

Functional Analysis of the Two Alternative Translation Initiation Sites of Foot-and-Mouth Disease Virus

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The effect of deletion of each of the two authentic polyprotein translation initiation sites of foot-and-mouth disease virus on viral protein synthesis and replication was analyzed. Deletion of either the first or the second initiation site led to the expression of only one form of the leader protein, L or L', respectively, but in vitro processing of the viral polyprotein and cleavage of eIF-4 γ were not affected by either deletion. Whereas RNA in which the first translation initiation site had been deleted led to the production of viruses in transfected BHK cells, deletion of the second translation initiation site abolished virus replication.

Translation initiation of picornavirus mRNA differs from the normal mechanism used in eucaryotic cells (11) in that it starts cap independently at an internal AUG codon (9, 13, 17). This different initiation mechanism allows the viruses to express their gene products when cellular protein synthesis has been turned off. The shutoff of cellular protein synthesis has been attributed in part to the proteolytic degradation of the eIF-4 γ component (p220) of the cellular cap-binding protein complex by the action of a virus-encoded protease (4, 5).

The protein synthesis of foot-and-mouth disease virus (FMDV) initiates at two start codons located 84 nucleotides apart in the same reading frame (representing the 11th and the 12th AUG codons in strain O₁K at positions 805 and 889, respectively) (7, 20). This leads to the synthesis of two alternative N-terminal processing products of the viral polyprotein, the leader proteins L and L' (1, 2). We have shown that the L' form of the leader is a proteinase which cleaves itself from the rest of the precursor polyprotein (19), and Devaney et al. (3) demonstrated that this protein induces the degradation of eIF-4 γ in FMDV-infected cells. The latter function is executed in other picornaviruses (e.g., poliovirus, coxsackievirus and rhinovirus) by the viral 2A gene product (12, 14). Medina and coworkers have recently shown that both the L and the L' forms of the FMDV leader protein exhibit comparable activities with respect to the cleavage of the L-P1 junction in the viral polyprotein as well as the degradation of the eIF-4 γ component of the cellular cap-binding protein complex (15).

Because the two forms of leader protein do not differ in proteolytic activity and specificity, as was also confirmed by our data presented here, the question arises what functional significance the two translation initiation sites of FMDV might have. The presence of these sites in all aphthovirus strains (18) implies an essential role, e.g., differential use of the two initiation sites by the translational apparatus in the course of the infection cycle.

In order to analyze possible functions of the alternative translation initiation sites of FMDV, mutants of our infectious cDNA clone (20) containing only one of the two AUG codons each were constructed and used to analyze the proteolytic

activity and specificity of the resulting L/L'-proteases and the effect on the viability of the corresponding mutant viruses.

The full-length cDNA clones pSPFMDVpolyC₄₀ and pSPFMDVpolyC₂₅ [containing poly(C) tracts of 40 or 25 cytidyl residues, respectively] were derived from the infectious FMDV cDNA clone pFMDV-YEP-polyC (20). Essentially, in these plasmids, the yeast shuttle vector part of pFMDV-YEP-polyC has been replaced by the RNA expression vector pSP65 (16). The mutant plasmids pFMDV-L and pFMDV-L', containing only one of the two authentic viral translation start sites each (Fig. 1), were derived from both pSPFMDVpolyC₂₅ and pSPFMDVpolyC₄₀ by site-directed mutagenesis according to the PCR method of Higuchi et al. (8). To eliminate the first start site, the AUG codon at positions 805 to 807 was changed into UGG, yielding plasmid pFMDV-L'. Similarly, the second start site (AUG codon at positions 889 to 891) was changed into a UUU codon in plasmid pFMDV-L.

In order to analyze the effect of these mutations on expression and processing of the viral gene products in vitro, the plasmids pFMDV-L and pFMDV-L' were each linearized at the *Hpa*I site downstream from the poly(A) tract and transcribed with SP6 RNA polymerase. Alternative cleavage with *Bam*HI at position 3000 led to transcripts encoding L/L' and part of the P1 region only. The RNA was translated in rabbit reticulocyte lysate in the presence of [³⁵S]methionine, and the resulting proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography. The leader proteins L and L' as well as the expressed portion of the P1 precursor (a 65-kDa protein consisting essentially of P1ABC) were identified by immunoprecipitation with specific antisera (Fig. 2). As expected, only the long leader protein, L, was expressed in the pFMDV-L RNA-programmed lysate (lanes 3 and 6) and only the short leader protein, L', was expressed in the pFMDV-L' RNA-programmed lysate (lanes 4 and 7), whereas translation of the wild-type RNA led to the production of both forms of the protein (lanes 2 and 5). Neither expression nor processing of the remainder of the viral polyprotein was found to be significantly affected by the different translation initiation sites, as exemplified in lanes 1 to 3 and 7 to 9 by the presence of the P1ABC protein in all three lysates. Even at very short periods of incubation (<20 min), no unprocessed fusions of L or L' with parts of the polyprotein precursor were detected (data not shown).

