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Theiler's Virus as a Vector for Foreign Gene Delivery

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DA strain and other strains of the TO subgroup of Theiler's murine encephalomyelitis viruses are members of the *Cardiovirus* genus of picornaviruses and produce a persistent demyelinating disease in mice. A recent study from our laboratory (W.-P. Kong, G. D. Ghadge, and R. P. Roos, Proc. Natl. Acad. Sci. USA 91:1796–1800, 1994) demonstrated that the leader, which is encoded at the N terminus of the Theiler's murine encephalomyelitis virus polyprotein, can be partially replaced by foreign sequences as well as completely deleted, with no loss of infectivity in BHK-21 cells. In this study, we have inserted up to 724 nucleotides into the leader coding region of an infectious DA clone. Recombinant viruses were produced, and the inserts were shown to be stable for at least three passages in BHK-21 cells.

Theiler's murine encephalomyelitis virus (TMEV) designates a group of serologically related mouse picornaviruses that are most closely related to members of the *Cardiovirus* genus (reviewed in reference 21). There are two subgroups of TMEV which differ in their biological activities. GDVII strain and other members of the GDVII subgroup of TMEV are highly neurovirulent for weanling mice. In contrast, DA strain and other members of the TO subgroup produce an early nonfatal neuronal infection associated with relatively large amounts of virus, followed by a persistent demyelinating infection in which only small amounts of infectious virus are detectable. The pathogenesis of DA-induced demyelinating disease is unclear, but a variety of experimental data suggests that the immune system contributes to this late disease. The present study involves the preparation and characterization of DA viruses with various recombinant genes inserted into the leader (L) coding region. This recombinant virus system may have value in neurobiological investigations and in investigations of the interactions of autoimmunogenic peptides with DA-induced disease.

There are several recent studies involving the use of picornaviruses as vectors for foreign gene expression. One approach has involved insertions within the capsid coding region. In the case of poliovirus (an enterovirus), recombinant genes have been inserted into the region coding for an exposed loop of a capsid surface protein, VP1 (4, 5, 8, 11, 14, 17, 18), and have also been inserted at the amino or carboxyl terminus of the region coding for all of the capsid proteins (P1 proteins) (3, 15). Recently, Altmeyer et al. (2) inserted 441 nucleotides (nt) coding for human immunodeficiency virus gp120 into the L coding region (at the N terminus of the polyprotein) of mengovirus, a cardiovirus similar to TMEV. In addition, Andino et al. (3) inserted up to 1,090 nt coding for human immunodeficiency virus type 1 p17 and $p24^{Gag}$ fusion protein at the start of the poliovirus polyprotein; although enteroviruses do not have an L coding region, this insertion is in a location analogous to the cardiovirus L coding region (i.e., upstream of P1). In other cases, bicistronic constructs have been generated by introducing an additional internal ribosome entry site in front of the foreign gene (1). In addition, poliovirus minireplicons have been used to express foreign proteins in combination with helper poliovirus or recombinant vaccinia virus (19).

Picornaviruses have been used as vectors primarily with an aim towards vaccine design for immunization purposes. In contrast, the goal of the investigations reported here was to determine the feasibility of using TMEV as a vector for studies directed at the clarification of the virus' interactions with the immune system and for studies of various neurologic diseases (e.g., Alzheimer's disease). We separately inserted two autoimmunogenic peptides, mouse myelin proteolipid protein (PLP) and the human acetylcholine receptor (AChR), into DA VP1 loop II in order to produce virus that might elucidate these virus-immune system relationships. For experiments involving insertions in the L coding region, we also used a peptide from the mouse amyloid precursor protein (MoAPP) and human β-amyloid peptide; this sequence contained two mutations that have been associated with familial Alzheimer's disease (13, 16).

