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Foot-and-mouth disease virus particles contain replicase protein 3D

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ABSTRACT **An antibody against the *Escherichia coli*-expressed RNA polymerase of foot-and-mouth disease virus (FMDV) reacts with the virus in ELISA and radioimmunoprecipitation experiments and with a protein of the disrupted virus particle in an immunoblot analysis. Treatment of the virus with trypsin, which cleaves capsid protein VP1 and a 56-kDa polypeptide present in trace amount in the particles, reduces the level of the reaction in ELISA and radioimmunoprecipitation and eliminates the immunoblot reaction. Electron microscopy showed that only ≈20% of the virus particles reacted with the anti-polymerase antibody, whereas most reacted with an antibody against the immunodominant G-H loop of the virus. In the presence of ammonium ions, the expressed polymerase degrades the RNA of the virus into molecules sedimenting at ≈12 S, indicating that it can act as a hydrolytic as well as a polymerizing enzyme. Moreover, the RNA in trypsin-treated virus particles is degraded when incubated at 37°C, suggesting that the cleaved 56-kDa protein still possesses hydrolytic activity. In addition, the anti-polymerase antibody, which inhibits the polymerase activity of the *E. coli*-expressed protein, also partially inhibits the hydrolytic activity of the previously described endonuclease of the virus particle, suggesting that this enzyme is identical with the polymerase or forms part of it.**

Although it is generally accepted that picornaviruses consist of one molecule of positive-sense single-stranded RNA of ≈2600 kDa, the four capsid proteins VP1–VP4 (each present in 60 copies), and one or two copies of VP0 (the precursor of VP4 and VP2), traces of other proteins of 40 kDa and 56 kDa have been found consistently in highly purified particles of foot-and-mouth disease virus (FMDV) (1, 2). J.F.E.N. (unpublished observation) has also found traces of proteins of similar size in poliovirus particles. The 56-kDa protein in FMDV is phosphorylated (3) and, with one of the two proteins of 40 kDa, is cleaved by treating the particles with trypsin (2). With the exception of VP0 (40 kDa), to our knowledge, the identity of these minor polypeptides has not been established with certainty. Biochemical mapping indicated that in addition to VP0 a second protein of 40 kDa was present. This protein was encoded downstream of the capsid proteins (2), and the 56-kDa protein was considered to be the virus-infection-associated (VIA) antigen (the viral RNA polymerase; refs. 4 and 5) on the basis of its molecular mass and its reaction with serum from convalescent and vaccinated animals.

Because of their failure to detect antibody to the VIA antigen in the sera of vaccinated animals, in contrast to its presence in convalescent sera, Cowan and Graves (4) concluded that its presence was associated with infection. However, recent work has demonstrated (6–8) the presence of antibodies against this antigen and other nonstructural proteins in the sera of vaccinated animals. It has been shown (2, 9) that antiserum produced in guinea pigs that had received highly purified inactivated virus particles reacted with VIA antigen isolated from virus. We have now obtained direct evidence that the VIA antigen is a component of at least 20–30% of the virus particles by showing that the *Escherichia coli*-expressed enzyme induces antibody in guinea pigs that reacts with the 56-kDa protein.

In addition, we show that the antibody inhibits the polymerase activity of the product and also partially inhibits the degradation of the viral RNA when it is released from virus particles (10, 11). The presence of the polymerase may thus account for the rapid loss of infectivity when the virus is incubated at 37°C (12), a finding that is correlated with *in situ* degradation of the genomic RNA (13). Moreover, this degradation is accelerated in the presence of NH_4^+ ions, which also enhance the hydrolysis of the isolated RNA by the *E. coli*-expressed polymerase.

