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In Vitro Morphogenesis of Foot-and-Mouth Disease Virus

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Foot-and-mouth disease virion RNA is translated efficiently and completely in a rabbit reticulocyte lysate cell-free system. Treatment of cell-free lysates with monospecific serum prepared against the individual viral structural proteins or with monoclonal antibodies prepared against the inactivated virus or against a viral structural protein precipitated all of the structural proteins, suggesting that structural protein complexes were formed in vitro. Sucrose gradient analysis of the cell-free lysate indicated that complexes sedimenting at 5, 14, 60 to 70, and ca. 110S were assembled in vitro. Structural proteins VP_0, VP_1, and VP_3 were the major polypeptides found in these complexes. The material sedimenting at 110S, i.e., containing VP_0, VP_1, and VP_3, was precipitated by a 140S-specific monoclonal antibody but not by a 12S subunit-specific monoclonal antibody, suggesting that this capsid structure contained at least one epitope present on the intact virus.

Picornaviruses are nonenveloped particles containing a single-stranded RNA molecule of positive polarity and 60 copies each of four capsid proteins (VP_1 to VP_4) (1). The morphogenesis of picornaviruses is not completely understood; however, it is believed to proceed via a number of subviral structures. Several of these particles have been isolated from infected cells and characterized with respect to sedimentation rate and protein composition (21).

The smallest detectable capsid structures found in many picornavirus-infected cells are 13S particles, which are pentamers of the capsid protein precursor (16, 17, 20). The precursor protein pentamer is cleaved to yield 14S particles composed of five copies each of VP_0, VP_1, and VP_3. Alternatively, it has been suggested that protomers consisting of one copy of VP_0, VP_1, and VP_3 may be formed by the cleavage of the capsid protein precursor (21). However, this structure, which would sediment at 5 to 6S, has only been isolated in bovine enterovirus-infected cells (22). Procapsids, also called empty capsids, are formed by the assembly of 14S particles and are found in most picornavirus-infected cells except for cardioviruses (20, 21). The entrance of an RNA molecule results in the formation of provirions, which are unstable intermediates (6). The cleavage of VP_0 to VP_2 and VP_4 is believed to be the final step in maturation.

The morphogenesis of foot-and-mouth disease virus (FMDV), an aphthovirus, has not been as well studied as have some other picornaviruses. However, procapsids have been characterized in infected cells and shown to be precursors of mature virions (23).

We and others have demonstrated that FMDV can be efficiently and completely translated in cell-free systems (5, 9, 11; M. J. Grubman, B. H. Robertson, D. O. Morgan, D. M. Moore, and D. Dowbenko, submitted for publication). specific antiserum against viral structural proteins or with monoclonal antibodies against inactivated virus or against VP_3 (the trypsin-sensitive protein also called VP_1 and located at the carboxy-terminal portion of the capsid protein precursor) results in the precipitation of all of the viral structural proteins and their precursors (Grubman et al., submitted for publication). This suggested that structural protein complexes were formed in the cell-free system. Palmenberg (18) recently reported the formation of some capsid intermediate structures in reticulocyte lysates programmed with encephalomyocarditis virus RNA.

In this report the structural protein complexes formed in FMDV RNA-programmed reticulocyte lysates have been characterized by sedimentation analysis and protein composition. These complexes sediment at ca. 5, 14, 60 to 70, and 110S. In addition, structural proteins VP_0, VP_1, and VP_3 present in the approximately 110S complex were precipitated by both polyclonal serum against inactivated virus and a monoclonal antibody specific for 140S virus but not by a monoclonal antibody specific for 12S viral subunits.

MATERIALS AND METHODS

Materials. [^{35}S]methionine (1,000 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Heat-killed Formalin-fixed *Staphylococcus aureus* cells (Cowan I strain) were purchased from Bethesda Research Laboratories, Gaithersburg, Md. Pancreatic RNase A was purchased from Worthington Diagnostics, Freehold, N.J., and micrococcal nuclease was purchased from P-L Biochemicals, Inc., Milwaukee, Wis.

