JOURNAL OF VIROLOGY, May 1987, p. 1507–1516
0022-538X/87/051507-10$02.00/0

Vol. 61, No. 5

# Analysis of the Complete Nucleotide Sequence of the Picornavirus Theiler's Murine Encephalomyelitis Virus Indicates That It Is Closely Related to Cardioviruses

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Received 15 September 1986/Accepted 19 January 1987

**Theiler's murine encephalomyelitis viruses (TMEV) are naturally occurring enteric pathogens of mice which constitute a separate serological group within the picornavirus family. Persistent TMEV infection in mice provides a relevant experimental animal model for the human demyelinating disease multiple sclerosis. To provide information about the TMEV classification, genome organization, and protein processing map, we determined the complete nucleotide sequence of the TMEV genome and deduced the amino acid sequence of the polyprotein coding region. The RNA genome, which is typical of the picornavirus family, is 8,098 nucleotides long. The 5′ untranslated region is 1,064 nucleotides long (making it the longest in the picornavirus family after the aphthoviruses) and lacks a poly(C) tract. Computer-generated comparison of the 5′ and 3′ noncoding regions and polyprotein revealed the highest level of nucleotide and predicted amino acid identity between the TMEV and the cardioviruses encephalomyocarditis virus (EMCV) and Mengo virus. The TMEV polyprotein, which appears to be processed like EMCV since the amino acids flanking the putative proteolytic cleavage sites have been conserved, begins with a short leader peptide followed by 11 other gene products in the standard L-4-3-4 picornavirus arrangement. Because of these similarities, we propose that the TMEV be grouped with the cardioviruses. However, since TMEV and EMCV have different biophysical properties and show no cross-neutralization, they most likely belong in a separate cardiovirus subgroup.**

Theiler's murine encephalomyelitis viruses (TMEV) are naturally occurring enteric pathogens of mice which constitute a separate serological group within the picornavirus family (26). The more than 20 TMEV reported in the literature since Theiler's original isolations in the 1930s (45) can be divided into two groups based on their neurovirulence after intracerebral inoculation (13, 25). Included in the first group are the two most virulent viruses (FA and GDVII), which cause rapidly fatal encephalitis in mice (46). All of the remaining isolates, which constitute the second group, are much less virulent but still cause central nervous system disease. The latter group produces central nervous system involvement in the form of acute poliomyelitis (early onset) followed by a chronic, inflammatory, demyelinating process (late onset). The demyelinating disease is due to persistent infection wherein low levels of infectious virus can be recovered for many months from the target organ, the central nervous system. Persistent TMEV infection in mice provides a relevant experimental animal model for the human demyelinating disease multiple sclerosis (24).

The picornavirus family contains four major groups, including the human enteroviruses, the human rhinoviruses, the aphthoviruses (foot-and-mouth disease viruses FMDV), and the cardioviruses, and potentially a fifth group, hepatitis A virus (38). Before this report, the exact classification of TMEV within this family has not been clear. Because of similarities in the epidemiology and pathogenesis of TMEV and the human enteroviruses and the apparent absence of a long poly(C) tract in the 5′ noncoding region of the TMEV RNA, it was previously felt that TMEV more closely resembled the human enteroviruses (37). Knowledge of the nucleotide sequences of picornaviruses representing the major groups has enabled precise comparisons among member viruses, more clearly indicating evolutionary relationships (7, 8, 15, 22, 30, 31, 34, 43). Therefore, determining the TMEV nucleotide sequence should provide important information about their classification, as well as their genome organization and protein processing map.

Because viruses from the two TMEV virulence groups produce distinctly different diseases in mice, TMEV are ideally suited for molecular pathogenetic studies of picornaviruses. Having the nucleotide sequence should greatly facilitate construction of intertypic infectious cDNA clones for this purpose.

We determined the complete nucleotide sequence of the TMEV (BeAn 8386 strain) genome and deduced the amino acid sequence of the polyprotein coding region. The untranslated 5′ end of the viral RNA was found to be 1,064 nucleotides long (making it the second longest in the picornavirus family) and was found to lack a poly(C) tract. Computer-generated comparison of the 5′ and 3′ noncoding regions and polyprotein revealed the highest level of nucleotide and predicted amino acid homology between TMEV and the cardioviruses, encephalomyocarditis virus (EMCV) (31), and Mengo virus (A. C. Palmenberg, unpublished data). The TMEV polyprotein, which appears to be processed like EMCV since the amino acids flanking the putative proteolytic cleavage sites have been conserved, begins with a short leader peptide followed by 11 other gene products in the standard L-4-3-4 picornavirus arrangement (39). Thus, TMEV are more closely related to the cardioviruses than to the human enteroviruses as previously reported (37).

## MATERIALS AND METHODS

**Materials.** The following reagents were obtained commercially: reverse transcriptase, Seikagaku; actinomycin D,

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Merck & Co., Inc.; RNasin, Promega-Biotec; [$^{35}$S]α-thio-dATP and [α-$^{32}$P]-dCTP, Amersham Corp.; deoxynucleoside and dideoxynucleoside triphosphates, Boehringer Mannheim Biochemicals; T4 DNA ligase, nuclease S1, oligo $(dT)_{12-18}$, large (Klenow) fragment of *Escherichia coli* DNA polymerase I, T4 DNA polymerase, RNase H, and terminal deoxynucleotidyltransferase, P-L Biochemicals, Inc.; oligo($dT_8dC$), New England BioLabs; restriction endonucleases, P-L Biochemicals and New England BioLabs.

**Virus and cell culture.** The passage history of the BeAn 8386 strain of TMEV and the techniques of plaque purification, growth in BHK-21 cell monolayers, and purification by isopycnic centrifugation in $Cs_2SO_4$ gradients were as previously described (36). BHK-21 cells were cultivated in Dulbecco modified Eagle medium as previously described (36).

**Virion RNA.** BeAn 8386 viral RNA was extracted from 2 mg of purified virions with an equal volume of a 7:3 mixture of phenol-chloroform, suspended in 20 mM Tris hydrochloride (pH 7.6)–140 mM NaCl–1 mM EDTA, and then centrifuged in a 5 to 25% (wt/vol) sucrose gradient (37). The integrity of the virion RNA was determined by electrophoresis in a 1% native agarose gel (20 mM Tris hydrochloride [pH 7.9], 5 mM sodium acetate, 1 mM EDTA).

