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The Foot-and-Mouth Disease Virus Leader Proteinase Gene Is Not Required for Viral Replication

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The foot-and-mouth disease virus (FMDV) leader (L) proteinase has only two known functions: (i) autocatalytic removal from the N terminus of the viral polyprotein and (ii) cleavage of the p220 subunit of the eukaryotic initiation factor 4F complex, which helps to shut off host protein synthesis. Cleavage of p220 appears to be important for picornavirus replication, since rhinoviruses and enteroviruses utilize a different proteinase (2A) to cleave p220. To explore the role of L in FMDV replication, we generated synthetic FMDV genomes lacking the L gene and tested their viability in cells. Genomes were constructed with the N-terminal Gly codon of VP4 positioned directly following either the first (Lab) or second (Lb) Met codon of the L protein. Cells transfected with synthetic RNAs lacking L and initiating with the Lab Met codon failed to produce viable virus, but cells transfected with RNAs that utilized the second AUG to drive translation of the viral polyprotein produced viable viruses. These leader-deleted viruses produced plaques on BHK cells that were slightly smaller than those produced by wild-type (WT) virus, grew to slightly lower titers than WT virus in BHK cells, shut off host protein synthesis more slowly than WT virus, and were slightly attenuated in mice. These studies indicate that the L proteinase is not essential for FMDV replication and show that in the cells and animals tested the L gene has a limited effect on virus replication.

The foot-and-mouth disease virus (FMDV) genome contains a single open reading frame about 7,000 bases in length that is initiated from an internal ribosome entry site (IRES) about 1,100 bases from the 5′ end of the RNA (9, 27). The viral polyprotein is processed by three viral proteinases co- and posttranslationally (Fig. 1). The leader (L) and 2A proteinases appear to cleave at only a single site in the polyprotein, whereas the 3C proteinase cleaves at multiple sites. The L gene is positioned at the 5′ end of the open reading frame and contains two potential in-frame initiation codons (84 nucleotides apart) which encode proteins defined as Lab and Lb (37), with predicted molecular weights of 23,025 and 19,861 (for type A_{12} [33]), respectively. Nucleotide sequence analyses have revealed that these two initiation codons are present in representatives of all seven serotypes of FMDV (13, 39), and although the first AUG (at the start of Lab) is not always in a context that should initiate efficiently (26), both Lab and Lb have been detected in in vitro translation reactions and in infected cells (13, 39).

The L protein autocatalytically removes itself from the N terminus of the elongating polyprotein (41), exposing the N terminus of the capsid precursor protein P1, permitting its N-terminal myristoylation (12); however, L can also accomplish this cleavage in *trans* (41). The L gene product shows sequence similarity to the papain family of proteinases (20), and inhibitor studies (24) and site-directed mutagenesis studies (34) have confirmed that it belongs to this class of proteinases. The 2A proteinase of FMDV cleaves the C-terminal end of the P1-2A product from the P1-P2 precursor, and 2A cleavage activity can be accomplished within a foreign protein by a segment of as few as 13 amino acid residues (38), consistent with the fact that 2A can function only in *cis*. The 2A proteinase of enteroviruses and rhinoviruses is considerably larger, and it cleaves the viral polyprotein between P1 and P2 (43). The 3C proteinase has been extensively studied for all five genera of the *Picornaviridae*. Several studies have noted sequence similarities between 3C and cellular serine proteinases (6, 19), and this sequence similarity and use of cysteine in the active site have been confirmed by recent structural studies on the 3C proteinases of hepatitis A virus (1) and human rhinovirus (29). Experiments with the FMDV 3C proteinase have confirmed the expected requirements of this cysteine for activity (21).

In addition to cleavage of virally encoded proteins, L and 3C are known to cleave host cell proteins. 3C cleaves histone H3, which could contribute to the shutoff of host cell transcription (18, 42), and L cleaves the p220 subunit of eukaryotic initiation factor 4F (eIF-4F) (14, 23), resulting in the shutoff of cap-dependent host cell protein synthesis, without affecting viral protein synthesis which can occur in the presence of cleaved p220. Although an active L proteinase is encoded only by viruses in the *Aphthovirus* genus of the *Picornaviridae* (16), the 2A proteinase of enteroviruses and rhinoviruses also cleaves p220, but at a different site in the protein (40). Viruses in the *Cardiovirus* genus encode an L protein that does not have any known protease function, although removal of the L gene from the genome of Theiler's murine encephalomyelitis virus produces viruses with altered host range in cell culture (10, 25) and virulence in mice (10).

Attempts to develop live-attenuated foot-and-mouth disease (FMD) vaccines by classical methods have met with limited success (3), and the well-known instability of the viral genome (15) strongly suggests that viruses attenuated by mutation at a limited number of sites could easily revert to virulence. We have previously reported attempts to attenuate FMDV by deletion of the poly(C) tract near the 5′ end of the genome (36), on the basis of the successful application of this type of genetic engineering to the production of attenuated cardioviruses (17). Although genetically engineered FMDV with poly(C) tracts of

