

Influence of the Theiler's Virus L* Protein on Macrophage Infection, Viral Persistence, and Neurovirulence

OLIVIER VAN EYLL AND THOMAS MICHIELS*

Christian de Duve Institute of Cellular Pathology, Université Catholique de Louvain, B-1200 Brussels, Belgium

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The genome of picornaviruses contains a large open reading frame (ORF) translated as a precursor polypeptide that is processed to yield all the proteins necessary for the viral life cycle. In persistent but not in neurovirulent strains of Theiler's virus, an overlapping ORF encodes an additional 18-kDa protein called L*. We confirmed previous work showing that the L* ORF of persistent strains facilitates the infection of macrophage cell lines, and we present evidence that this effect is due to the L* protein itself rather than to competition for the translation of the two overlapping ORFs. The introduction of an AUG codon to restore the L* ORF of the neurovirulent GDVII strain also enhanced the infection of macrophages, in spite of the divergent evolution of this protein. The presence or the absence of the L* AUG initiation codon had only a weak influence on the neurovirulence of the GDVII strain and on the persistence of the DA1 strain. The results obtained with DA1 in vivo contrast with the results reported previously for DAFL3, another molecular clone of the same virus strain, where the AUG-to-ACG mutation of the L* initiation codon totally blocked viral persistence (G. D. Ghadge, L. Ma, S. Sato, J. Kim, and R. P. Roos, *J. Virol.* 72:8605–8612, 1998). Thus, a factor that is critical for the persistence of a given clone of Theiler's virus is dispensable for the persistence of a closely related clone, indicating that different adjustments in the expression of persistence determinants occur in related viral strains.

Theiler's murine encephalomyelitis virus (Theiler's virus or TMEV) is a picornavirus that infects the central nervous system (CNS) of the mouse (30). Several Theiler's virus strains were isolated and classified into two subgroups on the basis of the diseases that they provoke. Neurovirulent viruses, such as GDVII or FA, cause an acute fatal encephalomyelitis. Persistent strains, such as DA or BeAn, sometimes referred to as Theiler's original strains, cause a persistent CNS infection and a chronic demyelinating disease considered an experimental model for multiple sclerosis (for a review, see references 7 and 20).

Upon intracerebral inoculation, persistent strains of Theiler's virus cause a biphasic disease in susceptible mice: first, the virus induces a mild encephalitis in the gray matter of the brain; subsequently, the virus is found predominantly in the white matter of the spinal cord, where it persists lifelong, causing inflammation and demyelination.

Macrophages appear to play an important role in the infection process. First, Lipton and coworkers (14) showed, by two-color fluorescence staining, that macrophages contained most of the viral load during the chronic stage of the disease. In agreement with these data, the depletion of infiltrating macrophages in vivo almost completely cleared the infection (23). Second, demyelination was suggested to be related to a bystander effect of activated macrophages infiltrating the lesions (4). Accordingly, upon macrophage depletion, the numbers of demyelinating lesions were drastically reduced, confirming the crucial role of macrophages in the pathology (23). However, there has been no evidence, until now, that macrophages are the reservoir cells that allow the virus to escape the immune response.

The genome of Theiler's virus is an 8-kb-long RNA molecule of positive polarity. It contains a large open reading frame

(ORF) translated as a long precursor polyprotein that undergoes autoproteolytic processing to yield all the viral proteins required to fulfill the viral life cycle. However, in persistent strains of Theiler's virus, an additional protein, called L*, was found to be translated from an alternative ORF starting 13 nucleotides (nt) downstream from the AUG codon of the main ORF and ending just upstream of the *cis*-acting replication element recently discovered in the VP2-coding region (11, 15). The L* ORF is conserved in all the persistent strains analyzed so far (18). In neurovirulent strains of Theiler's virus, an ACG codon is substituted for the AUG codon initiating the translation of the alternative ORF. The presence of the L* ORF in the persistent strain DAFL3 was found to enhance the infection of macrophage cell lines (21, 28) but not of other cell types (22), possibly via the inhibition of apoptosis (9). It is not known whether this antiapoptotic activity results from the expression of the L* protein itself or from the presence of the L* alternative ORF that might compete with the translation of some proapoptotic viral factor encoded by the main ORF.

In vivo, the L* ORF has been reported to be critical for viral persistence in the CNS of the mouse (9, 13). This effect could be correlated with the alteration of the H-2K-restricted cytotoxic T-lymphocyte response of the host (13). However, although recent works have reported the complete lack of persistence of a DAFL3 virus clone carrying an AUG-to-ACG mutation of the L* initiation codon, previous work done with the same virus as well as work performed with a different clone of strain DA or with persistent strain BeAn has shown the persistence of some recombinant viruses lacking the L* ORF: the DA/GDVII recombinants GD5'-1B/DAFL3 (8, 24) and R2 (17) and the BeAn/GDVII recombinants 3B and 41 (1).

The aim of this work was fourfold: (i) to revisit the role of the L* ORF in viral persistence, (ii) to check whether the expression of the L* protein itself or competition between the translation of the L* ORF and that of the main ORF is responsible for the enhancement of macrophage infection, (iii) to analyze whether the L* ORF of virus GDVII is functional upon reintroduction of a translation initiation codon, and (iv)

* Corresponding author. Mailing address: Christian de Duve Institute of Cellular Pathology, Université Catholique de Louvain, MIPA-VIRO 74-49, 74 ave. Hippocrate, B-1200 Brussels, Belgium. Phone: 32 2 764 74 29. Fax: 32 2 764 74 95. E-mail: michiels@mipa.ucl.ac.be.

to evaluate the influence of the lack of the L* ORF on the neurovirulence of virus GDVII.

MATERIALS AND METHODS

Cell culture. BHK-21 cells were cultured in Glasgow minimum essential medium (Gibco-BRL) supplemented with 10% newborn bovine serum (Gibco-BRL), 100 IU of penicillin per ml, 100 µg of streptomycin per ml, and 130 g of tryptose phosphate broth (Gibco-BRL) per liter. L929 cells were cultured in Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 IU of penicillin per ml, 100 µg of streptomycin per ml, and 100 mM sodium pyruvate. Raw264.7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 100 IU of penicillin per ml, 100 µg of streptomycin per ml, 100 mM sodium pyruvate, and 5% either Mycclone fetal calf serum (Gibco-BRL Mycclone Super Plus-Bovine Serum; catalog no. 10081-071, batch no. 30Q7351A) or standard fetal calf serum (Gibco-BRL; catalog no. 10270-031, batch no. 40F8550J). The use of different sera allows modulation of the activation and differentiation states of these cells as well as their susceptibility to Theiler's virus infection (26).

