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Picornavirus Morphogenesis

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SUMMARY

The *Picornaviridae* represent a large family of small plus-strand RNA viruses that cause a bewildering array of important human and animal diseases. Morphogenesis is the least-understood step in the life cycle of these viruses, and this process is difficult to study because encapsidation is tightly coupled to genome translation and RNA replication. Although the basic steps of assembly have been known for some time, very few details are available about the mechanism and factors that regulate this process. Most of the information available has been derived from studies of enteroviruses, in particular poliovirus, where recent evidence has shown that, surprisingly, the specificity of encapsidation is governed by a viral protein-protein interaction that does not involve an RNA packaging signal. In this review, we make an attempt to summa-

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doi:10.1128/MMBR.00012-14

rize what is currently known about the following topics: (i) encapsidation intermediates, (ii) the specificity of encapsidation (iii), viral and cellular factors that are required for encapsidation, (iv) inhibitors of encapsidation, and (v) a model of enterovirus encapsidation. Finally, we compare some features of picornavirus morphogenesis with those of other plus-strand RNA viruses.

INTRODUCTION

The *Picornaviridae* represent a large family of small plus-strand RNA viruses that cause a bewildering array of human and animal diseases ranging from severe (poliomyelitis, encephalitis, meningitis, and hepatitis) to mild (common cold). This family, which is still growing rapidly, consists of 46 species grouped into 26 genera, the best known of which are the genera *Enterovirus* (e.g., poliovirus [PV], rhinovirus, coxsackievirus, and echovirus), *Aphthovirus* (foot-and-mouth disease virus [FMDV]), *Cardiovirus* (encephalomyocarditis virus [EMCV] and Theiler's virus), and *Hepatovirus* (hepatitis A virus [HAV]) (1).

Picornaviruses are nonenveloped particles (27 to 30 nm in diameter) that consist of a capsid with icosahedral symmetry containing a tightly packaged, nonsegmented, single-stranded, positive-sense genomic RNA (~7,500 nucleotides [nt]). The X-ray structures of human rhinovirus 14 (HRV14) (2) and poliovirus type 1 (3) were already published in 1985. They were the first known structures of picornaviruses, but currently, the structures of many picornaviruses, most recently that of hepatitis A virus (4), have been described. Although the basic architectures of picornavirions are similar, there are many differences in their building blocks (e.g., the nature of the processed capsid precursors) and surface properties.

Enterovirus capsids are composed of 60 copies each of four viral polypeptides known as VP1 to VP4. They form capsids (Fig. 1A and B) that display 2-, 3-, and 5-fold symmetry axes (5). VP1, VP2, and VP3, the building blocks of the outer shell of poliovirus type 1 (Mahoney), can be presented as wedge-like structures (Fig. 1C) with major "neutralization antigenic sites" (N-Ags) (binding sites for neutralizing antibodies) that are displayed on the surface of the assembled poliovirions (discussed in Steps in Picornavirus Morphogenesis, below) (5). The small VP4 molecules reside inside the virion, but they can "breathe" into the virion surface even at physiological temperatures (6).

The naked protein shell of picornaviruses harbors the RNA genomes (~7.2 to 8.4 kb), which are covalently linked at their 5′ ends through a phosphodiester bond (Tyr-p-U) to the small viral peptide VPg (22 to 25 amino acids) (7). VPg is followed by a long (500- to 1,200-nt) 5′ nontranslated region (NTR) containing replication signals (the "cloverleaf") and the "internal ribosomal entry site" (IRES), a single large open reading frame (ORF) encoding the polyprotein, and, finally, a short (30- to 650-nt) 3′ NTR with a poly(A) tail (Fig. 2) (7). The genome has been organized by consensus into 1ABCD-2ABC-3ABCD units, where the numbers indicate three different functional domains and the letters identify proteins mapping to one of the three domains (e.g., $3D^{pol}$ is the RNA polymerase, with the superscript indicating a known enzymatic function). Members of some genera (e.g., *Cardiovirus* and *Aphthovirus*) also contain a "leader protein" (L) that is fused to the N terminus of the polyprotein (Fig. 2) (8).

The life cycle of picornaviruses starts with the binding of the virus to a cell surface receptor (9). This interaction leads to virus internalization and destabilization of the capsid, which allows the release of the genome from the endosome into the cytoplasm (9, 10). After release, the RNA serves as mRNA for the IRES-mediated translation of the polyprotein (7) that is cotranslationally processed by the viral proteinases $2A^{pro}$ and $3CD^{pro}$ into precursor and mature proteins (11). Since poliovirus (and presumably all picornaviruses) can replicate in enucleated cells (see reference 12 and references therein), the major target of viral genetic armor is aimed at the cytoplasm, which in 2 to 3 h is remodeled for their benefit (13). All nonstructural proteins participate in this task, but, as discussed below, $2C^{ATPase}$ and its precursor $2BC^{ATPase}$ are involved in viral replication and in modifying the cell architecture to a greater extent than any other viral protein. Particularly startling is the rearrangement of cellular membranes into structures required for the formation of the RNA replication complex (14, 15). Typical for single-stranded RNA viruses of plus polarity, virus-encoded proteins (RNA polymerase $3D^{pol}$, 3AB, VPg, $2C^{ATPase}$, and more) catalyze the synthesis of minus strands, which in turn are the templates for the synthesis of progeny plus strands. This involves VPg-dependent initiation of RNA synthesis by $3D^{pol}$ (16), a process now known to function in many RNA virus systems. Notably, the functions of the IRES in controlling both translation as well as genome replication are dependent upon the participation of numerous cellular proteins, the number of which seems to increase steadily. The newly made viral RNA can be used as either mRNA for translation, a template for replication, or a substrate of encapsidation into a protective coat as soon as enough genomes and capsid proteins have been synthesized (14, 15). The mature virus exits the host cell either after cell lysis or by a not yet fully understood mechanism, possibly involving autophagosomes (17, 18). Generally, the enteroviral life cycle is very fast, with the whole process being completed in about 8 h. Among the exceptions is, most notably, HAV, whose replication cycle in tissue culture cells requires at least a week (19) (see also below).

Morphogenesis is the least-understood step in the life cycle of *Picornaviridae*. The packaging of genomic RNA occurs in the cytoplasm of the infected cell, and it is a complex process that, just like in other viral systems, requires a high degree of precision and specificity to regulate the interactions between participating macromolecules. Indeed, no RNA other than genomic RNA is generally encapsidated into enterovirus virions. Although the basic steps of assembly, the formation of capsid precursors followed by the capture of genomic RNA, have been known for some time, very few details about the mechanism providing specificity and the factors that regulate this process are available. Most of the available evidence about picornavirus morphogenesis is derived from studies with PV, a member of the C-cluster enteroviruses.

The recent report that the encapsidation specificity of poliovirus (and related C-cluster coxsackieviruses) is based on protein-protein interactions ($2C^{ATPase}$-capsid proteins) was a breakthrough in research on enterovirus morphogenesis (20, 21). Other important results of picornavirus encapsidation include (i) the observation that glutathione (GSH) is an important host factor for enterovirus morphogenesis by providing stability to capsid precursors and the mature virus (22–24); (ii) the discovery that Aichi virus, a member of the recently discovered genus *Kobuvirus*, uses an RNA packaging signal to confer specificity to its encapsidation (25, 26); and (iii) a report that hepatitis A virus uniquely uses its very small nonstructural protein 2A (also called X) in the formation of a precursor of the mature virus particle (27). In addition, there is an exciting report on the production of an enveloped

