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A Tick-Borne Langat Virus Mutant That Is Temperature Sensitive and Host Range Restricted in Neuroblastoma Cells and Lacks Neuroinvasiveness for Immunodeficient Mice

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Langat virus (LGT), the naturally attenuated member of the tick-borne encephalitis virus (TBEV) complex, was tested extensively in clinical trials as a live TBEV vaccine and was found to induce a protective, durable immune response; however, it retained a low residual neuroinvasiveness in mice and humans. In order to ablate or reduce this property, LGT mutants that produced a small plaque size or temperature-sensitive (*ts*) phenotype in Vero cells were generated using 5-fluorouracil. One of these *ts* mutants, clone E5-104, exhibited a more than 10^3-fold reduction in replication at the permissive temperature in both mouse and human neuroblastoma cells and lacked detectable neuroinvasiveness for highly sensitive immunodeficient mice. The E5-104 mutant possessed five amino acid substitutions in the structural protein E and one change in each of the nonstructural proteins NS3 and NS5. Using reverse genetics, we demonstrated that a Lys_{46}→Glu substitution in NS3 as well as a single Lys_{315}→Glu change in E significantly impaired the growth of LGT in neuroblastoma cells and reduced its peripheral neurovirulence for SCID mice. This study and our previous experience with chimeric flaviviruses indicated that a decrease in viral replication in neuroblastoma cells might serve as a predictor of in vivo attenuation of the neurotropic flaviviruses. The combination of seven mutations identified in the nonneuroinvasive E5-104 mutant provided a useful foundation for further development of a live attenuated TBEV vaccine. An evaluation of the complete sequence of virus recovered from brain of SCID mice inoculated with LGT mutants identified sites in the LGT genome that promoted neurovirulence/neuroinvasiveness.

The antigenically related viruses of the tick-borne encephalitis virus (TBEV) complex of the *Flaviviridae* family that includes Langat virus (LGT) and Central European and Far Eastern tick-borne encephalitis, Kyasanur forest disease, Louping ill, Negishi, Omsk hemorrhagic fever, and Powassan viruses are endemic to the northern hemisphere, causing human disease of differing severity with up to 30% mortality (22). Since the amino acid identity among the structural envelope (E) glycoproteins of the tick-borne flaviviruses is 78% or greater, they share many protective E protein epitopes that can induce cross-resistance among members of this serogroup.

The naturally attenuated Malaysian TBEV, LGT strain TP21, is not associated with human disease under natural conditions. Infection with LGT induced cross-reactive neutralizing antibodies against TBEV and prevented disease caused by TBEV complex viruses in experimental animals (26, 29, 36, 38, 39, 41). Compared to more virulent members of the TBEV complex, LGT was found to be significantly attenuated with respect to neuroinvasiveness in mice as well as neurovirulence in monkeys (24, 27, 40). Subsequently, LGT TP21 (also known as the Yelantsev virus) was tested extensively as a live attenuated virus vaccine candidate in humans (14, 21, 37). This vaccine candidate was more effective in preventing tick-borne encephalitis in regions of endemicity than was an early version of formalin-inactivated TBEV vaccine (34, 37). However, further clinical study of the LGT vaccine candidate was stopped because of the rare occurrence of postvaccination neurological disease (1 per 20,000 vaccinations). Since LGT induced a durable protective immune response following a single immunization, an attempt to further attenuate this virus for humans appeared warranted. It was hoped that LGT attenuation for humans could be achieved by ablating the low level of residual neuroinvasiveness observed in mice.

A further-attenuated mutant of LGT, designated strain E5 (LGT E5), was selected at Johns Hopkins University by 42 consecutive passages of the wild-type (wt) LGT strain in embryonated chicken eggs (40). LGT E5 differed from the former LGT vaccine candidate by its lower neuroinvasiveness (ability of a virus to spread from a peripheral site of inoculation to the central nervous system [CNS] and cause disease) for mice and low neurovirulence in rhesus monkeys that were inoculated intracerebrally (i.c.) or intraspinally (24, 25, 27, 40). Based on the assessment of histological lesions in the CNS of rhesus monkeys, the attenuated LGT E5 exhibited a level of neurovirulence that was less than that of 17D yellow fever virus vaccine but greater than that of the attenuated poliovirus type 3 oral vaccine strain (24). Nevertheless, LGT E5 showed very little evidence of neuroinvasiveness in normal mice even when inoculated at a high dose; e.g., only 10 to 20% of adult Swiss mice inoculated intraperitoneally (i.p.) with 10^7 PFU of LGT E5 developed signs of encephalitis (27). However, LGT E5 retained a high level of neuroinvasiveness in highly susceptible severe combined immunodeficient (SCID) adult mice.

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In the present study, we sought to further attenuate LGT E5 by growing the virus in the presence of a mutagen and searching for progeny that exhibited a small plaque (*sp*) phenotype in Vero cells, a temperature-sensitive (*ts*) phenotype in Vero or neuroblastoma cells, or a host range (*hr*) phenotype characterized by decreased replication in murine or human neuroblastoma cells at 32°C. Small plaque or temperature-sensitive mutant viruses were sought first since such viruses are often associated with attenuation in animals and humans (6, 10, 15, 32, 35, 43). In addition, we attempted to identify LGT mutants with restricted replication in neuroblastoma cells since there is a correlation between reduction in replication in cells of neural origin and attenuation of virus in vivo as described for attenuated strains of poliovirus (1, 18), Japanese encephalitis virus (9), and TBEV chimeric viruses (33). In the present study, one LGT E5 progeny clone (E5-104) was identified which had the *sp*, *hr*, and *ts* phenotypes in cell culture and had decreased neuroinvasiveness in SCID mice. The genetic basis of this set of phenotypes was examined by introducing the mutations that were identified in E5-104 virus singly or in combination into LGT E5 by using reverse genetics and evaluating the recombinant viruses in vitro for the *sp*, *ts*, and *hr* phenotypes and in vivo for neuroinvasiveness in SCID mice.

MATERIALS AND METHODS

Cell cultures and viruses. Simian Vero cells (World Health Organization seed, passage 143) were maintained in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Cambrex, Walkersville, MD), 2 mM L-glutamine (Invitrogen), and 0.05 mg/ml of gentamicin (Invitrogen). Mouse Neuro-2A neuroblastoma cells were purchased from the American Type Culture Collection and maintained in MEM supplemented with 10% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (Quality Biological, Gaithersburg, MD). Human SH-SY5Y neuroblastoma cells (passage 26) were kindly provided by E. Dragunsky (CBER/FDA, Bethesda, MD) and maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% FBS and 0.05 mg/ml of gentamicin.

