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In Vitro Synthesis and Assembly of Picornaviral Capsid Intermediate Structures

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Cell-free translation of encephalomyocarditis RNA in extracts of rabbit reticulocytes results in the synthesis of viral proteins indistinguishable from those produced during virus infection of cells. The viral capsid proteins are produced in an active form capable of assembly into viral capsid intermediate structures. Protomers (5S), pentamers (14S), and shell-like structures (75 to 85S) can be detected after prolonged incubation in the extracts. Proteolytic cleavage of capsid precursor proteins appears to be a prerequisite for assembly, in apparent contrast to cell-associated assembly. Assembly of pentamers is also preceded by conversion of protein ε1 to ε in a step which may reflect an amino-terminal blocking reaction.

Picorna virions contain a single messenger sense RNA genome (molecular weight, 2.6 × 10^6) encapsidated by 60 protein subunits, each composed of four nonidentical polypeptide chains (β, δ, γ, and α) (20). Viral capsid and noncapsid proteins are derived by sequential cleavage of a large polyprotein precursor molecule, which represents nearly the entire coding capacity of the RNA (5). Figure 1 illustrates the processing scheme of encephalomyocarditis (EMC) polyprotein.

The process by which capsid proteins or their precursors assemble into virions is not well understood. Several subviral particles thought to be intermediates in capsid morphogenesis have been isolated from cytoplasmic extracts of infected cells. Of these, three structures identified by their sedimentation characteristics (5S, 13 to 14S, 70 to 80S) are most commonly implicated in virion formation (3, 7, 9, 20, 22).

Protomers (5S), the smallest assembly subunits, contain one copy each of ε, γ, and α or their cleavage precursors (e.g., D1 + α or A) (9). Pentamers (13 to 14S) contain five protomers and represent one-twelfth of an empty capsid (70 to 80S) or virion shell (15, 20). The process by which RNA is inserted into capsids is unknown. Also unclear is the role of the various proteolytic steps on the assembly scheme.

A viral protease, protein p22, is responsible for capsid maturation cleavages of protein A1 (to ε,γ,α), as well as self-cleavage reactions within the protease precursor molecules, proteins C and D (10, 11, 21). The agents which catalyze the initial cleavages of the polyprotein (to A1,F,C) and the final cleavage of ε (to δ,β) have yet to be identified. This latter cleavage is observed only during the last stages of viral morphogenesis and is presumably dependent upon RNA encapsidation (4).

Cell-free translation of EMC RNA in extracts of rabbit reticulocytes results in the synthesis of proteins indistinguishable from those produced during viral infection of cells (25). Protease p22 is synthesized in an enzymatically active form capable of catalyzing stepwise cleavage reactions directly analogous to those observed in vivo (12, 24, 25).

In view of the efficiency and fidelity of the cell-free processing, it was of interest to determine whether the capsid proteins produced by these cleavages could also exhibit activity and, like their in vivo counterparts, be capable of assembly into capsid or capsid-like structures. We now report the in vitro formation of 5S, 14S, and 75 to 85S EMC capsid intermediates and have determined that cleavage of A1 to ε,γ,α precedes assembly of protomers into pentamers.

MATERIALS AND METHODS

Synthesis of viral proteins. Translation of EMC viral RNA in extracts of rabbit reticulocytes was carried out as previously described (10, 13, 24). The standard protein synthesis mixture contained about 90 μM each of 19 unlabeled amino acids and about 1 μM [^{35}S]methionine (1,000 to 1,200 Ci/mmol). Incubation at 30°C was from 3 to 15 h as indicated. Reactions were terminated by addition of pancreatic RNase to a concentration of 100 μg/ml followed by incubation at 30°C for 30 min.

