JOURNAL OF VIROLOGY, Mar. 1986, p. 983–991
0022-538X/86/030983-09$02.00/0

Vol. 57, No. 3

Characterization of Foot-and-Mouth Disease Virus Gene Products with Antisera Against Bacterially Synthesized Fusion Proteins

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Received 26 August 1985/Accepted 14 November 1985

Defined segments of the cloned foot-and-mouth disease virus genome corresponding to all parts of the coding region were expressed in *Escherichia coli* as fusions to the N-terminal part of the MS2-polymerase gene under the control of the inducible λPL promoter. All constructs yielded large amounts of proteins, which were purified and used to raise sequence-specific antisera in rabbits. These antisera were used to identify the corresponding viral gene products in ^{35}S-labeled extracts from foot-and-mouth disease virus-infected BHK cells. This allowed us to locate unequivocally all mature foot-and-mouth disease virus gene products in the nucleotide sequence, to identify precursor-product relationships, and to detect several foot-and-mouth disease virus gene products not previously identified in vivo or in vitro.

The genome of foot-and-mouth disease virus (FMDV) consists of a single-stranded RNA molecule of about 8,300 nucleotides containing a single long open reading frame encoding a polyprotein of about 260 kilodaltons (kDa) (for review, see reference 23). Maturation of the virus involves processing of the polyprotein into some 15 mature gene products by virus- and host-specific proteases. The polyprotein region has been completely cloned and sequenced for two FMDV strains (4, 5, 9), and its deduced amino acid sequence was found to correlate quite well with a preliminary map predicted earlier for most FMDV gene products (23). However, except for the structural proteins and the viral replicase where detailed protein data exist (1, 14, 21, 25, 26), this map has been based mostly on indirect evidence, such as the relative order of the appearance of proteins, during biosynthesis and common peptide patterns (7, 10, 11, 17, 24), or on a comparative analysis (8) with the well-characterized gene products of poliovirus (12). In addition, most of these data had been obtained only by in vitro translation of viral RNA in reticulocyte lysates; consequently, several gene products predicted from the nucleotide sequence have not yet been identified.

To provide a more solid basis for the in vivo protein map of FMDV, we have produced segment-specific antisera by expressing distinct regions of the FMDV genome in *Escherichia coli* and used these as immunological tools. Thus, a set of antisera covering almost completely the viral polyprotein region was obtained. Immunoprecipitation by these antisera of radiolabeled proteins from virus-infected cells allowed us to identify all of the natural viral gene products predicted from the physical map.

MATERIALS AND METHODS

Construction of expression vectors pEx30 and pEx31. FMDV-specific gene products were synthesized in *E. coli* by using various derivatives of the expression vector pPLc24 (Fig. 1) (18), in which foreign proteins are produced fused to the N-terminal part of the MS2 polymerase and controlled by the inducible λPL promoter. There are only two restriction sites in pPLc24 (*Bam*HI, *Hin*dIII) available for cloning of foreign DNA next to the MS2 polymerase fragment. To construct expression vectors of more general use and suitable for oriented insertion of various restriction fragments, the vector pPLc24 was modified as follows (Fig. 1). First, a unique *Eco*RI site between the λ promoter region and the MS2 portion was deleted by cleaving with *Eco*RI and filling in the single-stranded ends before religation. To allow fusion of DNA fragments in all three reading frames to the MS2 polymerase, small deletions at the *Bam*HI site were then introduced by extended digestion with nuclease S1, and an *Eco*RI site was reintroduced by using a synthetic oligonucleotide. Further restriction sites were introduced by adding DNA fragments of different origin. The *Eco*RI-*Cla*I-*Hin*dIII fragment in pEx30 derives from pBR322 (27); the *Eco*RI-*Bam*HI-*Pst*I-*Bgl*II-*Hin*dIII fragment in pEx31 derives from pSV010 (16). Other combinations of restriction sites such as the *Hin*dIII-*Pst*I-*Xba*I-*Hin*dIII sequence in vector pEx30 were obtained by ligation of mixtures of different commercial oligonucleotide linkers. All of the variants thus obtained were characterized by sequence analysis of the new inserts and the junction sequences.

Construction of plasmids expressing FMDV genes. DNA fragments from the cDNA clones depicted in Fig. 2 were isolated and inserted into the appropriate expression vectors. The FMDV sequences expressed as fusion proteins from these plasmids are listed in Table 1.

Plasmid pEVP1 was constructed by inserting a *Bam*HI-*Hin*dIII fragment (positions 3000 to 3847) from cDNA clone pFMDV88 into the expression vector pPLc24. In this construct the translational stop codon is provided by the vector sequence. Thus the fusion protein EVP1 contains 15 vector-encoded amino acids at its C terminus.

pE34 is derived from the larger plasmid pE52 (not shown) which contained an *Hin*dIII-*Eco*RI restriction fragment (positions 3847 to 5149) by deleting an internal *Bal*I fragment (positions 3922 to 4463). To express the FMDV-specific reading frame in this construction, the *Hin*dIII restriction site was deleted by filling in with *E. coli* DNA polymerase I. The fusion protein expressed from the resulting construction, pE34, thus contains 98 N-terminal amino acids of the MS2 polymerase followed by 25 amino acids which are encoded by an unused open reading frame of the virus (nucleotide positions 3848 to 3922) and the C-terminal part of P2 delimited by the *Bal*I site (position 4463) and the *Eco*RI