The wt LGT strain TP21 and its further-attenuated E5 derivative were prepared as described previously (7). Both viruses were plaque purified three times in Vero cells under soft agar and finally amplified by two passages in Vero cells. The titers of virus in Vero cells were 2.4×10^8 PFU/ml for LGT strain TP21 and 1.2×10^8 PFU/ml for E5 as determined by an immunostaining plaque-forming assay (25). The recombinant E5 virus (clone E5-651) recovered from a full-length infectious cDNA was described previously (25) and had an additional passage in Vero cells. The nucleotide (nt) sequences of LGT TP21 and LGT E5, which is the parent of the 5-fluorouracil (5-FU) mutants, were determined previously and assigned GenBank accession no. AF253419 and AF253420, respectively (7).

Chemical mutagenesis of LGT E5. Confluent monolayers of Vero cells were infected with LGT E5 at a multiplicity of infection (MOI) of 0.01 and incubated for 1 h at 37°C. Infected cells were then overlaid with MEM supplemented with 2% FBS and 5-FU (Sigma, St. Louis, MO) at concentrations ranging from 10 μM to 10 mM. After incubation at 32°C for 5 days, cell culture medium was harvested, clarified by centrifugation, and frozen at −70°C. Clarified supernatants were then assayed for virus titers on Vero cells by using a plaque-forming assay in which foci of infection are identified by immunostaining as described previously (25). Virus titer in the supernatant from Vero cells treated with 1 mM 5-FU was 1,000-fold reduced compared to that of the virus derived from untreated cells. The virus suspension harvested from the 1 mM 5-FU-treated cells was terminally diluted to derive clones for phenotypic characterization. Briefly, 96-well plates of Vero cells were inoculated with the 5-FU-treated virus at an MOI that yielded 10 or fewer virus-positive wells per plate. After 5 days of incubation at 32°C, cell culture medium from the 96-well plates was removed and stored at −70°C, and the virus-infected cell monolayers were identified by immunostaining. Virus from each positive well was transferred to confluent Vero cells in six-well plates for amplification at 32°C. Cell culture medium was harvested from individual wells 6 to 7 days later, clarified by centrifugation, aliquoted, and frozen at −70°C. The titer of each virus clone was determined by plaque titration on Vero cell monolayers at 32°C. A total of 346 LGT E5 clones were prepared from the 1 mM 5-FU-treated virus suspension.

Identification of LGT 5-FU mutants with *sp* size and *ts* phenotypes. The plaque size of each of the 346 5-FU virus clones on Vero cell monolayers was compared to that of its LGT E5 parent. After virus adsorption for 1 h at 37°C, the infected cell monolayers in 24-well-plates were overlaid with MEM supplemented with 0.8% methylcellulose (EM Science, Gibbstown, NJ), 2% FBS, gentamicin, and L-glutamine. After incubation of replicate plates for 4 days at 37°C, plaques were visualized by immunostaining and counted. Twenty-seven 5-FU mutant viruses with mean plaque diameters of ≤0.5 mm in Vero cells (≤25% the size of parental E5 virus) were selected and designated as having the *sp* phenotype.

The 346 mutant virus clones were also compared with the E5 parent for their level of replication in mouse Neuro-2A cells at 37°C. Cell monolayers in duplicate wells of 24-well-plates were inoculated with virus at an MOI of 0.01 PFU/cell and incubated at 37°C for 4 days in L-15 medium (Quality Biological) supplemented with 2% FBS and gentamicin. The yield of virus in the culture medium was determined by plaque titration in Vero cells. Nineteen mutants that had an *sp* phenotype on Vero cells exhibited a 100-fold or greater reduction in titers in mouse Neuro-2A cells at 37°C compared to that of the E5 parent. These 19 selected viruses were terminally diluted three times and amplified in Vero cells at 32°C.

The 19 selected mutant clones were next screened for temperature sensitivity by assessing virus replication at 32° and 37°C in Vero, Neuro-2A, or SH-SY5Y cells. Cell monolayers in duplicate wells of 24-well-plates were inoculated with mutant virus at an MOI of 0.01 in MEM supplemented with gentamicin and 2% FBS. Cells were incubated at 32° or 37°C for 4 days, and the yield of virus in culture medium was determined by plaque titration in Vero cells at 32°C. Any mutant virus that exhibited a 100-fold-or-greater reduction in titer at 37°C relative to its titer at 32°C was designated as having a *ts* phenotype. We also identified mutants that were restricted in replication in neuroblastoma cell lines at a permissive temperature, and viruses that exhibited a 100-fold-or-greater reduction in titer in neuroblastoma cells at 32°C compared to that in Vero cells were designated as having an *hr* phenotype.

Generation of recombinant LGT E5 mutants containing single or combinations of mutations. A full-length infectious cDNA clone (pE5-651), derived from LGT E5-651 (25), was used in this study to generate the recombinant LGT mutants bearing mutations present in the 5-FU mutant virus E5-104, since an infectious clone with the exact consensus sequence of the E5 parent did not yield a viable virus. E5-651 differed in sequence from the E5 parent by eight nucleotides, of which three produced an amino acid substitution in the envelope protein E (Glu_{149}→Gly and Glu_{291}→Gly) and nonstructural protein NS4B (Ala_{183}→Val). Similar to its E5 parent, the recombinant E5-651 clone was not neuroinvasive in normal adult mice when a large dose (10^6 PFU) was used for intraperitoneal inoculation, but both E5 and E5-651 were highly neuroinvasive in SCID mice (25).

Each nucleotide substitution (G_{1325}→U, C_{1410}→A, $CA_{1324\text{-}5}$→AC, C_{1770}→U, or A_{1913}→G) that was identified in the E gene of the 5-FU mutant virus E5-104 and that produced an amino acid substitution (Val_{119}→Leu, Thr_{147}→Asn, Thr_{205}→Asn, Ser_{267}→Leu, or Lys_{315}→Glu, respectively) was introduced separately into the infectious pE5-651 cDNA clone by site-directed mutagenesis as follows. The cDNA fragment from nt 121 to 2784 of the E5-651 genome that was flanked with SfiI and XmaI cleavage sites was subcloned into vector pUC18. Each nucleotide substitution at the position in E indicated above was engineered in this fragment using the QuikChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) with primers that contained the targeted substitution. The mutant DNA fragment was cloned back into the pE5-651 cDNA clone. The presence of the introduced mutation in each construct was confirmed by sequence analysis. The five resulting cDNA plasmids that each encoded a unique mutation in E were designated p651/E-Leu_{119}, p651/E-Asn_{147}, p651/E-Asn_{205}, p651/E-Leu_{267}, and p651/E-Glu_{315}.

To generate cDNA plasmids encoding the single mutations in NS3 (Lys_{46}→Glu) or NS5 (Asn_{17}→Asp) from mutant virus E5-104, the PCR-amplified cDNA fragment from the E5-104 genome between the SpeI and ApaI sites (nt 3661 to 4801) or between the ApaI and AgeI sites (nt 4801 to 9737), respectively, was used to replace the corresponding sequence of the E5-651 genome. The presence of the introduced mutation in the NS3 or NS5 gene was confirmed by sequence analysis, and the final cDNA plasmids were designated p651/NS3-Glu_{46} and p651/NS5-Asp_{17}.

