Polyprotein Processing of Theiler's Murine Encephalomyelitis Virus

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To investigate polyprotein processing of Theiler's murine encephalomyelitis viruses, we analyzed in vitro translation reactions programmed by in vitro-derived transcripts from an infectious full-length cDNA clone of the DA strain of Theiler's virus. To help identify the proteinases that carried out the processing, we modified the DA cDNA clone transcription template by linearization with different restriction endonucleases that generate templates of different lengths or by constructing linker insertion or deletion mutations or both in putative proteinase-coding regions. Protein 3C carried out most of the cleavages of the polyprotein, as is true for the other picornaviruses that have been studied. A second proteinase also appeared active at the LP12A-2B junction. A protein of slightly faster mobility than the leader protein was seen with translation of transcripts derived from DA cDNA but not GDVII cDNA. This protein may be synthesized from an alternative initiation site in the DA leader-coding region out of phase with the polyprotein reading frame. Our findings are relevant to ongoing investigations of the abnormal virus expression seen in DA virus late demyelinating disease, since polyprotein processing is critical in regulating picornaviral gene expression.

Theiler's murine encephalomyelitis viruses (TMEV) are murine picornaviruses that are most similar on a sequence level to members of the cardiovirus genus (20, 22, 25, 26). TMEV strains can be divided into two subgroups on the basis of their different biological activities and reactivity with neutralizing monoclonal antibodies (15, 17). The GDVII strain and other GDVII subgroup strains produce an acute, fatal poliomyelitis. In contrast, the DA strain and other TO subgroup strains produce a persistent demyelinating infection in mice; additionally, there is evidence that the expression of TO subgroup strains is restricted in the chronically infected central nervous system tissue (3). A major goal of investigators of TMEV is the delineation of the molecular basis of the biological activities of the strains, such as the virus persistence and nonproductive restricted expression. Since viral polyprotein processing is a major determinant of the regulation of picornaviral gene expression, we studied TMEV polyprotein processing and the proteinases that mediate this process.

All picornavirus RNA genomes encode a single large precursor polypeptide that is sequentially cleaved by proteinases into functional proteins—the viral capsid proteins and nonstructural proteins. More than one proteinase is involved in these cleavages. Although the viral proteinase 3C performs most of the polyprotein cleavages for the picornaviruses that have been studied, picornaviruses differ with respect to the other specified proteinases and their substrate recognition sites (14). In poliovirus, a member of the enterovirus genus, peptide 2A rapidly cleaves at the P1-P2 junction while the polyprotein is still being translated on the ribosome, as well as within 3CD to produce 3C' and 3D' (31). The P1-P2 cleavage is mediated by polypeptide 3C in foot-and-mouth disease virus (FMDV) (an aphthovirus) and encephalomyocarditis virus (EMCV) (a cardiovirus) (11, 24, 32). The polyprotein of the latter two viruses demonstrates a very rapid cleavage between P12A-2B, not P1-P2, that may be mediated by 2A. FMDV and EMCV also encode a leader protein (L) at the N terminus of the polyprotein. The FMDV

L autocatalytically cleaves itself from the polyprotein (30), while in EMCV, the L-P1 site is cleaved by 3C (24). The EMCV L protein has not been shown to have cleavage activity on any viral substrates. In all picornaviruses studied, there is a putative autocatalytic activity which cleaves VP0 into VP2 and VP4 in the final stage of viral maturation (14).

The picornaviral proteinases are believed to play a role in the virally induced suppression of cellular translation as well as polyprotein processing. The inhibition of host-cell cap-dependent translation that follows enterovirus and aphthovirus infections is associated with cleavage and inactivation of p220, a component of the cap-binding complex (16); poliovirus 2A indirectly mediates the cleavage of p220 (13, 16), while FMDV L protein indirectly or directly cleaves p220 (7). Infection of cells by the cardioviruses and TMEV does not result in cleavage of p220 (16, 18).

We recently generated infectious cDNA clones of the DA strain of TMEV (see accompanying report [27]). We now present data on in vitro translation of in vitro-derived transcripts from wild-type and mutated full-length cDNAs to characterize TMEV polyprotein processing and the proteinases involved.

