

Key words: FMDV/leader protein/protein modification

Modification of the Leader Protein (Lb) of Foot-and-Mouth Disease Virus

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(Accepted 2 June 1988)

SUMMARY

Translation of foot-and-mouth disease virus RNA for extended periods in rabbit reticulocyte lysates results in the appearance of a previously undescribed protein. A protein with similar properties can also be detected in BHK cells at late times after virus infection. Specific immunoprecipitation has shown that this protein (Lb') is closely related to the smaller of the two leader proteins, Lb, although it migrates with an apparently higher *M_r* in SDS-polyacrylamide gels. The conversion of Lb to Lb' can be mimicked by treatment with carboxypeptidase B. It is suggested that C-terminal trimming of Lb to produce Lb' results in an increase in negative charge and is responsible for its slower migration in SDS-PAGE.

INTRODUCTION

Foot-and-mouth disease viruses (FMDV) form the genus aphthovirus in the family *Picornaviridae* which also includes the enteroviruses, rhinoviruses and cardioviruses. These viruses have genomes of positive sense ssRNA which, in the case of FMDV, is approx. 8.5 kb in length (Sangar, 1979; Clarke *et al.*, 1987). The genome is expressed as a single polypeptide which is cleaved into mature proteins. The structural protein precursor region (P1) of the entero- and rhinoviruses starts at the N terminus of the polypeptide (Dorner *et al.*, 1982) but in the cardio- and aphthoviruses P1 is preceded by a short leader protein (L) (Sangar, 1979). The aphthoviruses are further characterized by the presence of two independent translation initiation AUG codons in the same reading frame and located 84 nucleotides apart. Both of these codons initiate protein synthesis *in vivo* and *in vitro* to produce alternative forms of the leader protein (Clarke *et al.*, 1985; Sangar *et al.*, 1987). The larger of these (Lab) is initiated from the first functional AUG and contains all of the sequence of the shorter leader (Lb) plus an N-terminal extension of 28 amino acids.

Little is known about the function of the leader proteins in FMDV but it may be relevant that the presence of two functional initiation codons is a highly conserved feature in viruses of all seven serotypes of the aphthoviruses (Sangar *et al.*, 1987). The Lb has been shown to have a specific proteolytic activity (Strebel & Beck, 1986) but its only known function is to cleave the polypeptide at the Lb/P1 junction.

In this paper we describe a new protein (Lb') that can be detected both *in vitro* and *in vivo* and which is closely related to Lb. Although Lb' migrates more slowly than Lb on PAGE we believe that it is produced by proteolytic processing of Lb.

METHODS

Preparation of virus RNA. BHK cells were infected at an m.o.i. of approx. 100 (FMDV serotype A₁₀ strain 61). After 3 h the infected cells were harvested and the virus RNA was purified as described previously (Rowlands *et al.*, 1978).

Translation of RNA in vitro. The micrococcal nuclease-treated rabbit reticulocyte lysate of Pelham & Jackson (1976) was purchased from Amersham. RNA (1 µg) was added to 10 µl of lysate containing the appropriate radiolabel and the mixture incubated at 30 °C. Samples were treated with 10 µg RNase A before PAGE.

Labelling of virus-specific proteins in infected cells. Monolayers of BHK cells in 2 oz bottles were infected at an m.o.i. of approx. 100. Proteins were labelled at either 1 to 2 h or 2 to 3 h post-infection (p.i.) with the appropriate radioactive precursor. Cytoplasmic extracts for immunoprecipitation were made by lysing the cells in 1 ml of Eagle's medium containing 1% NP40 and 0.5% sodium deoxycholate. After 15 min on ice the nuclei were removed by centrifugation at 2000 g for 5 min and the supernatants were stored at -70°C .

Synthesis of peptides and coupling to carrier protein. Peptides were synthesized by the method of Houghten (1985). They were coupled to keyhole limpet haemocyanin through an additional cysteine at their C termini using *N*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Liu *et al.*, 1979). Rabbits were injected with approximately 50 μg of coupled peptide in complete Freund's adjuvant and boosted twice at monthly intervals with the same amount of peptide in incomplete Freund's adjuvant.

Immunoprecipitation. To 300 μl of cytoplasmic extract, 30 μl of 10% SDS was added and the sample was boiled for 1 min. The mixture was diluted 10-fold with cold Eagle's medium, undiluted antiserum was added (15 μl) and left for 17 h at 4°C . Precipitation with Protein A (Pansorbin; Calbiochem) and washing of the pellets were performed as described by Harris *et al.* (1981).

