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A Single Base Deletion in the 5′ Noncoding Region of Theiler's Virus Attenuates Neurovirulence

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Viral chimeras have been constructed through in vitro manipulations of the infectious cDNA clones of two prototypes of Theiler's murine encephalomyelitis virus: (i) the virulent GDVII strain and (ii) the less virulent BeAn and VL strains. Previous studies have suggested that the phenotypic differences in virulence between the BeAn and GDVII strains map to both the 5′ noncoding and the coat protein regions of these viral genomes. It is shown here that attenuation mapped to the 5′ noncoding region is due, at least in part, to an inadvertent deletion resulting from a cloning artifact of one C nucleotide out of four between positions 876 and 879 in the BeAn sequences. The in vitro growth characteristics in BHK-21 cells, however, do not reflect the large differences in neurovirulence between chimeras that are identical except for the deleted C. Another chimera with a mutation at position 877 and a deletion at 976 is also attenuated. The wild-type sequences from the less virulent strains BeAn and VL between nucleotides 1 and 933, in an otherwise GDVII chimera, do not attenuate virulence. Sequences of the 500 nucleotides of the 5′ noncoding region proximal to the translation initiation codon were obtained for nine additional Theiler's virus strains. The attenuating deletions are discussed in the context of these sequences and the proposed secondary structures for the 5′ noncoding region.

There are two major subgroups among the various strains of Theiler's murine encephalomyelitis virus (TMEV), a picornavirus that is an enteric pathogen of mice. Division into these two groups is based on neurovirulence characteristics after intracerebral inoculation in mice. The strains comprising the highly virulent group rapidly produce, at low dosages, fatal encephalitis in mice. The number of strains in this group available in the laboratory are few; only two, GDVII and FA, are addressed in this report. Members of the second group, which includes the less virulent strains BeAn, VL, and DA, are characterized by a 10^6-fold reduction in 50% lethal dose (LD_{50}) in comparison with the virulent group and are capable of producing a persistent infection in the central nervous system. This infection is associated with chronic, inflammatory demyelinating disease (14), and therefore these less virulent strains are used in an animal model system for human demyelinating disorders such as multiple sclerosis.

One goal of neurovirulence studies with TMEV is to determine how the sequence of the 1,064 nucleotides (nt) in the 5′ noncoding (5NC) region relates to RNA structure and function. Questions include how functions for this region, such as replication and translation, can affect neurovirulence and persistence. It is possible that persistence requires more than just attenuation of virulence (8, 16, 27), but the genetic elements responsible for these phenotypes are only partially understood (1, 9, 16, 27). In this study, we have further localized and characterized at the molecular level possible neurovirulence determinants in the 5NC region.

Previous studies have suggested the presence of an attenuating determinant in the 5NC region of the less virulent virus strains BeAn (1, 16) and DA (8, 27). Use of recombinant chimeras, constructed by exchanging corresponding genomic regions of virulent and less virulent strains, has shown that sequences from nt 1 to 1137 from a cDNA clone of BeAn, in a chimera that otherwise contains GDVII sequences, produce a virus as attenuated as the BeAn strain. Studies with other chimeras have shown that in addition to this region, the P1 region encoding the coat proteins probably contains a neurovirulence determinant (1, 9). The P2 (except for possibly P2A), P3, and 3′ noncoding regions of the less virulent BeAn virus do not significantly attenuate the chimeras (1).

These experiments have now been extended by the construction of additional chimeras which map on a finer scale the 5NC attenuation determinants. It has been determined that an attenuating element maps to a region which was discovered to contain an inadvertent deletion unknowingly introduced into the BeAn cDNA clone. This deletion was also in previous chimeras that had been used to map neurovirulence (1, 16), and therefore the conclusion that BeAn contains an attenuating determinant in its 5NC region must be reconsidered. Sequences of a number of strains from both groups of TMEV were obtained and compared in an effort to establish structure-function relationships. The sequence alignments identify regions which are highly conserved among the various strains and therefore probably are functionally significant.

MATERIALS AND METHODS

Cell culture. BHK-21 cells were grown in 60-mm-diameter plates in Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 10% tryptose phosphate broth (GIBCO), 100 μg of streptomycin and 100 U of penicillin per ml, and 5% iron-supplemented bovine calf serum (HyClone). Plaque size, virus growth, and temperature sensitivity of the chimeras were determined by standard plaque assay on BHK-21 cell monolayers as described previously (15). Cells were inoculated and incubated for 3 days at the indicated temperature, and plaques were identified by staining with 0.12% crystal violet.

