Direct Evidence of a Role for Amino Acid 101 of VP-1 in Central Nervous System Disease in Theiler's Murine Encephalomyelitis Virus Infection

ANDREAS ZURBRIGGEN,¹ COLIN THOMAS,¹ MASAHIITO YAMADA,¹ RAYMOND P. ROOS,²
AND ROBERT S. FUJINAMI^{1*}

Department of Neurology, University of Utah, Salt Lake City, Utah 84132,¹ and Department of Neurology, University of Chicago Pritzker School of Medicine, Chicago, Illinois 60637²

Received 16 August 1990/Accepted 15 January 1991

The DA virus, a member of the TO subgroup of Theiler's virus, invokes a chronic demyelinating disease in its natural host, the mouse. RNA transcripts from a cDNA clone, pDAFL3, are infectious, and the resulting virus, DAFL3, produces in mice a disease indistinguishable from that caused by the DA virus. Using oligonucleotide-directed site-specific mutagenesis, a single nucleotide, cytosine at position 3305 (viral genome), was changed in this infectious cDNA to a thymine. The mutated nucleotide is located in an area coding for a neutralizing epitope on loop II of VP-1. Virus OSM101, produced from the mutagenized plasmid pDA101, had the same growth characteristics and plaque phenotype *in vitro* as the virus DAFL3 produced from clone pDAFL3. However, *in vivo* in the mouse, virus OSM101 was markedly less neurovirulent than DAFL3. Central nervous system tissues from mice infected 4 to 6 weeks previously with the OSM101 virus contained less infectious virus and fewer infected cells than central nervous system tissues from animals infected with the control virus, DAFL3. Thus, we demonstrated that the single nucleotide change resulting in an amino acid substitution at position 101 (threonine to isoleucine) of VP-1 determines one aspect of Theiler's virus persistence and disease in mice.

Theiler's murine encephalomyelitis virus (TMEV) is a member of the family *Picornaviridae*. Based on different biological and pathological characteristics, TMEV can be divided into two subgroups. The GD-VII subgroup consists of the highly virulent GD-VII and FA strains, which cause an acute lethal polioencephalomyelitis. The most studied members of the other subgroup, TO (named after Theiler's original virus), are the WW, BeAn 8386, and DA strains, of which the DA strain was used in this study. Members of the TO subgroup can cause a persistent demyelinating disease after infection. Subsequent to intracerebral infection of susceptible mice, the DA strain produces a biphasic central nervous system (CNS) disease (25), an acute polioencephalomyelitis, and a chronic demyelinating disease primarily in the spinal cord (6, 26). This chronic stage of TMEV-induced demyelination is a favored animal model for human demyelinating disorders, such as multiple sclerosis. Immune-mediated mechanisms (27, 41) and direct virus-induced mechanisms (2, 4, 28, 44, 45) as well as a combination of both (1, 51) have been proposed to be responsible for the TMEV-induced demyelination.

Recently, antigenic escape mutants of various viruses have been successfully used for pathogenesis studies (7, 11, 13, 30, 46, 48). We previously described an altered and diminished pattern of disease in immunocompetent mice after infection with a neutralization-resistant DA virus variant, H7A6-2 (55). This variant virus was selected by using a neutralizing monoclonal antibody (MAb), H7, to DA virus VP-1. After intracerebral infection, the variant virus produced much less inflammation in the CNS than the parental DA virus. Additionally, the number of viral antigen- and viral nucleic acid-containing cells in the spinal cords of

variant virus-infected mice was decreased, and variant virus did not persist, in contrast to the DA virus. The exact epitope involved in the reduced neurovirulence of the variant virus was mapped by enzyme-linked immunosorbent assay (ELISA), neutralization assay, and direct RNA sequencing (56). The variant virus, H7A6-2, contained a single point mutation leading to an amino acid replacement within a neutralizing epitope of VP-1 located in an exposed loop on the surface of the virus. However, since only approximately 15% of the total viral genome was sequenced in that study (56), the possibility remained that other mutations were responsible for the reduced neurovirulence of the variant virus. In this study, we chose a different approach to access the importance of this amino acid within the structural protein VP-1 for the attenuation phenotype. This was accomplished by engineering the specific nucleotide substitution coding for this amino acid change by site-directed mutagenesis into an infectious cDNA clone.

The manipulation of infectious cDNA clones has become a very powerful tool in molecular structure-function and pathogenesis studies (16–18, 23, 35–37, 39, 49). Picornaviruses are small positive single-stranded RNA viruses and are therefore optimal for such infectious cDNA studies. Viral RNA transcripts generated *in vitro* from full-length cDNAs can be used to transfect cells and thereby produce infectious virus. Recently, infectious transcripts for different picornaviruses have been constructed: polioviruses (20, 38), human rhinovirus type 14 (31), coxsackievirus B3 (19), encephalomyocarditis virus (32), mengovirus (9), hepatitis A virus (5), and foot-and-mouth disease virus (53). Lately, infectious transcripts for members of both TMEV subgroups, the demyelinating TO subgroup containing the DA (29, 42) and BeAn (3) viruses and the highly virulent subgroup GD-VII (47), have been constructed. These infectious transcripts allowed recombinant studies between the highly virulent and

* Corresponding author.

the less virulent TMEV strains. With chimeric cDNAs made between GD-VII and DA with the subsequent production of GD-VII/DA recombinant viruses, a segment including the coding region of capsid proteins VP-2, VP-3, and VP-1 and nonstructural protein 2A appears critical for mouse lethality (14). Similar studies with GD-VII and BeAn recombinants identified a region encoding the leader and coat proteins as critical for mouse death (3). Thus, specific TMEV segments have now been associated with viral functions and altered pathogenesis.

In this study, we took advantage of the availability of an infectious TMEV cDNA clone to use oligonucleotide-directed site-specific mutagenesis to introduce a mutation at position 3305 (corresponding to the location of the nucleotide alteration of the variant virus, H7A6-2). In this way, we constructed an infectious TMEV clone having the exact point mutation as the variant virus, H7A6-2. This construction allowed us to determine the exact location of a neutralizing epitope of DA virus and its importance in determining viral growth and persistence in the mouse CNS.

