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RNA Binding Properties of Poliovirus Subviral Particles

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The mechanism of encapsidation of the RNA genome of poliovirus and other picornaviruses is unknown. To test whether any of the putative assembly intermediates of poliovirus could interact directly with the poliovirus RNA genome, poliovirus RNA was attached to magnetic streptavidin beads and incubated with partially purified extracts containing ^{35}S-labeled 14S pentamer and 75S empty-capsid subviral particles from infected cells. The amount of labeled protein bound to the beads was monitored, thus testing the RNA-binding activities of only the labeled viral proteins in the preparations. In this assay, nonspecific RNA-binding activity was displayed by the 14S pentameric particles and mature virions. 75S empty capsids displayed no propensity to associate with RNA. 14S pentamers were demonstrated to form rapidly sedimenting complexes and to undergo a conformational alteration upon RNA binding. These findings are consistent with a direct role for the 14S pentameric particles in RNA packaging during poliovirus morphogenesis.

For poliovirus and other RNA viruses to propagate, the viral assembly process must allow for the selective encapsidation of the viral RNA genome and result in a viral structure that can both protect the genome and allow later release of the genome into newly infected cells. The identity of the viral particle responsible for RNA packaging and the mechanism for specific packaging of the viral genome remain unknown. The current study was undertaken to identify the subviral particle of poliovirus whose physical properties were most consistent with a direct role in RNA packaging.

Poliovirus, a member of the *Picornaviridae* family, consists of a nonenveloped icosahedral capsid shell and a single-stranded positive-sense RNA genome. This 7.5-kb genome is polyadenylated and has a small viral protein, VPg or 3B (38, 52), covalently attached to its 5′ end. To initiate an infection, the virion binds to its cellular receptor (43) and undergoes a physical alteration of its capsid structure that includes the loss of capsid protein VP4 from the viral particle (21, 58) and a subsequent uncoating step that releases the genome from its protective capsid (28, 34). The uncoated RNA genome is translated into a polyprotein that is processed into smaller polypeptides by the virally encoded proteases 2A, 3C, and 3CD (29, 62, 66). The viral RNA is replicated in association with the surface of membranous vesicles that proliferate as a result of infection (10, 11, 15, 20). These vesicles may also be the site of assembly of new viral particles (53).

Although the structure of poliovirus is known in detail (25, 30), comparatively little is understood about the viral assembly process. Mutations resulting in defects in RNA packaging map to the amino terminus of VP1 (3, 34) and to arginine residues in VP1, VP3, and VP4, all located in the interior of the capsid (2). During poliovirus infection, many different subviral particles are assembled from the newly synthesized capsid proteins VP0, VP1, and VP3 (54, 59). The relationship of most of the subviral particles to mature virions has been deduced from their sedimentation behavior, protein composition, and antigenicity as well as their ability to aggregate or disassociate into other subviral particles in vitro. The subviral particles proposed to be intermediates in morphogenesis are protomers, pentamers, empty capsids, and provirions (54, 59). Protomers, particles that sediment at 5S, form after folding of the capsid domain of the polyprotein precursor (48, 49, 59). Pentamers, composed of five protomers and sedimenting at 14S, are stabilized at their fivefold axis by the myristate moiety at the amino terminus of VP0 (17, 25). The pentameric subviral particle has been found to be common to all picornaviruses (54). Twelve 14S pentamers can assemble into the next-higher-order particle, the empty capsid, both in vivo and in vitro (4, 33, 54). The empty-capsid particles sediment at 75S and contain no RNA (40). They accumulate in infected cells treated midinfection with guanidine-HCl to inhibit viral RNA synthesis and can be dissociated in vitro into 14S pentamers (32, 41, 47). The particle thought to be the direct precursor to the virion is the provirion, an icosahedron composed of 60 copies of the polypeptides VP0, VP1, and VP3 and containing the viral genome (18, 24, 28, 55). Cleavage of the VP0 proteins in the provirions would then give rise to the more-stable mature virions. It is not known which of the particles that lack RNA interacts directly with the viral RNA during RNA packaging; the empty capsids and the pentamers have both been suggested (32, 53, 54, 57, 59).

Although RNA encapsidation appears to be specific for poliovirus positive-strand RNA within infected cells, the mechanism of specific packaging has not yet been elucidated. In cells infected with both poliovirus and mengovirus, another picornavirus, no detectable cross-packaging was observed (1). Poliovirus negative strands represent less than 0.0025% of the encapsidated RNA population, although they are present in infected cells at approximately 2% the concentration of the viral positive strands (46). Sequence analysis of the 5′ ends of nucleic acids isolated from purified poliovirus virions has shown that if host RNAs are encapsidated, they represent only 5% or less of the total packaged RNA population (23). Specific RNA sequences that are responsible for the selective encapsidation of poliovirus positive strands have not yet been identified. If a unique packaging site exists, the successful packaging of various subviral RNAs argues that the site does not lie between nucleotides 1225 and 2708 in the type 1 Sabin poliovirus genome (36), between nucleotides 756 and 1805 in the type 3 Leon poliovirus genome (51), or between nucleo-

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tides 1175 and 2956 in the type 1 Mahoney poliovirus genome (46a).

The only traces of the viral RNA that have been resolved in the three-dimensional structure of the poliovirus virion are two purine residues stacked against Trp-38 and Tyr-41 of VP2. To be detected, these nucleotides must be present in a majority of the 60 possible such positions in the interior of the virion (25). Thus, there must be many sequences in the viral genome that interact with these residues. The recently determined three-dimensional structure of poliovirus empty capsids has revealed a trefoil-shaped depression that encircles the threefold axis (8). This feature is highly reminiscent of a structure within bean pod mottle virus that has been shown to complex with 33 nucleotides of RNA (16). The trefoil-shaped depression is expected to be present twenty times in the 75S empty capsids of poliovirus. Barring a large rearrangement of the capsid proteins in the conversion between 14S pentamers and 75S empty capsids, each 14S pentamer should contain five single depressions, each of which would constitute one-third of the complete trefoil. It is not yet known whether the hydrophobic depressions in the 75S empty capsids or in the 14S pentamers are capable of binding RNA.