A faint protein band that migrates slightly faster than L' was

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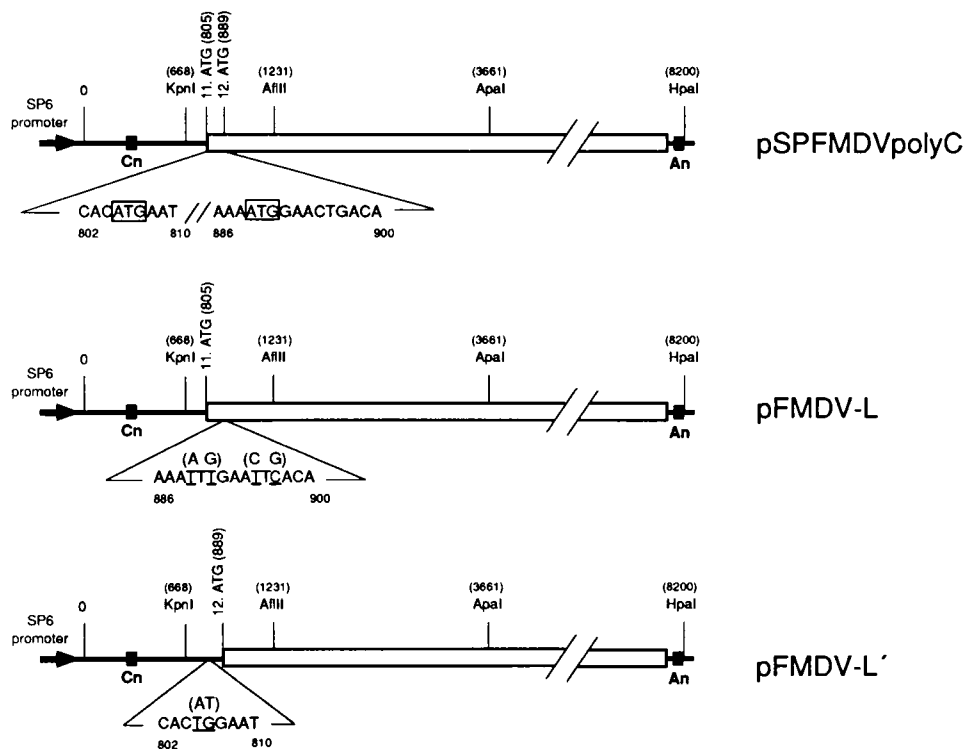


FIG. 1. Schematic diagram of wild-type plasmid pSPFMDVpolyC and mutant plasmids pFMDV-L and pFMDV-L'. The AUG codons at positions 805 and 889 (boxed in the top sequence) corresponding to the two translation initiation sites of FMDV O₁K have been mutagenized as shown. Exchanged nucleotides are underlined, and the original bases are given in parentheses. The number of C residues (n) in the poly(C) tract (Cn) is either 25 or 40 in all of the constructs.

detected in autoradiographs of the *in vitro* translation products of the L mutant, and this band was also immunoreactive with anti-L antiserum (Fig. 2, lane 5). It could be that this smaller protein represents a protease with the specificity of L' and that the long L form was only able to cleave itself from the

polyprotein but would not possess specificity for other substrates, e.g., eIF-4 γ . The occurrence of this protein in previous experiments (15) cannot be excluded by the published data. The short form could arise by proteolytic processing of the L form, or it could be due to internal translation initiation at or near the mutagenized start codon because of a signal structure on the RNA positioning the ribosome to this region. There is, in fact, a polypyrimidine stretch in front of the second translational start site which looks very similar to a sequence in front of the first start site.

In order to rule out AUG-independent internal translation initiation, we constructed an alternative version of plasmid pFMDV-L in which 6 of the 10 pyrimidine residues of the polypyrimidine region in front of the mutagenized start codon were replaced by purine residues. Corresponding exchanges in the polypyrimidine stretch of the first translational start site interfere with translation initiation, as has been shown by Kühn et al. (13). The mutagenesis led to the exchange of three amino acid residues in the N-terminal part of the protein which did not impair its autoproteolytic cleavage from the polyprotein precursor. However, expression of this variant of pFMDV-L led to the appearance of even more protein bands, all smaller than L' (data not shown). Although these results do not rule out processing of an active L'-like protease from the L precursor, we assume that the occurrence of multiple protein bands indicates unspecific degradation of L rather than activation of a smaller enzyme.