Separation of 14S complexes. Samples of translated lysates (60 μl) prepared as described above were

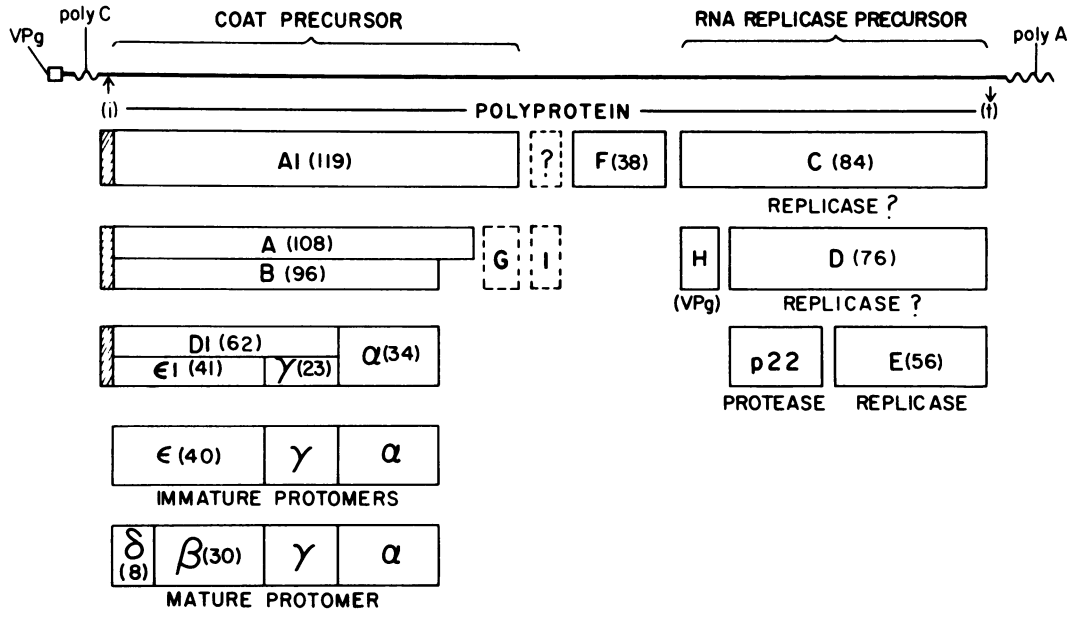

FIG. 1. EMC protein processing scheme. A proteolytic cleavage map of EMC polyprotein has been modified from reference 21. The numbers in parentheses represent molecular weights ($\times 10^3$) as approximated by gel electrophoresis (23). The map positions of proteins in dashed boxes (---) are unclear. This representation is not to scale.

diluted with 1 volume of buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 6.9]–200 mM potassium chloride–5 mM magnesium chloride–5 mM β-mercaptoethanol) and then layered onto 5 to 20% sucrose gradients (12.2 ml) prepared in the same buffer. Centrifugation was carried out in an SW41 rotor at 38,000 rpm for 15 h at 4°C. Gradients were collected by puncturing the bottom of the tube and collecting dropwise into fractions. Polioviral 14S pentamers labeled with [^{3}H]leucine were prepared as described previously (3) and used as sedimentation markers.

Separation of 75 to 85S complexes. Translation mixtures (120 μl) prepared as described above (12 to 15 h incubations) were diluted to 1 ml by addition of buffer containing 1% Nonidet P-40 and 0.5% deoxycholate. The samples were layered into SW60 polyallomer tubes which contained a CsCl buffer cushion (0.5 ml, 1.45 g/ml) overlaid by 15% sucrose in buffer (1.5 ml). Centrifugation was for 2 h at 50,000 rpm. The samples were fractionated dropwise by puncturing the bottom of the tube, and fractions representing the interface between the CsCl and 15% sucrose layers were pooled (150 μl) and then dialyzed for 1 h against 200 ml of buffer. The resulting solutions were layered onto 15 to 30% sucrose gradients (12.2 ml) and subjected to centrifugation in an SW41 rotor for 3.5 h at 36,000 rpm. All procedures were performed at 18 to 20°C.

