JOURNAL OF VIROLOGY, June 1991, p. 3395–3399
0022-538X/91/063395-05$02.00/0

Vol. 65, No. 6

Alternative Translation Initiation Site in the DA Strain of Theiler's Murine Encephalomyelitis Virus

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Polyprotein processing studies of Theiler's murine encephalomyelitis virus (TMEV), a group of mouse picornaviruses, demonstrated synthesis of a protein we have called l during in vitro translations from the RNA of DA, a demyelinating strain of TMEV, but not GDVII, an acute neurovirulent strain. We have proposed that l is synthesized from an alternative initiation site in the DA leader (L) coding area out of phase with the polyprotein reading frame (R. P. Roos, W.-P. Kong, B. L. Semler. J. Virol. 63:5344–5353, 1989). We now provide support for this proposal from experiments involving in vitro translation of three separate mutations of an infectious DA cDNA clone: DA"l"-1, which contains a base mismatch at the putative initiation codon of l, DAL-1, which contains a base mismatch at the presumed authentic initiation site of L at the beginning of the polyprotein; and DAL:NheI, which contains nucleotides coding for a four-amino-acid insertion in the L coding area with a termination codon in the l reading frame. Our results demonstrate that the DA strain uses an alternative initiation site and reading frame to in vitro synthesize l. l may have a role in the biological activity of the virus.

Theiler's murine encephalomyelitis viruses (TMEV) are a group of serologically related picornaviruses, most closely related to the genus *Cardiovirus*, that cause neurological and enteric disease in the mouse (13, 15, 18). TMEV strains are divided into two subgroups on the basis of their different biological activities (10, 11). The DA strain and other members of the TO subgroup cause a progressive demyelinating disease that is associated with persistent infection. In contrast, the GDVII strain and other members of the GDVII subgroup cause an acute lethal motor neuronal infection. The determinants for these varied diseases are in the process of being defined, partly on the basis of chimeric cDNA studies and site-directed mutagenesis of infectious TMEV cDNAs.

Translation of picornavirus RNA is said to "break the rules" of normal eukaryotic translation (7). In normal eukaryotic translation, ribosomes are believed to enter the 5′ capped end of the mRNA and then scan until an AUG with satisfactory context for translation initiation is reached (9). In contrast ribosomes bind to an internal ribosomal entry site in the 5′ noncoding region of the picornavirus genome and then proceed to the appropriate initiating AUG (8, 16). A number of other potential initiation codons are bypassed as a result of this internal binding. In the case of the DA strain of TMEV, eight AUGs are bypassed prior to initiation occurring at N1066 (14). The designation of the AUG at N1066 as the initiation codon is based on alignments from other picornaviruses, the presence of a good consensus sequence, and the prediction of a downstream long open reading frame. Following initiation, a long polyprotein is synthesized that is proteolytically cleaved into smaller structural and nonstructural proteins. The TMEV 3C gene product carries out most of these cleavages, as is the case with other picornaviruses (21).

During our investigations of the time course of TMEV polyprotein processing (21), we identified a small protein of about 18 kDa, which we called l, that was synthesized in in vitro translations in rabbit reticulocyte lysates programmed from in vitro-derived transcripts from the DA strain but not the GDVII strain cDNA. l is an extremely early product that appears 5 min after the start of translation, and it demonstrates no evidence of processing during translation. We wondered whether l might be generated from an AUG at N1079 in the L coding area out of phase with the polyprotein reading frame and downstream from the putative polyprotein initiation codon at N1066 (Fig. 1). This AUG at N1079 has a good consensus sequence for initiation and could synthesize a 17-kDa protein before reaching a termination codon (Fig. 1). To begin to clarify these issues, we constructed (20) a mutant cDNA, DAL:NheI, which contained an *Nhe*I linker insertion (coding for four amino acids) in the *Xho*I site at N1221 in the leader (L) coding area; this insertion has a termination codon for the l reading frame but not the reading frame of the polyprotein (Fig. 1). We have previously reported that in vitro translation of in vitro-derived transcripts of pDAL:NheI demonstrates disappearance of the full-sized l and synthesis of a protein of more rapid mobility, which we assume to be truncated l (20). In this translation, L was synthesized and polyprotein processing appeared normal; the pDAL:NheI in vitro-derived transcripts were infectious. These studies supported our localization of the initiation AUG of l at N1079. In the present study, we further investigated the origin of l by specifically mutating the presumed initiating AUG for l at N1079 and the putative wild-type polyprotein initiating AUG at N1066 and by investigating polyprotein processing of in vitro-derived transcripts from the mutant cDNAs.