Virus strains. BeAn 8386 virus was isolated from a feral

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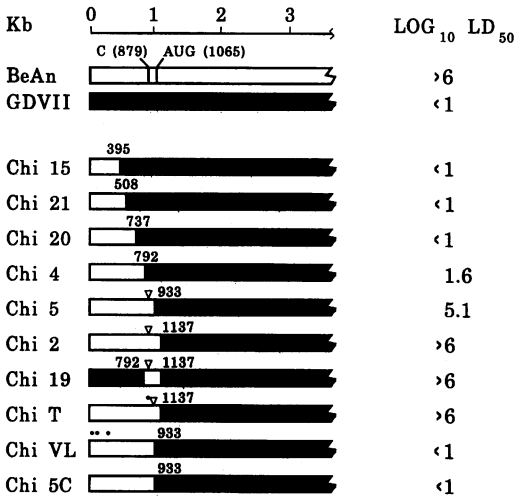

FIG. 1. Structures of the chimeric (Chi) constructs, derived from the recombinant cDNA clones, for BeAn (white) and GDVII (black). The nucleotide positions (numbered according to the BeAn sequence; see Fig. 2) of the restriction endonuclease sites (see Materials and Methods) used in the constructions are shown. The polypeptide begins at nt 1065 in the BeAn sequence. An inverted triangle and an asterisk denote a deletion and a mutation, respectively, relative to the BeAn wild-type sequence.

mouse in Belem, Brazil, in 1957 and was provided as a third-passage mouse brain stock by R. E. Shope (Yale University); WW was originally isolated in Philadelphia (32); VL was obtained from Pravin Bhatt (Yale University); TO(Yale) was obtained from the American Type Culture Collection. TO(B15) is a less virulent strain, but unlike the other less virulent strains, it is capable of establishing infection of the central nervous system via oral inoculation (7). The Vilyuisk strain was originally isolated in Siberia and has been partially characterized before (2, 17). Its virulence is not accurately known, since no one has reported a tissue culture-adapted isolate and quantitation of virus without a plaque-forming assay is difficult. However, Lipton et al. (17) note that the intracerebral adult mouse mean LD_{50} was $10^{9.3}$ per ml of brain stock, which is suggestive of a virulent virus. The virulent strains FA and GDVII and the less virulent strain TO(4) were from Theiler's original isolates (28). MGH, obtained from D. L. Huxsoll (Walter Reed Institute), was originally isolated from adult white rats (19).

Construction of recombinants. The full-length clones of BeAn and GDVII virus cDNA in the transcription vector pGEM-3 were constructed from subclones as previously described (1, 16). The numbered chimeras (Fig. 1; see below) were derived from the GDVII cDNA clone in pGEM-3 that was substituted, using designated restriction sites, with corresponding sequences from the BeAn cDNA clone. The relevant restriction sites and the corresponding nucleotide positions (BeAn coordinates) are *Bgl*II (395), *Sty*I (508), *Pvu*II (737), *Bgl*I (792), *Kpn*I (933), and *Ssp*I (1137). Chimera VL was constructed by using a GDVII cDNA clone in pGEM-4 substituted with corresponding sequences (obtained by polymerase chain reaction [PCR] amplification; see below) from virus strain VL. This construct was verified by sequencing PCR-amplified cDNA derived from the virus obtained from the brain of a dying mouse after transfection of RNA transcribed from the DNA clone and inoculation of virus into mice.

We inadvertently obtained another mutant, chimera T, that was missing a G at position 976 and contained the sequence ...CUCC... from nt 876 to 879 rather than the wild-type sequence ...CCCC.... These mutations, which arose during the cloning process, were discovered by sequencing the resulting DNA clone of chimera T before transfection and were verified again by sequencing the PCR-amplified cDNA from the virus obtained after transfection.

In vitro transcription and transfection. Clones were first linearized, and RNA transcripts were synthesized by using T7 RNA polymerase (Promega) as specified by the supplier. Subconfluent BHK-21 cell monolayers in 60-mm-diameter dishes were transfected with transcription reaction mixtures (approximately 10 to 30 μg of RNA) by using either DEAE-dextran (as described in reference 1) or Lipofectin (GIBCO-BRL) used as specified by the supplier. Progeny virus stocks were prepared after an additional passage in BHK-21 cells.

Animal inoculations and determination of LD_{50}. The number of PFU/LD_{50} was determined by using 6- to 12-week-old SJL mice inoculated in the right cerebral hemisphere with 10-fold dilutions (five to six mice per dilution) of parental and mutant viruses in 30 μl as previously described (1, 16). LD_{50} values of $>10^6$ could not be more precisely determined because of the limiting concentration of the viral stocks available.

