

# Molecular Cloning of the Complete Genome of Strain GDVII of Theiler's Virus and Production of Infectious Transcripts

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**We constructed a complete cDNA clone of strain GDVII of Theiler's virus in Bluescript plasmid. This recombinant plasmid, called pTMGDVII, was used to synthesize full-length RNA transcripts of the viral insert. This RNA was infectious for BHK cells. Virus R1-GDVII, obtained from transfected BHK cells, caused rapidly fatal encephalomyelitis in BALB/c mice. High amounts of viral antigens were present in neurons. No antigens were found in white matter. Therefore, the phenotype of R1-GDVII was indistinguishable from that of genuine GDVII Theiler's virus.**

Theiler's virus offers an ideal opportunity to identify viral genes responsible for persistence. Strains of this picornavirus fall into two classes with opposite phenotypes. Strains DA, BeAn, and WW persist for more than a year in the central nervous systems (CNS) of susceptible mice and cause primary demyelination (6, 9, 16). They infect oligodendrocytes (1), and their replication in the CNS is restricted (4, 5). Strains GDVII and FA, on the other hand, are highly virulent. They replicate permissively in neurons and kill their hosts in a matter of days (15). All Theiler's virus strains, however, are closely related. All of them replicate to high titers in BHK-21 or L cells and have common neutralization epitopes (10). The genomes of strains DA, BeAn, and GDVII have been sequenced (11, 13, 14) and were found to be more than 90% identical. At the amino acid level the polyproteins are ≈95% identical, and most of the differences are located in the P1 or capsid-coding region. Therefore, subtle differences may account for a dramatic change of phenotype. Mapping the regions responsible for neurovirulence and persistence will require the construction of recombinant genomes from infectious cDNA clones. In this article, we describe the construction of the first full-length cDNA clone of Theiler's virus. RNA transcripts of the cDNA were infectious when transfected into BHK-21 cells. The virus recovered after transfection caused acute fatal encephalomyelitis in mice. Capsid antigens were present in neurons and absent from white matter. Therefore, the phenotype of the virus obtained from the cDNA clone was indistinguishable from that of the genuine GDVII strain of Theiler's virus.

## MATERIALS AND METHODS

**RNA extraction and purification.** The GDVII strain of Theiler's virus was grown on BHK-21 cells in Dulbecco modified Eagle medium (DMEM). Viral RNA was extracted from CsCl-purified virions by proteinase K digestion and phenol-chloroform-sodium dodecyl sulfate extraction (3). The RNA was ethanol precipitated, and its structural integrity was checked by methylmercuric hydroxide agarose gel electrophoresis. This RNA was infectious upon transfection into BHK-21 cells, as described below.

**cDNA synthesis.** Purified viral RNA (5 μg) was denatured

at 80°C for 2 min and reverse transcribed at 42°C for 1 h with 60 U of reverse transcriptase by using 2 μg of oligo p(dT)<sub>12-18</sub> or a synthetic oligonucleotide (FT2) as a primer. The reaction contained (per 50 μl) 60 U of RNasin, 140 mM KCl, 100 mM Tris hydrochloride (pH 8.3), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, and 12 μCi of [α-<sup>32</sup>P]dGTP (Amersham Corp.). The RNA-cDNA hybrids were phenol extracted and purified by chromatography through a Sephadex G-50 column.

The second strand was synthesized by the RNase H-DNA polymerase I replacement method of Gübler and Hoffman (7, 12). RNA-cDNA hybrid (2 μg) was incubated successively for 1 h at 12°C and for 1 h at 22°C in a 150-μl reaction containing 20 mM Tris hydrochloride (pH, 7.5), 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6 mM MgCl<sub>2</sub>, 100 mM KCl, 300 μM each of dATP, dCTP, dGTP, and dTTP, 150 μM βNAD, 3.5 U of RNase H, and 100 U of DNA pol I.

Double-stranded cDNA was extracted with phenol-chloroform and size selected on a Biogel A-50 column. The fractions containing the largest cDNA were collected and ethanol precipitated.

