JOURNAL OF VIROLOGY, Oct. 1996, p. 6870–6875 Vol. 70, No. 10
0022-538X/96/$04.00+0
Copyright © 1996, American Society for Microbiology

# Mutational Analysis of the Encephalomyocarditis Virus Primary Cleavage

HARRY HAHN AND ANN C. PALMENBERG*

*Institute for Molecular Virology and Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, Wisconsin 53706*

Received 20 February 1996/Accepted 26 June 1996

**Sixteen substitution mutations of the conserved DvExNPGP sequence, implicated in cardiovirus and aphthovirus primary polyprotein cleavage, were created in encephalomyocarditis virus cDNA, expressed, and characterized for processing activity. Nearly all the mutations severely decreased the efficiency of the primary cleavage reaction during cell-free synthesis of viral precursors, indicating a stringent requirement for the natural sequence in this processing event. When representative mutations were tested in full-length genomic contexts, they were lethal and no revertants were observed. Not only were the primary cleavage reactions deficient in these polyproteins, but subsequent cleavage of P1 by endogenous or exogenous 3C$^{pro}$ was also impaired. This indicates that primary cleavage has a role in the proper processing of the viral capsid precursor.**

Encephalomyocarditis virus (EMCV) is a cardiovirus of the picornavirus family. Its single, positive-stranded RNA genome encodes a large open reading frame that, upon infection, is translated into a single polyprotein. This polyprotein is subsequently processed via a series of proteolytic events into its component proteins (11).

This proteolytic cascade is common to all picornaviruses and largely mediated by a virally encoded protease, 3C$^{pro}$. An exception is the first polyprotein scission, a cotranslational, autoproteolytic cleavage referred to as the primary cleavage. The primary cleavage can occur in one of two places: between 1D and 2A in enteroviruses and rhinoviruses or between 2A and 2B in cardioviruses and aphthoviruses. In enteroviruses and rhinoviruses, the cleavage is mediated by a self-encoded protease, 2A$^{pro}$, and does not require downstream viral proteins (20, 21). In cardioviruses and aphthoviruses, the cleavage also occurs cotranslationally but the 2A protein is not a protease and the reaction mechanism less understood (2, 7, 12, 19).

Previous reports have suggested that the primary cleavage of cardioviruses and aphthoviruses is not mediated by a virally encoded protease but rather that the scission occurs through a unique mechanism based on an inherent instability of the sequences that flank the primary cleavage site. With both EMCV and Theiler's murine encephalomyelitis virus, a related cardiovirus, primary cleavage can occur in the absence of large parts of the viral genome (2, 12). Studies of foot-and-mouth disease virus, an aphthovirus, further narrow the responsible sequences to a short viral segment no larger than 19 amino acids located at the 2AB junction (17). Furthermore, segments as short as 13 amino acids that contain this junction proved sufficient to mediate cleavage when transferred into a nonviral context (16).

The viral sequence implicated in these studies contains a stretch of eight amino acids highly conserved across cardioviruses and aphthoviruses. The consensus sequence is DvExN PGP, with uppercase letters denoting residues with absolute sequence conservation (11). The sequence found in EMCV is DIETNPGP. The primary cleavage occurs between the last two residues of this sequence (Gly-Pro) (12). The conserved sequence is extremely rare within current databases—the only examples are from cardioviruses, aphthoviruses, and group C porcine rotaviruses—and is always associated with a proteolytic cleavage activity (2, 9, 12, 17). The rarity of this sequence and its high degree of conservation suggest that this sequence is important to primary cleavage. We have thus targeted this sequence in EMCV for mutational analysis to determine the specific requirements of primary cleavage.

* Corresponding author. Mailing address: Department of Animal Health and Biomedical Sciences, University of Wisconsin, 1655 Linden Dr., Madison, WI 53706. Phone: (608) 262-7519. Fax: (608) 262-7420. Electronic mail address: acp@ahabs.wisc.edu.

## MATERIALS AND METHODS

**Nomenclature.** Standard single-letter codes are used to designate amino acids. In our convention, uppercase letters denote the EMCV strain Rueckert (EMCV-R) sequence while lowercase letters indicate sequences that differ from EMCV-R. For brevity, only the first (DIET) or second half (NPGP) of the conserved octamer is stated, and it may be assumed that the other four residues are of wild-type sequence. An asterisk following a viral gene indicates that only a partial sequence is present.