An in vitro assay in which immobilized RNA molecules select RNA-binding proteins from protein preparations (5, 14) was used to detect interactions between putative viral assembly intermediates and the poliovirus RNA genome. This assay allowed us to determine whether either 14S pentamers or 75S empty capsids isolated from cells infected with type 1 Mahoney poliovirus could bind to poliovirus RNA. We found that the 14S pentamers display nonspecific RNA binding properties in the immobilized RNA assay, form aggregates in the presence of RNA in solution, and undergo a conformational change after RNA binding. The 75S empty capsids, on the other hand, did not display any propensity to bind RNA or to alter their conformation in its presence. A subviral particle capable of packaging the entire poliovirus genome is expected to display nonspecific RNA binding properties, as well as possible sequence-specific binding properties that could confer specificity on the initiation of RNA packaging. Thus, although the observed binding of 14S pentamers to RNA was not observed to be specific for a particular RNA sequence, the data presented here argue that the 14S pentamer is likely to be the assembly intermediate responsible for encapsidation of the poliovirus genome.

MATERIALS AND METHODS

Cells and viruses. Stocks of wild-type Mahoney type 1 poliovirus and VP1-102 were prepared, and HeLa cells were maintained in suspension and plated before viral infection as previously described (35).

Preparation of ^{35}S-labeled empty capsids. Empty capsids were purified by sedimentation of infected HeLa cell lysates through sucrose gradients. For each gradient, monolayers of approximately 2×10^7 HeLa cells were infected with poliovirus at a multiplicity of infection of 50. After 30 min of adsorption, 10 ml of Dulbecco modified Eagle medium (GIBCO/BRL) lacking methionine was added to each 150-mm-diameter plate. The cells were labeled with 0.4 mCi of [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear) at 2 h 45 min postinfection, treated with 2.1 mM (200 μg/ml) guanidine-HCl (pH 7) at 3 h 45 min postinfection, and harvested by scraping at 5 h postinfection. The cells were washed in phosphate-buffered saline (PBS), resuspended in 0.5 ml of PBS, and frozen in a dry-ice–ethanol slurry. The samples were thawed on ice; 0.5 volume of cold PBS that contained 2% Nonidet P-40 (NP-40), 1% deoxycholate 40 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride was added; and incubation on ice was continued for 20 min. The lysates were centrifuged for 10 min at 1,600 × *g* to pellet the nuclei. The supernatants (0.5 ml per gradient) were layered onto 11-ml linear gradients of 15 to 30% sucrose solutions prepared in a buffer of PBS and 20 mM EDTA. The gradients were centrifuged at 31,000 rpm for 4 h at 4°C in an SW41 rotor (Beckman). Fractions (0.5 ml each) were collected from the bottom of the gradient and assayed for radioactivity by liquid scintillation counting. The fractions were frozen on dry ice and stored at −70°C. To purify the empty capsids further, 0.5 ml of the pooled peak was diluted with 0.5 ml of reticulocyte standard buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$) and 1.0 ml of lysing solution (PBS, 40 mM EDTA, 2% NP-40, 1% deoxycholate, 2 mM phenylmethylsulfonyl fluoride) and again subjected to centrifugation through 15 to 30% sucrose gradients as above. All manipulations of particle samples were done at 4°C, and all plasticware used with purified empty-capsid fractions was siliconized.

Preparation of ^{35}S-labeled 14S pentamers. A variation of the protocol for alkali dissociation of 75S empty capsids was used (41, 47). Aliquots (0.5 ml each) of pooled ^{35}S-labeled empty capsids were diluted with 0.5 ml of reticulocyte standard buffer. The pH of the sample was then increased to between 8.3 and 8.5 by the addition of 1.0 M Tris-HCl, pH 8.8. After a 30-min incubation on ice, the pH was adjusted to 7.5 by the addition of 0.2 M HCl and the sample was diluted 1:1 with lysing solution. The samples were frozen on dry ice, stored at −20°C for at least 24 h, thawed on ice, and subjected to centrifugation through linear gradients of 5 to 20% sucrose solutions prepared in a buffer of PBS and 20 mM EDTA for 20 h in an SW41 rotor at 27,500 rpm at 4°C. Fractions were collected, pooled, and stored as above.

Preparation of ^{35}S-labeled virions. Virions were purified by cesium chloride density gradient centrifugation as previously described (7). HeLa cell monolayers were infected and labeled with [^{35}S]methionine and [^{35}S]cysteine in the same manner as for the preparation of empty capsids.

RNAs. Poliovirus virion RNA was labeled with [^{3}H]uridine during infection of HeLa cells with wild-type poliovirus and prepared from virions purified on a CsCl gradient as previously described (7). The RNA concentration was determined by spectrophotometry. Transcripts of full-length poliovirus positive strands and negative strands were synthesized in vitro by T7 RNA polymerase from T7pGempolio (60) or T7oilop plasmid DNA, respectively. T7oilop contains the poliovirus cDNA inserted in reverse orientation in front of a T7 promoter so that a full-length negative-strand transcript can be synthesized in vitro. T7pGempolio was digested with *Eco*RI and T7oilop was digested with *Sac*I before the transcription reactions. Labeled and unlabeled nucleotides were removed by three ammonium acetate-ethanol precipitations or by centrifugation through a spin column (5 Prime→3 Prime). The amount of RNA synthesized was determined either from the amount of incorporation of radiolabeled nucleotides into the transcript or by spectrophotometry. The in vitro-made RNAs were synthesized in the presence of [^{3}H]UTP (Amersham) to a final specific activity of approximately 2×10^{-8} μCi/mol. Poly(C) (Pharmacia) had an average length of 500 bases and was radioactively labeled with [^{3}H]CTP (NEN) by extending the poly(C) with SP6 polymerase. All RNAs were labeled to a low specific activity to avoid interference in the ^{35}S channel when the double labeling was analyzed by scintillation counting.

All RNAs were photobiotinylated by incubating with photobiotin (biotin coupled to a photoreactive aryl azide group; Vector) in the presence of strong visible light, according to the Photobiotin Labeling System protocol (Bethesda Research Laboratories). Amounts of RNA (8 μg) and photobiotin (1.5 μg) that resulted in the covalent addition of approximately three photobiotin moieties per 1,000 nucleotides were used (27, 61). The amount of RNA bound to streptavidin beads was determined by scintillation counting.

