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Review

Molecular determinants of Theiler's murine encephalomyelitis-induced disease

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Theiler's murine encephalomyelitis virus (TMEV) strains are divided into two subgroups on the basis of their differing disease phenotypes. Members of the GDVII subgroup, such as GDVII strain, produce an acute lethal polioencephalomyelitis. In contrast, members of the TO subgroup, such as DA strain, induce a persistent infection with chronic demyelination; this white matter disease serves as an experimental model of multiple sclerosis (MS) due to their similar pathology and because the immune system in both diseases appears to contribute to the demyelination. The availability of full-length infectious TMEV clones, the relative simplicity of the TMEV genome, and the availability of the mouse as a host provide the opportunity to identify molecular determinants and disease mechanisms that are responsible for neurovirulence, demyelination and virus persistence, and makes this a valuable system for pathogenesis studies.

Keywords: Theiler's virus; picornavirus; neurovirulence; demyelination; molecular pathogenesis

Introduction

Theiler's murine encephalomyelitis virus (TMEV) constitutes a group of closely related picornavirus strains that infect the gastrointestinal tract and central nervous system (CNS) of mice. TMEV strains can be divided into the GDVII and TO subgroups based on their differing biological properties. The GDVII subgroup strains, which include GDVII and FA, are highly neurovirulent; one plaque forming unit (pfu) kills a weanling mouse with an acute polioencephalomyelitis. In contrast, over 10^6 pfu of the TO subgroup strains, which include DA and BeAn, induce an early non-fatal acute encephalomyelitis that is followed by a persistent infection with chronic demyelination in weanling mice; virus expression appears to be restricted during the late white matter disease, with the production of relatively little viral antigen and genome in glial cells and macrophages.

The ease with which one can work with this mouse disease and the powerful molecular tools available have made it an attractive model for molecular pathogenesis studies. The TO subgroup-induced demyelinating disease is of special interest because it serves as an experimental model of multiple sclerosis: both disease processes have similar white matter pathology and the immune system appears to contribute to demyelination in both.

The differences between the disease phenoypes caused by the TO and GDVII subgroup strains prompted investigators to attempt to identify molecular determinants for TO subgroup demyelination/persistence and for GDVII subgroup neurovirulence. In order to identify these disease determinants, investigators have taken several approaches: (1) the construction and subsequent manipulation of infectious cDNA clones of strains of TMEV from the two different subgroups to generate recombinant DA/GDVII or BeAn/GDVII viruses; (2) the production of mutant TMEV by growing virus in the presence of neutralizing monoclonal antibodies (mAbs), and the subsequent examination of the phenotype of the mutant viruses resistant to the mAbs; (3) direct site-specific mutagenesis of the infectious cDNA clone, and the subsequent examination of the phenotype of the mutant virus.

The interpretation of results from the study of recombinant viruses has proven difficult for several reasons. Some of the recombinant viruses have contained inadvertant mutations that have not initially been recognized. Also, there are shortcomings and limitations intrinsic to this approach that have led to difficulties in interpretation of the

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results and in the precise delineation of disease determinants. This review provides an overview and summary of recombinant virus studies and of the molecular determinants of TMEV-induced disease.

The TMEV genome

The genome of TMEV consists of a single stranded positive sense RNA of 8.1 kilobases that shares the typical features of picornaviruses (Figure 1; reviewed by Roos and Casteel, 1992). The virus is classified in the cardiovirus genus of picornaviruses because of its similarity in sequence and genome organization to other cardioviruses, such as Mengovirus and encephalomyocarditis virus; for example, TMEV has a leader (L) protein in the most amino part of the polyprotein, as is the case with cardioviruses (and also the aphthovirus genus of the picornaviruses). The RNA does not have a cap – instead there is a small protein covalently bound to the 5′ terminus called VPg that is encoded by 3B. There is a long 5′ untranslated region (5′ UTR), of 1065 nucleotides in the case of the DA strain, which has a complicated secondary structure. Ribosomes enter internally in the 5′ UTR, bypass eight AUGs (in the case of DA strain), and initiate translation at the ninth AUG at 1066 with the synthesis of a long polyprotein that is cleaved by viral proteases (encoded by 2A and 3C) into structural and nonstructural proteins. The structural proteins are encoded by P1; specifically, 1A, 1B, 1C and 1D encode the capsid proteins VP4, VP2, VP3 and VP1 respectively (reviewed by Roos and Casteel, 1992).

