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A Second Protease of Foot-and-Mouth Disease Virus

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Foot-and-mouth disease virus (FMDV) genes are expressed as a polyprotein which is rapidly processed into the four primary cleavage products L, P1, P2, and P3. In secondary cleavage reactions, these are further processed into the mature proteins. The FMDV L protein is located at the N terminus of the polyprotein and is the first gene product released from the nascent polyprotein. For analysis of its biological function, the L gene was mutated by site-directed mutagenesis of cloned cDNA. In vitro translation of in vitro transcripts of these DNAs and expression studies in *Escherichia coli* showed that the L mutants affect the processing of the viral polyprotein. The mutants isolated were partially or totally defective in processing the polyprotein at the L/P1 junction. These mutants could, however, be processed in the presence of the wild-type L protein. Furthermore, an antiserum directed against the L protein inhibited processing at the L/P1 cleavage site, so that the release of the L protein from the polyprotein was blocked. These data reveal that the L gene product represents a viral protease which catalyzes its own release from the nascent polyprotein.

Picornaviruses are plus-stranded RNA viruses which replicate in the cytoplasm of their host cells independent of the transcriptional machinery of the host. They therefore represent an ideal, simple model system for the analysis of eucaryotic regulation mechanisms at the level of translational initiation as well as for the analysis of posttranslational modification without the interference of transcriptional effects.

In general, translation of eucaryotic mRNAs initiates at the first AUG codon downstream of the capped 5′ end (10), and ribosomes do not restart within a RNA molecule. Initiation of translation for foot-and-mouth disease virus (FMDV) differs from this general scheme in that the viral RNA acting as mRNA is not capped (14) and translation probably starts at two internal AUG codons (representing the ninth and tenth AUG codon downstream of the genomic poly(C) tract in strain O_1K) located about 1,300 nucleotides downstream of the 5′ end of the RNA (1, 6).

The regulation of gene activation by posttranslational modification of polypeptides, which is commonly used in eucaryotic systems (e.g., the activation of proteolytic enzymes like trypsin or chymotrypsin or the release of neuropeptides from precursor proteins) is the fundamental principle used in picornaviruses to produce functional gene products by proteolytic cleavage of a single primary polyprotein. This processing probably involves the activity of both host-encoded and virus-encoded proteolytic enzymes (2). As another possible function, viral proteases might be involved in the shutoff of host protein synthesis, since it has been shown for poliovirus and rhinovirus 14 that this shutoff is mediated by a proteolytic degradation of the cap-dependent translation initiation complex (4, 5). To analyze the mechanisms involved in the processing of the FMDV polyprotein and in the FMDV-induced shutoff of host protein synthesis, it is essential to identify and characterize the viral gene products concerned in these processes. For FMDV and poliovirus, the localization of the viral protease P3C has recently been described, and its proteolytic activity has been demonstrated by expression in *Escherichia coli* (8, 11) or by site-directed mutagenesis (A. Zibert, Diplom thesis, University of Heidelberg, Heidelberg, Federal Republic of Germany, 1984). In this paper we provide evidence that the FMDV L gene represents a second virus-encoded protease concerned with the processing of the viral polyprotein. The release of the L protein from the polyprotein occurs already in statu nascendi and was recently demonstrated to be catalyzed not by the viral P3C protease but rather by a cellular protease (2). In this paper we provide evidence that this processing step does not depend on the action of an external protease, but is an intrinsic feature of the L gene product itself, catalyzing its own release from the polyprotein.

MATERIALS AND METHODS

Plasmids. Plasmid pE88 was constructed by the insertion of an *Eco*RI-*Hin*dIII fragment (FMDV positions 892 through 3835) isolated from a FMDV C1 cDNA clone (C1-9 [1]) into the expression vector pEx31B (20), which is a derivative of pP1c24 (15). Plasmid pE16 was derived from pE88 by deletion of a *Xba*I-*Hin*dIII fragment (positions 1812 through 3835) as described previously (20). Both plasmids express FMDV proteins as fusions to the N-terminal part of the MS2 polymerase under the control of the λp_L promoter. Plasmid pSP88 was constructed by insertion of the same *Eco*RI-*Hin*dIII fragment as used for the construction of pE88 into the expression vector pSP65 (13). Since the inserted DNA fragment does not contain an AUG codon for the initiation of translation of the FMDV polyprotein, a chemically synthesized linker fragment with the sequence AATTCCATGCATCCATGGC was inserted into the *Eco*RI site. The correct orientation was determined by sequence analysis. Plasmid pSP3000 was constructed by insertion of a *Hin*dIII-*Bam*HI DNA fragment isolated from the FMDV O_1K cDNA clone pFMDV2735 (6) into the expression vector pSP64. In this plasmid, translation initiates at the two authentic FMDV start sites. pE16-32 codes for a mutant L gene and is derived from pE16 by site-directed mutagenesis (see below and Fig. 3 and 4). pE88-32 was constructed by subcloning the *Eco*RI-*Xba*I fragment from the mutant plasmid pE16-32 into pE88.