Growth of FMDV and isolation of virion RNA. FMDV (type A_{12}, strain 119ab) was grown in BHK-21 cells and purified as previously described (10, 15). RNA was extracted from purified virus by the phenol-chloroform-isoamyl alcohol (50:50:2) procedure, and intact 37S RNA was isolated from sucrose gradients (8), precipitated with 2.5 volumes of ethanol, and stored in samples at −70°C.

Preparation of rabbit reticulocyte lysates and in vitro protein synthesis conditions. Rabbit reticulocyte lysates were prepared as previously described (9) and stored in 400- to 500-μl aliquots at −70°C. The conditions for in vitro protein synthesis were as described previously (9, 19) and included 5 mM dithiothreitol–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.5.

Sucrose gradient centrifugation. After translation, the lysates were incubated with either a solution containing 30 mM EDTA and 60 to 80 μg of pancreatic RNase A per ml for 15 min at 30°C or 0.1 mM aurintricarboxylic acid for 15 min at 30°C followed by a four- to ninefold dilution in 0.15 M NaCl–0.01 M Tris-hydrochloride (pH 7.5)–0.002 M EDTA (NET buffer). The diluted lysates were layered on various sucrose gradients: (i) 4.6 ml of 5 to 20% (wt/vol) sucrose in NET buffer and centrifuged in an SW65 rotor at 54,000 rpm for 19.5 h at 4°C; (ii) 11.2 ml of 5 to 20% (wt/vol) sucrose in NET buffer and centrifuged in an SW41 rotor at 35,000 rpm for 17

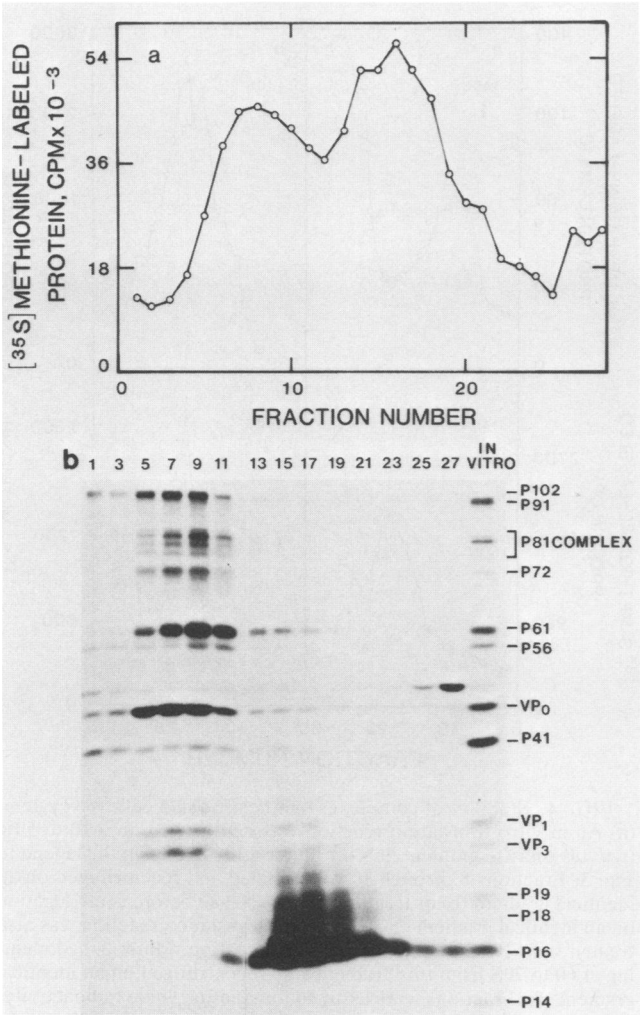

FIG. 1. Sucrose gradient (a) and PAGE (b) analysis of small complexes synthesized in a cell-free system. FMDV RNA was translated for 4 h in a reticulocyte lysate in the presence of [^{35}S]methionine. After translation, the lysate was treated with EDTA–pancreatic RNase A, diluted with NET buffer, and layered on a 5 to 20% (wt/vol) sucrose gradient in NET buffer. The gradients were centrifuged for 19.5 h at 54,000 rpm at 4°C in an SW65 rotor. Samples from each fraction were assayed for acid-insoluble radioactivity. Centrifugation is from right to left. Alternate fractions were precipitated with 4 volumes of acetone, suspended in sample preparation buffer, and analyzed by PAGE on a 20-cm 12.5% slab gel. Marker bovine serum albumin (4.2S) and chymotrypsinogen (2.6S) were centrifuged on similar gradients and monitored for protein concentration (3). The numbered lanes in the slab gel analysis refer to fractions from the sucrose gradient. The lane labeled in vitro is a lysate before centrifugation.