**Preparation of cDNA clones.** Purified virion RNA was transcribed into double-stranded (ds) cDNA for cloning. First-strand cDNA was synthesized with 800 U of reverse transcriptase per ml and 50 μg of TMEV RNA per ml in 50 mM Tris hydrochloride (pH 8.3)–8 mM $MgCl_2$–50 mM KCl–4 mM dithiothreitol–40 μg of actinomycin D per ml–800 μM each dATP, dCTP, dGTP, and dTTP–20 μg of oligo$(dT)_{12-18}$–1,000 U of RNasin per ml for 1 h at 41°C in the presence of 0.2 μCi of [α-$^{32}$P]dCTP per ml in 100 μl. After addition of EDTA to 20 mM, RNA-cDNA hybrids were extracted with phenol, fractionated by column chromatography, and then precipitated with ethanol. After alkaline denaturation of the RNA (130 mM NaOH at 65°C for 10 min) the first-strand cDNA was analyzed on 0.6% alkaline agarose gels and primarily found to be full length with several shorter fragments (data not shown). The overall cDNA synthesis corresponded to 45 to 60% copying of the template RNA (data not shown).

Second-strand cDNA was synthesized by using the large (Klenow) fragment of *E. coli* DNA polymerase I (0.02 U/ng of cDNA) in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 6.9)–10 mM $MgCl_2$–2.5 mM dithiothreitol–7 mM KCl–500 μM each dATP, dCTP, dGTP, and dTTP–10 μg of cDNA per ml for 20 h at 15°C in 50 μl. Second-strand cDNA synthesis was completed with reverse transcriptase under the same conditions as for the first strand.

After phenol extraction, column chromatography, and precipitation with ethanol, ds cDNA was treated with nuclease S1. The conditions of nuclease S1 digestion were determined to be optimum at 125 U/ml for 30 min at 37°C in 100 μl containing 800 ng of DNA, 200 mM NaCl, 50 mM sodium acetate (pH 4.5), 1 mM $ZnSO_4$, and 0.5% glycerol. Homopolymeric tails of 10 to 20 dCMP residues were added to ds cDNA with 270 U of terminal deoxynucleotidyltransferase per ml for 10 min at 37°C in 100 μl containing 400 mM sodium cacodylate, 50 mM Tris hydrochloride (pH 6.9), 2 mM $CoCl_2$, 2 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 80 μM dCTP, and 0.5 pmol of DNA ends.

Oligo(dC)-tailed ds cDNA was added to *Pst*I-digested, oligo(dG)-tailed pBR322 at approximately equimolar ratios and then annealed by incubation at 65°C for 5 min and cooling to 24°C overnight. *E. coli* DH1 was transformed with the annealed material as described by Hanahan (18).

**Restriction endonuclease digestion and mapping.** Preparations of recombinant plasmid DNAs were digested with restriction enzymes as directed by the manufacturers. The restriction fragments were analyzed by agarose or polyacrylamide gel electrophoresis.

**Hybridization probes.** cDNA clones containing specific viral sequences were selected by colony hybridization (16) to a cDNA probe randomly primed with calf thymus oligonucleotides by reverse transcription from the viral RNA.

**Nucleotide sequence analysis.** Nucleotide sequencing was performed with cDNA clones 1329, 1468, 1322, and 1316 by the dideoxynucleotide sequencing procedure of Sanger et al. (40) (see Fig. 1). Subclones in M13 mp18 and mp19 vectors used for the sequencing reactions were generated with the frequent-cutting restriction enzymes *Hpa*II, *Hae*III, *Rsa*I, *Sau*3A, and *Taq*I; other specific restriction enzymes; and also by unidirectional deletion of large cloned fragments to produce a series of overlapping clones as described by Dale et al. (10). To obtain unambiguous sequence data, we analyzed the entire sequence on both DNA strands. The 3' terminus of the genome was sequenced directly by primed synthesis from the viral RNA by the dideoxynucleotide method, using reverse transcriptase and oligo($dT_8dC$) as a primer (40).

**Computer analysis of nucleotide sequence.** The programs of Roger Staden were used to assemble the nucleotide sequence data and calculate the molecular weights of the predicted TMEV proteins (42). All other sequence manipulations were performed with the program library of the University of Wisconsin Genetics Computer Group on a VAX 11/780 super-minicomputer (11). To determine the percent similarity of the capsid proteins and 5' and 3' noncoding regions of the genome with those of other picornaviruses, we used the GAP program at a gap weight of 5.0 and a length weight of 0.3.

**Cloning the 5' end of the TMEV genome.** Primer extension with the DNA oligomer 3' GCCAA TGGGG GAAAG CTGCG 5', complementary to bases at the 5' end of clone p1329, as a primer and BeAn 8386 viral RNA was as previously described (23). Briefly, primer and viral RNAs mixed at a molar ratio of 10:1 were denatured by boiling at 100°C for 2 min, quenched in a dry-ice–ethanol bath, and then annealed at 42°C for 3 to 4 h in 400 mM NaCl–10 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] hydrochloride (pH 6.4)–2 mM EDTA in 20 μl. The conditions for primer extension with reverse transcriptase were identical to those described above for first-strand cDNA synthesis. These primer extension experiments revealed that the 5' noncoding region extends for about 30 to 50 bases beyond the end of clone p1329; however, cloning of this primer-extended fragment has not been successful to date. The 40 bases at the 5' terminus are from a cDNA clone which was obtained by the replacement synthesis method (17) as modified by Kandolf and Hofschneider (20) with the RNA of the GDVII strain of TMEV as a template. This cDNA clone contains the entire 5' untranslated region of GDVII virus and shares 94% sequence identity with nucleotides 41 to 1064 of the BeAn 8386 strain (unpublished data).

## RESULTS

**Analysis of cDNA clones.** As described in Materials and Methods, synthesized oligo(dC)-tailed ds cDNA to BeAn

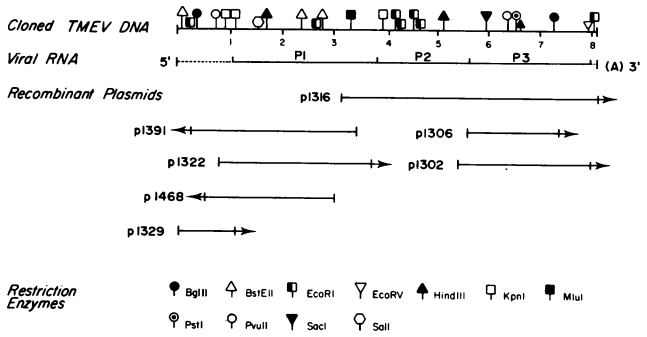


FIG. 1. A composite partial restriction endonuclease map of TMEV cDNA clones. The viral RNA, which was estimated to be 8.0 kilobases excluding the poly(A) tail, is shown directly below. The RNA genome is divided into noncoding regions and an open reading frame consisting of three parts based on the genome organization of EMCV (31). The positions of the virus-derived inserts from the seven clones are illustrated, and arrowheads indicate the orientation of the insert relative to the *Eco*RI site in pBR322. Experiments with eight other plasmids (data not shown) support this map.

