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Removal of the Genome-Linked Protein of Foot-and-Mouth Disease Virus by Rabbit Reticulocyte Lysate

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Rabbit reticulocyte lysate cleaves the genome-linked protein VPg from foot-and-mouth disease virus (FMDV) RNA. This activity could be reliably monitored since removal of the protein resulted in a change in migration in polyacrylamide gels of the small specific 5′ end fragment of the RNA (S fragment). The unlinking activity cleaved the bond between the tyrosine residue of VPg and the RNA to leave a 5′ phosphate on the RNA. The 5′ sequence of the RNA from which VPg had been removed by rabbit reticulocyte lysate was the same as that of FMDV mRNA isolated from infected cells. VPg released from the RNA was rapidly degraded by the rabbit reticulocyte lysate to material which eluted with the inclusion volume of a Sepharose 6B column and partitioned to the aqueous phase during phenol extraction. The unlinking activity was inhibited by heating the lysate to 56°C, by sodium dodecyl sulfate (SDS), EDTA, and Zn^{2+} ions but was unaffected by reducing agents, a translation inhibitor, and a number of protease and RNase inhibitors.

The genome of foot-and-mouth disease virus (FMDV), in common with other members of the Picornaviridae, consists of a linear single-stranded RNA with a molecular weight of about 3×10^6. This RNA can act as an efficient messenger in vitro (17). However, unlike the vast majority of eucaryotic mRNA's, the 5′ end of the RNA is covalently linked to a small protein, VPg (4, 7, 11, 18). With poliovirus and encephalomyocarditis virus, this linkage is through a tyrosine residue of VPg and the 5′ terminal uridylic acid residue of the RNA (1, 15, 19). There is strong circumstantial evidence which suggests that the same is true for FMDV RNA (8). The function of the VPg of picornavirus RNAs is not clear; however, it has been shown that all nascent RNA strands on the replicative intermediate of poliovirus contain VPg (4, 12), and this has led to the suggestion that it acts as a primer in RNA synthesis. In contrast to virion RNA, the mRNA isolated from poliovirus polyribosomes lacks VPg. This is the only difference between virion RNA and mRNA since both terminate with the same nonanucleotide pUUAAAACAG (13, 14) and have identical T1 fingerprints (11).

Since all nascent poliovirus RNA strands are linked to VPg molecules, whereas mRNA's isolated from polyribosomes lack this protein, it follows that infected cells must contain a mechanism for its removal. Such an activity has been detected in a variety of infected and uninfected cells (2, 3). Nuclear wash preparations were shown to cleave the protein-RNA link, leaving both moieties intact.

There is less information concerning the role of VPg in FMDV. It is not known whether all the strands of the replicative intermediate possess VPg; however, it has been shown that there is a VPg molecule on every encapsidated virion RNA strand (6), whereas some of the RNA isolated from polyribosomes lacks VPg (D. V. Sangar, unpublished data).

FMDV RNA is efficiently translated in the rabbit reticulocyte lysate system (17; T. J. R. Harris, F. Brown, and D. V. Sangar, Virology, in press), and we have now examined this system for an activity capable of cleaving VPg from the input virion RNA. In this paper we describe such an activity which efficiently removes VPg. The effect of a number of potential inhibitors on the unlinking activity has also been investigated.

MATERIALS AND METHODS

Preparation of virus RNA. The method has been described in detail in previous publications from this Institute (16). In the present experiments FMDV, type A_{10} (strain A_{61}) was used, and labeled virus preparations were produced by growing them in the presence of [^{3}H]uridine (30 Ci/mmol; 50 μCi/ml), [^{3}H]cytidine (0.5 Ci/mmol; 50 μCi/ml), [^{14}C]uridine (0.5 Ci/mmol; 2.5 μCi/ml), or [^{3}H]tyrosine (50 Ci/mmol; 50 to 250 μCi/ml). The amino acid-labeled viruses were grown in Eagle medium lacking the appropriate amino acid. The RNA was extracted from purified virus particles

with phenol-chloroform-8-hydroxyquinoline (50:50: 0.1) and was then precipitated with 2 volumes of ethanol at −20°C with 50 μg of *Escherichia coli* tRNA as carrier.