DA VP1 loop II insertions. In order to investigate the use of TMEV as a vector, we initially inserted a foreign sequence into loop II of DA VP1 (nt 3271 to 3339), since this region contains a TMEV neutralization epitope (6, 7) and because the coding sequences for small peptides have been successfully inserted in an antigenic loop (the BC loop) of poliovirus (4, 5, 8, 11, 14, 17, 18). The following DA VP1 loop II constructs were engineered into an infectious clone of DA, pDAFL3 (23) (Fig. 1). pDAVP1AchR(×1) was generated by inserting a complementary pair of synthetic 45-mer oligonucleotides (Operon Technologies, Alameda, Calif.) corresponding to the coding sequence for a myasthenogenic peptide (12) of the AchR α-chain (amino acids 130 to 140); pDAVP1AchR(×2) contained two copies of this peptide. A silent *Sna*BI restriction enzyme recognition site was engineered into the oligonucleotides for screening purposes. pDAVP1PLP was prepared by ligating a complementary pair of synthetic 42-mer oligonucleotides corresponding to the coding region of an encephalitogenic peptide (25) of mouse myelin PLP (amino acids 139 to 151) into *Bst*XI-*Bsp*HI-digested DAFL3 (which deleted nt 3282 to 3318); this peptide therefore replaced amino acids 93 to 103 of VP1.

The constructs mentioned above were linearized with *Xba*I (which cuts 3′ to the end of the DA genome) and then transcribed in a T7 RNA polymerase reaction, and the resultant RNAs were transfected into BHK-21 cells, as previously described (23). Transcripts derived from both pDAVP1AchR and pDAVP1PLP were not infectious, despite several separate

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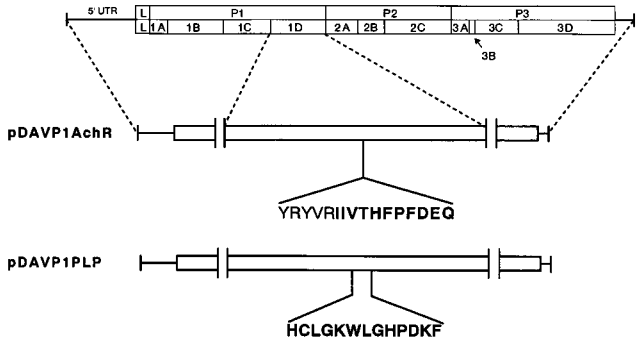

FIG. 1. Map of DA with inserts of recombinant genes. pDAVP1AchR has an insertion of the human AchR α-chain, amino acids 130 to 140 (in boldface), in the *Aat*II site of the loop II region of VP1 (1D). pDAVP1PLP was prepared by substituting the coding region of PLP, amino acids 139 to 151, for VP1 amino acids 93 to 103. UTR, untranslated region.

transfections into BHK-21 cells. These findings indicated that perturbation in DA VP1 loop II can interfere with infectivity. This conclusion was supported by the lack of infectivity of a construct that contained a deletion of nucleotides coding for VP1 amino acids 90 to 99 in this loop (data not shown). Work with poliovirus has similarly shown constraints on the size and structure of this loop (17, 18).

DA L recombinant viruses. Because the DA VP1 loop II insertions led to noninfectious virus, we targeted other regions of the polyprotein, specifically L, which is encoded at the N terminus of the polyprotein of members of the *Cardiovirus* and *Aphthovirus* genera of picornaviruses. The L coding region of DA virus is completely dispensable for infection of BHK-21 cells and can be partially substituted with nonviral sequence (10). In order to explore the use of the L region as an insertion site and its capacity to accommodate insertions of various sizes, several sequences of up to 724 nt were ligated into the full-length L coding region of the infectious DA clone (Fig. 2). For three of the constructs, we inserted a part of our previously prepared VP1 loop II clones [pDAVP1AchR(×1), pDAVP1AchR(×2), and pDAVP1PLP], which included the coding sequence for the foreign peptide as well as adjacent VP1 sequence. For these three constructs, a fragment of the chimeric VP1 region (nt 3052 to 3668) obtained after digestion with *Sal*I was blunt ended with Klenow fragment and then ligated into the *Xho*I site of DA L (nt 1221), which had been calf alkaline phosphatased and blunt ended following *Xho*I digestion. The resultant constructs were called pDAL/VP1AchR(×1), pDAL/VP1AchR(×2), and pDAL/VP1PLP, respectively (Fig. 2). A third construct, pDAL/APP, contained coding sequence for MoAPP amino acids 590 to 596, followed by the human β-amyloid peptide (40 amino acids) and then by MoAPP amino acids 637 to 654 (9) in this same *Xho*I site.

