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Foot-and-Mouth Disease Virus and Poliovirus Particles Contain Proteins of the Replication Complex

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Nonstructural proteins 2C, 3CD, 3C, and 3D, and the cellular protein actin, are present in highly purified preparations of foot-and-mouth disease virus (FMDV) and poliovirus. They remain bound in variable amounts to the RNAs when the RNAs are extracted from the viruses with phenol or phenol-sodium dodecyl sulfate (SDS) and, for FMDV, when the RNA is released from the particles by a lowering of the pH below 7. RNA prepared by these methods is rapidly degraded at 37°C, particularly in the presence of NH_4^+ ions, but hydrolysis can be prevented by antibody against *Escherichia coli*-expressed 3D, indicating that it is the RNA polymerase that has nuclease activity. In contrast, virion RNA from which the nonstructural proteins and actin have been removed by extraction with guanidine thiocyanate-phenol-chloroform or proteinase K-phenol is stable at 37°C, although its specific infectivity is lower than that of the RNA extracted with phenol or phenol-SDS. The possible implications of the close association of replication complex proteins with the RNA in virus particles are discussed.

Picornaviruses consist of one molecule of positive-sense single-stranded RNA with a molecular weight of 2,600,000 (2,600K); four capsid proteins, VP1 through VP4 (60 copies of each); and a small but variable number of copies of VP0. In addition to the copy of VPg covalently linked to the RNA, traces of other proteins have been found consistently in highly purified preparations of foot-and-mouth disease virus (FMDV) (12, 16, 17, 20, 25) and in poliovirus and bovine enterovirus (19b). One of the proteins in FMDV particles has been considered to be actin on the basis of its molecular weight and isoelectric point (12). A 56K protein has been postulated to be viral RNA polymerase (25) on the basis of its molecular weight and its reaction with serum from convalescent and vaccinated animals.

In a recent study we provided further evidence that 3D is a component of FMDV particles by showing that these particles react with antibody against the *Escherichia coli*-expressed enzyme (20). Moreover, we showed that antibody against the enzyme partially inhibited degradation of the RNA, when it was released from FMDV particles, by lowering the pH to 5, suggesting that the RNA polymerase has nuclease activity. This observation provides an explanation for the degradation of the RNA within FMDV particles when they are kept at 37°C (5). Poliovirus (28) and rhinovirus (11) also lose infectivity under similar conditions, probably by the same mechanism, although rather more slowly. Furthermore, an endoribonuclease activity was described in poliovirus (14), and we recently obtained preliminary evidence that poliovirus and encephalomyocarditis virus contain the corresponding enzyme (20). In situ degradation of the RNA in FMDV and poliovirus particles is greatly enhanced by NH_4^+ ions (26, 28) and is almost certainly related to the increase in enzyme activity that they induce (20, 21, 24, 25). These observations are suggestive of the close spatial relationship between the enzyme and the RNA in the virion.

Antibody against the RNA polymerase of FMDV reacts with virus particles in the enzyme-linked immunosorbent assay and the radioimmunoassay (RIA). Interestingly, immunoelectron microscopy with anti-3D antibody and gold-labeled antispecies antibody revealed that the reaction occurred at only one site on the particles. This contrasts with the reaction with antibody against the immunodominant site on VP1, where several molecules attach to the surface of each particle. The observation that trypsin cleaves virion 3D provides further evidence that part of it is on or close to the surface of the particles. However, trypsin-treated particles still react with anti-3D antibody and retain their nuclease activity (20), suggesting that the cleavage products of 3D are within the virus particles.

In this paper we describe further experiments with the RNA polymerase of the FMDV particle and extend our studies to other minor polypeptides which are present in both FMDV and poliovirus. It has been suggested that other nonstructural polypeptides of the replication complex may be involved in the infectious process or encapsidation of the RNA (1).

MATERIALS AND METHODS

Virus growth and assay. FMDV of serotype A, subtype 12, strain 119, was grown in BHK 21 monolayer cells in Eagle's medium. Poliovirus of serotype 1, Mahoney strain, was grown in Vero monolayer cells in Eagle's medium. The viruses were titrated by measuring the cytopathic effect in BHK 21 cells or Vero cells on microtiter plates.

