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The Leader Polypeptide of Theiler's Virus Is Essential for Neurovirulence but Not for Virus Growth in BHK Cells

MIRIAM A. CALENOFF,[1] CYRUS S. BADSHAH,[1] MAURO C. DAL CANTO,[2] HOWARD L. LIPTON,[3] AND M. KATHLEEN RUNDELL[1]*

Departments of Microbiology-Immunology[1] and Pathology,[2] Northwestern University School of Medicine, Chicago, Illinois 60611, and Department of Neurology, Evanston Hospital, Evanston, Illinois 60201[3]

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A leader polypeptide of unknown function is encoded by cardioviruses, such as Theiler's murine encephalomyelitis virus. Although the deletion of this polypeptide has little effect on the growth of parental GDVII virus in baby hamster kidney (BHK) cells, the mutant virus is completely attenuated and fails to kill mice receiving intracerebral inoculations of high doses of the virus.

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus belonging to the cardiovirus subgroup (23). Strains of TMEV fall into two groups on the basis of their growth in culture and their neurovirulence in intracerebrally inoculated, susceptible strains of mice. One group produces a rapidly fatal encephalitis in mice (31); the GDVII and FA strains are members of this group. The second group, which resembles Theiler's original isolates, is much less virulent. In contrast to mice infected with GDVII virus, those infected with high doses of BeAn or DA viruses survive initial infections but frequently develop progressive demyelinating disease, characterized by inflammatory lesions in the central nervous system (13, 14, 17, 18). Demyelination is associated with viral persistence (1a), a property that appears to depend upon the structural protein composition of the virus (7, 10, 25, 32, 34, 35).

In tissue culture, the demyelinating strains grow more slowly (2, 6) and produce less virus than does GDVII. The demyelinating strains form minute plaques at 33°C and fail to form plaques at 39°C (2). They also tend to remain more cell associated, with virions present within membranous structures of the cells (6). The GDVII virus forms intracellular crystalline arrays which are readily released from cells.

Infectious cDNA clones have been obtained for several isolates of TMEV (22, 23, 27). Sequence comparisons of strains belonging to the two subgroups show that one of the most divergent polypeptides is a small protein known as the leader, L (22). Among picornaviruses, leader polypeptides are encoded by cardioviruses and by foot-and-mouth disease virus (FMDV) (28). In FMDV, the leader has been shown to be an autocatalytic protease (30) which can cleave p220 of the cap binding protein complex. Consequently, the FMDV leader is involved in the shutoff of host protein synthesis (5). The cardiovirus leader protein, an extremely acidic protein, is unrelated to that of FMDV, and its function is unknown. The leader in murine encephalomyocarditis virus, another cardiovirus, is released from the viral polyprotein by the principal viral protease, 3C (21). Neither the murine encephalomyocarditis virus leader (21) nor the TMEV (26) leader has detectable autocatalytic activity.

In addition to encoding the leader protein, L, the less-virulent DA strain of TMEV encodes a smaller alternate leader protein, l, derived from translation with an AUG in a second reading frame (12). To study the role of the leader in TMEV infection without the complication of an alternate leader, we chose to study the virulent GDVII virus. We have generated a panel of GDVII leader deletion mutants in full-length infectious cDNA clones of the virus. Our studies indicate that the GDVII leader is dispensable for the growth of the virus and for plaque formation on BHK-21 cell monolayers. However, such a deletion results in complete attenuation of the virus.