Following transfection of BHK-21 cell monolayers with RNA transcripts derived from these chimeric DA L constructs, a cytopathic effect appeared but was delayed compared with that seen with wild-type DA, only becoming maximal after 4 to 6 days. The recombinant viruses were harvested, plaque purified, and passaged a total of three times on BHK-21 cells. The recombinant viruses achieved levels of infectivity that were generally comparable to those seen with the wild type, although some had a slightly decreased growth at some time points (Fig. 3). The plaque phenotype of recombinant viruses was the same as that seen with DA (data not shown).

In order to confirm the presence of the insert and test the stability of DA L recombinant viruses, RNA was extracted from unpurified stocks of recombinant viruses and used as a template for reverse transcription-PCR (RT-PCR). Total cellular RNA was prepared from virus-infected cells with RNAzol B, by using the manufacturer's protocol (Biotecx Laboratories, Inc., Houston, Tex.). The RT reaction mixture was incubated at 45°C for 30 min in a final volume of 20 μl containing 200 U of Moloney murine leukemia virus RT (GIBCO BRL, Grand Island, N.Y.), first strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphates, and 50 pmol of a negative-sense primer (GGGGGGCGTCGCCAGCATTGCCAC) which specifically binds 3′ to the L region (nt 1409 to 1432). In order to amplify L, 5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 50 pmol of a positive-sense primer which binds just 5′ to L at nt 1044 to 1064 (CCTTTTTATTACTATTGACAC) and 10 μl of 10× PCR buffer (Perkin-Elmer Cetus) were then added to make a final volume of 100 μl; 30 cycles of PCR were performed, at 95°C for 1 min, 55°C for 30 s, and 72°C for 30 s. The PCR amplification products were purified by a Centricon 100 concentrator (Amicon, Inc., Beverly, Mass.) and then either analyzed by agarose gel electrophoresis or sequenced by a double-stranded DNA cycle sequencing system (GIBCO BRL). Figure 4 demonstrates that the RT-PCR products from wild-type and recombinant viruses that had been