MATERIALS AND METHODS

The following reagents were obtained commercially: restriction endonucleases (New England BioLabs, Inc., Beverly, Mass., and Boehringer Mannheim Biochemicals, Indianapolis, Ind.); synthetic linkers (New England BioLabs); T7 RNA polymerase (Pharmacia, Inc., Piscataway, N.J.); RNasin (Promega Biotech, Madison, Wis.); and rabbit reticulocyte lysate (Promega Biotech).

Cells and viruses. L929 cells (obtained from J. Perrault) were used for plaque assays and transfections, while BHK-21 cells (obtained from J. Holland) were used for the growth of stock virus.

Construction of plasmids. The construction of pDAFL3, a DA full-length infectious cDNA clone in the transcription vector Bluescript SKII-, has been described elsewhere (see accompanying report in this issue [27]). pDAFL3 has the 5'

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FIG. 1. Diagram of the transcription template, pDAFL3, linearized at different sites by restriction endonucleases and a proposed TMEV polyprotein processing map. The upper part of the figure shows varied lengths of pDAFL3 generated by digestion with particular restriction endonucleases, as described in the text. The restriction endonuclease site is given in parentheses; *XbaI* cuts after the 3' end of the DA viral genome, generating a full-length template and transcript. The cross-hatching shows the coding regions for proteinases proposed or proved to be active in other picornaviruses (L, 2A, 3C). The translation of transcripts derived from these linearized templates is shown in Fig. 6 and 7. A processing map identifying polypeptide cleavages that have been described with other picornaviruses is presented in the lower part of the figure. Nucleotide positions relevant to the cleavages are given on the DA viral RNA genome.

end of the DA genome two nucleotides downstream from the bacteriophage T7 promoter.

pDAFL3 was linearized with different restriction endonucleases to generate transcription templates of different lengths (Fig. 1). To generate a full-length template, pDAFL3 was linearized with *XbaI*, which cuts after the 3' end of the DA genome. The templates that were generated by digestion with other restriction endonucleases are designated lin followed by the restriction endonuclease used in parentheses. pDAFL3(lin *SspI*) and pDAFL3(lin *XhoI*) were linearized in the L-coding region, pDAFL3(lin *HindIII*) was linearized in the VP2-coding region, pDAFL3(lin *BamHI*) was linearized in the 2A-coding region, and pDAFL3(lin *StuI*) was linearized in the 3C-coding region. There were two additional linearization studies not shown in Fig. 1 that involved *NcoI* digestion of a DA subgenomic cDNA clone (producing a transcript of 1,961 nucleotides) and a GDVII subgenomic cDNA clone (producing a transcript of 1,964 nucleotides) (J. Fu, S. Stein, L. Rosenstein, T. Bodwell, B. L. Semler, and R. P. Roos, manuscript in preparation).

A variety of linker or deletion mutations or both were constructed in regions of pDAFL3 that encode proteinases in other picornaviruses to determine the effect on polyprotein processing (Fig. 2). All the mutations were engineered to

maintain the downstream reading frame of the polyprotein.

pDAL:NheI contains a 4-amino-acid insertion (Ser-Arg-Leu-Ala) in L. This mutation was constructed by inserting an *NheI* linker (G-G-C-T-A-G-C-C) into the *XhoI* site at 1221.

pDALΔ*SspI*XhoI:NheI contains a 3-amino-acid insertion (Trp-Leu-Ala) with a deletion of 83 nucleotides in the L-coding region. This mutation was constructed by first inserting an *NheI* linker at the *SspI* site at 1138 of pDAL:NheI and then deleting the fragment between the *NheI* linkers at the *SspI* site and the *XhoI* site.

pDA2A:Clal contains a 4-amino-acid insertion (Pro-His-Arg-Trp) in 2A. This mutation was constructed by inserting a *Clal* linker (G-A-T-C-G-A-T-G) in the *BamHI* site of pDAFL3 at 3926.

pDA3CΔ*HpaI* contains a deletion of 213 nucleotides from 3C. This mutation was constructed by ligating the large fragment of pDAFL3 that was left after *HpaI* digestion at nucleotides 6249 and 6462.