The same strategy was used to generate a cDNA plasmid encoding the set of five mutations newly identified in the E protein of mutant virus E5-104. In this case, the PCR-derived cDNA fragment from the E5-104 genome between the SfiI site (nt 133) and the SpeI site (nt 3661) was used to replace the correspond-

ing sequence of the E5-651 genome. The full-length cDNA plasmid encoding the set of five mutations in E was designated p651/E_{box}.

Recovery and in vitro characterization of recombinant mutant viruses in cell culture. Full-length RNA transcripts were prepared from the above-described cDNA plasmids by in vitro transcription with SP6 RNA polymerase and used for the transfection of Vero cells as described previously (25). Recovered viruses were terminally diluted two times and amplified in Vero cells at 32°C. The complete sequence of the resulting recombinant mutant viruses was determined, and the presence of the introduced mutations was confirmed.

The recombinant mutant viruses as well as E5, E5-651, and E5-104 were evaluated in a comparative study for temperature sensitivity by assessing virus replication at 32, 34, 35, 36, 37, 38, or 39°C in Vero, Neuro-2A, or SH-SY5Y cells. Cell monolayers grown on 24-well-plates were infected with virus at an MOI of 0.01 PFU/cell for 1 h at room temperature, after which fresh medium was added. Infected cells were incubated at the indicated temperature for 4 days, and the yield of virus in culture medium was determined by plaque titration in Vero cells at 32°C. Mutant virus that exhibited a 100-fold-or-greater reduction in titer at 37°C compared to that for its titer at 32°C was designated as having a *ts* phenotype.

Multicycle growth of LGT E5, E5-104 mutant, and recombinant E5 viruses in Vero, Neuro-2A, and SH-SY5Y cells. The kinetics and level of replication of the biologically derived LGT parents (TP21 and E5), E5-104 5-FU mutant, and the recombinant cDNA-derived E5 viruses were compared in Vero, Neuro-2A, and SH-SY5Y cell lines at permissive and nonpermissive temperatures. Cells grown on 24-well plates were infected with virus at an MOI of 0.01 PFU per cell and were allowed to adsorb for 1 h at room temperature, after which fresh medium was added. Infected cells were incubated at 32 or 37°C. Virus in culture medium from two wells was harvested daily, and the titer in each well was determined in Vero cells. Plaques were enumerated after 4 days of infection using the immunostaining plaque-forming assay (25).

Evaluation of LGT in mice. The neurovirulence of parental E5 and the E5-104 mutant was assessed in suckling Swiss Webster mice (Taconic Farms, Germantown, NY) by intracerebral inoculation. Three-day-old mice in groups of 10 were inoculated with decimal dilutions of E5-104 mutant ranging from 10 to 10^4 PFU, 10 or 10^2 PFU of E5 parent, or MEM. Mice were monitored for 21 days for the development of encephalitis or death. Moribund mice were humanely euthanized.

The LGT E5, E5-104, cDNA-derived E5 clone 651, and recombinant mutant viruses generated in this study were analyzed for neuroinvasiveness by intraperitoneal inoculation of 3-week-old SCID (*ICRSC-M*) mice (Taconic Farms) in groups of 5 or 10. Mice were inoculated with a dose of 10^3 or 10^5 PFU of virus and were observed for 28 days for the development of encephalitis.

Sequence analysis of viral genome. The nucleotide sequence of the genome of E5 5-FU or recombinant mutants recovered from cDNA was determined as described previously by sequence analysis of four overlapping RT-PCR fragments which were derived directly from virus RNA (25).

The sequence of the LGT isolates from the brains of moribund mice was determined as follows. Brains of moribund mice, which exhibited signs of advanced encephalitis after intracerebral or intraperitoneal inoculation, were harvested and individually homogenized in a 10% suspension of phosphate-buffered Hanks' balanced salt solution containing 7.5% sucrose, 5 mM sodium glutamate, 5 μg/ml gentamicin, 2.5 μg/ml amphotericin B (Quality Biological) and 60 μg/ml clindamycin (Pharmacia & Upjohn, Kalamazoo, MI). Total RNA from 100 μl of 10% brain suspension was extracted and used for reverse transcription and PCR, and the consensus sequence of the RT-PCR products was determined.

Molecular structure modeling of the LGT E protein. The three-dimensional structure of soluble ectodomain fragment of the TBEV envelope protein (Protein Data Base entry code, 1SVB) was used for modeling of LGT E protein as a starting point (31). The amino acid sequence of LGT E5 envelope protein was submitted to the Swiss-Protein automatic comparative modeling server (http://swissmodel.expasy.org), where the energy-minimized homology modeling was performed, and the data obtained were visualized by Deep View Swiss-Pdb Viewer version 3.7 software (http://swissmodel.expasy.org/spdbv/). High quality pictures were generated using Pov-Ray v3.6 software (http://www.povray.org/).

RESULTS

Generation and selection of LGT 5-fluorouracil mutants. LGT E5 was mutagenized using 1 mM 5-FU in Vero cell cultures that were maintained at 32°C. The 346 E5 virus clones that were generated were compared with parental E5 virus for plaque size on simian Vero cell monolayers at 37°C and level of replication at 37°C in mouse Neuro-2A cells. Twenty-seven mutants with mean plaque diameters that were ≤25% of the size of parental E5 virus on Vero cells were selected and designated as having an *sp* phenotype. Nineteen mutants that had an *sp* phenotype on Vero cells exhibited a 100-fold-or-greater reduction in replication in murine neuroblastoma cells at 37°C compared to that of the E5 parent. After cloning by terminal dilution, these selected mutants were evaluated for temperature sensitivity by assessing the virus replication in Vero, Neuro-2A, or SH-SY5Y cell lines at 32°C or 37°C. Each of the 19 mutants tested had a *ts* phenotype on Vero or Neuro-2A cells, and only one clone (E5-104) was *ts* in SH-SY5Y cells. Next, we identified viruses that had alterations in their host range, i.e., they were restricted in replication in neuroblastoma cells at permissive temperature (32°C) but replicated efficiently in Vero cells at that temperature. This *hr* phenotype was defined as a 100-fold-or-greater reduction in virus titer in neuroblastoma cells at 32°C compared to that for Vero cells. The 19 5-FU viruses which had *ts* phenotypes in both Vero and Neuro-2A cells did not significantly differ from parental E5 virus in the titer of Vero cells at 32°C. Three of the 19 had an *hr* phenotype in murine neuroblastoma cells, but only 1 clone (E5-104) among the 3 *hr* mutants was found to have an *hr* phenotype in human neuroblastoma cells.

