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Infectious cDNA Clones of the DA Strain of Theiler's Murine Encephalomyelitis Virus

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The DA strain and other members of the TO subgroup of Theiler's murine encephalomyelitis viruses cause a persistent demyelinating infection, whereas the GDVII strain and other GDVII subgroup strains cause an acute lethal polioencephalomyelitis. We generated an infectious DA cDNA clone inserted into a transcription vector. Virus derived from transfection of transcripts produced a demyelinating disease indistinguishable from that of wild-type virus. The infectious clone provides a critical reagent for the production of interstrain recombinant viruses to help identify genetic loci responsible for the biological activities of the strains.

Although strains of Theiler's murine encephalomyelitis viruses (TMEV) belong to one serotype, they can be divided into two subgroups on the basis of their different biological activities (2, 3) as well as reactivity with neutralizing monoclonal antibodies (4, 6). DA and other TO subgroup strains cause a persistent demyelinating infection in mice. In contrast, GDVII and other GDVII subgroup strains are more neurovirulent and produce an acute, fatal polioencephalomyelitis with no persistence or demyelination. A major goal for investigators of this virus group is the delineation of molecular determinants of the varied biological activities—neurovirulence, persistence, demyelination—of the strains.

A powerful tool in molecular pathogenesis studies of picornavirus has been the use of infectious cDNA clones of viral RNAs. In poliovirus, infectious clones of neurovirulent and attenuated strains have been recombined to produce chimeric cDNAs and recombinant viruses to delineate genome segments and nucleotides responsible for neurovirulence (5). Investigation of the TMEV model system has several potential advantages over poliovirus studies. (i) The mouse is both the natural and experimental host for TMEV; for this reason, the effect of TMEV interstrain recombinants or mutated TMEV on disease in the natural host can be easily and effectively studied. (ii) The varied biological activities of strains from the two subgroups represent a natural comparative system for investigations with TMEV recombinant viruses. (iii) TO subgroup strains manifest biological activities that are unusual for picornaviruses—persistence and demyelination.

To generate a full-length clone, previously described DA subgenomic cDNA clones in pUC9 (9) were digested with restriction enzymes and then, in a number of steps, ligated together (Fig. 1). pDAFL1 contains the full-length cDNA clone of DA (including 13 adenylate residues at the 3′ end of the heteropolymeric sequences) inserted into the *Pst*I and *Bam*HI sites of the transcription vector Bluescript pSK− (Stratagene).

We were concerned that the 69 nucleotides of the polylinker between the T7 promoter and the 5′ end of the DA genome in pDAFL1 might interfere with infectivity. A previous report demonstrated that the elimination of nucleotides between the T7 promoter and the 5′ end of the viral genome increases infectivity of transcripts derived from an infectious poliovirus cDNA (13). For this reason, we reengineered the 5′ end of the DA genome in pDAFL1 two nucleotides downstream from the T7 promoter to generate pDAFL3 (Fig. 2). To bring the 5′ end of the DA genome in pDAFL1 nearer the T7 promoter, we synthesized paired complementary oligonucleotides to replace the part of DAFL1 that extended from a *Bgl*I site (N472) in pSK to N14 in the DA genome. The paired oligonucleotides (Fig. 2B) contained the T7 promoter 2 nucleotides upstream from the first 14 nucleotides of the DA genome; one end of the paired oligonucleotides was one-half of the *Bgl*I recognition site at pSKN472 followed by a unique *Cla*I site (to facilitate future manipulations), while the other end was one-half of the *Sma*I recognition site at N12. The N14-N8093 fragment from *Sma*I-*Xba*I-digested pDAFL1 was ligated to the annealed paired oligonucleotides and the two pSKII-fragments to finally yield pDAFL3 (Fig. 2A).