In vitro transcription. The transcription template was linearized with the appropriate restriction endonuclease and transcribed in a T7 RNA polymerase reaction as described previously (34). After transcription, the sample was extracted with phenol-chloroform (1:1) and precipitated in a

FIG. 2. Diagram of linker insertion and deletion mutations in putative proteinase-coding areas and a map of the TMEV-coding region. The restriction endonucleases used in generating the mutations are shown, and the sites are given in parentheses. The construction of the mutants is detailed in the text. The translation of transcripts derived from these mutants is shown in Fig. 7 and 8.

final concentration of 2.5 M ammonium acetate with 2 volumes of ethanol.

In vitro translation. Translation reactions were done at 30°C in a rabbit reticulocyte lysate as described previously (33), except for the presence of a final concentration of 10 mM potassium isothiocyanate (KSCN) and the absence of a postmitochondrial extract from uninfected cells. The use of potassium isothiocyanate was suggested by R. J. Jackson on the basis of his data from in vitro translation of EMCV RNA (11). Generally, 50 to 100 ng of in vitro-transcribed RNA was used in each 10-μl (final volume) translation reaction. The translation reaction was usually terminated after 3 h with the addition of Laemmli sample buffer. In some cases, 5 μg of cycloheximide per ml and 10 μg of pancreatic RNase per ml were added to the translation reaction for 20 min at 30°C followed by a further incubation for 1 h at 30°C with 3 μl of a postmitochondrial S-10 extract from DA-infected BHK21 cells prepared as described previously (1); the reaction was then diluted with Laemmli sample buffer. Samples of translation reactions were subjected to electrophoresis on 10 to 18% gradient or 12.5% polyacrylamide gels containing sodium dodecyl sulfate. The gels were fluorographed and then exposed to XAR-5 film.

Immunoprecipitation and antisera. Immunoprecipitation was performed as previously described (9, 29). Antisera used for immunoprecipitation included TMEV anti-VP1, anti-VP2, and anti-VP3 sera (6); monoclonal TMEV anti-VP1 (21); TMEV anti-3D serum kindly provided by M. Brahic (23); and poliovirus anti-2C serum (9).

RESULTS

Overall strategy and construction of different transcription templates. Our overall strategy related to the analysis of DA virus polyprotein processing is shown in Fig. 3. The pDAFL3 transcription template was modified either by linearization with restriction endonucleases that produce templates of different lengths or by engineering mutations into putative proteinase-coding regions. The template was then used in an in vitro transcription reaction with T7 RNA polymerase to generate RNA. Finally, the in vitro-derived transcripts were used as mRNAs in in vitro translations in a rabbit reticulocyte lysate. After translation, infected extracts were added to determine whether incomplete processing could be corrected in *trans*. The products of translation and

processing were then analyzed by polyacrylamide gel electrophoresis.

As shown in Fig. 1 and specified in Materials and Methods, pDAFL3 was digested with different restriction endonucleases to generate transcription templates of varied lengths. The different-sized templates were transcribed in vitro, and the synthetic mRNAs that were produced corresponded to the sizes of the individual linear cDNAs when analyzed by agarose gel electrophoresis. The transcripts were then used in an in vitro translation reaction to determine the effect of the truncated transcript (and the consequent shortened polyprotein) on polyprotein processing.

Our second approach involved engineering mutations into putative proteinase-coding regions of pDAFL3. As shown in Fig. 2 and specified in Materials and Methods, linker or deletion mutations or both were placed in the coding regions of L, 2A, and 3C. The mutated pDAFL3 cDNA was then transcribed in vitro, and the RNA was used in an in vitro translation reaction to determine the effect of the mutation on polyprotein processing.