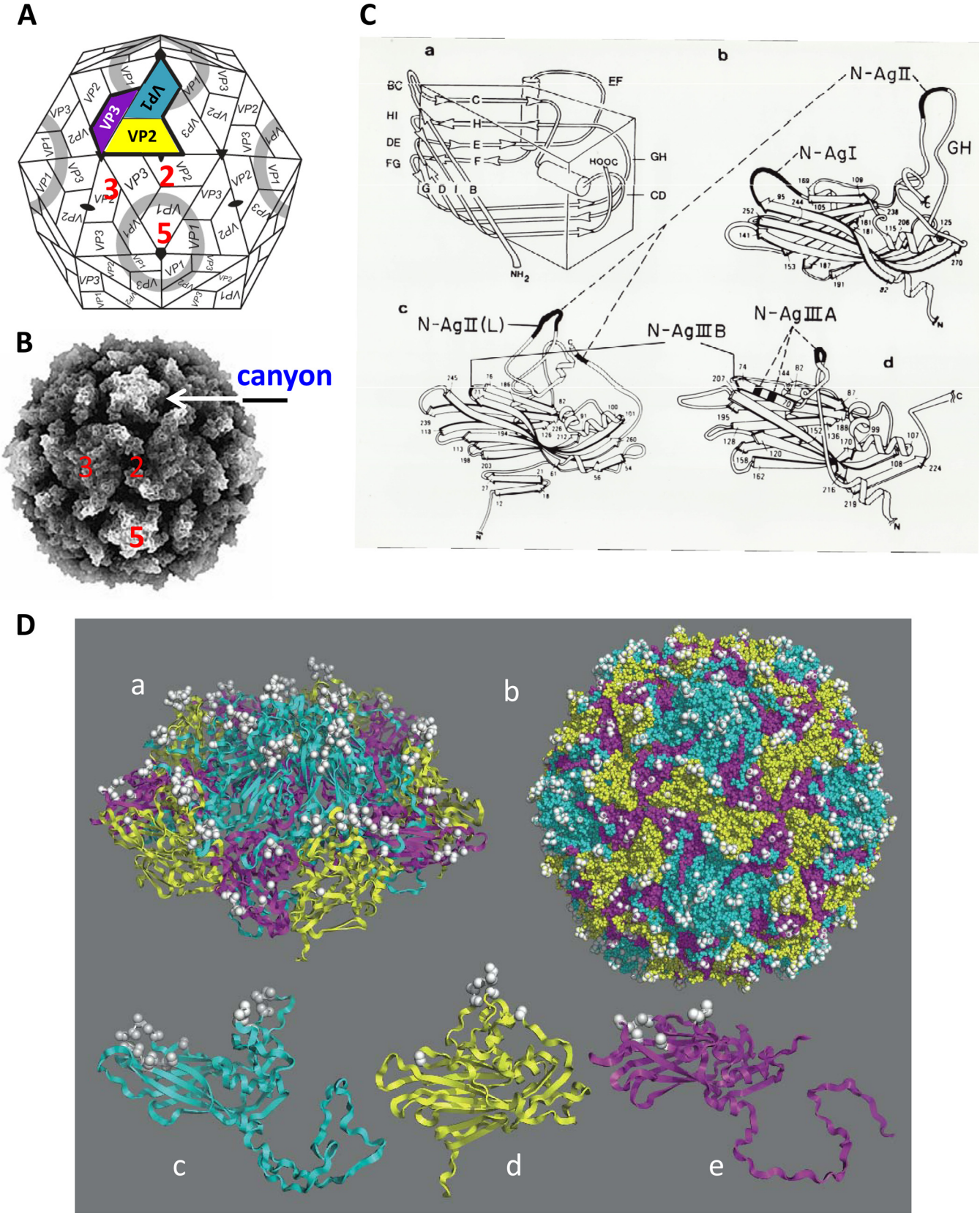


FIG 1 Structure of poliovirus. (A) Schematic diagram of the structure of poliovirus with icosahedral symmetry (3, 219). The 5-fold, 3-fold, and 2-fold axes of symmetry are indicated. The capsid proteins VP1 (blue), VP2 (yellow), and VP3 (magenta) make up the outer surface of the particle, whereas VP4 is located internally. The structure shown in color is the processed protomer of which VP0 has already been cleaved into VP4 and VP2. The canyon around the 5-fold axis

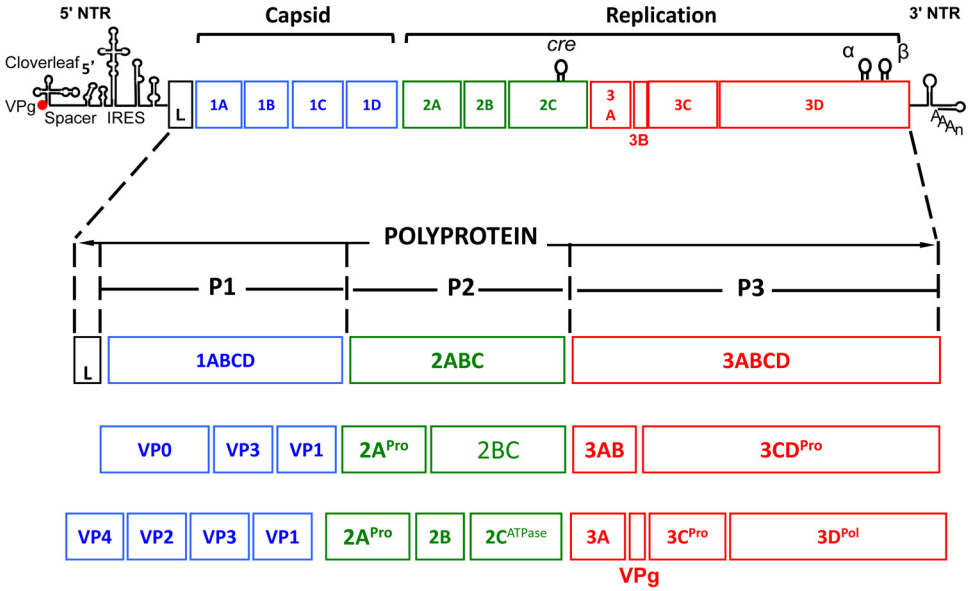


FIG 2 Genome structure of picornavirus and polyprotein processing. The picornavirus genome is a linear single-strand RNA ranging from 7.1 to 8.9 kb in length. The RNA has a covalently linked viral protein (VPg) at its 5′ end and poly(A) at its 3′ end. The ORF is flanked by a long 5′ nontranslated region (NTR) and a short 3′ NTR. The long 5′ NTR contains an internal ribosome entry site (IRES) that directs polyprotein translation. Besides the highly structured 5′ NTR and 3′ NTR, other essential RNA secondary structures have been identified in the open reading frame. For poliovirus, these structures are the *cis* replication element (*cre*) located in the $2C^{ATPase}$ coding sequence (106) and two RNA elements, α and β, located in the $3D^{pol}$ coding region (110). All known picornavirus genomes contain a *cre* element, but these structures may map to different locations in the genomes of different viruses. The first *cre* discovered maps to the P1 coding region of human rhinovirus 14 (HRV14) (174). No analyses of α and β elements in picornavirus genomes other than poliovirus have been published. The single open reading frame is organized as 1ABCD-2ABC-3ABCD, with the numbers indicating the three different domains and each letter representing a protein. The P1 region encodes the capsid structural polypeptides. The P2 and P3 regions encode the nonstructural proteins associated with replication. The polyproteins of member viruses of a large number of picornavirus genera (*Aphthovirus*, *Erbovirus*, *Kobuvirus*, *Cardiovirus*, *Teschovirus*, *Sapelovirus*, and *Senecavirus*) other than *Enterovirus* or *Hepatovirus* have an additional protein, the L protein, attached to the N terminus (8).

("membrane-cloaked") form of hepatitis A virus ("eHAV") that emerges from infected cells both *in vitro* and *in vivo* (28).

The aim of this review is to summarize what is currently known about the morphogenesis of picornaviruses, including steps in encapsidation, the specificity of encapsidation, viral and cellular factors required for morphogenesis, and inhibitors of morphogenesis. Emphasis is placed on the enteroviruses simply because of the greater abundance of information on these viruses. We conclude by describing an updated model of enterovirus morphogenesis, which is compared with the mechanism of particle assembly of other human and animal plus-strand RNA viruses. Unfortunately, due to the limited scope of this article, it is not possible to discuss every publication that deals with this subject in animal RNA virology or to give proper credit to everyone who has worked on this extremely complex process. However, we hope that the topics that we have selected for this review will provide the reader with an overview of the achievements and unsolved problems of picornavirus morphogenesis research that will stimulate further studies on this fascinating topic.

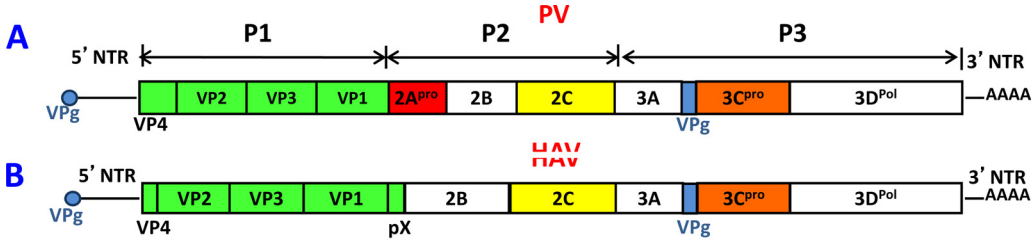
STEPS IN PICORNAVIRUS MORPHOGENESIS

Many of the individual steps in picornavirus morphogenesis were first studied with PV and are summarized in Fig. 3. Below, we describe them briefly and note differences that exist among different picornavirus species.

Polyprotein Processing, Myristoylation, and Interaction of P1 with Heat Shock Protein Hsp90

Enteroviruses. The capsid precursor domain (P1) of poliovirus is separated by the proteinase $2A^{pro}$ from the rest of the polyprotein

of symmetry is indicated with a ring. (Modified from reference 171 with permission of the publisher. Copyright 1989 Annual Reviews.) (B) Computer model of poliovirus. The 5-fold, 3-fold, and 2-fold axes of symmetry and the canyon are visible on the structure. (Reprinted from reference 172 with permission.) (C) Schematic representation of the three large poliovirus capsid proteins, each of which forms an eight-stranded, wedge-like, antiparallel β-barrel core (a) (3, 219). The antiparallel strands are connected by loops (BC, HI, DE, FG, GH, and CD). In panels b to d, the large capsid proteins are represented with ribbon diagrams (219). The four major neutralization antigenic sites (N-Ags) of poliovirus (type 1) map to surface loop extensions, as shown. N-AgI is a linear antigenic site that maps to the BC loop (amino acids 95 to 105) of VP1. All other major sites are discontinuous in nature: N-AgII (dotted line) spans VP1 and VP2 (amino acids 221 to 226 of VP1 and amino acids 164 to 172 of VP2). N-AgIII presents as two independent sites: N-AgIIIA consists of amino acids 58 to 60 and 71 to 73 of VP3 (dotted line), whereas N-AgIIIB contains amino acids 72 of VP2 and 76 to 79 of VP3. (Adapted from reference 3 with permission of AAAS.) (D) Localization of all major neutralization antigenic sites on the poliovirion indicating the density of possible neutralizing antibody-binding sites. (a) Band diagram of a pentamer containing the apex of the 5-fold symmetry axis. N-Ags are shown as white balls surrounding the mesa. Binding of antibodies to N-AgI, located on the rim of the canyon, leads to the neutralization of the virus by preventing attachment of the virus to the cellular receptor. (b) Dense distribution of N-Ags throughout the poliovirus capsid. (c to e) Band diagrams and neutralization antigenic sites of the capsid proteins VP1, VP2, and VP3. (Reprinted from reference 173 with permission of the publisher.)