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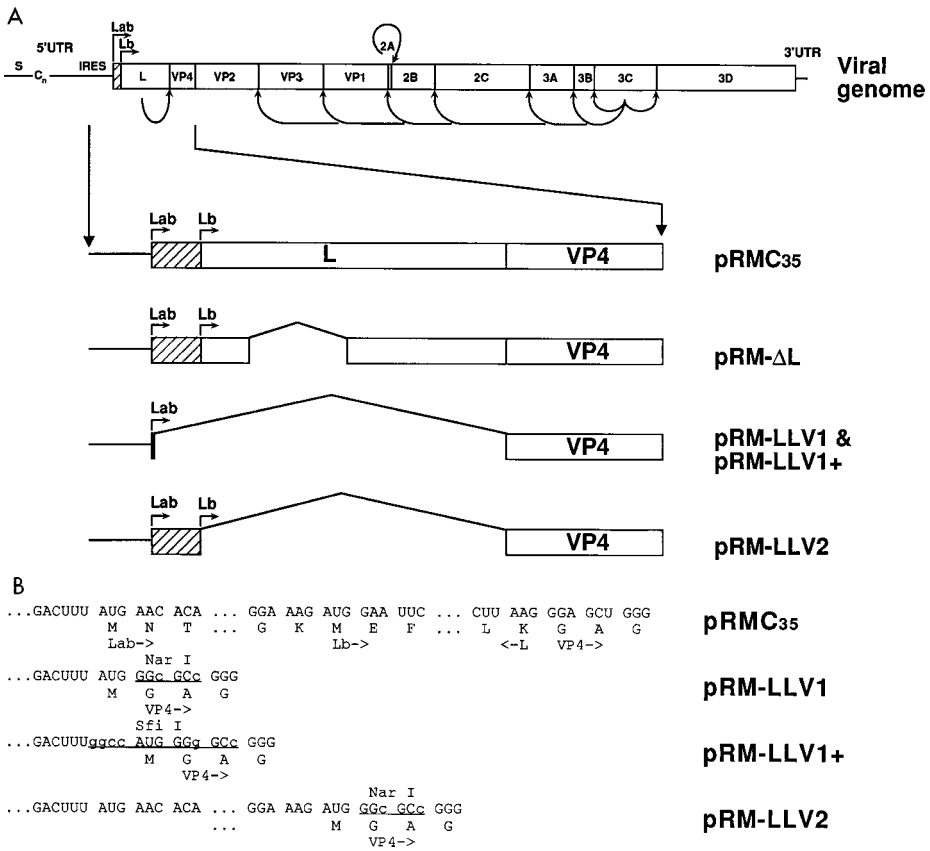

FIG. 1. (A) Diagram of the FMDV genome and L-deleted genomes. The viral open reading frame is boxed, and the shaded box corresponds to the 84-nucleotide region between the two in-frame initiation codons for Lab and Lb (see text). Abbreviations: C_n, poly(C) tract; S, 5′ portion of the genome or small fragment; UTR, untranslated region. (B) Sequences of the WT and mutant RNAs surrounding the initiation codons for Lab and Lb and the cleavage site between L and VP0. Dots indicate sequences left out for clarity; underlining indicates positions of restriction endonuclease sites in the cDNA; lowercase letters indicates nucleotides added or changed by site-directed mutagenesis.

only two nucleotides maintained their shortened poly(C) tracts in cell culture and in vivo, they were virulent in mice (36).

To investigate the role of the FMDV L proteinase in viral maturation, host cell protein synthesis shutoff, and viral pathogenesis, and to generate leader-deleted viruses that could serve as live-attenuated FMD vaccines, synthetic full-length RNAs lacking the L gene were constructed and transfected into eukaryotic cells. In the studies reported here, we describe the construction and characterization of a leaderless virus.

MATERIALS AND METHODS

Cell lines, viruses, and plasmids. Virus stocks were prepared and titrated by plaque assay in baby hamster kidney (BHK) cells (strain 21, clone 13) as described previously (36), and a bovine kidney cell line (BK-LF) was used to assay p220 cleavage activity (24). Wild-type (WT) FMDV type A_{12} used in all studies was derived from the infectious clone $pRMC_{35}$ (36). Plasmid pRM-KGE, which contains a mutation in the sequence encoding the cell binding site, has been described elsewhere (28). RNAs transcribed from this cDNA are translated and processed at the same efficiency as WT ($pRMC_{35}$) RNA, but cells transfected with pRM-KGE RNA produce virus particles incapable of binding to or infecting cells (28), thus eliminating their ability to initiate a second cycle of infection in transfection experiments. Plasmid pRM-ΔL (previously named $pRMC_{35}$-ΔL), which has an in-frame deletion of 192 nucleotides from the center of the L gene, has been described elsewhere (33), and all plasmids used to produce the mutant cDNAs described in this report were derivatives of $pRMC_{35}$.

Construction of cDNAs lacking L-coding sequences. Plasmids harboring full-length cDNAs containing complete L deletions were constructed by using PCR site-directed mutagenesis protocols (22) to introduce novel restriction sites at the junction of the IRES and the VP4 gene. Briefly, plasmids pRM-LLV1 and pRM-LLV2 were constructed by addition of a *Nar*I site encoding Gly-Ala (corresponding to the first two amino acids of VP4) to the initiation codons of Lab or Lb, respectively, and then ligating these segments to a VP4 cDNA containing silent mutations in the first and second codons (Gly-Ala) to produce a second *Nar*I site (Fig. 1). pRM-LLV1+, which contains the Lab initiation codon preceded by an additional four bases (GGCC), added to produce a better context for translation initiation (26), was created by addition of an *Sfi*I site at the IRES/VP4 junction (Fig. 1 and 2). Following mutagenesis, all plasmid DNAs were sequenced through the entire amplified region by using Sequenase (Amersham, Arlington Heights, Ill.).