Virus labeling and purification. Conventional methods were used for growing the viruses in the presence of [^{3}H]uridine in Eagle's medium or [^{35}S]methionine in the appropriately depleted medium. Harvests were pelleted at 200,000 × *g* for 1 h, and the resuspended pellets were clarified, made 1% with respect to sodium dodecyl sulfate (SDS), and centrifuged at 200,000 × *g* for 2 h in a 15 to 45% (wt/vol) sucrose gradient layered over 1 ml of saturated CsCl. After fractionation into 0.5-ml aliquots, 20-μl samples were assayed for radioactivity. The peak radioactive fractions were combined for further analysis. In some experiments the sucrose-gradient-purified FMDV was also centrifuged in a preformed CsCl (1.40 to 1.50 g/ml) or Nycodenz (Sigma, St. Louis, Mo.) gradient.

Antisera. Antibodies to *E. coli*-expressed FMDV 2C and 3D, encephalomyocarditis virus 3D, and FMDV VPg were raised in guinea pigs. Antisera against poliovirus 2C, 3C, and 3D and FMDV 3C were raised in rabbits. Convalescent guinea pig antiserum was obtained from animals infected at this laboratory with virus of serotype A, subtype 12. The antibody against poliovirus, type 1, was a mouse monoclonal preparation. Antiactin antiserum was purchased from Boehringer-Mannheim, Indianapolis, Ind.

RIA. Fifty microliters of purified [^{35}S]methionine-labeled virus was incubated with 50 μl of serum dilution, either for 2 h at 37°C or for 18 h at 4°C. The mixture

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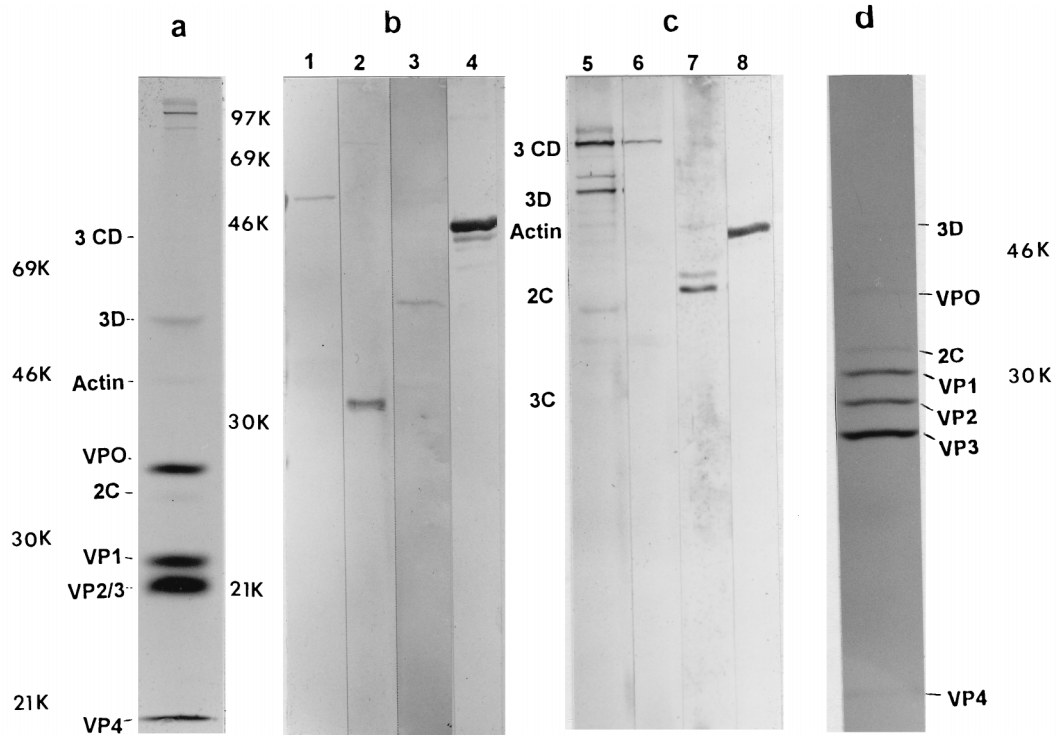

FIG. 1. SDS-PAGE analysis of the proteins of [^{35}S]methionine-labeled FMDV (a) and poliovirus (d) particles. (b and c) Western blot analysis of FMDV (lanes 1 to 4) and poliovirus (lanes 5 to 8) proteins by using specific antibodies: lanes 1 and 5, anti-3D; lanes 2 and 6, anti-3C; lanes 3 and 7, anti-2C; lanes 4 and 8, antiactin.

was then incubated with 50 μl of 10% protein A (IgG Sorb; The Enzyme Center, Malden, Mass.) for 1 h at 4°C, and the precipitate was collected by centrifugation and washed three times with high-salt RIPA (10 mM Tris [pH 7.6], 500 mM NaCl, 5 mM EDTA, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS). The precipitate was then resuspended in RIPA containing 150 mM NaCl for counting.