Polyacrylamide gel electrophoresis. Samples were analysed on 10% or 15% polyacrylamide slab gels using the discontinuous system of Laemmli (1970). After electrophoresis the gels were treated with Amplify (Amersham), dried and exposed to X-ray film.

Two-dimensional separation of proteins. Samples were treated for isoelectric focusing in the manner described by King *et al.* (1980). Proteins were separated in the first dimension on tube gels using 5% (v/v) Ampholines (pH 3.5 to 10). The second dimension was run on 10% gels using the system of Laemmli (1970).

Synthesis of FMDV subgenomic RNA transcripts from recombinant clones. Recombinant clones were constructed in order to clarify the modification of the leader proteins by removing the background of non-structural proteins. Three clones were used in this study. Plasmid pTC6 was constructed by ligating a 3120 bp *Xba*I-*Sac*I fragment from an FMDV, serotype A₁₀, genomic subclone into the SP6 transcription vector SP64. RNA transcripts derived from this plasmid contain 27 bases of linker sequence, 400 bases of 5' untranslated sequence and 2720 bases of coding sequence from FMDV. These transcripts should produce both leader proteins (Lab and Lb), and a truncated P1. Construction of the clone pRA6 is described in detail elsewhere (Clarke & Sangar, 1988). Briefly, it and pDS8 produce RNA transcripts which can only initiate translation at the second of the two AUG codons (i.e. Lb) of FMDV A₁₀. Thereafter pRA6 codes for the whole of P1, part of 2B, the third VPg, P3C and the N terminus of 3D. The construct pDS8 codes for the entire region from Lb through to the N terminus of 3D.

Production of RNA transcripts was essentially as described by Melton *et al.* (1984). Plasmid DNA (10 μg) was linearized with *Sac*I (pTC6) or *Hind*III, extracted with phenol/chloroform and ethanol-precipitated. *In vitro* transcription was then carried out with 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 (Amersham). 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 .

Enzyme treatment. *In vitro* translations were stopped by the addition of RNase A (10 μg) and incubating for 5 min at 30°C . Enzyme treatment of this mixture was performed directly as described in Results.

RESULTS

Detection and characterization of a post-translationally modified FMDV protein

After *in vitro* translation of FMDV (A₁₀) RNA for 40 min the two leader proteins Lab and Lb, corresponding to the two alternative initiating AUGs, were detected. Both leader proteins were also present in infected BHK cells (Fig. 1). However, if *in vitro* incubation was extended to 4 h an additional protein, Lb', appeared on SDS-PAGE which migrated more slowly than Lb. Furthermore the migration of Lb' was dependent on gel concentration; in 10% gels it migrated slower than Lab but in 15% gels it migrated faster than Lab (Fig. 1).

Kinetics of Lb' appearance

In order to study the kinetics of appearance of this new protein recombinant plasmids pRA6 or pDS8 were used (see Methods) to produce subgenomic RNAs which translated extremely well *in vitro* (B. E. Clarke & D. V. Sangar, unpublished data). These clones were constructed to eliminate the initiation codon for Lab and therefore initiation occurred at the Lb AUG. The absence of Lab from the expressed protein profile facilitated the visualization of Lb'.

