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Neurovirulence determinants of genetically engineered Theiler viruses

(picornavirus/recombinant viruses/Theiler murine encephalomyelitis viruses)

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ABSTRACT **Theiler murine encephalomyelitis viruses (TMEVs) are picornaviruses that cause enteric and neurological disease in mice. The GDVII strain and other members of the GDVII subgroup are highly virulent and cause an acute, fatal polioencephalomyelitis following intracerebral inoculation, whereas the DA stain and other members of the TO subgroup cause a persistent, demyelinating infection. We previously produced a full-length, infectious DA cDNA clone. We now describe the generation of a full-length, infectious GDVII cDNA clone and the subsequent production of intratypic chimeric cDNAs and intratypic recombinant viruses. Inoculation of the recombinant viruses into mice demonstrated that a major determinant of TMEV neurovirulence is within the GDVII 1B (capsid protein VP2)–2C coding region, most likely in the GDVII 1B (VP2)–2A coding region. Genomic sequences 5′ to this region of GDVII RNA also contribute to expression of the full neurovirulence phenotype. These data demonstrate the multigenic nature of TMEV neurovirulence, as has been reported for other viruses.**

Neurovirulence, the ability of a virus to kill following central nervous system infection, has been a topic of considerable interest for virologists. Infections with picornaviruses, such as poliovirus, have provided useful systems for the study of neurovirulence. For over three decades investigators have sought a molecular basis for the attenuation of neurovirulence seen in poliovirus vaccine strains. The recent development of recombinant viruses generated from infectious chimeric parental and vaccine poliovirus cDNA clones, as well as the use of site-directed mutagenesis of infectious clones, have provided the means to delineate the molecular determinants of poliovirus neurovirulence (reviewed in refs. 1 and 2). Investigations have identified specific nucleotides in the 5′ noncoding region and elsewhere in the genome as critical for poliovirus neurovirulence. These manipulations have been especially informative given the relatively small size of the picornavirus genome and the large amount of molecular and structural information that is presently available regarding poliovirus and other picornaviruses.

The study of Theiler murine encephalomyelitis viruses (TMEVs), a group of mouse picornaviruses most similar at the sequence level to cardioviruses, provides a valuable model system to investigate neurovirulence. One important feature of TMEVs is that the mouse serves as both the natural and the experimental host. Another special advantage of the TMEV model relates to the natural division of TMEV strains into two subgroups on the basis of their differing biological activities (3, 4). The GDVII strain and other members of the GDVII subgroup of TMEVs are highly neurovirulent; intracerebral inoculation of a weanling mouse with one plaque-forming unit (pfu) causes an acute, fatal polioencephalomyelitis. In contrast, the DA strain and other members of the TO subgroup of TMEVs are less neurovirulent and produce a different disease following intracerebral inoculation; a dose of 10^6 pfu does not kill a mouse but causes a persistent, demyelinating infection with restricted virus expression (5). It should be mentioned, however, that TMEVs generally produce an enteric infection in nature, with rare paralysis; it is only after experimental intracerebral inoculation that central nervous system disease commonly occurs. The diversity of disease phenotypes is striking given the sequence identity of over 90% at the nucleotide level and over 95% at the deduced amino acid level between strains from both subgroups (6–8).

We previously described (9) the generation of an infectious cDNA clone of the DA strain. We now describe the production of an infectious GDVII cDNA clone and the subsequent construction of intratypic chimeric cDNAs and intratypic recombinant viruses. A major genetic component of TMEV neurovirulence lies within a segment of the GDVII RNA that includes regions 1B (encoding capsid protein VP2) through 2C; the nucleotide sequences 5′ to this region also appear to contribute to the neurovirulence phenotype.

MATERIALS AND METHODS

Cells. L929 cells (mouse fibroblasts) were used for plaque assays and transfections, and BHK-21 cells (hamster kidney cells) for growing stock virus.

Molecular Cloning of GDVII cDNA. Double-stranded cDNA was prepared from purified GDVII viral RNA and then treated with Klenow fragment of DNA polymerase and ligated to *Sma* I-digested, calf intestinal alkaline phosphatase-treated vector, pBluescript KS(−) (Stratagene), by previously described methods (6, 10). Transformation, screening of colonies, and sequencing were as described (6). Inserts were localized on the GDVII genome by comparing the sequence with that published (7). The following clones were produced: pGX548 [with an insert that runs from nucleotide (nt) 274 to nt 2114], pGG152 (nt 1596–2541), pGG300 (nt 2201–4341), pGG366 (nt 4097–5410), and pGC9 (nt 5220–8139).

