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Capsid Intermediates Assembled in a Foot-and-Mouth Disease Virus Genome RNA-Programmed Cell-Free Translation System and in Infected Cells

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Structural protein complexes sedimenting at 140S, 70S (empty capsids), and 14S were isolated from foot-and-mouth disease virus-infected cells. The empty capsids were stable, while 14S complexes were relatively short-lived. Radioimmune binding assays involving the use of neutralizing monoclonal antibodies to six distinct epitopes on type A_{12} virus and polyclonal antisera to A_{12} structural proteins demonstrated that native empty capsids were indistinguishable from virus. Infected cell 14S particles possessed all the neutralizing epitopes and reacted with VP_2 antiserum. Cell-free structural protein complexes sedimenting at 110S, 60S, and 14S containing capsid proteins VP_0, VP_3, and VP_1 are assembled in a rabbit reticulocyte lysate programmed with foot-and-mouth viral RNA. These structures also contain the six epitopes, and cell-free 14S structures like their in vivo counterparts reacted with VP_2 antiserum. Capsid structures from infected cells and the cell-free complexes adsorbed to susceptible cells, and this binding was inhibited, to various degrees, by saturating levels of unlabeled virus. These assays and other biochemical evidence indicate that capsid assembly in the cell-free system resembles viral morphogenesis in infected cells. In addition, epitopes on the virus surface possibly involved in interaction with cellular receptor sites are found early in virion morphogenesis.

The morphogenesis of picornaviruses is a complex process that proceeds via a number of subviral structures. Some of these structures have been isolated from infected cells and characterized with respect to sedimentation rate, isoelectric point, and protein composition. Other analyses have included surface labeling and treatment with cross-linking reagents (36). Specific polyclonal antisera have been used to distinguish between native and mildly denatured virus particles (26, 36), and monospecific polyclonal antisera to viral structural proteins have been used to identify proteins present on the surface of certain precursor particles (18, 24, 30).

Monoclonal antibody technology has greatly facilitated our understanding of picornavirus antigenic determinants, mechanisms of neutralization (5, 8, 9, 11, 20, 31, 33, 40), and analysis of various picornavirus intermediate structures (9, 11, 19, 27). It is now feasible to follow the development of "native virus conformation" by examining the various subviral structures for the presence or absence of epitopes found on mature virus.

Foot-and-mouth disease virus (FMDV), an aphthovirus of the picornavirus family, contains the equivalent of 60 copies each of four capsid proteins, VP_1, VP_2, VP_3, and VP_4, encapsidating a single-stranded RNA molecule of positive polarity (1). Analogies to other picornaviruses have been made, but relatively little is known about the morphogenesis of FMDV. Experiments by Yafal and Palma (42) suggested that empty capsids, subviral particles lacking RNA, appear to be precursors of mature virions. However, little direct evidence has been presented concerning other FMDV capsid intermediate structures.

Recently Grubman et al. demonstrated that incubation of an FMDV RNA-programmed rabbit reticulocyte cell-free translation system resulted in the efficient synthesis and processing of virus-specific polypeptides and the assembly of various structural protein complexes (13, 17). Similar results have been reported in an encephalomyocarditis virus RNA-programmed cell-free system (34). The FMDV structural protein complexes were characterized by sedimentation and protein analyses and were found either to be enriched in or to contain only capsid proteins. In addition, a conformation-specific neutralizing monoclonal antibody was shown to react with the largest of the cell-free complexes.

In the present communication, structural protein complexes have been isolated from FMDV-infected cells and the reticulocyte cell-free system. These capsid structures have been characterized and compared by antibody and cell binding assays. By these criteria, capsid structures formed in the cell-free system resemble those present in infected cells.

MATERIALS AND METHODS

Growth and purification of FMDV and isolation of virion RNA. FMDV (type A_{12}, strain 119ab) was grown and purified, and intact 37S virion RNA was isolated as previously described (14, 16, 23). Virus labeled with [^{3}H]uridine (ICN Pharmaceuticals Inc., Irvine, Calif.) was prepared 5 to 7 h after infection and purified as previously described (23).

In vitro protein synthesis and isolation of capsid structures. The preparation of rabbit reticulocyte lysates and conditions for in vitro protein synthesis were as previously described (13, 15). After translation, the lysates were incubated with a solution containing EDTA and pancreatic RNase A and diluted in 0.15 M NaCl–0.01 M Tris hydrochloride (pH 7.5)–0.002 M EDTA (NET buffer), and the various capsid structures were collected after sucrose gradient centrifugation (13).

