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A Three-Nucleotide Insertion in the H Stem-Loop of the 5′ Untranslated Region of Theiler's Virus Attenuates Neurovirulence

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The highly structured 5′ untranslated region (5′ UTR) of Theiler's murine encephalomyelitis virus is involved in cap-independent translation of the viral RNA. Previously, we reported that the bicistronic mRNA chloramphenicol acetyltransferase–5′ UTR-luciferase (Luc) efficiently expressed Luc both in a rabbit reticulocyte lysate and when transfected into BHK-21 cells. Insertion of 3 nucleotides at position 665 in the 5′ UTR of this bicistronic mRNA resulted in greatly reduced Luc expression in BHK-21 cells but had little effect on expression of Luc in rabbit reticulocyte lysate. This mutation was also introduced into a virulent Theiler's murine encephalomyelitis virus chimera, Chi-VL. The kinetics of viral RNA and protein synthesis and virus production in BHK-21 cells were slower for the mutant chimera [Chi-VL(IN668)] than for Chi-VL; however, the final virus yields were comparable. Intracerebral inoculation of mice with the chimeras revealed that Chi-VL(IN668) was completely attenuated in neurovirulence. The reduced neurovirulence of Chi-VL(IN668) may be ascribed to its reduced growth in the central nervous system, most likely due to an impaired ability to synthesize viral proteins.

Theiler's murine encephalomyelitis viruses (TMEV) are positive-strand RNA viruses that belong to the *Cardiovirus* genus of the family *Picornaviridae* (10, 11). TMEV isolates can be broadly classified into two neurovirulence groups on the basis of their lethality following intracerebral (i.c.) inoculation of mice: a highly virulent group (e.g., GDVII, FA, and Ask-1 strains), which causes a rapidly fatal encephalitis due to lytic infection of neurons, and a less virulent group (e.g., BeAn and DA strains), which produces chronic demyelinating disease due to persistent central nervous system (CNS) infection (8, 23). Like other picornaviruses, the TMEV have an unusually long 5′ untranslated region (5′ UTR) of approximately 1,060 nucleotides (nt). We have recently shown that nucleotide sequences in the 5′ UTR can function as an internal ribosome entry site and that deletion of sequences that contain predicted secondary structures seriously impairs this function (1). In addition to containing signals for protein synthesis, the 5′ UTR probably also contains determinants of RNA replication and virus packaging. In the case of poliovirus, small insertions, deletions, and point mutations in the 5′ UTR dramatically affect the efficiency of virus replication (6, 14, 17). However, mutations in the poliovirus 5′ UTR may also lead to a defect in translation but not in virus RNA replication (19). Poliovirus translational deficiency with attenuation has been associated with a single base change in the 5′ UTR (21, 22). The other murine cardioviruses, mengo virus and encephalomyocarditis virus, have long homopolymeric poly(C) tracts within the 5′ UTR. It has been demonstrated by Duke et al. (3–5) that changes in the length of the poly(C) tract, while having no effect on the growth of the virus in cell culture, drastically attenuate virulence after intraperitoneal or i.c. inoculation of mice.

In this paper, we describe the properties of a TMEV mutant which contains an insertion of 3 nt at position 665 (BeAn virus coordinates) in the 5′ UTR. This insertion was inadvertently synthesized during a polymerase chain reaction (PCR) amplifying the 5′ UTR of BeAn with primers P-1, 5′ gagagcatgcTTGAAAGGGGGCCCGGGGGA 3′, and P-2, 5′ TCCATGTTccatgGCCATATTGACA 3′. P-1 corresponds to sequences at the 5′ end of the 5′ UTR, and P-2 corresponds to sequences complementary to the 3′ end of the 5′ UTR, where lowercase letters represent sequences not present in BeAn. The 3-nt insertion is part of the stem-loop structure, designated H in the 5′ UTR (Fig. 1) (12). Suggestive evidence of involvement of stem-loop H in BeAn internal ribosome entry site function has been obtained from deletion analysis of expression of a reporter gene, luciferase (Luc), in a bicistronic expression vector (1).