To obtain clones representing the 5′ end of the genome, a polymerase chain reaction was employed on pGX548 cDNA, using a primer complementary to nt 800–820 and another primer corresponding to nt 1–18. There were 25 cycles (denaturing at 94°C for 10 min, annealing at 37°C for 2 min, extension at 72°C for 6 min) and the last cycle had extension at 72°C for 14 min. The amplified 800-base-pair cDNA was

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Abbreviations: TMEV, Theiler murine encephalomyelitis virus; pfu, plaque-forming unit(s); nt, nucleotide(s).

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digested with *Apa* I and *Bgl* II, ligated to *Apa* I/*Bgl* II-digested pGX548, and used to transform XL-1 cells. The resultant clone, which contains an insert that runs from nt 14 to nt 2114, is designated pGX548-4.

To construct a full-length GDVII cDNA clone, we used the reengineered 5′ end of pDAFL3, a full-length infectious DA cDNA clone that contains the T7 promoter through the first 13 nt of DA [which are identical to the first 13 nt of GDVII (6, 7)]. The cloning scheme used for the generation of full-length infectious GDVII cDNA is outlined in Fig. 1.

Construction of Chimeric cDNA Clones. A number of chimeric cDNA clones were constructed by replacing part of the infectious DA or GDVII cDNA clone with the corresponding fragment from the other TMEV clone (9). The junctional regions of the DA/GDVII chimeric cDNAs were sequenced to verify that the insertions had the expected borders. The chimeras are named by first listing the TMEV strain that makes up the minority of the chimeric genome followed by the 5′ and 3′ boundaries of this segment, then a slash, and finally the other strain that contributed the remainder of the genome (Fig. 2): pDA2C-3′/GDVIIFL2 contains a DA segment from the *Stu* I site at nt 4834 to the 3′ end; pGD1B-2C/DAFL3 contains a GDVII segment from the *Nco* I site at 1964 to the *Stu* I site at 4843; pGD5′-1B/DAFL3 contains a GDVII segment from the 5′ end of the *Nco* I site at 1964; pGDNC/DAFL3 contains a GDVII segment from the *Eco*RI site at 280 to the *Kpn* I site at 936; pGD2A-2C/DAFL3 contains a GDVII segment from the *Aat* II site at 3918 to the *Stu* I site at 4843; pGD2C-3C/DAFL3 contains a GDVII segment from the *Stu* I site at 4843 to the *Stu* I site at 6518; pGD3C-3′/DAFL3 contains a GDVII segment from the *Bst*EII site at 5971 to the 3′ end.

***In Vitro* Transcription and Transfection.** The transcription template was linearized with *Xba* I, which cuts 3′ to the end of the TMEV genome, and then transcribed in a T7 RNA polymerase reaction and transfected onto Petri dishes of L cells, as previously described (9). Dishes that had an overlay of liquid medium and showed viral cytopathic effect were harvested, and/or well-isolated plaques from dishes with an agarose overlay were picked when visible under the microscope. The virus was passed in BHK-21 cells to produce a stock.