**Plasmid construction.** Standard recombination methods were used for plasmid construction (1, 18). Plasmid pE51D2A contains cDNA from the EMCV-R internal ribosome entry site followed by a contiguous viral segment encoding proteins 1C (partial), 1D, 2A, and 2B (partial) (13). The viral fragment between the *Eco*RI and *Bam*HI sites of this construct was excised and transferred to pBS-SK+ (Stratagene) to create pSK+1D2A (Fig. 2A). This plasmid was used as a template for mutagenesis of the DIETNPGP sequence according to the Transformer Site-Directed Mutagenesis System (Clontech). Sixteen substitutions, encoding at least one replacement at each location of the DIETNPGP sequence, were obtained and confirmed by sequencing. Four mutations, encoding nIET, DIdT, kPGP, and NPaP, were also analyzed in longer viral contexts. The pSK+1D2B plasmids were created by replacing the *Xcm*I-to-*Sac*II restriction fragment of pSK+1D2A with the analogous viral fragment flanked by those sites (Fig. 2B). This construct differs from pSK+1D2A by including all of 2B and a portion of 2C. The same four mutations were additionally transferred to full-length viral cDNAs by replacing the *Bsu*36I-to-*Sac*II fragment of pE-$C_9$ (4) with the analogous fragment from pSK+1D2B (Fig. 2C).

**In vitro translation.** Translation and primary cleavage of the mutant sequences were assayed in rabbit reticulocyte lysates by one of two methods. In the first, RNA was transcribed by T7 RNA polymerase from a DNA template (2 μg) linearized 3′ of the viral sequence of interest. The RNA (1 to 2 μg) was then used to program translation by reticulocyte lysate in a final volume of 15 or 30 μl (8, 14). In the second, a circular DNA template (0.5 to 1 μg) was used to program coupled transcription-translation reactions in a volume of 12.5 or 25 μl (3) (Novagen's Single Tube Protein System). All reactions proceeded at 30°C for 60 min and then were terminated by addition of RNase A and cycloheximide, each to 0.4 mg/ml. When appropriate, exogenous, purified recombinant mengovirus 3C$^{pro}$ was added to a final concentration of 12 μg/ml (5). Primary cleavage activity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Laemmli or 5 to 20% Laemmli gradient gels. Densitometric analysis was conducted with a PhosphorImager and ImageQuant software (Molecular Dynamics).

**Tissue culture infectivity.** RNA infectivity studies were conducted in HeLa

```
                                          primary
                                          cleavage
                                             |
FMD         nFdllkLag.DVEsNPG | Pf
EMC         gYfadlLih.DIEtNPG | Pf
Mengo       gYfsdlLih.DVEtNPG | Pf
TMEbean     dYyrqrLih.DVEtNPG | Pv
TMEGd7      dYykqrLih.DVEmNPG | Pv
RotaC/C     fqidkiLisgDVElNPG | Pd
RotaC/S     fqidriLisgDIElNPG | Pn
Consensus   ------L---DVE-NPG | P-
                      consensus
                       octamer
```

FIG. 1. Natural NPGP sequences. Representative polyprotein and consensus sequences near the region of the primary cleavage sites of aphthoviruses (foot-and-mouth disease virus [FMD]), cardioviruses (EMCV [EMC], mengovirus [Mengo], and Theiler's murine encephalomyelitis virus [TMEbean and TMEGd7]), and group C rotaviruses (RotaC/C and RotaC/S) are shown. The primary cleavage occurs between the glycine and proline dipeptide pair (G-P) (12).

cells as previously described (4, 6). Briefly, pE-$C_9$ and mutant derivatives were cleaved 3′ of viral sequences at a *Sal*I site (Fig. 2C). T7 RNA polymerase transcripts (5 to 500 ng) were transfected into confluent HeLa cell monolayers (60-mm-diameter plates) with dimethyldioctadecylammonium bromide (15). The monolayers were incubated at 37°C under 5% $CO_2$, and plaque formation was assayed at 30, 48, or 72 h posttransfection.

## RESULTS

**Primary processing of mutant sequences.** For each position in the EMCV octapeptide (DIETNPGP), one to four amino acid substitutions were engineered (Fig. 3B). Some substitutions were selected because of expected functional conservation (e.g., I to V, E to D, and G to A). Others were chosen to test permissible limits of radical substitution (e.g., D to H, P to R, and G to W). The primary cleavage was assayed in the absence of other processing events by expressing each pSK+1D2A sequence in reticulocyte extracts via coupled transcription-translation in the context of a truncated polyprotein (Fig. 3A). Conversion of precursor 1C*1D2A2B* into product 1C*1D2A by the wild-type sample was taken as benchmark of an efficient reaction. The smaller cleavage product, 2B*, was not visible on these gels.

The importance of certain positions within the octamer sequence was immediately apparent from this study. The three most active sequences had substitutions in the octamer 2, 3, and 4 positions (DvET, DIdT, and DIEa), although location alone did not predict the reactivity, as an alternative substitution at the 2 position (DfET) was completely inactive. Also inactive were certain substitutions at the 1, 6, and 7 positions (hIET, NlGP, and NPxP). The remaining mutations, at the 1, 5, 6, and 8 positions, had low but detectable activity.