Quantitation and analysis of proteins. To determine which proteins were ^{35}S labeled in the preparations of subviral particles, fractions from sucrose gradient sedimentation were trichloroacetic acid precipitated and visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37). Capsid proteins VP0, VP1, VP3, and VP2 were identified by using ^{35}S-labeled poliovirus virions as markers on some gels. To detect unlabeled proteins, Coomassie blue or silver staining of polyacrylamide gels was performed as described elsewhere (13). The concentration of 14S pentamers in the preparation used for the experiments in Fig. 3 and 8 was approximated from the amount of Coomassie blue staining in viral proteins relative to standards of known concentration; the extent of staining by Coomassie blue is proportional to the amount of most proteins (65).

Particle capture assay. Streptavidin-coupled magnetic beads (25 μl per binding reaction; Dynal) were prepared by washing three times in RNase-free PSE (PBS, 5% sucrose, 20 mM EDTA [pH 6.5]), resuspending in PSE, and incubating in the presence or absence of photobiotinylated RNA for 25 min at 4°C. The beads were collected by using a magnetized rack (Dynal). To block the sites of nonspecific binding of subviral particles to the surface of the magnetic beads, the beads were then incubated with solutions containing gelatin and NP-40 as well as the subviral-particle preparations. Unless otherwise indicated, the beads with or without bound RNA were incubated with a solution containing PSE, 23 mM $ZnSO_4$, 0.05% NP-40, 0.001% gelatin (pH 4.5), and either ^{35}S-labeled 14S pentamer or empty-capsid preparations in a total volume of 100 μl. Interactions between 14S pentamers and RNA were, in some preparations, dependent on the addition of exogenous Zn^{2+} (data not shown). The variability of this requirement between preparations possibly reflects a variable final concentration of this divalent cation in the 14S pentamer preparations. After this variability was observed, Zn^{2+} in a molar excess over EDTA was included routinely in the 14S pentamer-RNA binding reactions. The binding reactions were incubated for 30 min with occasional gentle mixing, and the magnetic beads and the supernatants were subsequently collected. The beads were then washed twice in binding buffer, and the wash supernatants were reserved. The washed

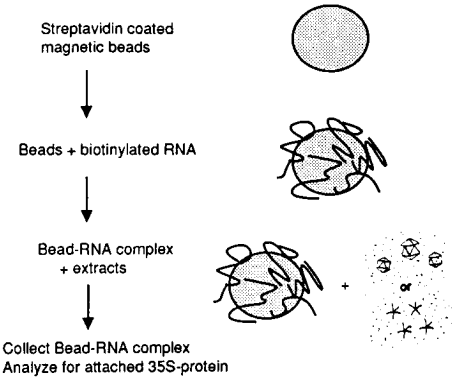

FIG. 1. Particle capture assay to monitor the binding of ^{35}S-labeled subviral particles to RNA. Full-length positive-strand poliovirus RNA was synthesized in vitro, modified by photobiotinylation, and bound to streptavidin-coated magnetic beads. The association of labeled proteins with the magnetic beads was then used to monitor the RNA binding properties of ^{35}S-labeled subviral particles. Any RNA binding properties of the unlabeled cellular proteins also present in the preparations of subviral particles were thus not detected.

beads were then resuspended in PSE, and the amounts of radioactivity in the bead-bound and supernatant (wash) fractions were determined by scintillation counting. All binding experiments were performed at 4°C.

Sucrose gradient analysis of particle integrity. The particles were incubated either at pH 4.5 in PBS–20 mM EDTA–23 mM $ZnSO_4$–2.5% sucrose–0.1% NP-40 (the buffer used in most RNA binding assays) or at pH 7.0 in PBS–20 mM EDTA–2.5% sucrose–0.1% NP-40 (the buffer used for sucrose gradient sedimentation) for 30 min at 4°C. The following sedimentation conditions were used for the analysis of the particles by sucrose gradient centrifugation. All gradients were composed of 11 ml of various percentages of sucrose in PBS with 20 mM EDTA and were centrifuged in an SW41 rotor at 31,000 rpm for 4 h at 4°C. The 14S pentamers were sedimented on linear 5 to 20% sucrose gradients, the 75S empty capsids were sedimented on linear 5 to 30% sucrose gradients, and the virions were sedimented on linear 5 to 40% sucrose gradients.

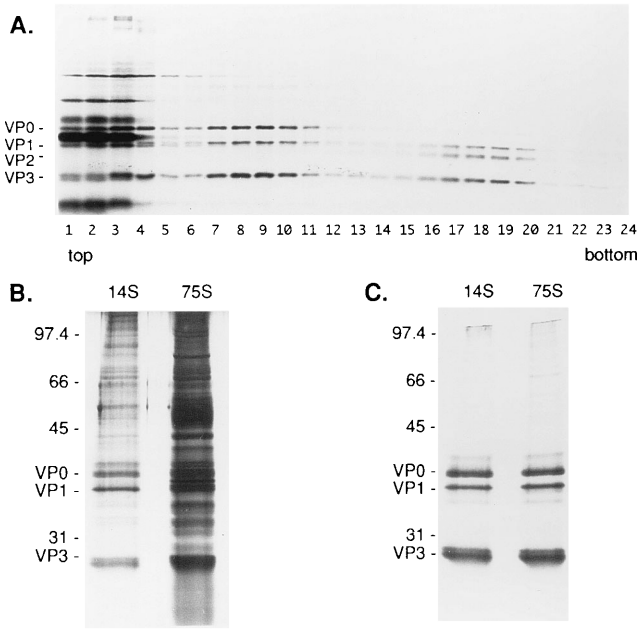

FIG. 2. Preparations of 14S pentamers and 75S empty capsids used in binding experiments. (A) Autoradiogram of aliquots (25 μl each) from 0.5-ml fractions across a 15 to 30% sucrose gradient used for the preparation of empty capsids. The 75S empty-capsid peak is in fractions 7 to 10. These peak fractions were pooled and used to prepare 14S pentamer and purer 75S empty-capsid preparations. (B) The protein composition of trichloroacetic acid-precipitated fractions of 14S pentamers and 75S empty capsids (after sedimentation through two sucrose gradients) was visualized by SDS-PAGE and silver staining. (C) Autoradiogram of the gel in panel B to visualize the ^{35}S-labeled proteins in the 14S pentamer and 75S empty-capsid preparations.