Wild-type pDAFL3 translations and time course study. Our initial experiments were directed at optimizing conditions for pDAFL3 translation. We first analyzed in vitro translation products from full-length transcripts of the pDAFL3 tem-

FIG. 3. Strategy for analyzing TMEV polyprotein processing.

plate that had been linearized by *Xba*I after the 3' end of the DA genome. In EMCV, a cardiovirus, translation efficiency is enhanced with the addition of KSCN to a final concentration of 5 to 10 mM to the translation reaction (11). We similarly found that the efficiency of translation of pDAFL3 in vitro-derived transcripts as well as DA virion RNA was markedly enhanced in the presence of comparable concentrations of KSCN, with an increase in the synthesis of authentic translation products (data not shown); for this reason, we used KSCN in all subsequent translation reactions. In poliovirus, the addition of a postmitochondrial extract from uninfected HeLa cells to rabbit reticulocyte lysates increases the efficiency of translation as well as the accuracy of translation initiation at the polyprotein initiation codon (8). In contrast, there was no improvement in translation of pDAFL3-derived RNA with the addition of an uninfected HeLa or BHK-10 extract (data not shown). The efficiency and accuracy of TMEV RNA translation in rabbit reticulocyte lysates that are not supplemented with extracts is similar to that described for EMCV and FMDV (11).

We immunoprecipitated the TMEV translation reaction mixture to identify the viral proteins. Figure 4 shows immunoprecipitation of translated proteins from a pDAFL3 transcript. The identity of proteins P12A, P1, 1ABC, 1AB, VP1, VP3, 3D, and 3CD in this figure was made possible by immunoprecipitation with specific antisera and consideration of the predicted molecular weights of the proteins (22). Since VP0 cleavage into VP2 and VP4 requires virus assembly and does not occur with in vitro translation, VP2 was located on the gel by immunoprecipitation of in vivo-labeled viral proteins with anti-VP2 antiserum (data not shown). Immunoprecipitation with poliovirus anti-2C antiserum produced a barely detectable signal at the position noted on the gel shown in Fig. 4. However, its location with respect to VP0 and VP1 tended to vary somewhat in other gels (data not shown). The identity of the other viral proteins was made possible by examining shifts in the mobility of proteins of pDAFL3 mutants (involving L, 2A, 3C; see below) and from predictions of molecular weights of the proteins.

We performed a time course study of in vitro translation of the pDAFL3 transcripts to study TMEV polyprotein processing. Samples of the translation reaction were collected at various times after the start of translation and were then diluted in Laemmli sample buffer and electrophoresed. Large products were made by 30 min after the beginning of the translation reading, and these polypeptides were then processed over the course of translation into smaller structural and nonstructural proteins (Fig. 5). Substantial amounts of 1ABC were produced during the in vitro translation reactions and more VP1 was synthesized than VP3, suggesting that the VP3-VP1 (1C-1D) bond is cleaved more efficiently than the VP0-VP3 (1AB-1C) bond. This latter observation has been observed previously for in vitro translation of EMCV RNA in reticulocyte lysates (10). A protein larger than P12A and P1 was tentatively identified as LP12A by analogy with EMCV because the protein changed in electrophoretic mobility after L mutation (see below) and because the electrophoretic mobility of the protein corresponded to a predicted molecular size of 117 kilodaltons (kDa) (22).

One interesting feature of the time course relates to the presence of two bands which are labeled in Fig. 5 as L because of their altered mobility or disappearance after mutations were made in the L-coding region (see below). Longer exposures of the gel showed that the lower, more prominent band appeared as early as 5 min after the start of

FIG. 4. Immunoprecipitation of proteins produced from in vitro translation of transcripts derived from pDAFL3. Translation reaction conditions and immunoprecipitations were as described in the text. Samples were diluted in Laemmli sample buffer and electrophoresed. Lane 1 is a translation reaction programmed from transcripts derived from wild-type (WT) pDAFL3. These translation products were immunoprecipitated with anti-TMEV VP2 antiserum (lane 2), anti-TMEV VP3 antiserum (lane 3), monoclonal TMEV anti-VP1 antibody (lane 4), and anti-TMEV 3D antiserum (lane 5). The marker (M) lane in this figure displays [³⁵S]methionine-labeled proteins from an extract of DA virus-infected BHK-21 cells harvested 14 h after infection.