For mutagenesis studies, we made use of pDAFL3 (which we will refer to as pDA), a full-length infectious cDNA clone of DA strain RNA in the transcription vector Bluescript SKII- (Stratagene, La Jolla, Calif.). The production and characterization of pDA have been previously described (22). Two mutants, pDA"l"-1 and pDAL-1, were generated by in vitro, site-directed mutagenesis, using slight modification of published techniques (6, 12). The mutagenesis was initially performed in a subclone of pDA in Bluescript SKII-

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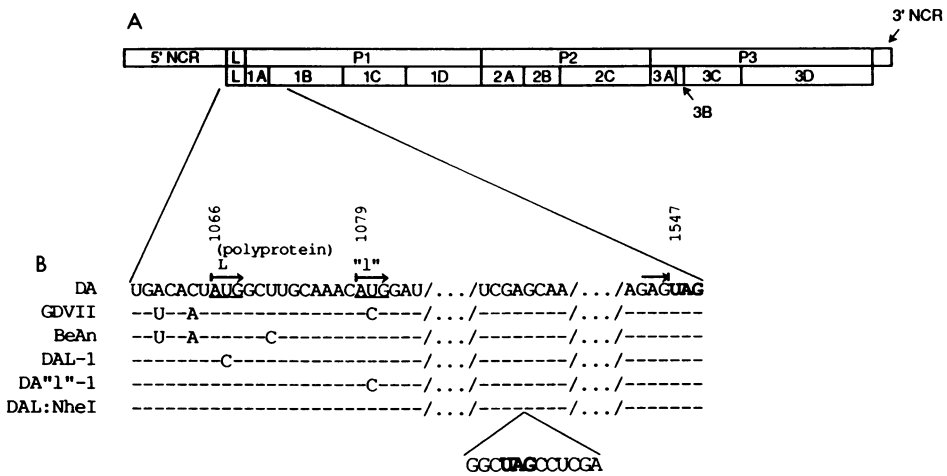

FIG. 1. Translation initiation sites of different strains of TMEV and mutants. (A) Coding region of the viral genome flanked by the 5′ noncoding region (5′NCR) and 3′ noncoding region (3′NCR). The viral protein products after polyprotein processing are indicated. (B) A portion of the RNA genome sequence surrounding the translation initiation site of the wild-type TMEV from different strains (DA, GDVII, and BeAn) and the mutants (DAL-1, DA"l"-1, and DAL:NheI). The nucleotide sequence of this region is shown for the DA strain; only differences between this sequence and those of the other strains and mutants are indicated. The putative polyprotein translation initiation site AUG at N1066 in DA is underlined in the DA genome. This AUG is not present in the mutant DAL-1 (in which the AUG is changed to ACG). The out-of-frame putative alternative translation initiation site AUG at N1079 for l is underlined in the DA genome. Note that this AUG is not present in the genomes of GDVII and mutant DA"l"-1 (in which the AUG is changed to ACG). The stop codon UAG at N1547 in frame with the alternative translation initiation site for l is indicated in bold type. In the case of DAL:NheI, an additional stop codon, UAG, in frame with the alternative initiation site for l was introduced into the genome by inserting an *Nhe*I linker in the *Xho*I site at N1221. The sequence of the *Nhe*I linker and the sequence surrounding the insertion are shown.