PCR amplification of cDNA and sequencing. Total cellular RNA was isolated from virus-infected BHK-21 tissue culture cells or mouse brains essentially by the method of Chomczynski and Sacchi (3). Avian myeloblastosis virus reverse transcriptase was used to synthesize cDNA from the RNA as described previously (16) except that it was found that no added viral sequence primers were necessary in the reverse transcriptase reaction, possibly because of the presence of both negative- and positive-strand viral RNA or self-priming. PCR amplifications using 1 to 2 μl of the cDNA synthesis reaction mixture were carried out as described previously (16) except that only 25 cycles were used. PCR products were purified by acrylamide gel electrophoresis followed by electroelution and then sequenced directly as described elsewhere (31). For most of the sequences shown in Fig. 2, the synthesized primers used for both amplification and sequencing corresponded to BeAn nt 527 to 546 and the complements corresponded to nt 1104 to 1086.

Nucleotide sequence accession numbers. GenBank/EMBL accession numbers of the new sequences presented here are M80883 for FA, M80884 for MHG, M80885 for TO(4), M80886 for TO(B15), M80887 for VL, M80888 for Vilyuisk, M80889 for WW, and M80890 for TO(Yale).

RESULTS

Recombinant virus chimeras of BeAn and GDVII. In previous studies, a recombinant virus, chimera 2 (1, 16), constructed with BeAn sequences from nt 1 to 1137 and GDVII in the rest of the viral genome, was shown to have an LD_{50} of $>10^6$ PFU. To further localize the suggested attenuation determinant in the 5NC region or the beginning of the leader peptide of the BeAn strain, a series of chimeras was constructed in which BeAn sequences progressively replaced the GDVII genome from the extreme 5′ end of the viral sequences. As shown in Fig. 1, chimeras 15, 21, 20, and 4 with BeAn sequences up to nt 395, 508, 737, and 792, respectively, exhibited no significant decrease in virulence compared with the GDVII phenotype. The equivalent fragment replacement for chimera 20 was done by using a *Pvu*II

restriction endonuclease site at nt 737, while a *BglI* site at nt 792 was used in the construction of chimera 4. However, the nucleotide sequences of the BeAn and GDVII viruses are identical between these two sites, and therefore these two chimeras should have identical sequences and properties. For verification, the nucleotide sequences of these viral genomes were determined between positions 568 and 975 by PCR amplification of cDNA derived from RNA obtained from infected tissue culture cells (see Materials and Methods). Both chimeras 4 and 20 had the expected sequences, but we cannot absolutely rule out the possibility that one of these chimeras has a mutation elsewhere in the genome. We do not consider the difference in their LD_{50} values to be significant.

Further replacement of GDVII sequences with the equivalent region from BeAn downstream of approximately nt 800 resulted in a dramatic decrease in virulence. Chimera 5, which has BeAn sequences up to nt 933, had an LD_{50} of 2.5×10^5 PFU, and the LD_{50} of chimera 2, with BeAn sequences up to nt 1137, was $>10^6$ PFU, as previously reported. Both of these chimeras also caused early-onset flaccid paralysis between 1 and 10 days postinfection (16), typical of the less virulent TMEV. Furthermore, chimera 19, in which only the sequences between nt 792 and 1137 are from the BeAn viral genome, was also attenuated. These results show that there is a strong attenuating sequence element or elements between nt 792 and 1137 in the BeAn genome.

Nucleotide sequences. To help establish structure-function relationships in this region and to attempt to identify potential attenuating determinants, nucleotide sequences for a number of virulent and attenuated strains were determined and compared (Fig. 2). It was hoped that at a few important positions in this region, all of the virulent strains would be uniquely distinguished from all of the nonvirulent strains; these specific sites would then be investigated directly by site-specific mutagenesis. With a total of 11 strains, only 2 of them definitely virulent (GDVII and FA; the virulence of Vilyuisk is not known; see Materials and Methods), the sequence alignments proved to be valuable in revealing highly conserved areas and features that were common to all viral genomes (see Discussion). In general, it can be noted that there are very few insertions or deletions in the wild-type sequences obtained, but it was discovered that our full-length laboratory cDNA clone of the BeAn virus contained a deletion between nt 876 and 879; the wild-type BeAn sequence contained four C residues, but the laboratory cDNA clone contained only three. Further sequencing revealed that the original cDNA subgenomic clones did not have the deletion but that during the construction of the full-length clone, the deletion was inadvertently introduced.

Other viral chimeric constructs. Since none of the wild-type strains sequenced contain a deletion in this region, the possible importance of the deleted C as a phenotypic determinant was investigated further by the construction of other recombinant virus chimeras. From nt 1 to 933 (to the *KpnI* site), chimera VL contains sequences from the viral genome of strain VL (Fig. 2). The VL sequences were used because they are nearly identical to those of BeAn in the 5NC region and do not contain the C deletion at position 879. VL is different from BeAn at nt 33, 41, and 236 (Fig. 1 and 2). The VL strain belongs to the less virulent subgroup, but as shown in Fig. 1, there is no attenuating element in its first 933 nt, in contrast to the equivalent BeAn sequence with the deleted C.