The lack of available enzymes that can cleave RNA was a major impasse in molecular manipulations of RNA viruses. This problem was bypassed by Racaniello and Baltimore (1981) who prepared cDNAs from poliovirus, ligated these cDNAs into a full-length cDNA clone, and demonstrated that the latter clone was infectious. The availability of the infectious clone permitted manipulation of the picornavirus genome as a cDNA using DNA modifying enzymes. As shown in Figure 2, the cDNA can be transcribed *in vitro* to generate the viral RNA genome, which is then transfected into cells; picornaviruses are positive sense RNA viruses, and therefore the transfected RNA is infectious and will produce virus. To date, in-

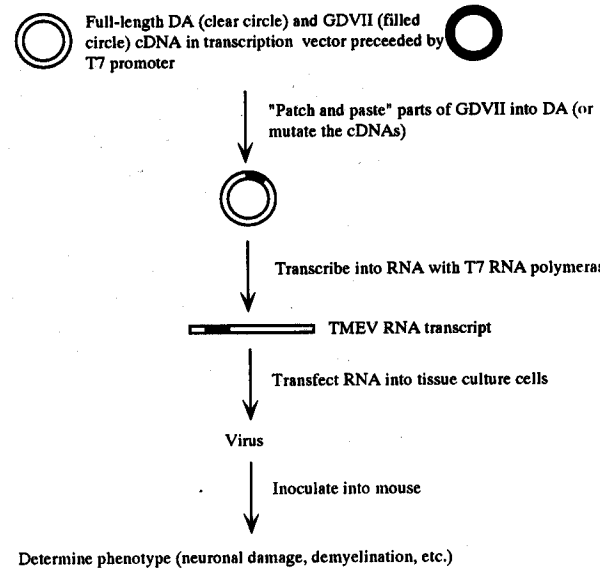

Figure 2 Diagram showing *in vitro* transcription of TMEV cDNA and transfection of the resultant RNA to produce virus. The T7 RNA promoter is present just upstream of full-length TMEV cDNA, which is cloned ino a transcription vector; addition of T7 RNA polymerase results in *in vitro* transcription of full-length TMEV cDNA. Subsequent transfection of the resultant RNA into cells generates infectious virus. In this way, the genome of RNA viruses such as TMEV can be manipulated at the cDNA level to produce mutations or chimeras with the subsequent production of mutated or recombinant viruses.

