepatitis C	HCV	Humans	Parenteral, transfusion	Hepatitis, liver cancer	Worldwide
Pestivirus					
Classical swine fever	CSFV	Swine	Contact	Fever, acute gastroenteritis	Europe, Americas
Bovine viral diarrhea	BVDV	Cattle	Contact	Usually none[b]	Worldwide

[a] Including humans.
[b] Calves infected *in utero* develop persistent infections that can lead to mucosal disease.

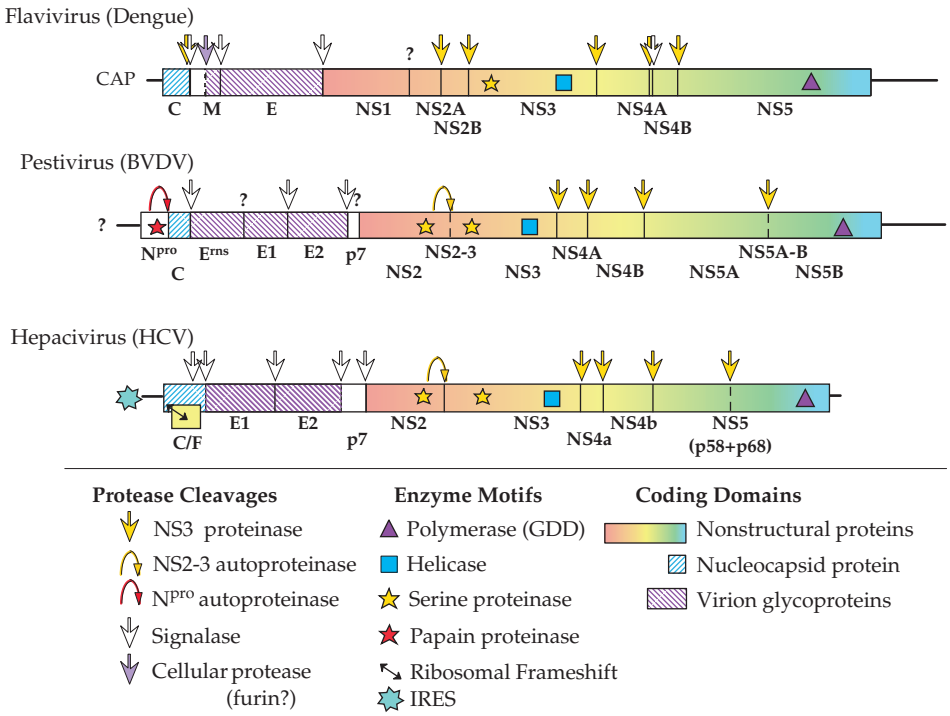


FIGURE 3.27 Genome organization of representatives of the three genera within the *Flaviviridae*. The cleavage sites indicated with dashed lines have not been precisely localized. Adapted from Figures 2, 4, 5 of the *Flaviviridae* in Fauquet *et al*. (2005) pp. 981–998. The data on the F protein of hepatitis C virus, which is produced from the core protein sequence by ribosomal frameshifting, came from Xu *et al*. (2004).

region called NS3, just upstream of a helicase. The crystal structure of the dengue virus protease and of the hepatitis C virus (HCV) protease has been solved to atomic resolution and they possess a fold similar to chymotrypsin, as is the case for other viral serine proteases whose structures have been solved. The enzyme is interesting in that a second polypeptide is required for activity, NS2B in flaviviruses and NS4A in HCV. From the atomic structure of the HCV protease complexed with the region of NS4A required for activity, it is clear that NS4A forms an integral part of the folded protease. Thus, it is puzzling that the protease consists of two cleaved products rather than one continuous polypeptide chain.

Flaviviruses encode only the NS3 protease. Hepaciviruses and pestiviruses encode a second protease in the NS2 region that cleaves between NS2 and NS3. Pestiviruses also encode a third protease at the N terminus of the polyprotein whose only known function is to cleave itself from the polyprotein precursor.

Flaviviruses have capped genomes whose translation is cap dependent. In contrast, the hepacivirus and pestivirus genomes are not capped and have an IRES in the 5′ nontranslated region. Members of family *Flaviviridae* do not have a poly(A) tail at the 3′ end of the RNA. Instead, a stable stem-loop structure is present at the 3′ end of the genome that is required for replication of the genomic RNA and for its translation, as described in more detail later. No nucleotide or amino acid sequence identity can be detected between members of different genera except for isolated motifs that are signatures of various enzymatic functions.

Viruses in the family are enveloped. As described later and in Chapter 2, members of the genus *Flavivirus* have a structure that is related to that of alphaviruses. Details of the structures of pestiviruses and hepaciviruses are lacking. Flaviviruses mature at intracytoplasmic membranes rather than at the plasma membrane.

Genus *Flavivirus*

There are about 53 species of flaviviruses currently recognized, and many species have important subtypes that are also named. A representative sample is given in Tables 3.11 and 3.12. The relationships of the viruses to one another is illustrated by the dendrogram in Fig. 3.28. All members of the genus are closely related and share significant amino acid sequence identity in their proteins, which results in serological cross-reactivity. Historically, members of this genus were assigned to it on the basis of these cross-reactions. Most are arthropod-borne, and they were once referred to as Group B arboviruses. They can be divided into three major groups

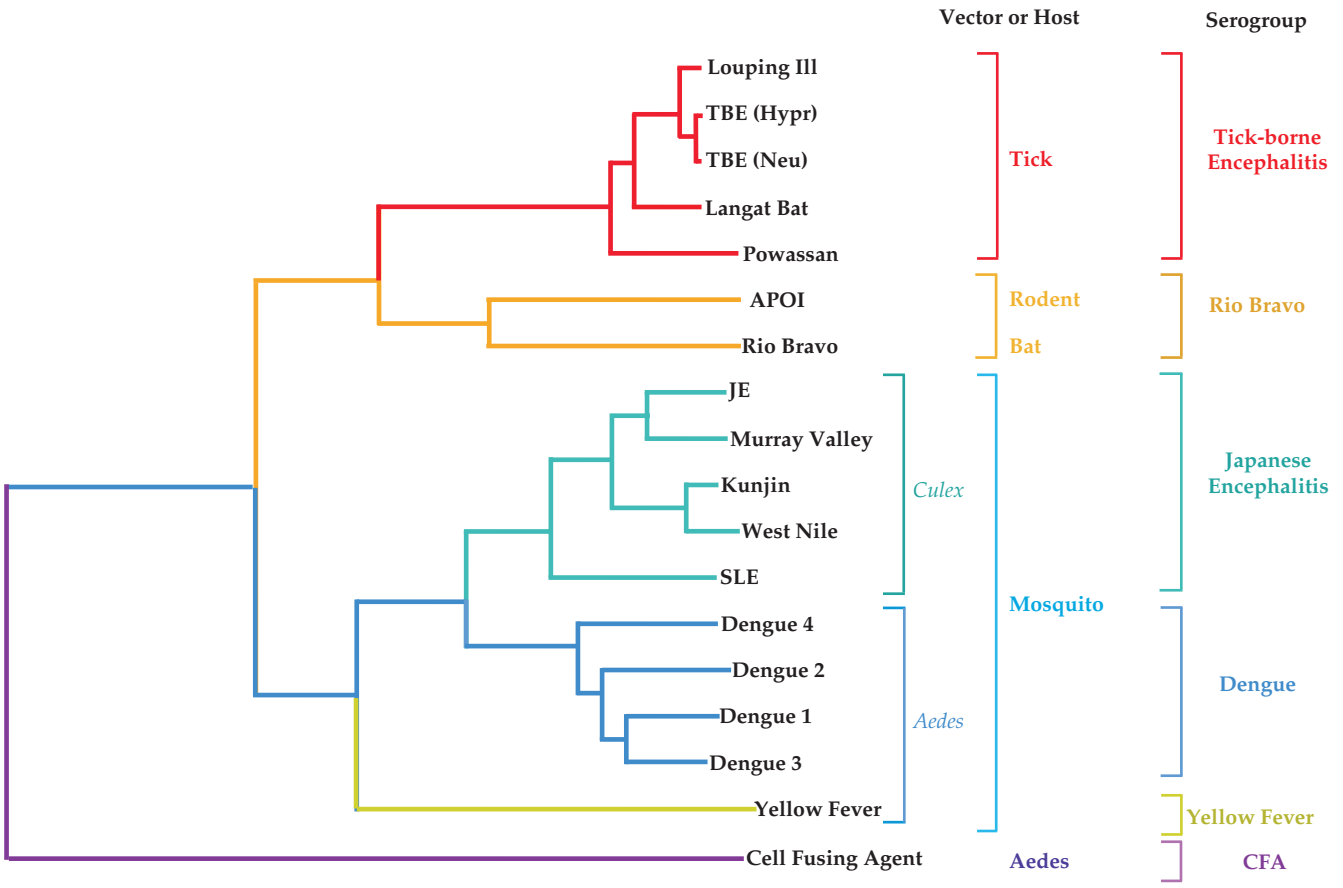


FIGURE 3.28 Phylogenetic tree of the flaviviruses based on NS3 polyprotein region using the neighbor-joining method. Data are from Billoir *et al*. (2000).

based on the vector utilized: the mosquito-borne group (which includes yellow fever, the dengue complex, and the Japanese encephalitis complex), the tick-borne encephalitis group (the TBE complex), and a group that lacks an arthropod vector. The last are of limited medical importance. Notice that in the phylogenetic tree in Fig. 3.28, the tick-borne viruses and the mosquito-borne viruses belong to different lineages. These viruses are adapted to a tick vector or to a mosquito vector, and interchange of vectors does not occur. Further, the tree indicates that the mosquito-borne viruses separate out into a lineage vectored primarily by mosquitoes belonging to the genus *Culex* and lineages vectored by mosquitoes belonging to the genus *Aedes*. However, in these lineages the restriction on the mosquito vector is not firm and mosquitoes belonging to other genera may vector many of these viruses. As two examples, the ancestral yellow fever virus in Africa is vectored by *Aedes* mosquitoes in both sylvan and urban cycles, but in the Americas it is vectored by *Hemagogous* mosquitoes in a sylvan cycle and *Aedes* mosquitoes in an urban cycle, as described later. West Nile virus, recently introduced into the United States, is vectored by a very wide variety of mosquitoes, and this has been in part responsible for the rapid spread of the virus across the United States.

Expression of the Viral Genome

The genome organization of a typical flavivirus is illustrated in Fig. 3.27. As for all plus-strand RNA viruses, the genomic RNA is a messenger and in the case of flaviviruses serves as the messenger for all of the virus encoded proteins. The RNA is capped but lacks 3′ poly(A). There is a stem-loop structure at the 3′ end which serves the same function as poly(A) in other messengers. This structure increases the efficiency of translation of the RNA by about 10-fold and will substitute for poly(A) in model systems. Viral proteins are not required for this effect and therefore cellular proteins must interact with this structure in order to increase the efficiency of translation. It is known that during translation of mRNAs that are capped and polyadenylated, there is an initiation complex formed that contains both cap-binding

protein and poly(A)-binding protein. Thus, the complex interacts with both ends of the mRNA to initiate translation. It is assumed that a cellular protein binds the 3′ stem-loop of flaviviruses and interacts with the initiation complex so as to perform the same function as the poly(A)-binding protein. Formation of this complex in the case of flavivirus RNAs could be enhanced by cyclization of the viral RNA described later, although the primary function of cyclization appears to be in the replication of the viral RNA.

The processing of the long polyprotein produced from the genome is complicated and is illustrated in Fig. 3.29 as an example of complex processing events that can occur in viral polyproteins associated with lipid bilayers. The nucleocapsid protein is 5′ terminal in the genome and is removed from the precursor polyprotein by the viral NS2B–NS3 protease. Two envelope proteins, prM (precursor to M) and E (envelope), follow. Both are anchored in the endoplasmic reticulum by C-terminal membrane-spanning domains and are usually, but not always, glycoproteins. A series of internal signal sequences is responsible for the multiple insertion events required to insert prM, E, and the following protein, NS1, into the endoplasmic reticulum. After separation of these three proteins by signalase, prM and E form a heterodimer. prM is cleaved to M by furin during transport of the heterodimer or during virus assembly. Assembly of virions is described in more detail later.

Following the E protein is NS1 (NS for nonstructural). NS1 is a glycoprotein and has multiple functions that are only poorly understood. It is found as dimers and higher multimers in three locations in mammalian cells: intracellular; anchored in the plasma membrane by a GPI (glycosyl-phosphotidylinositol) anchor; and as a soluble protein secreted from the infected cell. It is required for RNA replication, presumably a function of the intracellular form of the protein. For this function, it interacts with NS4A. The cell surface-anchored form is capable of antibody-induced signal transduction that may play a role in cell activation. The function of the secreted form is unknown but it has

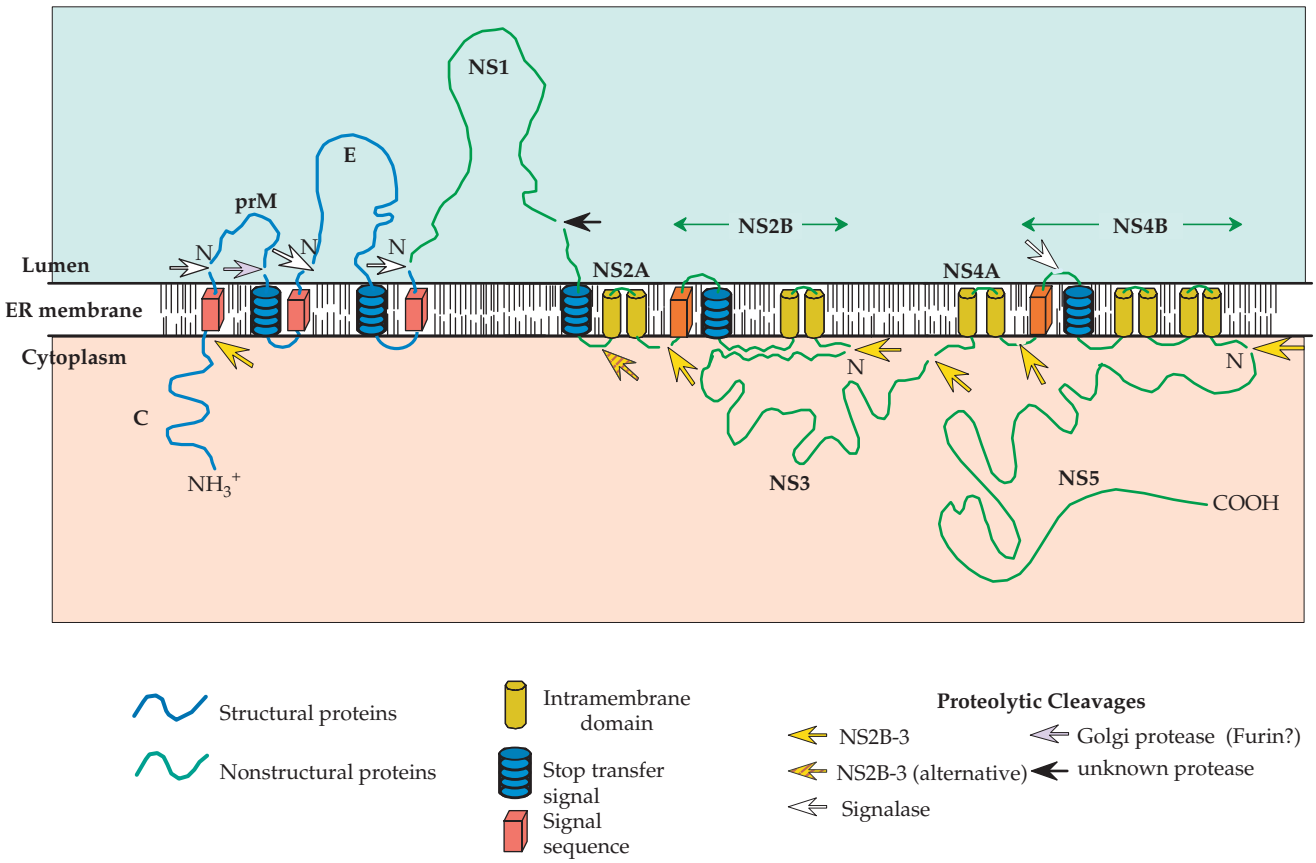


FIGURE 3.29 Processing of the flavivirus polyprotein into the structural and nonstructural proteins of the virus. The structural proteins (blue) at the N terminus of the polyprotein are processed primarily by signalase, with one late cleavage in prM due to furin. The nonstructural proteins (green) are mostly processed by the viral NS2B–NS3 protease. As indicated in the figure, the central 40 amino acids of NS2B interact with NS3, tying NS3 to the membrane, and this interaction is essential for proteolytic function. The striped arrow shows the alternative site of cleavage within NS2A that may lead to an anchored form of NS1. Adapted from Figures 3, 4, and 6 in Strauss and Strauss (1996).

been speculated that it has a role in counteracting immune responses to the virus.