8386 virus RNA was inserted into the *Pst*I site of oligo(dG)-tailed pBR322 for transformation of *E. coli*. Of 414 $Tet^r$ $Amp^s$ colonies, 90% showed strong hybridization signals by colony hybridization with an [$\alpha$-$^{32}$P]-dCTP-labeled cDNA probe made from BeAn 8386 virus RNA. The clones hybridizing with the probe were screened for insert size by digestion with *Pst*I and sizing of the released DNA by agarose gel electrophoresis. The large inserts within 14 cDNA clones were oriented relative to each other by restriction enzyme mapping. They were found to overlap to produce a contiguous stretch of TMEV-derived DNA approximately 8,000 nucleotides long (Fig. 1). Dideoxynucleotide sequencing revealed that one end of clone p1316 contained a poly(A) tail, indicating that this cloned fragment extended to the 3′ end of the viral genome. The predicted *Eco*RI and *Eco*RV sequences at the extreme 3′ end of this clone were identified within the viral RNA by dideoxynucleotide sequencing with BeAn 8386 virus RNA as a template and oligo($dT_8dC$) as a primer. All other restriction endonuclease cleavage sites predicted by the sequence were confirmed upon dideoxynucleotide sequencing of cDNA clones.

**Nucleotide sequence and genome organization.** With the notable absence of a poly(C) tract in the 5′ noncoding region, the organization and sequence of the TMEV genome (Fig. 2) was found to be remarkably similar to that of the cardiovirus EMCV (31). Since there is only one long open reading frame and the TMEV amino acid sequence so clearly matches that of EMCV (see below), we were readily able to localize the polyprotein coding region in the TMEV genome. The polyprotein initiates at the AUG codon at nucleotide 1065 and extends for 6,909 bases (or 2,303 codons) ending at the single UGA termination triplet at base 7972 (Fig. 2 and 3). The polyprotein coding region is flanked by 5′ and 3′ noncoding sequences of 1,064 and 125 nucleotides, respectively. As in most picornaviruses, the 5′ noncoding region contains a stretch of 11 pyrimidines interrupted by a single purine before the AUG at nucleotide 1065. Before the putative start site, eight other apparently unused initiation codons, including two in the third or open reading frame, were identified. A poly(A) tail of indeterminate length is present at the 3′ end of the viral genome; we observed 14 adenosine residues at the 3′ end of clone p1316.

**Deduced amino acid sequence of the polyprotein coding region.** The 2,303-amino-acid polyprotein has a calculated molecular weight of 255,990. The processing scheme follows the standard L-4-3-4 picornavirus polypeptide arrangement, i.e., the leader peptide (L), four capsid polypeptides in part one (P1) of the genome, three polypeptides in P2, and four polypeptides in P3 (39). The coding limits of individual polypeptides were predicted by analogy with those of EMCV, since the only confirmation to date of the deduced sequence is that of the N terminus of 1D (Raymond Roos, personal communication). The eight amino acids flanking the putative cleavage sites were found to be highly conserved for the two viruses (Table 1). The nonidentical amino acids in these locations generally represent functionally similar residues. By analogy with EMCV, all of the cleavage sites except two, 1A/1B and 2A/2B, are believed to be processed by the viral protease 3C (32, 33). The TMEV 3C protease therefore processes the Q-G, as well as the Q-S and Q-A, dipeptides and, in addition, the E-N dipeptide at the 1D/2A cleavage. However, only 6 of 9 Q-G, 2 of 13 Q-S, and 1 of 7 Q-A dipeptides in the polyprotein are cleaved by 3C, indicating that involvement of secondary, tertiary, or both types of structure is also important for recognition of these particular dipeptides. Whereas the 2A/2B site is also a Q-G dipeptide, there is evidence that it is cleaved autocatalytically in EMCV and not by the 3C protease (A. C. Palmenberg, personal communication).

A more complete TMEV protein map could be established from the deduced amino acid sequence than was available from previous pulse-chase and pactamycin mapping experiments (27, 28) (Fig. 3). In some instances, the sequence-derived molecular weights differed from earlier determinations based on electrophoretic mobility in polyacrylamide gels. Cleavage of the polyprotein gave rise to three primary products, the first of which (117,557 molecular weight [MW]) contains the leader protein (8,593 MW), the P1 capsid proteins, and the first P2 polypeptide, 2A (16,509 MW). Thus, the initial precursor released from the polyprotein is like that of the cardioviruses and differs from that of other groups of picornaviruses (6). The capsid proteins are arranged in the following order: 1A (VP4; 7,102 MW), 1B (VP2; 29,433 MW), 1C (VP3; 25,463 MW), and 1D (VP1; 30,457 MW). The second processing precursor (2BC) is 50,681 MW and gives rise to 2B (13,836 MW) and 2C (36,845 MW). The P2 proteins 2A, 2B, and 2C have not been assigned functions as yet for the cardioviruses. The third or carboxy-terminal precursor protein is 87,950 MW and is processed into the four mature proteins 3A (9,934 MW), 3B (2,169 MW), 3C (23,612 MW), and 3D (52,235 MW). Protein 3B, also called VPg, is a small protein which is 20 amino acids in size and is found covalently linked to the 5′ end of viral RNAs. This peptide may be important in viral replication. By analogy with other viruses, the 3C polypeptide is a viral protease and 3D is the viral polymerase.

**Nucleotide and polyprotein comparisons with other picornaviruses.** By computer-generated comparison, the sequence of the TMEV polyprotein was shown to be more similar to EMCV than to representative members of other picornavirus groups, e.g., human poliovirus type 1 (Sabin), FMDV A10, or hepatitis A virus (Fig. 4). Since the human polioviruses show extensive amino acid similarity with the human rhinoviruses, TMEV was only compared with the former. Considerable identity was seen in the nonstructural proteins 2C, 3C, and 3D for all five picornaviruses. Although the TMEV leader protein shares only 13% similarity with the EMCV leader, a high degree of identity was found for the capsid proteins of the two viruses. TMEV 1A, 1B, 1C, and