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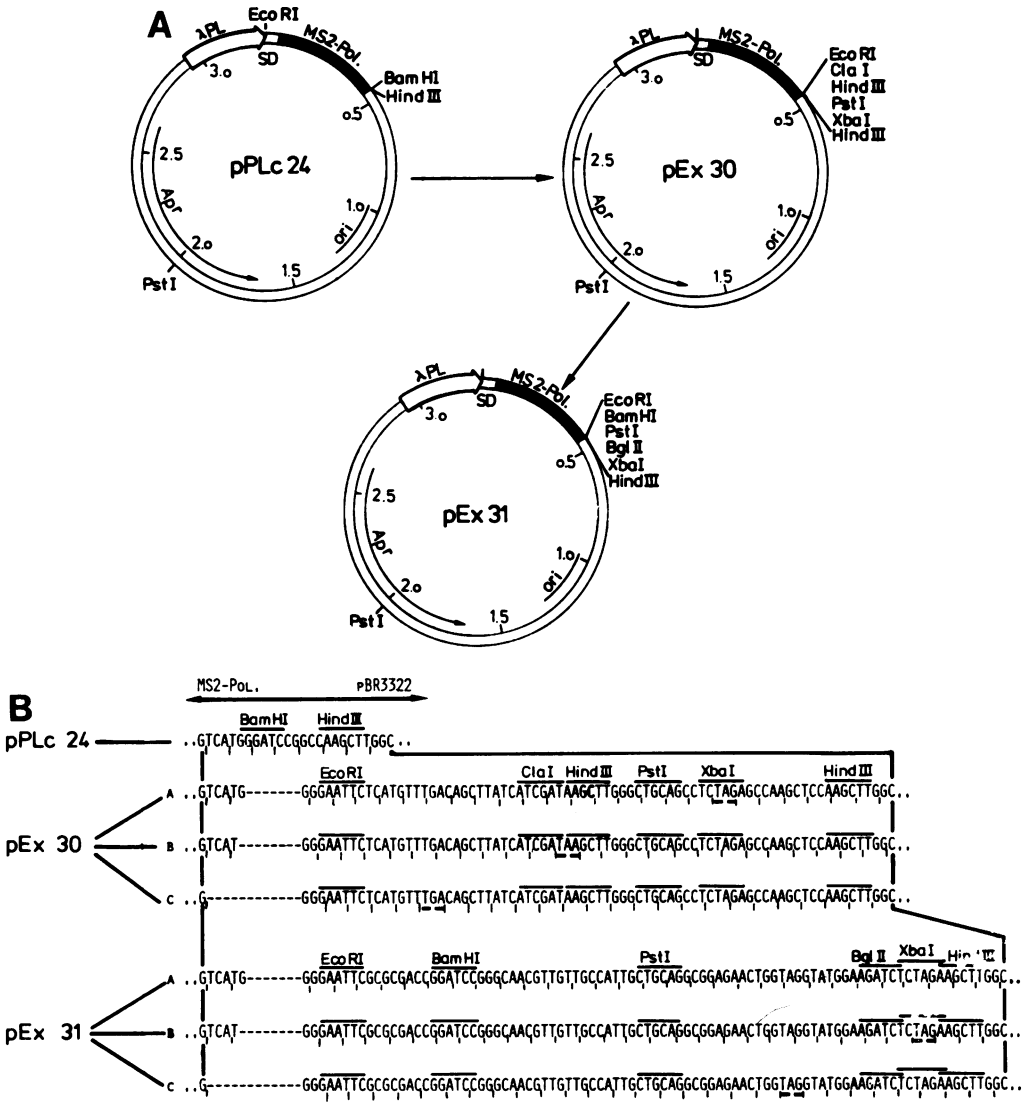


FIG. 1. Construction of expression vectors. (A) All expression vectors used in this work are derivatives of vector pPLc24 (18). The original *Eco*RI site was deleted, and different polylinkers or adaptors were introduced into the *Bam*HI-*Hin*dIII sites, resulting in vectors pEx30 and pEx31, respectively (see Materials and Methods). By deleting the *Bam*HI site of pPLc24 a set of vectors was obtained, providing the cloning sites in all three reading frames relative to the MS2 polymerase frame. Abbreviations: λPL, leftward promoter of bacteriophage lambda; SD, Shine-Dalgarno sequence of the MS2 polymerase; MS2-pol, N-terminus of the MS2 polymerase (99 amino acid residues). (B) Nucleotide sequence of the linker region in vectors pPLc24, pEx30, and pEx31. Restriction sites are indicated. Dashed lines represent deletions introduced to change reading frames (A-C). The resulting reading frames in the individual vectors are indicated. Translational stop codons are marked by bars below the sequence.

site (position 5149). At its C terminus E34 contains in addition nine amino acids encoded by the vector sequence.

In plasmid pEProt* an *Eco*RI-*Pst*I fragment from cDNA clone pFMDV100 was inserted into pEx31b. The fusion protein expressed from this plasmid contains 16 vector-encoded amino acids at its C terminus.

The plasmids pE12, pE12VPg, pEVPg, and pE20 are derivatives of pEProt*. To construct pE12 an *Sph*I restriction site in pEProt* (position 5479) was deleted by the exonucleolytic activity of *E. coli* DNA polymerase I, resulting in a 4-base-pair deletion and a frameshift that terminates translation at a stop codon in position 5485.

Plasmid pE12VPg is derived from plasmid pEProt* by deleting a *Dde*I-*Pst*I fragment (positions 5732 to 6296) from its C terminus.

Plasmid pEVPg contains the coding sequence for the three VPgs (P3Bs) and 19 amino acids from the carboxy terminus of the P3A gene. The plasmid was constructed by deleting the *Eco*RI-*Sph*I fragment (positions 5149 to 5482) from plasmid pE12VPg (Fig. 2). The fusion proteins E12VPg and EVPg both contain seven vector-encoded amino acids at their C termini.

Plasmid pE20 represents an N-terminal deletion mutant of pEProt*. This plasmid was constructed by deleting an *Eco*RI-*Sst*I fragment (positions 5149 to 5809) from pEProt*. Sticky ends were eliminated by using *E. coli* DNA polymerase I and religating the blunt ends. With this strategy the *Eco*RI site was reconstituted.