The PCR-amplified DNA was cloned in M13mp18 (25) and sequenced by the dideoxy method (16), and an M13mp18 5′ UTR clone containing the 3-nt insert at position 665 was used for further study. Sequences of the 5′ UTR which contain this insertion are referred to as IN668 here. Recombinant expression vectors that contain either the wild-type 5′ UTR (pPB310 [1]) or the IN668 (pIN668) sequence inserted between two reporter genes, chloramphenicol acetyltransferase (Cat) and Luc, were constructed (Fig. 2A). A *Bgl*II site at viral nt 398 enabled construction of similar recombinant DNAs in which nt 1 to 398 were deleted from the 5′ UTR (pPB310Δ398 and pIN668Δ398). The DNAs, pPB310 and pIN668, were transcribed in vitro with T7 RNA polymerase to obtain bicistronic mRNAs. Different dilutions of the mRNAs were electrophoresed in a 1% formaldehyde agarose gel, and their relative amounts were estimated by Northern (RNA) blot analysis (15) using ^{32}P-labeled nick-translated Luc DNA for hybridization. Proteins labeled with ^{35}S were synthesized from comparable amounts of RNA with rabbit reticulocyte lysate (Promega) and Expre^{35}S^{35}S

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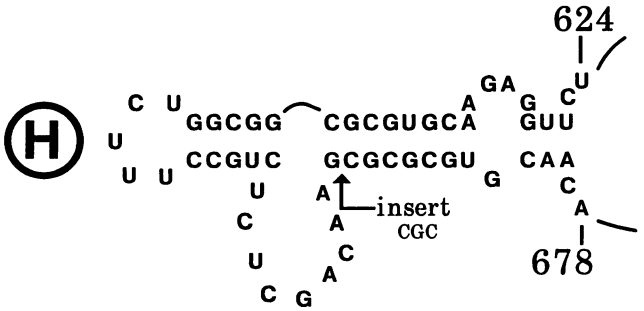

FIG. 1. H loop of the predicted secondary structure of the 5′ UTR of TMEV. The arrow shows the position of the insert.

protein labeling mix (NEN). The translation reaction was carried out at 30°C for 50 min with nonsaturating concentrations of RNA. The translation product was analyzed by electrophoresis in a sodium dodecyl sulfate–12% polyacrylamide gel (7). Figure 2B shows that both the wild-type 5′ UTR and IN668 efficiently support in vitro translation of Luc.

Five to ten micrograms of mRNA was also used to transfect BHK-21 cells with Lipofectin (GIBCO-BRL) (1). The RNA-Lipofectin mixture was incubated with the cells for 6 h, cell lysates were then prepared, and the levels of Cat and Luc activities were determined as described previously (2, 18). The maximum level of expression of the transfected RNA was observed under these conditions (1). In contrast to the results obtained with in vitro translation, Luc activity of transfected pIN668 RNA was reduced to about 3% of that of pPB310 (Fig. 2C). To control for efficiency of transfection in different experiments, the ratio of Luc activity to Cat activity was determined. In four independent experiments, BHK-21 cells were transfected with bicistronic mRNAs. The Luc activity/Cat activity ratio of the bicistronic mRNA Cat-IN668 5′ UTR-Luc(A_n) was normalized to that of mRNA Cat-BeAn 5′ UTR-Luc(A_n), and that ratio was 0.03, 0.02, 0.035, and 0.02 for the four experiments. These results show that pIN668 was highly inefficient in initiating Luc synthesis. Similar results were obtained when both the in vitro translations and the transfection studies were repeated with mRNAs prepared from pPB310Δ398 and pIN668Δ398 (data not shown). We previously found that deletion of nt 1 to 398 does not adversely affect the internal ribosome entry site function of the 5′ UTR (1). To determine whether the inefficiency of Luc expression was a reflection of RNA instability, the amount of the transfected RNA present in cells 6 h into the transfection process was determined. Transfection was done with ^{32}P-labeled RNA synthesized in the presence of [α-^{32}P]UTP (specific activity, 0.12 μCi/μmol). Cytoplasmic RNA was isolated as described by Sambrook et al. (15). In brief, cells were washed with ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions, scraped into microcentrifuge tubes and washed twice more, resuspended in 100 μl of buffer containing 140 mM NaCl–1.5 mM $MgCl_2$–10 mM Tris-HCl (pH 8.0)–0.5% Nonidet P-40 (Sigma Chemicals)–1.0 mM dithiothreitol–100 U of RNasin (Promega), vortexed briefly, and incubated on ice for 10 min. Unlysed cells, nuclei, and debris were pelleted by centrifugation, and the supernatant was extracted with phenol-chloroform and precipitated with ethanol. The precipitate was dissolved in loading buffer containing 6.6% formaldehyde, 50% formamide, 20 mM MOPS (morpholinepropanesulfonic acid)–8 mM sodium acetate (pH 7.0), and 1 mM EDTA (pH 8.0). An appropriate dilution of the transfecting RNA was also made in the loading buffer, and the RNA was electrophoresed in a 1% agarose gel containing formaldehyde. Figure 2D shows that at most twice as much full-length RNA was recovered from cells transfected with pPB310 as from cells transfected with pIN668. However, there was extensive degradation of input RNA; the degradation products are too small to expect active Luc synthesis. The difference in RNA recovery which may be assumed to parallel the stability of the transfected RNA does not account for the inefficient Luc expression by mRNA synthesized from pIN668. Thus, the defect in Luc expression by pIN668 is probably at the level of protein synthesis.