translation. The early appearance of this band with no subsequent change in size throughout translation (Fig. 5, lanes 3 to 8) initially raised the possibility that it represented L that autocatalytically cleaved itself from the polyprotein or that was cleaved by a host protein. However, studies involving linearization of the pDAFL3 cDNA template and L mutations (see below) suggested that the lower band, which we call l, is the product of translation from an alternative initiation site at nucleotide 1079 in the L-coding region, just downstream from the authentic polyprotein initiation codon at 1066 (22). The AUG at 1079 has a reasonable context (12) which could be used to translate a protein of about 17 kDa,

FIG. 5. Time course of in vitro translation of transcripts derived from pDAFL3. Samples were removed at the time intervals noted (', minutes). Translations and the preparation of the samples and marker (M) are described in the legend to Fig. 4. The L marker shows the location of l (see text) and, at a slightly slower mobility, L.

out of phase with the polyprotein reading frame, before encountering a stop codon at nucleotide 1547. An additional AUG at 1091 in this same reading frame could also be used, but the neighboring nucleotides substantially differ from the optimal consensus context for initiation (12). Authentic L is probably the faint band that is of slightly slower mobility than band l and that appears at a later time during translation. L seems to decline in amount during translation, perhaps related to degradation from instability, as reported for EMCV L (2). The mobility of L is slower than expected for a protein with a predicted molecular size of 9 kDa; EMCV L also demonstrates an aberrantly slow electrophoretic mobility during polyacrylamide gel electrophoresis (2, 10).

Translation of truncated pDAFL3 transcription templates. We linearized the pDAFL3 transcription template with different restriction endonucleases to produce cDNAs of varied lengths (Fig. 1). In some cases, a DA virus-infected BHK

extract was added after translation to determine the effect on processing of viral proteinases that were in the extract but were not synthesized by the truncated transcript and to verify that a modified polyprotein was still able to serve as a substrate for processing. Lanes in this and subsequent figures that contain translation reactions with an added infected extract are marked with a +.

In vitro translation of full-length transcripts derived from pDAFL3 templates that had been linearized with *Xba*I, which cuts after the 3' end of the DA genome, showed a number of viral structural and nonstructural proteins (Fig. 6A, lanes 14 and 15). In contrast, translation of transcripts derived from a pDAFL3 template linearized with *Ssp*I, pDAFL3(lin *Ssp*I), demonstrated no obvious translation product (Fig. 6A, lanes 2 and 3), presumably because the polyprotein-coding region was truncated shortly after the initiation codon. Similarly, translation of pDAFL3(lin *Xho*I) transcripts only synthesized a small truncated protein, presumably the amino-terminal part of L or l (Fig. 6A, lanes 4 and 5).

Translation of pDAFL3(lin *Hind*III) transcripts, which were derived from a pDAFL3 template linearized downstream from the L-coding region in VP2 (Fig. 6A, lane 6), demonstrated two products. The mobility of the larger product corresponded to that expected for the truncated VP1 plus L. A protein of approximately L size was detectable (lane 6), once more raising the possibility that L cleaves itself from P1. However, the absence of a protein product corresponding to the size of the truncated VP0 cleaved from L makes this unlikely and suggests that the smaller protein is l, the product of an alternative initiation. The addition of infected extract (Fig. 6A, lane 7) resulted in a somewhat smeared lower band which probably represents l and L, as well as the truncated VP0 without L.

To confirm the above conclusions, we performed an additional digestion of a DA cDNA subgenomic clone to produce a transcript of 1,961 nucleotides. Translation of this transcript produced protein products that were easily identifiable after polyacrylamide electrophoresis. Figure 6B shows the result of translation of a transcript derived from this subgenomic clone digested with *Nco*I, which cleaves in the VP0-coding region, 231 nucleotides downstream from the *Hind*III site. Two products are detectable in lane 2. The mobility of the upper band corresponded in size to that expected for L plus truncated VP0 (31 kDa), while the lower band, which we have tentatively identified as l, had a slightly faster mobility than L. No band was detectable that corresponded in mobility to that expected for the truncated VP0 without L (23 kDa). The addition of infected extract to L plus truncated VP0 (Fig. 6B, lane 3) showed an increase in intensity of the lowest band, which probably represents l and adjacent L, and the appearance of a new band corresponding in mobility to that expected for truncated VP0 from which L had been cleaved. Similar findings were also obtained from experiments with *Nco*I digestion of the full-length clone (data not shown). These results suggest that L is not autocatalytically cleaved from the polyprotein but that it can be cleaved by the addition of infected extract. Figure 6B also shows the results of translation of an in vitro-derived transcript of a GDVII subgenomic cDNA clone which was linearized with *Nco*I at nucleotide 1964. Interestingly, translation produced only a single protein that corresponded to the mobility expected for L plus truncated VP0 (Fig. 6B, lane 4). The addition of infected extract (Fig. 6B, lane 5) showed three bands: the upper band corresponds to L plus truncated VP0; the middle band corresponds to truncated