Next in the polyprotein precursor are two hydrophobic polypeptides called NS2A and NS2B. These proteins are cleaved by the viral NS2B–NS3 protease. They are associated with membranes and may serve to anchor parts of the replication machinery to internal membranes in the cell. NS2A has multiple functions. It inhibits the production of interferon-α/β by infected cells. As described in more detail in Chapter 10, the interferons are potent inhibitors of virus replication and most, perhaps all, viruses encode products to block interferon action. NS2A also has a role in the production of infectious particles from the infected cell, since certain mutations in this protein block virus assembly but do not affect other aspects of the virus life cycle. These mutants can be suppressed by changes in the NS3 helicase domain, suggesting an interaction between NS2A and NS3. NS2B also interacts with NS3, but with the protease domain. It is a cofactor required for the NS3 protease activity and the central domain of NS2B forms a complex with NS3, which follows NS2B in the polyprotein precursor. The NS2B–NS3 serine protease cleaves many bonds in the polyprotein. NS3 also has at least two other activities—the middle domain of NS3 is a helicase, required for RNA replication, and the C-terminal domain has RNA triphosphatase activity, an activity required for the capping of the viral genome.

NS4A and NS4B are hydrophobic polypeptides that are associated with membranes. They may function in assembly of the viral replicase on intracellular membranes. Both the viral NS2B–NS3 protease and cellular signalase are required to produce the final cleaved products.

NS5 is the viral RNA polymerase. It appears to be a soluble cytoplasmic protein that associates with membranes through association with other viral peptides. It also has methyltransferase activity and thus is the capping enzyme that caps the viral genome. Thus, capping requires two flaviviral proteins, NS3 (RNA triphosphatase) and NS5 (capping enzyme). Note the similiarity to alphaviruses where the RNA triphosphatase activity is also on the helicase-protease protein (nsP2, the analogue of flaviviral NS3), and the methyltransferase or capping activity is a different protein (nsP1). However, in alphaviruses the capping enzyme and the RNA polymerase (nsP4) are distinct proteins, whereas in flaviviruses they are present in the same polypeptide.

Replication of the Viral RNA

RNA replication is associated with the nuclear membrane. The composition of the replicase complex is not understood but is assumed to consist of many (most? all?) of the viral nonstructural proteins with associated cellular proteins. Cyclization of the RNA is required for replication. Sequences from the 5′ and 3′ regions of dengue virus RNA that form a number of stem-loop structures and that also cyclize the RNA are illustrated in Fig. 3.30, where two possible structures are shown. Experimental data have shown that the RNA sequence in the capsid protein downstream of the AUG start codon is involved in cyclization (region marked CS1). Sequences upstream of the start codon are also known to be required for cyclization, and the two structures show different ways that these might be used for cyclization. This figure also illustrates the long stem-loop structure at the 3′ end of the RNA, discussed earlier. A stem-loop structure in the 5′ region just upstream of the CS1 region has also been shown to be important in translation of the RNA, in this case for recognition of the AUG start codon, which is found in a poor context for a start codon.

The sequences surrounding CS1 are illustrated for a number of mosquito-borne flaviviruses in Fig. 3.31. This eight nucleotide sequence is invariant among the mosquito-borne flaviviruses, and experimental studies have shown that this sequence is important for cyclization and replication of the RNA. The 3′ sequences complementary to this region are found in the 3′ nontranslated region (see also Fig. 3.30). Changes in these sequences that eliminate cyclization prevent the RNA from replicating, even in model systems in which translation of the RNA is not required for expression of the replicase. Compensating mutations in the partner sequence that restore cyclization restore the ability of the RNA to replicate. Thus, cyclization is required for RNA replication.

The identities of the promoters recognized by the RNA replication machinery are as yet unknown, but the requirement for cyclization suggests that sequences at both ends of the RNA are required. The conservation of the 8-nucleotide core sequence suggests that these sequences might be part of the promoter recognized by the RNA replicase.

Formation of the Virion

Most flaviviruses mature at intracellular membranes. Budding figures have been described only rarely and assembly may be associated with the complex processing of the polyprotein. West Nile virus is an exception to this general rule. It grows to higher titers in cultured cells than other flaviviruses and budding of preassembled nucleocapsids at the plama membrane is readily seen. Even in this case, however, intracellular assembly of virions is also seen.

The processing of the structural proteins from the precursor polyprotein was described earlier. prM and E form a heterodimer shortly after synthesis. The assembly of flaviviruses has clear parallels with that of alphaviruses, as described in Chapter 2. E of flaviviruses and E1 of alphaviruses are homologous proteins, having the same structure and function (see Fig. 2.17). A heterodimer is first formed, between prM and E in flaviviruses and PE2 and E1 in alphaviruses. Immature virus particles can be isolated that have uncleaved PE2 or prM whose infectivity is very low. The trimeric spikes

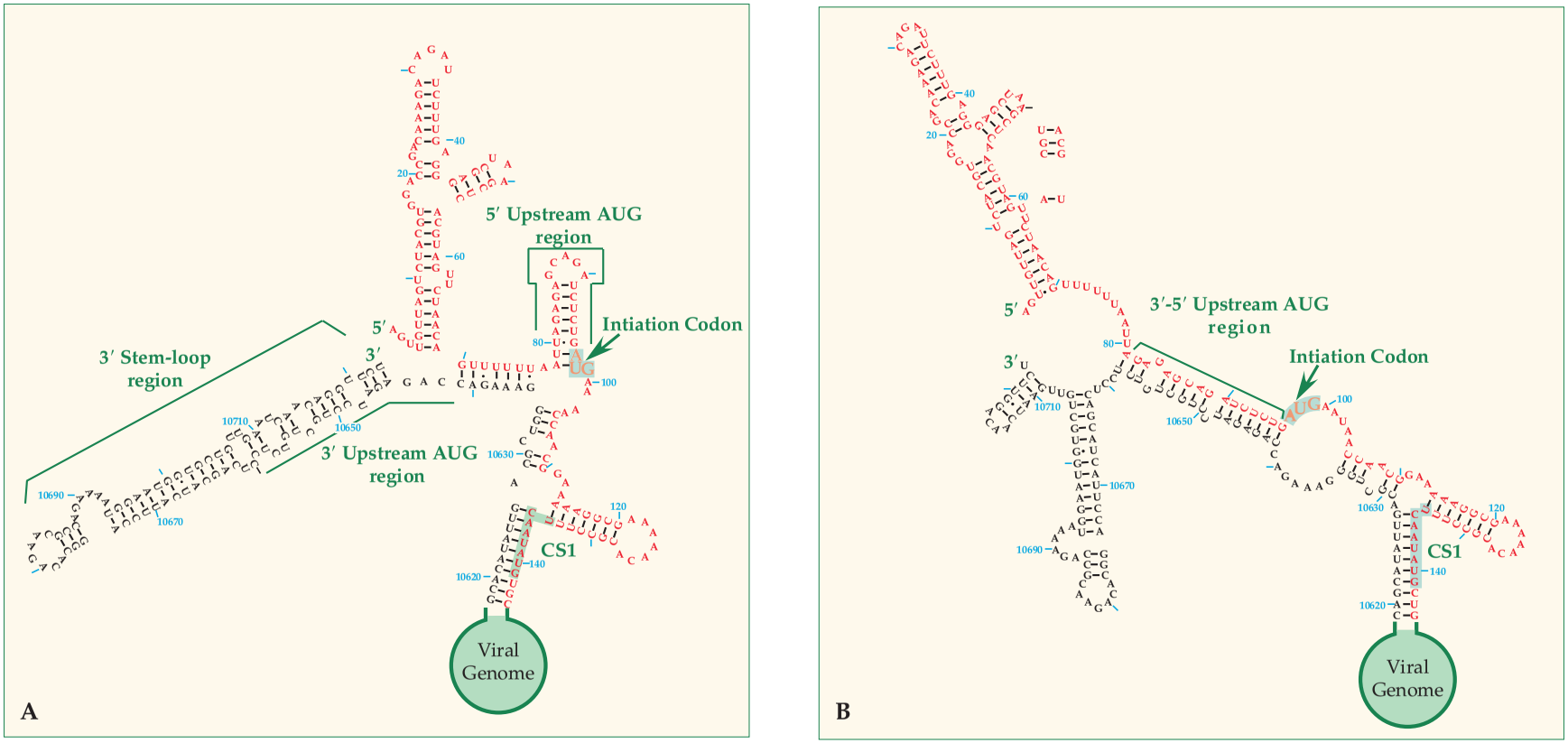


FIGURE 3.30 Computer-generated secondary structures of the last 106 nucleotides (nts 10618–10723 in black) and the first 144 nucleotides (in red) of the dengue 2 genome of strain 16681. The rest of the genome is indicated schematically by the green circle. The two predicted secondary structures shown in (A) and (B) are those with the lowest ΔG. The 3′ stem-loop structure, the 3′ and 5′ upstream AUG regions, the initiating AUG codon, and the conserved sequence (CS1, illustrated in Fig. 3.30) are labeled. Note that the stem-loop structure just upstream of CS1 (nt 114ff) is important for initiation of translation at the AUG start codon. This figure is adapted from Figure 5 in Alvarez *et al.* (2005).

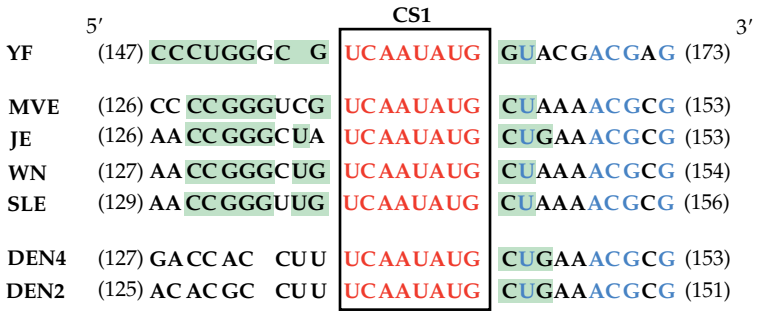


FIGURE 3.31 Conserved nucleotide sequence elements in the 5′ region encoding the capsid protein in six different mosquito-borne flaviviruses. The number of the first and last nucleotides shown is given in parentheses. The boxed nucleotides in red are those postulated to be important for cyclization of the RNA. Residues shaded in green are complementary to sequences at the 3′ end and those given in blue are conserved but probably not involved in cyclization. Adapted from Figure 7 of Hahn *et al*. (1987).

in these immature particles are quite similar in structure (see Fig. 2.15). Flaviviruses have a triangulation number of 3 and therefore 60 trimeric spikes each consisting of 3 heterodimers of prM and E. Alphaviruses have a triangulation number of 4 and therefore there are 80 trimeric spikes each consisting of 3 heterodimers of PE2 and E1. After cleavage of prM or of PE2, however, the structures are quite different. Alphaviruses retain 80 trimeric spikes with E2-E1 heterodimers. In flaviviruses, however, the M-E heterodimer dissociates and there is a dramatic rearrangement whereby 90 E-E homodimers are formed and the particle shrinks from 60 nm in diameter to 50 nm. Upon infection of a cell and exposure of the mature flavivirion to acidic pH there is another dramatic rearrangement and 60 E-E-E homotrimers are formed that tilt up so that the fusion peptide at the extremity of domain 2 is inserted into the cellular target membrane and fusion results. It seems clear that the structures of alphaviruses and flaviviruses had a common origin, and that recombination during their evolution resulted in this common structure becoming associated with different suites of RNA replication enzymes. It is possible that this structure, which allows enveloped viruses to have a regular icosahedral structure, evolved only once. It is also of note that both groups of these viruses are primarily arboviruses and perhaps this common structure is important for this.

The flavivirus nucleocapsid is thought to be icosahedral in symmetry, perhaps having a triangulation number of 3. There appears to be no interaction between the envelope proteins and the capsid proteins in flaviviruses, however, unlike the situation for alphaviruses, so that the icosahedral structure of the nucleocapsid, if it exists, is not coordinated with the icosahedral arrangement of the glycoproteins forming the outer surface of the virion.

Yellow Fever Virus

Many flaviviruses are important pathogens of humans. Different viruses may cause encephalitis, hemorrhagic fever with shock, fulminant liver failure, or disease characterized by fever and rash. Several important viruses and their diseases are listed in Tables 3.11 and 3.12. We begin the discussion of these viruses with yellow fever virus, the prototype flavivirus and a virus whose history was important in the development of the science of virology and of vaccinology.

Yellow fever virus (YFV) was once greatly feared and is still capable of causing large epidemics. The virus is viscerotropic in primates, the only natural hosts for it. The growth of the virus in the liver, a major target organ, causes the major symptoms of disease and the symptoms from which the name of the virus derives, jaundice following destruction of liver cells. The virus also replicates in other organs, such as kidney and heart, and causes hemorrhaging. Illness is accompanied by high fever. Death occurs in 20–50% of serious infections, usually on days 7–10 of illness and usually as a result of extensive liver necrosis.

YFV is present today in Africa and Latin America. It originated in Africa and spread to the Americas with European colonization and the introduction of slaves. The virus is maintained in two different cycles. In an endemic or sylvan cycle, it is maintained in *Aedes africanus* and other *Aedes* mosquitoes in Africa and in *Haemogogus* mosquitoes in the Americas. Monkeys form the vertebrate reservoir. In this cycle, forest workers and other humans who enter deep forests are at risk. Infection of humans can lead to the establishment of an epidemic or urban cycle in which the virus is transmitted by the mosquito *Aedes aegypti* and humans are the vertebrate reservoir. In this cycle, all urban dwellers are at risk. *Aedes aegypti* is a commensal of man, breeding around human habitation. It is widespread in the warmer regions of the world, including the southern United States, Central America and the Caribbean, large regions of South America, sub-Saharan Africa, the Indian subcontinent, Southeast Asia, Indonesia, and northern Australia.

History of Yellow Fever

In the 1800s, YFV was continuously epidemic in the Caribbean region, where it had a pronounced influence on

the development and settlement of the Americas by the Europeans. Caucasians and Native Americans are very sensitive to yellow fever, usually suffering a serious illness with a high death rate. Black Africans, who were brought as slaves to the New World to replace Native American slaves who had died in large numbers from European diseases, in general suffer less severe disease following yellow fever infection, presumably having been selected for partial resistence by millennia of coexistence with the virus. Their relative resistance to yellow fever resulted in the importation of even more black slaves into yellow fever zones. The high death rate among French soldiers sent to the Caribbean region to control black slaves was probably responsible for the decision by Napoleon to abandon the Louisiana territory by selling it to the United States, by which the United States underwent a huge territorial expansion. The high death rate among French engineers and workers in the 1880s under de Lesseps, who had previously supervised the construction of the Suez Canal, led to the abandonment of the attempt by the French to build a canal through Panama. The Panama Canal through Panama was built by the United States only after yellow fever was controlled.

From its focus in the Caribbean, yellow fever regularly spread to port cities in the southern and southeastern United States and as far north as Philadelphia, New York, and Boston. Epidemic yellow fever even reached London. The virus also spread up the Mississippi River from New Orleans. The virus was transported from its focus in the Caribbean by ships, which carried freshwater in which mosquitoes could breed. If there was yellow fever on the ship, the disease was maintained and could be transmitted by the mosquitoes or by infected individuals to ports at which the ships called. Yellow fever epidemics could afflict most of the population of a city and result in death rates of 20% or more of the city's original population.

One telling account of an epidemic in Norfolk, Virginia, in 1855 is described in the report of a committee of physicians established to examine the causes of this epidemic. Quarantine procedures to prevent the introduction of yellow fever were often thwarted by captains who concealed the presence of the disease to avoid a lengthy quarantine, even going to the extreme of secretly burying crew members who died while in quarantine. On June 6, 1855, the steamer *Ben Franklin* arrived from St. Thomas and anchored at the quarantine ground. There had been three cases of yellow fever on the ship during the voyage, of whom two died and were buried, one on land and one at sea. There was yet another case on board during quarantine who died and was buried ashore. Yet when the health officer, Dr. Gordon, visited the ship, he was told by the captain that there was no disease on the ship. The captain did admit that there had been two deaths during the voyage but ascribed them to other causes. After 13 days in quarantine and continued inspection by Dr. Gordon, who finding nothing amiss believed the captain's report that there was no disease aboard, the ship was allowed to dock. Yellow fever soon appeared in Norfolk. A number of early cases among the citizens of the town were ascribed to the ship passing within a half mile of their homes, and it is possible that infected mosquitoes were blown ashore, although it is also possible that workmen visiting the ship while laid up for repairs may have brought the disease into the town. The disease then spread in all directions at a uniform rate of about 40 yards per day until it encompassed the whole city. The epidemic peaked at the end of August and died out after a frost in October. During the epidemic, an estimated 10,000 cases of yellow fever occurred in a population of 16,000, and 2000 died of the disease. The report established two other facts about the disease: Persons who had had yellow fever previously were immune, and the epidemic was not spread by person-to-person contact.

The Walter Reed Investigation

At the turn of the twentieth century, there was much debate as to the mechanism by which yellow fever spread. The Department of the Army sent an expedition, under the command of Walter Reed, to Cuba, recently acquired by the United States from Spain, to study the disease. The commission undertook to test the thesis that the virus was transmitted by mosquitoes, using themselves as human volunteers. Mosquitoes were allowed to feed on yellow fever patients and then on members of the commission. At first there was a lack of understanding about the fact that mosquitoes are infected only by feeding on patients early in their disease, before an effective immune response arises, and about the necessity for an extrinsic incubation period in the mosquito, during which the virus establishes an infection in the salivary glands, before it can transmit the virus. Ultimately, however, the investigation team did succeed in infecting themselves by mosquito transmission and one member of the commission, Dr. Jesse Lazear, died of it. Fortunately, his was the only death recorded in these experiments. It is of note that in the days before the introduction of a vaccine, most researchers who studied yellow fever in the field or in the laboratory ultimately contracted the disease and many of them died.

