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Multiple Proteases in Foot-And-Mouth Disease Virus Replication

J. N. BURROUGHS,[1] D. V. SANGAR,[1]†* B. E. CLARKE,[1]† D. J. ROWLANDS,[1]† A. BILLIAU,[2] AND D. COLLEN[3]

Animal Virus Research Institute, Pirbright, Woking, Surrey GU24 ONF, United Kingdom,[1] and Rega Institute[2] and Center for Thrombosis and Vascular Research,[3] University of Leuven, Leuven, B-3000 Belgium

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Translation of foot-and-mouth disease virus RNA in a rabbit reticulocyte lysate for short time intervals resulted in the production of the peptides P20a, P16, and P88 (Lab, Lb, and P1) (R. R. Rueckert, *Recommendations of the 3rd European Study Group on Molecular Biology of Picornavirus*, Urbino, Italy, 1983). If further translation was prevented, the structural protein precursor P88 was not cleaved, even after prolonged incubation. This result indicates that the mechanism of the cleavage between P20a-P16 and P88 and of that between P88 and P52 (P2) differs from the mechanism of the secondary cleavages which produce the structural proteins. Furthermore, treatment of foot-and-mouth disease virus-infected cells with the protease inhibitor D-valyl phenylalanyl lysyl chloromethyl ketone prevented the in vivo cleavage between P20a-P16 and P88 but had no effect on any of the other cleavage events. These results suggest that the cleavage of the foot-and-mouth disease virus polyprotein utilizes two different host proteases.

Foot-and-mouth disease virus (FMDV) comprises the genus *Aphthovirus* of the family *Picornaviridae*. Although the basic strategy of replication is common to the entire family, there are a number of important differences between FMDV and poliovirus, a member of the genus *Enterovirus* and the most completely studied picornavirus. For example, in contrast to poliovirus, FMDV has a polycytidylate tract in the 5′ noncoding region of the genome (2), there are three tandem genes for the genome-linked protein VPg (8), leader proteins are coded to the 5′ side of the structural protein coding region of the genome (20), and many of the proteolytic processing sites on the polyprotein show no similarity to the analogous regions in poliovirus (1).

The genomic RNA of picornaviruses is ca. 8,000 nucleotides in length and serves directly as the messenger for protein synthesis (20). It is well documented that the initial product of picornavirus RNA translation is a polyprotein with a molecular weight of ca. 250,000 (3, 12). This is then cleaved into either three (poliovirus) (12) or four (FMDV) (20) primary products. These primary products are themselves then cleaved into the functional proteins found in infected cells. There is evidence that more than one protease is used in the posttranslational cleavage. Korant has shown with isolated poliovirus polyprotein that the initial cleavages to form the primary products can be produced by lysates of uninfected cells, whereas the further processing of these primary products requires infected cell extracts (14). Similarly, it has been shown that in an in vitro translation system programmed with encephalomyocarditis (EMC) virus RNA, the cleavage to yield protein A′, which is the precursor of the structural proteins, occurs very rapidly. However, this protein is not further processed unless translation is allowed to continue for longer times (9, 18). It has been shown that the protease required for cleavage of the precursor into the mature structural proteins is coded for by a portion of the RNA near the 3′ end of the genome (17). Thus, it appears that for EMC virus, at least two proteases are involved. Sequence studies on poliovirus have shown considerable uniformity of cleavage points, the majority of which occur at glutamine-glycine bonds, and it has been shown that antibody to P3-7C, the putative protease, inhibits cleavage at these sites (10). However, sequence data for FMDV have shown no such uniformity of cleavage sites (1), and it is possible that within the *Picornaviridae* family there are important differences in the cleavage strategies.

In this paper, we show that the FMDV polyprotein product is processed by at least two proteases.

MATERIALS AND METHODS

Purification of virus RNA. FMDV type A, subtype 10, strain 61, was grown in monolayers of BHK-21 cells and purified by differential centrifugation and sucrose gradient sedimentation. RNA was extracted from the virus and purified further as described previously (11).