MATERIALS AND METHODS

Preparation of Purified Virus, Trypsin-Treated Virus, and the Pentameric 12S Subunit. Virus of serotype A, subtype 12, was grown in monolayers of baby hamster kidney cells, in the presence of [^{3}H]uridine to label the RNA or in the presence of [^{35}S]methionine in methionine-free medium to label protein. The virus was purified essentially as described by Brown and Cartwright (14), with the final treatment of the pellet before sucrose gradient centrifugation in a 15–45% gradient being with either 1% SDS or 1% Nonidet P-40. Trypsin-treated virus was prepared by incubating purified particles with trypsin at 100 μg/ml for 30 min at 37°C. The 12S subunit was prepared by lowering the pH of the purified virus suspensions to 6.8. The pH was then returned to 7.6, and the suspension was made 1% Nonidet P-40 and centrifuged in a 15–45% sucrose gradient at 200,000 × *g* for 18 hr.

Viral RNA. RNA was extracted from purified virus by shaking with phenol in equilibrium with 0.1% SDS/100 mM sodium acetate, pH 5.0. The aqueous layer was mixed with 100 μg of tRNA and precipitated with 2 vol of ethanol at −20°C. The precipitate was collected by centrifugation, dissolved in 0.1% SDS 100 mM sodium acetate, pH 5.0, and centrifuged for 18 hr at 60,000 × *g* in a 5–25% sucrose gradient prepared in the same buffer.

Expression of FMDV Polymerase in *E. coli* Cells. Sequences encoding the polymerase were PCR-amplified from plasmid pMR15 (15) by using oligonucleotides OR42 (5′-GGGGAGATCTCTGGGTTGATTGTGGAC-3′) and OR43 (5′-GGGGAATTCTTACGCGTCACCGCACAC). The PCR product was digested with *Bgl* II and *Eco*RI and inserted into

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Abbreviations: FMDV, foot-and-mouth disease virus; VIA antigen, virus-infection-associated antigen; RIP, radioimmunoprecipitation.
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the expression plasmid pGEX-3X (Pharmacia) digested with *Bam*HI and *Eco*RI. Plasmid pMF7 encodes a glutathione *S*-transferase–factor Xa cleavage site–polymerase fusion protein. After cleavage with factor Xa, the cleavage product has three additional residues at the N terminus (Gly-Ile-Ser).

A culture of *E. coli* JM109 transformed with pMF7 was grown overnight at 37°C in Luria broth (LB) containing ampicillin (100 μg/ml). This culture was used to inoculate 1 liter of LB containing ampicillin and then incubated at 37°C, with agitation, for 4 hr. Isopropyl β-D-thiogalactopyranoside was added to 0.1 mM and the culture was incubated for a further hour. Cells were harvested by centrifugation, and the cell pellet was resuspended in phosphate-buffered saline (PBS, 10 ml/liter of culture) and disrupted by sonication. Triton X-100 was added to 1% and the sonicate was clarified by centrifugation (12,000 × *g*, 30 min). The supernatant was incubated with a 10-ml slurry of washed glutathione-Sepharose beads (Pharmacia) at 4°C overnight. The beads were pelleted (1500 × *g*, 1 min) and resuspended in PBS/10% (vol/vol) Triton X-100. Beads were washed three times by resuspension in wash buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl) and then resuspended in factor Xa cleavage buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM $CaCl_2$). The polymerase was cleaved from the glutathione *S*-transferase by the addition of bovine factor Xa (Boehringer Mannheim) to 97 μg/ml. The reaction mixture was incubated at 4°C for 48 hr with gentle stirring, and the beads were removed by centrifugation. Analysis of the purified product by PAGE showed that >95% migrated at 56 kDa.

Polymerase Assay. Polymerase activity was determined in a poly(A) polymerase assay as described by Sankar and Porter (16). Briefly, a 50-μl reaction mixture containing 1–8 μg of recombinant polymerase, 2.5 μg of poly(A), 1 μg of oligo (U), 10 μM UTP, 5 μCi of [^{3}H]UTP (1 Ci = 37 GBq), 5 mM dithiothreitol, 3.5 mM magnesium acetate, and 6 μM zinc chloride in 50 mM Hepes (pH 8) after 60 min at 30°C was precipitated with 10% (wt/vol) trichloroacetic acid with 100 μg of carrier tRNA. The precipitates were divided into two aliquots and collected on nitrocellulose filters (Millipore).