Plasmid pE56 has an *Hin*dIII fragment (positions 6451 to 8000) inserted into the vector pPLc24. Translation of the

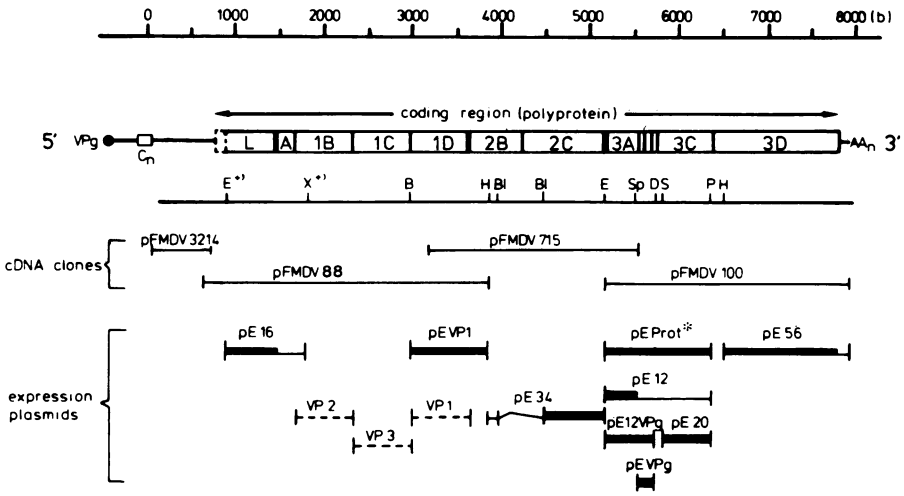


FIG. 2. Regions of the FMDV genome expressed in *E. coli*. cDNA clones used for construction of the expression plasmids and restriction sites important for subcloning of individual restriction fragments into appropriate expression vectors are depicted (X, *Xba*I; B, *Bam*HI; Bl, *Bal*I; H, *Hin*dIII; E, *Eco*RI; Sp, *Sph*I; D, *Dde*I; S, *Sst*I; P, *Pst*I; E^+ and X^+, resctriction sites present in strain C_1O, not in O_1K). Regions expressed as virus-specific proteins in *E. coli* are indicated by heavy bars. The broken line in pE34 indicates the deletion of an internal DNA fragment within construct pE52 (not shown). The protein expressed from plasmid pE16 is shortened in *E. coli*. In this case the heavy bar represents the approximate position of the processed product. The positions of the structural proteins P1B (VP2), P1C (VP3), and P1D (VP1) used for the production of antisera are indicated by dashed lines.

cloned gene terminates at the original stop codon of the FMDV polyprotein (position 7800).

All expression plasmids described so far were constructed from FMDV O_1K cDNA clones. They were checked by restriction analysis and in some cases also by sequence analysis.

To express a protein corresponding to the L' protein (Fig. 2), we used an *Eco*RI-*Xba*I restriction fragment (positions 892 to 1812) from a cDNA clone of serotype C_1 (clone C1-9 [2]). This fragment contains not only the complete L' region (missing only the putative start codon for this gene product) but also the region coding for the capsid protein P1A (VP4) and parts of the P1B (VP2) region. The *Eco*RI-*Xba*I fragment was inserted into pEx31b, resulting in plasmid pE16 (Fig. 2).

Expression of proteins in *E. coli*. Expression plasmids (Fig. 2) were transferred into competent *E. coli* C600 cells containing a temperature-sensitive mutant of the lambda repressor gene cI on a kanamycin resistance plasmid (pcI857 [19]). Cells were grown under selective pressure at 28°C to high density. To induce synthesis of fusion proteins, cells were diluted with 4 volumes of prewarmed culture medium without antibiotics and then incubated for 2.5 h at 42°C under good aeration (13). Bacteria from 0.5-ml samples were pelleted, suspended in an equal volume of sample buffer (4% sodium dodecyl sulfate [SDS], 125 mM Tris hydrochloride [pH 6.8], 10% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue), and heated to 95°C for 5 min. Proteins were analyzed on SDS-polyacrylamide gels (Fig. 3).

TABLE 1. Expression of plasmids and fusion proteins

Plasmid	Vector	FMDV map position[a]	No. of amino acids FMDV	Total no. of amino acids	Mol wt	
					Calculated[b]	Observed[c]
pE16(C1)	pEx31B	892 to 1812	307	418	46,725	32,000
pEVP1	pPLc24	3001 to 3849	283	394	44,250	45,000
pE34	pPLc24	4465 to 5151	229	367	40,900	40,000
pE12	pEx31B	5149 to 5481	111	212	23,700	26,000
pE12VPg	pEx31B	5149 to 5730	194	298	33,250	38,000
pEVPg	pEx31B	5482 to 5730	83	187	20,500	27,500
pEProt*	pEx31B	5149 to 6297	383	498	55,100	57,000
pE20	pEx31B	5812 to 6297	162	277	30,450	31,000
pE56	pPLc24	6451 to 7800	450	549	61,350	59,000

[a] Numbers represent limits of the genomic region expressed in *E. coli*.
[b] Calculated by computer analysis of the amino acid sequence deduced from the nucleotide sequence.
[c] Estimated from SDS-polyacrylamide gels as shown in Fig. 3.

Purification of fusion proteins. Purification of fusion proteins was done essentially as described by Küpper et al. (13). Cells from a 200-ml culture were washed once with 30 ml of 50 mM Tris hydrochloride (pH 8.0)–0.1 M NaCl and suspended in 1.6 ml 10% sucrose, 50 mM Tris hydrochloride (pH 8.0), 0.4 ml of lysozyme (5 mg/ml), and 0.4 ml of 0.5 M EDTA. The mixture was incubated at 37°C for 30 min, and then 4 ml of Triton lytic mix (0.1% Triton X-100, 50 mM Tris hydrochloride [pH 8.0], 62.5 mM EDTA) was added. Incubation was continued for 15 min on ice, followed by 30 min at 37°C. Cells were finally broken by sonification. Insoluble material was recovered by centrifugation (30 min, 20,000 × *g*) and extracted sequentially with 5 ml of 1 M urea and 5 ml of 7 M urea each for 30 min at 37°C. For SDS-gel electrophoresis, extracts were heated to 95° for 5 min in sample buffer. The 7 M urea fractions containing the fusion proteins were further purified by preparative SDS-polyacrylamide gel electrophoresis. After electrophoresis gels were stained for about 10 min with Coomassie blue (0.06% Coomassie brillant blue, 50% methanol, 10% acetic acid) to visualize the protein bands. The fusion protein was excised from the gel. To elute the protein the polyacrylamide was crushed and incubated in approximately 1 volume of phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate [pH 7.5])–0.1% SDS for 10 to 20 h at 42°C. Polyacrylamide was removed by centrifugation, and the supernatant was concentrated by lyophylization to about 1 mg of fusion protein per ml. The purity of extracted proteins was checked on analytical gels (Fig. 3B, lanes e).