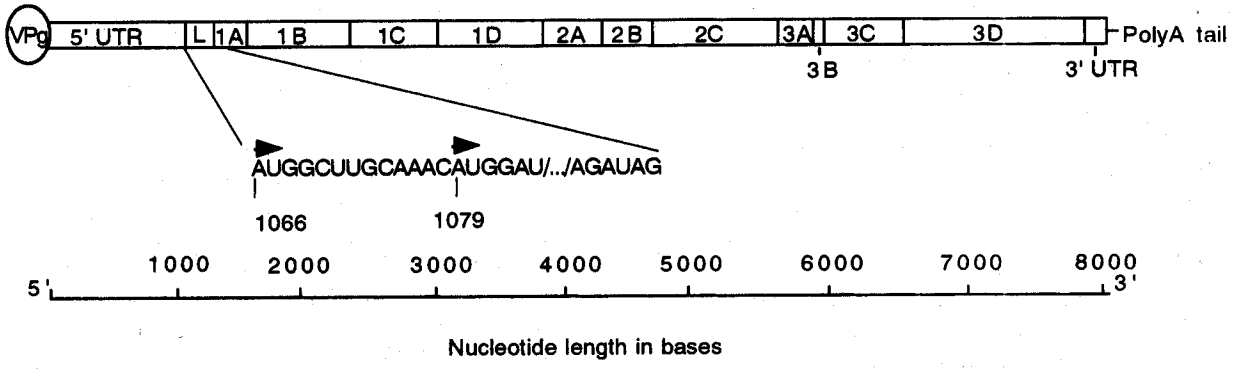

Figure 1 The TMEV single-stranded RNA genome of approximately 8.1 nucleotides. The 5′-untranslated region (5′-UTR) preceeds the polyprotein coding region, which has the leader (L) coding region as its most amino terminus; a 3′ UTR is at the 3′ end of the genome. The genomic region of P1 is divided into 1A, 1B, 1C and 1D coding regions which synthesize respectively the structural proteins VP4, VP2, VP3 and VP1. P2 (which includes 2A, 2B, 2C and 2D) and P3 (which includes 3A, 3B, 3C and 3D) encode various non-structural proteins. The polyprotein and L initiation codon is at nucleotide 1066, while the initiation codon for L* (see text) is at nucleotide 1079. There is a stop codon (UAG) for L* at nucleotide 1547.

fectious cDNA clones have been generated from three TMEV strains: DA (Roos *et al*, 1989; McAllister *et al*, 1989) BeAn (Calenoff *et al*, 1990) and GDVII (Calenoff *et al*, 1990; Fu *et al*, 1990b; Tangy *et al*, 1989). Sequence studies of these strains of TMEV have demonstrated that there is approximately 90% identity at the nucleotide level and 95% identity at the amino acid level, irrespective of the strain's subgroup.

TMEV genes important for neurovirulence

As soon as full-length clones of DA, BeAn and GDVII strains were available, recombinant intratypic DA/GDVII (and BeAn/GDVII) viruses were prepared in order to elucidate the viral genes responsible for the neurovirulence of GDVII. The parental GDVII strain prepared from the full-length cDNA of Roos and colleagues (Fu *et al*, 1990b) was highly neurovirulent, as defined by the death of a weanling mouse within a month following intracerebral inoculation, i.e., one plaque forming unit (pfu) of GDVII was equivalent to one 50% lethal dose (LD_{50}): in contrast, DA strain was not neurovirulent, i.e., the LD_{50} was greater than 10^6 pfu. Recombinant studies demonstrated that substitution into DA of a segment of the GDVII genome from the middle of 1B (VP2) to the middle of 2C was necessary for neurovirulence, i.e., recombinant viruses were not neurovirulent unless they contained this segment from GDVII (Fu *et al*, 1990a). In order to observe the *full* neurovirulence of GDVII, the recombinant virus had to contain additional upstream sequence, extending from the GDVII 5′ terminus all the way to 2C; however, it is important to note that the enhancement of neurovirulence by addition of this region was relatively minor compared to that derived from a segment that just contained the GDVII 1B-2C (or GDVII 1B-2A) region. The GDVII 1B-2C region appears to be critical for neurovirulence because it permits efficient replication of virus in certain regions of the CNS – death of mice correlates with elevated levels of infectious virus and with the presence of viral antigen within neurons of the brain stem and gray matter of the spinal cord (Rodriguez and Roos, 1992). These studies also suggested that neurovirulence of GDVII was multigenic.

Using different infectious DA and GDVII cDNA clones in recombinant virus studies of neurovirulence, Brahic and colleagues reported findings similar to those found by the Roos laboratory. McAllister *et al* (1990) reported that substitution of GDVII L to the 2A coding region into the DA genome was important for neurovirulence phenotype. Unfortunately, a more precise comparison with the results of Fu *et al* was not possible since these studies did not include calculation of LD_{50}s, but only determined the percentage of animals that died at a fixed dose of virus (McAllister *et al*, 1990).