Proteolytic degradation of eIF-4 γ by the two forms of the leader protein was determined by mixing HeLa cell extract as a source of eIF-4 γ with rabbit reticulocyte lysate previously incubated with either pFMDV-L or pFMDV-L'-derived RNA. Western blotting (immunoblotting) with a human eIF-4 γ -

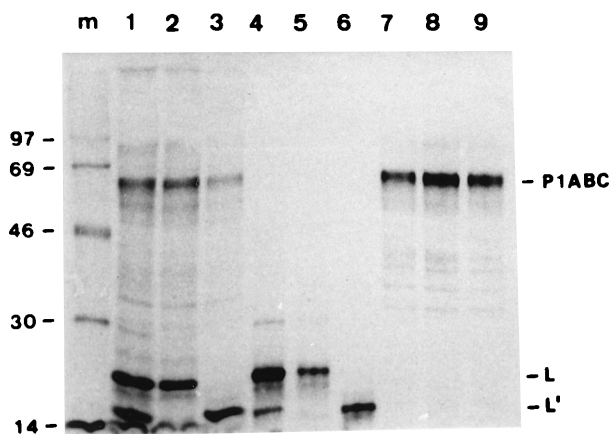


FIG. 2. *In vitro* translation products of the wild type (lanes 1, 4, and 7) and mutants pFMDV-L (lanes 2, 5, and 8) and pFMDV-L' (lanes 3, 6, and 9). *In vitro*-synthesized RNAs encoding the N-terminal part of the polyprotein (L/L' and P1ABC) were incubated in rabbit reticulocyte lysates in the presence of [³⁵S]methionine for 1 h at 30°C, and samples were analyzed by electrophoresis on 12.5% polyacrylamide gels and autoradiography. Lanes 1 to 3 show the total translation products, lanes 4 to 6 show proteins immunoprecipitated with antiserum anti-E16 (specific for L/L' proteins), and lanes 7 to 9 show proteins immunoprecipitated with anti-VP2 antiserum (recognizing the precursor P1ABC). Lane m is a protein standard (molecular mass in kilodaltons).

specific rabbit antiserum revealed that both forms of the protease led to the same complete cleavage of eIF-4 γ (data not shown), confirming recent results obtained by transfecting HTK-143 cells with a similar set of mutants (15).

The finding of identical proteolytic activities of the L and L' proteases was not fully unexpected. Although the lengths of the two proteins are conserved in different strains of FMDV, the sequence encoded by the region between the two start codons is highly variable. Only a few amino acid residues are conserved in all viruses, which implies that this part of the protein does not play an essential role. Replacing the L-specific N-terminal extension of the L'-protease by several amino acid residues of the *Escherichia coli lacZ* α fragment (unpublished results) or even by about 100 residues from the phage MS2 RNA polymerase (19) did not interfere with the autocatalytic processing activity of the enzyme. Therefore, we think that the active protease is represented by the L' peptide and that the N-terminal extension present in the L leader does not modify the proteolytic activity.

In order to analyze the effect of the deletion of one of the two translational start sites on viral replication, BHK-21 cells were transfected with equal quantities (0.05 to 5 μ g) of in vitro-synthesized full-length RNAs from plasmids pFMDV-L, pFMDV-L', and pSPFMDVpolyC as a wild-type control by using the calcium phosphate coprecipitation technique (10). Five to six hours after transfection, monolayers were overlaid with semisolid medium consisting of Dulbecco's modified Eagle's medium, 10% newborn calf serum, and 0.7% agarose. Upon incubation for 2 to 3 days at 37°C, cells were stained with neutral red. With wild-type RNA, plaques became visible after 45 h, whereas with pFMDV-L' RNA, a similar result was obtained only after 70 h. The recombinant wild-type viruses formed plaques of normal size, in contrast to the pFMDV-L'-derived viruses which led to very small plaques. No plaques at all were obtained upon transfection with RNA from the L mutant in six independent approaches, not even after 1 week of incubation. Furthermore, attempts to infect BHK-21 cells with the supernatant culture medium collected 3 days after transfection with L RNA or with lysates from transfected cells did not lead to an apparent cytopathic effect.

To exclude an additive negative effect of the deletion of one of the two translation initiation sites and a limited transfection efficiency of the plasmids containing poly(C) tracts of only 25 C residues, which in our hands was found to be the minimal length for efficient transfection, the transfections were repeated with RNAs derived from poly(C)₄₀-carrying cDNA clones. This extension of the poly(C) tract renders plasmid-derived FMDV RNA almost as infectious as viral RNA (6). No differences from the results presented above were observed, except for an earlier appearance of the plaques (30 and 55 h after transfection for pSPFMDVpolyC- and pFMDV-L'-derived RNA, respectively) and a slightly larger plaque size, as summarized in Table 1. The L mutant remained noninfectious even under these conditions and with as much as 5 μ g of RNA per transfection.