RESULTS

Particle capture assay to determine the RNA binding properties of poliovirus subviral particles. Two types of subviral particles, 75S empty capsids and 14S pentamers, have been suggested as candidates for the particles that directly package poliovirus RNA during viral assembly (32, 53, 54, 57, 59). To detect associations between RNA molecules and these putative assembly intermediates, we employed a strategy that utilizes radiochemically pure subviral particles from poliovirus-infected cells. Soon after infection with poliovirus, translation of the vast majority of host proteins is inhibited (22), and poliovirus protein synthesis proceeds by a cap-independent mechanism (50). Thus, viral proteins are preferentially labeled after poliovirus infection, and it is straightforward to obtain fractions of subviral particles in which poliovirus capsid proteins are the only labeled species. However, these labeled proteins still represent only a fraction of the total protein in such preparations, and the unlabeled proteins can provide background in assays that detect the binding of radioactive RNA molecules to the total proteins in a preparation.

To circumvent this problem, immobilized RNA was used to capture labeled subviral particles from a biochemically heterogeneous solution (Fig. 1). Full-length positive-strand poliovirus RNAs were transcribed in vitro and modified by photobiotinylation. These biotinylated RNAs were incubated with magnetic streptavidin-containing beads, and unbound RNAs were removed by collecting the beads and removing the supernatant. The RNA-bound beads were then incubated with ^{35}S-labeled preparations of subviral particles from poliovirus-infected cells. The amounts of ^{35}S-labeled protein in the supernatant, wash, and bead-bound fractions were measured and used to calculate the amount of bound subviral particles under the conditions of the assay.

Radiochemically labeled 75S empty capsids (40, 59) were prepared by infecting HeLa cells with poliovirus and labeling with [^{35}S]methionine and [^{35}S]cysteine at a time postinfection when host protein synthesis was inhibited. Guanidine, an inhibitor of poliovirus RNA synthesis, was added to increase the concentration of labeled subviral particles that lack RNA (32). Labeled cellular lysates were fractionated by sedimentation in a 15 to 30% sucrose gradient as shown in Fig. 2A; the 75S empty-capsid peak containing poliovirus proteins VP0, VP1, and VP3 could be clearly distinguished from the mature-capsid peak containing VP1, VP2, and VP3; VP4 could not be seen under the electrophoretic conditions used. The pooled empty-capsid-containing fractions from such gradients were further purified by sedimentation through a second 15 to 30% sucrose gradient. The ^{35}S-labeled 14S pentamers were prepared by dissociation of the purified empty capsids followed by sedimentation through 5 to 20% sucrose gradients and collection of the appropriate fractions.

The total protein contents of the 75S empty-capsid and 14S pentamer preparations are shown in Fig. 2B. As expected, these fractions contained many cellular proteins in addition to viral capsid proteins. However, Fig. 2C shows the radiochemical purities of the 75S empty-capsid and 14S pentamer preparations; the ^{35}S-labeled proteins present were almost exclusively the viral capsid proteins VP0, VP1, and VP3. The viral capsid proteins in these 14S pentamer and 75S empty-capsid

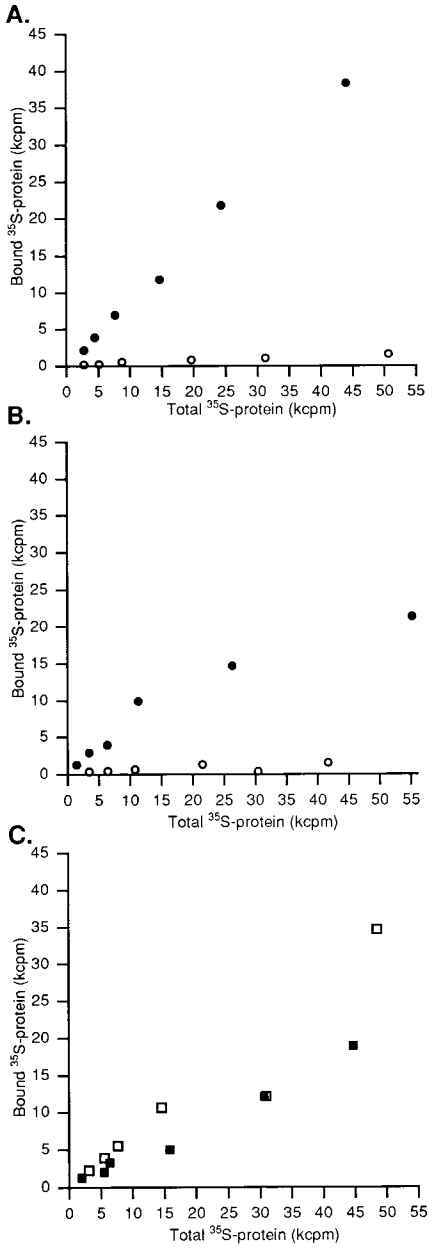

FIG. 3. Association of 14S pentamers (A and B) and empty capsids (C) with immobilized RNA in particle capture assay. Streptavidin beads with or without 68 ng (A and C) or 8 ng (B) of biotinylated positive-strand poliovirus RNA attached were incubated with increasing amounts of preparations of ^{35}S-labeled subviral particles. The amount of radioactivity retained on the magnetic beads is shown for pentamers (○, beads without RNA; ●, beads with RNA) and for empty capsids (□, beads without RNA; ■, beads with RNA).

preparations were estimated to compose 10 to 30% of the total proteins.

Pentamers, but not empty capsids, bind to immobilized RNA. To determine if either 14S pentamers or 75S empty capsids bind to poliovirus RNA, we incubated increasing concentrations of the labeled subviral particle preparations with full-length poliovirus RNA transcripts immobilized on magnetic streptavidin beads. As shown in Fig. 3A, when increasing amounts of a ^{35}S-labeled 14S pentamer-containing preparation were incubated with magnetic streptavidin beads to which 68 ng of poliovirus RNA had been immobilized, increasing amounts of ^{35}S-labeled protein bound to the beads. The amount of labeled 14S pentamers bound to the RNA-containing beads was much greater than the amount bound to the beads in the absence of RNA at every concentration of protein tested. Thus, the RNA was responsible for the increased binding of the labeled 14S pentamers with increasing extract concentrations.

When a smaller amount of RNA (8 ng) was immobilized on the beads, the binding of 14S particles (Fig. 3B) was observed to saturate over the same range of extract concentrations as that in Fig. 3A. Saturation occurred at a protein/RNA ratio that corresponded to 3 to 10 pentamers bound to each 7,500-nucleotide RNA molecule.