h at 4°C; and (iii) 11.2 ml of 10 to 50% (wt/vol) sucrose in NET buffer (or other buffers as indicated in the figure legends) and centrifuged in an SW41 rotor at 17,000 rpm for 17 h at 4°C. After centrifugation, fractions were collected, and samples were assayed for hot trichloroacetic acid-precipitable radioactivity. Samples from alternate fractions were diluted with an equal volume of water, 10 μg of bovine serum albumin was added as the carrier, and the solution was precipitated with 3 to 4 volumes of acetone. The precipitates were suspended in sample preparation buffer (0.063 M Tris-hydrochloride [pH 6.8], 2% sodium dodecyl sulfate, 0.68 M 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and analyzed by polyacrylamide gel electrophoresis (PAGE).

Immunoprecipitation. Immunoprecipitation of fractions from sucrose gradients with *S. aureus* cells bearing protein A was performed as described previously (11, 13) with modifications. To eliminate nonspecific immunoprecipitation, fractions were incubated with washed 10% *S. aureus* cells bearing protein A for 15 min at room temperature, and bacterial complexes were pelleted by centrifugation in an Eppendorf Micro Centrifuge for 5 min (12). The supernatant was then incubated with antiserum and *S. aureus* cells bearing protein A as previously described (11). The supernatant obtained after elution of the bacteria with sample preparation buffer and centrifugation was analyzed by PAGE.

PAGE. Samples were analyzed on 1.5-mm-thick, 20-cm 12.5% (wt/vol) or 10 to 20% polyacrylamide slab gels containing a 5% stacking gel in a discontinuous Tris-glycine

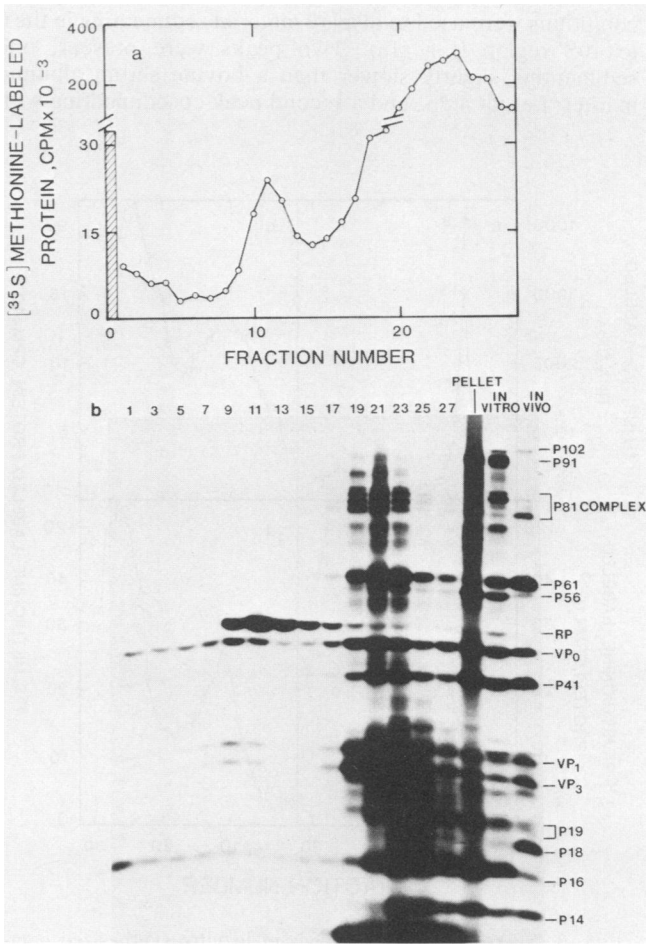

FIG. 2. Sucrose gradient (a) and PAGE (b) analysis of 14S complexes synthesized in a cell-free system. Virion RNA was translated in a reticulocyte cell-free system for 28 h (4-h pulse, 24-h chase), treated with EDTA–RNase A–1% NP-40, and centrifuged on a 5 to 20% (wt/vol) sucrose gradient in NET buffer at 35,000 rpm for 17 h at 4°C in an SW41 rotor. Samples from each fraction were assayed for acid-insoluble radioactivity, and alternate fractions were acetone precipitated and analyzed by PAGE on a 20-cm 12.5% slab gel. The lane in the slab gel analysis labeled in vivo is a [^{35}S]methionine-labeled FMDV-infected bovine kidney cell lysate. RP, Reticulocyte protein.

