Proteolytic Processing of Foot-and-Mouth Disease Virus Polyproteins Expressed in a Cell-Free System from Clone-Derived Transcripts

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All picornaviral genes are expressed as a single, large polyprotein, which is proteolytically processed into the mature proteins. Cell-free translation of foot-and-mouth disease virus (FMDV) RNA in a rabbit reticulocyte system produces functional proteins, including viral protease 3C, which plays a major role in processing the precursor proteins. To study the function of the two putative proteases 3C and leader (L) in processing, we constructed several cDNA plasmids encoding various regions of the FMDV type A₁₂ genome. These plasmids, containing FMDV cDNA segments under the control of the T7 promoter, were transcribed in vitro by using T7 RNA polymerase and then translated in rabbit reticulocyte lysates. The expressed FMDV gene products were identified by immunoprecipitation with specific antisera and analyzed by gel electrophoresis. The results demonstrate the following: (i) the leader protein, L, is processed from the structural protein precursor, P1, in the absence of any P2 or P3 region proteins; (ii) protein 2A remains associated with the structural protein precursor, P1, rather than the precursor, P2; (iii) the processing of the P1-2A/P2 junction is not catalyzed by 3C or L; (iv) the proteolytic processing of polyproteins from the structural P1 region (except VP4/VP2) and the nonstructural P2 and P3 region is catalyzed by 3C.

Foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family, contains plus-stranded RNA that is translated into a polyprotein encoded by a single, long open reading frame. Synthesis of the polyprotein begins at either of two initiation sites in the same reading frame, resulting in two possible leader proteins (L' or L) of molecular weights (MW) 20,000 and 16,000, which have different amino-terminal sequences but a common carboxy-terminal sequence (2). Following the leader region, the P1 region contains the capsid proteins VP0, VP3, and VP1, which are produced by proteolytic processing of the P1 precursor (14, 44). The P2 region contains two nonstructural proteins of unknown function, 2B and 2C (14). At the junction of the P1-P2 region is a sequence of 16 amino acids that has been referred to as the X region of FMDV (41). This oligopeptide may represent a partial deletion of a third nonstructural protein of the P2 region that is found in other picornaviruses, the 2A protein. The P3 region includes four nonstructural proteins: 3A, of unknown function; 3B (3B1, 3B2, and 3B3), the three genome-linked VPg proteins; 3C, a protease; and 3D, the RNA polymerase (14). The stable nonstructural proteins of FMDV are produced by the proteolytic processing of the precursor proteins P2 and P3 (14). The cleavages at leader-P1, P1-P2, and P2-P3 junctions appear to occur co-translationally because the full-length polyprotein is not normally observed in infected cells or during in vitro translation of FMDV RNA in the reticulocyte cell-free system (12).

The proteolytic processing of FMDV and other members of the *Picornaviridae* family has been examined by using pulse-chase experiments (3, 22, 25, 39, 44, 46). Processing

can be inhibited by adding zinc, iodoacetamide, or *N*-ethylmaleimide to the infected cells or viral RNA-directed reticulocyte lysates (5, 30, 39). On the basis of nucleotide sequencing and microsequencing of the amino and carboxy termini of radiolabeled proteins, it was shown for poliovirus that proteolytic cleavages occur between glutamine-glycine, tyrosine-glycine, and asparagine-serine pairs (27, 35). The exact conditions required for a peptide pair to serve as a processing site are not known, but it is clear that the presence of the peptide pair alone is not sufficient for cleavage, because several peptide pairs that would be expected to be cleaved are not used by the processing proteases (27). In FMDV, several of the 13 sites that must be cleaved to give rise to mature proteins occur at amino acid sequences that bear similarity to the glutamine-glycine sites of poliovirus. However, for FMDV, either glutamic acid or glutamine may be present and serine or threonine may be substituted for glycine. Other cleavage sites in FMDV show even greater diversity from poliovirus sites (6, 9, 41).

The 3C protein has been identified as a protease in encephalomyocarditis virus (EMC virus) (37, 38), poliovirus (18, 19, 23), and FMDV (29). For poliovirus, 3C has been shown to be required for processing of glutamine-glycine sites (18). Because of the lack of homology of many of the FMDV processing sites to the analogous poliovirus and EMC virus sites, cellular and other viral proteases have been suggested as being required for FMDV maturation. Recently, the leader protein of FMDV type O1 has been identified as a protease and has been shown to process the L-P1 junction (45). In the present study, several cDNA clones of FMDV (type A₁₂, strain 119ab) were constructed. In vitro transcription and translation of these clones has allowed determination of the role of the two putative FMDV

