Processing and Assembly of Foot-and-Mouth Disease Virus Proteins Using Subgenomic RNA

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(Accepted 2 June 1988)

SUMMARY

Recombinant DNA clones were constructed in order to study the mechanisms of proteolytic processing and assembly in foot-and-mouth disease virus (FMDV). RNA transcripts from these clones were synthesized using SP6 polymerase and translated in rabbit reticulocyte lysates. Efficient translation occurred in the absence of all 5' untranslated sequences and processing of the structural proteins occurred in the presence of functional 3C protease which can function in trans. The specificity of 3C protease activity is not limited to Glu-Gly bonds. Translation of correctly processed structural proteins leads to assembly of subviral structures resembling 'empty' particles. Further studies on the processing of the FMDV genome show that the primary cleavage (P1-P2) is mediated neither by 3C nor the second FMDV protease L. Preliminary evidence suggests that an initial very rapid cleavage occurs between 2A and 2B with subsequent cleavage of the P1/2A junction probably being carried out by 3C.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a member of the family Picornaviridae (genus aphthovirus), containing a single-stranded positive sense RNA genome which is translated to yield a single polyprotein. This is then cleaved by proteases to yield the final mature proteins found in infected cells. The first cleavages occur while the nascent polyprotein is still being translated. The mechanism of proteolysis was originally thought to involve host proteases but recent work with poliovirus (PV) has established that all cleavage activities can be accounted for by virus-coded proteins (for review see Nicklin et al., 1986). Complete proteolysis requires the activity of three proteases: 3C which cleaves at Gln-Gly sites (Hanecak et al., 1982), 2A which cleaves at Tyr-Gly sites (Toyoda et al., 1986) and an autocatalytic activity which cleaves the structural protein VP0 into VP2 and VP4 in the final stage of virus maturation (see Nicklin et al., 1986). Resolving the proteolytic pathways of poliovirus has been considerably helped by the ability to produce infectious virus from cloned full-length genomic cDNA (Racaniello & Baltimore, 1981: Kaplan et al., 1985; van der Werf et al., 1986). Recently, however, more information has been gained by transcribing specific regions of the genome into functional mRNAs using SP6- or T7-based systems and analysing the products by in vitro translation (Ypma-Wong & Semler, 1987a, Nicklin et al., 1987).

Parallel studies on the cardioviruses and aphthoviruses have been complicated in several respects. First, the genomic organization is slightly different from PV in that an additional protein, L, is encoded at the N terminus of the polyprotein. This protein has been shown to possess autocatalytic protease activity in FMDV (Strebel & Beck, 1986). Furthermore, although FMDV does produce a protease corresponding to 3C of PV (Klump et al., 1984) the region of the genome corresponding to 2A is very small and can code only for a 16 amino acid polypeptide (Carroll et al., 1984). Second, whereas for PV and encephalomyocarditis virus (EMCV) the actual proteolytic cleavage sites are fairly well conserved there appears to be little homology in the analogous FMDV sites which has led to the suggestion that cellular and other viral proteases
may be involved. The presence of a poly(C) tract within the genome has so far prevented the
construction of an infectious DNA clone and, consequently, it has been impossible to study
FMDV processing by this route.

Recently Vakharia et al. (1987) have reported a study on the A_{12} serotype of FMDV which,
using the T7-based in vitro system, has helped to clarify some of the proteolytic cleavage events.
In this paper we confirm their findings using another subtype of the virus, A_{10}, and extend the
analysis by examining the specificity of the 3C protease, the possible proteolytic activity of 2A,
the ability of 3C to function in trans and the effect of substrate conformation on proteolysis.
Furthermore we have been able to use the products of in vitro translation to study the mechanism
of morphogenesis in FMDV. This is an area that has been studied extensively in picornaviruses
but is still not totally understood.

Particles can be found in infected cells with sedimentation coefficients of 5S, 12S, 75S and
125S and it is generally considered that these are morphogenetic intermediates of FMDV
(Grubman et al., 1985; Boege et al., 1986). In vitro translation systems programmed with
 genomic RNA also produce subviral particles (Grubman, 1984) thus making it possible to study
morphogenesis in vitro.

In this paper we report the use of subgenomic RNA species prepared using SP6-based
recombinant plasmids to investigate in vitro assembly of subviral particles. The implications of
our findings in terms of vaccine development and viral morphogenesis will be discussed.