MATERIALS AND METHODS

Construction of mutants. A series of leader deletion mutants was constructed in a previously described full-length GDVII cDNA clone (2). The 76-amino-acid leader protein is encoded by the DNA segment between nucleotides 1069 and 1296 on the GDVII map (22). The mutant *dl*-23 was generated by excising a 67-bp fragment between restriction sites *Ssp*I (position 1141) and *Aat*II (position 1208). To preserve the reading frame, a 10-bp *Cla*I linker was ligated between the blunted ends of these two restriction sites. This resulted in the deletion of leader codons 25 through 47, with the introduction of four new linker-derived codons. This central deletion in *dl*-23 was extended towards the amino or carboxyl terminus of the leader sequence to generate the mutants *dl*-45N and *dl*-49C, respectively. These were constructed by replacing genomic sequences of *dl*-23 with smaller fragments generated by PCR. To make *dl*-45N, the DNA sequence from the *Mlu*I restriction site (position 640) through the second leader codon was amplified by PCR. The reverse primer used introduced a *Cla*I site immediately following the second codon of the leader. The amplified fragment was cleaved with *Mlu*I and *Cla*I and then introduced into *dl*-23, resulting in the deletion of codons 3 through 47 of the leader. The polypeptide encoded by *dl*-45N also contains three amino acids from the *Cla*I linker. *dl*-49C was constructed similarly, by replacing the segment of *dl*-23 between the linker *Cla*I site and an *Nco*I site (position 1964) with a smaller PCR-amplified segment. The amplified fragment contained an engineered *Cla*I site just before the last three leader codons. *dl*-NC was constructed by ligating the *Cla*I sites of *dl*-45N and *dl*-49C and encodes a leader with only 5 of the 76 leader amino acids, plus 2 amino acids from linker sequences. To generate the complete leader deletion mutant *dl*-L, the mutant *dl*-NC was the template in PCRs with oligonucleotides that lacked all leader sequences. These oligonucleotides hybridized to sequences in the 5′ noncoding region, including the initiating ATG, and to sequences in VP4. Consequently, in *dl*-L, the initiating ATG is followed immediately by the first codon of VP4.

In vitro transcription and transfection. Plasmid DNAs were linearized with *Xba*I, and RNA transcripts were synthesized with T7 RNA polymerase (Promega) as described previously (2). Subconfluent BHK-21 cell monolayers in 60-mm-diameter dishes were transfected with transcription reaction mixture (approximately 10 to 30 μg of RNA) with Lipofectin (GIBCO/BRL) as specified by the supplier. The transfected monolayers were incubated at 37°C until full cytopathic effect was observed (24 to 48 h), and then the viral lysates were harvested by freezing and thawing. The viral stocks used for experiments were derived by passaging the initial lysate once or twice in BHK-21 cells to amplify the virus.

Viral RNA analysis. For two of the mutant viruses, *dl*-L and *dl*-NC, viral RNA was isolated from infected BHK-21 cells by standard RNA isolation procedures

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University School of Medicine, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-5923. Fax: (312) 503-1339. Electronic mail address: mkr389@lulu.acns.nwu.edu.

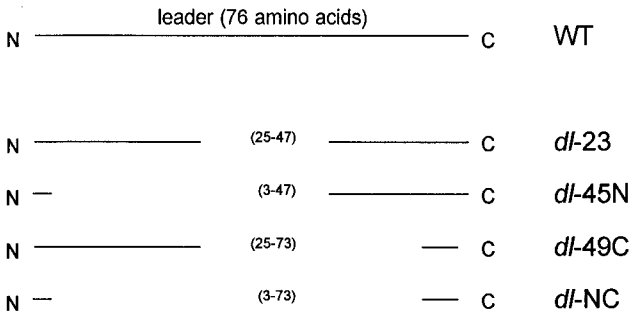

FIG. 1. Leader proteins expressed by mutant viruses. The leader proteins predicted to be made by the mutant viruses are indicated by the solid lines; gaps reflect the deletions present, with the numbers of the deleted amino acids given in parentheses. The leader proteins of *dl*-23, *dl*-45N, and *dl*-49C contain two to four nonleader amino acids from the *Cla*I linker sequences.