Analysis of proteins by SDS-polyacrylamide gel electrophoresis (PAGE). The method described by Laemmli (15) was used.

Analysis of viral RNA. RNA was extracted from purified [^{35}S]methionine or [^{3}H]uridine virus preparations by several methods: (i) by reducing the pH of the suspension to 5.0 with 0.1 M acetate in the presence of 0.1% SDS; (ii) by extracting the preparation with phenol (molecular biology grade; Sigma) which had been saturated with water; (iii) by reducing the pH to 5.0 with 0.1 M acetate–0.1% SDS and extracting with phenol saturated with water; (iv) by treatment with TRI reagent LS (guanidine thiocyanate-phenol; Molecular Research Center, Cincinnati, Ohio) followed by extraction with chloroform; and (v) by treatment with 0.5% SDS, 5 mM EDTA, (pH 8) and 0.5 mg of proteinase K per ml for 30 min at 37°C followed by extraction with phenol. In method i, the RNA was centrifuged directly in a 5 to 25% sucrose gradient prepared in 0.1 M acetate–0.1% SDS, pH 5.0. In methods ii, iii, iv, and v, the RNAs were mixed with 200 μg of tRNA and precipitated at −20°C with one volume of isopropanol. The precipitates were resuspended in 0.1 M acetate–0.1% SDS, pH 5.0, for further analysis by gradient centrifugation. After fractionation into 0.5-ml aliquots, 100 μl (for the [^{35}S]methionine preparations) or 20 μl (for the [^{3}H]uridine preparations) was counted. RNA preparations were tested for infectivity by mixing the dilutions with 2% Lipofectamine (Life Technologies, Gaithersburg, Md.) for 30 min and then diluting 10-fold with 0.04 M phosphate buffer, pH 7.6, containing 0.2% Lipofectamine before adding them to monolayers of BHK 21 cells (for FMDV) or Vero cells (for poliovirus). After 15 min at 37°C, the monolayers were washed with Eagle's medium at 37°C for 30 min and then overlaid with 0.5% agarose and incubated at 37°C in a 5% CO_2 incubator for 2 days before staining with crystal violet.

Electron microscopy. Sucrose-gradient-purified preparations were adsorbed to Formvar-coated carbon-stabilized grids, which were then blocked with 1% bovine serum albumin, 0.2% gelatin, 50 mM Tris-HCl, and 150 mM NaCl (pH 7.4) for 30 min. Guinea pig antiserum against the polymerase (20) diluted 1:100 in blocking buffer was added, and the mixtures were incubated overnight at 4°C. Then, either the grids were stained with 2% phosphotungstic acid (pH 7.4) or the virus-antipolymerase complexes were reacted with goat anti-guinea pig antiserum conjugated with 10-nm colloidal gold (Zymed, San Francisco, Calif.) diluted in blocking buffer before staining. The grids were then examined in a Philips 410 electron microscope at 80 kV.

RESULTS

Presence of minor polypeptides in FMDV and poliovirus. SDS-PAGE of the proteins of highly purified [^{35}S]methionine-labeled FMDV and poliovirus particles revealed several minor bands besides the well-characterized capsid proteins VP1 through VP4. In addition to VP0, the precursor of VP4 and VP2 which is present in a small but variable number of copies, in some experiments in which the gels were exposed to X-ray film for a prolonged period, other bands, of 68, 56, 43, 30, and 22K for FMDV (Fig. 1a and 2b, lane 3) and 70, 56, 43, and 35K for poliovirus, were detected. By using 20 μg of purified virus, these protein bands were identified by Western blotting with specific antisera as 3CD, 3D, actin, 2C, and 3C, respectively, for FMDV (Fig. 1b) and 3CD, 3D, actin, and 2C for poliovirus (Fig. 1c). In poliovirus, 3CD and 3D were present in approximately equal proportions, whereas there was a preponderance of 3D in FMDV.