Expression of FMDV-specific proteins in *E. coli*. Expression of FMDV-specific proteins in *E. coli* was performed as described previously (20). In short, DNA fragments isolated

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from cloned FMDV cDNA were subcloned into the expression vector pEx31B and transferred into competent *E. coli* 537 cells (12). Cells were grown to high density at 28°C, and then synthesis of fusion proteins was induced by shifting the temperature to 42°C for 2.5 h. Proteins were analyzed on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Bisulfite mutagenesis. The mutagenesis procedure (see Fig. 3) is a modification of the method described by Kalderon et al. (9). A 1-pmol portion of plasmid pE16 DNA linearized with *Pst*I and 1 pmol of the same plasmid DNA cleaved with *Eco*RI and *Ava*I (large fragment preparatively eluted from an agarose gel) were mixed in a total volume of 20 μl and denatured by the addition of 4 μl of 1 N NaOH. After 10 min of incubation at room temperature, 8 μl of 1 M Tris hydrochloride, (pH 8.0) and 40 μl of 0.1 N HCl were added, and the DNA was allowed to reanneal for 2 h at 65°C. The DNA was precipitated with ethanol and dissolved in 50 μl of 10 mM Tris hydrochloride (pH 8.0). A 280-μl portion of a freshly prepared bisulfite solution (156 mg of sodium metabisulfite [$Na_2S_2O_5$] and 64 mg of sodium sulfite [Na_2SO_3] dissolved in 340 μl of H_2O) and 10 μl of 0.1 M hydroquinone (prepared freshly) were added, and the reaction mixture was incubated in the dark under paraffin for 1 h at 37°C. The bisulfite was removed by chromatography on a Sephadex G150 column (bed volume, 2 ml) which had been equilibrated in 0.2 M Tris hydrochloride (pH 9.2)–50 mM NaCl–2 mM EDTA. The void volume (400 μl) was collected and incubated overnight at 37°C. The DNA was recovered by precipitation with ethanol. The mutagenized single-stranded gap was filled- in by incubation for 10 min at room temperature with 4 U of *E. coli* DNA polymerase I in the presence of the four deoxynucleoside triphosphates (100 μM each), 10 mM $MgCl_2$, and 20 mM Tris hydrochloride (pH 7.2) in a total volume of 50 μl. A 10-μl portion of this solution was used for transformation of competent *E. coli* 537 cells.

In vitro transcription. In vitro transcription was essentially performed as described by Melton et al. (13). DNA fragments were cloned into the expression vectors pSP64 or pSP65. Linearized DNA templates (5 μg) were transcribed in a solution containing 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM Spermidine, 10 mM dithiothreitol, 50 U of RNAsin (Promega Biotec Madison, Wis.), the four ribonucleoside triphosphates (500 μM each) and 10 U of SP6 polymerase (Boehringer Mannheim Biochemicals) in a total volume (500 μM each) of 50 μl. After 60 min of incubation at 40°C, the reaction mixture was extracted twice with phenol-chloroform and precipitated with ethanol. The RNA was dissolved in 40 μl of H_2O and used for in vitro translation in rabbit reticulocyte lysate (Amersham Corp.). For a standard in vitro translation assay (see below), 10% of this RNA was used without any further purification.

In vitro translation. RNA extracted from FMDV O_1K virus particles and in vitro transcripts were translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. In a standard reaction mixture, 0.5 to 1 μg of RNA was translated in 40 μl of reticulocyte lysate containing 50 μCi of [^{35}S]methionine (Amersham) in a total volume of 50 μl. After 60 min of incubation at 30°C, the reaction was stopped by addition of 100 μg of pancreatic RNase per ml. In vitro translation products were analyzed on a 12.5% SDS-polyacrylamide gel as described previously (20).

FMDV-specific antisera. The production of antisera against FMDV-specific proteins by the use of bacterially synthesized fusion proteins has been described previously (20). The anti-L and anti-P3C antisera used for inhibition of processing were purified by ion exchange chromatography on DEAE-cellulose by using the batchwise procedure of Stanworth (19). Antiserum (5 ml) was mixed with an equal volume of saturated ammonium sulfate solution, and the mixture was incubated for 10 min on ice. The precipitate was pelleted (10 min at 10,000 × *g*), suspended in 3.5 ml of phosphate-buffered saline (total volume, 5 ml), and again precipitated with ammonium sulfate. The pellet was then suspended in a minimal volume of 50 mM sodium phosphate (pH 6.5), dialyzed, and added to DEAE-cellulose (2 g/ml of serum) which had been equilibrated in the same buffer. The mixture was carefully stirred on a magnetic stirrer for 60 min at room temperature, and then the DEAE-cellulose was removed by centrifugation and washed once with 5 ml of 50 mM sodium phosphate (pH 6.5). The supernatants were pooled, concentrated by ammonium sulfate precipitation, resuspended in 5 ml of phosphate-buffered saline, and dialyzed against the same buffer.