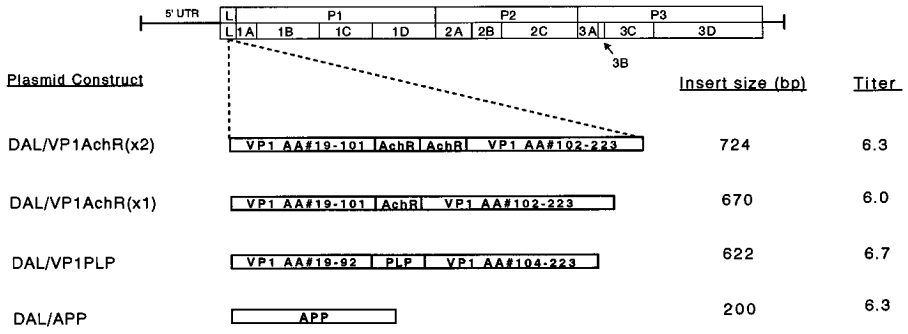

FIG. 2. Chimeric DA constructs with inserts in the *Xho*I site in the L coding region. The predicted amino acids (AA) inserted in L are noted, followed by the name of the construct. DAL/VP1AchR, DA VP1 AA 19 to 101 followed by either one (×1) or two (×2) copies of the human AchR α-chain AA 130 to 140, followed by VP1 AA 102 to 223; DAL/VP1PLP, DA VP1 AA 19 to 92 followed by the mouse PLP AA 139 to 151 followed by VP1 AA 104 to 223; DAL/APP, MoAPP 695 AA 590 to 596 followed by the human β-amyloid peptide followed by MoAPP AA 637 to 654 containing mutations associated with familial Alzheimer's disease. These DNAs were transcribed in vitro, and the resultant RNAs were transfected into BHK-21 cells to produce virus. The size of the insert in base pairs (bp) and the infectivity titer (log_{10} PFU/ml) of passage 3 of the viruses are shown. UTR, untranslated region.

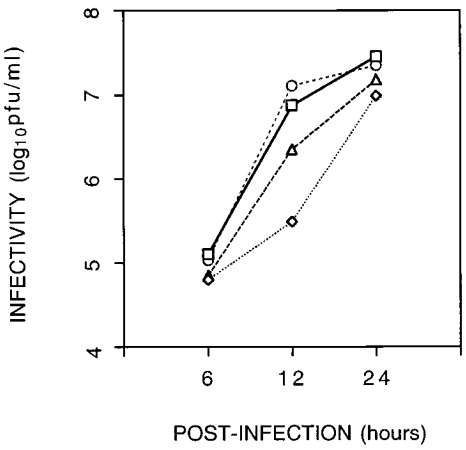

FIG. 3. Growth curves of recombinant DA viruses. Virus was adsorbed onto BHK-21 cells at a multiplicity of infection of 10 PFU per cell for 1 h with passage 3 virus stocks. Monolayers were then washed and scraped at different postinfection times. Results shown are the mean titers of three samples from each time point. □, DAFL3; ◇, DAL/VP1AchR; ○, DAL/VP1PLP; △, DAL/APP.

passaged one and three times in BHK-21 cells were of the appropriate size after amplifying nt 1044 to 1409, a region predicted to include the following inserted sequences: 365 bp for DAFL3, 1,089 bp for DAL/VP1AchR(×2), 1,035 bp for DAL/VP1AchR(×1), 987 bp for DAL/VP1PLP, and 565 bp for DAL/APP. Sequencing of purified RT-PCR products showed that the junctions of each insert had the expected sequence (data not shown). In some additional experiments, primers that were specific for the inserted sequence were used for RT-PCR; no amplified product was seen when wild-type DA RNA was tested, whereas recombinant viral RNAs demonstrated products of the expected size for the appropriate insert (data not shown). The results indicate that the recombinant viruses were stable after three passages in BHK-21 cells, with maintenance of the insert.

To study expression and processing of the recombinant proteins, in vitro-derived transcripts were translated in a rabbit

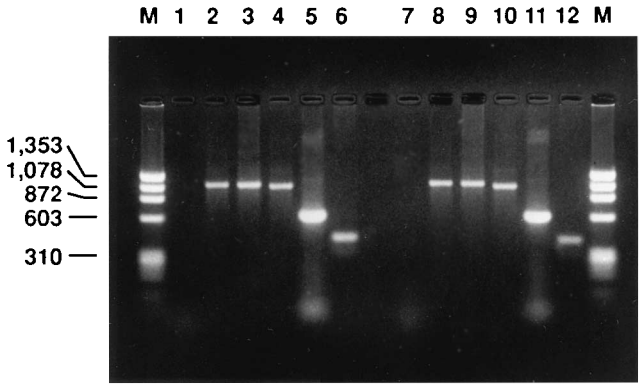

FIG. 4. RT-PCR of RNA extracted from BHK-21 cells infected with stock virus. RT followed by PCR amplification was carried out on RNA extracted from stocks of wild-type and recombinant viruses from passages 1 (lanes 1 to 6) and 3 (lanes 7 to 12) with DA specific primers that amplify the L region between nt 1044 and 1409. The reaction mixtures were then analyzed by agarose gel electrophoresis. M, ΦX174-*Hae*III digest. Lanes 1 and 7, negative control for RT-PCR which contains no template; 2 and 5, DAL/VP1AchR(×2); 3 and 9, DAL/VP1AchR(×1); 4 and 10, DAL/VP1PLP; 5 and 11, DAL/APP; 6 and 12, DAFL3 wild type without insert.