The results from both of these groups were at odds with some results found by Lipton *et al* (1991) involving BeAn/GDVII recombinant viruses. The latter investigators found that the presence of the BeAn 5′ UTR in a BeAn/GDVII recombinant virus led to attenuated disease even if the rest of the genome was derived from GDVII; this clearly was in conflict with findings of Fu *et al* (1990b) who showed that the presence of the GDVII 1B-2A segment in the DA genome led to a neurovirulent phenotype. This issue was clarified by recognizing that the full-length BeAn clone used in these studies had a single base deletion in the 5′ UTR that had been inadvertantly introduced in the initial cloning, and that the presence of this mutation in the recombinant viruses led to their attenuation (Pritchard *et al*, 1992). These observations demonstrate the potential for unknowingly introducing mutations into parental or recombinant cDNA clones of TMEV and the danger of such errors. Pritchard *et al* (1992) further hypothesized that this single base deletion disrupted the secondary structure of the BeAn 5′ UTR and led to an attenuated phenotype because of a decrease in translational efficiency; changes in the 5′ UTR of the RNA genome of Sabin strains of poliovirus have similarly been implicated in their attenuated phenotype (reviewed by Nomoto and Koike, 1992). It remains unclear in the study of Pritchard *et al* (1992) whether attenuation of the recombinant viruses' neurovirulence resulted from the mutation in the BeAn 5′ UTR because of a specific defect in the CNS growth of the virus, or merely a consequence of a general crippling of the virus characterized by poor growth both *in vivo* as well as *in vitro* (e.g. with a slowed one step growth curve).

To delineate the determinant(s) within the GDVII 1B-2C region, Roos and colleagues further divided the GDVII 1B-2C segment into two parts, 1B-2A and 2A-2C, and separately substituted each into the genome of DA to produce recombinant viruses (Zhang *et al* 1993) (Figure 3). Surprisingly, neither recombinant virus was neurovirulent, i.e., the LD_{50}s were characteristic of the parental DA virus. We presume that the attenuated phenotype of these recombinant viruses is a result of disrupted interactions between DA or GDVII genes or their gene products, i.e., these interactions are intact in the parental strains (and in the GDVII1B-2C/GDVII recombinant virus) and critical for the neurovirulence phenotype. Pritchard *et al* (1993) similarly noted limitations in recombinant virus studies, and demonstrated that there are assembly defects in some of the recombinant viruses that result from improper alignments between the chimeric proteins; these incongruent interactions between coat proteins in the recombinant viruses may mask identification of disease determinants. The studies of Zhang *et al* (1993) also suggested a redundancy of neurovirulence determinants, i.e., several regions of the genome can lead to equivalent degrees of