RESULTS

Processing of the L fusion protein in *E. coli*. The N-terminal part of the FMDV polyprotein was expressed in *E. coli* by fusion with the N terminus of the bacteriophage MS2 polymerase in vector pEx31B (20). Expression in this system is controlled by the inducible λp_L promoter (15). Two plasmids, pE16 and pE88, were constructed that contained an *Eco*RI-*Xba*I and an *Eco*RI-*Hin*dIII cDNA fragment, respectively, of FMDV strain C_1O (Fig. 1). Although the regions encoded by the two plasmids significantly differ in size (pE16 encodes the L region plus P1A and parts of the P1B region; pE88 encodes the L region plus the entire P1 region and some 80 amino acids of the P2 region), expression of these plasmids in both cases leads to the synthesis of stable 32-kilodalton (kDa) proteins (Fig. 2). Since the primary translation products have a predicted size of 47 and 120 kDa, respectively, it can be assumed that both proteins are subjected to processing in *E. coli*, probably at a common site. The length of the processed products correlates well with the size predicted for a fusion protein of 32 kDa, consisting of the MS2 polymerase portion (11 kDa) and the FMDV L gene (21 kDa) (6). Thus it seems likely that the two fusion proteins are processed at or near the natural L/P1 cleavage site. In Western blot experiments, the 32-kDa fusion protein was recognized by an antiserum against the L protein, but failed to react with an antiserum against P1B (not shown).

The possibility of a nonsense mutation in these plasmids could be excluded by sequence analysis of the inserted DNA (data not shown). The C-terminal processing products of both fusion proteins seem to be rapidly degraded, since they could not be detected in the cellular extracts even by serological methods (unpublished data).

An obvious mechanism that explains the processing of these fusion proteins involves the activity of a bacterial protease that might recognize the same (or a nearby) cleavage site as that used in the infected cell; this had been postulated to be accomplished by a cellular protease (18). Alternatively, this processing could be due to a proteolytic activity encoded in this part of the viral genome, analogous to the autocatalytic processing of the cloned P3C protease of poliovirus in *E. coli* (8).

As a working hypothesis we assumed that the L gene represents a second virus-encoded proteolytic enzyme able to catalyze its own release from the nascent FMDV polyprotein. To prove or disprove this, we designed the following series of experiments.

Mutagenesis of the L protein. To demonstrate that the

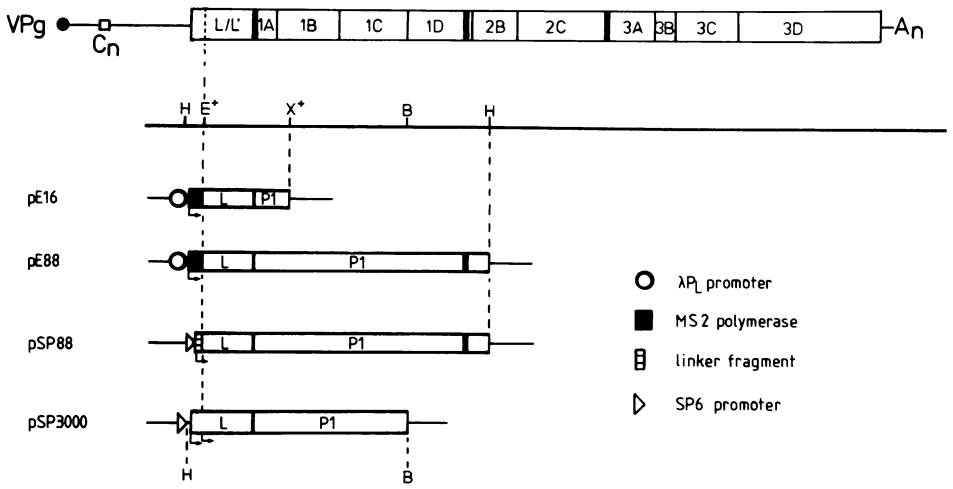

FIG. 1. Plasmids encoding the N-terminal sequences of the FMDV polyprotein. A map of the FMDV genome is given at the top of the figure. The primary cleavage sites of the polyprotein are indicated by heavy lines. The nomenclature is that used by Rueckert and Wimmer (17). Plasmids used for the expression of fusion proteins in *E. coli* (pE16 and pE88) contain an inducible λp_L promoter and some 100 amino acid residues of the MS2 polymerase, as indicated by the symbols. Plasmids for in vitro synthesis of virus-specific RNA contain the SP6 promoter and, in the case of pSP88, a linker fragment providing a translational start signal. Restriction sites important for the construction of the plasmids are integrated into the figure: B, *Bam*HI; H, *Hin*dIII; E^+ and X^+, *Eco*RI and *Xba*I restriction sites only present in strain C_1O. Arrows indicate translational start sites.

processing of the fusion proteins in *E. coli* at or near the C terminus of the L protein was a function of the L protein itself, we constructed a series of point mutants of the L gene by using site-specific mutagenesis by bisulfite (9). This method was preferred to deletion or insertion of DNA fragments to avoid major changes in the protein sequence.