Chimera 5C was constructed to show that the only attenuating element was the deleted C in the BeAn sequences in chimera 5. Chimera 5C is identical to chimera 5 (BeAn sequence up to the *KpnI* site at nt 933) except that the C at nt 879 was added so that the BeAn part of the sequence is now identical to that of the wild-type strain. Chimera 5C was as virulent as GDVII.

Chimera T contains wild-type BeAn sequences up to position 1137 except for a U rather than a C at position 877 and a deletion at position 976. The deletion occurred at the *PmlI* restriction site that was used in the cloning. This construct was also attenuated, with an LD_{50} of $>10^6$ PFU (Fig. 1), but produced early-onset paralysis in mice more often than did chimera 5. This result is another example of a small variation in the 5NC region sequence which causes a large attenuation in the virulence.

In vitro growth characteristics. Plaque sizes, temperature sensitivities, and one-step growth curves were determined for the parental GDVII and BeAn strains and for chimeras 5 and 5C. Table 1 compares the temperature sensitivities and plaque sizes for these strains grown at 33, 37, and 39.8°C. The only one showing a temperature sensitivity was the parental BeAn strain (which lacks the C at nt 879), whose titer at 39.8°C was reduced by a factor of over 10^4 compared with that at 33°C; the GDVII, chimera 5, and chimera 5C titers at the higher temperature were reduced by a factor of only 0.6 to 0.8. The BeAn plaque size was small at all temperatures (regardless of the presence or absence of C-879; data not shown), while the GDVII and chimera plaque sizes were larger but varied with temperature. The plaque sizes of chimera 5C were nearly indistinguishable from those of GDVII at all temperatures, but plaque sizes of chimera 5 were smaller than those of chimera 5C at 33 and 39.8°C. There appear to be slight differences between chimeras 5 and 5C, but it is doubtful that these differences can explain the large difference in neurovirulence between these two chimeras. It should be noted that these results do not support an absolute correlation between neurovirulence attenuation of TMEV chimeras, small plaque size, and temperature sensitivity. Such a general correlation has been observed previously (1), but an exception has been noted. The less virulent chimera 2, which also has C-879 deleted, has large plaque size and is not temperature sensitive (1).

One-step growth curves (not shown) also revealed only slight differences among GDVII, chimera 5, and chimera 5C. Growth of chimera 5C appeared faster at early time points, but the growth curves appeared to converge at 11 h.

DISCUSSION

Previous reports have demonstrated the use of interstrain recombinant viruses in mapping the neurovirulence and persistence phenotypes of different strains of TMEV to the 5NC and P1 regions of the genome (1, 8, 9, 16, 27). Further refinement of this technique together with nucleotide sequencing has now shown that neurovirulence attenuation by the 5NC region of our BeAn virus (1, 16) is due, at least in part, to a deletion in our laboratory cDNA clone of one C out of four in the wild-type strain between positions 876 and 879. This deletion occurred during construction of the full-length cDNA clone from subgenomic clones, as revealed by DNA sequencing of the various subclones used, rather than during passage of the virus in tissue culture cells or mice. We have further shown that the less virulent virus strain VL, which is very similar to BeAn, does not contain an attenuating element in its 5NC region up to the *KpnI* site at nt 933. The