buffer system (14). After electrophoresis, the gels were either dried and directly exposed to Kodak Blue Brand X-ray film (Eastman Kodak Co., Rochester, N.Y.) or fluorographed with sodium salicylate (4). After fluorography, the gels were dried and exposed to X-ray film at −70°C (2).

RESULTS

Identification of cell-free synthesized complexes sedimenting at ca. 5S. We have previously shown that structural protein complexes appear to be formed in a reticulocyte lysate cell-free system programmed with FMDV RNA, since monospecific antisera against three structural proteins and monoclonal antibodies prepared against inactivated virus, VP_3 (the trypsin-sensitive protein also called VP_1) or a cyanogen bromide fragment of VP_3 precipitated all of the virion structural proteins from a cell-free translation system (Grubman et al., submitted for publication).

To identify complexes formed in the cell-free system, FMDV RNA-programmed lysates labeled with [^{35}S]methionine were centrifuged on sucrose gradients. Centrifugation conditions were used to display material sedimenting in the 0 to 10S region (Fig. 1a). Two peaks were present, one sedimenting slightly slower than a bovine serum albumin marker, i.e., at 4.2S, and a second peak cosedimenting with a chymotrypsinogen marker, i.e., at 2.6S. Analysis of the protein composition of these peaks revealed that capsid proteins VP_0, VP_1, and VP_3 were greatly enriched in the 4 to 5S peak (Fig. 1b). Capsid precursors P91 and P72 and some nonstructural polypeptides also sedimented in this region. Treatment of the lysate with Nonidet P-40 (NP-40) before centrifugation resulted in essentially the same profile on a sucrose gradient (data not shown). Gel analysis revealed that the structural proteins were still enriched in the 5S region (data not shown). After detergent treatment, P41 and P14, polypeptides coded for by the P2 region of the genome and membrane associated in infected cells (Grubman et al., submitted for publication), were present in the smaller peak, suggesting that these proteins may also be membrane associated in the cell-free system.