**Processing kinetics of mutant sequences.** Four of the mutations spanning the octapeptide and representative of the high-activity (DIdT), low-activity (nIET and kPGP), and inactive (NPaP) sequences were transferred to a larger viral context, pSK+1D2B, encoding the viral 2B and 2C (partial) sequences. Cell-free expression showed the primary reactivities to be the same as for the smaller plasmids, with both product bands evident (Fig. 4, lanes I). The wild type and the DIdT mutant cleaved efficiently, nIET and kPGP cleaved only partially, and NPaP did not cleave at all. Overnight incubation left the patterns unchanged, indicating that the uncleaved precursor (1C*1D2A2B2C*) was stable (Fig. 4, lanes II), as has been reported for other primary reactions (2, 12, 17).

These uncleaved proteins were not functionally inert, however, and new bands appeared when $3C^{pro}$ was added (Fig. 4,

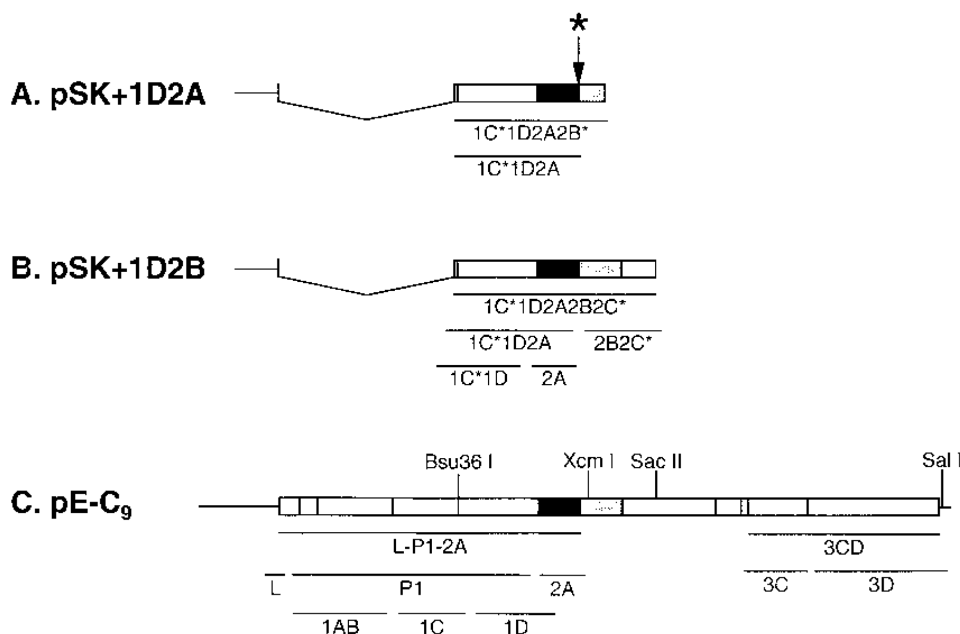


FIG. 2. NPGP-containing EMCV cDNA clones. (A) pSK+1D2A is derived by transfer of the *Eco*RI-to-*Bam*HI fragment of pE51D2A, previously described (13), into pBS+SK and contains a portion of 1C, all of 1D and 2A, and a portion of 2B. This construct was used as a template for site-directed mutagenesis. The primary cleavage site is marked with an arrow. (B) pSK+1D2B was created by transferring the *Xcm*I-to-*Sac*II fragment from pE-$C_9$ into pSK+1D2A. pSK+1D2B contains a portion of 1C; all of 1D, 2A, and 2B; and a portion of 2C. (C) Mutations were engineered into a full-length viral cDNA context by transferring the mutation-containing *Bsu*36I-to-*Sac*II fragment from pSK+1D2B to pE-$C_9$.