Preparation of intracellular capsid structures. Bovine kidney (BK) cells were infected with FMDV at a multiplicity of approximately 100 PFU per cell and labeled with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) after the appearance of cytopathic effects (17). After

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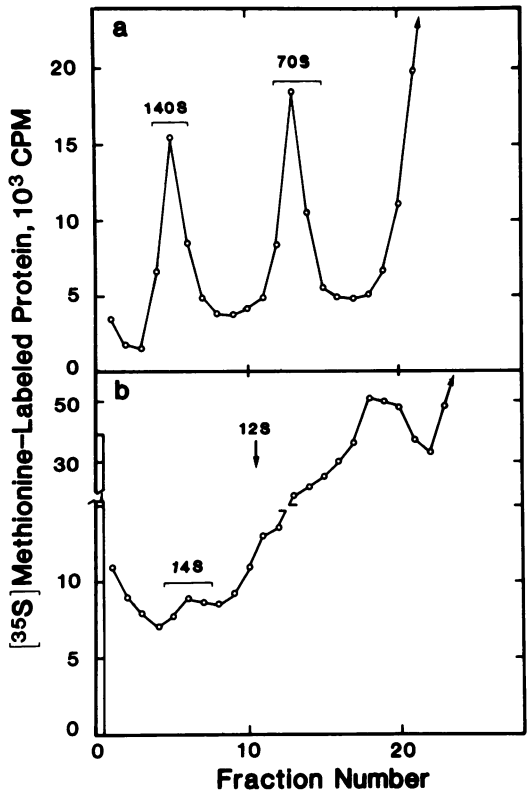

FIG. 1. Sucrose gradient analysis of structural protein complexes from FMDV-infected cells. Cytoplasmic extracts were prepared from FMDV-infected BK cells which had been labeled for 60 min with [^{35}S]methionine. (a) Intracellular 140S and 70S structures were isolated from cytoplasmic extracts by sucrose gradient centrifugation as described in Materials and Methods. Samples from each fraction were assayed for acid-insoluble radioactivity. (b) Intracellular 14S structures were isolated from the cytoplasmic extract in panel a by sucrose gradient centrifugation as described in Materials and Methods. Samples were assayed as in panel a. The box at the bottom (left) of the gradient represents the acid-precipitable counts in the pellet. Acid-derived ^{125}I-labeled 12S subunits were centrifuged in a parallel gradient, and their position is indicated in panel b by the arrow. Centrifugation is from right to left.

labeling, the cells were washed and lysed, and intracellular capsid structures were obtained as described by Yafal and Palma (42). The 140S and 70S structures (empty capsids) were isolated by centrifugation of infected cell cytoplasm on a 10 to 50% (wt/vol) sucrose gradient in NET buffer in an SW41 rotor at 17,000 rpm for 17 h at 4°C. The 14S structures were isolated by centrifugation of infected cell cytoplasm on a 5 to 20% (wt/vol) sucrose gradient in NET buffer in an SW41 rotor at 35,000 rpm for 18 h at 4°C.

Radioimmunoassay (RIA). Radioactively labeled antigens were incubated with antibody for 1 h at room temperature. A 10% suspension of *Staphylococcus aureus* cells bearing protein A was added, and the mixture was incubated for an additional 15 min at room temperature. The bacteria were pelleted and washed three times with NET–0.05% Nonidet P-40 (NP-40). The bound antigen-antibody complex was eluted in 0.063 M Tris hydrochloride (pH 6.8)–2% sodium dodecyl sulfate–0.68 M 2-mercaptoethanol–10% glycerol–0.005% bromophenol blue at 37°C for 10 min. The protein A-bearing *S. aureus* suspension was pelleted, and the supernatant was precipitated with trichloroacetic acid.

Hybridomas. The production and characterization of hybridomas from mice immunized with various antigens has been described previously (32, 37; C. A. Timpone, M.S. thesis, Cornell University, Ithaca, N.Y., 1982). Cloned monoclonal antibodies were purified from culture fluids of growing hybridoma cells by ultrafiltration followed by affinity chromatography on protein A-Sepharose 4B columns. Purified antibodies were iodinated by the Iodogen procedure, and the labeled antibodies were separated from free iodine by Sephadex G25 gel filtration (37).

PAGE. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) on 12.5% slab gels (13, 22) that were treated with sodium salicylate, dried, and exposed to Kodak Blue Brand X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C (6, 7). Viral proteins were identified according to their map coordinates (38).

Adsorption studies. BK cells were removed from 2-liter roller bottles by method 2 of Baxt and Bachrach (4). Radioactively labeled capsid structures were incubated at 4°C with 100 μl of cells at 5×10^7 cells per ml. At various times tubes were removed, cells were washed twice with phosphate-buffered saline, and trichloroacetic acid precipitates were prepared to determine cell-associated radioactivity. Competition experiments were performed by adsorption of unlabeled virus, at a ratio of 10^5 particles per cell, to 100 μl of cells for 1 h at 4°C before addition of the various labeled capsid structures. Samples were processed as described above.