To determine the effect of this mutation on TMEV virulence, the mutation was introduced into a virulent TMEV clone, Chi-VL (Fig. 3A) (13), which consists of the 5′ 933 nt of BeAn virus (except for nt 33 and 41) and the remaining sequences from GDVII. The nt 33 and 41 do not influence the virulence of Chi-VL (13). Assembly of Chi-VL(IN668) was accomplished by replacing the *Bst*EII (nt 71)-to-*Bst*XI (nt 848) DNA fragment in Chi-VL with the corresponding region from pIN668. T7 RNA polymerase transcripts of Chi-VL(IN668) were then used to transfect BHK-21 cells. Progeny virus sequences were verified by isolation of RNA from Chi-VL(IN668)-infected BHK-21 cells, followed by cDNA synthesis, PCR amplification of cDNA between nt 527 and 1104, and sequencing of both DNA strands directly (13).

The growth properties of Chi-VL(IN668) were studied in both BHK-21 cells and 6-week-old CD-1 mice after i.c. inoculation. Plaque size and kinetics of growth of Chi-VL(IN668) in BHK-21 cells were not significantly different from those of the virulent Chi-VL or GDVII viruses (data not shown). The extensive secondary structure present in the 5′ UTR suggests that structural disruptions in this region may lead to temperature sensitivity. In the case of poliovirus, insertions in the 5′ UTR leading to temperature-sensitive virus have been reported (14, 23). The presence of additional nucleotides in the 5′ UTR raises the possibility that Chi-VL(IN668) is temperature sensitive. In fact, Chi-VL(IN668) was minimally temperature sensitive—the efficiency of plating (ratio of titer at 39°C to titer at 33°C) of Chi-VL was 0.84 and that of Chi-VL(IN668) was 0.018, while the corresponding values for GDVII and BeAn were 0.5 and $<10^{-5}$, respectively.

The temporal course of viral RNA and protein synthesis at early times postinfection in BHK-21 cells for Chi-VL(IN668) and Chi-VL at a multiplicity of infection of 15 was analyzed. Figure 3B shows that viral RNA synthesis was reduced in Chi-VL(IN668)-infected cells by threefold. Since actinomycin D-sensitive viral RNA synthesis has been reported for poliovirus mutants containing changes in the 5′ UTR (14, 24), virus-specific RNA synthesis in infected cells was monitored in the presence of actinomycin D (14). The ratio of the yield of virus in the presence of actinomycin D (actinomycin D present/actinomycin D absent ratio) for Chi-VL was 0.64, and that for Chi-VL(IN668) was 0.33. Thus, the reduced RNA synthesis levels observed for the mutant are due partly to its inherent actinomycin D sensitivity and partly to an RNA synthesis defect. As shown in Fig. 3C, the kinetics of accumulation of viral proteins were also slower in the case of Chi-VL(IN668), suggesting that reduced growth of Chi-VL(IN668) in BHK-21 cells is probably a reflection of reduced viral protein synthesis.

Chi-VL(IN668) was also relatively avirulent following i.c.