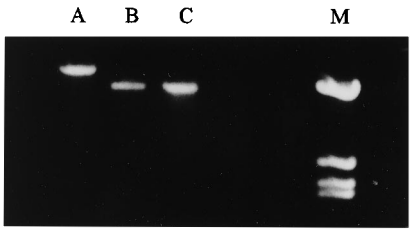

FIG. 2. Presence of deletions in viral RNA. Viruses obtained from the transfection of BHK cells with T7-generated transcripts were used to infect additional BHK cells and to isolate total intracellular RNA for reverse transcription and PCR amplification. Primers were used to generate a WT product of 1,357 bp, shown in lane A. Lanes B and C show the shorter products obtained from *dl*-NC and *dl*-L, respectively. The largest marker in lane M is a 1,193-bp *Hin*fI fragment from pGEM4.

(3) and then was used for reverse transcription and PCR with RNase H-deficient Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) according to the manufacturer's directions. The primer used for reverse transcription was from nucleotides 1977 to 1956 of GDVII; the forward primer was from nucleotides 619 to 638.

Cell culture and plaque assays. BHK-21 (baby hamster kidney cells) and L-929 cells (mouse fibroblasts) were grown in 60-mm-diameter plates in Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 7.5% fetal calf serum. Plaque morphology and virus titers were determined for BHK-21 cells as described previously (15). Plaquing on L-929 cells was done by the same method.

Viral protein synthesis and analysis. Plasmids were transcribed and translated in vitro in a TNT rabbit reticulocyte lysate (Promega) according to the manufacturer's protocol. Typically, 1 μg of nonlinearized plasmid DNA and 30 μCi of [^{35}S]methionine (SJ 1515; Amersham) were used in a 25-μl reaction. After 3 h at 30°C, 3 to 5 μl of each reaction mixture was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (29). Intracellular viral protein synthesis was analyzed by infecting BHK-21 cells in 35-mm-diameter dishes with 10 PFU per cell. At various time points, infected cells were incubated in methionine-free medium for 1 h and then pulsed for 30 min with 50 μCi of [^{35}S]methionine (Translabel; ICN). Viral proteins were extracted in buffer containing 20 mM Tris (pH 7.4), 100 mM sodium chloride, and 0.5% Nonidet P-40 and then were analyzed by SDS-PAGE.

Animal inoculations. Four- to five-week-old SJL/J mice were inoculated intracerebrally with virus in a volume of 30 μl as described previously (2). At the indicated time intervals, animals were sacrificed. The brains and spinal cords of infected mice were weighed and then suspended in phosphate-buffered saline at 10% (wt/vol) final concentrations for sonication. Plaque assays were done with 0.1-ml volumes of the sonicated 10% suspension and further dilutions of the sonicated preparations.

RESULTS

Viability of leader deletion mutants. To determine whether the leader protein was required for viability, various deletions were introduced into leader coding sequences. Proteins expressed by the various mutant constructs are diagrammed in Fig. 1. The first mutation made, *dl*-23, contained an internal deletion of 23 amino acids. The transcripts made from *dl*-23 were infectious and resulted in cytopathic effects with approximately wild-type (WT) efficiency on transfected cells. This deletion was then expanded by introducing *Cla*I sites near the amino- or carboxyl-terminal leader sequences, as described in Materials and Methods, and then combining these with the *Cla*I site of *dl*-23. One of the resulting constructs, *dl*-45N, gave rise to infectious virus. *dl*-45N should express a leader that contains the first 2 leader amino acids but lacks the next 45. No infectious virus was obtained from transcripts of *dl*-49C, a construct which lacks most of the 3′ end of the leader sequences. Interestingly, expanding this carboxyl-terminal deletion by combining it with *dl*-45N restored viability (and yielded *dl*-NC). This showed that the carboxyl-terminal residues deleted in *dl*-49C were not intrinsically required by the virus. The polypeptide made from *dl*-NC should contain only five natural leader amino acids, two from the N terminus and three from the C terminus.