FIG. 6. In vitro translation of transcripts derived from pDAFL3 or TMEV subgenomic cDNA clones linearized at different lengths. Details regarding the linearizations are given in Fig. 1 and Materials and Methods. Translations and the preparation of the samples and marker (M) are described in the legend to Fig. 4. The L marker shows the location of L (see text) and, at a slightly slower mobility, L. Reactions that were incubated with a DA virus-infected BHK-21 cell extract after translation are marked with a +. (A) Translation reactions programmed by transcripts derived from the pDAFL3 template linearized by the restriction endonucleases listed above the lanes and depicted in Fig. 1; *XbaI* linearizes after the 3' end of the DA genome, producing a full-length template and transcript. (B) Translation reactions programmed by transcripts derived from a DA subgenomic cDNA clone (lanes 2 and 3) and a GDVII subgenomic cDNA clone (lanes 4 and 5) that were linearized with *NcoI*; the positions of the viral proteins and molecular weight markers were obtained from samples run in other lanes of the gel. K, 10^3 .

VP0 cleaved of L; and the lower band corresponds to L. These results indicate that GDVII strain RNA does not have an alternative initiation site that leads to the production of L; in fact, there is no AUG in the GDVII genome that corresponds to the AUG in the DA genome at position 1079.

The pDAFL3(lin BamHI), pDAFL3(lin StuI), and pDAFL3(lin HpaI) templates were linearized before or within the 3C-coding region (Fig. 6A, lanes 8 to 13). There was incomplete processing of translated proteins programmed from transcripts of these templates. For example, no production of capsid proteins was observed in lanes 8 to 13. These findings suggested that 3C is a major TMEV proteinase. I was detectable in all these latter translation reactions, presumably because of the maintenance of the alternative initiation site at nucleotide 1079. The most prominent translation products had mobilities smaller than expected for the polyprotein. This latter finding suggests that some processing occurred from a proteinase in addition to 3C. It is possible that this additional proteinase is 2A (or has 2A-like activity) because 2A has been proved or implicated

to have proteolytic activity in other picornaviruses (4, 31). When an infected extract was added to the translation products from pDAFL3(lin StuI) and pDAFL3(lin HpaI) transcripts, processing was fairly complete, with the appearance of authentic L (Fig. 6A, lanes 11 and 13). This result suggests that 3C was the active proteinase for most of the TMEV polyprotein cleavages, including L-P12A, and that this proteinase functioned in *trans*. In contrast, the addition of an infected extract to the translation products of pDAFL3(lin BamHI) did not improve processing (Fig. 6A, lane 9), suggesting that a proper LP12A size or conformation or both are necessary for proper processing of this fragment into capsid proteins by 3C. In FMDV, a full-length P1-2A is not required for P1 processing (32), although a full-length poliovirus P1 is necessary for P1 processing (19, 33).

Translation of pDAFL3 linker and deletion mutants. Linker or deletion mutations or both were engineered in the pDAFL3 transcription template in regions that encode proteinases in other picornaviruses (L, 2A, 3C). Translation of transcripts programmed by pDA3CΔHpaI (Fig. 7A, lane 3),