In the case of the empty capsids, however, the amount of ^{35}S-labeled protein associated with the magnetic beads was higher in the absence of RNA than in the presence of RNA at every protein concentration tested. The reason for the nonspecific binding of the 75S particles to the magnetic beads is not known. Under many different experimental conditions, including those in which the nonspecific binding of 75S empty capsids to the magnetic beads was reduced (Fig. 4B), the presence of RNA on the beads did not lead to an increase in the association of empty capsids. Therefore, there was no indication that the 75S particles bound to the immobilized RNA, and the presence of the RNA seemed to interfere with the nonspecific binding of the 75S empty capsids to the beads.

The RNA-mediated association of the ^{35}S-labeled 14S pentamers with beads was found to be pH dependent, occurring only below pH 5.5 (Fig. 4A). The empty capsids did not exhibit RNA-mediated binding at any pH tested (Fig. 4B and data not shown). Since the outer surfaces of virions and 75S empty capsids are structurally nearly identical (8), we tested whether labeled virions could bind to immobilized RNA in this assay (Fig. 4C). Surprisingly, virions were observed to display RNA-mediated binding to the beads. Although association of the virions with RNA was not dependent on pH over the same pH range as 14S pentamer binding to RNA (Fig. 4C), virions were not observed to bind to RNA above pH 6.0 (data not shown). Virions did not display the high nonspecific binding to beads shown by 75S empty-capsid preparations.

14S pentamers display RNA-dependent changes in sedimentation behavior. Since the conditions of the particle capture assay are different from those under which the particles are isolated, it was important to test the integrity of the particles under the conditions of the assay. ^{35}S-labeled pentamers, empty capsids, and virions were incubated for 30 min at 4°C in binding buffer under either the pH 4.5 RNA binding conditions or the pH 7.0 isolation conditions and subsequently sedimented through sucrose gradients (Fig. 5). To test the effect of RNA upon the sedimentation behavior of these particles, preparations of each particle were incubated with 6 μg of poliovirus positive-strand RNA, transcribed in vitro, under both RNA binding conditions and isolation conditions (Fig. 5).

Pentameric subviral particles did not display an alteration in their sedimentation properties after incubation at pH 4.5 in the absence of RNA (Fig. 5A) or at pH 7 in the presence or absence of RNA (Fig. 5B). However, in the presence of RNA at pH 4.5, the conditions under which RNA binding was observed, the sedimentation of 14S pentamers was altered in two potentially interesting ways; both faster-sedimenting and more slowly sedimenting species were seen (Fig. 5A). The faster-sedimenting species toward the bottom of the gradient is quite likely to be a complex between the poliovirus RNA and the 14S pentamers. The presence of the slower-sedimenting

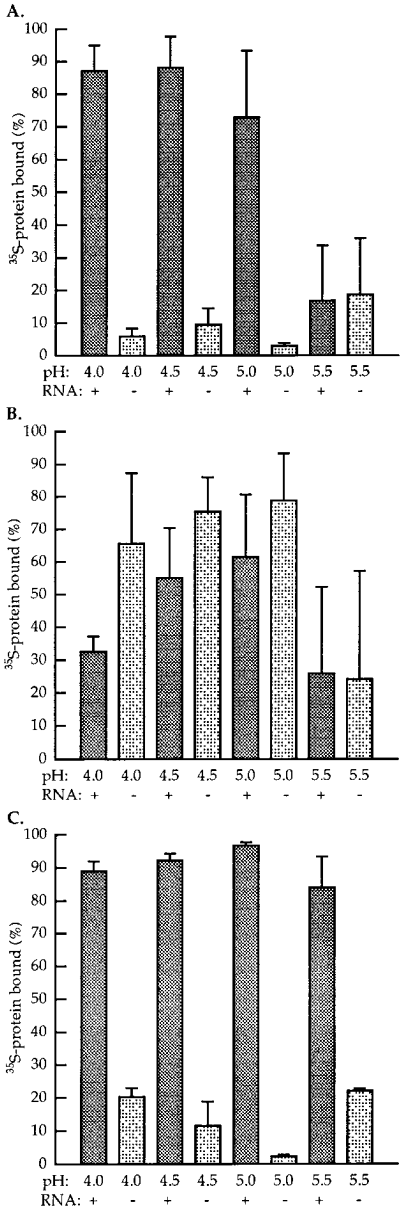

FIG. 4. pH dependence of RNA binding of pentamers, empty capsids, and virions. The percentages of ^{35}S-labeled protein in preparations of pentamers (A), empty capsids (B), and virions (C) that bound to the beads in the presence and absence of RNA under different pH conditions are shown. The data are averages and standard deviations for duplicates.

species suggests that RNA binding caused a conformational change in the 14S pentamer structure; this possibility will be addressed further below.

The empty capsids, on the other hand, displayed altered sedimentation behavior under the pH 4.5 conditions but not under the pH 7 isolation conditions. The partial dissociation of the 75S particles under conditions of the RNA binding assay may be responsible for the high nonspecific binding of these particles to magnetic beads (Fig. 2C and 3B). The presence of RNA had no discernible effect on the sedimentation of the particles under either set of conditions tested, in agreement with the apparent lack of interaction with RNA in the particle capture assay (Fig. 2C and 3B).

The sedimentation of the virions remained unchanged following incubation under all conditions tested, as one might expect for an infectious virus that must survive the extreme conditions of the alimentary tract. Despite the RNA binding properties of virions observed in the particle capture assay (Fig. 3C), the presence of RNA had no affect on their sedimentation behavior.

To investigate further the state of the nominally 14S pentamers after RNA binding, the particle capture assay was used to isolate ^{35}S-labeled material derived from 14S pentamer preparations that was bound to RNA (Fig. 6). The ^{35}S-labeled pentamers were incubated with RNA bound to magnetic beads, the unbound proteins were removed, and the bead-bound material was released by digestion with RNases at 4°C. The supernatant from the RNase digestion was sedimented through a linear 5 to 20% sucrose gradient for 20 h to resolve the 14S pentamers from the more slowly sedimenting species in Fig. 5A. As a control, ^{35}S-labeled pentamer preparations were incubated with beads that lacked RNA, treated with RNases, and subjected to sedimentation through identical sucrose gradients. The ^{35}S-labeled protein released from the formerly RNA-coated beads was quantitatively converted from the 14S pentamers to material that sedimented similarly to 5S protomers, whereas only a small amount of this conversion occurred in the presence of the control beads that lacked RNA (Fig. 6). When the proteins constituting these particles were released from the beads by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE, the capsid proteins were intact and VP0 was not cleaved (data not shown). Thus, it seems that pentamers undergo a conformational change upon RNA binding. In the presence of RNA, a rapidly sedimenting species is formed. When the RNA is removed, the pentamers no longer sediment as 14S pentamers but instead as much smaller particles.