Production of T7 transcripts, in vitro translation, and transfection into cells. T7 transcripts of *Not*I-linearized plasmid DNA were produced by using a MegaScript kit (Ambion, Inc., Austin, Tex.). Transcripts were translated in vitro by using reticulocyte lysates as described by Vakharia et al. (44) or transfected into BHK cells by using Lipofectin (GIBCO-BRL, Gaithersburg, Md.) (36) or electroporation (28).

Radioimmunoprecipitation and gel electrophoresis. Radioimmunoprecipitates were prepared from [^{35}S]methionine (New England Nuclear [Boston, Mass.] or Amersham)-labeled in vitro translation reaction mixtures, transfected cells, or infected cells, using standard techniques. Immunoprecipitations were performed with monoclonal antibody 2PD11, which recognizes conformational determinants on the viral capsid (4, 5), or a bovine convalescent serum to FMDV. Radiolabeled cell lysates, translation reaction mixtures, or immunoprecipitates were resolved by electrophoresis on 15% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) and fluorographed.

RESULTS

Construction of cDNAs lacking all or a portion of the L gene. Plasmids containing the full-length type A_{12} cDNA molecules lacking all or a portion of the L gene were constructed by standard techniques and are shown schematically in Fig. 1A (see Materials and Methods). The exact nucleotide sequences

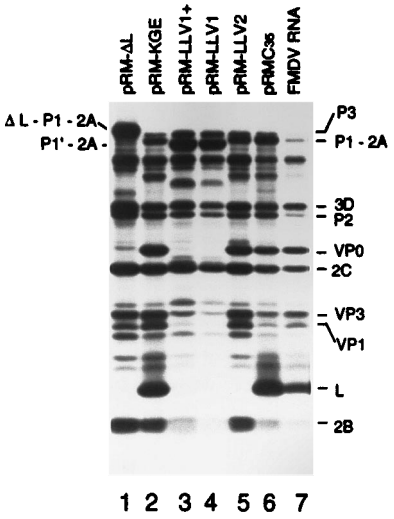

FIG. 2. In vitro translation of FMDV mutant transcripts. Transcripts from cDNAs shown in Fig. 1 were translated in a cell-free system, and the radiolabeled products were examined by SDS-PAGE on a 15% gel. The transcripts used are indicated above the lanes. Equal amounts of trichloroacetic acid-precipitable protein were loaded in each lane.

surrounding the initiation codons positioned in front of the VP4 coding regions of the three cDNA clones containing complete deletions of L are shown along with WT sequences in Fig. 1B. pRM-ΔL has an in-frame deletion within the L gene, which abrogates L function (33). pRM-LLV1 contains the Lab AUG positioned in front of the codon corresponding to the N-terminal Gly of VP4. pRM-LLV1+ was constructed by using the Lab AUG found in pRM-LLV1, but with modifications of the sequence 5′ of the AUG to produce a more favorable context for translation initiation (26), and pRM-LLV2 contains the Lb AUG positioned in front of the same Gly codon of VP4. The genome-length cDNAs present in all of these plasmids were derived from the infectious cDNA clone, $pRMC_{35}$, and were preceded by a synthetic T7 RNA polymerase promoter and followed by a unique *Not*I site (36).

In vitro translation of RNAs lacking the L gene. T7 RNA transcripts derived from pRM-KGE, pRM-ΔL, pRM-LLV1, pRM-LLV1+, and pRM-LLV2 were checked for the ability to function as templates for protein synthesis in reticulocyte lysates. Transcripts from pRM-KGE, pRM-ΔL, and pRM-LLV2 were translated in vitro at least 10 times more efficiently than transcripts from pRM-LLV1 and pRM-LLV1+ (results not shown). Qualitatively, the translation products produced from transcripts of pRM-KGE and pRM-LLV2 were identical to the products produced by virion RNA except that the reaction mixtures programmed with pRM-LLV2 RNA did not contain L (Fig. 2). The translation products obtained from pRM-LLV1 and pRM-LLV1+ transcripts differed dramatically from those produced by the other transcripts. Neither P1-2A nor VP0 was observed, and there were reduced amounts of VP3 and VP1; instead, a product that migrated more rapidly than P1-2A was observed (P1′-2A), suggesting that its synthesis had initiated at a site within the VP4 coding region (Fig. 2). Since 3C-mediated cleavage of P1/2A products generated in vitro is inefficient (2, 44), exogenous *Escherichia coli*-produced 3C (2) was added to help clarify the nature of the aberrant product generated in the pRM-LLV1 or pRM-LLV1+ translation reaction. These studies showed that the band that migrated more quickly than P1-2A could be cleaved to products that comigrated with VP1, VP3, and a third product that migrated faster than VP0 (results not shown).

TABLE 1. Characteristics of synthetic RNAs in BHK monolayers transfected by using Lipofectin

Source of RNA	Specific infectivity (10^4)[a]	Plaque size (mm)
$pRMC_{35}$	3.6	4–6
pRM-LLV1	ND	
pRM-LLV1+	ND	
pRM-LLV2	3.3	2–4
pRM-ΔL	ND	
pRM-KGE	ND	

[a] PFU per microgram of synthetic RNA obtained with the indicated plasmids; average of two preparations. ND, none detected in 250 ng.