C

	PV		HAV
I	P1 is myristoylated and cleaved from the polyprotein by $2A^{pro}$.	I	P1-pX is cleaved from the polyprotein by proteinase $3C^{pro}$. (P1-pX myristoylation is not observed).
II	P1 adopts a processing-competent conformation by association with Hsp90, P1*.		Hsp90 is not required for HAV production.
III	Cleavage of P1* by $3CD^{pro}$ to (VP0, VP3, VP1) , 5S "Protomer"	II	Cleavage of P1-pX by $3C^{pro}$ but a "Protomer" is never observed.
IV	$(VP0, VP3, VP1)_5$, 14S "Pentamer"	III	$(VP0, VP3, VP1\text{-}pX)_5$, "Pentamer"
V	$[(VP0, VP3, VP1)_5]_{12}$, 75S "Empty capsid, Procapsid"	IV	$[(VP0, VP3, VP1\text{-}pX)_5]_{12}$, "Empty capsid"
		V	$[(VP0, VP3, VP1\text{-}pX)_5]_{12}$**RNA**, "Preprovirion"
VI	$[(VP0, VP3, VP1)_5]_{12}$**RNA** , 150S "Provirion"	VI	$[(VP4, VP2, VP3, VP1\text{-}pX)_5]_{12}$**RNA**, "eHAV", Enveloped infectious particles
VII	$[(VP4, VP2, VP3, VP1)_5]_{12}$**RNA**, 150S Virion	VII	$[(VP4, VP2, VP3, VP1)_5]_{12}$**RNA**, "HAV" naked virion

FIG 3 Genome organization of poliovirus (PV) and hepatitis A virus (HAV) and steps in virion formation. The general PV/HAV genome organization is the same: a VPg-linked genome; a long 5′ NTR harboring replication signals and the IRES; a single ORF divided into P1 (capsid region [green]) and P2 plus P3, encoding nonstructural proteins; and 3′-terminal RNA structures terminated by poly(A). (A) PV encodes two proteinases (red and orange), of which $3C^{pro}$ functions mostly as a processing precursor of $3CD^{pro}$. The proteinase $2A^{pro}$ cleaves P1 from P2-P3 *in statu nascendi*. In addition, it cleaves numerous cellular proteins to the advantage of viral proliferation. The highly complex $2C^{ATPase}$ (yellow) is involved in numerous steps of viral replication, including morphogenesis. (B) HAV lacks $2A^{pro}$ and instead retains a sequence (pX) that is involved in morphogenesis. HAV uses $3C^{pro}$ to catalyze the release of P1-pX (green) from the polyprotein. (C, left) Individual steps in PV morphogenesis. We speculate that pentamers engage in genome packaging via interactions between capsid proteins and $2C^{ATPase}$. (Right) HAV morphogenesis is different from that of PV in many aspects. Nonmyristoylated HAV P1-pX (green) is cleaved and processed by $3C^{pro}$ (VP0, VP3, and VP1-pX). Hsp90 is not involved in HAV morphogenesis. We speculate that pentamers interact with genomic RNA to form "preprovirions." The events following the formation of the preprovirion are extraordinarily different from those of all other picornaviruses: they involve "cloaking of the particles" in host cell membranes (28), which predominantly leads to infectious "eHAV" particles still carrying VP1-pX. The site and mechanism of the switch to naked virions lacking pX and VP4 are still under investigation (28). For further details, see the text.

by cotranslational cleavage at a Y/G site (29) (Fig. 3C, PV step I). The N-terminal glycine of the newly formed poliovirus P1 capsid precursor is rapidly modified (perhaps also cotranslationally) by covalent linkage to myristic acid (30–32), after which it becomes a client of the cellular chaperone Hsp90 to attain a processing-competent conformation (referred to as P1* in Fig. 3C, step II) (33, 34). It is not known whether myristoylation of PV P1 is a prerequisite for its interaction with the chaperone Hsp90. However, only P1* can be effectively cleaved by the proteinase $3CD^{pro}$ into VP0, VP3, and VP1 (Fig. 3C, PV step III) (33, 35). After proteolytic cleavage by $3CD^{pro}$, the capsid proteins dissociate from Hsp90, an observation which indicates that subsequent steps of morphogenesis are independent of the chaperone. Instead of separating and dispersing in the cytoplasm, VP0, VP3, and VP1 immediately assemble into the 5S protomer, which functions as the fundamental building block of the capsid (see below). Notably, myristoylation of the N-terminal domain of the polyprotein is not conserved among all picornaviruses; in particular, it is absent in hepato- and parechoviruses (36, 37).

Hepatovirus. Unlike enteroviruses, cardio-, aphtho-, and hepatoviruses follow a different cascade for the processing of their polyproteins. In the case of HAV, the primary cleavage at the 2A/2B junction by $3C^{pro}$ yields the capsid precursor P1-2A, also referred to as P1-pX (Fig. 3C, HAV step I) (38). The small HAV 2A protein (pX) (8 kDa) has no enzymatic activity, but it is instead an essential component of the capsid precursor P1 for morphogene-

sis (38). HAV P1-pX is further processed by $3C^{pro}$ into VP0, VP3, and VP1-pX (Fig. 3C, HAV steps II and III). Neither HAV P1 nor VP0 is myristoylated (36), and Hsp90 is not required for HAV production (39). During later steps in HAV morphogenesis, pX is finally cleaved off from VP1 by an unidentified proteinase (see also below). Cardio- and aphthoviruses, in turn, evolved a very unusual processing mechanisms leading to the severance of P1 from the polyprotein (11). Briefly, it occurs by autocatalytic cleavage that does not involve a proteinase. Instead, it is catalyzed at a conserved motif (DxExNPG/\P) by a "CHYSEL" (*cis*-acting *hy*drolyase *el*ement) mechanism. For cardioviruses, the resulting P1 capsid precursor is linked to a 2A polypeptide lacking enzymatic activity; for aphthoviruses, it is linked to a minute "2A peptide" (only 18 amino acids long). Both of these appendices are later removed from the P1 precursors by the corresponding viral $3C^{pro}$ proteinases (11).

Formation of Small Capsid Precursors: the 5S Protomer (VP0, VP1, VP3) and the 14S Pentamer $(VP0, VP1, VP3)_5$

After the processing of the capsid precursor P1, the capsid proteins VP0, VP3, and VP1 of all known picornaviruses remain associated and immediately form the "protomer" (VP0, VP3, VP1) (Fig. 1A and 3C, PV step III). Five protomers then assemble to form a 14S "pentamer," $(VP0, VP3,VP1)_5$ (Fig. 1D and 3C, PV step IV). As discussed below (see "Myristic Acid"), the myristate moiety at the N terminus of the PV P1 precursor (VP4) enhances the assembly of protomers into pentamers (40–42). It should be noted that the temperature-sensitive (*ts*) growth phenotype of the PV type 3 Sabin vaccine strain is related to a defect at the nonpermissive temperature in the early assembly process, specifically at the protomer/pentamer step (43). In the case of HAV, 5S particles are not observed in infected cells, presumably because of the highly efficient association of protomers into pentamers (Fig. 3C, HAV steps II and III) (44), which is enhanced by the presence of pX (2A) in VP1-pX (45).

The exact role of pentamers in the assembly of the mature virus particle is still controversial. According to the currently accepted model, pentamers condense around the viral RNA during the assembly process to form a provirion. The alternate model proposes that pentamers first assemble into an empty capsid, into which the viral RNA is inserted to yield the provirion. Several lines of evidence favor the first model: (i) only 14S pentamers but not empty capsids display RNA-binding activity *in vitro*, and 14S pentamers undergo a conformational change upon RNA binding (46); (ii) during *in vitro* cell-free synthesis of mature poliovirus, only 14S pentamers interact with the newly made viral RNA to form mature virus (47); (iii) 14S pentamers can be found in cells infected with all picornaviruses (48, 49); (iv)14S pentamers can be made to accumulate in infected cells by using a temperature block, and after removal of the block, they can be chased into mature viruses (50); and (v) 14S pentamers are associated with the replication complex in infected cells and can be cross-linked to viral RNA but not to the replicative intermediate (RI) (51). Our recent studies with L-buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, also suggest that 14S pentamers are critical for the formation of mature virus particles and that the 75S empty capsids may not be functional intermediates in virus morphogenesis (22) (see below).

Formation of 75S Empty Capsids: $[(VP0, VP1, VP3)_5]_{12}$

Empty capsids, also known as procapsids, contain 12 pentamers but no viral RNA and sediment at 75S in sucrose gradients (Fig. 3C, PV step V). They have been known for decades to be formed during poliovirus infection (52, 53) but are not present in cells infected with mengovirus, a species of *Cardiovirus* (49). Early studies suggested the existence of two functional states of procapsids in PV-infected cells, one active and one inactive (48, 54, 55). The active procapsid can be disassembled into pentamers and can be reassembled into stable procapsids. Two different forms of procapsids, similar to those found *in vivo*, can also be observed during *in vitro* assembly of 14S particles (48, 56). It was proposed that the active particle exists *in vivo*, while the inactive particle is only a by-product of isolation procedures or conditions used during *in vitro* assembly. Putnak and Phillips (48) reported that the conformation of empty capsids made from 14S particles is enhanced by a "morphopoietic factor" present in infected cell extracts, but the identity of such a putative factor, if it exists, has never been elucidated.

As noted above, the role of the procapsid in viral morphogenesis is not yet clear. Some early studies suggested the possibility that it plays a role as a precursor. The addition of 1 to 3 mM guanidine hydrochloride (GnHCl) at midinfection, which inhibits RNA synthesis, caused the accumulation of procapsids, which could be chased into virus particles (57, 58). From our studies with BSO, we found that 75S particles, although they are made in normal amounts during infection in GSH-depleted cells, do not progress into forming mature virus, an observation which suggests that 75S may not be a functional capsid intermediate (22).

The Provirion: $[(VP0, VP1, VP3)_5]_{12}RNA$

In poliovirus morphogenesis, the provirion contains viral RNA in an "immature shell" composed of VP0, VP1, and VP3 (58, 59) (Fig. 3C, PV step VI) and sediments at 150S in sucrose gradients (60). The RNA in provirions is resistant to degradation by pancreatic RNase. However, in contrast to mature poliovirus, treatment of the particles with EDTA or 1% SDS results in the release of the viral RNA, yielding an empty capsid (60). The encapsidation of the viral RNA requires compact condensation of the RNA molecule and neutralization of the negatively charged phosphate groups by cations (61).

Using BSO, the inhibitor of poliovirus morphogenesis, we observed that GSH depletion leads to the accumulation of "provirion-like" particles, which migrate slightly faster on sucrose gradients than the 150S particles made under normal growth conditions and contain essentially no infectious virions, suggesting that GSH is required for the final maturation of viral particles (22).

In HAV, there is yet another intermediate, the "preprovirion," $[(VP0, VP3, VP1\text{-}pX)_5]_{12}RNA$, that still contains polypeptide X linked to VP1 (Fig. 3C, HAV step V) (62).

