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Biochemical Map of Polypeptides Specified by Foot-and-Mouth Disease Virus

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Pulse-chase labeling of foot-and-mouth disease virus-infected bovine kidney cells revealed stable and unstable viral-specific polypeptides. To identify precursor-product relationships among these polypeptides, antisera against a number of structural and nonstructural viral-specific polypeptides were used. Cell-free translations programmed with foot-and-mouth disease virion RNA or foot-and-mouth disease virus-infected bovine kidney cell lysates, which were shown to contain almost identical polypeptides, were immunoprecipitated with the various antisera. To further establish identity, some proteins were compared by partial protease digestion. Evidence for a membrane association of the polypeptides coded for by the middle genome region is also presented. A biochemical map of the foot-and-mouth disease virus genome was established from the above information.

Foot-and-mouth disease virus (FMDV), an aphthovirus of the Picornaviridae family, contains a single-stranded RNA molecule which acts directly as an mRNA. To initiate translation of FMDV and other picornaviruses, ribosomes attach, presumably at one site, close to the 5′ end of the RNA and synthesize a polyprotein which is processed during translation into four primary cleavage products, i.e., P0, P1, P2, and P3 (there are three primary cleavage products for some picornaviruses). Subsequently, the polyprotein is further cleaved via a number of steps into viral structural and nonstructural proteins (1, 14, 19, 42).

The cleavage pathway for the primary product coding for the structural proteins of FMDV has been fairly well defined (14); however, the processing of primary products from the middle and 3′ end of the genome is still unclear. Furthermore, the function of most FMDV-induced nonstructural proteins is unknown.

It has been previously demonstrated that FMD virion RNA is accurately and completely translated in cell-free systems into virus-specific proteins (11, 19, 43). Furthermore, it has been shown that an active virus-specific proteolytic processing enzyme was synthesized in the cell-free system (19).

This report presents additional information concerning the identity and genome position of both structural and nonstructural virus-specific proteins synthesized in the rabbit reticulocyte cell-free system and in FMDV-infected bovine kidney (BK) cells. Utilizing antisera against a number of virus-specific proteins, we mapped polypeptides from the structural protein, middle, and 3′ regions of the genome. The cellular location of the middle genome polypeptides was also determined.

MATERIALS AND METHODS

Materials. [^{35}S]methionine (>1,000 Ci/mmol), [^{3}H]methionine (80 Ci/mmol), and ^{14}C-labeled amino acids were purchased from New England Nuclear Corp., Boston, Mass. Heat-killed, Formalin-fixed *Staphylococcus aureus* cells (Cowan I strain) were purchased from Bethesda Research Laboratories, Gaithersburg, Md. Pancreatic RNase A and tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin were purchased from Worthington Diagnostics, Freehold, N.J., micrococcal nuclease was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., and *S. aureus* V8 protease was obtained from Miles Laboratories, Inc., Elkhart, Ind.

Growth of FMDV and isolation of virion RNA. FMDV (type A_{12}, strain 119ab) was grown in baby hamster kidney (BHK-21) cells and purified as described previously (20, 30). RNA was extracted from purified virus by the phenol-chloroform-isoamyl alcohol (50:50:2) procedure, and intact 37S RNA was isolated from sucrose gradients (18), precipitated with 2.5 volumes of ethanol, and stored in aliquots at −70°C.

Lysates from virus-infected BK cells. Monolayers of secondary or tertiary BK cells were infected with FMDV and labeled with radioactive amino acids, upon the appearance of virus-induced morphological alterations, for 15 or 60 min as previously described (19). In pulse-chase experiments, infected cells were labeled for 5 min with [^{35}S]methionine, the radioisotope was removed, and incubation was continued in the presence of 1 mM methionine for various periods of time.

Preparation of a membrane-enriched subcellular fraction from infected cells. BK cells were infected with FMDV and labeled with [^{35}S]methionine as described above. At the termination of labeling, the cells were washed twice with phosphate-buffered saline, suspended in reticulocyte standard buffer (RSB) (0.01 M NaCl, 0.01 M Tris-hydrochloride [pH 7.5], 0.0015 M $MgCl_2$), and allowed to swell for 15 min at 4°C. The cells were lysed by Dounce homogenization, the nuclei were removed by centrifugation at 1,500 rpm for 3 min, and the cytoplasm was centrifuged at 11,000 rpm for 30 min in an HB-4 rotor (21). The pellet, termed the membrane-enriched cytoplasmic pellet, was suspended in RSB; the supernatant was termed the cytoplasmic supernatant. The cytoplasm was also treated with RSB containing 1% Nonidet P-40 (NP-40) and 1% sodium deoxycholate and centrifuged as described above; the pellet was then resuspended in RSB. The various samples were mixed with an equal volume of

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twice-concentrated sample preparation buffer (0.125 M Tris-hydrochloride [pH 6.8], 4% sodium dodecyl sulfate (SDS), 1.36 M 2-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue) or precipitated with acetone and suspended in sample preparation buffer and analyzed by polyacrylamide gel electrophoresis (PAGE).

Antisera. Sera from guinea pigs (1) recovered from infection with type A_{12} strain 119 (hyperimmune serum) (2) injected with inactivated type A_{12}, strain 119ab and (3) injected with structural proteins VP_1, VP_2, or VP_3 (type A_{12}, strain 119) isolated from SDS-urea PAGE were prepared as previously described (13, 23, 32). Antiserum to virus infection-associated antigen (VIAA) was prepared in guinea pigs (33), whereas normal serum was obtained from untreated guinea pigs.