that extended from the *Kpn*I site at N934 to the *Hin*dIII site at N1730. This recombinant plasmid was introduced into the *Escherichia coli dut ung* strain CJ236. A uracil-containing single-stranded plasmid DNA was produced from these cells by superinfection with the helper phage M13K07. The uracil-containing DNA was purified and annealed with either (i) a mutated 23-base oligonucleotide (GCTTGCAAACACG GATACCCAGA) in which the underlined nucleotide changed the putative initiation AUG for l at N1079 to ACG to generate pDA"l"-1 or (ii) a 23-base oligonucleotide (TATTTGACACTACGGCTTGCTTGC) in which the underlined nucleotide changed the wild-type putative polyprotein initiation codon at N1066 to ACG to generate pDAL-1 (Fig. 1). The nucleotide change at N1079 was not predicted to change the amino acid sequence of L. With the oligonucleotide used as a primer, the complementary strand was synthesized and ligated to form covalently closed circular DNA. The latter DNA was used to transform *E. coli* MV1190, which has a functional uracil *N*-glycosylase, thus selecting against the parental, uracil-containing strand. Candidate mutant plasmids from the MV1190 cells were analyzed by dideoxynucleotide sequencing as described previously (14). The mutated subclone was then digested with *Kpn*I and *Hin*dIII; to generate the full-length cDNA clone, this insert was ligated to a fragment which extended from the *Hin*dIII site at N1730 to the *Aoc*I site at N4628 and to a fragment which contained the SKII- vector attached to N1-N934 and N4628 to the end of the DA genome. Dideoxynucleotide sequencing (17) confirmed the appropriate changes in the two mutants shown in Fig. 1.

Analysis of pDA"l"-1. pDA"l"-1 and the parental pDA and pGDFL2 (the full-length clone of GDVII in Bluescript SKII- [5], which we will refer to as pGDVII) were digested 3′ to the TMEV genome with *Xba*I and then in vitro transcribed as described previously (26). The in vitro-derived transcripts were used to program a rabbit reticulocyte in vitro translation system. The translation was performed at 30°C in a rabbit reticulocyte lysate with final concentrations of 10 mM potassium isothiocyanate and 0.7 mM magnesium ion, using published methods (26). After 3 h of translation, Laemmli sample buffer was added to half of each sample, and the rest of the sample was treated with 5 μg of cycloheximide per ml and 1 mg of pancreatic RNase per ml for 20 min at 30°C and then incubated for 1 h at 30°C with a postmitochondrial S-10 extract from DA strain-infected BHK-21 cells prepared as described previously (1). This latter reaction was then terminated with Laemmli sample buffer. The translation samples were then subjected to electrophoresis on 15% polyacrylamide gels containing sodium dodecyl sulfate. The gels were fluorographed with Amplify (Amersham, Arlington Heights, Ill.) and exposed to XAR-5 film.

In vitro translation of wild-type pDA cDNA, as shown in Fig. 2 (lanes 2 and 3), led to the synthesis of a number of structural and nonstructural proteins as well as an 18-kDa protein that we have designated l. Translation of in vitro-derived transcripts of pDA"l"-1 (lanes 6 and 7) also demonstrated a number of the viral proteins with apparently normal polyprotein processing. However, no l was synthesized. The apparently normal processing of this mutant was supported by the observation that the transcripts were infectious (unpublished data). Translation of transcripts derived from pGDVII (Fig. 2, lanes 14 and 15) did not demonstrate the 18-kDa l protein, as previously noted (21); L tended to run at a mobility slightly different from that seen with L of pDA.

As further confirmation that the mutation in the AUG at N1079 had interfered with the synthesis of l, wild-type pDA and pGDVII as well as the mutant pDA"l"-1 cDNA templates were digested with *Nco*I, which cuts in the VP0 coding area (Fig. 3). As expected (and diagrammed in Fig.