Preparation of Anti-Polymerase Antiserum. A first inoculation of 10 μg of the polymerase was made subcutaneously into guinea pigs as a 50:50 mixture (vol/vol) with an oil adjuvant. Second and third inoculations, each of 100 μg, were again given subcutaneously with oil 28 and 56 days later. Blood was collected by heart puncture 14 days after the second and third inoculations.

RNase-free IgG was prepared by filtration through DEAE-Sephacel (Pharmacia) in 10 mM sodium phosphate, as described by Brown and Stewart (17), with an additional filtration through Bio-Gel P-100 (Bio-Rad). The IgG was checked for its freedom from RNase activity by showing that it did not hydrolyze virus RNA when the mixture was incubated overnight at 37°C.

Serology. *ELISA*. Purified recombinant polymerase (0.1 μg per well in 15 mM sodium carbonate/35 mM sodium bicarbonate, pH 9.6) was adsorbed on to 96-well microtiter plates overnight at 4°C. The plates were then washed with 40 mM sodium phosphate/15 mM NaCl/0.05% Tween 20. Blocking was done with 40 mM sodium phosphate/3% (wt/vol) gelatin/1% Tween 20 for 1 hr at 37°C. Anti-polymerase antiserum raised in guinea pigs was added to the plates in 1:2 dilutions in 40 mM sodium phosphate/1% gelatin/1% Tween 20 and incubated for 1 hr at 37°C. After washing the plates, alkaline phosphatase-conjugated goat anti-guinea pig IgG (Kirkegaard & Perry Laboratories) in PBS/1% gelatin was added for 1 hr at 37°C. Excess conjugate was removed by washing as before. Reaction products were quantified by adding *p*-nitrophenyl phosphate, disodium salt (Sigma) for 30 min at 37°C and another 30 min at room temperature. The reaction was stopped with 3 M NaOH, and the color was read at 405 nm with an ELISA reader (Molecular Devices). To test the reactivity of the anti-polymerase antiserum against the virus particle, plates were coated with (*i*) purified virus particles, (*ii*) disrupted virus obtained by acidification at pH 6.8, (*iii*) virus particles treated with trypsin, or (*iv*) disrupted particles obtained by acidification at pH 6.8 of trypsin-treated virus particles. The ELISA was performed as described above.

Radioimmunoprecipitation (RIP). Virus particles labeled with [^{35}S]methionine were used. As described above, the virus was treated with trypsin or the pH was lowered to 6.8 and the same amount of radioactivity was used for each experiment. Twenty microliters of diluted serum was mixed with the antigens and the volume was brought to 250 μl with TNEN (50 mM Tris·HCl, pH 7.5/150 mM NaCl/5 mM EDTA/0.05% Nonidet P-40)/0.05% normal guinea pig serum and incubated for 2 hr at 37°C. Fifty microliters of a 10% suspension of *Staphylococcus aureus* ghosts (The Enzyme Center, Malden, MA), washed three times in TNEN, was added to each sample and the mixture was incubated for 1 hr at 4°C. The pellets were washed three times in TNEN/0.05% normal guinea pig serum and dissolved in a solubilizer solution, and radioactivity was measured by liquid scintillation counting.

Immunoblot analysis. Proteins were separated by SDS/PAGE and electrophoretically transferred to an Immobilon-P membrane (Millipore) as described by Towbin *et al.* (18). After transfer, the membrane was blocked with 5% (wt/vol) nonfat milk in 20 mM Tris·HCl, pH 7.6/300 mM NaCl for 1 hr to saturate any remaining binding sites. Blots were then treated sequentially with guinea pig anti-polymerase antibody, followed by alkaline phosphatase-conjugated goat anti-guinea pig IgG. Reacting bands were visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt (Pierce).