Mature Virions: $[(VP4, VP2, VP3, VP1)_5]_{12}RNA$

With most picornaviruses, the final step of morphogenesis is accomplished by the cleavage of VP0 into VP2 and VP4 (Fig. 3C, PV step VII). This triggers a rearrangement of the capsid proteins and confers stability to the resulting icosahedral particle (3). There is no evidence that the RNA is structured within the mature virus particle (63). In contrast to provirions, mature poliovirus particles are resistant to SDS or EDTA treatment and are not permeable to

Cs^+ ions (64). It should be noted that the mature virus particles of Aichi virus (65) and echovirus 22 (66) contain VP0 but no VP4.

The maturation cleavage of PV VP0 during the last step of virion formation is efficient, yet 1 or 2 VP0 molecules out of 60 remain uncleaved (67). The mechanism of VP0 cleavage is most likely autocatalytic and dependent on RNA encapsidation (3, 67). An asparagine and a serine form the VP4/VP2 cleavage sites (N/\S) of most picornaviruses (68). It was originally speculated that a serine (VP2 S10) near the N terminus of VP2 was activated during virion maturation to catalyze VP0 cleavage (69). However, mutation of this conserved serine to either cysteine (S10C) or alanine (S10A) yielded no maturation phenotypes of the respective virus variants (68). In contrast, a histidine (H195) in VP2, conserved in all picornaviruses, has been identified as being essential for efficient cleavage at the VP4/VP2 junction (70). An H195T or H195R mutation in PV VP2 resulted in the assembly of highly unstable 150S particles that contain uncleaved VP0 (70). It was suggested that the histidine activates local water molecules and initiates a nucleophilic attack on the scissile bond.

There are several amino acids in the capsid proteins that appear to directly affect morphogenesis, uncoating, or both. For example, two determinants of mouse adaptation of PV, the VP1 T22I and VP2 S32T mutations, affect both uncoating and particle assembly (71). Similarly, a deletion of residues 8 and 9 in VP1 (mutant 101) affects RNA release, while a deletion of residues 1 to 4 in VP1 (mutant 102) leads to defects in both encapsidation and uncoating (72). Finally, a *ts* mutant of PV (VP2-103), containing a single amino acid change, Q76R, accumulates provirions but no mature virus at the nonpermissive temperature (73).

Structural analyses have indicated that there is a major difference between empty capsids and mature viruses in the network formed by the N-terminal extensions of capsid proteins on the inner surface of the shell (3). On the inner surface of the mature capsid, the NH_2-terminal strands of VP1, VP2, and VP3 form an extensive network, which links the five protomers together to form a pentamer (3). The NH_2 termini of five VP3 molecules intertwine around the 5-fold axis and form a twisted tube, which was proposed to contribute to the stability of pentamers. The small capsid protein VP4 has a more extended structure than the other three capsid proteins, and it is similar to the NH_2-terminal strands of VP1 and VP3 in its position (3).

Other factors also affect virus maturation. As noted above, myristoylation of VP4 is important for virus maturation (36). As discussed below (see "3CD and VPg"), virus maturation is also enhanced by the viral protein 3CD in an *in vitro* translation/RNA replication system (74, 75). Moreover, we have recently shown that GSH is required for the production of infectious 150S particles (22). Finally, Richards and Jackson (18) proposed that virion maturation occurs in a cellular compartment known as the acidic autophagosome, but whether these structures are essential for poliovirus replication remains uncertain. Those authors observed that inhibitors of vesicle acidification have no effect on the production of 150S virus particles but inhibit the cleavage of VP0 to VP2 and VP4 and the accompanying formation of infectious virions. Nevertheless, it should be remembered that poliovirus RNA can translate→replicate→encapsidate in the cell-free environment of a cytoplasmic extract of HeLa cells (76), and although membranous components are absolutely required (77), it is unlikely that highly organized membrane structures would survive the preparation of the cell extract.

With the maturation of the viral particle, there is a mesa formed at the top of the 5-fold axis of the poliovirion that is surrounded by a cleft that has been termed "canyon" (Fig. 1A and B) (2). All enteroviruses (including rhinoviruses) have this canyon (78). Although canyons of several enteroviruses function as the receptacle of their respective receptors (e.g., PV, HRV14, and coxsackie B virus 3 [CBV3]), a large number of enteroviruses use structures other than this cleft to attach to the cell (HRV2, some echoviruses, and coxsackie B viruses) (9, 79–82).

Of those picornaviruses analyzed, VP1, the largest capsid protein, forms at the bottom of canyons a peculiar hydrophobic pouch or "pocket," first observed by Hogle and colleagues (3). VP1 pockets found in a large number of picornavirions are occupied by a "pocket factor" of cellular origin whose identity and function are still ill defined. In general, they have been described as fatty acid-related, hydrophobic compounds, but some enteroviruses (HRV14 and HRV3) and some cardioviruses (EMCV and mengovirus) do not appear to carry pocket factors altogether (78, 82). Although there is no proof that pockets and pocket factors are involved in morphogenesis or uncoating, it is highly unlikely that their existence is just an accident.

The so-called WIN compounds are among the best-known inhibitors of picornavirus replication. They invade and lodge inside the hydrophobic pocket of enteroviruses beneath the canyon floor of the capsid (78, 83, 84). Because the binding sites of the viral receptor and the pocket factor overlap, only one WIN compound fits into the space. As was found for human rhinoviruses, binding of these compounds can have two distinct consequences: they may inhibit either receptor binding, as in the case of HRV14 (85, 86), or uncoating, as with HRV1A (87).

Mature poliovirions display on their surface an impressive web of neutralization antigenic sites (N-Ags), which are defined as structures (linear or nonlinear) to which neutralizing antibodies can bind. The positions of the N-Ags on the individual large capsid proteins are shown in Fig. 1C and Dc to e. The entire web of N-Ags on virions is shown in Fig. 1Db. With the use of specific monoclonal antibodies, these epitopes offer convenient ways to identify mature virions in tissue culture cells or by light microscope imaging experiments. Generally, poliovirions express major (N-AgIA, N-AgIIA, N-AgIIIA, and N-AgIIIB) and minor (N-AgIB and N-AgIIB) neutralization antigenic sites. Altogether, polioviruses express three unique sets (in sequence and/or structure) of all these sites; hence, poliovirus exists as three serotypes. We note that if poliovirus should attempt to evolve a fourth serotype, it would have to change sequences and/or structures of all N-Ags, thereby generating an entirely new set. Fortunately, no new poliovirus serotypes have been discovered since the three serotypes were originally identified in 1951 (88). Thus, changing of an entire set of N-Ags appears to be extremely difficult, even over a time of, say, 1 century. In contrast, human rhinoviruses, whose crystal structures are very similar to those of the polioviruses, have evolved dozens of unique sets of N-Ags; hence, human rhinoviruses exist as dozens of serotypes.

Special Case of a Picornavirus: the Naked and Membrane-Cloaked Particles of Hepatitis A Virus

A very special case among the picornaviruses is HAV. HAV is released from tissue culture cells and its target tissue *in vivo* (in infected humans) in not only in a nonenveloped (naked) form (Fig. 3C, step VII) but also in an enveloped form (eHAV) (Fig. 3C,

step VI) (28). HAV released from cells is cloaked in host-derived membranes, which protect the virus from neutralization by antibody. Using a pathway involving the endosomal sorting complexes, the preprovirions envelop themselves into membranes (1, 2, or even 3 particles per vesicle) and egress cells as enveloped particles called eHAV (Fig. 3C, step VI) (28). During this process, VP0 undergoes maturation cleavage, processing VP0 into VP2 and VP4 (23 amino acids long), presumably by RNA-induced autocatalysis. pX is retained as a component of eHAV (60 copies of VP1-pX) until the eHAVs shed the membranes prior to fecal egress from a patient, a step still poorly understood (Fig. 3C, step VII) (28) but leading to the maturation of naked virions in stool. During the last step of morphogenesis, pX is cleaved off from all VP1 proteins of the particle by an unknown cellular proteinase (19).

DO PICORNAVIRUSES USE RNA PACKAGING SIGNALS IN MORPHOGENESIS?

Morphogenesis of progeny viral genomes is highly specific, and RNA viruses have evolved different elaborate mechanisms to discriminate against nucleic acids as substrates other than their own. The best-known mechanism, used by many RNA viruses, is an RNA packaging signal that is recognized by one or more capsid proteins to provide specificity to the encapsidation process (89–91). Another mechanism has been suggested (without experimental proof), that specific interactions between capsid proteins and the nonstructural proteins of the replication complex are involved (92). This would place the progeny RNA near the site of assembly. As we will show, picornaviruses have evolved to use both strategies: encapsidation specificity by protein-protein interactions (enteroviruses) and encapsidation by recognition of packaging signals (Aichi virus of the genus *Kobuvirus*).

All Searches for RNA Packaging Signals in Enterovirus Genomes Have Failed

As outlined below, numerous studies aimed at identifying RNA-based encapsidation signals in the 5′ and 3′ NTRs of enteroviral genomes have been unsuccessful. These searches generally included the construction of chimeric genomes in which various segments were exchanged between genomes of different enteroviruses, followed by assaying for the nature of the progeny virus (if there was one). Alternatively, the polyprotein of enterovirus was radically recoded by changing codon pairs without altering the codon usage or the amino acid sequence of the target protein. By the latter strategy, it was assumed that RNA packaging signals would be destroyed without interference with genome replication, provided that essential replication signals, like *cre*(2C), were not modified.

The 5′ NTR. The first evidence against the 5′ NTR carrying an RNA packaging signal was provided by Semler and colleagues, who exchanged nearly the entire 5′ NTR of PV with that of CBV3 (nt 1 to 627) without losing the PV-specific replication-and-encapsidation phenotype (93, 94). Subsequently, numerous PVs with exchanges of IRESs from different viruses (including that from hepatitis C virus [HCV]) were constructed and analyzed; none of the chimeras showed significant translation/replication/morphogenesis phenotypes (7, 95). Similarly, the cloverleaf at the 5′ end of the PV genome was exchanged with that of the quite distantly related human rhinovirus 2 (HRV2). This chimera also expressed an adequate proliferation phenotype (96). Combined, these data strongly suggest that the PV genome, and perhaps all enterovirus genomes, does not contain a packaging signal in the 5′ NTR.