To examine whether the reduced efficiency of mutant pFMDV-L' was a consequence of the presence of only one translational initiation site or whether additional effects at the translational or posttranslational level were to be observed in vivo, synthesis and processing of the viral polyprotein in infected cells were monitored by ³⁵S labelling and viral proteins were compared with those of the wild type. As can be seen in Fig. 3 from the total protein pattern (lane 2) as well as from the specifically immunoprecipitated proteins (lanes 3 and 4), there is no difference between wild-type and mutant gene products, except for the absence of the long L form of the

TABLE 1. Characteristics of FMDV mutants

Clone	Length of poly(C) (no. of C residues)	Start codon ^a	Phenotype ^b
pSPFMDVpolyC ₄₀	40	1st and 2nd	Large plaques
pFMDV-L ₄₀	40	1st	Nonviable
pFMDV-L' ₄₀	40	2nd	Small plaques
pSPFMDVpolyC ₂₅	25	1st and 2nd	Medium-sized plaques
pFMDV-L ₂₅	25	1st	Nonviable
pFMDV-L' ₂₅	25	2nd	Very small plaques

^a The first start codon is at position 805 and the second start codon is at position 889 of the nucleotide sequence of FMDV O₁K (7).

^b Plaque morphology observed upon transfection of BHK-21 cells under agar overlay.

leader protein in the L' mutant (compare lanes 2 and 3 with lanes 5 and 6, respectively), reflecting the results of the in vitro translation in reticulocyte lysates (Fig. 2).

In vitro increase in the Mg²⁺ concentration was found to enhance translation initiation at the first start codon of FMDV, but not at the second start codon (18). However, it is not known how this relates to the in vivo situation. By pulse-labelling at different times during the infection cycle, a shift from preferential translation initiation at the second start codon to the first start codon was observed later in infection, although the L/L' ratio did not change by more than a factor of 2 to 3 (data not shown). Both the in vitro and in vivo data could point to a differential use of the two translation initiation sites by the virus. The small difference in the expression levels of the two forms of the leader peptide during the infection cycle alone cannot explain the inability of the L mutant to establish virus replication. We rather suppose that an additional effect, e.g., missing interaction of a regulatory factor with the mutagenized second translation initiation site, may interfere with the replication of this mutant. Transfection of IBRS cells with the two mutant RNAs led to the same results (data not shown),

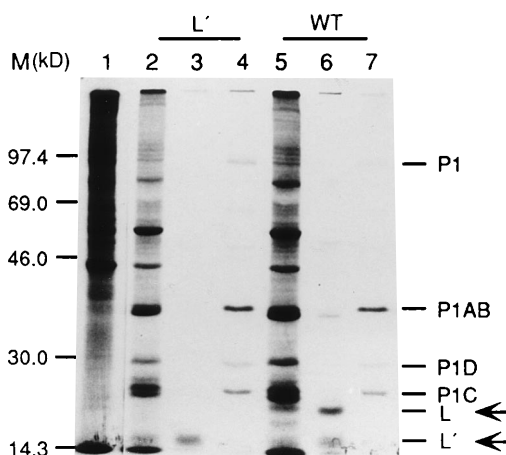


FIG. 3. Polypeptides in wild-type (WT) and L' mutant FMDV-infected BHK-21 cells. Cells were labeled with [³⁵S]methionine 3 h postinfection for 1 h (wild type) or 2 h (L' mutant). The proteins were analyzed by electrophoresis on a 12.5% polyacrylamide gel and autoradiography. Lanes: 1, mock-infected cells; 2 and 5, total proteins induced by L' mutant virus or wild-type virus; 3 and 6, proteins immunoprecipitated with anti-L antiserum; 4 and 7, proteins immunoprecipitated with anti-VP2 antiserum. Molecular mass markers (M [in kilodaltons]) are given on the left, and several viral proteins are indicated on the right. The arrows point to the L and L' proteins.

indicating that the inability of the L mutant to replicate was not restricted to BHK cells.

Translation initiation of the viable L mutant resembles that of the rhinovirus/enterovirus group of picornaviruses, in particular that of poliovirus. There is a cryptic AUG codon at the 3'-terminal end of the internal ribosomal entry site of these viruses which is essential as a structural element but does not function as a translational start signal. The initiation site of protein synthesis is localized further downstream, comparable to the second initiation site of FMDV. The two types of internal ribosomal entry site elements found in picornaviruses may thus have evolved from a prototype in which the ribosomal entry site and translation initiation site were separated. Aphthoviruses may have acquired the ability to use this essential structural element of the ribosomal entry site as an additional translation initiation codon.

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