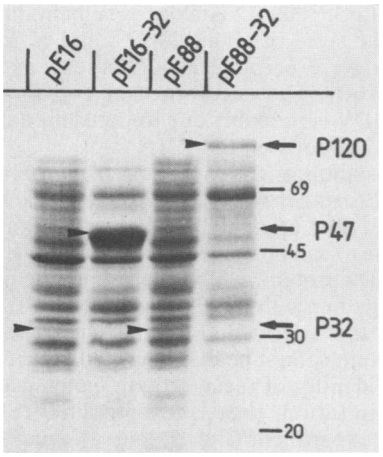

FIG. 2. Expression of the wild-type and mutant FMDV L gene in *E. coli*. Plasmids pE16 and pE88 were constructed as shown in Fig. 1 and described in Materials and Methods. Plasmid pE16-32 was obtained by bisulfite mutagenesis of plasmid pE16 (see Fig. 4). Plasmid pE88-32 was constructed by introducing the *Eco*RI-*Xba*I restriction fragment from pE16-32 into plasmid pE88. Protein synthesis from these plasmids was induced as described in Materials and Methods. The proteins were analyzed on a 12.5% SDS-polyacrylamide gel and stained with Coomassie blue. Plasmids pE16 and pE88 both direct the expression of a 32-kDa protein (not present in lanes pE16-32 and pE88-32), whereas the mutant plasmids pE16-32 and pE88-32 express proteins of 47 and 120 kDa, respectively, which cannot be found with the wild-type plasmids. Proteins expressed are indicated by arrows. The positions of marker proteins are indicated.

Since the effect of bisulfite is strictly limited to single-stranded DNA, we constructed plasmids that were partially single-stranded in the region to be mutagenized. For the mutagenesis procedure we used plasmid pE16. The strategy applied is schematically shown in Fig. 3 and is described in detail in Materials and Methods. A mixture of mutants was obtained, and the mutants were screened with regard to an altered processing on SDS-polyacrylamide gels. Three different types of protein patterns were observed (Fig. 4A): (i) the wild-type protein, corresponding to plasmids expressing only the 32-kDa protein; (ii) a 47-kDa protein corresponding to the full length of the encoded sequence, as well as a 32-kDa protein (e.g., mutants 33, 36, and 45), probably representing mutants that partially lost their ability to be processed; and (iii) the 47-kDa protein alone (from one plasmid, pE16-32), corresponding to mutant 32, whose translational product totally failed to be processed.

To test whether the mutation of the gene encoding the L protein would affect the expression of the E88 fusion protein as well, we constructed plasmid pE88-32, which is identical to plasmid pE88 (Fig. 1), except that the *Eco*RI-*Xba*I fragment from mutant pE16-32 was inserted into this plasmid. The mutant plasmid pE88-32 directs the synthesis of a 120-kDa protein, whereas the 32-kDa protein expressed by the nonmutagenized plasmid pE88 could not be detected (Fig. 2).

Since the mutagenesis reaction had been limited to a region corresponding to amino acid positions 30 to 74 of the L protein, the altered processing should not be due to a modified processing site. Nevertheless, we confirmed this for mutant 32 by nucleotide sequence analysis of the complete FMDV insert. For mutants 33, 36, and 45, only the mutagenized region was sequenced (Fig. 4B).

In mutant 32, three C-to-T transitions were found, of which only one resulted in an amino acid exchange (Thr to Ile at position 55 of the L protein; Fig. 4B). The other mutants, which have only partially lost their ability to be processed, carry up to two amino acid exchanges in different positions (Fig. 4A).