Radiolabeled protein assay. Samples to be tested were spotted onto filter disks (2.5 cm, Whatman 3MM) which had been pretreated with 1% sodium dodecyl sulfate and 3% Casamino Acids solution. The disks were allowed to air dry and then were washed successively with: (i) 10% trichloroacetic acid containing 3% Casamino Acids solution and 1% sodium dodecyl

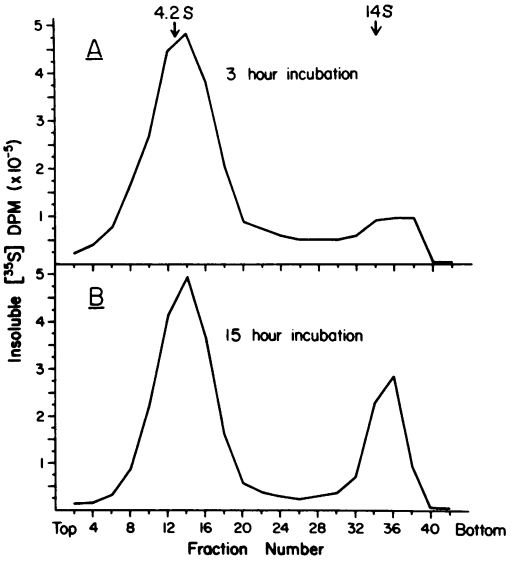

FIG. 2. Sucrose gradient profiles of in vitro-synthesized EMC proteins, 0 to 20S in size. In vitro translation of EMC RNA and fractionation on sucrose gradients was carried out as described in the text. The samples in panels A and B were incubated for 3 (A) and 15 h (B), respectively, at 30°C before fractionation. Portions (50 μl) from each gradient fraction were analyzed for acid-insoluble radioactivity as described. ^{3}H-labeled disrupted polio virions were sedimented in a parallel gradient as 14S marker. All gradients contained rabbit hemoglobin (4.2S) marker (10).

sulfate (10 min, 20°C) (ii) 5% trichloroacetic acid solution containing 1.5% Casamino Acids solution (10 min, 100°C); (iii) twice with 5% trichloroacetic acid (5 min, 20°C); (iv) ethanol (2 min, 20°C); and then (v) anhydrous ether (2 min, 20°C). After drying, radioactivity was detected by liquid scintillation counting.

Gel electrophoresis. Polyacrylamide slab gel electrophoresis has been described previously (10). All gels contained 5 to 8 M urea, as indicated, to aid in the separation of ε1 from ε and p22 from γ. Samples were subjected to an acetone precipitation step before electrophoresis. Treatment of gels and detection of radiolabeled proteins by autoradiography was exactly as described previously (10, 24).

RESULTS

Picorna virion assembly intermediate structures are identified by their sedimentation velocities and protein compositions (20). Accordingly, EMC proteins synthesized in reticulocyte extracts were fractionated on sucrose gradients to determine their sedimentation characteristics. The samples were not denatured before sedimentation except for treatment with high concentrations of RNase, which reduced interference on the gradients by RNA and ribosome-bound protein.

Typical gradient profiles within the sedimentation range of 0 to 20S are illustrated in Fig. 2. The upper panel (A) shows the result of a 3-h translation reaction, and the lower panel (B) represents an overnight (15 h) reaction. In both samples, the majority of the radioactive protein cosedimented near the 4.2S hemoglobin marker.

Figure 2 also shows a second protein peak whose sedimentation velocity is indistinguishable from that of a polio pentamer marker (14S) derived from disrupted virions. The 14S peak is considerably larger in panel B, where it represents about 25% of the in vitro-synthesized protein. Since the translation reactions are complete within 2.5 to 3 h of incubation (25), enhancement of the 14S peak at longer times represents a slow, non-translation-dependent conversion of 4 to 5S to 14S material. Maximum conversion took 13 to 15 h. Longer incubation (up to 24 h) did not further increase the size of the peak (data not shown).