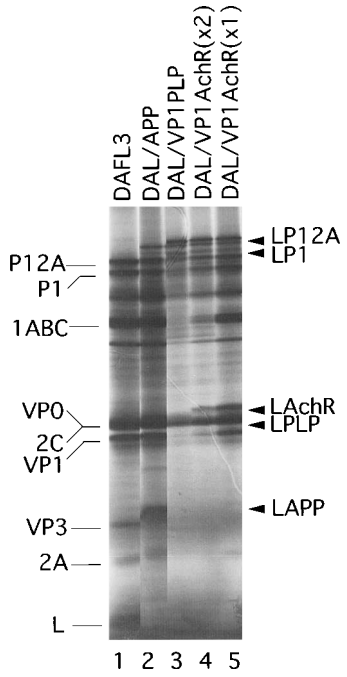

FIG. 5. In vitro translation of in vitro-derived transcripts derived from wild-type pDAFL3, pDAL/APP, pDAL/VP1PLP, pDAL/VP1AchR(×2) and pDAL/VP1AchR(×1). Translations were performed at 30°C in a rabbit reticulocyte lysate with 3 h of incubation. Samples were diluted in Laemmli sample buffer and electrophoresed. Gels were dried and exposed to X-ray film for autoradiography. L fusion proteins are indicated by arrowheads on the right. Processing products are shown on the left.

reticulocyte lysate at 30°C as previously described (22) and terminated after 3 h with the addition of Laemmli sample buffer. The recombinant protein was expressed as a fusion protein with L, which was cleaved from the polyprotein during processing (Fig. 5). The identity of these products as L fusion proteins was determined by immunoprecipitation (24) of the translation reaction mixtures with anti-L antiserum (Fig. 6, even-numbered lanes). To produce the antiserum, a fragment of pDAFL3 from the *Dra*II site (nt 1001) to the *Aoc*I site (nt 1332) was blunt ended and ligated to two *Bam*HI linkers. This fragment was then ligated into the *Bam*HI site of the pMAL-C vector (New England Biolabs, Beverly, Mass.). Restriction endonuclease digestion and sequencing confirmed that the resultant construct contained the coding sequence for a fusion protein that included the L protein. The fusion protein was expressed, isolated, and purified according to the manufacturer's protocol, and used for the immunization of rabbits.

Figure 5 demonstrates that the recombinant constructs synthesized structural and nonstructural proteins in vitro that were generally similar in amount to those synthesized by wild-type DA; however, some precursors with a slower mobility than P12A tended to accumulate to a greater extent in the case of the translated lysates programmed by the chimeric constructs (Fig. 5, lanes 2 to 5) than for those programmed by wild-type DA (Fig. 5, lane 1). These precursors were tentatively identified as LP12A and LP1 in immunoprecipitation experiments (Fig. 6, lanes 4, 6, 8, and 10). The persistence of these precursors suggests that proteolytic processing of the recombinant viruses was less efficient than that of the wild type. The presence of LP1 suggests that protein processing was aberrant, since LP12A and P12A rather than LP1 are processing products of the wild-type DA polyprotein (22).