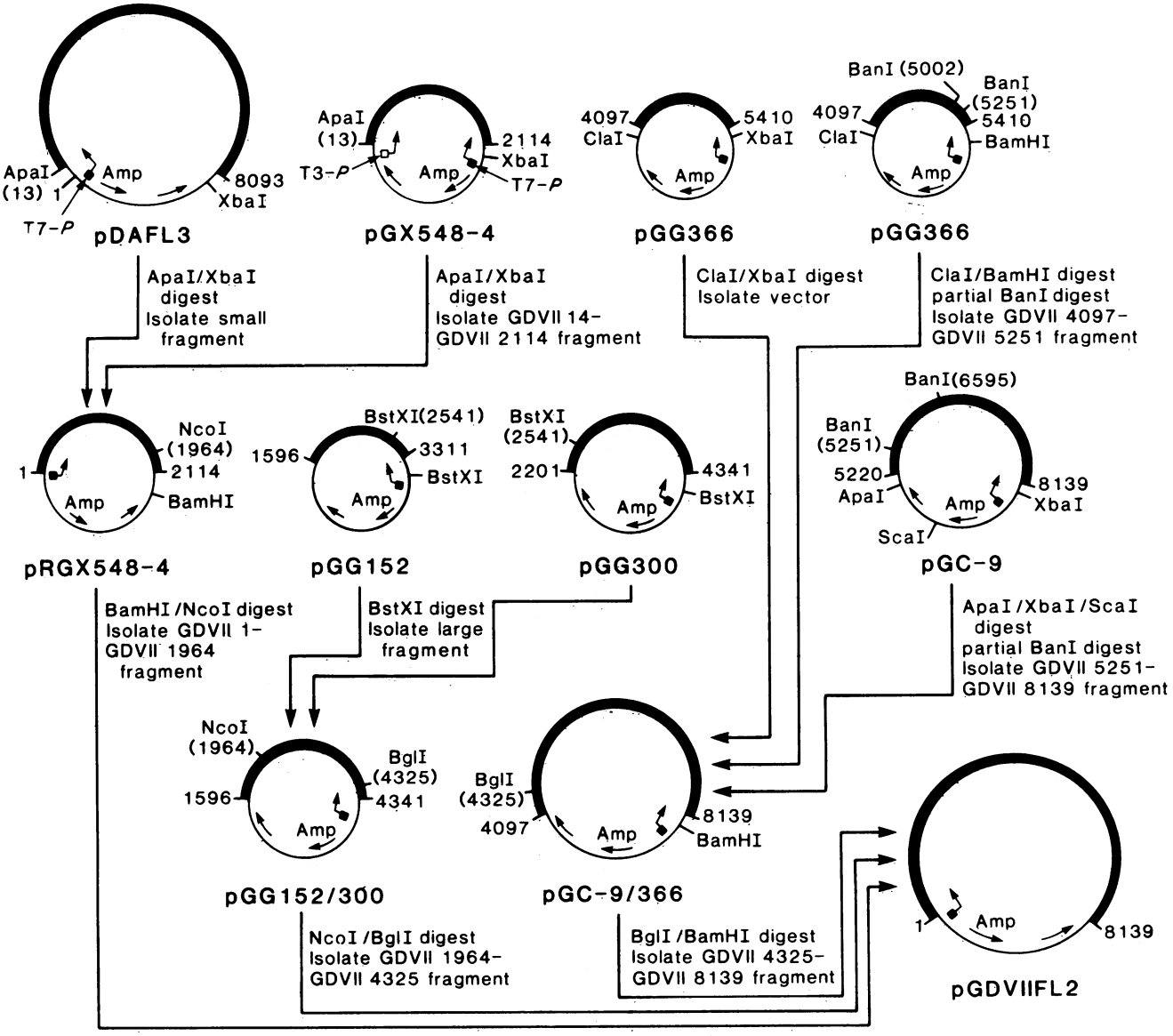

FIG. 1. Construction of the full-length, infectious GDVII cDNA, pGDVIIFL2. Numbers refer to the nucleotide number of the GDVII genome and numbers in parentheses correspond to the restriction enzyme sites on the GDVII genome. The T7 promoter (T7-*P*) and the ampicillin-resistance gene (Amp) are shown; the origin of replication is the unlabeled arrow. Details regarding the construction of the clone are given in *Materials and Methods*.

Animal Inoculations. Five to 10 weanling DBA/2 mice (Charles River Breeding Laboratories) were inoculated intracerebrally with 30 μl of undiluted virus or 10-fold dilutions of virus. The mice were observed for 4 weeks to arrive at a 50% lethal dose (LD_{50}) according to the method of Karber (11).

RESULTS

Construction of Full-Length Infectious GDVII cDNA Clone. A series of overlapping GDVII subgenomic cDNA clones was generated that ran from nt 14 to the 3′ end of the genome, including 27 adenylate residues at the 3′ end. pDAFL3, a full-length infectious DA cDNA clone, was used as a source of the transcription vector (pSKII−) and of the first 13 nt of the TMEV genome (which are located 2 nt downstream from the T7 promoter; Fig. 1). The subgenomic cDNA clones were digested with restriction enzymes, and after a number of ligation steps a full-length cDNA copy of the GDVII genome inserted into pSKII− was generated. *In vitro* transcripts of *Xba* I-digested pGDVIIFL2 had an infectivity of $\approx 10^{2.7}$ pfu/μg of RNA.