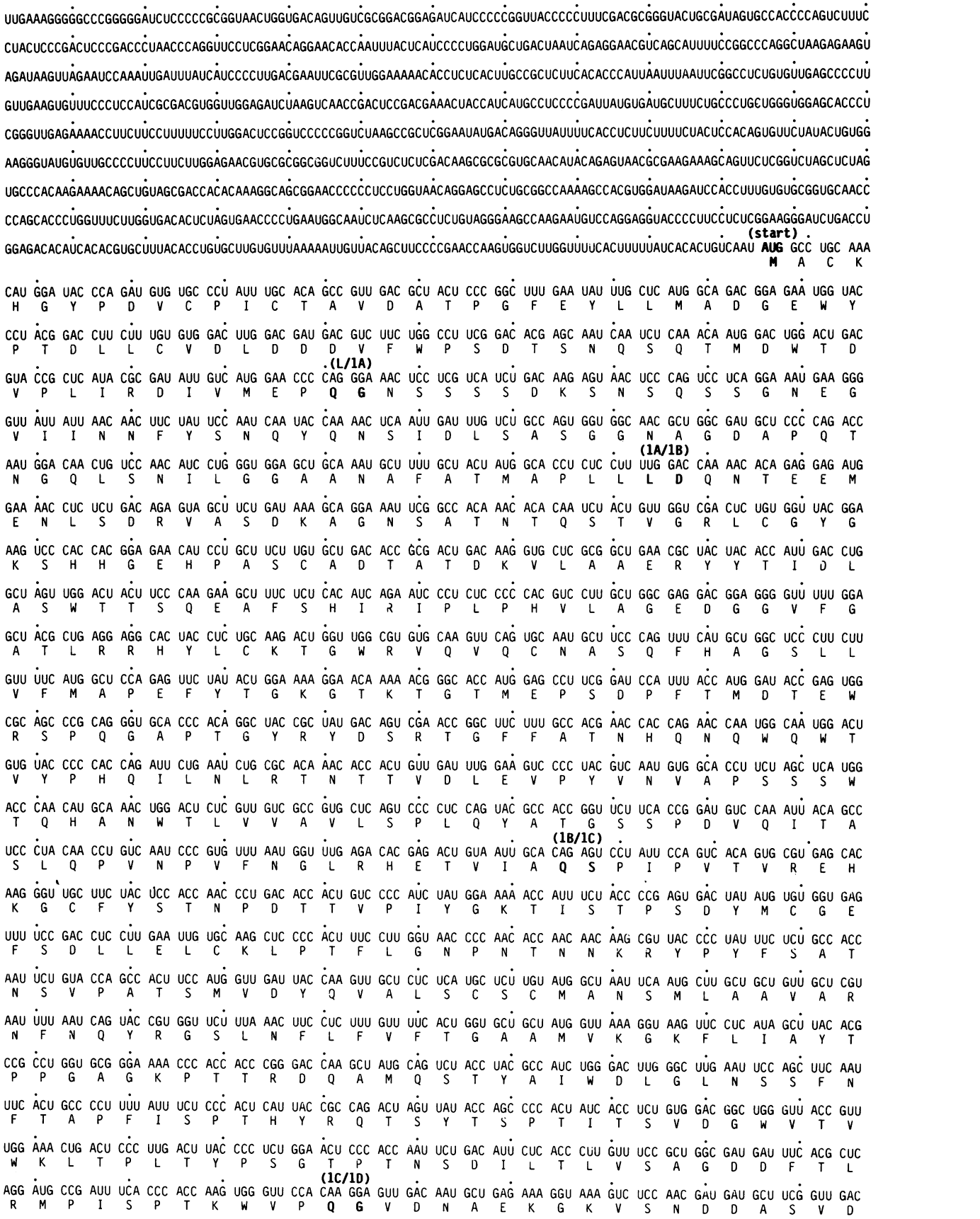


FIG. 2. The nucleotide sequence of the BeAn 8386 strain of TMEV with the predicted amino acid sequences of the virus-specific proteins.

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3057  UUU GUC GCC GAA CCA GUC AAG CUG CCC GAG AAC CAA ACC CGG GUG GCC UUC UUC UAU GAC AGA GCU GUC CCU AUA GGU AUG UUG AGG CCC
        F   V   A   E   P   V   K   L   P   E   N   Q   T   R   V   A   F   F   Y   D   R   A   V   P   I   G   M   L   R   P

3147  GGC CAA AAU AUG GAA ACC ACU UUU AAC UAC CAA GAA AAU GAU UAC CGC CUC AAU UGU CUU UUG CUA ACC CCU CUC CCU UCC UUU UGU CCU
        G   Q   N   M   E   T   T   F   N   Y   Q   E   N   D   Y   R   L   N   C   L   L   L   T   P   L   P   S   F   C   P

3237  GAC AGU UCU UCC GGG CCC CAA AAA ACA AAG GCU CCC GUU CAA UGG CGA UGG GUG CGA UCU GGC GGC GUC AAC GGC GCC AAC UUU CCG CUC
        D   S   S   S   G   P   Q   K   T   K   A   P   V   Q   W   R   W   V   R   S   G   G   V   N   G   A   N   F   P   L

3327  AUG ACU AAG CAG GAC UAU GCC UUC CUU UGU UUU UCU CCC UUU ACC UUU UAC AAG UGU GAC CUU GAG GUC ACA GUC AGU GCC CUG GGC AUG
        M   T   K   Q   D   Y   A   F   L   C   F   S   P   F   T   F   Y   K   C   D   L   E   V   T   V   S   A   L   G   M

3417  ACA CGG GUU GCC UCC GUG CUC CGU UGG GCC CCU ACC GGC GCC CCU GCG GAU GUU ACU GAC CAG CUA AUA GGU UAC ACA CCC AGC CUU GGU
        T   R   V   A   S   V   L   R   W   A   P   T   G   A   P   A   D   V   T   D   Q   L   I   G   Y   T   P   S   L   G

3507  GAG ACG CGU AAU CCG CAU AUG UGG CUC GUU GGU GCU GGC AAC UCA CAA GUU UCU UUU GUG GUU CCC UAU AAC UCC CCU CUC UCC GUC CUC
        E   T   R   N   P   H   M   W   L   V   G   A   G   N   S   Q   V   S   F   V   V   P   Y   N   S   P   L   S   V   L

3597  CCU GCC GCU UGG UUU AAU GGA UGG UCC GAC UUU GGA AAC ACU AAG GAC UUU GGA GUC GCC CCU AAC GCA GAC UUC GGG CGU CUC UGG AUC
        P   A   A   W   F   N   G   W   S   D   F   G   N   T   K   D   F   G   V   A   P   N   A   D   F   G   R   L   W   I

3687  CAG GGC AAU ACC UCU GCC UCC GUC CGG AUC AGG UAC AAG AAA AUG AAG GUC UUC UGC CCC CGC CCG ACC CUC UUC UUC CCC UGG CCU ACG
        Q   G   N   T   S   A   S   V   R   I   R   Y   K   K   M   K   V   F   C   P   R   P   T   L   F   F   P   W   P   T
                                                                                    (1D/2A)
3777  CCC ACC ACC ACC AAG AUC AAU GCU GAC AAC CCA GUC CCC AUU CUU GAA CUC GAG AAU CCC GCU GCU CUC UAC CGC AUU GAU CUC UUC AUC
        P   T   T   T   K   I   N   A   D   N   P   V   P   I   L   E   L   E   N   P   A   A   L   Y   R   I   D   L   F   I