**Cloning of cDNA.** Homopolymeric tails of 15 to 20 dCMP residues were added to the cDNA with 20 U of terminal deoxynucleotidyl transferase (Amersham) in a 50-μl reaction containing 500 ng of cDNA, 2.5 μM dCTP, and the cobalt buffer of the manufacturer. The mixture was incubated at 30°C for 5 min. The reaction was stopped by raising the EDTA concentration to 20 mM. The cDNA was extracted with phenol-chloroform and ethanol precipitated. Double-stranded dC-tailed cDNA (350 ng) was annealed to 700 ng of *Pst*I-cut dG-tailed pBR322 DNA in a 50-μl reaction containing 100 mM NaCl, 10 mM Tris hydrochloride (pH 7.5), and 1 mM EDTA. The mixture was heated for 10 min at 65°C and slowly cooled to room temperature.

The annealed DNA was used to transform competent *Escherichia coli* DH-5 as described by Hanahan (8).

**Analysis of cDNA clones.** Ampicillin-resistant clones were analyzed by restriction enzyme mapping. Clones containing the 5' extremity of the viral genome were detected by colony filter hybridization. A specific oligonucleotide (FT1) labeled with polynucleotide kinase and [γ-<sup>32</sup>P]ATP was used as the probe. Clones displaying strong hybridization signals were also studied by restriction enzyme mapping.

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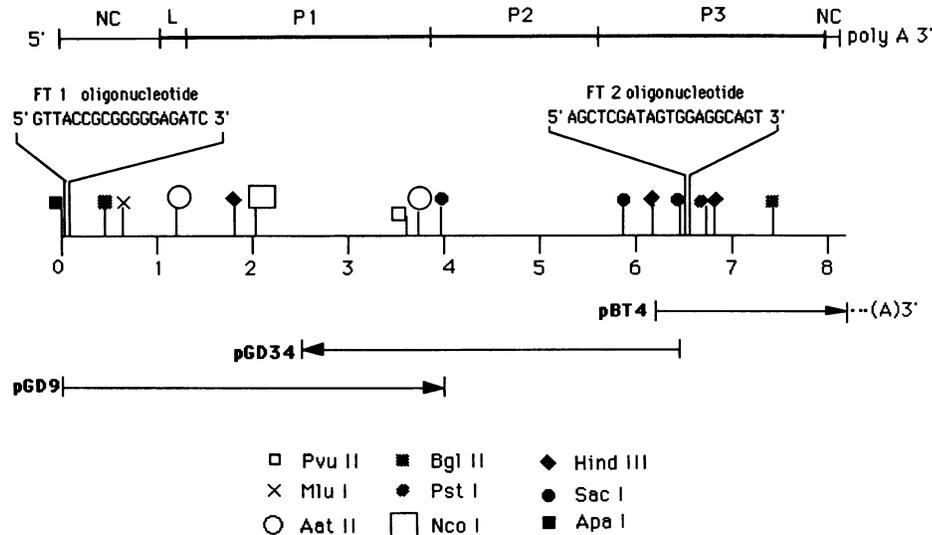


FIG. 1. Organization of the GDVII virus genome and partial restriction map of the three overlapping cDNA clones used for the full-length construction. The genome contains a 5' and a 3' non-coding region. The large open reading frame consists of a leader peptide and regions P1, P2, and P3. Oligonucleotides FT1 and FT2, which were used for cloning, are also indicated. Arrows show the orientation of inserts with respect to the *EcoRI* restriction site of plasmid pBR322. Other restriction sites are also indicated.

**Subcloning in Bluescript vector.** Plasmids were purified by centrifugation in CsCl density gradients followed by RNase A and proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Purified plasmids were digested with the appropriate restriction enzymes. The fragments to be subcloned were purified by agarose electrophoresis and NaI-glass beads extraction (Gene Clean), and ligated to the Bluescript vector (Stratagene) in a 10- $\mu$ l reaction containing 50 mM Tris hydrochloride (pH 7.6), 10 mM MgCl<sub>2</sub>, 5% (wt/wt) PEG 8000, 1 mM ATP, 1 mM dithiothreitol, 300 ng of DNA, and 2.5 U of T4 DNA ligase (Amersham). The

reactions were incubated for 1 h at 14°C and used to transform competent XL blue cells as described by Hanahan (8).

**Sequencing.** The *PstI-EcoRI* fragment containing the first 300 nucleotides of the 5' end of clone pGD9 was subcloned in Bluescript. Plasmids were sequenced with the Klenow fragment of DNA pol I by using the specific Bluescript primers KS and SK (Stratagene).