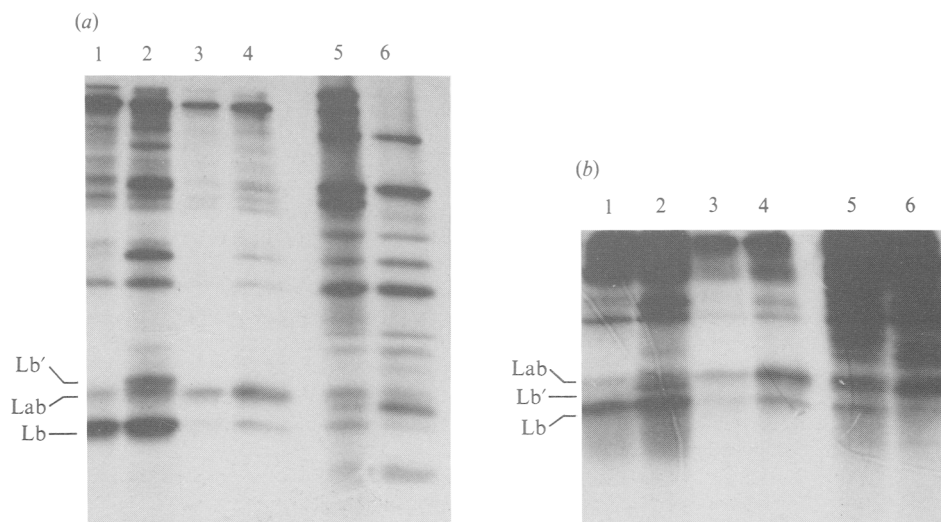


Fig. 1. SDS-PAGE of proteins synthesized *in vitro* and *in vivo*. Separation was on 10% (a) or 15% (b) gels. Conditions for *in vitro* translation of FMDV RNA were as follows: 40 min at 2 mM-Mg²⁺ (lane 1), 240 min at 2 mM-Mg²⁺ (lane 2), 40 min at 4 mM-Mg²⁺ (lane 3) and 240 min at 4 mM-Mg²⁺ (lane 4). Lane 5 represents proteins from FMDV-infected BHK cells after 5 min pulse and lane 6 shows the same proteins after 5 min pulse and 30 min chase.

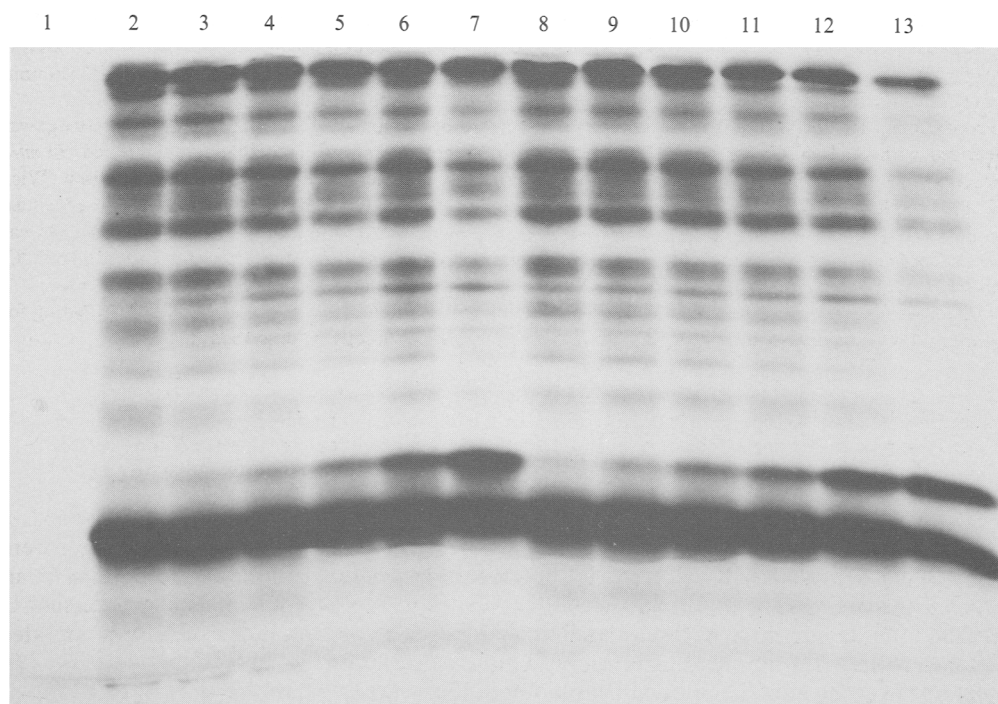


Fig. 2. Time course of the appearance of Lb' during *in vitro* translation. RNA obtained from clone pDS8 was translated for 25 min in a rabbit reticulocyte lysate. Translation was either stopped (lanes 2 to 7) by the addition of 1 μ l RNase A (10 mg/ml) or allowed to continue (lanes 8 to 13). The incubation was stopped after 0 min (lanes 2 and 8), 10 min (lanes 3 and 9), 30 min (lanes 4 and 10), 60 min (lanes 5 and 11), 120 min (lanes 6 and 12) or 240 min (lanes 7 and 13). Lane 1 shows the translation products when RNase was added together with the RNA and incubated for 4 h. After RNase treatment the samples were separated by 10% PAGE and exposed to X-ray film.