METHODOLOGY

Construction of recombinant DNA subclones of FMDV serotype A_{10}. Recombinant plasmids were constructed
using standard cloning procedures (Maniatis et al., 1982). The strategy for producing the clones used in this study
is outlined in Fig. 1. Initially a 3-05 kb EcoRI–XhoI fragment from recombinant plasmid pFA76. p16 was purified
from plasmid sequences by gel electrophoresis and subcloned into the transcription vector pSP65 using the
EcoRI–SalI sites. The FMDV sequence coded by this fragment is derived from serotype A_{10} and extends from the
EcoRI site just downstream of the leader protein sequence (Lb) (Carroll et al., 1984) through the
coding sequence of Lb, P1, 2A and terminates at a XhoI site located 285 nucleotides into the 2B region. This clone,
 pBC6, was extended to include the region coding for protease 3C by digesting with XhoI and HindIII and adding
by ligation a corresponding fragment derived from clone pFA206 (Boothroyd et al., 1981). This construct does not
contain the portion of the genome coding for the C terminus of 2B, 3A or two of the three 3B (VPg) proteins but the
translational reading frame is preserved so that one VPg protein as well as 3C and the N terminus of 3D should also
be expressed. However, in order for these transcripts to be expressed, translational initiation codons must be
present. Initially a 270 bp EcoRI fragment was isolated from a shotgun cDNA clone S_{Lb} A_{10} (A_{12} serotype).
This fragment was then cloned into EcoRI-digested and dephosphorylated pBC7. The orientation of the fragment in
the resultant clone, pRA6, was checked by primer extension sequencing.

A further series of experiments was carried out to remove the leader proteins in an attempt to initiate translation
within the structural region. These constructions made use of the presence of a Neo site at an in-phase AUG
within the 1B (VP2) protein. pRA6 was digested with NeoI and EcoRI, sticky ends were then filled in with DNA
polymerase (Klenow fragment) and religated. The resulting clone, pRA6D1, therefore codes for the same proteins
as pRA6 except for the absence of Lb, 1A (VP4) and a truncated 1B (VP2). The 5′ (N) and 3′ (C) sequences of the
clones used in this study are indicated in Fig. 2.

Synthesis of FMDV subgenomic RNA transcripts from recombinant clones. Production of RNA transcripts was
especially as described by Melton et al. (1984). Plasmid DNA (10 μg) was linearized with specific restriction
enzymes as indicated in Results, extracted with phenol/chloroform and ethanol-precipitated. In vitro transcription
was then carried out using 45 units of SP6 RNA polymerase (Promega Biotech, Madison, Wis., U.S.A.) for 2 h at
37 °C in the presence of 40 mm-Tris–HCl pH 7-5, 6 mm-MgCl2, 2 mm-spermidine, 0-5 mm-rNTPs, 10 mm-
dithiothreitol and 5 units of RNasin (Promega Biotech). After transcription, template DNA was removed by incubation
with 10 units of RNase-free DNase (RQ1 DNase; Promega Biotech) for 15 min at 37 °C. Transcripts were then
phenol/chloroform-extracted, ethanol-precipitated and stored at −70 °C.

was purchased from Amersham. RNA (1 μg) was added to 10 μl of lysate containing [35S]methionine (10 μCi) and
the mixture was incubated at 30 °C for the required length of time. Samples were treated with 10 μg of RNase A
before polyacrylamide gel electrophoresis by standard methods (Laemmli, 1970). After electrophoresis gels were
treated with Amplify (Amersham), dried and exposed to X-ray film.

Preparation of cytoplasmic extracts. Monolayers of BHK-21 cells in 4 oz (approx. 120 ml) bottles were infected
with virus at a high m.o.i. (>100 p.f.u./cell). At 2-5 h post-infection (p.i.), the cells were washed in cold phosphate-
buffered saline and 10 ml of 20 mM-Tris–HCl pH 7.6 was added. The cell monolayers were detached from the glass by gentle rocking and cytoplasmic extracts were prepared by Dounce homogenization in 2 ml of 20 mM-Tris–HCl pH 7.6. Cell debris was removed by centrifugation at 2000 g for 2 min and the supernatant (50 µl) was added to a translation mixture which had been incubated for 2 h at 30 °C. Incubation was then continued for a further 2 h at the same temperature and the products were analysed by PAGE. Extracts of uninfected cells were treated similarly.

Viruses used in this study were A10 (61), O1 Kaufbeuren and SAT3 Bec 1/65.

