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Location of the Initiation Site for Protein Synthesis on Foot-and-Mouth Disease Virus RNA by In Vitro Translation of Defined Fragments of the RNA

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An mRNA-dependent reticulocyte lysate has been used to translate foot-and-mouth disease virus RNA in vitro. Polypeptides P16, P20a, and P88, which have been shown to be derived from the 5′ end of the RNA by pactamycin mapping experiments with infected cells, were preferentially synthesized in vitro. Removal of VPg, the small protein covalently linked to the 5′ end of the genome RNA, had no effect on the translation of the RNA. The two RNA fragments (L and S) produced by specific digestion of the polycytidylic acid [poly(C)] tract with RNase H were also translated in vitro. The L fragment, consisting of RNA to the 3′ side of the poly(C) tract and including the polyadenylic acid [poly(A)] tract, directed the synthesis of the same products as those made by full-length RNA. However, no small defined products were produced when the S fragment, which contains the 5′ end of the RNA, was translated. These results show that the major initiation site for protein synthesis on foot-and-mouth disease virus RNA is to the 3′ side of the poly(C) tract. Furthermore, the use of *N*-formyl [^{35}S]methionine $tRNA_f^{Met}$ as a label for the initiation peptides showed that the major polypeptide labeled in lysates primed with both full-length RNA and the L fragment was P16, i.e., the protein nearest the initiation site for translation as deduced from pactamycin mapping experiments. Fragments of RNA were also translated in vitro. Those containing the poly(C) tract gave products similar to those produced when full-length RNA was translated. The polypeptides synthesized when fragments containing the poly(A) tract were used, however, did not resemble those made from full-length RNA.

Foot-and-mouth disease virus (FMDV) is a member of the family Picornaviridae. These viruses contain a single-stranded RNA molecule that acts directly as the mRNA and has a tract of polyadenylic acid [poly(A)] at the 3′ end; the 5′ end is blocked by VPg, a small covalently linked protein (15, 22, 24). In addition to the poly(A) (4), FMDV RNA contains a homogeneous tract of cytidylic acid [poly(C)] 100 to 200 nucleotides long near the 5′ end of the RNA (1, 3, 9, 10). Digestion of FMDV RNA with RNase H in the presence of oligodeoxyguanylic acid [oligo(dG)] specifically digests the poly(C), leaving two fragments of RNA (22). The short fragment (S) is about 400 nucleotides long and contains VPg, showing that the poly(C) tract is not at the 5′ terminus of the RNA (22). The long fragment (L), which is about 7,500 nucleotides long, contains the remainder of the RNA, including the poly(A) tract at the 3′ end (22).

Studies of the in vivo translation directed by picornavirus RNAs have shown that they possess only one major initiation site for protein synthesis, located near the 5′ end of the RNA (13). This mechanism of protein synthesis has allowed the order of the genes on the RNA to be determined by using pactamycin to block the initiation of protein synthesis (21). With FMDV, we have shown that there are three major primary products synthesized in infected BHK-21 cells; these were ordered 5′ P88-P52-P100 3′ (8, 23). Primary product P88 was shown to be the precursor of the virus structural proteins, and P100 was shown to be the precursor to a component of the virus-specific RNA polymerase (P56a) (16a).

There have been several recent reports of the complete translation of picornavirus genomes in micrococcal nuclease-treated cell-free systems (18, 25, 26). We have used this system to investigate the ability of FMDV RNA to direct protein synthesis in vitro, and the products have been compared with the virus-specific proteins found in FMDV-infected cells. In addition, we have added the L and S fragments, prepared by RNase H treatment of the virus RNA, to the in

vitro system in an attempt to locate the position of the initiation site of protein synthesis and to define the role of the poly(C) in translation.

MATERIALS AND METHODS

Labeling of virus-induced proteins in vivo. Monolayers of BHK-21 cells in 4-ounce (ca. 120-ml) bottles were infected at 37°C at a multiplicity of infection of about 100. Virus was allowed to attach and absorb for 30 min, after which the monolayers were washed with methionine-free Eagle medium. The cells were incubated for a further 90 min before they were pulsed with [^{35}S]- or [^{3}H]methionine (910 and 780 Ci/mmol, respectively; obtained from the Radiochemical Centre, Amersham, United Kingdom) for 5 or 10 min. In chase experiments the medium containing the [^{35}S]methionine was replaced with Eagle medium supplemented with 1 mM methionine, and the incubation was continued for a further 30 min. For mapping, pactamycin at a final concentration of 1.5×10^{-7} M was added to the medium 2 min before the addition of [^{35}S]methionine. In some experiments, zinc acetate or iodoacetamide, at a final concentration of 5×10^{-4} M, was added 10 min before the radioactive label to inhibit the secondary proteolytic cleavages.