In vitro translation of virus RNA. Virus RNA was translated in the presence of [^{35}S]methionine (>6,000 Ci/mmol; Amersham International) in micrococcal nuclease-treated rabbit reticulocyte lysate (Amersham International) (19). When further incubation was required to test for proteolytic processing, translation was terminated by the addition of cycloheximide and RNase I (both at 100 μg/ml). For direct analysis of polypeptides by polyacrylamide gel electrophoresis, an equal volume of 4% sodium dodecyl sulfate (SDS)–4% β-mercaptoethanol–20% glycerol–0.125 M Tris-hydrochloride (pH 6.8) was added, and the samples were heated at 100°C for 5 min.

Cytoplasmic extracts of BHK cells. Monolayers of BHK-21 cells in 4-oz (ca. 120-ml) bottles were infected with virus at a high multiplicity (>100 PFU per cell). At 2.5 h after infection, the cells were scraped from the glass and cytoplasmic extracts were prepared as described by Lowe and Brown (16). Extracts of mock-infected cells were prepared similarly.

Polyacrylamide gel electrophoresis. Samples were analyzed on polyacrylamide slab gels by the method of Laemmli (15). After electrophoresis, gels were dried and exposed to Fuji RX film.

Pulse-chase labeling of virus polypeptides. Pulse-chase experiments were performed as described by Harris et al. (11). In some experiments, 10^{-7} M pactamycin (a gift from Upjohn Ltd.) and, in other experiments, the protease inhibitor D-valyl phenylalanyl lysyl chloromethyl ketone (DVal-Phe-Lys-CH_2Cl) was added 3 or 15 min, respectively, before the addition of [^{35}S]methionine.

* Corresponding author.
† Present address: Wellcome Biotechnology Limited, Pirbright, Woking, Surrey GU24 ONF, U.K.

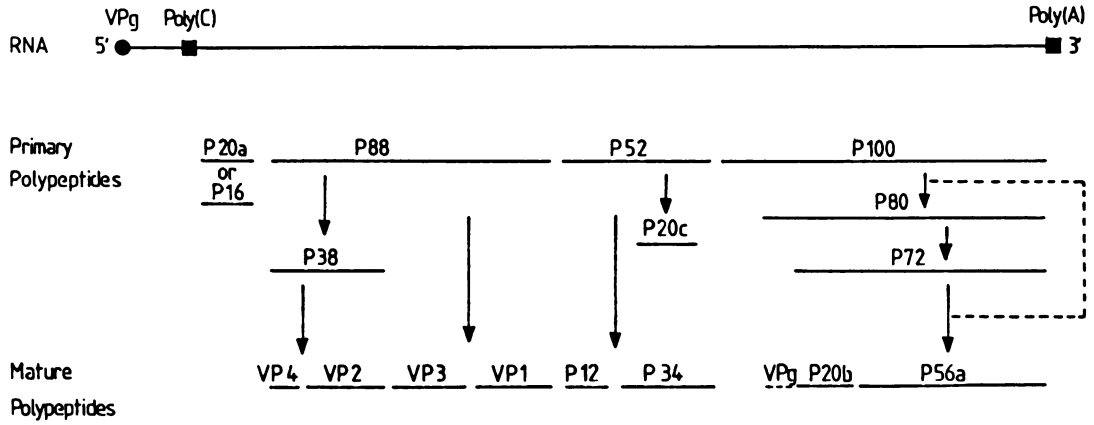

FIG. 1. Biochemical map of FMDV (7, 13). Poly(C), Polycytidylate; poly(A), polyadenylate.

Immunoprecipitation of virus-induced polypeptides. FMDV-infected BHK cells were treated at 105 min postinfection with 1 mM DVal-Phe-Lys-CH_2Cl for 15 min. [^{35}S]methionine (100 μCi) was added for 10 min and then chased with excess unlabeled methionine for 30 min. A cytoplasmic extract was made with Nonidet P-40–deoxycholate as described by Harris et al. (11). The cytoplasmic extract was treated in two ways. (i) The extract was diluted 10 times with phosphate-buffered saline, and 30 μl of antiserum was added. The reaction was allowed to proceed at 4°C for 18 h, after which, 30 μl of anti-species antibody was added. After a further 18 h, the precipitate was collected by centrifugation and washed three times. The pellet was dissolved in 2% SDS–2% β-mercaptoethanol–10% glycerol–0.125 M Tris (pH 6.8) and analyzed on 10% Laemmli polyacrylamide gels. (ii) The cytoplasmic extract was made 1% SDS and 1% β-mercaptoethanol and boiled for 2 min. The extract was then diluted 10 times in phosphate-buffered saline and treated as in (i).