3867  ACC UUC ACU GAU GAG UUC AUC ACC UUU GAU UAC AAG GUU CAC GGA CGU CCU GUG CUU ACC UUC CGG AUC CCA GGC UUC GGC CUG ACC CCG
        T   F   T   D   E   F   I   T   F   D   Y   K   V   H   G   R   P   V   L   T   F   R   I   P   G   F   G   L   T   P

3957  GCA GGU AGG AUG CUC GUG UGC AUG GGC GAA CAA CCC GCA CAU GGU CCG UUC ACC UCC UCU AGA UCC CUC UAU CAU GUC AUU UUU ACU GCU
        A   G   R   M   L   V   C   M   G   E   Q   P   A   H   G   P   F   T   S   S   R   S   L   Y   H   V   I   F   T   A

4047  ACU UGC UCU UCC UUU AGC UUU AGU AUC UAC AAG GGG CGG UAC CGC UCC UGG AAA AAG CCC AUC CAU GAC GAG CUU GUG GAU CGU GGU UAC
        T   C   S   S   F   S   F   S   I   Y   K   G   R   Y   R   S   W   K   K   P   I   H   D   E   L   V   D   R   G   Y

4137  ACC AUU UUC GGC GAG UUC UUC AAG GCU GUG CGC GGA UAC CAU GCU GAC UAU UAC AGA CAG AGA CUC AUA CAC GAU GUG GAA ACA AAU CCA
        T   I   F   G   E   F   F   K   A   V   R   G   Y   H   A   D   Y   Y   R   Q   R   L   I   H   D   V   E   T   N   P
                                    (2A/2B)
4227  GGC CCU GUG CAG UCG GUU UUU CAG CCA CAA GGU GCG GUG CUA ACU AAA UCC CUA GCA CCC CAG GCA GGA AUU CAA AAC CUC CUU CUA CGC
        G   P   V   Q   S   V   F   Q   P   Q   G   A   V   L   T   K   S   L   A   P   Q   A   G   I   Q   N   L   L   L   R

4317  CUC CUC GGC AUA GAC GGU GAC UGU UCA GAA GUU AGU AAA GCA AUC ACA GUC GUC ACC GAC UUA GUU GCU GCA UGG GAA AAG GCA AAA ACC
        L   L   G   I   D   G   D   C   S   E   V   S   K   A   I   T   V   V   T   D   L   V   A   A   W   E   K   A   K   T

4407  ACC CUG GUU UCC CCU GAA UUC UGG UCA AAA CUC AUA UUA AAA ACC ACC AAA UUC AUU GCU GCC UCU GUG CUU UAC CUA CAC AAC CCU GAU
        T   L   V   S   P   E   F   W   S   K   L   I   L   K   T   T   K   F   I   A   A   S   V   L   Y   L   H   N   P   D

4497  UUU ACC ACC ACU GUU UGU CUU UCA UUG AUG ACU GGU GUG GAC CUC CUC ACC AAC GAU UCU GUU UUU GAU UGG CUC AAA CAA AAA UUG UCU
        F   T   T   T   V   C   L   S   L   M   T   G   V   D   L   L   T   N   D   S   V   F   D   W   L   K   Q   K   L   S
                                                                                (2B/2C)
4587  UCC UUC UUU CGC ACC CCU CCC CCA GCU UGC CCC AAU GUU AUG CAG CCU CAG GGA CCU CUA CGC GAG GCC AAU GAA GGC UUC ACC UUU GCU
        S   F   F   R   T   P   P   P   A   C   P   N   V   M   Q   P   Q   G   P   L   R   E   A   N   E   G   F   T   F   A

4677  AAG AAC AUU GAG UGG GCU AUG AAA ACC AUC CAG UCU GUU GUU AAU UGG CUU ACU AGU UGG UUU AAA CAA GAA GAG GAU CAC CCC CAA UCA
        K   N   I   E   W   A   M   K   T   I   Q   S   V   V   N   W   L   T   S   W   F   K   Q   E   E   D   H   P   Q   S

4767  AAA UUA GAC AAA CUG CUC AUG GAA UUC CCC GAC CAU UGC AGG AAC AUC AUG GAU AUG AGA AAC GGU CGA AAG GCC UAU UGU GAG UGC ACU
        K   L   D   K   L   L   M   E   F   P   D   H   C   R   N   I   M   D   M   R   N   G   R   K   A   Y   C   E   C   T

4857  GCU UCC UUU AAG UAU UUU GAU GAA CUU UAC AAU CUU GCU GUU ACU UGC AAA AGA AUU CCA UUG GCC UCC CUA UGU GAA AAA UUU AAG AAU
        A   S   F   K   Y   F   D   E   L   Y   N   L   A   V   T   C   K   R   I   P   L   A   S   L   C   E   K   F   K   N

4947  AGA CAU GAU CAC UCU GUC ACU AGA CCC GAG CCG GUG GUU GUC GUU CUU CGC GGC GCC GCU GGG CAA GGU AAG UCU GUG ACC AGC CAA AUU
        R   H   D   H   S   V   T   R   P   E   P   V   V   V   V   L   R   G   A   A   G   Q   G   K   S   V   T   S   Q   I

5037  AUC GCC CAA UCC GUG UCA AAG AUG GCC UUU GGU CGU CAG UCU GUC UAU UCA AUG CCC CCC GAU UCG GAA UAU UUU GAU GGC UAU GAA AAU
        I   A   Q   S   V   S   K   M   A   F   G   R   Q   S   V   Y   S   M   P   P   D   S   E   Y   F   D   G   Y   E   N

5127  CAA UUU UCU GUG AUU AUG GAU GAU CUA GGA CAA AAU CCU GAU GGC GAA GAC UUC ACU GUC UUU UGU CAA AUG GUU UCU AGC ACA AAU UUU
        Q   F   S   V   I   M   D   D   L   G   Q   N   P   D   G   E   D   F   T   V   F   C   Q   M   V   S   S   T   N   F

5217  CUU CCG AAU AUG GCU CAC CUG GAA AGA AAA GGC ACU CCU UUU ACC UCU AGC UUC AUU GUU GCU ACA ACA AAU UUG CCC AAA UUC CGC CCU
        L   P   N   M   A   H   L   E   R   K   G   T   P   F   T   S   S   F   I   V   A   T   T   N   L   P   K   F   R   P

5307  GUU ACG GUU GCC CAC UAC CCC GCU GUU GAU AGG CGA AUC ACC UUU GAU UUU ACU GUU ACU GCU GGA CCC CAC UGC AAA ACG CCU GCU GGA
        V   T   V   A   H   Y   P   A   V   D   R   R   I   T   F   D   F   T   V   T   A   G   P   H   C   K   T   P   A   G

5397  AUG UUG GAU GUU GAG AAA GCU UUU GAU GAG AUA CCU GGC UCC AAA CCU CAG CUU GCC UGC UUC AGU GCU GAU UGC CCC CUC CUA CAC AAA
        M   L   D   V   E   K   A   F   D   E   I   P   G   S   K   P   Q   L   A   C   F   S   A   D   C   P   L   L   H   K