Separation and isolation of virus-specified polypeptides. The labeled polypeptides were separated and isolated essentially as described previously (8). The gel system employed was either a 7.5 or 10% discontinuous gel as described by Laemmli (14) or a 15% polyacrylamide gel, using the Laemmli buffer system but incorporating 4 M urea in the stacking and running gels. The polypeptides were visualized by either autoradiography or fluorography (2).

Peptide mapping. Peptides were eluted from the gel segments, carboxymethylated, digested with trypsin, and analyzed by ion-exchange chromatography as described previously (7).

Preparation of virus RNA. FMDV type A_{10} (strain A_{61}) was grown in monolayers of 10^8 BHK-21 cells in Roux bottles. Large amounts of virus containing undegraded RNA were prepared from about 4×10^9 cells which had been infected at a high multiplicity of infection. Cells were infected in 20 ml of Eagle medium containing at least 2×10^9 PFU of virus. Virus was allowed to absorb for 30 min at 37°C, the cells were washed with 20 ml of warm Eagle medium, and 20 ml of fresh Eagle medium was added. As soon as the cells left the glass (about 3 to 4 h after infection), the bottles were cooled to 4°C, and the virus was purified from the medium by $(NH_4)_2SO_4$ precipitation and centrifugation through 15 to 45% sucrose gradients in NTE (140 mM NaCl, 20 mM Tris-hydrochloride, 5 mM EDTA, pH 7.6) (22). The RNA was extracted as previously described (22) and purified further by centrifugation on 5 to 25% sucrose gradients in NTE containing 0.1% sodium dodecyl sulfate (SDS) (60,000 × *g*, 20°C, 16 h, Beckman SW42.1 rotor). The peak fractions of RNA sedimenting at 35S (relative to 28S and 18S rRNA markers run in a parallel gradient) were detected by absorbance at 260 nm, pooled, and precipitated at −20°C with 2 volumes of ethanol. With this growth and purification procedure, at least 75% of the virus RNA was recovered in the 35S peak.

Enzymatic and chemical digestion of the RNA. The poly(C) tract in FMDV RNA was specifically digested with *Escherichia coli* RNase H (EC 3.1.4.34) to produce the L and S fragments, essentially as described previously (22). Enzyme was obtained commercially (Enzobiochem, New York, N.Y.; catalog no. ERN-H) at a specific activity of 1,000 to 2,000 U/ml and as a gift from J. L. Darlix (Département de Biologie Moléculaire, Geneva, Switzerland) at a specific activity of 500 U/ml. Digestion conditions were adjusted to produce the L and S fragments without concomitant endonuclease fragmentation. The standard incubation mixture in 30 μl of RNase H buffer (50 mM Tris-hydrochloride [pH 7.9], 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 10% glycerol) consisted of the following: 30 μg of RNA, 1 μg of oligo(dG_{12-18}) (Collaborative Research Inc., Waltham, Mass.), and 2 to 4 U of enzyme, added in that order. Larger amounts of RNA were digested at the same ratio in proportionately larger volumes. The reaction mixtures were incubated at 30°C for 45 min; after the addition of 0.47 ml of NTE containing 0.1% SDS, the L and S fragments were separated by centrifugation on 5 to 25% sucrose gradients as described above. The amount of the poly(C) tract remaining in the L fragment was checked by using [^{3}H]cytidine or [^{32}P]RNA (22).

To obtain random fragments of RNA, we made use of the fact that virus grown at 37°C overnight after infection of cells at a low multiplicity produced randomly fragmented RNA when purified as described above (see reference 6; T. J. R. Harris, unpublished data). In contrast to the peak of RNA at 35S, obtained from virus grown after infection at a high multiplicity, a broad band of RNA sedimenting from 10 to 35S was found. This band was precipitated with 2 volumes of ethanol at −20°C and further fractionated into molecules containing poly(C) or poly(A) by affinity chromatography on 100-mg columns of oligo(dG)- or oligodeoxythymidylic acid [oligo(dT)]-cellulose, respectively (Collaborative Research, Inc.; T_5 or T_3), as described (11). Fragments were centrifuged on 5 to 25% sucrose gradients in NTE containing 0.1% SDS, and the RNAs sedimenting at 10 to 20S, 20 to 30S, and 30 to 37S were pooled and precipitated with ethanol. To remove the VPg, we digested the RNA with 1 mg of pronase per ml in 0.1 M Tris (pH 7.6) containing 1% SDS for 60 min at 37°C at an enzyme/RNA ratio of approximately 100:1.