With the discovery that the virus was mosquito borne, the U.S. Army began a campaign in Havana to eliminate mosquito breeding places by eliminating sources of water around human habitation. It was (and still is) common for drinking water to be stored around houses throughout Latin America in large pots that served as excellent breeding places for *Ae. aegypti*. The campaign, which included smashing such water containers, succeeded in breaking the mosquito transmission cycle and yellow fever as an epidemic agent disappeared from Havana within months. This approach was later exported to other areas with great success, including Panama. These successes led to the belief that yellow fever could be eradicated, but the discovery of the endemic cycle of yellow fever dispelled this idea. Forest workers who cut

down trees and brought the mosquitoes down from the upper canopy, where they transmit the disease to monkeys, were particularly at risk. Once infected, a person is able to bring the disease back to town where it can get into the *Ae. aegypti* population and start an urban epidemic.

The Yellow Fever Vaccine

In the late 1920s, yellow fever virus was successfully propagated in rhesus monkeys, in which it causes a lethal disease and in which it can be experimentally passed from monkey to monkey. One such strain was derived from an infected human named Asibi. Theiler and Smith passed the Asibi strain of yellow fever in chicken cells, and after approximately 100 passages, it was found that the resulting virus was no longer virulent for rhesus monkeys. After additional passages, this virus, called 17D, was ultimately used as a live virus vaccine in humans and has proved to be one of the best and most efficacious vaccines ever developed. The vaccine virus has been given to about 350 million people. It causes very few side reactions, although three recent vacinees developed full-blown yellow fever and died. The vaccine is essentially 100% effective in providing long-lasting protection against yellow fever. This vaccine is routinely given to travelers to regions where yellow fever is endemic and is used to control the spread of epidemic yellow fever in Latin America and, with less success, in Africa. The success of this vaccine has served as a model for the development of other live virus vaccines, namely, passing the virus in cultured cells from a nonnative host. Recent sequencing studies have found that the 17D vaccine differs from the parental Asibi strain at 48 nucleotides that result in 22 amino acid substitutions. The substitutions responsible for the attenuation of the virus are not known, but it is suggestive that 8 of the amino acid substitutions are found in the E protein, where they might alter host range.

Yellow Fever Today

Although not as wide ranging as previously, yellow fever continues to cause epidemics in Africa and South America as illustrated in Fig. 3.32. On an annual basis, 50–300 cases are officially reported in South America and up to 5000 cases in Africa, but these figures are significantly underreported and the World Health Organization estimates that there are 200,000 cases of yellow fever each year with 30,000 deaths. Between 1986 and 1991, annual outbreaks of yellow fever occurred in Nigeria that probably resulted in hundreds of thousands of cases. An intense campaign beginning in 1992 to vaccinate the population of Nigeria has resulted in the virtual disappearance of yellow fever in Nigeria, but epidemics

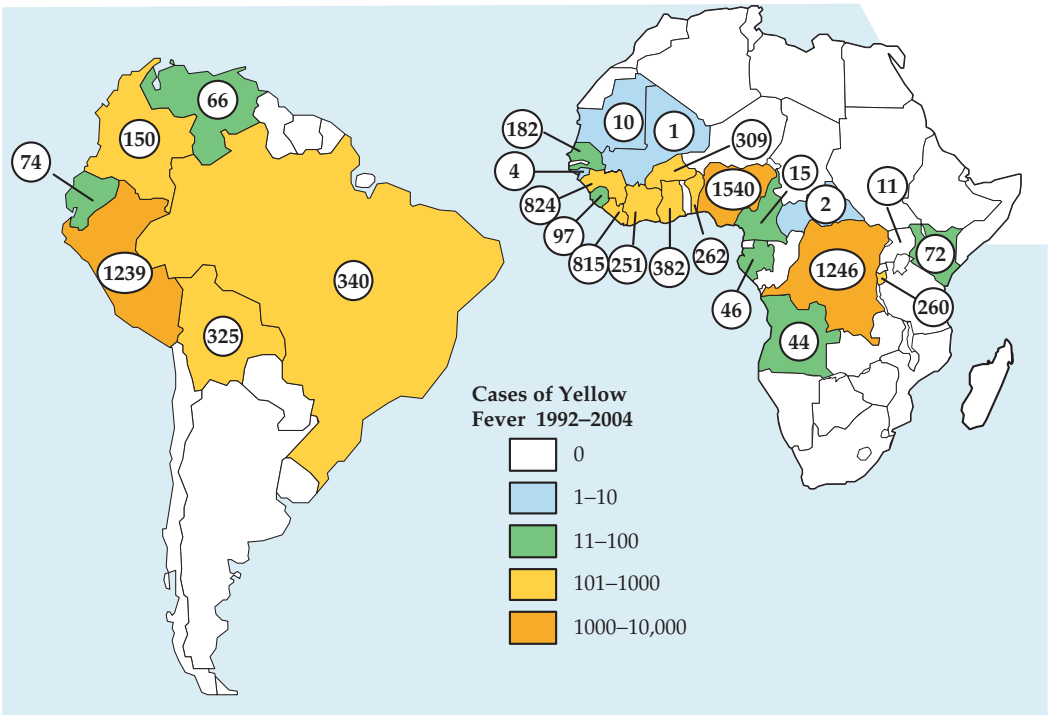


FIGURE 3.32 Cumulative number of cases of yellow fever reported to the World Health Organization for the years 1992 through 2004, by country. It is suspected that cases in Africa may be underreported by a factor of 10 or more. Immunization coverage in Africa has remained low and the disease has continued to spread. Major epidemics (>250 cases) have occurred in Liberia, Burundi, and Peru in 1995, Guinea in 2000, Burkina Faso in 2002, and the Democratic Republic of Congo in 2004. Note that while Nigeria had 19,891 cases between 1980 and 1991, since 1994 there have been only 12 cases. Data from: http://www.who.int/immunization_monitoring/data/data_subject/en/index.html.

continue to occur in other African countries. Epidemics of yellow fever also continue to occur in Peru, Bolivia, Brazil, Ecuador, Columbia, and Venezuela, perhaps in part due to the reemergence of *Ae. aegypti* in South America as described in more detail later. There was one imported case of yellow fever in the United States in 1996, in which an American who visited the jungles of Brazil along the Amazon River without being immunized returned to the United States with yellow fever and died of the disease. Because of the endemic cycle in which monkeys are the reservoir, it is probably impossible to eradicate the virus as has been done with smallpox and as is planned for poliovirus and measles virus.

Dengue Viruses

The four dengue viruses, now considered by the ICTV to be serotypes of a single viral species, have recently undergone a dramatic expansion in range. The incidence of dengue fever is estimated to have increased 30-fold over the last 40 years and dengue viruses now infect an estimated 50–100 million humans each year. Infection may be subclinical or may result in dengue fever, which is usually uncomplicated but which can progress to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Uncomplicated dengue fever is characterized by headache, fever, rash, myalgia (muscle pain, from *myo* = muscle and *algia* = pain), bone pain, and prostration. The disease may be mild or it may be extremely painful (an old name for the disease is break-bone fever which dramatically describes the joint pain that can occur), but it is almost never fatal. However, progression to DHF or DSS is associated with a significant mortality rate. In the absence of medical care, mortality can be as high as 20%, but with good medical care the mortality rate is a few percent. Up to 250,000 cases of DHF and DSS are recorded each year, most of them in children, and in Southeast Asia DHF and DSS are a leading cause of mortality in children. DHF and DSS have also become important in Latin America. It is thought that DHF and DSS are caused by immune enhancement in which infection by one serotype of dengue virus expands the population of cells that can be infected by a second serotype. Many antibodies induced by the four dengue viruses are cross-reactive, reacting not only with the infecting virus but with the other dengue viruses as well. Immediately following infection, in fact, a person is immune to all four serotypes. With time this cross protection fades, and in less than a year the person remains immune only to the infecting virus, probably for life. After this time, infection with another serotype can occur and cause disease. Still present, however, are cross-reactive antibodies that can react with the newly infecting virus but which cannot neutralize the virus to provide protection. The nonneutralizing antibodies are thought to enable the virus to infect a larger number of lymphocytes by means of Fc receptors (see Chapter 10) than would otherwise result from infection only of cells expressing the dengue receptor. This expanded pool of infected cells results in increased cytokine production and can result in capillary leakage and shock. Although second infections are common, infection by a third serotype is rare. Evidently the boost to the immune system from the second infection results in an increase in the amount and avidity of cross-reactive antibodies.

Although infection by a second serotype is important for the development of DHF, it is also known that the probability of contracting DHF is in part a function of the virulence of the virus that is responsible for the second infection. Some dengue strains grow better than others and are more likely to cause DHF upon a second infection than other strains of the same virus. As one example, there was very little DHF in Sri Lanka before 1989 despite the continuing circulation of all four dengue serotypes. In 1989, however, a new strain of DEN-3 appeared in Sri Lanka that caused a large number of cases of DHF. A second example, DEN-2 in the Americas, is described in Chapter 8.

Because second infections by a different serotype are much more likely to lead to DHF than primary infections, the development of vaccines against dengue has progressed slowly. The possibility is real that immunizing against one serotype might put a person at risk for a more serious illness. Current efforts in Thailand are directed toward developing a quadrivalent attenuated virus vaccine that would immunize against all four serotypes simultaneously. U.S. scientists are independently attempting to develop vaccines for the viruses, based either on attenuated dengue viruses or on the development of chimeric flaviviruses that express dengue envelope antigens in a yellow fever vaccine background (see Chapter 11). These various vaccine candidates are in clinical trials as of this writing. A major problem has been the tendency of vaccinated humans to respond strongly to one of the four serotypes in live virus vaccines, often to DEN-3, while responding only weakly or not at all to other serotypes. Changing the ratios of the four viruses in the mix and use of multiple inoculations are being tested as possible ways to overcome this problem.

Dengue viruses are maintained in *Ae. aegypti* in urban settings in most of the world, but also in *Ae. albopictus* in Asia, and humans are the vertebrate reservoir. Part of the difficulty in developing vaccines is that there is no animal model for the disease. The virus will infect monkeys but does not cause disease in them. Small animal models of infection exist but the infection process is artificial and the resulting disease is not dengue fever (DF) or DHF. DF and DHF are exclusively human diseases and the dengue viruses that infect humans are exclusively human viruses.

The Spread of Dengue Viruses

The four serotypes of the dengue viruses arose in Old World monkeys and jumped to humans an estimated 200–1000 years ago. As noted before, the human viruses are now strictly human viruses. Although they will infect monkeys

under laboratory conditions (without causing disease), the reservoir in nature is exclusively humans. However, the monkey viruses still exist as monkey viruses in a sylvatic cycle in Asia and Africa. The human viruses have been continuously active over large areas of Asia and the Pacific region for many years. In areas of Thailand where the viruses are endemic, for example, most people are infected by multiple serotypes in childhood and DHF is a leading cause of mortality in children.

The viruses have recently dramatically expanded their range in the Americas. Before 1970 there was very little dengue activity in the Americas, probably because of mosquito control efforts that were abandoned about that time. Following this, dengue activity increased dramatically, especially upon the introduction of new strains of the virus from Asia. By the 1990s there occurred widespread epidemics that affected many millions of people every year. Epidemics have resulted in an estimated 100 million cases of dengue infection in Brazil alone. The introductions of Asian viruses included more virulent strains of dengue that together with the circulation of multiple serotypes led to epidemics of DHF. Dengue virus is now a major health problem in Latin America. Dengue has also become more active in the Pacific region. Recent epidemics in Hawaii, the first in 50 years, have resulted in more than a hundred documented cases of dengue fever. This topic of the origin and spread of dengue to the Americas is discussed in more detail in Chapter 8.

Japanese Encephalitis Virus

The Japanese encephalitis (JE) complex of flaviviruses includes a large number of related viruses, many of which cause encephalitis in humans. For these viruses, the majority of human infections are inapparent and, for most, fewer than 1% of infections result in neurological disease. However, when encephalitis develops it is often serious with case fatality rates as high as 50% and neurological sequelae are frequent among survivors. In addition to JE, these include St. Louis encephalitis (SLE), Murray Valley encephalitis (MVE), and West Nile viruses. The close relationships of these viruses are illustrated in Fig. 3.28. Some of these viruses are widespread whereas others are much more local in their distribution. West Nile virus, for example, is now virtually worldwide (the Australian strain is often called Kunjin virus), whereas MVE virus is only found in Australia. Thus, circulation of at least some of these viruses has occurred over widespread areas, as is the case for the dengue viruses described in the preceding section. For most of the viruses in this lineage, birds form the major vertebrate reservoir and culicine mosquitoes are the major vectors.

JE virus is distributed throughout Asia, including Japan, India, Southeast Asia, Indonesia, the Philippines, and Borneo (Fig. 3.33). Reported cases of JE encephalitis average 30,000–50,000 per year with 10,000 deaths, but the disease is greatly underreported. Only one JE virus infection in 200 or 300 results in encephalitis, with children and the elderly being at higher risk. The fatality rate following JE encephalitis is 2–40% in different outbreaks, but 45–70% of survivors have neurological sequelae. In endemic areas, virtually all people have been infected by the time they reach adulthood. Bird–mosquito–bird transmission is the normal transmission cycle, but domestic pigs are particularly important amplifying hosts for transmission to humans because they are found in proximity to their human owners. Various species of *Culex* mosquitoes transmit the virus. During peak transmission seasons, up to 1% of *Culex* mosquitoes around human habitations may be virus infected. Travelers to endemic regions have a probability of about 10^{-4}/week of contracting JE, and 24 cases of JE encephalitis in travelers were reported between 1978 and 1992. Inactivated virus vaccines are in use in different regions of Asia. The Japanese have long used such a vaccine to eliminate JE encephalitis from their population, and the Chinese have recently developed a vaccine that is being used in China and Thailand. The Japanese vaccine is available in the United States for travelers to endemic regions. Of considerable interest is the finding that JE virus infection may reactivate in mice after the immune system first damps it out. Reactivation in other animals may also occur and could be important for persistence of the virus in nature.

For the dengue viruses, immune enhancement is important for the disease caused in humans. There is no evidence that immune enhancement plays a role in the disease caused by JE virus or other flaviviruses such as MVE virus. It is interesting, however, that in a mouse model system, prior treatment with subneutralizing concentrations of anti-JE serum resulted in an increase in virus growth and in mortality in the mice following infection by MVE virus. This suggests that the potential for immune enhancement exists for other flaviviruses but does not occur in humans other than the dengue viruses because the immune reaction to flaviviruses is normally strong and not cross-reactive and subneutralizing concentrations of antiviral antibodies do not exist. It does raise a warning flag for vaccines, however, and vaccines that increase the seriousness of disease caused by subsequent infection have occurred in the case of measles virus and respiratory syncytial virus (see Chapters 4 and 10).

As indicated before, most human infections by JE virus do not result in invasion of the nervous system and encephalitis does not occur. Two laboratory experiments are of interest in this regard. In one experiment, JE virus variants were selected that failed to bind to mouse brain membrane receptor preparations. These mutants were attenuated for neuroinvasiveness and neurovirulence because their receptor-binding preferences were altered. In a second experiment, passage of JE virus and of MVE virus in cultured cells selected for variants that bound to glycosaminoglycans (GAG). These

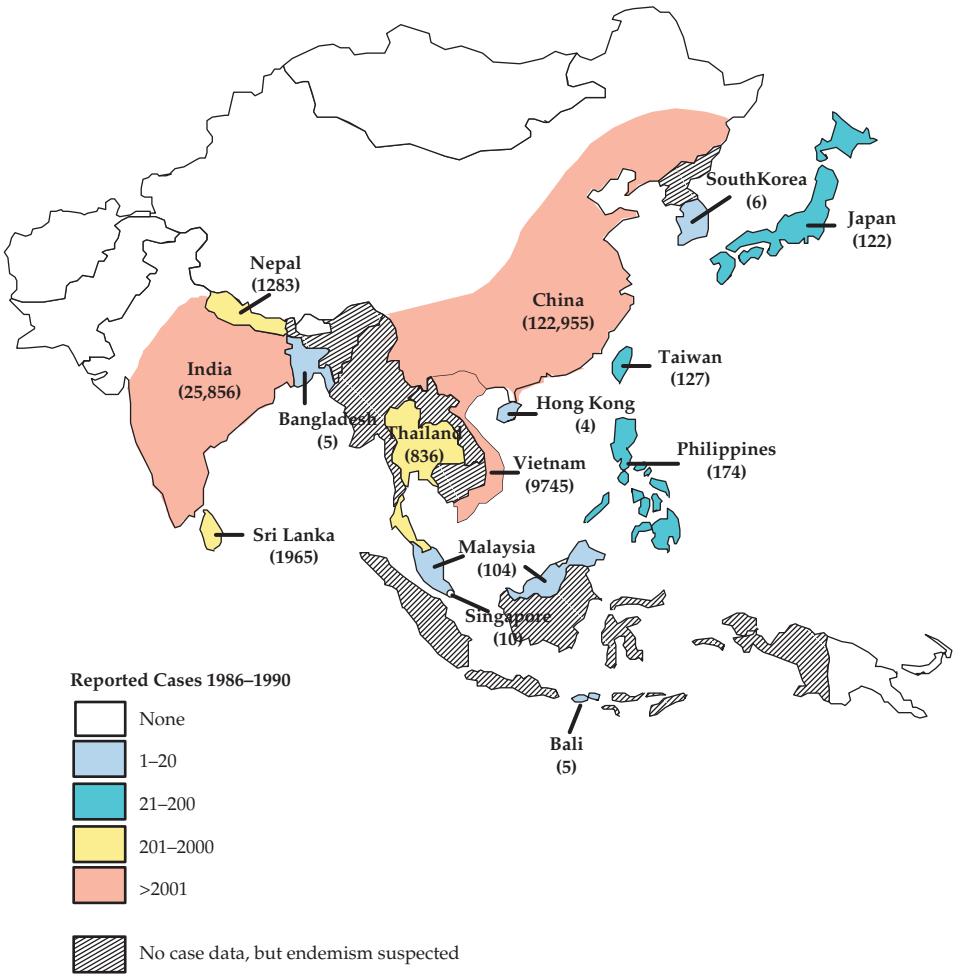


FIGURE 3.33 Range and reported cases of Japanese encephalitis, 1986–1990. Adapted from *MMWR* (1993) Vol. 42, RR-1, p. 2. Since this detailed report, the first human cases were reported in Papua New Guinea in 1997, there were two fatal cases on islands in the Torres Strait in 1995, and the virus was detected in mainland Australia (the Cape York Peninsula) in 1998.

variants were rapidly removed from the bloodstream when inoculated into mice and were attenuated. It was suggested that GAGs present on cells and extracellular matrices result in the removal of these variants from blood and tissues before replication and neural invasion can take place. Evidence was presented that the attenuation of the live JE virus used as a vaccine in China, called SA14-14-2, may have resulted, at least in part, from such an effect.