Once it became apparent that significant portions of the leader region could be deleted without affecting viability, leader sequences were deleted completely in the construct *dl*-L by PCR mutagenesis with *dl*-NC as the template. In this construct, VP4 sequences immediately follow the initiating AUG codon at GDVII nucleotide 1069. Transcripts of *dl*-L gave rise to infectious virus.

To confirm that virus isolated after transfection carried deletions in its leader sequences, RNA was isolated from infected cells and the region containing the leader sequences was amplified by PCR. As shown in Fig. 2, the product amplified between position 619 and 1977 on the GDVII map was smaller when RNA was obtained from *dl*-NC- and *dl*-L-infected cells, compared with that obtained from WT-infected cells. The sizes of the amplified products were consistent with those predicted for these variants. The PCR products shown in Fig. 2 were also directly sequenced, beginning 80 nucleotides upstream of the initiating AUG and reading through the leader sequences and into VP4. No alterations other than the predicted deletions were detected (data not shown).

Viral protein synthesis in vitro. Before an attempt was made to derive virus from the mutant constructs, translation of viral proteins in vitro from T7 polymerase-generated transcripts was used to determine whether significant alterations in polyprotein processing occurred in the absence of the leader polypeptide. As shown in Fig. 3, the profiles of polypeptides made in reticulocyte lysates from the *dl*-L (lane E) and *dl*-NC (lane F) mutant constructs were very similar to those of the WT GDVII virus (lane D). Polypeptides made from transcripts of *dl*-NC included a protein that migrated slightly more slowly than VP0 (authentic VP0 is shown in lane A). It seemed likely that this protein was related to VP0 but that leader-derived amino acids at its amino terminus could not be removed by the viral protease. Confirmation that the altered protein was related to VP0 came from immunoprecipitation of radiolabeled infected cell extracts with an anti-VP2 monoclonal antibody. This antibody precipitates VP0 from infected cell extracts which do not contain significant amounts of VP2, a protein found only in mature virus. Immunoprecipitates of *dl*-NC-infected extracts contained a more-slowly-migrating VP0 (lane H) than that of *dl*-L (lane I)- or WT (lane G)-infected cells.

Translation of transcripts derived from the nonviable mutant *dl*-49C showed an accumulation of precursor proteins and reduced levels of capsid proteins and other mature viral products (data not shown). The juxtaposition of N-terminal leader sequences with VP0 may have changed the conformation of the nascent polypeptide dramatically enough to reduce proteolytic

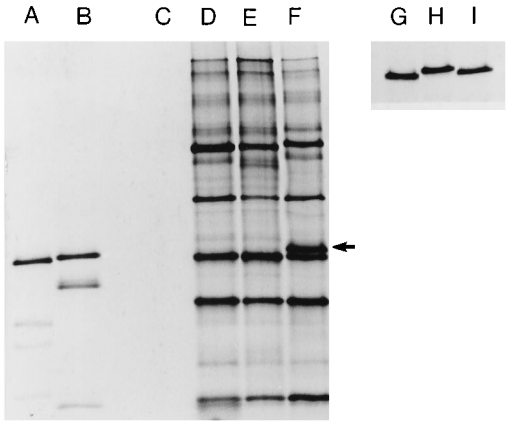

FIG. 3. In vitro transcription-translation of mutant plasmids. Plasmids containing full-length WT GDVII (lane D), *dl*-L (lane E), and *dl*-NC (lane F) were transcribed and translated with T7 polymerase and reticulocyte lysate in a coupled system. The results of a translation performed in the absence of RNA are shown in lane C. For immunoprecipitation, infected BHK cells were labeled with [^{35}S]methionine between 10 and 11 h postinfection; VP0 proteins were then immunoprecipitated from WT (lane G)-, *dl*-NC (lane H)-, and *dl*-L (lane I)-infected cell extracts by using an anti-VP2 (anti-VP0) monoclonal antibody. Altered VP0 expressed by *dl*-NC is marked with an arrow (lane F); its relationship to VP0 was confirmed by immunoprecipitation (lane H). For marker proteins, VP0 and 2C, with initiating AUG and terminating codons, were cloned into plasmid pGEM4 and then expressed by transcription-translation of pGEM4 derivatives (VP0 and 2C marker proteins are shown in lanes A and B, respectively).

processing. This may account for the nonviability of *dl*-49C. As noted above, *dl*-49C and *dl*-46N were ligated together to generate *dl*-NC, a viable variant. Thus, proper processing was restored by the removal of residual leader sequences present in *dl*-49C, indicating that no other defect was present in this construct.

