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Picornaviral Structure and Assembly

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INTRODUCTION

Because of their simple composition, picornaviruses provide an excellent model system for studying the assembly of biologically (and medically) important structures. In recent years, a considerable amount of progress has been made in determining the structure and replication of these viruses. Moreover, exciting breakthroughs appear imminent. For example, the development of techniques for the rapid sequencing of deoxyribonucleic acid and, more recently, ribonucleic acid (RNA) will permit the entire sequencing of picornaviral RNA molecules in the near future. Such information will have an enormous impact on our understanding of the functioning of picornaviral RNAs. However, fundamental questions remain concerning virion fine structure and morphogenesis. We are just beginning to get some insights into the topology of the viral capsid proteins, although their specific roles in such things as cell receptor recognition, RNA protection, uncoating, and antigenic expression remain obscure, and little is known about the configuration of the viral RNA in the viral particles or the significance of minor capsid proteins. In the

absence of answers to these and other questions, it is impossible to construct an accurate model of the picornavirion. In the area of morphogenesis, the exact sequence of assembly reactions leading to infectious virus particles and the mechanisms governing these reactions remain unknown. In fact, there is controversy concerning the roles of several subviral intermediates isolated from infected cells under a variety of experimental conditions. More to the point, we cannot be certain that all of the crucial intermediates have been isolated, much less characterized in detail. These unresolved problems not only reveal our ignorance but, more importantly, define the research areas that must be investigated.

The aim of this review was to collate much of the recent information on the structure and morphogenesis of picornaviruses and integrate it with established concepts. We view structure and morphogenesis as two facets of a common problem; to understand one aids in conceptualization of the other. We hope that this review is of interest to those working with a variety of RNA and deoxyribonucleic acid viruses and that it contributes to our knowledge of the structure and assembly of such diverse structures as ribosomes, RNP particles (messenger RNA [mRNA]-protein complexes), and chromosomes in eucaryotic cells. Certainly, the converse has proven to be true; i.e., experimental approaches used to define the structure and function of other ribonucleoproteins, such as ribosomes, have been applied successfully to the study of picornaviruses. We also hope that this review presents a more accurate description of picornavirions and a detailed description of the morphogenetic process.

Finally, we attempted to provide a reasonably thorough bibliography from which interested readers can gain easy access to the primary literature. In this regard, Sangar (200) recently has reviewed the replication of picornaviruses. Earlier reviews on this subject were published in 1972 by Phillips (177) and in 1976 by Rueckert (198). Also, many of the recent experimental advances are described in a compilation of seminars given at a North Atlantic Treaty Organization-sponsored conference (170). However, we recognize and take responsibility for the omission of much relevant work simply because its inclusion would have been beyond the scope of this review. To simplify our task, we used the polioviral terminology to describe viral structural and nonstructural (noncapsid) proteins, and for purposes of clarity we integrated the experimental findings obtained with different picornaviruses in the belief that a set of common mechanisms underlies the replication and morphogenesis of all picornaviruses. Thus far, experimental results overwhelmingly support this view.

FINE STRUCTURE OF PICORNAVIRIONS

Virus Particles

General description, classification, and biophysical characteristics. The picornaviruses are currently classified into five groups (or genera), based on buoyant density, acid lability, and viral serology. In addition, there is some expectation that RNA fingerprint analyses reflecting sequence homologies may be used in future classification schemes. Of the frequently studied viruses, polioviruses, coxsackieviruses (subgroups A and B), and the echoviruses are enteroviruses, mengovirus, Maus-Elberfeld virus, and encephalomyocarditis (EMC) virus are cardioviruses, and foot-and-mouth disease virus (FMDV) is the prototype of the aphthovirus group. Finally, the human and equine rhinoviruses are placed in separate genera. The distinguishing characteristics of these viruses are shown in Table 1. There are no common intergeneric antigens.

All picornaviruses are quite similar in composition, structural features, and replication schemes. Where notable differences exist and are pertinent, they are described below.

The elucidation of the fine structures of picornaviruses has been much more difficult than might have been presupposed from the composition of these viruses. Physicochemically, picornavirions are spherical ribonucleoprotein particles exhibiting icosahedral symmetry (21, 74). The dry diameter of these particles is 27 to 28 nm (205), and they are composed of 70% protein and 30% RNA (28); the particle molecular weight is about 8.5×10^6 (25, 205). These virions lack lipids, and there seems to be general agreement that the viral structural proteins are not glycosylated (28, 64; Phillips, unpublished data), although one publication reports the isolation of glucosamine from capsids (90). The sedimenta-

TABLE 1. *Distinguishing properties of picornavirus genera*

Genus	Acid stability	Density in CsCl (g/ml)	$s_{20,w}$[a]
Enterovirus	Yes	1.34	155
Cardiovirus	No	1.34	155
Rhinovirus	No	1.40	155
Aphthovirus	No	1.44	155

[a] $s_{20,w}$, Sedimentation coefficient corrected to water at 20°C.

tion coefficients and the buoyant densities of the particles appear to differ among certain groups (Table 1). We should point out that the apparent buoyant density of a virus is influenced by the hydrodynamic properties of the particles in solution which, in turn, are dependent on the degree to which the capsids are permeable to the dissolved solute (e.g., Cs^+) and the amount of solvation. Recent experiments have demonstrated that the buoyant density of poliovirions increases from 1.34 to 1.40 g/ml as a consequence of replication in the presence of CsCl and that this permits the entrapment of cesium atoms during particle morphogenesis (147). Under these conditions, approximately 4,200 Cs atoms are bound per virus particle. In contrast, rhinovirions are freely permeable to Cs and bind about the same number of Cs ions (25). Thus, density differences between groups seem to be due in large part to Cs binding to the RNA genome (153), which is the same size for all of the picornaviruses (see below). Poliovirus "dense" particles, which are often detected in partially purified virus preparations as a minor component banding at 1.44 g/ml in CsCl, may arise as a consequence of damage to the capsid, thus making it permeable to Cs ions (241). Likewise, glutaraldehyde treatment of FMDV results in an increased buoyant density (from 1.43 to 1.46 g/ml), probably as a consequence of the RNA in fixed particles becoming more accessible to Cs ions (202).

Chemical composition and stoichiometry. More than any other single technique, polyacrylamide gel electrophoresis used for the fractionation of proteins dissociated with sodium dodecyl sulfate and a reducing agent paved the way for determining the polypeptide compositions of viruses (140). By determining the relative molar amounts of the intrinsically radiolabeled polypeptides extracted from purified virions, it became possible to derive empirical formulas for picornaviral particles (29, 140, 197). These studies showed that viral capsids were composed of four distinct polypeptides. Table 2 shows the remarkable similarity among the capsid proteins of different picornaviruses. This similarity is even more striking when the methods of viral protein synthesis and processing are examined (see below). Many analyses of the structural proteins (some of which are cited in Table 2) and compositional analyses of structural mutants (68) and different serotypes (180) have shown clearly that considerable variations in major capsid polypeptides are compatible with infectivity. In fact, such alterations may lead to increased capsid stability (68). Most studies indicate the presence of 60 copies of each of the four capsid polypeptides (143, 150, 215), although discrepancies have been reported. A bovine enterovirus was shown to contain one-half (ca. 30) of the expected number of VP4 polypeptides (110), and analyses performed on type 1 poliovirus also showed aberrant molar ratios (68). In the former study, failure to recover VP4 quantitatively from gels after fixing and staining may have influenced the results. Finally, several investigators have reported multiple species of

TABLE 2. *Properties of the structural proteins of picornaviruses*[a]

Viral polypeptide	Mol wt ($\times 10^3$)	pI[b]	N terminus[c, d]	C terminus[c]	Unusual characteristics
VP1 (α)	35.5 ± 1.5	Alkaline or acidic	Glycine	Leucine or glutamine	None
VP2 (β)	30.0 ± 1.0	Acidic	Aspartic acid	Glycine or glutamine	None
VP3 (γ)	25.0 ± 2.0	Acidic	Serine or threonine	Leucine (glutamine)[e]	None
VP4 (δ)	8.0 ± 2.0	Neutral or acidic	Blocked	Alanine	No free -SH (no cysteine); rich in aspartic acid (or asparagine) and glutamine

[a] Data were compiled from the following sources: mengovirus, references 132, 243, and 245; FMDV, references 7 and 145; poliovirus, references 30, 31, 138, 175, 177, and 233; EMC virus, references 26 and 31; echovirus 12, reference 119; rhinovirus 1A, reference 31.

[b] pI determinations for polioviruses (89, 90) and mengovirus (42) were made in 9 M urea; pI's determined in urea were reduced by 0.5 U. The pI determined for the VP0 polypeptide of poliovirus empty capsids (procapsids) was 6.6, a value midway between the values for VP2 and VP4, the polypeptides to which VP0 is a precursor (89).

[c] Amino acid composition and partial sequencing information are pertinent only to mengovirus (243, 245) and FMDV (145). The poliovirus compositional analysis is from reference 139.

[d] Three N-terminal amino acids were detected from poliovirus capsid polypeptides but have not yet been identified as belonging to specific proteins (30); their assignment here is arbitrary and is based on the presumption that they are the same as the N termini found for other picornaviruses.

[e] The amino acids in parentheses have not been identified with certainty.

some of the capsid proteins, as determined by one-dimensional gel electrophoresis (68) and two-dimensional gel electrophoresis (91, 92); in one case, charge modification of VP2 as a consequence of storage of the virus in phosphate-buffered solutions was given as an explanation for some of the reported heterogeneity (92). Interestingly, temperature-sensitive mutants of mengovirus also exhibit multiple VP2-like components (17). It is possible that altered structural polypeptides arise from ambiguous processing of precursor proteins (49; see below).

Preparations of highly purified picornavirus particles invariably contain small amounts (one or two copies per particle) of a fifth polypeptide, VP0 (or ϵ). The capsid polypeptides VP2 and VP4 are produced by cleavage of VP0 (Fig. 1). However, it is not clear whether every particle possesses one or two VP0 molecules. For example, the relative amounts of VP0 in virion preparations could be accounted for by assuming contamination with 1 to 2% naturally occurring empty capsids (i.e., procapsids), which are themselves composed of 60 copies each of VP0, VP1, and VP3 (141). This is approximately the amount of empty shell contamination observed in preparations of poliovirus (19; Phillips, unpublished data).

Empty capsids may arise by damage to virions, but artificially produced empty shells do not contain detectable VP0 (141). If VP0 is a necessary component of infectious virus particles, what role this molecule plays in capsid stability, infectivity, or viral morphogenesis remains a mystery.

Degradation studies. Controlled degradation of virus particles has been a useful method for elucidating viral fine structure. More recently, degradation and electron microscopy have been used together with cross-linking reagents of defined size and reactivity to provide additional information concerning the organization of viral components. We should mention that caution is necessary in extrapolating information obtained by this experimental method to morphogenetic models because virion-derived "subunits" do not exist as such in vivo; this unique situation is discussed below.