RESULTS

Isolation of structural protein complexes from infected cells. Cytoplasmic extracts from infected cells analyzed by sucrose gradient centrifugation contained both 140S virus and 70S empty capsids (Fig. 1a) or 14S structures (Fig. 1b). The 140S peak contained structural proteins VP_1, VP_2, VP_3, and VP_4 and RNA. The 70S material lacked RNA, but contained VP_0, VP_1, VP_3, and small amounts of VP_2 and VP_4 (data not shown). A small peak or plateau is clearly present in the 14S region of the FMDV-infected cell extract (Fig. 1b). Similar results were obtained for cytoplasmic extracts treated with sodium deoxycholate (Fig. 1b), sodium deoxycholate plus NP-40, or Sarkosyl. In each case structural proteins VP_0, VP_1, and VP_3 were the predominant polypeptides present (data not shown). The small amount of 14S material found during continuous labeling contrasts with the relatively large amounts of such structures in poliovirus-infected cells (9, 35, 36).

A pulse chase experiment was used as an alternate method for detecting 14S structures. Infected cells were either pulsed for 10 min or pulsed for 10 min and chased for 20 min. The cytoplasmic extracts were analyzed by sucrose gradient centrifugation (Fig. 2a and b), and fractions from the 12S to 14S region were examined by PAGE (Fig. 2c). A shoulder of labeled material present in the 12S region during the pulse contained structural proteins VP_0, VP_1, and VP_3 as well as some structural protein precursor, P1 (P91). After the 20-min chase, a peak containing only VP_0, VP_1, and VP_3 was present in the 12S to 14S region (Fig. 2c).

Additional evidence for 14S structures was obtained by incubation of cytoplasm from infected cells with a ^{125}I-labeled purified monoclonal antibody which is reactive with capsid structures (see following section and Table 1). The reaction mixture analyzed by sucrose gradient centrifugation contained free-labeled antibody (7S) and a peak of radioactive material containing antibody presumably bound to 14S structures sedimenting at approximately 16S (Fig. 3a). This antibody also reacted with 140S and 70S structures in a

similar assay (data not shown; note the pellet, shown as the hatched area at the bottom of the gradient in Fig. 3a). Radioactivity in the 16S peak and the pellet size were reduced by prior incubation of the cytoplasm with unlabeled, purified monoclonal antibody; a concomitant increase in free antibody was noted (Fig. 3b). Incubation of the cytoplasm with a ^{125}I-labeled monoclonal antibody to purified bluetongue virus did not result in a 16S peak (Fig. 3c).

Antigenic comparison of capsid structures from infected cells. The capsid structures isolated from infected cells were reacted with monoclonal antibodies in a RIA (Table 1). Neutralizing antibodies used include those which recognize six different epitopes on the virus particle (37; Timpone, M.S. thesis; M. Grubman, unpublished observations), i.e., 2PD11, 2FF11.11, 6EE2, 7SF3, 6FF5, and 6HC4. Antibodies 2PC5 and 6HE4 neutralize but apparently react with the same epitopes as 2PD11 and 7SF3, respectively, while 2FF11.1 and 6AE9 react only with acid-derived 12S subunits (5, 32, 37; Timpone, M.S. thesis).