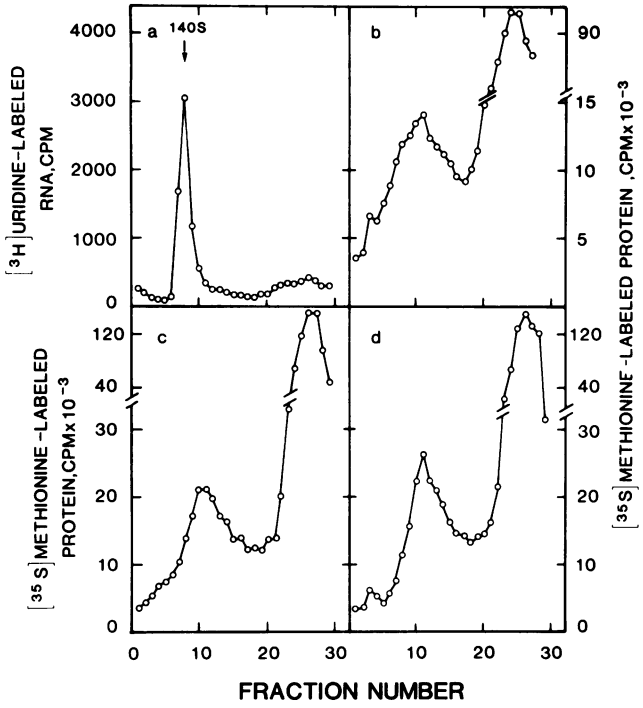

FIG. 3. Sucrose gradient analysis of in vitro-synthesized complexes under various ionic conditions. Virion RNA was translated for 30 h as described in the legend to Fig. 1, treated with EDTA–pancreatic RNase, diluted with various buffers, and layered on 10 to 50% (wt/vol) sucrose gradients in various buffers. Centrifugation was at 17,000 rpm for 17 h at 4°C in an SW41 rotor. Each fraction was assayed for acid-insoluble radioactivity. (a) [^{3}H]uridine-labeled purified 140S FMDV in NET buffer; (b) in vitro-synthesized lysate in NET buffer; (c) in vitro-synthesized lysate in NT buffer (0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.5); (d) in vitro-synthesized lysate in 0.15 M KCl–0.005 M $MgCl_2$–0.01 M Tris-hydrochloride, pH 7.5.

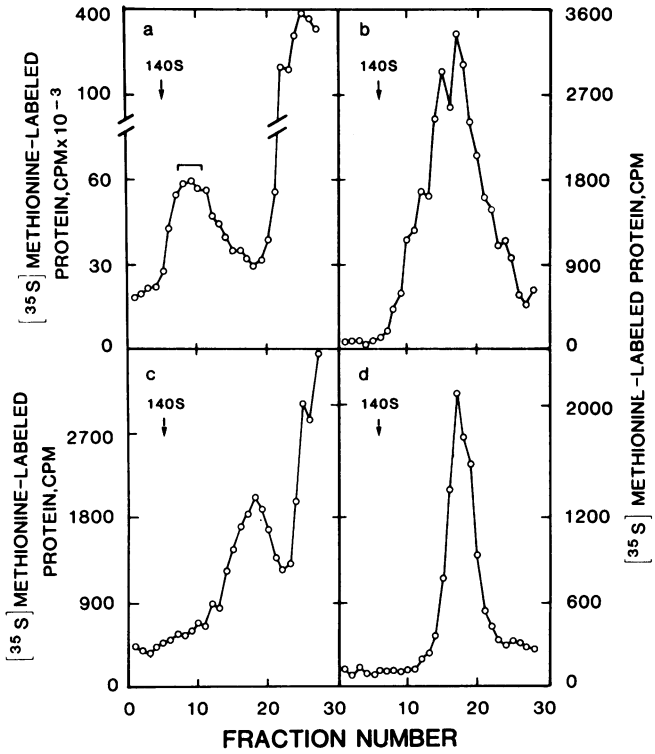

FIG. 4. Stability of complexes synthesized in a cell-free system. (a) An in vitro translation reaction was centrifuged on a 10 to 50% (wt/vol) sucrose gradient in NET buffer as described in the legend to Fig. 3. Fractions 8 through 10 were pooled and recentrifuged on an identical gradient (b) or treated with 1% NP-40 before centrifugation on an identical gradient (c). An in vitro translation reaction was also treated with 1% Sarkosyl before centrifugation. Material sedimenting at 60 to 70S from this gradient was recentrifuged on an identical gradient (d). Fractions were assayed for acid-insoluble radioactivity. [^{3}H]uridine-labeled 140S FMDV was centrifuged in a parallel gradient.

Identification of 14S complexes. Formation of 14S complexes is one of the initial steps in picornavirus morphogenesis. Thus, a labeled cell-free translation system was centrifuged on a sucrose gradient under conditions which would allow detection of complexes sedimenting in this region. The results depicted in Fig. 2a indicate that a labeled complex sediments at ca. 14S (12S subunits prepared by acid disruption of the intact virus were used as a sedimentation marker in similar gradients). The major polypeptide in this peak is a reticulocyte-coded protein of ca. 50,000 daltons which is