Chimeric cDNA Clones and Recombinant Viruses. The generation of infectious cDNA clones of GDVII and DA provided reagents for the preparation of chimeric cDNAs. Each of the chimeric cDNAs (Fig. 2) was linearized with *Xba* I and transcribed *in vitro* with T7 polymerase. The transcripts were then separately transfected into L cells to generate virus. Some of the titers of recombinant viruses appeared lower than normally seen with the parental virus (see Fig. 2 legend), suggesting that they may be impaired in growth.

The recombinant viruses maintained the plaque size phenotype of the parental strain (3) that contributed the 1B (VP2)–2C fragment. GD1B-2C/DAFL3 and DA5′-1B/GDVIIFL2 viruses had large plaques as did GDVIIFL2 (Fig. 3), while GD1B-1C/DAFL3 had small plaques (data not shown) as did DAFL3. The latter two recombinant viruses involve constructs that are still under study.

Transfection-derived viruses were then inoculated into mice to test for neurovirulence, which we operationally defined as death of a weanling mouse within 4 weeks of intracerebral virus inoculation. DAFL3 and GDVIIFL2 viruses had an LD_{50} of $>10^6$ pfu and ≈1 pfu, respectively (Fig. 2). It should be noted that although 10^6 pfu of the DA strain does not kill a mouse within 4 weeks and is considered "not neurovirulent" by our operational definition, the DA strain does produce a progressive demyelinating paralytic disease that can last for the life-span of the mouse (9). Results of inoculation with several recombinant viruses were of note, as detailed below.

GDVII 1B (VP2)–2C Segment Contains a Major Neurovirulence Determinant. Recombinant GD1B-2C/DAFL3 virus had an LD_{50} of $10^{2.7}$. The LD_{50} titration had a somewhat "jagged" appearance because occasional deaths occurred over a wide range of dilutions. Interestingly, GD1B-2C/DAFL3 virus frequently caused nonlethal paralysis, in contrast to GDVIIFL2 virus, which always killed mice that showed paralysis. The LD_{50} result identifies a fragment that runs from GDVII nt 1965 in the VP2 (1B) coding area to GDVII nt 4843 in the 2C coding area as containing a major neurovirulence determinant. A number of other recombinant viruses shown in Fig. 3 that contained portions of the GDVII genome but did not contain the GDVII 1B–2C coding regions were attenuated and did not kill mice.

The Important Neurovirulence Determinant in the GDVII 1B (VP2)–2C Segment Probably Lies in the 1B–2A Coding Area. The neurovirulence determinant within the GDVII 1B–2C (nt 1965–4843) segment was more finely delineated by