West Nile Virus

West Nile (WN) virus was first isolated in 1937 from the blood of an infected woman in the West Nile province of Uganda. Until 1999 it was not considered an important pathogen, causing only sporadic cases of encephalitis in parts of Africa, Asia, and Europe and having little effect on wildlife. But with the recent occurrence of outbreaks of encephalitis in Europe, the Middle East, and North America the situation changed. Not only has infection by the virus resulted in large numbers of cases of neurological disease in humans, but domestic animals, especially horses, and wildlife, particularly birds, have been severely impacted. The effect of the virus in North America has been especially dramatic, as described in more detail in Chapter 8. Since its arrival in 1999, more than 20,000 Americans have become ill from West Nile infection and more than 800 have died. The virus has also had severe effects upon horses and many species of birds.

Two lineages of WN virus are recognized. The lineage present in North America, Europe, the Middle East, India, Australia (where the subtype present has been called Kunjin virus), and parts of Africa, called lineage 1, contains both

virulent and attenuated strains and is responsible for WN disease. Lineage 2 is present only in sub-Saharan Africa and Madagascar and is mostly maintained in an enzootic cycle. The association of the virus with significant outbreaks of disease in humans, domestic animals, and birds, and the widespread dispersion of the virus in Eurasia and the Americas, appears to be the result of the emergence of a more virulent strain of the virus.

Various species of culicine mosquitoes are the principal vectors of WN virus, although the virus has also been isolated from species of *Aedes*, *Coquillettidia*, *Culiseta*, and *Ochlerotatus*, among others. In Europe and Africa the principal vectors are *Culex pipiens*, *Cx. univittatus*, and *Cx antennatus*, in India *Cx. vishnui*, in Australia *Cx. annulirostris*, and in North America *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. tarsalis*, among others. During epidemics, from 0.1% to as many as 15% of *Culex* mosquitoes were found to be infected.

WN virus infects a wide spectrum of vertebrates. The reservoir of the virus is various species of birds. More than 150 species of birds were shown to be infected by WN virus and only in birds, with few exceptions, does the virus produce high enough viremia titers to infect mosquitoes. Laboratory studies showed that viremia titers of 10^5 to 10^7 are required to infect mosquitoes; at the lower titer fewer than 15% of feeding mosquitoes become infected, whereas at the higher titer more than 70% become infected. Mammals in general do not generate such high viremia titers after infection by WN virus. The maximum viremic titer in humans and horses, for example, appears to be about 10^3. Among birds, grackles, corvids (crows, ravens, jays, magpies), house finches, house sparrows, shorebirds, hawks, and owls are most susceptible to the virus and show high mortality rates upon infection (25–100% in various studies). They develop sufficiently high viremia to efficiently infect mosquitoes that feed upon them. WN virus has also been isolated from amphibians and reptiles, and the lake frog of Russia develops sufficiently high viremia that it might serve as a reservoir.

There are reports that WN virus can persist in infected animals for a considerable time. Virus could be isolated from experimentally infected ducks and pigeons for more than 3 months, for example. Such persistence could be important in the persistence of the virus in nature. Also important is transovarial transmission, which occurs in many flaviviruses, and West Nile is no exception. During transovarial transmission, the eggs laid by the mosquito are infected and the emergent mosquito is thus infected. This mechanism is especially important in temperate climates where adult mosquitoes die off during the winter and the species persists as diapausing embryos or larvae.

WN virus can also be spread by means other than by mosquitoes, although the importance of such spread in the maintenance and spread of the virus is unknown. Birds excrete virus in their feces, which can serve as a source of infection of contacts. Alligators were accidentally infected by feeding them infected birds. In humans, transmission of the virus by blood transfusion or via breast milk has occurred.

Pathology of West Nile Disease

About 80% of human infections appear to be asymptomatic. In the 20% of infections that result in clinical disease, most result in a self-limited illness characterized by fever, headache, fatigue, malaise, muscle pain, and weakness. In fewer than 1% of infected humans does the virus cross the blood–brain barrier and cause neurological disease such as meningitis, encephalitis, or paralysis. In about 13% of patients experiencing neurological disease, infection of anterior horn cells of spinal motor neurons causes an acute flaccid paralysis very similar to poliomyelitis resulting from poliovirus infection. The fatality rate for neuroinvasive disease is about 10%, and many survivors, especially those with poliomyelitis type disease, never fully recover.

Infection with WN virus may be serious because the virus interferes with the innate immune system (see Chapter 10). Several of the nonstructural proteins of the virus interfere with phosphorylation of the Janus kinases JAK1 and Tyk2. This prevents the activation of the transcription factors STAT1 and STAT2 and their transport to the nucleus. Activation of these factors is required for the cell to respond to the signaling of the interferons, normally a first line of defense against viral infection.

Vaccines to protect horses have been developed. One is an inactivated virus vaccine. The other is a recombinant vaccine that uses canarypox virus to express WN virus antigens. Two viruses for human use are also being developed. The first is an inactivated virus vaccine. The second is a chimeric YF–WN live virus vaccine in which the prM and E proteins of the 17D YF virus vaccine strain have been replaced with those of WN virus (see Chapter 11).

Other Flaviviruses of the JE Complex

MVE virus is an Australian virus that is closely related to JE virus. It causes encephalitis in humans, but the number of cases is small. Birds are the primary vertebrate reservoir, and epidemics of MVE have been associated with wet years when the mosquito population expands and nomadic waterfowl invade regions that are normally too dry to support them. *Culex annulirostris* is the primary vector for MVE.

SLE virus is a North American virus belonging to the JE complex that causes regular epidemics of encephalitis in the United States. The virus is widely distributed and cases of SLE encephalitis have been recorded in every state, with the majority of cases occurring in the Mississippi River valley, Texas, California, and Florida. Data for the years 1964–2003 are shown in Fig. 3.34. In the epidemic year 1975 there were 1815 cases of SLE encephalitis officially reported in the United States, but in nonepidemic years there may be fewer

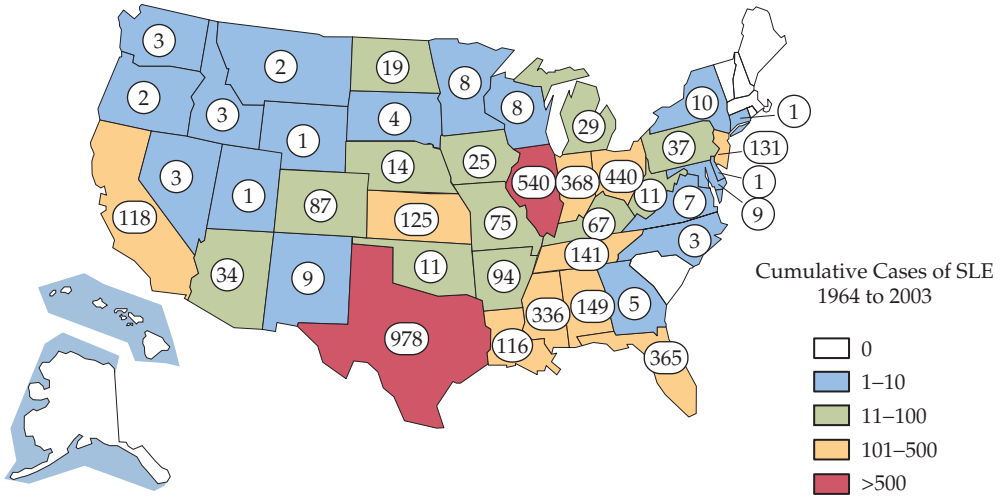


FIGURE 3.34 Distribution of cases of St. Louis encephalitis occurring between 1964 and 2003 in the United States, shown by state. The large number of cases in Florida includes the most recent U.S. epidemic, which occurred in 1990, during which Florida reported 223 cases and 11 deaths. Data came from Fields *et al*. (1996) p. 981, and *MMWR*, *Summary of Notifiable Diseases, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003*. St. Louis encephalitis was not a notifiable disease nationwide until 1998. Recent reported cases were 24 in 1998, 4 in Florida in 1999, 2 in Texas in 2000, 79 in 2001 of which 71 were in Louisiana, 28 in 2002, and 41 in 2003. These have been incorporated into the state totals shown.

that 50 cases. The most recent epidemic occurred in 1990 in Florida with 223 cases and 11 deaths. The case fatality rate is about 7% overall, but is higher in the elderly. Most infections by SLE are inapparent, as is the case for many encephalitis viruses. The ratio of inapparent to clinical infection is age dependent and varies from 800 to 1 in children to 85 to 1 in the elderly. The virus is transmitted by *Culex* mosquitoes, and the primary vertebrate reservoirs are wild birds.

Tick-Borne Encephalitis Viruses

The tick-borne encephalitis (TBE) viruses are important pathogens in Europe and Asia, and there is also a representative in North America. The viruses include Central European encephalitis (CEE), louping ill, Russian spring-summer encephalitis (RSSE), Kyasanur Forest disease, Omsk hemorrhagic fever, and Powassan viruses. Members of the TBE complex form a distinct group within the flaviviruses (Fig. 3.28), but share 40% amino acid sequence identity with the mosquito-borne flaviviruses, showing their close relationship to other flaviviruses. Most TBE viruses are transmitted by *Ixodes* ticks and can cause a fatal encephalitis in humans. An inactivated virus vaccine is widely used in Central Europe to protect people exposed to ticks. Even so, several thousand cases of TBE encephalitis occur each year. The case fatality rate is 1–2%, with 10–20% of survivors having sequelae in the RSSE form. RSSE, and perhaps other TBE viruses, can also be contracted by drinking raw goat's milk and possibly other forms of raw milk. The virus has a tendency to set up persistent infection in experimental animals and possibly in humans as well. Although *Ixodes* ticks are the primary vector, *Dermacentor* ticks and ticks of other genera are also capable of transmitting the virus. The distributions of two species of *Ixodes* ticks that are important vectors of TBE are shown in Fig. 3.35 together with the geographic range of endemic TBE disease.

Powassan virus is a member of the complex found in North America and in Russia. In North America, 20 cases of Powassan encephalitis have been reported since 1958.

All known TBE viruses cause encephalitis in humans with the exception of Omsk hemorrhagic fever virus, which causes hemorrhagic fever in humans, as its name implies, in the absence of encephalitis. Two other members of this complex, Kyasanur Forest disease virus and Alkhurma virus, which are closely related and may represent isolates of the same virus, also cause hemorrhagic fever in humans but it is associated with encephalitis. Omsk hemorrhagic fever virus also differs from other TBE viruses in that its principal tick vector is *Dermacentor reticulates* rather than an *Ixodes* tick.

Cell Fusing Agent

A flavivirus called cell fusing agent was discovered in laboratory cultures of *Ae. aegypti* cells in 1975. The relationship of this virus to other flaviviruses is shown in Fig. 3.28.

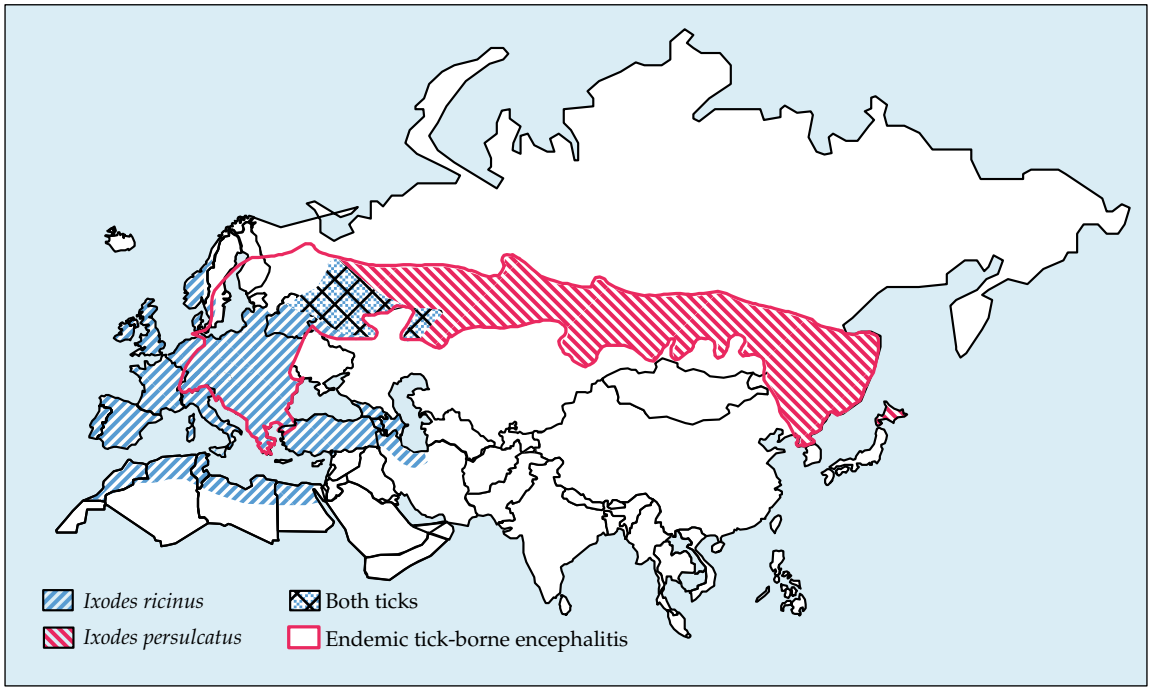


FIGURE 3.35 Geographic distribution of two major tick vectors of tick-borne encephalitis. Also shown is the major region in which TBE is endemic. Adapted from Porterfield (1995) p. 207.

This virus is an insect only virus and is not known to infect vertebrates. Very recently, new isolates of a strain of cell fusing agent have been made from wild-caught mosquitoes in Puerto Rico belonging to at least two genera, *Aedes* and *Culex*. Remarkably, DNA sequences related to cell fusing agent have been identified in the genomes of wild-caught mosquitoes, presumably having integrated into the mosquito genome at some time in the distant past. This and other recent isolates of new flaviviruses has led to the suggestion that there are as yet many flaviviruses in nature that remain to be identified.

Genus *Hepacivirus*

Hepatitis C virus (HCV) forms a second genus in the *Flaviviridae*. The virus was discovered in 1989 as a causative agent of nonA-nonB hepatitis in humans. Despite the inability to grow the virus in culture or in a small animal model, the complete genome sequence of the virus was established using the methods of modern biotechnology and verified by injection of viral RNA produced from cDNA clones into the liver of a chimpanzee, the only animal other than humans that is infectible by the virus. The HCV genome, which is slightly smaller than those of the flaviviruses and pestiviruses, has an organization similar to those of the other members of the family (Fig. 3.27). It has a number of important differences from the genome of members of the genus *Flavivirus*, some of which are illustrated in the figure. One is the presence of two proteases rather than one. The NS3 protease is shared with the flaviviruses but requires NS4A as a cofactor rather than NS2B. The second protease, which has a catalytic cysteine, is present in NS2 and its only known cleavage function in viral replication is to cleave the NS2–NS3 bond.

A second difference is the lack of a 5′ cap and the possession instead of an IRES, so that initiation of translation of the plus-strand genome is not cap-dependent but uses an IRES as does poliovirus. A third difference is the production of a small (17 kDa), short-lived protein called F (for frame shift) or ARFP (for ***a***lternative ***r***eading ***f***rame ***p***rotein) that is encoded within the C protein gene in a different reading frame. Translation of this protein requires initiation at the 5′ end of the polyprotein followed by a frameshift near residue 11 of the capsid protein. There is evidence that it is produced in infected humans but it is not known if this protein plays a role in virus replication. Of note is the fact that no other plus-strand RNA virus is known to produce two different proteins from two different reading frames in the same nucleotide sequence, although this phenomenon occurs in several other classes of viruses.