In vitro translation of virus RNA and RNA fragments. The mRNA-dependent micrococcal nuclease-treated rabbit reticulocyte lysate system was used in all experiments (20). The standard protein-synthesizing mixture (20 μl) contained 17 μl of nuclease-treated lysate, 0.3 μl of pig liver tRNA (6 μg/μl), 1.2 μl of [^{35}S]methionine (910 Ci/mmol), and 1.5 μl of distilled water. This was incubated with the appropriate RNA (0.5 to 2 μg) at 30°C for various times, and the extent of protein synthesis was determined by trichloroacetic acid precipitation (20). In the experiments with *N*-formyl [^{35}S]methionine $tRNA_f^{Met}$, excess unlabeled methionine (1.25 mM) was added to prevent the incorporation of [^{35}S]methionine into internal positions in the polypeptide. Reactions were terminated by incubating the lysate with 5 μl of an

RNase mixture (T_1, 500 U/ml; A, 700 U/ml; T_2, 33 U/ml) at 37°C for 15 min. After the addition of 5 volumes of 0.125 M Tris (pH 6.8)–2% SDS–2% 2-mercaptoethanol, the mixture was boiled for 2 min and the polypeptides were separated by polyacrylamide gel electrophoresis as described above.

RESULTS

Extending the in vivo-derived genetic map. In addition to the three primary products, P88, P52, and P100, which we have previously described in FMDV-infected cells (8, 23), we have recently detected two considerably smaller polypeptides, P20a and P16, which are efficiently labeled when infected cells are given a short pulse of [^{35}S]methionine (Fig. 1, lane A). These polypeptides were also found in infected cells treated with iodoacetamide, a compound which inhibits many of the secondary cleavages which give rise to functional proteins, suggesting that P20a and P16 are primary products. Pactamycin mapping was used to determine the map positions of these additional primary products. Figure 1, lane B, shows the polypeptides labeled with [^{35}S]methionine for 5 min, 2 min after the addition of pactamycin to infected cells. Compared with the control, which lacked pactamycin (Fig. 1, lane A), there was a substantial decrease in the amount of P88, whereas P52 and P100 were relatively unaffected. However, labeling of P20a and P16 was almost completely eliminated, placing them nearer the 5′ end of the genome than P88. Tryptic peptide maps of P20a and P16 have shown that they are not related to any of the other primary products but are closely related to each other and are probably derived from the same region of the RNA (D. V. Sangar and T. R. Doel, unpublished data). Pulse-chase experiments failed to show any precursor-product relationship between P20a and P16, suggesting that they may arise by an alternative cleavage mechanism, as demonstrated for other picornavirus proteins (8, 16). However, after a chase period, one and sometimes two new products with molecular weights of approximately 20,000 were also detected. These are thought to be cleavage products of P100 and P52. For ease of reference, we refer to the primary product as P20a.

In vitro translation of FMDV RNA. In vitro translation of FMDV RNA was studied with the mRNA-dependent rabbit reticulocyte lysate of Pelham and Jackson (20). The incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material in response to FMDV RNA was approximately 40-fold greater than that in the lysate incubated without added RNA (Table 1). The polypeptides labeled with [^{35}S]-methionine during the 40-min incubation were analyzed on a 10% polyacrylamide gel. The major products synthesized were the polypeptides P88, P20a, and P16 found in infected cells (Fig. 2). Furthermore, tryptic peptide mapping experiments showed that the in vitro and in vivo products were very closely related (Fig. 3). The major products made in vitro after a short incubation are, therefore, those polypeptides coded for by the 5′ end of the RNA. However, polypeptides comigrating with both P52 and P100 were also detected, indicating that at least a proportion of the ribosomes was traversing the entire genome (Fig. 2). When the in vitro incubation was extended to 135 min, additional polypeptides, corresponding to those proteins found in infected cells that are derived by cleavage of the primary products, were found. For example, polypeptides comigrating with P38 (VP0) and P34, derived from P88 and P52, respectively (23), were readily detected (Fig. 2). However, we were able to detect only trace amounts of P56a,

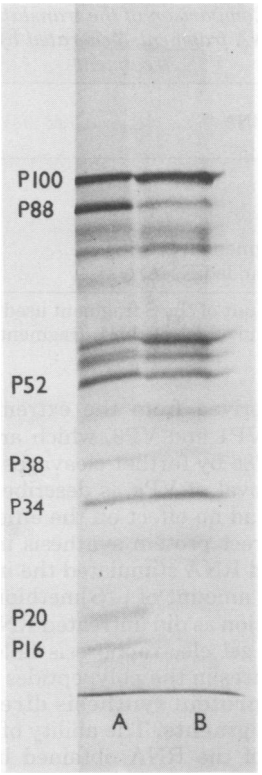

FIG. 1. *Pactamycin mapping of the induced proteins of FMDV. Virus-infected BHK-21 cells were labeled for 5 min with [^{35}S]methionine either at 120 min postinfection (lane A) or at 122 min postinfection after the addition of 1.5 × 10^{-7} M pactamycin 2 min previously (lane B). The labeled polypeptides were analyzed on 7.5% polyacrylamide gels.*

TABLE 1. *Comparison of the translation efficiency of the RNA fragments generated by RNase H treatment*

RNA	Incorporation (cpm)
Full length	64,167
L fragment	65,778
S fragment[a]	7,223
Random fragments (4 to 8S)	7,546
Control (no added RNA)	1,717

[a] The amount of the S fragment used was five times the molar equivalent of the L-fragment or full-length RNA.

which is derived from the extreme 3′ end of P100, and VP1 and VP3, which are derived in vivo from P88 by further cleavages (8, 23).