Growth characteristics of mutant viruses. Viruses from the third passage posttransfection were compared with WT virus for similarities in growth rates and yields. Equivalent multiplicities (10 PFU per cell) of WT GDVII, *dl*-NC, and *dl*-L were used to initiate the growth curves. As shown in Fig. 4, both

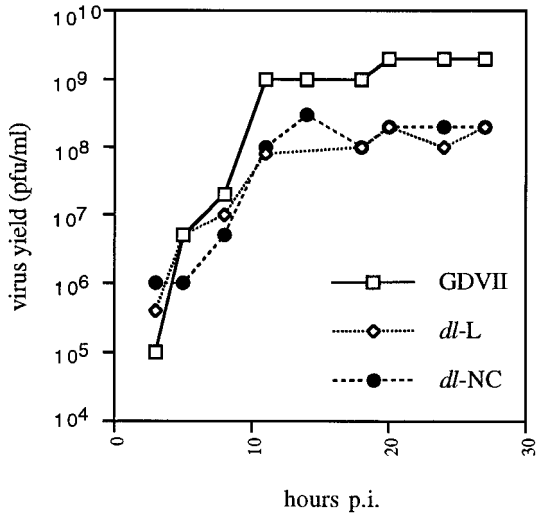

FIG. 4. Growth of WT GDVII, *dl*-NC, and *dl*-L in BHK cells. Cells were infected with virus (10 PFU per cell) at 37°C and then lysates were prepared at various times postinfection. Virus titers were determined by plaque assays with BHK cells.

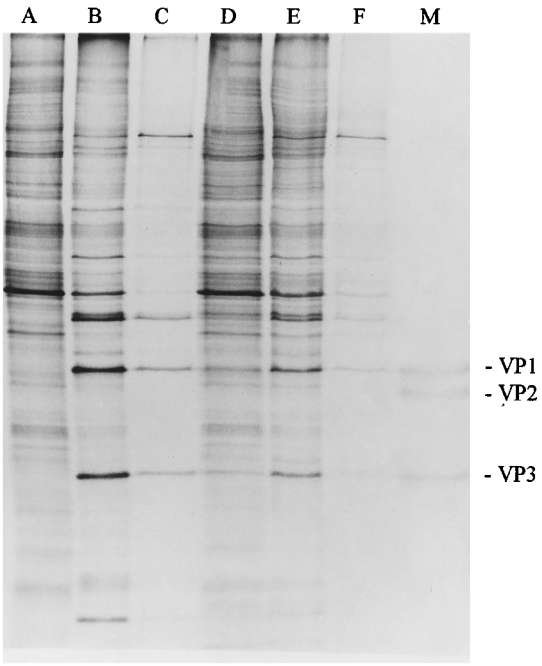

FIG. 5. Intracellular viral protein synthesis by mutant *dl*-L. Cells were infected with 10 PFU of WT GDVII or *dl*-L per cell and then labeled at various times postinfection for 1 h with [^{35}S]methionine as described in Materials and Methods. Patterns shown are proteins synthesized by mock-infected cells (lane A), cells infected with WT GDVII for 5 and 8 h (lanes B and C, respectively, and cells infected with *dl*-L for 5, 8, and 12 h (lanes D to F, respectively). The marker lane (M) shows the positions of VP1, VP2, and VP3 obtained from isolated virions.

mutant viruses grew in BHK cells but showed a delay in growth relative to WT GDVII. The delay in the time course of the infection was even more apparent when viral protein synthesis in infected BHK cells was examined (Fig. 5). Although viral proteins were readily observed by 5 h postinfection with WT GDVII (lane B), they were barely detectable in cells infected with *dl*-L until 8 h postinfection (lane E). The reduced amounts of viral proteins present in lanes C (WT, 8 h postinfection) and F (*dl*-L, 12 h postinfection) reflected extensive cytopathic effects at these times.