MATERIALS AND METHODS

Cells and viruses. BHK-21 cells were cultured in Dulbecco modified Eagle medium (Cell Culture Facility, University of California, San Diego) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), nonessential amino acids, glutamine, sodium pyruvate, penicillin, streptomycin, and amphotericin B (Irvine Scientific, Santa Ana, Calif.). Cells were passaged twice weekly. The DA strain of TMEV (the parental strain) used in this study was originally obtained from J. Lehrich and B. Arnason (24). Passage history and variant virus selection has been previously described (55). Production and construction of DAFL3 and OSM101 are described below. A pool of the various virus strains was grown in BHK-21 cells, and the virus titers were determined by plaque assay (55).

Viral infectious cDNA. Construction of pDAFL3, an infectious cDNA for the TMEV DA strain, has been previously described in detail (42). Mice infected with virus derived from plasmid pDAFL3 developed an inflammatory demyelinating disease indistinguishable from the disorder produced by the parental DA strain (42).

Growth of M13K07 helper bacteriophage. M13K07 helper phage was grown in *Escherichia coli* DH5 α F' by the procedure of Vieira and Messing (50). Single plaques were picked and grown overnight in YT (50) medium containing kanamycin. The following day, the cells were pelleted by centrifugation and the supernatant fluid was used as the inoculum of M13K07 helper phage.

Preparation of single-stranded DNA. Single-stranded DNA was prepared by the method of Kunkel et al. (21). The desired DNA fragment isolated from the plasmid pDAFL3 covering the region 2433 to 3926 (according to the numbering of Ohara et al. [33]) was first cloned into the *Bam*HI site of pUC119, using *E. coli* DH5 α F' as a host, and selection was performed with ampicillin. Overnight cultures of transformed *E. coli* CJ236 cells were infected with M13K07 helper phage at a multiplicity of infection of 2 to 10 and incubated for 90 min. Then kanamycin was added to select for the M13K07 helper phage, and cultures were grown overnight at 37°C. After 12 h, single-stranded DNA was purified from the cell supernatant fluid. A small aliquot of the purified single-stranded DNA was size analyzed on a 1% agarose gel.

Oligonucleotide. A 20-base-long oligonucleotide, d(CTTT TAACCACIATGGCGGC), was synthesized on a gene as-

sembler (Pharmacia, Uppsala, Sweden) that corresponded to the sequence of the variant virus, H7A6-2, and was complementary to the single-stranded DNA except for a mismatch in the center (a thymine instead of the cytosine that the variant virus, H7A6-2, has at position 3305). This nucleotide change was predicted to change VP-1 amino acid residue 101 to isoleucine.

High-efficiency oligonucleotide-directed site-specific mutagenesis. Oligonucleotide-directed mutagenesis was performed by the method described by Kunkel et al. (21). This procedure is outlined in Fig. 1. The selected plasmid was named pDA101.

In vitro transcription. pDAFL3 and the mutated pDA101 (2 μ g of each) were linearized with *Xba*I. The in vitro transcription was performed with 30 U of T7 RNA polymerase in a 100- μ l reaction mixture containing 40 mM Tris (pH 7.5), 6 mM MgCl₂, 10 mM NaCl, 10 mM dithiothreitol, 5 U of Rnasin (Promega), 0.5 mM each ATP, CTP, UTP, and GTP (Pharmacia LKB Biotechnology, Piscataway, N.J.), and 2 mM spermidine for 30 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, a small aliquot of the resulting RNA was analyzed on a 0.8% formaldehyde-agarose gel.

Transfection of cells. BHK-21 cells (about 60% confluent) were transfected with 1 to 2 μ g of RNA from pDAFL3 or pDA101 by using Lipofectin Reagent (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.) as recommended by the vendor. Virus obtained from plasmid pDAFL3 was named DAFL3, and virus from the mutated plasmid pDA101 was designated OSM101. From both viruses a pool was grown up, and the titer was determined by plaque assay on BHK-21 cells (56). RNA from DAFL3 and OSM101 was prepared, and the mutated region and adjacent areas were sequenced as described below.

Preparation of infected cellular RNA templates. BHK-21 cells (3×10^8) were infected with parental DA, DAFL3, H7A6-2, or OSM101 virus and harvested 22 h postinfection. Under these conditions, all viruses grew to similar titers. After being washed with phosphate-buffered saline (PBS), the infected cells were resuspended in 1 ml of 25 mM sodium citrate (pH 7.0) (Fisher Scientific Co., Pittsburgh, Pa.). The cells were then lysed by the addition of 8 ml of GTC solution (4 M guanidinium thiocyanate [International Biotechnologies, New Haven, Conn.], 25 mM sodium citrate, 0.5% sodium lauroyl sarcosine [Sigma Chemical Co., St. Louis, Mo.], 0.1 mM β -mercaptoethanol [Fisher]). The DNA was sheared by vortexing. The lysate was then overlaid onto a 2-ml 5.7 M cesium chloride (Sigma) cushion and centrifuged at 30,000 rpm in a Beckman SW41 rotor at 15°C for 20 h. The resulting RNA pellet was dissolved in diethyl pyrocarbonate (Behring Diagnostics, La Jolla, Calif.)-treated water and phenol-chloroform extracted.

RNA sequencing. A dideoxynucleotide RNA sequencing technique previously described (56) was used. The corresponding DNA sequences were read and compared with the earlier published DA virus sequence (33).

ELISA. The ELISA was performed as described earlier by Rice and Fujinami (40). For viral antigen, cytoplasmic extracts from cells infected with DAFL3, OSM101, the H7A6-2 variant, or the parental DA virus were used. In this study, two antibodies were used, MAb H7 and rabbit polyclonal anti-DA virus. Both antibodies have been previously characterized in detail (54).