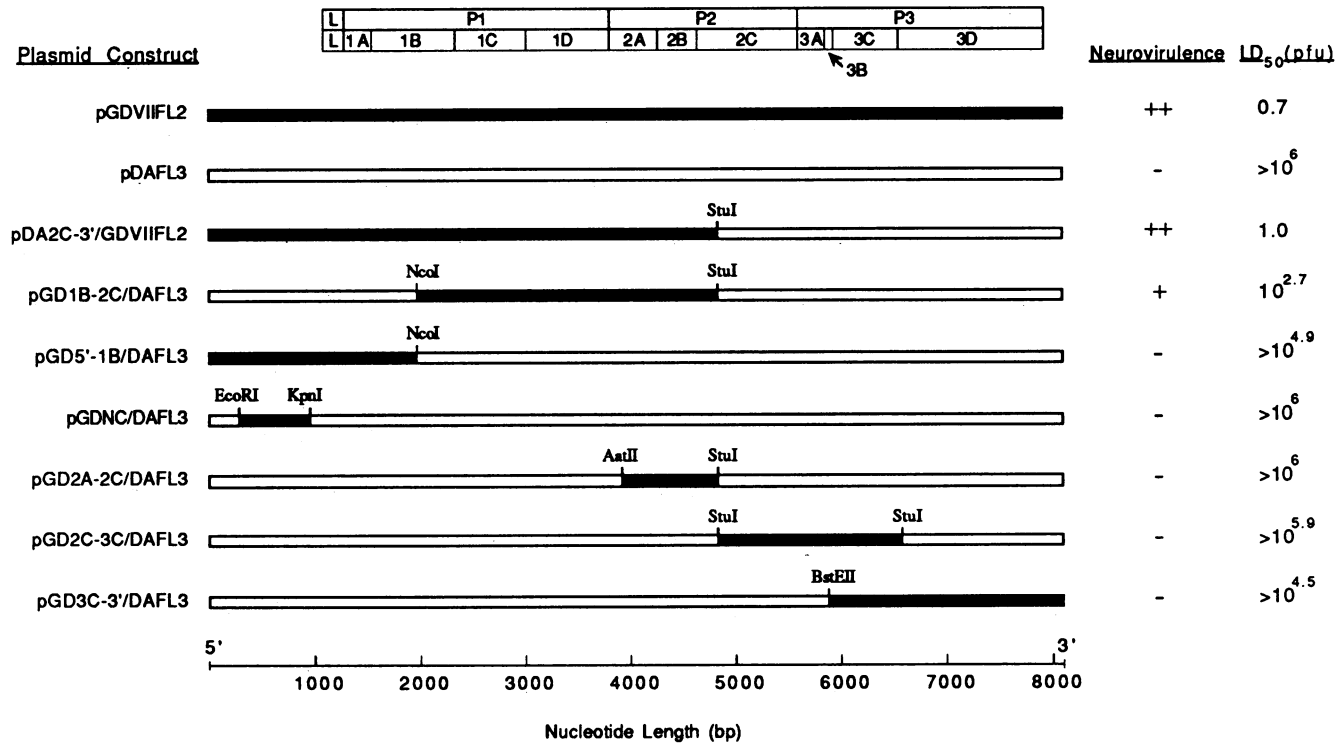

FIG. 2. TMEV chimeric intratypic cDNAs and their neurovirulence phenotype. Restriction sites used to generate the intratypic chimeric cDNAs are noted above the individual plasmid constructs. GDVII segments are shown as black bars, while DA segments are shown as the open bars. The positions of the restriction sites are aligned with the TMEV coding area shown at the top, and the nucleotide position in the genome is shown at the bottom. In the coding-region map, L encodes the leader protein; P1 encodes the precursor for capsid proteins 1A (VP4), 1B (VP2), 1C (VP3), and1D (VP1). P2 and P3 encode precursors for nonstructural proteins. The neurovirulence phenotype is given as the LD_{50} (pfu) as well as graded: −, attenuated; +, somewhat neurovirulent; ++, highly neurovirulent. Note that recombinant viruses GD5′-1B/DAFL3, GD2C-3C/DAFL3, and GD3C-3′/DAFL3 are listed as attenuated because no mice died after inoculation with undiluted virus; however, the LD_{50} could not be designated as $>10^6$ because of the relatively low titer of these viruses.

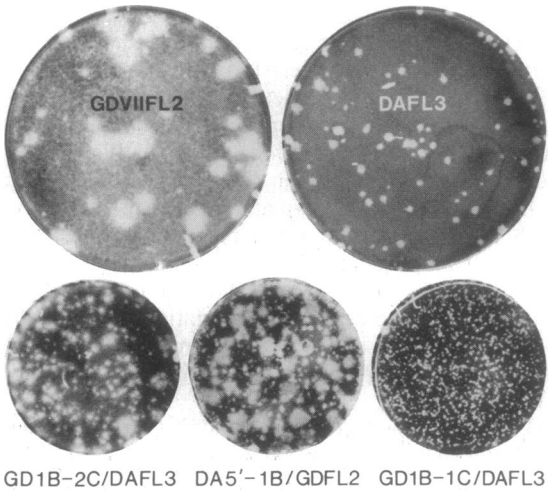

FIG. 3. Plaque phenotype of TMEV parental strains and selected recombinant viruses. L929 cells were transfected with *in vitro*-derived transcripts or infected with virus derived from DAFL3 (small plaque size), GDVIIFL2 (large) and GD1B-2C/DAFL3 (large). See text regarding viruses DA5'-1B/GDVIIFL2 (large) and GD1B-1C/DAFL3 (small).

noting that substitution of GDVII nt 3918–4843 into the DA genome produced a recombinant virus (GD2A–2C/DAFL3) that had an attenuated phenotype and did not kill mice. This finding indirectly suggests that GDVII nt 1965–3917, a region that extends from the coding region of VP2 (1B) to the amino-terminal half of 2A, comprises a major neurovirulence determinant.