It is highly unlikely that these minor polypeptides are present adventitiously, because two cycles of sucrose gradient centrifugation after treatment with SDS failed to remove them. An additional step, in which the sucrose-gradient-purified virus was centrifuged in a CsCl or Nycodenz gradient, also failed to remove them, in agreement with the results of others (12, 25). Since the nonstructural L protein of FMDV was not detected, nonspecific attachment of the other minor polypeptides seems improbable. Furthermore, virus grown in the presence of [^{3}H]choline did not contain any radioactivity, indicating that the nonstructural proteins are not bound to the virus particles by lipid membranes.

Location of the minor polypeptides on FMDV RNA. [^{35}S]methionine-labeled FMDV was adjusted to pH 5 with 0.1 M acetate–0.1% SDS and either centrifuged directly in a sucrose gradient prepared in 0.1 M acetate–0.1% SDS (pH 5) or

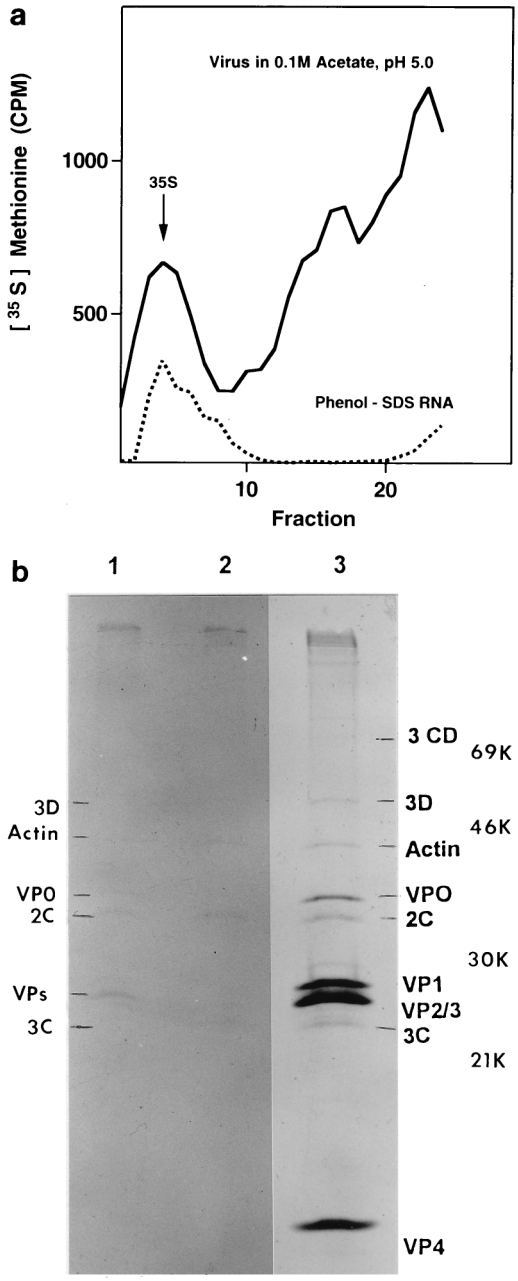

FIG. 2. (a) Sucrose gradient analysis of RNA released from [^{35}S]methionine-labeled FMDV by lowering the pH to 5.0 or by extraction with phenol-SDS at pH 5.0. (b) SDS-PAGE analysis of proteins associated with the extracted RNA. Lane 1, acid-released RNA; lane 2, phenol-SDS-extracted RNA; lane 3, virus particle proteins.