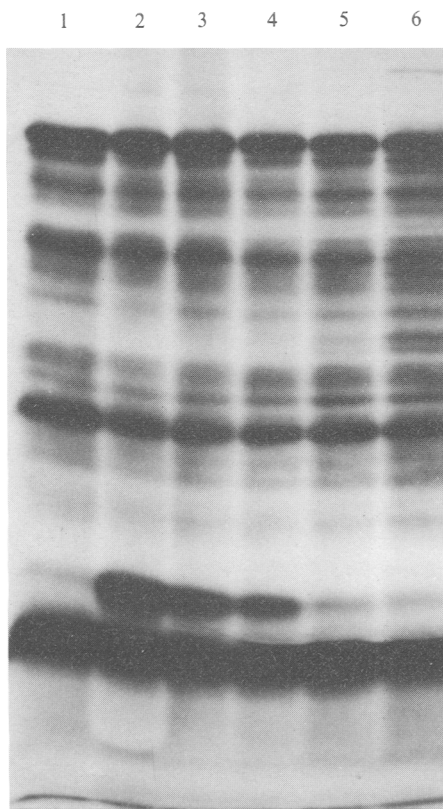


Fig. 3

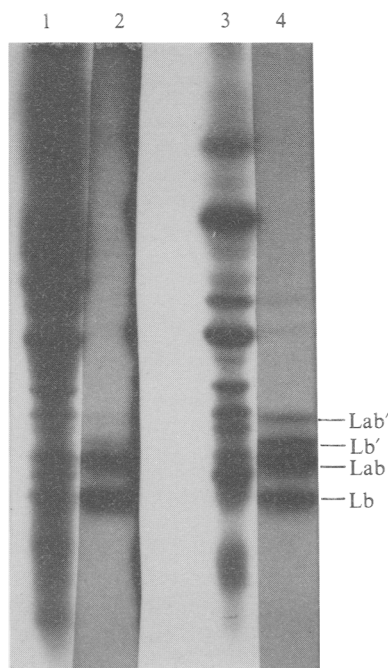


Fig. 4

Fig. 3. The effect of dilution on the formation of Lb'. RNA from clone pRA6 was translated for 25 min in a rabbit reticulocyte lysate. Translation was then stopped by the addition of different volumes of RNase containing the same amount of enzyme. Lane 1, 5 min incubation after RNase addition (1 μ l of 10 mg/ml); lane 2, 240 min incubation with no RNase added; lane 3, 240 min incubation with RNase (1 μ l of 10 mg/ml); lanes 4, 5 and 6, 240 min incubation with 5 μ l of 2 mg/ml, 25 μ l of 0.4 mg/ml or 75 μ l of 0.1 mg/ml, respectively. Samples were then analysed on 10% gels.

Fig. 4. Autoradiograph of PAGE gel showing immunoprecipitation of virus proteins. BHK cells were labelled with [35 S]methionine for 1 h and the cell lysate was immunoprecipitated with peptide antiserum. Lane 1, cells were labelled from 1 to 2 h p.i. and immunoprecipitated using peptide antiserum (lane 2). Lane 3, cells were labelled from 2 to 3 h p.i. and immunoprecipitated using peptide antiserum (lane 4). Bands representing Lb, Lab and Lb' are indicated; a fourth band is present and tentatively labelled Lab'.

Although Lb' was not detected after short periods of translation (Fig. 2, lanes 2, 8), continued protein synthesis was not required for its production. This was shown by inhibiting translation of pDS8 after 25 min with RNase A and incubating for increasing times. Lb' appeared with similar kinetics in the absence of concomitant protein synthesis (Fig. 2). The production of Lb' could, however, be greatly inhibited by dilution of the lysate at the time of RNase A addition. This is shown in Fig. 3 using a different clone, pRA6, which is structurally identical to pDS8 in the region coding for Lb/Lb'.

Translation in the presence of 4 mM-Mg $^{2+}$ alters the ratio of leader proteins so that Lab is the major product formed; under these conditions Lb' was not observed (Fig. 1) thus suggesting that Lb is the precursor of Lb'. This was supported by the finding that [35 S]methionine-labelled Lb and Lb' produced identical tryptic maps (data not shown) and also by the observation that subgenomic RNA that could code for Lb but not Lab still produced Lb'.

