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Flaviviridae: The Viruses and Their Replication

Brett D. Lindenbach ***Heinz-Jürgen Thiel*** ***Charles M. Rice***

INTRODUCTION

The first human virus was discovered over one century ago when Walter Reed demonstrated that yellow fever could be experimentally transferred via the filtered serum of an

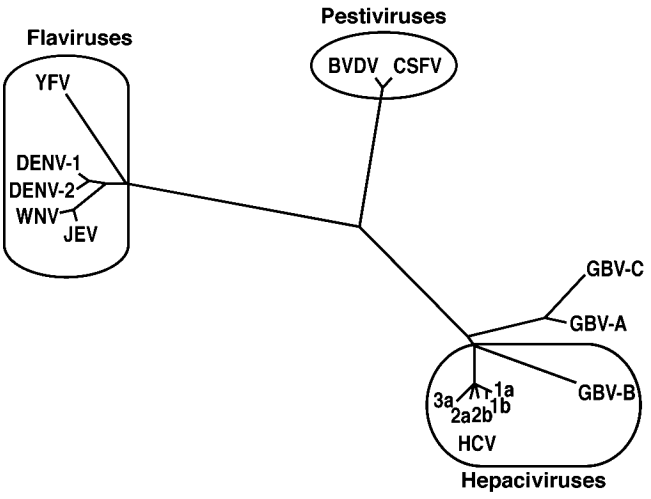

Figure 33.1 The family *Flaviviridae*. Phylogenetic tree based on analysis of NS3 helicase regions. Shown are members of the flavivirus genus: yellow fever virus (*YFV*), dengue-1 (*DENV*-1), dengue-2 (*DENV*-2), West Nile virus (*WNV*), and Japanese encephalitis (*JEV*); the pestivirus genus: bovine viral diarrhea virus (*BVDV*) and classical swine fever (*CSFV*); several hepacivirus (*HCV*) isolates, including *GBV-B*; and the unclassified viruses *GBV-A* and *GBV-C*. (Figure adapted from ref. 653, with permission.)

infected individual, and that this infectious agent was transmitted to humans by mosquitoes (682). It is now appreciated that yellow fever virus (YFV) is but one representative of a large family of related positive-strand RNA viruses, the *Flaviviridae* (from the Latin flavus, "yellow") (Fig. 33.1; Table 33.1). This family currently consists of three genera: *Flavivirus*, *Pestivirus* (from the Latin pestis, *plague*), and *Hepacivirus* (from the Greek hepar, hepatos, *liver*) (710). In addition to these genera, two groups of unassigned viruses, GBV-A and GBV-C, await formal classification within the family. As detailed below, members of this family share similarities in virion morphology, genome organization, and replication strategy, but exhibit diverse biological properties and a lack of serological cross-reactivity. The increasing significance of *Flaviviridae* as human and animal pathogens (Chapters 34 and 35) emphasizes that their study remains no less pertinent than in Reed's time.

Family Classification

Positive stranded RNA viruses have been classified into three superfamilies, based on the evolutionary relatedness

TABLE 33.1
MEMBERS OF THE FAMILY *FLAVIVIRIDAE*

Taxonomic Unit	Representative Examples
Genus *Flavivirus*	
Tick-borne viruses	
Mammalian tick-borne group (15)	Tick-borne encephalitis virus, European subtype (TBEV-Eu)
	Tick-borne encephalitis virus, Far Eastern subtype (TBEV-FE)
Seabird tick-borne group (4)	Tyuleniy virus
Mosquito-borne viruses	
Aroa virus group (4)	Aroa virus
Dengue virus group (5)	Dengue virus, types-1 to 4 (DENV-1 to DENV-4)
	Kedougou virus
Japanese encephalitis group (10)	Japanese encephalitis virus (JEV)
	West Nile virus (WNV)
Kokobera virus group (2)	Kokobera virus
Ntaya virus group (6)	Ntaya virus
Spondweni virus group (2)	Spondweni virus
Yellow fever virus group (9)	Yellow fever virus (YFV)
Viruses with no known vector	
Entebbe bat virus group (3)	Entebbe bat virus
Modoc virus group (6)	Modoc virus
Rio Bravo virus group (7)	Rio Bravo virus
Unclassified (3)	Cell fusing agent virus
Genus *Pestivirus*	Bovine viral diarrhea virus 1 (BVDV-1), four serotypes
	Bovine viral diarrhea virus 2 (BVDV-2), two serotypes
	Border disease virus, two serotypes
	Classical swine fever virus (CSFV), four serotypes[a]
	Pestivirus of giraffe (unclassified)
Genus *Hepacivirus*	Hepatitis C virus (HCV), six genotypes
	GB virus B (GBV-B)
Unclassified (2)	GB virus A (GBV-A), GBV-A-like viruses
	GB virus C (GBV-C), Hepatitis G virus (HGV)

[a]CSFV was formerly called hog cholera virus (HCV). The name was changed to avoid confusion with hepatitis C virus. Numbers in parentheses refer to the number of virus species recognized within each group.

of their RNA-dependent RNA polymerases (RdRP). The *Flaviviridae* are members of superfamily 2, bearing distant similarity to coliphages and the plant-infecting carmo-, tombus-, diantho-, and subgroup I luteoviruses (354). The *Flaviviridae* also encode RNA helicases in the helicase superfamily 2, which share the sequence *DExH/D* in the Walker B motif that coordinates divalent cations within the catalytic core (231). Before the era of molecular biology, members of the family *Flaviviridae* had been previously classified as *Togaviridae* (411).

Family Characteristics and Replication Cycle

This chapter is organized around common features of the family *Flaviviridae* life cycle (Fig. 33.2). Enveloped virions are composed of a lipid bilayer with two or more species of envelope (E) glycoproteins surrounding a nucleocapsid, which consists of a single-stranded, positive-sense genome RNA complexed with multiple copies of a small, basic capsid (C) protein. Binding and uptake are believed to involve receptor-mediated endocytosis via cellular receptors specific for viral envelope proteins. The low pH of the endosomal pathway induces fusion of the virion envelope with cellular membranes. Following uncoating of the nucleocapsid, the RNA genome is release into the cytoplasm. The viral genome serves three discrete roles within the life cycle: as the messenger RNA (mRNA) for translation of all viral proteins, a template during RNA replication, and genetic material packaged within new virus particles. The organization of the genome RNA is similar for all genera. Viral proteins are produced as part of a single long polyprotein of more than 3,000 amino acids that is cleaved by a combination of host and viral proteases. The structural proteins are encoded in the N-terminal portion of the polyprotein with the nonstructural (NS) proteins in the remainder. Sequence motifs characteristic of a serine protease, RNA helicase, and an RdRP are found in similar locations in the polyproteins of all three genera (489). Although helicase activities have been demonstrated or predicted for numerous positive-strand RNA viruses, their precise role in RNA replication remains speculative. Possible functions include melting regions of RNA secondary structure involved in template recognition, increasing polymerase processivity by eliminating secondary structures, resolving duplexes formed during the process of replication, or acting as a translocase to remove or exchange proteins bound to viral RNA. The cleavage products containing these regions are believed to form the enzymatic components of the RNA replicase. RNA replication occurs in cytoplasmic replication complexes that are associated with perinuclear membranes, and also via synthesis of a genome-length minus strand RNA intermediate. Progeny virions are thought to assemble by budding into an intracellular membrane compartment, most likely the endoplasmic reticulum (ER), then transited through the host secretory pathway and released at the cell surface.

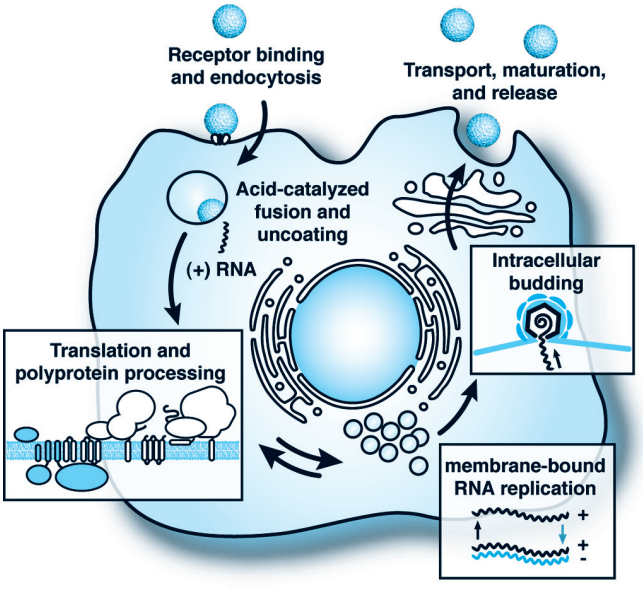

Figure 33.2 Virus life cycle. See the text for further details.

FLAVIVIRUSES

Background and Classification

The *Flavivirus* genus consists of more than 70 viruses, many of which are arthropod-borne human pathogens (Table 33.1). Flaviviruses cause a variety of diseases, including fevers, encephalitis, and hemorrhagic fevers (Chapter 34). Entities of major global concern include dengue virus (DENV) with its associated dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), Japanese encephalitis virus (JEV), West Nile virus (WNV), and YFV, which have been reviewed elsewhere (450). Other flaviviruses of regional or endemic concern include Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and tick-borne encephalitis virus (TBEV). Decreases in mosquito control efforts during the latter part of the 20th century, coupled with societal factors (e.g., increased transportation and dense urbanization) have contributed to the re-emergence of Flaviviruses such as DENV in South and Central America. Following an outbreak in New York City in 1999, WNV has spread throughout much of North America and Central America (see Chapter 34).

The development of the first live-attenuated flavivirus vaccine, YFV strain 17D, which has been described elsewhere (682), led to Max Theiler's recognition by the Nobel Prize committee in 1951. Only a limited number of flavivirus vaccines are available, including inactivated TBEV and JEV for use in humans and inactivated WNV for use in animals (577). Development of effective DENV vaccines that exhibit cross-protection, thought to be important for

preventing subsequent dengue-associated immunopathogenesis (see below), are proving to be particularly challenging. The ability to genetically manipulate flaviviruses, described below, is being used to develop novel approaches, including live attenuated chimeric vaccines to other flaviviruses based on the YFV-17D backbone (see Chapter 34).

Viruses within the genus are categorized into antigenic complexes and subcomplexes based on classic serological criteria or into clusters, clades, and species, according to molecular phylogenetics (95). These latter methods have permitted the classification of viruses such as YFV, which lacks close relatives. The salient features of *Flavivirus* taxonomy are illustrated in Table 33.1. Mosquito-borne and tick-borne flaviviruses, although distinct, appear to have evolved via a common ancestral line that diverged from nonvector borne viruses (i.e., for which no arthropod vectors are known). Additional points should be clarified with regard to this genus organization. First, the name *tick-borne encephalitis virus* is commonly applied to either central European encephalitis virus or Far Eastern encephalitis virus, although they clearly represent distinct viruses with differences in vector species, geographical distribution, and sequence relatedness (165). Some reports have appeared of intertypic recombination among DENV isolates, although the taxonomic status of these isolates is currently unclear (Chapter 34).

Structure and Physical Properties of the Virion

Flavivirus particles are small (≈50 nm) and contain an electron dense core of ≈30 nm, surrounded by a lipid envelope (508). Viruses sediment between 170 and 210S, and have buoyant densities of 1.19 to 1.23 g/cm^3, depending on the lipid composition, which can vary by host (619). Electron micrographs of virus particles are presented in Figure 33.3. The surface of virus particles contains two viral proteins, E (envelope) and M (membrane). The E glycoprotein, the major antigenic determinant on virus particles, mediates binding and fusion during virus entry. The M protein, produced during maturation of nascent virus particles within the secretory pathway, is a small proteolytic fragment of the

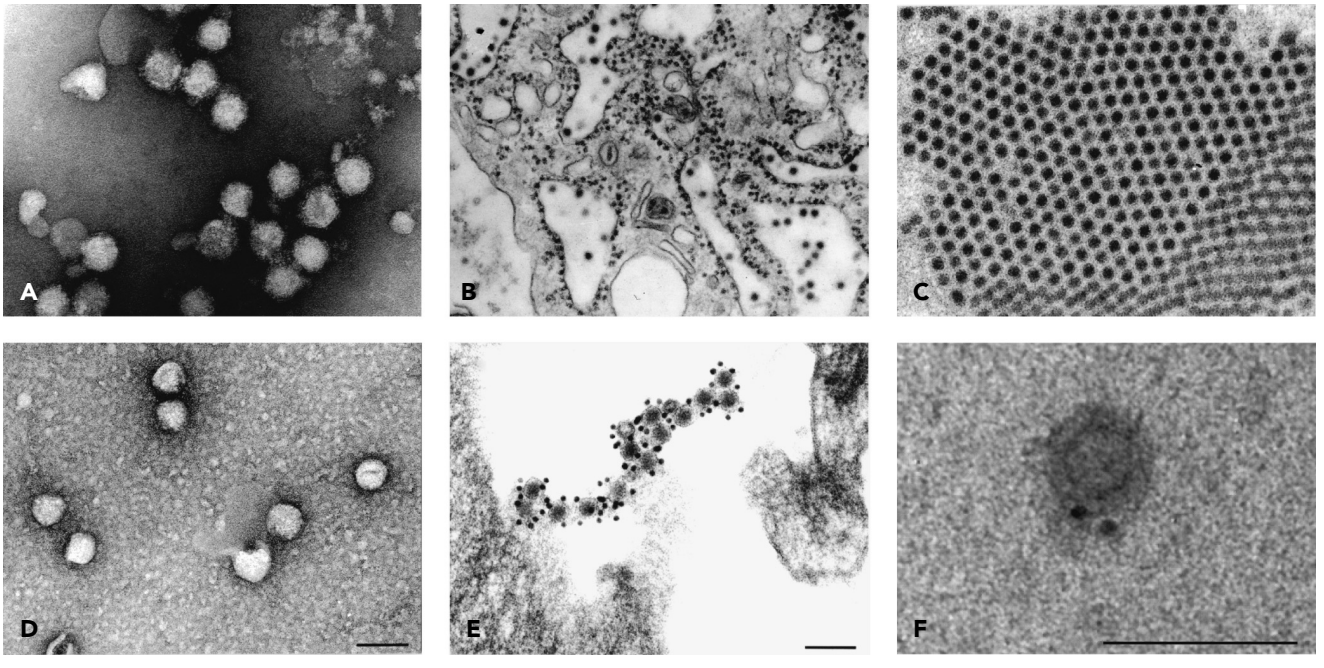

Figure 33.3 Electron micrographs of virions and infected cells. **A:** Purified St. Louis encephalitis virus (SLE) negatively stained with ammonium molybdate (508). Surface projections appear as a very thin, indistinct layer. (Courtesy of Dr. Frederick A. Murphy.) **B:** Thin section of a baby hamster kidney (BHK)-21 cell at 48 hours after infection showing SLE virions in the cisternae of the endoplasmic resticulum (765). (Courtesy of Frederick A. Murphy, Sylvia G. Whitfield, and A. K. Harrison.) **C:** Paracrystalline array of SLE virus in a *Culex pipiens* mosquito salivary gland cell 25 days after blood meal feeding on an infected suckling mouse. (Courtesy of Sylvia G. Whitfield, Frederick A. Murphy, and W. Daniel Sudia). **D:** Classical swine fever virus (CSFV) virions negatively stained with uranyl acetate (Courtesy of Dr. Frank Weiland). **E:** Ultrathin section of STE cells infected with CSFV and immunostained with E^{rns}-specific monoclonal antibodies 24/16 and colloidal gold. Bar, 100 nm. (From ref. 750, with permission.) **F:** A hepatitis C virus in cell culture (HCVcc) particle was detected by immunogold labeling (10-nm gold particle) for E2 (738). (Courtesy of Drs. Ralf Bartenschlager and Fredy Huschmand.)

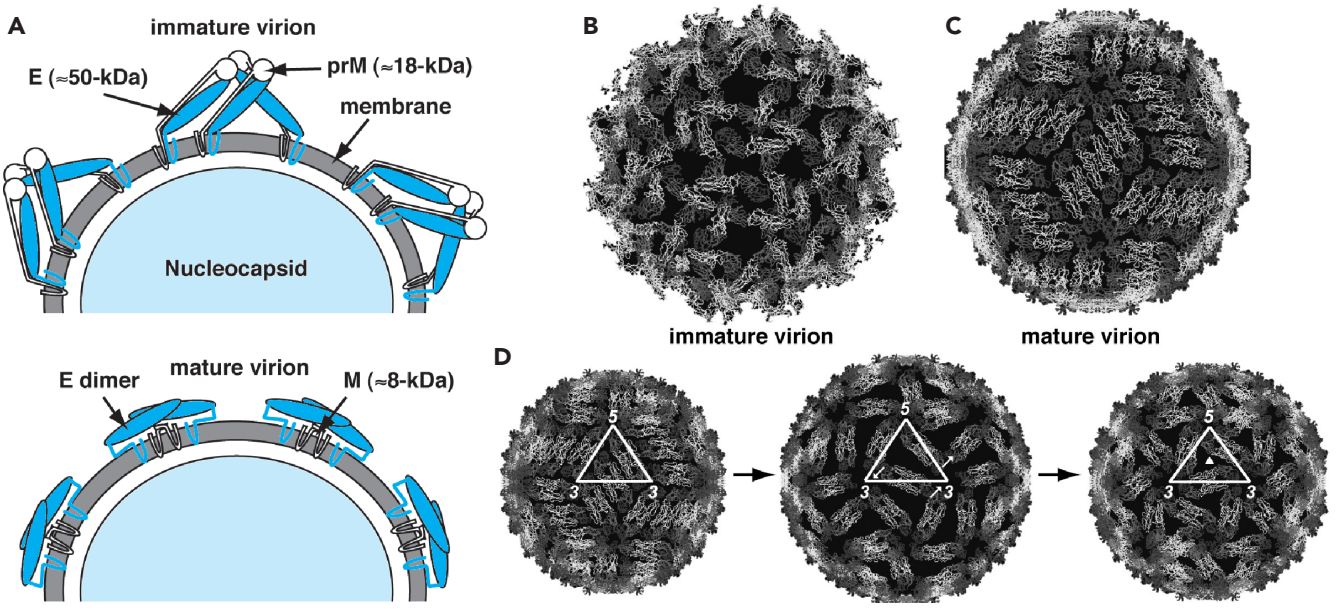

Figure 33.4 Structures of flavivirus particles. **A:** Envelope proteins of mature and immature virions. **B:** Cryo-electron microscopy (Cryo-EM) reconstruction of immature dengue virus 2 (DENV-2) particles, with the best fit of E fit into the electron density. prM protein density is not represented in this panel. **C:** Cryo-EM reconstruction of mature DENV-2 particles, with the best fit of E fit into the electron density. **D:** Model of the low pH-induced fusogenic state. (Courtesy of Dr. Richard Kuhn.)

precursor prM protein. Removal of the lipid envelope with nonionic detergents reveals discrete nucleocapsids (120 to 140S; 1.30 to 1.31 g/cm^3), which consist of C (capsid) protein and genomic RNA (gRNA), which has been reviewed elsewhere (619). Isolated nucleocapsids become unstable under high salt conditions, disassembling into capsid protein dimers (332).

Cryoelectron microscopy and image reconstruction have recently provided a wealth of information on flavivirus structure (504). Purified, mature DENV-2 particles display a relatively smooth outer surface (361), consistent with the head-to-tail configuration of E protein dimers lying parallel to the lipid bilayer (602). Fitting the E protein structure into this model indicates that 90 E dimers are tightly packed in an unusual herringbone pattern of icosahedral symmetry (Fig. 33.4C). These results suggest that E dimers may undergo rotational rearrangements around three- and fivefold axes of symmetry to form fusogenic trimeric complexes (Fig. 33.4D). Interestingly, E trimerization has been observed during conversion of E to the fusogenic form by low pH (13,266,675). Higher resolution structures revealed the transmembrane domains of E and M and the membrane proximal stem-anchor region of E (807). Remarkably, although electron density is observed below the membrane envelope, the nucleocapsids do not appear to have discernible symmetry. Immature DENV-2 and YFV particles are larger (60 nm) and display T = 1 symmetry, with 60 spikes each composed of three E monomers surrounding a putative trimer of prM (808). Thus, the arrangement of viral glycoproteins in immature virions is radically different than in mature virions and, as expected, prM covers the protruding fusion peptide in E domain II. Following prM cleavage and release of the pr peptide, 60 E trimers must dissociate, rotate, and reform into 90 antiparallel dimers seen in mature virions (Fig. 33.4).