The removal of VPg as described by Sangar et al. (24) had no effect on the efficiency of the RNA to direct protein synthesis in vitro. Pronase-treated RNA stimulated the incorporation of a similar amount of [^{35}S]methionine in a 40-min incubation as did untreated RNA, and polyacrylamide gel electrophoresis failed to detect any differences in the polypeptides synthesized.

In vitro protein synthesis directed by the L and S fragments. The ability of the L and S fragments of the RNA obtained by RNase H digestion to direct the incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material was determined. The radioactivity incorporated in response to full-length RNA and the L fragment was similar (Table 1). Although the S fragment produced a slight stimulation in the incorporation of label, this was no greater than that produced by random fragments of RNA sedimenting at about 4 to 8S (i.e., of a size similar to the S fragment) generated by digestion of FMDV RNA with RNase H in the absence of oligo(dG) (Table 1).

A comparison of the proteins synthesized in vitro during a 40-min incubation in response to the L fragment and to full-length RNA by polyacrylamide gel electrophoresis showed that qualitatively similar polypeptides were made, with proteins P16, P20a, and P88 derived from the 5′ end being particularly evident (Fig. 4a). To compare the smaller polypeptides, we also analyzed the translation products on 15% gels in the presence of 4 M urea. Figure 4b shows that the same polypeptides were made from both L-fragment and full-length RNAs.

Figure 4 also shows that the translation of the L fragment led to an apparent increase in the synthesis of polypeptides with molecular weights of 35,000 and 30,000. This increase was not always as pronounced as that shown in Fig. 4, and its significance, if any, is unclear at present. In addition, the amount of P88 produced in some experiments in response to L-fragment RNA was considerably lower than the control incubation with full-length RNA. This decrease, however, was not observed consistently, the same preparation of L-fragment RNA producing variable amounts of P88 when translated at different times in the same batch of reticulocyte lysate. It is unlikely that the incorporation of label directed by the L fragment was due to full-length RNA molecules which had escaped RNase H digestion since an analysis of L-fragment RNA by RNase T_1 digestion indicated that less than 3% of the poly(C) remained (see reference 22). This view was confirmed by showing that the specific activity of the L fragment was

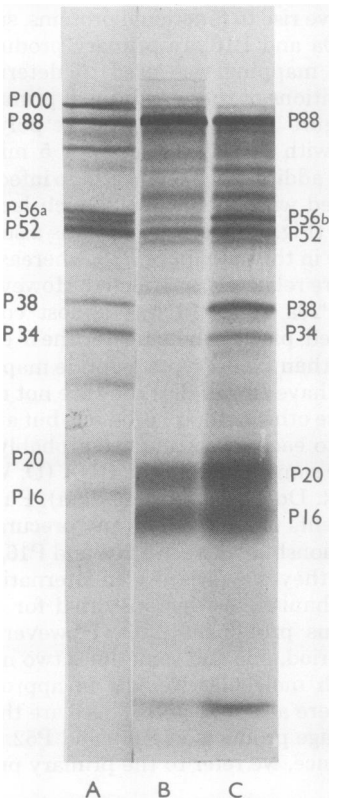

FIG. 2. *Polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro in response to FMDV RNA. The rabbit reticulocyte lysate was incubated with the RNA in the presence of [^{35}S]methionine for either 40 min (lane B) or 135 min (lane C). The polypeptides labeled with [^{35}S]methionine in virus-infected BHK-21 cells between 120 and 125 min postinfection are shown in lane A for comparison. The polypeptides were analyzed in nonadjacent lanes of a 10% polyacrylamide gel.*