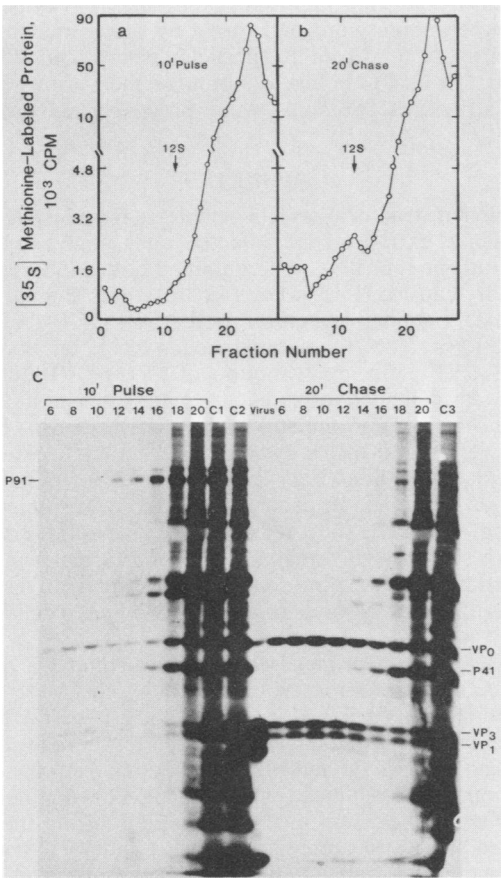

FIG. 2. Sucrose gradient (a, b) and PAGE (c) analysis of a pulse chase experiment in infected cells. FMDV-infected BK cells were labeled with [^{35}S]methionine for 10 min and then chased with a 100-fold excess of unlabeled methionine for 20 min. Cytoplasmic extracts were prepared and centrifuged on 5 to 20% (wt/vol) sucrose gradients as described in the legend to Fig. 1b. Samples from each fraction were assayed for acid-insoluble radioactivity, and alternate fractions were acetone precipitated and analyzed by PAGE. The numbers above the gel in panel c refer to the gradient fraction numbers of the various samples; C1, C2, C3, and Virus refer to cytoplasmic extracts before centrifugation after a 10-min label, 20-min chase, or 60-min label and purified FMDV, respectively.

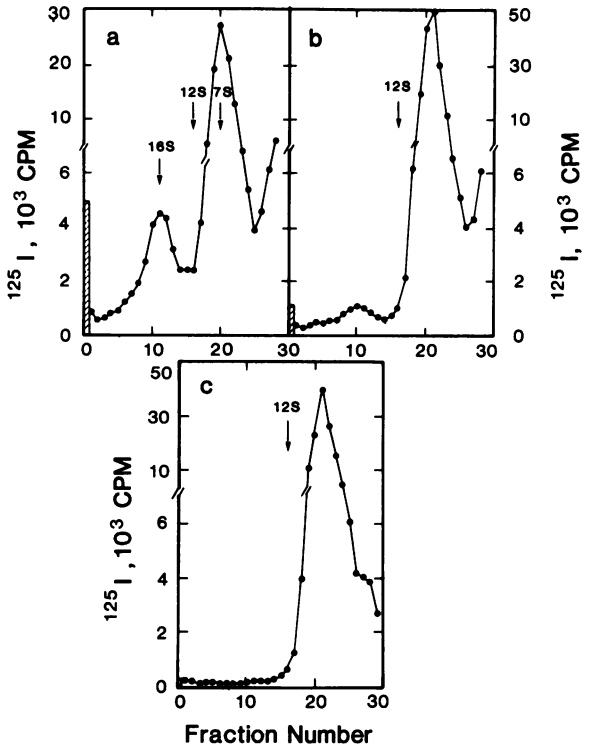

FIG. 3. Identification of 14S structural protein complexes by reaction of an infected cell cytoplasm with a labeled monoclonal antibody. BK cells were infected with FMDV, and a lysate was prepared as described in Materials and Methods (approximately 10^7 cells per ml). The lysate (20 μl) was incubated for 60 min at room temperature with either (a) NET–0.05% NP-40 buffer, (b) approximately 54 ng of purified cloned monoclonal antibody 6EE2, or (c) buffer. Approximately 50 ng of ^{125}I-labeled 6EE2 was added to the systems shown in panels a and b, while an approximately equivalent amount of a purified ^{125}I-labeled bluetongue virus monoclonal antibody was added to the system shown in panel c. Incubation was done as described above. Each reaction mixture was diluted to 1 ml with NET buffer and centrifuged on a 5 to 20% (wt/vol) sucrose gradient in NET buffer at 30,000 rpm for 17.5 h at 4°C in an SW41 rotor. Fractions were collected, and radioactivity was counted in a gamma counter. Acid-derived ^{125}I-labeled 12S subunits, centrifuged on a parallel gradient, and ^{125}I-labeled immunoglobulin G which has a sedimentation coefficient of 7S, were used as markers. The hatched box at the bottom (left) of the gradients represents the acid-precipitable counts in the pellet.

Intact FMDV characteristically reacts with antisera against VP_1, but lacks reactivity with antisera against VP_2 or VP_3 (2, 18, 30). Intracellular virus and empty capsids react essentially the same as purified virus with antisera against these three capsid polypeptides and with the monoclonal antibodies used (Table 1). Furthermore, titration of the neutralizing monoclonal antibodies with intracellular virus and empty capsids demonstrated almost identical avidity to that of purified virus (data not shown). However, monoclonal antibody 6FF5 had a lower avidity for virus and empty capsids than did the other antibodies (37; data not shown). The 14S subunits also reacted similarly to virions and empty capsids with the monoclonal antibodies, but were distinguishable from the larger structures because of their reactivity with antisera against VP_2. The 14S particles containing VP_0, VP_1, and VP_3 also showed different binding characteristics than acid-derived 12S structures which contain VP_1, VP_2, and VP_3 (Table 1).