By far the most informative experiments of this type have been performed on the cardioviruses, particularly EMC virus and mengovirus, because of the acid lability of these viruses. Similar experiments have been done with FMDV. In contrast, the enteroviruses generally are stable to a wide range of salt and pH conditions and thus require rather drastic denaturing

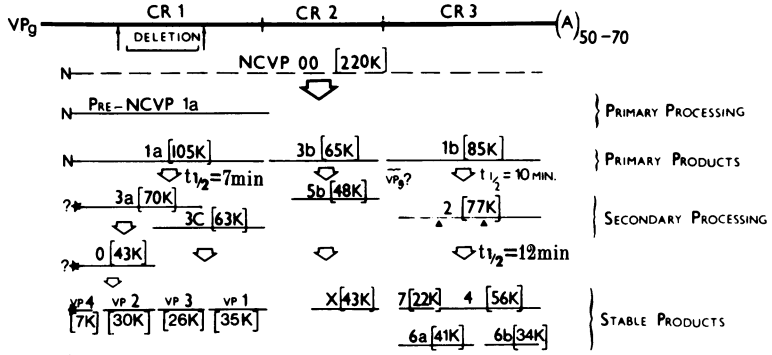

FIG. 1. *Synthesis and processing of the picornaviral precursor polyprotein NCVP 00. The picornaviral genomic RNA is shown subdivided into three coding regions (CR 1, CR 2, and CR 3). The structural proteins are encoded by CR 1, the replicase activities are encoded by CR 3, and CR 2 encodes the stable nonstructural protein NCVP X. The deletion indicated in CR 1 is present in poliovirus defective-interfering particles; consequently, these particles are not able to make structural proteins, and their formation is dependent upon replication in cells infected with wild-type virus (179). The broken line representing NCVP 00 indicates the nascent cleavage of this molecule, which is probably catalyzed by host cell proteases (116, 220). A precursor to NCVP 1a, designated pre-NCVP 1a, is sometimes observed, most notably in reticulocyte lysates translating EMC virus RNA. N-terminal processing appears to eliminate this polypeptide in vivo. The amino-terminal processing reactions also include the addition of a blocking group (indicated by stars) to VP4 and, perhaps, to its immediate precursors. Secondary processing, which is probably mediated by a virus-encoded protease(s) (NCVP 7 or NCVP X? or both), occurs in vivo and also in reticulocyte lysates. However, the cleavage of VP0 occurs only in vivo and may be linked intimately to RNA encapsidation. Some precursor polypeptides can be cleaved in more than one way; this is demonstrated by the alternate cleavage products of the replicase precursor NCVP 2 (see text). The small, 5'-terminal, genome-linked protein VPg has been mapped tentatively at or near the N terminus of the replicase precursor NCVP 1b (113, 163). $t_{1/2}$ values indicate the approximate half-lives of the precursors in vivo at 37°C. 220K, 220,000 daltons.*

conditions to disrupt their particles.

When exposed to mild acid in the presence of Cl^- or Br^-, mengovirus or Maus-Elberfeld virus yields particles that sediment at 13.4S and are composed of five molecules each of VP1, VP2, and VP3 [i.e., $(VP1\text{-}VP2\text{-}VP3)_5$] (66, 144). VP4 precipitates under these conditions (150) (Fig. 2). In the presence of 2 M urea, which disrupts hydrogen bonds and hydrophobic interactions, the 13.4S pentamers are degraded into 5S components containing only one molecule of each protein (143, 150). In an electron microscope, the 13.4S pentamers appear as concave disks or skullcaps measuring 17 by 14 nm, and the 5S structures appear as 6.8-nm spheres (150). In situ treatment of poliovirions (19) or FMDV (229) on supporting membranes with very high salt concentrations dissociates each capsid into 10 to 12 skullcaps which contain the three large capsid proteins and are 16 to 18 nm in diameter and ca. 4 nm thick. It is certainly tempting to postulate that the pH-dissociable 13.4S cardiovirus pentamers and the poliovirus and FMDV skullcaps are analogous subunits held together by different kinds of bonds. These structures and their interrelationships are shown diagrammatically in Fig. 2.

Reconstitution experiments. Although there are many reports of successful reconstitution of plant viruses (11), the results of attempts to reassemble animal viruses from virion-derived subunits have been equivocal at best. Some degree of reconstitution of infectivity of poliovirus degraded in the presence of 9 M urea and 0.09 M 2-mercaptoethanol has been reported (63). The infectivity of the reconstituted virus approached that of infectious RNA assayed in the presence of diethylaminoethyl dextran and was resistant to ribonuclease (RNase). However, the obvious experiment showing neutralization of infectivity with antiserum was not done, leaving some question as to the degree of renaturation achieved.

Picornaviruses treated with alkali, concentrated urea, or heat could be converted into fibrillar nucleoproteins (15, 237); residual empty shells were destroyed selectively with sodium dodecyl sulfate (228). Fibrils were also generated by treating a reconstituted mixture of artificially produced empty shells and viral RNA with alkali (237). Not surprisingly, fibrils are composed of RNA plus VP1, VP2, and VP3; the relative ratios of these components were different in different preparations. It seems likely that fibrils arise secondarily as a consequence of the denaturation of empty shells in the presence of viral RNA, and thus it remains to be shown that they resemble any viral structure present in vivo.

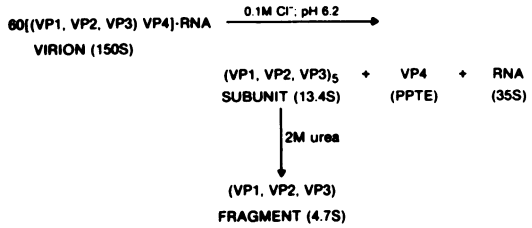

FIG. 2. *Scheme showing the dissociation of the acid-labile mengovirions into 13.4S subunits, virion RNA, and a precipitate (PPTE) containing VP4 (from Mak et al. [143]). The 13.4S subunit is degraded by urea into 4.7S mature protomers. In addition to VP4, the precipitate also contains RNA, minor amounts of $(VP1\text{-}VP2\text{-}VP3)_5$ pentamers, and the capsid protein precursors VP0 and NCVP 3c (see Fig. 1), perhaps present as immature pentamer forms (D. Scraba, personal communication). Although poliovirus terminology is used here, it should be noted that enteroviruses are not acid labile.*

Topological features. The spatial relationships among the individual polypeptides comprising capsids have been explored by using the following three experimental approaches: (i) surface labeling of intact particles, (ii) treatment of particles with cross-linking reagents of defined size and reactivity, and (iii) reactions with specific antibodies. The first two approaches probe for the specific reactive groups in the viral proteins that are available (or exposed) to the reagent. Possible denaturing effects are inherent in most surface-labeling techniques and may cloud the interpretation. The results of antibody studies (unless monoclonal antibody is used) must also be interpreted with caution because of the undefined and possible multiplicity of antigenic determinants. Still, meaningful information has been obtained by using these techniques.

Generally, external iodination of picornavirions labels only the VP1 polypeptide (39, 132, 137, 233) unless degradation occurs before or concurrent with the iodinating reaction. If viral degradation occurs, all four viral polypeptides are labeled. The fact that similar results have been obtained with procedures that label tyrosine residues or free amino groups (N-terminal or lysine residues) provides some support for the conclusion that VP1 occupies an exposed position on capsids. However, mildly denatured viral particles possess quite different reactivities. Poliovirions exposed to heat or rhinovirions exposed to acid become "A" particles, which contain RNA but have the following characteristics: (i) loss of VP4, (ii) altered sedimentation coefficient, (iii) inability to attach to susceptible cells, and (iv) changed antigenicity (50, 70, 125). Sur-

face labeling of A particles with acetic anhydride (which labels free amino groups) results in labeling primarily VP2. When subjected to external iodination, naturally occurring empty capsids (i.e., procapsids) were labeled mainly in VP0 and VP3 (39). The implication of this work is that the topography of virions is fundamentally different from that of A particles and empty shells.

Hordern et al. (102) examined the topological relationships of mengovirus capsid proteins by using reversible cross-linkers, such as dimethyl suberimidate, dimethyl adipidate, and dithiobis(succinimidyl proprionate), in conjunction with controlled degradation of derivatized particles. These workers found that only dithiobis(succinimidyl proprionate), which had a linking distance of 1.5 nm, was able to form VP1-VP2 dimers and prevent acid dissociation of reacted particles into 13.4S pentamers (see above) (Fig. 2). In contrast, dimethyl suberimidate, which had a shorter cross-linking distance, did not stabilize the particles to acid dissociation and did not cause the formation of VP1-VP2 dimers. All three reagents caused VP1 dimer formation, and both dimethyl suberimidate and dithio-bis(succinimidyl proprionate) generated larger complexes of VP1. $(VP2)_n$, $(VP3)_n$, or VP4-linked polypeptides were not detected in any experiment. Similar results were obtained by Wetz and Habermehl (233) by using dimethyl suberimidate- and dimethyl adipidate-treated poliovirus particles. Unfortunately, enteroviruses are not acid labile, so it was not possible to repeat the controlled degradation experiments of Hordern et al.; in any case, if poliovirus is constructed in a similar manner, as we suspect it is, then the nature of pentamer-pentamer bonds must differ, perhaps relying more on hydrophobic bonding or hydrogen bonding or both, in contrast to the electrostatic interactions which hold together the pentamers of cardioviruses.

These results are consistent with a model in which VP1-VP2 bonds hold adjacent pentamers together [i.e., $(VP1\text{-}VP2\text{-}VP3)_5$], whereas the primary stabilizing contacts within pentamers arise from VP1-VP1 bonds. Two bonding networks compatible with this model are shown in Fig. 3 (65).

Antigenic properties. Intact picornavirions exhibit D (or N) antigenicity; anti-D antibody effectively neutralizes infectivity (82). Mild denaturation of enteroviruses with heat, urea, alkali, or various combinations of these agents causes the release of the viral RNA as well as VP4 (and perhaps limited amounts of other structural polypeptides), leaving behind empty shells possessing C (or H) antigenicity. Anti-C antibody is not neutralizing (82) and is nonreactive with intact virions, but this antibody specifically agglutinates artificially produced and naturally occurring empty capsids (105). However, there is evidence from electron microscopic studies that conversion from D to C reactivity may not be absolutely dependent on the release of the viral genome (111). More recently, Hasegawa and Inouye (94) have shown that echovirus pro-

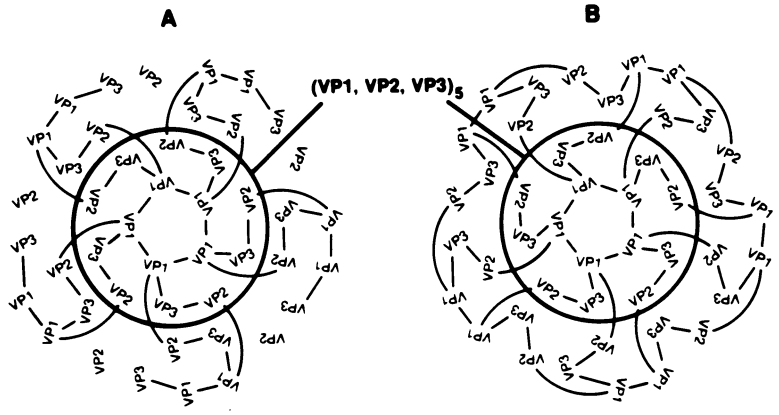

FIG. 3. *Bonding net models that account for the cross-linking data obtained for mengovirus (102). Cleavage of the bond between VP1 and VP2 generates $(VP1\text{-}VP2\text{-}VP3)_5$ pentamers (indicated by circles) in both models. Cleavage of VP1-VP1 contacts leads to $(VP1\text{-}VP2\text{-}VP3)_2$ subunits for model A and $(VP1\text{-}VP2\text{-}VP3)_5$ subunits for model B. Mature protomers, i.e. $(VP1\text{-}VP2\text{-}VP3)_1$, would be generated if both VP1-VP2 and VP1-VP1 contacts were broken. Both bonding nets are consistent with VP1-VP2 being the acid-dissociable bond linking the 12 pentamers of the capsid and VP1-VP1 being the stabilizing linkage within individual pentamers. VP4 is not cross-linked to any of the other capsid polypeptides and is precipitated when the mengovirion is dissociated with acid. Its domain within the capsid is not known. Modified from Dunker (65).*

capsids exhibited D antigenicity, but that after mild heat treatment, which was not sufficient to alter the sedimentation properties or polypeptide composition of the procapsids, they became C-reactive particles. The naturally occurring empty shells of FMDV also possess D antigenicity and attach to susceptible cells as efficiently as virions (196). There is evidence that an analogous situation may exist among the rhinoviruses (134). Another finding that emphasizes the role of capsid conformation in antigenic expression is the interconvertible viral species (conformers) with differing isoelectric points present in poliovirus populations (146). The A form (pI 7) is D antigenic, whereas the B form (pI 4.5) is C antigenic. Since both species are infectious, it follows that both contain infectious viral RNA. Finally, reactions of enteroviruses with specific neutralizing antibody or specific cellular receptors also cause the antigenic change from D to C; in the latter case, VP4 seems to be lost (50).