HCV also differs in the way that RNA replication is anchored to a membrane. RNA replication in plus-strand RNA viruses occurs in association with membranes. In flaviviruses, the RNA polymerase is thought to associate with membranes by means of its association with membrane bound proteins such as NS4A or NS4B. In HCV, the RNA

polymerase NS5B is itself anchored in the membrane by a C-terminal transmembrane anchor. Interestingly, this anchor is required for RNA replication and the HCV sequence cannot be substituted with that from the pestivirus bovine viral diarrhea virus. Thus, this anchor plays a role in RNA replication other than simply anchoring the polymerase in the membrane.

Another interesting difference is the cleavage of the N-terminal capsid protein from the polyprotein precursor. The capsid protein is anchored in the membrane by a C-terminal transmembrane anchor, as described earlier for flaviviruses. In flaviviruses the capsid protein is cleaved from this signal sequence anchor by the NS3 protease, but in HCV it is cleaved by a cellular protein, signal peptide peptidase.

Because of its importance as a human disease agent, HCV has been the subject of intensive study. Progress has been relatively slow because the only animal model for the disease is the chimpanzee, which are rare and expensive, limiting the number of experiments that can be performed, and because until very recently there was no cell culture system in which the virus would undergo a complete replication cycle and release infectious virus. Studies in cultured cells have relied upon the expression of parts of the genome in expression vectors, and more recently upon the replication of truncated versions of the genome called replicons. Replicons encode all of the genes required for RNA replication but lack the genes encoding structural proteins. Thus, only part of the virus life cycle can be studied using these reagents. The very recent development of systems using cultured cells that allow a complete virus replication cycle with the release of infectious virus will allow more rapid progress in the future. A second advance has been the use of immunodeficient mice (***s***evere ***c***ombined ***i***mmuno***d***eficiency or SCID mice) into which have been grafted human liver cells. These can be infected by HCV and although the numbers of such animals is limiting, they possess obvious advantages over the use of chimps.

Natural History of HCV

HCV is a causative agent of blood-borne hepatitis in man. In the United States, HCV was once spread primarily through transfusion of contaminated blood, but the development of a diagnostic screen for the virus has virtually eliminated this source of infection in the developed world. However, the virus continues to be transmitted through the sharing of needles by drug users. The virus can also be transmitted sexually or from mother to child but these mechanisms are inefficient. There are additional mechanisms of transmission that are not well understood. In some developing countries, circumcision or scarification practices may be important in the spread of the virus.

HCV is worldwide in distribution, as illustrated in Fig. 3.36. It has been estimated that ~3% of the world's population, 170 million people, are infected by the virus. The highest infection rate found was among Egyptian blood donors, where up to 19% were seropositive for HCV, which may have resulted in part from past treatment for bilharziasis

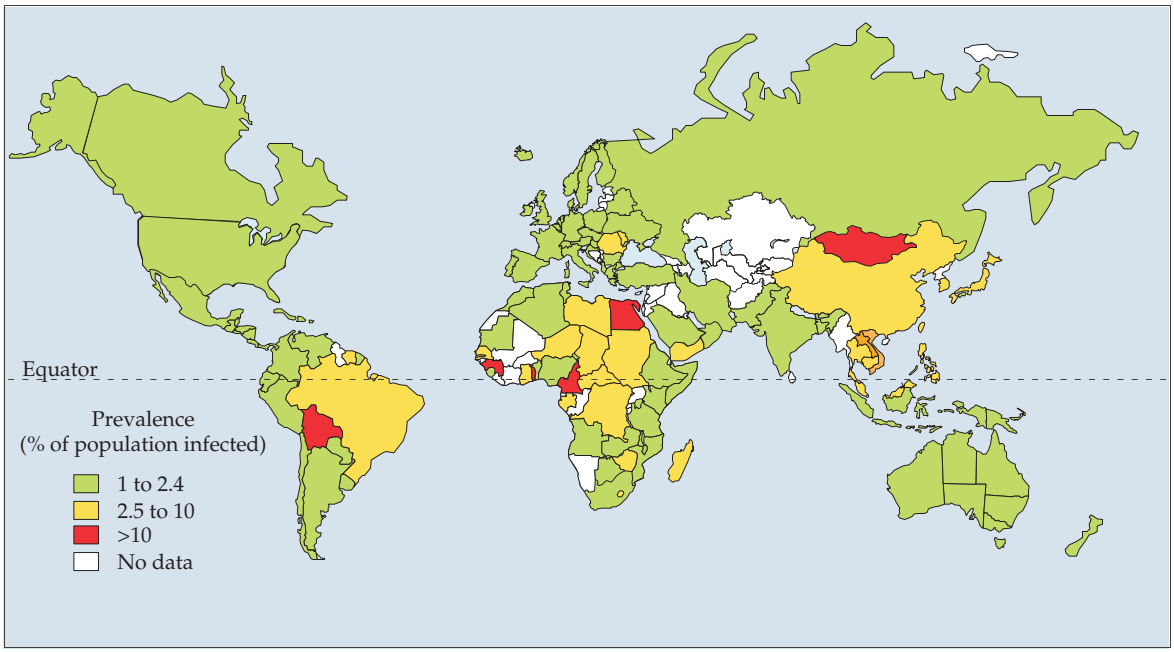


FIGURE 3.36 Worldwide prevalence of hepatitis C as of April 2003 based upon published data. Map was found at: http://www.reliefweb.int/rw/RWB.NSF/db900LargeMaps/SKAR-64GDV4?OpenDocument.

using inadequately sterilized needles. There are six different clades or genotypes of the virus, which differ by more than 30% in nucleotide sequence and are numbered from 1 to 6. In turn, each clade has many isolates that may differ by up to 25% in nucleotide sequence, so that the clades can be subdivided into subclades called a, b, etc. These different viruses all cause the same disease but differ in the severity of the disease caused and in their ease of cure. Genotype 1 is the clade commonly found in the United States and probably became widespread only with the introduction of blood transfusion in the 1940s. In Africa and Asia, the virus has been endemic for a long time, and the different clades have different geographic distributions. Thus, for example, clade 5 is commonly found only in South Africa, clade 4 is widely distributed in the Middle East, clade 6 in eastern Asia, and clade 2b in the Mediterranean and the Far East.

HCV Disease and Its Treatment

Infection with HCV can be extremely serious. The initial infection may cause no disease or may result in hepatitis accompanied by jaundice, but fulminant liver failure is rare. However, in 70–80% of infections, the infection becomes chronic. During chronic infection, up to 10^{12} viruses are produced each day and turn over with a half-life of about 3 hours, and the more or less constant viral load in the blood is 10^3–10^7 per ml. This chronic infection is well tolerated by some and in a minority of cases spontaneous remission may occur in the absence of medical intervention. However, in many persons chronic hepatitis results. Most seriously, in about 20% of chronic infections liver cirrhosis develops after a long lag, usually more than 20 years, and hepatocellular carcinoma develops in up to 2.5%. Liver failure due to HCV infection is the leading cause of liver transplantation in the United States.

The current treatment for chronic HCV infection is injection of interferon-α conjugated to polyethylene glycol, which increases its stability, together with the inhibitor ribavirin. This treatment results in curing the infection in about half the cases but the cure rate depends upon the genotype of the virus. In one trial, 42% of patients chronically infected with genotype 1 HCV were cured whereas patients chronically infected with genotype 2 or 3 virus exhibited a cure rate of 80%. This treatment is not only expensive but relatively toxic and many patients tolerate it poorly. This consideration, as well as the fact that half the patients show no effect or only transient relief from this treatment, has led to intense efforts to develop new treatments. These include efforts to develop vaccines as well as efforts to develop antiviral agents that will interrupt virus replication or prevent the virus from interfering with the host defenses against the virus. Antivirals currently in clinical trials include nucleoside analogues that when incorporated into viral RNA result in chain termination, two compounds that bind to the viral RNA polymerase NS5B and inhibit its activity, two inhibitors of the viral NS3–4A protease, and three compounds that modulate the immune system. Other drugs are also being studied as possible antivirals.

HCV Suppression of the Immune Response

In order to establish a chronic infection, HCV interferes with many aspects of both the innate and adaptive immune responses of the host. The importance of such interference for chronicity and the persistence of the virus in nature is illustrated by the fact that the virus interferes in so many different ways. The immune system is described in some detail in Chapter 10. Here we note that the first line of defense against viral infection is the production of type 1 interferons (IFN) α and β, components of the innate immune system. The NS3–4A protease of HCV interferes with the induction of IFN by cleaving two intermediates, called MAV5 and TRIF, in two different but overlapping activation pathways. MAV5 is required in the pathway that starts from an intracellular sensor of double-strand RNA called RIG-1, whereas TRIF is required in the pathway that starts from a membrane bound sensor of double-strand RNA called Toll-like receptor 3 (see Chapter 10). The result is that both pathways are disabled.

The HCV core protein interferes with the activity of any IFN that might be produced. It induces the expression of cellular proteins called SOCS1 and SOCS3. These block the JAK–STAT pathway by which IFN induces the production of hundreds of proteins required for defense against viral infection (Chapter 10). Protein NS5A independently interferes with the IFN system in at least two ways. It induces the production of IL-8, which attenuates the expression of genes induced by the activity of IFN. It also binds to a protein called PKR that is induced by IFN, thereby inhibiting its activity. Protein E2 also inhibits PKR. Other HCV proteins are also known to interfere with the activity of IFN.

HCV also interferes with the adaptive immune system. Interestingly, instead of a general interference with the adaptive system, as happens with HIV, for example, that cripples immune responses against all pathogens, the modulation by HCV is limited to HCV-specific responses, leaving the immune system free to control other viral infections. The mechanisms by which this occurs are incompletely understood. What is known is that successful clearance of HCV infection in humans is associated with a strong T-cell response, both $CD8^+$ and $CD4^+$, and that immunologic memory results such that although reinfection by HCV can occur, it does not lead to chronic infection. In humans in which the infection becomes chronic, $CD8^+$ cytotoxic T cells are relatively few and these T cells recognize fewer epitopes. In one study, $CD4^+$ helper T cells from persistent infections recognized very few epitopes whereas those from humans who cleared the infection recognized up to 14 different epitopes.

HCV-Related Viruses

Viruses related to HCV, called GB viruses (from the initials of a surgeon with hepatitis from which they were first isolated), are known. GBV-A and GBV-B viruses have a genome organization very similar to that of HCV, but share little amino acid sequence identity with HCV or with each other. They may eventually be classified as new genera within the *Flaviviridae*, more closely related to genus *Hepacivirus* than to genus *Flavivirus* or genus *Pestivirus*. A third virus, GBV-C, also called hepatitis G virus or HGV, is related to GBV-A. These three viruses appear to be widely distributed and establish chronic infections in humans, but there is no evidence that they cause disease.

Genus *Pestivirus*

Three closely related viruses belonging to the genus *Pestivirus* are important pathogens of domestic animals and have been well characterized. These are bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV) (also called hog cholera virus), and border disease virus of sheep (BDV). These three viruses share more than 70% amino acid sequence identity and exhibit extensive serological cross-reactivity. Their genome organization is similar to those of other viruses in the family (Fig. 3.37). Pestiviruses have also been isolated from a number of other mammals including giraffe, deer, bison, bongo, alpaca, and reindeer. The taxonomic status of these isolates is still unclear. Some are classified as strains of one of the three viruses just listed but some, at least, may represent other species of pestivirus

BVDV exhibits an important and interesting disease syndrome in cattle. Animals infected as adults by the virus may exhibit no disease or may have symptoms that include diarrhea, but they recover uneventfully. However, when a pregnant cow is infected by the virus, infection of the fetus may cause the fetus to become immunologically tolerant to the virus, resulting in a chronic infection that lasts for the life of the animal. Such *in utero* infection may lead to developmental abnormalities or runting in the calf, and may render the calf sensitive to infection by other microorganisms, all of which have serious economic effects. A more interesting effect of the chronic infection, however, is the development in some animals of fatal mucosal disease at the age of 1–2

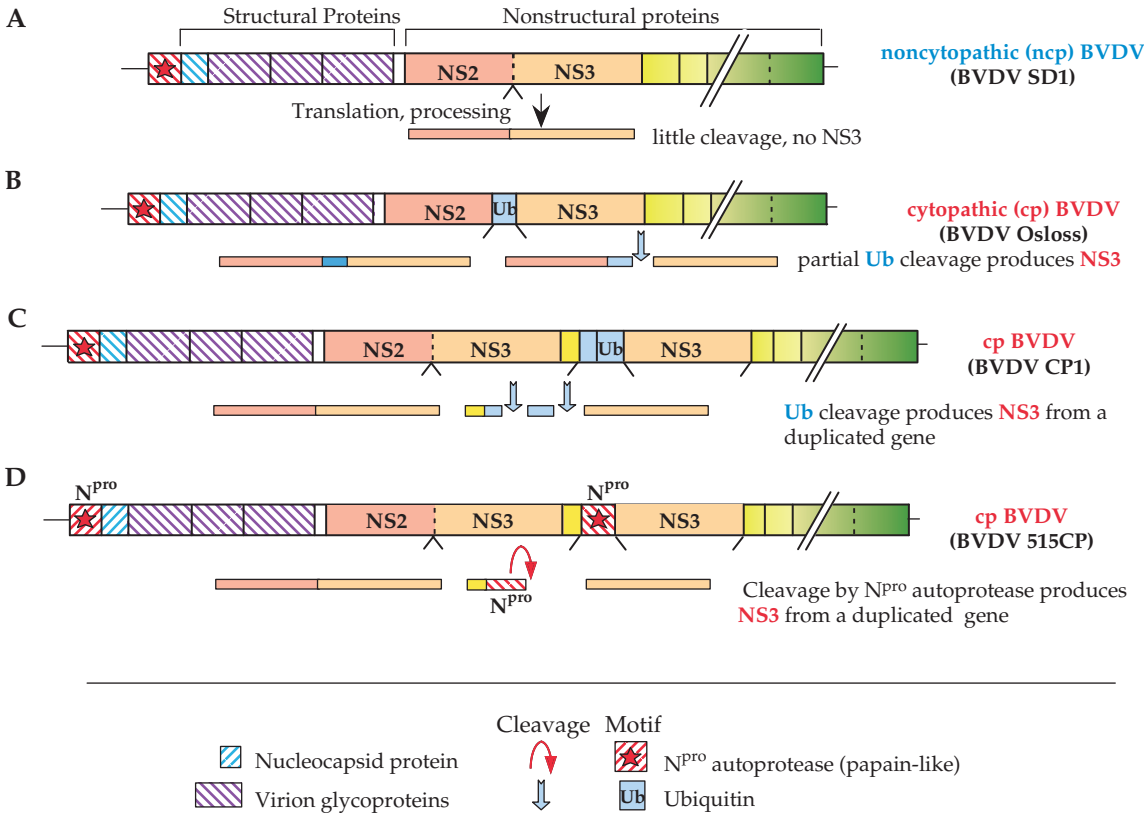


FIGURE 3.37 Genome organization of cytopathic and noncytopathic strains of the pestivirus BVDV. (A) In noncytopathic (wild-type) strains little cleavage occurs between NS2 and NS3. In cytopathic strains, NS3 is produced either by an upstream insertion of ubiquitin (see B), insertion of multiple ubiquitin sequences plus duplication of NS3 sequences (see C), or duplication of the N^{pro} proteinase and its insertion immediately upstream of a duplicated NS3 (see D). Data for this figure came from Meyers and Thiel (1996).

years; once symptoms appear, the animal dies within weeks. Animals that die of this disease are found to be infected by two types of BVDV. One is the normal wild-type virus, which is noncytopathic in cultured cells. The second type of BVDV is a new strain that is cytopathic in cultured cells. The cytopathic BVDV strain is derived from the wild-type strain by recombination, which occurs during the persistent infection. Several different cytopathic BVDV strains have been sequenced, and they all have in common that NS2–3 (formerly called p125) is cleaved to produce NS3 (also called p80). It is the production of NS3 that renders the virus cytolytic in culture and causes lethal mucosal disease in cattle. As illustrated in Fig. 3.37, the cleavage to produce NS3 can be induced in several different ways. In at least three cytopathic BVDV strains, cellular ubiquitin sequences were inserted (in different ways) within the sequence encoding this protein, such that a cellular enzyme that cleaves specifically after ubiquitin cleaves the BVDV polyprotein to produce NS3. Another mechanism to produce NS3 was the insertion of the BVDV N^{pro} autoprotease immediately upstream of the NS3 sequence. A third mechanism, not illustrated in the figure, is the insertion of cellular sequences derived from a protein called Jiv.