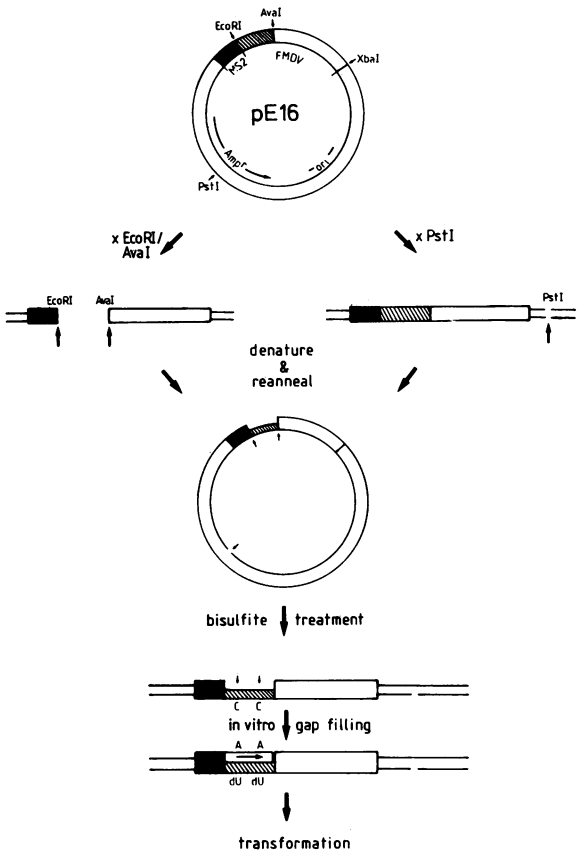

FIG. 3. Strategy applied for site-directed mutagenesis of the FMDV L gene. Plasmid pE16 was linearized either by cleavage with *Pst*I or by a double digest with *Eco*RI and *Ava*I, thereby deleting a portion of the L gene region. The two plasmids were mixed, denatured, and then reannealed. Partially single-stranded circular hybrid molecules were subjected to the mutagenesis procedure. The mutagenized single-stranded gap was filled in by using *E. coli* DNA polymerase I prior to transformation of the plasmids into competent *E. coli* cells. Details are described in Materials and Methods. Abbreviations: C, cytidine; A, adenine; dU, deoxyuridine. Arrows point to cleavage sites used for linearization of the plasmid DNA.

Expression of the L protein in the rabbit reticulocyte system. To test whether the processing of the MS2-L fusion protein was only an artifact occurring in *E. coli*, we analyzed the effect induced by the L gene mutants in a rabbit reticulocyte extract which has been shown to express and process FMDV gene products in a way similar to that in infected cells (18). In the following discussion, the proteins expressed by pSP3000 and pSP88 are designated as wild type, although the L gene product expressed from pSP88 starts at the second translational start site (giving rise to protein L′, according to Rueckert and Wimmer [17]) and has four artificial amino acids at its N terminus (see Materials and Methods). The efficiency of processing of the viral polyprotein nevertheless seems to be unaffected by N-terminal modifications of the L protein.

For expression of L gene mutants in the rabbit reticulocyte system, RNA specific for the N-terminal part of the polyprotein was prepared by in vitro transcription of FMDV sequences under the control of the SP6 promoter. The *Eco*RI-*Hin*dIII insert DNA of both the wild-type and the mutant plasmid pE88 (pE88 and pE88-32, respectively) was subcloned into pSP65 (13). Since the cloned FMDV insert does not contain either of the two translation start sites of the polyprotein, we introduced into the *Eco*RI site a synthetic DNA fragment providing an AUG start codon (together with four additional amino acid residues) in the appropriate reading frame. The resulting plasmids pSP88 and pSP88-32 are shown in Fig. 1.

In vitro translation of the wild-type SP6 RNA resulted in the production of two proteins, with molecular sizes of about 20 and 85 kDa, which were identified by immunoprecipitation as proteins L and P1, respectively (Fig. 5). In contrast, translation of the mutant SP6 RNA resulted in the synthesis of only one protein, of about 100 kDa, which is recognized both by anti-L antiserum as well as anti-P1B antiserum and thus represents the unprocessed precursor protein L/P1.

From this experiment it is obvious that translation of the N-terminal part of the FMDV polyprotein alone leads to the release of the L protein, indicating that no gene products encoded in the downstream portion of the viral genome are necessary to catalyze this reaction.

Cotranslation of wild-type and mutant L gene. Although all experiments described so far point to a proteolytic function of the L protein, mere steric hindrance of a cellular protease by a conformational change of the mutated polyprotein could not be entirely excluded. This objection could, however, be solved if the mutant polyprotein were able to be processed by the wild-type L protein in a *trans* reaction.

To allow discrimination between the translational products encoded by the wild-type and mutant RNAs in a cotranslation experiment, we prepared RNAs encoding different portions of the FMDV genome. The wild-type L gene product was expressed with RNA derived from plasmid pSP3000 (Fig. 1), which contains the two authentic FMDV translational start signals. This plasmid also codes for a part of the P1 region corresponding to P1A, P1B, and P1C. Translation of pSP3000 RNA alone results in three proteins, with molecular sizes of about 20, 25, and 65 kDa, respectively, which were serologically identified as L′, L, and P1ABC (Fig. 6B). The occurrence of two forms of the L protein in FMDV is probably due to translational start at two different AUG codons on the RNA (1).

The cotranslation of RNA derived from the mutant plasmid pSP88-32 together with RNA from pSP3000 is shown in Fig. 5, lane e. The original 100-kDa translation product of pSP88-32 (lane a) can no longer be recognized. Instead, there is a new 85-kDa protein, which represents the processed P1 precursor, together with the 65-kDa protein P1ABC and the double band L/L′ derived from the wild-type RNA. The mutant L protein cannot be distinguished from the wild-type protein. The identity of these protein bands was proven by immunoprecipitation (not shown). The result of the cotranslation experiment (Fig. 5, lane e) clearly shows that the wild-type L gene product is capable of processing the mutant polyprotein in *trans*, indicating that there is no steric masking of the processing site in the mutant but that there is a loss of the biological activity. Cotranslation of pSP88 RNA and pSP88-32 RNA shows the same effect (not shown).

Inhibition of the L/P1 processing by anti-L antiserum. As another direct indication of inherent proteolytic activity of the L protein, we could demonstrate that cleavage of the polyprotein at the L/P1 junction was inhibited by an anti-L antiserum. This was true in the translation of both viral RNA as well as in vitro-synthesized RNA (Fig. 6).