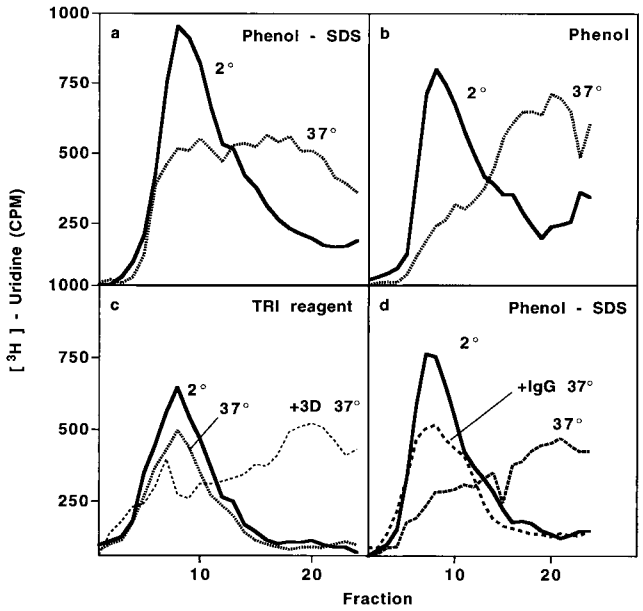

FIG. 3. Sucrose gradient analysis of RNA extracted from [^{3}H]uridine-labeled FMDV by different procedures, showing the effect of heating at 37°C for 4 h in the presence of NH_4^+ ions on the sedimentation rate. RNA was extracted with phenol-SDS (a), phenol (b), or TRI reagent (c). Panel c also shows the effect of added *E. coli*-expressed 3D, and panel d shows the inhibitory effect of anti-3D immunoglobulin G on the degradation of phenol-SDS-extracted RNA heated at 37°C for 6 h in the presence of NH_4^+ ions.

extracted with phenol before centrifugation of the RNA in the aqueous layer. The 35S RNA peak, which in each case was radioactive (Fig. 2a), was mixed with 200 μg of tRNA and precipitated with 2 volumes of ethanol. The radioactivity was not due to the presence of VPg on the RNA, since methionine is not present in this protein in the FMDV strain used. Analysis of the precipitates by SDS-PAGE showed the presence of faint radioactive bands corresponding to the minor bands found with the virus particles, together with traces of capsid proteins VP1 through VP3 (Fig. 2b) in the case of virus that had not been extracted with phenol.

Effect of 3D on virion RNA. It was shown several years ago that the RNA extracted from FMDV with phenol or phenol-SDS loses infectivity rapidly at 37°C (3, 4). The loss was at least as rapid as that found with virus particles. We have now shown that the loss of infectivity of isolated RNA is caused by degradation of the molecule and is particularly rapid in the presence of NH_4^+ ions. After 4 h at 37°C in 250 mM NH_4Cl in HEPES buffer, pH 8, most of the RNA sediments more slowly than 35S, the sedimentation coefficient of the intact molecule (Fig. 3a and b). Degradation was greatly reduced by mixing the RNA with RNase-free anti-3D immunoglobulin G, indicating that the 3D on the RNA is responsible for its hydrolysis (Fig. 3d).

The minor polypeptides can be removed with the TRI reagent and with proteinase K (see Materials and Methods). RNA obtained by these procedures is more stable than the phenol- or phenol-SDS-extracted RNA and still sediments at 35S (Fig. 3c) after being heated under the same conditions. However, addition of *E. coli*-expressed 3D degraded the RNA rapidly. Interestingly, RNA extracted with TRI reagent or proteinase K has a specific infectivity 10-fold lower than that of phenol-SDS-extracted RNA (Table 1). However, the infectivity of the TRI reagent- or proteinase K-phenol-extracted RNA is retained after it is heated at 37°C in 250 mM NH_4Cl, whereas that of the phenol- or phenol-SDS-extracted RNA is considerably lower after incubation under the same conditions. Parallel experiments with RNA of poliovirus extracted with TRI reagent or phenol-SDS gave essentially the same results, although the rate of degradation of the latter was much slower than that of FMDV RNA prepared by phenol-SDS extraction.

Location of the nonstructural proteins in the virus particles. In a previous study we had shown that antibody against 3D of FMDV reacted with virus particles and that this reaction occurred at a single site (20). We have now confirmed by RIA

TABLE 1. Effect of heating for 4 h at 37°C on the infectivity of RNA extracted with various reagents from FMDV and poliovirus particles[a]

RNA source	Extraction reagent(s)	Infectivity (PFU/ml) at:	
		2°C	37°C
FMDV	Phenol	3.2×10^4	30
	Phenol-SDS	2.8×10^4	2.7×10^2
	TRI	4.0×10^3	2.5×10^3
	Phenol-SDS	1.0×10^4	—[b]
	Proteinase K	1.3×10^3	1.2×10^3
Poliovirus	Phenol-SDS	1.1×10^4	30
	TRI	40	70