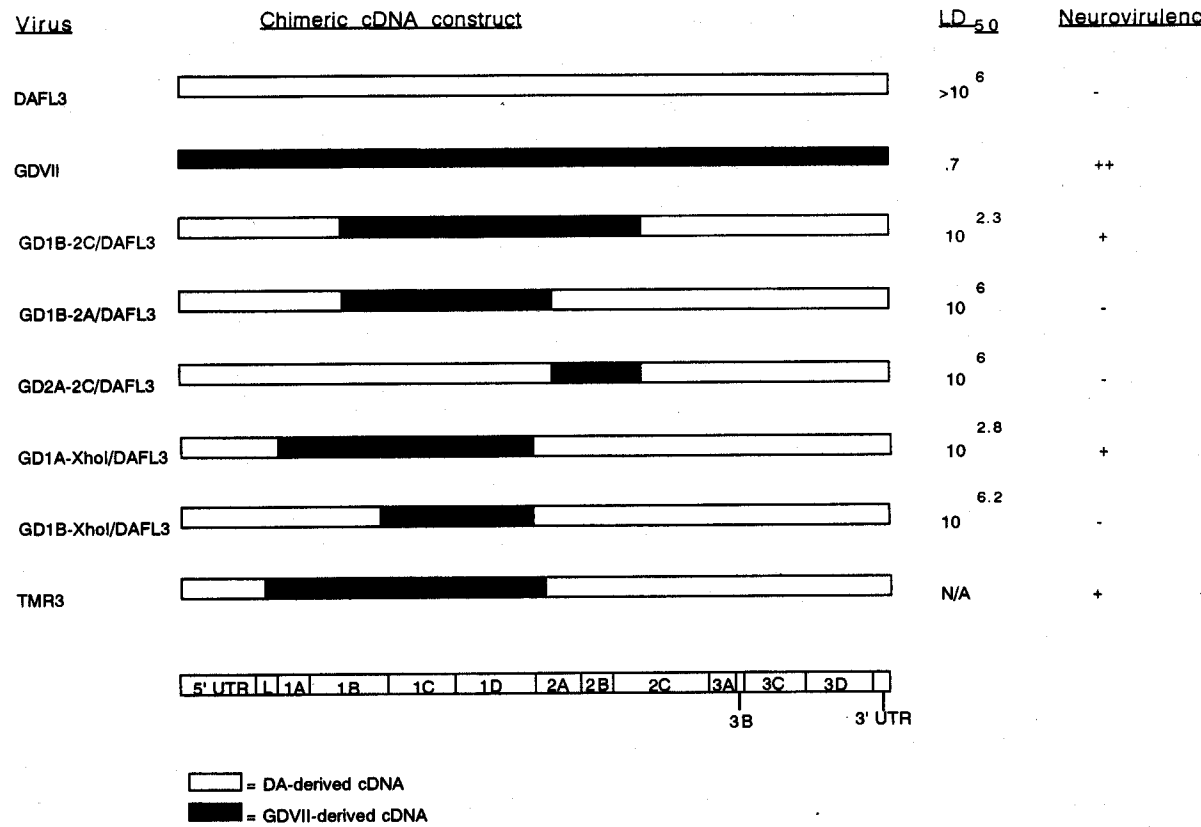

Figure 3 Chimeric TMEV cDNAs and the neurovirulence phenotype of the recombinant viruses. GDVII segments are shown as the dark, shaded areas; DA segments are shown as the open, light areas of the genome. The chimeric cDNAs are transcribed *in vitro*, the resultant RNAs are transfected into tissue culture cells to generate virus and the virus stock is then inoculated into mice to determine neurovirulence. The corresponding regions of the viral genomic and the polyprotein products are shown below the chimeric cDNAs. The genomic coding regions 1A, 1B, 1C and 1D synthesize the structural proteins VP4, VP2, VP3 and VP1 respectively. The neurovirulence phenotype is given as the number of plague-forming units (pfus) that kills 50% of mice (LD_{50}), as well as being graded −(attenuated), +(somewhat neurovirulent) or ++(comparable in neurovirulence to GDVII strain). Note that the presence of the GDVII 1B-2C segment (in GD1B-2C/DAFL3 virus) contains a major neurovirulence determinant; however, there is surprisingly little or no neurovirulence when either GDVII 1B-2A or GDVII 2A-2C is separately substituted into the DA genome. This result suggests that interactions between GDVII genes or gene products are critical for neurovirulence, and that these are disrupted in the latter two constructs. In order to study this further, a 'silent' XhoI site was engineered at the 3′ end of P1. GD1A-XhoI virus was neurovirulent, suggesting that sequences upstream from P2 are sufficient for neurovirulence. The loss in neurovirulence of GD1B-XhoI virus may also be related to disruptions in critically important interactions of TMEV genes or gene products. Data about TMR3 virus is from McAllister *et al* (1990).

neurovirulence. Although this redundancy is not unexpected – since a virus is likely to contain multiple means of inducing disease – it does serve to further complicate the interpretation of recombinant virus studies and the identification of neurovirulence determinants. This inability to delineate genomic determinants of disease more finely emphasizes the fact that intratypic TMEV recombinant virus studies appear to demonstrate more shortcomings and limitations than have been found in the case of poliovirus wild type-Sabin recombinant studies; this is presumably because Sabin strains were derived after relatively few passages of the parental strain (with the subsequent generation of a limited number of point mutations), while there is substantial evolutionary divergence of DA and GDVII strains. Despite the limitations of the TMEV recombinant studies, Zhang *et al* (1993) were able to narrow down the region critical for the majority of neurovirulence to a region of P1, from the GDVII 1B through the 1D capsid protein coding region (GD1A-Xho1/DAFL3 virus, see Figure 3).

Recombinant viruses and the molecular determinants for DA demyelination and persistence