**In vitro transcription.** Plasmid pTMGDVII was linearized with *XhoI*, treated with proteinase K, extracted with phenol-chloroform and precipitated with ethanol. RNA transcription was performed with T7 RNA polymerase (Stratagene) in a reaction mixture containing 40 mM Tris hydrochloride (pH 8), 10 mM MgCl<sub>2</sub>, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 0.4 mM each of ATP, CTP, UTP, and GTP, 100 U of RNasin, 4  $\mu$ g of linearized pTMGDVII, and 40 U of T7 RNA polymerase. The reaction was incubated at 37°C for 30 min. The size of the RNA synthesized was analyzed by methylmercuric hydroxide agarose electrophoresis. Synthesis of minus-strand RNA was identical, except that pTMGDVII was linearized with *BamHI* and transcription was performed with T3 RNA polymerase (Stratagene).

**Transfection of cells with RNA.** Monolayers of BHK-21 and L cells grown in DMEM supplemented with 10% newborn calf serum were used for transfection experiments. Transfections were carried out as described by Van Der Werf et al. (17). RNA was diluted in Hanks balanced salt solution (5 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 8 g of NaCl, 0.37 g of KCl, 0.125 g of Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, and 1 g of glucose per liter, pH 7), mixed with an equal volume of DEAE-Dextran (1 mg/ml in Hanks

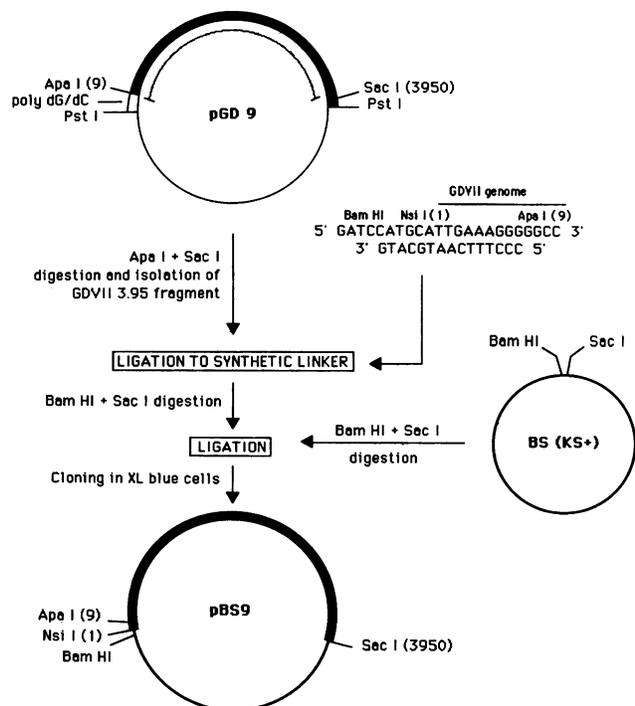


FIG. 2. Cloning of the 5' end of GDVII genome. Thick lines represent the viral cDNA. Thin and intermediate lines represent pBR322 and Bluescript vector, respectively. The inner arc underlines the DNA fragment which was subcloned. Nucleotide numbers for *SacI* (3950, 5900, 6450) and *PstI* (8190) restriction sites were obtained by physical restriction mapping and therefore are only approximate.