Construction of mutant viruses. Viruses mutated in the L* region were obtained by site-directed mutagenesis by the method of Kunkel (12). Mutations were generated on subclones carrying the appropriate region. The restriction fragment carrying the mutation was sequenced to ensure that no unwanted mutation occurred and was then cloned back in a full-length cDNA clone.

The two AUG-to-ACG mutations were introduced in virus DA1 with oligonucleotide TM97 (CAATAGGGGCACAGTCTGGGTATCCGTGTTTGCA AGCCAT). Mutagenesis was performed on pTM410, a pTZ19R (Pharmacia) derivative containing the 5' end (nt 1 to 1729) of the DA1 genome. The *BbrPI*-*BsiWI* fragment (nt 804 to 1265 of DA1) was then used to replace the corresponding fragment of pTM410 (16, 19). The recombinant plasmid obtained, carrying the two AUG-to-ACG mutations, was called pOV23. The virus produced from that construct is called OV23 (Fig. 1).

A stop codon at codon 39 of the L* ORF was introduced into pTM410 with oligonucleotide TM223 (GACGTCATCGTCTAGGTCCACACAAA). The *BbrPI*-*BsiWI* fragment was cloned into pTM598, a pTM410 derivative carrying a mutation in the corresponding fragment. The recombinant DA1 virus carrying the Trp-to-stop mutation at codon 39 of L* was called OV41 (on plasmid pOV41).

To introduce a stop codon at codon 93 of the L* ORF, mutagenesis of pTM410 was performed with oligonucleotide TM224 (GAATAGAAAGTTGTT TATAATGACCCCTT). The *BsiWI*-*MscI* fragment (nt 1265 to 1705 of DA1) carrying the mutation was then used to replace the corresponding fragment of pTM533, a pTM410 derivative carrying a deletion in the same fragment (19). The recombinant DA1 virus carrying the Leu-to-stop mutation at codon 93 of L* was called OV42 (on plasmid pOV42).

The ACG-to-AUG mutations restoring the L* initiation codon in the genome of virus GDVII were introduced with oligonucleotide TM98 (ATGGCTTG CAAACATGGATACCCAGATGTGTGCCCTATTGTG). Mutagenesis was performed on pTM427, a pTZ18R (Pharmacia) derivative containing the 5' end (nt 1 to 1733) of the GDVII genome. The *BbrPI*-*AocI* fragment (nt 807 to 1334 of GDVII) was then ligated to *AocI*-*SgrAI* and *SgrAI*-*BbrPI* fragments of pTMGDVII (29) derivatives to form pOV28. This plasmid contains the full-length genome of the GDVII virus with the ACG-to-AUG mutations at the first and fifth codons of L*. The virus produced from that construct is called OV28 (Fig. 1).

Virus production. Viruses were produced as described previously (19) by transfection of BHK-21 cells with genomic RNAs transcribed in vitro from plasmids carrying the corresponding cDNAs: pTM410 (16, 19), pTMGDVII (29), and pOV23, pOV28, pOV41, and pOV42 (this work).

Culture supernatants were collected after the cytopathic effect was reached (generally between 48 and 72 h after transfection). The culture supernatants were frozen, thawed, and centrifuged at 4,000 × g for 10 min. The supernatants were then collected and stored in aliquots at -70°C. Viruses were titrated by a standard plaque assay on BHK-21 cells.

In vitro translation. In vitro coupled transcription-translation was performed with rabbit reticulocyte lysates (Promega TNT) according to the manufacturer's recommendations. Samples were run on standard Tris-glycine-sodium dodecyl sulfate-11 or 12.5% polyacrylamide gels.

Metabolic labeling. BHK-21 cells (2 × 10⁵) grown in a 1.5-cm well were infected in serum-free medium at a multiplicity of infection of 10 PFU per cell. After 1 h of incubation at 37°C, actinomycin D was added to a final concentration of 2 µg/ml and newborn calf serum was added to a final concentration of 2%. Ten hours after infection, cells were washed and cultured for 1 h in methionine-deficient minimum essential medium (Gibco-BRL) containing 1% newborn calf serum. Twenty microcuries of a ³⁵S-labeled methionine-cysteine mixture (Promix; Amersham-Pharmacia Biotech) was added to the culture. After 10 h of incubation, cells were collected and resuspended in sample buffer (62.5 mM Tris [pH 6.8], 2% β-mercaptoethanol, 3% sodium dodecyl sulfate, 10% glycerol, 0.1% bromophenol blue). Samples were then run on Tris-Tricine-sodium dodecyl sulfate-13% polyacrylamide gels.

Analysis of mixed viral infections. The proportions of DA1 and OV23 viruses in a mixture were estimated by restriction analysis of reverse transcription (RT)-

DA1:	Met Ala Cys Lys His Gly Tyr Pro Asp Val	⇒ L
	<u>AUG</u> GCU UGC AAA <u>CAU</u> GGA UAC CCA <u>GAU</u> GUG...	
	Met Asp Thr Gln Met Cys	⇒ L*
	1 2 3 4 5 6	
OV23:	Met Ala Cys Lys His Gly Tyr Pro Asp Val	⇒ L
	<u>AUG</u> GCU UGC AAA <u>CAC</u> GGA UAC CCA <u>GAC</u> GUG...	
	Thr Asp Thr Gln Thr Cys	⇒ /
	1 2 3 4 5 6	
GDVII:	Met Ala Cys Lys His Gly Tyr Pro Asp Val	⇒ L
	<u>AUG</u> GCU UGC AAA <u>CAC</u> GGA UAC CCA <u>GAC</u> GUG...	
	Thr Asp Thr Gln Thr Cys	⇒ /
	1 2 3 4 5 6	
OV28:	Met Ala Cys Lys His Gly Tyr Pro Asp Val	⇒ L
	<u>AUG</u> GCU UGC AAA <u>CAU</u> GGA UAC CCA <u>GAU</u> GUG...	
	Met Asp Thr Gln Met Cys	⇒ L*
	1 2 3 4 5 6	
DA1:	Thr Asp Leu Leu Cys Val Asp Leu Asp Asp	⇒ L
	...ACG GAC CUU UGU UGU GAC <u>CUG</u> GAC GAU...	
	Arg Thr Phe Phe Val Trp Thr Trp Thr	⇒ L*
	32 33 34 35 36 37 38 39 40	
OV41:	Thr Asp Leu Leu Cys Val Asp Leu Asp Asp	⇒ L
	...ACG GAC CUU UGU UGU GAC <u>CUA</u> GAC GAU...	
	Arg Thr Phe Phe Val Trp Thr <u>AMB</u>	⇒ L*/
	32 33 34 35 36 37 38 39 « STOP »	
DA1:	Ser Gly Asn Glu Gly Val Ile Ile Asn Asn	⇒ VP4
	...UCA GGU AAC GAA GGG GUC AUU <u>AUU</u> AAC AAC...	
	Gln Val Thr Lys Gly Ser Leu Leu Thr	⇒ L*
	86 87 88 89 90 91 92 93 94	
OV42:	Ser Gly Asn Glu Gly Val Ile Ile Asn Asn	⇒ VP4
	...UCA GGU AAC GAA GGG GUC AUU <u>AUA</u> AAC AAC...	
	Gln Val Thr Lys Gly Ser Leu <u>OCH</u>	⇒ L*/
	86 87 88 89 90 91 92 93 « STOP »	