Neutralization assay. Approximately 200 PFU of virus (DAFL3 and OSM101) in 100 μ l were incubated with various dilutions of MAb H7 and rabbit polyclonal anti-DA virus in

FIG. 1. Construction of the mutagenized infectious clone pDA101 from clone pDAFL3. A segment containing the area of interest was excised from pDAFL3, cloned into pUC119, mutagenized, and cloned back into pDAFL3.

100 μ l for 1 h on ice. The virus-antibody solution was allowed to adsorb onto BHK-21 cell monolayers for 1.5 h at room temperature. The cell monolayers were then overlaid with 0.5% agarose in medium 199 (GIBCO Laboratories, Grand Island, N.Y.)–2% fetal bovine serum and incubated for 4 days at 37°C. After fixation and staining with 1% crystal violet, plaques were enumerated (15). Neutralization endpoint was defined as a 50% reduction in the number of plaques.

Animal tissue. Four- to 6-week-old SJL/J mice (Jackson Laboratories) were used. Mice were infected intracerebrally with a concentration of 3×10^5 PFU of DAFL3 or OSM101 virus in 50 μ l of PBS. For immunohistochemistry and in situ hybridization studies, five animals in each group were perfused 1, 4, and 6 weeks postinfection (p.i.) with 4% PBS-buffered paraformaldehyde (J. T. Baker Chemical Co., Phillipsburg, N.J.). Brains and spinal cords were removed and processed for paraffin embedding. Sagittal sections (4 μ m) were cut and used for immunohistochemistry and in situ hybridization.

For virus isolation, TMEV was quantified by plaque assay

on BHK-21 cells as described by Zurbriggen and Fujinami (54).

Immunohistochemistry. For immunohistochemistry, an anti-DA antibody raised in rabbits by intravenous immunization with purified virus was used. Preparation and characterization of this polyclonal rabbit anti-DA antibody have been described elsewhere (54). This serum reacted with VP-1 and VP-2 and weakly with VP-3 by Western immunoblotting. Immunohistochemistry was performed as previously described (54).

In situ hybridization. (i) **Preparation of DNA probes.** For this study, the TMEV clone pDA8 was used. This probe is approximately 1,600 bp long and complementary to the structural viral proteins VP-1 and VP-3 of the DA strain. The specificity of pDA8 has been confirmed by Northern (RNA) blot analysis and by sequencing (52). The viral insert was enzymatically cleaved from the vector and gel purified. Purified insert cDNA was radiolabeled by a random hexanucleotide primer method (12) with [α -thio- 35 S]5'-dCTP and [α -thio- 35 S]5'-dATP (Dupont, NEN Research Products,

Boston, Mass.). The probes were labeled to 10^8 cpm/ μ g of DNA.

(ii) **Tissue preparation for in situ hybridization.** Tissue sections were processed as described earlier (54). Briefly, after rehydration, tissue sections were rinsed twice in H_2O , incubated for 10 min with 0.2 M HCl, rinsed twice in H_2O , and immersed in 1% Triton X-100 for 1.5 min. After being washed with PBS, the slides were treated for 20 min at 37°C with proteinase K at 1 μ g/ml in 20 mM Tris HCl–2 mM $CaCl_2$. The slides were then postfixed with 4% PBS-buffered paraformaldehyde for 4 min and washed with PBS. After dehydration through ascending concentrations of ethanol, slides were hybridized with 0.5 μ g of probe per ml in 50% formamide–5 $\times$ hybridization salts–5 $\times$ Denhardt's solution–500 μ g of salmon sperm DNA per ml–250 μ g of HeLa cell RNA per ml–0.1% Triton X-100–10 mM dithiothreitol.

Hybridization was done overnight at 37°C. Excess ^{35}S -cDNA was diluted by several changes of 2 $\times$ and 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Washed slides were dehydrated, air dried, and immersed in NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.). After 4 days of exposure, the slides were developed with Kodak D19 and fixed with Kodak standard fixer. The slides were counterstained with hematoxylin, coverslipped, and examined by light microscopy.

RESULTS

Construction of infectious mutant plasmid pDA101. To modify the single nucleotide 3305 (of the DA virus genome), we used oligonucleotide-directed site-specific mutagenesis. The construction of the mutagenized clone, pDA101, is outlined in Fig. 1. A segment of DNA which contained the location to be altered was excised from the infectious cDNA, pDAFL3, and cloned into pUC119 for the preparation of single-stranded DNA by using M13K07 helper phage. This single-stranded DNA was used as a template for oligonucleotide-directed site-specific mutagenesis. Colonies were screened by restriction mapping with *Kpn*I. The insert from pDAFL3 cloned into the above vector pUC119 contained a unique restriction site for *Kpn*I. By modifying nucleotide 3305 (of the virus genome), this unique restriction site was lost. An insert missing this *Kpn*I site and therefore containing a mutation within the specific area was placed back into the infectious clone pDAFL3 and was designated pDA101. To examine the orientation of the insert and to confirm the specific nucleotide replacement of clone pDA101, we sequenced an area covering the whole insert and spanning both ligation sites in parallel with pDAFL3. A cytosine at 3305 was replaced in pDA101 by a thymine. No other nucleotide changes or mutations were observed within the sequenced area.