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proteases, 3C and L, in catalyzing each of the cleavages required for maturation.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases, *Escherichia coli* DNA polymerase, *E. coli* DNA ligase, and T4 DNA ligase were from New England BioLabs, Inc. The large fragment of DNA polymerase (Klenow fragment), calf intestinal alkaline phosphatase, and polynucleotide kinase were from Boehringer Mannheim Biochemicals. Reverse transcriptase and RNasin were from Promega Biotec. *E. coli* RNase H, micrococcal nuclease, and mung bean nuclease were from Pharmacia, Inc. Pancreatic ribonuclease A was from Worthington Diagnostics. All enzymes were used as specified by the manufacturers. T7 RNA polymerase was produced from the cloned gene and purified by the published procedure (7). The protein synthesis inhibitor sparsomycin was a gift of J. Douros, National Cancer Institute, Bethesda, Md. [α - 32 P]dCTP (800 Ci/mmol) and [35 S]methionine (>1,000 Ci/mmol) were obtained from New England Nuclear Corp. Oligonucleotides were synthesized by using β -cyanoethyl phosphoramidites from American BioNuclear and a Microsyn 1450 DNA synthesizer from Systec, Inc.

Construction of plasmids. Recombinant plasmids were constructed by standard cloning procedures (33). All transformations were performed with *E. coli* JM83 for pUC-derived plasmids and with *E. coli* DH1 for pBR322-derived plasmids. Construction of DNA plasmids pCT220, pE7, pT465, and pCT27, spanning most of the FMDV genome, was reported previously (41). The DNA plasmid pT416 (positions 4290 through 6660) was constructed by using techniques previously described (10, 28, 41, 48). Construction of plasmid pVVLP1 was carried out by the published procedure (16). Briefly, phosphorylated *Bam*HI linkers were ligated to the ends of double-stranded cDNA and then double digested with *Bam*HI and *Eco*RI. The resulting fragment, containing an *Eco*RI site of FMDV (position 88), was then cloned between *Eco*RI and *Bam*HI sites of pBR322. Thus plasmid pVVLP1 contains all of the L (except the major initiation codon) and P1 (except the terminal 16 amino acids of VP1) regions of the FMDV genome. Plasmids were prepared and purified by the published procedure (17). FMDV (type A₁₂, strain 119ab) was grown and purified, and virion RNA was isolated as previously described (11, 13).

To construct plasmid pTLP1, DNA from plasmid pVVLP1 was digested with *Eco*RI and then treated with Klenow fragment in the presence of dATP and dTTP (1 mM each). Phosphorylated synthetic adaptors of the sequence AATTC CATATGG were ligated to the *Eco*RI blunt-ended plasmid pVVLP1 and then digested with restriction enzymes *Nde*I and *Bam*HI. The resulting fragment, containing a unique *Nde*I site with AUG initiation codon and a restored *Eco*RI site, was then inserted between the *Nde*I and *Bam*HI sites of a modified T7 expression vector, pET-3c (8; A. H. Rosenberg, B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier, Gene, in press), that lacked an *Eco*RI site. Transformants were screened by colony hybridization (15) and by restriction enzyme analysis. One representative clone, encoding all of the L (from the major initiation site) and P1 regions of the FMDV genome, was chosen and designated pTLP1.

To construct plasmids pTLP1A, pTLP12, pTLP123, and pTLP13C, the parent plasmid pTLP1 was digested with *Eco*RI and *Bam*HI, dephosphorylated with calf intestinal alkaline phosphatase, and then used as a vector. DNA from

plasmids pVVLP1, pT465, pCT27, and pT416 was digested with appropriate restriction enzymes, and the purified fragments were then used for cloning. Thus plasmid pTLP1A was constructed by using the *Eco*RI-*Pst*I fragment of pVVLP1 (FMDV positions 88 through 2192 [41]) and the *Pst*I-*Bgl*II (partial) fragment of pT465 (FMDV positions 2192 through 3186) with the *Eco*RI-*Bam*HI-cut vector. Similarly, plasmid pTLP12 was constructed by using the *Eco*RI-*Pst*I fragment of pVVLP1 and the *Pst*I-*Bgl*III (complete) fragment of pT465 (FMDV positions 2192 through 4274) with the *Eco*RI-*Bam*HI-cut vector. Plasmid pTLP123 was constructed by using the *Eco*RI-*Pst*I fragment of pVVLP1 (positions 88 through 2192), the *Pst*I-*Cla*I fragment of pT465 (positions 2192 through 4012), the *Cla*I-*Xho*I fragment of pCT27 (positions 4012 through 4753), and the *Xho*I-*Bam*HI fragment of pT416 (positions 4753 through 6404) with the *Eco*RI-*Bam*HI-cut vector (pTLP1). Plasmid pTLP13C was constructed essentially as pTLP123, except that a shorter *Pst*I-*Xho*I fragment of pT465 (positions 2192 through 3142) was used and the *Cla*I-*Xho*I fragment of pCT27 (positions 4012 through 4753) was omitted.