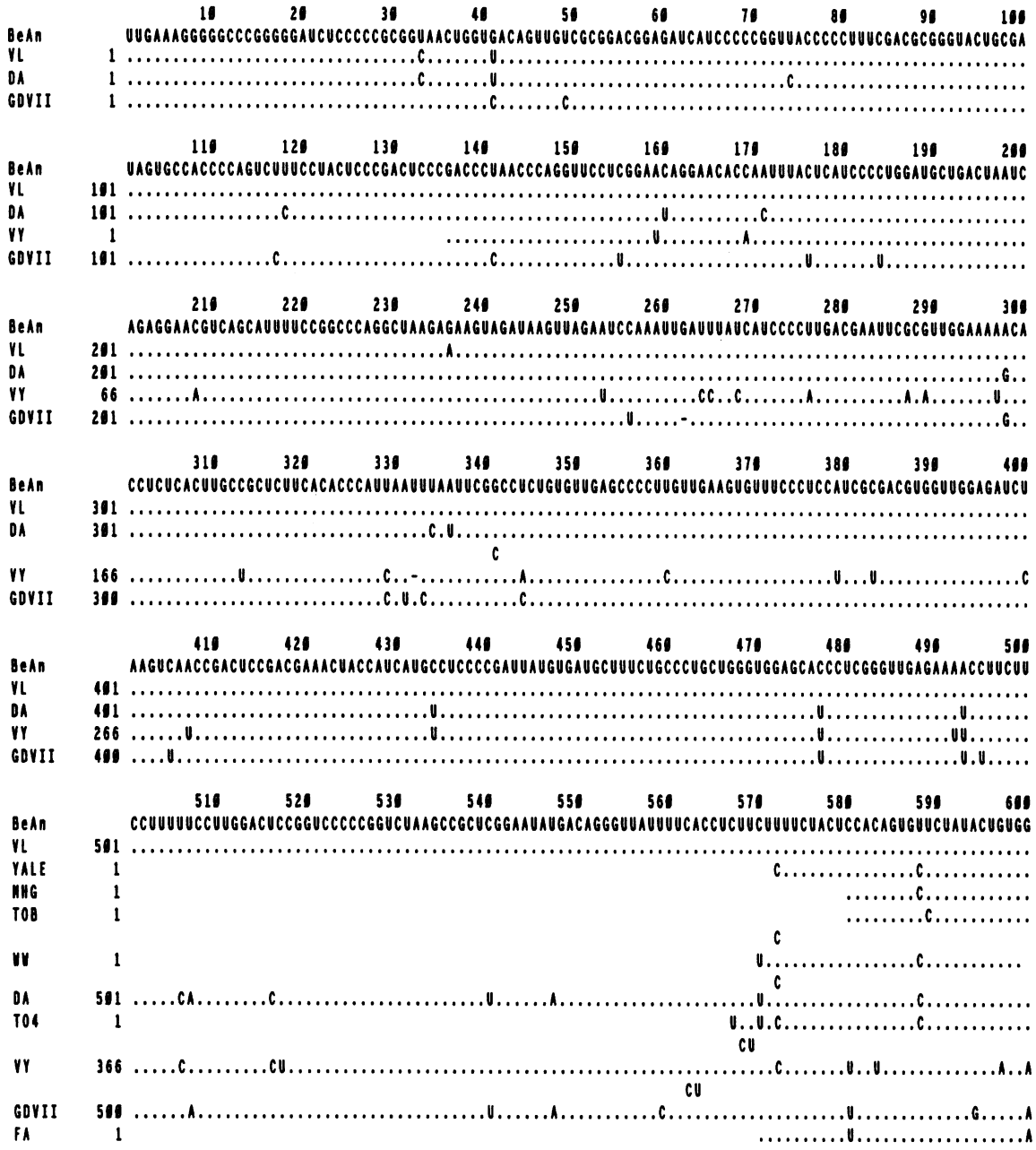

FIG. 2. Aligned nucleotide sequences of parts of the 5NC region for TMEV strains. Nonstandard abbreviations: TOB, TO(B15); YALE, TO(Yale); TO4, TO(4); VY, Vilyuisk. Identity at each position with the printed BeAn sequence is indicated by a dot, differences are indicated with a letter, deletions are shown as a dash, and insertions are shown as letters above the sequence at the point of insertion. Previously published sequences include those for BeAn (22), GDVII (21), and DA (20). The published GDVII sequence has been corrected by deleting one of the A residues in the stretch following position 1000, so that all strains are now identical in this region. The Y at position 1091 indicates a pyrimidine which was used as an ambiguous base in the primer for PCR amplification of this region.

previous conclusion that BeAn contains an attenuating determinant in its 5NC region must be reconsidered.

For unknown reasons, our attempts to obtain a viral chimera which has wild-type BeAn sequences up to nt 1137 (chimera 2 with the deleted C replaced; Fig. 1) have not yielded viable virus so far. It is therefore not yet possible to prove that there are no attenuating determinants in BeAn sequences between nt 933 and 1137. In the part of the leader peptide that is encoded up to nt 1137, only amino acid 22 is different between GDVII (Asp) and BeAn (Gly). There is at least a 1-log-unit difference in LD_{50} between chimera 5 (BeAn sequences up to nt 933) and chimera 2. This difference may not be significant, since the LD_{50} values of chimeras 4 and 20 are about 1 log unit apart even though their sequences appear to be identical (see Results). Further experiments are needed to evaluate the possibility of attenuating determinants between nt 933 and 1137 in BeAn.