In vitro transcription and transfection. Clone pDA101 and pDAFL3 as a control were linearized and transcribed in vitro. The sizes of the RNA transcripts from both pDA101 and pDAFL3 were analyzed on denaturing formaldehyde-agarose gels. RNAs from both transcripts had the same size, approximately 8,000 bases. The transcripts were not treated with DNase before BHK-21 cell transfection, since pDAFL3 DNA by itself was not infectious and DNase treatment of the transcription product did not affect the efficiency of BHK-21 cell transfection and virus production (42). However, RNase treatment of the transcripts from pDAFL3 resulted in a total loss of infectivity (42). BHK-21 cells were transfected with the transcription reaction mix to examine whether the mutated DA virus cDNA clone, pDA101, was still infectious

FIG. 2. (a) Sequence comparison between the original DA virus and the virus variant, H7A6-2. The sequences read from the gels correspond to the viral genome sequences. The variant virus differs at one nucleotide (★). The loading order of the reaction was AGCT. (b) Sequence comparison between the virus DAFL3 and the mutagenized virus OSM101. RNA from OSM101 virus-infected cells had the same nucleotide change at position 3305 (★) as RNA from the variant virus, H7A6-2. Autoradiograms of the gels from the sequencing reactions indicated that a cytosine (DA and DAFL3) was replaced by a thymine (H7A6-2 and OSM101). The loading order of the reaction in all instances was AGCT.

and, if so, to a similar extent as the original pDAFL3. Transcription products from both plasmids, pDAFL3 and pDA101, yielded comparable virus titers after transfection of BHK-21 cells. Virus recovered from plasmid pDAFL3 was designated DAFL3 virus, and virus produced from plasmid pDA101 was named OSM101 virus. Both viruses DAFL3 and OSM101 had similar growth properties and reached comparable high titers within the same time when grown in BHK-21 cells. The DAFL3 virus stock had a titer of 9.5×10^7 PFU/ml, and the OSM101 virus stock had a titer of 8.2×10^7 PFU/ml, as determined by a plaque assay. No variation in the plaque phenotype between DAFL3 and OSM101 viruses was observed. The plaques produced in BHK-21 cells by either virus were identical in size, shape, and turbidness.

Viral RNA sequencing. To confirm the modified mismatch nucleotide within the OSM101 virus, we compared the nucleotide sequence of the coding region of structural protein, VP-1, of RNAs from the parental DA virus, the variant virus H7A6-2, DAFL3, and OSM101 (Fig. 2). No differences were observed between DA and DAFL3 virus RNAs within the sequenced area. OSM101 virus had the expected single nucleotide difference at position 3305 from DA and DAFL3 viruses (Fig. 2). A cytosine was replaced in OSM101 virus by a thymine, resulting in a change of amino acid 101 of VP-1 from a threonine to an isoleucine in OSM101. This mutation was exactly the same as previously described for the virus variant H7A6-2 (56).

ELISA. The epitope modified in the virus variant H7A6-2 was determined to be the recognition site of MA b H7. To ensure the exact location of this epitope responsible for

TABLE 1. Antibody reaction and comparison of the mutagenized OSM101 virus with the control DAFL3 virus, the variant virus H7A6-2, and its parental DA virus^a

Virus antigen	ELISA ^b		Neutralization assay ^c	
	Anti-DA	MAb H7	Anti-DA	MAb H7
DA	18.8	21.7	+	+
H7A6-2	19.3	2.2	+	—
DAFL3	19.7	21.3	+	+
OSM101	19.2	1.5	+	—

^a Antibodies used were anti-DA, a rabbit hyperimmune antiserum, and MAb H7, which was used to select the virus variant H7A6-2.

^b Relative titer in log₂.

^c Neutralization was scored as + when infectivity was neutralized at a dilution of 1/500 (see Materials and Methods).

continued viral growth and persistence in the CNS, we performed ELISAs using two different antibodies, a rabbit polyclonal anti-DA antibody and MAb H7, which had been originally used to select the virus variant H7A6-2 (Table 1). The rabbit anti-DA antibody recognized all four viruses, DA, the variant H7A6-2, DAFL3, and the mutated OSM101, tested at the same dilutions (Table 1). However, MAb H7 reacted only with antigen from DA virus- and DAFL3 virus-infected cells and did not bind to the variant virus H7A6-2 or OSM101 virus (Table 1).

Neutralization assay. To further ensure the biological importance of the mutated nucleotide in modifying the epitope recognized by MAb H7, we performed neutralization assays with the same antibodies as the ELISA. We studied the ability of the two antibodies to neutralize the mutated virus, OSM101. As positive controls, the parental DA and DAFL3 viruses were used, and results were compared with those for the mutagenized virus, OSM101. The results from the neutralization assay are presented in Table 1. The rabbit anti-DA antibody neutralized all virus strains effectively at dilutions of 1/1,000 and 1/2,000 and partially at 1/10,000. In contrast, MAb H7 neutralized only the positive control viruses DA and DAFL3 at both tested dilutions, 1/100 and 1/500 (Fig. 3). The mutated OSM101 virus was not neutralized by MAb H7. Analogous to the variant virus H7A6-2, the virus OSM101 was neither recognized nor neutralized by MAb H7 as determined by ELISA and neutralization assay (Table 1). Yet, both virus variants H7A6-2 and OSM101 were recognized and neutralized by the rabbit polyclonal anti-DA antibody (56).

Infection of mice with the mutated virus, OSM101. The results from ELISAs and neutralization assays demonstrated that the mutated virus, OSM101, was comparable to the variant virus, H7A6-2, in its ability to be recognized and neutralized by MAb H7 (Table 1). In addition, RNA sequencing demonstrated the same mutation in the OSM101 virus as in the variant virus, H7A6-2 (Fig. 2). To test the hypothesis that this mutation in the neutralizing epitope in the structural protein VP-1 was responsible for a diminished late disease and persistence, we initiated *in vivo* studies. SJL/J mice were infected with the mutagenized virus, OSM101, and, as a control, with the original virus, DAFL3. To determine the amount of infectious virus recovered from the CNS, we performed plaque assays using homogenized brain and spinal cord tissue from DAFL3- and OSM101-infected mice at 1, 2, 4, and 6 weeks p.i. The results from the plaque assay are presented in Table 2. During the acute disease (1 week p.i.), the CNS from both groups of mice contained comparable amounts of infectious virus. Mice

FIG. 3. Neutralization assay performed as described in Materials and Methods. The rabbit polyclonal anti-DA antibody neutralized all three viruses, DA, DAFL3, and OSM101. MAb H7 neutralized only DA and DAFL3 viruses and did not neutralize the mutagenized virus, OSM101.