Construction of plasmids pTRP12 and pTRP123 was essentially the same as described for pTLP12 and pTLP123, except that the *Xba*I-*Bam*HI-cut pET-3c vector was used. Besides the fragments used for the construction of plasmids pTLP12 and pTLP123, additional *Xba*I-*Kpn*I fragment from pCT220 (FMDV positions -400 through -136 in the noncoding region) and *Kpn*I-*Eco*RI fragment from pE7 (FMDV positions -136 through 88) were used. Representative clones, containing an additional 400 nucleotides of the noncoding region and 88 nucleotides of the coding region of the FMDV genome, were selected and designated pTRP12 and pTRP123, respectively.

To construct plasmid pTP12, DNA from plasmid pTLP1 was digested with *Hind*III, treated with mung bean nuclease, and digested with *Kpn*I. The purified fragment was inserted into pTLP12 that was made blunt ended at the *Eco*RI site and cut with *Kpn*I. Similarly, plasmid pTP123 was constructed by inserting the *Nde*I-*Kpn*I fragment from pTP12 into the *Nde*I-*Kpn*I-cut vector pTLP123. Representative clones, lacking all of the L region of the FMDV genome, were chosen and designated pTP12 and pTP123, respectively (see Fig. 1).

In vitro transcription. In vitro transcription reactions were carried out as described previously (34) with modifications. Purified plasmid DNAs were linearized by digestion with restriction enzyme *Eco*RV or *Ssp*I, phenol extracted, and ethanol precipitated. A typical reaction mixture (100 μ l) contained 40 mM Tris hydrochloride (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1 U of RNasin per μ l, 1 mM each ATP, CTP, GTP, and UTP, and 2.5 μ g of plasmid DNA as template. Synthesis was initiated by addition of T7 RNA polymerase (5 μ g), and the reaction mixture was incubated at 37°C for 1 h. A portion of the above mixture was used to initiate in vitro translation.

In vitro translation. The preparation of rabbit reticulocyte lysates and conditions for in vitro protein synthesis were as previously described (12), except that the lysates contained 1 μ Ci of [35 S]methionine per μ l and the level of added Mg²⁺ was lowered to 120 μ M. The final Mg²⁺ concentration was brought to 1.5 mM by the addition of 15 μ l of transcription product (see above) to 50 μ l of lysate, thus initiating translation. Incorporation of ³⁵S was determined as previously described (12). Use of purified FMDV RNA (2 μ g) led to incorporation of 8 to 9 μ Ci under these conditions.

Antisera. Polyclonal antisera against VP1, 2B, 2C, 3A, and

FIG. 1. Schematic presentation of the plasmids encoding FMDV-specific polyproteins. A map of the FMDV genome is given at the top of the figure. The coding region of the genome is drawn to scale, and the proposed nomenclature is used (43). The major and alternate translation initiation sites are marked by a solid and a broken arrow, respectively. VPg is the viral protein covalently linked to the 5' end of the viral RNA. Selected restriction sites important for the construction of the plasmids are incorporated in the figure: B, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; M, *Bam*HI; O, *Xho*I; V, *Eco*RV; X, *Xba*I. Parentheses indicate the restriction site being lost or created in construction of the plasmid. The bridged area on pTLP13C represents deletion of the coding region between *Xho*I sites. All plasmids for the in vitro synthesis of virus-specific RNA contain a T7 promoter at their 5' end. All pTL series of plasmids contain the coding sequence of the L (from the major initiation site followed by the *Eco*RI site) and the P1, P2, and P3 regions of the FMDV genome, as shown. The unique *Eco*RV site present in the coding sequence of the viral protease 3C is shown for clone pTLP123, which was linearized at that site. All pTR series of plasmids contain 400 bases of the noncoding sequence (from the *Xba*I site) and the coding regions L, P1, P2, and P3 of the genome. However, all pTP series plasmids differ from the pTL and pTR series in that they lack the coding region of L'/L.

3C were prepared in rabbits (14). Antiserum against inactivated FMDV (type A₁₂, strain 119ab) was made in guinea pigs (20). Antiserum against 2A (previously called X) was prepared by subcutaneously injecting guinea pigs with 200 µg of the synthetic peptide Asn-Phe-Asp-Leu-Leu-Lys-Leu-Ala-Gly-Asp-Cys, which was coupled to keyhole limpet hemocyanin by using *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester (32) and emulsified with complete Freund adjuvant. Boosters were given at 3-week intervals, using alum as the adjuvant. Serum samples were collected 4 days after the fifth injection. The peptide represents the first 10 of the 16 amino acid residues making up the X region (41), which is now referred to as 2A.

Immunoprecipitation. In vitro translation lysates were preincubated with *Staphylococcus aureus* bearing protein A to minimize nonspecific protein binding and then immunoprecipitated as described previously (21). Guinea pig antisera were used at a final dilution of 1:40, and rabbit antisera were used at a 1:4 dilution. When indicated in the figure legends, lysates were denatured by the addition of sodium dodecyl sulfate (SDS) and dithiothreitol to final concentrations of 1% (wt/vol) and 1 mM, respectively, heated to 100°C for 5 min, and then diluted 10-fold with buffer (21) before being preincubated with *S. aureus* bearing protein A. The eluted immunocomplexes were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (14, 31).