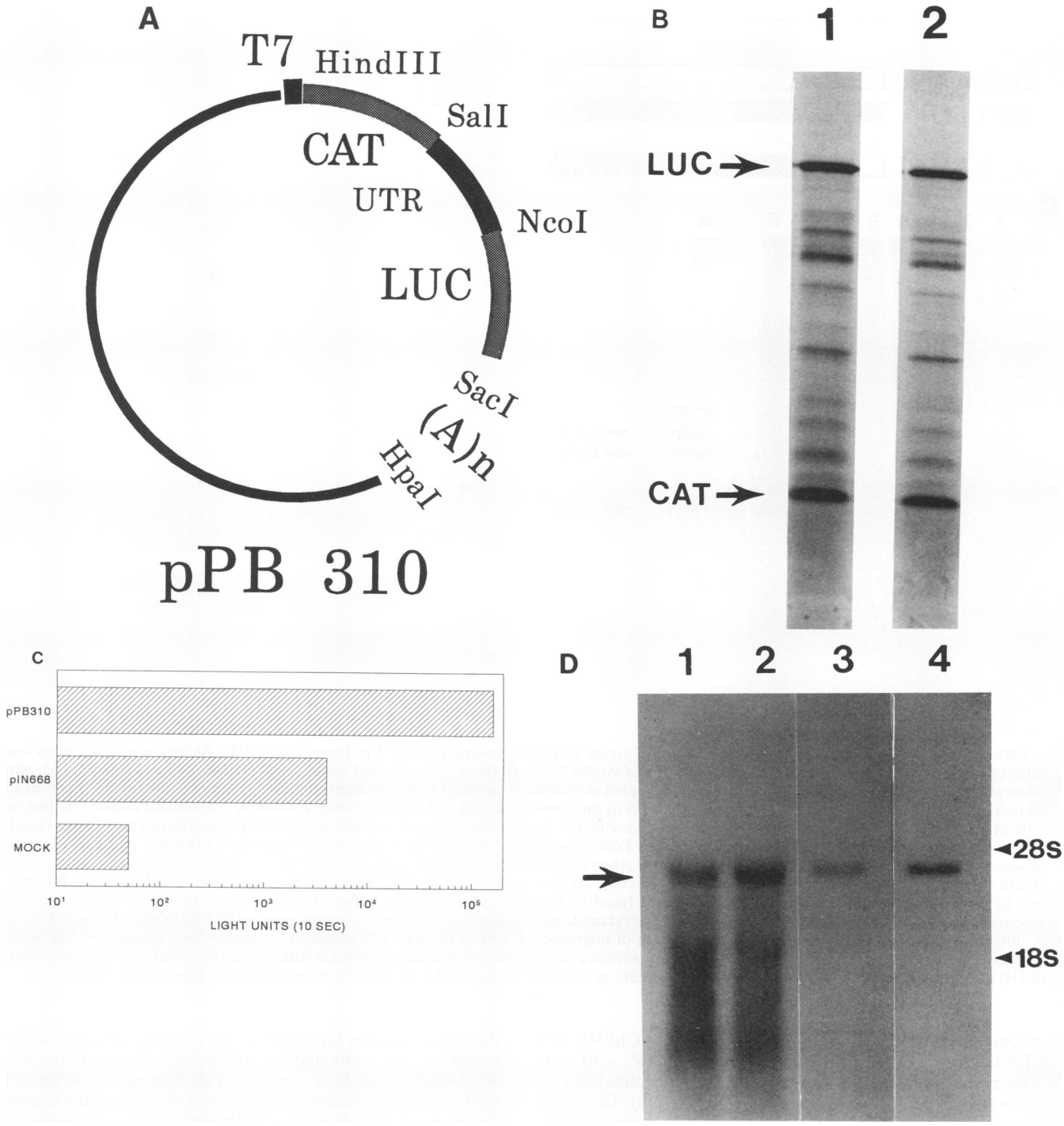

FIG. 2. (A) Bicistronic vector for the expression of Luc and Cat constructed in pGEM4. UTR represents 5′ UTR sequences from either BeAn (pPB310) or IN668 (pIN668). $(A)_n$, poly(A) sequences at the 3′ terminus of the T7 RNA polymerase transcript Cat-UTR-Luc. (B) Autoradiogram showing electrophoretic profile of [^{35}S]methionine-labeled protein synthesized in a rabbit reticulocyte lysate by bicistronic mRNAs Cat-5′ UTR(BeAn)-Luc (lane 1) and Cat-5′ UTR(IN668)-Luc (lane 2). The mobilities of Cat and Luc were previously determined with antibodies to Cat and Luc. (C) Luc activity in lysates of BHK-21 cells transfected with bicistronic mRNAs Cat-5′ UTR(BeAn)-Luc from pPB310 and Cat-5′ UTR(IN668)-Luc from pIN668. (D) Autoradiogram of ^{32}P-labeled RNA recovered from BHK-21 cells transfected with T7 RNA polymerase transcripts of pIN668 (lane 1) and pPB310 (lane 2), a 1/5,000 dilution of transcribed, input pIN668 RNA (lane 3), and a 1/5,000 dilution of transcribed, input pPB310 RNA (lane 4) electrophoresed in the same 1% agarose-formaldehyde gel. The arrow at left indicates the position of full-length mRNA. The arrowheads at right indicate positions of 28S and 18S rRNAs.