In vitro characterization of E5-104. The plaque diameter of E5-104 (0.18 mm) on Vero cell monolayers (Fig. 1A) was approximately 12-fold smaller than that of its E5 parent virus. Since E5-104 was decreased in replication in murine neuroblastoma cells during an initial screening of the 5-FU mutant viruses at 37°C, its temperature sensitivity in cells of neural origin was evaluated in more detail. First, the magnitude of replication of wt LGT, E5, and E5-104 were compared in murine and human neuroblastoma cells as well as in Vero cells on day 4 postinfection over a broad range of temperatures (32° to 39°C) (Fig. 1B). LGT grew efficiently in each of the three cell cultures, reaching a titer ranging from 7.0 to 8.0 $\log_{10}$ PFU/ml with less than a 100-fold reduction in titer at any elevated temperature. E5 replicated efficiently and yielded a high virus titer in each cell line at temperatures less than 38°C; however, it manifested a reduction in replication of greater than 1,000-fold in the Neuro-2A cell line at 38°C, and virus replication was not observed in each of the cell lines at 39°C. Since E5 was not 100-fold restricted in replication at 37°C in any of the three cell lines, it was considered not to have the *ts* phenotype. In contrast, E5-104 was *ts* in each of the three cell lines and exhibited a greater degree of temperature sensitivity compared to that of E5, with complete restriction of replication observed at ≥35°C in murine neuroblastoma cells and at ≥37°C in Vero or human neuroblastoma cells (Fig. 1B). For that reason, the kinetics of replication of E5-104 at 32°C and 37°C were compared to those of wt LGT and E5 by infecting Vero or neuroblastoma cells at an MOI of 0.01 PFU/cell and measuring virus yield at 24-h intervals (Fig. 1C). LGT and E5 replicated efficiently in Vero or neuroblastoma cells at 32°C or 37°C with virus titers reaching 6.8 to 7.9 $\log_{10}$ PFU/ml on day 5 postinfection. The growth of E5-104 virus at 37°C in each of the three cell lines was dramatically restricted. At the permissive temperature of 32°C, E5-104 replicated moderately efficiently in Vero cells, attaining a titer of 5.7 $\log_{10}$ PFU/ml that

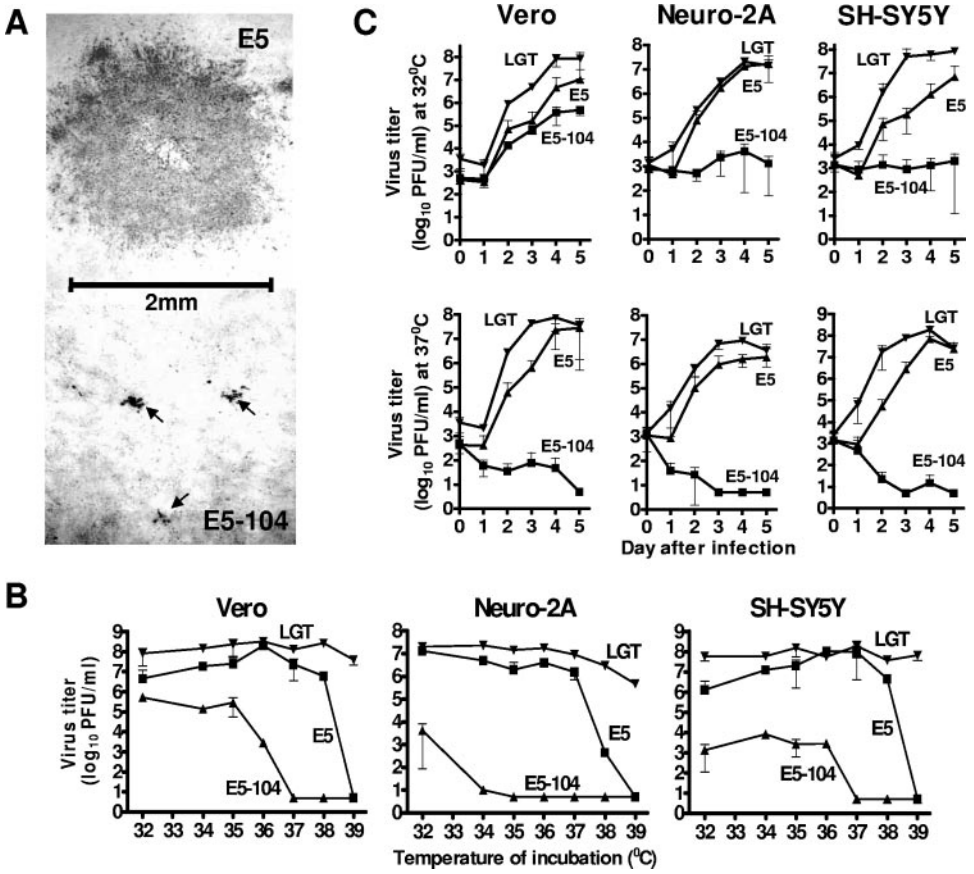

FIG. 1. Plaque morphology and growth analysis of parental E5 virus and its E5-104 derivative in Vero cells and growth in neuroblastoma cells of human or murine origin. (A) Plaques of E5 and E5-104 on Vero cell monolayers were visualized using immunoperoxidase staining on day 4 postinfection. The mean plaque diameter of 11 randomly selected E5 plaques was 2.2 mm. Plaques of E5-104 (mean diameter of 0.18 ± 0.02 mm; $n = 9$) are indicated by arrows. (B) Growth of the wt LGT, E5, and E5-104 viruses in Vero and neuroblastoma cell cultures at temperatures ranging from 32°C to 39°C. Confluent cell monolayers in 24-well plates were infected with parental or mutant virus at an MOI of 0.01 and incubated at the indicated temperature for 4 days. Virus titers in cell culture medium were determined by plaque assay in Vero cells incubated at 32°C. The limit of detection was 0.7 $\log_{10}$ PFU/ml. Bars represent the standard error of three to four separate experiments. (C) Replication kinetics of wt LGT, E5, and E5-104 viruses in Vero cells or in Neuro-2A or SH-SY5Y neuroblastoma cells following incubation at 32°C or 37°C. Virus was inoculated at an MOI of 0.01, and the virus titer in cell culture medium was determined in Vero cells cultivated at 32°C.

was 20 times lower than that of E5. E5-104 was highly restricted in replication at 32°C in either murine or human neuroblastoma cells compared to its replication in Vero cells, indicating that its replication was both host range restricted and temperature sensitive in both neuroblastoma cell lines.