Detection of subviral particles following in vitro translation of subviral RNA. RNA (1 µg) was translated for 5 h in 10 µl of rabbit reticulocyte lysate and diluted to 500 µl with 0.1 M-NaCl, 0.1 mM-Tris–HCl pH 7.6 containing 1%
Fig. 2. (a) Sequence arrangement at the 5' end of pRA6 and pRA6δL. Initiator methionine codons are underlined and the number in brackets indicates the amino acid number within VP2 at which this codon occurs. The point at which transcriptional initiation is thought to occur is indicated by *. (b) Sequence arrangement at the 3' end of both clones used in this study. Horizontal lines indicate the length of transcripts when linearized with the specific enzymes indicated and the status of P3C activity in each case is indicated. The location of the histidine residue implicated in the active site of the protease is indicated by *.
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NP40. Subviral particles were separated by centrifugation for 2 h at 150000 g in a 15 to 45% linear sucrose gradient containing 0.1 M-NaCl, 0.1 M-Tris-HCl pH 7.6. After centrifugation the gradient was fractionated and 10 μl aliquots were measured directly for radioactivity using liquid scintillation.

RESULTS

In vitro translation of RNA transcripts from subgenomic FMDV clones

FMDV RNA is translated well in rabbit reticulocyte lysates and after prolonged incubation proteolytic processing of the precursor proteins into the expected mature viral proteins can be seen (Fig. 3, lanes 5 and 6). Similarly in vitro translation of FMDV RNA lacking the untranslated region to the 5' side of the poly(C) tract yields the same spectrum of proteins as does full-length RNA (Sangar et al., 1980). We therefore investigated the effects of eliminating all of the 5' non-coding sequence on translation and protein processing in vitro.

Recombinant DNA clones were constructed in which FMDV serotype A10 sequences were placed under the transcriptional control of the SP6 polymerase promoter. The two clones used in this study are indicated in Fig. 1 in which their alignment with the FMDV genome is also shown. Due to the convenience of construction each clone possesses a deletion in the middle portion of the genome but translational reading frames are preserved. pRA6 encodes the smaller leader protein (Lb), the entire structural precursor P1, 2A, truncated 2B, one VPg protein, the 3C protease and terminates after 19 amino acids of 3D. Translation of RNA from pRA6 (linearized by HindIII) for 40 min (Fig. 3, lane 2) produces three major proteins corresponding to

![Fig. 3. RNA transcripts from pRA6 and pRA60L linearized with specific restriction enzymes were translated in rabbit reticulocyte lysates and the products analysed on a 12.5% polyacrylamide gel. Lanes 1 and 2, pRA6 (HindIII); lanes 3 and 4, pRA6 (PstI); lanes 5 and 6, FMDV RNA; lanes 7 and 8, pRA60L (HindIII) translated for 4 h or 40 min respectively. The positions of the major translation products are indicated.](image-url)
P1, Lb and a spurious protein derived from the engineered part of the genome. The rapid appearance of Lb confirms the very rapid autocatalytic cleavage of Lb from the structural precursor. Furthermore the concomitant appearance of authentic P1 suggests a rapid processing event at the C terminus. When translation of this RNA was allowed to continue for 4 h (Fig. 3, lane 1) substantial processing of the structural precursor occurs and processed products representing 1CD (VP1 and VP3), 1AB (VP0), 1C (VP3) and 1D (VP1) are clearly visible.

It is well documented for PV and EMCV that protein 3C is responsible for cleavage of the structural precursor. This protease has been characterized as a cysteine protease (Argos et al., 1984) and by sequence comparison of several different picornaviruses two conserved amino acids have been implicated as reactive residues in the active site (Werner et al., 1986). Furthermore these amino acids have been shown to be directly involved in the active site of 3C from PV (Ivanoff et al., 1986). Sequence analysis of the 3C–3D junction (Fig. 2) reveals the presence of three unique restriction sites one of which (PstI) is immediately adjacent to one of these residues (His 182). We therefore examined the possibility that RNA transcripts derived from PstI-digested pRA6 might be protease-negative. Translation of this RNA for 40 min or 4 h is shown in Fig. 3 (lanes 4 and 3 respectively) and as before short periods of translation resulted in Lb, P1 and the hybrid protein. However prolonged incubation did not result in further processing of P1 into the mature structural proteins. One new protein was observed after extended incubation (lane 3) but this has been recently identified as a modified version of Lb (Sangar et al., 1988) and is also observed in the protease-positive translation (lane 1). Therefore we can conclude that removal of the C terminus of 3C adjacent to His 182 inactivates the protease and is further indirect evidence that this residue is involved in the catalytic function. These data also allow us to assign definitely the cleavages between 1AB (VP0)/1C (VP3) and 1C (VP3)/1D (VP1) of FMDV to 3C protease. Subsequent experiments using SalI-linearized pRA6 indicated that the absence of eight amino acids from the C terminus of 3C did not inhibit its activity although the rate of cleavage was lower (not shown).