RNA sequences in the polyprotein-encoding region. (i) The P1 capsid domain. PV proliferation can lead to the evolution of defective interfering (DI) particles, in which a portion (of various lengths) of the P1 domain encoding the capsid proteins has been deleted (97, 98). DI particles recruit their capsid proteins in *trans* from coinfecting wild-type (*wt*) PV, thereby reducing (interfering with) the replication of the *wt* virus. Kuge and colleagues subsequently made the important observation that the deletions in the P1 domain of DI particles must be in frame with the rest of the polyprotein, or else there is no PV genome replication and encapsidation (99); they concluded correctly that translation of the ORF of the polyprotein (regardless of whether it contains P1 sequences or not) is required in *cis* for genome replication. Novak and Kirkegaard later provided strong support for this hypothesis (100) (see below). Kajigaya et al. proposed that the P1 region is unlikely to harbor an RNA packaging signal since the genomes of DI particles can be encapsidated in *trans* (101).

A number of investigators then replaced part or all of the capsid coding sequence in P1 with foreign genes, thereby generating replicons suitable for studies of PV replication and encapsidation, even for gene therapy. Porter and colleagues expressed a multitude of different genes in PV replicons, with firefly luciferase being the most suitable for studies of encapsidation (102). Barclay and colleagues used chloramphenicol acetyltransferase (CAT) inserted into the P1 domain as a foreign marker gene (103). These replicons carrying foreign genes could be encapsidated in *trans* by coinfection with either a vaccinia virus expressing the poliovirus capsid proteins (104) or PV (105). The exquisite specificity of encapsidation was revealed when enteroviruses other than PV were used in coinfections: the PV replicons could not be packaged in *trans* into capsids of bovine enterovirus, C-cluster coxsackievirus A21, B-cluster coxsackieviruses B3 and B4, rhinovirus 14, or enterovirus 70 (102, 103).

(ii) The P2-P3 nonstructural domains. A computational analysis of conserved RNA structural elements in genomes of PV and related C-cluster coxsackie A viruses (C-CAVs) identified an RNA element mapping to the coding region of PV $2C^{ATPase}$, termed *cre*(2C) (106). *cre*(2C) plays an essential role in genome replication; equivalent *cre* elements have been found in the genomes of all picornaviruses (107, 108). Using all enterovirus genome sequences known in 2001, Witwer et al. identified a highly conserved stem-loop structure in the C-terminal coding region of the PV RNA polymerase $3D^{Pol}$ (109), subsequently called the beta stem-loop (110). However, no evidence has been uncovered to suggest that either *cre*(2C) or the beta stem-loop is involved in morphogenesis (107, 110).

Recently, we used a genome-wide scan involving large-scale recoding of the ORF of the PV polyprotein in combination with synthetic biology. This allowed us to search for functional RNA elements in enterovirus RNAs that had escaped previous discovery (111, 112). A computer algorithm, called "Scrambled" (SD), shuffled synonymous codons in different domains of the PV polyprotein, thereby introducing hundreds of silent mutations into the ORF without altering the amino acid sequence or codon usage. Scrambling of the P1 capsid-encoding region had no effect on PV proliferation, although 934 mutations were introduced into the 2,642-nt P1 coding domain (112). Scrambling of the P2 domain

plus a small segment of the P3 domain (701 mutations in 2,214 nt), however, killed the virus, as expected, because *cre*(2C) in P2 was destroyed. The virus was revived when *cre*(2C) was rebuilt into the otherwise scrambled sequence (110). Genetic analyses provided no evidence for *cre* as a packaging signal (113). Similarly, the scrambled sequence (on average, every fourth nucleotide was changed) is not expected to support morphogenesis. Scrambling of P3 (625 mutations in 1,764 nt) revealed the existence of 2 closely spaced, functionally redundant RNA elements (α and β) in the close vicinity of the C terminus of the $3D^{pol}$-encoding region (Fig. 2), which we found are required for RNA replication but not for encapsidation (110). This leads us to the conclusion that it is highly improbable that there are RNA sequence elements essential for particle assembly in the coding region of the PV polyprotein.

The 3′ NTR. Several studies have shown the importance of the PV 3′ NTR for genome replication. For example, Pilipenko et al. provided genetic evidence that "kissing" between stem-loops X and Y in the 3′ NTR was required for adequate genome synthesis (114). Similarly, an intact PV 3′ NTR was essential for efficient PV RNA synthesis followed by encapsidation (115). While there is no controversy about these data (116), the following surprising observations clearly show that the PV 3′ NTR is not required for PV replication and morphogenesis. First, the entire 3′ NTR can be replaced with structurally very different 3′ NTRs from HRV14 or B-cluster CBV4 without significant impairment of PV replication and PV encapsidation (117). Second, Todd et al. reported the astounding observation that the entire PV 3′ NTR can be deleted [except for poly(A)], yet the resulting variant replicated and packaged albeit with reduced efficiency (118).

Encapsidation of enterovirus negative-strand RNAs under certain circumstances. Under normal conditions of infection, only plus-strand RNAs are encapsidated. As an exception, the encapsidation of minus-strand RNAs has also been observed in noncytopathogenic CVB3 that was isolated from persistently infected murine hearts (119). The RNA genomes of these viruses contain 5′-terminal deletions in the cloverleaf, and when replicated, the amount of progeny minus strand present is much increased relative to the amount of plus strand. Since minus and plus strands have complementary rather than identical sequences, these results argue against an RNA signal determining the specificity of encapsidation (see Model of Enterovirus Morphogenesis, below).

Together, we conclude that there is no evidence that the genome of enterovirus harbors an RNA packaging signal essential for morphogenesis.

RNA Packaging Signal in the Genome of Aichi Virus

The *Picornaviridae* are a huge family of viruses that have evolved specialties in different stages of their replication, although generally, they follow a common proliferation plan. It therefore may not be surprising that morphogenesis of Aichi virus of the genus *Kobuvirus* involves an RNA packaging signal. The 5′ NTR of Aichi virus contains a hairpin at the very 5′ end that has been reported to play a role in particle assembly (25). Mutations constructed into the stem of the hairpin lead to the production of mostly empty capsids and very few mature virus particles. In addition to this hairpin, the leader (L) protein of Aichi virus harbors a possible function in virus assembly (26).

SPECIFICITY OF ENTEROVIRUS ENCAPSIDATION IS PROVIDED BY A SPECIFIC INTERACTION BETWEEN CAPSID PROTEINS AND NONSTRUCTURAL PROTEIN $2C^{ATPase}$

During the life cycle of picornaviruses, the plus-stranded full-length genomic RNA serves three main functions: (i) as mRNA for translation, (ii) as the template for RNA replication, and (iii) as the genome for encapsidation. As first suggested by K. Kirkegaard, the three fundamental processes of translation, replication, and morphogenesis in picornavirus proliferation are so tightly linked that they serve as a "higher-order" proofreading mechanism: without adequate translation, there is no genome replication, and without genome replication, there is no encapsidation (92, 95). It follows that ready-made genomic RNA (VPg-linked genomic RNA, infectious mRNA, or genomic transcripts) present in the cytoplasm has no chance to serve as the substrate for the assembly of virions, as was observed in studies of a cell-free replication system developed by Molla et al. (76). Thus, a positive identification of an encapsidation signal is possible only if modifying that signal has no detrimental effect on RNA translation or RNA replication (20). This observation and the lack of apparent RNA packaging signals in enterovirus genomes raised the possibility that the specificity of encapsidation of poliovirus, and perhaps of all enteroviruses, is provided solely by an interaction between capsid and nonstructural proteins, a mechanism that would be quite unique in virology.

Multiple Roles of Enterovirus $2C^{ATPase}$ in the Virus Replication Cycle

$2C^{ATPase}$ is a complex nonstructural polypeptide that expresses numerous functions: (i) it has been proven to be crucial for RNA replication and binding (120–124); (ii) it contains a nucleoside triphosphate (NTP)-binding domain (125) and possesses ATPase activity (discovered *in vitro*), which is inhibited by guanidine hydrochloride (GnHCl) (126); (iii) it has been implicated (weakly) in virus uncoating (127); (iv) together with its processing precursor $2BC^{ATPase}$, it is involved in cellular membrane rearrangements and the formation of the cytoplasmic vesicle "web" typical of all enterovirus replication (13, 128–131); and (v) drug (hydantoin) inhibition and genetic studies have provided convincing genetic evidence that $2C^{ATPase}$ is involved in encapsidation (20, 21, 132).

Analyses of the linkage map of nonstructural proteins revealed that $2C^{ATPase}$ has binding affinity for 3AB (133, 134) or $3C^{pro}$ (135). For enterovirus 71 infections, it has been reported that $2C^{ATPase}$ binds the host protein reticulon-3 (136), but this was not found in a yeast two-hybrid scan with poliovirus $2C^{ATPase}$ (C. Wang and E. Wimmer, unpublished data). In a recent study, the interaction of $2BC^{ATPase}/2C^{ATPase}$ with VCP/p97, a cellular ATPase, was demonstrated, which was strongly enhanced by GnHCl (137). The significance of this observation, however, has not been elucidated. Considering the multitude of $2C^{ATPase}$ functions in various phases of enterovirus replication combined with the functional linkage between translation, replication, and morphogenesis, deciphering a role of $2C^{ATPase}$ in morphogenesis seemed hopeless.