Antisera against P41, P19, and P14 were prepared with a ^{14}C-labeled amino acid membrane-enriched cytoplasmic pellet from FMDV-infected BK cells which was electrophoresed on preparative 1.5-mm thick, 20-cm long, 12.5% polyacrylamide slab gels. The gels were dried and exposed to X-ray film. Protein bands of 41,000, 19,000, and 14,000 daltons were excised and eluted from the gel in an Isco electrophoretic sample concentrator as described by Semler et al. (47), except that carrier protein was not used during the elution. The eluted proteins were assayed for purity by analysis on PAGE and stained by the silver stain technique (50).

A fusion protein composed of 17 amino acids from the *Escherichia coli* tryptophan leader gene-E protein gene, P18, lacking 21 amino acids from its amino terminus and 21 amino acids from the amino terminus of VIAA, was produced by transformation of *E. coli* with an expression plasmid utilizing the tryptophan promoter-operator (28). The expressed polypeptide (molecular weight [MW], ca. 25,000) was isolated from gels and eluted as described above.

Rabbit antisera to P41, P19, P18, and P14 were elicited by injection of various amounts of the polypeptides emulsified with complete Freund adjuvant into two sites on the back of New Zealand White rabbits. Booster injections were given at 3-week intervals, and sera were obtained 10 days after the third injection. The blood was incubated at 37°C for 30 min and left at 4°C overnight. The serum was obtained by centrifugation at 1,300 rpm for 30 min and tested for reactivity with radiolabeled FMDV-infected cells or with radiolabeled reticulocyte lysates programmed with FMDV RNA by immunoprecipitation and analysis by PAGE. Additional injections were administered in cases in which no antibody reactivity could be demonstrated.

Before immunoprecipitation of a cell-free lysate with anti-P19 serum, the lysate was precleared with anti-P14 serum, because the antigen used for preparation of anti-P19 was contaminated with P14.

Preparation of rabbit reticulocyte lysates and in vitro protein synthesis conditions. Rabbit reticulocyte lysates were prepared by the procedure of Schimke et al. (45) and stored in 400- to 500-μl fractions at −70°C. The conditions for in vitro protein synthesis were as described previously (19, 38). In addition, the translation system contained 5 mM dithiothreitol and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5).

Partial protease digestion. The procedure of Cleveland et al. (12) was used for partial protease digestion. Gel slices of [^{35}S]methionine-labeled polypeptides were excised from 12.5% polyacrylamide slab gels, rehydrated, and placed in the wells of a 15%, 20-cm polyacrylamide slab gel. The wells were overlaid with various concentrations of *S. aureus* V8 protease, and electrophoresis was performed until the bromophenol blue dye front approached the stacking gel-resolving gel interface. The current was then turned off for 30 min. Electrophoresis was continued at 75 to 85 V for 17 to 18 h; the gels were then fixed, fluorographed, dried, and exposed to X-ray film at −70°C.

Immunoprecipitation. Immunoprecipitation of reticulocyte lysates or cytoplasmic extracts from infected cells with *S. aureus* cells bearing protein A (SAPA) was performed as described previously (24, 26) with modifications. To eliminate nonspecific immunoprecipitation, lysates were incubated with washed 10% SAPA for 15 min at room temperature, and bacterial complexes were pelleted by centrifugation in an Eppendorf microfuge for 5 min (25). The supernatant was then incubated with antiserum as described previously (24). Control experiments to check for proteolysis were performed as described above, except that buffer was used instead of antiserum. The supernatant obtained after pelleting of the sample preparation buffer-eluted bacteria was analyzed by PAGE.

PAGE. Samples were analyzed on 1.5-mm thick, 20-cm 12.5% (wt/vol) polyacrylamide slab gels containing a 5% stacking gel in the discontinuous Tris-glycine buffer system (29). After electrophoresis, the gels were either dried and directly exposed to Kodak Blue Brand X-ray film or fluorographed with sodium salicylate (10). After fluorography, the gels were dried and exposed to X-ray film at −70°C (4).

RESULTS

Identification and comparison of polypeptides synthesized in infected cells with those synthesized in a cell-free system. Infected BK cells were pulse-labeled with [^{35}S]methionine for 5 min and chased with unlabeled methionine for various periods of time; the lysates prepared from these cells were analyzed by PAGE. The results shown in Fig. 1, lanes 1 through 4, show that P102, P91, P81 complex, P56, and a protein slightly larger than VP_1 are unstable and are chased into lower-MW proteins such as VP_0, P41, VP_1, VP_3 (the trypsin-sensitive protein also called VP_1 and located at the carboxy terminus of the P1 region), P19, P18, and P14, which increase during the chase. A stable polypeptide of 16,000 daltons (P16) has been shown to be a primary cleavage product in vivo and in vitro and is derived from the 5' end of the genome coding region (19, 43). Labeling of FMDV-infected cells in the presence of iodoacetamide, which inhibits processing of the primary cleavage products, also results in the buildup of P102, P91, and P56, suggesting further that these polypeptides are primary products (data not shown).