infected with DAFL3 virus had approximately 3.5×10^5 PFU of infectious virus per g in brain tissue and 1.2×10^5 PFU/g in the spinal cord. OSM101 virus-infected mice had 3.8×10^5 PFU of infectious virus per g in brain tissue and 3.3×10^5 PFU/g in the spinal cord (Table 2). At 2 weeks p.i., OSM101 virus-infected mice had approximately five times less infectious virus in the brain and about 10 times less in the spinal cord than DAFL3 virus-infected animals (Table 2). By 4 weeks p.i., mice infected with the DAFL3 virus contained significantly higher virus titers in the CNS than animals infected with the mutated OSM101 virus. Three of the five mice infected with OSM101 virus had no detectable virus by 4 weeks p.i. (Table 2). One mouse of these five had 7×10^2 PFU/g of brain tissue, and another mouse had 1.6×10^3 PFU/g of spinal cord. At 6 weeks p.i., no virus was recovered by plaque assays from the CNS of any OSM101 virus-infected animals (Table 2). However, CNS tissues from mice infected with the control virus, DAFL3, still had detectable titers of infectious virus (2.43×10^3 PFU/g in brain tissue and 1.63×10^4 PFU/g in spinal cord tissue).

The *in vivo* disease phenotype of both viruses, DAFL3 and OSM101, was compared by examining the number of infected cells and virus distribution by immunohistochemistry and *in situ* hybridization of brain and spinal cord from DAFL3- and OSM101-infected mice. *In situ* hybridization results were similar to the results obtained by immunohistochemistry. Similar numbers of viral nucleic acid-containing cells and viral antigen-positive cells were found in the CNS. Both viruses induced a similar acute disease. The number and size of inflammatory lesions as well as the number of infected cells in the CNS were comparable between the two groups of infected mice at 1 and 2 weeks p.i. Both groups of mice infected with either virus, DAFL3 or OSM101, contained single infected cells and had small foci of infected cells in the brain stem. In the spinal cord, a few infected cells were observed within the grey and white matter of animals

TABLE 2. Comparisons of infectious virus isolated from CNS tissues from DAFL3- and OSM101-infected mice

Time p.i. (wks)	Mouse no.	PFU/g of tissue			
		DAFL3		OSM101	
		Brain	Spinal cord	Brain	Spinal cord
1	1	2.97×10^5	1.23×10^5	2.41×10^5	1.83×10^5
	2	7.33×10^5	2.79×10^5	2.69×10^5	3.14×10^5
	3	1.17×10^5	6.46×10^4	1.52×10^5	8.00×10^4
	4	3.83×10^5	8.76×10^4	1.03×10^6	1.00×10^6
	5	2.33×10^5	5.80×10^4	2.37×10^5	7.53×10^4
2	Avg	3.52×10^5	1.22×10^5	3.85×10^5	3.30×10^5
	1	1.28×10^4	2.91×10^5	5.67×10^3	1.08×10^4
	2	1.73×10^4	1.55×10^5	9.06×10^3	8.45×10^4
	3	1.51×10^4	3.58×10^5	4.22×10^3	1.61×10^4
	4	2.23×10^4	7.07×10^5	4.46×10^3	3.85×10^4
4	5	1.32×10^4	3.26×10^5	2.78×10^3	9.15×10^4
	Avg	1.61×10^4	3.67×10^5	5.23×10^3	4.82×10^4
	1	4.08×10^3	4.25×10^5	— ^a	—
	2	7.39×10^3	9.75×10^4	—	—
	3	1.50×10^3	1.57×10^4	7.03×10^2	—
6	4	4.92×10^3	1.14×10^4	—	1.60×10^3
	5	9.97×10^2	2.16×10^4	—	—
	Avg	3.77×10^3	1.14×10^5	NC ^b	NC
	1	2.13×10^3	2.25×10^4	—	—
	2	—	1.81×10^4	—	—
	3	1.28×10^3	8.43×10^3	—	—
	4	5.60×10^3	2.53×10^4	—	—
	5	3.14×10^2	7.33×10^3	—	—
	Avg	2.43×10^3	1.63×10^4	NC	NC

^a —, Undetectable (lower limit of detection = 1.25 PFU of virus per g of tissue).

^b NC, Not calculated.

infected with either virus strain. In contrast to the early acute disease, at later times p.i. an obvious difference in the number of infected CNS cells between DAFL3- and OSM101-infected mice was clearly observed. By 6 weeks p.i. (chronic phase), very few viral antigen (Fig. 4a)- and viral nucleic acid-positive cells were detected in the CNS from animals infected with the mutagenized virus, OSM101. Three of the five mice had no demonstrable virus-infected cells in the spinal cord, and in only one of these three animals was virus found in the brain. However, at this same time point, 6 weeks p.i., brain and primarily spinal cords from DAFL3 virus-infected animals contained large numbers of viral antigen- and viral nucleic acid-positive cells. Most of the cells infected with the DAFL3 virus were observed in the white matter of the spinal cord (Fig. 4b). There was a clear difference in the number of infected cells within the spinal cord between DAFL3 virus- versus OSM101 virus-infected mice. Thus, spread, replication, and/or persistence of the mutagenized OSM101 virus in the mouse CNS was markedly diminished compared with the DAFL3 virus.

DISCUSSION

In this study, we clearly defined amino acid 101 in loop II of VP-1 as an important determinant of DA virus neurovirulence in its natural host, the mouse. This was accomplished by oligonucleotide-directed site-specific mutagenesis (21). A single nucleotide (3305, viral genome) was changed from a

FIG. 4. (a) Spinal cord from mouse 6 weeks after infection with the mutagenized virus, OSM101. Immunohistochemistry was done with rabbit anti-DA virus. (b) Spinal cord from mouse 6 weeks after infection with the progenitor virus, DAFL3. Immunohistochemistry was done with rabbit anti-DA virus.

cytosine to a thymine within an infectious cDNA, pDAFL3 (42), of the DA virus strain, thus generating an infectious clone, pDA101, with the exact nucleotide mismatch as the variant virus H7A6-2 (56).