Treatment of RNA with rabbit reticulocyte lysate. Ethanol precipitates of virus RNA (~2 μg) were washed with ethanol and dried under vacuum. The precipitates were dissolved in 10 or 20 μl of rabbit reticulocyte lysate (obtained either from the Radiochemical Centre, Amersham, England, or as a gift from R. Jackson, University of Cambridge) and incubated for 30 min at 30°C. In the majority of experiments a lysate treated with micrococcal nuclease to remove endogenous message was used. In the experiments testing the effect of divalent cations, however, it was necessary to use an untreated lysate. The reaction was stopped by the addition of 500 μl of 0.15 M NaCl-10 mM Tris-hydrochloride-1 mM EDTA (pH 7.5; NTE) containing 0.2% SDS, and the RNA was extracted with phenol-chloroform and precipitated with 2 volumes of ethanol for subsequent analysis.

When unlinking of VPg from RNA was measured by partition on phenol extraction, samples of the phenol and aqueous phases were dried onto glass fiber paper and counted in toluene-based scintillant. Compounds to be tested for inhibitory effects on the unlinking of VPg were added to the rabbit reticulocyte lysate, and the mixture was incubated at 30°C for 10 min before adding the RNA.

Treatment of virus RNA with RNase H. This method was described in detail by Rowlands et al. (16). RNase H purified from *E. coli* was obtained from Enzo Biochem at a concentration of 1,000 U per ml. Precipitates of RNA were washed with ethanol, dried, and redissolved in 40 μl of RNase H buffer (50 mM Tris-hydrochloride [pH 7.9]-10 mM $MgCl_2$-1 mM EDTA-10 mM dithiothreitol-10% glycerol), and 0.5 μg of oligodeoxyguanylate$_{12-18}$ [oligo(dG); Miles Laboratories, Inc.] was added, together with 0.2 U of RNase H. Digestion was for 30 min at 30°C, and the reaction was stopped by the addition of 10 μl of 150 mM EDTA (pH 7.6)-2.5% SDS.

Treatment of virus RNA with RNases or proteinase K. FMDV RNA was incubated with RNases T_1 (500 U/ml), T_2 (33 U/ml), and A (700 U/ml) in 50 μl of buffer (0.05 M sodium acetate, pH 5.0) for 60 min at 37°C. Digestion was stopped by the addition of 0.5 ml of NTE-SDS followed by phenol-chloroform extraction. Virus RNA (approximately 1 μg) was digested with proteinase K (1 mg/ml) in 50 μl of NTE-SDS for 60 min at 37°C. Digestion was stopped as above.

Polyacrylamide gel electrophoresis. The products of RNase H digestion were analyzed on 10% polyacrylamide gels by the method of Laemmli (9) with the following modifications: a stacking gel was omitted, and the gel buffer was raised to pH 8.95 and included 2 mM EDTA. Electrode buffer was 0.38 M glycine-0.05 M Tris (pH 8.3)-0.1% SDS-2 mM EDTA. An equal volume of 0.01 M Tris (pH 6.8)-4 mM EDTA-1% SDS-20% glycerol-0.04% bromophenol blue-xylene cyanol was added to the RNase H-digested RNA, and the samples were heated for 5 min at 56°C before loading onto the gel. Electrophoresis was at 100 V until the dye markers (bromophenol blue and xylene cyanol) reached the bottom of the gel. Slab gels were analyzed by fluorography, and cylindrical gels were sliced into 1.5-mm sections and digested with NCS before counting in toluene scintillant as described previously.

Estimation of size of VPg by gel filtration. RNA labeled in the VPg moiety with [^{3}H]tyrosine was incubated with rabbit reticulocyte lysate for 30 min at 30°C. The sample was diluted to 500 μl with 0.1 M Tris (pH 8.8) containing 6 M guanidine hydrochloride and marker proteins (ovalbumin, 5 mg; cytochrome *c*, 2 mg; insulin, 8 mg). Mercaptoethanol (5 μl) was added, and the sample was incubated for 4 h at 37°C. The proteins were carboxymethylated by adding 48 mg of iodoacetamide and incubating for 1 h at 37°C, after which the reaction was stopped by the addition of 50 μl of mercaptoethanol. Phenol red was added, and the sample was made 10% with respect to glycerol. The proteins were separated on a column (80 by 1.5 cm) of Sepharose 6B using 6 M guanidine hydrochloride-0.1 mM dithiothreitol as eluant. Marker proteins were detected by their absorption at 280 nm, and VPg was detected by counting the samples in Triton-toluene (1:2 by volume) scintillant. It was necessary to dilute each sample with an equal volume of water before adding scintillant to prevent crystallization of the guanidine hydrochloride.