The gradient fractions depicted in Fig. 2B were subjected to gel electrophoresis for protein identification (Fig. 3). The peak at the top of the gradient (4 to 5S) contained numerous viral capsid and noncapsid proteins. Precursor proteins A1, A, B, C, and D, when present (e.g., 1-h incubation samples), also sedimented in this peak (10). Their absence in this sample reflects proteolytic processing during the 15-h incubation period. Figure 3, as well as other gradient experiments (10), have never revealed any capsid proteins sedimenting more slowly than 4 to 5S. This suggests that proteins arising from cleavage of a single capsid precursor molecule probably do not dissociate but remain together as a protomer unit throughout assembly.

Compared to the peak at the top of the gradient, the 14S peak contained fewer proteins. In this gel, small amounts of protein E contaminated the 14S region. However, in most experiments a protein pattern similar to that shown in Fig. 4 was observed. As can be seen, the 14S region of this experiment contained only pro-

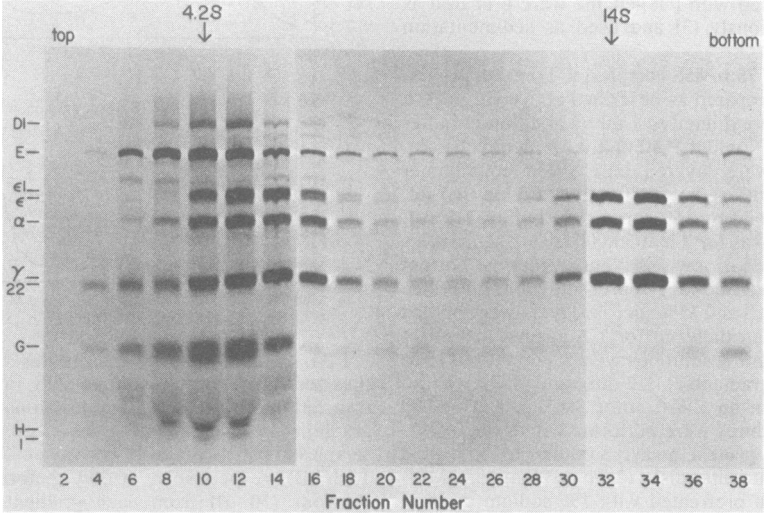

FIG. 3. Viral protein identification of the sucrose gradient fractions. Portions (50 μl) of the gradient fractions depicted in Fig. 2B were treated by acetone precipitation and then layered on polyacrylamide slab gels containing 5 M urea. After electrophoresis, the gels were dried, and the protein bands were visualized by autoradiography.

teins ε, γ, and α. A densitometer tracing of the autoradiogram depicted in Fig. 4 (lane 4) indicates that the relative molar ratios of 14S proteins was 1:1:1 (ε:γ:α) (Fig. 5).

Taken together, the sedimentation velocity (14S), protein composition (ε,γ,α), and molar ratio are strong evidence that the 14S peak represents a pentameric association of capsid protomers. Thus, capsid proteins synthesized in vitro possess at least some assembly activity and are demonstrably capable of undergoing one of the first steps of virion formation.

In contrast to pentamers isolated from infected cells (9), no capsid precursors (A1, A, B, D1) have ever been observed in 14S peaks formed in lysates, even when short-term translation samples were fractioned. This indicates that complete cleavage (to ε,γ,α) precedes or occurs simultaneously with assembly in vitro.

The formation of pentamers in lysates appears to occur spontaneously and efficiently. In some experiments, as much as 75% of the capsid protein sedimented at 14S after prolonged incubation (15 h). It was therefore somewhat surprising when initial attempts to detect capsid assembly intermediate structures larger than 14S were decidedly unsuccessful.

However, a recent report by Marongiu et al. (8) suggested that polio virion shells in their native conformation are inherently unstable and can be disrupted by variations in pH and temperature. Accordingly, care was taken in subsequent experiments to maintain uniformity of conditions (18°C, pH 6.9) throughout lysate fractionations.