[a] [^{3}H]uridine-labeled virus was added to unlabeled virus to allow assessment of the specific infectivity of the RNA in each preparation.
[b] —, noninfectious.

that FMDV reacts with anti-3D antiserum at 4°C as well as at 37°C. However, there is no reaction between the virus particles and antibodies against actin and the other nonstructural proteins (Table 2). Some FMDV particles, when combined with anti-3D antibody, showed a protuberance at a single site (Fig. 4). Besides providing evidence in support of the results which indicated that only a single molecule of 3D is present in the virus particle, this result allows speculation about the release of the RNA when the virus infects cells (see Discussion).

In contrast to FMDV, the antibodies against poliovirus 3D did not react with intact particles, although they reacted with the protein when it had been released from the particles (Fig. 1c). These results indicate that FMDV 3D is exposed on the surface of about 20% of the particles, possibly because of the mobility of the capsid proteins of this virus.

DISCUSSION

We have shown consistently that nonstructural proteins 2C, 3C, 3CD, and 3D, components of the replication complex, and the cell protein actin are present in highly purified preparations of FMDV and poliovirus. The crucial issue initially was to determine whether these proteins were merely contaminants which had not been removed by the rigorous purification procedure. The method we used, namely, SDS treatment followed by sucrose gradient centrifugation, almost certainly removes any cellular material on the surfaces of the particles. Moreover, we have found that the addition of a CsCl or Nycodenz gradient step did not remove the minor polypeptides. There is already considerable evidence that the nonstructural protein 3D and the cellular protein actin are not contaminants but are located within FMDV particles (8, 12, 20). Particularly convincing evidence was provided by the observation that whereas only VP1 and 3D were labeled when FMDV was iodinated, up to 11 proteins were labeled when disrupted particles were iodinated (12). Moreover, it had been shown that FMDV particles did not hydrolyze added rRNA, but gently disrupted particles did so (8).

TABLE 2. RIAs of the reactions between FMDV and poliovirus particles and several specific antisera at 37°C

Antiserum	% Precipitated in presence of excess antiserum	
	FMDV[a]	Poliovirus[b]
FMDV 3D	16	1.5
Poliovirus 3D	27	3
EMCV[c] 3D	18	ND[d]
Actin	0	3
FMDV 3C	0	0
FMDV 2C	1.5	0
FMDV VPg	2.5	ND
Convalescent-phase FMDV	88	2
Poliovirus	0	87

[a] Values are means of nine assays.
[b] Values are means of two assays.
[c] EMCV, encephalomyocarditis virus.
[d] ND, not determined.

We had shown previously (20), using anti-3D antibody, that at least part of the RNA polymerase of FMDV is located at the surface of the virus particle, confirming the conclusions reached by others (12, 25). Using immunoelectron microscopy, we showed that antibody to 3D reacted at a single site on about 30% of the virus particles, in marked contrast to antibody against the immunodominant surface loop, which reacts at several sites. In confirmation of our earlier finding, only about 20% of the particles were precipitated by anti-3D antibodies in RIA. Moreover, virion 3D was cleaved by treatment with trypsin, but its ability to react with antibody or to hydrolyze the RNA within the virus particle was retained. This also suggests that only a part of the protein is exposed on the surface of the virus particle. Although these results could be interpreted as showing that only about 30% of the particles contain the enzyme, when the virus is incubated at 37°C all of the RNA is hydrolyzed. This suggests that all of the particles contain the enzyme, but it is exposed on only 20 to 30% of the particles. In contrast, actin is located within the particles and could not be iodinated until the particles were disrupted (12). In support of these observations, we have found that only anti-3D antibody reacts with intact particles in RIA whereas antibodies to 2C, 3C, 3D, and actin react with disrupted particles (Table 2 and Fig. 1b). We have now shown that poliovirus also contains 2C, 3C, 3CD, and 3D, and actin although, unlike FMDV, the virus particles are not precipitated with anti-3D antiserum (Table 2).

It is striking that antiserum against 3D of poliovirus and encephalomyocarditis virus also precipitated FMDV. However, comparison of the sequences of the 3D of each virus shows that there are several regions of amino acid sequence homology.