Unique Assay Separating Replication from Morphogenesis in Tissue Culture Experiments

A key for these studies was a novel reporter virus that has allowed us to distinguish the function of $2C^{ATPase}$ in RNA replication from that in encapsidation in a two-step experiment (20) (Fig. 4). The

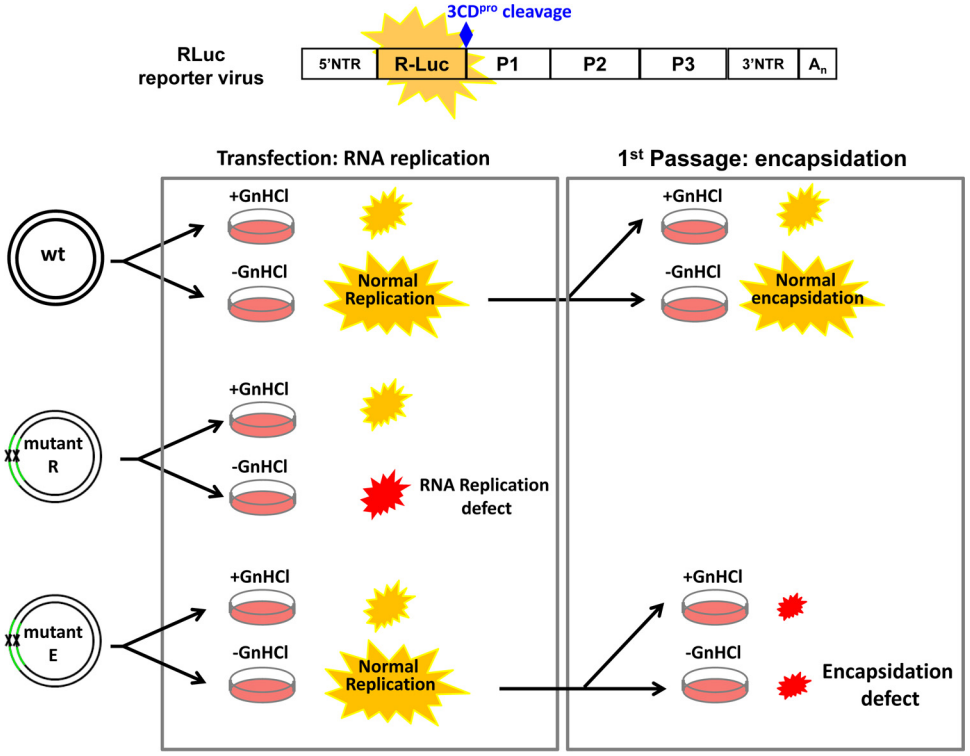


FIG 4 Use of renilla luciferase (RLuc) reporter virus to distinguish a defect in RNA replication from a defect in encapsidation. The genome of the RLuc reporter virus contains an RLuc gene fused to the N terminus of the PV polyprotein coding sequence. The RLuc reporter virus can distinguish a defect in RNA replication (mutant R) from that in encapsidation (mutant E) in a two-step experiment. In the first step, the reporter transcripts are transfected into HeLa cells in the absence and presence of guanidine hydrochloride (GnHCl), a potent inhibitor of RNA replication. After transfection, luciferase activity produced in the absence of the drug is a measure of RNA replication, while in the presence of the drug, only translation of the transfecting RNA is measured. In the second step, lysates of cells transfected in the absence of the drug are used to infect fresh HeLa cells. Luciferase activity in the absence of the drug is a measure of encapsidation in the first HeLa cells that were transfected. Mutant R has a defect in genome replication; hence, only a small Luc signal derived from translation of the transfected RNA is observed. Mutant E has a defect in encapsidation. It produces a robust Luc signal after transfection (first HeLa cell monolayer), but no Luc signal is detected in infected HeLa cells because no infectious progenies are formed during transfection.

reporter virus genome contains the renilla luciferase (RLuc) gene fused to the N terminus of the polyprotein that is cleaved off from the polyprotein by $3CD^{pro}$ after translation. RNA replication and encapsidation are measured by luciferase activity after (i) transfection into HeLa cells (assay for replication) and (ii) the first passage of virus (if morphogenesis took place) into new naive HeLa cells (assay for morphogenesis). Specifically, RNA transcripts of the reporter cDNA are transfected into HeLa cells, in the absence and presence of GnHCl, a potent inhibitor of RNA replication (124, 138, 139). The luciferase activity produced in the absence of the drug is a measure of both translation and RNA replication of genomes (and, hence, a measurement of replication), while in the presence of the drug, only protein translation is assayed. In the second step, lysates of cells transfected in the absence of the drug are used to infect fresh HeLa cells. For the *wt* reporter virus, which is normal in both RNA replication and encapsidation, luciferase activity is produced during both transfection and infection after passage in new cells (Fig. 4). With a mutant defective in RNA replication ("mutant R"), only luciferase activity from protein translation of the transfected viral RNA is produced (Fig. 4). However, encapsidation-defective mutants ("mutant E"), even though luciferase activity can be observed from RNA replication in transfected cells, does not produce any significant levels of luciferase activity after passage into fresh HeLa cells (Fig. 4). This is because no intact infectious virus capable of infecting fresh cells upon passage is produced after transfection. Using this strategy, we have discovered that at least one of the functions of $2C^{ATPase}$ in assembly is to interact with one of the capsid proteins (VP3 and/or VP1) (20, 21).

Analyses of Chimeras between C-Cluster Coxsackie Viruses and Poliovirus Suggest That $2C^{ATPase}$-Capsid Protein Interaction Provides Specificity of Morphogenesis

In the course of the global eradication of poliovirus with the live, oral Sabin vaccine (OPV), the observation was made that OPV can readily recombine in the field with closely related C-CAVs (140). The recombinants evolved rapidly into viruses that were as neurovirulent as the *wt* polioviruses targeted for eradication, and they caused small epidemics of poliomyelitis in many parts of the world. All neurovirulent recombinants were found to have capsid domain P1 of PV (and, hence, the virulence identity of PV) and various parts of downstream domains P2 and P3 of C-CAV. We abbreviate the recombinants "P-C-C," indicating the three-domain polyproteins from two different viruses. In our studies of this highly important phenomenon for polio eradication, we generated P-C-C viruses in the laboratory and compared them with their C-P-P counterparts, e.g., consisting of the capsid domain of C-CAV and the two nonstructural domains of PV. C-P-P has the

identity of a coxsackievirus because of its interaction with ICAM-1 as a receptor and pathogenesis resembling that of the common cold. An unexpected observation was made, that all P-C-C constructs grew very well, whereas most of the C-P-P constructs did not grow at all (141). Importantly, the genomes of C-P-P translated and genome replicated with *wt* kinetics, but as Luc chimeras, they did not produce virions in our two-step assay. However, blind passage of a C-P-P variant on HeLa cells generated individually two poorly growing small-plaque suppressor mutants, with the first having a single amino acid substitution in C-CAV VP3 (C*-P-P) and the second having a single amino acid exchange in PV $2C^{ATPase}$ (C-P*-P). The poor-growth phenotype of C*-P-P or C-P*-P in tissue culture cells changed to a variant growing with *wt* kinetics and a large-plaque phenotype when both mutations were engineered into the chimeric genome, yielding the C*-P*-P genotype (20). These results suggested a direct interaction between $2C^{ATPase}$ and the capsid protein VP3, which was subsequently proven by coimmunoprecipitation assays.

Alanine Scanning Analysis of $2C^{ATPase}$ Confirms the Linkage between the Nonstructural Protein and Capsid Proteins in Morphogenesis

The protein-protein mechanism for encapsidation specificity has been further supported by alanine scanning mutagenesis of $2C^{ATPase}$, a strategy involving the sequential replacement of clusters of charged amino acids in a polypeptide with alanine residues (21). Of many mutants that were generated, one was "quasi-infectious" (*qi*), a phenotype with greatly debilitated replication such that progeny virions could not be isolated. However, blind passage of the *qi* variant yielded second-site suppressor mutations leading to new variants that could be grown and plaque titrated. In the case of the $2C^{ATPase}$ *qi* mutant, the second-site suppressor mutations mapped to capsid proteins, a result supporting rescue by regeneration of the $2C^{ATPase}$-capsid protein interaction. Surprisingly, in two independently rescued variants, the second-site suppressor mutations each mapped to either VP3 or VP1. These genetic results are strong evidence that $2C^{ATPase}$ and capsid proteins must communicate for morphogenesis to occur, most likely by direct binding (20).

Other Nonstructural Proteins Involved in Picornavirus Morphogenesis

3CD and VPg. The PV proteinase $3CD^{pro}$, a multifunctional polypeptide, is the precursor of the RNA polymerase $3D^{pol}$ (lacking RNA polymerase activity) as well as of the proteinase $3C^{pro}$. The $3CD^{pro}$ protein, an important proteinase itself (Fig. 3C, PV step III), is also an important RNA-binding protein during RNA replication. It interacts with both the 5′ cloverleaf structure of the viral RNA, in conjunction with 3AB or PCBP2 (142, 143), and the *cre*(2C) RNA element during the protein-priming step of RNA synthesis (144, 145). The best-known function of $3CD^{pro}$ in encapsidation is the proteolytic processing of P1. It appears that $3CD^{pro}$ can cleave all designated Q/\G scissile bonds in the PV polyprotein, whereas $3C^{pro}$ cannot: the latter is insufficient for processing P1 (146). Thus, $3CD^{pro}$ is for as-yet-unknown reasons exclusively responsible for the cleavage of the PV capsid precursor P1 into VP0, VP1, and VP3.

Another role for the 3CD polypeptide in encapsidation, which is independent of its protease activity, was discovered with studies of an *in vitro* translation/RNA replication system that produces viable poliovirus (76). It was observed that proteolytically inactive 3CD (designated 3CD instead of $3CD^{pro}$) strongly stimulated mature virus production in the *in vitro* system (74, 75). Stimulation was dependent on the RNA-binding activity of the protein, residing in the 3C domain, and the integrity of "interface I" in the 3D domain. Interestingly, 3CD only stimulates the encapsidation of a VPg-linked viral RNA template (74, 75). A T7 RNA polymerase-generated transcript RNA could not replace VPg-linked RNA in the stimulation reaction, even if the two 5′-terminal G's were removed by ribozyme. Thus, it appears that 3CD has an affinity for the VPg-linked 5′ end of the poliovirus genome that may aid in encapsidation, an observation that also suggests a role for VPg in encapsidation.