5487  AGA GGA GUC AUG UUC ACC UGC AAU CGC ACC CAG ACC GUU UAC AAC CUC CAA CAA GUU GUA AAA AUG GUC AAC GAC ACC AUU ACC CGC AAG
        R   G   V   M   F   T   C   N   R   T   Q   T   V   Y   N   L   Q   Q   V   V   K   M   V   N   D   T   I   T   R   K
                                                (2C/3A)
5577  ACU GAA AAU GUG AAG AAA AUG AAU AGC CUG GUU GCU CAG UCU CCA CCA GAC UGG GAG CAC UUU GAG AAC AUC CUC ACC UGU CUC CGU CAG
        T   E   N   V   K   K   M   N   S   L   V   A   Q   S   P   P   D   W   E   H   F   E   N   I   L   T   C   L   R   Q
```

FIG. 2—*Continued*

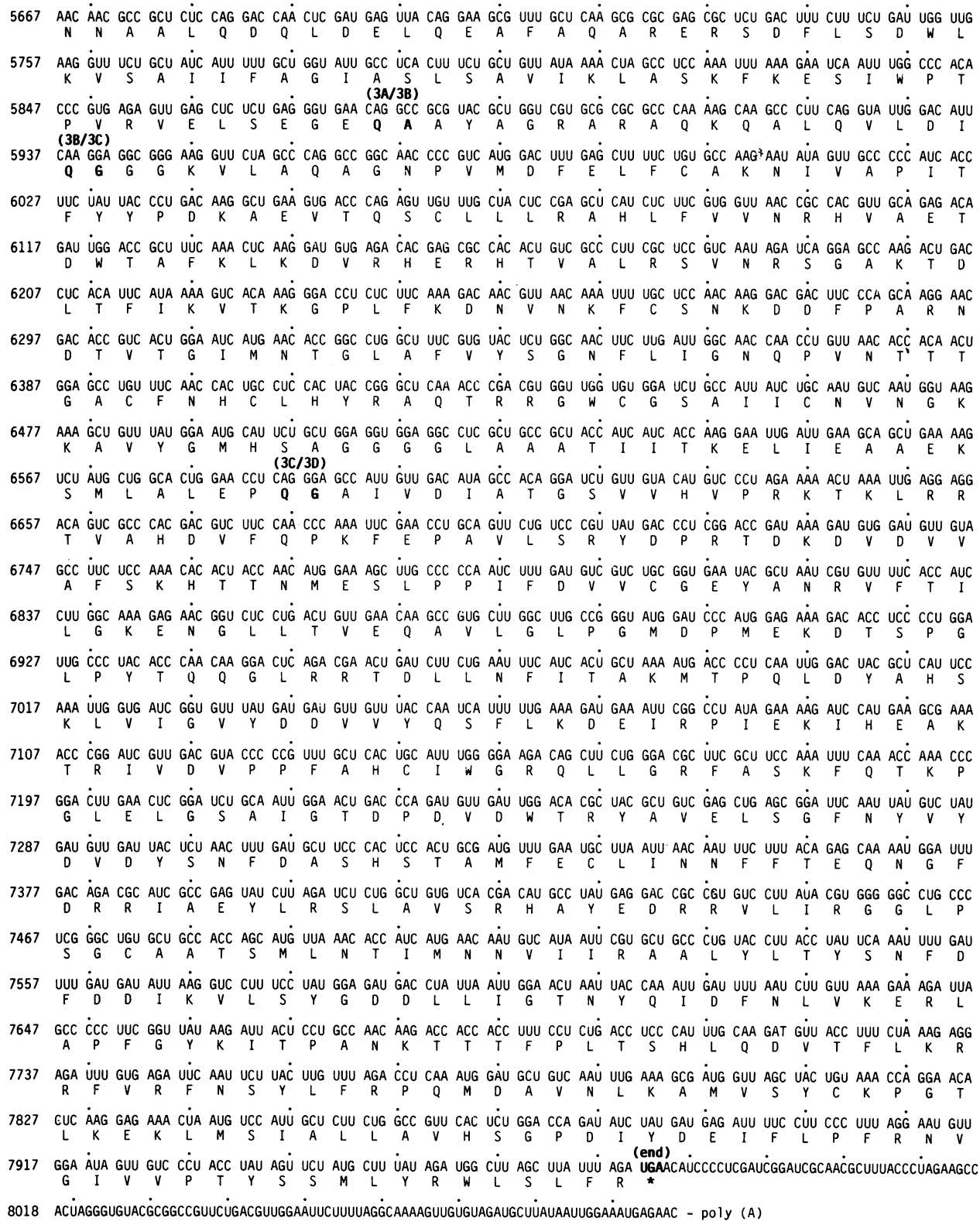


FIG. 2—*Continued.*

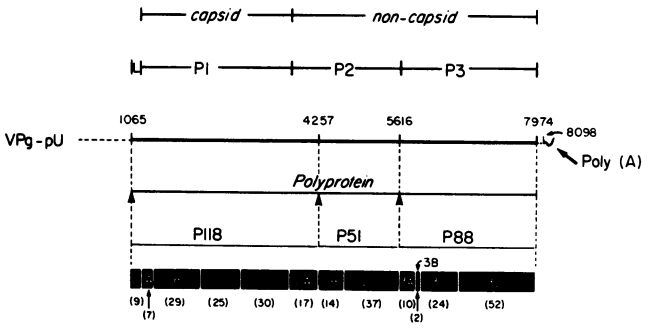


FIG. 3. TMEV-specific protein cleavage scheme. L, P1, P2, and P3 represent the leader protein and the three main processing regions of the viral polyprotein. The numbers in parentheses are the molecular weights (in thousands) of each of the proteins as calculated from their predicted amino acid sequences. The nomenclature of the proteins is that of Rueckert and Wimmer (39).

1D showed 69, 71, 66, and 46% amino acid identity, respectively, with the EMCV capsid proteins, as compared, for example, with only 15, 33, 27, and 14% homology with the respective human poliovirus type 1 capsid proteins.

The TMEV 5′ noncoding region differs markedly from that of EMCV since it is 231 nucleotides longer and has no poly(C) tract. However, nucleotide sequence comparison showed 68% identity of the 450 bases 5′ to the polyprotein start codon for the two viruses (bases 612 to 1064 of TMEV versus 385 to 833 of EMCV; Fig. 5). Except for the seven nucleotides at the 5′ end of TMEV which were identical to those bases in EMCV, no more identity of nucleotides 1 to 611 was found with EMCV than with any of the other picornaviruses (<39%). The 3′ noncoding region of TMEV was found to be more similar to EMCV in length (126 bases) and nucleotide identity (48%) than with poliovirus type 1 (72 bases; 38%), FMDV A10 (96 bases; 35%), or hepatitis A virus (64 bases; 33%). In contrast to EMCV, which has two

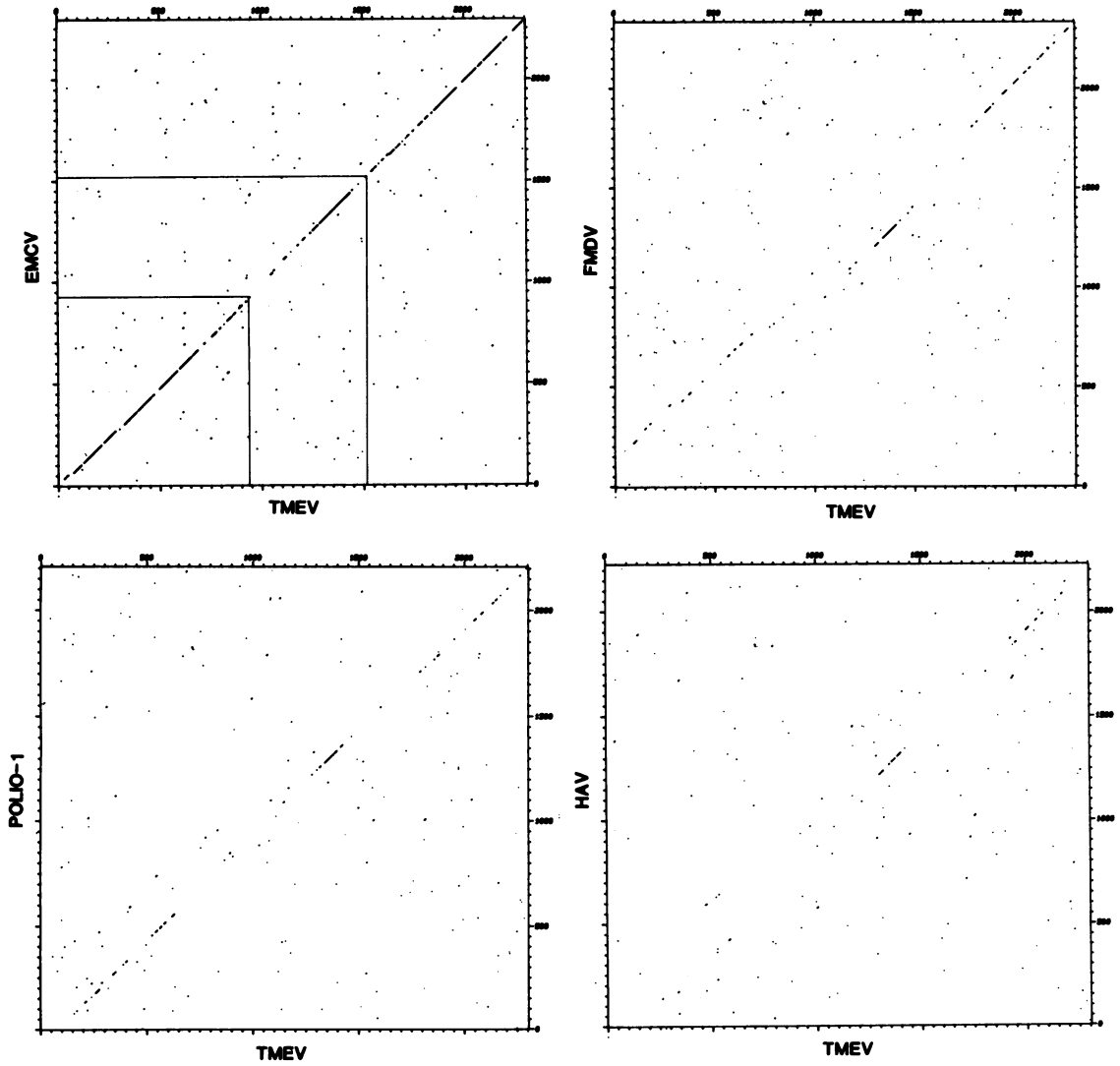


FIG. 4. Computer-generated dotplot comparisons of the TMEV polyprotein with those of four other picornaviruses. The TMEV polyprotein is located on the ordinate, and the polyproteins of EMCV, FMDV A10, human poliovirus type 1 (Sabin), and hepatitis A virus are located on the axis. A dot was placed on the plot whenever five amino acids within a window of nine residues matched exactly between the two sequences (11).

TABLE 1. Comparison of the predicted proteolytic cleavage sequences of the TMEV polyprotein with those of the EMCV polyprotein

| Proteins | Cleavage sites[a] | |
|---|---|---|
| | TMEV | EMCV |
| Leader/1A | MEPQ/GNSS | FELQ/GNST |
| 1A/1B | PLLL/DQNT | PLLA/DQNT |
| 1B/1C | VIAQ/SPIP | LSRQ/SPIP |
| 1C/1D | WVPQ/GVDN | WSPQ/GVEN |
| 1D/2A | LELE/NPAA | LMLE/SPNA |
| 2A/2B | FQPQ/GAVL | FQTQ/GAAV |
| 2B/2C | MQPQ/GPLR | FQQQ/SPLK |
| 2C/3A | LVAQ/SPPD | LVAQ/GPVD |
| 3A/VPg (3B) | EGEQ/AAYA | EQEQ/GPYN |
| 3B/protease (3C) | LDIQ/GGGK | LDIQ/GPNP |
| 3C/polymerase (3D) | LEPQ/GAIV | FEPQ/GALE |

[a] The proteolytic cleavage sites are indicated by a /, and the TMEV amino acids which are the same as those of EMCV are underlined.

stop codons at the end of the polyprotein reading frame, the TMEV polyprotein terminates at a single UGA triplet.