Treatment of FMDV with trypsin reduces the infectivity of most strains by about 3 log units. Capsid protein VP1 is cleaved at the GH loop and near the C terminus. Moreover, the minor polypeptides can no longer be detected, suggesting that the cleavage exposes the minor polypeptides to the enzyme. Nevertheless, trypsin-treated virus is still precipitated by anti-3D antibodies, indicating that the enzyme has been cleaved and not hydrolyzed into small fragments. The observation that the RNA extracted from trypsin-treated virus particles, in which the minor polypeptides can no longer be detected, is still infectious also suggests that intact minor polypeptides are not essential for retaining the infectivity of the RNA (4). Similarly, virus particles treated with chymotrypsin are infectious although the minor polypeptides are no longer detectable (25).

How the replication complex proteins could become encapsidated is suggested by recent work with poliovirus. It has been proposed that 2C has a role in virus structure and may function by facilitating assembly around newly synthesized RNA (19). More recently, it has been shown (2) that the replication complex proteins have an affinity for the 5′ end of newly synthesized sense RNA and membranes, and it has also been suggested (6) that 2C of the same virus is necessary for assembly.

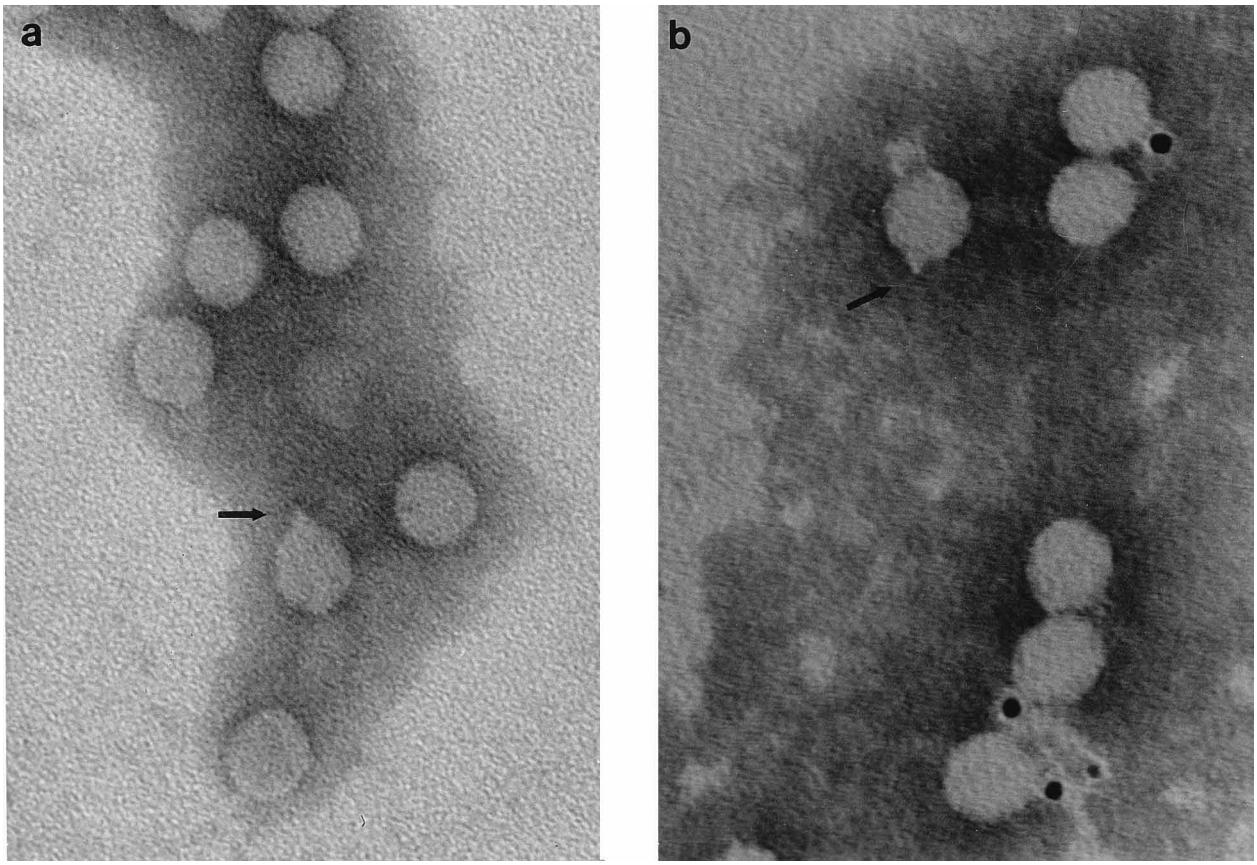

FIG. 4. Electron micrographs of FMDV particles incubated with anti-3D antibody alone (a) or with anti-3D antibody followed by antispecies antibody linked with gold particles (b). Note the single protuberances on the particles (arrows).