Despite the longer growth period of the *dl*-L mutant virus, the overall profile of the viral polypeptides was similar to that of WT-infected cells, suggesting, as did the in vitro translation analyses, that the elimination of the leader sequences did not interfere with normal polyprotein processing. It was also apparent that extensive shutoff of the host cell occurred in *dl*-L-infected cells, although this may have been slightly less efficient than in WT infections.

The virus *dl*-23 grew to titers comparable to those of GDVII and had a WT plaque size. *dl*-46N, *dl*-NC, and *dl*-L showed approximately 1-log reductions in final titers, and plaque sizes were also reduced (Fig. 6). However, these viruses formed larger plaques than did the demyelinating strains of TMEV, e.g., BeAn, which forms plaques less than 1 mm in diameter. Also in contrast to BeAn, plaques were apparent at both 33 and 39°C. Recently, it was reported that the leader sequences of a demyelinating strain of TMEV, DA, were required for low-multiplicity growth of the virus in mouse L-929 cells (11). As shown in Fig. 6, this was also the case with the neurovirulent GDVII virus. Plaquing efficiencies of the WT virus were similar in L-929 and BHK-21 cells, but the *dl*-L strain of GDVII gave plaques only in the BHK-21 cell line.

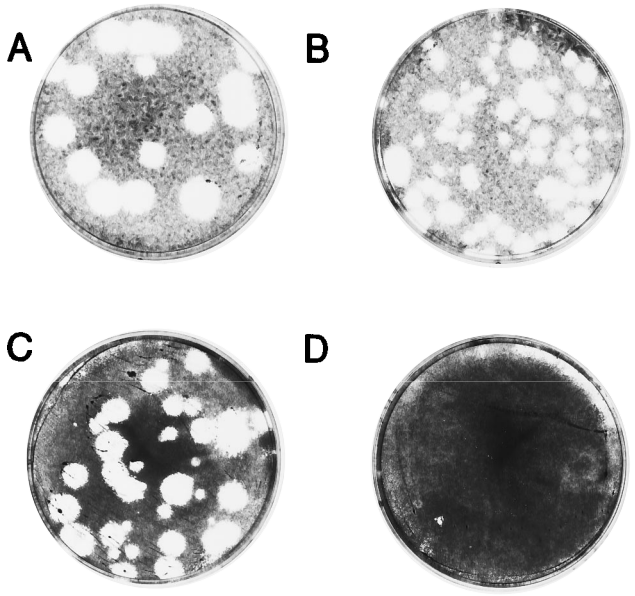

FIG. 6. Plaque formation on BHK-21 and L-929 cells. The WT GDVII virus and the *dl*-L mutant virus were diluted and used to infect either BHK-21 cells or mouse L-929 cells. Results shown were obtained with a 10^{-7} dilution of a GDVII lysate and a 10^{-6} dilution of a *dl*-L lysate. (A) GDVII virus, BHK-21 cells; (B) *dl*-L virus, BHK-21 cells; (C) GDVII virus, L-929 cells; (D) *dl*-L virus, L-929 cells.