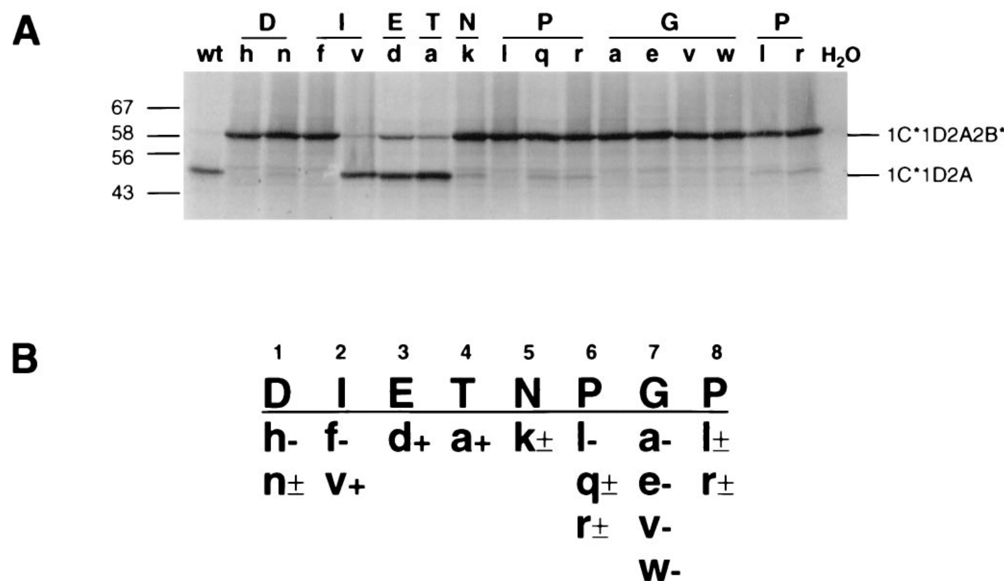


FIG. 3. Primary cleavage of mutant sequences. (A) Plasmid pSK+1D2A cDNAs containing wild-type or mutant sequences were added to coupled transcription-translation reactions (12.5 μl). After 1 h at 30°C, loading buffer (12.5 μl) was added and the samples were boiled and then electrophoresed through an SDS–12% PAGE gel. wt, wild type. Molecular weights (in thousands) are indicated on the left. (B) The extent of primary cleavage was quantitated by densitometry with Molecular Dynamic's PhosphorImager and ImageQuant software. +, >80% cleavage efficiency; ±, 10 to 30% cleavage efficiency; and −, <10% cleavage efficiency, relative to the uncleaved precursor band.

lanes III). Mutant precursor (1C*1D2A2B2C*) from inefficient primary reactions (nIET, DIdT, and NPaP) was cleaved into 1C*1D and 2A2B2C*, suggesting that $3C^{pro}$ recognition of the 1D/2A site was not unduly affected by the inability to execute a proper primary cleavage. In contrast, $3C^{pro}$ activity at the 2B/2C site was influenced by presentation. Although this site is usually among the most reactive in the polyprotein (5), it was not cleaved within any tested sequence, perhaps because the 2C* segment of the substrate was synthesized in a truncated context. Likewise, cleavage at the 1C*/1D site did not occur in these reactions. Again, the truncated substrate (only 17 amino acids of 1C are present) may have influenced $3C^{pro}$ reactivity. Abnormal context may have also influenced the unexpected 40-kDa bands in the wild-type and DIdT samples, which may represent aberrant $3C^{pro}$ events within the 1C*1D2A precursor.

Additionally, a time course for the reaction was performed to establish the kinetic profile of primary cleavage activity (Fig. 5). For the wild-type and DIdT sequences, cleavage occurred rapidly, with products appearing at 20 min. Very little, if any, uncleaved precursor was observed. The small amount of uncleaved product seen for these sequences appeared to be stable, as no additional cleavage of this product was observed, even after overnight incubation. Similarly, the other, poorly cleaving sequences (data not shown for kPGP or NPaP) displayed some cleavage that appears to have occurred cotrans-