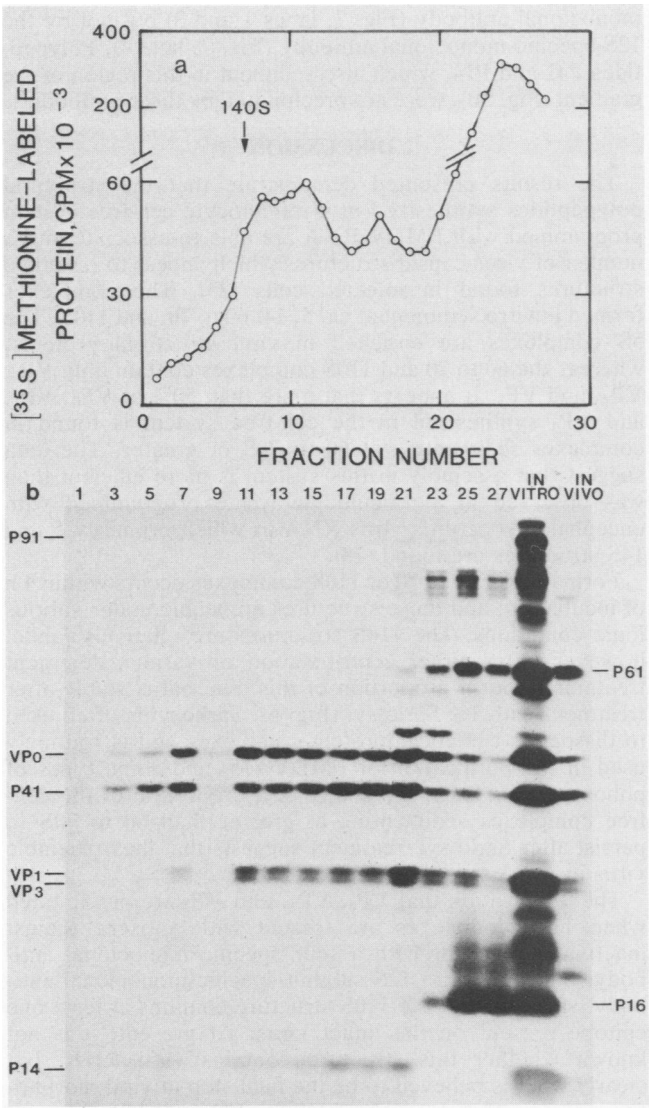

FIG. 5. Sucrose gradient (a) and PAGE (b) analysis of large complexes synthesized in a cell-free system. An in vitro translation reaction programmed with FMDV RNA and treated with 10^{-4} M aurintricarboxylic acid after 4.5 h of incubation was centrifuged on a 10 to 50% (wt/vol) sucrose gradient in NET buffer as described in the legend to Fig. 3. Fractions were assayed for acid-insoluble radioactivity. Alternate fractions were acetone precipitated and analyzed for protein composition on a 20-cm 12.5% slab gel. [^{3}H]uridine-labeled 140S FMDV was centrifuged on a parallel gradient as a marker.

synthesized in a cell-free system in the absence of exogenous RNA (unpublished data) (Fig. 2b). Structural proteins VP_0, VP_1, and VP_3 and a small amount of nonstructural polypeptide P16 were the only virus-specific polypeptides present in this peak. The reticulocyte-coded protein and P16 are not components of the 14S structural protein complex, since treatment of a total cell-free system with antisera against various structural proteins does not precipitate these two polypeptides (Grubman et al., submitted for publication).

Formation of procapsids and larger structures. Precipitation of structural proteins VP_0, VP_1, and VP_3 from a cell-free translation system with a monoclonal antibody that is 140S specific suggested that capsid structures larger than 14S may be formed in vitro (unpublished data). Centrifugation of cell-free translation reactions under conditions which would detect procapsids or virus particles demonstrated that a broad peak of material sedimented at ca. 110S and was observed under various ionic conditions (Fig. 3). Material sedimenting at 60 to 70S was sometimes also observed (see Fig. 5 and 6). The majority of material sedimenting at 110S was unstable, since recentrifugation resulted in most of this material sedimenting at ca. 60 to 70S (Fig. 4b). Treatment of the 110S material with 1% NP-40 (Fig. 4c) or 1% Sarkosyl (data not shown) also resulted in the breakdown of the 110S peak; in addition, a significant amount of material was released to the top of the gradient. In contrast, material isolated from the 60 to 70S region of a gradient on which an in vitro translation reaction treated with 1% Sarkosyl had been layered was stable upon recentrifugation (Fig. 4d).