In addition to mature virions, smaller, noninfectious particles are released from flavivirus-infected cells (664). These particles are termed slowly sedimenting (70S) hemagglutinin (SHA) because, as with virions, they can agglutinate red blood cells at low pH. SHA are smaller than virions (≈14 nm diameter) but have a similar buoyant density (1.23 g/cm^3). These particles contain E and M proteins, but lack nucleocapsids (664). Cells that express prM and E alone can produce a related type of particle, the recombinant subviral particle (RSP) (13,434,630). RSPs are 30 nm in diameter, slightly less dense than virus particles (1.14 g/cm^3), and can undergo acid-catalyzed fusion similar to virions (630). Cryo-image reconstruction of TBEV RSPs suggests a T = 1 icosahedral arrangement of 30 E protein dimers (190), markedly different from virions but spatially similar to their proposed fusogenic form (361). In addition, virion-sized particles have been observed with these expression systems (15,434).

Binding and Entry

Several cell surface molecules have been shown to interact with flavivirus particles, as has been recently reviewed (504), but only a few receptors have been characterized. Flaviviruses can utilize multiple receptors for

different cell types and in different host species. Infection of dendritic cells (DC) is particularly important because these intradermal cells can be primary targets early in infection. DC infection with DENV depends on target cell expression of the C-type lectin DC-SIGN (516,698). Cryo-image reconstruction reveals that the carbohydrate recognition domains of DC-SIGN bind glycans on adjacent molecules within the E dimer on intact DENV-2 particles (567). DC-SIGN is thought to function as an attachment receptor, because DC-SIGN internalization is not necessary for DENV infectivity (436). Thus, other molecules are presumably needed for virion endocytosis. Interestingly, WNV virus appears to utilize the related lectin DC-SIGNR for DC infection (146). Furthermore, YFV-17D, which lacks glycan modifications on E, can infect DC cells in a lectin-independent way (31). Other proteins tentatively identified as flavivirus receptors include $\alpha_v\beta_3$ integrin, GRP78 (BiP), and CD14 or a related molecule (504). In addition, highly sulfated glycosaminoglycans (e.g., heparan sulfate) have been shown to play an important role in the initial attachment of several flaviviruses to target cells (109,358). Also, virus particles opsonized with immunoglobulins show enhanced binding and infection of cells expressing immunoglobulin Fc receptors (548,635). It is widely suspected that antibody-enhanced infection may be relevant to the pathogenesis of DF and DHF, which occur more frequently in people previously exposed to other DENV serotypes (see Chapter 34).

Flaviviruses are internalized via clathrin-coated pits and trafficked to a prelysosomal endocytic compartment where low pH induces fusion between the virus and host cell membranes to release the virus nucleocapsid (115,225,226). During this transition, E protein dimers dissociate into monomers, and then reform into homotrimers (13,674,675). The efficiency of fusion is influenced by the lipid composition of target membranes: Cholesterol and oleic acid enhance fusion, whereas lyophosphatidylcholine inhibits fusion (677,678). Furthermore, lipid composition can influence the pH threshold of fusion (355). Perhaps because of the fluid nature of the flavivirus nucleocapsid structure (361,807), it appears that viral genomes are directly accessible for translation after membrane fusion (355).

Genome Structure

As for all positive-strand RNA viruses, the flavivirus genome is infectious (549). Full-length infectious complementary DNA (cDNA) clones have been constructed for several flaviviruses, allowing flavivirus biology to be dissected through reverse genetics (412,613). Flavivirus genomes consist of a single, positive-strand RNA of ≈11 kb (sedimentation, 42S) with a 5′ type I cap, $m^7GpppAmpN_2$ (121,760). Additional methylation of the N_2 residue (type II cap) has also been detected in RNA from infected cells. Unlike cellular messenger RNA (mRNA), flavivirus genomes lack a 3′ polyadenylate tail (760). Genomes encode a single long open reading frame (ORF) flanked by 5′ and 3′ noncoding regions (NCR) of ≈100 nucleotide (nt) and 400 to 700 nt, respectively (454) (Fig. 33.5A).

The sequence of the 5′ NCR is not well conserved between different flaviviruses, although common secondary structures have been found within this region (77,714). These structures influence the translation of the genome. Indeed, morpholino oligos complementary to the 5′-stem loop abolished DENV RNA translation and virus replication (151,277,341). Another major function of the 5′ NCR probably resides in the complementary region of the negative strand, which serves as a site of initiation for positive-strand synthesis during RNA replication. Deletions in this region were lethal for DENV-4 replication, although they had minimal effects on translation of the mutant RNA (92). One of the viable mutants exhibited a limited host-range growth phenotype, suggesting that host-specific factors interact with this RNA region. Indeed, several human proteins, including La and TIAR, can bind to the 3′ NCR of minus strand RNA (396,645,792). Further studies have shown that WNV replication was inhibited in a TIAR-knockout cell line, supporting the functional importance of this interaction (396).

The organization of the 3′ NCR differs greatly between mosquito-borne and tick-borne viruses. Although the 3′ NCR of flavivirus genomes exhibit great variability, similar patterns of conserved sequences and structures have been found among flaviviruses (454), and are illustrated in Figure 33.5A. The greatest structural similarity is a long (90 to 120 nt) 3′ stem-loop (3′ SL) that differs in primary sequence between mosquito-borne and tick-born flaviviruses (714). Mutational analysis of the DENV-2 and WNV revealed essential virus-specific and host-specific functional regions within the 3′ SL (174,715,798,802). The 3′ SL enhances translation of reporter mRNAs containing the DENV 3′ NCR (111,276). Furthermore, DENV-2 translation and replication were inhibited by a morpholino oligo complementary to the top loop of the 3′ SL (277). The 3′ SL can interact with several proteins of functional relevance, including the viral replicase proteins NS3 and NS5 (108,144). The 3′ SL of WNV and DENV-4 was also shown to bind translation elongation factor 1A (EF1A) (56,148). These results are intriguing, because EF1A and its prokaryotic homolog EF-Tu have been shown to function in the replication of several positive-strand RNA viruses (64,259,310,801). In addition to EF1A, the human La autoantigen (148,219) and murine Mov34 protein (689) were found to bind to the 3′ SL of DENV-4 and JEV viruses, respectively, although the functional relevance of these interactions is presently unknown.

Just upstream of the 3′ SL, a 25-nt region (CS1) that is well conserved among mosquito-borne flaviviruses was found to basepair with a complementary sequence (5′ CS) in the beginning of the capsid gene, more than 10 kilobase (kb) upstream (251). Similar long-distance basepairing

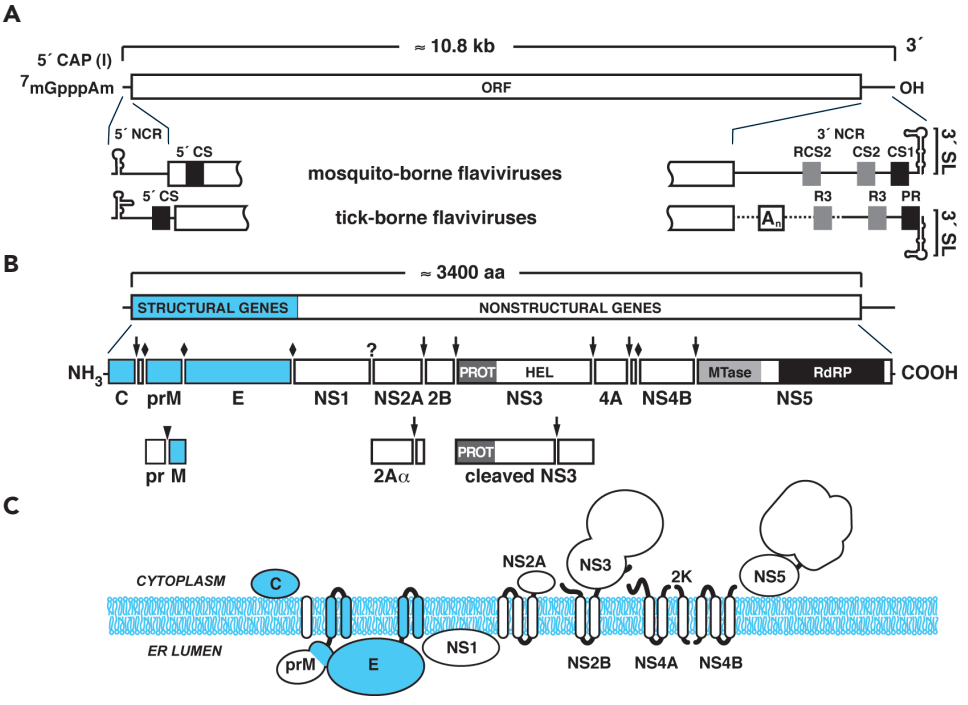

Figure 33.5 Flavivirus genome structure and expression. **A:** Genome structure and RNA elements. The viral genome is depicted with the structural and nonstructural protein coding regions, the 5′ cap, and the 5′ and 3′ noncoding regions (NCR) indicated. Models of functionally important secondary and tertiary structures within the 5′ and 3′ NCR and the coding region are shown with predicted hairpin loops indicated by letters. (See the text for further details.) **B:** Polyprotein processing and cleavage products. *Boxes* below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Structural proteins are colored cyan, whereas nonstructural (NS) proteins are white. Cleavage sites for host signalase ♦, the viral serine protease (*downward arrow*), furin or related protease (*triangle*), or unknown proteases (*?*) are indicated. **C:** The proposed topology of the flavivirus polyprotein cleavage products with respect to the endoplasmic reticulum (ER) membrane is shown. The proteins are approximately to scale (areas are proportional to the number of amino acids) and arranged in order (left to right) of their appearance in the polyprotein. Mature structural proteins are shaded and C-terminal membrane spanning segments of M and E are indicated.

also occurs between conserved sequences in the 5′ and 3′ NCR of tick-borne viruses (327,346). Complementarity between these cyclization sequences was shown to be essential for flavivirus replication (327,346), and is likely to be required for selection of RNA templates for replication (794). In contrast, genome cyclization is apparently not involved in regulating genome translation (18,111,151,277).

In mosquito-borne flaviviruses, the latter half of the CS1 can basepair with an internal loop in the stalk of the 3′ SL, forming a small pseudoknot (645), and the 3′-CS-A of TBEV actually overlaps with the 3′ SL. Thus, the formation of the critical 3′ SL structures and genome cyclization are interrelated and may act as a conformational switch between different uses of the RNA template. Given the role of the 3′ SL in enhancing translation and the importance of genome cyclization in selecting templates for RNA replication, it is tempting to speculate that these structures may regulate the use of flavivirus RNA as templates for translation versus RNA replication.

Copies (one or two) of a second conserved sequence (CS2 and RCS2) are also found among some mosquito-borne flaviviruses (251). These regions are predicted to fold into bifurcating stem-loops that can potentially form pseudoknots with adjacent sequences (531). Flavivirus genomes and replicons containing deletions in CS2 or RCS2 are viable but highly attenuated (18,422,472).

As mentioned, tick-borne flaviviruses share a unique 3′ NCR organization, with a 350-nt 3′-conserved region preceded by a variable domain (740). Interestingly, a polyadenylate sequence is found in the variable region of some TBEV isolates (740). The variable region is dispensable for virus replication, whereas deletions that extend into the conserved region are progressively deleterious (453).

Translation and Proteolytic Processing

The efficiency of genome translation can be a primary determinant of flavivirus infectivity (166). Therefore, flaviviruses use several mechanisms to facilitate translational competence, including specialized structures within the 5′ and 3′ NCR, as described above. Translation is cap dependent and initiates by ribosomal scanning. Nevertheless, it

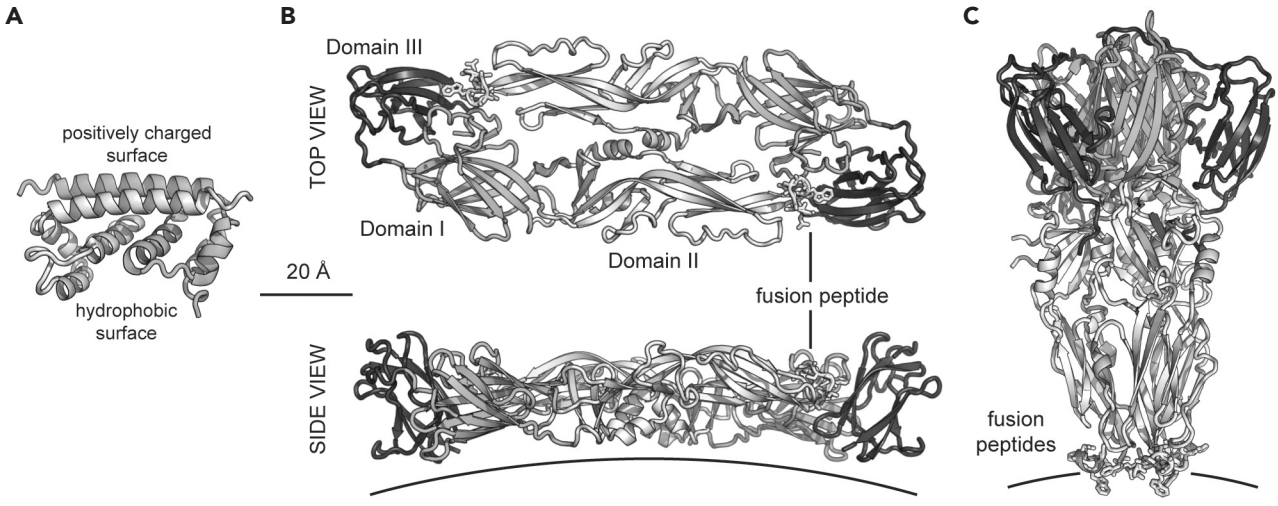

Figure 33.6 Structures of flavivirus C and E proteins. **A:** Dengue virus 2 (DENV-2) capsid protein dimer, with putative RNA- and membrane-binding surfaces indicated. Individual subunits are shaded white and cyan, rendered from pdb 1R6R (442). **B:** The structure of the DENV-2 E glycoprotein dimer is represented in this ribbon diagram, as viewed perpendicular (**top**) or laterally (**bottom**) with respect to the lipid bilayer. Individual E monomers are colored cyan (Domain I), white (Domain II), or dark gray (Domain III). The amino acid side chains of the fusion peptide are shown. Rendered from pdb 1OAN (494). **C:** TBEV E protein trimers, with subunits are colored as in **C**. Rendered from protein database (pdb) coordinates 1URZ (73). All figures rendered with PyMOL (http://pymol.sourecforge.net/).

appears that DENV can resist inhibition of cap-dependent translation by an unknown mechanism (166). Furthermore, the initiation codon for many mosquito-borne flaviviruses lacks a consensus Kozak motif, often with multiple in-frame AUG nearby. To help ensure proper start site selection, DENV apparently utilizes a small hairpin in the capsid gene to induce ribosomal pausing over the authentic AUG (124).

Translation of the single, long ORF produces a large polyprotein that is co- and post-translationally cleaved into at least 10 proteins (Fig. 33.5B). The N-terminal one fourth of this polyprotein encodes the structural proteins (C-prM-E), followed by the nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5) (98,99,605,756). Host signal peptidase is responsible for cleavages between C/prM, prM/E, E/NS1, and 2K-NS4B. A virus-encoded serine protease, discussed below, is responsible for cleavages between NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K, and NS4B/NS5 junctions. The enzyme responsible for NS1-2A cleavage is presently unknown. The expected topology of the flavivirus polyprotein is depicted in Figure 33.5C.

Features of the Structural Proteins

C Protein

Capsid (C) protein is a highly basic protein of ≈11 kd. Charged residues are clustered at the N- and C-termini, separated by an internal hydrophobic region that mediates membrane association (442). Nascent C (anchC) also contains a C-terminal hydrophobic anchor that serves as a signal peptide for ER translocation of prM. This hydrophobic domain is cleaved from mature C by the viral serine protease (424). C protein folds into a compact dimer with each monomer containing four alpha helices (156,305,442). Based on the asymmetric distribution of positively charged and hydrophobic residues, RNA binding and membrane interaction surfaces have been tentatively assigned (Fig. 33.6A). The TBEV C protein can tolerate deletions of up to 16 amino acids (aa) from the central hydrophobic membrane interaction helix, albeit with increased production of empty particles (345). Mutants containing larger deletions are not viable, but can be rescued by second-site changes that increase the hydrophobicity of downstream sequences (347). Thus, it is not yet clear how C protein dimers are organized within nucleocapsids, but interaction with RNA or DNA can induce isolated C protein dimers to assemble into nucleocapsidlike particles (332).

Membrane Glycoprotein prM

As mentioned above, the glycoprotein precursor of M protein, prM (≈26 kd), is translocated into the ER by the C-terminal hydrophobic domain of C. Signal peptidase cleavage is delayed, however, until the viral serine protease cleaves upstream of this signal sequence to generate the mature form of C protein (19,424,782). This strategy seems to result from the combination of a fairly short (14 to 22 aa) signal sequence, suboptimal residues at the signalase cleavage site, and downstream regions of prM (425,680). In addition, E protein expression influences the rate of this signalase cleavage (432). Optimization of the prM signalase cleavage site so that it is no longer regulated by the viral serine protease leads to increased production of empty virions

and lower levels of infectious virus (386,425). Thus, this coordinated anchC/prM cleavage serves to delay structural protein processing and virus production until viral serine protease levels are sufficiently high, late in infection.

The N-terminal region of prM contains one to three N-linked glycosylation sites (101) and six conserved cysteine residues, all of which are disulfide linked (524). The prM protein folds rapidly and assists in the proper folding of E protein (351,432). The C-terminal TM domains of prM and E act as ER-retention signals and may assist in their heterodimerization (406,532,534). A major function of prM is to prevent E from undergoing acid-catalyzed rearrangement to the fusogenic form during transit through the secretory pathway (247,266). The conversion of immature virus particles to mature virions occurs in the secretory pathway and coincides with cleavage of prM into pr and M fragments by the Golgi-resident protease furin or a related enzyme (667) (Fig. 33.4A). Following cleavage, prM-E heterodimers dissociate, the pr fragment is released, and E homodimers form (675,757) (compare Figs. 33.4B and C). Blocking prM cleavage by ammonium chloride treatment or by mutation of the furin-like cleavage site leads to the release of noninfectious, immature virions (175,247). Interestingly, cleavage site mutations can be partially suppressed by second-site changes that alter the number of Cys residues in the adjacent pr segment and the resulting virus exhibits a temperature sensitive (ts) phenotype (176). Thus, these double mutant virions contain intact prM that may be misfolded.

Envelope Glycoprotein

E protein (≈53 kd), the major protein on the surface of flavivirus virions, mediates receptor binding and membrane fusion. E is synthesized as a type I membrane protein containing 12 conserved cysteines that form disulfide bonds (525) and, for some viruses, E is N-glycosylated (102,767). As mentioned, proper folding, stabilization in low pH, and secretion of E depends on coexpression with prM (351,432).

The native form of E folds into an elongated structure rich in β-sheets and forming head-to-tail homodimers that lie parallel with the virus envelope (602), which has also been reviewed elsewhere (504). Each E protein subunit is composed of three domains: I, which forms a β-barrel; II, which projects along the virus surface between the transmembrane regions of the homodimer subunits; and III, which maintains an immunoglobulin-like fold (Fig. 33.6B). The putative fusion peptide, which mediates insertion into the target cell membrane, is located at the tip of domain II, distal to the transmembrane region (12,602). Domain III appears to be involved in receptor binding and is a major target of neutralizing antibodies (reviewed in Chapter 34). Between the ectodomain of E and the membrane is a short but functionally important stem-anchor region composed of two α-helices that lie parallel to the plane of the membrane (14,807).

On exposure to low pH, E protein dimers dissociate into their monomeric subunits, which then form trimers (13,675,676). Although the structure of the fusogenic intermediate has not been yet been discerned, the postfusion form of the trimeric E ectodomains interact in a parallel fashion, with their fusion peptides fully extended back toward the fused membrane (73,495) (Fig. 33.6C). Although the overall E subdomain organization is preserved, domain III must rotate and fold back more than 30 Å in relation to domain I for this conformational switch to occur. Indeed, neutralizing antibodies against domain III can inhibit a postattachment step of viral entry (526), and a soluble form of domain III can act as a potent dominant-negative inhibitor of this conformational change (399). In addition, domain II rotates relative to domain I (73,495). Similar displacement of domain II is seen in crystals of native E protein grown in the presence of the detergent β-octylglucoside, which binds in a small hydrophobic pocket in the domain I/II hinge region (494,809). Residues that influence the pH threshold for membrane fusion surround this pocket (494). The stem-anchor region may further stabilize the trimeric structure by fitting into a groove on the surface of the trimer (675,679), and lateral interactions between E trimers suggest that membrane fusion may be cooperatively activated (676).

Features of the Nonstructural Proteins

NS1

The NS1 glycoprotein (≈46 kd) is translocated into the ER during synthesis and cleaved from E protein by host signal peptidase, whereas an unknown ER-resident host enzyme cleaves the NS1/2A junction (185,186). The eight C-terminal residues of NS1 and greater than 140 amino acids of NS2A are required for this processing event (186,281). In addition, truncated and elongated forms of NS1, which presumably differ in their C-terminal cleavage sites, have been observed for JEV and MVEV (62,458). NS1 is largely retained within infected cells but can localize to the cell surface and is slowly secreted from mammalian cells (412).