A mutation in the amino terminus of VP1 does not interfere with RNA binding. The three-dimensional structure of poliovirus has shown that the amino termini of VP1, VP2, and VP3, as well as the entire VP4 polypeptide, are located in the interior of the virion, potentially interacting with the viral RNA (30). A few poliovirus mutants whose mutations map to these regions and which display defects in RNA packaging have been characterized (2, 3, 34). One of these mutants, VP1-102, bears a deletion of the first four amino acids of VP1. At elevated temperatures in both HeLa and CV1 cells, VP1-102-infected cells accumulate 14S and 75S particles at the expense of the larger, RNA-containing provirion and virion particles, indicating a specific block in RNA packaging. Complementation and dominance experiments showed that this block resulted from a defect in the mutant protein rather than the mutant RNA (34). To test whether this mutation affected the RNA binding properties of 14S pentamers in the particle capture assay, ^{35}S-labeled 14S pentamers were prepared from cells infected with VP1-102 at a restrictive temperature. The RNA binding properties of 14S pentamers derived from VP1-102 virus were compared with those of wild-type 14S pentamers in the particle capture assay (Fig. 7). No difference between the RNA binding of mutant particles and that of wild-type particles was observed. Therefore, the RNA binding properties of the 14S pentamers in the particle binding assay are conferred by additional determinants besides the wild-type amino terminus of VP1. It will be interesting to test additional poliovirus mutants with phenotypic defects in RNA packaging for altered RNA binding properties in this assay.

Binding of pentamers to immobilized RNA is nonspecific for RNA sequence. To test for any specificity of the interactions of 14S particles with poliovirus positive-strand RNA, we investi-

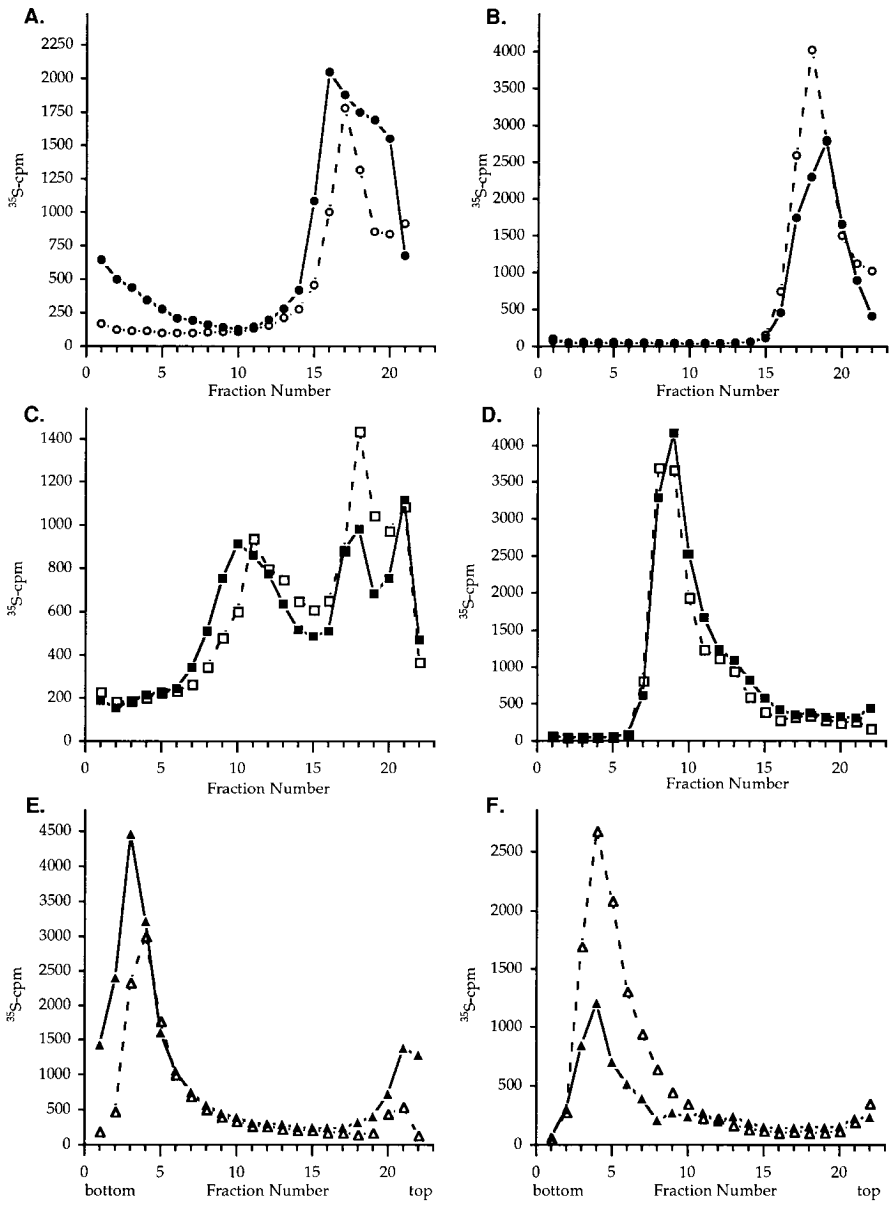

FIG. 5. Sucrose gradient centrifugation of ^{35}S-labeled particles after incubation with and without RNA at pH 4.5 and 7.0. (A) Pentamers incubated at pH 4.5 under RNA binding conditions with RNA (●) or without RNA (○); (B) pentamers incubated at pH 7.0 under isolation conditions with RNA (●) or without RNA (○); (C) empty capsids incubated under pH 4.5 conditions with RNA (■) or without RNA (□); (D) empty capsids incubated under pH 7.0 conditions with RNA (■) or without RNA (□); (E) virions incubated under pH 4.5 conditions with RNA (▲) or without RNA (△); (F) virions incubated under pH 7.0 conditions with RNA (▲) or without RNA (△).