The proteins synthesized in a cell-free system programmed with FMD virion RNA comigrate with most of the proteins found in virus-infected cells (Fig. 1, lanes 5 through 7 [19]) and also are very similar to these polypeptides, as demonstrated by tryptic peptide analysis and partial protease digestion (19; data not shown). The in vitro-synthesized polypeptide of slightly higher MW than VP_0 (Fig. 1, lane 7) is a reticulocyte-specified protein (data not shown).

However, a number of differences do exist. Polypeptide P72 is found in an in vitro translation (migrating just below P81 complex; Fig. 1, lane 7), but not in infected cells (Fig. 1, lanes 1 through 6) (24). Furthermore, the relative amount of a number of polypeptides, i.e., P102, P91, P18, and P16, varies between the cell-free system and infected cells. An explanation for these differences is not clear but may be related to the efficiency of translation of the full-length RNA

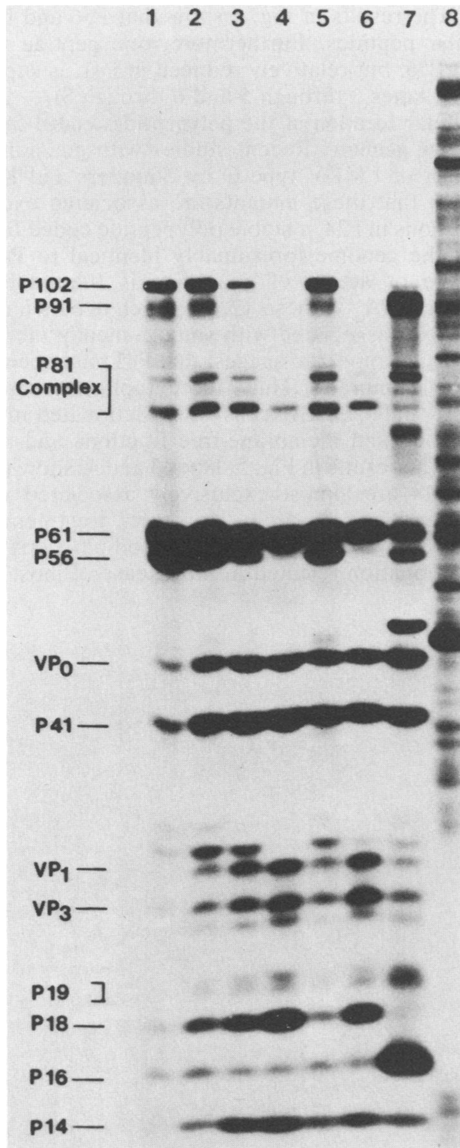

FIG. 1. Pulse-chase labeling of FMDV-infected BK cells. BK cells were infected with FMDV at a multiplicity of infection of about 100 PFU and labeled with [^{35}S]methionine for 5 min. After removal of the isotope, the cells were washed twice with media and incubated for various periods of time with media containing 1 mM methionine. Lysates were prepared as described in the text, and electrophoresis was carried out on a 20-cm 12.5% polyacrylamide slab gel. Lane 1, Infected BK cells labeled for 5 min; lane 2, infected BK cells labeled for 5 min and chased for 5 min; lane 3, infected BK cells labeled for 5 min and chased for 20 min; lane 4, infected BK cells labeled for 5 min and chased for 60 min; lane 5, infected BK cells labeled for 15 min; lane 6, infected BK cells labeled for 60 min; lane 7, [^{35}S]methionine-labeled FMDV RNA-directed cell-free lysate incubated for 120 min; lane 8, mock-infected BK cells, labeled for 60 min.

or the efficiency of protein processing in the two systems or both.

Strategy in mapping the genome. The approach used to determine the precursor-product relationship and genome position of the polypeptides specified by FMDV was to immunoprecipitate the in vitro translation products or infected cell lysates with monospecific antisera prepared against FMDV structural and nonstructural polypeptides. Following the convention recommended previously (Annual Meeting of the American Society of Virology, Ithaca, N.Y., 1982) for the organization of the picornavirus genome, evidence is presented below that demonstrates the relationship between polypeptides located in three of the four coding regions of the picornavirus genome.

Precursor-product relationship of the structural proteins. The in vitro translation products of FMDV RNA were treated with monospecific antisera prepared against structural proteins VP_1, VP_2, and VP_3; the immune complexes were precipitated with SAPA and analyzed by PAGE.

The results shown in Fig. 2, lanes 1, 3, and 5, demonstrate that the antisera described above precipitated all the viral structural proteins (VP_0, VP_1, VP_3) and their presumed precursors P91, P72, and P60. Antiserum against inactivated virus also immunoprecipitated the same proteins (Fig. 2, lane 7). The polypeptides slightly smaller than P91, which are also precipitated in the lanes described above, are derived from the structural protein region and are not related to the P81 complex since they are slightly larger than the P81 complex and are not precipitated by antiserum against VIAA (data not shown [see Fig. 6B, lane 5]).