Sequence determination from the 5′ end of rabbit reticulocyte-treated RNA. Samples (20 μg) of FMDV RNA were incubated with 200 μl of rabbit reticulocyte lysate or with 40 μl of NTE for 30 min at 30°C. The RNA was recovered from both samples by phenol-chloroform extraction, ethanol precipitation, and sucrose gradient centrifugation, followed by a second ethanol precipitation. The RNA precipitate from half of each sample was recovered by centrifugation, washed with ethanol, dried, and dissolved in 100 μl of 0.1 M Tris (pH 7.9)-10 mM $MgCl_2$ and incubated with 0.5 U of calf alkaline phosphatase (Boehringer Mannheim Corp.) which had been purified by filtration through Sephadex G-100. The reaction was stopped by phenol-chloroform extraction, and the RNA was precipitated with ethanol at −20°C overnight. The two alkaline phosphatase-treated RNA samples and the two left untreated were recovered by centrifugation, washed with ethanol, dried, and dissolved in 90 μl of denaturation buffer (5 mM Tris [pH 7.5]-0.1 mM EDTA-1 mM spermidine). The samples were heated at 60°C for 3 min, chilled in ice, and 10 μl of 10× kinase buffer (0.5 M Tris [pH 7.6]-0.1 M $MgCl_2$-0.05 M DTT) was added. The samples were transferred to plastic tubes in which 25 μCi of [^{32}P]ATP (400 Ci/mmol) had been dried. One unit of polynucleotide kinase (New England Biochemical) was added to each, and the samples were incubated at 37°C for 30 min. The reactions were stopped by filtration through Sephadex G-100, in NTE-SDS, and the RNA was recovered by ethanol precipitation. The RNA was treated with RNase H in the presence of oligo(dG) as described above. The S fragments were separated on slab gels as described above, and the wet gels were exposed to X-ray film (Fuji RX) overnight at 4°C. The S fragment band seen in the rabbit reticulocyte lysate-alkaline phosphatase-treated sample was cut out, and the RNA was eluted by fragmentation of the gel in NTE-0.2% SDS and incubation at 37°C

for 6 h. The eluate was filtered through a membrane filter (Millipore Corp.), and the RNA was precipitated with ethanol using tRNA as carrier. Rapid RNA sequencing was performed as described previously (6).

RESULTS

In vitro cleavage of VPg from virion RNA. The removal of VPg from virion RNA was detected by the change in electrophoretic mobility in polyacrylamide gels of the specific S fragment containing the 5′ end of the RNA. The S fragment is produced by digestion of the polycytidylic acid tract, which is present about 400 bases from the 5′ end of FMDV RNA, with RNase H in the presence of oligo(dG) (16). The S fragment can be separated into two closely migrating bands (6) which appear to represent identical RNA fragments linked to either of the two differently charged species of VPg found in FMDV (8; T. J. R. Harris, unpublished data). Digestion with proteinase K produces a single band which migrates faster than before protease digestion (6). Virus-specified RNA isolated from infected cells yields three separate S fragments (6); the two more slowly migrating fragments correspond to those obtained from virion RNA, whereas the faster migrating band lacks VPg. The migration of S fragment can therefore be used as an indicator for the presence or absence of VPg on FMDV RNA.

The result of incubating [^{3}H]uridine-labeled virus RNA with rabbit reticulocyte lysate is shown in Fig. 1. The two S fragments obtained by RNase H treatment of untreated RNA were converted into a single band which migrated slightly faster than the S fragment produced from proteinase K-treated virion RNA. This result indicates that most of the VPg is removed from the RNA by incubation with rabbit reticulocyte lysate, but it does not prove that the phosphodiester link between VPg and RNA is cleaved.