FIG. 1. Constructs with mutated L* ORFs. The sequences of the mutated regions in OV23, OV28, OV41, and OV42 are shown under the corresponding segment of the parental strain. Translation of the main ORF is shown above and translation of the L* ORF is shown below the nucleotide sequence. Amino acids of L* are numbered. The AUG codons of the main and L* ORFs and the codons subjected to mutagenesis are underlined.

PCR products. To this end, RNA was extracted from infected cells or tissues, and a 1.1-kb fragment of the viral genome spanning the leader region was amplified by RT-PCR using primers TM4 and TM132 (Table 1). This fragment was subsequently digested with enzyme *AflIII*, which allowed discrimination between the wild-type and mutant genomes. Upon gel electrophoresis, the proportions of DA1 and OV23 viruses could be estimated by the relative intensities of bands specific for each virus. This approach is very sensitive for detecting small differences between two viruses, since it is independent of sample-to-sample variations and of any technical bias related to virus quantification, RNA extraction, cDNA synthesis, or PCR amplification.

The same procedure was applied for the GDVII-OV28, DA1-OV41, and DA1-OV42 mixtures. The restriction enzymes used to digest the PCR products were *AflIII*, *BfaI*, and *MseI*, respectively.

Infection of mice. Three-week-old female SJL/J mice were inoculated intracranially in the right hemisphere with 40 µl of a virus suspension. For histological

TABLE 1. Primers and PCR conditions

Primers	Gene fragment	Fragment length (bp)	No. of cycles	Annealing temp (°C)
TM4 and TM132	Virus	1,125	30-40	55
TM87 and TM88	β-Actin	1,060	25	58
TM217 and TM218	IL-1β	381	35	55
TM215 and TM216	IL-6	283	35	55
TM221 and TM222	IL-12 (p40)	1,160	35	55
TM213 and TM214	TNF-α	383	30	55
TM233 and TM234	IFN-γ	496	35	55

examination, tissues from four mice were embedded in paraffin after 4% paraformaldehyde fixation and tissues from two mice were embedded in O.C.T. compound (Tissue-tek) for cryosectioning. Longitudinal sections (8- μ m thick) of the spinal cord were examined. Viral antigen was detected by immunohistochemical analysis with a polyclonal rabbit antibody directed against the viral capsid (kindly provided by Michel Brahic) and a secondary antibody coupled to horseradish peroxidase (Envision; Dako). Diaminobenzidine (Sigma) was used as the chromogenic substrate. Inflammatory foci were detected after hematoxylin staining. Demyelinating lesions were detected by standard Luxol fast blue staining.

Dot blot and RT-PCR. For dot blot or RT-PCR analysis, RNA was prepared from cells or tissues (brain and spinal cord) using the technique of Chomczynski and Sacchi (3). Dot blotting and RT-PCR to detect viral RNA were performed as described previously (27). The PCR conditions used are presented in Table 1. The primers were as follows: TM4, 5'-TTC CCT CCA TCG CGA CGT GGT; TM87, 5'-ATG GAT GAC GAT ATC GCT GC; TM88, 5'-GCT GGA AGG TGG ACA GTG AG; TM132, 5'-GTG CCA TAG TAG CAA AAG CA; TM213, 5'-GTT CTA TGG CCC AGA CCC TCA CA; TM214, 5'-TCC CAG GTA TAT GGG CTC ATA CC; TM215, 5'-TCC ATC CAG TTG CCT TCT TG; TM216, 5'-CCA GTT TGG TAG CAT CCA TC; TM217, 5'-GCA ACT GTT CCT GAA CTC A; TM218, 5'-CTC GGA GCC TGT AGT GCA G; TM221, 5'-GCA CAT CAG ACC AGG; TM222, 5'-CAA CGT TGC ATC CTA GGA TGG; TM233, 5'-GAC AAT CAG GCC ATC AGC AAC; AND TM234, 5'-CGC AAT CAC AGT CTT GGC TAA.

RESULTS

Construction of a DA1 mutant lacking L* and of a GDVII mutant expressing L*. Two AUG codons, conserved in persistent strains, could initiate the translation of the L* ORF (Fig. 1). The first AUG codon (at nt 1079) is in a good context for translation initiation and is likely to be the actual L* initiation codon. Indeed, a mutant virus lacking this AUG was reported to lack the expression of the L* protein (11). However, a second AUG codon, located four codons downstream from the first one and in the same phase, might be able to initiate low levels of L* translation when the first AUG codon is lacking. Strikingly, in the genome of the neurovirulent GDVII virus, the L* ORF is conserved, but both AUG codons are replaced by ACG codons.

We used site-directed mutagenesis to replace, in DA1, the two AUG codons with ACG codons. These mutations did not affect the amino acid sequence of the L protein encoded by the main ORF. The DA1 recombinant lacking the L* AUG codons was called OV23. We also constructed a mutant of GDVII in which two AUG codons were substituted for the ACG codons in order to restore the L* ORF. This GDVII derivative, expressing L*, was called OV28.

As expected, translation in reticulocyte lysates of the RNAs transcribed from pTMDA1 and pOV28 but not from pTMGDVII and pOV23 yielded a protein corresponding to L* (Fig. 2A). The L* protein expressed by pOV28 migrated slightly faster than that of pTMDA1, although the predicted molecular masses of these proteins are 18.230 and 17.863 kDa, respectively. The L* protein was also detected in extracts from BHK-21 cells infected with viruses DA1 and OV28 but not with viruses OV23 and GDVII (Fig. 2C). The production and plaque size of the OV23 and OV28 viruses on BHK-21 cells did not differ from those of their corresponding parental wild-type viruses.

The L* ORF of viruses DA1 and OV28 (a GDVII derivative) facilitates macrophage infection. In previous work done with the DAFL3 molecular clone of strain DA, the absence of the L* ORF dramatically reduced the level of infection of different macrophage cell lines (21, 22, 28). To verify that this result also applied to the DA1 clone of strain DA and to analyze whether the restored L* ORF of the GDVII variant (OV28) could also facilitate infection of macrophages, we compared the growth kinetics of viruses DA1 and GDVII with those of their counterparts lacking or expressing the L* ORF.