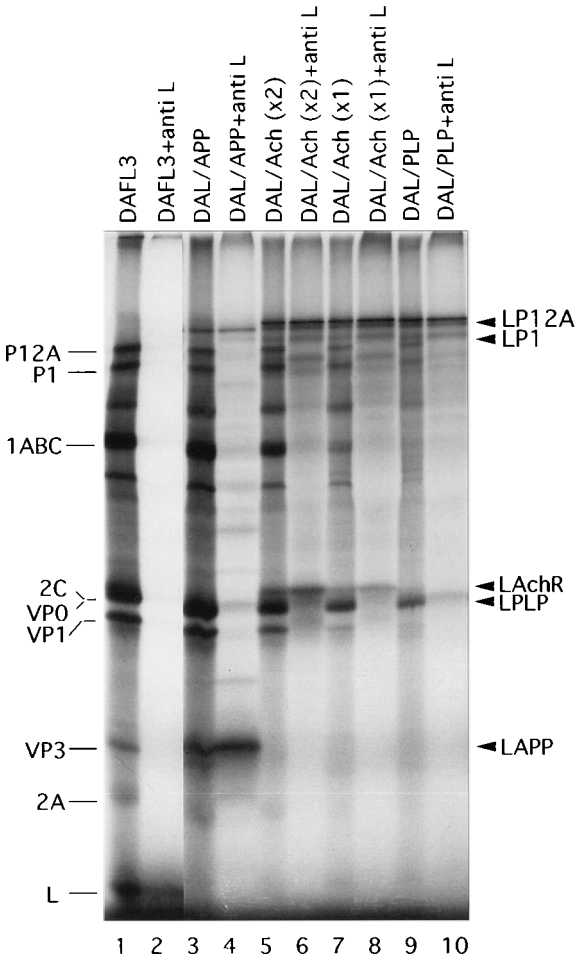

FIG. 6. Immunoprecipitation of in vitro-translated lysates from in vitro-derived transcripts of wild-type pDAFL3, pDAL/APP, pDAL/VP1PLP, pDAL/VP1AchR(×2), and pDAL/VP1AchR(×1). The translated proteins shown in odd-numbered lanes were tested for immunoprecipitation (shown in the adjacent even-numbered lanes), using anti-L antiserum. L fusion proteins are indicated by arrowheads on the right. Processing products are shown on the left. The preparation of the samples is described in the legend to Fig. 5.