A basic question investigators sought to answer was whether DA had unique determinants for its demyelinating and persistent infection phenotype that were not shared by GDVII *or* whether GDVII shared determinants for this phenotype with DA, but failed to induce the late white matter disease because its significant neurovirulence led to the early death of mice, leaving no survivors to develop the late demyelination. Inoculation of a

number of different DA-GDVII recombinant viruses demonstrated that viruses with substitutions of different DA segments that spanned the whole DA genome, into the GDVII 'backbone' caused demyelination and evidence of persistent virus in survivors (Fu *et al*, 1990a; Rodriguez and Roos, 1992). This result suggested that both GDVII and DA contain genetic determinants for late demyelination and persistence; it should be mentioned, however, that one cannot completely rule out an alternative possibility, namely that the determinants of demyelination and persistence are multiple and redundant and only present in DA – and that GDVII has no determinants for demyelination. Even if there are determinants for demyelination and persistence present on all TMEV strains, it is clear that there may be additional strain-specific determinants for the late disease that are present on TO subgroups strains, but not GDVII subgroup strains; this possibility is supported by finding substantially different degrees of demyelination among groups of mice inoculated with various DA-GDVII recombinant viruses (Rodriguez and Roos, 1992).

Our findings that both GDVII and DA contain determinants for late demyelination and persistence were challenged by Brahic and colleagues. The latter group reported that DA VPI is necessary for TMEV demyelination and persistence since DA-GDVII recombinant viruses that do not contain DA VP1 fail to demyelinate or persist, i.e., chimeras that contain VP1 from GDVII do not cause the late white matter disease (Tangy *et al*, 1991). We decided to exchange viruses and reagents with Brahic and colleagues and uncovered the basis for this difference – their DA clone (derived from a plasmid called pTMDA) has an asn as VP2 141, while our DA clone (derived from pDAFL3) has a lys. In other words, a recombinant virus that has asn as VP2 141 (in the TMDA 'backbone') on the same genome as the 1B(VP2)-2A segment of GDVII fails to demyelinate or persist, while a recombinant virus that has lys as VP2 141 (in the DAFL3 'backbone') that is present on the same genome as the 1B-2A segment of GDVII demyelinates and persists (Jarousse *et al*, 1994) (see Figure 4). One should note that this VP2 141 residue is present in the DA backbone of the recombinant virus, and not in the GDVII substituted region (see Figure 4). Interestingly, an asn is also present as VP2 141 in the parental virus derived from the infectious clone (pTMDA) of Brahic and colleagues and this virus *induces* demyelinating disease – while our pDAFL3 infectious clone, which also generates a demyelinating virus, contains a lys at VP2 141. Recent studies by Roos and colleagues have shown that placement of asn rather than lys at VP2 141 in DAFL3 virus leads to a significant attenuation of its demyelinating activity (Zhang *et al*, 1995). These results suggest that there is an amino acid(s) in the 1B-2A