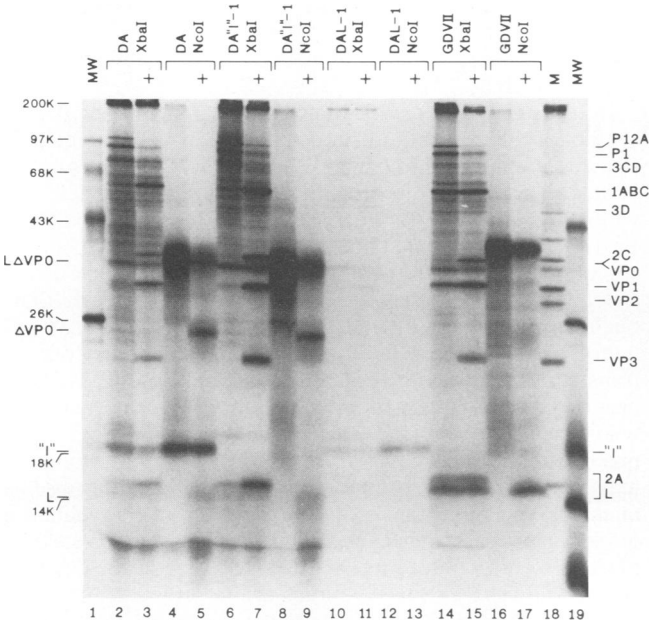

FIG. 2. In vitro translation of wild-type pDA, pGDVII, and mutants. Shown are translation of in vitro-derived transcripts derived from cDNA clones linearized by *Xba*I or *Nco*I. Translations and the preparation of the samples are described in the text. +, reaction mixtures that were incubated with a DA virus-infected BHK-21 cell extract after translation. Lanes 1 and 19 contain molecular weight (MW) markers, and lane 18 (M) contains [^{35}S]methionine-labeled proteins from an extract of DA virus-infected BHK-21 cells harvested 14 h after infection. The cDNA templates used to program in vitro transcription for in vitro translation are as follows: lanes 2 and 3, *Xba*I-digested pDA; lanes 4 and 5, *Nco*I-digested pDA; lanes 6 and 7 *Xba*I-digested pDA''l''-1; lanes 8 and 9 *Nco*I-digested pDA''l''-1; lanes 10 and 11 *Xba*I-digested pDAL-1; lanes 12 and 13, *Nco*I-digested pDAL-1; lanes 14 and 15, *Xba*I-digested pGDVII; lanes 16 and 17, *Nco*I-digested pGDVII. The positions of truncated LVP0 (LΔVP0), truncated VP0 (ΔVP0), L, and l are noted.

3), translation of transcripts derived from *Nco*I-cut pGDVII demonstrated truncated LVP0 with no evidence of l (Fig. 2, lane 16). Following addition of infected extract (Fig. 2, lane 17), the truncated LVP0 was incompleted cleaved to L and truncated VP0; the truncated VP0 is not well seen in this particular gel. Translation of transcripts derived from *Nco*I-cut pDA, as previously described (21), produced a truncated LVP0 and a protein that we presume to be l of about 18 kDa (Fig. 2, lane 4). The addition of an infected S-10 extract (lane 5) led to the cleavage of L from truncated VP0 (which runs at about 39 kDa); L runs aberrantly (and not very clearly in the gel shown in Fig. 2), considering its molecular mass, at about 9kDa. Translation of transcripts derived from *Nco*I-digested pDA''l''-1 led to the synthesis of truncated LVP0 with no l apparent (lane 8); after the addition of infected extract, LVP0 was cleaved to L and truncated VP0, again without apparent synthesis of l (lane 9).

Analysis of pDAL-1. As a control for the construct described above and to confirm the presumed localization of the initiation codon for the polyprotein at N1066, the latter AUG was changed to an ACG in the generation of pDAL-1. Translation of transcripts derived from *Xba*I-digested pDAL-1 demonstrated synthesis of an 18-kDa l protein but virtually no synthesis of the rest of the viral protein (Fig. 2, lanes 10 and 11). l was synthesized presumably because of the continuing presence of the AUG at N1079. Mutation of the polyprotein's AUG at N1066 abolished synthesis of the full-length polyprotein; the minimal synthesis of other proteins that was seen on long exposures presumably resulted from an alternative AUG in frame with the N1066 initiation codon, perhaps from the AUG at N1243. As expected, in vitro-derived transcripts derived from pDAL-1 were not infectious.