FIG. 2. In vitro translation of FMDV-specific synthetic RNA and the processing of the leader protein. RNA derived from in vitro transcription of pTLP1 and pTLP1A DNA was translated in a rabbit reticulocyte system, and the products were then analyzed by PAGE on a 12.5% slab gel. The marker lane M represents a standard cell-free translation sample programmed with FMD virion RNA, as described in Materials and Methods. The truncated P1' nearly comigrates with P1-2A; it contains 20 amino acids from the vector, which compensates for the deleted 16 amino acids at the carboxy terminus of VP1. The minor band between P2 and VP0 results from the labeling of a protein present in the reticulocyte lysate by a [³⁵S]methionine degradation product. It can be seen because the specific activity of the clone-derived lysates is lower than that of the purified FMDV RNA marker lysate.

RESULTS

Expression of FMDV cDNA clones in vitro. To study the proteolytic processing of the FMDV polyprotein, we constructed several plasmids that contained cDNA segments of the FMDV type A₁₂ genome placed between a T7 ϕ 10 promoter and a T ϕ termination sequence. The regions of polyprotein encoded by these plasmids are schematically represented on the biochemical map of the FMDV genome (Fig. 1). For all of the pTL plasmids, the encoding of the polyprotein initiated from the major initiation site in the L region (an *Nde*I site followed by an *Eco*RI site) and terminated after 20 codons of gene 10 sequence of T7 DNA. For example, plasmid pTLP12 encoded the L region, the entire P1 and P2 regions, and an additional 20 amino acids of the expression vector. In plasmids pTRP12 and pTRP123, 400 nucleotides of the noncoding region were included. Although five AUG codons are present in the noncoding region of these plasmids, none of them was used as a start signal (41), and the encoding of the polyprotein could initiate from the major as well as the alternate initiation site in the L'/L region. In plasmids pTP12 and pTP123, the entire coding

FIG. 3. In vitro translation of FMDV-specific RNA and the processing of the P1-2A/P2 junction. RNA derived from in vitro transcription of plasmid pTP12 and pTLP12 was translated, and the products were immunoprecipitated with various antisera and analyzed by PAGE on a 12.5% slab gel. Lanes: M, standard cell-free translation sample programmed with FMD virion RNA; RL, translation products from reticulocyte lysate; IV, immunoprecipitation with anti-inactivated virus; 2B, immunoprecipitation with anti-2B protein; 2C, immunoprecipitation with anti-2C protein.

region of L'/L was deleted and the encoding of the polyprotein initiated near the L-P1 junction (Fig. 1).

For the expression of FMDV cDNA clones, plasmid DNA was transcribed in vitro and the RNA transcripts thus obtained were used to direct translation reactions in a rabbit reticulocyte cell-free system. The in vitro translation products were analyzed by SDS-PAGE either directly or after being identified by immunoprecipitation.

Processing of the leader/P1 junction. Analysis of the translation products derived from clones pTLP1 and pTLP1A yielded a band at MW 16,000 that comigrated with the leader protein, L (Fig. 2). Similarly, the translation products derived from clones pTRP1 and pTRP1A also yielded the same products as the corresponding pTLP clones, except for the appearance of an additional minor protein band at MW 20,000 that corresponded to the alternate leader protein, L' (data not shown). These results indicate that L' and L are processed from the structural protein precursor, P1, in the absence of any P2 or P3 region proteins and support the conclusion of Strebel and Beck (45) that the leader gene product is a protease which catalyzes its own release from the nascent polyprotein.

Processing of the P1-2A/P2 junction. The translation products of two clones, each containing the P1 and P2 regions but either including or lacking L (pTLP112 and pTP12, respectively), were examined for processing. The labeled products derived from each clone were identified by immunoprecipitation of the lysates with antisera to inactivated virus (directed against all structural proteins) or to individual nonstructural proteins 2B and 2C. The leader-containing and leader-deleted transcripts yielded protein bands at MW 91,000, consisting of the P1-2A region (discussed below),

and MW 58,000, representing the P2 region plus the additional 20 amino acids of the vector (Fig. 3). A trace band at MW 149,000 corresponds to unprocessed P1-2A/P2. As expected, the expression of clone pTLP12 produced a leader protein, L, which was absent in the leader-deleted clone pTP12 (Fig. 3, lanes pTLP12-RL and pTP12-RL). The results demonstrate that the processing of the P1-2A/P2 junction is catalyzed by neither L nor 3C.