Electron microscopy. Sucrose-gradient purified virus was adsorbed to Formvar-coated carbon-stabilized nickel grids, which were then blocked with 1% bovine serum albumin/0.2% gelatin/50 mM Tris·HCl/150 mM NaCl, pH 7.4, for 30 min. Either guinea pig antiserum raised against a peptide corresponding to the immunodominant region of VP1 (residues 141–160) or anti-polymerase antiserum diluted 1:100 in blocking buffer was added, and the mixtures were incubated overnight at 4°C. Antibody binding was detected by goat anti-guinea pig antisera conjugated with 10-nm colloidal gold (Zymed) diluted in blocking buffer. Grids were stained with 2% (wt/vol) phosphotungstic acid (pH 7.4) and examined in a Philips 410 electron microscope at 80 kV. In some experiments, convalescent swine serum (diluted 1:1000) was adsorbed to the grid and allowed to dry, and the virus preparation was then added. This procedure enabled us to complex a greater number of virus particles, albeit aggregated, with the two antibodies and at the same time offset any differences in adsorption to the grids of particles bearing or lacking the polymerase. The occurrence and density of gold label on well-dispersed virions were assessed by photographing random fields at low magnification. The occurrence of gold label on clumps was assessed by photographing areas of similar size with each antibody preparation and then counting the number of gold particles.

RESULTS

ELISA and RIP of the Minor Polypeptides in the Virus Particle. The anti-polymerase antiserum had a titer of $\log_{10}$ 1.95 (50% end point) with a polymerase preparation that had been adsorbed to a microtiter plate. After absorption with disrupted *E. coli* cells, the antiserum had a titer of $\log_{10}$ 1.84, showing that it contained specific anti-polymerase antibody.

Purified virus particles adsorbed to a microtiter plate reacted with the antiserum in ELISA (Fig. 1*a*). Trypsin-

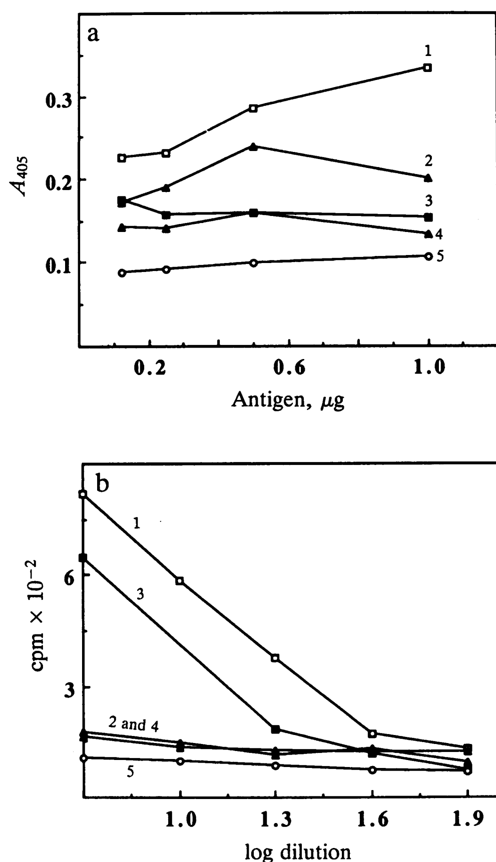

FIG. 1. ELISA (*a*) and RIP (*b*) of virus and trypsin-treated virus at pH 7.6 and at pH 6.8. In the ELISA, anti-polymerase antiserum (diluted 1:40) and normal guinea pig serum (diluted 1:40, as control) were used. For the RIP experiments, the sera were used at dilutions of 1:5, 1:10, 1:20, 1:40, and 1:80. Curves: 1, virus at pH 7.6; 2, virus at pH 6.8; 3, trypsin-treated virus at pH 7.6; 4, trypsin-treated virus at pH 6.8; 5, control.