**FMDV 3C protease can function in trans and its activity is dependent on substrate conformation**

Since Lb has been shown to catalyse the L/P1 cleavage we investigated its potential role elsewhere in the proteolytic pathway. We therefore constructed a deletion derivative of pRA6 in which the coding regions for Lb, 1A (VP4) and 125 amino acids of 1B (VP2) were missing, with translation initiation occurring at a natural methionine residue in VP2 (see Methods). Translation of this RNA (pRA6ΔL HindIII) proceeded as efficiently as the parent construction and the expected truncated P1 protein was the major protein observed after 40 min (Fig. 3, lane 8). However, despite the presence of the entire 3C region prolonged incubation failed to result in processing of the truncated P1 protein. Nevertheless, the fact that the truncated P1 protein can be observed is evidence that the P1/P2 cleavage does occur in the absence of the Lb protease. Also, the observation of this cleavage occurring in protease-negative pRA6 translations precludes the involvement of 3C in this cleavage.

The failure of pRA6ΔL to process the truncated P1 protein may have been due to two reasons. First, the 3C protease itself may require the presence of Lb for activation or, second, the truncated P1 may adopt an unsuitable conformation for proteolysis. In order to address these questions RNA from pRA6ΔL (HindIII) and pRA6 (PstI; i.e. protease-negative) were translated alone and as a mixture. As can be seen in Fig. 4 (lane 3) an active 3C protease was clearly present in the mixed sample since significant amounts of 1C (VP3) and 1D (VP1) were detectable. Furthermore, the fact that the truncated P1 protein (ΔP1) is not processed clearly favours the unsuitable template hypothesis. This result does not totally exclude the possibility that Lb may be releasing active 3C from pRA6ΔL but previous work in *Escherichia coli* (Klump et al., 1984) indicates that this is unlikely. However, this experiment clearly shows that FMDV 3C protease can function *in trans*.

**Studies on the cleavage specificity of FMDV 3C protease**

The 3C protease from PV has been shown to cleave exclusively between Gln and Gly residues (Hanecak et al., 1982). However cleavage does not occur between every Gln–Gly pair in the
polyprotein, suggesting that some other element is involved in recognition. Similarly, for EMCV 3C cleavage normally occurs between Gln/Gly or Gln/Ser amino acid pairs which are usually flanked by Pro residues (Palmenberg et al., 1984). With FMDV, on the other hand, little homology is apparent around the known cleavage sites although the cleavages carried out by 3C indicate a preference for Glu/Gly bonds. Amino acid sequence analysis of the known cleavage sites in the structural region from four different strains of the virus are shown in Table 1. Apart from the maturation cleavage of 1A/1B (VP4/VP2) all of the cleavages are thought to be made by 3C and clearly there is a significant variation, particularly at the P1/P2 junction. The ability of 3C to function in trans in our assay allowed us to assess whether FMDV 3C from other strains of FMDV could cleave the structural precursor P1 from serotype A_{10}. Unprocessed substrate P1
Fig. 5. RNA transcripts derived from pRA6 (PstI) were translated in rabbit reticulocyte lysates under various conditions and analysed by 12.5% PAGE. Lane 1, translation for 2 h; lane 2, translation for 4 h; lane 3, translation for 2 h and then incubation for 2 h with uninfected cell extract; lane 4, translation for 2 h and then incubation for 2 h with extract from A10-infected cells; lane 5, translation for 2 h and then incubation for 2 h with extract from O1-infected cells; lane 6, translation for 2 h and then incubation for 2 h with extract from SAT3-infected cells.