Neuroinvasiveness and neurovirulence of E5-104 in mice. Next, the neuroinvasiveness and neurovirulence of E5-104 in mice were determined by an i.p. or i.c. route of inoculation, respectively. As observed previously (27), E5 exhibited extremely low residual neuroinvasiveness for adult Swiss mice (i.p. 50% lethal dose [LD_{50}] of $>10^7$ PFU) but remained highly virulent for SCID mice (i.p. LD_{50} of 0.06 PFU). Unlike its E5 parent, E5-104 was significantly attenuated in SCID mice (i.p. LD_{50} of $>10^5$ PFU). Specifically, groups of 10 adult SCID mice that were inoculated i.p. with E5-104 in a dose ranging from 10^2 to 10^5 PFU remained free of disease. The E5-104 mutant was at least $10^{6.2}$-fold-less neuroinvasive in SCID mice than was E5. Since E5-104 possessed the *sp*, *ts*, and *hr* phenotypes in vitro, it was not possible to determine which of the three phenotypes correlated with the attenuation (*att*) phenotype in mice, characterized by loss of neuroinvasiveness.

Newborn mice are sensitive animal models for measuring the neurovirulence of neurotropic flaviviruses, i.e., the ability to cause CNS disease following direct intracerebral inoculation (2, 28). E5 was previously shown to be highly neurovirulent with an i.c. LD_{50} of 20 PFU in suckling Swiss mice (27). In the present study, a substantial decrease in neurovirulence of the E5-104 virus compared to that of its E5 parent was not observed when assayed by i.c. inoculation of 3-day-old Swiss mice (data not shown). None of the mice inoculated with 100 PFU of E5 or its E5-104 mutant survived, and only 10 to 20% of mice inoculated with 10 PFU of either virus survived during a 21-day observation period. Nevertheless, the onset of encephalitis or death of mice inoculated with 10 or 100 PFU of E5-104 mutant virus was delayed by 3.9 or 4.1 days compared to that of mice inoculated with a similar dose of E5, suggesting that this mutant replicated more slowly in the brain of newborn mice. This increase in an average survival time of mice inocu-

TABLE 1. Sequence differences among E5, E5-104, and brain-passaged E5-104

Gene	Nucleotide position (genome)	Nucleotide in indicated virus				Amino acid change
		E5[a]	E5-104	E5-104 isolate from mouse brain[c]		
				Isolate 1	Isolate 2	
E	1306	C	U	U	U	None
	1325	G	U	U	U	Val_{119}→Leu
	1410	C	A	A	A	Thr_{147}→Asn
	1584–1585	CA	AC	AC	AC	Thr_{205}→Asn
	1708	G	U	U	U	None
	1770	C	U	C[b]	C[b]	Ser_{267}→Leu
	1913	A	G	G	G	Lys_{315}→Glu
NS3	4733	A	G	G	G	Lys_{46}→Glu
NS5	7712	A	G	G	G	Asn_{17}→Asp
	7801	A	G	G	G	None

[a] Results show the nucleotide sequence of E5 (GenBank accession no. AF253420) at the indicated positions in the genome.

[b] The underlined sequence change represents the difference between E5-104 and its mouse brain-passaged isolate.

[c] Virus isolate 1 was obtained on day 13 from the brain of a moribund mouse that was inoculated i.c. with 10 PFU of E5-104. Virus isolate 2 was harvested on day 12 from the brain of a moribund mouse that was inoculated i.c. with 100 PFU of E5-104.

lated i.c. with E5-104 mutant is consistent with its decreased neuroinvasiveness in SCID mice.

Nucleotide sequence analysis of E5-104. An examination of the genetic basis for the *sp*, *ts*, and *hr* phenotypes of E5-104 as well as its attenuation for SCID mice was initiated by the determination of its complete nucleotide sequence, which was compared with the previously published consensus sequence of the E5 genome (7) (Table 1). For the two viruses, there were 11 nucleotide differences in the sequences that were located within the coding regions of the genomes. Seven of these mutations resulted in amino acid substitutions, five in E (Val_{119}→Leu, Thr_{147}→Asn, Thr_{205}→Asn, Ser_{267}→Leu, and Lys_{315}→Glu) and one each in NS3 (Lys_{46}→Glu) or NS5 (Asn_{17}→Asp).

Mutations in brain isolates of E5-104. The delay in the onset of encephalitis and death of mice infected i.c. with 10 or 100 PFU of E5-104 versus that in mice infected with E5 suggested that mutations that permitted more efficient replication in the brain might have developed in E5-104 during its replication in this organ. In an attempt to better understand this delay, the brains of two moribund mice that exhibited signs of encephalitis were harvested, and the complete sequence of their genome was determined (Table 1). Both brain isolates differed from the E5-104 virus used to initiate infection by the nucleotide mutation (U_{1770}→C) located in structural protein E gene that resulted in the reversion of Leu_{267} to Ser. Because of the low dose of the biologically cloned preparation of E5-104 used for inoculation (one mouse received 10 PFU, and the other received 100 PFU), it is likely that the Leu_{267}→Ser mutation developed independently in the brains of the two mice rather than being present in the inoculum. This independent reversion to the sequence of the E5 parent suggests that the amino acid at position 267 in E protein contributes to the neurovirulence of LGT in mice, and the results reported below are consistent with this possibility.

Introduction of E5-104-specific mutations into the full-length infectious cDNA clone of E5 and recovery of viable virus progeny. To determine which of the seven amino acid substitutions identified in E5-104 (Table 1) specified the *sp*, *ts*, or *hr* phenotype in vitro and the *att* phenotype in vivo, each of the E5-104-specific mutations was introduced individually into the infectious full-length cDNA of E5 (clone pE5-651) (25). Since an infectious cDNA clone with the exact sequence of E5 was not available, pE5-651 was used in its place. As shown in Table 2, recombinant E5-651 differed from E5 virus by eight nucleotide changes (all located within the coding region of the genome) of which three resulted in an amino acid substitution in E (Glu_{149}→Gly and Glu_{291}→Gly) or NS4B (Ala_{183}→Val). Despite this difference in sequence, E5-651 retained the full set of in vitro and in vivo properties of the E5 parent (25), and these three amino acid substitutions are therefore considered noncontributory to the properties of the E5-651 recombinant derivatives bearing one or more of the introduced E5-104 mutations. The seven individual mutations in E5-104 were introduced into E5-651, but only five recombinant derivatives were recoverable and these are designated 651/E-Leu_{119}, 651/E-Asn_{147}, 651/E-Asn_{205}, 651/E-Glu_{315}, and 651/NS3-Glu_{46}. Sequence analysis of the genomes of these five recombinant viruses confirmed the presence of the introduced mutations. Only recombinant 651/E-Glu_{315} has an additional adventitious mutation, a G_{4866}→A change in NS3 that resulted in Ser_{90}→Asn substitution. This spontaneous change was not present in the plasmid DNA from which the virus was derived, suggesting that it occurred during virus recovery or amplification in Vero cells.

Viable recombinant virus containing either amino acid substitution Ser_{267}→Leu in E or Asn_{17}→Asp in NS5 was not obtained despite several attempts at recovery, suggesting that these mutations are lethal in the E5-651 background. Also, recombinant virus was not recovered when a combination of two (Lys_{46}→Glu in NS3 and Asn_{17}→Asp in NS5) or six (Val_{119}→Leu, Thr_{147}→Asn, Thr_{205}→Asn, Ser_{267}→Leu, and Lys_{315}→Glu in E and Asn_{17}→Asp in NS5) mutations identified in E5-104 virus were introduced into the E5-651 cDNA clone.