pDAFL1 was digested with *Xba*I and then transcribed in vitro with T7 polymerase by previously published methods (14). The products of this transcription were of the appropriate size when analyzed by electrophoresis on a 1% agarose gel (data not shown). In vitro-derived transcripts were quantitated by comparing a sample with dilutions of a known control RNA and by using 10 μCi of [α-^{32}P]GTP (specific activity, 3,000 Ci/mmol) per reaction. The in vitro-derived transcripts of *Xba*I-digested pDAFL1 had a maximum infectivity of 1.5×10^3 PFU/μg of RNA when transfected into L cells (1) (Table 1). In contrast, transcription of pDAFL3, which contained only two nucleotides between the T7 promoter and the 5′ end of the DA genome, produced RNA with an increased infectivity averaging 1.8×10^4 PFU/μg (Table 1). This infectivity was approximately 20-fold less than the infectivity of transfected virion RNA. The infectivity was a result of RNA since there was a complete loss of infectivity after treatment with 10 μg of RNase for 30 min, while there was no significant change in titer after treatment with 5 U of DNase for 30 min (data not shown). Virus produced from transfected pDAFL1 was neutralized by neutralizing monoclonal antibodies directed against TO subgroup strains (4) (data not shown). The sequence of the 5′ end of RNA extracted from virus produced from transfected

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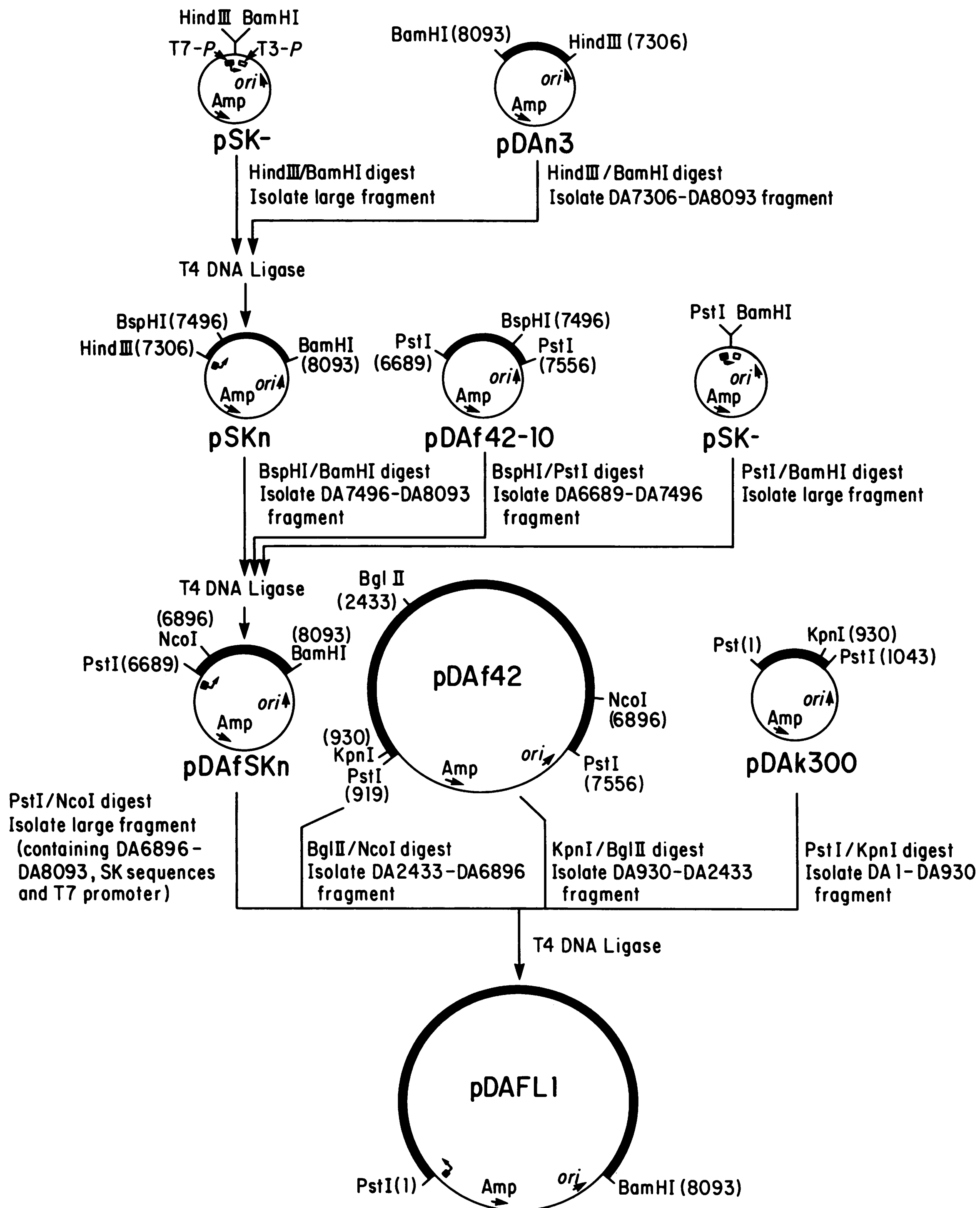