Table 1. Proteolytic cleavage sites within the structural proteins of various FMDV serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>VP4/VP2</th>
<th>VP2/VP3</th>
<th>VP3/VP1</th>
<th>VP1/P2A</th>
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<tbody>
<tr>
<td>A10</td>
<td>Ala/Asp</td>
<td>Glu/Gly</td>
<td>Gln/Thr</td>
<td>Leu/Asn</td>
</tr>
<tr>
<td>C1</td>
<td>Ala/Asp</td>
<td>Glu/Gly</td>
<td>Gln/Thr</td>
<td>Leu/Asn</td>
</tr>
<tr>
<td>O1</td>
<td>Ala/Asp</td>
<td>Glu/Gly</td>
<td>Gln/Thr</td>
<td>Leu/Asn</td>
</tr>
<tr>
<td>SAT3</td>
<td>Ala/Asp</td>
<td>Gln/Gly</td>
<td>Gln/Thr</td>
<td>Cys/Asn</td>
</tr>
</tbody>
</table>

was produced by translation of pRA6 (PstI) as before and mixed with either uninfected cell extract or extracts from cells infected with other strains. The results of this experiment are shown in Fig. 5. Clearly there is no processing of P1 in any of the control samples (lanes 1 to 3) but in the presence of extracts from A10, O1 or SAT3 the structural precursor is efficiently processed. This suggests that the proteases from these viruses have a fairly non-specific sequence requirement in terms of the actual peptide bond cleaved and may require a more complex recognition signal.

In vitro assembly of subviral particles derived from subgenomic RNA

In vitro assembly of subviral particles of picornaviruses is well documented particularly, for FMDV, by Grubman et al. (1985). We therefore examined the ability of correctly processed
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Fig. 6. Sucrose gradient sedimentation profile of protein products from pRA6 (HindIII). RNA transcripts were translated for 5 h in the presence of [35S]methionine and products were analysed by sucrose gradient centrifugation (a). The shoulder of material sedimenting at 60S to 75S, i.e. similar to 'empty' particles, was removed from the gradient and recentrifuged under similar conditions (b).

Fig. 7. PAGE (12.5%) of material isolated from the 75S peak shown in Fig. 6(b) (lane 1). Lane 2 shows the presence of other viral proteins in a fraction from the top of the gradient.

structural proteins, derived from subgenomic RNA, to assemble into subviral particles in the absence of other viral proteins and the complete genome. RNA from pRA6 (HindIII) was translated in vitro for 5 h and the products were analysed by sucrose gradient centrifugation. FMDV 'empty' particles have a sedimentation coefficient of 75S and a shoulder of labelled material was seen at that position in the gradient (Fig. 6a). When this material was recycled on a second sucrose gradient a peak at 75S was still evident (Fig. 6b). When analysed by PAGE this 75S material showed the expected profile of empty particles, i.e. 1C, 1D and 1AB (Fig. 7). These
particles also contained significant amounts of 1CD which has not been observed previously in subviral particles of FMDV. Clearly a population of subviral particles resembling ‘empty’ particles can be assembled in vitro using this system. However only 10% of the radioactivity sedimenting at 75S in the first gradient was found in the 75S peak in the second gradient, suggesting that these particles were unstable. This instability may be a consequence of the presence of unprocessed 1CD (see Discussion).

DISCUSSION

Despite being members of the same family it is becoming clear that there are significant differences between the proteolytic mechanisms of picornaviruses. In PV all cleavages can be assigned to proteolytic activity present in 3C (or its precursors) or 2A, apart from the maturation cleavage of VP0 which is believed to be an autocatalytic reaction associated with virus maturation (Hogle et al., 1984; also Rossmann et al., 1985 for human rhinovirus). The majority of cleavages are carried out between Gln/Gly pairs by 3C but the cleavages catalysed by 2A are associated with Tyr/Gly pairs and include the autocatalytic cleavage of P1/P2 (Toyoda et al., 1986; Nicklin et al., 1987). Thus the bond between P1 and 2A is cleaved very rapidly by 2A itself, with 3C then releasing 2A from the remainder of the polyprotein by cleaving between 2A and 2B. In EMCV the situation is reversed because the cleavage between 2A and 2B is very rapid with 3C finally releasing 2A from the structural proteins by cleavage at the N terminus of 2A (Jackson, 1986). Furthermore, preliminary evidence (A. C. Palmenberg, personal communication) suggests that the 2A/2B cleavage of EMCV is the result of autocatalytic cleavage by 2A at its C terminus. In contrast 2A of FMDV is an oligopeptide only 16 amino acids long (see Table 2) which has been considered to be a vestigial sequence and has been largely ignored.