gated the binding of labeled 14S pentamers to other RNA molecules. Increasing amounts of full-length, positive-sense poliovirus RNA, made in vitro, were immobilized onto beads and incubated with a fixed amount of ^{35}S-labeled 14S pentamers. Figure 8A shows that, at low RNA concentrations, the percentage of ^{35}S-labeled 14S pentamer bound increased with increasing amounts of immobilized RNA. In this experiment, this binding became saturated when approximately 10 ng of RNA was present on the magnetic streptavidin beads. Approximately 3 to 7 ng of 14S pentamers was present in these binding assays (see Materials and Methods), resulting in an estimated binding stoichiometry of two to five pentamers per 7,500-nucleotide poliovirus RNA molecule on the beads. This stoichiometry was comparable to that observed when 14S pentamer binding was titrated as a function of protein concentration (Fig. 3B).

Figure 8B shows the binding curves of two other poliovirus-derived RNA molecules of the same size as the full-length poliovirus transcript. Poliovirus RNA prepared from isolated virions is nearly identical to the in vitro RNA transcripts (60) but also contains VPg, a 22-amino-acid protein, covalently linked to its 5′ end and a longer stretch of poly(A) at its 3′ terminus. The virion RNA showed 14S binding properties similar to those of the positive-sense transcript in the particle capture assay; the same amount of binding at low RNA concentrations and the same saturation level were observed. Similarly, a transcript corresponding to sequences of the poliovirus negative strand gave rise to a binding curve super-

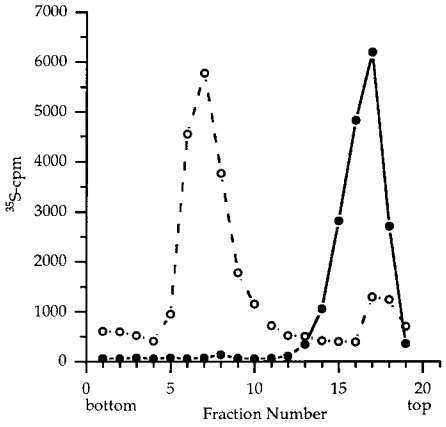

FIG. 6. Sucrose gradient centrifugation of nominally 14S pentamers after removal from RNA on magnetic beads. ^{35}S-labeled pentamer preparations were incubated with RNA-coated beads (●), the unbound supernatant was removed, and the beads were treated with RNase A and RNase T_1 to remove the bound protein. The material liberated by RNase digestion was sedimented through linear 5 to 20% sucrose gradients in PBS containing 20 mM EDTA at 27,500 rpm for 20 h in an SW41 rotor. ^{35}S-labeled pentamer preparations were also incubated with beads that did not contain immobilized RNA (○), and then RNase A and RNase T_1 were added to the incubation mixture. The supernatant from the beads was then removed and centrifuged as above.

imposable on those of the positive-strand RNAs. In addition, 14S pentamers bound to poly(C) with an average length of 500 bases in a manner indistinguishable from the heteropolymeric poliovirus sequences (Fig. 8B). Thus, in the particle capture assay, 14S pentamers bound to long single-stranded RNA molecules without apparent sequence specificity.

DISCUSSION

Two competing hypotheses for the assembly pathway have emerged from physical studies of subviral particles present in poliovirus-infected cells. One hypothesis (32), that viral RNA is threaded into intact 75S empty capsids after their assembly, is analogous to the known mechanism for packaging DNA into

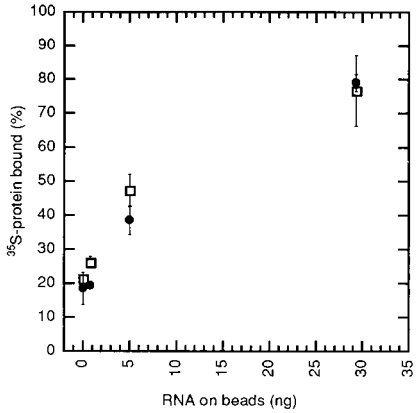

FIG. 7. Particle capture of pentamers prepared from wild-type and packaging-defective VP1-102-infected cells. The percentages of ^{35}S-labeled protein in wild-type (●) and VP1-102 (□) 14S pentamer preparations that bound to streptavidin magnetic beads are shown. Increasing amounts of full-length positive-strand poliovirus RNA were immobilized on the beads and incubated with a constant amount of 14S extract. Unlabeled extracts of infected cells were used as the blocking agents in this experiment. The 14S pentamer preparations from wild-type and VP1-102-infected cells were made under the same labeling conditions and had comparable specific activities.

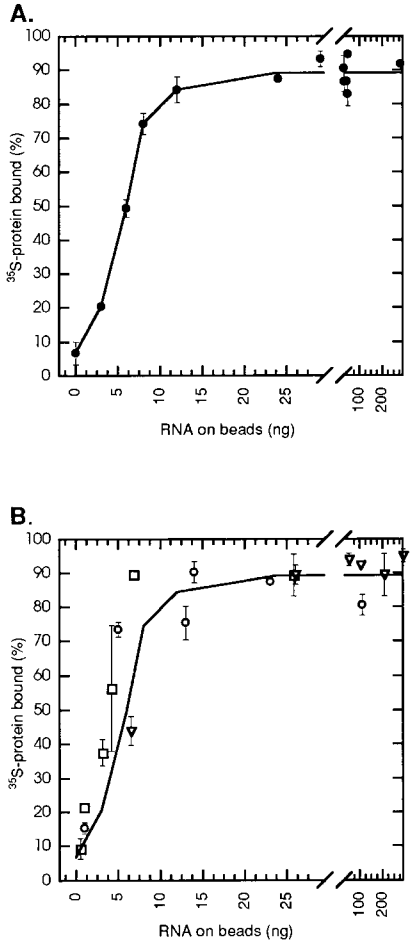

FIG. 8. Particle capture of 14S pentamers by different RNAs. (A) Various amounts of full-length poliovirus RNA, transcribed in vitro, were bound to beads and incubated with a constant amount of a ^{35}S-labeled 14S preparation. The data are percentages of ^{35}S-labeled protein in the 14S preparations that bound to the beads. (B) The percentages of ^{35}S-labeled protein in 14S preparations bound to various amounts of immobilized RNA extracted from poliovirus virions (○), full-length negative-strand poliovirus RNA made in vitro (□), and poly(C) RNA (▽) are shown. The binding curve of full-length positive-strand RNA made in vitro from panel A is shown for comparison. Approximately 3 to 7 ng of capsid proteins was present in each binding assay.

many bacteriophages (12, 42) as well as into parvoviruses (19). For poliovirus, this hypothesis was prompted by data showing that radioactivity accumulated in empty capsids during guanidine treatment could be chased into mature virus particles upon release of the guanidine block (32). However, since empty capsids can disassemble into 14S pentameric particles (41, 47), the empty capsids may or may not have been direct precursors to virions in these experiments.