TABLE 1. RIA of FMDV antibodies with capsid structures isolated from infected cells[a]

Antibody[b]	Dilutions	% bound[c]				
		Purified virus[d]	Intracellular 140S[d]	Empty capsids[d]	14S[d]	Acid derived 12S[d]
aVirus	1:20 guinea pig serum	51.4	44.8	36.2	43.0	40.1
aVP_1	1:20 guinea pig serum	42.3	47.6	38.0	43.5	23.5
aVP_2	1:20 guinea pig serum	1.1	0.8	1.5	12.2	23.2
aVP_3	1:20 guinea pig serum	0.3	0.7	0.0	0.8	4.0
2PD11.12.8.1	1:10 MAF[e]	48.7	36.9	30.0	26.0	23.6
2PC5.6	1:10 MAF	48.2	38.8	33.7	14.0	27.1
2FF11.1.6	1:10 MAF	0.0	1.1	0.6	0.0	20.8
2FF11.11.2	Undiluted TCF[f]	45.3	46.2	31.9	18.5	25.0
6EE2	Undiluted TCF	44.3	38.1	31.2	21.0	24.5
6HE4	Undiluted TCF	42.3	43.9	33.3	22.5	20.4
6HC4	Undiluted TCF	38.3	38.5	33.2	17.1	24.2
6AE9	Undiluted TCF	0.5	1.1	0.7	3.3	21.2
6FF5	Undiluted TCF	27.7	30.3	30.9	18.5	4.9
7SF3.1.H3	Undiluted TCF	36.6	38.9	32.7	24.2	13.7

[a] RIA was performed as described in Materials and Methods.

[b] aVirus, Antisera produced by injection of ethylenimine-inactivated type A_{12} virus, strain 119ab (3), into guinea pigs; aVP_1, antisera produced by injection of VP_1 into guinea pigs; etc. Monoclonal antibodies 2PD11.12.8.1, 2PC5.6, 2FF11.1.6, 2FF11.11.2, and 7SF3.1.H3 are referred to in the text as 2PD11, 2PC5, 2FF11.1, 2FF11.11, and 7SF3, respectively. Monoclonal antibodies 6EE2, 6HE4, 6HC4, and 6AE9 used in this experiment and that shown in Table 2 were not cloned, but identical results were obtained after two clonings. Antibody 6FF5 has not been cloned.

[c] A monoclonal antibody against bluetongue virus type 17 and normal guinea pig serum were included as controls for each antigen, and the average counts per minute bound were subtracted from both the input counts per minute and counts per minute bound of the particular antigen. The percent bound was then calculated by dividing the corrected counts per minute of an antigen bound by the corrected input counts per minute for this antigen and multiplying by 100.

[d] Purified virus, intracellular 140S, empty capsids, and 14S particles were purified as described in Materials and Methods. Acid-derived 12S subunits were prepared by treating virus with NET buffer (pH 5.5) and heating at 55°C for 15 min, and 12S material was purified by sedimentation on a 5 to 20% (wt/vol) sucrose gradient.

[e] MAF, Mouse ascitic fluid.

[f] TCF, Tissue culture fluid from growing hybridoma cells.

Antigenic analysis of cell-free assembled capsid structures. Capsid intermediate structures sedimenting at 110S, 60S, 14S, and 5S were isolated from cell-free protein-synthesizing systems and reacted in RIA with the antibodies described in Table 1. Neutralizing monoclonal antibodies 2PD11, 2FF11.11, 6EE2, 7SF3, 6FF5, and 6HC4 all reacted with the three largest cell-free capsid structures (Table 2), as had the infected cell structures (Table 1). The reduced binding of 110S structures compared with 60S and 14S particles may be caused in part by the cosedimentation with 110S structures of viral proteins 2A (P14) and 2C (P41) (13). PAGE analysis revealed that the only viral proteins from the 110S region immunoprecipitated by antiserum against inactivated virus (Fig. 4b) and by the above monoclonal antibodies (data not shown) were structural proteins VP_0, VP_1, and VP_3. The cosedimenting nonstructural proteins 2A (P14) and 2C (P41) were not precipitated by these antibodies (13; Fig. 4b).

In contrast to their lack of reactivity with virus and empty capsids, VP_2 and VP_3 antisera reacted with 110S and 60S structures from the cell-free system. Similarly, 6AE9 and 2FF11.1, which bound only acid-derived 12S subunits (Table 1), reacted very well with cell-free structures. To examine the relative binding of the monoclonal antibodies with the cell-free structures, various antibodies were titrated by RIA (Fig. 5). At a dilution of 1:100 or 1:1,000, VP_2 and VP_3 antisera showed little binding to 110S structures, while at these dilutions the binding of VP_1 antiserum was hardly affected (Fig. 5b). Similarly, dilution of 6AE9 and 6FF5 resulted in a rapid decrease in binding (Fig. 5c), while neutralizing monoclonal antibodies 2PD11, 2FF11.11, 6EE2, and 7SF3 bound well to the antigen even at high dilutions (Fig. 5b and c). The neutralizing antibodies, including 6FF5, behaved almost identically when titrated with purified virus (data not shown). However, 2FF11.1 still bound to cell-free 110S structures even at a 1:1,000 dilution. Similar results were obtained with 14S cell-free structures (unpublished observations).