It has not been possible to associate antigenic determinants with specific capsid polypeptides. The fact that VP4 seems to be lost quantitatively during early attachment to cell receptors (50) at first suggested that it might be the D antigenic determinant, a hypothesis supported by the finding of Breindl that anti-D antibody reacted with VP4 (22). However, the topological studies cited above indicate that VP4 probably is not located at the capsid surface. More recently, Lund et al. (137) used a panel of antibodies prepared against all four isolated capsid polypeptides of mengovirus and found that only anti-VP1 possessed neutralizing activity. Anti-VP2 antibodies reacted with mengovirions, as detected by complement fixation, but were not neutralizing. Anti-VP3 and anti-VP4 antibodies reacted with their respective polypeptides but not with intact virions. Therefore, D is most likely a conformational antigen which is determined at least in part by VP1 (137); VP4 may be required for correct conformation of this determinant.

Purified virus degraded with guanidine, which originally was described as S antigen (204), raises an antibody in rabbits which fails to neutralize virus infectivity (i.e., react with D antigen), yet agglutinates the 14S particle, a morphogenetic intermediate (Phillips, unpublished data). Anti-S antibody was reported to react with empty capsids (C antigen) (204), and we have obtained evidence that this antibody reacts with procapsids assembled in vitro (Phillips and Putnak, unpublished data).

Cell receptors and viral capsids. The presence of cell surface receptors appears to be the primary factor in determining the susceptibility of a cell or tissue to picornavirus infection (98, 99, 101, 127, 149). Many types of cells lacking receptors are infected readily by free RNA (149). Work conducted with mouse-human hybrid cells established that human chromosome 19, which ostensibly encodes a human plasma membrane protein, is essential for the susceptibility of heterokaryons to poliovirus infection (155). Likewise, the human group G chromosomes are required for coxsackie B virus attachment and penetration (112). The conservation of viral receptor sites indicates that these sites must be of general importance to cells.

The nature of the receptors is still largely unknown, and attempts to solubilize and purify them have met with only limited success. Poliovirus receptor activity appears to exist as a complex of glycoproteins and lipoproteins (149). Certain soluble polysaccharides inhibit hemagglutination by certain enteroviruses (129), and attachment of human rhinovirus type 2 and poliovirus type 2 are both inhibited by concanavalin A, which reacts with α-D-mannose constituents (131), suggesting the involvement of a sugar moiety of the glycoproteins in receptor recognition. Experiments involving the interaction of various human rhinovirus types with HeLa cells have indicated that there are about 10^4 receptor sites per cell (133). Both the rate and the temperature dependence of binding were different among the various types, and in some cases the receptor appeared to be type specific.

Early studies showed that the initial attachment of poliovirus particles to susceptible cells is reversible if it is carried out in the cold (70). At temperatures approaching 37°C, eclipse of virus infectivity rapidly ensues (98). The irreversible step appears to involve conformational changes in the virus capsid (125), along with the concomitant loss of VP4 (50) and an increased sensitivity to proteolytic enzymes (57, 70). Most of the altered particles apparently are eluted into the medium as the noninfectious A particles described above. Isolated plasma membrane fragments also effect eclipse (41), and this activity is abolished by proteases and detergents (56, 88). Plasma membrane fractions incubated in the presence of virus at 25°C have no activity, but between 30 and 37°C, the reaction has an activation energy of 60 kcal/mol (56), providing support for the hypothesis that there is multiple receptor binding in a fluid membrane (88). Interestingly, microsomal membrane fragments have receptor activity but do not promote uncoating (99).

The mechanisms governing attachment and uncoating remain controversial. Some reports suggest that viruses are uncoated outside cells and that the released genomes penetrate the cell membranes (89), whereas other reports propose

that modified virions are engulfed and uncoated within the cytoplasm (145, 192), perhaps with the aid of intracellular proteases. Recently, a membrane fraction prepared with the aid of nonionic detergents was shown to modify poliovirions to a form similar if not identical to A particles. Further incubation of the modified particles with membranes or treatment with chymotrypsin caused additional modification (i.e., loss of VP2 and sensitivity to RNase) (58). These data suggest a multistep uncoating process.

The fact that VP1 seems to occupy an external position on the capsid and the fact that, of the antibodies prepared against the purified capsid proteins, only anti-VP1 effectively neutralizes infectivity of virions (see above) make this capsid polypeptide a prime candidate for cell receptor recognition. However, other capsid proteins undoubtedly play a role in this reaction, because particles possessing VP1, such as A particles or empty shells, usually do not attach to susceptible cells. Again, the conceptual crutch of particle conformation appears to be important. In the final analysis, it remains to be shown which capsid proteins recognize or react with cell receptors.

State of the ribonucleic acid (RNA) within capsids. The nature of the packing of the viral RNA into virions remains an extremely important problem about which little definitive information is available. Formaldehyde cross-linking studies of mengovirions have shown that primarily VP2 and VP3 can be attached chemically to viral RNA (102). This is a particularly interesting result because in virions these polypeptides, especially VP2, possess amino and sulfhydryl groups resistant to external labeling with a variety of reagents (see above). On the other hand, similar studies suggest an internal location for VP4, yet this polypeptide is never found linked to viral RNA. Urea treatment of poliovirions and incubation of the dissociated products in isotonic NaCl resulted in the isolation of a 45S product that was sensitive to RNase and contained VP1 and traces of VP2 and VP3 (234). However, it is not clear whether this complex reflected an in situ association between RNA and VP1 or formed as a consequence of particle dissociation and reaggregation.

The hypothesis that viral RNA exists within a capsid in a "ball of yarn" conformation is supported by its resistance to external nucleases and its reported visualization by electron microscopy as an electron-dense corelike structure in poliovirus preparations examined in the presence of CsCl (18). No core was observed in empty shells. Vasquez and co-workers (personal communication) have isolated a 16-nm core from FMDV particles. Frisby et al. (78) compared the hyperchromicity of capsid RNA with that of RNA free in solution and concluded that EMC virus RNA in situ has less secondary structure; the implication was that RNA-protein interactions in situ prevent base pairing to a degree. Circular dichroism spectra and formaldehyde treatment of phenol-extracted EMC virus RNA suggest that about 60% of the nucleotides are involved in base pairing at 25°C. However, it is still not clear what the precise configuration of picornavirus genomes is when they are complexed with their capsid proteins or what the nature of the interactions is between the viral RNA and specific capsid proteins. Particularly perplexing is the role of VP4 and the possible role of the small protein VPg, which is attached covalently to the 5′ terminus of the viral RNA (see below).

We have already examined evidence that Cs^+ ions can replace Na^+ and K^+ and bind to virion RNA. However, in most cases this cannot account for neutralization of all 7,700 phosphate groups. Presumably, the basic amino acids (ca. 5,200 molecules) of each capsid contribute to charge neutralization.

Biological activities associated with virions. Finally, the possibility that picornavirions possess enzymic activities should be mentioned. Holland et al. (100) reported that a proteolytic activity copurified with mengovirions. Later, Lawrence and Thach (123) described a proteolytic activity which copurified with EMC virus capsid protein VP3. Recently, evidence has been obtained suggesting that this protease activity actually is associated with a protein that coelectrophoreses with VP3 (163) (see below). In 1974, Gauntt (79) found that the RNA of purified rhinovirus type 14 particles was hydrolyzed in situ during incubation at 34.5°C. Suspecting a similar phenomenon with FMDV, Denoya and colleagues characterized both the external and the internal endonuclease activities associated with purified FMDV particles (54, 55). Although it is thought that the external nuclease may be an adsorbed activity, the internal activity is more interesting, although its origin and significance remain unknown.

Fine Structure of Picornavirion RNA

Below, we discuss the unique physical features of picornavirion RNA and the possible functional significance of these features in replication, translation, and encapsidation. It is noteworthy that picornaviruses are convenient sources of large amounts of highly defined and purifiable mRNA molecules that can be used in eucaryotic protein-synthesizing systems.

Physical and biological properties. The

molecular weights of virion single-stranded RNAs have been measured by comparative electron microscopy (85, 158, 224), analytical ultracentrifugation (69), and electrophoresis on polyacrylamide or polyacrylamide-agarose gels in the presence of a variety of denaturants to minimize the effects of secondary structure on mobility (10, 130). More recently, Lee et al. (127) used nucleolytic digestion (with RNase T1) of ^{32}P-labeled RNA in conjunction with two-dimensional fingerprint analysis to estimate the molecular weights of the RNAs isolated from poliovirus types 1 and 2. Given this impressive array of experimental approaches, it seems safe to assign a molecular weight of $2.6 \times 10^6 \pm 0.2 \times 10^6$ to picornavirion RNA. A recent analysis of a bovine enterovirus genome yielded a molecular weight of 2.9×10^6 (225); we do not consider this RNA to be significantly larger than other picornavirus RNAs, given the range of experimental error.

The RNAs extracted from virions are intrinsically infectious (2, 45) and can be translated in cell-free systems (166, 210, 230). This fact defines the virion RNA as having positive polarity. Our discussion of the structural features of virion RNA begins at the ends of the RNA and works toward the middle, because there is far more known about the termini and also because the termini seem to be the most interesting since the 5′ end would be expected to contain the initiation site for ribosomes and the 3′ end would be expected to contain the termination site(s), as well as the binding site for initiation of negative-strand RNA synthesis (Fig. 4).