Under appropriate conditions (see above), a small 75 to 85S radiolabeled protein peak could be detected in EMC-translated samples (Fig. 6). Detection of this peak was facilitated by sedimenting the reaction mixtures onto CsCl cushions before the sucrose gradient step. In the absence of prefractionation, the 5S and 14S radiolabeled complexes tended to overwhelm and obscure the small peak further down the gradient. The 75 to 85S peak was generally broader and sedimented faster than a polio natural top component marker sample (75 to 80S). In most experiments, the peak represented about 2% of the total viral protein synthesized in vitro. Like the 14S peak, the 75 to 85S peak contained exclusively proteins ε, γ, and α (Fig. 7). A densitometer tracing of the autoradiogram (Fig. 7, lane 5) gave peaks identical to those of Fig. 5, again indicating that these proteins occurred in molar ratios of 1:1:1.

A second cycle of gradient sedimentation showed that the 75 to 85S material could be converted to 14S by either chilling the sample or lowering the ionic strength (data not shown). This result is similar to that reported for polio shells in their native conformation (8). Although low yields and instability have hampered efforts to further characterize the 75 to 85S peak, its properties seem consistent with those expected for (unstable) empty capsid-like structures.

Cleavage of ε to δ,β, which marks the emergence of infectious virions (4), has yet to be detected in lysates. It is therefore unlikely that viral RNA is packaged into shells under these experimental incubation conditions.

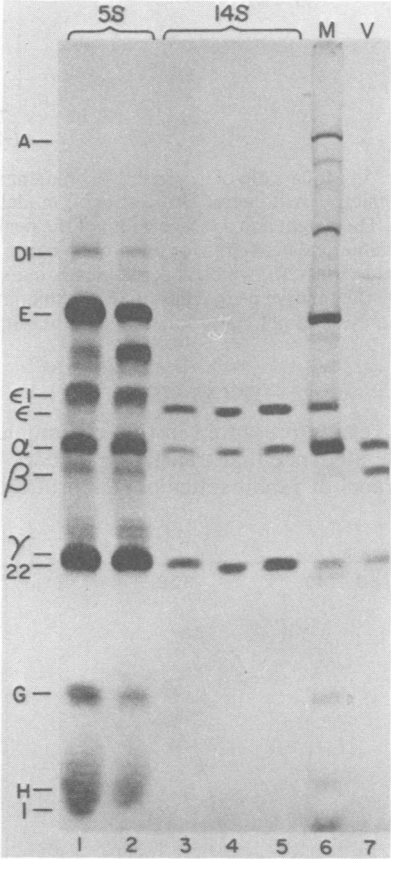

FIG. 4. Resolution of proteins ε1 and ε. EMC RNA was incubated in reticulocyte extract (30 μl) for 15 h at 30°C. The sample was fractionated on a sucrose gradient in a manner identical to that described in the legend to Fig. 2B. Portions of gradient fractions (50 μl) corresponding to the 5S (lanes 1 and 2) and 14S peak regions (lanes 3, 4, and 5) were treated by an acetone precipitation step and then subjected to electrophoresis on a polyacrylamide slab gel containing 8 M urea. Electrophoresis was carried out at 3 V/cm for 36 h, after which the gel was dried, and the proteins were detected by autoradiography. Lane 6 (M) contains [^{35}S]methionine-labeled viral protein markers from an EMC-infected HeLa cell extract. Lane 7 (V) contains [^{35}S]methionine-labeled EMC capsid proteins from disrupted virions.