FIG. 1. Construction of an infectious cDNA clone of DA strain in a transcription vector. pDAn3, pDAf42-10, pDAf42, and pDAk300 are DA subgenomic cDNA clones in pUC9 that have been previously described (9). In this figure and Fig. 2, numbers refer to the nucleotide number of the DA genome or SK, a transcription vector; numbers in parentheses correspond to the restriction enzyme site on the DA genome. The T3 promoter (T3-*P*) and T7 promoter (T7-*P*), the ampicillin-resistant site (Amp), and the origins of replication (*ori*) are shown.

pDAFL3 showed a terminus indistinguishable from that of DA wild-type viral RNA (data not shown).

After transfection of L cells, virus was picked from a plaque and grown for one and two passages on BHK-21 cells to produce stock virus for inoculation of nine 3-week-old SJL/J mice (Jackson Laboratory, Bar Harbor, Maine). Histopathological evaluation of the central nervous system of all the infected mice showed prominent inflammatory, demyelinating disease of the spinal cord 3 and 4 months after inoculation. Figure 3 shows a section of a spinal cord from a

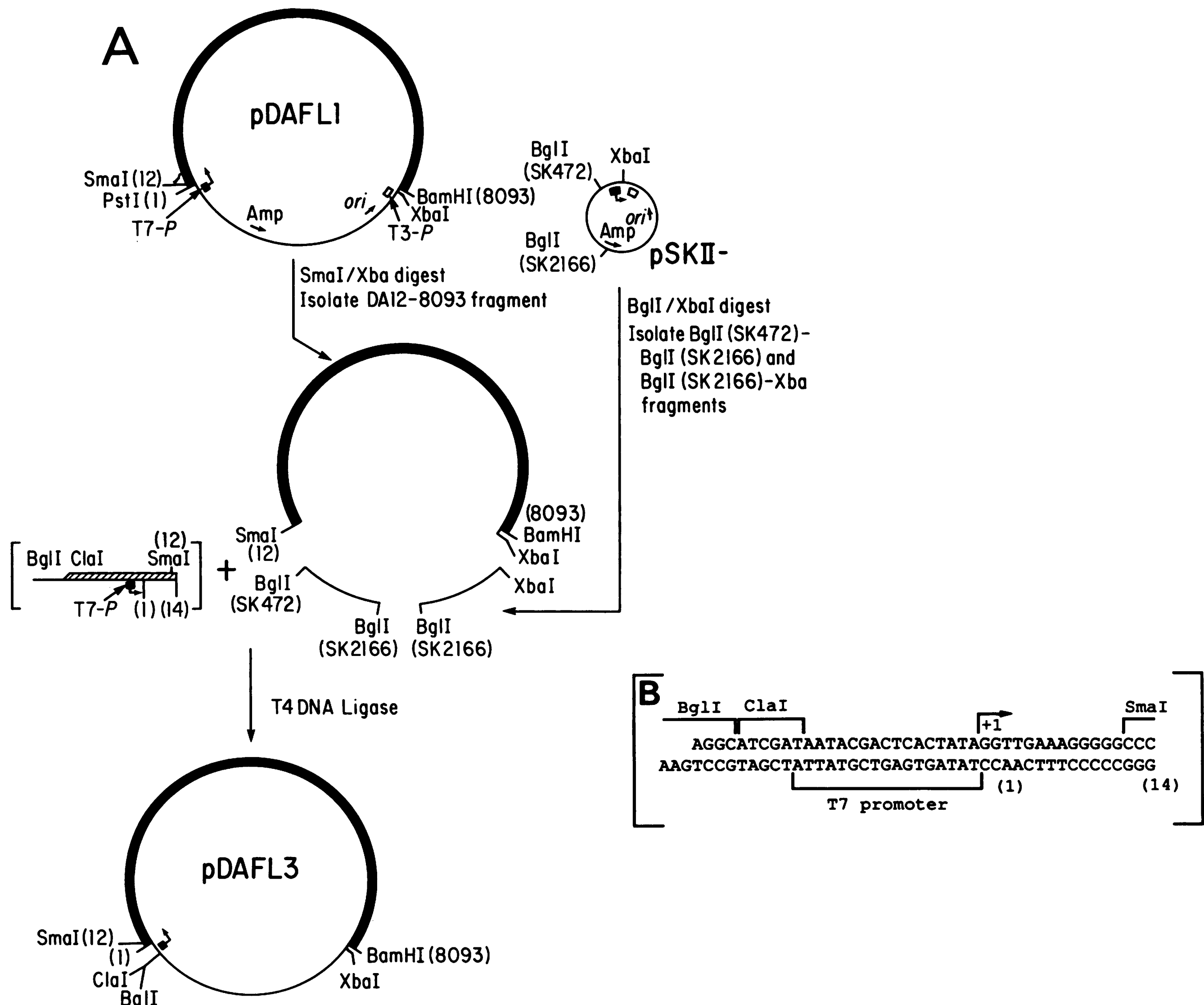

FIG. 2. Construction of pDAFL3, an infectious cDNA clone DA strain in a transcription vector, in which pDAFL1 was reengineered so that the 5′ end of the DA genome was placed two nucleotides downstream from the T7 promoter. (A) Outline of the construction of pDAFL3. The brackets represent paired oligonucleotides and