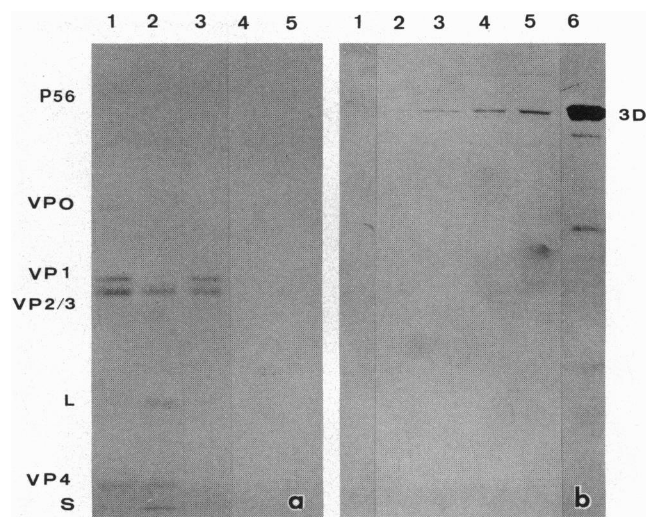

FIG. 2. (*a*) PAGE analysis of [^{35}S]methionine-labeled proteins of FMDV. Lanes: 1, intact virus; 2, trypsin-treated virus; 3, 12S pentameric subunit; 4 and 5, precipitate and supernatant from RIP experiment, respectively. (*b*) Western blot analysis of PAGE-separated proteins. Lanes: 1, trypsin-treated virus; 2, 12S subunit; 3, intact virus; 4 and 5, 200,000 × *g* pellet and supernatant from virus harvest, respectively; 6, recombinant polymerase.

treated virus particles gave a weak reaction with the antiserum, although a 56-kDa protein could not be detected by PAGE (Fig. 2*a*). Virus that had been incubated at pH 6.8 (conditions that disrupt the particles into VP4, 12S pentamers of VP1, VP2, and VP3, and RNA) reacted ≈65% as well with the antiserum as did intact virus particles. Trypsin-treated virus incubated at pH 6.8 and enzyme-treated particles maintained at pH 7.6 reacted similarly (Fig. 1*a*).

To study the location of the polymerase molecule, RIP experiments were done with similar preparations. Irrespective of the virus/antiserum ratio, only ≈30% of the radioactive virus was precipitated. After trypsin treatment, the amount of radioactivity precipitated was somewhat reduced (Fig. 1*b*). Since the 56-kDa protein cannot be detected by PAGE of the trypsin-treated particles, these results suggest that cleavage products are able to react specifically with the anti-polymerase antibody.

In contrast, virus particles incubated at pH 6.8 reacted essentially indistinguishably with antiserum to the polymerase and with normal serum. Moreover, the isolated 12S pentamers were not precipitated by the antiserum (Fig. 2*a*, lanes 4 and 5). These results, which differ from those obtained by ELISA, and results from RIP experiments with unfractionated acid-treated virus, in which the 12S pentamers are not separated from VP4 and the polymerase, can be accounted for by the release of the polymerase from the capsid proteins.

PAGE and Immunoblot Analysis of the Virus Proteins. The results of PAGE analysis of the proteins from purified and trypsin-treated virus particles and the 12S pentameric subunit are shown in Fig. 2*a* (lanes 1–3). Whereas the intact virus particles gave a faint but clear band at 56 kDa, neither the trypsin-treated particles nor the 12S subunit showed a band at this position. Immunoblot analysis of the separated proteins of the untreated virus also showed a fine band at 56 kDa, (Fig. 2*b*, lane 3), but no band was obtained with trypsin-treated virus particles (lane 1) or the 12S subunit (lane 2). As expected from the observations by Cowan and Graves (4) that virus harvests contained considerable amounts of the VIA antigen, both the pellet and 200,000 × *g* supernatant fractions from virus harvests showed a clear band at 56 kDa (Fig. 2*b*).