Why the production of NS3 renders the virus cytopathic and capable of causing lethal disease in cattle is a fascinating story of self-imposed limitation on virus growth not unlike the story of alphavirus downregulation described earlier in this chapter. For BVDV to persist in nature it must be able to establish persistent infection because persistently infected animals that continue to shed the virus are an important reservoir for the virus. Cytopathic viruses are not able to establish persistent infection and come to a dead end when they arise. It turns out that cleavage to form NS3 is essential for virus replication, and cleavage occurs early after infection by all BVDV strains, noncytopathic as well as cytopathic. Cleavage is effected by a protease in NS2, and this protease, like the NS3 protease of all members of the family, requires a cofactor for function. This cofactor is a cellular protein, however, not a virally encoded protein. This cellular cofactor is the protein Jiv, which forms a stable (but noncovalent) complex with NS2–3. The amounts of Jiv in the infected cell are limited; however, and it is soon titrated out. Once no free Jiv remains, NS2–3 cleavage cannot occur and no further increase in viral replication is possible, allowing the establishment of persistent infection with only limited amounts of virus being produced. If NS2–3 cleavage continues to occur because new protease sites have been introduced or because the virus encodes its own Jiv, virus replication continues to accelerate until it overwhelms the cell and the cell dies. As an aside, it is possible that the virus host range is controlled by the presence or absence in cells of sufficient Jiv able to act as a cofactor.

Thus, pestiviruses encode three proteases. The NS3 protease common to all *Flaviviridae* makes many cleavages in the polyprotein and functions both *in cis* and *in trans*. This protease requires a virally encoded cofactor, NS2B in flaviviruses and NS4A in pestiviruses and hepaciviruses. N^{pro} is an autoprotease whose only known cleavage in normal infection is to release itself from the polyprotein. The NS2 protease described here also functions as an autoprotease that makes only one cleavage, that between NS2 and NS3.

CSFV is epidemic in pig populations and causes serious illness, with different isolates differing in their virulence. Infection of pregnant sows can lead to abortion or to birth of persistently infected piglets, which soon die. BDV also can cause congenital infection, which can lead to abortion or to birth of animals that display a number of defects.

FAMILY *CORONAVIRIDAE*

The name *Coronaviridae* comes from the Latin word meaning crown, from the appearance of the array of spikes around the enveloped virion. The family is composed of a number of RNA-containing animal viruses currently classified into two genera, the genus *Coronavirus* (whose members will here be called coronaviruses) and the genus *Torovirus* (whose members will be referred to as toroviruses). A representative listing of viruses in the two genera is found in Table 3.13. The family is classified together with the *Arteriviridae* and the *Roniviridae* (described later) in the Order *Nidovirale*s, after the Latin word *nido* meaning nest, because they produce a nested set of mRNAs. Coronaviruses are somewhat larger in size (120–160 nm) than the toroviruses (120–140 nm) and have a larger genome (about 30 kb compared to 20 kb). In contrast to other (+)RNA viruses, the nucleocapsids of *Coronaviridae* are constructed using helical symmetry. The coronaviruses have a helical nucleocapsid 10–20 nm in diameter, whereas the toroviruses have a tubular nucleocapsid that appears toroidal in shape in the virion. The coronavirus virion is roughly spherical, whereas the torovirus virion is disk shaped or rod shaped. The viruses mature by budding through intracytoplasmic membranes. The coronaviruses have been well studied, whereas the toroviruses, which are composed of one pathogen of horses, one pathogen of cattle, a presumptive human torovirus, and a possible torovirus of swine, have attracted less attention.

Genus *Coronavirus*

The coronaviruses have the largest RNA genome known, 27–32 kb in size. The genome size of RNA viruses is thought to be limited by the mutation rate during RNA synthesis. Because there is no proofreading during RNA synthesis, an inherent mistake frequency results that is in the order of 10^{-4}. Thus, error-free replication of an RNA genome becomes impossible once the genome becomes too large. The 30-kb genome of coronaviruses may represent this upper limit. It is

TABLE 3.13 *Coronaviridae*

Genus/members	Virus name abbreviation	Usual host(s)	Transmission	Disease	World distribution
Coronavirus					
Group 1					
Transmissable gasteroenteritis	TGEV	Swine	Contact	Gastroenteritis	United States, Europe
Human coronaviruses 229, NL63	HCoV	Humans	Aerosols	Common cold	Americas, Europe
Group 2A					
Human coronaviruses OC43, HKU-1	HCoV	Humans	Aerosols	Common cold	Americas, Europe
Murine hepatitis	MHV	Mice	Aerosols, contact	Gastroenteritis, hepatitis	Laboratory mouse colonies worldwide
Group 2B					
Severe acute repiratory syndrome	SARS	Bats[a], Humans	Aerosols, contact	Fever, pneumonia, severe respiratory disease	Asia, Americas
Group 3					
Infectious bronchitis	IBV	Birds	Mechanical, oral–fecal	Bronchitis	Worldwide
Torovirus					
Berne (equine torovirus)	EqTV	Horses	Oral–fecal	Diarrhea	Europe, Americas
Breda (bovine torovirus)	BoTV	Cattle	Oral–fecal	Diarrhea	?
Human torovirus	HuTV	Humans	?	Diarrhea	?

[a] Bats have been identified as the vertebrate reservoir, but disease is primarily in humans.

also possible that because the coronaviruses undergo high-frequency recombination, as described later, they may be able to accommodate these large genomes because recombination offers a possible mechanism for correcting defective genomes. Intriguingly, coronaviruses and other members of the *Nidovirales* encode a number of RNA-processing enzymes including a 3′-to-5′ exonuclease that could conceivably make proofreading possible during RNA replication. However, there is as yet no evidence that the mutation frequency during coronaviral RNA replication is less than that occurring during replication of other RNA viruses.

The coronaviruses are grouped into three clades called groups 1, 2, 3, and examples are given in Table 3.13. Assignments were first based on serological cross-reactivity but more recently on sequence relatedness. Group 1 viruses include porcine epidemic diarrhea virus, porcine transmissible gastroenteritis virus, canine coronavirus, feline infectious peritonitis virus, and two human viruses, human coronaviruses 229E and NL63. Group 2 viruses are subdivided into two clades. Group 2A contains murine hepatitis virus (MHV), bovine coronavirus, rat sialodacryoadenitis virus, porcine hemagglutinating encephalomyelitis virus, canine respiratory coronavirus, equine coronavirus, and one human virus, human coronavirus OC43. Group 2B contains severe acute respiratory syndrome coronavirus (SARS HCoV). Group 3 contains a number of avian viruses, avian infectious bronchitis virus, turkey coronavirus, and recently described viruses of geese, pigeons, and ducks. Where known, the viruses in these different groups use different receptors to enter cells (see Table 1.3). A number of group 1 viruses use aminopeptidase N, also called CD13. Several group 2A viruses are known to use carcinoembryonic antigen-related adhesion molecules, which are members of the Ig superfamily. SARS virus uses angiotensin-converting enzyme 2.

Translation of the Viral Genome: The Nonstructural Proteins

The coronavirus genome is, as in the case of all plus-strand RNA viruses, a messenger, and the naked RNA is infectious. The organization of the 27.6-kb genome of avian infectious bronchitis virus (IBV) is shown in Fig. 3.38 as an example for the genus. The RNA, which is capped and polyadenylated, is translated into two polyproteins required for the replication of the viral RNA and the production of subgenomic mRNAs. The first polyprotein terminates at a stop codon 12.4 kb from the 5′ end of the RNA. Ribosomal frameshifting occurs frequently, however, and in the shifted frame, translation continues to the end of the RNA replicase-encoding region at 20.4 kb. The resulting polyproteins are cleaved by virus-encoded proteases, as illustrated in

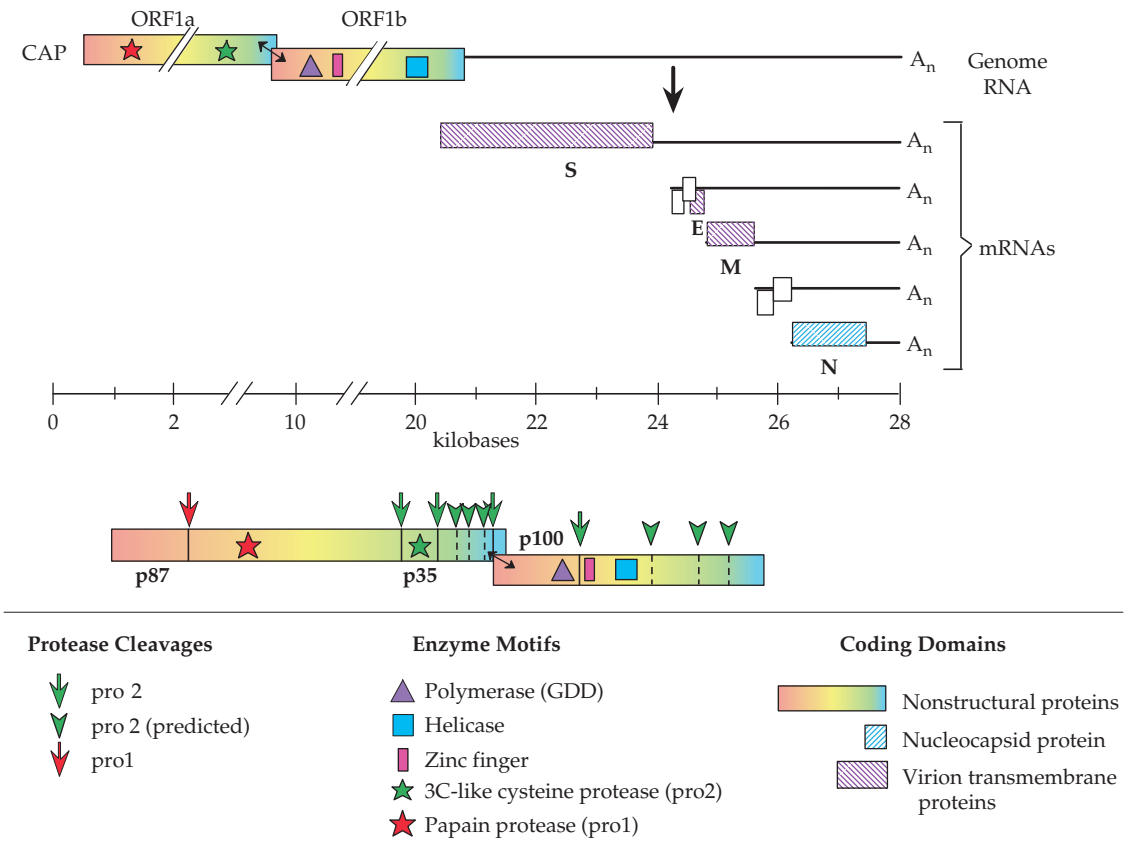


FIGURE 3.38 Upper panel: genome organization of the coronavirus, avian infectious bronchitis virus (IBV). ORF1a and ORF1b encode components of the viral replicase, and are translated as two polyproteins, with ribosomal frameshifting at the double-headed arrow. The remaining viral components are encoded in a nested set of mRNAs. The hatched proteins are polypeptides found in virions. White boxes are open reading frames of unknown function. E is a minor virion component, but essential for virus assembly. Lower panel: proteolytic processing of the IBV ORF1ab polyprotein. Motifs of papain-like proteases (pro1), 3C-like cysteine protease (pro2), RNA polymerase (GDD), zinc finger, and helicase are indicated with various symbols. Arrows at cleavage sites are color coded according to the protease responsible. Green arrowheads are predicted cleavage sites for pro2. Adapted from de Vries *et al*. (1997) with permission.

Fig. 3.38B. All coronaviruses possess at least two proteases, one papain-like and the other serine-like (but with cysteine at the active site as in poliovirus), and some encode a second papain-like protease so that they encode three proteases. Processing is complicated, as indicated in the figure.

Nidoviruses differ fundamentally from other RNA viruses in the number of nonstructural enzymes that they encode for the synthesis of the viral RNAs or for the purpose of enabling vigorous viral replication. The size of the RNA devoted to encoding these proteins in the coronaviruses is 20–30 kb, larger than the entire genome of other RNA viruses, and the number of cleaved products produced from the polyprotein precursors is large, on the order of 16. Perhaps the large size of the genome requires this. It is known that at least some of these proteins are devoted to countering host defenses against viral infection, which is surely important for the persistence of the viruses in nature.

The nonstructural proteins encoded in this domain of the genome include the two or three proteases described before, an RNA polymerase, an RNA helicase, and enzymes involved in capping that must perform functions similar to the corresponding enzymes in other (+)RNA viruses. The papain-like protease, however, has another function in addition to processing some of the sites in the nonstructural polyprotein. It is a deubiquitinating enzyme (DUB) whose precise role in virus infection is unknown. Ubiquitin and ubiquitin-like proteins (UBLs) are small proteins that are covalently attached to other proteins by ubiquitinating enzymes, either as single molecules or as branched chains. The role of ubiquitination is only incompletely understood but plays an important role in many cellular processes. One role of ubiquitination is to target proteins for degradation by the proteosome, a well-studied phenomenon. Ubiquination is also involved in membrane protein trafficking, in the activation of the transcription factor NFκB, in DNA repair, and in autophagy, a response to starvation in which double membrane structures are assembled that might serve as viral replication sites. Thus a viral DUB might stabilize proteins that enhance viral replication,

or might be important for the induction of NFκB, an important transcription factor during viral infection, or it might be important for constructing viral replication sites. There are also at least 10 UBLs derived from the same common ancestor as ubiquitin that are also conjugated to proteins to control cellular activities in ways that are but incompletely understood. One of these is the product of interferon-stimulated gene 15 (ISG15). This protein is induced by interferon and plays an unknown role in regulating the immune response to viral infection. It is not known if the viral DUB might also remove conjugated ISG15, but it is known that some other viruses target ISG conjugation. Influenza B virus produces a protein that binds to ISG, preventing the ISGlation of proteins. In addition, African swine fever virus, a large DNA virus, has a DUB that is thought to block the production of interferon by unknown mechanisms.

In addition to these gene products, nidoviruses encode distant homologues of at least five cellular enzymes associated with RNA processing. These are an endoribonuclease that cleaves after uridine residues, the 3′-to-5′ exonuclease mentioned earlier, a methyltransferase that might be part of the capping complex, an adenosine diphosphate-ribose 1′-phosphatase, and cyclic phosphodiesterase. Most coronaviruses encode all five of these enzymes whereas roniviruses encode only three and arteriviruses only one. The functions of these enzymes in the virus life cycle are unknown.

Production of Subgenomic RNAs

The members of the *Nidovirales* produce a nested set of subgenomic mRNAs (Fig. 3.38), which are capped and polyadenylated. The number produced depends on the virus but is 5 to 8 for most. Each subgenomic RNA is a messenger that is translated into one to three proteins from the 5′ ORF(s) in the mRNA. The five subgenomic mRNAs of IBV and the proteins translated from them are illustrated in Fig. 3.38A. Four of the subgenomic mRNAs are translated into the structural proteins in the virion, S, E, M, and N, found in that order in the genomes of all coronaviruses. Four small accessory proteins of unknown function are also produced, two from the E mRNA and two from RNA 5. Coronaviruses encode variable numbers of such accessory proteins which are not conserved as to sequence or to number among the various members of the family and whose function in unknown. It is also not known how multiple proteins are translated from a single mRNAs in the case of the coronaviruses.

Two mechanisms have been proposed for the production of these subgenomic RNAs. The first mechanism proposed was primer-directed synthesis from the (−)RNA template (i.e., from the antigenome produced from the genomic RNA). In this model, a primer of about 60 nucleotides is transcribed from the 3′ end of the template, which is therefore identical to the 5′ end of the genomic RNA. The primer is proposed to dissociate from the template and to be used by the viral RNA synthetase to reinitiate synthesis at any of the several subgenomic promoters in the (−)RNA template. Evidence for this model includes the fact that each subgenomic RNA has at its 5′ end the same 60 nucleotides that are present at the 5′ end of the genomic RNA, and that there is a short sequence element present at the beginning of each gene that could act as an acceptor for the primer (this sequence, e.g., is ACGAAC in the SARS CoV). A recent model proposes that the bulk of the subgenomic mRNAs are produced by independent replication of the subgenomic RNAs as replicons. Such replication is thought to be possible because the mRNAs contain both the 5′ and 3′ sequences present in the genomic RNA, and therefore possess the promoters required for replication. Evidence for this model includes the fact that both plus-sense and minus-sense subgenomic RNAs are present in infected cells. The model favored is that the subgenomic RNAs are first produced during synthesis of minus-strand RNA from the genomic RNA. In this model, synthesis initiates at the 3′ end of the genome and then jumps to the 5′ leader at one of the junctions between the genes. Once produced, the subgenomic RNAs begin independent replication.

Coronaviruses undergo high-frequency recombination in which up to 10% of the progeny may be recombinant. It is proposed that the mechanism for generation of the subgenomic RNAs, which requires the polymerase to stop at defined sites and then reinitiate synthesis at defined promoters, may allow the formation of perfect recombinants at high frequency.

Envelope Glycoproteins

Coronaviruses possess three envelope proteins—a spike protein (S), a membrane protein (M), and an envelope protein (E). The spike protein is a large protein (e.g., 1255 residues in the SARS CoV) that is heavily glycosylated (more than 10 carbohydrate chains attached) and anchored in the membrane of the virion by a transmembrane domain near the C terminus, with a C-terminal cytoplasmic tail of about 40 residues. It forms trimers that project from the surface of the membrane and give coronaviruses their characteristic corona. These spikes possess the receptor-binding activity, the major neutralizing epitopes, and the fusion activity of the virion. S contains two domains of about equal size called S1 (N terminal) and S2 (C terminal), and in some, but not all, coronaviruses these two domains are separated into different proteins by proteolytic cleavage of S. S1 contains the receptor-binding region and S2 contains the fusion domain. S is not well conserved, with only about 30% sequence identity among S proteins of coronaviruses belonging to different groups.