The effect of the DAL-1 mutation on synthesis of the authentic polyprotein and its products was most dramatically seen following translation of transcripts derived from *Nco*I-digested pDAL-1 (Fig. 2, lanes 12 and 13). In this case, the only protein synthesized with and without addition of infected extract was l. LVP0 was not produced, indicating no translation initiation from the putative authentic initiation site at N1066. Although the amount of l synthesized in this translation and in the translation of *Xba*I-digested pDAL-1 appears similar or less than the amount of l synthesized from transcripts derived from the other constructs shown in Fig. 2, this was not always the case; in one experiment, transcripts derived from *Nco*I- and *Xba*I-digested pDAL-1 had an efficient translation and led to the synthesis of abundant l (data not shown).

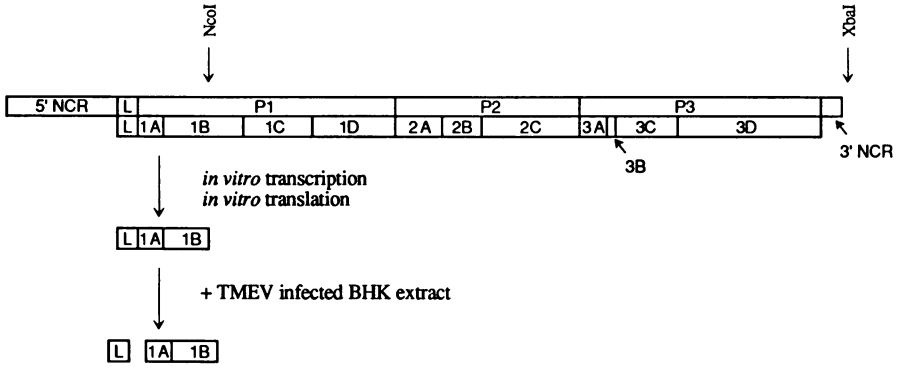

FIG. 3. Diagram of pGDVII transcription template linearized by *Nco*I and the expected translation products. After in vitro translation of the in vitro-transcribed viral cDNA template that is linearized by *Nco*I, a truncated LVP0 protein is expected. The addition of a DA virus-infected BHK-21 cell extract is predicted to cleave L from truncated VP0. The *Xba*I site, located downstream of the 3′ noncoding region (3′NCR) of the viral genome, is cleaved to generate a full-length cDNA template.

Consensus Sequence	5'...GCC**A/G**CCAUG**G**...3'
L	...GAC**A**CUAUG**G**...
"l"	...GCA**A**ACAUG**G**...

FIG. 4. Sequence of the region that includes the putative translation initiation site of DA virus polyprotein (L) and the putative alternative initiation site for l. The initiation codon (AUG) is underlined, and the surrounding sequences are compared with the consensus initiation sequence (9). The favorable nucleotides at the −3 and +4 positions are in bold type.

In summary, translation of transcripts of pDA cDNA templates with a mutation of the AUG at N1079 demonstrated no evidence of l production, although polyprotein processing appeared undisturbed; transcripts of this mutant were infectious. Translation of transcripts from pDA templates with a mutation in the AUG at 1066 had defective polyprotein production, although l was still synthesized; the latter result confirms the localization of the start site of polyprotein translation to N1066 and supports the separate initiation of l. Our previously published results (21) and data from this study indicate that two initiation sites are used with in vitro translation of pDA; the second initiation site is utilized to synthesize l. The data make unlikely other possible explanations for the origin of l, such as frameshifting.