Assay of VPg removal by phenol extraction. It should be possible to follow the cleavage of the phosphodiester bond between VPg and the RNA, and thus the removal of VPg from the RNA, by phenol extraction of lysate-treated RNA which is labeled in VPg (3). RNA labeled with [^{3}H]tyrosine was incubated with either rabbit reticulocyte lysate, RNases T_1, T_2, and A, proteinase K, or NTE-SDS as described above. After the addition of NTE-SDS to stop the reaction, the samples were extracted with phenol, and the radioactivity in the phenol and aqueous phases was determined. Table 1 shows that after incubation with NTE-SDS most of the label remained in the aqueous phase since the protein was still attached to RNA. As expected, the same result was obtained after proteinase K treatment since the enzyme leaves a stub of undigested amino acids, including the tyrosine, linked to the RNA (D. V. Sangar, unpublished data). After treatment of the RNA

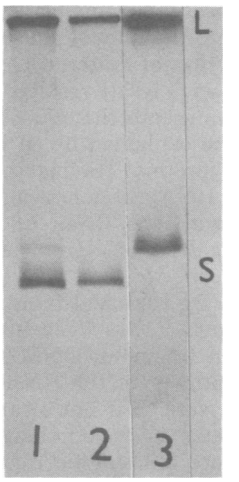

FIG. 1. *Effect of incubation of FMDV RNA with rabbit reticulocyte lysate or proteinase K on the migration of the S fragment in polyacrylamide gels. The RNA was recovered by phenol-chloroform extraction in the presence of SDS and subsequent ethanol precipitation. The polycytidylic acid tract was digested with RNase H in the presence of oligo(dG) to yield and L and S fragments which were then separated on a 10% polyacrylamide slab gel. Lane 1, RNA incubated with 20 μl of rabbit reticulocyte lysate for 30 min at 30°C; lane 2, RNA incubated with 1 mg of proteinase K per ml for 60 min at 37°C; lane 3, RNA treated with NTE-SDS for 30 min at 30°C.*

TABLE 1. *Estimation of removal of VPg from ^{3}H-tyrosine-labeled FMDV RNA by phenol extraction*[a]

Treatment	Phase	% Radioactivity
NTE-SDS	Aqueous	92
	Phenol	8
Lysate	Aqueous	96
	Phenol	4
Proteinase K	Aqueous	97
	Phenol	3
RNases A, T_1, and T_2	Aqueous	14
	Phenol	86

[a] Virus RNA was incubated with (A) 50 μl of NTE-SDS for 30 min at 30°C; (B) 10 μl of rabbit reticulocyte lysate for 30 min at 30°C; (C) 50 μl of NTE-SDS containing 50 μg of proteinase K for 60 min at 37°C; and (D) 50 μl of 0.05 M sodium acetate (pH 5) containing 500 U of T_1 per ml, 33 U of T_2 per ml, and 700 U of A per ml for 60 min at 37°C. The reactions were stopped by adding 500 μl of NTE-SDS per ml followed by phenol-chloroform extraction. Samples of the phenol and aqueous phases were dried onto glass fiber paper for counting.

with ribonuclease, the majority of the counts partitioned into the phenol phase. Surprisingly, phenol extraction of material treated with the rabbit reticulocyte lysate resulted in most of the label partitioning into the aqueous phase. This result could be explained by (i) incomplete removal of the protein, leaving the tyrosine attached to the RNA; (ii) noncovalent association of the VPg with the RNA; or (iii) extensive proteolytic digestion of VPg to yield hydrophilic residues.

Assay of VPg removal from dual-labeled RNA. Although it has been shown that the migration of S fragment of FMDV RNA is affected by treatment of the RNA with a rabbit reticulocyte lysate, it is not clear whether this treatment breaks the bond linking the protein to the RNA, since proteolytic digestion of VPg could give a similar result. To resolve this question, RNA labeled in VPg with [^{3}H]tyrosine was mixed with [^{14}C]cytidine-labeled RNA, and the mixture was incubated with either NTE-SDS or lysate, and the RNA was digested with RNase H and oligo(dG) as before. The fragments were separated on cylindrical gels which were sliced and counted in a liquid scintillation cocktail. The sample that had been incubated with NTE-SDS gave two major ^{14}C peaks corresponding to the L and S fragments. The L fragment is the remainder of the FMDV genome after removal of the polycytidylic tract and S fragment. Tyrosine counts were detected only in the S fragment. In contrast, no [^{3}H]tyrosine counts were found in the lysate-treated sample, presumably because VPg is not precipitated by 67% ethanol when cleaved from the RNA (Fig. 2). This result clearly shows that rabbit reticulocyte lysate contains an activity which can completely remove VPg from RNA and that S fragment migration in polyacrylamide slab gels is an accurate and sensitive method for detecting this activity.