In all experiments (viral RNA sequencing, ELISA, neutralization assay), the mutagenized virus, OSM101, appeared identical to the variant virus, H7A6-2, and the DAFL3 virus appeared identical to the DA virus (56). Therefore, the mutated nucleotide at position 3305 codes for an amino acid that constitutes part of a neutralizing epitope on VP-1 recognized by MAb H7.

The ability of the mutagenized virus, OSM101, to infect the CNS and produce CNS disease was investigated. In the previous publication, the variant virus, H7A6-2, was compared with the original DA virus (55, 56). In this study, the OSM101 virus was compared with the DAFL3 virus instead of with the DA strain for the following reasons. First, the mutated virus, OSM101, was created by mutagenesis of clone pDAFL3, the source of the DAFL3 virus. Second, both viruses, DAFL3 and OSM101, were generated in parallel by using the same molecular biological techniques. Third, the sequence of clone pDAFL3 (33) differs slightly from that of the DA virus, which was used as the parent for selection of the variant virus, H7A6-2 (56). However, these nucleotide differences do not account for the difference in the pattern of disease, since both the DA virus and the transcription products derived from the cDNA pDAFL3 produce an indistinguishable disease in mice (42, 56). The

results presented here are consistent with and extend the findings of other investigators who have demonstrated that infectious RNA transcripts from cDNAs induce in their host a disease indistinguishable from that caused by the original virus (3, 29, 42, 47).

Our data indicate that both viruses, DAFL3 and the mutagenized OSM101, invoked a comparable acute disease in mice. Virus titers and numbers of infected cells in the CNS were similar at 1 week after infection with either virus. However, the chronic disease (4 weeks and later) differed markedly between the two viruses. OSM101 virus replication and/or spread within the CNS was limited, and by 4 weeks p.i. only two of five mice contained detectable infectious virus. At 6 weeks p.i., no infectious virus could be isolated in OSM101 virus-infected animals. In contrast, CNS tissues from DAFL3-infected mice contained approximately 2.4×10^3 PFU/g in the brain and 1.6×10^4 PFU/g in the spinal cord at 6 weeks p.i.

The observed differences in the amount of infectious virus and infected CNS cells between DAFL3 and OSM101 virus were at the level of CNS pathology. No differences between the two clones, pDAFL3 and the mutagenized pDA101, were observed following in vitro transcription and transfection of BHK-21 cells. Both viruses, DAFL3 and OSM101, spread and replicated in tissue culture to equivalently high virus titers. No variations in plaque size, shape, or turbidity were observed. Therefore, the mutagenized virus did not have a general defect in virus replication. The same observation was made in the previous study, in which we could not demonstrate differences in plaque morphology between DA and H7A6-2 viruses (56).

In contrast to the above observation, other investigators have found a correlation between plaque size and virulence in various DA variants (34). We suspect that determinants of plaque morphology are localized to several different areas on the viral genome distinct from the region we mutated. Fu et al. (14) and Calenoff and co-workers (3) found a correlation between plaque size and the parental origin of the VP-2-2A and L/P1 regions, respectively. For the OSM101 virus, the phenotype did not change despite the marked differences in CNS disease.

We observed a minor difference between the virus titers in the CNS following inoculation with the variant virus, H7A6-2 (56), and OSM101 virus. No infectious virus was isolated from the CNS following infection with the variant virus, H7A6-2, 4 weeks p.i., whereas infectious virus was detected in two OSM101 virus-infected animals. However, the titers of infectious virus following inoculation with DAFL3 were also higher compared with titers following infection with DA virus (55). Experiments are ongoing to confirm these putative differences.

Our observations furnish direct evidence that amino acid 101 on loop II of VP-1 is at least one factor determining DA virus growth, spread, and/or persistence in the CNS. An additional neutralizing epitope at the carboxyl end of VP-1 of the DA strain has been proposed to be important in virus persistence and chronic demyelinating disease (43). Pathogenic features of picornavirus disease have been mapped to different areas of the viral genome including regions encoding the structural proteins as well as the 5' noncoding region. For example, increased neurovirulence of the Sabin type 3 poliovirus was associated with a single nucleotide change within the 5' noncoding region (10). However, host range of the poliovirus type 2 Lansing strain in mice was mapped to a site within VP-1 (22). The virulence of coxsackievirus B4, another member of the *Picornaviridae*, was defined to the P1

region (39). This area includes the 5' noncoding region and the genome encoding the structural proteins of the virus. Even with mengovirus, a closely related picornavirus, virus attenuation was mapped to the 5' noncoding poly(C) tract (8). Interestingly, TMEV does not have a poly(C) tract in its 5' noncoding region.

Recently, chimeric viruses have been used to study important viral functions and structures for pathogenesis. These constructs were made between virulent and less virulent infectious cDNA transcripts. For example, recombinant chimeras between the highly virulent GD-VII (producing an acute fatal disease) and DA virus and between GD-VII and the less virulent BeAn virus (a member of the TO subgroup, inducing a chronic demyelinating disease) were constructed by exchanging corresponding gene regions (3, 14). In this way, these investigators mapped the mouse neurovirulence within the VP-2-2A region and within the leader and coat proteins. However, this region was still very large and covered more than 30% of the viral genome (2,700 nucleotides). In our studies, we had the advantage of knowing that the specific nucleotide 3305 of the viral genome was responsible for CNS disease and persistence from previous observations using escape mutants and comparisons of sequence with that of the parental virus strain (56). The studies presented here compare a member of the TO subgroup with its variant virus, whereas the previous studies (3, 14) compared a virulent and nonvirulent strain.

In this study, we modified a single nucleotide within an infectious cDNA corresponding to nucleotide 3305 of the viral genome by using oligonucleotide-directed site-specific mutagenesis. The mutagenized OSM101 virus appeared to have the same properties as the variant virus, H7A6-2, and differed from the progenitor virus, DAFL3, in an analogous manner as the variant virus, H7A6-2, differed from its parental DA virus. Both viruses, the variant virus, H7A6-2, as well as the mutagenized virus, OSM101, vary from their initial viruses, DA and DAFL3, respectively, at one nucleotide at the same position. This nucleotide mismatch resulted in an amino acid modification. Thus, our results directly demonstrate that the single amino acid substitution at position 101 of the structural protein, VP-1, determines one aspect of TMEV neurovirulence in mice. These experiments also provided evidence for the importance of the specific neutralizing epitope in initiating chronic disease.