Partial protease mapping. The polypeptides Px, Py, and P20a were separated on 10% polyacrylamide gels, and their positions were identified by autoradiography. The regions containing these proteins were cut from the dried gels, and the proteins were eluted into 0.125 M Tris (pH 6.8)–2% SDS–2% β-mercaptoethanol and recovered by acetone precipitation in the presence of 25 μg of bovine serum albumin. The pellets were dried and treated as described by Cleveland et al. (6) by using 200 μg of chymotrypsin per ml for 30 min at 37°C. The products were analyzed on 15% polyacrylamide gels.

RESULTS

The biochemical map for FMDV is shown in Fig. 1.

In vitro translation in rabbit reticulocyte lysates. FMDV RNA was translated for different time intervals in the rabbit reticulocyte lysate system, and the protein products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Within 10 min of the initiation of translation, proteins P20a and P16 could be clearly seen, and at this time, only very small amounts of the other viral proteins were visible. P20a and P16 shared common sequences and were coded for by the extreme 5′ end of the translated portion of FMDV RNA. They are derived from independent translation initiation sites (B. E. Clarke, D. J. Rowlands, J. N. Burroughs, D. V. Sangar, and A. R. Carroll, manuscript in preparation). After a 20-min incubation, P88, the precursor of the structural proteins, was the major product. When translation was terminated shortly after the appearance of P88 (20 min), negligible processing of this protein occurred during prolonged incubation (16 h) at 30°C (Fig. 3). Furthermore, the addition of cytoplasmic extract from uninfected cells to the 20-min translated product failed to cleave P88 during prolonged incubation (Fig. 4). However, addition of the cytoplasmic extract from the infected cells resulted in the cleavage of P88 to VP0, VP1, and VP3 (Fig. 4).

When translation was allowed to proceed until proteins from the 3′ region of the RNA were produced, then some processing of P88 into the structural proteins VP0, VP1, and VP3 was observed (Fig. 2). When translation was terminated at this time (30 to 45 min) and the sample was incubated for 16 h at 30°C, the majority of P88 was processed into structural proteins (Fig. 3).

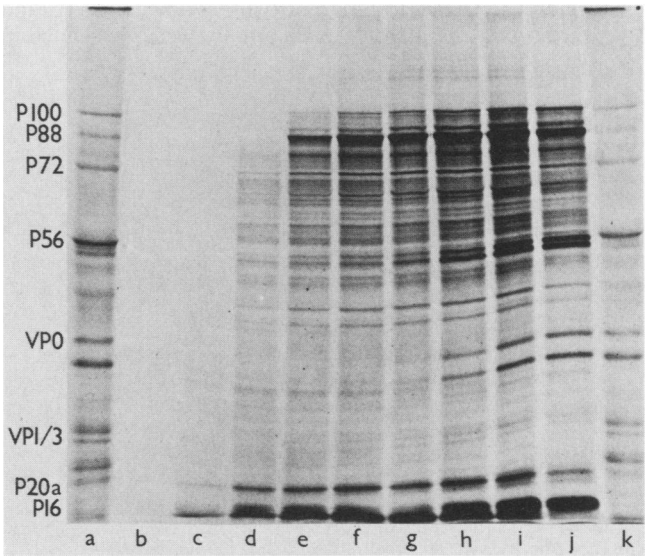

FIG. 2. Sequential synthesis of FMDV proteins in a rabbit reticulocyte lysate. Incorporation of [^{35}S]methionine was arrested at 5-min intervals by adding cycloheximide and pancreatic RNase. The samples were analyzed on a polyacrylamide gel. Lanes b through j correspond to samples incubated for 5, 10, 15, 20, 25, 30, 35, 40, and 45 min, respectively. Lanes a and k, Marker FMDV proteins synthesized in vivo.