Cultures of BHK-21 cells (hamster fibroblasts) or Raw264.7

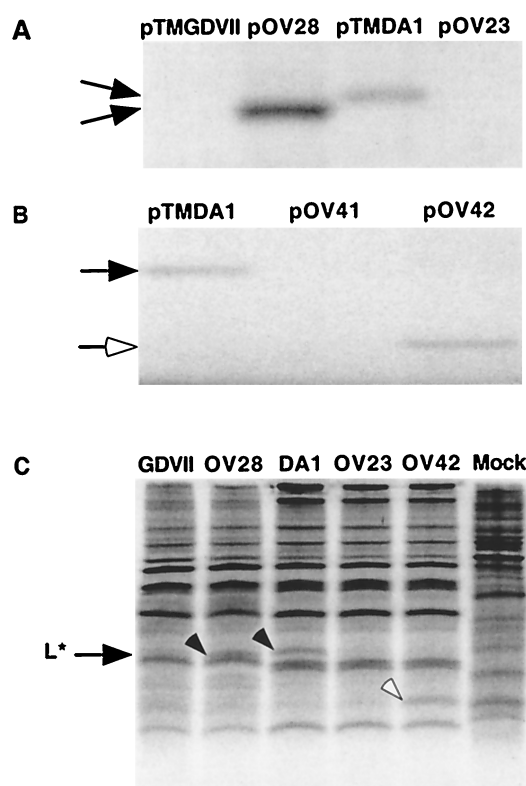


FIG. 2. Wild-type and mutant L* protein expression in rabbit reticulocyte lysates and in BHK-21 cells. (A and B) Detection of the L* protein in coupled transcription-translation reactions programmed with the indicated plasmids. L* (black arrows) was detected when the clones contained the AUG initiation codon (pTMDA1 and pOV28) but not when ACG codons replaced AUG codons (pOV23 and pTMGDVII). pOV42 contains a stop codon introduced at codon 93 of L* and produced a truncated L* protein (white arrow) of the expected molecular mass (10.532 kDa). The truncated L* protein expressed by pOV41 was too small (4.375 kDa) to be seen on the gel. (C) Extracts of [35 S]methionine-labeled BHK-21 cells infected for 21 h with viruses GDVII, OV28, DA1, OV23, and OV42 or mock infected. Black arrowheads indicate wild-type L* proteins. The white arrowhead indicates the truncated L* protein produced by OV42.

cells (mouse macrophages) were infected with the DA1, OV23, GDVII, and OV28 viruses, and viral replication was monitored by dot blot hybridization (Fig. 3). The data suggested a weak influence of the presence of the L* AUG codons. This influence was noted in the neurovirulent strain background but was less clear in the DA1 background. Similar conclusions were obtained when the production of infectious viruses by infected macrophages was measured by plaque assay (data not shown). Since the effect observed in this study appeared to be much weaker than that reported in previous studies for strain DAFL3 (21, 22), we wanted to confirm the influence of L* on the infection of macrophages by a more sensitive assay. Therefore, we used mixed infections, a strategy that turned out to be much more sensitive for detecting minor differences between viruses.

Cultures of BHK-21 cells, L929 cells (mouse fibroblasts), or Raw264.7 cells were infected with a 1:1 mixture of DA1 and OV23 viruses or with a 1:1 mixture of GDVII and OV28 viruses. After one to five passages of the viruses, the proportions of the two viruses were evaluated (Fig. 4A and B). In BHK-21 and L929 cells, the proportions of wild-type and mutant viruses remained about 1:1, even after up to five passages. In contrast, in Raw264.7 macrophages, the proportion of virus lacking the L* ORF clearly dropped after the first or the second passage. The same effect was visible when the multiplicity of infection

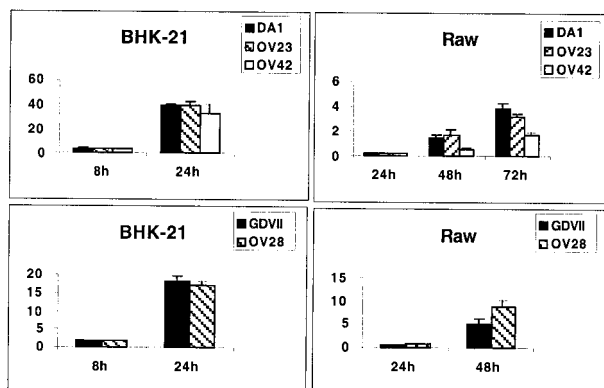


FIG. 3. Infection of macrophages and BHK-21 cells by L^* mutant and wild-type viruses. RNA was extracted from BHK-21 cells (left panels) or Raw264.7 cells (right panels) infected for the indicated times with 2 PFU of virus DA1, OV23, or OV42 (upper panels) or of virus GDVII or OV28 (lower panels) per cell. The amount of viral RNA was measured by dot blot hybridization and normalized to the amount of β -actin RNA (arbitrary units). The histograms show the means and standard deviations of data from an experiment done in triplicate. Similar data were obtained when the experiment was repeated.

was kept below 1 PFU per cell to rule out, in the case of BHK-21 cells, systematic coinfection of cells by wild-type and mutant viruses.

This experiment confirms the implication of the L^* ORF in the infection of macrophages. In addition, it shows that, in spite of some divergent evolution, the L^* ORF of the neurovirulent virus GDVII can play the same role when translation initiation is restored by the introduction of the AUG codon.

Competition for translation of the two overlapping ORFs or effect of the L^* protein? Enhancement of macrophage infection has been proposed to occur via an antiapoptotic effect of L^* (9). On one hand, this could be due to the activity of the L^* protein itself. On the other hand, it could be caused by a subtle competition between the translation of the main ORF encoding the viral polyprotein and of the ORF encoding L^* . In the latter hypothesis, the presence of the L^* ORF would decrease the translation of a proapoptotic factor expressed by the main ORF.

To discriminate between these possibilities, we constructed two additional L^* mutants of virus DA1 by introducing a stop codon in the L^* ORF, either at codon 39 (OV41) or at codon 93 (OV42), without affecting the amino acid sequence of the polyprotein translated from the main ORF (Fig. 1). These mutants thus retained the initiation codons for the two ORFs but produced truncated L^* proteins (Fig. 2B). The growth of OV42 in Raw264.7 cells appeared to be significantly restricted compared to that of the wild-type virus (Fig. 3). Moreover, in mixed-infection experiments, the OV41 (data not shown) and OV42 (Fig. 4C) mutant viruses were clearly affected in their ability to infect macrophage cell lines. Since these mutants retained the translation initiation codons of the two overlapping ORFs, enhancement of macrophage infection appears to be due to the L^* protein itself.