ACKNOWLEDGMENTS

We thank J. L. Whitton and J. Lipsick for excellent suggestions and Peggy Farness, Susan McClanahan, and Pamela Lewis for technical expertise.

This work was supported by National Multiple Sclerosis Society grants GR2087, GR1780, and FG 755-1 (A.Z.), and Public Health Service grants NS23162 (R.S.F.) and NS24575 (R.P.R.) from the National Institutes of Health. A.Z. is a Fellow of the NMSS and Schweizerische Stiftung fuer medizinisch biologische Stipendien. R.S.F. is a Javits Neuroscience Scholar, NINDS.

REFERENCES

1. Aubert, C., M. Chamorro, and M. Brahic. 1987. Identification of Theiler's virus infected cells in the central nervous system of the mouse during demyelinating disease. *Microb. Pathogen.* 3:319-326.
2. Brahic, M., W. G. Stroop, and J. R. Baringer. 1981. Theiler's virus persists in glial cells during demyelinating disease. *Cell* 26:123-128.
3. Calenoff, M. A., K. S. Faaberg, and H. L. Lipton. 1990.

- Genomic regions of neurovirulence and attenuation in Theiler murine encephalomyelitis virus. *Proc. Natl. Acad. Sci. USA* **87**:978–982.
4. **Chamorro, M., C. Aubert, and M. Brahic.** 1986. Demyelinating lesions due to Theiler's virus are associated with ongoing central nervous system infection. *J. Virol.* **57**:992–997.
 5. **Cohen, J. I., J. R. Ticehurst, S. M. Feinstone, B. Rosenblum, and R. H. Purcell.** 1987. Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. *J. Virol.* **61**:3035–3039.
 6. **Dal Canto, M. C., and H. L. Lipton.** 1977. Animal model of human disease: multiple sclerosis. *Am. J. Pathol.* **88**:497–500.
 7. **Dalziel, R. G., P. W. Lampert, P. J. Talbot, and M. J. Buchmeier.** 1986. Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. *J. Virol.* **59**:463–471.
 8. **Duke, G. M., J. E. Osorio, and A. C. Palmenberg.** 1990. Attenuation of mengo virus through genetic engineering of the 5' noncoding poly(C) tract. *Nature (London)* **343**:474–476.
 9. **Duke, G. M., and A. C. Palmenberg.** 1989. Cloning and synthesis of infectious cardiovirus RNAs containing short, discrete poly(C) tracts. *J. Virol.* **63**:1822–1826.
 10. **Evans, D. M. A., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, G. Stanway, J. W. Almond, K. Currey, and J. V. Maizel.** 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature (London)* **314**:548–550.
 11. **Evans, D. M. A., P. D. Minor, G. S. Schild, and J. W. Almond.** 1983. Critical role of an eight-amino acid sequence of VP1 in neutralization of poliovirus type 3. *Nature (London)* **304**:459–462.
 12. **Feinberg, A., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6–13.
 13. **Fleming, J. O., M. D. Trousdale, F. A. K. El-Zaatari, S. A. Stohlman, and L. P. Weiner.** 1986. Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. *J. Virol.* **58**:869–875.
 14. **Fu, J., S. Stein, L. Rosenstein, T. Bodwell, M. Routbort, B. L. Semler, and R. P. Roos.** 1990. Neurovirulence determinants of genetically engineered Theiler viruses. *Proc. Natl. Acad. Sci. USA* **87**:4125–4129.
 15. **Fujinami, R. S., and M. B. A. Oldstone.** 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* **230**:1043–1045.
 16. **Gething, M. J., R. W. Doms, D. York, and J. White.** 1986. Studies on the mechanism of membrane fusion: site-specific mutagenesis of the hemagglutinin of influenza virus. *J. Cell Biol.* **102**:11–23.
 17. **Hahn, C. S., and J. H. Strauss.** 1990. Site-directed mutagenesis of the proposed catalytic amino acids of the Sindbis virus capsid protein autoprotease. *J. Virol.* **64**:3069–3073.
 18. **Ivanoff, L. A., T. Towatari, J. Ray, B. D. Korant, and S. R. Petteway, Jr.** 1986. Expression and site-specific mutagenesis of the poliovirus 3C protease in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **83**:5392–5396.
 19. **Kandolf, R., and P. H. Hofschneider.** 1985. Molecular cloning of the genome of a cardiotropic coxsackie B3 virus, full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. *Proc. Natl. Acad. Sci. USA* **82**:4818–4822.
 20. **Kaplan, G., J. Lubinski, A. Dasgupta, and V. R. Racaniello.** 1985. In vitro synthesis of infectious poliovirus RNA. *Proc. Natl. Acad. Sci. USA* **82**:8424–8428.
 21. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
 22. **LaMonica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. *J. Virol.* **57**:515–525.
 23. **Lee, C. K., and E. Wimmer.** 1988. Proteolytic processing of poliovirus polypeptide: elimination of 2Apro-mediated, alternative cleavage of polypeptide 3CD by in vitro mutagenesis. *Virology* **166**:405–414.
 24. **Lehrich, J. R., B. G. Arnason, and F. H. Hochberg.** 1976. Demyelinating myelopathy in mice induced by the DA virus. *J. Neurosci.* **29**:149–157.
 25. **Lipton, H. L.** 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. *Infect. Immun.* **11**:1147–1155.
 26. **Lipton, H. L., and M. C. Dal Canto.** 1979. Susceptibility of inbred mice to chronic central nervous system infection by Theiler's murine encephalomyelitis virus. *Infect. Immun.* **26**:369–374.
 27. **Lipton, H. L., and F. Gonzalez-Scarano.** 1978. Central nervous system immunity in mice infected with Theiler's virus. I. Local neutralizing antibody response. *J. Infect. Dis.* **137**:145–151.
 28. **Lipton, H. L., J. Kratochvil, P. Sehti, and M. C. Dal Canto.** 1984. Theiler's virus antigen detected in mouse spinal cord 2 1/2 years after infection. *Neurology* **34**:1117–1119.
 29. **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. Pathogen.* **7**:381–388.
 30. **Minor, P. D., D. M. A. Evans, G. C. Schild, M. Ferguson, and J. W. Almond.** 1984. Identification of an antigenic site in the neutralization of type 3 poliovirus. *Rev. Infect. Dis.* **6**:S516–S518.
 31. **Mizutani, S., and R. J. Colonno.** 1985. In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. *J. Virol.* **56**:628–632.
 32. **Naviaux, R. K., S. H. Cohen, K. M. Vanden Brink, and G. W. Jordan.** 1990. Construction and characterization of two infectious molecular clones of encephalomyocarditis virus. *J. Virol.* **64**:913–917.
 33. **Ohara, Y., S. Stein, J. Fu, L. Stillman, L. Klamman, and R. P. Roos.** 1988. Molecular cloning and sequence determination of DA strain of Theiler's murine encephalomyelitis viruses. *Virology* **164**:245–255.
 34. **Oleszak, E. L., J. L. Leibowitz, and M. Rodriguez.** 1988. Isolation and characterization of two plaque size variants of Theiler's murine encephalomyelitis virus (DA strain). *J. Gen. Virol.* **69**:2413–2418.
 35. **Omata, T., M. Kohara, S. Kuge, T. Komatsu, S. Abe, B. Semler, A. Kameda, H. Itoh, M. Arita, E. Wimmer, and A. Nomoto.** 1986. Genetic analysis of the attenuation phenotype of poliovirus type 1. *J. Virol.* **58**:348–358.
 36. **Packham, G., A. Economou, C. M. Rooney, D. T. Rowe, and P. J. Farrell.** 1990. Structure and function of the Epstein-Barr virus BZLF1 protein. *J. Virol.* **64**:2110–2116.
 37. **Quinn, C. O., and G. R. Kitchingman.** 1986. Functional analysis of the adenovirus type 5 DNA-binding protein: site-directed mutants which are defective for adeno-associated virus helper activity. *J. Virol.* **60**:653–661.
 38. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**:916–919.
 39. **Ramsingh, A., A. Hixson, B. Duceman, and J. Slack.** 1990. Evidence suggesting that virulence maps to the P1 region of the coxsackievirus B4 genome. *J. Virol.* **64**:3078–3081.
 40. **Rice, G. P. A., and R. S. Fujinami.** 1986. Measles virus, p. 370–383. *In* H. V. Bergmeyer (ed.), *Methods and enzymatic analysis*. Verlag Chemie, Berlin.
 41. **Roos, R. P., S. Firestone, R. Wollmann, D. Variakojis, and B. G. W. Arnason.** 1982. The effect of short term and chronic immunosuppression on Theiler's virus demyelination. *J. Neuroimmunol.* **2**:223–234.
 42. **Roos, R. P., S. Stein, Y. Ohara, J. L. Fu, and B. L. Semler.** 1989. Infectious cDNA clones of the DA strain of Theiler's murine encephalomyelitis virus. *J. Virol.* **63**:5492–5496.
 43. **Roos, R. P., S. Stein, M. Routbort, A. Senkowski, T. Bodwell, and R. Wollmann.** 1989. Theiler's murine encephalomyelitis virus neutralization escape mutants have a change in disease phenotype. *J. Virol.* **63**:4469–4473.
 44. **Roos, R. P., and R. Wollmann.** 1984. DA strain of Theiler's murine encephalomyelitis virus induces demyelination in nude