Sequence of the 5′ end of lysate-treated virion RNA. Having shown that incubation with rabbit reticulocyte lysate removes VPg from FMDV RNA, we next examined the integrity of the exposed 5′ terminus by end labeling and RNA sequencing. Untreated and rabbit reticulocyte-treated RNA were labeled with [^{32}P]ATP and polynucleotide kinase with or without previous alkaline phosphatase treatment and analyzed by RNase digestion and polyacrylamide gel electrophoresis as described above. Although several bands were detected, indicating some random breakdown of the RNA, the only sample giving an authentic labeled S fragment was that which had been treated with both rabbit reticulocyte lysate and calf alkaline phosphatase (Fig. 3). This confirms that virion RNA cannot be 5′ end labeled, even after treatment with calf alkaline phosphatase, because of the presence of VPg (6) and indicates that rabbit reticulocyte lysate removes VPg, leaving a phosphate at the 5′ end which can be removed with phosphatase to leave a free 5′ OH available for end labeling. The ^{32}P-labeled S fragment was recovered from the gel and used for RNA sequencing by partial enzyme digestion (Fig. 4). The sequence of the first 22 bases was the same as that obtained for mRNA (6), indicating that the rabbit reticulocyte lysate removes VPg without extensive degradation of the 5′ end of the RNA.

Estimate of size of VPg removed by lysate. VPg released from [^{3}H]tyrosine-labeled FMDV RNA by incubation with rabbit reticulocyte lysate was analyzed by filtration through a column of Sepharose 6B in the presence of 6 M guanidine as described above. Most of the ^{3}H label eluted with phenol red marker dye in contrast to VPg prepared by digestion of RNA with RNase T_1, T_2, and A when most of the counts eluted with the heavy chain of insulin, molecular weight 3,500 (Fig. 5). It is clear, therefore, that the lysate degrades VPg released from the RNA.

Effect of potential inhibitors on the cleavage of VPg from FMDV RNA. The finding that the migration of S fragment in polyacrylamide gels is a reliable and sensitive method for detecting cleavage of VPg from RNA enabled us to investigate a number of potential inhibitors of the cleavage of VPg from RNA. The result of one such experiment is shown in Fig. 6. The effect of all of the potential inhibitors we have examined so far is shown in Table 2.

The cleavage enzyme in rabbit reticulocyte lysate is sensitive to heating at 56°C, is inhibited by 1% SDS and 5 mM zinc acetate, and appears to require magnesium ions since EDTA has a marked inhibitory action. In experiments using zinc, cobalt, or magnanese ions as potential inhibitors, a lysate not pretreated with micrococcal nuclease was used since these cations activate the nuclease, leading to extensive breakdown of the added RNA. Tyrosine phosphate, a possible competitive inhibitor, was slightly inhibitory. The enzyme, however, is resistant to several protease and RNase inhibitors and is not affected by reducing agents or sparsomycin. Since sparsomycin completely inhibits translation at the levels used in this experiment (data not shown), it follows that the unlinking activity is of cellular origin and not virus specified.

DISCUSSION

We have shown an activity in a rabbit reticulocyte lysate, using FMDV RNA as substrate, which cleaves the phosphodiester linkage between VPg and the RNA. The 5′ terminal se-