2A of hepatitis A virus. In contrast to most members of the *Picornaviridae*, HAV expresses only one proteinase ($3C^{pro}$) for the proteolytic cleavage of its polyprotein (147). The initial cleavage by HAV $3C^{pro}$ is between 2A and 2B, thereby producing a capsid precursor, P1-2A, which is subsequently cleaved into VP0, VP3, and VP1-2A. The 2A protein of HAV, also called pX, is a C-terminal extension of the structural protein VP1, and it lacks proteinase activity. Interestingly, pX (2A) functions in both particle assembly, by promoting the formation of pentamers, and the subsequent particle maturation step (38, 62, 147). Using genetic analyses, Morace and colleagues (27) showed that the C terminus of the 2A protein is important for the liberation of VP1-pX from the polyprotein, while a basic residue at the C terminus of VP1 is required for efficient particle assembly. The pX protein is removed from VP1-pX by an unknown host proteinase(s) so that mature nonenveloped HAV particles lack pX (28). However, in eHAVs, which are the dominant forms of virus released from infected cells, pX is still attached to VP1, but VP0 is already cleaved into VP2 and VP4 (28).

Leader protein of Aichi virus. The Aichi virus genome encodes a 170-amino-acid-long L protein upstream of the capsid-encoding region (Fig. 2). This polypeptide exhibits no sequence similarity to the L proteins of other picornaviruses, such as that of aphthovirus, cardiovirus, or teschovirus (26). A deletion analysis of the Aichi virus L protein revealed that this polypeptide is important for both RNA replication and encapsidation. Specifically, a deletion of the C-terminal 50 amino acids of the L protein resulted in efficient RNA replication, but the virus yield was strongly reduced compared to that of the *wt* virus. A sedimentation analysis of the mutant virus showed that it has a severe defect in the formation of mature virions but not in that of empty capsids (26), indicating that L protein is involved in virus morphogenesis.

CELLULAR FACTORS INVOLVED IN PV MORPHOGENESIS

Heat Shock Proteins Hsp70 and Hsp90

Heat shock proteins are a group of functionally related proteins that aid in the folding or unfolding of proteins. Their expression levels are increased when cells are exposed to elevated temperatures or other stresses. An interaction between the PV P1 capsid precursor and Hsp70 was first observed by Macejak and Sarnow (34). Subsequently, the involvement of the cellular chaperone Hsp90 in picornavirus assembly was discovered with the use of geldanamycin, a specific inhibitor of Hsp90, to inhibit poliovirus growth in tissue culture cells (33). In uninfected cells, Hsp70 delivers proteins to Hsp90 in a partially folded state (148, 149). It was proposed that Hsp90 aids in the proper folding of the PV P1 capsid

precursor, maintains it in a processing-competent conformation, and protects it from proteasomal degradation (33). A recent report suggests that the role of Hsp90 in FMDV morphogenesis is to stimulate the formation of pentamers from protomers rather than to promote P1 processing (150).

Myristic Acid

Myristic acid, also called tetradecanoic acid, is a saturated fatty acid cotranslationally linked to the N terminus of the PV P1 precursor (30–32). The myristic acid moiety functions in particle assembly, possibly both early and late in the process (151, 152). Two possible functions of myristoylation in the early particle assembly process have been proposed. First, myristoylated VP0 targets P1 to the membranous replication complex (42). Second, the myristic moiety facilitates protomer-protomer interactions for pentamer assembly (40, 41). Similar functions were proposed for myristoylation during pentamer formation of FMDV (36). VP0 myristoylation also appears to be important in late stages of PV assembly during virus maturation (40, 151, 152).

Glutathione

Glutathione (GSH) (γ-L-glutamyl-L-cysteinylglycine) is an important reducing agent in human and animal cells, which helps maintain the redox potential of cells and is important for immune function. It also has additional functions in processes such as signal transduction, apoptosis, gene expression, and regulation of protein function (153). The thiol group of GSH is responsible for its biological activity. GSH exists in cells either in a reduced form (GSH) or, to a lesser extent, in an oxidized state, glutathione disulfide (GSSG).

Glutathione synthesis in cells involves two consecutive steps. In the first step, glutamate and cysteine are linked to form a dipeptide by the enzyme glutamylcysteine synthase. In the second step, glutathione synthase catalyzes the addition of glycine to the dipeptide. L-Buthionine sulfoximine (BSO) is a potent inhibitor of glutamylcysteine synthase and of GSH biosynthesis (154). Depletion of GSH by BSO completely inhibits the growth of enteroviruses at the stage of virus morphogenesis (22, 24), but it has no effect on translation or genome replication. Moreover, PV or C-CAV20 variants resistant to depletion of GSH can readily be isolated, with the mutations mapping exclusively to viral capsid polypeptides (VP3 and VP1), an observation suggesting that GSH is needed for steps in morphogenesis downstream of RNA synthesis. We have recently shown that GSH depletion inhibits the accumulation of pentamers in PV-infected cells and that GSH directly interacts *in vitro* with capsid precursors and mature virus (22). Using a different inhibitor, TP219, Thibaut and colleagues also made a similar observation that GSH is required for the morphogenesis of coxsackie B virus (23). Indeed, GSH pulldown assays have shown that the compound interacts directly with 14S, 75S, provirions, and mature virus particles (22). In separate experiments, we have found that GSH protects mature virus from heat inactivation *in vitro* (22). We propose that the function of GSH in enterovirus morphogenesis is to stabilize the pentamers and the mature virus during virus assembly.

Cellular Membranes

The rearrangement of cellular membranes into specific structures associated with RNA replication/encapsidation is a characteristic of the plus-strand RNA virus life cycle in eukaryotic cells. Several cellular pathways, such as the secretory pathway, autophagy, and lipid biosynthesis, are involved in the formation of replication organelles (155–158). The PV-induced structures appear as vesicles of different sizes (50 nm to 400 nm in diameter) that are associated with the endoplasmic reticulum (ER) and later are clustered in the perinuclear region (159, 160). RNA replication of enteroviruses occurs in association with tubular structures, where the replication proteins are localized on external membranous surfaces facing the cytoplasm (158, 159). Early in infection, the tubular structures have a single membrane, but a very large fraction of them are later converted into double-membrane vesicles (155, 158, 161).

Relatively little is known about the requirement for membranes in morphogenesis. However, it was shown that membrane-associated PV replication complexes synthesizing viral RNA contain 5S protomers and 14S pentamers (162). These subviral particles and each of the capsid proteins (VP0, VP1, and VP3) can be UV cross-linked to progeny viral RNA. It was proposed that 14S pentamers associate with the viral RNA as the first step in encapsidation (51). A recent study suggested that blocking of autophagosome formation reduced PV RNA replication, while vesicle acidification was required only for the final maturation cleavage of the virus particles (163, 164). Specifically, the ratio of uncleaved VP0 to VP3 in 150S sucrose gradient peaks of PV-infected cell lysates was found to be increased about 2-fold in cells treated with vesicle acidification inhibitors such as ammonium chloride or bafilomycin A1. So far, the available evidence suggests that autophagosomes are supportive of but not required for PV replication and maturation.

INHIBITORS OF MORPHOGENESIS

Hydantoin

The small organic compound hydantoin [5(3,4-dichlorophenyl) methylhydantoin] strongly inhibits enterovirus morphogenesis in tissue culture by aborting maturation at an 110S intermediate (132). The effect of hydantoin is reversible since the 110S particles can be chased into mature virions after removal of the drug. PVs resistant to hydantoin were readily isolated, and the mutations were found to map to the $2C^{ATPase}$ protein. In subsequent studies with hydantoin in a cell-free translation/RNA replication system (76), inhibition of protein processing was observed, but no 110S particles were found (47).

Guanidine Hydrochloride

GnHCl reversibly inhibits viral RNA synthesis and encapsidation when used at concentrations where cellular macromolecular synthesis is not affected. When GnHCl is added to cells halfway through PV infection, the newly made RNA is trapped in 80S particles, called the guanidons, which accumulate (57). After the removal of the drug, the amount of 80S particles decreases, along with increases in the amounts of provirions and virions. Subsequent studies showed that GnHCl inhibits the ATPase activity of purified $2C^{ATPase}$ (126). Whether or not the ATPase activity of the $2C^{ATPase}$ protein is directly involved in encapsidation is not yet clear.

L-Buthionine Sulfoximine

As noted above (see "Glutathione"), BSO is a drug that inhibits glutamylcysteine synthase during the biosynthesis of the cellular

TABLE 1 Comparison of morphogenesis of *Picornaviridae* to those of other plus-strand RNA virus families

Morphogenesis trait	Presence of trait in virus family (reference[s])[a]					
	Picornaviridae		*Flaviviridae*		*Togaviridae* (SINV, SFV, rubella virus, Mayaro virus, VEEV)	*Coronaviridae* (MHV, SARS-CoV)
	PV, CAV, CBV, FMDV	Aichi virus	HCV, YFV, WNV	DENV		
RNA replication and encapsidation linked	+	+ (25)	+ (168, 175–181)	+ (182)	+ (183)	NA
Assembly associated with membrane	+ (51, 162)	NA	+ (184–187)	+(182)	+	+ (188–190)
Interaction between capsid and NS protein required	+ (20, 21)	NA	+ (191–199)	NA	+ (200)	NA
RNA encapsidation signal	−	+ (25, 26)	−	+ (201)	+ (202, 203)	+ (204–206)
Stable capsid intermediates	+ (63)	NA	−	−	−	−
Involvement of host factors	+ (22–24, 30, 32, 33)	NA	+ (207–215)	NA	+ (216–218)	NA

[a] PV, poliovirus; CAV, coxsackie A virus; CBV, coxsackie B virus; FMDV, foot-and-mouth disease virus; HCV, hepatitis C virus; YFV, yellow fever virus; WNV, West Nile virus; DENV, dengue virus; SINV, Sindbis virus; SFV, Semliki Forest virus; VEEV, Venezuelan equine encephalitis virus; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; NA, not available.

reducing agent glutathione (24, 154). GSH depletion by BSO treatment has no effect on protein translation or RNA replication of enteroviruses. In particular, BSO inhibits the accumulation of 14S particles and finally affects the production of mature virus in infected cells. Our recent studies with GSH pulldown assays have shown that GSH interacts directly with 14S, 75S, and mature virus particles. Drug-resistant mutants, which contained mutations in VP1 and VP3 at protomer-protomer interfaces, evolved during passaging, suggesting that the role of GSH during enterovirus morphogenesis is to stabilize the pentamers and mature virus particles both during and after the viral assembly.