Presence of polyadenylic acid. Like practically all eucaryotic mRNA's, picornaviral genomes possess a region of polyadenylic acid [poly(A)] residues at their 3′ termini (5, 139, 243). These poly(A) tails have been reported to contain anywhere from 10 or fewer to more than 150 residues; not surprisingly, there are reports that the poly(A) regions of cloned plaque isolates are heterogeneous (83, 214). It may be that some of this variation reflects different methodologies (27, 77). However, the length of the poly(A) region is longer in the nonencapsidated RNA molecules found in infected cells than in the RNA molecules isolated from virus particles (244).

There is conflicting evidence about whether the poly(A) region is necessary for infectivity. Originally, it was reported that it was (103, 213); more recent findings show that RNA molecule populations extracted from virions contain subpopulations that possess ≤10 to 12 residues but have the same specific infectivity as molecules containing longer poly(A) regions (12, 86). Therefore, it is not entirely clear that a certain minimum length of poly(A) is required for infectivity and, if so, whether that critical length is the same for different picornaviruses.

There are several important differences between picornaviral mRNA and eucaryotic mRNA's with respect to their synthesis and processing. First, the picornaviral poly(A) region is transcribed from a polyuridylic acid sequence at the 5′ terminus of a viral minus-strand template (59, 245), whereas the poly(A) regions of cellular mRNA's are added post-transcription-

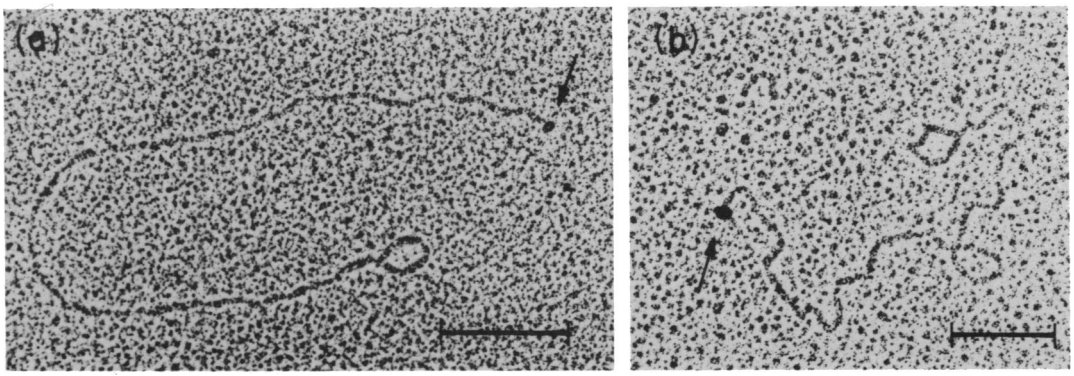

FIG. 4. *(a) Electron micrograph of poliovirion RNA spread by the benzyl-dimethylalkyl ammonium chloride method after treatment with dinitrophenyl fluorobenzene and rabbit anti-2,4-dinitrophenol immunoglobulin G to label VPg at the 5′ terminus and with the 3′ poly(A) end annealed to circular trypanosome mitochondrial deoxyribonucleic acid tailed with polybromodeoxyuridine. The arrow indicates the 5′-terminal protein VPg. (b) Cytochrome c spread of poliovirion RNA treated with dinitrophenyl fluorobenzene, rabbit anti-2,4-dinitrophenol immunoglobulin G, and the Fab fragment of goat anti-rabbit immunoglobulin G. The arrow indicates the 5′-terminal VPg. The 3′ poly(A) region was labeled by a trypanosome circle, as described above. Bar = 1 kb. From Wu et al. (239).*

ally by a terminal adenylate transferase (203). In addition, picornaviral transcription takes place in the cytoplasm, and processing of the transcripts to produce functional mRNA probably requires few, if any, of the reactions necessary to convert intranuclear cellular transcripts to functional cytoplasmic mRNA's.

It has been postulated that the poly(A) sequences of cellular mRNA's play some role in processing reactions, nuclear-cytoplasmic transport of mRNA's or their precursors, translation, and mRNA stability (23). However, it seems unlikely that the poly(A) region of picornaviral mRNA is necessary for translation, because most or all of it can be removed without gross alterations in the proteins synthesized from the poly(A)-deficient template in vitro (213). The physiological role of the poly(A) region remains to be determined, for both cellular and picornaviral mRNA's.

Existence of VPg. Picornavirion RNAs differ from eucaryotic mRNA's in the absence of a $m^7G^{5'}ppp^{5'}Np$ cap structure at the 5′ terminus (161, 235). In lieu of a cap, there is a small, virus-encoded (83), covalently linked protein designated VPg (104, 128, 201) (Fig. 4). Viral RNA from which VPg is removed by protease treatment remains infectious (160, 201). Interestingly, uninfected and virus-infected cells contain an enzymatic activity that readily cleaves VPg from added virion RNA molecules (4), and this may explain why viral RNA isolated directly from polysomes lacks VPg and terminates in pUp (71, 160). In cell-free protein-synthesizing systems, viral RNA lacking VPg forms initiation complexes (F. Golini, B. L. Semler, and E. Wimmer, submitted for publication). Thus, there is reason to believe that VPg is not crucial for translation. On the other hand, it is tempting to postulate a role for VPg in the ability of infected cells to translate only viral messages, even in the presence of large amounts of cellular mRNA's. The translation of cellular mRNA's seems to rely upon the recognition of the cap structure by a eucaryotic initiation factor(s) (8, 9, 190, 206, 207, 211), at least one of which is not necessary for the translation of picornaviral RNA (or other uncapped mRNA's) (212). The ability of picornaviruses to inhibit selectively the translation of cellular mRNA's while they synthesize their own proteins remains largely unexplained, and it is possible, but not likely, that different picornaviruses have evolved different mechanisms for accomplishing this.

The snake venom phosphodiesterase-sensitive linkage between VPg and the 5′ terminus of the poliovirus genome is O^4-(5′-uridylyl)tyrosine, a high-energy (−9.6 kcal/mol) bond (193). The 5′-terminal sequence is VPg(tyr-O)-pUUAAAACAG- (174).

The finding that VPg is linked to the 5′ polyuridylic acid terminus of negative-strand RNA in the replicative intermediate and replicative forms, as well as to the 5′ ends of nascent positive RNA strands, prompted speculation that this molecule functions as a primer for RNA replication (173). Although this remains possible, other roles include (i) a nonprimer role in RNA replication, (ii) a role as a regulatory protein determining which newly synthesized RNA molecules are to be encapsidated, and (iii) a role in protecting the 5′ end from exonucleases. None of these hypotheses is entirely satisfying. For example, the fact that VPg is attached to negative strands is difficult to reconcile with its action as a "pilot" protein involved in the morphogenetic process. On the other hand, minus strands are only produced very early in the infectious cycle, at a time when there are few structural intermediates.

Internal sequences. The existence of a large polycytidylic acid-rich region in some picornavirion RNAs was first reported by Porter et al. (183) for EMC virus. This region was found as a large RNase T1-resistant digestion product. Subsequently, other cardioviruses and FMDV (24, 94) were shown to possess large cytosine-rich regions of from 100 to more than 200 nucleotides. In the case of FMDV RNA, the polycytidylic acid region is located about 400 nucleotides from the 5′-terminal VPg (195). Since no polyproline-rich protein has ever been reported as a translation product of any picornavirus RNA, it seems that there are sizable noncoding regions at the 5′ termini of aphthovirus and cardiovirus RNAs. (Recently, a proline-rich 23-kilodalton polypeptide was detected in reticulocyte lysates programmed with EMC viral RNA, and this may represent at least a partial translation of the polycytidylic acid-rich sequence in vitro [162].) Enteroviruses apparently do not possess internal polycytidylic acid regions, and the function and evolutionary significance of such sequences are not known.

Considerable progress toward sequencing entire picornavirus genomes has been made (67, 184, 185, 246), but the details of this work are beyond the scope of this review. Fellner (67) has examined these sequences critically in light of sequence homologies and RNA secondary structure; interested readers are encouraged to consult his review. Nevertheless, the following preliminary conclusions can be drawn from the data already accumulated for a number of different picornaviruses: (i) enterovirus and aphthovirus RNAs are lacking a 5′-AAUAAAA-3′ sequence

proximal to the 3′ terminus (this sequence is thought to act as a signal for the post-transcriptional polyadenylation of eucaryotic mRNA's); (ii) within the sequence of 33 nucleotides next to the poly(A) regions, cardioviruses contain seven nonsense (termination) codons in various reading frames (poliovirus type 1 contains six of these codons within the last 72 nucleotides); and (iii) the minimum number of untranslated nucleotides next to the poly(A) region is 26 for EMC virus (246) and 43 for FMDV (184), if the appropriate reading frame is utilized. Much additional information should be available soon, after the sequences of entire picornavirus genomes have been determined.

SYNTHESIS AND PROCESSING OF VIRAL PROTEINS

Evidence for the Monocistronic Nature of Viral RNA

All eucaryotic mRNA's appear to be functionally monocistronic; i.e., they direct the synthesis of a single continuous polypeptide chain from a 5′-proximal initiation site. So far, little direct evidence to the contrary has been found (121). Therefore, we would expect that the mRNA's of mammalian viruses would function in the same manner. For picornaviruses, whose single-stranded RNA genomes are capable of mRNA functions, most of the experimental evidence supports the hypothesis that a single polypeptide chain is synthesized. However, these viruses direct the syntheses of discrete capsid proteins, as well as other proteins presumed to be responsible for viral RNA replication, proteolytic activities, suppression of host cell macromolecular synthesis, cell lysis, etc. The intracellular synthesis of these viral proteins was first demonstrated by Summers et al. (219), who showed that poliovirus-infected HeLa cells synthesized at least 14 different viral proteins. More recent analyses of some picornavirus-infected cells have revealed as many as 22 different proteins (199). Clearly, this amount of protein requires more than twice the genetic information that can be encoded in a picornavirus genome, assuming single initiation and termination sites and a single reading frame. It was quickly discovered (126) and confirmed (97, 142) by pulse-chase experiments in vivo that certain viral proteins are precursors to others. By treating infected cells with amino acid analogs (107, 109), zinc (32), which can act reversibly (119), or inhibitors of proteolytic enzymes (116, 220), a large "polyprotein" (NCVP 00) having an apparent molecular weight of ca. 220,000 was detected. It is now believed that NCVP 00 may be the single protein product from which all of the other virus-specific polypeptides are derived. NCVP 00 can be thought of as possessing at least three domains (corresponding to the coding regions shown in Fig. 1), which become unique polypeptides by proteolytic cleavage reactions. These function in capsid formation, in RNA replication and, probably, in the subsequent proteolytic processing of other primary products (Fig. 1).

Cleavage of Viral Proteins

By using an mRNA-dependent reticulocyte lysate system (168) programmed with poliovirus (208) or EMC virus (166, 209) RNA, the synthesis and processing of virus-specific proteins were shown to occur in vitro, except for the cleavage of VP0 to VP2 plus VP4, which is thought to be tightly coupled to RNA encapsidation (see below). The results obtained in vivo and in vitro suggest that the primary processing reaction(s) (i.e., cleavage of NCVP 00) occurs by autocatalysis or by a ubiquitous host enzyme acting on the nascent chain (33, 107), whereas subsequent cleavages reflect the action of a virus-induced protease(s) (115, 118, 163, 166).