Since the expressed protein pattern for the viral RNA is rather complex, the translation products were characterized by immunoprecipitation with anti-L and anti-P1B antiserum.

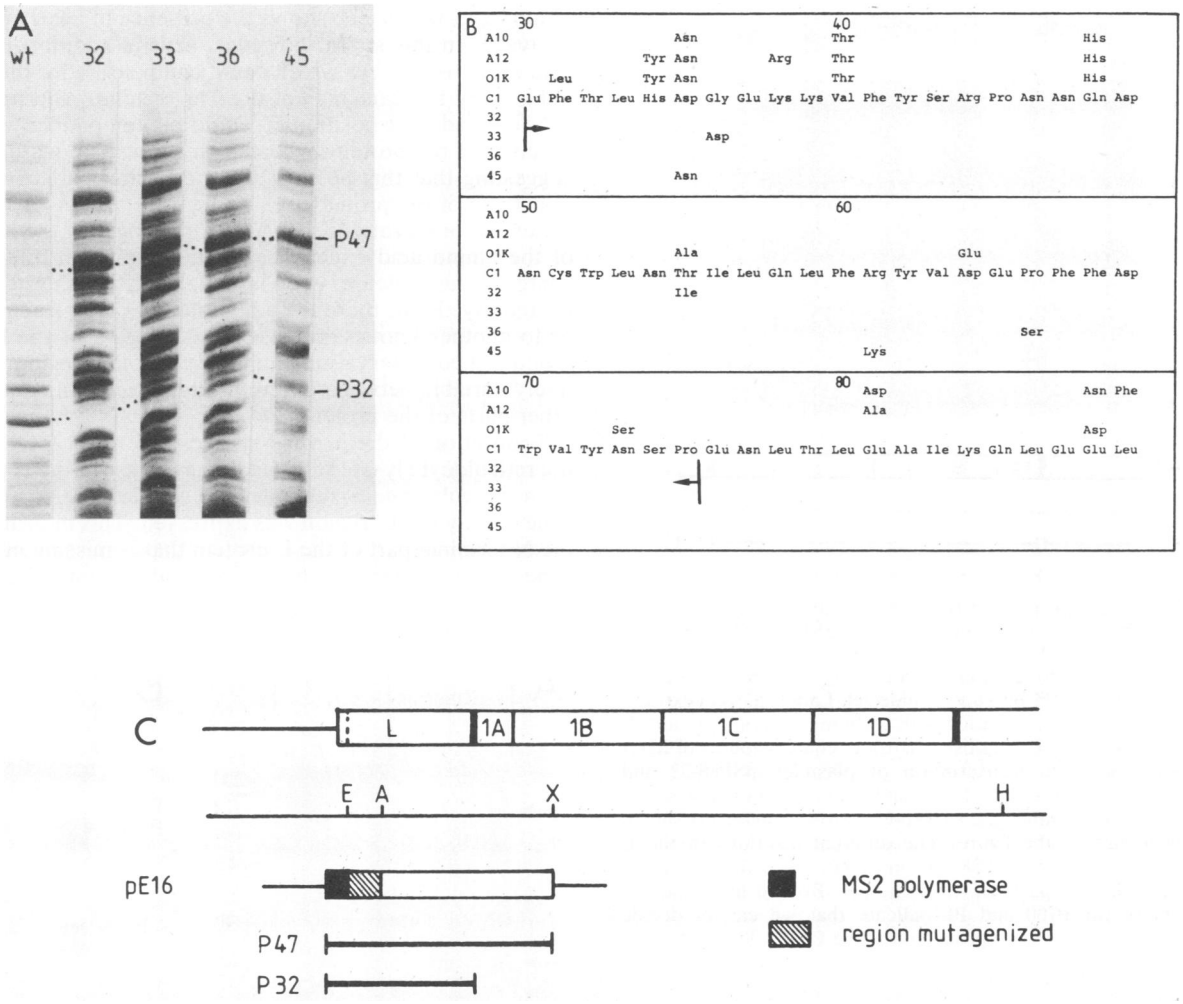

FIG. 4. Identification and analysis of L gene mutants. (A) Plasmid pE16 (wild type) and mutants were expressed in *E. coli* as described in Materials and Methods and analyzed on a 12.5% SDS-polyacrylamide gel. Proteins induced by the expression plasmids are indicated by dotted lines and are discussed in the text. (B) Mutant plasmids were analyzed by sequence determination, and the deduced amino acid sequence was compared with the L gene sequence of wild-type C_1O and other FMDV strains as indicated. The region mutagenized is delimited by arrows. The numbers represent amino acid positions of the L protein, starting at the first methionine residue (FMDV O_1K nucleotide sequence position 802). Amino acid sequences of the L gene were deduced from the cDNA sequences, as described by references 1, 6, 3, and 16 for C10, O1K, A10, and A12, respectively. (C) Schematic presentation of proteins expressed from plasmid pE16. The hatched region corresponds to the region subjected to mutagenesis. Proteins P47 and P32 were identified by their reaction with anti-L and anti-P1B antisera. Abbreviations: A, *Ava*I; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

Processing of L and P1 is complete in the absence of anti-L antiserum, whereas in the presence of this serum the major product precipitated is an unprocessed 120-kDa protein (Fig. 6). The precipitation of this precursor with preimmune serum (Fig. 6A, + anti-L, lane b) is due to the presence of anti-L antiserum in the translation assay.