To investigate further the relationship between Lab, Lb and Lb', antiserum was raised to a synthetic peptide representing the 20 amino acids at the N terminus of Lb. This antiserum specifically immunoprecipitated [³⁵S]methionine-labelled proteins corresponding to Lab, Lb and Lb' produced by translation both *in vitro* (data not shown) and *in vivo* (Fig. 4). The use of the antiserum was essential to distinguish the leader proteins synthesized *in vivo* from other viral and host proteins which otherwise obscured this region of the gel. This was particularly evident in lysates of cells labelled from 1 to 2 h p.i. The immunoprecipitated samples clearly showed proteins migrating in the positions of Lab, Lb and Lb' plus a minor band migrating with an apparently higher M_r (Fig. 4).

The ratio of Lab:Lb was similar in samples labelled from 1 to 2 h or from 2 to 3 h but the proportion of Lb' was greater in samples labelled from 2 to 3 h. However, Lb' was not seen in samples from cells labelled for 15 min at 2.5 h p.i. indicating that the production of Lb' was relatively slow (data not shown).

What is the modification of Lb?

As described above the modification of Lb to Lb' was more easily detected in translation products of SP6 transcripts from recombinant subgenomic clones. In order to study the nature of the modification a further clone, pTC6, was used for translation. *In vitro* transcripts from this plasmid contain the coding sequences for both leader proteins (Lab and Lb) and a truncated P1 only. In practice little of the truncated P1 protein is observed probably due to instability of the product; only the leader proteins are seen in significant amounts. Labelled translation products from pTC6 were therefore synthesized and analysed by two-dimensional gel electrophoresis (Fig. 5). The product (Lb') that appeared after prolonged incubation had a more negative charge than Lb. The product Lab also resolved into two species; however, the relative amounts of these did not alter with different times of translation and the charge change was less than that observed for the Lb to Lb' transition. We do not know the nature of the modification of Lab. The charge and gel migration differences between Lb and Lb' could be indicative of a chemical derivatization of Lb (Obijeski *et al.*, 1974) which did not influence the profile of the tryptic maps of the two proteins. To investigate this possibility, a variety of radiolabelled precursors were added to both *in vivo* and *in vitro* translation systems. There was no detectable incorporation of any of the following precursors into either Lb or Lb': [³²P]orthophosphate, [γ -³²P]ATP, [α -³²P]ATP, [α -³²P]UTP, [³H]myristic acid, [³H]palmitic acid or [¹⁴C]acetyl-coenzyme A.

However, the addition of 2 μ g of carboxypeptidase B to an *in vitro* lysate which contained mainly Lb resulted in the rapid loss of Lb and the appearance of Lb' (Fig. 6). This effect could be prevented by the addition of 1,10-phenanthroline (a known inhibitor of carboxypeptidase B; Folk *et al.*, 1960) along with the protease (Fig. 6). The 'natural' conversion of Lb to Lb', i.e. that produced by extended incubation of the *in vitro* lysate, was not inhibited by 1,10-phenanthroline (data not shown).

DISCUSSION

Viruses from all FMDV serotypes initiate protein synthesis at two separate AUGs to produce alternative leader proteins Lab and Lb (Sangar *et al.*, 1987) but it is not known whether these two proteins have separate functions. By using antisera raised to a synthetic peptide we have been able to show that the ratio of Lab:Lb is the same at 1 to 2 h and 2 to 3 h p.i. Thus it appears unlikely that these proteins are made in different amounts during the course of an infection.

In addition to Lab and Lb we have now detected another species of leader protein, Lb', which is produced during prolonged *in vitro* translation or during virus replication in BHK cells. The amount of Lb' relative to Lb increases late in infection. Tryptic peptide maps and immunoprecipitation with specific antiserum showed that the two proteins are closely related. We therefore investigated the possibility that Lb' is derived from Lb by chemical modification, by translating in the presence of a number of potential precursors of modification. All these experiments were negative including labelling with acetyl-coenzyme A, although it has previously been suggested that Lb is blocked by an acetyl group (Robertson *et al.*, 1985).