Transfection of RNAs lacking the L gene into cells by using Lipofectin. T7 RNA transcripts derived from $pRMC_{35}$, pRM-ΔL, pRM-LLV1, pRM-LLV1+, and pRM-LLV2, were transfected into monolayers of BHK cells by using Lipofectin, and the monolayers were stained 72 h later to reveal plaques. Under these conditions, pRM-LLV2- and $pRMC_{35}$-derived transcripts showed essentially identical specific infectivities, whereas no plaques were detected in cells transfected with pRM-ΔL, pRM-LLV1, or pRM-LLV1+ RNAs (Table 1). Furthermore, the pRM-LLV2 RNA produced plaques slightly smaller than those produced by the L gene-containing RNA from $pRMC_{35}$ (Table 1).

Transfection of leaderless RNAs into cells by electroporation. Five to ten micrograms of T7 RNA transcript derived from pRM-KGE, pRM-ΔL, pRM-LLV1, pRM-LLV1+, or pRM-LLV2 was transfected into 1.6×10^7 BHK cells by electroporation, and the cells were incubated overnight in the presence or absence of radiolabel as described by Mason et al. (28). Cells transfected with transcripts from pRM-KGE, pRM-ΔL, and pRM-LLV2 showed cytopathic effect (CPE) in over 95% of the cells within 16 h of transfection, whereas cells transfected with pRM-LLV1- and pRM-LLV1+-derived transcripts showed CPE that was indistinguishable from that of cells electroporated in the absence of RNA. Since the viral particles produced by pRM-KGE RNA are not infectious (28), and hence unable to spread between cells following transfection, the extensive CPE observed in these cultures indicated that highly efficient transfection was achieved under these conditions.

Plaque assays performed with freeze-thawed lysates of transfected cultures revealed high titers of virus in cells transfected with RNA from pRM-LLV2, but no virus was detected in cells transfected with RNAs from pRM-KGE, pRM-ΔL, pRM-LLV1, or pRM-LLV1+. Fresh BHK cells were incubated with the freeze-thawed lysates of cells transfected with each of the latter four RNAs and incubated for an additional 48 h at 37°C. The cells remained indistinguishable from uninfected cells, and plaque assays performed on freeze-thawed lysates from these blind passages did not reveal any virus capable of forming plaques on BHK cells. Our inability to recover live virus from lysates of cells transfected with the pRM-KGE RNA was expected, since the viral particles produced from this RNA cannot bind to or infect cells (28). The inability to rescue virus from lysates of pRM-ΔL RNA-transfected cells was also expected, since the truncated L proteinase cannot remove itself from the N terminus of the P1 molecule, thus preventing capsid assembly.

SDS-PAGE of immunoprecipitates prepared from radiolabeled, transfected cells confirmed that viral proteins were

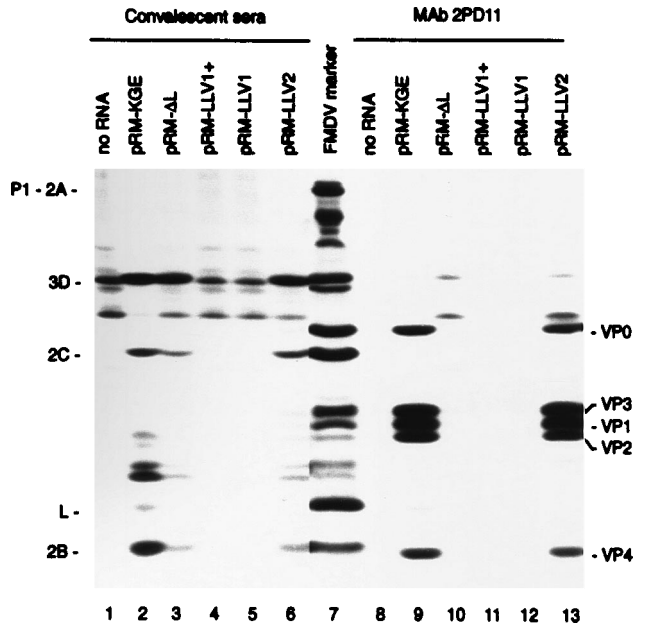

FIG. 3. FMDV proteins immunoprecipitated from lysates of cells transfected with RNA transcripts. BHK-21 cells were electroporated with mutant RNAs (as indicated above the lanes) and radiolabeled with [^{35}S]methionine. Cell extracts were immunoprecipitated with bovine convalescent serum (lanes 1 to 6), while vision proteins present in the culture fluid were precipitated with monoclonal antibody (MAb) 2PD11 (lanes 8 to 13), and the samples were analyzed by SDS-PAGE on a 15% gel. Lane 7 contains in vitro translation products of FMD virion RNA.

present in cells transfected with RNAs from pRM-KGE, pRM-ΔL, and pRM-LLV2 (Fig. 3). However, no viral products were detected in cells transfected with RNAs from pRM-LLV1 or pRM-LLV1+ (Fig. 3). Furthermore, analysis of the culture fluid revealed that virions were efficiently released only by cells transfected with pRM-KGE or pRM-LLV2 RNAs (data not shown). These data strongly suggest that the major initiation site used for production of P1-2A from the pRM-LLV2 RNA was the Lb AUG, since only the product initiated at this site could produce the N-terminal Gly-X-X-X-Ser/Thr consensus sequence for myristoylation (12) (Fig. 1B), and myristoylation is important for many steps of picornavirus assembly (8, 32).