These results suggest that structural protein complexes may be formed in cell-free translations, thus confusing presumed precursor-product relationships (17a). To address this question, complexes were dissociated with heat and SDS (49), and immunoprecipitations were performed with monospecific antisera against VP_1, VP_2, VP_3, and inactivated virus antiserum. Immunoprecipitation of treated lysates with the antisera described above demonstrated that the complexes were dissociated since the monospecific antisera only reacted with the structural proteins for which they were specific (Fig. 2, lanes 2, 4, and 6). It is possible to order the precursor structural proteins with the antisera described above, since the relative position of the mature structural proteins is known (1, 14, 42). Polypeptide P91 was precipitated by all the monospecific antiserum, demonstrating that this polypeptide is the precursor of all the mature structural proteins (Fig. 2, lanes 2, 4, and 6). Polypeptide P72 was precipitated by anti-VP_1 and anti-VP_2 serum, but not by anti-VP_3 serum (Fig. 2, lanes 2, 4, and 6), placing this polypeptide at the amino terminus of P91, whereas P28 was only precipitated by anti-VP_3 serum (Fig. 2, lane 6), locating it at the carboxy terminus of P91. Only anti-VP_1 and anti-VP_3 sera react with P60 (a faint band in Fig. 2, lane 6, but more distinct in other experiments [data not shown]), whereas anti-VP_2 serum precipitates a polypeptide of 19 kilodaltons termed P19 structural or P19s (Fig. 2, lanes 2, 4, and 6).

It is interesting that inactivated virus antiserum or monoclonal antibodies prepared against inactivated virus or VP_3 do not precipitate any virus-specific polypeptides in treated lysates (Fig. 2, lane 8; data not shown), suggesting that these antisera only recognize epitopes present on intact virus or subviral particles.

Precursor-product relationship of the polypeptides coded for by the middle of the genome. To determine the relationship of the nonstructural polypeptides with the FMDV primary cleavage products, antiserum against a number of nonstructural proteins was prepared. The polypeptides of interest were isolated from infected BK cells by preparative PAGE and injected into rabbits. The results (Fig. 3A, lanes 4 and 5) show that antisera against P41, a stable nonstructural polypeptide, precipitates P56, an unstable protein, and P41 from infected cell lysates. Antiserum against P14 (Fig. 3B, lanes 3 and 4) precipitates P56 and P14. Normal rabbit serum

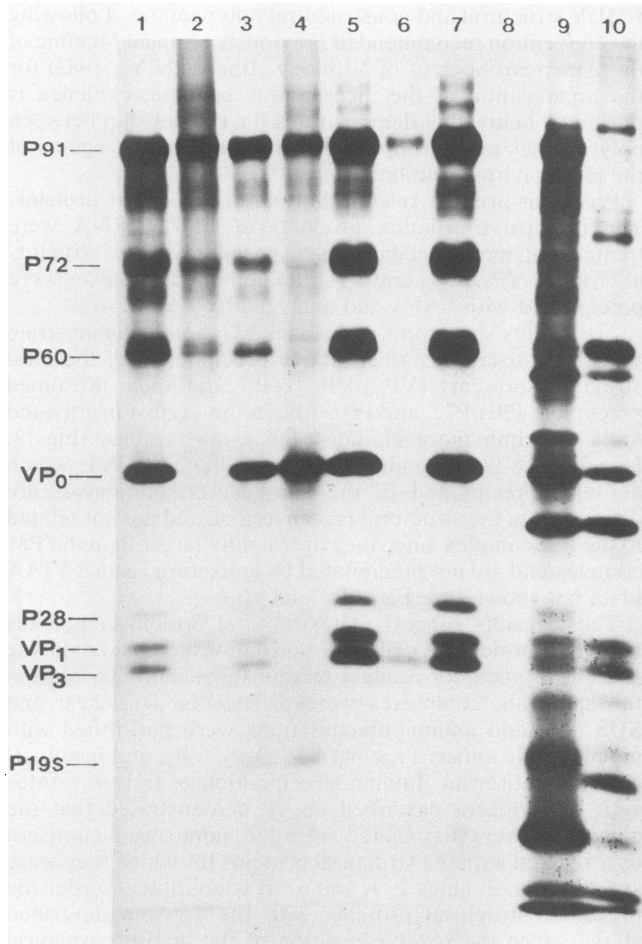

FIG. 2. Immunoprecipitation of viral-specific polypeptides with monospecific antisera against the viral structural proteins. A [^{35}S]methionine-labeled in vitro lysate programmed with FMD virion RNA was boiled for 5 min in the presence of 1% SDS–1 mM dithiothreitol–0.05% NP-40 and diluted 10-fold with 0.15 M NaCl–0.005 M EDTA–0.05 M Tris-hydrochloride (pH 8.2)–0.05% NP-40–1 mg of bovine serum albumin per ml (49). The treated lysate and a control, untreated lysate were immunoprecipitated with various antisera, eluted, and analyzed on a 20-cm 12.5% polyacrylamide slab gel. Lanes 1 and 2, Control and treated lysate immunoprecipitated with a 1:15 dilution of antiserum against VP_1; lanes 3 and 4, control and treated lysate immunoprecipitated with a 1:15 dilution of antiserum against VP_2; lanes 5 and 6, control and treated lysate immunoprecipitated with a 1:15 dilution of antiserum against VP_3; lanes 7 and 8, control and treated lysate immunoprecipitated with a 1:15 dilution of guinea pig serum against inactivated virus; lane 9, control in vitro lysate; lane 10, [^{35}S]methionine-labeled lysate from FMDV-infected BK cells.

does not precipitate any of these polypeptides (Fig. 3A and B, lanes 2 and 3 and lanes 1 and 2, respectively). Identical results were obtained with translation products from a cell-free system programmed with FMDV RNA or from infected cells labeled with [^{35}S]methionine for 15 min (a short pulse results in the presence of a larger amount of P56 [Fig. 1, lanes 5 and 6]).