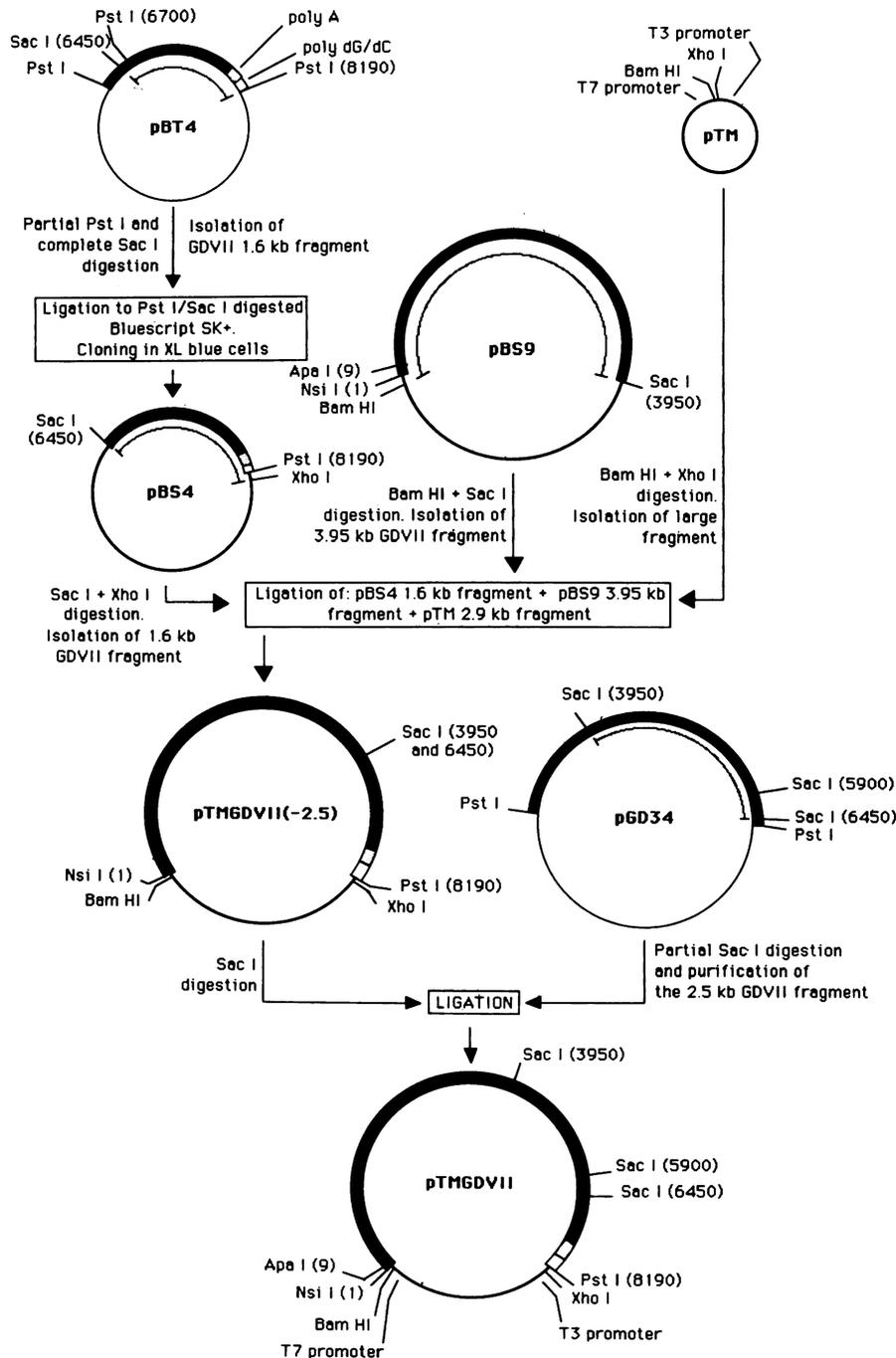


FIG. 3. Construction of the full-length pTMGDVII clone. Thick lines represent the viral cDNA. Thin and intermediate lines represent pBR322 and Bluescript vector, respectively. pTM plasmid was derived from Bluescript by deleting part of the polylinker in order to place the *Bam*HI site next to the T7 promoter. The inner arcs underline the DNA fragments which were subcloned. Nucleotide numbers for *Sac*I (3950, 5900, 6450) and *Pst*I (8190) restriction sites were obtained by physical restriction mapping and therefore are only approximate.

balanced salt solution) and placed at 0°C for 30 min. Confluent cells were washed three times with DMEM and incubated for 30 min at room temperature with 0.2 ml of the RNA DEAE-Dextran mixture per 35-mm plate. DMEM containing 2.5% fetal calf serum was added, and the plates were incubated for 5 h at 37°C. Cells were then washed three times with DMEM and incubated at 37°C in DMEM without serum.

**Detection of viral antigens in infected cells.** The recombinant virus (R1-GDVII) obtained by RNA transfection was grown on BHK-21 cells, and the titer was determined by endpoint dilution (eight wells per dilution).