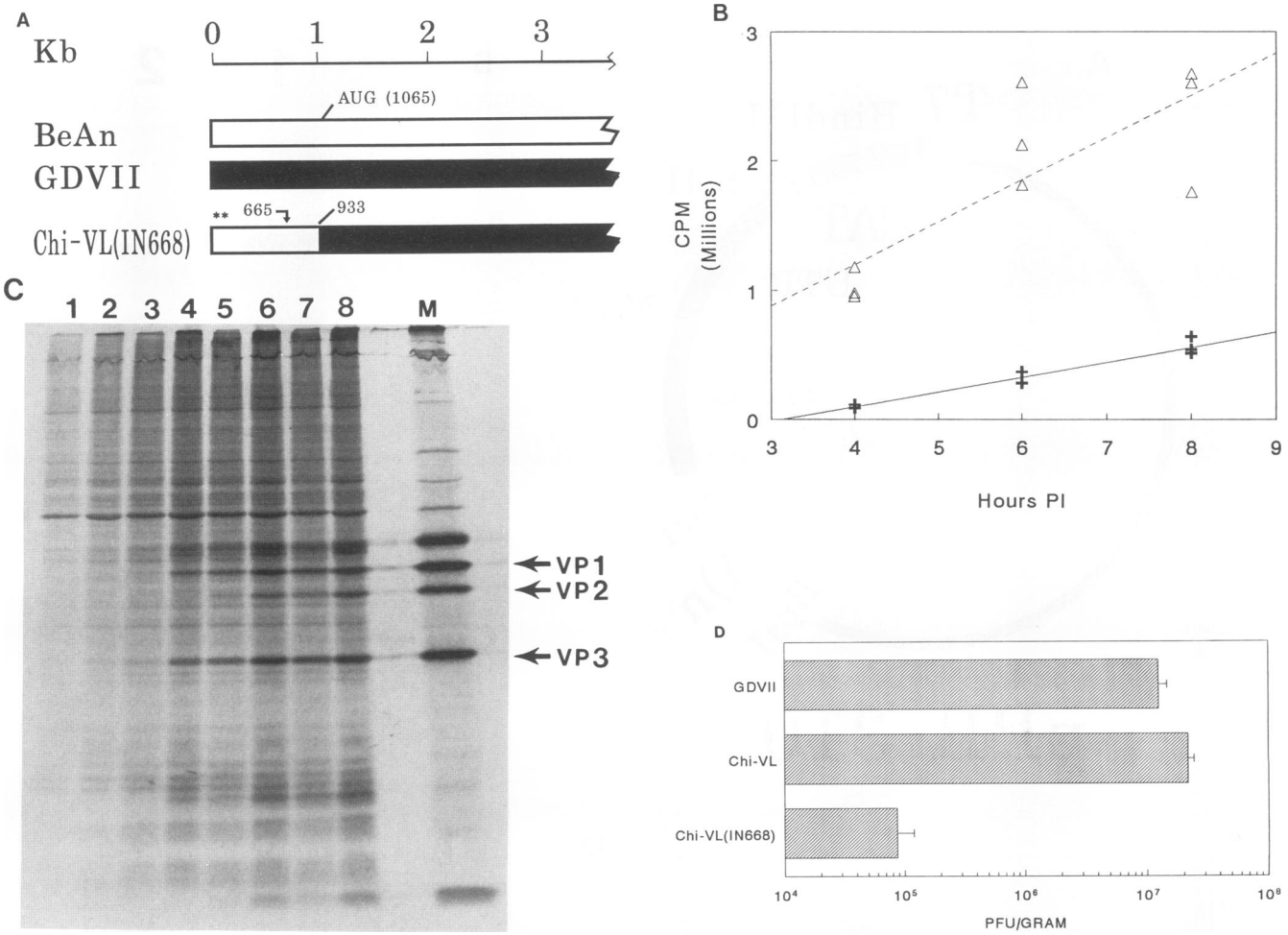

FIG. 3. (A) Structure of virus Chi-VL(IN668) with arrow indicating position of the 3-nt insert in Chi-VL. Asterisks indicate nucleotide differences between Chi-VL and BeAn. (B) Synthesis of viral RNA in BHK-21 cells infected with Chi-VL(IN668) (+) and Chi-VL (△). After adsorption of virus for 45 min at 22°C, cells were incubated in Dulbecco modified Eagle medium containing 5 μg of actinomycin D per ml and 20 μCi of [^{3}H]uridine per ml. Cells were washed in PBS at the times indicated and lysed in buffer containing 1% sodium dodecyl sulfate and 0.01 M EDTA (pH 7.5), nucleic acids were precipitated with 10% trichloroacetic acid, and the radioactivity was determined. Three samples were used for each time point. PI, postinfection. (C) Protein synthesis in BHK-21 cells infected with Chi-VL(IN668) and Chi-VL. After adsorption of virus for 45 min at 22°C, the cells were washed and Dulbecco modified Eagle medium without Met, supplemented with 150 μCi (1,130 Ci/mmol) of [^{35}S]Met per ml, was added to the cells. At 3 h (lanes 1 and 2), 5 h (lanes 3 and 4), 7 h (lanes 5 and 6), and 9 h (lanes 7 and 8) postinfection, cells were washed in PBS and lysed in 400 μl of immune lysis buffer. Ten microliters of lysate was analyzed by electrophoresis in a sodium dodecyl sulfate–12% polyacrylamide gel (7). Lanes 1, 3, 5, and 7, infection with Chi-VL(IN668); lanes 2, 4, 6, and 8, infection with Chi-VL. Lane M shows the position of migration of VP1, VP2, and VP3 obtained by immunoprecipitation of ^{35}S-labeled lysates of GDVII-infected BHK-21 cells. (D) CNS virus titers at day 5 after i.c. inoculation of mice with viruses derived from infectious clones of GDVII, Chi-VL, and Chi-VL(IN668). The mean titers ± standard errors of the mean for five animals in each group are shown.

inoculation of mice compared with the virulent Chi-VL and GDVII viruses (PFU/50% lethal dose were $>10^5$, <10, and <10, respectively). CNS virus titers were determined on day 5, the time of peak CNS virus growth (Fig. 3D) (8). The mean virus titer for GDVII- and Chi-VL-infected mice was 10^7 PFU/g of brain, whereas for Chi-VL(IN668)-infected mice, the titer was $<10^5$ PFU/g. While