Alternative initiation sites have been described in the case of foot-and-mouth disease virus (FMDV), another picornavirus. FMDV has two initiation sites in the leader coding area that are in the same reading frame, resulting in the synthesis of a polyprotein (and L protein) with varying amino termini (3, 23). The frequency of use of the first initiation site depends on the particular FMDV strain and whether there is in vitro or in vivo synthesis of viral proteins. Kozak (9) has noted that an AUG codon can be leaky, permitting initiation from the subsequent AUG if the first AUG has a suboptimal sequence context or if the reading frame of the first AUG terminates before the second AUG. In the case of FMDV, the more upstream AUG is frequently in a suboptimal sequence context, perhaps partly explaining initiation at the downstream AUG. However, there are rare examples of functionally bicistronic messages in which the 5′ proximal AUG is in good context and has a reading frame that does not terminate before the second AUG is reached (9). In the case of TMEV, the AUGs at both N1066 and the N1079 are in favorable sequence context (Fig. 4). It may be that following internal binding of the ribosome, translation initiation occurs at the AUG at 1066, with some leaky scanning leading to alternative initiation at 1079. When the AUG at 1066 is mutated (in pDAL-1), the majority of the ribosomes presumably continue to scan past 1066 to 1079, leading to efficient translation with an enhanced l synthesis compared with pDA (data not shown).

Alternative initiation sites have also been described for poliovirus in vitro translation (2, 4, 19). These initiation sites are thought to be an artifact of the in vitro translation system because the proteins synthesized from the alternative sites are not detectable following viral infection of HeLa cells and because the addition of HeLa cell postmitochondrial S-10 extracts to the rabbit reticulocyte lysate increases the amount of authentic viral proteins and decreases the production of aberrantly initiated proteins (4, 19). The S-10 is presumed to supply a factor that binds to the viral RNA and improves the quality and fidelity of the translation in in vitro systems. It remains a possibility, however, that certain neural cells, like the rabbit reticulocyte, are deficient in the HeLa cell factor(s) and lead to the production of aberrantly initiated proteins from poliovirus (24, 25) (or other picornaviruses, such as TMEV).

Does TMEV l have any biological significance or is it an artifact of the in vitro translation system? Immunoprecipitation and immunostaining will be important to clarify whether in fact l is produced after infection of tissue culture cells and within the infected central nervous system. The fact that BeAn, a demyelinating strain of TMEV, has an AUG corresponding to the N1079 position present in DA while GDVII, a nondemyelinating strain, does not (Fig. 1) suggests that l might play a role in TMEV demyelinating disease. Of interest is the recent finding that virus obtained from the transfection of in vitro-derived transcripts of pDAL:NheI produced little if any demyelination compared with DA wild-type virus following inoculation of mice (8a). This latter observation suggests that truncation of l or mutation of L may be important for production of this white matter disease in mice. The importance of l vis-à-vis L in demyelinating disease may be clarified by inoculating virus derived from pDA"l"-1, which does not synthesize "l" but does synthesize a wild type L. These studies are in progress.

We thank Lee Baksas for secretarial assistance and Bert Semler and Richard Jackson for review of the manuscript and helpful suggestions.

This work was supported by NIH grant PO1 NS21442-06 and by grant RG1512-C-4 from the National Multiple Sclerosis Society to Raymond P. Roos.

REFERENCES

1. **Brown, B. A., and E. Ehrenfeld.** 1979. Translation of poliovirus RNA *in vitro*: change in cleavage patterns and initiation sites by ribosomal salt wash. Virology **97**:396–405.
2. **Celma, M. L., and E. Ehrenfeld.** 1975. Translation of poliovirus RNA *in vitro*: detection of two different initiation sites. J. Mol. Biol. **98**:761–780.
3. **Clarke, B. E., D. V. Sangar, J. N. Burroughs, S. E. Newton, A. R. Carroll, and D. J. Rowlands.** 1985. Two initiation sites for foot-and-mouth disease virus polyprotein *in vitro*. J. Gen. Virol. **66**:2615–2626.
4. **Dorner, A. J., B. L. Semler, R. J. Jackson, R. Hanecak, E. Duprey, and E. Wimmer.** 1984. In vitro translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. J. Virol. **50**:507–514.
5. **Fu, J., S. Stein, L. Rosenstein, T. Bodwell, M. Routbort, B. L. Semler, and R. P. Roos.** 1990. Neurovirulence determinants of genetically engineered Theiler's viruses. Proc. Natl. Acad. Sci. USA **87**:4125–4129.
6. **Geisselsoder, J., F. Witney, and P. Yuckenberg.** 1987. Efficient site-directed *in vitro* mutagenesis. BioTechniques **5**:786–791.
7. **Jackson, R. J.** 1988. Picornaviruses break the rules. Nature (London) **334**:292–293.
8. **Jang, S. K., H. G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.