Processing by the 3C protease. To study the role of 3C protease in the proteolytic processing of the FMDV polyprotein, we selected clone pTLP123, which contains the coding capacity for the L, P1, P2, and P3 regions up to and including 20,000 daltons of the amino-terminal segment of the polymerase (3D). Linearization of this clone at the unique *SspI* site downstream from the cloned FMDV sequence yielded a polyprotein that has a functional 3C protease. However, linearization of this clone at the unique *EcoRV* site located in the coding region of the 3C protease (MW 22,993 [41]) made it possible to prepare a polyprotein that lacked the carboxy-terminal portion (MW 9,000) of 3C protease. On the basis of the conservation in FMDV 3C of cysteine and histidine residues that have been shown to be involved in the active site of poliovirus 3C (23), translation of RNA transcripts from *EcoRV*-linearized pTLP123 would yield a truncated 3C missing these residues and thus lacking proteolytic activity.

The translation products from clone pTLP123 linearized at the *SspI* site were processed into mature proteins indistinguishable from those produced in an FMDV RNA-directed translation, except that the full-length 3D and 3BCD complexes were not produced, owing to the truncation of 3D in this clone (Fig. 4A, lane RL). This analysis was confirmed by immunoprecipitation of this lysate with antisera against inactivated virus (lane IV) or against nonstructural proteins 2B, 2C, 3A, and 3C (Fig. 4A, lanes 2B to 3A, and data not shown).

The protein products of clone pTLP123 linearized at the *EcoRV* site, however, were processed into just three bands: the leader protein, P1-2A, and P2-P3 up to the truncated 3C (designated P2-P3') (Fig. 4B, lane RL). The interpretation of these results, confirmed by immunoprecipitation (lanes IV to 3C), is that the putative leader protease and the unidentified protease that cleaves the P1-2A/P2 junction are active but 3C is inactive. Polypeptide P1-2A was the only band immunoprecipitated with antisera against inactivated virus (Fig. 4B, lane IV), indicating that 3C is responsible for the processing of P1-2A into VP0, VP3, and VP1. Antisera against 2B, 2C, 3A, and 3C each precipitated only the fused P2-P3' precursor (Fig. 4B, lanes 2B to 3C), demonstrating that 3C is required for the processing of P2 and P3 into mature nonstructural proteins and for the cleavage of the P2-P3 junction.

It should be noted that in pTLP123 (*SspI*) lysates and in FMDV RNA-directed control lysates, the nonstructural proteins 2B, 2C, 3A, and 3C tended to participate in multiprotein complexes even after they had been proteolytically processed. This conclusion is based on the observation that antisera against 2B, 2C, and 3A all precipitated the same group of precursor and mature proteins from the lysate unless the lysates were denatured by treatment with SDS, dithiothreitol, and heat prior to reaction with antisera, as described in Materials and Methods. This denaturing treatment diminished precipitation of nonspecific mature proteins, retaining reactivity with the specific mature protein and their nonproteolytically processed precursors. (Lysates denatured prior to reaction with antisera are indicated with a plus sign in Fig. 4.) However, denaturation of the lysate

FIG. 4. Expression and processing of FMDV proteins in vitro. A cell-free translation system programmed with FMDV-specific RNA derived from in vitro transcription of pTLP123 DNA linearized with *SspI* (panel A) or *EcoRV* (panel B) and pTLP13C DNA (panel C) was immunoprecipitated with various antisera and analyzed by PAGE on a 12.5% slab gel. Lanes: M, standard cell-free translation sample programmed with FMD virion RNA; RL, translation products from reticulocyte lysate; IV, immunoprecipitation with anti-inactivated virus; 2B, immunoprecipitation with anti-2B protein; 2C, immunoprecipitation with anti-2C protein; 3A, immunoprecipitation with anti-3A protein; 3C, immunoprecipitation with anti-viral protease 3C; 1D, immunoprecipitation with anti-VP1. Lysates denatured prior to immunoprecipitation are denoted by a plus sign. Products unique to the cloned FMDV sequences are identified for panels B and C. Truncated proteins are indicated by a prime, as in 2B'-3BC. These products are explained in the text.

eliminated reactivity with anti-3C antibody, suggesting that its binding may require a native protein.

Clone pTLP13C, which contains coding capacity for L, P1-2A, the MW-11,000 amino-terminal sequence of 2B (designated 2B'), the carboxy terminus of 3B1 (MW 1,964), 3B2, 3B3, 3C, and the MW-20,000 amino-terminal sequence of the polymerase (designated 3D'), was used to assess the possible role of proteins 2B, 2C, and 3A in proteolytic processing. The [35 S]methionine-labeled translation lysate of pTLP13C was processed to L, mature VP0, VP3, VP1, and a number of other products (Fig. 4C, lanes RL and IV). In addition, VP1 (1D) antiserum was able to precipitate structural proteins VP1, VP0, and VP3 along with their precursors when the lysate was used as an antigen, but just VP1 and its precursors when the denatured lysate was used as antigen (Fig. 4C, lanes 1D and 1D +). This indicates the ability of the structural proteins derived from this clone to participate in protein complexes. Antisera against 2B precipitated two precursors: 2B'-3BCD' (MW 64,000) and 2B'-3BC (MW 42,000). The final processed 2B' fragment was too small to be resolved on this gel and migrated with the dye front. Antisera against 2C and 3A did not react with any products (data not shown), which was expected owing to deletion of

these coding areas. Protease 3C was precipitated by antisera against 3C (Fig. 4C, lane 3C), as were the precursors identified by 2B antiserum (see above). A precursor at an apparent MW of 25,000 was precipitated by 3C, but not by 2B antiserum; it may represent 3BC.