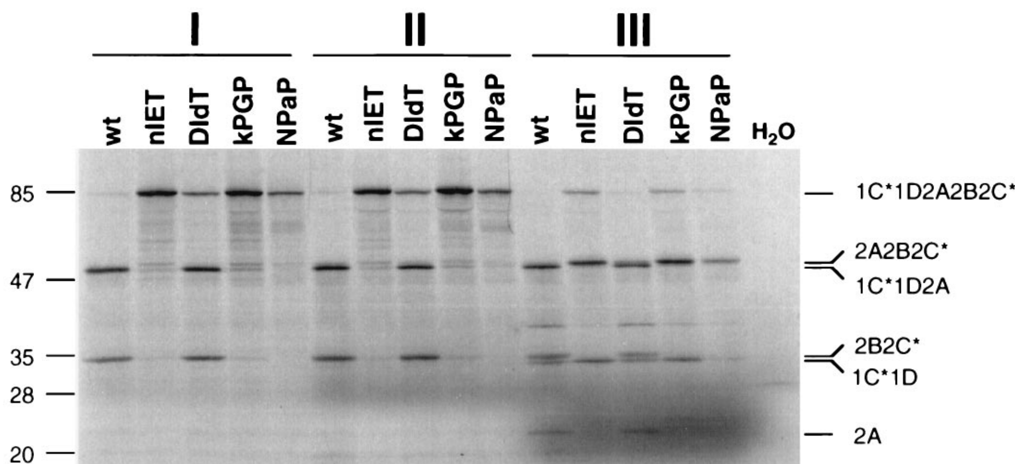


FIG. 4. Primary cleavage and $3C^{pro}$ processing of mutant sequences. Plasmid DNA (0.5 μg) was used to program coupled transcription-translation reactions (volume, 25 μl). After 1 h at 30°C, the reaction volume was split three ways. One third was placed at −70°C (lanes I). To another third, RNase A and cycloheximide were added, and the reaction mixture was incubated at 30°C overnight (lanes II). RNase A, cycloheximide, and $3C^{pro}$ were added to the final third before incubation at 30°C overnight (lanes III). All samples were then collected, and loading buffer (8 μl) was added, boiled, and electrophoresed through a 5 to 20% gradient polyacrylamide gel. wt, wild type. Molecular weights (in thousands) are shown on the left.

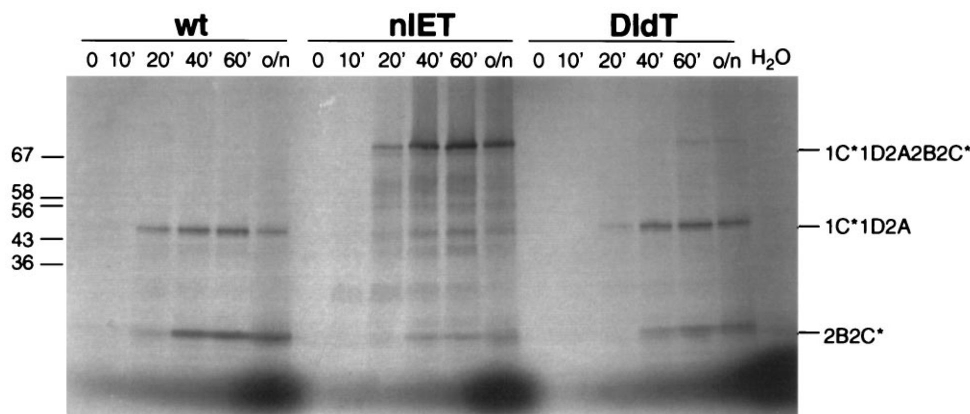


FIG. 5. Time course of primary cleavage activity. Wild-type (wt), nIET, and DIdT pSK+1D2B DNA (2 μg each) was linearized with *Sac*II and transcribed by T7 RNA polymerase. The RNA (2 μg) then programmed 30-μl translation reactions at 30°C. Aliquots (5 μl) were removed at the indicated times, reacted with RNase A and cycloheximide, and placed at −70°C until the time course was complete. Samples were then analyzed as described in the legend to Fig. 4. o/n, overnight. Molecular weights (in thousands) are shown on the left.

lationally. The uncleaved product did not appear to cleave over time, although a general degradation of protein products was observed after overnight incubation. This indicates that the bond was stable if cleavage was not cotranslational.

**Processing of full-length sequences.** To examine the effect of primary cleavage reactions on the processing of P1 precursors, pE-$C_9$ plasmids containing wild-type, DIdT, kPGP, and NPaP sequences were linearized within their 2B sequences with *Bsa*HI. Transcripts from these templates directed equivalent synthesis of P1-containing protein (Fig. 6A, lanes I). For the wild-type and DIdT sequences, the prominent product was the expected L-P1-2A, whereas the poorly cleaving sequences, kPGP and NPaP, produced larger products corresponding to L-P1-2A2B*. Overnight incubation showed that these products were largely stable, and only a slight amount of general degradation was observed (Fig. 6A, lanes II).

However, when exogenous $3C^{pro}$ was added to these samples, dramatic differences between the wild-type and mutant sequences became apparent (Fig. 6A, lanes III). The wild-type L-P1-2A product was processed to capsid proteins 1AB, 1C, and 1D. Intermediate precursor 1ABC was also visible. Surprisingly, all of the mutant sequences were defective in this processing, even if primary cleavage had occurred efficiently. The DIdT precursor was processed to P1, but subsequent processing steps were impaired. Although some 1ABC was produced, little if any of the fully processed product 1AB, 1C, or 1D was visible. The poorly cleaving mutant sequences kPGP and NPaP were even more impaired. Processing to P1 was less efficient than with the wild type or DIdT, and no further processing of P1 was evident.

To determine the effect of the primary cleavage mutations on translation of the entire EMCV genome, full-length RNAs were transcribed from *Sal*I-linearized pE-$C_9$ cDNAs containing wild-type, nIET, DIdT, kPGP, and NPaP sequences. When translated in cell extracts, these RNA transcripts also directed equivalent and efficient synthesis of the entire polyprotein (Fig. 6B, lanes I). Mutant sequences gave no evidence that overall synthesis of completed polyprotein was affected, as P3 proteins, notably 3CD and 3D, were present in all samples. However, as expected, some differences in the protein patterns of the poorly cleaving mutant sequences were apparent. Specifically, a number of high-molecular-weight species, putatively identified as P1-region polypeptides, were visible in the nIET, kPGP, and NPaP samples, suggesting that their defective primary reactions had impacted on subsequent cascade events. The DIdT sample, on the other hand, processed similarly to the wild type.