Processing of the translation product of RNA derived from pSP3000 is clearly inhibited by the anti-L antiserum and results in an 85-kDa protein (Fig. 6B). In the absence of this antiserum (lane a) or in the presence of a control antiserum (lane c), this protein is cleaved to the 65-, 25-, and 20-kDa products.

The anti-L antiserum used in these experiments was raised in rabbits and acted against the bacterially synthesized fusion protein E16 described above. It could be sufficiently purified from contaminating RNases by chromatography on DEAE-cellulose, as described in Materials and Methods.

DISCUSSION

Processing of the FMDV polyprotein involves the activity of several proteases which might be virus specific as well as host specific. The FMDV P3C gene product has been postulated to have a proteolytic function, analogous to the poliovirus P3C protein, which is better characterized in terms of substrate specificity (7). This was recently confirmed both by expression in *E. coli* (11) and by site-directed mutagenesis of the P3C gene (Zibert, Diplom Thesis). More recently, Burroughs et al. (2) demonstrated that translation of FMDV RNA in vivo in the presence of the protease inhibitor D-Val-Phe-Lys-CH_2Cl results in the inhibition of processing of the polyprotein at the L/P1 junction, while all other processing steps, including the two remaining primary cleavages, were not affected. The authors concluded that this protease inhibitor specifically inhibits a cellular protease responsible for processing at the L/P1 junction.

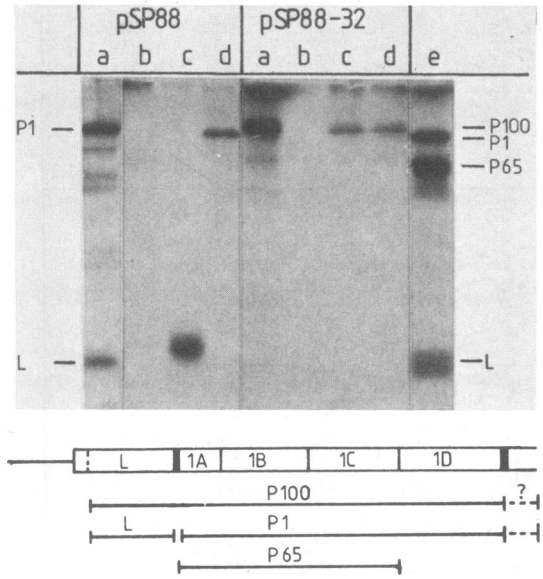

FIG. 5. Expression of wild-type and mutant L genes in vitro. RNA derived from in vitro transcription of pSP88 (wild type) and pSP88-32 (mutant) was translated in a rabbit reticulocyte system, and the translation products were identified by immunoprecipitation and analyzed on a 12.5% polyacrylamide gel. Lanes: a, total extract; b through d, immunoprecipitation with preimmune serum (b), anti-L antiserum (c), and anti-P1B antiserum (d); e, equal amounts of RNA derived from in vitro transcription of plasmids pSP88-32 and pSP3000 (Fig. 1) were cotranslated, and the translational products were analyzed on the same gel. All proteins are schematically shown in the lower part of the figure. The different positions of the L proteins (lanes a and c; pSP88) are artificial, since the two lanes derive from different parts of the same gel. Broken lines and the question mark for P100 and P1 indicate that we cannot decide whether these proteins were processed at the P1/P2 cleavage site in this experiment.

Our data now provide convincing evidence that processing of the FMDV polyprotein at the L/P1 junction is not catalyzed by a cellular protease but is an inherent property of the FMDV L gene product itself. This finding is especially interesting, since an L gene located upstream of the structural proteins is not present in other picornaviruses.

A proteolytic function of the L protein is further supported by the observation that there are two blocks of homology between the L gene and the P3C protease, in which 5 of 10 amino acids (positions 74 through 83 in L and positions 130 through 139 in P3C) and 11 of 21 amino acids (positions 126 through 146 in L and positions 56 through 79 in P3C) of the L protein are identical.

The exchange of a single or, as in the case of mutant 45, two amino acid residues in the L protein leads to a partial or total inhibition of the processing activity at the L/P1 junction, whereas the junction was cleaved in the nonmutagenized proteins. These variations in the amino acid sequence, which appear to be located in a region of the L gene not highly conserved between different strains of the virus, may not significantly influence the structure of the processing site and thus should not inhibit the activity of a cellular protease. Therefore, the reduction in processing activity must be due to a change in the biological activity of the L gene product itself.

Interestingly, the Thr residue in FMDV strain C_1O, mutated to Ile in mutant 32, is changed to an Ala residue in strain O_1K, which also shows the presence of an active L/P1 cleavage. In this strain, however, several additional amino acid exchanges occur which could compensate for the effect induced by the exchange of the Thr residue. Alternatively, an Ala residue at position 55 could be compatible with the function of the protein, whereas an Ile residue may not be, suggesting that this position is an essential part of a functional site of the protease.