## DISCUSSION

In the present study, ds cDNA to the viral RNA of a demyelinating disease strain of TMEV (BeAn 8386 virus) was molecularly cloned and sequenced, and the polyprotein amino acid sequence was deduced. The fact that the TMEV polyprotein was found to be highly similar to that of the well-characterized cardiovirus EMCV was instrumental in determining the TMEV genome organization and protein map and indicated that the two viruses are closely related. Nucleic and amino acid computer analyses showed that the two viruses have related 5′ and 3′ noncoding regions and short leader peptides and that the capsid and nonstructural proteins have similar sizes and sequences (Fig. 3). Between picornavirus groups, the capsid proteins are generally least conserved. However, a high degree of homology exists between the capsid proteins of TMEV and EMCV (Fig. 4). For example, proteins 1B and 1C of TMEV shared 70% identity with those proteins in EMCV as compared with the only 55% identity found between the human enteroviruses poliovirus type 1 and coxsackievirus B3. Protein 1D is the least-conserved capsid protein between TMEV and EMCV (46%). Nonetheless, proteins 1D of these viruses were as similar as the comparison of 1D proteins between human poliovirus type 1 and coxsackievirus B3 (43%). Based on these similarities, we propose that the TMEV be grouped with the cardioviruses. However, since TMEV and EMCV have different biophysical properties and show no cross-neutralization (9, 38), they most likely belong in separate cardiovirus subgroups.

A distinct difference between EMCV and TMEV is the absence of a poly(C) tract in the 5′ noncoding region of the TMEV RNA. The RNAs of the cardioviruses (and aphthoviruses) differ from those of other picornaviruses in containing a long poly(C) tract located near the 5′ end of the RNA. Its length is variable, with EMCV isolates having poly(C) tracts as long as 250 bases, and its function is unknown (3, 4). Not only does TMEV lack a poly(C) tract (37), the first 611 nucleotides in the TMEV 5′ noncoding region [the site where the poly(C) tract would be located] show no more identity with EMCV than with any other picornavirus. In contrast, the 450 nucleotides upstream of the polyprotein start codon were found to be 68% homologous between TMEV and EMCV (Fig. 5). This sharp break in homology, which occurs just downstream from the EMCV poly(C) tract, may provide a clue to the origin of TMEV. TMEV and EMCV may have evolved from one another as a result of deletions [including the poly(C) tract] or insertions near the 5′ end of the untranslated region. Since intertypic recombination has been demonstrated among aphthoviruses (21) and among the three human poliovirus types (1, 14), it is possible that cardioviruses have arisen by recombination. Thus, differences in the beginning of the 5′ noncoding region may be important in the evolution of cardioviruses.

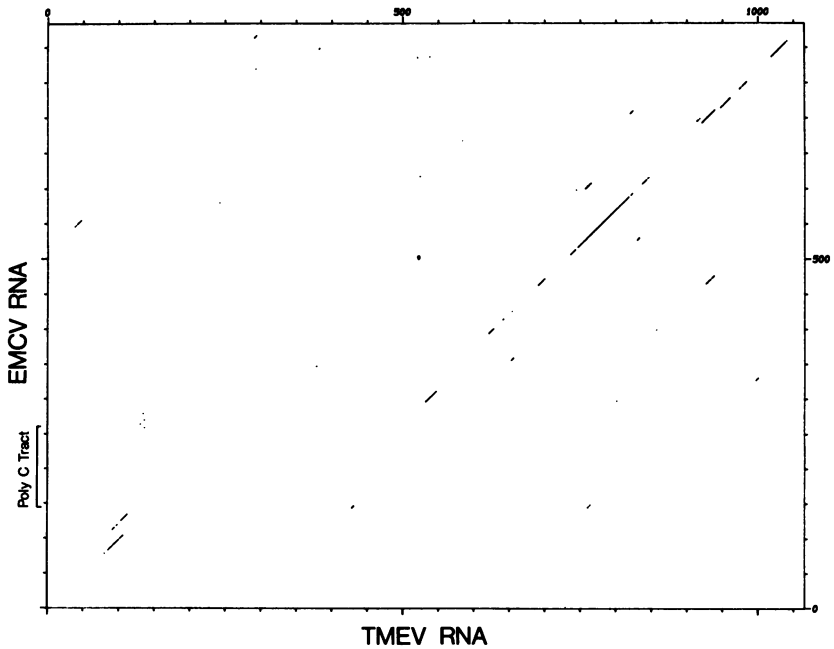


FIG. 5. Computer-generated dotplot comparison of the 5′ noncoding regions of TMEV and EMCV RNAs. The Compare computer program used a stringency of 14 nucleotides within a window of 21 nucleotides to determine regions of similarity.