Additional evidence that mutations in the 3D of the structurally similar echovirus 12 lead to the production of unstable particles has also been provided (19a). Consequently, unless there is a mechanism to remove the replication complex proteins, it is easy to visualize how they could become encapsidated with the RNA to form particles. It is also possible, because of their intimate association with the RNA, that they form some or all of the RNA packaging signal. Recent studies indicate that the initial event in poliovirus encapsidation occurs in the replication complex (23). The empty particles present in virus harvests would be formed on membranes by complexing capsid proteins with the replication complex proteins in the absence of RNA. We have evidence that empty particles of FMDV also contain replication complex proteins, but 12S pentamers do not, which supports the observations that poliovirus 14S pentamers and membranes from infected cells would form empty particles, whereas they would not do so with membranes from uninfected cells (22).

The observations that poliovirus isolates which have mutations in either 2C (19) or 3D (9, 13a) are defective in uncoating argue for their presence in virus particles. Exit of the RNA from a single site on the virion has been proposed in the infectosome model (18), and the electron microscope studies on Flock House virus (13) provide further evidence for this idea. We have found that some FMDV particles complexed with antibody against 3D have a small protuberance at a single site (Fig. 4). This suggests that this is the site from which the RNA is released, particularly since we have shown in this study that 3D is attached to the RNA. This model is similar to that which is usually accepted for RNA phage and parvovirus, with the single copy of 3D taking the place of the A protein of MS2 (27) and the NS-1 protein of the minute virus of mice (7).

Significantly, the nonstructural proteins and actin are still bound to the RNA of FMDV when it is released at pH 5 or is prepared by extraction with phenol or phenol-SDS, although they can be removed with the TRI reagent or proteinase K-phenol. This would account for the observation, made many years ago, that the RNA of FMDV extracted with phenol or phenol-SDS loses infectivity rapidly at 37°C or much lower temperatures, even in the presence of SDS (3, 4). The presence on the RNA of 3D, acting as a nuclease, accounts for these losses. In contrast, the infectivity of the naked RNA obtained by extracting the virus particles with the TRI reagent or proteinase K-phenol is stable at 37°C, although its specific infectivity is significantly lower than that of the phenol-extracted RNA (Table 1). The fact that the naked RNA, like cDNA and RNA transcripts, is infectious shows that the minor polypeptides associated with the phenol-SDS-extracted RNA are not essential for infectivity. Nevertheless, the difference in specific infectivity raises the question of whether the replication complex proteins in the virus particle have a role in the process of infection. It is perhaps significant that protein-free RNA transcripts from virus cDNA have a lower specific infectivity than viral RNA (18).

The lower infectivity of the naked RNA cannot be attributed to hidden breaks in the molecule, because it sediments as a single peak at 16S in the presence of 6% formaldehyde (data not shown). This S value corresponds to a molecular weight of

2.5×10^6 (10). Nor can the higher specific infectivity of the phenol-SDS-extracted RNA be attributed to a protective effect of the minor polypeptides, because it was hydrolyzed by 10 pg of pancreatic RNase per ml, the lowest concentration of enzyme which degraded the naked RNA (data not shown). We have also found that there is no difference between the two RNAs in their affinity for membranes nor in their mRNA activity in vitro (data not shown).

Nevertheless, the difference in specific infectivity between phenol-extracted and naked RNA raises the question of whether the replication complex proteins in the virus particle have a role in the process of infection. It is perhaps significant that protein-free RNA transcripts from virus cDNA have a lower specific infectivity than viral RNA (18). Our present findings indicate that we should not ignore the possibility that viral RNA, entering the cell with replication complex proteins, may be involved in an early round of RNA synthesis. Indeed, if this does occur with some RNA molecules in multiply infected cells, there would be no need to switch from translation to replication with the same RNA template.

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