Characterization of virus recovered from cells transfected with the pRM-LLV2 RNA. Virus recovered from cells electroporated with pRM-LLV2 RNA (BHK passage 1; BHKp1) was passaged two additional times in BHK cells and compared with a BHKp3 virus obtained from cells transfected with the WT infectious clone pRMC$_{35}$. As expected, PCR analysis of the genome of the BHKp3 virus recovered from pRM-LLV2-transfected cells revealed that it lacked the L gene. Side-by-side comparison of plaque sizes of these viruses on BHK cells showed that the pRM-LLV2-derived virus (LLV2) formed plaques slightly smaller than those formed by WT virus (2 mm versus 4 mm at 40 h postinoculation), consistent with data from Lipofectin transfection assays (Table 1; see above). Furthermore, although LLV2 clearly caused CPE on BHK and BK-LF cells, the appearance of CPE was delayed relative to WT virus infections (results not shown), and one-step growth curves of WT virus- and LLV2-infected cells revealed that LLV2 was released from cells slightly more slowly than WT virus (Fig. 4).

The ability of LLV2 to shut off host cell protein synthesis was evaluated by radiolabeling BHK cells at various times postinfection and evaluating total protein synthesis by trichloroacetic

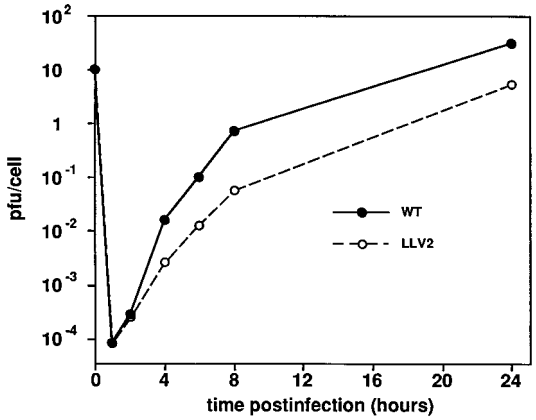

FIG. 4. One-step growth curve of WT virus and LLV2. BHK-21 cell monolayers were infected with WT virus or LLV2 at a multiplicity of infection of 10 (shown at the 0-h time point), rinsed with a pH 6 buffer at 30 min postinfection (to remove residual input virus [36]), and then incubated at 37°C. The amount of virus released into the culture fluid was determined at each time point by plaque assay.

acid precipitation and specific protein synthesis by SDS-PAGE. Evaluation of total protein synthesis showed that like WT virus, LLV2 reduced the total amount of protein synthesized by infected cells. In the experiment shown in Fig. 5, at the

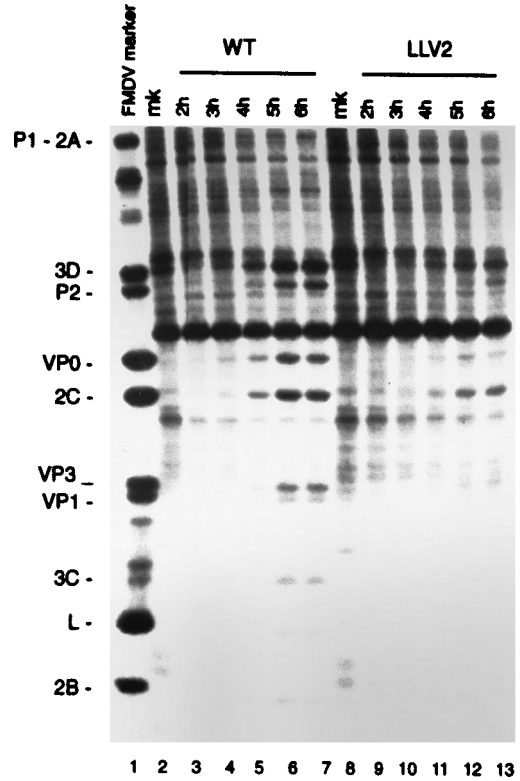

FIG. 5. Qualitative analysis of protein synthesis in WT virus- and LLV2-infected cells. WT virus- or LLV2-infected BHK cells were radiolabeled with [^{35}S]methionine for 1 h at various times after infection (lanes 3 to 7 or 9 to 13, respectively). Cytoplasmic extracts were prepared, protein synthesis was quantitated by measuring radioactivity incorporated into trichloroacetic acid-precipitable material, and equal amounts of radiolabeled protein were resolved by SDS-PAGE on a 15% gel. Lane 1 represents the in vitro translation products of FMD virion RNA; lanes 2 and 8 contain radiolabeled mock-infected (mk) cell extracts.

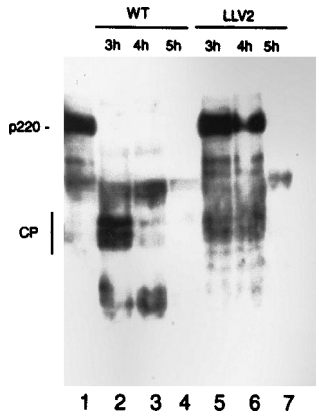

FIG. 6. Analysis of p220 in WT virus- and LLV2-infected cells. Cytoplasmic extracts from uninfected BK-LF cells (lane 1), WT virus-infected BK-LF cells (lanes 2 to 4), or LLV2-infected BK-LF cells (lanes 5 to 7) at 3, 4, and 5 h postinfection were prepared and separated by SDS-PAGE. Western blot analysis was performed with a polyclonal serum specific for p220. The positions of p220 and p220 cleavage products (CP) are indicated.