These results indicate that the primary cleavage between P20a or P16 and P88 is different from the secondary cleavages of P88 which result in the formation of the structural proteins. It seems reasonable from these data to suggest that the enzyme(s) responsible for primary cleavage between P20a or P16 and P88 is host coded and that further processing of P88 involves a virus-coded enzyme.

The effect of the protease inhibitor DVal-Phe-Lys-CH_2Cl on virus protein processing in FMDV-infected BHK-21 cells. FMDV rapidly and efficiently inhibits cell protein synthesis; from about 2 h postinfection, only virus-induced proteins are made in significant quantities. After a 10-min pulse with [^{35}S]methionine, all the primary products were visible as well as many of their cleaved products (Fig. 5, lane a). During a chase with excess unlabeled methionine, the primary products disappeared and were replaced by their cleavage products (Fig. 5, lane c). Treatment of the cells with pactamycin for 3 min before the addition of label allowed the gene order to be ascertained; the decrease in the amount of radioactivity was P20a > P88 > P52 > P100 (L > P1 > P2 > P3 [Fig. 5]).

The effect of addition of the protease inhibitor DVal-Phe-Lys-CH_2Cl (5) 15 min before a 10-min pulse with [^{35}S]methionine is shown in Fig. 5, lane b. There was almost complete elimination of the polypeptides P88, P20a, and P16, which were replaced by a new protein P108, not seen in untreated cells. On lower-percentage gels, P108 was resolved into two polypeptides, P108 and P104 (data not shown). The other primary products, P100 and P52, appeared unaffected by the inhibitor and were produced in amounts similar to those found in untreated cells. The use of pactamycin clearly showed that these new proteins, P108 and P104, were located near the 5′ end of the genome (Fig. 5). The effect of a chase on the protease inhibitor-treated cells is shown in Fig. 5, lane d. The primary products P100 and P52 were processed normally to P72 and P34 and the proteins P108 and P104 were also cleaved. There was an increase in VP1 and VP3, which are normal products of P88; there was only a small increase in P38 (VP0), and two new proteins, Px and Py, were seen. The identity of Px and Py was determined in two ways.

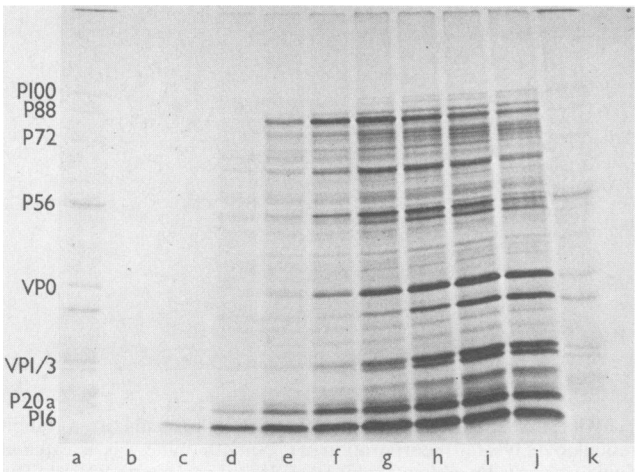

FIG. 3. Same procedure as in Fig. 2, but in addition, after inhibition of translation at the times indicated in the legend to Fig. 2, samples a through i were incubated at 30°C for a further 18 h before they were analyzed on a polyacrylamide gel.