After overnight incubation, some additional processing occurred in all samples, presumably through low-level activity of the endogenously synthesized $3C^{pro}$ (Fig. 6B, lanes II). The 3D band was darker in all samples, for example, but there was little evidence of change in the high-molecular-weight species. The addition of exogenous $3C^{pro}$, however, again produced marked differences in the processing patterns (Fig. 6B, lanes III). In the wild-type sample, the P1-region precursor was cleaved completely into component capsid products (e.g., 1AB, 1C, and 1D). The high-molecular-weight species in the mutant samples also disappeared when $3C^{pro}$ was added and were replaced with more-dense P1 bands, suggesting that the P2 and P3 moieties of these products were processed. As before, however, none of the mutant sequences, including DIdT, seemed capable of further P1 processing. $3C^{pro}$ was again inactive on these substrates if the polyprotein contained a mutant sequence, regardless of whether the sequence underwent primary cleavage (i.e., DIdT). This suggests that the sequence surrounding the primary cleavage had ramifications beyond the local effect on primary cleavage.

Another unexpected observation in this experiment was the presence of a protein species in the nIET, kPGP, and NPaP reactions that comigrated with the L-P1-2A band of wild-type and DIdT reactions. Authentic L-P1-2A was not expected in these samples, as all other evidence suggested that the primary cleavage did not occur to an appreciable extent with these mutations. We are currently examining whether this product is the result of spurious $3C^{pro}$-mediated cleavage near the primary cleavage site or a fortuitous comigration of an altogether different precursor of similar mass (such as P1-2A2B).

**Tissue culture infectivity.** To assess the effect of the primary cleavage mutations on viral replication, full-length wild-type and mutant RNAs (up to 500 ng) were transfected into HeLa cells. pE-$C_9$ RNA produced medium-size plaques after 30 h and had a specific infectivity of approximately 1,000 PFU/μg (data not shown), consistent with previous reports (4). The mutant RNAs, however, gave no plaques under any of the conditions described. The transfected cells were lysed, replated, and reincubated for up to 72 h with no indication of viral infectivity, even from the DIdT mutant (data not shown).