To detect viral antigens, BHK-21 cells were infected with 10<sup>2</sup> 50% tissue culture infective doses (TCID<sub>50</sub>) of R1-GDVII per cell. Cells were harvested 4 h later and deposited on glass microscope slides by cytocentrifugation. Fixation in

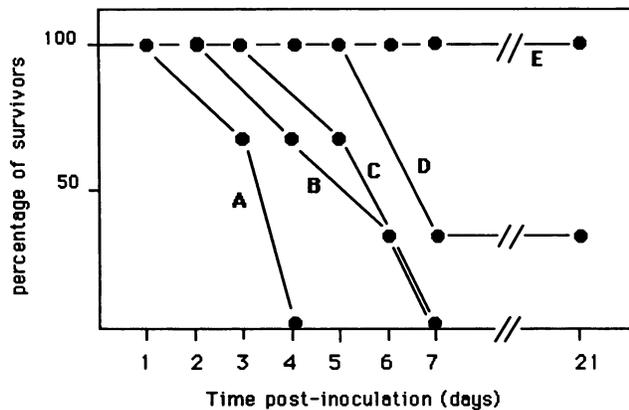


FIG. 4. Mortality of BALB/c mice inoculated intracranially with the following decreasing dilutions of R1-GDVII:  $4 \times 10^5$  TCID<sub>50</sub> (A),  $4 \times 10^4$  TCID<sub>50</sub> (B),  $4 \times 10^3$  TCID<sub>50</sub> (C),  $4 \times 10^2$  TCID<sub>50</sub> (D), and  $4 \times 10^1$  TCID<sub>50</sub> (E). As a control, BALB/c mice were inoculated also with  $10^5$  TCID<sub>50</sub> of original GDVII virus. The mortality curve obtained was superimposable to curve A. Three mice were inoculated for each dilution. Mice which survived after 7 days did not show neurological symptoms.

paraformaldehyde-glutaraldehyde and detection of GDVII TMEV capsid antigens by immunocytochemistry were performed as previously described (2). The immune serum against virus has also been previously described (2). This serum binds strongly to capsid proteins VP<sub>1</sub> and VP<sub>2</sub> and weakly to capsid protein VP<sub>3</sub>.

**Animal studies.** BALB/c mice (3 to 4 weeks old) were purchased from the Institut Pasteur. They were inoculated intracranially with 50  $\mu$ l of phosphate-buffered saline containing dilutions of R1-GDVII. To detect viral antigens, moribund mice were perfused under anesthesia with 20 ml of phosphate-buffered saline followed by 20 ml of cold paraformaldehyde-glutaraldehyde fixative. Postfixation dissection of the CNS, paraffin embedding, and sectioning were performed as described previously (2). The detection of GDVII capsid antigens by immunocytochemistry was performed on CNS paraffin sections as previously described (2) with the same serum used for BHK cells.

## RESULTS

**Cloning GDVII Theiler's virus genome.** We produced a series of cDNA clones which collectively covered the entire GDVII genome. Their restriction enzyme map is presented in Fig. 1.

Clone pBT4, which contains the 3' extremity of the genome, was described previously (13). All other clones were obtained by priming the cDNA synthesis with a specific oligonucleotide (FT2) corresponding to a sequence located close to the 5' extremity of clone pBT4 (Fig. 1).

Several large inserts (3 to 4 kilobases [kb]) were oriented relative to one another by restriction enzyme mapping. They covered most of the viral genome, but none reached its 5' extremity.

Clones covering the 5' extremity of the viral genome were identified by colony hybridization with an oligonucleotide (FT1) synthesized according to the sequence of Theiler's virus GDVII RNA (14) (Fig. 1). Of  $\approx 17,000$  colonies tested, 61 hybridized with FT1 under stringent conditions. The sequence of clone pGD9 showed that it contained the first nucleotide of the 5' extremity of the viral RNA.

**Construction of full-length Theiler's virus cDNA.** Three overlapping clones (pGD9, pGD34, and pBT4) that covered the entire genome were chosen to reconstruct a full-length cDNA.