- mice. *Ann. Neurol.* **14**:494–499.
45. Rosenthal, A., R. S. Fujinami, and P. W. Lampert. 1986. Mechanism of Theiler's virus induced demyelination in nude mice. *Lab. Invest.* **54**:515–522.
 46. Spriggs, D. R., and B. N. Fields. 1982. Attenuated reovirus type 3 strains generated by selection of haemagglutinin antigenic variants. *Nature (London)* **297**:68–70.
 47. 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.
 48. Thomas, A. A., F. Vrijksen, and A. Boeye. 1986. Relationship between poliovirus neutralization and aggregation. *J. Virol.* **59**:479–485.
 49. Vialard, J., M. Lalumiere, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. Richardson. 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the beta-galactosidase gene. *J. Virol.* **64**:37–50.
 50. Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* **153**:3–11.
 51. Yamada, M., A. Zurbriggen, and R. S. Fujinami. 1990. Monoclonal antibody to Theiler's murine encephalomyelitis virus defines a determinant on myelin and oligodendrocytes, and augments demyelination in experimental allergic encephalomyelitis. *J. Exp. Med.* **171**:1893–1907.
 52. Yamada, M., A. Zurbriggen, and R. S. Fujinami. 1990. The relationship between viral RNA, myelin specific mRNA's, and demyelination in the central nervous system disease during Theiler's virus infection. *Am. J. Pathol.* **137**:1467–1479.
 53. Ziebert, A., G. Maass, K. Strebel, M. M. Falk, and E. Beck. 1990. Infectious foot-and-mouth disease virus derived from a cloned full-length cDNA. *J. Virol.* **64**:2467–2473.
 54. Zurbriggen, A., and R. S. Fujinami. 1988. Theiler's virus infection in nude mice: viral RNA in vascular endothelial cells. *J. Virol.* **62**:3589–3596.
 55. Zurbriggen, A., and R. S. Fujinami. 1989. A neutralization-resistant Theiler's virus variant produces an altered disease pattern in the mouse central nervous system. *J. Virol.* **63**:1505–1513.
 56. Zurbriggen, A., J. M. Hogle, and R. S. Fujinami. 1989. Alteration of amino acid 101 within capsid protein VP-1 changes the pathogenicity of Theiler's murine encephalomyelitis virus. *J. Exp. Med.* **170**:2037–2049.