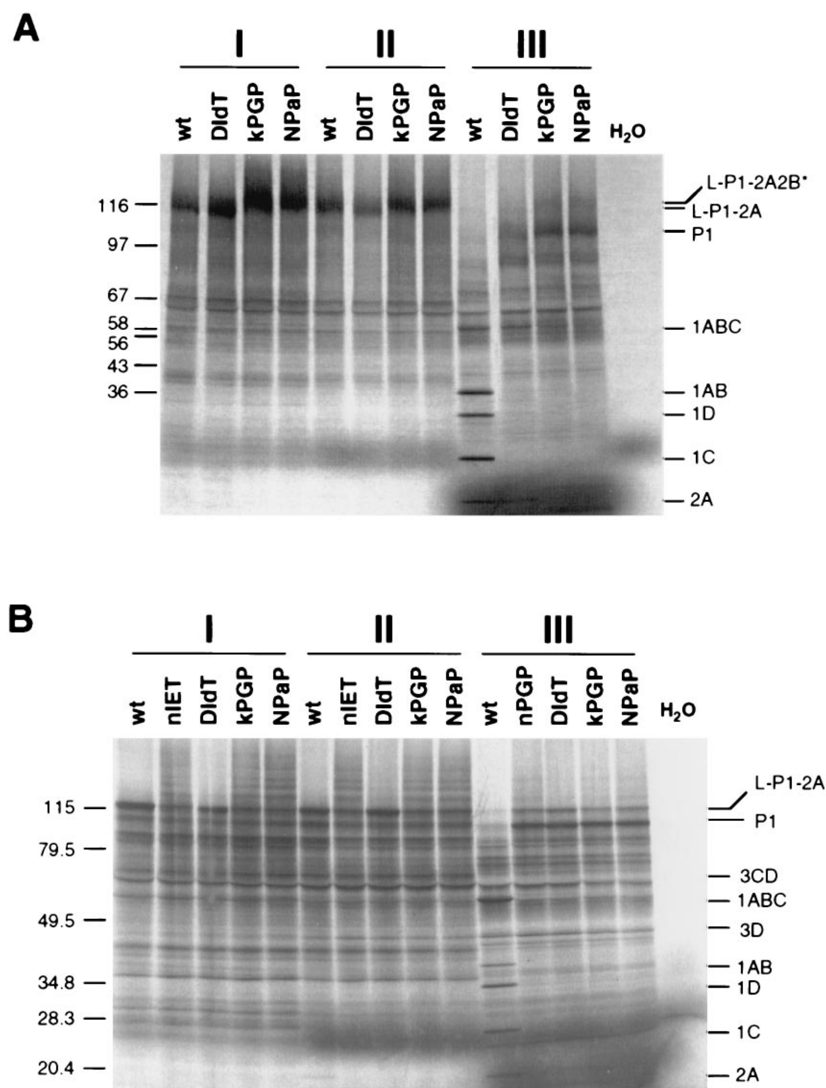


FIG. 6. Processing from full-length cDNA. Plasmid pE-$C_9$ RNA transcripts derived from cDNAs linearized with *Bsa*HI (A) or *Sal*I (B) were translated and processed as described in the legend to Fig. 4: I, 1-h reaction; II, overnight processing; III, overnight processing with exogenous $3C^{pro}$; wt, wild type. Molecular weights (in thousands) are shown on the left.

## DISCUSSION

A variety of indirect evidence has indicated that the highly conserved DvExNPGP sequence found at the primary cleavage site of EMCV is important for primary cleavage (11, 12, 17). Through mutational analysis, we have now shown that stringent sequence requirements do indeed exist for this octamer in EMCV and that even apparently conservative substitutions can have dramatic effects on primary cleavage and viral replication.

Specifically, we discovered that only 3 of the 16 substitutions that we created in this octapeptide were able to cleave efficiently at the primary cleavage site. One of these substitutions, DvET, mutates the EMCV sequence to one found naturally in mengovirus, Theiler's murine encephalomyelitis virus, foot-and-mouth disease virus, and rotaviruses. Val at this position is more prevalent than Ile, which is found only in EMCV and one rotavirus strain. A second acceptable substitution, DIEa, occurs at a position where octamer identity is unusually lax: Thr, Ser, Met, and Leu are all found naturally at this position. The third efficient cleaver, DIdT, is a conservative Glu-to-Asp mutation. Though a consensus Glu at this position is a defining characteristic of the octamer, replacement with Asp resulted in a strong functional sequence in cell-free assays, suggesting that this location does not participate fully in autoproteolysis. All of the remaining 13 mutations effectively crippled the primary cleavage reaction. The seventh position, Gly, on the amino side of the processed bond, was putatively identified as having the highest mutational sensitivity, and even the most conservative replacement, NPaP, was inactive. We conclude that the DvEx NPGP segment, and especially the second quartet (NPGP), is probably highly tuned for biological function and little sequence variation can be tolerated.

In addition to furthering the definition of the sequence requirements for primary cleavage, we have uncovered a putative role for this cleavage in EMCV. Poliovirus primary cleavage appears to have a role in processing of the capsid precursor in that primary cleavage between 1D and 2A in that system must precede processing of P1. Mutations that prevent primary cleavage result in a P1 precursor resistant to $3C^{pro}$ processing

(10). We have uncovered a similar role for the primary cleavage of EMCV. Although the location and mechanism for primary cleavage are entirely different from those of poliovirus, EMCV primary cleavage, carried out by the natural sequence, was also a prerequisite for P1 processing. Of the proteins synthesized by our mutant sequences, most $3C^{pro}$ cleavage sites should have remained accessible for posttranslational processing. The primary reaction, however, and the specific sequences used to carry it out left an indelible imprint on the P1 precursor and determined its ability to be subsequently cleaved into mature capsid proteins. Surprisingly, this effect was also generally true for the DIdT sequence, which, although not a natural viral sequence, was still about 80% as reactive in vitro as the wild type. The lack of reactivity of mutant P1 precursors to further processing presumably explains why these sequences were not infectious. The results also suggest that primary cleavage itself, though necessary, is not sufficient to ensure $3C^{pro}$-mediated P1 reactivity and that other functional requirements, highly sensitive to changes, exist within the DIETNPGP sequence.

Although we presently understand very little about the primary cleavage mechanism, two obvious models might explain these sequence consequences for the P1 precursors. In the first model, primary cleavage might be presumed to play an obligate role in the folding of the nascent polypeptide. Folding of the picornaviral polypeptide must be tightly regulated, and the primary cleavage, occurring where and when it does, ensures proper folding of the L-P1-2A precursor, making it a suitable substrate for $3C^{pro}$-mediated processing. The behavior of the DIdT mutation further suggests that the carboxy-terminal portion of this precursor is of particular importance, not only to the primary cleavage itself but to folding of the released polypeptide.