Since virus containing amino acid substitution Ser_{267}→Leu in E protein was not viable, the contribution of this mutation to the *sp*, *ts*, or *hr* phenotype of the E5-104 mutant could not be directly evaluated. For this reason, the recombinant 651/E_{box} virus that contains five amino acid changes observed in the E5-104 E protein, including the Ser_{267}→Leu substitution, was recovered. There were 15 nt differences between the consensus sequence of 651/E_{box} and E5-651 (Table 2) that resulted in nine amino acid substitutions in only the envelope glycoprotein E. These amino acid differences had three distinct origins. First, five changes (Val_{119}→Leu, Thr_{147}→Asn, Thr_{205}→Asn, Ser_{267}→Leu, and Lys_{315}→Glu) were the desired amino acid substitutions present in the E5-104 mutant. Two spontaneous amino acid changes (Asn_{135}→Asp and Asn_{470}→Ser) occurred during the 651/E_{box} virus recovery, while the remaining two amino acid changes (Gly_{149}→Glu and Gly_{291}→Glu) were the result of cloning the E gene region of E5-104 into E5-651 cDNA, since the E protein sequences of E5-651 and E5-104 differ at these two amino acids as indicated above. Thus, phenotypic differences between 651/E_{box} and E5-651 need to be interpreted in the context of the presence of these additional sequence differences.

TABLE 2. Sequence differences among E5, E5-651, E5-104, and recombinant cDNA rescued mutants[a]

Gene	Nucleotide position (genome)	Nucleotide in indicated virus[c]									Amino acid change
		E5	E5-651	E5-104	Recombinant E5-651 mutant derivative						
					651/E-Leu_{119}	651/E-Asn_{147}	651/E-Asn_{205}	651/E-Glu_{315}	651/NS3-Glu_{46}	651/E_{box}	
prM	673[b]	C								U	None
E	1306	C		U						U	None
	1325	G		U	U					U	Val_{119}→Leu
	1373[b]	A								G	Asn_{135}→Asp
	1410	C		A		A				A	Thr_{147}→Asn
	1416	**A**	**G**		**G**	**G**	**G**	**G**	**G**		**Glu_{149}→Gly**
	1584–1585	CA		AC			AC			AC	Thr_{205}→Asn
	1708	G		U						U	None
	1770	C		U						U	Ser_{267}→Leu
	1842	**A**	**G**		**G**	**G**	**G**	**G**	**G**		**Glu_{291}→Gly**
	1913	A		G				G		G	Lys_{315}→Glu
	2379[b]	A								G	Asn_{470}→Ser
NS1	**3046**	**U**	**C**		**C**	**C**	**C**	**C**	**C**		**None**
	3154	**U**	**C**		**C**	**C**	**C**	**C**	**C**		**None**
NS3	**4528**	**U**	**C**		**C**	**C**	**C**	**C**		**C**	**None**
	4733	A		G					G		Lys_{46}→Glu
	4866[b]	G						A			Ser_{90}→Asn
NS4B	**6955**	**C**	**U**		**U**	**U**	**U**	**U**	**U**	**U**	**None**
	7294	**G**	**A**		**A**	**A**	**A**	**A**	**A**	**A**	**None**
	7455	**C**	**U**		**U**	**U**	**U**	**U**	**U**	**U**	**Ala_{183}→Val**
NS5	7712	A		G							Asn_{17}→Asp
	7801	A		G							None

[a] The sequence differences between cDNA-derived E5-651 and biological E5 parent (GenBank accession no. AF253420) are in bold letters. Mutations that were engineered in the recombinant viruses are indicated by underlined letters.
[b] Mutation that was present in only the recombinant E5-651 derivative.
[c] An empty cell indicates no change from the E5 virus sequence.

In vitro phenotypes of recombinant E5-651 mutants. The recombinant E5-651 mutants were evaluated for *sp* (Fig. 2A), *hr* (Fig. 2B and Table 3), and *ts* (Table 3) phenotypes in vitro and for neuroinvasiveness in SCID mice (Table 4). Results of this analysis are summarized in Table 5. The plaque size of recombinant mutant viruses was consistently smaller in Vero cells at 32° or 37°C than that observed for parental E5-651 virus. As illustrated in Fig. 2A, three of the six recombinant mutants had an *sp* phenotype in Vero cells since their plaque diameter was <25% of the plaque size of E5-651. Among these mutants, 651/E-Glu_{315}, 651/E_{box}, and 651/NS3-Glu_{46} exhibited the greatest reductions in plaque diameter (Fig. 2A) and the lowest efficiencies of virus replication in Vero cells at the permissive temperature of 32°C (Fig. 2B). Two of these mutants (651/E-Glu_{315} and 651/E_{box}) showed decreases in replication at 32°C in murine Neuro-2A cells, whereas replication levels of 651/E-Leu_{119}, 651/E-Asn_{147}, and 651/E-Asn_{205} were comparable in Vero and Neuro-2A cells (Fig. 2B). The 651/NS3-Glu_{46} virus containing a single Lys_{46}→Glu substitution in NS3 showed a 13-fold reduction in replication in Neuro-2A cells at 32°C compared to that in Vero cells. Since only 651/E-Glu_{315} and 651/E_{box} carrying a common Lys_{315}→Glu mutation in E have an *hr* phenotype in murine neuroblastoma cells, it would appear that the Lys_{315}→Glu mutation in E is the major contributor to the *hr* phenotype of E5-104 in this cell line. None of the cDNA-derived mutants displayed the *hr* phenotype in SH-SY5Y cells (Fig. 2B; Tables 3 and 5), suggesting that the set of mutations identified in the structural and nonstructural proteins of the E5-104 mutant is required to manifest the *hr* phenotype in human neuroblastoma cells. However, two mutants, 651/E-Glu_{315} and 651/E_{box}, grew slowly in SH-SY5Y cells (Fig. 2B), suggesting a possible contribution of the Lys_{315}→Glu mutation in E protein to the *hr* phenotype of the E5-104 mutant in human neuroblastoma cells.

The *ts* phenotype of each recombinant mutant and its E5-651 parent was examined in more detail by measuring its efficiency of replication in Vero and neuroblastoma cells at various temperatures (Table 3). Temperature sensitivity was defined as a reduction in virus titer at 37°C that was greater than 2.0 log_{10} PFU/ml compared to the titer at the permissive temperature of 32°C on day 4 postinfection. 651/E-Leu_{119} and 651/E-Glu_{315} were *ts* in each of the three cell lines, whereas the other E5-651 recombinants were *ts* in only one or two cell lines. However, the recombinant viruses were less restricted in replication in each cell line compared to E5-104, indicating that the *ts* phenotype, like the *hr* phenotype, is a composite phenotype with contributions from more than one of the individual mutations present in E5-104.