NS1 contains two or three N-linked glycosylation sites and 12 conserved cysteines that form disulfide bonds (388,458,664). The pattern of disulfide pairing has been recently solved (63,739). Around 30 minutes after synthesis, NS1 forms highly stable homodimers and acquires an affinity for membranes (768,769). Because this protein is largely hydrophilic in amino acid content and lacks transmembrane domains, the nature of this membrane association remains unclear. One possibility is that dimerization creates a hydrophobic surface for peripheral membrane association. Alternatively, one group has reported that DENV-2 NS1 exhibits many properties of a GPI-anchored protein (301), although efficient GPI-anchoring of this protein seems inconsistent with the C-terminal peptide sequencing data of WNV NS1 (761).

NS1 has an important yet unclear role in RNA replication. NS1 localizes to sites of RNA replication (447,763), and mutation of the N-linked glycosylation sites in NS1 can lead to dramatic defects in RNA replication (512) and virus production (139,512). Furthermore, a ts mutation in YFV NS1 profoundly inhibits RNA accumulation under nonpermissive conditions (513). Trans-complementation studies reveal that NS1 functions at a very early stage in RNA replication (328,329,409), and that an interaction between NS1 and NS4A is required for replicase function (410).

The function of the extracellular forms of NS1 is not yet clear. During infection, strong humoral responses are made against this protein, and antibodies against the cell surface form can direct complement-mediated lysis of virus-infected cells (412). In addition, some NS1-specific protective antibodies apparently can function in a complement- and FcR-independent manner (119). Antibody-based stimulation of surface NS1 can induce tyrosine phosphorylation of several unidentified proteins in DENV-2-infected cells (301), and it has been proposed that NS1 may mimic important human molecules (104,184). The secreted form of NS1 was originally characterized as the soluble complement-fixing antigen present in the serum and tissues of infected animals (70), which peaks during the acute phase of infection (8,444). Although an earlier study suggested that dimerization was necessary for NS1 secretion (575), a Kunjin virus (KUNV) mutant that produces unstable NS1 dimers still secretes NS1 (252). Interestingly, this mutant grows poorly and is attenuated *in vivo*. Secreted NS1 assembles into soluble hexameric particles of ≈11 nm that appear to be three dimers held together by hydrophobic interactions (141,192). This form of NS1 is taken up by hepatocytes and trafficked to late endosomes, where they accumulate (9). Although the function of this compartmentalization is not yet clear, pretreatment of hepatoma cells with secreted NS1 can enhance the level of subsequent flavivirus infection (9).

NS2A and NS2B Proteins

NS2A is a relatively small (≈22 kd) hydrophobic protein. Its N-terminus is generated via NS1–2A cleavage by an unknown ER-resident host enzyme (186). Cytosolic cleavage at the NS2A/2B junction by the NS2B-NS3 serine protease, therefore suggests a transmembrane topology for NS2A. In addition, the serine protease can cleave at an internal site in NS2A to generate a C-terminally truncated form, NS2Aα (102,520). Mutations at the YFV NS2Aα cleavage site provided evidence that NS2A is involved in virus assembly, and a mutation in NS2A that blocks virus production can be suppressed by a second mutation on the surface of the NS3 helicase domain (363). An upstream mutation in KUNV NS2A has also been shown to block virus assembly (417). The involvement of nonstructural proteins, in particular the NS2-3 region, in virus assembly appears to be an emerging theme for all three genera of the family *Flaviviridae*. KUNV NS2A localizes to subcellular sites of flavivirus RNA replication (448) and interacts with replicase components NS3 and NS5, as well as the 3′ NCR of genome RNA (448). Thus, NS2A is an attractive candidate for coordinating the shift between RNA packaging and RNA replication, processes that have been shown to be linked (330).

Besides its apparent roles in RNA replication and virus assembly, DENV-2 NS2A has also been shown to act as an interferon (IFN) antagonist by inhibiting IFN signaling (507) and specific mutations in KUNV and WNV NS2A have been identified that diminish this inhibitory activity (418,420) and attenuate WNV virulence in mice (420). Interestingly, these mutations are adaptive and enhance the ability of KUNV replicons to establish persistent replication in IFN competent cell lines (418). Remarkably, NS2A of the tick-borne flavivirus Langat does not share this property, which appears to be carried out instead by NS5 (52).

NS2B is also a small (≈14 kd) membrane-associated protein (123). NS2B forms a stable complex with NS3 and acts as a cofactor for the NS2B-NS3 serine protease (187). The cofactor activity lies in a central peptide that intercalates within the fold of the serine protease domain (179), similar to the hepatitis C virus (HCV) NS4A cofactor. Mutation of conserved residues in NS2B can have dramatic effects on autoproteolyic cleavage at the NS2B/NS3 junction and transcleavage activities (100,523).

NS3 Protein

The NS3 is a large (≈70 kd) multifunctional protein, containing several activities required for polyprotein processing and RNA replication. The N-terminal third of the protein is the catalytic domain of the NS2B-NS3 serine protease complex (40,103,230). In addition to cleaving the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions, the protease generates the C-termini of mature capsid protein (19,782) and NS4A (402), and can cleave at internal sites within NS2A and NS3 (see above and below). The protease preferentially cleaves after adjacent basic residues (101) and crystal structures for the DENV-2 NS3 protease lacking the NS2B cofactor, with or without a substrate inhibitor, have been solved (510,511). These studies confirm the overall similarity to other members of this enzyme family, but reinforce an unusually flexible mode of substrate binding in the S1 pocket. Single chain proteases have been recently created by genetically fusing the NS2B cofactor region with the NS3 protease domain (106,800). The structures of the WNV and DENV-2 scNS2B-3 proteases reveal that the cofactor region of NS2B contributes a β-strand to forming the chymotrypsin-like fold (Supplemental Fig. 33.1A), similar to what has been seen with HCV (179).

As for other members of the family, the C-terminal region of NS3 encodes regions of significant homology to supergroup 2 RNA helicases (232). RNA-stimulated nucleoside triphosphatase (NTPase) activity (758) and RNA unwinding activity have been demonstrated for flavivirus NS3 proteins (747) and mutagenesis of helicase-active sites

has confirmed an essential role for this enzyme in viral replication (460), as has been reviewed (204). The x-ray structures of the helicase–NTPase domain of DENV and YFV NS3 have recently been solved (773,777). The helicase structure consists of three subdomains, two domains conserved among helicase family members and involved in nucleoside triphosphate hydrolysis, and a unique C-terminal domain that may be involved in virus-specific RNA and protein recognition activities (Supplemental Fig. 33.1C).

Flavivirus NS3 also encodes an RNA triphosphatase activity (RTPace) proposed to dephosphorylate the 5′ end of genome RNA before cap addition (759). Recent studies suggest that the flavivirus RTPase requires NS3 C-terminal sequences but utilizes the Walker B motif in the helicase–NTPase catalytic core for phophodiester bond hydrolysis (33,51). Thus, all three nucleic acid-modifying activities of NS3 (NTPase, helicase, and RTPase) rely on a common active center. In this regard, NS3 was shown to bind to the 3′ SL in association with NS5, and the NTPase activity of NS3 was enhanced in the presence of NS5 (108,144).

Truncated forms of NS3 have been observed for DENV-2 and TBEV, which result from an alternative serine protease cleavage site in the helicase domain (25,578,709). It is unclear what the roles of these cleavages are, although it is possible that the products could have a distinct function. It was recently found KUNV replicons bearing large in-frame deletions in the helicase domain could be complemented in *trans*, in contrast to deletions in the serine protease domain (306,329,419).

Flaviviruses are often cytopathic in mammalian cells, killing via apoptotic mechanisms. The NS3 proteins of Langat, DENV-2, and WNV have been shown to induce apoptosis (573,585,643), in some cases through activation of caspase-8 (573,585). Whether this is the normal pathway for flavivirus-induced cell killing requires further study.

The NS4A and NS4B Proteins

The NS4A and NS4B are small (16 kd and 27 kd, respectively) hydrophobic proteins. As mentioned, an important genetic interaction exists between NS1 and NS4A (410). A role for NS4A in RNA replication is supported by the colocalization of this protein with replication complexes (448). As mentioned for C protein, processing at the 2K/NS4B junction by signal peptidase requires NS2B-NS3 serine protease cleavage at a site just upstream of the 2K internal signal peptide (402,572). Overexpression studies show that regulated NS4A/2K/4B cleavage is necessary for induction of membrane rearrangements by NS4A (609). NS4B can associate with membranes independently of the 2K signal peptide and appears to be a polytopic membrane protein (402,490). NS4B colocalizes with NS3 and viral double stranded RNA (dsRNA) in ER-derived membrane structures presumed to be sites of RNA replication (490,764). NS4B is also posttranslationally modified to a form that migrates faster on sodium dodecyl sulfate-polyacrylamide gel elecrophoresis (SDS-PAGE) (102,572), although the identity and function of this modification remain to be determined. As noted above for the NS2A protein, DENV NS4A and NS4B can also block type I IFN signaling (507). NS4B has the strongest antagonistic effect, which requires either proper processing of the NS4–NS4B polyprotein by the NS2B–NS3 serine protease or expression of NS4B with an N-terminal signal peptide (506).

NS5 Protein

The NS5 is a large (103 kd), highly conserved, multifunctional protein with methyltransferase (MTase) and RdRP activities. Originally, the N-terminal region of NS5 was found to exhibit homology with S-adenosyl-methionine (SAM)-dependent MTase, suggesting that this protein is involved in modification of the 5′ cap (353). It was subsequently shown that the purified N-terminal domain of DENV-2 NS5 could transfer methyl groups from SAM to capped RNA substrates (168). The subsequent 2.4 Å crystal structure of this domain complexed with S-adenosyl-homocysteine revealed structural similarity with other MTases (168). Biochemical and biophysical studies demonstrated that guanosine triphosphate (GTP) is bound with high specificity via an unusual set of contacts (168). Mutations disrupting these contact sites ablated GTP binding in vitro (168) and were lethal for DENV-2 replication (255). This GTP binding site, which presumably mimics the 5′ cap structure, can also accommodate ribavirin triphosphate (50) (Supplemental Fig. 33.2A). Ribavirin inhibits the NS5 MTase activity, perhaps providing at least one mechanism for the antiflaviviral action of this nucleoside (50).

The C-terminus of NS5 contains significant homology to RdRPs of other positive-strand RNA viruses (354,605). Polymerase activity of this protein has been confirmed with recombinant NS5 (1,249,692). The major product of these *in vitro* reactions is often a self-primed copy-back RNA. However, *de novo* initiated RNA synthesis has been demonstrated for DENV-2 NS5 (1) and probably reflects the mode of initiation used for authentic flavivirus replication. Mutagenesis of the KUNV polymerase active site motif confirmed that it is essential for virus replication, and that polymerase activity could be supplied in trans from a KUNV replicon (326).

Because NS5 has been shown to function as an RdRP, it must localize to sites of viral RNA synthesis, although this remains to be demonstrated. NS5 forms a complex with NS3 (303,318) and can stimulate both the NTPase and RTPase activities of NS3 (144,793). Cross-linking studies have shown that both proteins bind to the 3′-SL of genome RNA (108). NS5, and the phosphorylated form in particular (318), has been shown to localize to the nucleus (83,318). Interestingly, the interdomain region that mediates interaction with NS3 was recently shown to contain nuclear localization signals recognized by importin $\beta 1$ and importin α/β (80,303). New roles, other than in RNA replication, have recently emerged for flavavirus NS5. DENV-2 NS5

induces IL-8 transcription, and secretion, which may enhance viral spread or disease by recruiting inflammatory cells to the site of infection (470). In addition, TBEV NS5 blocks signaling of IFN-α/β and IFN-γ by binding to their receptors and inhibiting phosphorylation of both Janus kinases, Jak1 and Tyk2, and hence downstream activation of signal transducer and activator of transcription 1 (STAT1) (52).

RNA Replication

The flavivirus replicase associates with membranes through interactions involving the small hydrophobic NS proteins, viral RNA, and presumably some host factors. Replication begins with the synthesis of a genome-length minus strand RNA, which then serves as a template for the synthesis of additional plus strand RNA. Minus strand RNA has been detected as early as 3 hours after infection (409). Viral RNA synthesis is asymmetric, with plus strands accumulating in around tenfold excess over minus strands (122,512). Flavivirus replication can be followed by metabolic labeling of virus-specific RNA in the presence of actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Three major species of labeled flavivirus RNA have been described, including genome RNA, a double-stranded replicative form (RF), and a heterogeneous population of replicative intermediate (RI) RNA that most likely contain duplex regions and recently synthesized plus strand RNA displaced by nascent strands undergoing elongation (118,122). Pulse-chase analyses indicate that RF and RI RNA are precursors to genome RNA (118,122). This mode of replication, with minus strands serving as templates for the production of multiple plus strands, can be described as semiconservative and asymmetric (118).

Membrane Reorganization and the Compartmentalization of Flavivirus Replication

Several studies have described ultrastructural changes to membranes of flavivirus-infected cells, predominately in the perinuclear region (764). In general, the earliest event is the proliferation of ER membranes, followed by the appearance of smooth membrane structures around the time of early logarithmic virus production. Smooth membrane structures are small clusters of 70- to 200-nm vesicles, or vesicle packets, containing electron dense material within the lumen of smooth ER. These structures continue to accumulate during later times of infection, when they become adjacent to newly formed, convoluted membranes. Convoluted membranes appear to be contiguous with the ER as randomly folded membranes or highly ordered structures that are sometimes described as paracrystalline arrays (382,509). The subcellular sites of RNA replication have been probed by metabolic labeling of nascent RNA, by immunolabeling with sera reactive to anti-dsRNA, which presumably recognizes RF and RI RNA, and by *in situ* hybridization (764). Viral RNA accumulates in association with vesicle packets in the perinuclear region of mammalian cells. Surprisingly, NS2B and NS3 localize with distinct convoluted membranes leading to the hypothesis that membrane reorganization in infected cells might give rise to adjacent, but distinct subcellular structures dedicated to viral polyprotein processing versus RNA replication. It is worth noting, however, that vesicle packets have been described only at late times after infection and the subcellular sites and structures involved in early RNA synthesis remain to be defined.

Replicase activity is concentrated in dense membrane fractions that are enriched for most viral nonstructural proteins (117). Most NS5 can be physically separated from membrane fractions (117,244) or removed by proteolysis (727) without affecting replicase activity. This suggests that only a small fraction of NS5 (and NS3) resides in active replication complexes, a result that is consistent with recent data for HCV (491,583). Recent biochemical analyses suggest that the flavivirus replicase resides in double-layered membrane compartments that can be distinguished based differential sensitivity to proteases and nucleases after detergent treatment (726). Most replicase products formed *in vitro* appear to involve elongation of endogenous templates rather than *de novo* synthesis, although reinitiation of RNA synthesis has been described (116). Initiation from exogenous RNA requires templates containing the 5′ and 3′ cyclization sequences (794), which are also required for replication in cells, as described above. The major product of these reactions, however, arises from self-primed copy-back synthesis rather than *de novo* initiation.

Assembly and Release of Particles from Flavivirus-infected Cells

Ultrastructural studies indicate that virion morphogenesis occurs in association with intracellular membranes (508). Electron microscopic studies of flavivirus-infected cells have consistently observed fully formed virions within the lumen of a compartment believed to be the ER (Fig. 33.3B). In many studies, virions appear to accumulate within disorderly arrays of membrane-bound vesicles (Fig. 33.3C). Budding intermediates and clearly distinguishable cytoplasmic nucleocapsids have not been frequently observed, suggesting that the process of assembly is rapid. Nascent virions appear to be transported by bulk flow through the secretory pathway and released at the cell surface (449). Budding at the plasma membrane has been occasionally observed, but does not appear to be a major mechanism for virion formation. These ultrastructural observations, together with studies on structural protein biosynthesis, oligomer formation, and the properties of intracellular and released virions, suggest the following model for virion assembly and maturation, which has been reviewed by others (265,504). As described above, proper folding of E requires cosynthesis with prM (351,432), and both proteins remain associated as detergent-stable heterodimers

(265,266,757) that are retained in the ER via sequences in their C-terminal transmembrane anchors (534). The highly basic C protein interacts with the viral genome RNA in the cytoplasm to form a nucleocapsid precursor that acquires an envelope by budding into the ER lumen. Recent studies suggest that the basic building block of the flavivirus nucleocapsid is likely to be a C protein dimer (332).

Later stages in virion maturation include glycan modification of E (for some viruses) and prM by trimming and terminal addition (102,138,256,458), implying that virions move through an exocytosis pathway similar to that used for synthesis of host plasma membrane glycoproteins. Although differences in the efficiency of prM cleavage have been noted, this cleavage generally distinguishes released virus from intracellular virus particles (644). Intracellular M-containing virions have not been detected, suggesting that prM cleavage occurs just before release of mature virions. This cleavage can be inhibited by elevating the pH in intracellular compartments (266,586,644) or by introducing mutations at the basic pr/M cleavage site (175). The cleavage site specificity (101) and biochemical data (667) indicate that furin is the enzyme responsible for prM cleavage. Although inhibiting prM cleavage does not impair virus release, studies on prM-containing particles suggest that furin cleavage (667,757) or a major structural alteration in prM (176) is required to generate highly infectious virus. As discussed above, the current hypothesis is that uncleaved prM prevents E from undergoing an acid-catalyzed conformational change during transit of immature virions through an acidic intracellular compartment. Cleavage of prM by furin and release renders the mature virion ready for acid-catalyzed rearrangements required for productive entry.

More complex interactions are also involved in flavivirus assembly and egress. As mentioned, assembly is coupled to RNA replication (330) and emerging evidence links determinants in NS2A and NS3 to the assembly process, independent of their roles in proteolytic processing and RNA replication (363,417,419). An RNA packaging signal in the flavivirus genome has yet to be identified but the ability of subgenomic replicons lacking the structural region to be packaged in trans (306,331) suggests that an obligate packaging signal does not reside in this region of the genome. Finally, inhibitor and RNA interference studies implicate the Src family kinase c-Yes in WN virion egress from the ER (274). Further studies are needed to define the roles of this and other cellular pathways in flavivirus morphogenesis and release.

Host Resistance to Flavivirus Infection

In the last several years, it has become increasingly clear that positive-strand RNA viruses go to great lengths to inhibit the antiviral action of interferons. As mentioned, flavivirus nonstructural proteins inhibit IFN-induced signaling through the IFN receptor and the JAK-STAT pathway (52,248,307,418,420,421,506,507,638). Also important is the ability of these viruses to prevent the induction of IFN through activation of IFN regulatory factor 3 (IRF-3). As will be discussed for HCV, the toll-like receptor 3 (TLR3) and retinoic-acid-inducible protein I (RIG-I) pathways are designed to sense extracytoplasmic or cytoplasmic structured RNA leading to IRF-3 and nuclear factor-kappaB (NF-κB activation and induction of interferon-β. Studies have demonstrated that, although WNV infection does induce IFN-β (105,203), induction is delayed because of the ability of the replicating virus to avoid early activation of IRF-3 through both RIG-I dependent and independent pathways (202). The ability of flaviviruses to control these intrinsic cellular antiviral response pathways may well explain differences in replication efficiency in cell culture and pathogenesis *in vivo*.

The long search for the murine flavivirus-resistance gene, Flv, uncovered yet another link between flavivirus replication, pathogenesis, and the IFN system (78,728). Beginning with classic studies in the 1920s on innate resistance to flaviviruses, it was recognized that resistance in mice was conferred by inheritance of an autosomal dominant trait. Resistant mice (Flv^r) can be infected with different flaviviruses but spread is impaired and viral tissue titers much reduced. These *in vivo* observations could be recapitulated in cultured cells from congenic resistant and susceptible mice. In 2002, two groups (457,556) independently identified the murine Flv gene as 2′, 5′-oligoadenylate synthetase 1B (Oas1b). OAS1b is an IFN-induced enzyme that is activated by dsRNA, synthesizing 2′, 5′-oligoadenylic acid, which in turn activates the latent ribonuclease, RNase L, leading to nonspecific degradation of RNA (621). The Oas1b allele found in all susceptible mouse strains contained a single C-to-T transition in the fourth exon, leading to a premature stop codon and a truncated form of OAS1b lacking the domain required for oligomerization and enzyme activity. This work identified OAS1b as a key IFN-induced host cell factor controlling flavivirus replication and mortality in mice, presumably via RNaseL-mediated degradation of viral RNA leading to delayed or diminished viral replication (312,542,632). Remarkably, a single nucleotide polymorphism in exon 2 of human OasL was found to be enriched in patients hospitalized with WNV infection (779). This polymorphism is predicted to enhance splicing and expression of OASL, which is enzymatically inactive and might act as a dominant negative inhibitor of active OAS forms.