EMCV and FMDV also differ from PV in the possession of a leader protein which, for FMDV, has been shown to possess proteolytic properties and to cleave at the L/P1 junction. In this respect EMCV and FMDV differ because this cleavage is carried out by 3C in EMCV (Parks et al., 1986). It has been suggested that the L proteins of FMDV may substitute for the lack of a functional 2A. However the data presented here, together with those recently reported by Vakharia et al. (1987) have discredited this suggestion. Vakharia et al. (1987) showed that the primary cleavage P1/P2 for FMDV resembles EMCV in that it occurs between 2A/2B and not 1D/2A. Our data show that this cleavage occurs in the absence of either functional Lb or 3C. From the structure of the deletion mutant pRA66L the activity must be associated with the remaining structural proteins (1C or 1D), the single VPg, the oligopeptide 2A or a host protein present in the lysate. We would speculate that the most obvious candidate must be 2A itself for two reasons. First, 2A sequences from several different serotypes of FMDV have now been determined (Table 2) including the most evolutionarily distant South African Territory strains and show that 2A is the most highly conserved protein in the genome. This implies that the sequence does have some crucial role in replication since a truly vestigial protein would be expected to show considerable variability. Second, since the mechanism of P1/P2 cleavage resembles that of EMCV we have compared the amino acid sequences of 2A and EMCV and discovered a 16 amino acid motif within the EMCV 2A which is homologous to FMDV (Table 2). Furthermore, preliminary evidence for EMCV (A. Palmenberg, personal communication)

<table>
<thead>
<tr>
<th>Serotype</th>
<th>PID (VP1)</th>
<th>P2A</th>
<th>P2B</th>
</tr>
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<tbody>
<tr>
<td>A (_{12})</td>
<td>LL</td>
<td>NFDLLKLAGDVESNPG</td>
<td>PFFFDADV</td>
</tr>
<tr>
<td>A (_{10})</td>
<td>LL</td>
<td>NFDLKLAGDVESNLG</td>
<td>PFFFDADV</td>
</tr>
<tr>
<td>C (_{1})</td>
<td>LL</td>
<td>NFDLKLAGDVEPNG</td>
<td>PFFFDADV</td>
</tr>
<tr>
<td>SAT (_{3})</td>
<td>MC</td>
<td>NFDLKLAGDVEPNG</td>
<td>PFFFDADV</td>
</tr>
<tr>
<td>O (_{1})</td>
<td>TL</td>
<td>NFDLKLAGDVEPNG</td>
<td>PFFFDADV</td>
</tr>
<tr>
<td>EMCV</td>
<td></td>
<td>FADLL - I H - D1ETNPG</td>
<td></td>
</tr>
<tr>
<td>2A (119-132)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
indicates that it is possible to delete sequences from the N or C termini of 2A and still retain activity in vitro. It will be extremely interesting to see whether this 16 amino acid motif is the catalytic unit of EMCV 2A. Similarly deletion and site-directed mutagenesis of FMDV 2A should establish its true role in the 2A/2B cleavage event.

The N-terminal cleavage of 2A, i.e. P1/2A for FMDV, has been ascribed to 3C (Vakharia et al., 1987). This cleavage is difficult to observe because of the rapid cleavage of 2A/2B and the very small difference in M, between P1 and P1/2A. However when the truncated P1 construct was translated in the presence of active 3C, processing of the smaller P1 could be seen after prolonged incubation (Fig. 3, lanes 7 and 8), in keeping with the removal of the oligopeptide 2A.

Analysis of the sequences from various serotypes of FMDV at the junctions now known to be cleaved by 3C, including P1/2A, show a degree of degeneracy. Recently, Parks & Palmenberg (1987) have used site-directed mutagenesis to study the 3C-mediated cleavage sites in EMCV and found that 3C could process Gln/Ala as well as the natural Gln/Gly but could not cleave Gln/Val, Gln/Glu, Lys/Gly, Lys/Ala, Lys/Val, Lys/Glu or Pro/Gly. In view of the ability of 3C to cleave in trans (Nicklin et al., 1987; Vakharia et al., 1987) as well as the sequence variation between serotypes we were able to study the specificity of FMDV 3C directly. We have shown that FMDV 3C can cleave at Gln/Gly, Glu/Gly, Gln/Thr, Glu/Thr, Cys/Asn and Leu/Asn. However, as in PV and EMCV the 3C protease does not cleave at every occurrence of these sequences and the recognition site must involve more complex features.