Production and test of antisera. Rabbits were primed with

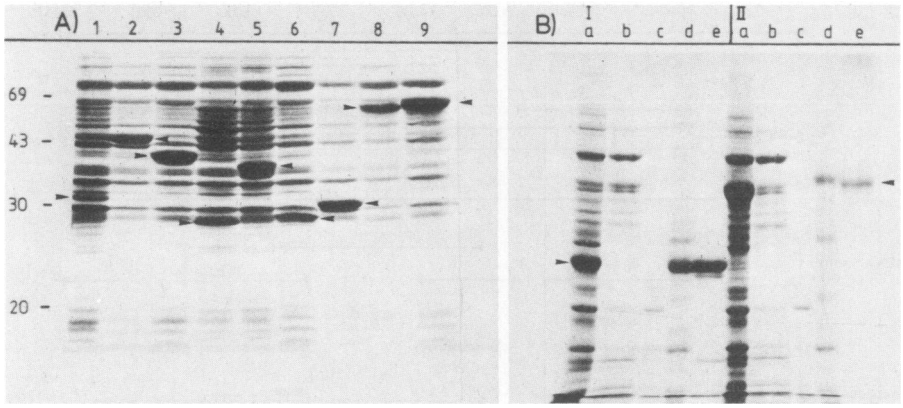


FIG. 3. Analysis and purification of fusion proteins synthesized in *E. coli*. *E. coli* cells contained the following plasmids: 1, pE16; 2, pEVP1; 3, pE34; 4, pE12; 5, pE12VPg; 6, pEVPg; 7, pE20; 8, pEProt*; 9, pE56. Cells were grown and induced as described in Materials and Methods. (A) Total cell extracts corresponding to about 10^8 cells were analysed on a 12.5% SDS–polyacrylamide gel. Proteins were stained with Coomassie blue. The positions of marker proteins are indicated. (B) Purification of fusion proteins demonstrated for protein E34 (I) and protein E56 (II). Lanes a, total cell extract; b, soluble fraction; c, 1 M urea eluate; d, 7 M urea eluate; e, protein from a 7 M urea eluate, further purified on a preparative SDS-polyacrylamide gel. Analysis was done on a 10% SDS–polyacrylamide gel; minor protein bands visible in lanes e represent degradation products of the fusion proteins rather than contaminations with *E. coli* proteins. The positions of the fusion proteins in the gel are indicated by arrows.

about 150 μg of fusion protein suspended in Freund complete adjuvant and boosted at intervals of 3 to 4 weeks with the same amount of protein in Freund incomplete adjuvant. Sera were titrated by enzyme-linked immunosorbent assay and Western blotting with the purified antigen (used for immunization) as well as unpurified fusion proteins (total cell extracts). All antisera used in this work were positive to a dilution of about 10^{-4} to 10^{-5}. In general, no significant reaction with *E. coli*-specific proteins could be detected. If desired, cross-reaction between the antisera, due to the N-terminal MS2 sequence shared by all fusion proteins, could be eliminated by competing with an *E. coli* extract containing the MS2 polymerase fragment produced by the vector pPLc24 without the FMDV insert. For immunoprecipitation of proteins from virus-infected BHK cell extracts this cross-reaction was of no importance.

Labeling of virus-specific proteins in infected cells. Monolayers of BHK cells grown in 25-cm^2 tissue culture flasks were infected with FMDV O_1K at a high multiplicity of infection (>100). Infection was allowed to proceed for 1 h at 37°C, and then excess virus was removed by washing cells with Earle salt solution (free of amino acids). [^{35}S]cysteine or [^{35}S]methionine (or both) was added at different times after infection (see Results and Discussion) to final concentrations of 0.6 mCi/ml each. Double labeling was used to enhance specific radioactivity of proteins. Labeling was stopped by lysing cells in 5 ml of RIPA (150 mM NaCl, 10 mM sodium phosphate [pH 7.5], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) after washing once with Earle buffer. Extracts were incubated for 10 min at 65°C to denature viral particles. The membrane fraction was pelleted by centrifugation and suspended in 7 M urea by sonification. The membrane fraction and the soluble fraction were then pooled and stored in 50% glycerol at −20°C.

Immunoprecipitation. A constant amount of preswollen protein A-Sepharose (50-μl bed volume) was incubated with antiserum (20 μl) for 1 h at room temperature in a total volume of 220 μl of RIPA. Protein A-Sepharose was washed once with 1 ml of RIPA to remove unbound antibodies and then incubated with 150 μl of the radioactive antigen in a total volume of 600 μl for 45 min at room temperature. Unbound material was removed by washing twice with RIPA. In some cases (anti-EVP1 and anti-VP1 sera), two additional washing steps were added to prevent coprecipitation of P1B and P1AB, probably as aggregates with P1D. Immunoprecipitated proteins were solubilized by heating to 95°C for 15 min in an equal volume of sample buffer and analyzed on SDS-polyacrylamide gels.

RESULTS AND DISCUSSION

Construction of plasmids expressing FMDV genes and expression of fusion proteins in *E. coli*. Specific DNA fragments from cloned FMDV cDNA preferentially corresponding to single genes, as predicted by Forss et al. (9), were cloned into appropriate expression vectors, which were modified and improved for this purpose (see Materials and Methods). Information on the segments chosen for expression in *E. coli* is presented in Fig. 2, and the proteins obtained and some of their characteristics are listed in Table 1.

In general, cDNA from FMDV strain O_1K was used, except for the construction of pE16, which contains cDNA from FMDV serotype C_1 (clone C1-9 [2]), because this strain provided appropriate *Eco*RI and *Xba*I restriction sites that were not present in serotype O_1K cDNA. Since serotypes O_1K and C_1O are 96% homologous at the protein level in this genomic region (2), the C_1O proteins were expected to cross-react serologically with the corresponding O_1K gene products. Gel electrophoretic analysis of extracts from induced *E. coli* cells (Fig. 3A) revealed that all constructs expressed high yields of proteins not present in the *E. coli* host and whose molecular weights correlated well with the values calculated from the nucleotide sequences (Table 1).