An alternative hypothesis is that 14S pentameric particles are responsible for packaging the RNA genome and that capsid assembly and RNA packaging occur simultaneously. 14S pentameric particles, unlike empty capsids, can be isolated from cells infected with any known picornavirus (59). Radioactivity accumulated in 14S pentamers during poliovirus infection at a low temperature was shown to chase into mature virus particles upon raising of the temperature (57). However, neither of these observations precludes the possibility that morphogenesis proceeds through a short-lived empty-capsid intermediate. Initiating the assembly of an RNA virus by a

small subviral particle has precedent in bacteriophage R17 (which initiates assembly with dimers of the phage coat protein [9]), turnip crinkle virus (whose assembly is initiated by three coat protein dimers [64]), and tobacco mosaic virus (whose assembly is initiated by a 20S aggregate of the capsid protein [63]).

In the work reported here, we have used a particle capture assay to test the RNA binding properties of the two subviral particles of poliovirus proposed to be involved in viral assembly and of intact virions. We demonstrate that 14S pentamers and intact virions, but not 75S empty capsids, can bind to RNA and that the 14S pentamers undergo an alteration of their conformation upon RNA binding. These data suggest that the pentamer, or possibly the protomer, is the subviral particle that initiates encapsidation of the viral genome. This is the first report of an RNA binding activity for a subviral particle of poliovirus as well as for the mature poliovirus virion.

In the assembly of an infectious virus particle, the viral genome must first be recognized and then the entire genome must be encapsidated. This initial recognition of the viral genome may be accompanied by a conformational alteration in the RNA-associated capsid proteins which allows additional capsid protein subunits to be added efficiently. We have shown that 14S pentameric particles display nonspecific RNA binding properties in our assay. In the presence of RNA, 14S particles form more rapidly sedimenting aggregates and undergo a conformational change that can be detected when the RNA is removed by treatment with RNases. Empty capsids did not display an ability to bind RNA or to alter their conformation in its presence. The recently determined structure of 75S empty capsids has revealed a trefoil-shaped depression, centered around each threefold axis on the interior of the icosahedral structure, that is hypothesized to be an RNA binding site (8). Binding of RNA to the portions of the suggested RNA binding site present in the 14S pentamers could bring three 14S pentamers into proximity to assemble the three-lobe trefoil structure. It is possible that the new interactions formed upon RNA binding could disrupt the interactions that had previously stabilized the 14S pentamers, which would now require the continued presence of RNA for stability.

The low pH requirement of RNA binding by the pentamers may indicate that modulation of the particle charge or conformation is necessary to reveal their capacity to bind RNA. Assembly of poliovirus virions in vivo may be associated with host constituents such as heat shock protein 70 (39) and the membranous vesicles on which the viral RNA replication complex assembles (53). The effect of cellular proteins or structures on either the conformation of 14S pentamers or the pH of their local environment is not known.

The observed binding of intact virions to RNA was unexpected, given the similarity in the structure and antigenicity of the external surfaces of 75S empty capsids and virions (8, 31, 56). Furthermore, RNA binding by virions seems unlikely to be related to viral assembly. However, several conformational changes that lead to loss of infectivity and RNA release during the entry of polioviruses into cells have been observed for intact virions but not for 75S empty capsids (reviewed in reference 26). It is possible that the ability of intact virions to bind RNA reflects a conformational change involved in RNA release during cell entry; this possibility remains to be investigated.

Poliovirus virions purified from infected cells contain very small amounts, if any, of host or negative-strand RNA (23, 46), suggesting that the encapsidation process is selective for poliovirus positive strands. VPg, attached to the 5′ end of the encapsidated positive strands, cannot be the sole packaging determinant, because it is also attached to the 5′ end of viral negative strands (45, 52). In the particle capture assay, 14S pentamers did not discriminate between RNA substrates as might have been expected if there were a 14S pentamer binding site with an extremely high affinity on the poliovirus positive strand. In addition, the recognition of poly(C) by 14S pentamers suggests that, under the conditions of the assay, the RNA-pentamer interaction is dependent on neither the sequence nor the structure of the RNA. However, the RNAs tested in this study were quite long. Given the demonstrated nonspecific affinity of 14S pentamers for RNA, it is very possible that a high-affinity RNA sequence or structure would not have been detected when imbedded in these long RNA molecules. Shorter regions of the poliovirus RNA genome will be tested individually in the particle capture assay to search for RNA sequences or structures with increased affinity for the 14S pentamers.

If poliovirus contains specific packaging sites, they are not sufficient for packaging the genome. During poliovirus infection, only newly synthesized positive strands are packaged; previously synthesized, complete positive strands present in the cytoplasm following inhibition of RNA synthesis could not be chased into mature particles (6). Therefore, at least some of the observed specificity of poliovirus packaging results from coupling between poliovirus replication and packaging. Circumstantial evidence for a link between RNA synthesis and packaging was provided by Pfister et al. (53), who showed by immunoelectron microscopy that antigenic determinants characteristic of 14S pentamers, but not of empty capsids, associate with poliovirus replication complexes. The association suggests that 14S subviral particles bind to the replication complex or to associated cellular structures. A recent report that purified mature virions of foot-and-mouth disease virus, another picornavirus, often remain associated with the viral RNA-dependent RNA polymerase (44) argues that the interaction between capsid structures and the replication complex may be direct. Thus, some or all of the specificity of poliovirus RNA packaging might be generated through the colocalization of replication and assembly, with the 14S pentameric subviral particles localized near the replication complex in position to bind newly synthesized positive-strand RNA on the pathway to viral assembly.

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