Electron Microscopy. Immune complexing of virus particles with antiserum against the polymerase provided a striking contrast to the result obtained with antiserum against a peptide corresponding to the immunodominant loop (aa 141–160) on VP1. Approximately 20% (61 of 311 virions) of the dispersed virions were labeled with the anti-polymerase antibody and in most of these complexes only a single gold particle was associated with each virion (Fig. 3*a*). In contrast, most of the virions were decorated with the anti-peptide antibody and an average of 4.2 gold particles per virion was seen (Fig. 3*b*). The distribution of gold label on the virions aggregated on the antibody-coated grids was similar to that obtained with the dispersed virions for both the anti-polymerase antibody (18%, 22 particles per 120 virions) and the anti-peptide antibody (4.6 particles per virion). The size of the aggregate did not appear to influence the density of labeling in either case. This observation correlates with the results of RIP experiments that showed that the anti-polymerase antiserum precipitated ≈30% of the radioactive virus particles, irrespective of the virus/antiserum ratio. Antiserum against the peptide corresponding to aa 141–160 of the G-H loop of the virus precipitated all the radioactivity.

Inhibitory Activity of the Anti-Polymerase Antibody. The IgG fraction of the anti-polymerase antiserum inhibited the activity of the *E. coli*-expressed enzyme by ≈90%. In addition, the activity of the antibody in inhibiting the endonuclease activity of the virus particle was also investigated.

Virus radiolabeled in the RNA with [^{3}H]uridine and purified using Nonidet P-40 before separation by sucrose gradient

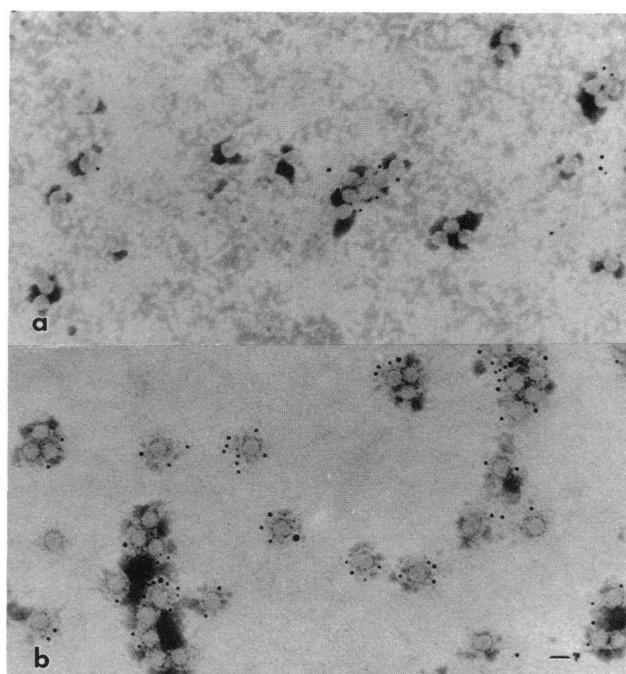

FIG. 3. Electron micrographs of virus particles complexed with guinea pig antiserum against the polymerase (*a*) or the peptide corresponding to the immunodominant loop region of the virus (*b*). Antibody binding was detected by goat anti-guinea pig serum conjugated with 10-nm colloidal gold. (Bar = 50 nm.)

centrifugation was brought to pH 5 for 10 min to expose internally located RNase activity and then restored to pH 7.6 (11). The acid-disrupted virus was then incubated for 17 hr at 4°C or 37°C with RNase-free IgG from either anti-recombinant RNA polymerase serum or normal serum. The mixtures were centrifuged on sucrose gradients in 100 mM sodium acetate, pH 5.0/0.1% SDS. The distribution of radioactivity in Fig. 4*a* shows the partial enzyme inhibitory activity of the specific anti-polymerase IgG. The degradation that is observed may be due to the inability of the antibody to completely block the activity of the enzyme or to the presence of another nuclease in the virus particle.