Specific binding of capsid structures to susceptible cells. Capsid structures from infected cells and the cell-free system were assayed for binding to susceptible BK cells. In a typical binding assay about 40 to 50% of purified virus bound to BK cells over a 30 min period (data not shown). When cells were first saturated with unlabeled virus, the subsequent addition of labeled virus was substantially inhibited, demonstrating that the binding was specific (Table 3). Similarly, the binding of intracellular 140S, empty capsids, and acid-derived 12S subunits to BK cells were also specific, based on the inhibition of their binding to cells by unlabeled virus (Table 3).

Cell-free 110S and 14S capsid structures both bound to BK cells, and binding increased with time (Table 3; data not shown). The binding of these structures was inhibited by unlabeled virus, although not as completely as the binding of control virus (Table 3).

DISCUSSION

Our results indicate that (i) various capsid intermediate structures are present in FMDV-infected cells, (ii) 14S particles in infected cells are relatively short lived, (iii) virus particles and empty capsids from infected cells are antigenically indistinguishable by the criteria used, and (iv) structural protein complexes assembled in an FMDV RNA-programmed cell-free translation system resemble comparable structures from FMDV-infected cells in S rates, protein

composition, and their ability to bind specifically to both antisera and susceptible cells.

The morphogenesis of FMDV has not been as well studied as that of other picornaviruses, but it has been suggested that empty capsids are precursors of intact virions (42). The present study demonstrates that relatively stable 70S empty capsids and 140S virions are present in FMDV A_{12}-infected cells (Fig. 1a). In contrast, the 14S particles which we have detected and which have not been previously described in FMDV-infected cells, are relatively short lived. Whether they are precursors of empty capsids has not been demonstrated. However, in size and protein composition they resemble precursor 14S particles present in other picornavirus-infected cells (9, 28, 29, 35, 36, 41).

Monoclonal antibodies elicited with either inactivated virus, VP_1, or a cyanogen bromide fragment of VP_1 as well as polyclonal antisera against VP_1, VP_2, and VP_3 were used in RIA against various capsid structures. Two of the monoclonal antibodies, 2PD11 and 2FF11.11, appear to be conformation site specific, since by Western blot analysis they do not react with VP_1 or any other viral structural proteins (unpublished observations). The other four epitopes reactive with 6EE2, 6FF5, 6HC4, and 7SF3, appear to be continuous sequences because these monoclonal antibodies bind to various fragments of VP_1 (37). Also, antibodies 6EE2 and 6HC4 inhibit the adsorption of virus to susceptible cells, perhaps by reacting with the A_{12} virus cell attachment site (5) or by altering virus conformation (10, 25).

Conformation-dependent epitopes may play an important role in eliciting protective responses against FMDV in animals, since the amount of virus-derived VP_1, biosynthetically synthesized VP_1, or fragments of VP_1 required to elicit immunity are significantly greater than the equivalent amount of VP_1 present in inactivated whole-virus vaccines (2, 21, 32). Although epitopes involved in neutralization are present on intact VP_1 and fragments of this protein (37), it appears that these epitopes are not as effective as conformation-dependent epitopes, at least for type O_1 FMDV, in eliciting neutralizing antibodies which can protect cattle and swine against challenge (D. O. Morgan, unpublished observations).

RIAs demonstrated that the six distinct neutralizing epitopes present on virions are also on empty capsids and 14S particles from infected cells (Table 1). The antibodies to these epitopes have a similar avidity for virions and empty capsids. Because of the difficulty in obtaining 14S particles, the avidity of the antibodies for these particles could not be determined. Although it appears that the six neutralization epitopes described are present on an early putative assembly

TABLE 2. RIA of FMDV antibodies with cell-free assembled structural protein complexes[a]

Antibody[a]	Dilutions	% bound[a]			
		110S[b]	60S[c]	14S[d]	5S[e]
aVirus	1:20 guinea pig serum	27.4	34.5	47.0	4.3
aVP_1	1:20 guinea pig serum	33.0	87.2	38.4	3.7
aVP_2	1:20 guinea pig serum	20.0	77.1	44.2	0.6
aVP_3	1:20 guinea pig serum	14.8	40.5	3.3	1.3
2PD11.12.8.1	1:10 MAF[a]	15.2	63.3	37.5	0.3
2PC5.6	1:10 MAF	11.0	51.0	42.8	0.3
2FF11.1.6	1:10 MAF	9.1	51.5	3.3	0.1
2FF11.11.2	Undiluted TCF[a]	27.5	90.2	35.0	0.4
6EE2	Undiluted TCF	33.9	131.9	34.2	0.7
6HE4	Undiluted TCF	26.5	86.6	50.9	0.4
6HC4	Undiluted TCF	33.0	112.8	39.2	0.5
6AE9	Undiluted TCF	35.3	94.7	35.9	1.7
6FF5	Undiluted TCF	31.0	111.9	33.9	1.5
7SF3.1.H3	Undiluted TCF	30.6	94.9	44.6	0.3