When formyl-[^{35}S]methionyl transfer RNA was added to a reticulocyte lysate translating only EMC virus RNA, the only labeled molecules were pre-NCVP 1a, NCVP 1a, and, to a limited extent, NCVP 3a (166) (Fig. 1). These results suggest that in EMC virus, the N terminus of NCVP 00 is conserved until the formation of VP0 or VP4. In contrast, Lawrence and Thach (123, 248) found that the N terminus of the pre-NCVP 1a protein was degraded in EMC virus-infected cells. Although the reason for the discrepancy between the in vitro and the in vivo findings is unclear, it is possible that the introduction of formyl methionine groups, which normally are not found in eucaryotic cells, may inhibit N-terminal processing. In poliovirus-infected cells, pre-NCVP 1a molecules have not been found (210), nor have they been detected in in vitro translating systems (163, 166, 208). In vitro translation of poliovirus RNA in the presence of radioactive formylmethionyl transfer RNA labeled NCVP 1a (40), indicating conservation of the N terminus of NCVP 00.

As Table 2 shows, the N terminus of VP4 is blocked, although the nature of the blocking group is unknown. It is not known whether VP0, the immediate precursor of VP4, also possesses a blocked N terminus. However, if the in vitro translation experiments reflect events occurring in vivo, the introduction of the blocking group must occur concomitant with or after the proteolytic processing of the capsid precursor, NCVP 1a. This blocking group must serve an important function, since current information

suggests that it is present in many, if not all, picornaviruses.

Number of Initiation Sites

How convincing is the evidence that picornaviruses possess a single initiation site? The supporting evidence can be summarized as follows.

(i) Under conditions of cleavage inhibition, a 220-kilodalton protein (NCVP 00) is synthesized. The molecular weight of NCVP 00 is within 10% of the maximal coding capacity of the viral genome, presuming a single reading frame is used.

(ii) In vitro translation systems that contain ^{35}S-labeled formylmethionyl transfer RNA and are programmed with FMDV RNA or the larger of the two fragments remaining after polycytidylic acid region removal result in the majority of label being recovered in a single polypeptide (200), which is consistent with a single initiation site 400 to 600 nucleotides from the 5′ terminus (67, 184, 246). Similar experiments with EMC viral RNA labeled only the capsid precursor or its precursors (166).

(iii) The in vivo molar ratio of the products made from the three coding regions (Fig. 1) is 1.0. Although there have been many reports of the asymmetric production of viral proteins (135, 164), Rueckert and co-workers (152, 199) maintain that such findings reflect (i) the failure to score certain polypeptides as intermediate cleavage products and (ii) possible preferential degradation of products derived from the right-hand portions of the viral genomes.

(iv) The inability to detect complementation between picornavirus temperature-sensitive mutants (47) is understandable if the picornaviral RNA is a single gene and mutations have a high probability of exerting pleiotrophic effects because of altered processing of NCVP 00.

Nonetheless, the existence of a second initiation site in picornaviral genomes has some support. Abraham and Cooper (1) reported that there were no common tryptic digest fragments in NCVP X and NCVP 00, a finding consistent only with a second cistron model. However, Rueckert and co-workers (199) have shown that in rhinovirus-infected cells NCVP X and NCVP 1a come from a common precursor having a molecular weight of approximately 146×10^3, a result which supports strongly the hypothesis that a common cistron generates both of these polypeptides. An additional discrepancy in the single-initiation-site model comes from experiments in which two reproducible formyl-[^{35}S]methionyl-labeled tryptic digest fragments were detected in an in vitro system translating poliovirus RNA (40, 114). These fragments possessed dissimilar penultimate amino acids, indicating that they were from two unique initiation sequences. The relative amounts produced in vitro were dependent on Mg^{2+} concentration. One of the peptides represented the N terminus of NCVP 1a because (i) a polypeptide of this molecular weight was labeled in vitro and (ii) a correspondingly smaller polypeptide was detected in lieu of NCVP 1a when lysates were programmed with the RNA from a deletion mutant (defective-interfering particle) unable to synthesize a complete capsid precursor molecule. This smaller polypeptide, which was approximately 60% the mass of NCVP 1a, presumably was the fragment synthesized from the mutant CR 1 region of the defective-interfering particle (Fig. 1). The second formyl methionine-labeled polypeptide has not been identified yet (106), but it was produced in lysates programmed with defective-interfering particle RNA, indicating its lack of relatedness to NCVP 1a.

Finally, we should point out that, especially at relatively high concentrations of viral RNA, reticulocyte lysates synthesize a number of proteins for which no in vivo equivalents are detected (163, 166, 208; Phillips, unpublished data), and it is not clear at this time whether these proteins arise by premature termination, faulty processing, or the utilization of alternate initiation sites, perhaps due to limited hydrolysis of the viral RNA.

Although some controversy concerning the single-initiation-site model remains, the existence of two different sites does not a priori conflict with the monocistronic nature of the picornaviral genome. For example, Pelham (167) obtained evidence that the 1.3×10^6-dalton M RNA of cowpea mosaic virus, an uncapped message like picornavirus RNAs, binds two ribosomes at two distinct, in-phase initiation sites. Thus, it is possible to envision the synthesis of two polypeptides which are identical, except that one contains a short additional N-terminal sequence. Such heterogeneity among the large primary translation products of picornaviruses in vivo might go unnoticed.

The history of surprises encountered in the study of RNA bacteriophages should alert picornavirologists to a number of additional surprises that could lay ahead. These include the synthesis of read-through proteins at low frequencies (80, 165, 232), some of which are essential for the production of viral progeny (96); also, not many virologists anticipated the existence of overlapping genes in this simple group of viruses (6, 13). Thus, although the monocistronic, single-initiation-site model provides an explanation for much of the data collected concerning the synthesis and processing of picornaviral proteins,

some anomalous findings need explanation, and healthy skepticism is not unwise.

Mapping of Viral Proteins

The use of genetic recombinational analysis provided the first, albeit crude, map of picornaviral proteins. These experiments suggested that structural proteins were synthesized from one end of the viral genome, whereas the proteins responsible for certain aspects of viral RNA replication were derived from the other end (46, 47).

Later, it was theorized that if the single-initiation-site hypothesis was correct, then a specific inhibitor of protein synthesis could be used to map the relative distances of each protein from the initiation site (i.e., the 5′ terminus). The basis for this technique is that the farther away a protein product is from the initiation site, the more efficiently it is labeled with radioactive amino acids added at different times after initiation is inhibited. Pactamycin (43) treatment of virus-infected cells has been used successfully to show the relative locations of the viral proteins (31, 188, 218, 221). This information provided the basis for the scheme shown in Fig. 1. Pelham (166) used an EMC virus RNA-programmed reticulocyte lysate to which [^{35}S]methionine was added at progressively later times after initiation began. The resulting data further substantiated the conclusions drawn from the pactamycin experiments. Finally, a comparative analysis of the tryptic digest peptides obtained from most of the viral proteins established the relatedness of the polypeptides common to a particular coding region of the RNA (107, 109, 199). It is gratifying that there was excellent agreement between genetic analyses and various biochemical approaches aimed at determining a genetic map and elucidating precursor-product relationships.

Functions of Viral Nonstructural Proteins

Relatively little is known about the precise functions of the viral proteins other than the structural or capsid proteins. Several problems confront investigators who study these functions. First, there is some evidence that both intermediate precursors and stable products carry out crucial viral functions. Second, conditional lethal mutants do not exhibit complementation, thus eliminating one of the most powerful tools available for this kind of work. Finally, most of the picornavirus temperature-sensitive mutants that have been isolated and characterized exhibit some degree of pleiotrophism, suggesting that structural proteins may play a role in RNA synthesis (48) and that the products thought to function in RNA synthesis may influence virion structure, possibly through effects on morphogenetic events (see below). Below, we examine the functions of the viral nonstructural proteins in the order of their synthesis (Fig. 1). Insofar as they are known, the roles of the capsid proteins have already been discussed (see above).

Proteolytic activities. NCVP X, which is coded proximal to the capsid precursor NCVP 1a, may be a protease. Two independent groups of workers recently reported that EMC virus RNA-programmed reticulocyte lysates induce the appearance of a proteolytic activity only after ribosomes traverse the CR 1 region, as shown in Fig. 1 (166, 208, 209). More recently, Korant et al. (115, 118) purified a protease from poliovirus-infected cells acting on viral precursors and found that NCVP X was the major viral component. No such activity appeared in uninfected cells. The possibility that structural polypeptides were responsible was eliminated because the activity appeared in cells infected with defective-interfering particles (115). However, a second viral polypeptide with a molecular weight of approximately 22×10^3 (our estimate from the data of Korant et al. [115]) also was present in small amounts. More recently, Palmenberg et al. (163) purified a putative viral protease from reticulocyte lysates programmed with EMC virus RNA. Using sucrose gradient fractionation followed by precipitation of structural contaminants with antiviral antibody, these investigators identified a small 22-kilodalton protein as the protease that acted on structural precursors. They used a method similar to that of Pelham (166), in which limited translation products (composed mainly of pre-NCVP 1a and NCVP 1a molecules) were used as the substrates for proteolysis assays. It is not clear whether the 22-kilodalton protein corresponds to the NCVP 7 protein shown in Fig. 1, but this is consistent with the fact that the 22-kilodalton protein shares tryptic peptides with NCVP 1b and NCVP 2 (163). These findings are not necessarily contradictory; both NCVP X and NCVP 7 may possess proteolytic activity. In fact, since neither protein is a primary translation product, one or both must arise autocatalytically or through the action of a reticulocyte enzyme. A role for NCVP X as a membrane-associated protein (34) has been proposed because of its presence in fractions enriched in smooth endoplasmic reticulum from virus-infected cells (226).

Origin of RNA-replicating enzymes. There is now substantial evidence that the polypeptides encoded in the CR 3 region are active in the replication of viral RNA molecules. A number of workers have found that purified replicase preparations are highly enriched with

NCVP 4 (76, 136, 138, 226). More recently, replicase preparations able to initiate complementary strands were shown to contain NCVP 2 as well as NCVP 4 (52, 76), suggesting that NCVP 2, perhaps in conjunction with other host proteins or viral proteins (53) or both, acts to initiate RNA synthesis, whereupon it is cleaved to form NCVP 4, which then elongates the polyribonucleotide chain. This scheme could explain the finding that restrictive temperature-induced degradation of NCVP 2 in cells infected with a type 3 strain of poliovirus correlated with inhibition of RNA synthesis (18). Conversely, Korant (117) isolated a mutant with a very stable replicase activity; the rate of processing of its NCVP 2 was significantly slower than that of wild-type virus. Drescher (61) found that only RNA^- temperature-sensitive mutants exhibited gross alterations in the synthesis and processing of the NCVP 1b and NCVP 2 precursors.

What continues to perplex many picornavirologists are the other well-documented virus-induced functions for which neither a mechanism nor a specific viral protein has been found. These include the shutoff of cellular RNA and protein synthesis, the induction (or derepression) of smooth membrane biogenesis, and the cause of cytopathology.

Regulation

It is clear that an outstanding feature of picornavirus replication is post-translational proteolysis (32, 109). Post-translational processing occurs in uninfected eucaryotic cells, where it regulates secretory proteins (222) and activates zymogens (159). More recently, proteolytic processing has been implicated in the morphogenesis of the cytochrome *c* oxidase complex (186). Thus, post-translational processing seems to be a general feature of the biology of eucaryotic cells.