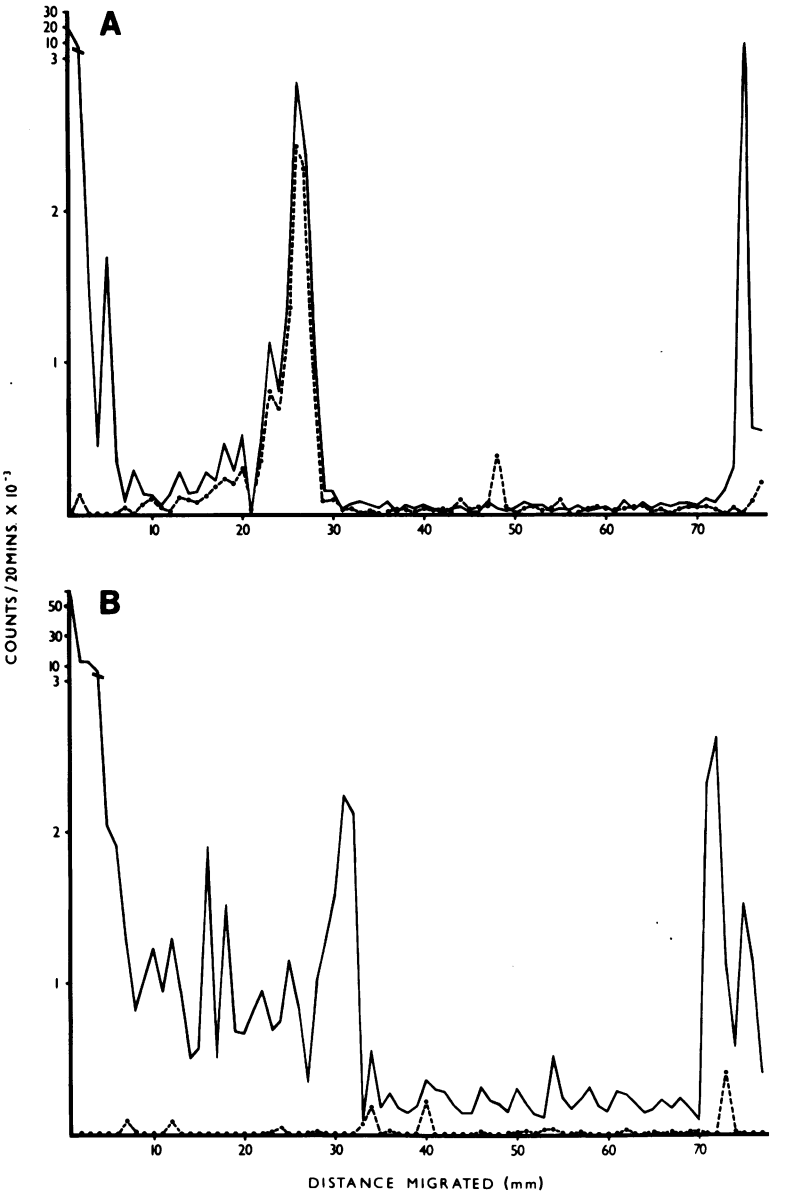

FIG. 2. *Effect of incubation of FMDV RNA with rabbit reticulocyte lysate on the migration of the S fragment in polyacrylamide gels. A mixture of RNA preparations labeled with [^{14}C]cytidine or [^{3}H]tyrosine was treated as described in Fig. 1, except that separation was on cylindrical gels (15 by 0.8 cm). The samples were co-electrophoresed, and the gels were sliced into 1.5-mm segments. Radioactivity was determined by digestion with NCS and counting in a liquid scintillation counter. (A) RNA incubated with NTE-SDS; (B) RNA incubated with lysate. Symbols: ---, [^{3}H]tyrosine; —, [^{14}C]cytidine.*

quence of RNA which had been incubated with rabbit reticulocyte lysate to remove VPg was found to be the same as that of FMDV mRNA extracted from infected cells.

The method which we used routinely to assay for the removal of VPg from RNA was the change in migration of the S fragment of FMDV RNA on polyacrylamide gel electrophoresis. This method is relatively sensitive since approximately 5% of the radioactivity in uniformly labeled RNA is present in the S fragment and allows several experiments to be compared on a