HEPATITIS C VIRUSES

Background and Classification

As detailed in Chapter 35, hepatitis C virus was identified in 1989 through expression cloning of immunoreactive cDNA derived from the infectious non-A, non-B hepatitis agent (114), which had already become recognized as the

major cause of transfusion-acquired hepatitis. The identification of HCV led to the development of diagnostic tests to screen blood supplies, which dramatically decreased the incidence of posttransfusion hepatitis. Nevertheless, more than 170 million people, roughly 3% of the human population, are infected with HCV (Chapter 35), and virus transmission remains a significant public health concern. Much has been learned about the virus, however, including the biochemical characterization of several critical viral enzymes, the elucidation of high-resolution structural information for more than 40% of the viral proteins, and the development of systems to study all aspects of the viral life cycle.

Hepatitis C virus typically causes persistent hepatotropic infections, although it is challenging to reliably detect viral antigens in infected liver tissues (35). In addition, evidence for replication exists in extrahepatic reservoirs, including peripheral blood lymphocytes (411), gut epithelial cells (152), and the central nervous system (199). Based on the difficulty in detecting HCV *in vivo*, it was originally thought to replicate poorly. Yet mathematical models of HCV dynamics indicate that a chronically infected patient produces approximately 10^{12} virions per day, with a virion half-life of 2 to 3 hours (555).

Hepatitis C virus is the type member of the *Hepacivirus* genus, which also includes GB virus B (710). For historical reasons, this latter virus will be described with the other GB viruses later in this chapter. Hepaciviruses share many features in common with the pestiviruses, including genome organization, a similar mechanism of translational control, and limited sequence relatedness. Based on its evolutionary history as inferred from phylogenetic analysis, HCV is currently classified into six major genotypes, which differ from each other by more than 30% at the nucleotide level, as well as numerous subtypes (655). HCV genotypes show differences in worldwide distribution, transmission, and disease progression. HCV genotype 1, which is the most common, is also the most difficult to treat with current therapies (275). In addition to the six major genotypes, intergenotypic and intersubtype recombinant HCV genomes have also been described (655).

Experimental Systems

Major limitations in the ability to study HCV replication have been the inability to efficiently culture the virus and the lack of small animal models. As will be discussed below, recent progress has been made in both of these areas. Much of the early work on HCV utilized clinical samples to define the physical properties of the virus and examine pathogenesis induced during experimental infection of chimpanzees. The chimpanzee remains the only animal model that reproduces many aspects of HCV infection in humans, including a high rate of viral persistence and the development of HCV-specific innate and adaptive immune responses (reviewed in Chapter 35). Currently, the most useful small animal model for HCV replication is the uPA-SCID mouse bearing human liver grafts (474). Nevertheless, the uPA-SCID mouse remains a technically challenging system with which to work, and does not currently model the human adaptive immune response or HCV pathogenesis.

For more than 25 years, attempts have been made to propagate the non-A, non-B agent, and later HCV, in various cell cultures. Continuous hepatoma, B-cell and T-cell lines, primary hepatocytes from humans and chimpanzees, and peripheral blood mononuclear cells (PBMC) have all been reported to support HCV replication following infection with clinical samples or after isolation of primary cells from chronically infected patients, which has been reviewed elsewhere (36). In some cases, HCV RNA has been detected in these cultures for more than 1 year, although the viral genome levels in these culture systems are extremely low. None of these early culture systems have been sufficiently robust, however, to permit classic virologic, biochemical, or genetic dissection of the virus life cycle. One laboratory described the isolation of a B-cell lymphoma line from a chronically infected patient that seems to express some markers of HCV replication (687). This line, termed SB, reportedly produces virions that are infectious in primary hepatocytes and other B cells, and induces mutations in the host cellular genome via activation of type II nitric oxide synthase and cytidine deaminase (445,446).

Shortly after the HCV genome sequence was completed, the first functional cDNA clones of HCV were constructed and shown to produce RNA transcripts that were infectious by direct intrahepatic inoculation into chimpanzees (348,783). These infectious clones have been used to show that all viral enzymes, the p7 gene, and correct 3′ end are essential for HCV replication *in vivo* (349,620,784). Furthermore, the ability to use genetically defined inocula to initiate clonal infections provided a useful tool to study virus evolution and immune responses to infection (86). Despite their demonstrated utility *in vivo*, infectious transcripts failed to replicate after transfection into cultured cells.

The first broadly useful cell culture system for studying HCV RNA replication was developed in 1999 by Lohmann et al. (429). These investigators engineered selectable subgenomic genotype 1b HCV replicons by replacing most of the structural region in the genome of the Con1 strain with the neomycin-resistance gene (429). The HCV nonstructural genes were expressed in a bicistronic configuration via an internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV). Following RNA transfection into the human hepatoma line Huh-7, rare G418-resistant cells were selected and shown to express HCV proteins and harbor persistently replicating HCV RNA at a copy number of 500 to 5,000 genomes per cell. Thus, the replicon system provides an important tool to study HCV RNA replication in cell culture as well as a functional cell-based system for screening antiviral compounds.

Sequencing of replicon RNA after selection revealed cell culture-adaptive mutations that increase RNA replication

and the rate of Neo-transduction by up to 10,000 times (59). The adaptive mutations that were originally identified were single codon changes in the NS5A gene, although adaptive changes were later mapped to several other nonstructural genes, as reviewed elsewhere (35). Although only limited information is available on how adaptive mutations alter protein function (described later), adaptive mutations have been mapped to surface residues on NS3 and NS5B, suggesting that they modulate protein interactions. Furthermore, adaptive mutations can be grouped into different functional classes. Adaptive mutations in NS4B, NS5A, or NS5B strongly enhance RNA replication on their own but are mutually incompatible with each other, whereas adaptive mutations in NS3 tend to be weaker but can synergize with stronger adaptive changes (35). Initially, efficient RNA replication was limited to the Con1 subgenomic replicon in Huh-7 cells. Subgenomic replicons were subsequently constructed from genotype 1a and 2a isolates, and replicons were found to replicate in additional cell lines, including HeLa, 293, HepG, and even a mouse hepatoma line (35). A particularly interesting replicon is the genotype 2a JFH-1 isolate, which efficiently replicates in several cell types without adaptive mutations (322).

Subgenomic replicons allow RNA replication to be studied at the level of genetics and biochemistry, although these systems only model the intracellular aspects of the virus life cycle. Full-length genotype 1a and 1b genomes bearing adaptive mutations were shown to replicate in cell culture, but virus particles were not produced (60,292,563). Heterologous systems, therefore, were developed to examine the role of HCV glycoproteins in virus entry. These include glycoprotein-dependent fusion assays with cells or liposomes, formation of virus-like particles produced in insect cells, and pseudotyped rhabdoviruses and retroviruses (35,735). As will be described below, retrovirus pseudoparticles expressing HCV-glycoproteins (HCVpp) have proved particularly useful for modeling HCV entry.

Recent progress has led to the production of infectious HCV in cell culture (HCVcc). Although adaptive changes strongly enhance RNA replication in cell culture, full-length genomes containing them were highly attenuated in chimpanzees (90). In cultured cells, transient overexpression of an unadapted genome led to the production of HCV-like particles (267), although infectivity was not reported. Thus, it appears that adaptive mutations that enhance replication in culture might interfere with virus production. Based on this hypothesis, full-length genomes were constructed from the genotype 2a JFH-1 replicon, which does not require adaptive changes for efficient replication. Full-length JFH-1 initially produced low levels of infectious HCVcc in cell culture, but infectivity increased with passage (738,811). Alternatively, a chimeric genome based on JFH-1 and a related genotype 2a strain were constructed and shown to be highly infectious (407). Infectivity was demonstrated by the ability of HCVcc to initiate replication in naïve Huh-7 cells and by the neutralization of this infectivity with antibodies against HCV. Depending on the culture conditions and viral genetic factors, HCVcc grows to titers of approximately 10^5 infectious units/mL (407,562,811). Importantly, HCVcc is infectious in chimpanzees (408,738) and uPA-SCID mice bearing human liver grafts (408). Unlike other clinical isolates of HCV, virus recovered from infected animals is highly infectious in cell culture (408). Curiously, another group recently reported a cell culture-adapted genotype 1a genome that produces infectious HCVcc, albeit with very low infectivity (791). Thus, it remains unclear how adaptive mutations affect virion production. Nevertheless, these genotype 1a and 2a HCVcc systems provide powerful tools to dissect the complete life cycle of HCV.

Structure and Physical Properties of the Virion

Hepatitis C virus particles are estimated to be between 30 and 80 nm, based on filtration and electron microscopic (EM) examination (68,261,799). Unambiguous visualization of HCV particles in patient samples, however, remains a challenge, as previously reviewed elsewhere (411). EM images of HCVcc reveal spherical virions, 50 nm in diameter, with an electron dense core and lacking surface features (Fig. 33.3F).

Hepatitis C virus exhibits an unusually low buoyant density for a small, enveloped RNA virus. Initial measurements in sucrose gradients indicated that infectious HCV has a buoyant density of 1.10 g/mL (67). Furthermore, in highly infectious acute-phase chimpanzee serum, HCV-specific RNA was detected in fractions of low buoyant density, 1.03 to 1.10 g/mL, with less infectious samples exhibiting heterogeneity and increased buoyant densities (271). The low buoyant density of HCV may be related to the ability of the virus to interact with serum lipoproteins. Indeed, HCV RNA-containing particles from patient sera cofractionate with β-lipoproteins in density gradients (20,522,713) and size-exclusion chromatography (574) and can be immunoprecipitated with antibodies against apolipoprotein B (352,522,713) or apolipoprotein AI (352). Furthermore, the heterogeneity and increased particle density observed in some samples may come from immunoglobulin binding and the existence of nonenveloped nucleocapsids (21).

Similar to what has been reported for clinical isolates, the peak of HCVcc RNA has a buoyant density approximately 1.15 g/mL (94,407,738,791,811), whereas the peak infectivity is near 1.10 g/mL (94,407,811). Furthermore, a greater fraction of virus particles recovered from HCVcc-infected animals have a buoyant density of 1.10 g/mL and a higher specific infectivity in cell culture (408). Thus, infectivity of the genotype 2a HCVcc correlates well with particles of a lower buoyant density. This correlation was not observed with a genotype 1a HCVcc (791), which might be due to genotype-specific differences in lipoprotein association (352). The potential interaction with lipoproteins is likely to have important functional consequences for virus neutralization and in virus entry. Clearly, further work is

needed to define the nature of the infectious HCV particle. Although the protein composition of virions has not yet been determined, it is known that HCV encodes a basic C protein and two envelope glycoproteins, which will be described below.

Binding and Entry

A number of candidate HCV receptors have been identified. Although some of these molecules have been shown to participate in HCV entry, no single molecule is sufficient. Thus, HCV is likely to utilize a receptor complex or series of coreceptors that probably includes one or more unidentified liver-specific molecules.

One important receptor component is the tetraspanin family member CD81, which binds tightly to the HCV structural glycoprotein E2 via residues in the CD81 large extracellular loop (268,559,565), and is essential for target cell infection with HCVpp (136,381,804) and HCVcc (94,407,738). It is unlikely, however, that CD81 is a primary attachment receptor, because it is widely expressed in many cell types and HCVpp entry can be inhibited by antibodies against CD81 even after virus adsorption, suggesting that CD81 functions during a postattachment step during virus entry (136). Although this role has not yet been defined, it is interesting to note that related tetraspanins mediate membrane fusion, as has been reviewed elsewhere (395). In addition to its role in virus entry, CD81 can direct the HCV glycoproteins to be secreted in exosomal vesicles compartment (456). Furthermore, it has been suggested that interaction of HCV particles with lymphocyte-expressed CD81 may contribute to the disregulated immune response seen in patients with HCV (142,610, 722).

Increasing evidence indicates that scavenger receptor class B type I (SR-BI) is involved in HCV entry. SR-BI, which is highly expressed on hepatocytes, plays important physiologic roles in the selective uptake of cholesterol and cholesterol esters from lipoproteins. SR-BI can mediate binding of HCV E2 to cultured hepatoma cells, suggesting a role for SR-BI in primary attachment (628). SR-BI, however, also appears to function in a postattachment role. Specifically, high-density lipoproteins can enhance HCVpp entry via an unknown mechanism that requires cellular expression of SR-BI, an intact E2 HVR1 region, and apolipoprotein C1 (37,476,734). Oxidized low-density lipoprotein (LDL), which binds SR-BI in a distinct manner, is a potent inhibitor of HCVcc and HCVpp entry at a postattachment step (737). Furthermore, the ability of SR-BI to enhance HCVpp entry is HCV strain dependent (381).

Other cell surface molecules implicated in binding HCV include C-type lectins, glycosaminoglycans, and the LDL receptor (129). Although the C-type lectins DC-SIGN, L-SIGN, and DC-SIGNR can all bind the HCV E2 glycoprotein, it is likely that they function in disseminating HCV to new host cells rather than in the infection of lectin-expressing cells. The role of LDL receptor is largely based on the interaction between HCV and β-lipoprotein particles. Although this molecule can mediate binding and internalization of HCV, it has not yet been demonstrated that LDL receptor-mediated entry leads to productive infection.

Following attachment, HCV is internalized, presumably via clathrin-mediated endocytosis. Productive entry depends on acidification of the endosomal compartment, most likely to trigger rearrangement of the HCV glycoproteins into their fusogenic form (284,721). Yet, HCVcc particles are resistant to inactivation by low pH, and the time- and temperature-dependent ability of HCVcc particles to undergo acid-mediated entry suggests that interactions at or near the cell surface may prime HCV for low pH activation (721). Whereas flavivirus particles are primed during maturation via the furin-mediated cleavage of flavivirus prM (described above), similar acid-resistance has been observed for pestiviruses. In contrast, low pH alone is capable of inactivating HCVpp (284,535) or inducing HCVpp fusion with cholesterol-rich liposomes (380). These discordant results may reflect structural or compositional differences between pseudotyped retroviral particles and authentic HCV particles.

Genome Structure

The HCV genome is an uncapped, 9.6-kb RNA containing highly structured 5′ and 3′ ends. The 5′ NCR is a well-conserved, 341-nt sequence element that folds into a complex structure consisting of four major domains and a pseudoknot (Fig. 33.7A) (411). The first 120 nt serves as a minimal replication element, although nearly the entire 5′ NCR is needed for efficient RNA replication (208,238,339, 441,601). As will be described later, the 5′ NCR also directs the cap-independent translation of a single large ORF of approximately 3,011 codons.

A number of interesting RNA features have been described within the HCV ORF. Conserved secondary structures have been predicted within the C and NS5B genes based on sequence covariation, observed rates of synonymous substitution, and computerized predictions of RNA secondary structure (725). Mutation of the stem-loop structure within the C gene (428 to 508 nt) demonstrated that it is important for infectivity (469). Furthermore, one of the predicted stem-loop structures in NS5B, 5BSL3.2, was confirmed by biochemical probing and shown to be essential for RNA replication (207,387,795). In addition, the HCV ORF contains fewer UA and UU dinucleotides than expected by chance (254). This is significant because these dinucleotides are the preferred cleavage sites of the IFN-stimulated RNase L. Based on the large-scale computerized folding of numerous positive-strand RNA viral genomes, the HCV ORF is also predicted to contain an unusually high rate of internal base pairing (656). Intriguingly, this feature is common only among viruses that cause persistent infections. Thus, the observed bias in nucleotide

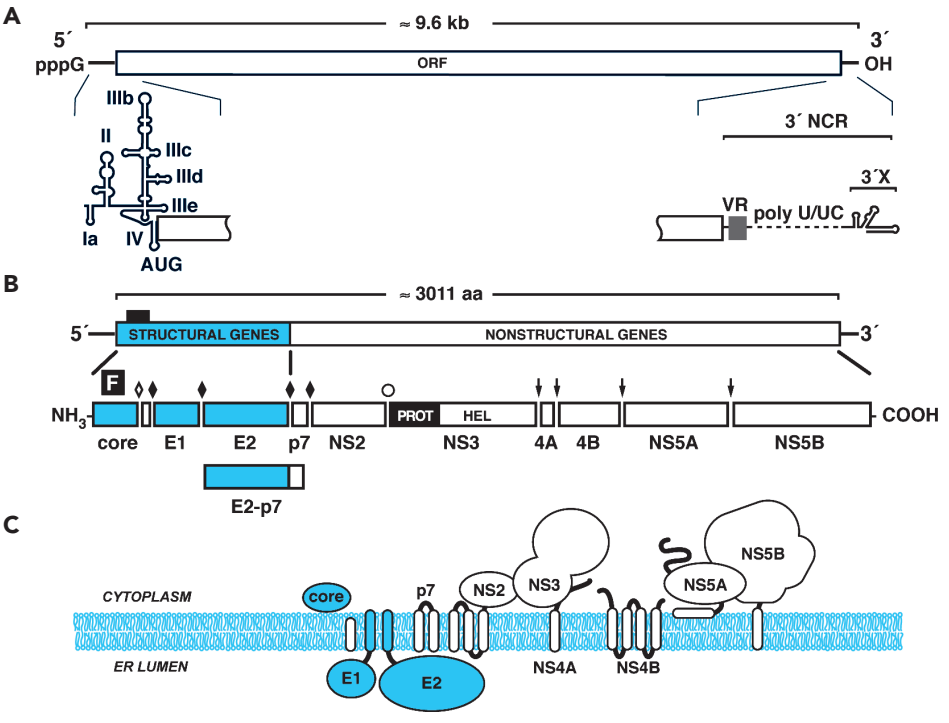

Figure 33.7 Hepacivirus genome structure and expression. **A:** Hepatitis C virus (HCV) genome structure and RNA elements, with important RNA elements indicated as in Figure 33.5. **B:** Polyprotein processing and cleavage products. Cleavage sites are indicated as in Figure 33.5, except the NS2/3 cleavage is mediated by the NS2 cysteine autoprotease (*open bullet*). **C:** Polyprotein membrane topology. See the legend of Figure 33.5 for symbol definitions and the text for further details.

composition and propensity for internal base pairing within the HCV ORF may reflect adaptation to evolutionary pressure exerted by innate antiviral pathways.

The 3′ NCR was originally thought to terminate in polyadenosine or polyuridine. Improved methods for producing 3′ terminal cDNA later revealed, however, that the HCV 3′ NCR actually consists of a short (~40 nt) variable domain and a polyuridine/polypyrimidine (polyU/UC) tract, followed by a highly conserved 98-nt 3′X domain (693). Mutagenesis studies revealed that the 3′X domain is essential for RNA replication and that the polyU/UC tract must be at least 26 nt (206,784,789). Furthermore, RNA replication requires long-distance base pairing between the 3′X domain and the 5BSL3.2 element within the ORF (207). The function of this kissing interaction and whether any trans -acting factors are involved remain to be determined. A number of cellular factors have been found to bind to the HCV 3′ NCR, including RNA binding proteins polypyrimidine-tract binding (PTB) protein, heterogeneous ribonuclear protein C, glyceraldehyde-3-phosphate dehydrogenase, HuR, and La autoantigen, which have been reviewed elsewhere (411). The functional relevance of these interactions remains an ongoing area of investigation.

Translation and Proteolytic Processing

The 5′ NCR of HCV contains an internal ribosome entry site to direct cap-independent translation of the viral genome. The IRES is encoded by 5′ NCR domains II through IV, although flanking sequences can influence IRES activity (35). In the current model of HCV IRES function, free 40S ribosomal subunits directly bind to the 5′ NCR domains IIId–f and adopt an mRNA-bound conformation (536,558,665). The IRES·40S complex then binds the large initiation factor eIF3, and probably also the ternary complex eIF2·GTP·Met-tRNA$_i$, to form a 48S intermediate complex in which the initiating AUG codon at nt 342 is positioned within the ribosomal P-site (302,536). The HCV IRES interacts with eIF3 via determinants in domain IIIb (134) and thereby functionally and structurally mimics the 5′ cap-binding complex eIF4F (660). Following GTP hydrolysis and recruitment of a 60S ribosomal subunit, the 48S intermediate is converted into a translationally active 80S complex (302,536,558). This schema highlights important differences from canonical translation initiation of cellular mRNA. Furthermore, under some conditions, the HCV IRES does not require eIF3 or ternary complex and is capable of directing translation from non-AUG initiation sites (374).

A number of trans-acting cellular factors have been shown to participate in HCV IRES function. The human La protein stimulates IRES activity via binding near the initiator AUG and recruiting the 40S ribosomal subunit (137,576), and inhibiting La activity strongly decreases HCV translation and replication (137,157). Other host factors that stimulate HCV IRES activity include proteosome α-subunit PSMA7 (359) and nucleolin (300). In addition, polycytidine binding proteins 1 and 2 and PTB were shown to bind the 5′ NCR, although their functions in the virus life cycle remain to be defined (35). IRES-mediated translation can be inhibited by long-distance base pairing of 5′ NCR nt 24 to 38 to one of the aforementioned stem-loop regions

(nt 428 to 442) within the core region (340). Interestingly, this part of the 5′ NCR overlaps with the binding site of an abundant and liver-specific microRNA (miRNA), miR-122, which is critical for an early event in RNA replication (308).