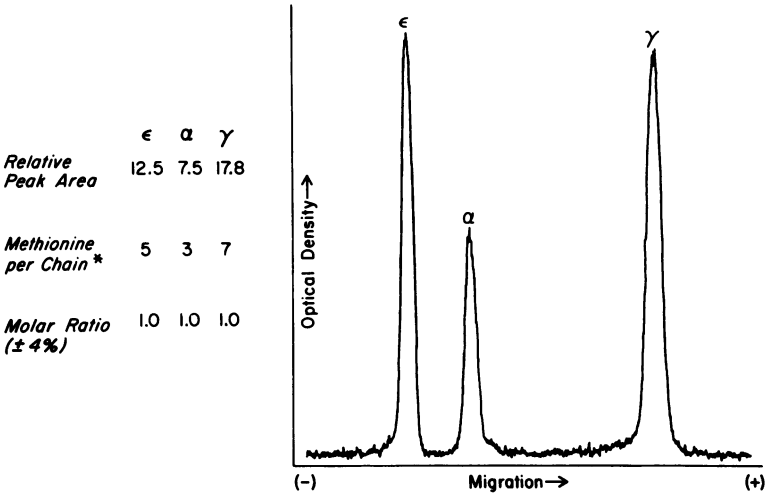

FIG. 5. Molar ratio of ε:γ:α in 14S gradient peak. Lane 4 of the autoradiogram depicted in Fig. 4 was scanned with a microdensitometer (Joyce-Loebl, model MKIII C) to give a graphic representation of the protein band profile. The height and average width of the peaks within the profile were measured and combined (multiplied) to give a numerical peak area for the protein bands. The methionine per chain values (*) are those reported for Maus Elberfeld virus (20), which is serologically indistinguishable from EMC (20). The molar ratio was determined by dividing the relative peak areas by the methionine per chain and then normalizing the lowest number to a value of one. Analysis of gel lanes 3 and 5 gave the same results to within 4%.

DISCUSSION

The results presented in this communication provide evidence that translation of EMC RNA in extracts of rabbit reticulocytes produces active capsid proteins capable of assembly into virion intermediate structures. When the translation reactions are terminated after a relatively short period of incubation (3 h), capsid protein sequences are represented primarily by 5S precursors and protomeric associations of their cleavaged products.

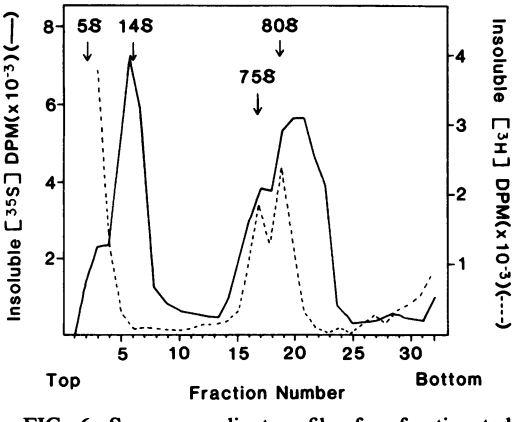

FIG. 6. Sucrose gradient profile of prefractionated EMC protein complexes formed in vitro. EMC proteins synthesized in reticulocyte extract were sedimented onto a CsCl cushion and then fractionated on a sucrose gradient as described in the text. Portions (50 μl) from each gradient fraction were analyzed for acid-insoluble radioactive protein as described. ^{3}H-labeled polio natural top component (virion shells), representing a mixture of N and H antigenic forms (18; A. Mosser, personal communication), was isolated from infected HeLa cells (17) and run as an internal sedimentation marker (75 to 80S).

Longer incubation (up to 15 h) produces a distinct peak of 14S protein on sucrose gradients. The 14S material is identified as a pentameric association of capsid protomers on the basis of protein composition, protein molar ratios, and cosedimentation with authentic pentamers from disrupted polio virions.

It is unclear why the complete formation of pentamers in reticulocytes requires 15 h. During viral infection of cells, virion formation is virtually complete within 5 to 6 h (2). One possible rate limiting step in vitro may be proteolytic processing of capsid precursors. Although p22 cleaves with fidelity in lysates, the precursors have longer apparent half-lives than in infected cells (1, 11). The absence of precursors in lysate-formed pentamers suggests that cleavage, at whatever the rate, is a prerequisite to assembly.