6- to 7-h labeling period, a 61% shutoff for WT virus and a 57% shutoff for LLV2 were observed. However, the reduction in protein synthesis in LLV2-infected cells was delayed relative to WT virus infection; for example, at 2 to 3 h postinfection, the shutoff was 25% for WT virus and 6% for LLV2 infection. Furthermore, analysis of the specific protein products produced by infected cells showed that for the WT virus, there was a rapid increase in viral protein synthesis relative to cellular protein synthesis, which was not as pronounced in LLV2-infected cells (Fig. 5). Similar results were obtained for a second cell line (BK-LF; results not shown).

Direct demonstration of differences in the stability of p220 in BK-LF cells infected with the WT virus and LLV2 was evaluated by Western blot (immunoblot) assay with a p220-specific antibody (24) (Fig. 6). These experiments showed that in contrast to LLV2-infected cells, intact p220 rapidly disappeared from cells infected with WT virus, and characteristic cleavage products were observed at 3 h postinfection (Fig. 6, lane 2). At later times postinfection, no products that reacted with this serum in Western blot assays were detected in WT virus-infected cells (Fig. 6, lane 4). However, at later times postinfection, intact p220 also disappeared from cells infected with LLV2, suggesting that factors other than the presence of L in infected cells altered the steady state levels of p220. The time of loss of p220 in LLV2-infected cells correlated well with the appearance of CPE and the shutoff of host protein synthesis, although it is unclear if the apparent degradation of this translation initiation factor resulted in these changes or was a result of the global changes in cellular metabolism that result from virus infection. Interestingly, the degradation of p220 in LLV2-infected cells could be inhibited by treatment of the culture with the cysteine proteinase inhibitor E-64d (results not shown).

To test further the effect of L on virus biology, we compared the virulence of WT virus and LLV2 in mice. Dilutions of WT virus and LLV2 were inoculated into litters of suckling mice, and the 50% lethal doses (LD_{50}s) were established by using standard techniques (35). In the first experiment (10-day-old mice), LLV2 was approximately 40-fold less virulent by this criterion (Table 2). However, we noted that LLV2 behaved very differently from WT virus in these experiments. Specifically, the 100% lethal dose for LLV2 corresponded to over 60,000 LD_{50}, whereas the uniformly lethal dose for WT virus was approximately 44 LD_{50} (Table 2). To investigate this phenomenon, a second experiment was performed with slightly younger (7-day-old) mice. This experiment revealed nearly identical LD_{50}s for the two viruses, but once again the 100% lethal dose for LLV2 was very high, corresponding to approximately 10,000 LD_{50}, whereas for WT virus it was approximately 20 LD_{50} (Table 2). These results are consistent with the possibility that there was a delay in spread of the LLV2 virus, allowing recovery in some animals, consistent with our observation that CPE and virus replication observed in LLV2-infected cells in culture were delayed relative to WT virus.

DISCUSSION

Among the picornaviruses, only members of the *Aphthovirus* genus contain an active leader (L) proteinase gene. The L proteinase has only two known functions, its autocatalytic removal from the N terminus of the viral polyprotein and cleavage of the p220 subunit of eIF-4F. Cleavage of p220 is thought to be responsible for enhancing the translation of viral genes in infected cells, by inhibiting translation of capped host cell mRNAs in the presence of viral translation which occurs by a cap-independent mechanism. Cleavage of p220 appears to be important for picornavirus replication, since the rhinoviruses and enteroviruses utilize a different proteinase (2A) to cleave p220 (16). Furthermore, analysis of genetically engineered poliovirus genomes with mutations in the 2A gene have suggested additional roles for this gene in poliovirus-infected cells (30, 45). However, virus-specified p220 cleavage is not essential for picornavirus replication or host protein shutoff, since cardioviruses do not appear to have a proteinase that cleaves p220 (31).

To explore the role of the L proteinase in viral replication, we have generated synthetic viral genomes lacking the L gene and tested their viability in cells. To produce leaderless viruses, we designed genomes that initiated translation of the VP4 protein from both of the possible leader initiation codons (see the introduction). Interestingly, only the genome that initiated polyprotein synthesis at the second L AUG (Lb AUG; cDNA clone pRM-LLV2) produced a live virus. The inability to generate leaderless viruses from RNAs in which the polyprotein was positioned behind the first AUG of L (Lab AUG; cDNA clones pRM-LLV1 and pRM-LLV1+) appears to have been due to the greatly reduced ability of these RNAs to efficiently initiate synthesis of the truncated viral polyprotein at the Lab AUG. Although we did not exhaustively test all of the possible

TABLE 2. Virulence of the WT virus and LLV2 in suckling mice

Virus	Expt 1 (10-day-old mice)			Expt 2 (7-day-old mice)		
	PFU/LD_{50}	100% lethal dose (PFU)	0% lethal dose (PFU)	PFU/LD_{50}	100% lethal dose (PFU)	0% lethal dose (PFU)
WT	2	44	0.04	0.9	20	0.02
LLV2	76	$>6 \times 10^4$	<0.06	0.5	1.1×10^4	<0.01

sequences preceding the AUG for the ability to improve translation efficiency, the two sequences tested, the WT UUUAUG (pRM-LLV1) and GCCAUG (pRM-LLV1+), displayed similar low translational efficiencies in vitro, and synthetic RNAs with either sequence failed to produce viable viruses.