[a] RIA, % bound, antibody, MAF, and TCF are defined in Table 1 footnotes.
[b] Cell-free lysate programmed with FMDV RNA and not treated with detergent was centrifuged on a sucrose gradient, and the material sedimenting at 110S was used in the assay.
[c] Cell-free lysate programmed with FMDV RNA treated with 1% NP-40 was centrifuged on a sucrose gradient, and the material sedimenting in the 60S region was isolated and recentrifuged on a second sucrose gradient. The peak of material sedimenting at 60S was pooled and used in the assay.
[d] Cell-free lysate programmed with FMDV RNA treated with 1% NP-40 was centrifuged on a sucrose gradient, and 14S structures were isolated and used in the assay.
[e] These structures were isolated as described in reference 13.

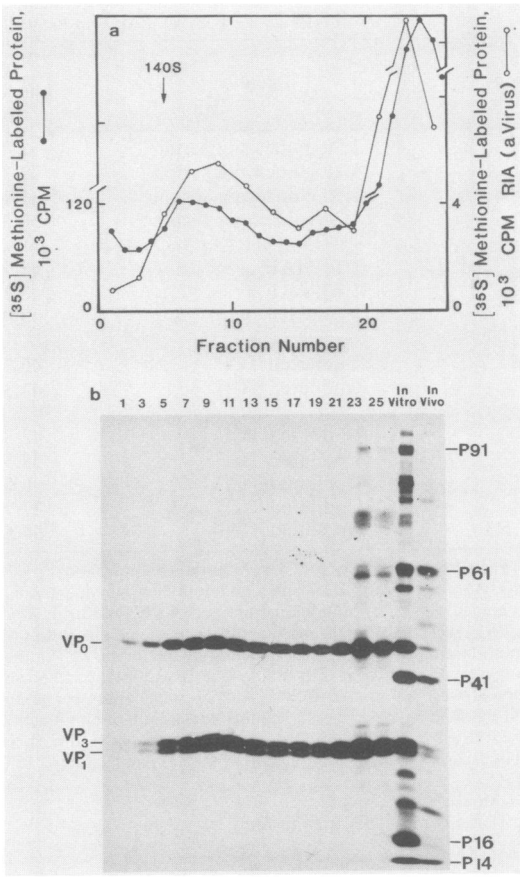

FIG. 4. Sucrose gradient (a) and PAGE (b) analysis of immunoprecipitation with inactivated virus antisera of large complexes synthesized in a cell-free system. FMDV RNA was translated in a cell-free system in the presence of [^{35}S]methionine for 4 h. The lysate was incubated with EDTA-pancreatic RNase A, diluted with NET buffer, and centrifuged on a 10 to 50% (wt/vol) sucrose gradient as described in the legend to Fig. 1a. Portions of each fraction were assayed for hot acid-insoluble radioactivity (●). Alternate fractions were immunoprecipitated with a 1:20 dilution of antisera elicited with inactivated virus and protein A-containing *S. aureus* cells. A portion of the eluted material was assayed for hot acid-insoluble radioactivity (○), and the remainder was analyzed by PAGE. [^{35}S]methionine-labeled 140S FMDV was centrifuged on a parallel gradient as a marker. 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, and the in vivo labeled lane is an infected cell lysate.