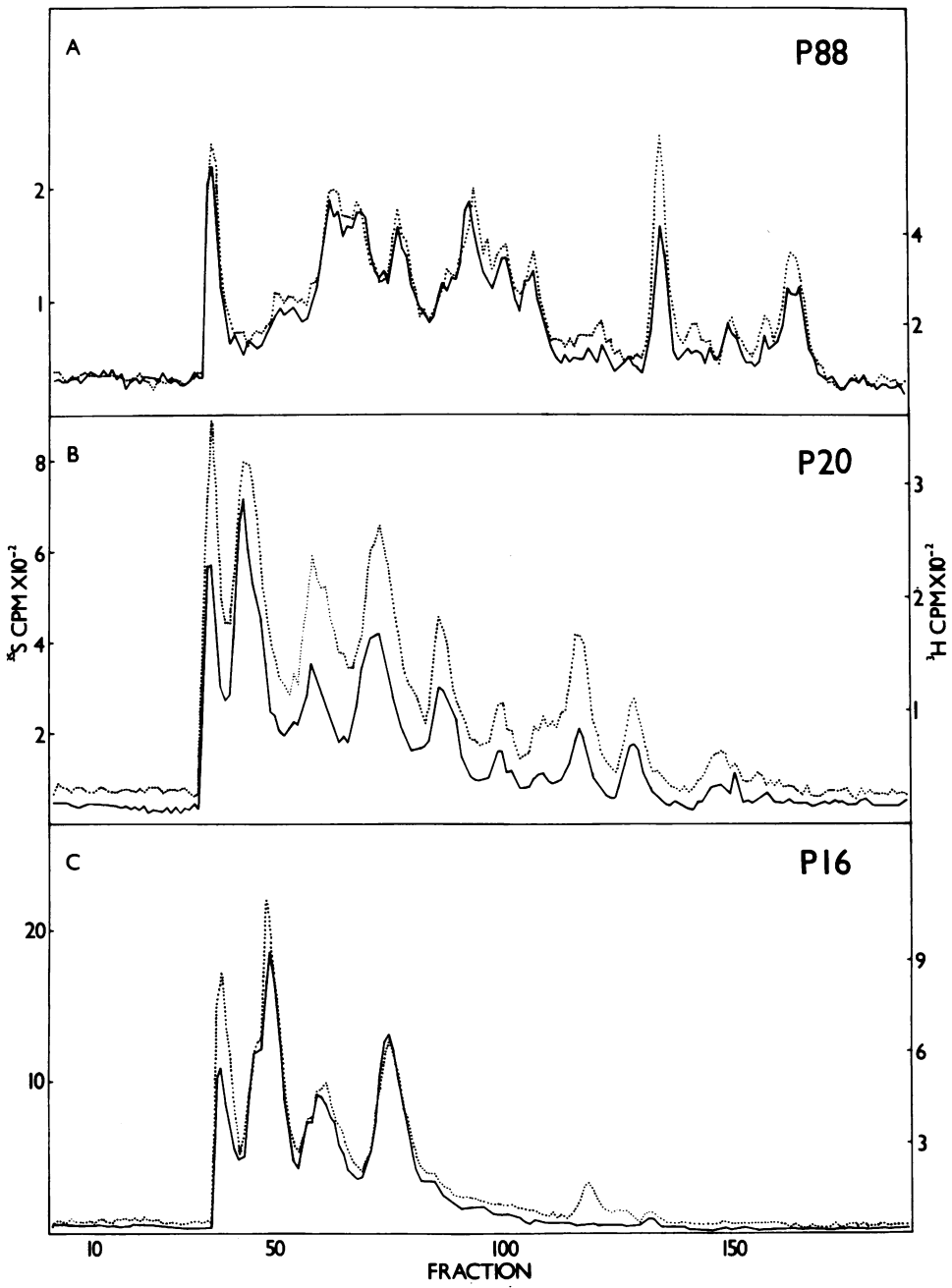

FIG. 3. *Comparison of the polypeptides synthesized in vivo and in vitro by ion-exchange chromatography of their tryptic peptides: (A) P88; (B) P20; (C) P16. Polypeptides made in vivo were labeled with [^{3}H]-methionine (---), and those in vitro were labeled with [^{35}S]methionine (—).*

similar to that of the full-length RNA (Table 2).

An analysis of the products synthesized in vitro in response to the S fragment showed the same spectrum of polypeptides as produced by the L fragment, but in much smaller amounts (Fig. 4a and b). Furthermore, it was not possible to demonstrate the synthesis of a polypeptide with a molecular weight of less than 16,000, the expected size for a translation product coded only by the S fragment (Fig. 4b).