Only in the cases of pE16, pEVPg, and pE12VPg were significant deviations observed. The protein expressed by plasmid pE16 (Fig. 3A) had an apparent molecular mass of 32 kDa instead of the expected value of 46 kDa. However, in Western blotting experiments with the same extract, a minor 46-kDa protein was detected both with anti-E16 and anti-(O_1K)VP2 serum, whereas P32 reacted only with anti-E16 serum, indicating that a primary 46-kDa product was syn-

thesized and processed after the translation of the major 32-kDa protein (data not shown). The predicted second processing product of 14 kDa could not be detected and is probably rapidly degraded.

The other abnormal products (proteins EVPg and E12VPg) migrate at 27 and 38 kDa instead of the expected values of 20 and 33 kDa, respectively. The reason for this aberrant migration is not clear. The highly positive charge of the P3B gene products (+10) may have contributed to this result, although the net charge of the fusion protein is only +4.

Purification of fusion proteins and preparation of antisera. All fusion proteins analyzed were found in the particulate fraction of the bacterial cell extracts and were insoluble in 1 M urea, a property which facilitated their purification from the bulk of the cellular proteins (see Materials and Methods) (Fig. 3B). Only a few *E. coli* proteins copurified with the FMDV fusion proteins in the 7 M urea eluate. These were largely eliminated by preparative SDS-polyacrylamide gel electrophoresis. FMDV proteins eluted from the gels were used for immunization of rabbits. Thus, high-titer antisera against nine MS2-FMDV fusion proteins were obtained (Fig. 2 and Table 1). In addition, antisera against the structural proteins P1B (VP2), P1C (VP3), and P1D (VP1) were prepared by immunization with proteins purified from viral particles. Altogether, these antisera cover almost completely the coding region of the FMDV genome (Fig. 2).

Immunoprecipitation of virus-specific gene products. To characterize all viral proteins present at different times after infection, we decided to use continuous labeling rather than pulse-chase labeling, which would eliminate most of precursors and processing intermediates. Three different extracts were prepared (Fig. 4). and analyzed by immunoprecipitation with the antisera described above. Extract I was obtained from cells labeled between 1.5 and 2 h postinfection (p.i.). In extract II proteins were labeled between 1.5 and 3 h p.i., whereas in extract III cells had been labeled between 2 and 4 h p.i. An extract from mock-infected cells (extract IV) was prepared analogously to extract II. In contrast to extracts I, II, and III, where proteins had been labeled both with [^{35}S]methionine and [^{35}S]cysteine to enhance their specific activity, proteins in extract IV were labeled only with [^{35}S]methionine.

Virus infection results in the production of a series of FMDV-specific proteins, the pattern of which changes with time (Fig. 4). Extract II consists mainly of large precursor proteins, whereas extract III is more complex, containing mostly smaller processing products. Only low amounts of cellular proteins were labeled under the conditions used (lane IV). Extract I did not contain significant amounts of labeled viral proteins and thus was neglected in the following analysis.

The labeled proteins from extracts II and III were analyzed by immunoprecipitation for their assignment to the FMDV sequence. All antisera specifically recognized one or several viral proteins. In addition with all antisera, including the preimmune sera, a typical set of proteins with molecular masses between 45 and 90 kDa was precipitated unspecifically. In competition experiments the precipitation of these proteins was not affected (see Fig. 6). No proteins were specifically precipitated with preimmune sera (Fig. 5, lane O).

Typical protein patterns found by immunoprecipitation of FMDV proteins from extracts II and III and their correlation to the viral genome are shown in Fig. 5. Viral proteins were characterized by their reaction with individual antisera as well as their molecular masses estimated from SDS-polyacrylamide gels. As a general feature, all precursor proteins and processing intermediates observed were recognized by all antisera directed against the respective genomic region; e.g., all six antisera which are directed against proteins from the P3 region recognized the primary cleavage product P3 (Fig. 5B, lanes 7 to 12).

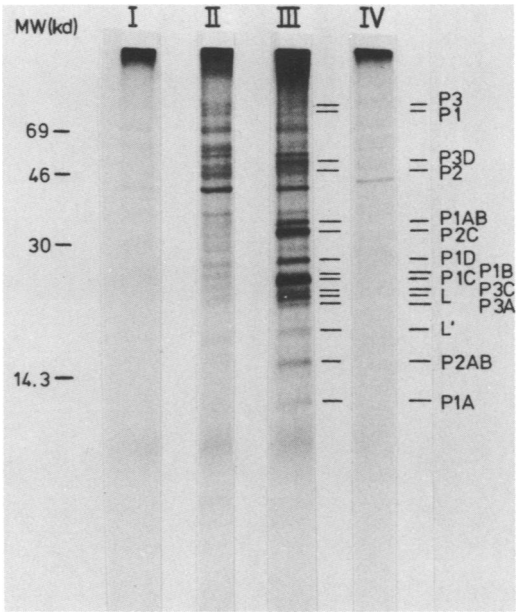
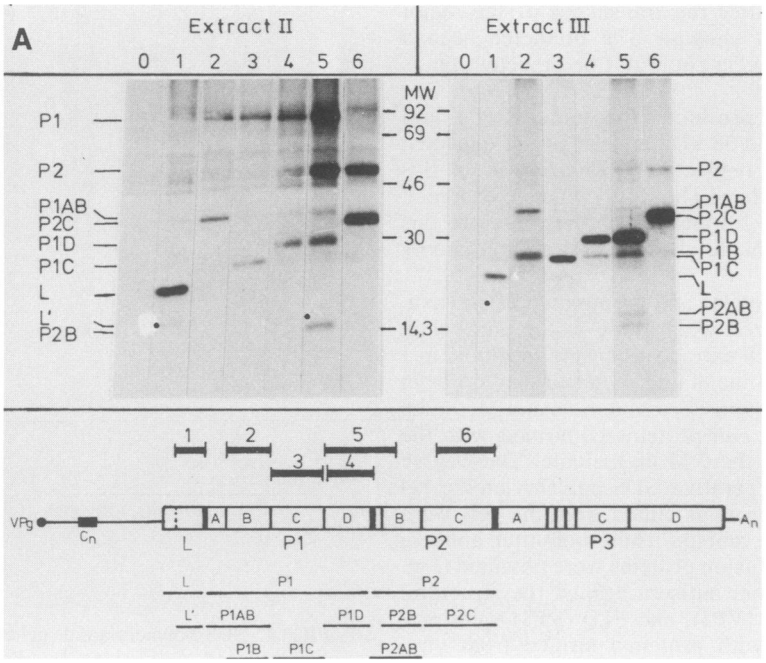