To further examine the relationship of P41 and P14 to P56, the [^{35}S]methionine-labeled polypeptides from a cell-free system were excised from a polyacrylamide slab gel and subjected to limited protease digestion with *S. aureus* V8 protease. The results in Fig. 4 show that P56 and P41 have very similar peptides. Furthermore, one peptide which is present in P56, but relatively reduced in P41, is supplied by P14 (Fig. 4, lanes 3 through 5 and 6 through 8).

Intracellular location of the polypeptides coded for by the middle of the genome. Recent studies with guanidine-resistant mutants of FMDV type O by Saunders and King (44) have shown that these mutants are associated exclusively with alterations in P34, a stable polypeptide coded for by the middle of the genome (presumably identical to P41). The primary site of action of guanidine is the inhibition of picornavirus RNA synthesis (2, 7), which occurs on replication complexes associated with smooth membranes (8, 16).

These results appear to suggest that P41 might be associated with the membrane. Thus, the cytoplasm from radiolabeled FMDV-infected BK cells was fractionated into membrane-enriched and membrane-free fractions and analyzed by PAGE. The results in Fig. 5, lanes 3 and 4, show that P56, P41, and P14 are almost exclusively associated with the membrane-enriched fraction. However, treatment of the cytoplasm with 1% NP-40 and 1% sodium deoxycholate before fractionation resulted in the release of most of these

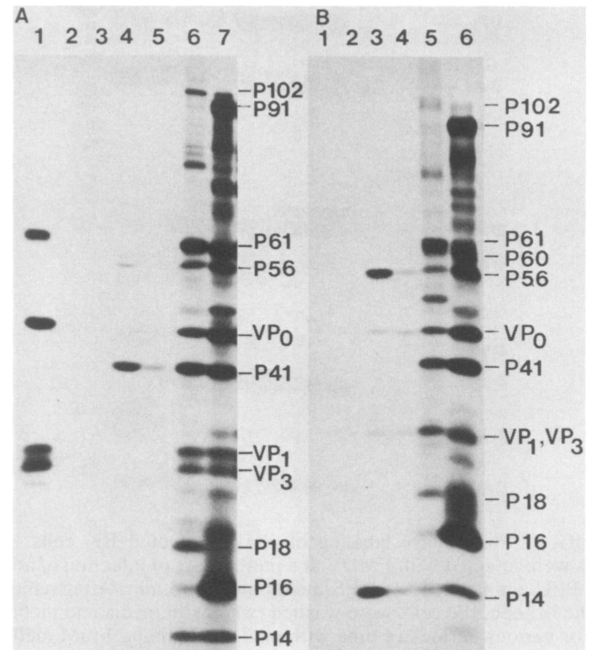

FIG. 3. Immunoprecipitation of FMDV nonstructural polypeptides coded for by the P2 region. Infected BK cells were labeled for 60 min with [^{35}S]methionine, and a detergent-treated cytoplasmic extract was prepared as described in the text. The extract was immunoprecipitated with various antisera and SAPA, and the eluted material was analyzed by PAGE on a 20-cm 12.5% slab gel. (A) Lane 1, Lysate immunoprecipitated with a 1:15 dilution of guinea pig hyperimmune serum; lane 2, lysate immunoprecipitated with undiluted normal rabbit serum; lane 3, lysate immunoprecipitated with a 1:5 dilution of normal rabbit serum; lane 4, lysate immunoprecipitated with undiluted rabbit serum against P41; lane 5, lysate immunoprecipitated with a 1:5 dilution of rabbit serum against P41; lane 6, untreated lysate; lane 7, [^{35}S]methionine-labeled FMDV-RNA-directed cell-free lysate. (B) Lanes 1 and 2, same as (A) lanes 2 and 3; lane 3, lysate immunoprecipitated with undiluted rabbit serum against P14; lane 4, lysate immunoprecipitated with a 1:5 dilution of rabbit serum against P14; lanes 5 and 6, same as (A) lanes 6 and 7.

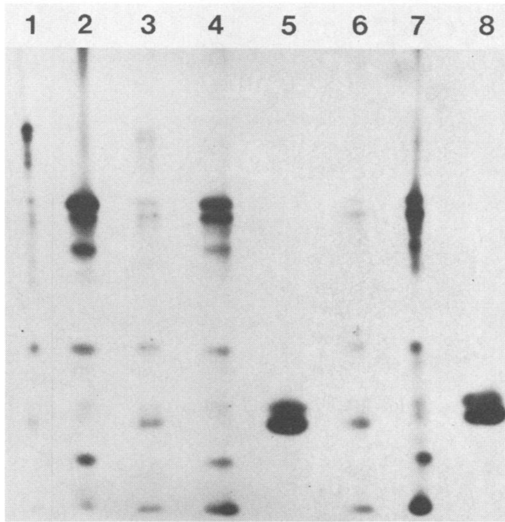

FIG. 4. SDS-PAGE analysis of the products of partial protease digestion of P56, P41, and P14. [^{35}S]methionine-labeled P56, P41, and P14 were synthesized in an FMDV RNA-directed cell-free system and separated on a 12.5% slab gel. The individual polypeptides were excised, partially digested with various amounts of *S. aureus* V8 protease, and resolved by electrophoresis on a 15% polyacrylamide slab gel. Lanes 1 and 2, P56 and P41 digested with 1.25 μg of enzyme per ml, respectively; lanes 3 through 5, P56, P41, and P14 digested with 10 μg of enzyme per ml, respectively; lanes 6 through 8, P56, P41, and P14 digested with 25 μg of enzyme per ml, respectively.

three polypeptides into the membrane-free supernatant (Fig. 5, lanes 5 and 6).