The GDVII Genome 5′ to the GDVII 1B–2C Segment Influences Neurovirulence When Present Along with the 1B–2C Segment, but Not When Present Alone. Although GDVII 1B–2C (nt 1965–4843) contains a neurovirulence determinant, the LD_{50} of the GD1B-2C/DAFL3 recombinant virus ($10^{2.7}$) suggests that this virus is somewhat attenuated compared to wild-type GDVII. Additional recombinant studies noted below provided evidence that the GDVII genome 5′ to nt 1965–4843 influences neurovirulence when present along with this segment, but not when present alone. The substitution of GDVII nt 1–1964 along with GDVII nt 1965–4843 into the DA genome gave the recombinant virus (DA2C-3′/GDVIIFL2) a neurovirulence phenotype equivalent to that seen with GDVII, although substitution in the DA genome of only GDVII nt 1–1964 or 281–934 (in GD5′-1B/DAFL3 and GDNC/DAFL3 viruses, respectively) did not kill mice.

DISCUSSION

We have described the generation of genetically engineered TMEVs from infectious cDNA clones of genomic RNA from virus strains with markedly different biological properties. Our goal was to identify genetic loci responsible for TMEV neurovirulence. It will be of interest to also study surviving mice inoculated with the recombinants in order to identify genome segments critical in determining DA virus persistence and demyelinating activity.

Substitution of GDVII nt 1965–4843 from the 1B (VP2)–2C coding region into the DA genome enhanced the neurovirulence of DA virus. The 1B–2C segment includes scattered nucleotide differences coding for 80 amino acid differences between GDVII and DA. Studies involving GD2A-2C/DAFL3 chimeric cDNA (from an *Aat* II site in 2A to the *Nco* I site in 2C) have shown that this recombinant virus is not neurovirulent, suggesting indirectly that GDVII 1B–2A contains this key neurovirulence determinant. Unfortunately, attempts to produce a chimera containing GDVII 1D (encoding VP1) within the DA genome have not been successful. We suspect that a site in VP1 (see below) or VP3, corresponding to a site important in poliovirus neurovirulence (2), may also be important in TMEVs.

By what mechanism(s) could a TMEV capsid protein affect neurovirulence? An amino acid segment located in GDVII capsid protein VP1 (and represented in the GD1B-2C/DAFL3 recombinant virus) may enable TMEV to efficiently bind to the mouse neuron, producing an acute fatal polioencephalomyelitis. The importance of a picornaviral capsid protein to neurovirulence has been demonstrated by studies with poliovirus. The substitution of a short amino acid segment that corresponds to a trypsin-sensitive neutralization site from mouse-adapted poliovirus type 2 VP1 for the corresponding fragment from poliovirus type 1 confers a mouse neurovirulence phenotype on poliovirus type 1 (12, 13). It is presumed that this change in VP1 alters the ability of poliovirus to bind to mouse neurons.

The importance of viral surface proteins and particular epitopes on these proteins to neurovirulence has also been demonstrated for a number of viruses outside the picornavirus group. Reassortant studies with reovirus types 1 and 3 have found that the S1 gene, which encodes the hemagglutinin and is believed to be important in binding to specific cellular receptors, determines which specific neural cell is targeted for infection and, more specifically, whether the virus will infect ependymal cells and cause a nonlethal hydrocephalus or infect neurons and cause a fatal encephalitis (14). A monoclonal antibody-resistant reovirus mutant with a single amino acid substitution in the hemagglutinin exhibits decreased neurovirulence, with decreased virus replication in the brain as well as

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                1C/1D
BeAn    ----v-----------------------------------------------------N-----Y--------------------q-------------
DA      -a---s-----------------------------------------------I-S--V-----L-----------------t-----------------
GDVII   -----i-----------------------------------------------S-----F------------y---------r-------------
CONSENSUS wvpqg-dnaekgkvsnddasvdfvaepvklpenqtrvaffydravpigmlrpgqnmettf-yqend-rlncllltplpsfcpdsssgpvktkapvqwrwvrsg