Our finding that viable viruses could be generated only when the polyprotein was initiated from the second AUG (Lb) is consistent with the finding that this site is the primary one used in infected cells (see the introduction) and argues against the suggestion of Belsham (7) that the Lb AUG is only slightly favored over the Lab AUG. Further support for our finding that a leaderless virus could be constructed only by utilizing the Lb AUG comes from recent work by Cao et al., showing that genetically engineered viruses with mutations in the Lb AUG were not viable whereas viruses with mutations in the Lab AUG were (11). Taken together, our results and those of Cao et al. (11) suggest that the RNA sequence between the two AUGs is required for efficient translational initiation at the second (Lb) AUG in the FMDV genome (e.g., this region could function as part of the IRES).

Characterization of the leaderless virus, LLV2, demonstrated that the L proteinase is not required for shutoff of host protein synthesis. Specifically, the leaderless virus formed clear plaques nearly as large as those formed by WT virus and grew nearly as well as WT virus in liquid culture. LLV2 also caused CPE in infected cells and shut off host protein synthesis, albeit more slowly and less completely than WT virus. Interestingly, late in infection, p220 was degraded in LLV2-infected cells, probably by a cellular cysteine proteinase since degradation was prevented by the inhibitor E-64d. Although LLV2 displayed different properties in mice, it was not dramatically attenuated relative to WT virus. However, since the disease caused by FMDV in baby mice does not resemble FMD, it is possible that LLV2 differs in virulence and pathogenicity relative to WT virus in livestock. Furthermore, studies in a natural host may help reveal why FMDV has maintained a gene that is apparently not needed for growth in tissue culture.

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REFERENCES

1. **Allaire, M., M. M. Chernaia, B. A. Malcolm, and M. N. G. James.** 1994. Picornavirus 3C cysteine proteinases have a fold similar to chymotrypsin-like serine proteinases. Nature (London) **369:**72–76.
2. **Bablanian, G. M., and M. J. Grubman.** 1993. Characterization of the foot-and-mouth disease virus 3C protease expressed in Escherichia coli. Virology **197:**320–327.
3. **Bachrach, H. L.** 1968. Foot-and-mouth disease virus. Annu. Rev. Microbiol. **22:**201–244.
4. **Baxt, B., D. O. Morgan, B. H. Robertson, and C. A. Timpone.** 1984. Epitopes on foot-and-mouth disease virus outer capsid protein VP1 involved in neutralization and cell attachment. J. Virol. **51:**298–305.
5. **Baxt, B., V. Vakharia, D. M. Moore, A. J. Franke, and D. O. Morgan.** 1989. Analysis of neutralizing antigenic sites on the surface of type A_{12} foot-and-mouth disease virus. J. Virol. **63:**2143–2151.
6. **Bazan, J. F., and R. J. Fletterick.** 1988. Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: structural and functional implications. Proc. Natl. Acad. Sci. USA **85:**7872–7876.
7. **Belsham, G. J.** 1992. Dual initiation sites of protein synthesis on foot-and-mouth disease virus RNA are selected following internal entry and scanning of ribosomes in vivo. EMBO J. **11:**1105–1110.
8. **Belsham, G. J., C. C. Abrams, A. M. Q. King, J. Roosien, and J. M. Vlak.** 1991. Myristoylation of foot-and-mouth disease virus capsid protein precursors is independent of other viral proteins and occurs in both mammalian and insect cells. J. Gen. Virol. **72:**747–751.
9. **Belsham, G. J., and J. K. Brangwyn.** 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. **64:**5389–5395.
10. **Calenoff, M. (Northwestern University).** 1995. Personal communication.
11. **Cao, X., I. E. Bergmann, R. Füllkrug, and E. Beck.** 1995. Functional analysis of the two alternative translation initiation sites of foot-and-mouth disease virus. J. Virol. **69:**560–563.
12. **Chow, M., J. F. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) **327:**482–486.
13. **Clarke, B. E., D. V. Sangar, J. N. Burroughs, S. E. Newton, A. R. Carroll, and D. J. Rowlands.** 1985. Two initiation sites for foot-and-mouth disease virus polyprotein in vivo. J. Gen. Virol. **66:**2615–2626.
14. **Devaney, M. A., V. N. Vakharia, R. E. Lloyd, E. Ehrenfeld, and M. J. Grubman.** 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. J. Virol. **62:**4407–4409.
15. **Domingo, E., J. Díez, M. A. Martínez, J. Hernández, A. Holguín, B. Borrego, and M. G. Mateu.** 1993. New observations on antigenic diversification of RNA viruses. Antigenic variation is not dependent on immune selection. J. Gen. Virol. **74:**2039–2045.
16. **Dougherty, W. G., and B. L. Semler.** 1993. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. Microbiol. Rev. **57:**781–822.
17. **Duke, G. M., J. E. Osorio, and A. C. Palmenberg.** 1990. Attenuation of Mengo virus through genetic engineering of the 5′ noncoding poly(C) tract. Nature (London) **343:**474–478.
18. **Falk, M. M., P. R. Grigera, I. E. Bergmann, A. Zibert, G. Multhaup, and E. Beck.** 1990. Foot-and-mouth disease virus protease 3C induces specific proteolytic cleavage of host cell histone H3. J. Virol. **64:**748–756.
19. **Gorbalenya, A. E., A. P. Donchenko, V. M. Blinov, and E. V. Koonin.** 1989. Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common fold. FEBS Lett. **243:**103–114.
20. **Gorbalenya, A. E., E. V. Koonin, and M. M.-C. Lai.** 1991. Putative papain-related thiol proteases of positive-strand RNA viruses: identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, α-, and coronaviruses. FEBS Lett. **288:** 201–205.
21. **Grubman, M. J., M. Zellner, G. M. Bablanian, P. W. Mason, and M. E. Piccone.** Unpublished data.
22. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of in vitro preparation and specific mutation of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:**7351–7367.
23. **Kirchweger, R., E. Ziegler, B. J. Lamphear, D. Waters, H.-D. Liebig, W. Sommergruber, F. Sobrino, C. Hohenadl, D. Blass, R. E. Rhoads, and T. Skern.** 1994. Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4γ. J. Virol. **68:**5677–5684.
24. **Kleina, L. G., and M. J. Grubman.** 1992. Antiviral effects of a thiol protease inhibitor on foot-and-mouth disease virus. J. Virol. **66:**7168–7175.
25. **Kong, W.-P., G. D. Ghadge, and R. P. Roos.** 1994. Involvement of cardiovirus leader in host cell-restricted virus expression. Proc. Natl. Acad. Sci. USA **91:**1796–1800.
26. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:**282–292.
27. **Kühn, R., N. Luz, and E. Beck.** 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **64:**4625–4631.
28. **Mason, P. W., E. Rieder, and B. Baxt.** 1994. RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway. Proc. Natl. Acad. Sci. USA **91:**1932–1936.
29. **Matthews, D. A., W. W. Smith, R. A. Ferre, B. Condon, G. Budahazi, W. Sisson, J. E. Villafranca, C. A. Janson, H. E. McElroy, C. L. Gribskov, and S. Worland.** 1994. Structure of human rhinovirus 3C protease reveals a trypsin-like polypeptide fold, RNA-binding site, and means for cleaving precursor polyprotein. Cell **77:**761–771.
30. **Molla, A., A. V. Paul, M. Schmid, S. K. Jang, and E. Wimmer.** 1993. Studies on dicistronic polioviruses implicate viral proteinase $2A^{pro}$ in RNA replication. J. Virol. **196:**739–747.
31. **Mosenkis, J., S. Daniels-McQueen, S. Janovec, R. Duncan, J. W. B. Hershey, J. A. Grifo, W. C. Merrick, and R. E. Thach.** 1985. Shutoff of host translation by encephalomyocarditis virus infection does not involve cleavage of the eucaryotic initiation factor 4F polypeptide that accompanies poliovirus infection. J. Virol. **54:**643–645.
32. **Muscofo, N., J. Simons, and M. Chow.** 1991. Myristoylation is important at multiple stages in poliovirus assembly. J. Virol. **65:**2372–2380.
33. **Piccone, M. E., S. Sira, M. Zellner, and M. J. Grubman.** 1995. Expression in Escherichia coli and purification of biologically active L proteinase of foot-and-mouth disease virus. Virus Res. **35:**263–275.
34. **Piccone, M. E., M. Zellner, T. F. Kumosinski, P. W. Mason, and M. J.**