Neurovirulence of mutant viruses. The parental GDVII virus is highly neurovirulent. In our study, the 50% lethal dose was about 20 PFU and nearly all mice died within 7 days following infection with 100 to 200 PFU (Table 1). In contrast, mice survived infections with more than 10^6 PFU of demyelinating viruses, e.g., BeAn. Similar experiments were performed with the various leader mutant viruses. The first virus isolated, *dl*-23, remained virulent, but it required about 10 times more virus to kill mice (data not shown). Interestingly, viruses that contained the larger deletions *dl*-NC and *dl*-L were not neurovirulent, and mice survived infections with doses as high as 10^6 PFU. The results shown in Table 1 are representative of four experiments, two of which are shown. No killing by *dl*-L or *dl*-NC has yet been observed.

Growth of mutant viruses in vivo. Because of the lack of any neurovirulence, it became of interest to determine the levels of the mutant viruses in the brains and spinal cords of infected mice. As shown in Table 2, parental GDVII virus was found 7 days postinfection at high levels in both brains and spinal cords, even though the inoculum used for infection was small (20 PFU). In contrast, neither *dl*-L nor the demyelinating BeAn strain reached levels equivalent to those of GDVII, and the levels were frequently lower than those of input viruses (10^6 PFU) by day 7 postinfection. As shown previously by several laboratories, low but detectable levels of the BeAn virus were found at days 17 and 35 postinfection (1, 10, 16, 19). This was not the case for the *dl*-L mutant, and no virus was recovered in several experiments after the first 2 weeks of infection. Although the data are not shown here, several animals infected with the *dl*-L virus were examined for histologic evidence of demyelination; no such evidence was obtained.

The levels of *dl*-L recovered 7 days postinfection were reduced relative to the inoculum of 10^6 PFU, raising the question of whether any virus replication was occurring in infected mice. To examine this, virus levels were determined at earlier time points postinfection. In experiments for which the results are not shown, levels of recovered virus were as low at days 1 and 3 as those shown for day 7 in Table 2. When virus yields were examined within the first day of infection (Table 3), an eclipse phase was apparent at 6 h postinfection, and then the levels of virus increased until 18 h. Thus, virus replication did occur following infection with *dl*-L, although the levels of this virus in the brain or spinal cord never reached those of WT GDVII.

TABLE 1. Survival of animals infected with TMEV variants[a]

Expt no.	TMEV strain	Inoculum (PFU)	Survival (no. survived/ no. infected)
1	GDVII	20	1/2
	GDVII	200	0/3
2	GDVII	20	2/4
	BeAn	10^6	4/4
	dl-L	10^6	4/4
	dl-NC	10^6	4/4

[a] SJL/J mice were infected intracerebrally as described in Materials and Methods. Survival was determined out to 21 days postinfection.

TABLE 2. Recovery of virus from infected SJL/J mice[a]

Inoculum	Day	Virus yield (plaques/g of tissue) from:	
		Brain	Spinal cord
GDVII (20 PFU)	7	10^6	10^6
	17	0	0
	35	0	0
dl-L (10^6 PFU)	7	10^4	10^5
	17	0	0
	35	0	0
BeAn (10^6 PFU)	7	10^4	10^4
	17	10^2	10^2
	35	10^2	10^2

[a] The brains and spinal cords of two mice were pooled before virus isolation.

DISCUSSION

The leader polypeptide found in cardioviruses such as TMEV has no known function. It is an unusual polypeptide which is extremely acidic (net charge, −16) and very small (76 amino acids) (22). In contrast to data for the leader of FMDV, no evidence for a proteolytic function of the TMEV leader has been obtained.

A genetic approach was taken in an effort to determine the effects of mutations in the leader polypeptide on virus growth. Results presented in this report indicate that the leader protein

TABLE 3. Eclipse and subsequent replication of *dl*-L virus in mice[a]

Time postinfection (h)	Virus yield (plaques/g of tissue) from:	
	Brain	Spinal cord
6	1.0×10^4	3.3×10^2
12	2.0×10^5	1.1×10^3
18	6.5×10^5	1.0×10^4