Sequence alignments shown in Fig. 2 reveal heterogene-

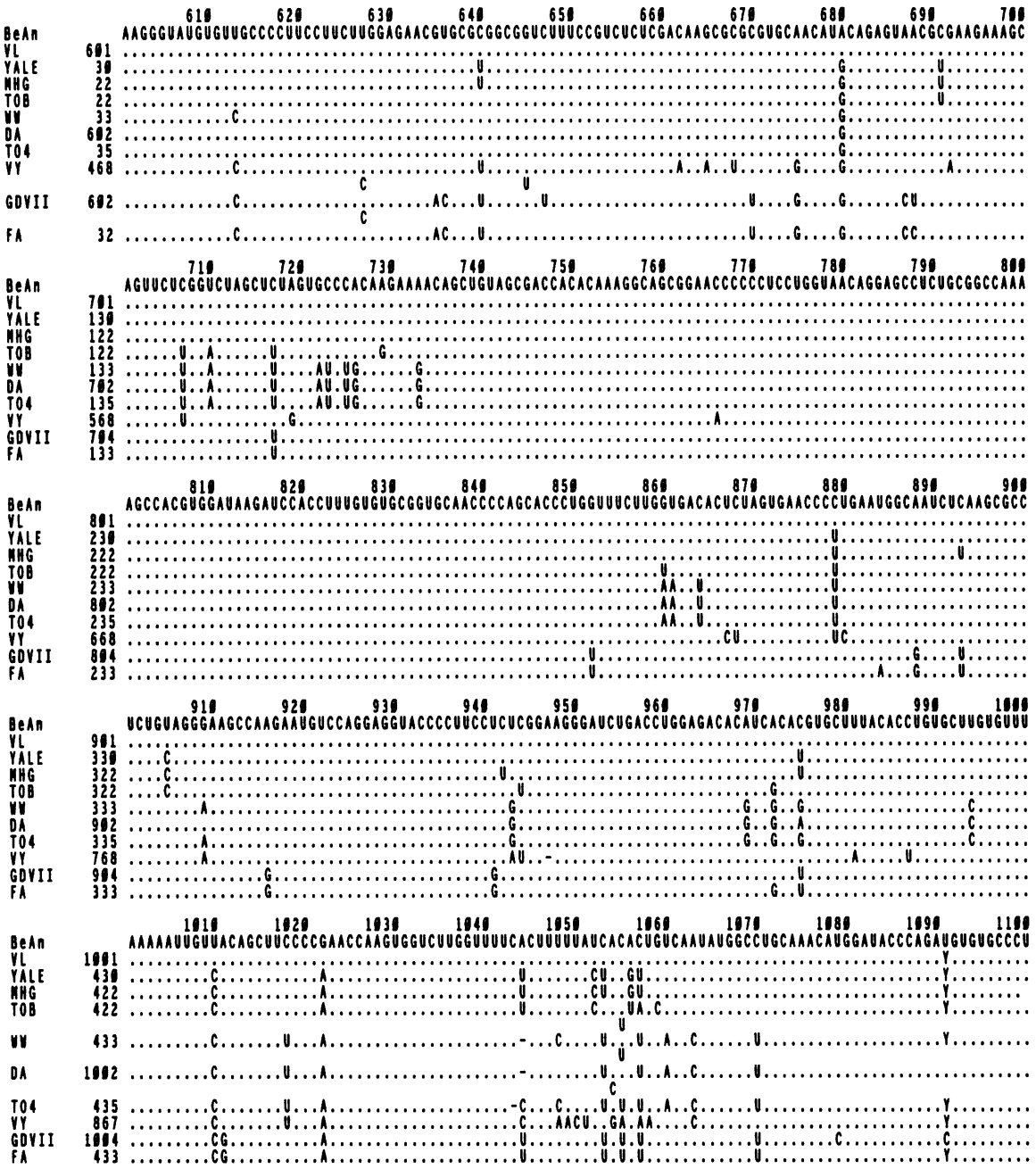

FIG. 2—*Continued.*

ity, either a C or U residue, among 11 strains at position 879 and no correlation between the particular nucleotide and the phenotype. However, a deletion near or at that position drastically attenuates neurovirulence (Fig. 1). A predicted secondary structure model for this region of the BeAn genome is shown in Fig. 3. This structure is based on both theoretical analyses of published sequences and some experimental verification using chemical and enzymatic probes (23). A similar structure has been proposed for encephalomyocarditis virus and aphthovirus RNAs (6, 23). The structure shown in Fig. 3 from domains G to L is taken from Pilipenko et al. (23), domain F is based on the equivalent structure predicted for encephalomyocarditis virus (6), and no ordered structure is shown for positions past domain L because of the extreme heterogeneity of sequences in this region (Fig. 2). In general, sequences of TMEV strains other than BeAn can also be folded into the structure shown in Fig. 3, but there are exceptions which suggest that further refinements of the predicted folding are necessary. In support of the structure shown are six positions, indicated by + marks between paired bases, where one or more strains have compensating mutations (A · U ↔ G · C) that conserve the base pairing shown. Many of the other variations in the sequences result in U · A-to-U · G changes in the base pairing or are present in non-base-paired loops and bulges. However, interstrain sequence differences in domains F and