Several observations, however, argue against the location of the amino acid exchanges of the mutants in the active center of the protease, even in mutant 32, which totally lost its activity. First, there is no homology to the protease P3C or to another known protease in this region of the L gene (unpublished observations); and second, this region is relatively variable between different viral strains, in contrast to other parts of the protein.

Translation of the in vitro-synthesized wild-type RNA in the reticulocyte lysate resulted in the release of the L protein from the polyprotein, regardless of whether the complete L gene or only the L′ region was expressed. This indicates that the N-terminal part of the L protein that is missing in the L′ gene is of no importance for correct and efficient processing.

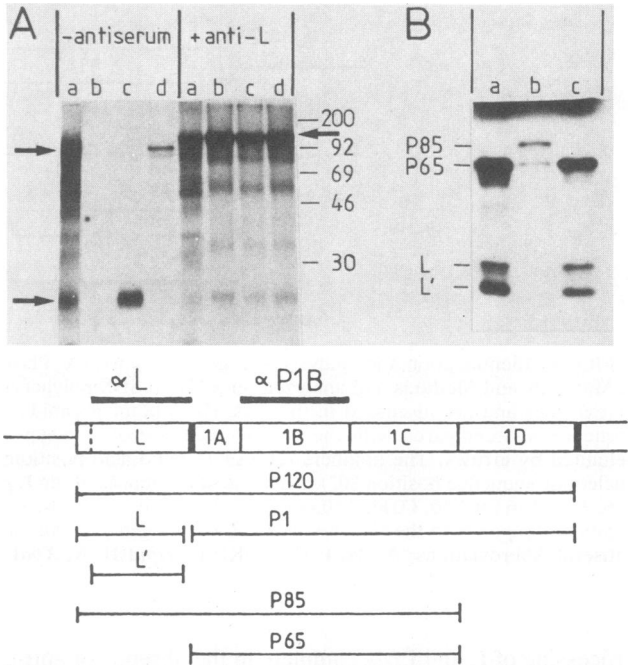

FIG. 6. In vitro translation of FMDV-specific RNA and inhibition of processing at the L/P1 cleavage site by anti-L antiserum. (A) FMDV O_1K RNA was translated in a rabbit reticulocyte lysate in a total volume of 60 μl containing 40 μl of lysate, as described in Materials and Methods, with and without 10 μl of anti-L antiserum. The extracts were analyzed on a 12.5% SDS-polyacrylamide gel either directly (lane a) or after precipitation with preimmune serum (lane b), anti-L antiserum (lane c), or anti-P1B antiserum (lane d). (B) RNA derived from in vitro transcription of pSP3000 DNA was translated in a rabbit reticulocyte extract homologous to that used for panel A. Total extracts were analyzed on a 12.5% SDS-polyacrylamide gel. Lanes: a, buffer (phosphate-buffered saline) added; b, anti-L antiserum added; c, controle serum (anti-P3C) added. The proteins expressed under these conditions were identified by immunoprecipitation (not shown). The location of the proteins in the FMDV genome is schematically shown in the lower part of the figure.

This interpretation is further supported by the fact that the FMDV gene product is expressed in *E. coli* in a fusion of approximately 100 N-terminal amino acid residues of the phage MS2 polymerase to the L′ protein. This extension, as well as a shorter fusion (pSP88) with four additional N-terminal residues expressed in the reticulocyte lysates, seems not to interfere with the proteolytic function of the protein.

The exact location of the cleavage site between L and P1 is not known. Processing of the fusion protein in *E. coli*, however, must occur directly at or very close to the authentic in vivo processing site, since the anti-L antiserum, which is directed against the bacterial fusion protein E16, cross-reacted neither with proteins from the P1 region expressed in vivo (20) nor with the remaining processing products of pSP3000 or pSP88 in the reticulocyte system, which both contain the N-terminal part of P1.

At present, our data show only a proteolytic function of the L protein catalyzing its self-release from the polyprotein, but they do not give any indications of a possible biological role of this protein during virus propagation. Since it is the first gene product occurring on translation of the FMDV RNA, it might be involved in catalyzing additional steps in the processing of the FMDV polyprotein. However, it seems unlikely that the L protease is involved in catalyzing the remaining primary cleavages, since these reactions seem not to be inhibited by the anti-L antiserum (unpublished data). This hypothesis is further emphasized by the observation of Burroughs et al. (2) that the L/P1 junction is the only processing site in the FMDV polyprotein that is inhibited by the protease inhibitor D-Val-Phe-Lys-CH_2Cl in vivo. As regards a possible function of the L protease, one can speculate on an involvement in the shutoff of the host cell protein synthesis, probably by a proteolytic degradation of the cap-dependent translation initiation complex, as demonstrated for other picornaviruses (4, 5). Our antiserum against the L protease should be a helpful tool in answering that question.

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