[a] SJL/J mice were inoculated with 10^6 PFU of *dl*-L. At each time point, two (18 h) or three (6 and 12 h) animals were sacrificed and levels of virus in the brain and spinal cord were determined for the individual animals. The values shown are means, and the variation from animal to animal was less than 1 log.

of GDVII, a neurovirulent strain of TMEV, is not required for growth in BHK cells. A mutant virus that completely lacks leader sequences grows nearly as well as the WT parent, showing an only slightly reduced rate of growth and a smaller plaque size. The mutant virus was fully capable of reducing host protein synthesis, which was of interest because this function has been attributed to the FMDV leader protein (5). Although virus yields of the leader deletion mutant were similar to those of a demyelinating strain of TMEV, BeAn, the plaques of the leader deletion mutant virus were significantly larger (diameter, 2 to 4 mm) than those of BeAn (diameter, <1 mm). While these studies were under way, it was shown by Kong et al. (11) that the deletion of leader sequences in another demyelinating strain, DA, did not impair the growth of DA virus in BHK cells. In contrast, deletion of the DA leader resulted in the failure of the mutant virus to form plaques in mouse L-929 cells or to spread through a culture when used at less than 1 PFU per cell. These same properties were found for the GDVII leader mutant. Thus, our studies support the finding of Kong et al. that the leader polypeptide plays a role in the host range of the virus, although the mechanism for cell-type specificity remains to be determined.

With the exception of the small deletion *dl*-23, which showed reduced but significant neurovirulence, all larger deletions led to complete inability of the mutant viruses to kill mice. The low yields of virus recovered even within the first week of infection suggested that replication of *dl*-L in vivo was extremely inefficient. We have been able to demonstrate a virus eclipse within the first day of infection, followed by an increase in virus yield. This suggested that replication, although impaired, did occur in vivo.

Although the leader is not required for growth of the virus in BHK cells, *dl*-L has failed to replicate in any mouse cells tested, including primary oligodendrocytes and established mouse cell lines such as NIH 3T3 (1). The failure of the *dl*-L variant to replicate or form plaques in mouse cell lines reflects its severely reduced virulence in animals and suggests that the growth of virus variants in mouse lines will provide a useful model for defining the properties relevant to pathogenesis. The growth restriction imposed by the absence of the leader polypeptide must differ from that apparent in demyelinating strains of TMEV, e.g., DA and BeAn, which form plaques in L-929 cells equivalent to the minute plaques formed in BHK cells, as long as the leader polypeptide is present.

Other approaches which reduced the neurovirulence of GDVII virus have been taken. These include alterations in the 5′ noncoding regions of infectious cDNA clones (16, 24) and the construction of chimeric viruses with BeAn or DA (2, 7, 8, 10, 25, 33–35). The *dl*-L virus described in this study is an important complement to existing variants, because the virus is attenuated even though it contains intact regulatory 3′ and 5′ regions of GDVII as well as the structural proteins.

The GDVII leader mutant *dl*-L was effectively cleared from infected mice within 2 weeks of infection. This also contrasts with the well-documented behavior of BeAn and other attenuated strains, which may persist throughout the lifetime of the animal (19). A key question in current studies of the TMEV viruses is the mechanism through which the virus persists, because studies of chimeric BeAn/GDVII or DA/GDVII viruses have shown a strong correlation between viral persistence and eventual demyelination (7, 10, 24, 25, 34, 35). The apparent failure of *dl*-L to persist suggests that poor replication of a variant virus alone is not sufficient to allow the virus to persist in animals.

A significant body of evidence exists which suggests that demyelination is immune mediated (for a review, see reference 20) and correlates with the class II-restricted responses to viral capsid antigens (4, 9, 20). The dominant epitope which has been identified for SJL/J mice is identical for GDVII and BeAn viruses (9). Thus, immune responses to this epitope would be expected following infection with either GDVII or BeAn. Despite the presence of this epitope in *dl*-L, demyelination has not been observed following infections with this variant, providing additional support for the hypothesis that viral persistence is required for demyelination to occur.

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