Precursor-product relationship of the polypeptides coded for by the RNA polymerase gene. Antiserum against VIAA (which is located at the carboxy terminus of the polyprotein [41]) precipitated P61 from an infected cell lysate (Fig. 6A, lane 4). Small amounts of P102 and P81 complex (faintly visible, but more apparent on other gels [data not shown]) were also precipitated. Similar results were obtained by immunoprecipitation of a cell-free translation system, except that P102 and P81 complex were more efficiently precipitated from the cell-free system (Fig. 6B, lane 5). The immunoprecipitation experiments with anti-VIAA serum support kinetic experiments and MW determinations that P61 is VIAA (P56 in 8 M urea) (19, 31, 33, 40). Furthermore, antiserum against VIAA inhibits FMDV RNA polymerase activity (34, 39).

Antiserum prepared in a rabbit against an *E. coli* fusion protein containing P18 precipitated mainly P81 complex and P18 from an infected cell lysate (Fig. 6A, lane 3). Small amounts of P102 and a number of other polypeptides were also precipitated by this serum. Significantly larger amounts of P102 were precipitated by anti-P18 serum from infected cell lysates labeled for 15 min (data not shown) (a short pulse results in the presence of a larger amount of P102 [Fig. 1, lane 5]).

Antiserum against P19 precipitated equal amounts of three low-MW polypeptides, including P19, from an infected cell lysate (Fig. 6A, lane 2). Small amounts of P102 and some other proteins were also visible. The major proteins precipitated from a cell-free translation system (Fig. 6B, lane 2) were P102, P19, and a polypeptide slightly larger than P19 (Fig. 6B, indicated with arrowheads). In addition, two high-MW proteins, slightly smaller than P102 (Fig. 6B, indicated with arrowheads) were also observed. Three other polypeptides precipitated by this serum were precipitated by normal serum (Fig. 6B, lane 1).

DISCUSSION

The results presented in this report have given us the necessary data to map the positions of most of the FMDV-specific proteins (Fig. 7). Microsequencing of various virus-specific polypeptides and nucleic acid sequencing of most of the genome of type A_{12}, strain 119ab has confirmed and expanded this information (B. H. Robertson, M. J. Grubman, G. N. Weddel, D. M. Moore, J. D. Welsh, T. Fischer, D. J. Dowbenko, D. G. Yansura, B. Small, and D. G. Kleid, manuscript in preparation).

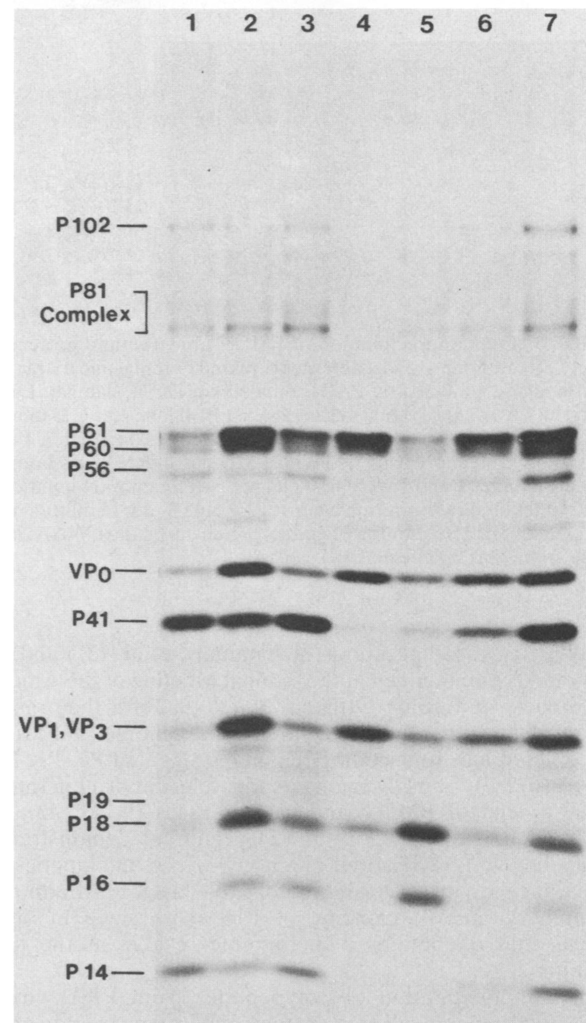

FIG. 5. Intracellular location of some FMDV nonstructural proteins. Infected BK cells were labeled for 60 min with [^{35}S]methionine, and nuclei were removed after lysis of cells in hypotonic solution. The cytoplasm was centrifuged at 11,000 rpm for 30 min or before centrifugation was treated with 1% sodium deoxycholate and 1% NP-40. In both cases, a pellet and a supernatant fraction were prepared and analyzed for FMDV-specific polypeptides by PAGE on a 20-cm 12.5% slab gel. Lane 1, Nuclear fraction; lane 2, cytoplasmic fraction; lane 3, membrane-enriched cytoplasmic pellet; lane 4, membrane-free cytoplasmic supernatant; lane 5, detergent-treated, membrane-enriched cytoplasmic pellet; lane 6, detergent-treated, membrane-free cytoplasmic supernatant; lane 7, total cell extract.