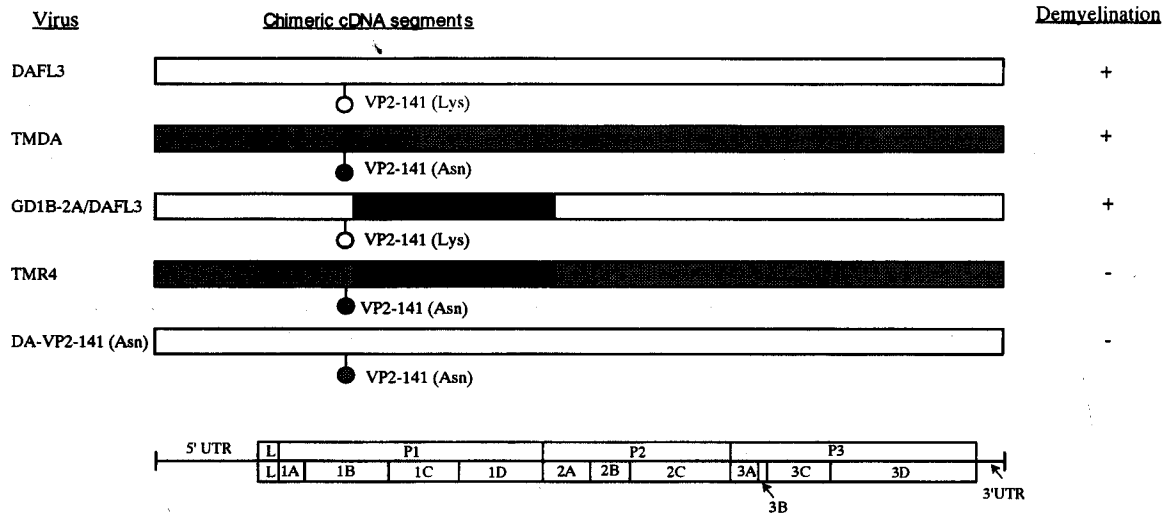

Figure 4 The importance of DA VP2 amino acid 141 to DA-induced demyelinating disease. Chimeric TMEV cDNAs and the neurovirulence phenotype of the recombinant viruses. GDVII segments (which were generated by Roos and colleagues) are shown as the dark, shaded areas; DAFL3 segments (which were generated by Roos and colleagues) are shown as the open, light area; TMDA segments (which were generated by Brahic and colleagues) are shown as densely cross-hatched. The chimeric cDNAs are transcribed *in vitro*, the resultant RNAs are transfected into tissue culture cells to generate virus, and the virus stock is then inoculated into mice to determine neurovirulence. The presence of an Asn or Lys as VP2 141 is noted, as is the demyelinating activity of the viruses. Note that the presence of VP2 141 Asn in TMR4 (which is a GDVII1B-2A/DAFL3-like recombinant virus produced by Brahic and colleagues) and in mutated DA virus (DA-VP2-141Asn virus) decreases the demyelinating activity of the virus; however, the demyelinating activity is maintained in the case of the TMDA parental virus despite the presence of Asn at VP2-141. The latter results suggest that the TMEV 1B-2A segment can modulate the effect of VP2-141 on demyelinating activity.

segment of DAFL3 different from the sequence of the 1B-2A segment of TMDA and that this residue(s) interacts with an asn at VP2-141 and modulates the demyelination phenotype.

These results demonstrate that an amino acid residue may have different effects when present in a parental rather than a recombinant virus; this can obviously be a source of confusion in the interpretation of studies of recombinant TMEV viruses. Similarly, a mutation in amino acid 270 in VP1 has varying neurovirulence depending on whether the mutation is present in parental GDVII or recombinant virus (Senkowski *et al*, 1995). One can easily understand how mutations may interact differently when present in different genome 'backbones', especially if there are multigenic contributions to disease phenotype, i.e., if several genes are important for a phenotype, then the viral genes present in a recombinant and the parental strain may manifest slightly different functions and interactions that influence disease induction and maintenance.

Neutralization escape mutant viruses and molecular determinants of TMEV-induced disease

Investigations of DA virus mutants that are generated in the presence of various neutralizing mAbs have proven valuable in the identification of molecular determinants of TMEV-induced disease. The initial interest in generating these neutralizing mAb resistant viruses was two-fold. First, sequence analysis of RNA of TMEV mutant viruses enabled investigators to identify mutations in amino acids, and thereby identify neutralization sites on the virus for the mAb (that were presumed critical for escape of the virus from the neutralizing mAb). Second, some of the neutralization escape mutant DA viruses had a change in desease phenotype, as has been found to be the case in other viruses; the critical mutation in these neutralizing mAb resistant viruses presumably corresponds to a disease determinant as well as a neturalization epitope.

Studies of neutralization escape mutant and other mutant viruses resistant to DA neutralizing mAbs identified the following locations for three separate neutralization sites of DA: amino acid 268 (in the carboxyl region) of VP1 [val to phe] (Senkowski *et al*, 1995), amino acid 101 of VP1 [thr to ile] (Zurbriggen *et al*, 1991a), and amino acid 141 of VP2 [lys to asn] (Zhang *et al*, 1995). Mutant viruses with these changes all demonstrated a decrease in demyelinating activity. Of interest is the fact that all of these mutations are located on or near the rim of the 'pit', a structure identified in crystallographic studies thought to be the putative receptor binding region of the virus (Grant *et al*, 1992; Luo *et al*, 1992). These findings suggest that antibody mediates neutralization by preventing the binding of virus to the cell receptor, and that these mutant viruses fail to demyelinate because of interference with binding of the mutant viral attachment site to receptors of particular cell types (e.g. oligodendrocytes).

To clarify whether the altered disease produced by the escape mutant viruses is due to a change in the immune response to the virus (as a result of the mutation in a neutralization site), investigators have compared disease caused by the escape mutant virus with that caused by wild type virus following inoculation of immunocompromised mice. For example, a neutralization escape virus with a mutation in VP1 101 has a diminished growth and spread compared to wild type virus following inoculation of nude mice, suggesting that the difference in disease phenotype of this particular mutant virus in immunocompetent mice cannot wholly be explained as a result of a change in the host's immune response (Zurbriggen *et al*, 1991b).