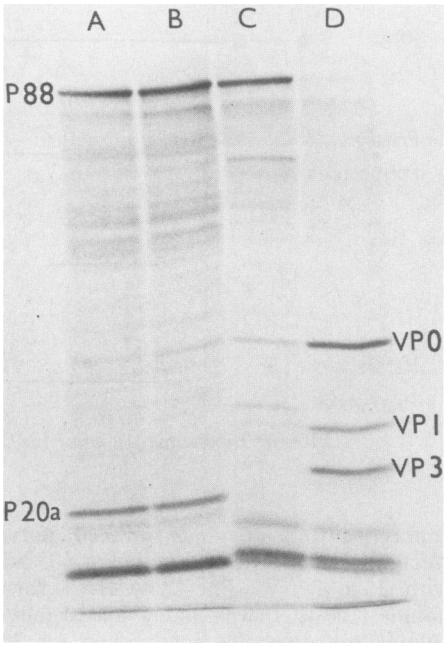

FIG. 4. The effects of noninfected or FMDV-infected cell extracts on the processing of in vitro translation products of FMDV RNA. In vitro translation programmed with FMDV RNA in the presence of [^{35}S]methionine was stopped after 20 min of incubation. One-half of the sample was mixed with cytoplasmic extract from noninfected BHK cells, and the other half was mixed with a similar extract made 2.5 h after infecting the cells with FMDV (>100 PFU per cell). One-half of each mixture was mixed with an equal volume of 2× Laemmli disruption buffer and heated to 100°C for 2 min. The remainder of each mixture was incubated at 37°C for 16 h before disruption. Samples were then analyzed on a 12.5% Laemmli gel; autoradiography followed. Lanes A and C, In vitro translate plus noninfected cell extract disrupted immediately; lanes B and D, in vitro translate plus infected cell extract disrupted after 16 h at 37°C.

In the first method, Px and Py were produced in BHK cells as described above. A cytoplasmic extract was made and immunoprecipitated (11) with antiserum raised to SDS-gel-purified VP1, VP2, or VP3. All three antisera efficiently precipitated Px and Py as well as VP1 and VP3. However, if the cytoplasmic extract was boiled in the presence of 1% SDS and 1% β-mercaptoethanol for 1 min, diluted 10 times with phosphate-buffered saline, and then immunoprecipitated, only VP2 antisera precipitated Px and Py, although with a much lower efficiency than with the nondenatured proteins (Fig. 6). We interpret this result as showing that Px and Py contained VP2 sequences but not VP1 or VP3 sequences; it should be noted that, as expected, P108-P104 was precipitated by all three antisera.

As an alternative approach to the identification of Px and Py, we performed partial Cleveland digests (6) on gel-purified proteins with chymotrypsin (Fig. 7). The partial proteolysis patterns produced from Px and Py were virtually identical to each other and similar to those produced from a mixture of VP0 and P20a.

These results indicated that the protease inhibitor DVal-Phe-Lys-CH_2Cl had blocked the cleavage between P20a and P88 and between P16 and P88, producing two new proteins, P88 + P20a = P108 and P88 + P16 = P104. The other primary cleavages between P88 and P52 and between P52 and P100 were unaffected as were all the secondary cleav-