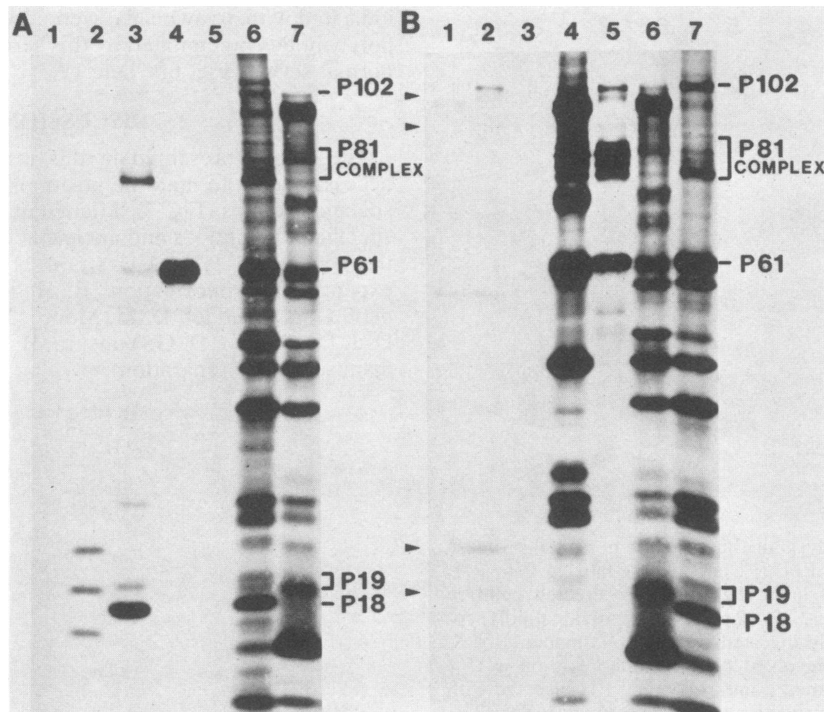

FIG. 6. Immunoprecipitation of FMDV nonstructural proteins coded for by the P3 region. (A) Infected BK cells were labeled for 60 min with [^{35}S]methionine, and a detergent-treated cytoplasmic extract was prepared. The extract was immunoprecipitated with various antisera as described and analyzed by PAGE on a 20-cm 12.5% slab gel. Lane 1, Undiluted normal rabbit serum; lane 2, undiluted rabbit serum against P19; lane 3, undiluted rabbit serum against P18; lane 4, a 1:15 dilution of guinea pig serum against VIAA; lane 5, a 1:15 dilution of normal guinea pig serum; lane 6, untreated 60-min labeled lysate; lane 7, [^{35}S]methionine-labeled FMDV RNA-directed cell-free lysate. (B) A cell-free translation system programmed with FMD virion RNA was immunoprecipitated with various antisera and analyzed by PAGE as in (A). The extracts in lanes 1 and 2 were first cleared by immunoprecipitation with undiluted serum against P14. Lane 1, Undiluted normal rabbit serum; lane 2, undiluted rabbit serum against P19; lane 3, a 1:15 dilution of normal guinea pig serum; lane 4, a 1:15 dilution of hyperimmune guinea pig serum; lane 5, a 1:15 dilution of guinea pig serum against VIAA; lane 6, untreated cell-free translation reaction; lane 7, [^{35}S]methionine-labeled lysate from FMDV-infected BK cells.

Following the suggestions of Kitamura et al. (27) and E. Wimmer (recommended at the Annual Meeting of the American Society of Virology, Ithaca, N.Y., 1982) for the organization of the picornavirus genome, the genome of FMDV was divided into four coding regions designated P0, P1, P2, and P3 (Fig. 7). The P0 region encodes a leader protein found at the 5′ end of FMDV and cardioviruses (9, 19, 43). In FMDV, P16 is coded for by the P0 region, as demonstrated by labeling with [^{35}S]formyl-methionyl tRNA and kinetics of appearance in vitro (19, 43; unpublished data). Furthermore, preliminary microsequencing of P16 synthesized in vitro reveals that it contains a methionine residue at the first position.

Immunoprecipitation of polypeptides from FMD virion RNA programmed cell-free systems with monospecific antisera against a number of structural proteins has confirmed the precursor-product relationship of the structural protein region, P1. Moreover, the order of presumed alternative cleavage products P60, P28, and P19s was determined with the above antisera.

Monospecific antisera against a number of nonstructural polypeptides has allowed us, for the first time, to determine the precursor-product relationship of polypeptides coded for by the middle genome region, P2. Furthermore, microsequencing of P56 and P41 isolated either from infected cells or cell-free translation systems and nucleic acid sequencing of this region, when correlated with the results obtained from immunoprecipitation experiments, have defined the order of the P2 region, i.e., P14-P41 (Robertson et al., manuscript in preparation).

The function of the P2 region of picornaviruses is unknown. However, by utilizing guanidine-resistant mutants of FMDV type O, Saunders and King (44) have demonstrated that only P34, a stable polypeptide from the P2 region (equivalent to P41), is altered. Since guanidine inhibits picornavirus RNA synthesis, they suggested that this polypeptide may be involved in virus-specific RNA synthesis. It has been demonstrated in poliovirus-infected cells that protein NCVPX (also termed P2-X), equivalent to P41, is found associated with membranous structures (5, 6, 48).