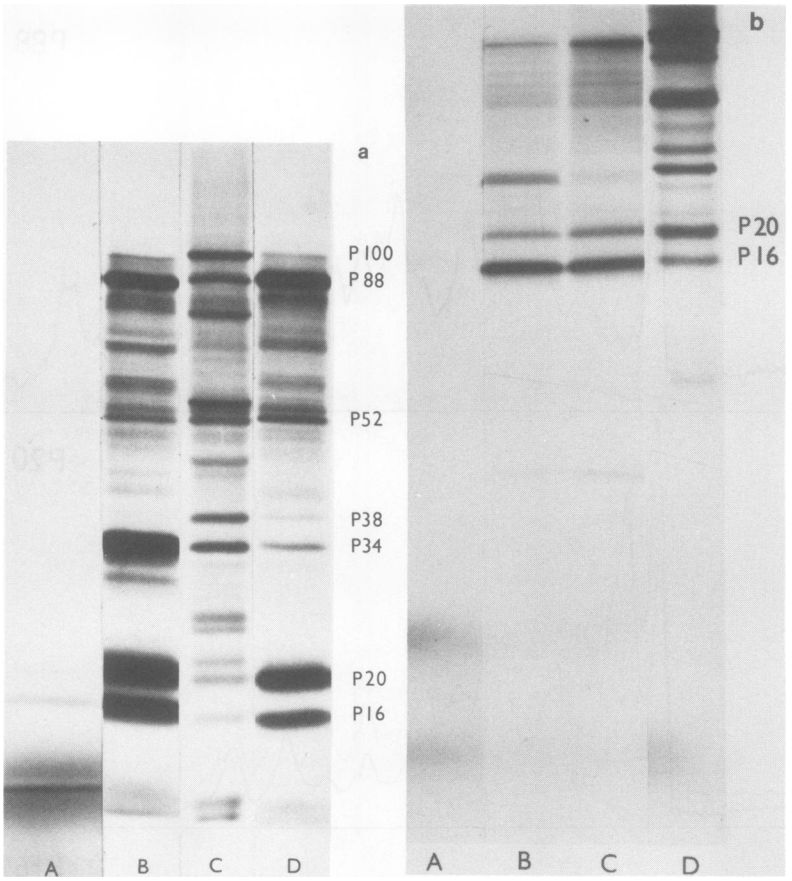

FIG. 4. *Comparison by polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro during a 40-min incubation wtih FMDV RNA and [^{35}S]methionine. (a) Full-length RNA (lane D); L fragment (lane B); S fragment (lane A). (b) Full-length RNA (lane C); L fragment (lane B); S fragment (lane A). The polypeptides labeled with [^{35}S]methionine in virus-infected BHK-21 cells between 120 and 130 min postinfection are shown in lane C of (a) and lane D of (b) for comparison. The polypeptides in (a) were analyzed in 10% gels; those in (b) were analyzed in 15% gels containing 4 M urea in the running and stacking gels.*

In vitro translation with 5′ or 3′ fragments of FMDV RNA. Fragments of FMDV RNA containing either the poly(C) or the poly(A) tract were isolated by oligo(dG)- or oligo(dT)-cellulose affinity chromatography. The fragments were fractionated by sucrose gradient centrifugation into three size classes (10 to 20S, 20 to 30S, and 30 to 37S), and RNA from each size class was added to the reticulocyte lysate. RNA from each size class containing the poly(C) tract directed the synthesis of protein. The 20 to 30S size class was translated more efficiently than the 30 to 37S size class, but the smallest size class (10 to 20S) was not translated as efficiently as the others (Table 3). The proteins synthesized in response to the poly(C)-containing fragments were qualitatively similar to those produced by full-length RNA (Fig. 5) although the synthesis of polypeptides P20a and P16, which are coded by the 5′ end of the RNA, was enhanced when the smaller size classes were used.

TABLE 2. *Comparison of the in vitro synthesis of [^{35}S]methionine-labeled, trichloroacetic acid-precipitable material directed by different amounts of virus RNA or the L fragment*

Sample	Incorporation[a] with following amt (μg) of template RNA:		
	2.0	0.67	0.22
Virus RNA	47,177	40,438	16,180
L fragment	41,258	30,912	15,308

[a] The numbers are ^{35}S counts per minute rendered acid insoluble after 30 min of incubation in the standard reticulocyte lysate system.

Similar experiments with the poly(A) fragments showed, unexpectedly, that all size classes

were translated efficiently, particularly fragments of 20 to 30S (Table 3). However, in contrast to the fragments containing poly(C), the major polypeptides produced by the poly(A)-containing fragments did not correspond in size to the polypeptides produced by untreated RNA (Fig. 5), presumably because, even in the 30 to 37S size class, most of the molecules lacked the 5′ end of the RNA.