Effect of L^* on the neurovirulence of virus GDVII. On the basis of phylogenetic data, we postulated that the GDVII strain of Theiler's virus evolved from a subset of persistent strains and selectively lost the L^* initiation codon as a way to gain neurovirulence (18). We were thus curious to see whether the OV28 variant expressing L^* would be attenuated compared to the parental GDVII virus. SJL/J mice were infected intracerebrally with 10^3 PFU of either virus. From 4 days after infection, signs of encephalitis were prominent in most of the

mice, irrespective of the virus with which they had been inoculated. Mice were sacrificed 5 days after inoculation, and the amounts of viral RNA present in the brains and spinal cords of individual mice were quantified by dot blot hybridization (Fig. 5A). No difference was observed between the amounts of viral RNA in the brains and spinal cords of the mice inoculated with the OV28 and GDVII viruses.

To confirm these data, we infected 10 SJL/J mice with 10^3 PFU of a 1:1 mixture of the GDVII and OV28 viruses. At 5 days postinoculation, mice were sacrificed and the proportions of the two viruses were evaluated by RT-PCR and restriction analysis as described for the infection of cultured cells (Fig. 5B). In the brain, the proportions of the two viruses did not differ significantly. Six mice had more GDVII, two mice had about equal amounts of the two viruses, and two mice had more OV28. In each mouse, the proportion of GDVII virus was higher in the spinal cord than in the brain, suggesting that the wild-type virus spread somewhat faster than did the L^* -expressing virus. However, the global influence of L^* on neurovirulence was very weak.

Effect of the L^* mutation on the persistence of virus DA1. Groups of four SJL/J mice were inoculated intracerebrally with 2.5×10^5 PFU of the DA1 and OV23 viruses. Mice were sacrificed 5 and 45 days after inoculation, and total RNA was prepared from brains and spinal cords. The amounts of viral

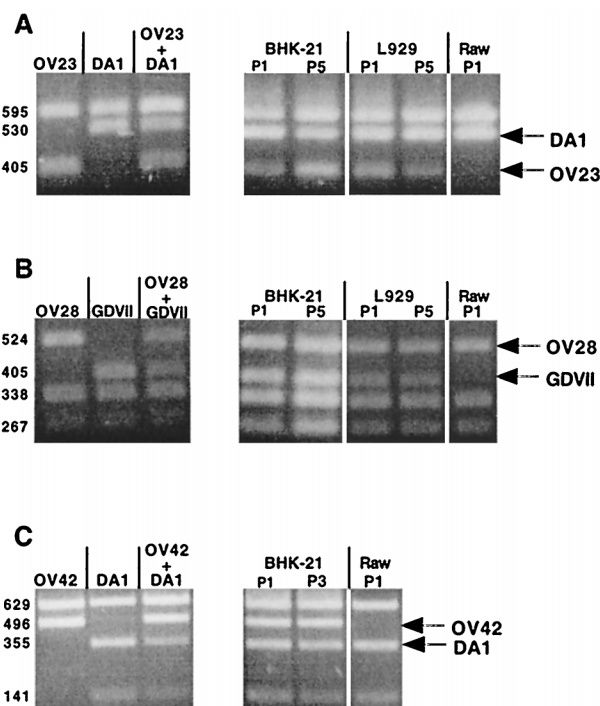


FIG. 4. Competition of wild-type and L^* mutant viruses for growth in fibroblast and macrophage cell lines. Mixtures of wild-type and mutant viruses (1:1) were prepared and used to infect BHK-21 cells, L929 cells (fibroblasts), or Raw264.7 (Raw) cells (macrophages). RNA was extracted from infected cells after one to five passages of the virus mixture. As a control, RNA was extracted from the virus mixture before infection and from the parental virus stocks. The L^* region was then amplified by RT-PCR and digested with a restriction enzyme diagnostic for the wild-type or mutant viruses. (Left panels) Control analysis of the parental viruses and of the mixtures used. (Right panels) Analysis of the virus contained in infected cells after one to five passages (P1 to P5). The fragments that are diagnostic of a given virus are indicated by arrows. (A) Afl III digests of PCR fragments from DA1 and OV23 infections. The sizes of the fragments are indicated in base pairs. (B) Afl III digests of PCR fragments from GDVII and OV28 infections. (C) Mse I digests of PCR fragments from DA1 and OV42 infections.

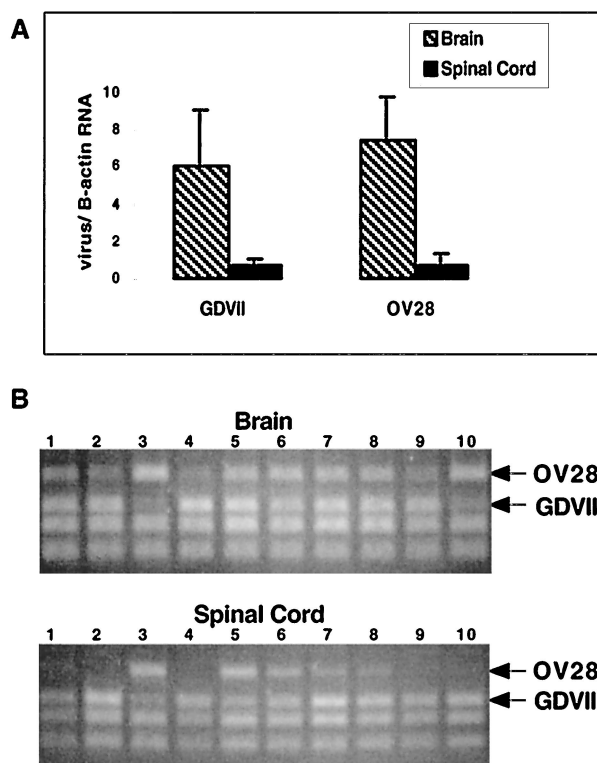


FIG. 5. Detection of GDVII and OV28 RNAs in the brains and spinal cords of infected mice. (A) RNA was extracted from the brains and spinal cords of mice 5 days after intracerebral inoculation of 10^3 PFU of GDVII ($n = 7$) and OV28 ($n = 6$) viruses. Viral RNA was detected by dot blot hybridization and normalized to the amount of β -actin RNA (arbitrary units). (B) *Afl*III cleavage of the PCR fragment amplified from the brains and from the spinal cords of mice infected for 5 days with 10^3 PFU of a 1:1 mixture of GDVII and OV28 viruses. Arrows indicate fragments specific for each virus.