which had a deletion in the 3C-coding region, showed incomplete processing. A large precursor polypeptide was barely detectable, although smaller proteins similar in mobility to those synthesized with the latter three linearized templates (described above) were prominent. As previously noted, the finding that only very small quantities of a large precursor polypeptide were present after *in vitro* translation and only on prolonged exposures of the gel suggested that another proteinase was active. The addition of an infected extract to the translation reaction produced nearly complete processing (Fig. 7A, lane 6), again demonstrating the role of 3C as a proteinase and its ability to cleave *in trans*. A band tentatively identified as I was detectable in Fig. 7A, lanes 3 and 6, presumably because of continuing translation from the alternative initiation site. The location of a small polypeptide with an electrophoretic mobility consistent with that expected from a deleted 3C and/or authentic L that was cleaved *in trans* is noted in Fig. 7A, lane 6, with an arrow. As expected, transcripts derived from mutant pDA3CΔHpaI were not infectious when transfected into L929 cells.

Translation of a transcript derived from pDAL:Nhe, which had a linker insertion in the L-coding region, is shown in Fig. 7A, lanes 2 and 5. Normal processing was seen, and on longer exposures, a band interpreted as L was detectable (Fig. 7B, lanes 3 and 4). The transcripts of this mutant were infectious when transfected into L929 cells, which also suggested that there was normal processing. However, no I was detected in this autoradiogram (Fig. 7A, lanes 2 and 5). If I is translated from an alternative initiation site at 1079, the *NheI* linker insertion at the *XhoI* site at 1221 would create a stop codon for this alternative reading frame and result in a protein of 47 amino acids. When the translation products were analyzed on 10 to 18% gradient polyacrylamide-sodium dodecyl sulfate gels, a small protein of the expected size of truncated I was consistently observed on prolonged exposures (Fig. 7B, lanes 3 and 4, arrow) that was not present with DA wild-type translations.

In contrast to the results with pDAL:NheI, the translation products from pDALΔSspIXhoI:NheI-derived RNA showed no detectable I or L (data not shown). We presume that I was too small to detect because the deletion at the *SspI* site at nucleotide 1138 is followed by the stop codon in the *NheI* linker insertion (see previous discussion of pDAL:Nhe). The mobility of the 1ABC of the deletion mutant appeared slightly but consistently slower than that of the 1ABC of the wild type, suggesting that the deleted L was not cleaved from 1ABC in the translation reaction. The incompleteness of L-P1 cleavage was also supported by the absence of VP0 and the fact that the transcripts of the L deletion mutant

FIG. 7. *In vitro* translation of transcripts derived from wild-type (WT) and mutated pDAFL3. Details related to the generation of the mutants are given in Fig. 2 and Materials and Methods. Translation reactions were programmed with transcripts derived from the template listed above the lane. Translations and the preparation of the samples and marker (M) are described in the legend to Fig. 4. (A) Translation reactions from transcripts derived from pDAFL3, pDAL:NheI, and pDA3CΔHpaI. The position of L and/or truncated 3C in lane 6 is shown with an arrow. Lanes 4 to 6 represent samples in which DA virus-infected extract was added after translation (+ inf BHK). (B) Detail of lanes showing translation reactions from transcripts of wild-type (WT) pDAFL3 and pDAL:NheI run on a 10 to 18% gradient polyacrylamide-sodium dodecyl sulfate gel. The autoradiogram was intentionally overexposed to show the position of a small protein in lanes 3 and 4 (arrow) that has been tentatively identified as a truncated I.

FIG. 8. In vitro translation of transcripts derived from wild-type and mutated pDAFL3. Details related to the translation and generation of pDA2A:Clal are given in Fig. 2 and Materials and Methods. Translations and the preparation of the samples and marker (M) are described in the legend to Fig. 4. Lanes 3 and 5 represent samples in which DA virus-infected extract was added after translation and are marked with a +. The position of the mutated 2A with slower mobility than authentic 2A is shown with an arrow.

were not infectious (data not shown). We presume that the L-P1 that is synthesized after the L deletion is no longer an appropriate template for 3C cleavage of L-P1 and that L stays attached to the capsid proteins and leads to a loss of infectivity. Longer exposures of the gel showed slightly increased mobility of LP12A, as expected from translation of transcripts from the L deletion mutant.