On the basis of the results with clone pTLP13C, it is possible to conclude that the carboxy end of 2B and all of 2C and 3A are not responsible for any of the processing of the P1 region, because this processing occurs in the absence of these gene products as long as full-length 3C is present.

The leader-deleted clone pTLP123, as predicted, lacked the leader protein band at MW 16,000. This clone was not as efficiently translated and processed, but was otherwise identical to pTLP123 (data not shown), indicating that the leader protein was not involved in the processing of the P1, P2, and P3 precursors into mature products.

Processing of pTLP123 (*EcoRV*) products by proteases from an FMDV RNA-directed lysate. As described above, clone pTLP123 linearized at the *EcoRV* site lacked the carboxy half of the 3C protease and hence the ability to process the precursor products derived from this clone. Therefore an assay was developed to demonstrate that the products of this clone can be used as a substrate by the viral protease, i.e.,

FIG. 5. Processing of pTLP123 (*EcoRV*) products by proteases from an FMDV RNA-directed lysate. [35 S]methionine-labeled products of pTLP123 (*EcoRV*) were prepared as described in Materials and Methods. After 2 h, 200 μ M sparsomycin was added to inhibit protein synthesis. An unlabeled FMDV lysate was prepared by translation of FMD virion RNA for 2 h in the presence of 2 mM methionine, while a mock lysate was prepared in the absence of virion RNA. One volume of the labeled pTLP123 (*EcoRV*) was mixed with an equal volume of unlabeled FMDV lysate or mock lysate and incubated at room temperature for 20 h. Samples were analyzed by PAGE on a 12.5% slab gel. Lanes: M, purified FMD virion RNA-directed translation; 1, pTLP123 (*EcoRV*) labeled lysate, translation terminated at 2 h and kept for 20 h at room temperature with no addition of unlabeled lysate; 2, pTLP123 (*EcoRV*) labeled lysate mixed with unlabeled mock lysate; 3, pTLP123 (*EcoRV*) labeled lysate mixed with unlabeled FMDV lysate; 4, pTLP1 labeled lysate mixed with unlabeled FMDV lysate; 5, pTLP1 labeled lysate, translation terminated at 2 h and kept for 20 h at room temperature with no addition of unlabeled lysate. Lanes 3, 4, and 5 were autoradiographed three times as long as lanes 1, 2, and M.

that they have not lost the ability to be processed as a result of improper folding due to truncation of the polyprotein. FMDV RNA was translated in the presence of nonradiolabeled methionine for 2 h to allow synthesis of viral proteases. Simultaneously, a [35 S]methionine-labeled translation of pTLP123 (*EcoRV*) was carried out for 2 h, and protein elongation was then terminated by the addition of 200 μ M sparsomycin. The pTLP123 (*EcoRV*) lysate was mixed with equal volumes of either the unlabeled FMDV lysate or a mock lysate translated in the absence of FMDV RNA. The mock lysate served as a control for the possibility that a protease contained in the reticulocyte lysate was responsible for processing. Sparsomycin was added to prevent synthesis of radiolabeled products from FMDV RNA during mixing of the lysates. After being mixed, the lysates were kept at room temperature for 20 h and then analyzed by SDS-PAGE.

The result of a mixing experiment is shown in Fig. 5. The [35 S]methionine-labeled lysate of clone pTLP123 (*EcoRV*) contained leader, P1-2A, and P2-P3' bands (lane 1). The

addition of unlabeled FMDV proteins resulted in the further processing of pTLP123 (*EcoRV*) precursor P1-2A into mature VP0, VP3, and VP1 and of P2-P3' precursor into P2, 2B, and 2C (lane 3). Polypeptides 3A and 3C' (carboxy terminus deleted) could not be identified in the lysate. When mock lysate was used in place of the FMDV lysate, no further processing of pTLP123 (*EcoRV*) product occurred (lane 2). Moreover, the addition of FMDV-infected cell lysates, but not control lysates, resulted in similar processing (data not shown). Therefore the specific proteins encoded by FMDV were necessary and sufficient to achieve processing of pTLP123 (*EcoRV*)-derived protein precursors, indicating the suitability of these precursors to serve as substrates for proteolysis despite the artificial truncation of the polyprotein encoded by this clone.