RNA in these organs were measured by dot blot hybridization. Slightly more OV23 than DA1 was detected in the brains and spinal cords 5 days after inoculation (data not shown). Forty-five days after inoculation, the amounts of the OV23 and DA1 viruses were similar (Fig. 6A and B). This observation was very surprising, since previous work reported a total lack of persistence of an L* mutant of DAFL3, another molecular clone of strain DA (9, 13). Hence, we wondered whether the virus that we detected in the CNS 45 days after inoculation might have reverted to the wild-type genotype. From the spinal cord RNA of the mice inoculated with OV23, we amplified, by RT-PCR, cloned, and sequenced a fragment containing the 5' end of the L* region (nt 933 to 1230). In all four mice, the two AUG-to-ACG mutations introduced to form OV23 were still present. In addition, no mutation appeared in the first 150 nt of the L* region that could restore the translation of the L* ORF by introducing a frameshift or a start codon in the sequence. Thus, the OV23 virus was able to persist in the CNS of the mouse in spite of the absence of the L* AUG initiation codon.

To confirm these data, we inoculated four SJL/J mice with 10^6 PFU of a 1:1 mixture of the DA1 and OV23 viruses. At 45 days after inoculation, the proportions of the two viruses in the brains and spinal cords were assayed by RT-PCR and restriction analysis as described above (Fig. 6C). In the spinal cord of one out of four mice (mouse 3), the proportions of the two viruses were equivalent. In three mice, the amount of wild-type virus (DA1) was slightly higher than that of OV23. The situa-

tion was different for the brains: three mice had similar amounts of the DA1 and OV23 viruses, while one mouse had more OV23 than DA1. Since the spinal cord contains most of the viral load during persistence, the data suggest that the L* mutation had, on average, a weak negative effect on viral persistence. Nevertheless, in all mice studied, the mutant virus readily persisted in amounts close to those of the wild-type strain.

Characterization of CNS infection by virus OV23. Since the OV23 mutant had a slightly impaired ability to infect macrophages in vitro and since macrophages are thought to contain the major viral load in vivo, we tested whether the persistence of OV23 in the CNS would generate an inflammatory demyelinating disease resembling the one generated by DA1. First, we used comparative RT-PCR to evaluate the production of proinflammatory cytokines (interleukin 1 β [IL-1 β], IL-6, IL-12, tumor necrosis factor alpha [TNF- α], and gamma interferon [IFN- γ]) in the spinal cords of infected mice (the same mice as those used for dot blotting and sequencing). The amounts of the various cytokine mRNAs were higher for DA1- and OV23-infected mice than for control mice but did not differ clearly between mice infected by these two viruses (Fig. 7).

Six additional SJL/J mice were then inoculated with 10^5 PFU of DA1 or OV23 for histological examination. Longitudinal

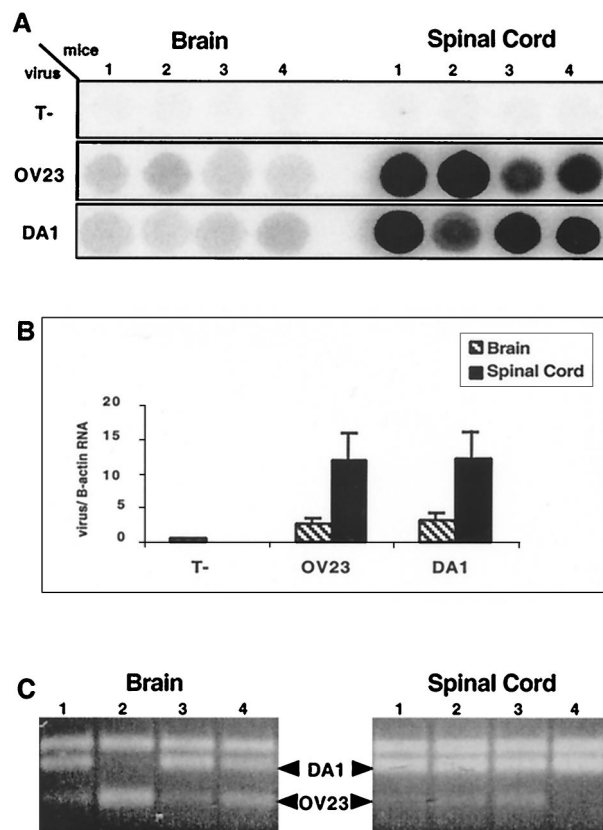


FIG. 6. Detection of viral RNA in the brains and spinal cords 45 days after inoculation. (A) Viral RNA was detected by dot blot hybridization in total RNA extracted from the brains and spinal cords of mice infected for 45 days with 10^5 PFU of DA1 and OV23 viruses. Control mice (T-) were inoculated with a nonpersistent DA1 virus mutant. (B) Quantification of the dot blot shown in panel A after normalization to the amount of β -actin RNA (arbitrary units). (C) *Afl*III cleavage of the PCR fragment amplified from the brains and spinal cords of four mice infected for 45 days with a 1:1 mixture of DA1 and OV23 viruses. Arrowheads indicate fragments specific for each virus.

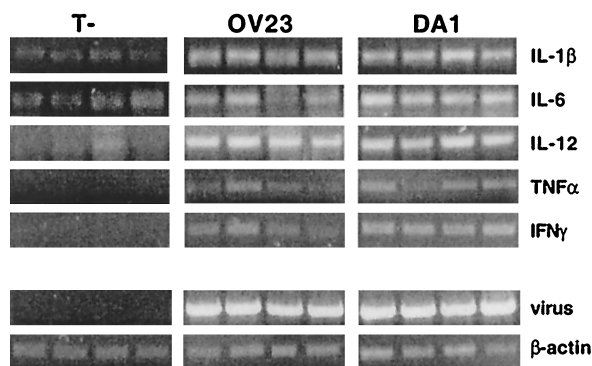


FIG. 7. Comparative RT-PCR detection of proinflammatory cytokine mRNAs in the spinal cords of mice 45 days after inoculation of 10^5 PFU of DA1 and OV23 viruses. Equal amounts of spinal cord RNA, processed in parallel, from the mice indicated in Fig. 6A were subjected to RT-PCR to compare the levels of IL-1 β , IL-6, IL-12, TNF- α , and IFN- γ mRNAs, and, as controls, of viral RNA and β -actin mRNA.

sections were examined for the presence of persistent virus by immunohistochemical analysis. Inflammation was detected after hematoxylin staining, and Luxol fast blue staining was performed to detect demyelination. In all six mice inoculated with OV23, viral antigen, inflammation, and demyelinating lesions were observed, as in DA1-infected mice (Fig. 8).