The M protein is smaller, 221 residues in SARS CoV, and spans the lipid bilayer three times such that it has only a small fraction of its mass exposed outside the bilayer. The E protein is quite small, only 76 residues in SARS CoV, and

has one membrane-spanning region. These two proteins are important for virion morphogenesis.

Some coronaviruses belonging to group 2 also possess a fourth envelope protein, a hemagglutinin-esterase (HE). Remarkably, this protein appears to be homologous to the H-E of influenza C virus (described in the next chapter). It appears that recombination between a coronavirus and an influenza C virus occurred that led to exchange of this protein. Because only some coronaviruses possess HE, whereas all influenza C viruses possess it, the simplest hypothesis is that HE was an influenza C protein that was acquired by a coronavirus. Presumably, this acquisition was maintained because it extended the host range of the coronavirus by allowing it to infect cells by binding to 5-*N*-acetyl-9-*O*-acetyl-*N*-neuraminic acid, a type of sialic acid, or to related sialic acids, depending on the specificity of the HE. Maintaining the HE protein has a cost for the virus. Mouse hepatitis virus loses HE when passed in culture, demonstrating that it is not needed for replication in cultured cells and that virus without the gene outcompete virus with the gene. In mice, MHV with HE is more virulent than virus without this gene and can spread more easily to the nervous system. Importantly, the HE gene is conserved in MHV strains isolated in the field, showing that this gene confers a selective advantage upon the virus.

Diseases Caused by Coronaviruses

Until recently, coronaviruses were considered to cause only mild disease in humans. Two human coronaviruses were known, HCoV OC43 (group 2A) and HCoV 229E (group 1). These viruses are responsible for about 25% of human colds and are spread by a respiratory route. Unlike rhinoviruses, they cause not only upper respiratory tract infections but sometimes lower respiratory tract infections as well, which are more serious. There is weak evidence that coronaviruses might also cause gastroenteritis in humans, because there have been reports of coronaviruses in the stools of people suffering from gastroenteritis. The status of coronaviruses as human disease agents changed with the recent isolation of two new human coronaviruses, NL63 (group 1) and HKU1 (group 2A), and with the 2003 epidemic of SARS (group 2B). NL63 is an important cause of severe lower respiratory tract infections in both adults and children. HKU1 has been isolated from adults with pneumonia. SARS causes an atypical pneumonia that carries a 10% fatality rate. It is a bat virus that jumped to humans in China, causing an epidemic of SARS that began in 2002. In 2003 it was spread around the world by air travelers, eventually causing more than 8000 cases of human disease and almost 800 deaths. It was eventually controlled by culling of animals that served as intermediates in passing the virus from bats to humans, and by quarantine procedures. There is concern that epidemics will recur since the virus is widely distributed in China. This topic is covered in more detail in Chapter 8.

Coronaviruses for many other animals are known, including mice, chickens, pigs, and cats. Diseases associated with various coronaviruses in these animals include respiratory disease, gastroenteritis, hepatitis, and a syndrome similar to multiple sclerosis of humans, as well as other illnesses. Mouse hepatitis virus has been particularly well studied as a model for the genus. Feline infectious peritonitis (FIP) coronavirus has also been intensively studied. This virus causes a severe infection of cats that is often fatal. It is immunosuppressive and the high fatality rate results from an inability to control the infection such that viral replication eventually reaches very high levels. Vaccination of cats with either structural proteins or nonstructural proteins did not protect the animals. In fact, vaccination with structural proteins made subsequent infection with live virus more severe. Persistent infection was observed in most animals, and there is evidence that virus remains even in animals that eventually control the infection since virus replication can resume if the animals are immunosuppressed. There are some parallels with SARS infection of humans, in that T-cell lymphopenia and viral persistence have been reported.

FAMILY *ARTERIVIRIDAE*

The family *Arteriviridae* contains four viruses, which are listed in Table 3.14. There are no known human viruses in the family, but it is of interest because it represents an intermediate between the coronaviruses and other enveloped (+)RNA viruses. The genome of equine arterivirus is illustrated in Fig. 3.39. The arteriviruses have a 13-kb genome that is very similar in organization and expression strategy to that of coronaviruses. The virion (60 nm) is enveloped, as are the coronaviruses, but the nucleocapsid, which is poorly defined, is probably icosahedral rather than helical. The arteriviruses could have arisen by the acquisition of new structural proteins by a coronavirus (or vice versa). The existence of this family, which appears to be a coronavirus with structural proteins that lead to icosahedral symmetry rather than helical symmetry, illustrates a problem for taxonomy. The ICTV has classified these viruses as a distinct family, but created the order *Nidovirales* to indicate their relation to the coronaviruses.

The four arteriviruses are lactate dehydrogenase-elevating virus of mice (LDV), equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and porcine reproductive and respiratory syndrome virus (PRRSV). The primary target cells in their respective hosts are macrophages, and all are associated with persistent, long-term infections. LDV causes a lifelong infection of mice that requires special care to detect. EAV causes epizootics of subclinical or mild respiratory diseases in adult horses. Infection can lead to abortions in pregnant mares, and infection of young horses causes a more serious illness. The virus persists for long

TABLE 3.14 *Arteriviridae* and *Roniviridae*

Genus/members	Virus name abbreviation	Usual host(s)	Transmission	Disease	World distribution
Arteriviridae					
Arterivirus					
Equine arteritis	EAV	Horses	Aerosols, contact	Fever, necrosis of arteries, abortion	Worldwide
Porcine reproductive and respiratory syndrome	PRRSV	Pigs	Oral–fecal?	Infertility, respiratory distress	?
Lactic dehydrogenase-elevating	LDV	Mice	Biting	?	?
Simian hemorrhagic fever	SHFV	Monkeys	Biting	Hemorrhage	?
Roniviridae					
Okavirus					
Gill-associated virus	GAV	Invertebrates (prawns)	Vertical, horizontal	Chronic subclinical, also acute necrosis of lymphoid organ	Asia and Australia

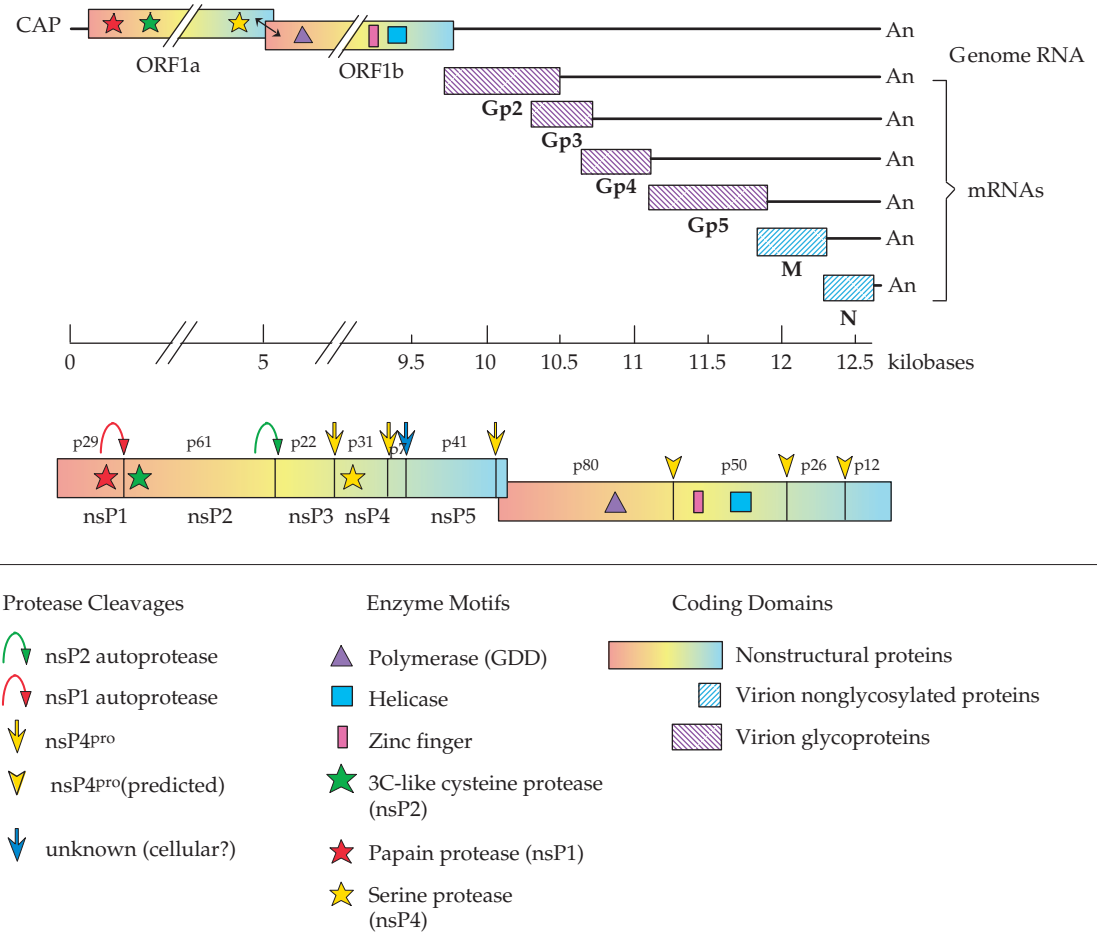


FIGURE 3.39 Upper panel: genome organization of an arterivirus, equine arteritis virus. ORF1a and ORF1b encode components of the viral replicase and are translated as a polyprotein with ribosomal frameshifting at the arrow. The remaining viral components are encoded in a nested set of mRNAs. The hatched proteins are polypeptides found in virions. Lower panel: proteolytic processing of the equine arteritis virus ORF1ab polyprotein. Positions of motifs of proteases, polymerase, zinc finger, and helicase are indicated with various symbols. Arrows are color coded to indicate cleavage by the corresponding protease. Arrowheads are predicted cleavages. Blue arrowhead is a cleavage site possibly cleaved by a cellular protease. Adapted from de Vries *et al*. (1997) and den Boon *et al*. (1991).

periods, and in stallions the virus may be secreted in semen for the life of the animal.

PRRSV causes respiratory distress in pigs of all ages and abortions and stillbirths in pregnant sows. SHFV is an African virus that causes persistent, inapparent infections in African monkeys. When introduced into colonies of Asian monkeys, however, it causes fatal hemorrhagic fever.

FAMILY *RONIVIRIDAE*

The *Roniviridae*, from ***ro***d-shaped ***ni***dovirus, are represented by a single known virus, gill-associated virus, which infects shrimp (Table 3.14). Its genome organization presents yet another permutation of how ancestral genes become associated with one another. The nonstructural genes, which occupy 20 kb, are translated from the genomic RNA by mechanisms that are very similar as those used by other members of the *Nidovirales* (Fig. 3.40). However, the structural proteins are translated from only two subgenomic mRNAs, one that is translated into the nucleocapsid protein, and one that is translated into a polyprotein precursor for the envelope proteins, which are separated from one another by signalase. The assembled virion is bacilliform in shape, 150–200 nm long and 40–60 nm in thickness. The virion thus resembles that of the rhabdoviruses (Chapter 4) rather than those of other nidoviruses.

THE PLUS-STRAND RNA VIRUSES OF PLANTS

Most plant viruses possess (+)RNA as their genome. Some have as their genome a single RNA molecule and produce subgenomic mRNAs, whereas in others the viral genome is divided into two or three or more segments. In plant viruses in which the genome is present in more than one segment, each segment is packaged separately into different particles and infection requires the introduction into the same cell of at least one of each genome segment. It is of interest that such an arrangement is common in plant viruses but nonexistent in animal viruses, presumably because of differences in the mechanisms by which plant and animal viruses spread and infect new cells or new hosts. Many (+)RNA plant viruses are rod shaped, formed using helical symmetry (e.g., tobacco mosaic virus, Fig. 2.2), while others are icosahedral (e.g., the comovirus cowpea mosaic virus, Figs. 2.5 and 2.7). No (+)RNA plant viruses are enveloped. Many of these viruses are major agricultural pathogens responsible for a great deal of crop damage worldwide. Although important as plant pathogens, plant viruses will not be covered here except for a description of the genomes of certain families that are of particular interest because of what they tell us about the evolution of viruses.

Several families of (+)RNA plant viruses share sequence identity with one another and with the alphaviruses. This collection of viruses, sometimes referred to as the Sindbis superfamily or the alphavirus superfamily, includes the alphaviruses, the tobamoviruses, the bromoviruses, and other families of plant viruses. The genomes of the tobamovirus tobacco mosaic virus (TMV), the bromovirus brome mosaic virus (BMV), and the alphavirus Sindbis virus are compared in Fig. 3.41. The genome of TMV is one molecule of (+)RNA and two subgenomic RNAs are produced. The genome of BMV consists of three molecules of (+)RNA and one subgenomic RNA is made. The alphaviruses have been described. Notice that a characteristic of this superfamily is that all viruses in it produce at least one subgenomic mRNA. The members of this superfamily all share three proteins (or protein domains) with demonstrable sequence homology, as indicated in the figure. These three are a viral

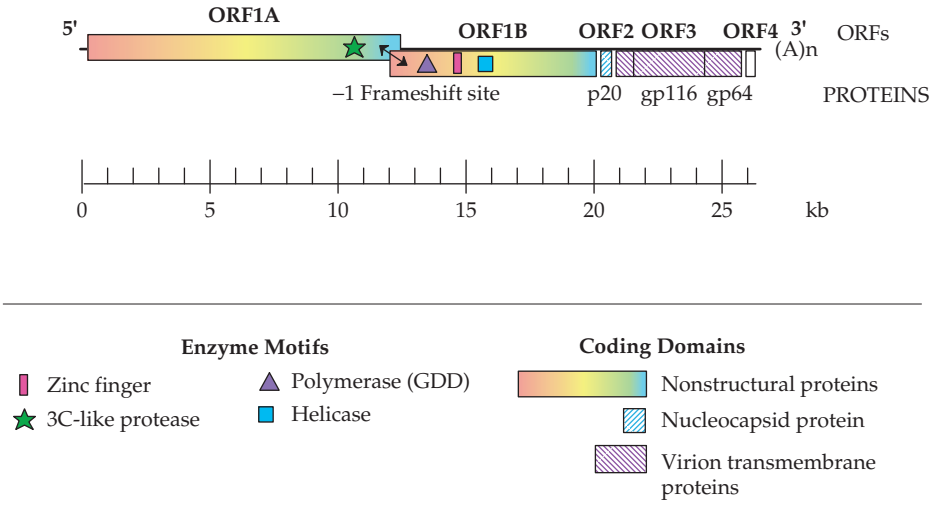


FIGURE 3.40 Genome organization of the *Roniviridae*. Redrawn from Cowley and Walker (2002).

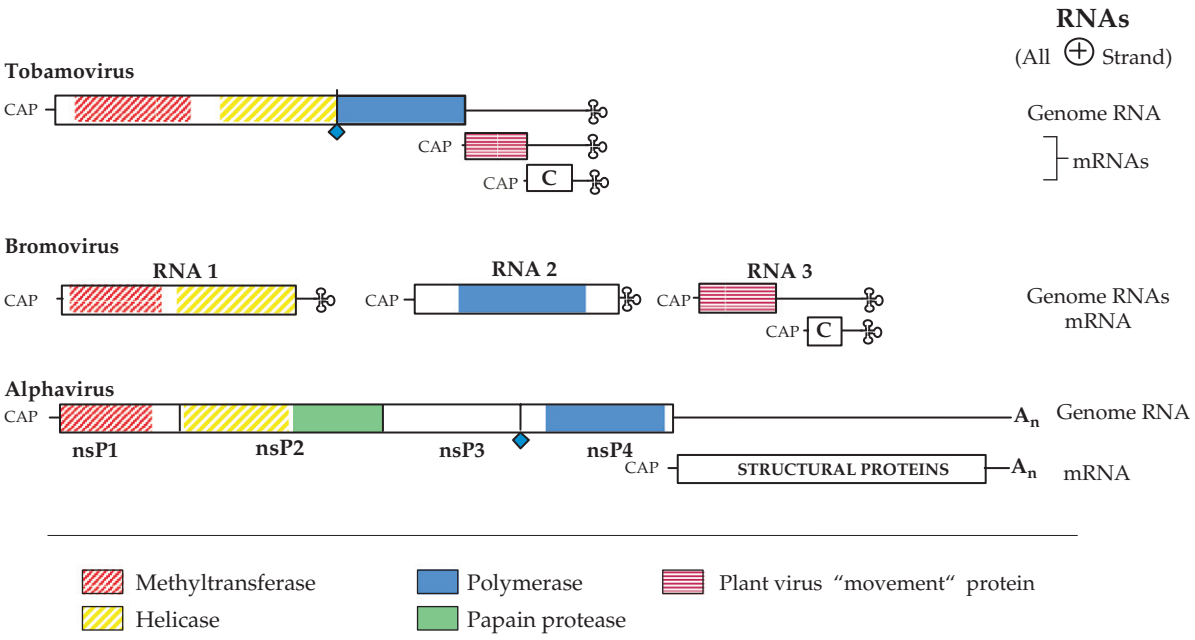


FIGURE 3.41 Comparison of the genome organization of alphavirus Sindbis with representatives of two plant virus families. Three shaded domains illustrate regions of low but significant sequence homology, which extend over hundreds of amino acids, within the methyltransferase, helicase, and polymerase proteins. The blue diamond is a leaky termination codon that is read through to produce the downstream blue-shaded domains in the tobamoviruses and the alphaviruses. C is the coat protein. The plant viruses have no module corresponding to the protease in nsP2 nor to protein nsP3. The alphaviruses have no domain corresponding to the "movement" protein of plant viruses. Adapted from Strauss and Strauss (1994), Figure 35.