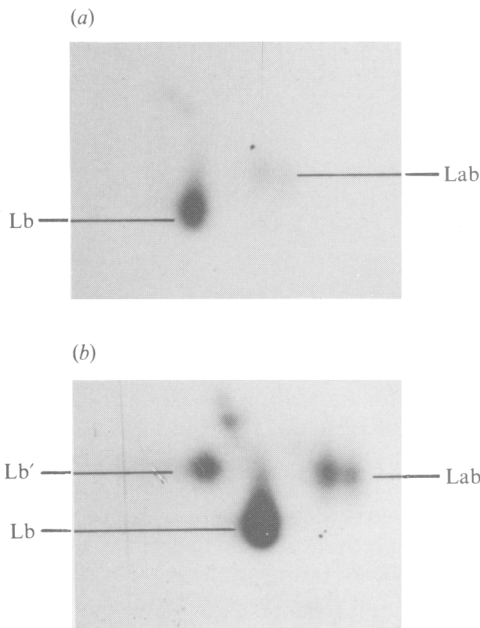


Fig. 5

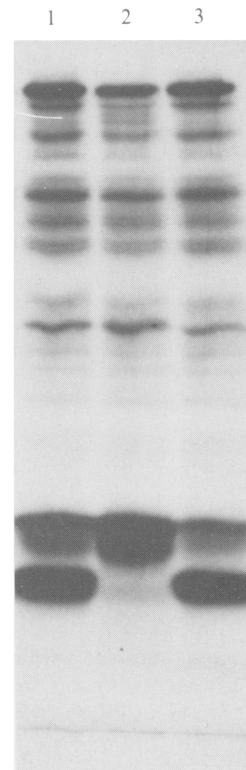


Fig. 6

Fig. 5. Two-dimensional gel electrophoresis of the translation products of pTC6 after 40 min (a) or 240 min (b) translation. The first dimension (horizontal) is isoelectric focusing and the second dimension (vertical) is SDS-PAGE.

Fig. 6. Autoradiograph following SDS-PAGE of proteins synthesized in a rabbit reticulocyte lysate. Lane 1, translation for 1 h; lane 2, translation for 1 h and 5 min incubation with 2 µg carboxypeptidase B; lane 3, translation for 1 h and 5 min incubation with a mixture of 2 µg carboxypeptidase and 3 µg 1,10-phenanthroline. Electrophoresis was in a 10% gel.

We were surprised to find that Lb could be converted into a protein with the characteristics of Lb' by treatment with carboxypeptidase B. This enzyme is specific for C-terminal basic amino acids (Wintersberger *et al.*, 1965) and could therefore remove the terminal lysine residue found at the C terminus of Lb (Chow *et al.*, 1987). Thus, despite migrating more slowly than Lb in 10% polyacrylamide gels, Lb' has a lower M_r . This kind of anomalous migration of proteins in polyacrylamide gels has been described previously (Curran & Kolakofsky, 1987). It is of interest that the electrophoretic mobility of Lab is not affected by carboxypeptidase B despite presumably having a C terminus identical to that of Lb. We do not know, however, whether the C terminus of Lab is resistant to proteolysis or whether cleavage of amino acids from the C terminus occurs but does not result in aberrant migration in gels.

Although carboxypeptidase B can mimic the natural modification of Lb it does not appear to be the enzyme responsible for the cleavage in rabbit reticulocyte lysates since this 'natural' modification cannot be inhibited by 1,10-phenanthroline. We speculate that the cleavage is mediated by the leader protein itself. Although the evidence is circumstantial, this suggestion is supported by the findings that the modification *in vivo* is a late event and therefore unlikely to be host cell-coded, and that Lb' is produced efficiently from transcripts prepared from pTC6, which produces almost exclusively Lab and Lb' thus making it unlikely that other virus proteins

are involved. However dilution experiments indicate that if the leader protein is the protease then the reaction is not intramolecular. The results presented here were obtained with virus of serotype A₁₀ but Western blots using peptide antiserum have indicated that a similar modification of Lb occurs with viruses of serotypes O and SAT₂ (R. P. Clark & D. V. Sangar, unpublished data).

The function of the leader protein is not clear although it has been demonstrated that it is a protease that cleaves itself from the remainder of the polyprotein (Strebel & Beck, 1986). This current work suggests that the leader protein may also possess an exopeptidase activity. Whether the trimming of Lb resulting from this activity has biological significance is at present not known.

We thank Dr F. Brown for critically reading the manuscript and for useful suggestions, Mr A. Syred for peptide synthesis, Mr P. Barnett and Mr G. Fox for valuable assistance, and Ms L. Joyce for typing the manuscript.

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