FIG. 4. SDS-polyacrylamide gel electrophoresis of proteins from virus-infected cell extracts. Proteins were labeled with [^{35}S]methionine or [^{35}S]cysteine or both as follows: I, 1.5 to 2 h p.i. (methionine plus cysteine); II, 1.5 to 3 h p.i. (methionine plus cysteine); III, 2 to 4 h p.i. (methionine plus cysteine); IV, mock-infected extract labeled as II (methionine only). Predominant viral proteins are designated as identified in this work by immunoprecipitation. P1A has not been characterized serologically, but is expected to run at the respective position in the gel. The positions of marker proteins are indicated.

The protein patterns resulting from processing of the primary products are rather complex and consist of precursors, processing intermediates, and mature viral proteins. Therefore, they will be discussed separately for the individual precursors. We use the new L434 nomenclature convention (22) to designate the proteins identified with our approach.

L-L' region. Two FMDV-specific proteins from the L-L' region were immunoprecipitated with the anti-E16 serum (Fig. 5A, lane 1) and were interpreted as overlapping leader proteins L and L'. Their molecular masses of 23 and 19 kDa, respectively, agree well with the values of 24 and 21 kDa predicted from the nucleotide sequence (9) and observed for the in vitro translation experiments (2). The in vivo data are thus consistent with the notion presented previously that L' does not represent a processing product of the L protein but derives from a second translation start at FMDV position 889 (2, 9).

P1 region. Four different antisera, anti-P1B, anti-P1C, anti-P1D, and anti-EVP1 (Fig. 2) were used to characterize the four capsid proteins derived from the P1 region of the FMDV polyprotein. As expected, all of these antisera recognize the 85-kDa precursor protein P1 (Fig. 5A, lanes 2 through 5) in addition to smaller processing products which were identified as P1AB, P1B, P1C, and P1D due to their



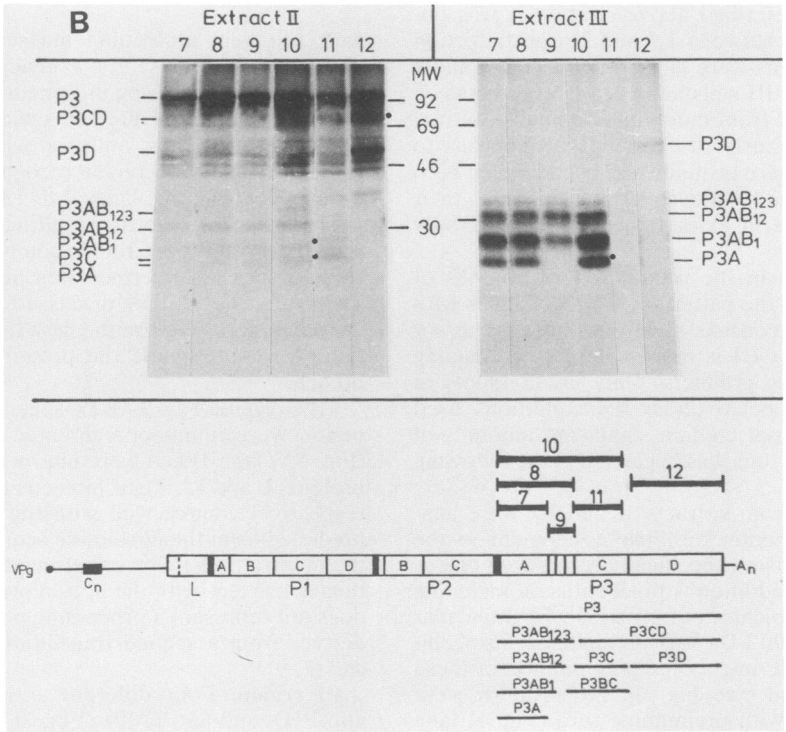


FIG. 5. SDS-polyacrylamide gel electrophoresis of immunoprecipitated viral proteins. Viral proteins were immunoprecipitated as described in Materials and Methods. The immunoprecipitation data were separated into two blocks, A and B. (A) Immunoprecipitation of viral proteins with antisera against the L, P1, and P2 regions. Lanes 0, preimmunserum; 1, anti-E16 serum; 2, anti-VP2 serum; 3, anti-VP3 serum; 4, anti-VP1 serum; 5, anti-EVP1 serum; 6, anti-E34 serum. (B) Analysis of viral proteins with antisera directed against the P3 region. Lanes: 7, anti-E12 serum; 8, anti-E12VPg serum; 9, anti-EVPg serum; 10, anti-EProt* serum; 11, anti-E20 serum; 12, anti-E56 serum; 13, total