TABLE 1. In vitro growth characteristics of temperature sensitivity and plaque size

Virus	Temp (°C)	Titer[a] (PFU/ml)	EOP[b]	Plaque size (mm)
BeAn parent	33	1.8×10^8	1	<0.25
	37	2.3×10^7	0.13	<0.25
	39.8	6.8×10^4	3.8×10^{-4}	0.25
GDVII parent	33	1.6×10^8	1	1–3
	37	1.2×10^8	0.75	5–6
	39.8	9.5×10^7	0.60	2–4
Chimera 5	33	1.1×10^8	1	0.5–1.5
	37	1.6×10^8	1.4	4–6
	39	8.8×10^7	0.80	1–2.5
Chimera 5C	33	3.0×10^8	1	1–3.5
	37	2.0×10^8	0.67	4–7.5
	39.8	1.7×10^8	0.57	3–5

[a] Standard plaque assays were performed on virus stocks, produced by one passage posttransfection in BHK-21 cells, at the indicated temperatures. The BeAn parent lacks the C at nt 879.

[b] Efficiency of plaquing (EOP) is the ratio of the titer at T°C to that at 33°C.

G and part of H have several indications of possible deviations from the structure shown in Fig. 3. Several strains have different bases at positions 588 and 613, in domains F and G, respectively, and an insertion between positions 627 and 628, in domain H, that would alter the base pairing shown (Fig. 2 and 3). In addition, for the FA, GDVII, and Vilyuisk strains, there appear to be compensatory mutations at nt 580 (C→U) concomitant with nt 600 (G→A) and at nt 613 (U→C) concomitant with nt 675 (A→G), suggesting base pairing not shown in Fig. 3. For example, the helix in domain F could be extended by 3 more bp (eliminating the bottom of the domain G helix) to include the apparent compensatory mutations at 580 and 600. Other positions with different bases (in at least one strain) that do not support the base pairing shown are nt 710 and 1010. Further analyses of these sequences will be useful in predicting a consensus secondary structure.

Other studies have implicated the region equivalent to that shown in Fig. 3 in cap-independent translation of cardiovirus RNA (10, 11). Of the four C residues between nt 876 and 879, the first two are shown in the structure as base paired in a helix and the other two are shown as part of a non-base-paired bulge. An explanation for the dramatic effect of the deletion on neurovirulence is not apparent from this structure. It is possible that a deletion of one of these C's could affect either tertiary interactions (mediated through pseudoknots, for example) in the RNA structure or protein-RNA binding interactions. As shown in Fig. 2 and 3, the deletion near nt 879 is not in a highly conserved region, but such regions are nearby: the end of the I domain, parts of the J and K domains, and the L domain. By analogy to the function of equivalent sequences in other cardioviruses (10, 11), it is likely that these sequences play important roles in the translation process, and therefore the deleted C attenuates by decreasing the efficiency of translation. It is also possible that other mechanisms, such as effects on RNA stability, packaging, or replication, are involved.

Attenuation of neurovirulence by deletions or mutations in the 5NC region of TMEV may be analogous to a similar mechanism in poliovirus vaccine strains. Two attenuation determinants have been identified in the type 3 vaccine strain, P3/Sabin, including a nucleotide mutation at position 472 in the 5NC region (30). Other studies have further shown that this mutation affects the translation efficiency of poliovirus RNA in vitro (26). Three regions of the type 1 vaccine