RNA polymerase, a helicase, and a capping enzyme (characterized by methyltransferase activity). In the case of the alphaviruses and the tobamoviruses, all three domains are found on one genome segment and readthrough is required to translate the polymerase. In the bromoviruses, the capping enzyme and the helicase are encoded on one segment, but the polymerase is encoded on a different segment. The alphaviruses encode a protease to separate the three domains from one another, but the plant viruses do not. While these three shared proteins have clearly diverged from a common ancestral source, other domains within the nonstructural proteins are different from family to family. The alphavirus protease and nsP3 are not shared with the plant viruses, while the plant viruses possess movement proteins that are not shared with the alphaviruses. The structural proteins of the different families are also distinct. These observations clearly point to the occurrence of extensive recombinational events during the evolution of this group of viruses from a common ancestral source. Recombination has brought together new combinations of genes appropriate to the different lifestyles of the various members of the superfamily. In addition, the structural proteins differ among these three families so that the structures of the virions are very different from one another. The alphaviruses are enveloped, icosahedral particles (Figs. 2.5, 2.14A, and 2.25C). TMV is rod shaped (Fig. 2.2). BMV is icosahedral but is not enveloped. Thus, recombination has brought together different RNA replication modules with different structural protein modules to give rise to the current families of viruses.

Similar considerations pertain to two families of plant viruses (the *Comoviridae* and the *Potyviridae*) and the animal picornaviruses, which are all related to one another and are sometimes referred to as the picornavirus superfamily. The *Comoviridae* have a bipartite genome, whereas the *Potyviridae* and the *Picornaviridae* have a single molecule of RNA as their genome. Characteristics of this superfamily include the absence of subgenomic RNAs, the presence of 5′ VPg and 3′ poly(A) on the viral RNAs, and the production of at least one protease. The genome organizations of two members of the *Comoviridae* that belong to different genera, tomato black ring virus (genus *Nepovirus*) and cowpea mosaic virus (genus *Comovirus*), are compared with that of picornavirus poliovirus in Fig. 3.42. The members of this superfamily have demonstrable homologies in their RNA polymerases, 2C helicases, and $3C^{pro}$ proteases. Further, the RNA genomes have a 5′VPg and are polyadenylated, as noted. Proteases, VPg's, and poly(A) are very unusual in plant viruses, found only in members of this superfamily. It is clear that these viruses are all related to one another, and that multiple recombination events have taken place to give rise to the current families.

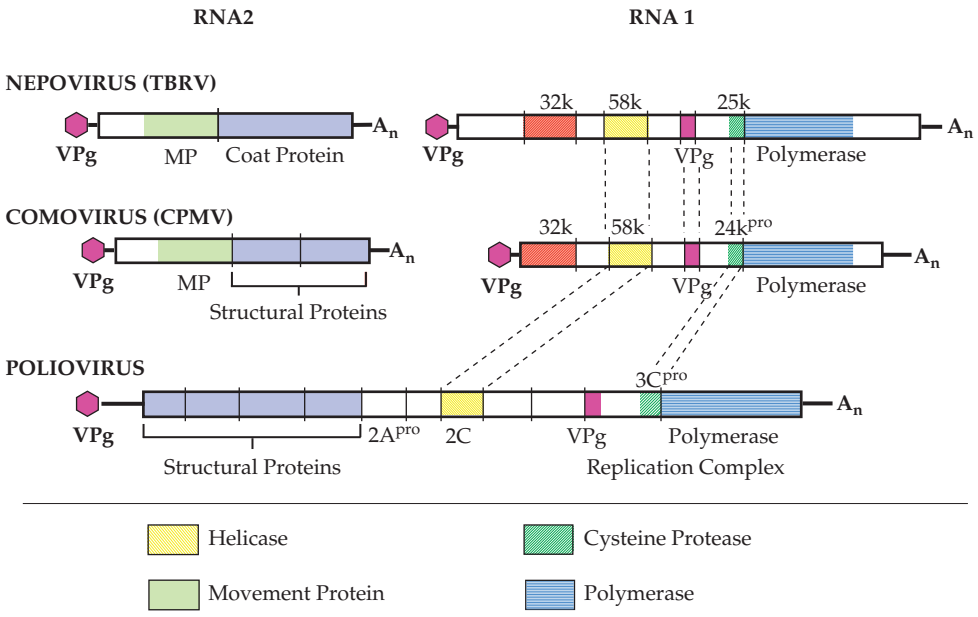


FIGURE 3.42 Comparison of the genomes of bipartite como- and nepoviruses and monopartite poliovirus. Domains in the helicase, polymerase, and protease that share sequence homology over long stretches of amino acids are identified with differently colored patterns. The related 32k proteins of como- and nepoviruses and the movement proteins encoded in RNA 2 (MP) have no counterpart in poliovirus. TBRV, tomato black ring virus; CPMV, cowpea mosaic virus. The structural proteins of the three viruses show no sequence similarity. Adapted from Strauss and Strauss (1997) Figure 2.12.

ORIGIN AND EVOLUTION OF PLUS-STRAND RNA VIRUSES

A reasonable hypothesis for the origin of the RNA viruses is that they began as an mRNA that encoded an RNA polymerase. The acquisition of an origin of replication that allowed the mRNA itself to be replicated by its encoded product would give rise to a self-replicating RNA and could have represented the first step in the development of a virus. Subsequent recombination with an mRNA encoding an RNA-binding protein that could be modeled into a capsid would give rise to a very simple virus. This protovirus could then evolve through continued mutation and recombination into something more complex. In support of this idea is the fact that the capsid protein of a large number of viruses, including bacterial viruses, plant viruses, and animal viruses that are otherwise unrelated to one another, share a common fold, suggesting that once a proper capsid protein arose it was retained during the evolution of many viruses while being modeled into new shapes.

Examples of the importance of recombination in the evolution of RNA viruses have been discussed. Computer-aided studies that have attempted to align the amino acid sequences of the proteins of different (+)RNA viruses have suggested that all these viruses share core functions that have common ancestral origins. These results are summarized in Fig. 3.43. All RNA viruses possess an RNA polymerase and these all appear to have derived from a common ancestral source. However, three lineages of RNA polymerases can be distinguished that probably diverged from one another early in the evolution of RNA viruses. Most RNA viruses also possess an RNA helicase that is required to unwind the RNA during replication. These helicases also appear to have diverged from a single source, but three lineages can be distinguished here as well. A third shared function in those RNA viruses with capped mRNAs is a methyltransferase gene (an activity required for capping), and two methyltransferase lineages can be distinguished. Finally there are the viral proteases that process polyproteins. The two distinct types of proteases with independent origins are the proteases derived from serine proteases (which may possess serine or cysteine at the active site) and the papain-like proteases. The different lineages of these four core activities have been reassorted in various ways during the evolution of the RNA viruses, as shown in the figure.

The second mechanism for divergence among viruses is mutation. Lack of proofreading in RNA replication means that the mistake frequency during replication is very high, on the order of 10^{-4}. Most mistakes are deleterious and do not persist in the population. However, because the mistake frequency is so high, many different sequences can be tried rapidly because of the rapid replication rate of viruses. The net result is that the rate of sequence divergence in RNA viruses is very high, up to 10^6-fold faster than their eukaryotic hosts. Three studies of the rate of sequence divergence in RNA viruses are illustrated in Fig. 3.44. In these studies, regions of the genomes of viruses isolated over a period of many years were compared. The rates of sequence divergence in a

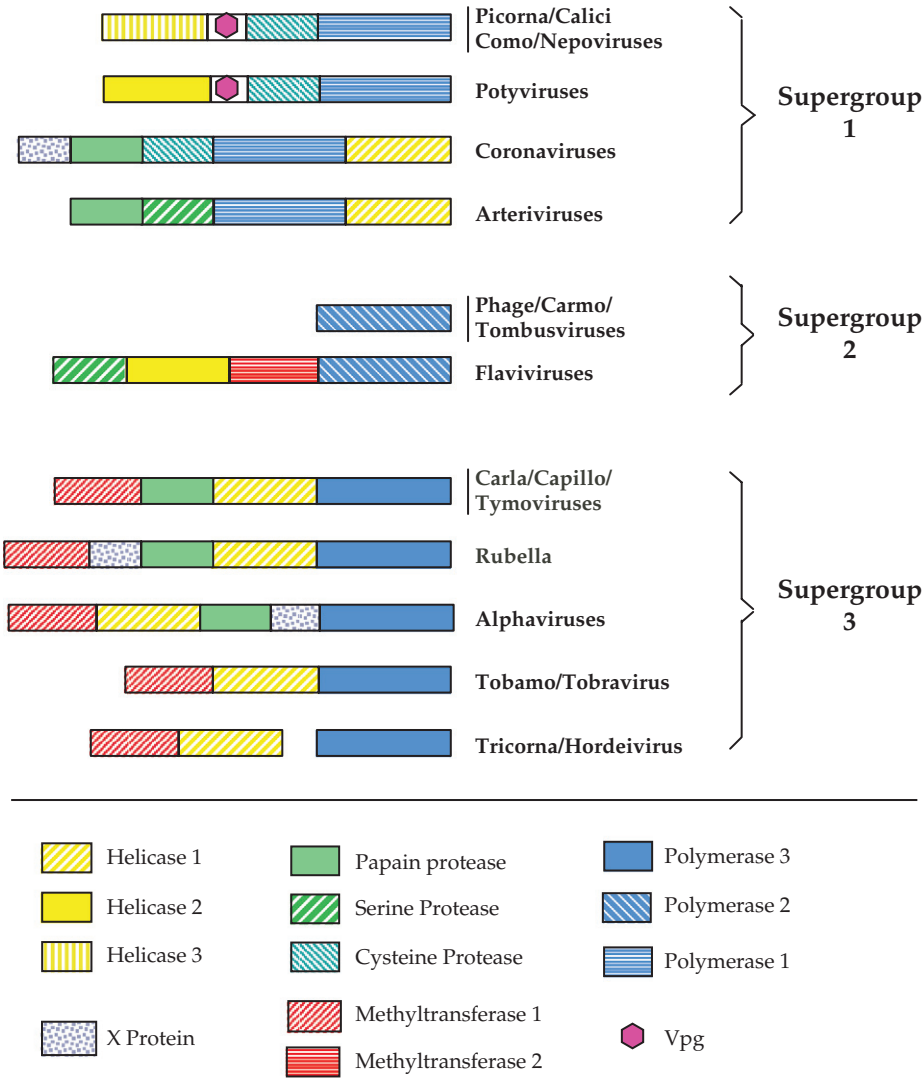


FIGURE 3.43 Genome organizations of plus-strand RNA viruses, grouped into three supergroups on the basis of sequence relationships among the nonstructral proteins. The RNA polymerases (blue), the proteinases (green), and the helicases (yellow) are each divided into three groups; the methyltransferases (red) are divided into two groups. From a relatively small number of building blocks, it is possible to arrive at the genomes of all of these viruses by divergence of individual domains and by recombination to reassemble them into different plans. Adapted from Strauss and Strauss (1994) Figure 36.

picornavirus and in influenza virus (Chapter 4) were found to be 0.5–1% per year. Changes in third codon positions, which are usually silent, occur more rapidly than changes in first or second codon positions, which usually result in an amino acid substitution. In alphaviruses, which alternate between insect and vertebrate hosts, the rate of divergence was significantly less, 0.03% per year, because changes that might be neutral or positively selected in one host are often deleterious in the other host. One of the apparent paradoxes of such studies is the observation that despite rapid sequence divergence, the properties of most viruses appear to remain stable for centuries or millennia. This is due in part to the fact that although the sequence may drift, the virus continues to fill the same niche and selection ensures that the properties of the virus change only slowly. A second factor is that different domains of the genome, or even different nucleotides or amino acids, diverge at very different rates because of differences in selection pressure. Studies of the rates of divergence of viruses perforce will measure the rates of domains that diverge most rapidly. There is no fossil record to tell us when currently extant viruses might have diverged from one another, and viruses in collections have all been isolated within the last 70 years. Thus, all studies of divergence in nature examine only the divergence that has occurred within the last 70 years. Such considerations have two practical implications. Vaccines developed against most viruses

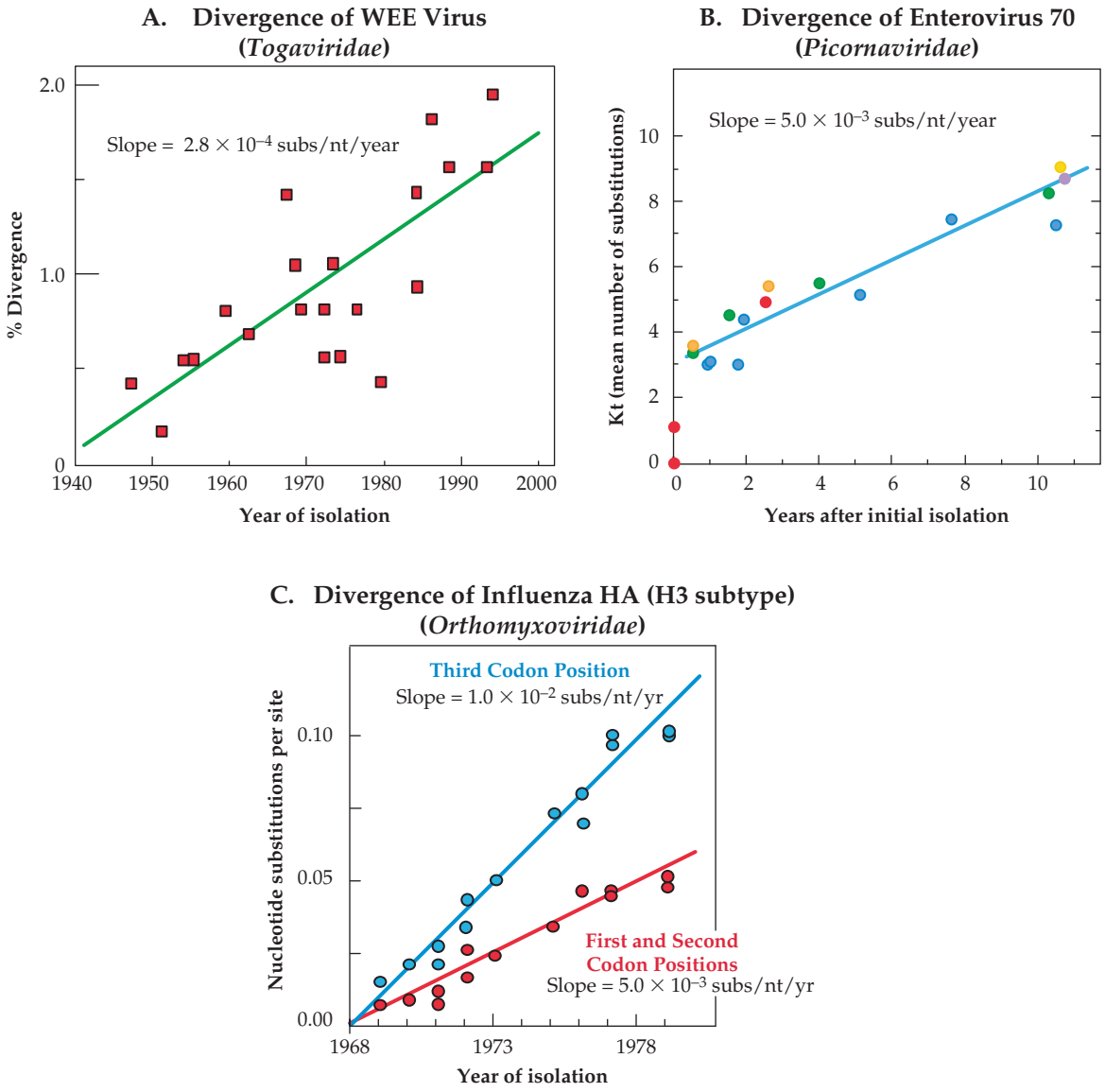


FIGURE 3.44 Divergence plots for two different plus-strand RNA viruses, which differ by more than an order of magnitude in their divergence rates, and a minus-strand virus. Data for (A) are from Weaver *et al*. (1997) for 477 nucleotides of the C terminus of the E1 protein-coding region, for (B) from Takeda *et al*. (1994) for the 918 nucleotides which encode the VP1 protein, and for (C) from Saitou and Nei (1986) for the ~1700 nt encoding the hemagglutinin. In (B) the dots are color coded by location of isolation: red, North Africa; orange, Europe; yellow, Pakistan; green, South East Asia; blue, Japan; purple, Honduras.

continue to be effective for long periods of time, but ultimately may have to be reformulated as the virus drifts. Second, viruses can in principle change very rapidly. Human immunodeficiency virus (Chapter 6) and influenza virus (Chapter 4) do change rapidly in response to immune pressure, and viruses that jump to a new host have been known to change rapidly (e.g., the DNA virus canine parvovirus, Chapter 7, and SARS coronavirus, this chapter and Chapter 8).

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