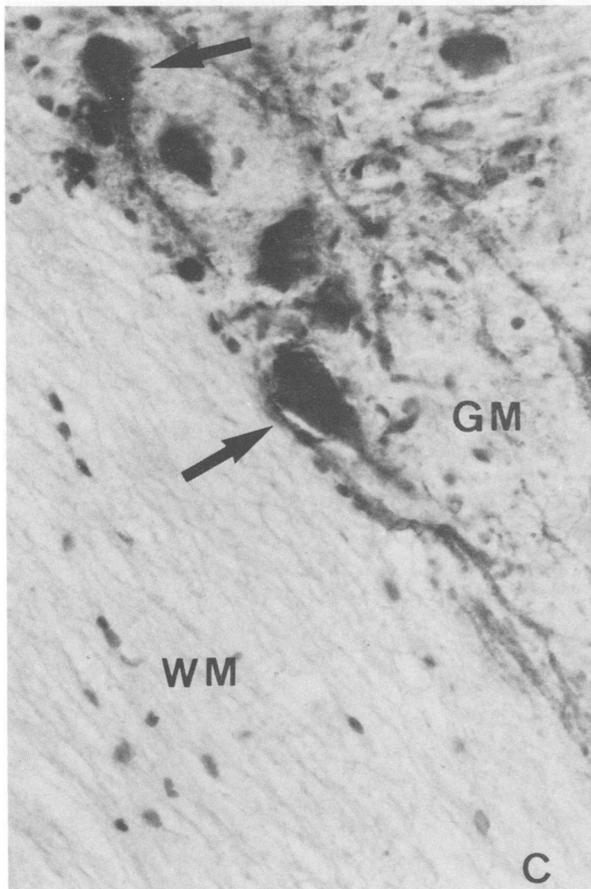
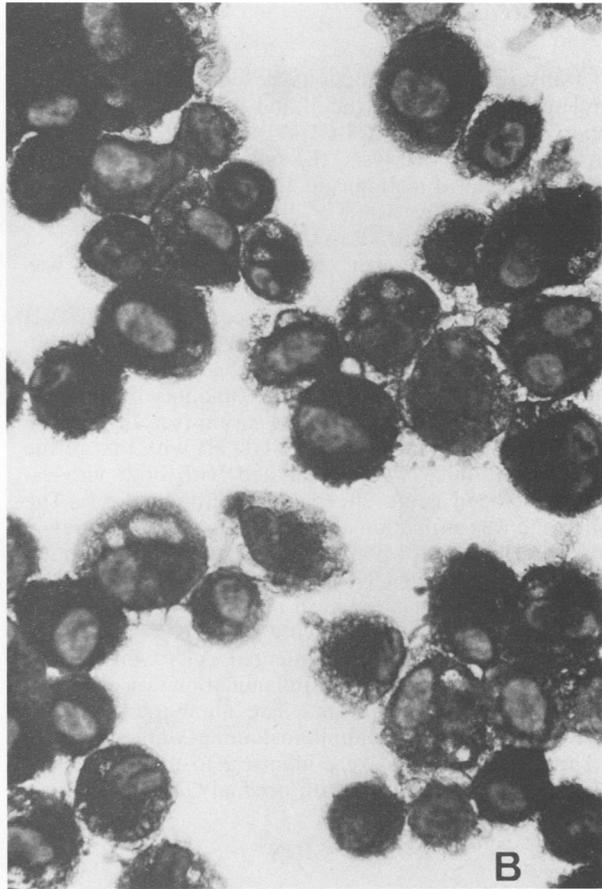
In order to eliminate the excess nucleotides left at the 5' end by the cloning procedure (dG-dC tail and polylinker), we synthesized a linker which extended from viral nucleotide 1 to the *Apa*I site at nucleotide 9. It also contained a *Nsi*I site, which would allow further trimming of the DNA if necessary for infectivity, and a *Bam*HI site for cloning. The linker was ligated to the *Apa*I-*Sac*I fragment of plasmid pGD9. The ligation product was introduced into Bluescript (KS<sup>+</sup>) to produce plasmid pBS9 (Fig. 2). The *Sac*I-*Pst*I insert ( $\approx 1.6$  kb) of pBT4, containing the 3' extremity of the viral genome, was isolated and subcloned in Bluescript (SK<sup>+</sup>). The new plasmid was called pBS4 (Fig. 3). The *Bam*HI-*Sac*I insert (4.0 kb) of pBS9 and the *Sac*I-*Xho*I insert (1.6 kb) of pBS4 were isolated (Fig. 3). These two fragments were ligated in a single reaction to a plasmid called pTM which had been previously digested by *Xho*I and *Bam*HI. Plasmid pTM was derived from Bluescript by deleting the *Sac*I and six other restriction sites from the polylinker and placing the *Bam*HI site next to the T7 promoter sequence.