are detailed below. (B) Paired oligonucleotides used in construction of pDAFL3 which contain one-half of a *Bgl*I restriction enzyme recognition site in SKII−, a *Cla*I site, the T7 promoter, and the first 14 nucleotides of the DA genome up to one end of a *Sma*I site.

TABLE 1. Results of transfection into L-929 cells[a]

Sample	Infectivity (PFU/μg)
pDAFL1 in vitro-transcribed RNA	2.5×10^1
	5.0×10^2
	1.5×10^3
pDAFL3 in vitro-transcribed RNA	3.6×10^4
	1.0×10^4
	1.4×10^4
	2.8×10^3
DA virion RNA	3.6×10^5

[a] Each infectivity result was obtained in a separate experiment.

mouse sacrificed 3 months after inoculation; there are lymphocytic infiltrates in the meninges and white matter perivascular spaces with macrophages in the disrupted white matter. The brain appeared normal, aside from a few perivascular inflammatory cuffs in the brain stem and occasional calcified foci in the hippocampus. The above findings were characteristic of disease seen after inoculation with wild-type DA virus.

While this manuscript was in preparation, Tangy et al. (12) published data concerning the preparation of infectious GDVII cDNA clones; however, infectivity titers of in vitro-derived GDVII transcripts were not presented. We have also produced an infectious GDVII cDNA clone as well as a series of DA-GDVII chimeric cDNA clones (J. Fu, S. Stein, L. Rosenstein, T. Bodwell, B. L. Semler, and R. P. Roos, manuscript in preparation). Our preliminary data (using