A labeled translation lysate of pTLP1 was also examined for processing by an unlabeled FMDV RNA-directed lysate. The addition of unlabeled FMDV proteins resulted in further processing of the P1' precursor into VP0, VP3, and VP1' (Fig. 5, lane 4). The truncated VP1' migrated slightly faster than full-length VP1 on SDS-PAGE, as expected. These results indicate that a full-length P1-2A precursor is not necessary to serve as a substrate for processing. It was recently shown that cloned P1' precursor of poliovirus lacking VP1 is not processed by exogenous protease (34a).

Mapping of the 2A region. Unlike other picornaviruses, FMDV has a very short 2A protein. From the nucleotide sequence and microsequencing of the VP1 carboxy terminus and the amino terminus of 2B, it was discovered that 16 amino acids occupy the region between these two proteins

FIG. 6. Immunoprecipitation of cell-free translation products directed by synthetic RNA transcripts from FMDV clones by anti-2A peptide antiserum. Cell-free translations and immunoprecipitations were carried out as described in Materials and Methods. Samples were analyzed by PAGE on a 12.5% slab gel. Lanes: 1, pTLP1; 2, pTRP12; 3, purified FMD virion RNA; 4, pTLP123; 5, pTLP13C; 6, pTLP12; M, lysate directed by purified FMD virion RNA.

(6, 41). The fate of this oligopeptide during proteolytic processing has not been studied previously.

Antiserum was prepared against a synthetic peptide corresponding to the 10 amino acids at the amino terminus of 2A. The antiserum, which was reactive with the eliciting peptide in a radioimmunoassay (data not shown), was used to immunoprecipitate the labeled proteins derived from various clones. The clone pTLP1, which lacks the 2A region (and also lacks the terminal 16 amino acids of VP1), was used as a control to demonstrate that the antiserum does not recognize possible cross-reacting sequences in the P1 region (Fig. 6, lane 1). In the lysates resulting from each of the longer clones, a band was precipitated at MW 91,000, corresponding to P1-2A (lanes 2 to 6). This indicates that in FMDV, 2A remains associated with the structural protein precursor during primary processing.

Microsequencing of the carboxy terminus of the MW-91,000 protein band was not performed; therefore it is not known whether all 16 amino acids of 2A remain associated with P1. We have not excluded the possibility that an immunoreactive piece of 2A is processed with P1 and that some portion of 2A, not detected by this antiserum, remains associated with P2.

In the lysates of clones containing an active 3C protease, a band at MW 28,000 is sometimes observed just above the structural proteins VP3 and VP1. This band reacts with both antisera against 2A and VP1 (Fig. 6, lane 5, and Fig. 4C, lane 1D) and presumably represents VP1-2A prior to proteolytic removal of 2A to yield mature VP1. This result is consistent with the mapping of 2A at the carboxy terminus of the P1-2A precursor, where during secondary processing of the P1-2A precursor by 3C protease, the VP3-VP1 cut is made prior to the VP1-2A cut.

DISCUSSION

Picornavirus replication is dependent upon proper proteolytic processing of a polyprotein to yield mature structural and nonstructural proteins. Recent experiments with various members of this family have revealed important differences in the processing strategies used by different viruses.

In poliovirus, the prototype for picornaviruses, the polyprotein is cleaved at three types of sites: glutamine-glycine, tyrosine-glycine, and asparagine-serine (35). Poliovirus contains 12 utilized cleavage sites, 9 of which are of the glutamine-glycine type (27, 35). The poliovirus-encoded protease 3C cuts the polyprotein at these glutamine-glycine sites (18). Antibodies against 3C inhibit cleavage at glutamine-glycine sites but not at the two utilized tyrosine-glycine sites, which occur at 1D-2A and within the 3D protein. This observation led to the identification of a second protease, 2A, which processes the tyrosine-glycine site within 3D and may also cleave the 1D-2A junction (47). The asparagine-serine site is utilized only once, at 1A-1B (the processing of VP0 into VP4 and VP2), and this cleavage occurs during final assembly of the virus (26). It has been suggested for another picornavirus, human rhinovirus 14, that this cleavage is catalyzed by nucleophilic attack of the Ser-10 β -hydroxyl of VP2, resulting in a reaction similar to that of a serine protease. Because there is no base analogous to the histidine of the serine proteases, it has been proposed that the base is provided by the entry of the viral RNA into the empty capsid (1, 42).

On the basis of nucleotide and protein sequences, FMDV would be expected to differ significantly from poliovirus in its processing. For FMDV, at least 13 cleavage sites are

utilized to produce the structural and nonstructural proteins. Many of the proteolytic processing sites on the FMDV polyprotein show no similarity to the analogous regions in poliovirus. Another important difference exists for the 2A protein (MW 34,000), which has been shown to have proteolytic activity for tyrosine-glycine sites in poliovirus, whereas 2A of FMDV is a 16-amino-acid oligopeptide and is unlikely to be a protease, because of its small size and because tyrosine-glycine sites are not utilized in processing. However, FMDV, in contrast to poliovirus, encodes leader proteins which have been implicated in processing the leader-P1 site (45). Because of these differences between poliovirus and FMDV, it was necessary to study the role of the two putative proteases of FMDV, 3C and L, in the processing of the FMDV polyprotein.