Transcripts from the pDA2A:Clal linker insertion mutation generated translation products that were nearly identical to those produced from wild-type transcripts (Fig. 8, lanes 2 and 4). In addition, DA2A:Clal in vitro-derived transcripts were infectious when transfected into L929 cells (data not shown). These results suggest that 2A is not a proteinase or that the linker insertion mutation did not inactivate its proteolytic activity. The 2A activity of EMCV is difficult to

inactivate, despite extensive mutations (A. Palmenberg, personal communication as cited in reference 4).

DISCUSSION

We provided data concerning in vitro translation of RNAs generated from wild-type and modified full-length clones of the DA strain of TMEV. For these investigations, we made use of transcription templates of different lengths obtained by linearization with different restriction endonucleases and of transcription templates that had been mutated in putative proteinase-coding regions.

Linearization and mutant studies showed that transcripts which failed to synthesize a complete 3C had incompletely processed proteins with no evidence of capsid proteins. Proteinase 3C can function *in trans* since the addition of DA-infected BHK extract caused complete and efficient processing of the polyprotein. These findings indicate that protein 3C is the major proteinase for TMEV, as is true for the other picornaviruses that have been studied.

Despite the absence of 3C activity in the translation reactions described above, certain cleavages of the polyprotein were still apparent. The mobilities of the major translated products were most compatible with proteins of the molecular weight of LP12A and 2BCP3 (refer to Fig. 7A), suggesting that an additional proteinase was active. We performed a linker insertion mutation of 2A because its demonstrated role in poliovirus polyprotein processing (31) and its presumed role in polyprotein processing of EMCV and FMDV make it a candidate for carrying out the LP12A-2B cleavage (4). Although we were unable to prove that 2A is a proteinase, the study of translation reactions programmed from transcripts of 2A deletion mutations may be helpful in future investigations. However, the 2A active peptide may be small and difficult to inactivate. Recent evidence suggests that FMDV 2A acts as a proteinase in subgenomic clones despite its small size (16 amino acids) (4).

Translation of the DA cDNA transcription template produced a protein of slightly faster electrophoretic mobility than L. Although the appearance of this protein early in translation suggested the possibility of autocatalytic cleavage of L from the polyprotein, linearization and mutation studies suggested that this was not the case and were consistent with the conclusion that the early protein was a product of an alternative initiation site. We suspect that this protein, I, is synthesized from an alternative initiation site at nucleotide 1079 in the L-coding region out of phase with the polyprotein reading frame. A stop codon at 1547 would predict I to have the mobility that we observed on gels, which corresponded to a protein of 17 kDa. Future immunoprecipitation studies with antiserum directed against a peptide synthesized from predicted amino acids of I and the use of *N*-formyl-[³⁵S]methionyl tRNA should help clarify this issue. FMDV has two independent translation initiation sites in the L-coding region that are used in vivo and in vitro (5, 28); in contrast to DA, these sites are in the same reading frame and produce alternative forms of L with different amino termini.

Is I an artifact of the in vitro reticulocyte lysate translation system or is it an authentic product of infection? It is possible that cellular factors bind to TMEV RNA to influence the efficiency and site(s) of initiation of translation and to determine whether I is synthesized. The absence of I in the labeled marker obtained from infected BHK-21 cells suggests that I is not normally produced in these cultured cells. However, it is possible that particular neural cells within the

central nervous system translate the TMEV genome in a manner similar to that of the reticulocyte lysate and lead to the synthesis of I. Of interest is the fact that the RNA genomes of both the DA and BeAn strains, the only TO subgroup strains that have been sequenced (22, 26), have an AUG at 1079 or the equivalent nucleotide position, while the GDVII genome does not (25). One question that arises is whether the presence of I is important in the pathogenesis of the different central nervous system disease that is caused by TO subgroup strains.

The continued study of TMEV polyprotein cleavages, translation initiation, and translation efficiency—processes key in regulating picornaviral gene expression—will hopefully clarify the pathogenesis of this virus infection and the molecular basis for the varied biological activities of the strains. Altered DA polyprotein processing, the production of I, or diminished translational efficiency may play a role in the restricted expression and persistence of DA in certain cell types. Our ability to generate mutations in the infectious pDAFL3 clone and then inoculate the mutated virus into the natural host, the mouse, will be important in these on-going investigations.

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