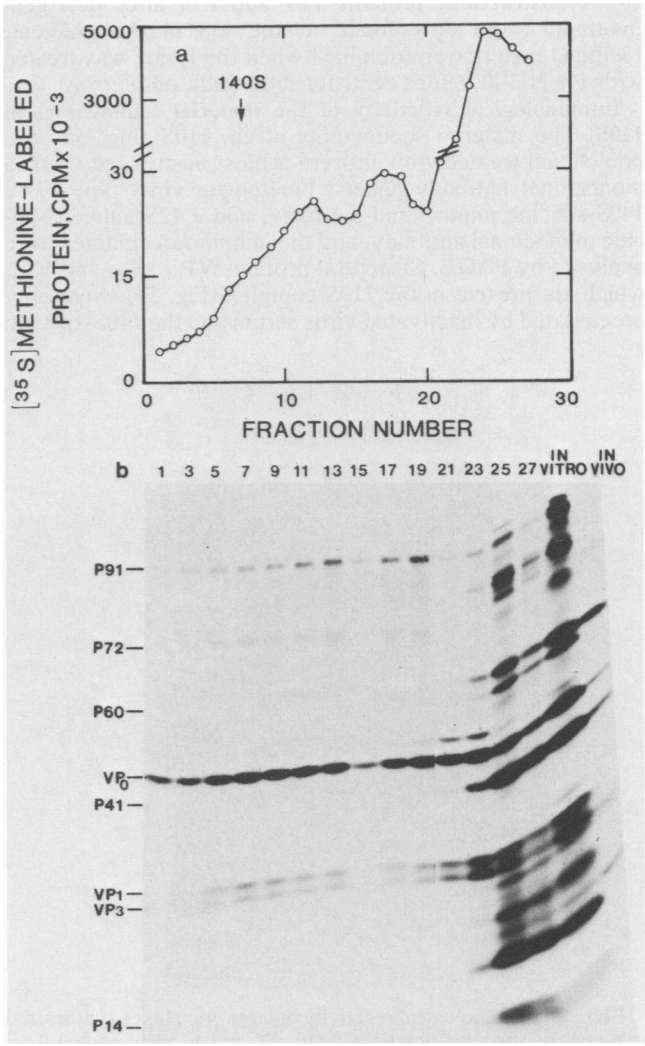

FIG. 6. Sucrose gradient (a) and PAGE (b) analysis of large complexes synthesized in a cell-free system after treatment with 1% Sarkosyl. An in vitro translation reaction programmed with FMDV RNA was treated with aurintricarboxylic acid–1% Sarkosyl and centrifuged on a 10 to 50% (wt/vol) sucrose gradient in NET buffer as described in the legend to Fig. 5. Fractions also were assayed as described in the legend to Fig. 5. Alternate fractions were acetone precipitated and analyzed on a 10 to 20% slab gel. [^{3}H]uridine-labeled 140S FMDV was centrifuged on a parallel gradient as a marker.

Analysis by PAGE of an in vitro translation reaction centrifuged to display procapsids and larger structures (Fig. 5a) revealed that structural proteins VP_0, VP_1, and VP_3 and nonstructural proteins P41 and P14 were the only polypeptides sedimenting in structures of 60 to 70S or larger (Fig. 5b). A small amount of structural protein precursor P91 was also present in complexes sedimenting at ca. 140S (Fig. 5b, lane 7). Of the structural proteins synthesized in the cell-free system, ca. 50% or more were found in complexes sedimenting at 60 to 70S or greater.

Treatment of the cell-free virion RNA-programmed lysate with 1% Sarkosyl and analysis by sucrose gradient centrifugation revealed two broad peaks, one at 60 to 70S and one at 110S (Fig. 6a). PAGE analysis demonstrated that only structural proteins VP_0, VP_1, and VP_3 and precursor proteins P91 and P72 were present in these two regions (Fig. 6b). Nonstructural proteins P41 and P14 after detergent treatment were only found at the top of the gradient. Identical results were obtained when the lysate was treated with 1% NP-40 before centrifugation (data not shown).

Immunological reactivity of the material sedimenting at 110S. The material sedimenting at ca. 110S (Fig. 5a) was pooled and treated with antisera against inactivated virus, a monoclonal antibody against bluetongue virus type 17, a 140S-specific monoclonal antibody, and a 12S subunit-specific monoclonal antibody, and the immunoprecipitates were analyzed by PAGE. Structural proteins VP_0, VP_1, and VP_3, which are present in the 110S complex (Fig. 5b), were only precipitated by inactivated virus serum and the 140S-specific monoclonal antibody (Fig. 7, lanes 1 and 3) but not by the 12S-specific monoclonal antibody (Fig. 7, lane 4). Polypeptides P41 and P14, which also sediment in this region of the gradient (Fig. 5b), were not precipitated by these antibodies.