The results of this study show that four different types of proteolytic processing occur in FMDV. The putative leader protease, L, is required for cleavage at the leader-P1 junction (45) (Fig. 2). Leader and alternate leader proteases are not needed for any of the other cleavages to generate mature FMDV proteins, as demonstrated by comparison of the leader-deleted clones (pTP12 and pTP123) with leader-containing clones (pTLP12, pTLP123, pTRP12, and pTRP123).

The second type of cleavage in FMDV is that catalyzed by 3C protease. The specificity of FMDV 3C has been determined to be far less stringent than that of poliovirus, which utilizes only glutamine-glycine bonds. All of the processing of FMDV proteins from the nonstructural P2 and P3 regions and between these regions has been attributed to 3C. It was not possible from this study to definitively assign the cleavages at the three VPg and 3C-3D sites. However, the glutamic acid-glycine bonds that are present at these sites also exist at the 3A-3B junction which is cleaved by 3C; therefore it is most probable that all processing of the P2 and P3 regions is catalyzed by 3C protease. The processing within the P1 region is catalyzed predominantly by 3C, the sole exception being the third type of cleavage, which occurs at VP4-VP2 (1A-1B) during viral assembly. The mechanism of this cleavage may be similar to that proposed for HRV-14 (see above), but because FMDV lacks the VP2 Ser-10 present in HRV-14, the agent of the nucleophilic attack remains to be elucidated.

The fourth and final type of cleavage in FMDV occurs at P1-2A/P2. Data presented here indicate that on initial processing, some, and possibly all, of the 2A oligopeptide is associated with the P1 region. This situation is different from that of poliovirus, in which processing occurs at P1-P2 and 2A remains associated with P2 after initial processing (35). Processing of FMDV at this site is more similar to that of EMC virus, which encodes 2A protein that remains associated with the P1 region after initial processing at P1-2A/2B (39). The protease responsible for cleaving P1-2A/2B in FMDV has not been identified, but neither L nor 3C is involved, because this cleavage occurs in clone pTP12, which lacks both proteases. The P1-2A/2B site is processed in clone pTLP13C, which lacks the carboxy terminus of 2B and all of 2C and 3A, ruling out the involvement of those deleted proteins in the processing. The results support the hypothesis that a host protease cleaves this site (4); however, we cannot exclude the possibility that the P1-2A/2B site is cleaved by an unidentified proteolytic activity associated with P1-2A or with the amino-terminal 11,000 daltons of 2B.

Despite the similarity of FMDV and EMC virus in the initial processing of 2A at the carboxy terminus of the P1-2A region, differences in processing do exist between the vi-

FIG. 7. Biochemical map of the FMDV genome. Viral RNA, encoded precursor proteins, and their mature proteins are schematically presented. The major and alternate translation initiation sites are indicated by solid and broken arrows, respectively. VPg is the viral protein covalently linked to the 5' end of the viral RNA.

ruses. Unlike FMDV, the leader-P1 junction in EMC virus is catalyzed by 3C and not by L (38). In EMC virus, the primary cleavage between 2A and 2B occurs at a glutamine-glycine pair (36), the site of cleavage commonly catalyzed by 3C protease. However, on the basis of recent experiments involving the use of synchronized in vitro translations of EMC virus RNA, the bond at 2A-2B is cleaved shortly after synthesis, prior to the synthesis of any detectable 3C or precursors of 3C (24). The protease responsible for 2A-2B processing in EMC virus therefore may be a protease different from 3C (24). The protease responsible for processing the tyrosine-proline site at 1D-2A in EMC virus (36) also remains unidentified, but it has been suggested that 3C is involved (24). If this turns out to be the case, then FMDV will again bear a closer similarity to EMC virus than to poliovirus in regard to proteolytic processing.

It is necessary to note that the activities that have been attributed to 3C are based in part on work with clone pTLP123 linearized at the *EcoRV* site. The RNA produced from these clones is deficient not only in the carboxy-terminal 9,000 daltons of 3C but also the amino-terminal 20,000 daltons of 3D that is present in clones linearized at the *SspI* site. Therefore it is possible that the amino terminus of 3D is responsible for the processing of sites we have attributed to 3C. This is highly unlikely, because 3D is associated with an RNA-dependent RNA polymerase activity (14, 40), whereas 3C has been shown to be a protease (29).

On the basis of the above results, a biochemical map of the FMDV genome is presented (Fig. 7), showing viral RNA, its encoded precursor proteins, and the processed mature proteins.

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