TABLE 3. *Comparison of the translation efficiency of RNA fragments containing either poly(A) or poly(C) isolated by affinity chromatography*

RNA	Incorporation (cpm)
Poly(A)	
30 to 37S	35,281
20 to 30S	58,818
10 to 20S	17,122
Poly(C)	
30 to 37S	28,291
20 to 30S	52,853
10 to 20S	4,310
Control (no added RNA)	1,518

Analysis of initiation peptides with *N*-formyl [^{35}S]methionine tRNA$_f^{Met}$. In eucaryotic cells, protein synthesis is initiated by a specific methionyl tRNA (tRNA$_f^{Met}$). Normally, the amino-terminal methionine residue in the new polypeptide is removed. However, if the amino-terminal group is blocked by formylation, then the methionine residue is not removed although it is incorporated normally. Thus, by adding FMDV RNA to a reticulocyte lysate and labeling with *N*-formyl [^{35}S]methionine tRNA$_f^{Met}$, it should be possible to identify the polypeptides containing an initiation site. The translation of both full-length and L-fragment RNAs in the presence of this radioactive label resulted in the incorporation of radioactivity mainly into P16, with a small amount being incorporated into P20a (Fig. 6a). An examination of the polypeptides labeled with *N*-formyl [^{35}S]methionine tRNA$_f^{Met}$ on 15% gels failed to

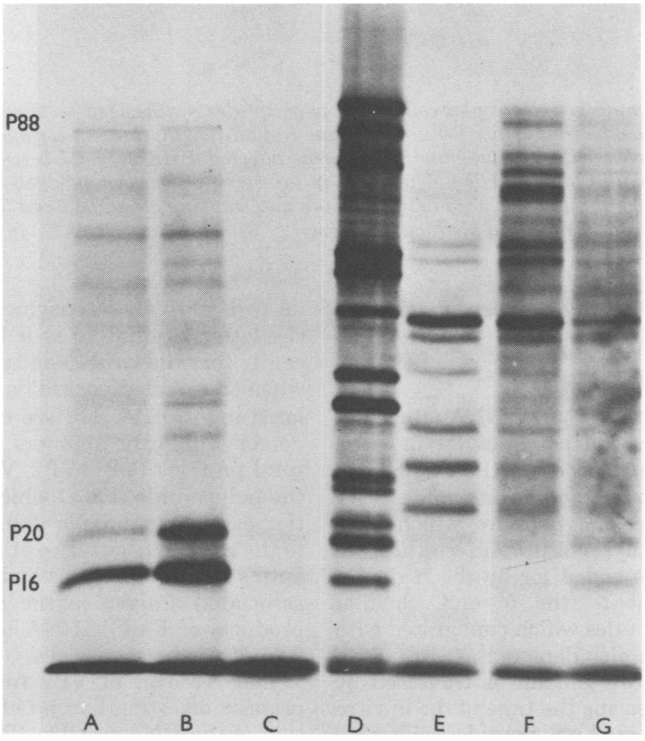

FIG. 5. *Polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro with fragments of RNA containing either poly(C) or poly(A). Fragmented RNA was separated by sucrose gradient centrifugation, and the molecules containing poly(C) or poly(A) selected by affinity chromatography were incubated with the reticulocyte lysate in the presence of [^{35}S]methionine. (Lanes A to C) Poly(C)-containing RNA: (A) 30 to 37S; (B) 20 to 30S; (C) 10 to 20S. (lanes E to G) Poly(A)-containing RNA: (E) 30 to 37S; (F) 20 to 30S; (G) 10 to 20S. Lane D contains the polypeptides labeled with [^{35}S]methionine in virus-infected BHK-21 cells between 120 and 130 min. The polypeptides were analyzed on 10% gels.*

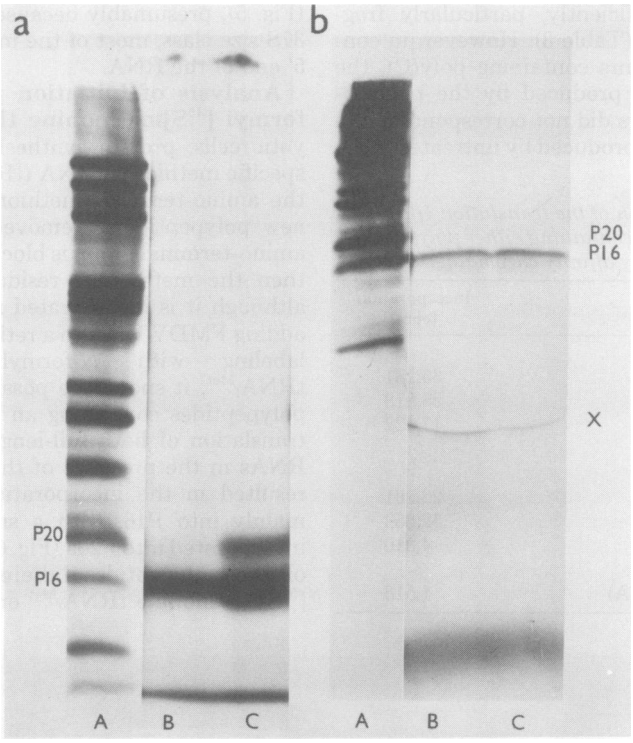

FIG. 6. *Polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro with N-formyl [^{35}S]-methionine tRNA$_f^{Met}$ as the radioactive precursor. (Lane B) Full-length; (lane C) L fragment. For comparison, the polypeptides labeled with [^{35}S]methionine in virus-infected BHK-21 cells between 120 and 130 min postinfection are shown in lane A. The polypeptides in (a) were analyzed in 10% gels, and those in (b) were analyzed in 15% gels containing 4 M urea in the running and stacking gels. The band labeled X is an artifact which is also found in a lysate incubated with N-formyl [^{35}S]methionine tRNA$_f^{Met}$ in the absence of added RNA.*

show the presence of any polypeptide smaller than P16 (Fig. 6b).