DISCUSSION

This study confirms work from other laboratories showing that the presence of the L* ORF in the genome of persistent Theiler's virus strains enhances the infection of macrophage cell lines (21, 22, 28). Given the close proximity of the AUG codon governing translation initiation of the two ORFs, it was tempting to speculate that the effect of the L* ORF could be mediated by modulating translation of the main ORF through competition for translation initiation. However, our data suggest that the L* protein itself plays a role, since mutant DA1 viruses in which the initiation codons were conserved but which produced a truncated L* protein were impaired in macrophage infection.

The sequences of the L* proteins of DA1 and GDVII vary at 31 of 156 positions (80% identity). The amino acid sequences of proteins encoded by the main ORF in the same genome region (L, VP4, and the N terminus of VP2) vary at only 13 of 156 positions (92% identity). This suggests that, in addition to the loss of the AUG codon, the L* region of GDVII has evolved divergently and could have become ineffective. Moreover, it was suggested that the GDVII internal ribosome entry site could be inefficient in promoting translation of the L* ORF in specific cell types (31). We observed that, after reintroduction of an AUG initiation codon, the L* ORF of the neurovirulent GDVII mutant virus was detectable in BHK-21 cells. The L* ORF of this virus enhanced the infection of macrophage cell lines, suggesting that the L* protein of GDVII is functional in spite of its divergent evolution. Moreover, these results indicate that the internal ribosome entry site of strain GDVII can also promote translation of the L* ORF in these cells.

It is noteworthy that an ACG codon was reported, in rare cases, to serve as an initiation codon (2, 5) so that, at this point, we cannot rule out the possibility that the wild-type GDVII virus (and the OV23 mutant) expresses small amounts of the L* protein. This would explain why the entire L* ORF was conserved in the genomes of neurovirulent viruses. This notion

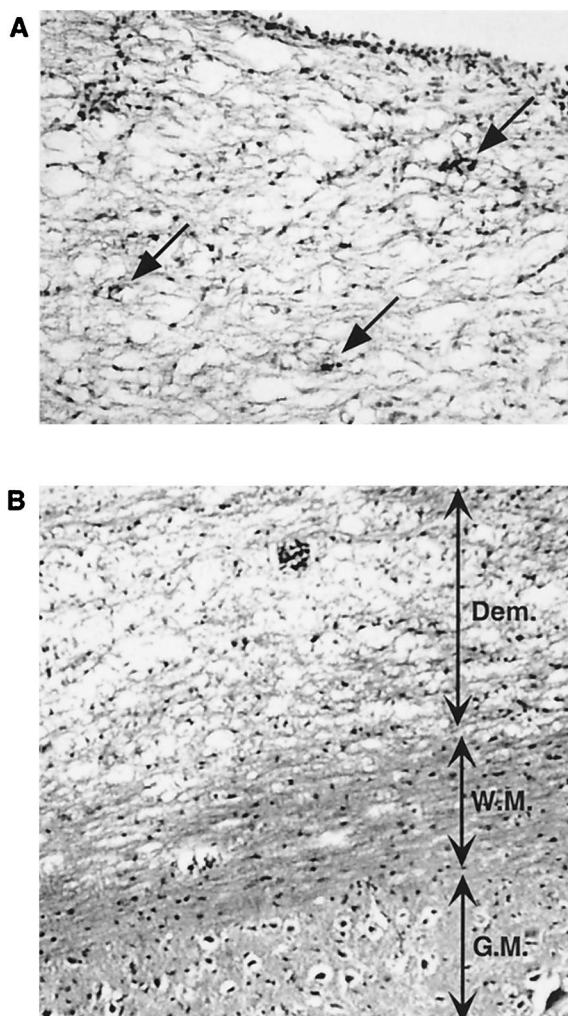


FIG. 8. Longitudinal sections of the paraffin-embedded spinal cord of a mouse infected for 45 days with the OV23 L* mutant virus. (A) Immunohistochemical analysis and hematoxylin staining. Arrows indicate viral antigen detected by immunohistochemical analysis. Hematoxylin staining reveals extensive meningoencephalitis and inflammation in the white matter. (B) Luxol fast blue-hematoxylin staining. An area with extensive demyelination (Dem.) is shown. Normal white matter (W.M.) and gray matter (G.M.) are indicated.

could also explain why the introduction of a stop codon in L* (OV42) had a more pronounced effect than the AUG-to-ACG mutation (OV23) with regard to infection of macrophages (Fig. 3). On the other hand, the AUG-to-ACG mutation was the only mutation of the start codon that could preserve the amino acid sequence of the L protein translated from the main ORF.

Phylogenetic data suggested that the neurovirulent viruses evolved from a subset of persistent strains and lost the two potential L* initiation codons during evolution, probably as a way to gain neurovirulence (18). However, we observed that introduction of the L* AUG codons in the GDVII virus hardly reduced neurovirulence. It is possible that the inactivation of the AUG codons really provides some advantage to a persistent strain to evolve toward neurovirulence but that this influence is too weak to be seen in the context of a highly neurovirulent strain.

Unexpectedly, in the DA1 virus background, inactivation of the L* AUG initiation codon only slightly impaired macro-

phage infection in vitro and hardly decreased persistence of the virus in the CNS of the mouse. Although the mixed-infection data showed a slight negative effect of the L* mutation on persistence, in all mice examined 45 days after infection, the OV23 virus readily persisted and the infection closely resembled that of the DA1 virus for any aspect examined: amounts of viral RNA, detection of viral antigen, inflammation, or induction of demyelination. These results are in strong contrast to those of Ghadge et al. (9) and Lin et al. (13), who failed to detect any persistence of an L* mutant virus by RT-PCR and by in situ hybridization, respectively.

A few parameters differed between the experiments. First, OV23 was constructed by mutating the two AUG codons likely to initiate the translation of L*, while only the first AUG codon was mutated to ACG in the DAFL3 mutant. Second, the molecular clones of the viruses differed, although they were derived from the same parental strain (DA) (6). However, SJL/J mice were used for all infection experiments.

Regarding the AUG-to-ACG mutations, it is difficult to correlate the lack of persistence of the DAFL3 L* mutant with the mutation of only the first AUG codon, since one would have expected exactly the opposite. Regarding the molecular clones of the DA strain, DAFL3 was used in the laboratory of R. Roos (25), while DA1 was used in our experiments. It is known that several point mutations differentiate the two clones. Notably, a single amino acid difference at VP2 (residue 141) was found to affect the persistence of a GDVII-DA chimeric virus (10). Since the L* ORF appears to be critical for the persistence of DAFL3 and dispensable for the persistence of DA1, one must assume that, in DA1, other factors can compensate for the absence of L* (or possibly for its very low level of expression from the ACG codon).