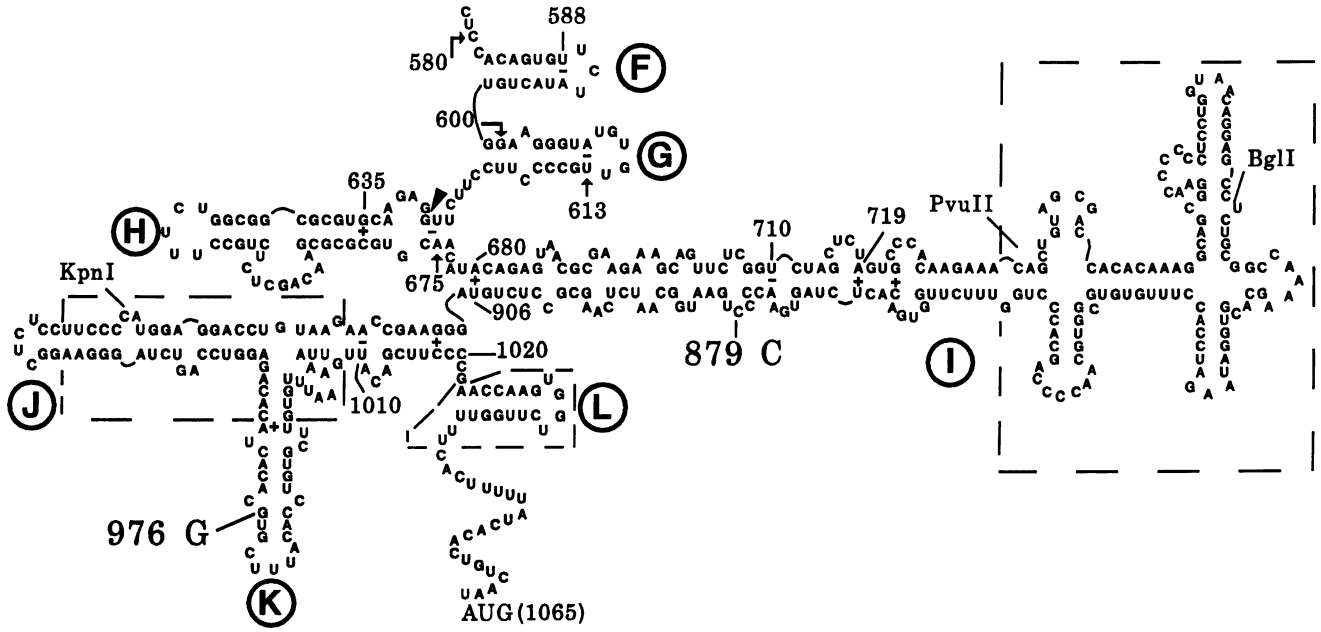

FIG. 3. Predicted secondary structure of part of the BeAn strain 5NC region based on previous publications (6, 23). The boxed regions are those showing little or no sequence heterogeneity in the Fig. 2 alignments. The + marks are between base pairs where the aligned sequences, for at least one virus strain, have compensating mutations (A · U ↔ G · C) that conserve the base pairing. The minus marks are between base pairs that are not always structurally conserved. The arrowhead indicates the position of an insertion for strains GDVII and FA. The two sets of bases with bent or straight arrows indicate suggested pairs of compensating mutations. Other important bases and restriction sites are also indicated. Domains of the structure are named by the circled letters F to J according to the nomenclature of Duke et al. (6).

strain, P1/Sabin, have been associated with attenuation elements, including position 480 (12), and recently position 481 has been shown to be one of the determinants that attenuate vaccine-related type 2 poliovirus (18, 24). In general, the region from nt 443 to 499 in the 742-nt 5NC region of poliovirus may be relatively intolerant of insertions or deletions, since several groups have been unable to construct such a viable mutation in this region (4, 13, 29), although several examples of viable point mutations in this region have been reported (25). A noninfectious poliovirus type 1 mutant, in which nt 461 and 462 are deleted, has recently been shown to have severely decreased translational efficiency, compared with the wild type, in an in vitro translation assay (5).

The in vitro growth characteristics of the TMEV chimeras in BHK-21 cells, however, do not reflect large differences in neurovirulence between the chimeras that are identical except for the deleted C (chimeras 5 and 5C; Table 1). The differences in the temperature sensitivities, plaque sizes, and growth curves are only slight. For these chimeras, the effects which lead to large differences of neurovirulence in mice are probably not detected in viral growth characteristics in BHK-21 cells. It is possible that there are host factors (translation enzymes present in neuronal cells, for example) which affect viral neurovirulence and are sensitive to the deletion of the C.

For all of the sequences shown in Fig. 2, all but one strain (the most divergent, Vilyuisk, with a deletion at position 947) have the same number of nucleotides between position 646 and the initiating AUG codon at position 1065, although there are several insertions or deletions upstream of nt 646. This size conservation suggests a functional significance. In particular, between position 1044 and the initiation codon there is extreme interstrain sequence heterogeneity but absolute conservation in size. This region probably acts as a spacer between sequence-specific protein-binding domains and regulatory signals, as suggested by Jackson et al. (10).

Another 5NC region mutant, chimera T, which contains wild-type BeAn sequences up to position 1137 except for a U rather than a C at position 877 and a deletion at position 976, is also attenuated, with an LD_{50} of $>10^6$ PFU. It is not clear whether either or both mutations cause the attenuation, but this result shows that other relatively small changes in this region can result in significant phenotypic changes. It is also possible, but we think unlikely, that an attenuating element in the wild-type BeAn sequences between nt 933 and 1137 contributes to this phenotype.

The results presented here have shown that a single nucleotide deletion in the 5NC region can attenuate the virulence of a virus by over 10^4-fold. The mechanism for this attenuation, possibly through reduced translational efficiency, remains to be elucidated. The newly determined sequences will be of use in assigning structure-function relations in the 5NC region.

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