It is noteworthy that the TMEV 5′ noncoding region is the longest in the picornavirus family next to that of the aphthoviruses. Among picornaviruses, the aphthoviruses and TMEV are the only viruses known to persist in immunocompetent animal hosts (5, 25, 43). It is possible that RNA sequences or secondary structures upstream of the polyprotein start site provide important controlling elements for viral replication, enabling picornaviruses to persist.

The recent discovery of the strong evolutionary conservation of the backbone of the structural proteins of icosahedral plant viruses and animal picornaviruses allows prediction of potential antigenic sites on animal picornavirus particles (19, 35). Computer-generated comparisons of the TMEV and EMCV capsid proteins (Fig. 4) have identified long regions of homology as well as shorter, more poorly conserved amino acid stretches. Interestingly, the four major neutralizing immunogenic sites on human rhinovirus 14, which reside on external protrusions, all lie within these poorly conserved regions (35, 41). These highly variable regions therefore represent potential immunogenic sites on the surface of the TMEV. The 11-amino-acid insertion at residues 136 to 146 of TMEV protein 1B relative to EMCV corresponds with the neutralizing immunogenic region on the human rhinovirus 14 1B external protrusion (puff; 35), making these residues a logical immunogenic site for TMEV. Two other immunogenic sites may reside on TMEV 1D at residues 50 to 100, 200 to 220, or both. The former corresponds to residues 80 to 95 in 1D on human rhinovirus 14, an external protrusion called the loop, which contains two neutralizing immunogenic sites (35, 41). Protein 1D residues 200 to 220 correspond to the FMDV loop at the carboxy end of FMDV protein 1D, which has been identified as an important neutralizing epitope for the aphthoviruses (2, 12, 29, 44). The atomic resolution of the structure of Mengo virus which has just been completed (28a), combined with capsid amino acid alignments of picornaviruses (Palmenberg, unpublished data), provides strong support for location of immunogenic sites at both the 1B puff and the 1D FMDV loop of TMEV. These regions in proteins 1B and 1D in Mengo virus and, by analogy, TMEV form large external protrusions from the virion surface. We are currently selecting escape mutants to neutralization by neutralizing monoclonal antibodies to identify the neutralizing immunogenic sites for TMEV.

Ultimately, it should be possible to exploit the close relationship of TMEV and EMCV by constructing intertypic cardiovirus recombinant cDNA clones. Constructs could be made between TMEV and EMCV as well as between viruses from the two virulence groups of TMEV. Such infectious cDNA clones would be useful in determining which regions in the genome are responsible for the vastly different pathogenetic properties of the two groups of TMEV and between the two subgroups of cardioviruses. It may also be possible to use this approach to elucidate the function of the poly(C) tract.

## ACKNOWLEDGMENTS

This research was suported by U.S. Public Health Service grant NS 21913 from the National Institutes of Health and National Multiple Sclerosis Society grant RG 1815 A-6. D.C.P. is a postdoctoral fellow of the National Multiple Sclerosis Society.

We thank Beth Ostrowski, Joseph Borkowski, and Steve Whittler for excellent technical assistance. We also thank Ann Palmenberg for enthusiastic support, helpful discussions, and review of the manuscript.

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