Picornaviral NCVP 00 is not detected in virus-infected cells or in reticulocyte lysates translating viral RNA. NCVP 00 can be detected only if proteolysis is inhibited or if analogs are used to produce aberrant molecules (32, 107, 109, 116, 119, 220). Therefore, cleavage of the nascent polypeptide chain must occur before the ribosome has traversed the entire genome. The mechanism operating to generate the primary translation products (Fig. 1) remains obscure, but either (i) it reflects the proteolytic activity of a ubiquitous cellular protein(s) (116, 220), which may be intimately involved in protein synthesis, or (ii) the hydrolysis of NCVP 00 is autocatalytic. The problem inherent in distinguishing between these two mechanisms is that proteolysis must be inhibited to detect NCVP 00, but under these conditions what is inhibited? Is it an exogenous protease, or is it a conformational change in NCVP 00 that makes this molecule resistant to exogenous or autogenous proteolysis?

In contrast, the protease(s) that catalyzes the secondary cleavages seems to be virus-specific enzyme (see above). It seems probable that if there is regulation in terms of modulating viral functions, such as RNA synthesis, then this regulation is exerted at the level of post-translational processing. Some support for this hypothesis comes from several kinds of experiments. As noted above, Korant (117) characterized a poliovirus mutant with a relatively stable RNA polymerase activity and a decreased rate of processing of NCVP 2. If NCVP 2 functions in the initiation of viral RNA synthesis and is cleaved immediately thereafter, its stoichiometric action would loosely couple initiation of RNA strands to the amount of processing enzyme needed to cleave capsid precursor molecules into capsid proteins, especially if the half-life of the putative protease (NCVP 7) is relatively brief. Another intriguing facet of processing that might have ramifications in the regulation of viral functions is the alternate processing of certain intermediates. For example, McLean et al. (152) showed that NCVP 2 of both polioviruses and rhinoviruses can be cleaved by either of two mutually exclusive alternate pathways (Fig. 1). Do these alternate protein products exert specific functions, or are they a mechanism for eliminating excess NCVP 2 molecules when RNA synthesis occurs at adequate levels? Obviously, we must await the results of further experiments, but the once prominent hypothesis that picornaviruses simply overproduce viral proteins without any regulation already is becoming suspect.

MORPHOGENETIC STRUCTURES

Structural Intermediates in Morphogenesis

Since we have discussed the morphology of picornavirions, the structure of picornavirion RNA, and the mechanisms operating in the synthesis and formation of virus-specific proteins, we can now analyze the information available concerning the morphogenetic process. Until recently, such an analysis would have been restricted entirely to the CR 1 region of the viral genome since it is here that all of the structural proteins are encoded. Now, it is appropriate to consider possible functions expressed by the products of both the CR 2 region and the CR 3 region because (i) one or both sequences may encode a viral protease acting on the capsid precursor NCVP 1a and (ii) the encapsidation of viral RNA may be linked intimately to the cleav-

age of VP0 (Fig. 5), as well as to the synthesis of progeny RNA strands. First, we consider the forms of structural proteins present in infected cells (Table 3).

Forms of the capsid precursor (NCVP 1a). The capsid precursor NCVP 1a apparently can exist in infected cells in soluble and aggregate forms, a conclusion drawn both from in vivo experiments (151) and in vitro experiments (208; Phillips, unpublished data). The earliest detectable, organized structure containing NCVP 1a is the 13S particle found in EMC virus-infected cells (150) and in rhinovirus-infected cells (151); the relatively slow turnover of this particle in these cells permitted its isolation and partial characterization. In poliovirus-infected cells, indirect evidence for the presence of this particle was derived from pulse-labeling experiments in which a 14S particle with a relatively high concentration of NCVP 1a was found (217). The 13S particle appears to be a pentamer of NCVP 1a molecules, and originally it was suggested that cleavage of the capsid precursor could take place only in this form (150). Unfortunately, the synthesis and processing of soluble NCVP 1a in vitro in the absence of particle formation (208; Phillips, unpublished data) make this attractive hypothesis unlikely. However, it is still reasonable to hypothesize that in vivo a significant amount of the capsid precursor forms 13S particles, whereupon the cleavage of NCVP 1a gives rise to 14S particles.

14S particles. The 14S particle was first described by Watanabe et al. (231) as a 10S particle that was labeled rapidly with radioactive amino acids in poliovirus-infected cells. Independently, Phillips et al. (181) isolated a particle (later corrected to 14S [175]) which was composed primarily of VP0, VP1, and VP3. Since the composition of this particle was similar to that of procapsids, it was presumed to be a structural subunit. Later, it was shown that the 14S particle

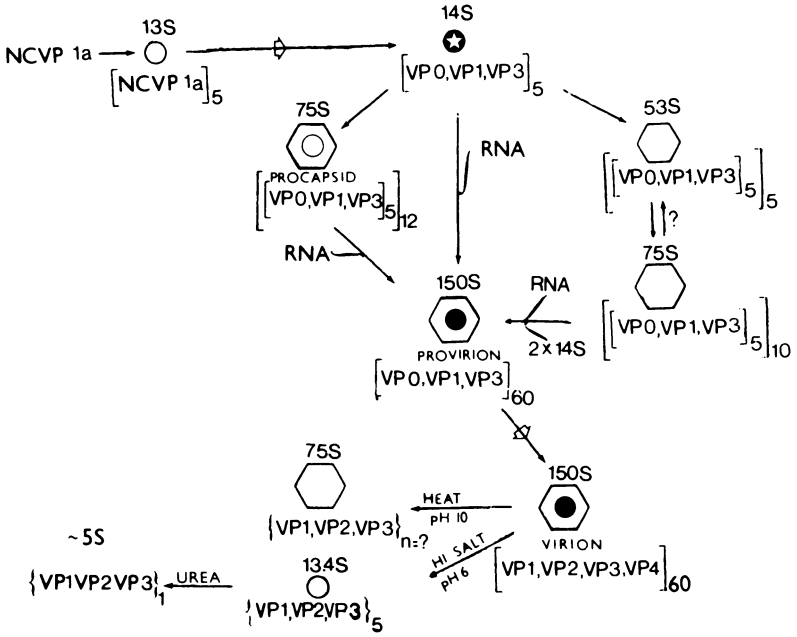

FIG. 5. *Picornavirus morphogenetic schemes showing the hypothetical pathways and structutal intermediates in virion assembly. This diagram shows three hypothetical pathways leading from 14S particles to mature, infectious virions. In the pathway on the left, 12 14S particles polymerize to form a procapsid (a 75S empty shell), which then interacts with viral RNA to form a 150S provirion. In the middle pathway, 14S particles interact directly with the viral RNA to form a provirion. However, in the absence of viral RNA, 14S particles would form 75S empty capsids. Empty capsids might also be generated as degradation products of provirions. In the pathway on the right, 14S particles first polymerize to form 53S half-procapsids (60, 124). This structure or its dimer (the 75S procapsid-like structure) reacts with viral RNA. Two 14S subunits are added after or concurrent with RNA encapsidation. A provirion-like particle is an intermediate common to all three pathways; a virion is formed when VP0 is cleaved to VP2 and VP4. The virion is represented as being devoid of VP0 polypeptides. However, the presence of a VP0 molecule(s) might result from failure to cleave one or two copies during virion formation. At the bottom, the degradation of virions to 75S empty capsids containing VP1, VP2, VP3, and 13.4S pentamers is shown. The 13.4S pentamers can be degraded to 5S protomers, as shown in Fig. 2.*

TABLE 3. *Putative structures in the morphogenesis of picornaviruses*

Particle	Probable polypeptide composition	RNA	Density in CsCl (g/ml)	Antigenicity	Labeling time in vivo (min)[a]
13S	$(NCVP\ 1a)_5$	−	ND[b]	ND	7
14S	$(VP0\text{-}VP1\text{-}VP3)_5$	−	ND	S(C?)	7–10
75S (procapsid)[c]	$[(VP0\text{-}VP1\text{-}VP3)_5]_{12}$	−	1.29	C(D?)	15–20
150S (provirion)	$[(VP0\text{-}VP1\text{-}VP3)_5]_{12}$	+	ND	ND	20?
150S (virion)	$[(VP1\text{-}VP2\text{-}VP3\text{-}VP4)_5]_{12}$	+	1.34	D	20–30

[a] The exact times in large part reflect experimental conditions and, particularly, the amount of isotope used.
[b] ND, Not determined.
[c] Not found in cardiovirus-infected cells.

possessed many properties consistent with a precursor role, including the ability to form 75S structures in vitro very similar to the procapsids synthesized in vivo (175, 176, 181). The fact that 14S particles isolated in sufficient concentrations could self-assemble in the apparent absence of exogenous factors (176, 182) permitted electron microscopic confirmation that the product was an empty shell (Fig. 6). Furthermore, Ghendon and co-workers (81) showed that poliovirus-infected MiO cells accumulated 14S particles when viral RNA synthesis was blocked reversibly by low concentrations of guanidine and that the 14S particles were converted to virions when the inhibitor was removed. The characteristics of 14S particles and the evidence supporting the precursor role of these particles can be summarized as follows: (i) they are always present in cells infected with different picornaviruses; (ii) they are labeled rapidly with radioactive amino acids; (iii) they require the synthesis of viral RNA and protein; (iv) they are composed of structural proteins; (v) pulse-chase results are consistent with a precursor role; (vi) under certain conditions, accumulation of these particles occurs upon blockage of viral RNA synthesis; and (vii) they form empty capsids very similar to the procapsids in vitro.

Procapsids. Procapsids are naturally occurring, stable empty shells found in cells infected with enteroviruses, rhinoviruses, and aphthoviruses but not in cells infected with cardioviruses. As determined by electron microscopic and serological studies, the appearance of procapsids in infected cells roughly parallels the production of progeny virus (177). Originally, procapsids were identified in poliovirus-infected cells as being either morphogenetic intermediates or products derived from an unidentified precursor because they possessed polypeptide VP0 (141). Later, it was thought that they play a precursor role because (i) VP0 was shown to be a precursor of viral capsid proteins VP2 and VP4 (108) (Fig. 1), (ii) the time required for labeling in vivo was approximately 15 min and procapsids were labeled before virions became labeled (170, 216), (iii) it was found that the addition of 1 to 3 mM guanidine at midinfection to inhibit RNA synthesis caused the accumulation of procapsids which could be chased into virus particles (75, 108), and (iv) pulse-chase experiments performed in the presence of protein synthesis inhibitors showed a flow of radioactivity from procapsids into virions (240). This last finding contrasts with earlier experiments with other picornaviruses (216) and differs from the results of Ghendon et al. (81) described above; these differences may reflect the cell types used or the particular viruses used, but they cannot be reconciled at this time.