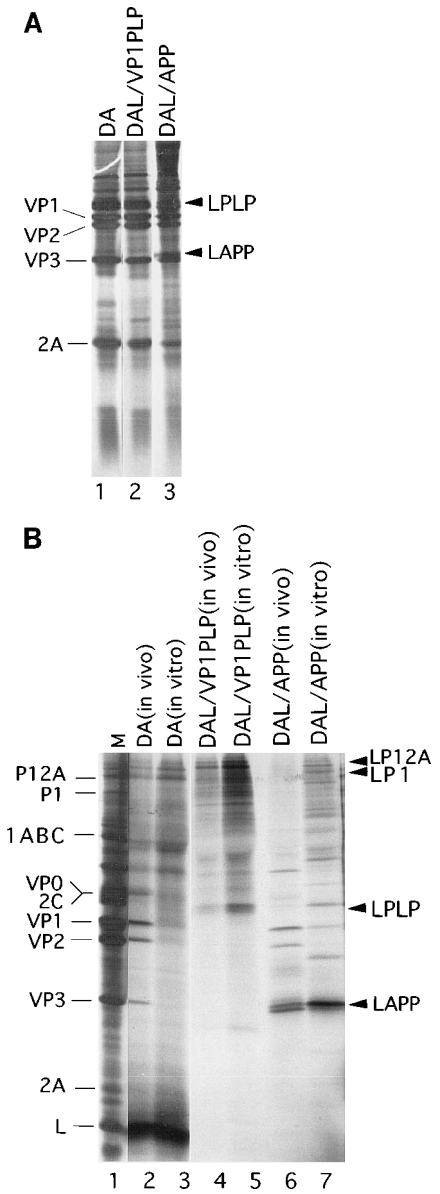

FIG. 7. Expression of L fusion proteins in BHK-21 cells infected with wild-type DA and recombinant viruses. The cells were radiolabelled and infected as described in the text. The preparation of the samples is described in the legend to Fig. 5. (A) [^{35}S]methionine-labelled BHK-21 cells infected with wild-type DAFL3 virus (lane 1), DAL/VP1PLP virus (lane 2), and DAL/APP virus (lane 3), harvested 20 h after infection. (B) Immunoprecipitation with anti-L antiserum of lysates of BHK-21 cells infected with wild-type DAFL3 virus, DAL/VP1PLP virus, and DAL/APP virus (lanes 2, 4, and 6, respectively) and in vitro-translated lysates programmed by transcripts from wild-type DAFL3, DAL/VP1PLP, and DAL/APP (lanes 3, 5, and 7, respectively). Lane 1 (M), [^{35}S]methionine-labelled BHK-21 cells infected with wild-type DAFL3 virus.

Recombinant viruses were then investigated to determine whether the L fusion protein was expressed in BHK-21 cells. Virus-infected cells were radiolabelled by previously published methods (22). Briefly, BHK-21 cells were infected by the DA wild-type, DAL/VP1PLP, or DAL/APP mutant virus at a multiplicity of infection of 10 PFU per cell. After 6 h, the cells were washed and methionine-free medium was added; after one additional hour, 54 μCi of [α-^{35}S]methionine (ICN Biomedicals, Costa Mesa, Calif.) was added to the medium. The cells were incubated for 20 h before washing and harvesting by lysis. Samples were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the gels were dried and then exposed to X-ray film for autoradiography. LPLP (Fig. 7A, lane 2) and LAPP (Fig. 7A, lane 3) fusion proteins were discernible at their predicted electrophoretic mobility; their identities were confirmed by immunoprecipitation studies with anti-L antiserum (Fig. 7B). Immunoprecipitation studies demonstrated that the fusion proteins were synthesized in vivo and that the electrophoretic mobility for LPLP as well as LAPP fusion proteins was the same in infected cells (Fig. 7B, lanes 4 and 6, respectively) and in in vitro-translated lysates (Fig. 7B, lanes 5 and 7, respectively). Similar results were also found in the case of LAchR (data not shown).

Our studies demonstrate that up to 724 nt (241 amino acids) can be inserted within DA L with no substantial effect on viral polyprotein processing or infectivity. The maximal size of an insertion that can be tolerated in the TMEV L coding region with maintenance of infectivity remains uncertain; as noted above, studies with poliovirus suggest that over 1,090 nt may be able to be accommodated (3).

The TMEV recombinant viruses have been inoculated into mice, and studies regarding the pathology of the experimental disease are in progress. The studies involving inoculation of recombinant DA virus that contains an insertion of an encephalitogenic PLP peptide (that is known to produce an autoimmune demyelinating disease in SJL mice [25]) may determine whether there is an enhanced capacity for demyelination and inflammation (i.e., antigenic mimicry [6]). Inoculation of recombinant virus that contains an insertion of a myasthenogenic peptide of the α-chain of the AChR may clarify the role of a virus and of antigenic mimicry in the initiation of a systemic autoimmune disease such as myasthenia gravis. Future studies examining the effect of inoculation of recombinant DA that contains the MoAPP insertion may be valuable in clarifying the pathogenesis of Alzheimer's disease, especially since transgenic studies using constructs containing the amyloid precursor protein have failed to produce pathology typical of Alzheimer's disease (20). A potentially important feature of DA as a virus vector is the presence of a significant initial focus of viral infection and pathology in the hippocampus, a major site of Alzheimer's disease pathology. The availability of a recombinant TMEV that is pathogenic for mice may have other advantages not available with a traditional transgenic mouse system, viz., TMEV has the potential to persistently express non-TMEV genes extracellularly and intracellularly within the central nervous systems of animals of various ages.

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