Translation of the HCV genome produces a large polyprotein that is proteolytically cleaved to produce ten viral proteins (Fig. 33.7B). The amino-terminal one third of the polyprotein encodes the virion structural components: core (C) protein and glycoproteins E1 and E2. Following the structural region is a small integral membrane protein, p7; it is not yet known whether p7 is a structural protein. The remainder of the genome encodes the NS proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. NS proteins are expressed within infected cells and coordinate the intracellular processes of the virus life cycle.

Mature forms of the HCV proteins arise via co- and posttranslational cleavage mediated by cellular and host-encodes proteases, which has been reviewed elsewhere (35). During structural region processing, cleavages between C/E1, E1/E2, and E2/p7 are mediated by signal peptidase. In addition, mature core protein is released from the E1 signal peptide via signal peptide peptidase. Within the NS region, signal peptidase also mediates cleavage of p7/NS2. The remainder of NS region processing occurs via two virus-encoded proteases. The NS2 cysteine autoprotease mediates cleavage at the NS2/3 junction, whereas the NS3-4A serine protease cleaves at all downstream junctions: NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B. The resulting polyprotein topology is shown in Figure 33.7C.

In addition to proteins encoded by the large ORF, it appears that small protein products can be produced from the +1 reading frame of the core gene during HCV infections (69). At least three different forms of alternative reading frame protein (ARFP) have been described, ARFP/F (frameshift), ARFP/DF (double frameshift), or ARFP/S (short form). It is thought that these products are produced via ribosomal frameshifting, although the use of alternate translational initiation sites may also be involved (69). It is equally unclear what role, if any, ARFP expression has on the virus life cycle. Mutant genomes with a disrupted +1 reading frame are highly attenuated *in vivo* (469). These mutations, however, also disrupt the conserved RNA structure within the C gene, as has been discussed above.

In the discussion that follows, the numbering of amino acids is based on the polyprotein of the first functional cDNA clone, H77 (GenBank accession number AF009606).

Features of the Structural Proteins

C Protein

The first protein encoded by HCV is alternately referred to as C, core, or capsid protein. It is well-conserved, rich in proline and basic residues, and thought to multimerize and bind the HCV genome to form the viral nucleocapsid. Proteolytic processing of C protein occurs in two steps. First, the C/E1 junction is cleaved by host signal peptidase to yield a 191 aa, 23 kd membrane-anchored form of C and the N-terminus of E1 (269). Subsequent cleavage within the C-terminal membrane anchor by signal peptide peptidase liberates a 21-kd mature form of C (468,530,563,622,787). The authentic signal peptide peptidase cleavage site has not yet been determined but likely occurs somewhere between residues 173 and 182 (290,415).

Following removal of the C-terminal membrane anchor, mature C protein consists of a hydrophilic 120 aa N-terminal domain I followed by a hydrophobic ≈50 aa domain II (66). Domain I contains many basic residues and can nonspecifically bind RNA (342,622). Interaction of C protein with the 5′ NCR can inhibit IRES function (650), and C protein can mediate dimerization of the 3′X region (140). In addition, C protein can apparently be phosphorylated in domain I by protein kinases A and C (437). Domain II mediates the association of C protein with membranes and lipid droplets (280) and is important for folding of detergent-solubilized C protein residues 1 to 169 into a compact, soluble dimer rich in α-helix (66). C protein dimers have also been detected during overexpression in Huh-7 cells (438). In the absence of detergents, a slightly longer form of recombinant C protein (residues 1 to 179) can multimerize into a β-sheet-rich structure (366), although it is not yet clear whether these represent native homooligomers or misfolded aggregates (66). Nevertheless, it is very likely that C protein does interact with itself during nucleocapsid assembly, and interacting regions have been mapped in both domains I and II (467). In addition to these homotypic interactions, C protein has been shown to interact with E1 (423) and can assist in its folding (475).

C protein is targeted to the cytoplasmic surface of the ER (500,611), cholesterol-rich detergent-resistant membranes (459), and lipid droplets (30,611). Lipid droplet association requires prior cleavage with signal peptide peptidase (468). It has been proposed that this interaction with lipid droplets may be related to the increased risk of liver steatosis in patients with HCV or the development of steatosis in certain transgenic mice that overexpress HCV C protein (Chapter 35). A few groups have also shown that C protein can localize to mitochondrial outer membranes (639), and to the nucleus. Nuclear localization, however, is not consistently observed and usually involves the overexpression of truncated forms of C, as been reviewed elsewhere (411).

In addition to its role in virus assembly, C protein has been implicated in numerous cellular pathways, including altered signaling, transcriptional control, apoptosis, and cellular transformation (467,708).

Envelope Glycoproteins

The HCV glycoproteins E1 (aa 192 to 383, ≈30 kd) and E2 (aa 384 to 746, ≈70 kd) presumably mediate HCV attachment and membrane fusion. It is believed that these

proteins may behave as a class II fusion protein complex (778), although structural data are lacking. As described above, E2 binds to multiple putative receptors, including CD81. It has also been noted that E1 residues 264 to 290 bear similarity to fusion peptides encoded by the flavivirus and paramyxovirus glycoproteins (194). Both are type I transmembrane glycoproteins, containing a large extracellular domain and a single C-terminal transmembrane domain (Fig. 33.7C). To generate this topology, the E1 and E2 transmembrane domains contain two short (<20 aa) hydrophobic stretches separated by charged residues, and adopt an intramembrane hairpin structure during translocation. Following signal peptidase cleavage, the lumenal C-termini reorient to face the cytoplasm, yielding tandem ER-resident proteins each with a single membrane anchor (127). Furthermore, these charged transmembrane domains mediate E1-E2 heterodimerization (533) and ER retention of the glycoprotein complex (125,126).

The ectodomains of E1 and E2 are heavily N-glycosylated (up to 5 and 11 sites, respectively) and contain multiple disulfide-linked cysteines. Glycoprotein folding, oxidation, and maturation take place via a slow, cooperative process involving ER-resident chaperones (35,735). Productive folding leads to the formation of noncovalently linked E1-E2 heterodimers that are protease resistant, no longer associated with chaperones, and display conformational epitopes that are also present on HCV virions (128,584). Extensive interactions form between the E1 and E2 ectodomains during folding of the native heterodimer (790). In addition, the membrane proximal linker region of E2 contains a heptad repeat (residues 675 to 699) that is important for heterodimerization and HCVpp entry (159). E2 folding is rapid; the rate-limiting step in heterodimer formation appears to be proper folding of E1, which requires coexpression of C, E2, or both, as has been reviewed (735). Other results, however, suggest that E1 assists in the folding of E2 (72). Nevertheless, when the E2 ectodomain (HCV residues 384 to 661) is expressed on its own, it is partially secreted in a monomeric form that is capable of binding CD81 and several conformation-specific antibodies, albeit with lower affinities than the native form of E2 (263,487,537). Thus, the E2 ectodomain is capable folding independently of other HCV proteins.

Characterization of the HCVpp-associated glycoproteins revealed that the N-linked glycans of E1 remain in the high mannose form and those of E2 are modified, suggesting that these proteins are differentially exposed during transit through the Golgi apparatus (193,535). Glycosylation of E1 depends on E2 coexpression and is important for proper E1-E2 heterodimer formation (160,477). Glycosylation is also important for the proper folding and secretion of E2 (662) and for the assembly and release of HCVpp (223). Interestingly, mutagenesis of E2 glycosylation site Asn-423 or Asn-448 still leads to the production of HCVpp, although their infectivity is blocked at the level of virus binding or entry (223).

The HCV glycoproteins are important humoral antigens that can be protective or lead to virus neutralization (Chapter 35). Antibody competition studies suggest that the E1 and E2 ectodomains contain two or three immunogenic domains, respectively (85,325,389,594). It is not yet clear how these immunogenic domains correspond to structural domains, although epitopes in E2 differ in their ability to neutralize HCVpp infectivity and in their conformation under low pH, suggesting that they exhibit functional and structural differences (324).

A major antigenic determinant of neutralizing antibody response is hypervariable region 1 (HVR1), encoded by the N-terminal 27 residues (aa 384 to 410) of E2 (321,753). HVR1-specific antibodies can protect chimpanzees from infection with HCV encoding the immunizing HVR1 (189), and early induction of HVR1-specific antibodies correlates with viral clearance in humans (11,816). HVR1 exhibits a high level of sequence variability even within a single patient, largely driven by immunogic selection (188,463,497,754). Despite this high level of diversity, HVR1 appears to play an important role in E2 function. Clear constraints on HVR1 heterogeneity exist, including a few conserved positions and an overall tendency to retain basic residues (96,272,463,553). Although an HCV genome with an HVR1 deletion was infectious in chimpanzees, the resulting virus was highly attenuated (198). This phenotype may reflect a defect in virus binding and entry, because HVR1-deleted E2 binds poorly to SR-BI (628) and HCVpp with mutated or deleted HVR1 have lower SR-BI–mediated and HDL-enhanced infectivity (37,38,96).

Beyond its role in HCV entry and as an important antigen, E2 may have additional functions. One group reported that E2 overexpression inhibits eIF2α phosphorylation by the dsRNA-activated protein kinase (PKR) or the ER-stress signaling kinase PERK (545,705).

p7 Protein

As stated above, it is not yet known whether p7 is virion associated. p7 is a small (aa 747 to 809, 7 kd) hydrophobic protein predicted to span the membrane twice, with ER-lumenal N- and C-termini and a short, positively charged cytoplasmic loop. Incomplete or delayed processing by host signal peptidase between E2, p7, and NS2 can lead to the production of E2-p7 and E2-p7-NS2 (368,403,493,563,641), although p7 processing appears to be complete *in vivo* (521). The localization of p7 is not yet clear, but overexpression of an epitope-tagged p7 lacking the upstream signal sequence from E2 has been localized to the ER and mitochondria (241).

The p7 protein is essential for infectivity *in vivo* (620). Chimeric genomes containing heterologous p7 genes indicate that it functions in a genotype-specific way and, therefore, likely interacts with other viral proteins. As subgenomic replicons do not include p7, it is not necessary for RNA replication (429). *In vitro*, p7 can multimerize and form cation-conductive membrane channels that are

inhibited by amantadine, iminosugar derivatives, and hexamethylene amiloride (242,546,571). It is not yet clear what role this activity plays in the HCV life cycle, although other viruses express similar viroporins that participate in virus release and entry.

Features of the Nonstructural Proteins

NS2 Protein

The NS2 (aa 810 to 1,026, 23 kd) is a membrane-spanning protein that contains a C-terminal cysteine protease domain (235,270,433). The membrane topology of NS2 has been a subject of recent debate. As described above, the N-terminus of NS2 is produced by signal peptidase cleavage, indicating that it is oriented toward the ER lumen. Yet *in vitro* translation in the presence of microsomes or overexpression in cell culture indicates that NS2 spans the membrane four times, resulting in ER-lumenal N- and C-termini (623,780). This topology is at odds, however, with the cytoplasmic localization of NS3, which is required for NS2-3 cleavage. Recent structural studies indicate that the fourth putative transmembrane domain in NS2 may actually be cytoplasmic, lying parallel to the plane of the lipid bilayer (433).

The C-terminal domain of NS2 (aa 907 to 1,026) contains the active site residues (His-952, Glu-972, and Cys-993) of a cysteine protease (235,270,433). The only known function of this enzyme is to cleave the NS2/3 junction. Autoprotease activity is remarkably resistant to single amino acid substitutions around the cleavage site, but requires expression of the N-terminal domain (aa 1,027 to 1,207) of NS3 (235,270,597). Although this includes the serine protease domain of NS3 (described below), NS2/3 cleavage does not require an active NS3 protease. The NS2/3 autoprotease is stimulated *in vitro* by the addition of microsomal membranes, detergents, and Zn^{2+}, which all contribute to the proper folding of NS2 and NS3 (35). It is not yet clear how the N-terminal region of NS3 regulates NS2/3 cleavage, but it should be noted that cleavage is inhibited by NS4A, which allows the N-terminal domain of NS3 to fold into the compact serine protease structure (145).

The structure of the C-terminal domain, which has been recently solved by x-ray crystallography, confirms that NS2 is a cysteine protease with the active site geometry of a serine protease (433). The C-terminus of NS2 is bound in the substrate-binding pocket, suggesting that protease activity will be blocked following NS2/3 cleavage. Interestingly, the cysteine protease domain homodimerizes, leading to the formation of two composite active sites at the dimer interfaces. This architecture reveals a mechanism that can control polyprotein processing, and explains the ability of some mutant NS2/3 molecules, inactivated by distinct mutations, to reform an active autoprotease in trans (235,597). Mutagenesis studies demonstrate that NS2/3 cleavage is required for HCV replication in cell culture and infectivity *in vivo* (349,755). Although uncleaved NS2/3 retains serine protease activity, it is rapidly degraded by the proteasome (235,270,755).

NS2 has an important, albeit unclear, role in producing infectious virions. Chimeric HCVcc genomes were found to produce the highest level of infectivity when genome fragments are fused between the first and second transmembrane domains of NS2 (562). Interestingly, a naturally occurring intergenotypic recombinant genome also contains a cross-over junction within NS2 (313). Thus, the N-terminal region of NS2 may interact with the structural proteins and p7, whereas downstream sequences in NS2 may interact with other NS proteins. Indeed, NS2 has been reported to interact with E2 (641) as well as other NS proteins (155,333,414,597).

A few cellular proteins also interact with NS2. NS2 binds to and inhibits the cellular proapoptotic molecule CIDE-B (180). NS2 can be phosphorylated on the conserved Ser-977 by casein kinase 2, leading to the rapid degradation of NS2 by the proteosome (200). Furthermore, overexpression of the N-terminal region of NS2 has been reported to downregulate host cellular transcription (162).

NS3 Protein

The HCV NS3 (aa 1,027 to 1,657, 70 kd) is a multifunctional protein, containing an N-terminal serine protease domain and a C-terminal RNA helicase/NTPase domain. Both enzyme activities are critical for viral replication, biochemically well characterized, and several high-resolution structures of NS3 have been solved (35,411).

For complete folding and enzyme activity, the serine protease domain of NS3 (aa 1,027 to 1,207) requires interaction with NS4A (34,183,404), as well as coordination of a structural Zn^{2+} ion. The serine protease domain is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites (671). The serine protease domain first cleaves the NS3/4A site in cis, whereas subsequent cleavages at downstream sites occur in trans with the preferred order NS5A/B > NS4A/B > NS4B/5A. Analysis of cleavage products has revealed that these cleavage sites are well conserved and conform to the sequence (Asp/Glu)XXXX(Cys/Thr)/(Ser/Ala). In addition, NS3 has been shown to cleave within the NS3 helicase domain, NS4B, and NS5A (35).

The HCV serine protease is similar to other chymotrypsinlike proteases, with active site residues (His-1083, Asp-1107, and Ser-1165) and a substrate-binding surface located in a cleft between two β-barrel domains (671). The first β-barrel domain includes an intercalated peptide from the central region of NS4A. In the absence of the NS4A cofactor, the N-terminal 28 residues of NS3 remain flexible (466) and NS3 is rapidly degraded (772). In addition to stabilizing the serine protease fold, NS4A also serves to anchor this enzyme complex to cellular membranes via its hydrophobic N-terminal domain (772). Interestingly, the structure of a single-chain, full-length NS3 shows the C-terminus coordinated by the serine protease, as would be

expected from the cis-cleavage reaction (786) (Supplemental Fig. 33.1B).

The structure of the NS3-4A serine protease reveals an unusually shallow and hydrophobic substrate-binding surface, with the S1 pocket partially occluded by NS3 residue Phe-1180, (411,554). Thus, preference for Cys at the P1 position of the NS4A/4B, NS4B/5A, and NS5A/5B cleavage sites can be understood on the basis of interactions that likely occur between the sulfhydryl of cysteine and the aromatic ring of phenylalanine (566). Additional substrate-binding energy comes from a network of interactions involving the P6 to P4′ positions. As a result of this substrate recognition mode, N-terminal cleavage products are slowly released from the substrate-binding surface and can inhibit subsequent catalysis (672). The discovery of this product-based inhibition led to the development of peptidomimetic compounds that potently inhibit NS3-4A serine protease activity, NS protein processing, and HCV replication (147,515). Interestingly, the preference for threonine at the P1 position of the NS3/4A cleavage site appears to reflect the selection of a substrate that is easily released following autocleavage (744,786).

In addition to its role in HCV polyprotein processing, NS3-4A serine protease activity antagonizes innate antiviral defenses by blocking activation of the transcription factors IRF-3 and NF-κB, as has been reviewed elsewhere (304) and (Chapter 35). This is accomplished in two ways. First, NS3-4A cleaves and inactivates the mitochondrial outer membrane protein alternately known as IPS-1, MAVS, VISA, or Cardif (397,486). IPS-1 transduces signals from RIG-I and MDA5, sensors of cytoplasmic dsRNA that lead to IRF-3 and NF-κB activation. This is important, because RIG-I activation has been shown to inhibit HCV replication (684), and a dominant negative mutation in RIG-I renders an Huh-7 subline highly permissive for HCV RNA replication (61,684). In addition, NS3-4A cleaves TRIF, a molecule that transduces signals from TLR3 leading to IRF-3 and NF-κB activation (397). TLR3 is a transmembrane Toll-like receptor that recognizes dsRNA located in lumenal and extracellular spaces. Thus, viral proteolysis of these cellular substrates subverts cellular antiviral defenses.

Similar to other *Flaviviridae*, the C-terminal domain of HCV NS3 (aa 1,209 to 1,657) encodes a superfamily 2 RNA helicase/NTPase. These enzymes utilize the energy derived from NTP hydrolysis to translocate along and unwind double-stranded nucleic acids (97). Although the precise role of the NS3 helicase is not yet known, helicase activity has been shown to be essential for HCV RNA replication and viral infectivity (349,373). Although the helicase has lagged behind other targets for HCV drug development, inhibitors of this activity have recently been described (204).

The HCV helicase domain contains two parallel α-β subdomains with active site residues lying at their interface (97) (Supplemental Fig. 33.1D). NS3 also contains a less-conserved C-terminal subdomain rich in α-helices. Cocrystallization of the NS3 helicase domain with 8- or 16-nt oligodeoxyuridine revealed a single-stranded DNA (ssDNA)-binding cleft formed between the first two subdomains and subdomain 3 (335,451).

The mechanism of helicase action is incompletely understood and an area of intense interest. NS3 has been shown to unwind RNA and DNA homo- and heteroduplexes by binding to an unpaired region of a template strand and translocating in a 3′ to 5′ direction (35). NS3 preferentially hydrolyzes adenosine triphosphate (ATP) to power this reaction, but can utilize any standard NTP or deoxynucleoside NTP (dNTP). NTPase activity is coupled to RNA binding, and is particularly stimulated by polyuridine or polydeoxyudridine (35). Recent kinetic studies have elegantly shown that the NS3 helicase domain progressively rips apart large duplex segments, up to 18 base pair (bp), in a step-wise fashion (161,393,394,642).

A number of protein–protein interactions have been shown to influence HCV helicase activity. Chief among these is dimerization of the helicase domain, which facilitates cooperative unwinding of long templates (394,426,451,642). Furthermore, optimal helicase activity on dsRNA templates requires full-length NS3-4A, implying that the serine protease and helicase activities may be coordinated in their physiologic state (246,283,360,540). Helicase activity of full-length NS3 may also be upregulated by NS5B, presumably by interacting with the serine protease domain (803). Likewise, the NS3 helicase activity facilitates NS5B-mediated RNA synthesis (561). Helicase activity can be downregulated by the cellular enzyme PRMT1, which methylates one or more arginines within the helicase motif VI involved in coordinating NTP (163,604). Interestingly, this same region of NS3 is also the target of an unknown cellular protease, which presumably destroys NTPase and helicase activities by proteolysis (652). In addition, many cell culture-adaptive mutations have been mapped to residues on the surface of the helicase domain and to the region linking the protease and helicase (35), suggesting that these are critical sites of protein interaction or conformation.

NS4A and NS4B Proteins

At 54 residues (aa 1658–1711, 8 kDa), NS4A is the smallest NS protein. Nevertheless, it has multiple functions in the virus life cycle. As described above, the central region of NS4A (aa 1678–1691) is a cofactor of the serine protease by completing the fold of the protease domain. Furthermore, NS4A facilitates recognition of RNA substrates by the full–length NS3 protease/helicase (540). The NS3–4A complex is anchored to cellular membranes via the hydrophobic N-terminal region of NS4A (695,772). NS4A can physically interact with NS4B, NS5A and uncleaved NS4B-5A (26,155,405). The C-terminal region of NS4A (aa 1697-1711) contains a large number of conserved acidic residues and is predicted to form an α-helix. This region has been implicated in NS4A's ability to promote NS5A hyperphosphorylation (314,344).