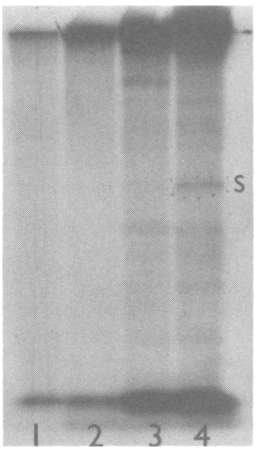

FIG. 3. *Removal of VPg from the 5' end of FMDV RNA by incubation with rabbit reticulocyte lysate. The RNA was incubated in vitro with ^{32}P γ-ATP and polynucleotide kinase after the appropriate treatments (see below), and the L and S fragments produced by digestion with RNase H-oligo(dG) were separated by polyacrylamide gel electrophoresis. Lane 1, RNA incubated with NTE; lane 2, RNA incubated with NTE and calf alkaline phosphatase; lane 3, RNA incubated with lysate; lane 4, RNA incubated with lysate and calf alkaline phosphatase. Labeling of S fragment only occurred in the RNA which had been treated with both lysate and phosphatase.*

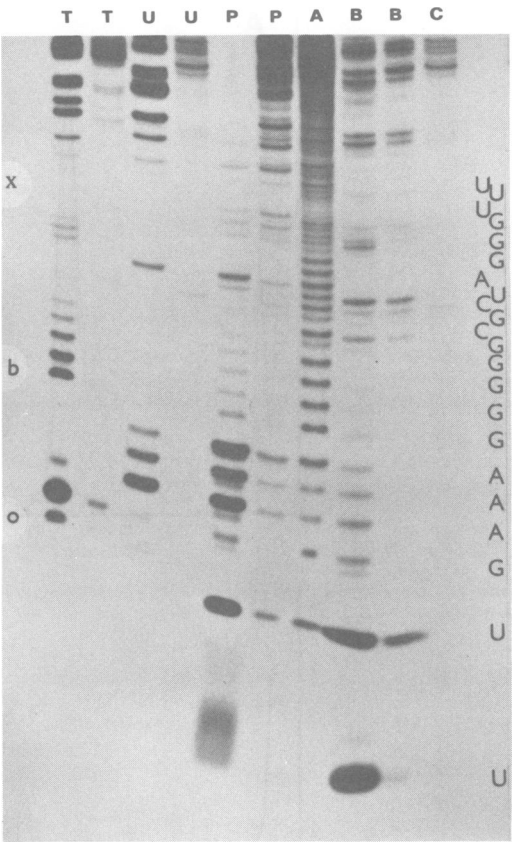

FIG. 4. *Autoradiograph of a 20% polyacrylamide gel of partial enzyme digests of RNA, ^{32}P end labeled after incubation in the reticulocyte lysate. The enzymes used are indicated at the top of each lane: T, RNase T_1; U, RNase U_2; P, Physarum nuclease; A, alkali; B, B. cereus enzyme; C, control, no enzyme. The position of the dye markers: x, xylene cyanol FF; b, bromophenol blue; o, orange G are also shown.*

single slab gel. This degree of sensitivity would not be possible with the small amount of radioactivity incorporated into VPg. Detecting the presence of VPg by measuring the mobility of the S fragment also appeared to be reliable, whereas anomalies were observed when the removal of VPg was monitored by phenol extraction. We found that VPg partitioned into the aqueous phase even when it had been removed from the RNA, a result which was explained by the finding that the rabbit reticulocyte lysate extensively degraded VPg to small hydrophilic peptides.

A similar degradation of poliovirus VPg by HeLa cell cytoplasmic extract and rabbit reticulocyte lysate has been described by Ambros, Pettersson, and Baltimore (3), and these workers were able to separate the enzyme activity responsible for cleaving the phosphodiester bond from the VPg degradative enzyme. Our results indicate that VPg is only degraded after it is cleaved from the RNA since in experiments where only a portion of the RNA had lost VPg, only two classes of S fragment were seen, one possessing a complete protein molecule and one lacking the protein. There was never any evidence of RNA molecules which contained a portion of VPg as judged by mobility of the S fragment.

The functional significance, if any, of the removal of VPg from picornavirus RNA is not known. It has been suggested that translation of the RNA requires the removal of VPg, but there is evidence that the initiation complex at least can be formed with VPg still present on the RNA (5). It is possible that VPg has a morphogenetic role in virus assembly and that its removal from RNA destined to become messenger is a controlling event in virus replication.