Despite these findings and the inherent stability of procapsids in vitro, we cannot rule out the possible existence in vivo of a labile structure from which procapsids are a degradation product. In addition, the morphology of procapsids has been examined recently by Lee and Colter (126), who isolated 53S particles from mengovirus-infected cells and showed that in high-salt solutions they were converted to 75S particles; the latter were not detected in virus-infected cells. Using exclusion chromatography, these investigators determined particle molecular weights of 2.2×10^6 and 4.4×10^6 for the 53S and 75S particles, respectively, suggesting that (i) the 75S particles were dimers of the 53S particles and (ii) the composition of the 75S particles was best represented by $(VP0\text{-}VP1\text{-}VP3)_{50}$ rather than the generally accepted $(VP0\text{-}VP1\text{-}VP3)_{60}$ (Fig. 5). Thus, Lee and Colter envisioned the mengovirus procapsid as an incomplete, yet stable shell (126). Whether these findings apply to naturally occurring procapsids remains to be determined. Procapsids contain no detectable RNA, nor do they associate with viral RNA in vitro (Phillips, unpublished data). They may possess traces of other noncapsid polypeptides (177, 227).

Provirions. Provirions were isolated origi-

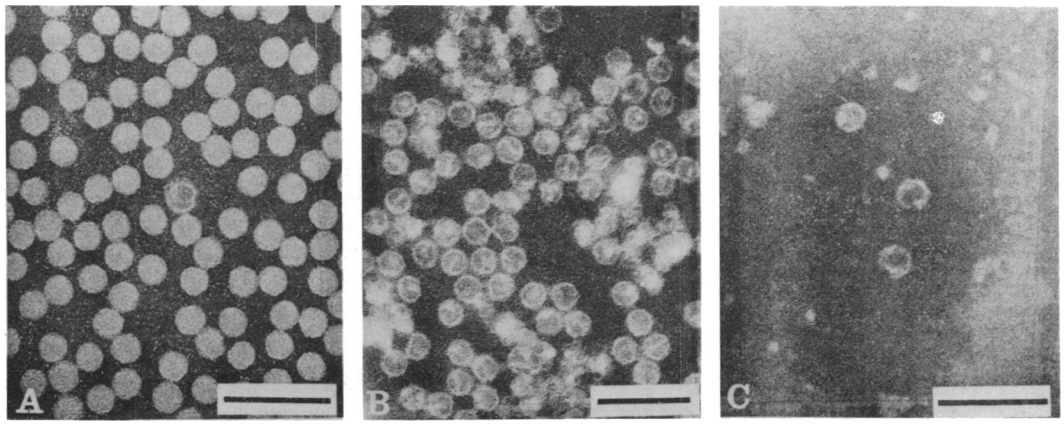

FIG. 6. *Electron micrographs of poliovirions (A), empty capsids (procapsids) isolated from infected cells (B), and empty shells formed by the self-assembly of 14S particles in vitro (C). All specimens were treated with phosphotungstic acid (pH 7.0). Bars = 0.1 μm. From Phillips (176).*

nally as discrete entities sedimenting at 120S in sucrose gradients, which permitted a direct examination of their chemical and physical properties (72, 73). Subsequently, they were reported to be inseparable from 150S virus particles (87) (see above). Provirion-like structures also were isolated from bovine enterovirus-infected cells (95). These structures sedimented at 145S and were composed of VP0, VP1, VP2, VP3, and VP4 polypeptides, suggesting arrest during cleavage of VP0 molecules. The distinguishing features of provirions are (i) the polypeptide composition of a procapsid, (ii) the presence of viral RNA resistant to RNase, (iii) sensitivity at neutral pH to sodium dodecyl sulfate and high salt concentrations, such as 3 M CsCl, and (iv) in some cases a susceptibility to disruption by ethylenediaminetetraacetate. Under physiological conditions, none of these treatments affects virions, and only sodium dodecyl sulfate disrupts procapsids. The relative instability of provirions is important because it exemplifies a possible intermediate which, under certain conditions, may dissociate into a procapsid and raises a question about the existence of still more labile intermediate structures. In addition, certain temperature-sensitive mutants, particularly RNA^- mutants, make 150S particles which have a very low ratio of plaque-forming units to particles and possess primarily VP0, VP1, and VP3 (61). An RNA^+ temperature-sensitive mutant with an altered VP0 also forms 150S particles containing large amounts of VP0, suggesting a low efficiency of cleavage of this polypeptide (62). It is tempting to suggest that these structures are mutant provirions, but this must await tests of sensitivity to RNase, ethylenediaminetetraacetate, sodium dodecyl sulfate, and high salt concentrations.

ASSEMBLY REACTIONS IN VITRO

Conversion of 14S Particles into Procapsids

Although all picornavirus-infected cells synthesize 14S particles, at this time only the 14S particles isolated from poliovirus-infected cells possess a demonstrable biological activity in vitro. As noted above, if 14S particles are obtained in sufficient concentrations or are concentrated by ultrafiltration, the formation of procapsids proceeds in the apparent absence of exogenous factors (182), thus permitting their identification by electron microscopy (Fig. 6).

Evidence for a Morphopoietic Factor

In contrast, 14S particle preparations too dilute to self-assemble are always assembled into procapsids upon incubation with extracts prepared from virus-infected cells, but not from uninfected cells (81, 181). Clearly, viral RNA and protein synthesis are required for expression of this assembly-promoting activity, but the assembly reaction itself occurs in the presence of RNase and inhibitors of protein synthesis (175). There is no difference between the time course for the self-assembly reaction and the extract-mediated assembly reaction (175) (Fig. 7); thus, the assembly-promoting activity may act by facilitating the self-assembly reaction at low 14S particle concentrations. Both the extract-mediated reaction and the self-assembly reaction are highly temperature dependent (optimal temperature, 37 to 40°C) and occur only at a neutral

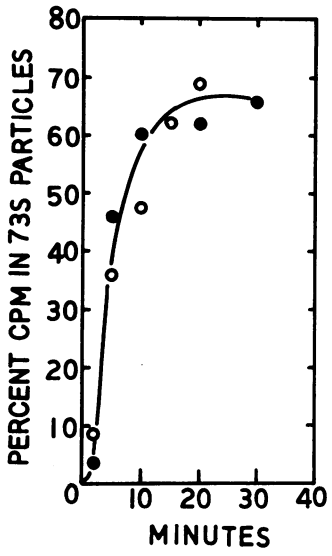

FIG. 7. *Normalized kinetics of the extract-mediated appearance of 75S empty shells as they are assembled from 14S particles in vitro at 37°C. The extent of 75S particle formation at any given time was determined by adding the amounts of radioactivity in the 75S peak and dividing the resulting value by the total amount of radioactivity recovered from the sucrose gradient. Each point represents the average of two determinations. The open and solid circles represent different 14S particle preparations. From Phillips (175).*

pH and low salt concentrations (181; Phillips, unpublished data).

The obvious explanation for the assembly-promoting activity of extracts (namely, their endogenous supply of 14S particles) became suspect when it was shown that (i) extracts preincubated to deplete endogenous precursors retained their assembly-promoting activity (171, 175), (ii) preincubated extracts could be diluted up to 40-fold and still exhibit activity (172), whereas the self-assembly of 14S particles into procapsids was very dilution sensitive (205), (iii) the activity was associated with a rough endoplasmic reticulum fraction containing few or no free 14S particles (membrane-bound particles were detected) (171), and (iv) the activity was totally resistant to doses of ultraviolet irradiation sufficient to inhibit the self-assembly reaction (182). This activity was sensitive to trypsin, deoxycholate, and sodium dodecyl sulfate (which disrupts 14S particles). Recently, Phillips et al. (179) reported that extracts from cells infected with defective-interfering particles contained no detectable assembly-promoting activity, which seems to indicate an association between this activity and structural proteins. On the other hand, two RNA^+ temperature-sensitive mutants containing altered VP0 or VP1 polypeptides both synthesized 14S particles which were defective in the ability to polymerize into procapsids yet retained the assembly-promoting activity of wild-type 14S particles (62). An RNA^+ mutant for which no identifiable alterations in structural or nonstructural polypeptides were detected but which showed a reduced rate of NCVP 1a processing produced polymerizable 14S particles, as shown by the assembly of these particles into procapsids in extracts from wild-type virus-infected cells, but exhibited greatly decreased assembly-promoting activity for wild-type 14S particles in vitro (61, 62). A similar RNA^+ temperature-sensitive mutant was described by Mikhejeva et al. (154). The nature of the cytoplasmic assembly factor(s) can be summarized as follows: (i) requires viral RNA and protein synthesis; (ii) not present in uninfected cells; (iii) associated with rough endoplasmic reticulum; (iv) sensitive to trypsin or 1% deoxycholate; (v) resistant to RNase; (vi) preincubation to deplete endogenous 14S precursor particles does not eliminate activity; (vii) absent in cells infected with defective-interfering particles; and (viii) may exist independently of polymerizable 14S particles. Either assembly activity arises from a form(s) of structural protein other than 14S particles, or, conceivably, it requires some unique conformational state of the 14S particle. In this regard, the finding that assembly activity was associated with rough endoplasmic reticulum containing bound 14S particles (171) suggests that such membranous structures may bind 14S particles, thus facilitating their assembly into procapsids in vitro and, perhaps, into virions in vivo (see below).

ROLE OF INTRACELLULAR MEMBRANES SYNTHESIZED (MODIFIED) IN RESPONSE TO VIRAL INFECTION

Picornavirus multiplication in host cells is known to cause quantitative and qualitative changes in the endoplasmic reticulum (3, 37, 51, 156). Much experimental evidence, particularly the association of viral RNA-synthesizing machinery with intracellular membranes, indicates an active role for membranes in picornavirus replication.

Electron micrographs of HeLa cells infected with poliovirus show extensive proliferation of smooth endoplasmic reticulum in the perinuclear region beginning about 3 h postinfection (3, 51). Concomitantly, there is an increased rate of synthesis of lipids and phospholipids, as shown by the uptake and incorporation of radiolabeled precursors (51, 157). [2-^{3}H]glycerol incorporation into rough membranes increased

fivefold about 3.5 h after infection. The majority of the label shifted to the smooth membrane fractions after a 60-min chase (157). Mosser et al. (156) found a decrease in the quantity and an increase in the density of HeLa cell rough endoplasmic reticulum after poliovirus infection. These changes appeared to parallel an increase in the amount of smooth endoplasmic reticulum and a decrease in the specific activity of various endoplasmic reticulum-associated cellular enzymes. This may occur partly as a result of virus-induced shutoff of host protein synthesis. The stimulus for membrane proliferation is not known.

Role of Membranes in RNA Synthesis

The association of picornaviral RNA replication complexes with smooth membrane vesicles (34, 37, 38, 124) suggests that membranes play a role in viral RNA replication. Poliovirus RNA replication in Hep-2 cells was linked temporally to membrane proliferation and vacuolization (14). In contrast, protein synthesis peaked well before any cytopathic changes could be observed (14). If guanidine is added to infected cells to inhibit virus-specific RNA replication (223), extensive membrane proliferation is not observed, even though phospholipid synthesis occurs at a rate greater than that in uninfected cells (157). Caliguiri and Tamm (37) have reported finding a 130S membrane-bound structure containing polioviral replicative intermediate RNA. On the basis of pulse-label experiments, they suggest that RNA replication takes place in association with smooth membranes. The newly formed mRNA then associates with ribosomes, forming patches of rough endoplasmic reticulum, where the majority of virus-specific protein synthesis occurs (194). The association of the polioviral RNA polymerase complex with membranes is most likely mediated by hydrophobic interactions between polymerase-associated proteins (NCVP 2, NCVP 4, and NCVP X?) and the hydrocarbon tails in the interior of the lipid bilayer (34). However, the active site of the RNA polymerase itself may not be associated intimately with lipids.