NS4B (aa 1712-1972, 27 kDa) is an integral membrane protein containing four central transmembrane domains separating cytoplasmic N- and C-terminal regions (172,287,439). However the membrane topology of NS4B is not yet fully understood, as the N-terminal region can also interact with membranes (172,439).

NS4B is likely to play a critical role in organizing the membrane-bound replication complex. Expression of NS4B is sufficient to induce the formation of the "membranous web" structures characteristic of HCV RNA replication complexes (167,233). In addition, overexpression of NS4A, NS4B, or NS4A-4B has been reported to induce an ER stress-mediated unfolded protein response, reduce ER-to-Golgi traffic, inhibit protein synthesis, and cause cytopathic effects (195,287,320,350,810).

Consistent with an important role in RNA replication, a number of cell culture-adaptive mutations have been mapped to NS4B (reviewed in [35]). NS4B also encodes a GTPase activity that is reportedly critical for RNA replication (169). However further work is needed to understand its function, as the putative GTP binding site is disrupted by some adaptive mutations that enhance replication (35). NS4B has also been reported to inhibit the RNA polymerase activity of NS5B (561).

The NS5A Protein

NS5A (aa 1,973 to 2,420, 56 to 58 kd) is a phosphoprotein with an important yet unclear role in RNA replication. NS5A localizes to active replication complexes (233,501), and a number of cell culture adaptive mutations that strongly enhance RNA replication have been mapped to NS5A (35). NS5A is a G/U-rich RNA binding protein with a high affinity for the polypyrimidine tract in the HCV 3′ untranslated region (UTR) (285). Although the precise role of NS5A in RNA replication is not yet known, it has been shown to interact with NS5B and inhibit its RNA polymerase activity (155,651). Mutations in NS5A that block these interactions are detrimental for RNA replication (648).

NS5A is a multidomain protein, containing an N-terminal Zn^{2+}-binding domain (aa 1,973 to 2,186), a central domain (aa 2,223 to 2,315) that may be helix-rich, and a natively unfolded C-terminal domain (aa 2,329 to 2,420) (706). Separating these domains are two flexible regions of low complexity sequence (LCS). The N-terminus of domain I contains an amphipathic helix that mediates the peripheral membrane association of NS5A (71,171). The structure of this membrane anchor was recently confirmed by NMR (552). X-ray crystallography of domain I residues 2,009 to 2,171 revealed a novel protein fold, a unique Zn^{2+}-binding motif, and a pair of disulfide-linked cysteines (707) (Supplemental Fig. 33.2B). Domain I forms a homodimeric claw, with a basic groove that is postulated to bind RNA. A highly conserved surface on the outside of the claw overlaps with one of the regions critical for NS5B binding (648). Structural information is not yet available for domains II or III. It is known, however, that domain III is poorly conserved and tolerant of large deletions and insertions (24,285,416,465,501).

NS5A is phosphorylated on multiple serine residues by cellular kinases, and is typically found in hypophosphorylated (56 kd) and hyperphosphorylated (58 kd) forms. Nevertheless, biochemical experiments indicate that NS5A can be phosphorylated by casein kinase II or a related member of the CMCG kinase family (286,291,334,599). In addition, a genome-wide screen for yeast kinases that phosphorylate NS5A *in vitro* identified homologs of the mammalian enzymes AKT, MEK1, MKK1, and p70S6K as putative NS5A kinases (130).

The identity of the relevant phophoacceptor sites is unclear. Mutagenesis of genotype 1b strain HCV-J indicated that basal phosphorylation involves aa 2,200 to 2,250 and 2,351 to 2,420 (696). Furthermore, Ser-2197, Ser-2201, and Ser-2204 are important determinants for NS5A hyperphosphorylation (696), although it is not clear whether these sites are actually phosphorylated. The major sites of NS5A phosphorylation have been biochemically mapped to the highly conserved Ser-2194 in genotype 1b strain BK (323) and to the nonconserved Ser-2321 in genotype 1a strain H77 (598). Thus, basal phosphorylation appears to target phosphoacceptor sites in domains II and III, whereas hyperphosphorylation sites may be clustered in LCS-I. For unknown reasons, hyperphosphorylation of NS5A requires coexpression of NS3-5A in cis (344,517), interaction with NS4A (26,314), or both.

Mutagenesis studies indicate that basal phosphorylation of NS5A is dispensable for RNA replication in cell culture (24,285). Furthermore, an inverse correlation is found between the levels of hyperphosphorylation and RNA replication. Highly adaptive mutations (e.g., Ser-2204-Ile in NS5A or Lys-1846-Thr in NS4B) tend to decrease the level of NS5A hyperphosphorylation (24,59). Pharmacologic inhibition of NS5A hyperphosphorylation increases RNA replication and bypasses the need for cell culture adaptive mutations (518). Surprisingly, these drugs inhibit replicons that contain adaptive mutations such as Ser-2204-Ile. One interpretation of these seemingly disparate results is that at least some level of phosphorylation is needed to initiate replication. One known effect of hyperphosphorylation is that it decreases interaction between NS5A and hVAP-A a SNARE-like vesicle sorting protein that may help to organize the HCV replication machinery (182). hVAP-A interacts with NS5A, NS5B, and localizes to putative sites of RNA replication (217,724). The importance of hVAP-A in HCV replication has also been confirmed by small interfering RNA (siRNA)-mediated knockdown and overexpression of a dominant-negative mutant form of this protein (217,805).

In addition to hVAP-A, numerous cellular interaction partners have been described for NS5A (603,708). Although the biology underlying some of these observations is not yet clear, a few particularly intriguing examples have

been noted. For instance, NS5A was shown to interact with the geranylgeranylated cellular protein FBL2 (741). Replication of genotype 1b subgenomic replicons is strongly inhibited by pharmacologic inhibition of protein geranylgeranylation, siRNA-mediated knockdown of FBL2, or expression of a dominant-negative form of FBL2 (317,741,788). Because FBL2 contains an F-box motif, it is likely to be involved in targeting specific proteins for ubiquitylation and degradation, although the identity of a relevant substrate is not yet known.

A number of studies suggest that NS5A is involved in resistance to IFN treatment (411). According to one line of evidence, treatment effectiveness correlates with high mutation rates in NS5A residues 2,209 to 2,248, a region termed the interferon sensitivity determining region (ISDR) (177,178). Furthermore, the ISDR was found to mediate interaction with, and inhibition of, the dsRNA-activated kinase PKR (211–213). The correlation between ISDR sequence change and IFN resistance, however, has not yet been confirmed in other studies (547), and this region of NS5A was shown to be dispensible for HCV RNA replication in cell culture (59,416). Moreover, it appears that mechanisms beyond PKR-mediated control of translation are critical for innate control of HCV replication (201,560). Nonetheless, it remains possible that NS5A contributes to IFN resistance via its reported ability to induce IL-8 expression (569), which can antagonize type I interferon responses, as has been reviewed elsewhere (603).

Overexpression of NS5A has been reported to induce a number of effects in cells, including oxidative stress; activation of signaling pathways, including STAT-3, PI3K, and NF-κB; and altered transcriptional regulation (227,262,681). Other reported NS5A interaction partners include apoliporotein A1, the major protein found on HDL; p53, a tumor suppressor; Grb-2, an adaptor protein involved in mitogen signaling; SRCAP, an adenosine triphosphatase (ATPase) that activates cellular transcription; karyopherin β3, a protein involved in nuclear trafficking; Cdk1/2, cyclin-dependent kinases that regulate cell cycle control; and Fyn, Hck, Lck, and Lyn, Src-family kinases (35,411,443,603,708).

NS5B

NS5B (aa 2,421 to 3,011, 68 kd) is the major enzyme of HCV RNA replication, the RdRP. This protein has been extensively characterized biochemically and structurally, and is a major target for development of HCV-specific antivirals. In addition, NS5B is a tail-anchored protein, containing a 21-aa C-terminal hydrophobic tail (781) that post-translationally inserts into the ER membrane (299,636). Mutations that interfere with this membrane association destroy RNA replication (390,499). Nevertheless, tail anchor deletion mutants retain RdRP activity and permit the efficient expression and purification of soluble, active, recombinant NS5B for biochemical and structural studies (781).

Similar to other polymerases, NS5B has a right hand structure, with distinct finger, palm, and thumb domains (6,75,391) (Supplemental Fig. 33.2C,D). HCV NS5B first revealed the closed structure of RdRP, containing extensive contacts between the finger and thumb domains surrounding a preformed active site. Subsequent studies showed that the closed, active structure is capable of opening into an inactive form via movement of the thumb domain (55). Conserved RdRP motifs and catalytic residues are primarily located in the palm domain and serve to properly align the RNA template strand, NTP substrates, and two divalent cations that catalyze nucleotide transfer (reviewed in [730]). Structures of NS5B in complex with divalent cations and NTP revealed an active site geometry remarkably similar to human immunodeficiency virus (HIV) reverse transcriptase (an RdDP) and the RdRP of the dsRNA bacteriophage ϕ6 (74,527). In addition, a low-affinity GTP binding pocket was identified 30 Å from the active site at the interface of the thumb and finger domains (74). Mutation of the low-affinity GTP binding site demonstrated that it is dispensable for RdRP activity *in vitro*, but critical for RNA replication in cell culture (93,589). RNA templates slide into a hydrophilic groove within the finger domain (338,527). A β-hairpin that protrudes from the thumb into the active site cavity is likely to be involved in correct positioning of the template 3′ end (279,527,589). This structure may act as a flap that is displaced during RNA synthesis to allow the dsRNA product to exit the polymerase core. Furthermore, a C-terminal regulatory loop wraps around the thumb and inserts into the active site, decreasing RNA binding and RdRP activity (392,589).

NS5B has been shown to elongate annealed primers or self-priming copy-back templates (48). RNA synthesis utilizes the divalent cations Mg^{2+} or Mn^{2+} to catalyze nucleotide incorporation at a rate of 150 to 200 nt/min (431,528). NTP analogs containing 2′C-methyl groups are potent chain terminating RdRP inhibitors of HCV and other *Flaviviridae* (147). As the purine 2′ position is not involved in catalysis, these compounds may impose steric constraints within the catalytic core. Resistance to these inhibitors is easily acquired in HCV by mutation of Ser-2703 to Thr, although this mutation in the NTP binding pocket has some adverse effects on RNA replication (488).

In vivo, HCV RNA synthesis is thought to initiate *de novo* (i.e., without a primer) (440,591,815). As for other RNA polymerases, NS5B initiates *de novo* with a purine nucleotide, which can be mono-, di-, or triphosphorylated (588,589,647). *De novo* initiation is destroyed by mutations that affect GTP binding in the catalytic core but do not disrupt NTP incorporation (593). Additional structures within NS5B that control *de novo* initiation versus primed synthesis include the β-hairpin and C-terminal regulatory loop, which limit dsRNA binding (110,392,589). RNA templates that are efficiently used for *de novo* initiation, at least *in vitro*, contain limited secondary structure and an unpaired 3′ end (316). NS5B can also utilize circular RNA templates

for *de novo* initiation, suggesting that a free 3′ end is not absolutely required and that RNA is loaded when NS5B is in the open conformation (590). Nevertheless, the above template requirements are notably different from the natural site of HCV minus-strand initiation, the 3′ NCR, which terminates with uridylate base paired in a stable stem-loop. When the HCV 3′ NCR is used as a template in *de novo* initiation reactions, only internally initiated minus strands are produced (316,336,529,686). Addition of a few unpaired 3′ nt, however, leads to the production of template-length minus strand products (529). Thus, authentic initiation of HCV minus strand synthesis may depend on the local unwinding of 3′ secondary structures, perhaps by the NS3 helicase.

NS5B RdRP activity is strongly dependent on higher-order interactions. Important contacts between NS5B domains were revealed through the study of non-nucleoside RdRP inhibitors (NNI), which can allosterically block NS5B activity (55,154,245,435,717,718,742). In addition to intramolecular interactions, oligomerization of NS5B leads to cooperative stimulation of RNA synthesis (581,743), and the NS3 helicase enhances primed RNA synthesis activity (561). As mentioned, both NS4B and NS5A inhibit RNA synthesis (561,651). Nevertheless, mutations in NS5A that inhibit interaction with NS5B are detrimental for RNA replication (648). Based on mutagenesis studies, it appears that NS5B residues involved in NS5A binding reside primarily on the back of the thumb and inner surface of the fingers (581).

Several host factors have been found to bind NS5B, some of which influence RdRP activity. One cellular factor shown to be critical for RNA replication is the proline cis-trans isomerase cyclophilin B (749). Although it is not yet known how this molecule enhances replication, it binds to the C-terminal 70 aa of NS5B. As described above, this region includes the C-terminal regulatory domain that interferes with template binding. Interestingly, interaction of cyclophilin B and NS5B is inhibited by cyclosporin A, which was previously shown to inhibit HCV replication (748). This suggests that nonimmunosuppressive analogs of cyclosporin A could be used to inhibit HCV replication *in vivo* (538). Cyclosporin analogs, however, seem to have the greatest effect on genotype 1b replication, with only modest effects on genotype 2a (295). Phosphorylation of NS5B by the cellular serine kinase PRK2 was reported to enhance HCV RNA replication (337). NS5B also interacts with the vesicle-sorting proteins hVAP-A and hVAP-B, which bind NS5A (253,724). Overexpression of hVAP-B increases RNA replication, whereas knockdown of this molecule decreases NS5B stability (253). Another cellular binding partner that affects NS5B turnover is hPLIC1, a protein that can couple the ubiquitylation machinery to the proteasome (218). Overexpression of hPLIC1 increases the turnover of NS5B and decreases RNA replication. Regulated turnover of NS5B may also be related to decreased levels of the retinoblastoma tumor suppressor, which remarkably appears to bind to the RdRP active site of NS5B (505). In addition, NS5B appears to interact with two nuclear proteins, nucleolin and the p68 RNA helicase (224,273). The functional consequences of these interactions remain unclear.

In addition to template-directed RNA synthesis, NS5B can also add one or a few untemplated nts to the 3′ end of an RNA substrate (48,587,647). It should be noted that several reports did not detect this terminal nucleotide transferase (TnTase) activity or showed that a cellular TnTase copurified with NS5B (319,428,528,781). Nevertheless, TnTase activity of a highly purified NS5B preparation was shown to depend on RdRP active site residues (587,593). Moreover, NS5B TnTase activity can convert an RNA lacking a 3′ initiation site into a useful template for *de novo* initiation (587). Thus, TnTase activity may be important for maintaining genome integrity.

RNA Replication

Translation and RNA replication are mutually exclusive processes, because they proceed in opposite directions on a given RNA template. Positive-strand RNA viruses, therefore, regulate the rate of genome translation versus replication. Assuming that an HCV particle is likely to resemble other members of the family *Flaviviridae*, approximately 200 copies of the structural proteins must be made for each nascent genome that is produced and packaged. Indeed, subgenomic replicon-bearing cells produce ≈1,000-to-1 molar ratio of viral proteins to viral RNA (583). Thus, translation of the HCV genome occurs more frequently than replication. One way this could be regulated is via crosstalk between the determinants that control translation and genome replication. For instance, the cellular PTB protein binds to the HCV 5′ NCR and core coding region where it may modulate IRES activity (23,298,716), and to the 3′ end where it may repress replication (298,723). Similarly, La protein was shown to bind to both HCV NCR (see above). For the related pestiviruses, several NF/NFAR proteins were shown to bind the 5′ and 3′ NCR and regulate genome circularization, and might also be involved in regulating HCV translation versus replication (296,297). It has been suggested that HCV translation can be autoregulated through product inhibition: low levels of HCV core protein can enhance HCV IRES-mediated translation, whereas high concentrations inhibits HCV translation (65,806). Finally, it is interesting to note that polycytidine-binding protein 2 (PCBP-2) binds to the HCV 5′ NCR (210,666). PCBP-2 interacts with the 5′ NCR and RdRP of another positive-strand RNA virus, poliovirus, to control the switch between translation and replication (216).

As for all positive-strand RNA viruses, the HCV genome is recruited out of translation and into a membrane-associated replication complex, or replicase. Specifically, NS proteins and HCV RNA associate with a dense

perinuclear matrix of ≈85-nm vesicles termed the membranous web (167). The membranous web is the site of RNA replication, containing HCV NS proteins and nascent RNA (170,233,501). Several studies have shown that the membranous web is likely to be derived from the rough ER (35), although one group has reported that these membranes are insoluble in nonionic detergents and may be Golgi-derived intracellular membrane rafts (7,646). Regardless of their origin, formation of the membranous web is induced by NS4B expression (167), and may activate ER stress pathways (697). The process of HCV replication also induces the expression of genes involved in lipid metabolism, including ATP citrate lyase and acetyl-CoA synthetase (317,683). Furthermore, HCV RNA replication is stimulated by increased availability of saturated and monounsaturated fatty acids, and inhibited by polyunsaturated fatty acids or inhibitors of fatty acid synthesis (317). These results suggest that membrane fluidity is important for the function of the membranous web. In addition, altering cholesterol metabolism pharmacologically can lead to the disassembly of the replicase and strongly inhibit RNA replication, primarily because of the reduced geranylgeranylation of FBL-2, which interacts with NS5A (317,788).

The HCV replicase can be accessed biochemically by using permeabilized cells, cell lysates, or membrane preparations isolated from HCV replicon-bearing cells (7,10,258,371,491,583,785). These *in vitro* systems allow the study of RNA elongation using endogenous templates but they do not accept exogenous templates. Nevertheless, the sensitivity of RNA synthesis to heparin (258) and the pulse-chase metabolic labeling of ssRNA into dsRNA (371) suggest that at least a limited amount of *de novo* synthesis can occur *in vitro*. Furthermore, HCV RNA is protected from nuclease degradation by a detergent-sensitive membrane (7,491,583,785), suggesting that RNA synthesis may be enclosed within the membranous web. Similarly, replicase activity is insensitive to protease digestion unless solubilized by detergent, whereas most of the NS proteins are digested by this treatment (491,583). These data support the hypothesis that active replicase is bound by a limiting membrane and demonstrate that a vast excess of NS proteins are produced. This enclosed replicase presumably includes a channel for the exchange of NTP with nascent RNA and pyrophosphate, similar to the spherule structures proposed for other positive-strand RNA viruses (583).

As for other *Flaviviridae*, HCV RNA replication initiates with the synthesis of a genome-length, negative-strand RNA. Similar to what has been described for the flaviviruses and pestiviruses, negative strands are found in partially double-stranded replicative intermediates or fully double-stranded replicative forms (10). Negative-strand RNA then serves as a template for multiple rounds of nascent, positive-strand synthesis, leading to the asymmetric accumulation of nearly 10 positive-strands for every negative strand (7,375,429,491,583). At least for cell culture-adapted genotype 1b replicons that do not make infectious virus, each cell contains approximately 100 negative-strands, 1,000 positive-strands, and 1,000,000 copies of each viral protein (583). A number of factors, however, influence the rate of HCV RNA replication. In Huh-7 cells, genotype 1b replication is robust in exponentially growing cells and repressed in growth-arrested cells (519,564,638). Interestingly, this block may be caused by reduced pyrimidine nucleotide pools, because replication can be restored in confluent cells by supplementing media with uridine and cytosine (519). In addition, cotransfected replicons interfere with each other, suggesting that they compete for limiting cellular factors (181,427). Given that replication-defective genomes also compete but that translation-defective genomes do not, one of these limiting cellular factors is likely to interact with an NS protein (427).

Virus Assembly

Similar to the assembly of flaviviruses, it is likely that HCV particles bud directly into the ER, transit the secretory pathway, and are released at the cell surface. Only specific aspects of virus assembly could be modeled before the development of infectious cell culture systems. HCV particle assembly presumably begins with interactions between viral RNA and C protein. Although packaging signals of the HCV genome are not yet characterized, packaging specificity may involve interactions between C protein and the 5′ NCR (650,694). Furthermore, interaction with structured RNA has been shown to induce the *in vitro* assembly of C protein into nucleocapsid-like structures (364). During this assembly process, C protein becomes protease resistant, suggesting that it acquires a compact fold (365). Surprisingly, only aa 1 to 124 are required for assembly into 60-nm particles. Nucleocapsidlike particles have also been observed when full-length C protein is expressed in the yeast *Pichia pastoris* (2,452), or by *in vitro* translation (342,343). Particles produced by these systems have diameters of 33 to 50 nm and exhibit biophysical and morphologic similarity to nucleocapsids isolated from HCV-infected sera. It is not yet clear whether these processes of virion budding and nucleocapsid assembly are coupled or whether preformed nucleocapsid intermediates are formed before budding initiates. It is also possible to assemble intact virus-like particles (VLP) by overexpressing the HCV structural proteins in mammalian (57,58,492) or insect cells (39). Although baculovirus-produced VLP are morphologically heterogeneous and retained within cells, such particles have been isolated and used to study HCV-specific immune responses and attachment to target cells (120,673,720).