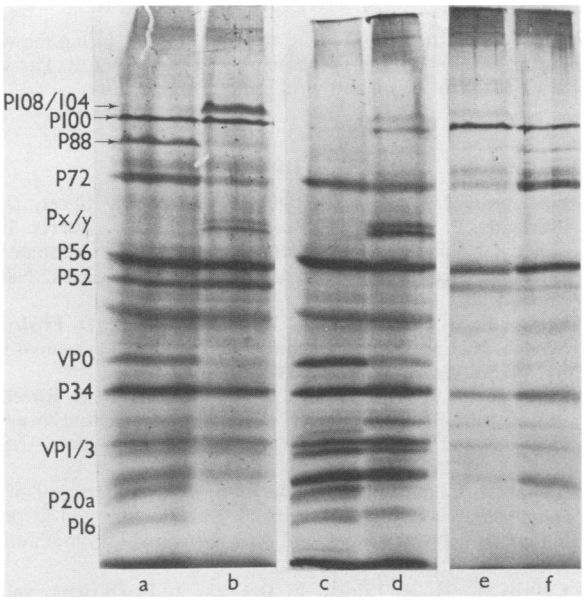

FIG. 5. Autoradiographs of 11% polyacrylamide gels of [^{35}S]methionine-labeled polypeptides made in FMDV-infected cells. Lane a, Polypeptides labeled with [^{35}S]methionine in a 10-min pulse. Lane b, Polypeptides labeled with [^{35}S]methionine in a 10-min pulse preceded by a 15-min treatment with the protease inhibitor DVal-Phe-Lys-CH_2Cl (1.0 mM). Lanes c and d, The same as lanes a and b, except for a 30-min chase with excess unlabeled methionine after the pulse. Lane e, Cells were treated with pactamycin (1.5×10^{-7} M) 3 min before being labeled with [^{35}S]methionine for 10 min. The protease inhibitor DVal-Phe-Lys-CH_2Cl (1.0 mM) was added 15 min before the addition of [^{35}S]methionine. Lane f, The same as lane e, except that no protease inhibitor was added.

ages. The proteins P108 and P104 were eventually cleaved to give VP1 and VP3, but VP0 was not seen. Instead, two new proteins were produced, Px and Py, which were presumed to be equivalent to VP0 + P20a and VP0 + P16, respectively. It can be seen from Fig. 5, lane d, that these proteins have apparent molecular weights greater than the predicted 58,000 and 54,000. This may be due to anomalous migration of the VP4 component of these proteins. VP4 is known to migrate at a slower rate in polyacrylamide gels than would be expected from its molecular weight. It is, however, possible that there is normally a posttranslational modification of P20a and P16 leading to a loss of material from the carboxyl terminal of the protein.

DISCUSSION

In this study, we examined the process by which the primary translation product of FMDV is cleaved to give the mature virus proteins. Translation of FMDV RNA for different times in a rabbit reticulocyte lysate showed that the primary product(s) P20a-P16, located nearest the 5′ end of the genome, was cleaved from the nascent polypeptides before the translation of detectable quantities of the other primary products. Furthermore, even when incubation was carried out for a time period sufficient to produce P88, the primary product was stable if translation was inhibited at this time by the addition of cycloheximide and RNase. These results suggest that the enzyme responsible for the proteolytic cleavage to release P20a-P16 is host coded in contrast to the enzymes that cleave VP0, VP1, and VP3 from P88, which are virus coded. It seems reasonable to assume that at least the primary cleavage between P20a-P16 and P88 is mediated by a host-coded protease. The evidence for the involvement of both host and virus proteases in the processing of the polyprotein is in agreement with previous work on poliovirus (14). However, recent work has shown that an amino-terminal leader protein in EMC which is equivalent to P20a-P16 of FMDV is cleaved from the structural precursor by a virus-specific protease (4).

The protease inhibitor DVal-Phe-Lys-CH_2Cl only inhibited the cleavage between P20a-P16 and P88, suggesting that this cleavage is different from all the other primary cleavages. Inhibition of the cleavage between P20a-P16 and P88 gave rise to two new proteins, the properties of which were consistent with their being P20a + P88 and P16 + P88. Since P20a and P16 are both labeled by using *N*-formyl-methionyl tRNA (20), this phenomenon implies that the synthesis is initiated from independent sites on the RNA genome. Nucleotide sequence data have confirmed this conclusion (manuscript in preparation).

The predicted composition of the novel proteins produced in the presence of the cleavage inhibitor was confirmed by Cleveland mapping and by immunoprecipitation with individual structural protein specific antisera. It is interesting that before SDS denaturation, antisera to VP1, VP2, or VP3 each precipitated the novel proteins Px and Py; only VP2 antisera, however, precipitated them after SDS denaturation. This implies that the VP0 component of Px and Py was complexed with VP3 and VP1 in the native state, despite the presence of uncleaved P16 and P20a.