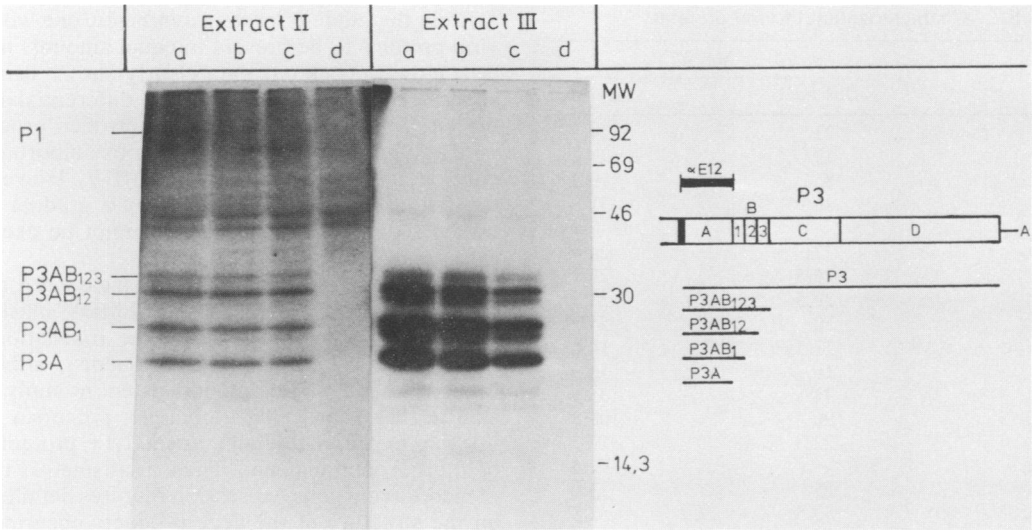


FIG. 6. Competition of immunoprecipitation of viral proteins with fusion proteins. Anti-E12 antibodies were bound to protein A-Sepharose. Then various amounts of bacterial fusion protein E12 (7 M urea extract) were added in 400 μl of RIPA and incubated at room temperature for 10 min before and 30 min after adding 150 μl of radiolabeled extract. Immunoadsorbed proteins were resolved by SDS-polyacrylamide gel electrophoresis. Details of the immunoprecipitation protocol are described in Materials and Methods. Lanes: a, no competing fusion protein added; b through d, increasing amounts of fusion protein E12 added (b, 0.1 μg; c, 1 μg; d, 25 μg). The correlation of proteins specifically immunoprecipitated to the genome is schematically shown; the genomic region recognized by the anti-E12 serum is indicated by a heavy bar.

reaction with the respective antisera. Precursor P1 was present only in extract II, demonstrating its efficient and rapid processing to the structural proteins P1AB, P1C, and P1D. P1AB is present in significant amounts even late in infection (Fig. 5A, lane 2), consistent with the notion that processing of P1AB into P1A and P1B is correlated with the maturation of virus particles. No stable processing intermediates other than P1AB from the P1 region were detected.

A 36-kDa protein and a 27-kDa protein were precipitated from extracts II and III, respectively, with the anti-VP1 and anti-EVP1 sera, in addition to the P1 and P1D proteins (Fig. 5A, lanes 4 and 5). The size of these proteins suggests that they represent P1AB (VP0) and P1B (VP2), probably precipitated as aggregates with P1D (VP1). This could be prevented by modifying the washing procedure after immunoprecipitation.

P2 region. In contrast to P1 and P3, the P2 precursor seems to be rather stable, since significant amounts of this protein were present even 4 h after infection (extract III). As identified with the anti-EVP1 and anti-E34 sera (Fig. 5A, lanes 5 and 6), processing of P2 results in three products with molecular masses estimated at 16, 17.5, and 34 kDa, respectively. Whereas the 50-kDa precursor P2 was recognized by both antisera, the 34-kDa protein was precipitated only by the anti-E34 serum, and the 16- and 17.5-kDa proteins were precipitated only by the anti-EVP1 serum.

Since anti-EVP1 serum recognizes only the first 78 amino acids of P2, p16 and p17.5 (each consisting of about 160 residues) cannot be encoded by two adjacent regions but must represent alternative cleavage products from the N terminus of P2. Our serological data do not discriminate whether this processing occurs at the boundary between P1 and P2 or between P2B and P2C, but observations made in in vitro translation experiments suggest a secondary cleavage site 16 amino acid residues downstream from the primary cleavage site between P1 and P2 (20). We therefore suggest that the 16- and 17.5-kDa proteins represent P2B and P2AB, respectively. Since there is no evidence for the existence of a P1D-P2A protein, we believe that P2B derives from a secondary processing event and not from an alternative primary cleavage of the polyprotein at two possible P1-P2 junctions. The hypothetical peptide P2A is too short (16 amino acids) to be detected in our analysis.

P3 region. Processing of the P3 region is very complex and seems to follow several parallel pathways, resulting in at least 10 different proteins. The specificity of immunoprecipitation of these proteins was demonstrated by competition experiments (Fig. 6) for the anti-E12 serum.

All P3-derived proteins were present in extract II, although in very different amounts. A protein of about 96 kDa corresponding to the P3 precursor was recognized by all antisera directed against this region. Its size is in good agreement with the predicted molecular mass of 100.8 kDa (Table 2). The P3 precursor was absent in extract III. Instead, a series of proteins with molecular masses between 22.5 and 33 kDa, recognized by the anti-E12, anti-E12VPg, anti-EVPg, and anti-EProt* sera, were the most prominent proteins (Fig. 5B, lanes 7 through 10). Of these, the 22.5-kDa protein was recognized only by the anti-E12, anti-E12VPg, and anti-EProt* sera but not by the anti-EVPg serum. It was therefore assigned to protein P3A, although its apparent molecular mass does not correlate too well with the predicted value of 17.3 kDa (Table 2) (9). Further proteins immunoprecipitated by the same antisera were identified as

extract III. The positions of weak protein bands to be seen only after overexposition (see the text) are indicated by dots. The positions of marker proteins are indicated. In the lower part of the figure genomic regions recognized by the individual antisera are designated by heavy bars. The correlation of the specifically immunoprecipitated proteins to the viral genome is schematically shown. The exposure time for extract II was 5 times longer than that for extract III.