the less virulent TMEV cause hind limb paralysis in mice after i.c. inoculation, Chi-VL(IN668)-infected mice remained normal. The stability of the mutation in Chi-VL(IN668) was confirmed by directly sequencing PCR-amplified virus-specific cDNA between nt 527 and 809 (13) synthesized from total RNA isolated from BHK-21 cells infected with brain homogenates of Chi-VL(IN668)-infected mice.

Chi-VL(IN668) codes for the same protein sequences as the highly virulent GDVII virus; yet, it is relatively avirulent following i.c. inoculation of mice. Survival of Chi-VL(IN668) animals is associated with lower virus titers in the brain and efficient virus clearance (9). One of the reasons for the poor growth of Chi-VL(IN668) in vivo is probably the inefficient expression of viral RNA and proteins due to the insertion of the 3 nt in the H stem-loop of the 5′ UTR. It has been previously shown that the GDVII virus promotes virus translation more efficiently than the less virulent BeAn and DA viruses (1, 20). On the basis of the studies of neurovirulence of GDVII and DA chimeras, Stein et al. (20) suggested that translational efficiency is necessary but not sufficient for neurovirulence. Zurbriggen et al. (26) investigated the reduced neurovirulence of a variant DA strain of TMEV which was resistant to neutralization by a neutraliz-

ing monoclonal antibody to TMEV capsid protein VP-1. The replications of wild-type and variant viruses in tissue culture were comparable; however, growth in the spinal cord was restricted. Alterations in tropism in the CNS were not apparent, and the reduced neurovirulence was ascribed to the reduced replication or spread of the virus in the CNS of the infected animal.

Although our results suggest that the poor growth of Chi-VL(IN668) is probably due to inefficient translation, the possibility that insertion in the H loop also results in direct inhibition of RNA synthesis or virus packaging is not excluded. Further studies of virus replication using virus replicons with essential virus proteins provided in *trans* will help to elucidate the role of TMEV 5′ UTR sequences in virus RNA synthesis and packaging.

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