Fractionation of FMDV-infected cells revealed that P56, P41, and P14 are mainly present in a membrane-enriched fraction. However, treatment of the cytoplasm with detergents before preparation of the membrane-enriched fraction resulted in the release of these three polypeptides from the membrane fraction. It is not clear, at present, whether these polypeptides have a role in RNA synthesis, such as anchoring the polymerase to membranes as has been suggested for poliovirus NCVPX (5), or whether they are functionally active in some other stage of RNA synthesis. The availability of monospecific antisera against these polypeptides now allows us to address these questions.

The polymerase (VIAA) has been positioned at the 3′ end of the genome by a combination of nucleic acid and amino

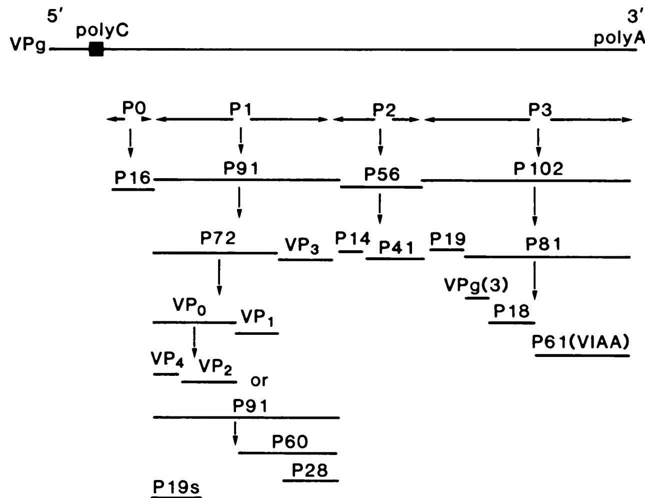

FIG. 7. Biochemical map of FMDV-induced polypeptides. The map is based on in vitro and in vivo observations (present results; 19, 42; Robertson et al., manuscript in preparation). MWs are those determined in gels with the discontinuous Tris-glycine buffer system described by Laemmli (29). VPg(3), The three different copies of VPg which are found in the FMDV genome (15); poly(A), polyadenylate; poly(C), polycytidylate.

acid sequencing (41). Antiserum against VIAA precipitates P102, a P81 complex consisting of four polypeptides and P61. The P81 complex is present both in FMDV-infected cells and cell-free translations (Fig. 1) and may be related to the three tandemly arranged, but distinct, VPg genes in the FMDV genome that have been recently demonstrated (15). The four polypeptides in this complex thus may consist of P81 lacking or containing one, two, or three copies of VPg. The observation that the major in vitro P81 polypeptide is larger than the major in vivo polypeptide may be the result of more efficient processing in vivo.

Antiserum against P18 precipitated mainly P102, P81 complex, and P18. The precipitation of small amounts of P61 by the antiserum is presumably due to the presence of 21 amino acids from P61 in the fusion protein used to inoculate rabbits. Polypeptide P102 was also precipitated by anti-P19 serum, confirming the position of P19 within the P3 region. The observation that anti-P19 serum did not precipitate P81 complex, even though anti-P18 and anti-VIAA sera did, suggests that the relative order of these polypeptides in the P3 region is P19-P18-VIAA (Fig. 7). However, it is not evident why equivalent amounts of two other low-MW polypeptides, in addition to P19, were precipitated by this serum. Nevertheless, microsequencing of P19 and P18 from cell-free systems and infected cells has also located these polypeptides in the P3 region and in the order suggested by the immunoprecipitation experiments (Robertson et al., manuscript in preparation).

Antisera against P19 and P18 precipitated a number of minor polypeptides of unknown origin. Some of these polypeptides may be nonspecifically precipitated, as has been observed with poliovirus monospecific antiserum and antiserum against VPg (22, 46, 48) and as observed in this report (Fig. 6B, lanes 1 and 2). Also, some proteins may represent alternate cleavage products of processing (3, 46, 48).

It has been demonstrated in an in vitro system that FMDV codes for an active proteolytic processing enzyme which is located distal to the structural protein gene (19). Likewise it has been demonstrated that both encephalomyocarditis virus and poliovirus code for a protease which is located in the P3 region directly 5′ of the RNA polymerase gene (17, 22, 36, 37). By analogy to other picornaviruses, P18 may be a virus-encoded protease.

Pallansch et al. (35) and Kitamura et al. (27) have shown that VPg from encephalomyocarditis virus and poliovirus is coded for by the P3 region and is processed from a polypeptide at the 5′ end of this region. Nucleic acid sequencing and microsequencing (Robertson et al., manuscript in preparation) demonstrate that the three tandem VPg genes in FMDV are located between P19 and P18, but immunoprecipitation experiments suggest that they are processed from P81 rather than P19 as shown for poliovirus (termed P3-9 [46]). Likewise, the pulse-chase experiment shown in Fig. 1, which demonstrates that the P81 complex is unstable, whereas P19 is stable, supports this view. Immunoprecipitation experiments with antiserum against VPg may clarify these relationships.

The organization of the FMDV genome has been established by a combination of techniques. Determination of the complete nucleotide sequence of FMDV will undoubtedly expand our knowledge of the FMDV genome. In addition, use of the various antisera raised against FMDV nonstructural polypeptides should be invaluable in elucidating the role of these proteins in the FMDV replication cycle.

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