The new HCVcc systems will allow virus assembly to be dissected at the genetic, biochemical, and cell biological levels. In HCVcc-producing cells, C protein predominantly localized to perinuclear membranes associated with lipid droplets, most likely the smooth ER (611). Surprisingly, expression of C protein did not overlap with the HCV

glycoproteins, which were retained in the rough ER. Thus, it is not yet clear how virus particles are assembled from these differentially localized proteins, and only a few electron-dense particles resembling a virus were observed. Interestingly, it was recently shown that a genetic interaction controlling the efficiency of virion release exists between the structural proteins and the first transmembrane domain of NS2 (562). Thus, another common feature among the family *Flaviviridae* is that the NS2, NS2A, or NS2-3 region plays an important yet unknown role in regulating virus assembly.

PESTIVIRUSES

Background and Classification

Pestiviruses are animal pathogens of major economic importance for the livestock industry. They include the type member, bovine viral diarrhea virus (BVDV), as well as classical swine fever virus (CSFV), and border disease virus (BDV) of sheep (710). Based primarily on nucleotide sequence and antigenic relatedness, two distinct species of BVDV have been identified: BVDV-1 and BVDV-2 (710). Newly described pestiviruses from giraffe and other wild ruminants indicate additional diversity within this genus (43,633,733); accordingly, the giraffe pestivirus has been included as tentative species within the *Pestivirus* genus (710). Within the family *Flaviviridae*, pestiviruses show greater similarity in genome structure and mechanism of initiating translation to the hepaciviruses than to the flaviviruses.

Pestivirus infections can be subclinical or produce a range of clinical conditions, including acute diarrhea, acute hemorrhagic syndrome, acute fatal disease, and a wasting disease, all of which has been reviewed elsewhere (711). CSFV, typically transmitted oronasally, leads to acute or chronic hemorrhagic syndromes with significant mortality. Ruminant pestiviruses, on the other hand, usually lead to inapparent infection or cause mild symptoms in adult animals. A notable exception is BVDV-2, which can be associated with a severe, acute hemorrhagic condition in cattle (135,551,595). In addition, diaplacental transmission of pestiviruses can cause fetal death, malformation and acute syndromes of the newborn, or it can lead to persistent infection of the offspring in a carrier state. Pestiviruses that cause persistent infection are noncytopathogenic (ncp) in cell culture. Persistently infected cattle can develop fatal mucosal disease. Interestingly, development of mucosal disease correlates with the occurrence of virus variants with a cytopathogenic (cp) phenotype. Live attenuated strains, inactivated virus preparations, and subunit vaccines are available for immunization against pestivirus-induced diseases (496); however, vaccination of persistently infected cattle with an attenuated cpBVDV strain can induce mucosal disease (46). Thus, a need exists for improved pestivirus vaccines, in particular, for preventing diaplacental infections.

In infected animals, pestiviruses can be detected in a variety of tissue types, including epithelial cells at the site of entry, endothelial cells, lymphoreticular cells, and macrophages. In persistently infected animals, BVDV can be detected in most tissues, including PBMC, the gastrointestinal tract, and neurons. Primary and continuous cell lines from natural host species are usually permissive for pestivirus replication in cell culture, although considerable differences in replication efficiencies have been noted (615). Highly permissive cell lines have been described for propagation of pestiviruses (498,615), and infectious cDNA clones have been constructed for many pestiviruses (411).

Structure and Physical Properties of the Virion

Pestiviruses have been difficult to purify because of modest growth in cell culture, inefficient release from infected cells, and association with serum components and cellular debris (379). Identification of efficient culture systems facilitated visualization of virus particles by electron microscopy (496,752) (Fig. 33.3D,E) and the characterization of the virion structural components (712). Virions are spherical enveloped particles, 40 to 60 nm in diameter, with an electron-dense inner core of about 30 nm (282). Pestivirus virions band at a buoyant density of 1.134 g/mL in sucrose and are inactivated by heat, organic solvents, and detergents (615). Unlike flaviviruses, which are rapidly inactivated by low pH, pestiviruses can survive over a relatively broad pH range (401). The chemical composition of highly purified preparations of pestivirus particles has not been determined; however, in addition to genome RNA and lipids, four structural proteins have been identified: the capsid protein (C) and three envelope glycoproteins: E^{rns} (for ribonuclease, secreted), E1, and E2 (618,712).

Binding and Entry

Binding and entry of pestiviruses is likely to be a multistep process involving initial attachment of virions, interaction with specific receptor(s), internalization, and membrane fusion. Whereas recombinant E2 and E^{rns} can bind independently to cell surfaces (288), E2 is the major determinant of cell culture tropism, at least for ruminant pestiviruses (398,600). Adsorption of CSFV E2 competitively inhibits infection with CSFV and BVDV, whereas inhibition by E^{rns} is seen for CSFV and selected BVDV strains but requires large amounts of E^{rns} (288). Given the observed binding of E^{rns} to cell surface glycosaminoglycans, this latter observation may be explained by high receptor density (293,294). Surprisingly, E1 and E2 are sufficient for entry of CSFV-pseudotyped HIV particles; E^{rns} is nonessential in this system (745). Bovine CD46 has been identified as a cellular receptor for BVDV-1 and BVDV-2, including primary clinical isolates (461). Experiments using chimeric CD46

molecules identified a discrete subregion within complement control protein repeat 1 as essential for BVDV binding and infection (356). The viral ligand for CD46 is probably BVDV E2, but this remains to be established. Other studies suggest a role for LDL receptor in both BVDV and HCV entry (5). After binding, BVDV enters target cells via clathrin-dependent endocytosis (357,385). Similar to HCV, BVDV must be primed to respond to low pH before fusion (357).

Genome Structure

The pestivirus genome consists of an ssRNA of approximately 12.3 kb in length (411). Similar to hepaciviruses, pestivirus genomes lack a 5′ cap and 3′ poly(A) tract (79,498). A long ORF of approximately 4,000 codons is flanked by a 5′ NCR of 372 to 385 nucleotides, and a 3′ NCR of 185 to 273 nucleotides (27,79,131). Two 5′-terminal stem-loop structures in the BVDV genome (domains Ia and Ib in Fig. 33.8A) are important for efficient RNA replication (209,797). A 5′-terminal GUAU sequence is essential for BVDV replication, perhaps via the function of its complement at the 3′ end of the minus strand, as part of a promoter for plus strand synthesis (209). Provided that this tetranucleotide sequence is retained, substitutions and deletions of hairpin Ia and part of Ib do not abolish replication (45). Thus, the 5′ signals essential for pestivirus genome replication are significantly shorter than for the hepaciviruses. Following the ORF, the 3′ NCR consists of a variable region followed by a conserved 3′-terminal stem loop preceded by a single-stranded region (153,796). Mutational analyses indicate that the terminal stem loop and the upstream single-stranded region harbor important primary and secondary structural elements that probably function in cis to direct minus-strand initiation, whereas deletions in the variable region are tolerated (297,541,796).

Translation and Polyprotein Processing

Cap-independent translation initiation of the pestivirus genome is mediated by an IRES that bears structural and functional similarity to that of HCV (compare Figs. 33.7A and 33.8A) (81,153,570,607). The minimal pestivirus IRES includes 5′ NCR domains II and III, and can be influenced by structured sequences downstream from the initiator AUG (113,514,570,607). As for HCV, the pestivirus IRES binds ribosomal 40S subunits without the need for translation initiation factors eIF4A, eIF4B, and eIF4F (557,558,661). Pestivirus proteins are translated as a single, large polyprotein, which is processed into individual viral proteins (498,579). The order of the cleavage products in the BVDV-NADL polyprotein is NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Fig. 33.8B) (132,133,483).

Unlike other *Flaviviridae*, the first pestivirus protein encoded in the long ORF is a nonstructural protein, N^{pro}, an autoprotease responsible for cleavage at the N^{pro}/C site (669,712,770). Processing in the pestivirus structural region appears to be mediated by at least three additional proteases. Host signal peptidase is believed to cleave at the C/E^{rns}, E1/E2, E2/p7, and p7/NS2 sites with incomplete cleavage at the E2/p7 site leading to accumulation of uncleaved E2-p7 (173,257,618). As for HCV, signal peptide peptidase mediates further processing of the pestivirus C protein in the transmembrane region to generate the C-terminus of mature C protein (264). The E^{rns}-E1 polyprotein (gp62) is processed slowly, presumably by a cellular protease that has not yet been identified (618). The

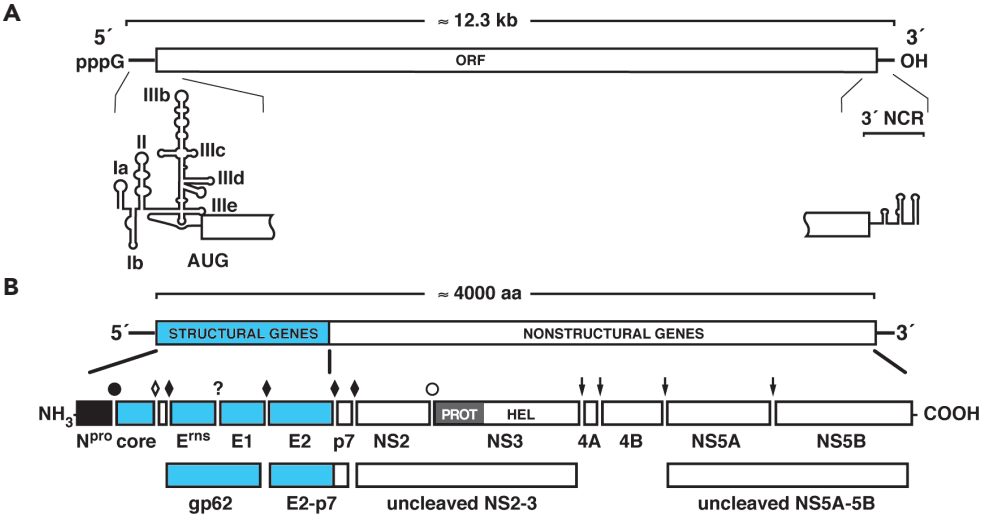

Figure 33.8 Pestivirus genome structure and expression. **A:** Genome structure and RNA elements. **B:** Polyprotein processing and cleavage products. Symbols identifying proteolytic cleavages for the cytopathogenic bovine viral diarrhea virus (cpBVDV) NADL strain are the same as those described in Figure 33.5, except for the proposed autocatalytic cleavage releasing the N-terminal nonstructural protein N^{pro} from the pestivirus polyprotein (669,770), which is indicated by a *closed bullet*.

mechanism of NS2-3 cleavage has only recently been elucidated by the identification and characterization of the NS2 autoprotease (369). NS2/3 cleavage can be incomplete, but is essential for viral RNA replication, and recent evidence suggests that uncleaved NS2-3 is required for virion production (4) (see also below). As detailed below, certain cp pestiviruses generate the authentic N-terminus of NS3 via several different mechanisms. The remaining cleavages in the polyprotein that generate NS4A, NS4B, NS5A, and NS5B are catalyzed by the pestivirus NS3-4A serine protease (699,771,776) (Fig. 33.8B).

Features of Pestivirus Proteins

N^{pro} Autoprotease

As mentioned earlier, N^{pro} is a nonstructural autoprotease that cleaves at a conserved site between Cys-168 and Ser-169 of the polyprotein (669,770). Site-directed mutagenesis identified residues Glu-22, His-49, and Cys-69 as important for catalysis, leading to the suggestion that N^{pro} may be an unusual subtilisin-like cysteine protease (616). N^{pro} can be functionally replaced by the cellular ubiquitin gene, which directs cellular hydrolases to cleave at the C-terminus of the ubiquitin monomer (47,719). Furthermore, N^{pro} is dispensable for autonomous viral replication in engineered and spontaneously derived subgenomic RNAs (47,502,700,719). N^{pro} also acts as an antagonist of IRF-3 activation and IFN production (612,614), in part by inhibiting IRF-3 transcription in CSFV-infected cells (367). Experiments with BVDV have shown that the protease activity of N^{pro} is not required for IFN antagonism (222). In accordance with these cell-based studies, N^{pro}-deletion mutants of CSFV are attenuated *in vivo* and have been proposed as live virus vaccine candidates (462).

Pestivirus Structural Proteins

The capsid (C) protein is a conserved, highly basic, 14-kd polypeptide consisting of about 21% Lys with a net charge around +12. The C-terminus of the CSFV C protein has been determined and is located in the hydrophobic portion of the internal signal peptide, which initiates translocation of E^{rns} into the ER lumen (264).

The E^{rns} glycoprotein (44 to 48 kd, formerly known as E0 or gp44/48) is heavily glycosylated at seven to nine potential N-linked glycosylation sites and forms disulfide-linked homodimers (378,712). Besides being associated with released virus via an unusual type of membrane anchor (191), E^{rns} is also secreted from infected cells (618,750,752). The most unusual feature of E^{rns} is a ribonuclease activity with specificity for uridine residues (260,637). Glycosylation and dimer formation are not required for this activity (766). Although the function of E^{rns} ribonuclease activity is not yet clear, it appears to be important. Antibodies that inhibit ribonuclease activity tend to neutralize virus infectivity (766), and mutations in E^{rns} that destroy ribonuclease activity give rise to viruses that are attenuated *in vivo* (478,479,736). Recombinant E^{rns} is toxic to lymphocytes *in vitro* (82), an observation that could be linked to the marked leukopenia seen in natural infections (688). Although cytotoxicity is a feature of other soluble ribonucleases (631), it is not yet clear whether the ribonuclease activity of E^{rns} is related to its toxicity. The C-terminal domain of E^{rns} can promote its translocation across the cell membranes, perhaps suggesting an intracellular target or function (377). Recombinant E^{rns}, however, can also bind strongly to the surface of cells, probably via interaction with glycosaminoglycans, and inhibit infection (293,752).

E1 and E2 are integral membrane proteins that contain two to three, and four to six N-linked glycosylation motifs, respectively (751). E1 and E2 are associated as disulfide-linked heterodimers that form slowly (618) and E2 is also present in homodimers (712,751). Recombinant CSFV E2 can bind to cells and block infection of CSFV and BVDV, suggesting a common E2 receptor or coreceptor for binding and entry of these pestiviruses (288). Although the precise roles of the viral glycoproteins in virus assembly and entry remain to be defined, monoclonal antibodies to E^{rns} (750) or E2 (158,543,731,751,762) can neutralize virus infectivity, and both antigens can elicit protective immunity (289,617,732).

p7 Protein

The p7 protein follows the virion structural proteins; it consists of a central charged region separating hydrophobic termini (173) and is required for production of infectious virus (257) but not RNA replication (47). As with the HCV p7 protein, pestivirus p7 is inefficiently cleaved from E2, probably via signal peptidase (173). Uncleaved E2–p7 is not required for replication in cell culture (257) and both E2–p7 and p7 appear to remain cell associated.

It is not yet clear if p7 is a structural or a nonstructural protein, although it has not been detected in purified virus (173). Similar to HCV p7, pestivirus p7 can form ion channels, suggesting that it could function in virus assembly and entry (242,243).

Pestivirus Nonstructural Proteins

The NS2 protein is a newly identified cysteine protease, distantly related to the NS2-3 autoprotease of HCV and GB viruses, which is responsible for processing of NS2-3 (~125 kd) (369,370). As will be discussed below, NS2-3 cleavage is essential for pestivirus RNA replication and the efficiency of NS2-3 cleavage is regulated by a cellular chaperone and can determine viral cytopathogenicity. Similar to other members of the family *Flaviviridae*, the NS2-3 region participates in virus assembly (4).

As for all members of the family *Flaviviridae*, pestivirus NS3 contains an N-terminal serine protease domain (41,230,771) and a C-terminal RNA helicase (232). As for HCV, the pestivirus NS3 serine protease requires NS4A as

a protein cofactor (776). The pestiviral NS3-4A serine protease cleaves between leucine and small uncharged amino acids: L↓(S/A/N) (699,776). Substitutions that eliminate serine protease activity abolish viral RNA replication, confirming its essential role in virus viability (236,776). Interestingly, protease activity is retained when threonine is substituted for the serine nucleophile (701). The NS3 protein of BVDV has been purified and shown to possess RNA helicase (746) and RNA-stimulated NTPase (691) activities. Site-directed mutagenesis of the conserved helicase and NTPase motifs inhibited or destroyed these activities and abolished viral replication (236).

The hydrophobic NS4A and NS4B proteins are similar in size, composition, and hydropathic properties to the NS4A and NS4B proteins of the hepaciviruses. NS4A acts as a cofactor for the NS3 serine protease (776) and as for HCV, cofactor activity involves interaction of a central domain of NS4A with the N-terminal region of NS3 (701). An analysis of recombinant ncpBVDV strains revealed that an insertion of viral sequences between the NS4A and NS4B genes did not interfere with efficient viral replication; thus the NS4AB precursor has no essential role, at least in cell culture (214).

The remaining two proteins, NS5A (~58 kd) and NS5B (~75 kd), are present as mature cleavage products as well as uncleaved NS5A-5B (132,133). Little is known about the function of NS5A. This protein is phosphorylated by a cellular serine or threonine kinase with properties similar to kinases that phosphorylate flavivirus NS5 and hepacivirus NS5A (596). Genetic analysis revealed that defects in the NS5A gene could be readily complemented in trans, whereas mutations in other NS genes could not (237).

The NS5B contains motifs characteristic of RdRP (131). The RNA polymerase activity of recombinant NS5B has been characterized *in vitro* and found to extend template-primed RNA into double-stranded copy-back products (372,430,670,812) or to catalyze *de novo* initiation from short, synthetic RNA or DNA templates (315,372). The structure of BVDV NS5B is similar to HCV NS5B and other RdRPs, containing a palm subdomain surrounded by finger and thumb subdomains (112). The pestivirus NS5B structure reveals a unique N-terminal region, which suggests a role for GTP in *de novo* initiation, and provides a framework for understanding the molecular mechanisms of small molecule inhibitors of the BVDV RdRP (28,539, 685).

RNA Replication

The basic aspects of pestivirus RNA replication are similar to those described for HCV: RNA replication is associated with cytoplasmic membranes and requires NS3 through NS5B as well as cellular components. Negative- and positive-strand pestivirus RNA have been detected from 4 to 6 hours after infection, followed by the asymmetric accumulation of additional minus- and excess plus-strand RNA (229). Accumulation of genome-length intracellular pestivirus RNA, which comigrate with virion RNA, generally follows the time course of infectious virus release, maximal virus titers being achieved about 12 to 24 hours after infection (229,498,579). Double-stranded RF RNA and partial duplex RI RNA have been tentatively identified (228,229,579).

Recently, new insights into the regulation of BVDV RNA replication and virus assembly have emerged. Originally, ncpBVDV was thought to express only uncleaved NS2-3, leading to the idea that NS2-3 could serve as a functional RNA replicase component. Consistent with this idea, cpBVDV isolates produce both uncleaved NS2-3 and NS3. Recent work has shown that NS2-3 cleavage is required for RNA replication and regulated by limiting quantities of the cellular chaperone Jiv (see below) (368). NS2-3 cleavage is nearly complete early in ncpBVDV infection, leading to efficient NS3 production and RNA replication. At later times when Jiv becomes limiting, NS2-3 autoprocessing becomes inefficient, NS2-3 levels increase and viral RNA synthesis rates decline (369). As for HCV, mutations that abolish BVDV NS2/3 processing and NS3 production block RNA replication. Jiv (J-domain protein interacting with viral protein) interacts with NS2 and acts as a cofactor for the autoprotease. The amount of Jiv in the infected cell controls pestiviral replication by limiting the generation of active protease molecules and replication complexes. In addition to viral template RNA and replicase proteins, a group of cellular NFAR proteins are recruited by the replication machinery of BVDV; the NFAR proteins associate specifically with the 5′ and 3′ termini of the viral RNA genome (296). The 3′ NCR also contains determinants that ensure efficient termination of translation, which is essential for efficient pestivirus RNA replication (297).

As described later, good evidence indicates that nonhomologous recombination can occur within pestivirus RNA, and between pestivirus RNA and host cellular RNA (reviewed in [485]). One likely mechanism is via copy-choice template recruitment during minus-strand synthesis, which is consistent with the coding orientation of cellular inserts. Alternatively, the existence of a viral RdRP-independent mechanism of RNA recombination has recently been demonstrated using a cell culture-based RNA recombination system in which homologous and nonhomologous recombination occurred between two overlapping transcripts, each lacking different essential parts of the viral RdRP (215).