DISCUSSION

We have shown that FMDV RNA efficiently directs the synthesis of polypeptides in vitro. Three of these in vitro products (P88, P20a, and P16) are made in large amounts and appear very similar to the three polypeptides which have been shown, by analysis of protein synthesis in infected cells, to be coded for by the region of the RNA adjacent to the 5′ end. Smaller amounts of polypeptides which comigrated with P52 and P100 were also detected in vitro, indicating that the entire genome is traversed by ribosomes. By increasing the time of the in vitro incubation, we detected polypeptides which comigrated with those polypeptides known to be produced in vivo by proteolytic cleavage of larger precursors. This result indicates either that there is an enzyme present in rabbit reticulocytes which is capable of processing FMDV proteins or that the virus genome can code for its own proteolytic enzyme in vitro. We favor the latter alternative as it has been shown recently that the virus-coded protease is produced when encephalomyocarditis virus RNA is translated in vitro (18, 26). We have been unable to detect significant amounts either of the structural proteins (VP1, VP2, VP3, and VP4) or of the polypeptide (P56a) which is the virus infection-associated antigen (23). This is in contrast to the work of Chatterjee et al. (5), who detected both structural proteins and the virus infection-associated antigen in the cell-free translation products of FMDV RNA in extracts prepared from Ehrlich ascites cells.

The removal of VPg from the genome by pronase digestion had no effect on the translation of the RNA in vitro. This is not surprising since it has been shown for poliovirus that the mRNA extracted from polysomes does not possess a covalently linked protein (12, 17). It appears unlikely, therefore, that VPg has a role in translation, although it should be appreciated that pronase digestion of FMDV RNA does not

completely remove the protein, but leaves a stub of amino acids still covalently attached to the RNA (T. J. R. Harris, unpublished data).

It has been shown previously that it is possible to digest the poly(C) tract of FMDV RNA with RNase H in the presence of oligo(dG) to yield two fragments, designated L and S (22). The polypeptides made by in vitro translation of the L fragment were similar to those produced from full-length RNA. Although there was some incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material when the S fragment was translated, the polypeptides synthesized were all made in much greater amounts by the L fragment. We believe that the apparent activity of the S fragment is due to contamination with random breakdown products of full-length RNA, particularly since the incorporation of radioactivity was similar and the same products were made when small nonspecific RNA fragments generated by RNase H digestion in the absence of oligo(dG) were translated.

The use of *N*-formyl [^{35}S]methionine $tRNA_f^{Met}$ as the radioactive precursor resulted in the majority of the label being incorporated into P16, although there was some labeling of P20a. These polypeptides have been shown to have similar tryptic peptide maps, suggesting that there is only one initiation site for protein synthesis on the RNA. As the pactamycin mapping experiments in vivo show that P16 is located nearest the initiation site, there is evidence that P16 is the initiation polypeptide in vivo, as well as in vitro. Since both P16 and P20a are produced when the L fragment is translated in vitro, we conclude that the ribosome-binding site on FMDV RNA lies to the 3′ side of the poly(C) tract.

We were surprised to find that small fragments of FMDV RNA, containing the poly(A) tract at the 3′ end, directed the incorporation of [^{35}S]methionine into protein when added to the in vitro system. The products obtained, however, were different from those synthesized in response to full-length RNA or to fragments containing the poly(C) tract. As there is one major initiation site near the 5′ end of the RNA, it appears that the minor sites exposed in fragmented RNA are blocked in full-length RNA and probably have no significance for protein synthesis in vivo (see reference 19).

The function of the S fragment and the adjacent poly(C) tract on the 5′ side of the ribosome-binding site remains unclear. It is possible that the translation of the S fragment requires conditions different from those needed for either full-length or L-fragment RNA. The most straightforward explanation for the lack of a polypeptide product specified by the S fragment is that the RNA on the 5′ side of the poly(C) tract is not translated, at least in vitro. If this is so, then the ribosome-binding site in FMDV RNA is farther from the 5′ end of the RNA than is generally found for other eucaryotic mRNA's. The nucleotide sequence at the 5′ end of FMDV RNA which is currently under study should give us a better understanding of its coding potential and function.

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