BeAn    -v----------------------f------------mtr-----------------------------------------v-----------------
DA      -..tt--------------------------------td-----------------------------------------t-----------------
GDVII   -a---------------------------------m-ag--s---------------------------------i--s-----------------
CONSENSUS g-nganfplmtkqdyaflcfspftyykcdlevtvsalg--tvasvlrwaptgapadvtdqligytpslgetrnphmwlvgagnsqisfvvpynsplsvlpaaw

                                                                                1D/2A
BeAn    ------------------------------------------------------------------------------------------------
DA      -------------l-----------------------------------------vs-rs-----------------f---------i-------------
GDVII   ----------------ts----i-----s-----------------------------------------------s-------------l----------
CONSENSUS fngwsdfgntkdfgvapnadfgrlwiqgntsasvrirykkmkvfcprptlffpwptptttkinadnpvpilelenpaalyridlfitftdefitfdykvhgr
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FIG. 4. Amino acid differences among three TMEV strains in part of the coding area that is suspected to contain a neurovirulence determinant (see *Discussion*). GDVII virus is neurovirulent, while DA and BeAn are not. Dashes represent amino acids that are identical to the consensus sequence shown in the bottom row, while differing amino acids are specifically noted. Dashes in the consensus sequence indicate positions at which all three strains differ. The two dots in the DA sequence represent a 2-amino acid deletion. The amino acids shown in uppercase letters refer to residues that are aligned with a trypsin-sensitive neutralization site of poliovirus and vary among the TMEV strains.

a change in neural cell tropism (15). Similarly, the importance of epitopes on a surface protein to neurovirulence has been noted for rabies virus (16), neurotrophic murine retroviruses (17, 18), and a coronavirus (19).

What sites on TMEV VP1 may be important to virus binding and neurovirulence? A previous study identified a trypsin-sensitive neutralization site on the carboxyl end of VP1 of TMEVs (20), and it was suggested that this epitope may affect virus binding. DA escape mutants resistant to monoclonal antibodies that are directed against this epitope do not produce the late demyelinating disease phenotype (21). By analogy with poliovirus, this site may be the critical one in the 1B (VP2)–2C coding area that appears responsible for neurovirulence. Another potentially important site in TMEV VP1 is the amino acid segment that is aligned with the trypsin-sensitive neutralization site of poliovirus. The residues in this aligned area of VP1 (22) that vary among different TMEV strains are shown in uppercase letters in Fig. 4.

Our results indicated that GDVII nt 1965–4843 from the 1B (VP2)–2C coding region is an important determinant of neurovirulence but that it does not achieve the full GDVII neurovirulence phenotype. A more neurovirulent recombinant virus was seen when GDVII nt 1–1964 in addition to GDVII nt 1964–4843 were substituted in the DA genome (to produce DA2C-3′/GDVIIFL2 recombinant virus). These additional segments may be critical to neurovirulence because of a key regulatory role of the 5′ noncoding region. The mechanism by which the poliovirus 5′ noncoding region enhances poliovirus neurovirulence is unclear, but it is presumed that changes in this segment influence viral RNA replication or translation. The presence of the GDVII leader protein may also be important in the DA2C-3′/GDVIIFL2 virus' neurovirulence because of potentially important differences noted between TMEV strains with respect to proteins encoded in the leader region (23). Interestingly, no increase in DA neurovirulence was seen with recombinant viruses generated when the DA genome was substituted with the GDVII 5′ segment without the GDVII 1B–2C segment. It may be that the GDVII 5′ noncoding region can only play a role in neurovirulence following the efficient binding of TMEV to the mouse neuron by means of a segment of VP1. Another possible explanation for our results is that a product synthesized from the GDVII 1B (VP2)–2C coding segment binds to the GDVII nt 1–1964 region and is important in viral expression and neurovirulence.

In summary, our TMEV studies demonstrate that neurovirulence has a multigenic origin, as has been shown with poliovirus (1, 2) and other viruses. For example, alterations in the herpes simplex virus thymidine kinase gene (24) and polymerase gene (25) and in at least two other sites on the viral genome (26–28) affect neurovirulence. Delineation of the TMEV sequences critical for neurovirulence and clarification of the mechanisms involved may improve our understanding of TMEV disease pathogenesis as well as neurovirulence in general.

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