The product of the three-fragment ligation, pTMGDVII (-2.5), contained the two extremities of the genome but lacked an internal *Sac*I-*Sac*I fragment of 2.5 kb. This fragment was obtained by a partial *Sac*I digestion of clone pGD34 and was introduced into the unique *Sac*I site of pTMGDVII(-2.5) (Fig. 3). This final step yielded plasmid pTMGDVII, which contained the T7 RNA polymerase promoter followed by a *Bam*HI and a *Nsi*I restriction site, the entire cDNA of the GDVII genome, a dG-dC tail, a polylinker, and the T3 RNA polymerase promoter. This plasmid was amplified in DH-5 bacteria grown on solid agar medium.

Additional residues at the 5' end of the RNA could have decreased its infectivity. The *Nsi*I restriction site would have allowed us to construct a plasmid with the T7 promoter directly attached to Theiler's virus genome (17). This step, however, was not necessary.

**Infectivity of RNA transcripts.** Plasmid pTMGDVII was linearized by digestion with *Xho*I and transcribed in vitro with T7 RNA polymerase. The plasmid was also opened at the *Bam*HI site and transcribed with T3 RNA polymerase to obtain control minus-strand RNA. Transcripts were used, without further purification, to transfect BHK and L cells. Plus-strand RNA (4  $\mu$ g/35-mm plate) gave a complete cytopathic effect in 48 h. The effect was indistinguishable from that produced by RNA purified from GDVII virions. Cells transfected with the same amount of minus-strand RNA were indistinguishable from control uninfected cells even after 1 week of incubation. It is interesting that the plus-

FIG. 5. Detection of Theiler's virus capsid antigens by immunocytochemistry. The primary serum was obtained by immunization of a rabbit with GDVII virus. (A) Uninfected BHK cells. (B) BHK cells infected with R1-GDVII virus and harvested 4 h postinfection. (C) Longitudinal section of spinal cord of a BALB/c mouse inoculated with R1-GDVII. WM, White matter; GM, grey matter. (D) Section of brain stem of a BALB/c mouse inoculated with R1-GDVII. In panels C and D, the arrows point to example of large neurons containing viral antigens. Antigens can also be seen in neurites. Original magnification,  $\times 620$ .



strand transcripts were infectious despite the presence of nonviral sequences at both the 5' and the 3' ends.

**Phenotype of the virus (R1-GDVII) obtained by transfection.** After complete cell lysis, the supernatant was clarified by low-speed centrifugation and used as a source of R1-GDVII virus. The titer was  $4 \times 10^8$  TCID<sub>50</sub> per ml. BHK cells were infected with R1-GDVII at a multiplicity of infection of 400 TCID<sub>50</sub> per cell, and complete lysis was obtained in 8 h.

BHK cells infected with 100 TCID<sub>50</sub> per cell of R1-GDVII were harvested 4 h after infection, cytocentrifuged onto microscope slides, fixed, and used for the detection of viral antigens. These cells contained large amounts of antigens that reacted with anti-GDVII capsid serum (see Fig. 5).

To compare the phenotype of R1-GDVII with that of the original GDVII, mice were inoculated with both viruses. Animals developed acute encephalitis within 1 week. The mortality rate was a function of inoculum size (Fig. 4). Seven animals were sacrificed when moribund, and the distribution of viral antigens in their CNS was studied by immunocytochemistry. Infection was found in numerous areas of brain and spinal cord grey matter. No viral antigens were found in white matter. The majority of infected cells were clearly identified as neurons (Fig. 5). No inflammation was observed except for some neuronophagia. The number of infected cells and the intensity of immunostaining were strikingly high. These observations were identical to those obtained with control mice inoculated with original GDVII virus.

#### DISCUSSION

We have succeeded in constructing a full-length cDNA of Theiler's virus GDVII strain by assembling three overlapping clones. Transcripts of this cDNA were infectious upon transfection into tissue culture cells. The virus recovered after complete cytopathic effect (R1-GDVII) produced acute encephalitis when inoculated into mice. Positive reaction of tissue sections with antiserum to GDVII demonstrated the same tropism for neurons and confirmed that R1-GDVII was identical to the genuine GDVII virus. This infectious cDNA will allow us to produce recombinant viruses with the persistent strain (DA) to segregate and identify the genes responsible for persistence, demyelination, and cell tropism.

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