Hydrolytic Activity of the Polymerase. Incubating the virus at 37°C leads to the rapid loss of infectivity and the concomitant degradation of the RNA *in situ*. Moreover, the RNA was degraded in trypsin-treated particles, even though the 56-kDa protein was no longer present, suggesting that the hydrolytic activity does not depend on the intact protein. To ensure that this degradation was not due to the adventitious presence of RNases, hydrolysis with trypsin was carried out in the presence of 0.02% SDS, conditions that allow the cleavage of VP1 and the 56-kDa protein to take place. The treated particles were then incubated for 17 hr at 37°C after adding 0.1% SDS. The RNA was still degraded under these conditions (Fig. 4*b*).

Scodeller *et al.* (19) showed that the degradation of the genomic RNA that occurs when FMDV particles are incubated at 37°C could be accelerated by the addition of NH_4^+ ions. We have now shown that the isolated virus RNA, which is degraded slowly by the *E. coli*-expressed polymerase, is hydrolyzed completely when 100–250 mM NH_4Cl is added (Fig. 4*c*). This result provides further evidence that the polymerase can act as a hydrolytic enzyme in the presence of NH_4^+ ions.

DISCUSSION

In 1966 Cowan and Graves (4) reported the presence of a group-specific protein (VIA antigen of 56 kDa) in FMDV-infected cells, which was later identified as the virus RNA polymerase (5, 20, 21). Rowlands *et al.* (9) demonstrated that antiserum raised in guinea pigs against purified inactivated virus particles also reacted with the VIA antigen and argued that the failure of Cowan and Graves (4) to detect the reaction with antiserum from vaccinated animals could have been because insufficient virus particles had been inoculated. An alternative explanation is the insensitivity of the serological methods used, since recent work (6–8) has shown that antibodies against several nonstructural proteins are present in the sera of vaccinated animals. Further evidence that the virus particle contained the polymerase came from PAGE separations of proteins from purified virus when a molecule was found consistently at 56 kDa (2). Furthermore, the protein from the virus particle had many of the properties of the polymerase molecule, including cross-reactivity in serological tests.

Our results show that the virus particle reacts with antibody raised against *E. coli*-expressed polymerase. With the expressed protein, the chances of producing antibody that