TABLE 2. Characterization of viral proteins

Protein		Mol wt	
		Observed[a]	Calculated[b]
L	(P20A)	23	24.5
L'	(P16)	19	21.2
P1	(P88)	85	79.3
P1AB	(VPO)	36	31.8
P1A	(VP4)		7.4
P1B	(VP2)	27	24.4
P1C	(VP3)	26.5	23.7
P1D	(VP1)	29.5	23.8
P2	(P52)	50	54.5
P2AB	(P14)	17.5	18.6
P2B		16	16.3
P2C	(P34)	34	35.9
P3	(P100)	96	100.8
P3A	(P12)	22.5	17.3
P3B	(VPg)		2.6
P3C	(P20B)	25	23.0
P3D	(P56)	53.5	52.8
$P3AB_1$		27	20.0
$P3AB_{12}$		31.5	22.6
$P3AB_{123}$		33	25.2
P3BC		29.5	25.6
P3CD		76	75.8

[a] Estimated from SDS-polyacrylamide gels as shown in Fig. 5.
[b] Calculated on the basis of the processing sites proposed by Forss et al. (9).

precursors $P3AB_1$, $P3AB_{12}$, and $P3AB_{123}$, respectively, i.e., proteins P3A plus one, two, or three VPgs (P3Bs). P3A and the P3AB intermediates accumulate late in the infection cycle, except for $P3AB_3$, which seems to be less stable. No free P3B (VPg) could be detected, probably because only one methionine and no cysteine residues are contained in P3B. All three P3AB intermediates appeared as double bands on SDS-polyacrylamide gels (best resolved in Fig. 6), whereas P3A did not show this effect. These double bands therefore probably originate from a modification such as uridylation of the P3B portion of the proteins, which has been shown to occur at the VPg of poliovirus (6, 28). Unspecific protease activity, although not to be excluded, seems to be unlikely since only the three P3AB intermediates show this typical double-band pattern.

P3C, representing a virus-specific protease, seems to be rather unstable and does not accumulate during virus propagation. In extract III it can only scarcely be detected on overexposed films (marked by a dot in Fig. 5B, lane 11). In addition to P3C a protein interpreted as $P3B_3C$ was precipitated by the anti-E20 serum from extract II (both bands marked by dots (Fig. 5B, lane 11). All proteins found with antisera against fusion proteins E12, E12VPg, and EVPg were equally detected by the anti-EProt* serum. Proteins precipitated by the anti-E20 serum, however, could only scarcely be detected by the anti-EProt* serum, suggesting that the corresponding region is not immunodominant in the EProt* fusion protein.

The viral polymerase gene product (P3D) recognized by the anti-E56 serum could be dected both in extract II and extract III (Fig. 5B, lanes 12) in amounts significantly exceeding the neighboring P3C protease, but again no accumulation of the protein with time was observed, in contrast to P3A and its precursors. Furthermore, a protein of about 76 kDa recognized by the anti-E56 serum and interpreted as P3CD was present in significant amounts only in extract II (marked by a dot in Fig. 5B, lane 12).

From the mode of protein synthesis, one would expect all viral proteins to be present in equal amounts in the infected cell. However, as our data clearly show, significant differences occur probably because of a differential degradation of the viral proteins. Besides the controlled processing of the viral polyprotein, this may be the most important mechanism controlling gene expression in FMDV. However, a down-regulation of 3'-proximal genes by a gradual or even controlled reduction of translation cannot be excluded by our experiments.

Concluding remarks. Having a single RNA molecule as a genome, picornaviruses such as FMDV must regulate the expression of their genome on the translational and post-translational levels. As a consequence, although initially thought to be a simple model system to study gene expression in mamalian cells, a complex precursor-product relationship exists, including autocatalytic proteolytic cleavage of internal peptide bonds. Thus, a satisfactory understanding of picornaviral gene expression requires detailed information on the structure of the gene products occuring during synthesis and processing of the viral polyprotein.

On the basis of the nucleotide sequence and some available protein data, a preliminary map of the FMDV genome had been established previously. In this study we have undertaken a detailed serological analysis of the viral proteins synthesized in the infected cell to confirm, to complete, or, if necessary, to correct this preliminary map.

To obtain sequence-specific antisera we used mainly bacterially synthesized virus-specific proteins (with the exception of the structural proteins P1B, P1C, and P1D, where authentic viral protein were used) to elicit the appropriate antibodies. This approach yielded antisera against all parts of the polyprotein and free from cross-reaction with other viral gene products, which is generally a problem with antigens from natural sources. The construction of derivatives of the expression vector pPLc24, providing many restriction sites for the insertion of heterogenous DNA fragments in each reading frame, significantly facilitated the experimental procedure. We have shown that with these expression vectors all regions of the FMDV genome could be stably expressed and the resulting proteins could be easily purified. Antisera against these proteins efficiently recognized the natural viral proteins in immunoprecipitations as well as in Western blotting experiments (data not shown).

Thus, as we have also demonstrated for other systems (3), bacterially synthesized proteins appear to be similarly useful as serological tools, as are chemically synthesized peptides (15). An advantage of the former may be the wide range of chain length and the absence of contaminating sequence variants, and a disadvantage may be the presence of trace amounts of cross-reacting *E. coli* proteins which would not contaminate synthetic peptides.

In summary, our in vivo data generally confirm the overall picture of the FMDV protein map derived mostly from in vitro translation studies. However, several processing products were detected that have not been described either in vivo or in vitro, in particular the P2-derived P2AB and P2B proteins as well as the three P3AB intermediates. In agreement with earlier results (23), we could not detect proteins spanning primary cleavage sites (e.g., P1D-P2A or P2C-P3A), indicating that processing at the primary cleavage sites in the polyprotein is very efficient and occurs probably already in statu nascendi. The following processing of the primary cleavage products seems to be by and large a statistical event. However, since the processing products exhibit different turnover rates, some of the processing

products accumulate in infected cells, whereas others (e.g., the viral protease P3C) can hardly be detected.

The detailed knowledge of the protein map now allows us to ask questions about the interaction of individual viral gene products, especially for those whose functions are still unknown. Our set of specific antisera should represent a useful tool for such analyses, since they allow the detection and purification of gene products for which there is at present no functional assay.

After completion of this manuscript the results from microsequencing of major FMDV A_{12} proteins were published in this journal (20). These data and their interpretation by Robertson et al. correlate very well with ours.

ACKNOWLEDGMENTS

We thank M. J. Grubman and coworkers for supplying protein sequence data before publication.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Forschergruppe Genexpression) and by the Fond der Chemischen Industrie.

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