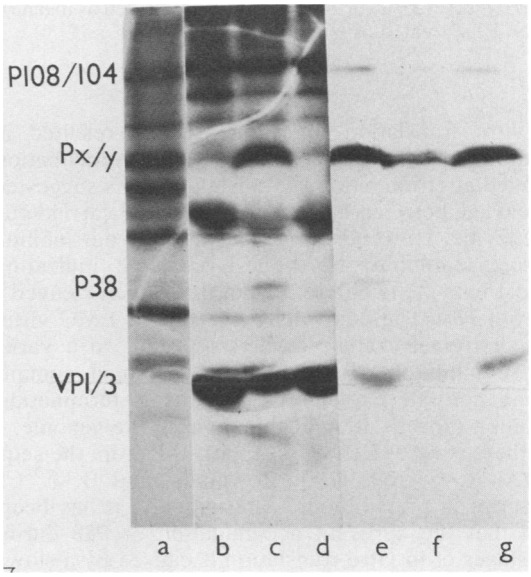

FIG. 6. Autoradiograph of 10% polyacrylamide gels of the cytoplasmic extract of BHK cells. Lane a, Polypeptides labeled in a 10-min pulse with [^{35}S]methionine, followed by a 30-min chase with unlabeled methionine. The cells were treated with 1 mM DVal-Phe-Lys-CH_2Cl for 15 min before the addition of radioactivity. Lanes b, c, and d, Immunoprecipitation of cytoplasmic extract after the extracts were boiled in 1% SDS–1% mercaptoethanol. Lanes e, f, and g, Immunoprecipitation of cytoplasmic extract. Immunoprecipitation was with antisera raised to denatured VP1, denatured VP2, and denatured VP3 for lanes b and e, lanes c and f, and lanes d and g, respectively. The figure is a compilation of two different exposure times: lanes b, c, and d were exposed for approximately five times longer than were the other lanes.

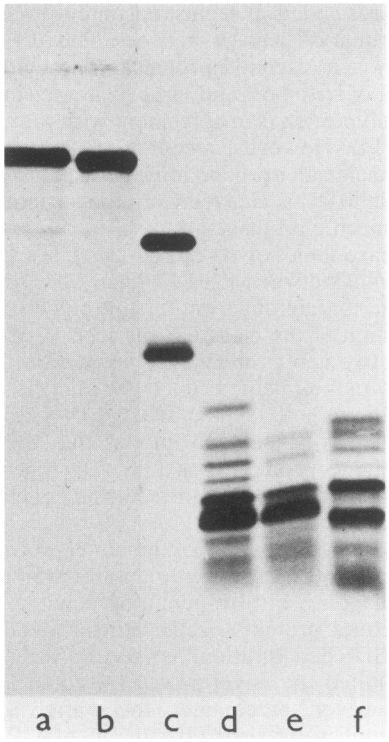

FIG. 7. Fluorograph of a 15% gel of [^{35}S]methionine-labeled virus polypeptides which had been extracted from a preparative polyacrylamide slab gel. Lane a, Untreated Px; lane b, untreated Py; lane c, untreated P38 + P20a; lane d, Px incubated with chymotrypsin (200 μg/ml) at 37°C for 30 min; lane e, Py treated as in lane d; lane f, P38 + P20a treated as in lane d.

In vitro translation for short periods resulted in the appearance of P88, which was stable when incubation was continued after inhibition of translation. This suggested that the cleavage between P88 and P52 was also carried out by a host enzyme. However, this cleavage was not inhibited by the protease inhibitor DVal-Phe-Lys-CH_2Cl, indicating that this host enzyme is different from that which cleaved P20a-P16 from P88. The equivalent cleavage in EMC virus also occurs extremely rapidly and is resistant to a variety of protease inhibitors (R. J. Jackson, personal communication). A possible explanation for this is termination and reinitiation of translation at this point in the genome. However, there is no evidence to support this from the sequence data (A. R. Carroll, D. J. Rowlands, and B. E. Clarke, manuscript in preparation). Alternatively, it has been suggested that the apparent accumulation of P88 during the early stages of in vitro translation is caused by a slowing of translation which is due to secondary structure properties of the RNA template in the region coding for the carboxy terminus of the structural protein precursor (21). However, measurements of protein chain elongation rates during in vitro translation of EMC RNA do not support this hypothesis (R. J. Jackson, personal communication). Since the proteolytic event concomitant with the final stage of virus morphogenesis, i.e., VP0→VP2 + VP4, appears to be distinct from all other posttranslational cleavages, there may be as many as four distinct proteases involved in FMDV replication.

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