One parameter which does affect the ability of 3C to process the structural proteins is the presence of the entire precursor P1, since the truncated P1 cannot be cleaved by active protease. This agrees with the data on PV (Nicklin et al., 1987; Ypma-Wong & Semler, 1987b) where precursors lacking 1A or 1D are poor substrates for 3C. In view of the intimate nature of the association between the structural proteins (Hogle et al., 1985; Rossmann et al., 1985) it seems reasonable to assume that 3C recognizes a conformational determinant which the truncated protein does not attain.

The data presented here and also by Vakharia et al. (1987) are in contrast to observations on PV in one important respect; Ypma-Wong & Semler (1987a) observed that almost the entire P3 region is required to generate an active 3C capable of cleaving P1 although 3C alone was sufficient to process P2 in PV. The data on FMDV, however, clearly differ in that 3C alone appears to be responsible for the P1 cleavages. In the A12 system (Vakharia et al., 1987) the active 3C transcripts do possess a 9 x 10^3 M, piece of 3D that cannot be excluded as having an effect on these cleavages. However our experiments show no difference in processing of P1 whether 19 amino acids of 3D are present or, indeed, if eight amino acids are missing from the C terminus of the protein. This difference between FMDV and PV may reflect the differences in the proteolytic pathways of the two viruses or possibly may be a consequence of factors present in the HeLa cell extracts necessary for efficient translation of PV RNA in vitro. We do not have data on the cleavages within P2 but Vakharia et al. (1987) clearly showed that all these cleavages are also performed by 3C with no involvement of other proteases, e.g. Lab or Lb.

The cleavages catalysed by 3C were thought to occur in a defined manner such that sequential release of Lb, 1D and finally 1C and 1AB should be observed. However Parks & Palmenberg (1987) observed for EMCV that translation of their subgenomic RNAs resulted in efficient 1AB/1C cleavage, a reaction normally preceded by 1C/1D scission (Parks et al., 1986). Similarly in all our translation experiments it appears that the 1AB/1C cleavage is at least as efficient as the 1C/1D scission. This is particularly obvious when the particles assembled in vitro are analysed. As can be seen in Fig. 7 there is a significant amount of unprocessed 1CD. Crystallographic evidence has suggested that interaction between processed N termini of the structural proteins is a critical step in viral morphogenesis (Arnold et al., 1987; Luo et al., 1987). Clearly in our particles there is a limitation in the number of free 1D termini and this, we feel, probably reflects the instability that we have observed.

The fact that we were able to assemble subviral particles from our subgenomic RNA has encouraging significance in terms of vaccine development. Many approaches towards subunit vaccines for FMDV have been made (e.g. Kleid et al., 1981; Bittle et al., 1982; Winther et al., 1986; Clarke et al., 1987; Francis et al., 1987) and, although significant progress has been made,
the most immunogenic particle apart from the virus itself is the 'empty' viral capsid (Rowlands et al., 1975). A major problem associated with FMDV vaccines has been outbreaks occurring as a result of improperly inactivated vaccines. Assuming the problems of instability can be overcome, e.g. by driving 1CD cleavage to completion, then it is feasible that expression of large amounts of 'empty' capsid could be produced from these non-infectious subgenomic clones.

The function of the leader proteins in FMDV remains an enigma. From the work of Strebel & Beck (1986) it is clear that this protein has proteolytic activity and can cleave the L–P1 bond in cis or in trans. However our data support those of Vakharia et al. (1987) showing that these proteins are not involved in any of the other events in the proteolytic pathway of FMDV in vitro. One possible function which these proteins may perform is in the modification of the C termini of the leader proteins which has recently been described (Sangar et al., 1988). If so, these proteins would be bifunctional in nature.

Finally, it is worth noting that in this system we have been able to initiate translation accurately and efficiently in the absence of any downstream untranslated region or cap proteins. Similar work using EMCV (Parks et al., 1986) or PV (Nicklin et al., 1987) have all used various amounts of 5' untranslated region although Nicklin et al. (1987) did show that truncation of this region improved translation in a reticulocyte system. However in FMDV the presence of significant amounts of the 5' untranslated region had little effect on translation.

We would like to thank Alan Brown for excellent technical assistance and also Dave Rowlands, Tony Carroll, Ed Westaway and Fred Brown for helpful discussions.

REFERENCES


Processing and assembly of FMDV proteins


(Received 17 March 1988)