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REFERENCES

- Adami, C., A. E. Pritchard, T. Knauf, M. Luo, and H. L. Lipton. 1998. A determinant for central nervous system persistence localized in the capsid of Theiler's murine encephalomyelitis virus by using recombinant viruses. *J. Virol.* **72**:1662–1665.
- Becerra, S. P., J. A. Rose, M. Hardy, B. M. Baroudy, and C. W. Anderson. 1985. Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. *Proc. Natl. Acad. Sci. USA* **82**:7919–7923.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Clatch, R. J., H. L. Lipton, and S. D. Miller. 1986. Characterization of Theiler's murine encephalomyelitis virus (TMEV)-specific delayed-type hypersensitivity responses in TMEV-induced demyelinating disease: correlation with clinical signs. *J. Immunol.* **136**:920–927.
- Curran, J., and D. Kolakofsky. 1988. Ribosomal initiation from an ACG codon in the Sendai virus P/C mRNA. *EMBO J.* **7**:245–251.
- Daniels, J. B., A. M. Pappenheimer, and S. Richardson. 1952. Observations on encephalomyelitis of mice (DA strain). *J. Exp. Med.* **96**:517–529.
- Drescher, K. M., L. R. Pease, and M. Rodriguez. 1997. Antiviral immune responses modulate the nature of central nervous system (CNS) disease in a murine model of multiple sclerosis. *Immunol. Rev.* **159**:177–193.
- Fu, J., M. Rodriguez, and R. P. Roos. 1990. Strains from both Theiler's virus subgroups encode a determinant for demyelination. *J. Virol.* **64**:6345–6348.
- Ghadge, G. D., L. Ma, S. Sato, J. Kim, and R. P. Roos. 1998. A protein critical for a Theiler's virus-induced immune system-mediated demyelinating disease has a cell type-specific antiapoptotic effect and a key role in virus persistence. *J. Virol.* **72**:8605–8612.
- Jarousse, N., R. A. Grant, J. M. Hogle, L. Zhang, A. Senkowski, R. P. Roos, T. Michiels, M. Brahic, and A. McAllister. 1994. A single amino acid change determines persistence of a chimeric Theiler's virus. *J. Virol.* **68**:3364–3368.
- Kong, W.-P., and R. P. Roos. 1991. Alternative translation initiation site in the DA strain of Theiler's murine encephalomyelitis virus. *J. Virol.* **65**:3395–3399.
- Kunkel, T. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492.
- Lin, X., R. P. Roos, L. R. Pease, P. Wettstein, and M. Rodriguez. 1999. A Theiler's virus alternatively initiated protein inhibits the generation of H-2K-restricted virus-specific cytotoxicity. *J. Immunol.* **162**:17–24.
- Lipton, H. L., G. Twaddle, and M. L. Jelachich. 1995. The predominant virus antigen burden is present in macrophages in Theiler's murine encephalomyelitis virus-induced demyelinating disease. *J. Virol.* **69**:2525–2533.
- Robert, P.-E., N. Escriviou, J. Ruelle, and T. Michiels. 1999. A coding RNA sequence acts as a replication signal in cardioviruses. *Proc. Natl. Acad. Sci. USA* **96**:11560–11565.
- McAllister, A., F. Tangy, C. Aubert, and M. Brahic. 1989. Molecular cloning of the complete genome of Theiler's virus, strain DA, and production of infectious transcripts. *Microb. Pathog.* **7**:381–388.
- McAllister, A., F. Tangy, C. Aubert, and M. Brahic. 1990. Genetic mapping of the ability of Theiler's virus to persist and demyelinate. *J. Virol.* **64**:4252–4257.
- Michiels, T., N. Jarousse, and M. Brahic. 1995. Analysis of the leader and capsid coding regions of persistent and neurovirulent strains of Theiler's virus. *Virology* **214**:550–558.
- Michiels, T., V. Dejong, R. Rodrigus, and C. Shaw-Jackson. 1997. Protein 2A is not required for Theiler's virus replication. *J. Virol.* **71**:9549–9556.
- Monteyne, P., J.-F. Bureau, and M. Brahic. 1997. The infection of mouse by Theiler's virus: from genetics to immunology. *Immunol. Rev.* **159**:163–176.
- Obuchi, M., Y. Ohara, T. Takegami, T. Murayama, H. Takada, and H. Iizuka. 1997. Theiler's murine encephalomyelitis virus subgroup strain-specific infection in a murine macrophage-like cell line. *J. Virol.* **71**:729–733.
- Obuchi, M., J. Yamamoto, M. N. Uddin, T. Odagiri, H. Iizuka, and Y. Ohara. 1999. Theiler's murine encephalomyelitis virus (TMEV) subgroup strain-specific infection in neural and non-neural cell lines. *Microbiol. Immunol.* **43**:885–892.
- Pena Rossi, C., M. Delcroix, I. Huitinga, A. McAllister, N. van Rooijen, E. Claassen, and M. Brahic. 1997. Role of macrophages during Theiler's virus infection. *J. Virol.* **71**:3336–3340.
- Rodriguez, M., and R. P. Roos. 1992. Pathogenesis of early and late disease in mice infected with Theiler's virus, using intratypic recombinant GDVII/DA viruses. *J. Virol.* **66**:217–225.
- Roos, R. P., S. Stein, Y. Ohara, J. Fu, and B. L. Semler. 1989. Infectious cDNA clones of the DA strain of Theiler's murine encephalomyelitis virus. *J. Virol.* **63**:5492–5496.
- Shaw-Jackson, C., and T. Michiels. 1997. Infection of macrophages by Theiler's murine encephalomyelitis virus is highly dependent on their activation or differentiation state. *J. Virol.* **71**:8864–8867.
- Shaw-Jackson, C., and T. Michiels. 1999. Absence of internal ribosome entry site-mediated tissue specificity in the translation of a bicistronic transgene. *J. Virol.* **73**:2729–2738.
- Takata, H., M. Obuchi, J. Yamamoto, T. Odagiri, R. P. Roos, H. Iizuka, and Y. Ohara. 1998. L* protein of the DA strain of Theiler's murine encephalomyelitis virus is important for virus growth in a murine macrophage-like cell line. *J. Virol.* **72**:4950–4955.
- Tangy, F., A. McAllister, and M. Brahic. 1989. Molecular cloning of the complete genome of strain GDVII of Theiler's virus and production of infectious transcripts. *J. Virol.* **63**:1101–1106.
- Theiler, M. 1937. Spontaneous encephalomyelitis of mice, a new virus disease. *J. Exp. Med.* **65**:705–719.
- Yamasaki, K., C. C. Weihl, and R. P. Roos. 1999. Alternative translation initiation of Theiler's murine encephalomyelitis virus. *J. Virol.* **73**:8519–8526.