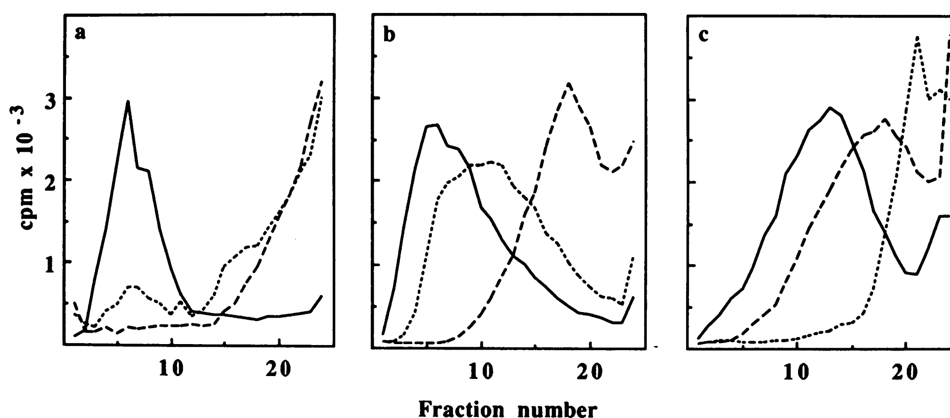

FIG. 4. Sucrose gradient analysis of virus RNA. (*a*) The effect of anti-polymerase IgG on the degradation of virus RNA by the endogenous endonuclease. Virus particles were brought to pH 5 and then restored to pH 7.6 before incubating with [^{3}H]uridine-labeled virus RNA for 18 hr at 4°C (solid line) or 37°C with IgG from normal guinea pig serum (dashed line) or with IgG from anti-polymerase antiserum (dotted line). (*b*) The effect of trypsin on the polymerase in the virus particle. Virus particles labeled with [^{3}H]uridine were incubated with trypsin (100 μg/ml) for 30 min at 37°C in the presence of 0.02% SDS. The solution was then made to 1% with SDS and the RNA was extracted either immediately (dotted line) or after incubation for 18 hr at 37°C (dashed line). RNA from untreated virus (solid line) served as control. (*c*) The effect of NH_4^+ ions on the degradation of the RNA. [^{3}H]Uridine virus RNA was incubated for 18 hr at 37°C either alone (solid line) or with 1 μg of recombinant RNA polymerase in the absence (dashed line) or presence (dotted line) of 250 mM NH_4Cl.

cross-reacts adventitiously with the virus capsid proteins or any BHK cell proteins that may be left on the virus particles are very small. Moreover, the anti-polymerase antiserum partially inhibits the endonuclease activity of the virus particles responsible for degradation of the RNA *in situ* and the consequent loss of infectivity when the virus is kept at 37°C.

Degradation of the viral RNA *in situ* when FMDV is heated at 37°C is accelerated in the presence of NH_4^+ ions (19). This phenomenon has also been observed with poliovirus (22) and encephalomyocarditis virus (unpublished observations). Provided our thesis is correct that this degradation is caused by activation of the hydrolytic activity of the polymerase, our observation that hydrolysis of the isolated virus RNA occurs by overnight incubation with the *E. coli*-expressed RNA polymerase in the presence of NH_4^+ ions is readily explained. It is of interest that Polatnick (23) found that NH_4^+ ions were required to optimize the polymerase activity of FMDV.

The fact that trypsin-treated virus particles are precipitated by the anti-polymerase antibody, despite our failure to detect any 56-kDa protein in the treated preparations, suggested that molecules of smaller molecular mass were still associated with the particles. Moreover, the RNA in the trypsin-treated virus particles was still hydrolyzed when these viruses were incubated at 37°C, thus providing further evidence that fragments of the polymerase molecule were retained in the particles. Our failure to detect these fragments in PAGE separations of the proteins of the enzyme-treated preparations may be because they migrate to the same position as the capsid proteins or are very small.

In preliminary experiments using ELISA and Western blot analysis, we have also detected similar molecules in poliovirus and encephalomyocarditis virus particles (unpublished observations). It is probably relevant that Ziola and Scraba (24) found one copy of a 57-kDa protein in Mengo virus, which is very closely related to encephalomyocarditis virus. Although these authors considered this protein to be the precursor of VP1 and VP3, our results indicate that in fact it may be the polymerase. These observations have clear implications for the stabilization of infectivity of picornavirus particles, in general, and for attenuated polio vaccines, in particular, where infectivity must be retained at an acceptable level.

It is possible that the incorporation of P56 is merely fortuitous. Nevertheless, it is tempting to ask whether the polymerase in the virus particle may have a functional role. The RIP and electron microscopic evidence show that only about one in five virus particles reacts with the anti-polymerase antibody but it is possible that the enzyme is located internally and is only exposed either in damaged particles or because of configurational mobility. The calculation made by Sangar *et al.* (2) on the basis of SDS/PAGE experiments with [^{35}S]methionine-labeled virus indicated that the virus particles contained one copy of the enzyme, and our unpublished experiments plus the additional knowledge of the number of methionine residues in the polymerase and capsid proteins support their conclusion. Moreover, incubation of the purified virus at 37°C results in the degradation of the genomic RNA in all the particles. The presence of one copy of the enzyme per virus particle suggests that it has a specific functional role.

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