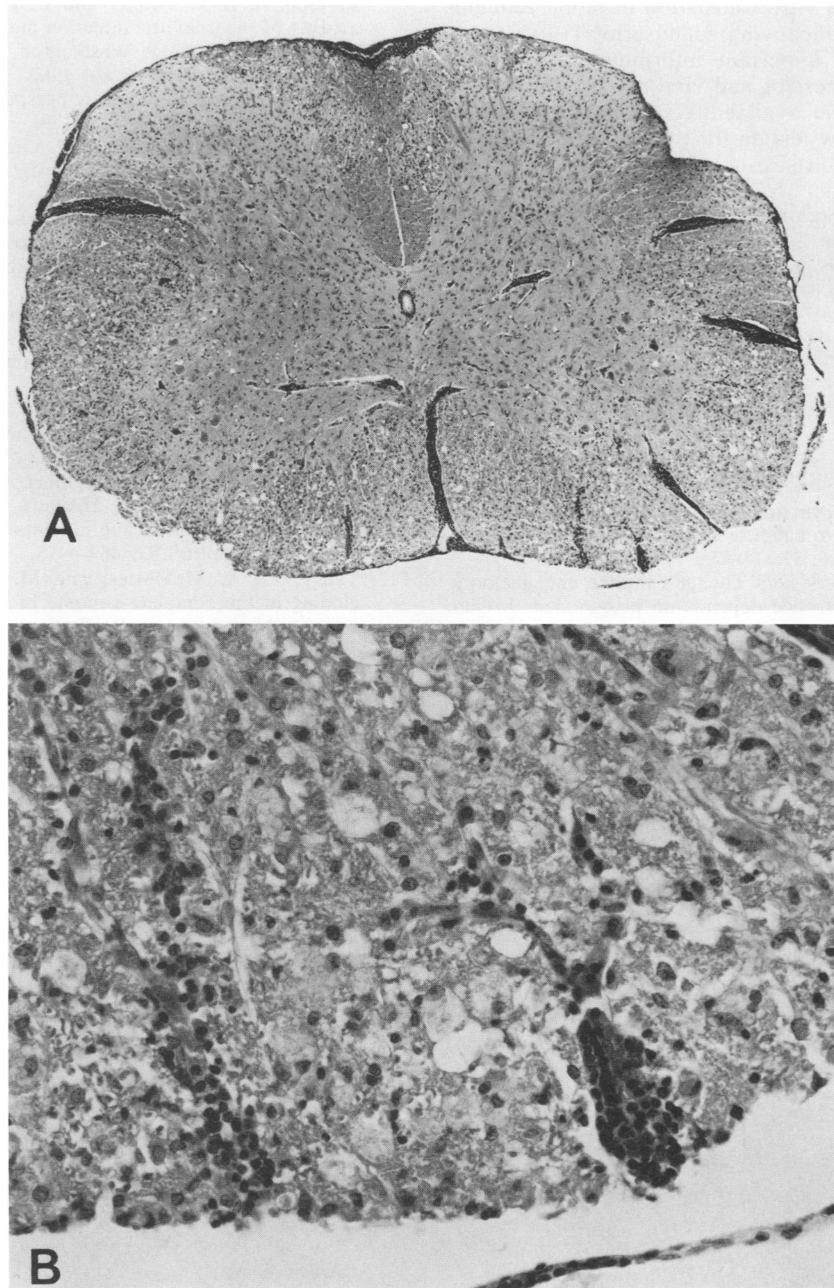

FIG. 3. Sections of spinal cord from mouse inoculated 3 month. previously with virus derived from transfected pDAFL3. See text for details. (A) Transverse section of spinal cord showing inflammatory cells in meninges and perivascular spaces with disruption of white matter (hematoxylin-eosin-stained section of paraffin-embedded tissue; ×40). (B) High-power view of white matter of above section (hematoxylin-eosin-stained section of paraffin-embedded tissue; ×300).

recombinant viruses generated from the chimeric cDNAs) have identified a segment from the polyprotein corresponding to the 1B-coding area to the 2C-coding area critical in determining the enhanced neurovirulence of the GDVII strain. These recombinant virus studies will also provide information concerning genome segments critical in producing demyelination and virus persistence. Site-directed mutagenesis of the infectious cDNA clones will provide a detailed localization of particular nucleotides key in producing specific biological effects. We are especially interested in mutating the carboxyl end of VP1, which contains a trypsin-sensitive, immunodominant neutralization site (8), because of its apparent role in the DA demyelinating disease phenotype (11).

Infectious clones of positive-strand viruses have allowed genetic manipulations to delineate the functions of specific viral gene products. For example, in vitro translation of in vitro-derived transcripts of wild-type and mutated full-length poliovirus infectious clones has provided information concerning viral polyprotein processing and the viral proteinases that carry out the processing (14). In a similar fashion, we used in vitro-derived transcripts from wild-type and

mutated pDAFL3 for in vitro translation in rabbit reticulocyte lysates (see accompanying manuscript [10]). These studies have provided important information concerning TMEV polyprotein processing and viral proteinases and, in addition, because of the availability of the mouse as an experimental host, allow testing for the effect of the mutations on demyelinating disease.

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