Neuroinvasiveness of recombinant mutant viruses in immunodeficient mice. The neuroinvasiveness of the E5-651 recombinant viruses bearing the E5-104 mutations was evaluated in SCID mice at a dose of 10^3 or 10^5 PFU. The development of encephalitis or the death of mice that were inoculated with recombinant mutants was observed for 28 days and compared

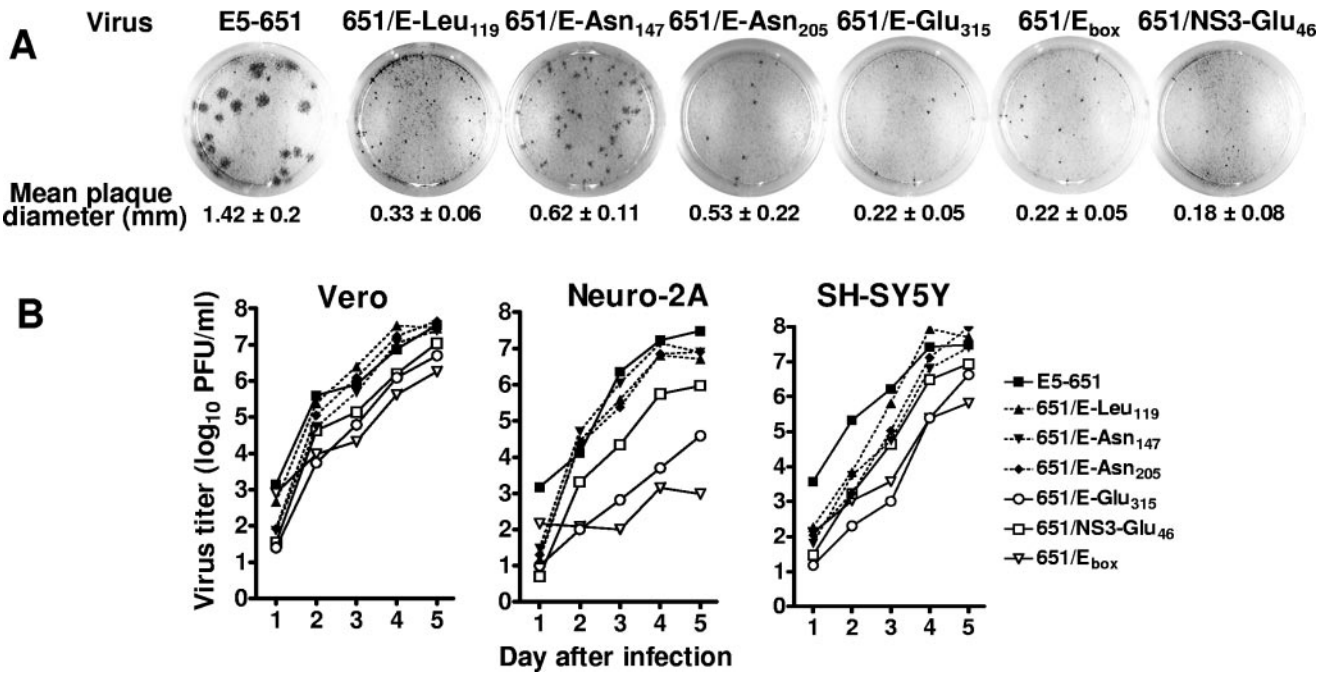

FIG. 2. Plaque morphology and growth analysis of cDNA-derived E5-651 virus and its recombinant mutants in Vero and neuroblastoma cells. (A) Plaque size of the E5-651 parent and its mutants on Vero cells monolayers. Monolayers of confluent cells were infected with the indicated viruses and incubated at 37°C for 4 days, and resulting plaques were visualized by immunostaining. The value below each well corresponds to the mean plaque diameter in mm ± standard error (n = 10 to 20 plaques per virus). (B) Analysis of growth of parental E5-651 and its recombinant mutant derivatives in Vero cells and in Neuro-2A or SH-SY5Y neuroblastoma cells following incubation at 32°C. Cells were infected with the indicated virus at an MOI of 0.01, and the virus titers in cell culture medium were determined as described in Materials and Methods.

to that of E5-651 and E5-104 viruses. Based on the moribundity rates and average survival times of SCID mice, mutant viruses that showed a >4-day delay in the onset of encephalitis or death and caused less mortality/morbidity in mice compared to E5-651 were considered to have an *att* phenotype (Table 4). Three mutant viruses, carrying single amino acid substitutions in E (namely, 651/E-Leu_{119}, 651/E-Asn_{147}, and 651/E-Asn_{205}), resembled their E5-651 parent in neuroinvasiveness, indicating that these mutations did not make independent contributions to the decreased neuroinvasiveness of the E5-104 parent virus. In contrast, 651/E-Glu_{315} and 651/NS3-Glu_{46} independently made contributions to attenuation, since increased survival and a delay in the onset of encephalitis or death were observed in groups of mice inoculated with these viruses. The mutant virus 651/E_{box} containing a combination of the five E5-104 mutations in E was more attenuated than either of these single mutation viruses, indicating that mutations in E other than the Lys_{315}→Glu substitution contribute to the decreased neuroinvasiveness of E5-104. SCID mice were completely resistant to i.p. inoculation with a dose of 10^3 PFU of 651/E_{box}, and 40% of mice survived at a higher dose of inoculation (10^5 PFU), with the average survival time being 25 days.

A summary of the phenotypes for recombinant viruses bearing the E5-104 mutations is presented in Table 5. The *sp*

TABLE 3. Temperature sensitivity of E5, E5-104, and recombinant E5-651 viruses in Vero and neuroblastoma cells