8a. **Kong, W., and R. P. Roos.** Unpublished data.

9. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
10. **Lipton, H. L.** 1980. Theiler's murine encephalomyelitis virus group includes two distinct genetic subgroups that differ physiologically and biologically. J. Gen. Virol. **46**:169–177.
11. **Lorch, Y., A. Friedmann, H. L. Lipton, and M. Kotler.** 1981. Theiler's murine encephalomyelitis virus group includes two distinct genetic subgroups that differ pathologically and biologically. J. Virol. **40**:560–567.

12. **McClary, J. A., F. Witney, and J. Geisselsoder.** 1989. Efficient site-directed *in vitro* mutagenesis using phagemid vectors. BioTechniques **7**:282–289.
13. **Nitayaphan, S., D. Omilianowski, M. M. Toth, S. Parks, R. R. Rueckert, A. C. Palmenberg, and R. P. Roos.** 1986. Relationship of Theiler's murine encephalomyelitis viruses to the cardiovirus genus of picornaviruses. Intervirology **26**:140–148.
14. **Ohara, Y., S. Stein, J. Fu, L. Stillman, L. Klaman, and R. P. Roos.** 1988. Molecular cloning and sequence determination of DA strain of Theiler's murine encephalomyelitis viruses. Virology **164**:245–255.
15. **Ozden, S. F., F. Tangy, M. Chamorro, and M. Brahic.** 1986. Theiler's virus genome is closely related to that of encephalomyocarditis virus, the prototype cardiovirus. J. Virol. **60**:1163–1165.
16. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334**:320–325.
17. **Pellett, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman.** 1985. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J. Virol. **53**:243–253.
18. **Pevear, D. C., M. Calenoff, E. Rozhon, and H. L. Lipton.** 1987. Analysis of the complete nucleotide sequence of the picornavirus Theiler's murine encephalomyelitis virus indicates that it is closely related to cardioviruses. J. Virol. **61**:1507–1516.
19. **Phillips, B. A., and A. Emmert.** 1986. Modulation of the expression of poliovirus proteins in reticulocyte lysates. Virology **148**:255–267.
20. **Roos, R. P., J. Fu, W. Kong, S. Stein, T. Bodwell, L. Rosenstein, M. Routbort, and B. L. Semler.** 1990. Infectious Theiler's virus cDNA clones: intratypic recombinants and viral polyprotein processing, p. 128–134. *In* M. A. Brinton and F. X. Heinz (ed.), New aspects of positive-strand RNA viruses. American Society for Microbiology, Washington, D.C.
21. **Roos, R. P., W.-P. Kong, and B. L. Semler.** 1989. Polyprotein processing of Theiler's murine encephalomyelitis virus. J. Virol. **63**:5344–5353.
22. **Roos, R. P., S. Stein, Y. Ohara, J. Fu, and B. L. Semler.** 1989. Infectious cDNA clones of the DA strain of Theiler's murine encephalomyelitis virus. J. Virol. **63**:5492–5496.
23. **Sangar, D. V., S. E. Newton, D. J. Rowlands, and B. E. Clarke.** 1987. All foot-and-mouth disease viruses initiate protein synthesis at two separate AUGs. Nucleic Acids Res. **15**:3305–3315.
24. **Svitkin, Y. V., S. V. Maslova, and V. I. Agol.** 1985. The genomes of attenuated and virulent poliovirus strains differ in their *in vitro* translation efficiencies. Virology **147**:243–252.
25. **Svitkin, Y., T. V. Pestova, S. V. Maslova, and V. I. Agol.** 1988. Point mutations modify the responses of poliovirus RNA to a translation initiation factor: a comparison of neurovirulent and attenuated strains. Virology **166**:394–404.
26. **Ypma-Wong, M. F., and B. L. Semler.** 1987. *In vitro* molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase. Nucleic Acids Res. **15**:2069–2088.