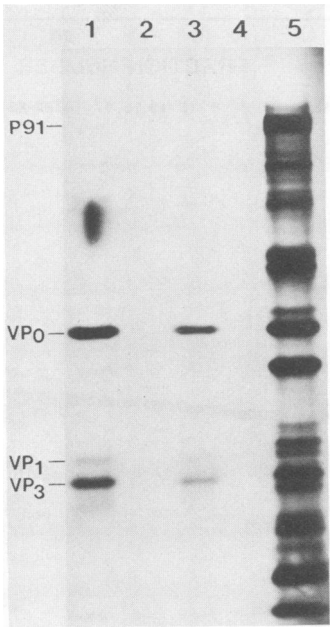

FIG. 7. Immunological reactivity of large complexes synthesized in a cell-free system. Fractions 8, 10, 12, and 14 were pooled from the sucrose gradient in Fig. 5 and immunoprecipitated with various antisera and *S. aureus* cells bearing protein A. The eluted material was analyzed on a 20-cm 12.5% slab gel. Lane 1, 1:20 dilution of guinea pig serum against inactivated FMDV A_{12} 119ab; lane 2, 1:5 dilution of mouse ascitic fluid from a monoclonal antibody against bluetongue virus type 17; lane 3, 1:5 dilution of mouse ascitic fluid from a monoclonal antibody reactive with 140S virus; lane 4, 1:5 dilution of mouse ascitic fluid from a monoclonal antibody reactive with 12S subunits prepared by acid disruption of the virus; lane 5, [^{35}S]methionine-labeled cell-free lysate programmed with FMDV RNA.

DISCUSSION

The results presented demonstrate that the structural polypeptides synthesized in a reticulocyte cell-free system programmed with FMDV RNA are able to associate into a number of virus capsid structures which appear to resemble structures found in infected cells (23). The complexes formed in vitro sediment at ca. 5, 14, 60 to 70, and 110S. The 5S complexes are enriched in viral structural proteins, whereas the 60 to 70 and 110S complexes contain only VP_0, VP_1, and VP_3. It appears that more than 50% of VP_0, VP_1, and VP_3 synthesized in the cell-free system is found in complexes sedimenting at 60 to 70S or greater. The data suggest that assembly in this system is more efficient than was observed in a reticulocyte lysate programmed with encephalomyocarditis virus RNA in which primarily 5 and 14S structures are found (18).

Formation of 60 to 70 or 110S complexes occurs within 4 h of incubation, and these structures are stable under various ionic conditions. The 110S structures are relatively labile, however, after either centrifugation or various detergent treatments, but a proportion of this material is stable after treatment with 1% Sarkosyl (Fig. 6). Sarkosyl is often used to dissociate ribonucleoprotein complexes and is routinely used in the purification of FMDV (15) and some types of poliovirus (7). Thus, the ability of a proportion of the cell-free complexes sedimenting at greater than 60 to 70S to persist after Sarkosyl treatment suggests that they resemble virus in their stability to Sarkosyl.

The observation that VP_0, VP_1, and VP_3 are precipitated when 110S complexes are treated with antisera against inactivated virus or with a 140S-specific monoclonal antibody, but not with a 12S subunit-specific monoclonal antibody, suggests that the 110S structure contains at least one epitope present on the intact virus. At present, it is not known whether this structure contains viral RNA, but clearly what is believed to be the final step in viral morphogenesis, i.e., the cleavage of VP_0 to VP_2 and VP_4, has not occurred.

Translation of FMDV RNA in a reticulocyte cell-free system appears to mimic, very closely, events occurring in FMDV-infected cells. Thus, in the cell-free system, structural and nonstructural proteins are synthesized and processed, two nonstructural polypeptides (P41 and P14) may be membrane associated, and various capsid structures are assembled. These various capsid intermediate structures require further physical and immunological characterization, but it would appear that the cell-free system may be useful in studying the various steps in picornavirus morphogenesis.

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