The role of the enzyme activity which degrades VPg in rabbit reticulocyte lysate is also unclear. The protease appears to be specific since virus proteins synthesized in vitro and the host proteins found in the lysate are stable for

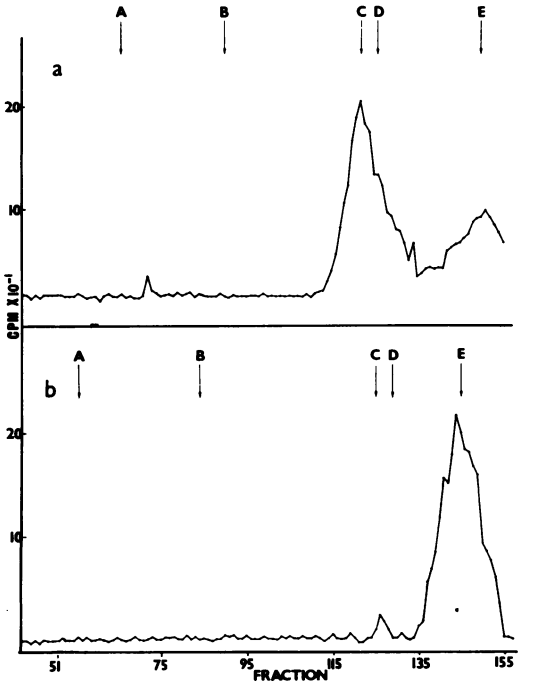

FIG. 5. *Estimation of size of VPg labeled with [^{3}H]tyrosine by filtration through Sepharose 6B in the presence of 6 M guanidine–0.1 mM dithiothreitol. VPg prepared from RNA by incubation with (a) RNase A, T_1, and T_2 for 12 h at 37°C; (b) rabbit reticulocyte lysate for 30 min at 30°C. Arrows indicate the position of marker proteins; A, ovalbumin; B, cytochrome c; C and D, insulin heavy and light chains. E indicates the position of phenol red.*

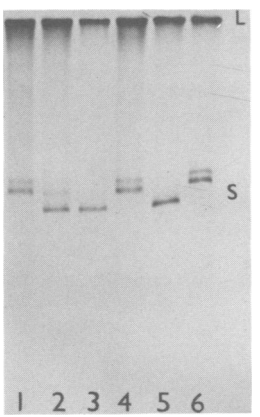

FIG. 6. *Effect of potential inhibitors on the removal of VPg from FMDV RNA. The rabbit reticulocyte lysate was treated with the potential inhibitor for 10 min at 30°C before adding the RNA. The RNA was then treated with RNase H, and the separations on polyacrylamide gels were made as in Fig. 1. Lane 1, lysate heated at 56°C for 30 min before addition of RNA; lane 2, 5 mM tyrosine phosphate; lane 3, 1% mercaptoethanol; lane 4, 1% SDS; lane 5, lysate only; lane 6, no addition to RNA.*

TABLE 2. *Effect of potential inhibitors on the cleavage of VPg from FMDV RNA*[a]

Treatment	Result
EDTA (5 mM)	+
56°C for 30 min	+
SDS (1%)	+
Zinc acetate (5 mM)	+
Cobalt chloride (5 mM)	±
Manganese chloride (5 mM)	±
Tyrosine phosphate (5 mM)	±
Sodium azide (0.1%)	−
Guanidine hydrochloride (200 μg/ml)	−
Phenylmethylsulfonyl fluoride (1 mM in 10% ethanol)	−
Trasylol (1 mM)	−
Sparsomycin (1 mM)	−
Pyrophosphate (8 mM)	−
Rat liver nuclease inhibitor	−
Heparin (500 μg/ml)	−
Ammonium acetate (pH 5.1) (1 mM)	−
Mercaptoethanol (1%)	−

[a] Rabbit reticulocyte lysate was treated for 10 min at 30°C, except where stated, with the compound under test. Virus RNA labeled with [^{3}H]uridine was added, and the incubation continued for a further 30 min at 30°C. Removal of VPg was monitored by the migration of S fragment, produced by digestion with ribonuclease H in the presence of oligo(dG) on polyacrylamide gels. Symbols: +, greater than 70% inhibition of cleavage activity; ±, a limited inhibition of cleavage activity (≃10%); −, no detectable inhibition.

several hours. The presence of such an activity could explain why free VPg is not detected in infected cells. The protease may be involved in control processes in the replication of picornaviruses.

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