Assembly and Release of Virus Particles

Other than the features of the virion structural proteins described above, little information is available on the assembly and release of pestiviruses from infected cells. Electron microscopic examination of virus-infected cells (53,239) suggests that pestiviruses mature in intracellular vesicles and are released by exocytosis. Consistent with intracellular budding, pestivirus envelope proteins are not

transported to the plasma membrane (240,752) and ER α-glucosidase inhibitors block infectious BVDV-1 particle formation (164,817). Interestingly, E^{rns} and E2 have been immunolocalized on isolated virus particles by electron microscopy, but E2 was not detected in particles undergoing secretion (or perhaps reattachment) at the cell surface (752). This suggests that E2 may be conformationally inaccessible to antibodies before maturation. As mentioned for the flaviviruses and HCV, nonstructural proteins also play a role in pestivirus virion assembly or release. These include uncleaved NS2-3, which is required for virus production (4) and more recently, NS5B (22).

Pathogenesis of Mucosal Disease and the Generation of Cytopathogenic Pestiviruses

Mucosal disease, the most severe outcome of BVDV infection, is usually fatal (485). This disease occurs only after *in utero* infection with an ncpBVDV strain between 40 and 125 days of gestation, leading to the birth of immunotolerant animals that remain persistently infected for life. In the case of a persistently infected animal exhibiting mucosal disease, both cp and ncp biotypes of BVDV can be isolated (464). The close serologic relatedness of ncp–cp pairs isolated from a mucosal disease-affected animal led to the suggestion that cpBVDV might arise from ncpBVDV by a rare mutational event. Genetic characterization of a number of these ncp-cp pairs has verified this hypothesis and led to the remarkable discovery that most cpBVDV strains are generated via RNA recombination, although a few cpBVDV strains lack obvious genome rearrangements (485,550). The presence of these genome rearrangements strongly correlates with increased NS3 expression, enhanced RNA replication, and cytopathogenicity in cell culture. Figure 33.9 illustrates a few of the remarkable cpBVDV genome alterations that have been discovered.

Common features of some cpBVDV variants include genome rearrangements or mutations that activate NS2-3 autoprotease activity, leading to increased production of NS3 and RNA replication. For instance, strain NADL (Fig. 33.9A) contains a fragment of the cellular Jiv gene inserted within NS2 (44). As described above, Jiv is an

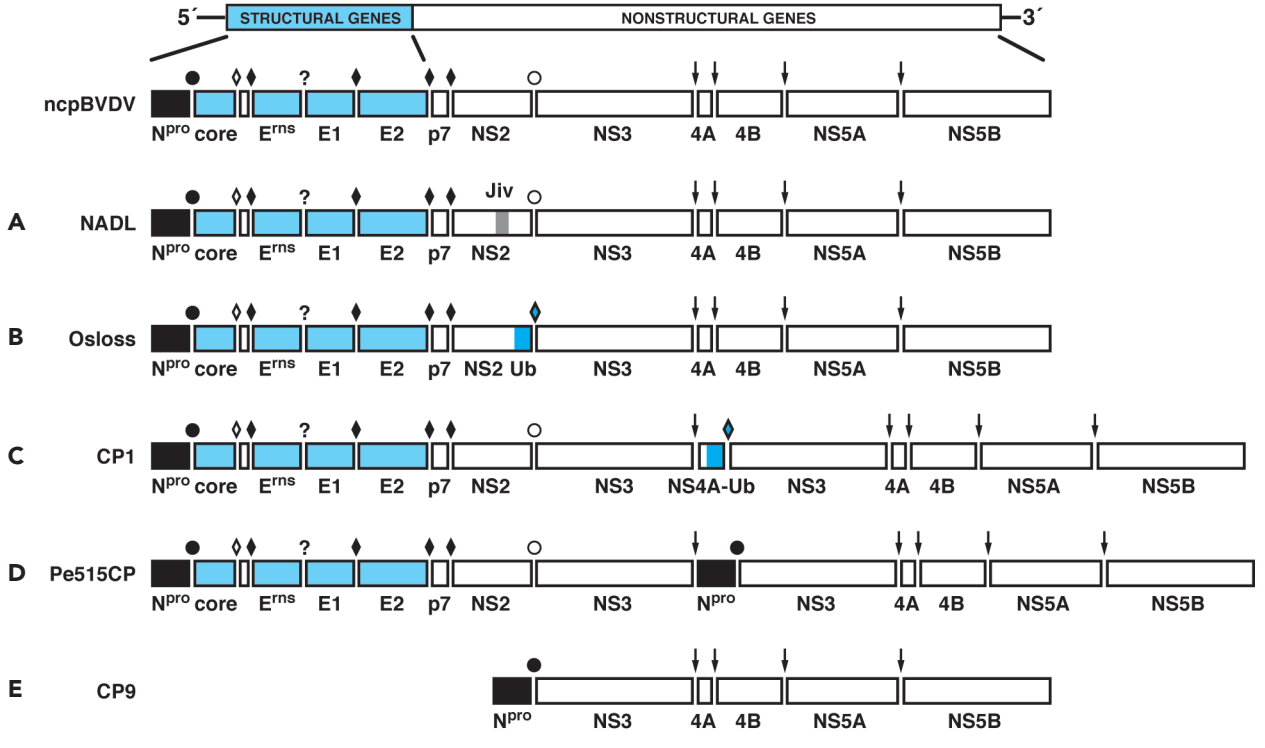

Figure 33.9 Genome rearrangements associated with the generation of cytopathogenic bovine viral diarrhea virus (cpBVDV). The **top** diagram indicates the polyprotein of a typical noncytopathogenic BVDV (ncpBVDV) isolate. **Below**, the polyproteins encoded by five different cpBVDV strains generated by RNA recombination are shown: NADL, Osloss, CP1, Pe515CP, and CP9. As discussed in the text, these cpBVDV polyprotein structures allow the production of both NS2-3 and NS3. In-frame insertions of host sequences (*cyan*) are present in NADL, Osloss, and CP1. The NS2 autoprotease is responsible for NS3 production in the NADL strain, but the inserted ubiquitin sequences in Osloss and CP1 provide sites for processing by host ubiquitin C-terminal hydrolase (*blue diamond*). For Pe515CP and the CP9 DI RNA, the N^{pro} autoprotease (*black box*) mediates the cleavage producing the NS3 N terminus. The nomenclature and organization of the cleavage products and the symbols for the normal processing enzymes are defined in Figures 33.5 and 33.8.

essential cofactor of the NS2 protease and overexpression of a critical 90 amino acid Jiv subdomain in the context of the viral genome enhances NS2-3 cleavage, regardless whether the fragment is provided in cis or in trans (369). Interestingly, a much smaller insertion is found in the NS2 gene of cpBVDV strain CP7, which contains a 27-nt duplication from an upstream region of the NS2 gene in an alternate reading frame (702). As with the Jiv fragment, this insertion also leads to increased NS2-3 processing and a virus that is cytopathic in culture (473,481). Other viral insertions at or very close to the same site have been described (29). For other cpBVDV isolates, increased NS2-3 autoprotease activity appears to result from point mutations that have accumulated within the NS2 gene (362).

Another common rearrangement in cpBVDV isolates involves the insertion of ubiquitin or ubiquitin-like genes immediately upstream of NS3 (32,482,580,703), leading to NS2/3 processing by ubiquitin C-terminal hydrolase or related enzymes (e.g., strain Osloss in Fig. 33.9B). In some cases (e.g., strain CP1 in Fig. 33.9C), this may be accompanied by additional genome rearrangements such as duplication of the NS3 and NS4A genes (483). Such strains will also express uncleaved NS2-3, which is important for virion production, as described above. A related type of insertion includes the light chain 3 gene of cellular microtubule-associated proteins, which is targeted for cleavage by a cellular protease (205,480).

A third type of cpBVDV genome rearrangement involves the repositioning of the N^{pro} autoprotease immediately upstream of NS3. In one such cpBVDV isolate, strain Pe515CP (Fig. 33.9D), the N^{pro} gene is duplicated together with the NS3 and NS4A genes (483). Alternatively, some cpBVDV isolates, such as CP9 (Fig. 33.9E), contain a precise deletion of the C-E^{rns}-E1-E2-p7-NS2 genes resulting in an in-frame fusion of N^{pro} and NS3 (704). Such subgenomic RNAs replicate autonomously, express NS3, and induce cytopathic effects within cells (47), but require ncpBVDV helper viruses to provide packaging functions in trans. For CSFV, cp subgenomic RNA have been isolated in which the entire coding sequence upstream of NS3 has been deleted (484).

Based on the common feature of increased NS3 expression by cp pestiviruses, it has been hypothesized that NS3 may be directly cytopathic. Alternatively, cytopathic effects could be a byproduct of enhanced viral RNA accumulation and activation of cellular pathways triggering apoptosis. Nevertheless, selection for ncp variants in cell culture yielded an ncpBVDV that still produces NS3 and viral RNA at levels comparable to those of the cp parent (582). Further analysis revealed that this variant encodes a point mutation in NS4B that attenuates the cytopathic effect of BVDV. Other ncpBVDV have been selected in culture with changes in NS4B (214). Given that NS4B is likely to be involved in membrane reorganization (based on functions of the HCV protein) and that cpBVDV can cause ER stress leading to apoptosis (309), it seems plausible that cytopathic effects may result from overcommitment of cellular membranes to replication complexes.

A larger question is how cytopathic effects on the cellular level are linked to mucosal disease in infected animals. Increased cell death could directly contribute to tissue injury and induce inflammation. However, animals with mucosal disease show increased numbers of infected cells, possibly because of differences in tropism, which could also contribute to disease (400).

GB VIRUSES

Discovery and Classification

In the early 1990s, a residual number of hepatitis cases were still not attributable to hepatitis A–E viruses. Efforts aimed at identifying additional hepatitis agents revealed three novel viruses that have been tentatively assigned to the family *Flaviviridae*. Two of these viruses, GBV-A and GBV-B, were cloned via representational difference analysis from the sera of tamarins experimentally infected with the GB hepatitis agent (659). The GB agent was originally derived from the serum of a 34-year old surgeon, "GB," who had active hepatitis, by serial passage in tamarins. Both viruses are similar to HCV, yet genetically quite distinct (503). Although originally derived from human hepatitis, subsequent work showed that GBV-A is an indigenous monkey virus that was likely acquired during passage in tamarins (87,383). Although some human cases that are not A–E hepatitis showed serologic reactivity to both GBV-A and GBV-B, reverse transcription polymerase chain reaction (RT-PCR) failed to detect either virus in human samples. Rather, a third related virus, GBV-C, was subsequently identified in human samples (658). Working independently, another group that was immunoscreening cDNA libraries from non-A, non-B hepatitis cases identified an agent, initially termed hepatitis G virus (HGV), which later turned out to be an independent isolate of GBV-C (413). Because no convincing link is found between this virus and any human disease, we will refer to it by its original designation, GBV-C.

Based on sequence relatedness and overall genome structure, GBV have been categorized within the family *Flaviviridae*. GBV-B was recently included within the genus *Hepacivirus* (710). GBV-A and GBV-C remain unclassified at the genus level because they share a number of unique features that distinguish them from other members of the family.

The inability to detect GBV-A or GBV-B in human samples led to investigation into their origins. Interestingly, GBV-A has been detected in several species of New World monkeys in the absence of experimental infection or overt disease (87,383). Viral sequences isolated from within a single primate species are highly related, whereas sequences

isolated from separate species show greater divergence, indicating that GBV-A has adapted to its primate hosts over extended periods of time (87,107,383). The distribution of GBV-B in nature is unknown because the only source of this virus is the original tamarin-passaged GB serum. Despite intensive efforts, it has never been isolated since.

Since its initial discovery, GBV-C infection has been found to be surprisingly common in the human population. Approximately 15% of healthy volunteer blood donors have markers of previous or ongoing infection with this virus (668). GBV-C is also found in chimpanzees (3,54). Phylogenetic analysis of GBV-C sequences has been complicated by an apparent bias against synonymous substitutions in some parts of the genome, leading to differences in inferred evolutionary relationships (654). The molecular basis for this bias in unclear; it may involve evolutionary constraints imposed by RNA structures (656) or cryptic ORFs (544,663). Nevertheless, GBV-C has been classified into four or five genotypes (654). Variation among GBV-C isolates remarkably reflects the geographic distribution of human migration, suggesting the long-term coevolution of this virus and its host. Given the rate at which RNA viruses typically evolve, this finding suggests that GBV-C is subject to unusual evolutionary constraints, as reviewed elsewhere (654).

Clinical Perspective

Although GBV-A and GBV-B were originally derived from a common source of human hepatitis, it is unclear whether either virus was the cause of hepatitis within patient GB. As described above, GBV-A is not associated with any known disease and is likely to have been acquired during tamarin passage. GBV-B can infect and cause hepatitis in New World monkeys such as tamarins, marmosets, and owl monkeys, but it does not infect chimpanzees (76,88,376). Because of this preference for lower primates, GBV-B is unlikely to be a human virus. Attempts to identify a GBV in the original GB clinical sample have failed, possibly because of degradation over prolonged storage (634).

Human infection with GBV-C is well documented, although direct association of this virus with any human disease has proved to be elusive (668). GBV-C frequently causes persistent infections that last for years without clinical effects (16,17). GBV-C appears to be primarily lymphotropic *in vivo*, although evidence also exists for hepatotropic isolates (196). Clearance usually correlates with the appearance of antibodies against the E2 viral glycoprotein (668). GBV-C is primarily transmitted parenterally or sexually, although a vertical transmission route is also likely (668). Because these routes also transmit many other human viruses, GBV-C coinfections with HBV, HCV, or HIV are not uncommon. Needless to say, coinfection with human hepatitis viruses has likely contributed to the confusing association of GBV-C with disease.

On the other hand, the interaction between GBV-C and HIV in coinfected people is intriguing. It has been noted that patients with HIV and GBV-C viremia tend to have higher $CD4^+$ T-cell counts, lower HIV titers, and slower HIV disease progression (668). Based on these observation as well as *in vitro* experiments, it has been proposed that GBV-C may interfere with HIV replication by altering expression of cytokines, chemokines, and chemokine receptors (568). It has also been argued, however, that because GBV-C is a lymphotropic virus, the GBV-C viremic status may simply reflect the higher $CD4^+$ counts in nonprogressors (729). Thus, the underlying reason for the correlation between GBV-C infection and slower HIV progression is not yet clear.

Experimental Systems

Little work has been done on GBV-A because it is an indigenous monkey virus that is not associated with disease. On the other hand, GBV-B is the closest relative of HCV and has been extensively studied as a surrogate model system. GBV-B can be readily cultured in primary tamarin or marmoset hepatocytes (42,76,376), but replicates poorly (if at all) in many immortalized cell lines (84). Full-length GBV-B cDNAs have been assembled and shown to be infectious and cause hepatitis in tamarins (89,455,627). Based on these functional clones, subgenomic GBV-B replicons have been constructed and shown to replicate in the human hepatoma lines Huh-7 and Hep3B, albeit with low efficiency (149,150). These cell culture systems and small primate models are being used to evaluate compounds that have antiviral properties against HCV and GBV-B.

GBV-C has been reportedly cultured in human hepatoma lines (640), primary human lymphocytes (220), peripheral blood mononuclear cells (197), and a derivative of the Daudi Burkitt's lymphoma line (649). Reminiscent of HCV, replication levels are low in these cell culture systems. Nevertheless, a GBV-C cDNA clone was constructed and shown to be infectious in primary human $CD4^+$ T-cells (775).

Virion Structure and Entry

Particles of GBV-A and GBV-B have not been characterized. Similar to HCV, GBV-C particles exhibit unusually low and heterogeneous buoyant density, with peaks near 1.07 to 1.09 g/mL and 1.17 g/mL (471,624,774). Also similar to HCV, interaction with lipoproteins may explain the low buoyant density of GBV-C particles (624). Treatment with detergents or organic solvents removes the viral envelope and shifts the peak of viral RNA to a higher density form that may represent nucleocapsids (471,624,774). This is interesting because, as described below, GBV-A and GBV-C do not encode an obvious capsid gene. Little is known about the entry mechanism of GBV,

although it has been proposed that GBV-C utilizes the LDL receptor (5).

Genome Structure and Expression

As with other *Flaviviridae*, the GBV encode a single long ORF containing structural genes followed by nonstructural genes, flanked by 5′ and 3′ NCR (384,503). As for HCV and the pestiviruses, GBV utilize an IRES to direct cap-independent translation (234,657). The GBV-A and GBV-C 5′ NCR, however, are much longer (>500 nt), sharing about 50% identity to each other, and can be folded into a similar structure that differs from other family members. At 445 nt, the GBV-B 5′ NCR is almost 30% longer than the HCV 5′ NCR, but the 5′ NCR of both viruses share significant similarity in primary and secondary structure (278). In fact, critical regions of the GBV-B and HCV IRESs have similar tertiary folds and can be functionally exchanged (311,606,608).

The GBV-A and GBV-C 3′ NCRs lack a poly(U/UC) tract and are highly conserved only within these virus groups, although they do terminate in conserved stem-loop structures (143,775). The GBV-B 3′ NCR is 361 nt long, containing a short poly(U) stretch 30 nt downstream of the stop codon, followed by 309 nt of a unique sequence (89,626). Although this region of the GBV-B genome does not contain sequence homology with HCV, the 3′ 82 nt of GBV-B can fold into a structure reminiscent of the HCV 3′ X region.

As with HCV, GBV-B contains a basic capsid protein followed by two envelope glycoproteins, E1 and E2. The genomes for GBV-A and GBV-C also contain E1 and E2 glycoproteins, but they lack any obvious capsidlike protein (413,503). The initiation codons utilized by the GBV-A and GBV-C IRES have not been firmly mapped, but appear to initiate translation at conserved AUG codons immediately upstream of the E1 gene (657). It has been observed, however, that people who are infected with GBV-C generate antibodies against a small basic peptide that can be translated from an in-frame upstream AUG, suggesting that such a protein is expressed *in vivo* (774). Alternative explanations for the lack of a capsid-like protein include the possibilities that GBV-A and GBV-C might usurp a capsid-like protein of the host cell or a coinfecting virus, or that additional GBV proteins may be involved. In this regard, a region of the GBV-C NS5A gene exhibiting a bias against synonymous mutation has been noted to potentially encode a small basic protein (10 kd, pI 11.5) in an ARF (544). Further characterization of GBV-A and GBV-C particles will be needed to demonstrate the nature of their nucleocapsid.

GBV-B encodes a 13 kd protein that shows partial homology to HCV p7 (221). This protein is predicted to span the membrane four times and can be processed by signal peptidase into two tandem p7-like genes (221,690). Remarkably, only the second half of p13, which has greater similarity to the HCV p7 gene, is needed for infectivity in tamarins (690).

The nonstructural proteins of GBV show the greatest similarity to HCV and the boundaries of cognate NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins have been proposed (384,503). Catalytic residues of the HCV NS2/3 autoprotease are conserved among GBV NS2 proteins, and this enzymatic activity has been demonstrated for GBV-C (49). Similarly, the GBV NS3 proteins encode N-terminal serine protease and C-terminal RNA helicases (384,503). The GBV-B serine protease activity shares substrate specificity with the HCV enzyme and requires the virus-specific NS4A cofactor (91,625,629). Consistent with this high degree of similarity, inhibitors of the HCV serine protease also inhibit the GBV-B protease (76). NTP-dependent RNA helicase activity has been demonstrated for NS3 proteins of GBV-B and GBV-C (250,813). The GBV-B NS5B has been shown to possess primer-dependent and *de novo* initiation RdRP and terminal transferase activities, albeit with different cation selectivity (592,814).

PERSPECTIVES

Research on the family *Flaviviridae* is progressing quickly. We sincerely apologize to the many authors whose papers could not be cited because of strict space constraints. Although much has been learned about the general lifecycle of the family *Flaviviridae*, large gaps in our knowledge exist for every step in the complex lifecycle of these viruses. The ongoing development of improved genetic and biochemical tools to study these viruses will certainly enable a more complete picture of their biology to emerge. Recent progress has been made in identifying host cell surface molecules that could be involved in binding and entry, although the details of such interactions, and how these control virus tropism and infection *in vivo*, are largely unknown. More effort is needed to understand the functional significance of polyprotein processing events for RNA replication, virus–host interaction, and virion formation. Furthermore, it is not understood how the competing processes of genome translation versus genome replication are regulated for this virus family. New insights have emerged regarding the enzymology of some viral nonstructural proteins, as well as the identity of a few host proteins that contribute to genome replication. The roles of several nonstructural proteins in this process are unknown, however. More description is needed for how all these components, together with viral RNA and subcellular membranes, combine to produce a functional replicase. Our understanding of virion formation and egress is still at an early stage, and it is not yet clear how structural proteins combine to form nascent virions, where this process occurs, or whether packaging is temporally regulated within the replication cycle. Answers to these and other pressing questions should reveal the unique aspects to the replication of this evolutionarily distinct family of viruses, and provide information useful for the development of effective immunization and therapeutic strategies

to control diseases caused by these diverse and important pathogens. Clearly, an exciting period of research into the family *Flaviviridae* lies ahead.

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