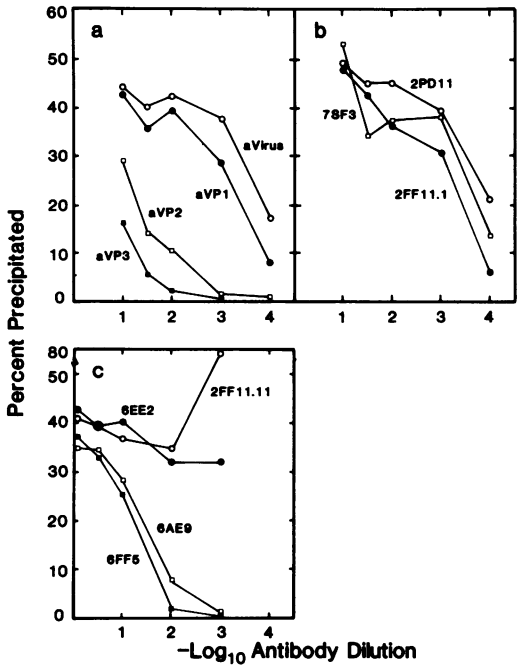

FIG. 5. RIA of antibodies with cell-free 110S antigen. Polyclonal and monoclonal antibodies were diluted with NET–NP-40 buffer and RIA with [^{35}S]methionine labeled 110S cell-free complexes isolated from sucrose gradients was performed as described in Materials and Methods. (a) Guinea pig antisera to virus, VP_1, VP_2, and VP_3; (b) mouse ascitic fluids from cloned monoclonal antibodies 2PD11, 2FF11.1, and 7SF3; (c) tissue culture supernatants from cloned monoclonal antibodies 2FF11.11, 6EE2, 6AE9, and uncloned 6FF5.

product in FMDV morphogenesis, it is also clear that the 14S particles are conformationally distinct from 140S and 70S particles because they are reactive with VP_2 antisera (Table 1).

Empty capsids appear antigenically identical to virions by the criteria used in this study. This is in accord with the observations of Rweyemamu et al. (39) that FMDV type A Pando empty capsids are as immunogenic as virions in guinea pigs and that antisera raised against empty capsid have the same serological specificity in neutralization tests as that of sera prepared against virions. Nevertheless, this apparent antigenic identity between FMDV virions and empty capsids may be a reflection of the particular set of monoclonal antibodies used in the present study. Thus, Ferguson et al. (11) and Emini et al. (9) have shown that certain poliovirus monoclonal antibodies, both neutralizing and nonneutralizing, can distinguish between virions and empty capsids.

TABLE 3. Specificity of binding of infected cell and cell-free isolated capsid structures

Structure used	% Binding[a]	% Inhibition[b]
Control virus	50.4	89.7
Intracellular 140S	31.6	81.7
Control virus	50.4	89.7
Empty capsids (70S)	30.0	82.7
Control virus	45.9	77.0
Acid-derived 12S	36.1	70.0
Control virus	58.6	78.2
Cell-free 110S	29.4	23.5
Control virus	65.3	65.0
Cell-free 14S	54.7	38.0

[a] Percent binding after 30 min.

[b] Percent inhibition of binding of labeled capsid structures by unlabeled virus after 30 min. The percent inhibition was determined by the following formula: % Inhibition = 100% − % binding in presence of unlabeled virus/% binding in absence of unlabeled virus.

RIAs also revealed that the six distinct neutralizing epitopes, including the two conformation site specific epitopes identified, are all present on previously described (13) cell-free assembled 110S, 60S, and 14S structures. Antibodies to these epitopes appear to have the same avidity for 110S and 14S structures as for virions and empty capsids. However, in contrast to virions and empty capsids, cell-free 110S and 60S structures appear to possess additional antigenic sites (Table 2). Titration of 110S structures with the various antibodies revealed, however, that the additional antigenic sites present at low antibody dilutions may simply be the result of antibody excess and low levels of antigen expressing these determinants (except for 2FF11.1) (Fig. 5).

All of the cell-free and infected-cell capsid structures tested bound to susceptible cells. Binding by intracellular 140S, empty capsids, and acid-derived 12S structures was competitively inhibited by unlabeled virus to the same extent as was binding by control virus (Table 3). Binding by cell-free 110S and 14S structures was also inhibited by unlabeled virus, but not to the same extent as for the isolated intracellular structures. This apparent difference may reflect, in part, the cosedimentation of membrane-associated nonstructural proteins 2A and 2C (17) with the 110S complex and of a reticulocyte protein with 14S structures (13). Perhaps proteins 2A and 2C may nonspecifically bind to cells, thus decreasing the apparent inhibition of binding of the cell-free structures by unlabeled virus.

The 110S particles formed in the cell-free system are unstable and, on recentrifugation or treatment with NP-40 or Sarkosyl, sediment mainly at approximately 60S (13). Under these conditions virions remain intact. Preliminary experiments indicate that the 110S particles are not infectious (unpublished observations), but it is not known whether they contain virion RNA. The 110S structure may be (i) an aggregate of more stable 60S particles, (ii) similar to provirions (12, 36), or (iii) an unstable intermediate lacking RNA.

The present results and previous biochemical evidence (13) indicate that the cell-free system appears to be useful for examining the assembly steps involved in FMDV morphogenesis, including the role of membranes (36), the mechanism and time of addition of the virion RNA, and the mechanism of cleavage of VP_0 into VP_4 and VP_2. The cell-free self-assembly of structural proteins into capsidlike structures antigenically similar to virions also suggests the possibility of constructing recombinant DNA vectors which could express, in an appropriate host, conformationally correct immunogens that lack nucleic acid and are effective and safe.

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