The importance of the L and L proteins*

Because of limitations in studies of recombinant viruses noted above and because there are relatively few neutralization sites on the virus that are available for mutation, investigators have also explored the effect on disease phenotype of genes in the infectious TMEV cDNA clones that are directly changed following site-specific mutagenesis. Kong *et al* (1994) demonstrated DA virus with a deletion in the L coding region grows well on BHK-21 cells, but not on L-929 cells. This suggests that the L protein is somewhat involved in a cell-specific restriction of virus growth and that this function may be important in disease pathogenesis. Calenoff *et al* (1995) confirmed the cell-specific restriction of TMEV L in studies with GDVII virus with a deletion of L. The latter investigators also reported that L was critical for the efficient growth of the virus in the weanling mouse brain. The actual function of L and its role in neurovirulence remain unclear.

Possibly relevant to the L story is the finding that an 18 kD protein, which we call L*, is synthesized from an AUG at nucleotide 1079 within the L coding region, 13 nucleotides downstream from the presumed authentic initiation codon of the polyprotein, and out of frame with the polyprotein (Kong and Roos, 1991; Chen *et al*, 1995) (Figure 1). We were initially interested in this observation because the AUG that synthesizes L* is present in DA and BeAn, the two demyelinating strains of TMEV that have been sequenced, and not in GDVII, the neurovirulent non-demyelinating strain of TMEV. We found that DAL*-1 mutant virus, which has an ACG rather than AUG at 1079 and therefore does not synthesize L*, induces little if any demyelination, suggesting that L* plays a critical role in the late demyelinating disease (Chen *et al*, 1995). The importance of L* is supported by our earlier studies that showed that a recombinant virus with GDVII sequence substituted for DA from the 5′ terminus to

the 1B coding region (and therefore with no L* initiation codon) produces relatively little demyelination (Rodriguez and Roos, 1992); however, the presence of demyelination in this recombinant virus demonstrates that other determinants beside L* contribute to the TMEV-induced white matter disease.

Although our data (Chen *et al*, 1995) demonstrate that L* is critical for the demyelinating activity of DA, we presently lack a more complete description of the role of L* in CNS disease and its mechanism of action. Of interest is the presence of a hydrophobic putative intramembrane region in the predicted amino acid sequence of L* and the finding that L* is membrane associated (Chen *et al*, 1995). It is also possible that cell-specific proteins differentially bind to the initiation codon of DA polyprotein *versus* the L* AUG and thereby affect the efficiency of initiation of translation of the polyprotein and the production of virion proteins and infectious virus. In this way, binding of oligodendrocyte- or macrophage-specific proteins could lead to relative decrease in translation of the viral polyprotein (with the release of little infectious virus) and an associated increase in the synthesis of L* (with the production of a restricted infection in oligodendrocytes or macrophages).

Summary
Recombinant virus studies have demonstrated that a region of the GDVII capsid proteins, from the 1B(VP2) through 1D(VP1) coding region, contains a major determinant for neurovirulence; however, the addition of upstream sequence from the GDVII 5′ UTR to 1B contributes to the full neurovirulence of the recombinant viruses. These studies were unable to more finely delineate the major neurovirulence determinant(s) within the IB-ID segment because of intrinsic limitations in this approach. In addition, the mechanism(s) by which this GDVII region causes an enhanced neurovirulence is unknown.

Recombinant virus studies suggested that all TMEV strains have determinants for demyelination and persistence, although one could not completely rule out the presence of multiple and redundant determinants of demyelination that are only present on TO subgroup strains. Studies of mutant viruses resistant to neutralizing mAbs have identified the importance of particular amino acids to the late disease phenotype. The similar localization of these mutations to the putative receptor attachment site of the virus suggests that these residues are critical for binding to particular cell receptors. Site directed mutagenesis studies have identified L as important in GDVII-induced encephalitic disease in mice and L* as critically important for demyelination.

Acknowledgements

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