Py-11

The nucleoside analogue Py-11 (2-amino-4,6-dichloropyrimidine) is a potent inhibitor of PV morphogenesis at the stage of cleavage of the capsid precursor P1, thus preventing the assembly of capsid proteins into pentamers (165). The effect of the drug can be antagonized by the joint addition of glutamine and cysteine during infection. The target of the drug has not yet been identified.

Geldanamycin

Geldanamycin is a benzoquinone ansamycin antibiotic, originally discovered in *Streptomyces hygroscopicus*, which binds to the ATP/ADP-binding pocket of Hsp90 and thereby inhibits its function (166, 167). Hsp90, a cellular chaperone, plays an essential role in the folding and function of protein kinases, steroid hormone receptors, and numerous cellular proteins involved in controlling the cell cycle and apoptosis. In 2007, Geller and coworkers discovered that geldanamycin inhibited the replication of three different picornaviruses in tissue culture, PV, HRV, and coxsackievirus (33). Those authors showed that the effect of geldanamycin is due to an inhibition of Hsp90-aided folding of its client, the viral capsid precursor P1. This folding, in turn, is essential for the processing of P1 by the proteinase $3CD^{pro}$ (see "Polyprotein Processing, Myristoylation, and Interaction of P1 with Heat Shock Protein Hsp90," above). Interestingly, attempts to isolate PV mutants resistant to geldanamycin inhibition failed.

COMPARISON WITH OTHER HUMAN AND ANIMAL PLUS-STRAND RNA VIRUSES

Besides picornaviruses, morphogenesis has been studied in any detail only with member viruses of three other families of enveloped human and animal plus-strand RNA viruses: *Flaviviridae*, *Togaviridae*, and *Coronaviridae*. These families encompass enveloped viruses with a nucleocapsid consisting of multiple copies of capsid protein complexed with the viral RNA. We summarize what is known about some important aspects of the morphogenesis of these viruses and compare them to those of picornaviruses in Table 1. Members of all four families exhibit a linkage of encapsidation to RNA replication, and they all use membranous structures for the site of morphogenesis. The majority of *Picornaviridae* and *Flaviviridae* do not possess any RNA-based encapsidation signal, or at least, none has been discovered so far. The exceptions are Aichi virus of the *Picornaviridae* and dengue virus (DENV) of the *Flaviviridae*, which have been grouped separately from the other members of the families in Table 1. On the other hand, for *Togaviridae* and *Coronaviridae*, there is strong evidence for RNA-based encapsidation signals for all members of the family examined so far. Stable capsid intermediates (except for empty capsids) have been observed only for the *Picornaviridae*. *Picornaviridae* and *Flaviviridae* are similar in that there are interactions between capsid and nonstructural proteins that are important for morphogenesis (20, 168). Based on genetic and biochemical experiments, we have recently proposed for PV and other enteroviruses that protein-protein interactions are sufficient to provide specificity to the encapsidation process (20). We suggest that the same mechanism might also apply to HCV and other viruses that do not possess specific RNA encapsidation signals.

MODEL OF ENTEROVIRUS MORPHOGENESIS

Any model of enterovirus morphogenesis has to take into account that only newly replicated RNAs are encapsidated, which indicates a tight linkage between RNA replication and encapsidation (76, 92). In Fig. 5, we propose a model of morphogenesis based on our studies of enteroviruses. Except for Aichi virus or HAV, it is possible that this model may be applicable to most picornaviruses, but the *Picornaviridae* are such a large family (169) that there may be different pathways of morphogenesis for the proliferation of member viruses of some genera.

As detailed in Fig. 3C and 5, we propose that the newly made capsid precursor P1 that is released from the polyprotein and myristoylated (Fig. 3C, PV stage I) interacts with the chaperone Hsp90 to achieve a conformation (PV stage II) competent for cleavage by

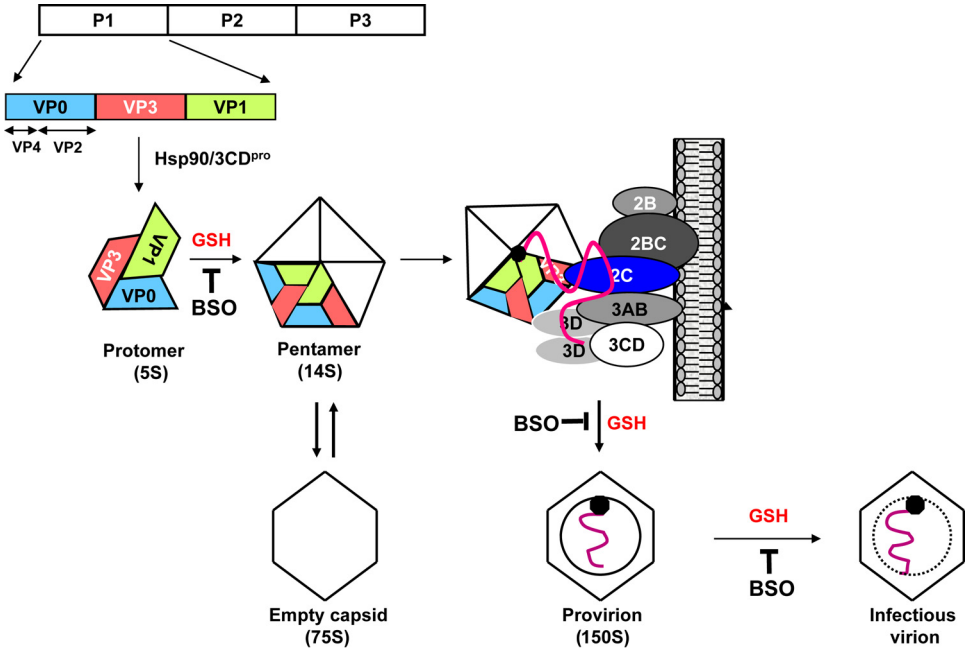


FIG 5 Model for enterovirus morphogenesis. After the newly made capsid precursor P1 is released from the polyprotein, it interacts with the chaperone Hsp90 to assume a conformation competent for cleavage by $3CD^{pro}$. After proteolytic processing, a protomer is formed spontaneously, consisting of one copy each of VP0, VP1, and VP3. In the presence of GSH, five protomers assemble to generate a pentamer, which, by interactions between VP3 and $2C^{ATPase}$ or between VP1 and $2C^{ATPase}$, is recruited to the replication complex to associate with the newly made VPg-linked plus-strand viral RNA. Subsequently, 12 pentamers condense around the RNA to produce a noninfectious provirion. The last step is the maturation cleavage of VP0, which is autocatalytic and RNA dependent and yields a stable particle containing 60 copies of VP1 and VP3, 59 copies of VP2 and VP4, and 1 copy of VP0. The steps at which GSH is required, as shown by inhibition with BSO, are marked.

$3CD^{pro}$ (33, 34). After proteolytic processing, assembly of the cleavage products occurs in a stepwise manner. Notably, processing of P1 does not result in the release of "free" polypeptides. Instead, a "protomer" is formed directly from P1, consisting of one copy each of VP0, VP1, and VP3 (PV stage III). Five protomers then assemble to generate a pentamer (PV stage IV). Based on the interaction between $2C^{ATPase}$ and the capsid protein VP3 or VP1 (20, 21), we suggest that $2C^{ATPase}$, a component of the membrane-associated replication complex, recruits the 14S capsid precursors to the site of particle assembly. Whether the 5′-terminal VPg that is likely to emerge first from the replication complex interacts with capsid proteins is not yet known. However, at this stage, 12 pentamers are likely to condense around the RNA to produce a noninfectious provirion (PV stage VI). Whether or not $2C^{ATPase}$ is also involved in the condensation of the viral RNA (via ATP hydrolysis) during packaging is not yet known. The last step is the maturation cleavage of VP0, which is autocatalytic and RNA dependent (67, 70, 170) and yields a stable particle containing VP1, VP2, VP3, and VP4 (PV stage VII). The role of the procapsids (PV stage V) in morphogenesis is still debated. Most likely, it is a by-product capable of reversible dissociation into pentamers. However, our evidence suggests that procapsids are not direct precursors of mature poliovirus (22). We propose that GSH is required for the stability of pentamers and mature virions during the early and late stages of assembly (22, 23).

In *wt* enterovirus infections, only plus-strand RNAs that emerge from the replication complex are encapsidated. This is most likely due to the fact that minus strands are normally sequestered from the replication complex through associations with plus strands in either the replicative form (RF) or replicative intermediate (RI) structures and not released. As pointed out above, the replication complex of special variants of coxsackie B virus can produce free minus-stranded RNAs, and in turn, those can be encapsidated (119). As noted above, the last steps of morphogenesis of HAV differ from those of enteroviruses (Fig. 3C, HAV steps I to VII).

CONCLUSIONS

Since the discovery of the basic steps of assembly more than 3 decades ago, progress in the field of picornavirus encapsidation has been slow. This may be the reason why the last full review on picornavirus morphogenesis was published in 1981 (49). Here, we have attempted to summarize older data collected decades ago, and we have detailed new discoveries made during last few years (20–23, 26, 28, 141). Comparison with other human and animal plus-strand RNA viruses reveals certain differences but also some unifying principles that link aspects of morphogenesis.

Among the many unsolved mysteries in picornavirus morphogenesis, questions that remain to be answered are as follows. (i) What is the precise mechanism by which provirions are formed—do pentamers condense around genomic RNA, or are genomes actively "inserted" into the empty capsids? (ii) What is the role of $2C^{ATPase}$ in the delivery of newly synthesized RNA from the replication complex to the capsid? (iii) What is the role of 3CD in enhancing virus maturation? (iv) What is the role of VPg in viral assembly? (v) What are the roles of host factors in encapsidation? Future studies using a combination of genetics and high-power microscopic imaging will hopefully extend and refine our

knowledge of the mechanism of picornavirus morphogenesis and enhance our ability for drug design to limit the toll of picornavirus diseases.

ACKNOWLEDGMENT

This work was supported by NIH grant AI 15122.

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