Role of Membranes in Morphogenesis

A possible role for membranes in capsid and virion morphogenesis has also been investigated. Virus particles associated with the poliovirus RNA replication complex have a ratio of radioactivity to infectivity three- to eightfold higher than virus particles from other cell fractions after a short pulse with [^{3}H]uridine (35). For this reason, it was postulated that virus RNA replication and virion formation are coupled processes which occur in association with smooth endoplasmic reticulum (35, 36). In contrast, the assembly of 14S particles into 73S empty capsids in vitro has been shown to be facilitated by rough membrane fractions (171), a finding which has been interpreted as supporting the hypothesis that there is a membrane-associated virus-specific assembly factor.

MORPHOGENETIC PATHWAYS

Figure 5 shows the prevailing hypothetical schemes concerning the morphogenesis of picornaviruses. Although it would be simpler and more satisfying to expect that only one of these pathways is operational in vivo, it is possible that alternate mechanisms exist in different picornavirus groups. None of these pathways can be eliminated at this time, although some have more direct experimental support and require less rationalization than others.

In assessing these schemes, it becomes necessary to reflect on the ambiguities regarding the precise structural features of virions. For example, what is the significance of the small amounts of VP0 molecules detected in virion preparations? Are they necessary for infectivity, or is their presence fortuitous? Provirions do not survive high-salt exposure, so CsCl-banded virions are not likely to contain these structures (87, 95). On the other hand, the nature and density of the salt-induced degradation products of provirions have not been well characterized. In any case, the main source of contamination, if indeed contamination is the origin of VP0 molecules in virion preparations, would most likely be procapsid-like structures. An indirect estimate of the degree of cross-contamination between procapsids and virions can be made by assuming that the former are devoid of VP2 and taking the amount of VP2 in banded procapsids as a measure of virion contamination. VP2 has been detected in empty shells purified by sucrose or CsCl gradients (141). Unfortunately, in these experiments this finding also could be explained by the presence of artificial empty shells (which contain VP2) or procapsid forms possessing VP2 (178, 227). To make matters worse, the ratio of particles to plaque-forming units in purified poliovirus preparations is at least 60:1 (147), and it can be argued that virus particles containing VP0 are noninfectious. Thus, it is difficult to find compelling evidence that bona fide virions contain VP0 molecules, but this possibility cannot be eliminated.

Another important consideration in evaluating the pathways leading to infectious virus particles is the morphology of the putative intermediates. Virtually nothing is known about the fine structures of the 13S and 14S particles. In

the case of the latter, sufficient purification has not been accomplished to permit an unequivocal identification by electron microscopy (Phillips, unpublished data). Molar ratio determinations revealed nonequimolar amounts of VP0, VP1, and VP3 (178), as well as small but reproducible amounts of other nonstructural polypeptides (182). These results probably reflect contamination with other viral proteins, as well as the presence of immature or defective 14S particles or both (172). Therefore, the most likely composition of biologically active particles is $(VP0\text{-}VP1\text{-}VP3)_5$ or five protomers, as suggested by Rueckert and co-workers (150, 151).

Until recently, procapsids were envisioned as empty shells composed of 12 14S particles (i.e., 60 protomers). However, Lee and Colter (126) have proposed that mengovirus procapsids are incomplete shells constructed of 10 14S particles and formed by the dimerization of two 53S particles (see above) (Fig. 5, right-hand pathway). The hypothesis that 53S particles are morphogenetic precursors is supported by the findings that they accumulate in temperature-sensitive mutant-infected cells at the nonpermissive temperature (60) and that they dissociate into 14S particles (127). Su and Taylor (216) partially characterized 45S particles obtained from bovine enterovirus-infected L cells and showed that they also formed 80S structures in the presence of high salt concentrations. It is interesting that in both cases the dimerization of 45S or 53S particles occurred at 4°C in the presence of high salt concentrations, in contrast to the assembly of 14S particles into procapsids, a reaction which occurs only at low ionic strengths and is very temperature dependent (181).

What is clearly evident from all of these results is the need for more detailed studies on the morphology of these putative intermediates. Electron microscopic examinations of virions and procapsids have been restricted almost exclusively to negative staining techniques (Fig. 6). No "half procapsid" or other partially assembled structures corresponding to the 45S or 53S structures have been identified. Thus, in the absence of a more detailed determination of the morphology of 45S and 53S particles, it is conceivable that the conversion of these particles to 75S to 80S structures represents a conformation change or aggregation with other cell or viral structures.

The hypothesis that procapsids represent partially enclosed shells made up of 10 14S particles may be one way of dealing with the conceptual dilemma associated with the encapsidation of a 2,500-nm RNA molecule into a fully enclosed shell, but it raises a number of equally perplexing questions. For example, what accounts for the relative stability of procapsids? What mechanisms prevent the formation of the theoretically more stable icosahedral (T = 1 class) shells formed by 12 14S particles, especially since 2 additional 14S particles must react later to form a provirion or virion (Fig. 5)? It seems that some directive factor (i.e., morphopoietic factor) is required. Phillips and co-workers have proposed that there is such a factor in poliovirus-infected cells, but that this factor acts to promote the formation of procapsids in vitro and, perhaps, the formation of virions in vivo.

Procapsids have been candidates for precursors of virus particles ever since they were shown not to be degradation products of virions (141). Much of the experimental evidence gathered so far has been interpreted as supporting this hypothesis (see above). Perhaps the main stumbling block is a conceptual one; i.e., if procapsids are icosahedral shells, as indicated by electron microscopy (Fig. 6), how are the viral RNA molecules encapsidated? In 1972, Phillips postulated that the RNA might be interpenetrated throughout the capsid and might not be stuffed into the center of a hollow shell (177). A more intriguing hypothesis is that the RNA molecule is synthesized into a procapsid. Yin (242) found that procapsids were associated with the viral RNA smooth membrane complexes isolated from poliovirus-infected cells and that this association promoted viral RNA synthesis. She also presented evidence that newly synthesized RNA became associated with a 135S provirion-like particle in vitro. Confirmation of these findings should have a high priority.

We suggest that the simplest morphogenetic pathway is the interaction of 14S particles directly with nascent viral polyribonucleotide chains (Fig. 5, center pathway), which protects the 5'-terminal VPg from cleavage (see above) and prevents ribosome attachment. This reaction might well proceed stepwise as the RNA molecule is synthesized. This mechanism presupposes that in vivo there is a single (or closely adjacent) site(s) at which both RNA synthesis and protein synthesis take place. Upon cell homogenization, this site might be artifactually separated on the basis of the densities of its component parts, thereby giving rise to smooth membranes containing most of the RNA-synthesizing machinery and rough membranes retaining most of the viral mRNA, ribosomes, and partially or fully assembled viral subunits (36–38, 156, 157, 171). Alternatively, there would have to be a mechanism for transporting protein subunits to the sites of viral RNA synthesis, where final maturation is thought to occur (35). In this regard, the RNA genomes of defective-interfering particles, which contain deletions

near their 5′ termini (Fig. 1), replicate their RNAs effeciently in the absence of structural proteins, yet their RNAs are encapsidated in cells coinfected with wild-type virus (44). Upon completion of a newly synthesized viral RNA molecule, a provirion-like particle is formed; subsequent cleavage of the VP0 polypeptides results in a virion. This model also explains the finding that viral RNA molecules are encapsidated relatively rapidly (5 to 10 min), as shown by uridine incorporation studies (170). It is not difficult to imagine a population of labile intermediates composed of variable numbers of 14S particles and varying lengths of nascent viral RNA, most of which are destroyed upon homogenization and subsequent attack by nucleases. The degradation of nearly completed provirion-like structures probably would give rise to procapsids or the 45S to 53S particles described above, whereas more primitive intermediate structures would dissociate into 14S particles. This might provide an alternate explanation for pulse-chase experiments that have been interpreted as supporting a direct role for procapsids in virion morphogenesis. Another pleasing aspect of this model is that it avoids the problems inherent in the encapsidation of an intact viral RNA molecule by a complete or incompletely enclosed shell. This model predicts that 14S particles ought to react with virion RNA molecules possessing VPg. Such experiments have been carried out, but recently an RNase activity has been detected in 14S particle preparations; this might explain why no evidence of an RNA-14S particle interaction was detected (Phillips, unpublished data).

A final consideration concerns the nature of the cleavage of VP0 to VP2 plus VP4, which is believed to be intimately associated with the final stages of virion maturation (Fig. 5). The isolation of provirion-like particles strongly suggests that VP0 is not cleaved until after the viral genome is encapsidated. Subsequent cleavage of VP0 must trigger a rearrangement of capsid subunits, and thus confer increased stability to the resulting virions, as shown by the sensitivity of provirions, but not virions, to anionic detergents and high salt concentrations. This cleavage reaction apparently is very efficient; thus, the enzyme responsible (assuming that the reaction is not autocatalytic) must be closely associated with the provirion during or after its formation. Lawrence and Thach (123) originally proposed that one of the capsid proteins (VP3) was a protease. The recent report of Palmenberg et al. (163) indicating that NCVP 7, which often comigrates with VP3 (Fig. 1), exhibits proteolytic activity makes the original finding suspect. On the other hand, preparations enriched in NCVP 7 or NCVP X fail to act on VP0; in fact, they only incompletely cleave NCVP 1a (115, 163). The hypothesis that one of the capsid proteins possesses proteolytic activity has much merit. Even the genome-linked protein VPg is a candidate for such a protease. It was noted above that undefined protease activities have been found associated with purified picornavirus preparations. It is also interesting that VP2 and VP4, the cleavage products of VP0, appear to occupy an internal (or at least unexposed) position in virions (see above), which may be a consequence of capsid rearrangement or may reflect the location of the VP0 polypeptide in provirions.

CONCLUDING REMARKS

We conclude by identifying those research areas in which continued progress will be needed in order to choose among the pathways shown in Fig. 5. The unpleasant fact is that although substantial progress has been made in elucidating picornavirus structure and characterizing putative precursor structures, we are no closer to understanding the configuration of viral RNA in virions or how it gets there than we were in 1972 (177). What is the first structural intermediate that interacts with the viral genome? What is the state of the genome at this time? (Is the genome a completed chain or is it still part of the replicative intermediate?) Do nonstructural viral or host cell proteins play a role? What is the origin and nature of the enzymes responsible for the various cleavage reactions, especially those processing NCVP 1a and VP0? In the next few years researchers will certainly provide answers to some of these questions and generate new insights into the morphogenesis of these viruses.

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ADDENDUM IN PROOF

Recently, we have found that the isoelectric point (pI) of the empty capsids formed by the self-assembly of poliovirus 14S particles in vitro is 5.0, whereas that of the empty capsids formed in the extract-mediated assembly reaction is 6.8. Procapsids, isolated from virus-infected cells, also focused at pH 6.8. Since all empty capsid and procapsid species possess similar, if not identical, polypeptide compositions, the different pI's seem to reflect different capsid conformations. Thus, the putative morphopoietic factor in poliovirus-infected cells may not only promote the formation of empty capsids, but may also direct their conformational state (Putnak and Phillips, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, T124, p. 258; manuscript submitted for publication).

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