Interestingly, protein ε1, the immediate precursor to protein ε (10, 11) was also completely absent from the pentamer peak (see Fig. 4). Although these proteins can be separated on polyacrylamide gels containing 5 to 8 M urea, the nature of the difference between ε1 and ε is unknown. Their tryptic peptides when labeled with methionine are indistinguishable (Mark Pal-

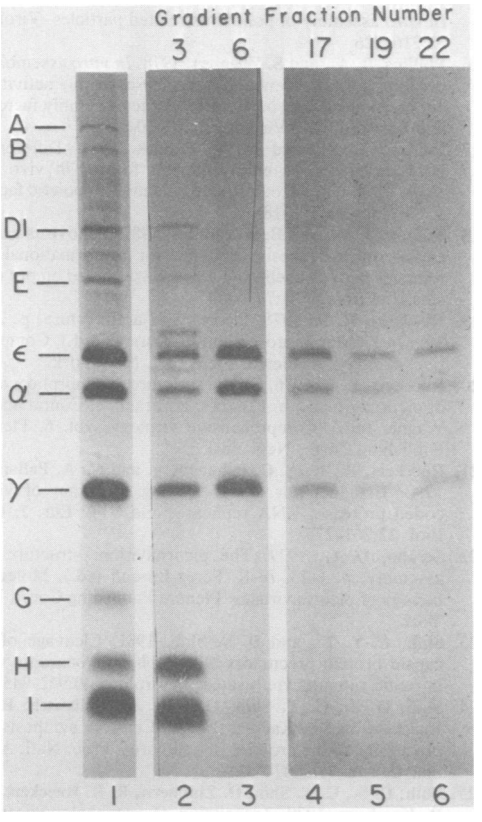

FIG. 7. Viral protein identification of the sucrose gradient fractions. Portions (50 μl) of gradient fractions depicted in Fig. 6 were treated by acetone precipitation and then layered onto polyacrylamide slab gels containing 5 M urea. After electrophoresis, the gels were dried and the protein bands detected by autoradiography. Lane 1 contains a portion of the CsCl 15% sucrose interface described in the text, which was used as starting material for the sucrose gradient. Lanes 2, 3, 4, 5, and 6 represent portions from sucrose gradient fractions 3, 6, 17, 19, and 22, respectively.

lansch, personal communication). Since δ, the eventual cleavage product of ε, has a blocked amino terminus (20), it is possible that the ε1-to-ε conversion reflects the addition of the blocking group. Kitamura et al. (6) have reported the blocked amino terminus of polio protein 1a (homolog to EMC protein A) isolated in vivo. If the in vitro EMC blocking reaction is not complete until the ε1-to-ε step, limited availability of properly modified ε might be expected to affect the rate of assembly. Although some modification other than blocking could also be responsible for the difference between these proteins, it is clear that the conversion is important for assembly in vitro, because ε1 is never observed in the 14S peak. We are currently examining the amino termini of these and other capsid precursors to determine the nature of the ε1-to-ε difference and how and when the terminal-blocking reaction occurs.

Another reason for the comparatively slow in vitro assembly rate might be the relatively low viral protein concentration. Even though every viral RNA molecule is translated an average of 8 to 10 times in this reticulocyte system (25), the total protein yield is about 10-fold lower than in a comparable volume of infected cells (unpublished observation). It has been reported that in vitro formation of polio shells is highly dependent upon the starting concentration of 14S precursors (14, 16). It follows, then, that formation of pentamers and shells in reticulocytes may also be adversely affected by low capsid protein concentrations.

Even though prolonged incubation does eventually allow a substantial conversion of protomers to pentamers in reticulocytes, the concentration effect may still be responsible for the low yield of 75 to 85S shell-like material. An equally plausible explanation for the very small 75 to 85S peak is the apparent instability of the particles in the present buffer system. Cardioviral capsids (EMC) are thought to be more labile than those of the enteroviruses (19). Shells may be formed efficiently in reticulocytes, only to break down during the gradient fractionation steps.

These alternatives (low rate of formation, particle instability) cannot presently be distinguished experimentally. However, the fact that any 75 to 85S material is observed in vitro is evidence that the 14S pentamers, once formed, are assembly competent. It is hoped that future experiments with stabilizing buffers or less labile viruses will produce increased yields of complete in vitro-synthesized picornaviral particles. Indeed, preliminary translation experiments with poliovirus have yielded 75 to 80S particles representing 5 to 10% of the available capsid protein (A. C. Palmenberg and S.-W. Hong, manuscript in preparation). Characterization of these particles is currently underway.

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