Grubman. 1995. Identification of the active-site residues of the L proteinase of foot-and-mouth disease virus. J. Virol. **69:**4950–4956.
35. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
36. **Rieder, E., T. Bunch, F. Brown, and P. W. Mason.** 1993. Genetically engineered foot-and-mouth disease viruses with poly(C) tracts of two nucleotides are virulent in mice. J. Virol. **67:**5139–5145.
37. **Rueckert, R. R., and E. Wimmer.** 1984. Systematic nomenclature of picornavirus proteins. J. Virol. **50:**957–959.
38. **Ryan, M. D., and J. Drew.** 1994. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO J. **13:**928–933.
39. **Sangar, D. V., S. E. Newton, D. J. Rowlands, and B. E. Clarke.** 1987. All foot-and-mouth disease virus serotypes initiate protein synthesis at two separate AUGs. Nucleic Acids Res. **15:**3305–3315.
40. **Sommergruber, W., H. Ahorn, H. Klump, J. Siepelt, A. Zoephel, F. Fessl, E. Krystek, D. Blass, E. Kuchler, H.-D. Liebig, and T. Skern.** 1994. 2A proteinases of coxsackie- and rhinovirus cleave peptides derived from eIF-4γ via a common recognition motif. Virology **198:**741–745.
41. **Strebel, K., and E. Beck.** 1986. A second protease of foot-and-mouth disease virus. J. Virol. **58:**893–899.
42. **Tesar, M., and O. Marquardt.** 1990. Foot-and-mouth disease virus protease 3C inhibits cellular transcription and mediates cleavage of histone H3. Virology **174:**364–374.
43. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45:**761–770.
44. **Vakharia, V. N., M. A. Devaney, D. M. Moore, J. J. Dunn, and M. J. Grubman.** 1987. Proteolytic processing of foot-and-mouth disease virus polyproteins expressed in a cell-free system from clone-derived transcripts. J. Virol. **61:**3199–3207.
45. **Yu, S. F., P. Benton, M. Bovee, J. Sessions, and R. E. Lloyd.** 1995. Defective RNA replication by poliovirus mutants deficient in 2A protease cleavage activity. J. Virol. **69:**247–252.