The second hypothesis suggests that the order of cleavage events is critical in L-P1-2A processing. In the natural P1 region proteolytic cascade, the preferred cleavage progression is 2A/2B (primary), then L/1A, 1D/2A, 1C/1D, and 1AB/1C (13). A failed or sequence-defective primary reaction may, through steric hindrance or improper product release, prevent sequential exposure of the other four internal sites in their usual turns. Such a mechanism might be especially inhibitory to the latter steps of the process, as observed with the in vitro reactions.

These models are not necessarily mutually exclusive nor inclusive of all possibilities. Common between them is the idea that a defective primary cleavage, or one carried out by an unacceptable sequence (e.g., DIdT) could have a dramatic effect on the suitability of L-P1-2A to serve as a substrate for $3C^{pro}$. We are planning additional mutations and sequence mapping to explore these possibilities.

## ACKNOWLEDGMENTS

We thank D. Hall for construction of DIdT, NlGP, and NPwP and providing $3C^{pro}$ and A. Kaminski for providing protein markers.

This work was supported by National Institutes of Health grant AI-17331 to A.C.P. and training grant GM-07215 to H.H.

## REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moor, J. G. Seidman, J. A. Smith, and K. Struhl.** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Batson, S., and K. Rundell.** 1991. Proteolysis at the 2A/2B junction in Theiler's murine encephalomyelitis virus. Virology **181:**764–767.
3. **Craig, D., M. T. Howell, C. L. Gibbs, T. Hunt, and R. J. Jackson.** 1992. Plasmid cDNA-directed protein synthesis in a coupled eukaryotic in vitro transcription-translation system. Nucleic Acids Res. **20:**4987–4995.
4. **Hahn, H., and A. C. Palmenberg.** 1995. Encephalomyocarditis viruses with short poly(C) tracts are more virulent than their mengovirus counterparts. J. Virol. **69:**2697–2699.
5. **Hall, D. J., and A. C. Palmenberg.** Mengo virus 3C proteinase: recombinant expression, intergenus substrate cleavage and localization *in vitro*. Virus Genes, in press.
6. **Hoffman, M. A., and A. C. Palmenberg.** 1995. Mutational analysis of the J-K stem-loop region of the encephalomyocarditis virus IRES. J. Virol. **69:**4399–4406.
7. **Jackson, R. J.** 1986. A detailed kinetic analysis of the in vitro synthesis and processing of encephalomyocarditis virus products. Virology **149:**114–127.
8. **Jackson, R. J., and T. Hunt.** 1983. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. Methods Enzymol. **96:**50–74.
9. **Langland, J. O., S. Pettiford, B. Jiang, and B. L. Jacobs.** 1994. Products of the porcine group C rotavirus NSP3 gene bind specifically to double-stranded RNA and inhibit activation of the interferon-induced protein kinase PKR. J. Virol. **68:**3821–3829.
10. **Nicklin, M. J. H., H. G. Kräusslich, H. Toyoda, J. J. Dunn, and E. Wimmer.** 1987. Poliovirus polypeptide precursors: expression in vitro and processing by exogenous 3C and 2A proteinases. Proc. Natl. Acad. Sci. USA **84:**4002–4006.
11. **Palmenberg, A. C.** 1990. Proteolytic processing of picornaviral polyprotein. Annu. Rev. Microbiol. **44:**603–623.
12. **Palmenberg, A. C., G. D. Parks, D. J. Hall, R. H. Ingraham, T. W. Seng, and P. V. Pallai.** 1992. Proteolytic processing of the cardioviral P2 region: primary 2A/2B cleavage in clone-derived precursors. Virology **190:**754–762.
13. **Parks, G. D., and A. C. Palmenberg.** 1987. Site-specific mutations at a picornavirus VP3/VP1 cleavage site disrupt in vitro processing and assembly of capsid precursors. J. Virol. **61:**3680–3687.
14. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67:**247–256.
15. **Rose, J. K., L. Buonocore, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10:**520–525.
16. **Ryan, M. D., and J. Drew.** 1994. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO J. **13:**928–933.
17. **Ryan, M. D., A. M. Q. King, and G. P. Thomas.** 1991. Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. J. Gen. Virol. **72:**2727–2732.
18. **Sambrook, J. E., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Shih, D. S., C. T. Shih, D. Zimmern, R. R. Rueckart, and P. Kaesberg.** 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. **30:**472–480.
20. **Sommergruber, W., M. Zorn, D. Blaas, F. Fessl, P. Volkmann, I. Maurer-Fogy, P. Pallai, V. Merluzzi, M. Matteo, T. Skern, and E. Kuechler.** 1989. Polypeptide 2A of human rhinovirus type 2: identification as a protease and characterization by mutational analysis. Virology **169:**68–77.
21. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45:**761–770.