Virus	Titer (mean $\log_{10}$ PFU/ml) of virus grown at selected temperatures (°C) in indicated cells[a]																				
	Simian Vero cells							Murine Neuro-2A neuroblastoma cells							Human SH-SY5Y neuroblastoma cells						
	32	34	35	36	37	38	Δ[b]	32	34	35	36	37	38	Δ	32	34	35	36	37	38	Δ
E5	6.7	7.2	7.4	8.3	7.4	6.8	−0.7	7.2	6.7	6.3	6.6	6.2	2.7	1.0	6.1	7.1	7.3	8.0	8.0	6.7	1.9
E5-651	6.9	6.6	7.4	7.5	7.5	6.0	−0.6	7.2	7.3	7.0	6.5	5.7	3.5	1.5	7.4	7.7	7.5	7.4	7.2	6.7	0.2
651/E-Leu_{119}	7.5	7.1	7.2	6.4	5.2	2.6	2.3	6.8	6.2	5.5	4.9	<0.7	<0.7	>6.1	7.9	7.5	6.7	6.4	5.7	5.0	2.2
651/E-Asn_{147}	7.1	7.2	7.2	7.2	5.9	3.5	1.2	7.2	6.5	5.7	5.3	3.2	<0.7	4.0	6.8	7.1	7.2	7.4	4.5	0.7	2.3
651/E-Asn_{205}	7.2	7.0	6.9	6.7	5.9	3.5	1.3	5.9	6.1	5.6	5.2	3.2	<0.7	3.7	7.1	7.9	7.4	6.8	6.8	2.9	0.3
651/E-Glu_{315}	6.1	6.0	5.6	5.4	3.7	1.0	2.4	3.7	3.0	3.1	<0.7	<0.7	<0.7	>3.0	5.4	6.1	6.4	5.4	3.1	<0.7	2.3
651/E_{box}	5.6	5.7	5.4	5.9	4.8	1.5	0.8	3.2	3.6	1.9	1.0	<0.7	<0.7	>2.5	5.4	5.9	5.6	4.5	1.6	<0.7	3.8
651/NS3-Glu_{46}	6.2	5.5	5.6	5.4	3.6	1.7	2.6	5.8	5.7	4.6	2.9	<0.7	<0.7	>5.1	6.5	7.2	7.1	6.7	5.6	<0.7	0.9
E5-104	5.7	5.2	5.5	3.4	<0.7	<0.7	>5.0	3.6	1.0	<0.7	<0.7	<0.7	<0.7	>2.9	3.1	3.9	3.4	3.5	<0.7	<0.7	>2.4

[a] Underlined values indicate a reduction of ≥2.0 $\log_{10}$ PFU/ml in virus titer at the indicated temperature compared to that of the titer at the permissive temperature (32°C).

[b] The Δ indicates a reduction in mean virus titer ($\log_{10}$ PFU/ml) at 37°C compared to that for the permissive temperature (32°C). Values of ≥2.0 $\log_{10}$ PFU/ml indicate the *ts* phenotype.

TABLE 4. Neuroinvasiveness of E5-651, E5-104, and recombinant E5-651 mutants in SCID mice

Virus (i.p. inoculation)	Dose (log_{10} PFU)	No. dead or moribund mice/no. mice tested	Average survival time of mice (days)	Harvest day for indicated mouse[a]	
				No. 1	No. 2
E5-651	3.0	5/5	15		13
	5.0	5/5	15.4	12	
651/E-Leu_{119}	3.0	5/5	16.6		18
	5.0	5/5	15.8	15	
651/E-Asn_{147}	3.0	5/5	16.6		18
	5.0	5/5	14.8	14	
651/E-Asn_{205}	3.0	5/5	16.8		
	5.0	5/5	13.8	18	13
651/E-Glu_{315}	3.0	3/5	24.2		21
	5.0	4/5	20.6	17	
651/E_{box}	3.0	0/5	>28		
	5.0	3/5	25	21	20
651/NS3-Glu_{46}	3.0	3/5	25		23
	5.0	5/5	20	17	
E5-104	3.0	0/5	>28		
	5.0	0/5	>28		

[a] The brain of a moribund mouse was harvested on the indicated day postinoculation.

TABLE 5. Biological properties of the cDNA-derived recombinant mutants

Virus	Phenotype of the recombinant viruses on indicated cells						Attenuation in mice[d]
	Vero		Neuro-2A		SH-SY5Y		
	sp[a]	*ts*[b]	*ts*[b]	*hr*[c]	*ts*[b]	*hr*[c]	
651/E-Leu_{119}	−	+	+	−	+	−	−
651/E-Asn_{147}	−	−	+	−	+	−	−
651/E-Asn_{205}	−	−	+	−	−	−	−
651/E-Glu_{315}	+	+	+	+	+	−	+
651/E_{box}	+	−	+	+	+	−	+
651/NS3-Glu_{46}	+	+	+	−	−	−	+
E5-104	+	+	+	+	+	+	+

[a] Virus with a mean plaque diameter that was ≤25% of the plaque size (1.42 mm, diameter) of parental E5-651 virus (Fig. 2A) on Vero cells was designated as having the *sp* phenotype.

[b] Temperature sensitivity was defined as a reduction of >2.0 log_{10} PFU/ml in virus titer in the indicated cells at 37°C compared to that for the titer achieved at the permissive temperature of 32°C. Data are derived from results presented in Table 3.

[c] *hr* phenotype was defined as a 100-fold-or-greater reduction in virus titer in neuroblastoma cells at 32°C on day 5 compared to that achieved in Vero cells (Fig. 2B).

[d] The mutant virus that showed a >4-day delay in the onset of encephalitis or death and caused less mortality/morbidity in SCID mice compared to that for E5-651 (Table 4) was considered as having an *att* phenotype.

phenotype in Vero cells and the restriction in replication in murine neuroblastoma cells of three recombinant viruses, 651/E-Glu_{315}, 651/NS3-Glu_{46}, and 651/E_{box}, correlated with their reduced neuroinvasiveness in immunodeficient mice. The attenuated 651/E-Glu_{315} and 651/E_{box} viruses, which had both *sp* and *hr* phenotypes, shared a common Lys_{315}→Glu substitution in E. The *sp* 651/NS3-Glu_{46} mutant, which contains a Lys_{46}→Glu substitution in NS3 and has a 13-fold reduction in replication in Neuro-2A cells at a permissive temperature (Table 3), was also found to have an *att* phenotype. However, each of these recombinant viruses was not as attenuating as E5-104 (Table 4), demonstrating that the *att* phenotype is a composite phenotype with the contribution of several point mutations in structural and nonstructural proteins.

It was possible to gain additional information about the genetic determinants of neuroinvasiveness/neurovirulence of the LGT by examining the genome sequence of the brain isolates for each recombinant virus indicated in Table 4, and results are summarized in Table 6. The prolonged replication of virus in SCID mice provides the opportunity for mutations that increase replication fitness to occur in individual animals. Mutations that independently arise in viruses from the brain of more than one animal suggest that they might play a role in neuroinvasiveness/neurovirulence. Each virus isolate from mouse brain differed from the virus used for inoculation by one to four nucleotide changes that were distributed throughout the genome, including structural and nonstructural protein genes as well as the 3′ noncoding region of the genome. Nine of the 14 observed amino acid changes were located in E, and 6 of them were mutations that were present in two to four isolates. Such mutations were also identified in C and NS3 genes.

Sequence analysis suggests that mutations at eight residues in C (Gln_{27}→Lys), E (Val_{43}→Ile, His_{130}→Tyr, Asp_{135}→Asn, Leu_{267}→Ser, Gly_{291}→Glu/Val, and Asp_{389}→Asn), or NS3 (Glu_{46}→Lys) (Table 6, boxes) likely contributed to neuroinvasiveness/neurovirulence. Four brain isolates from SCID mice inoculated with 651/E-Asn_{147}, 651/E-Asn_{205}, 651/E-Glu_{315}, or 651/NS3-Glu_{46} shared a coding change at nt 1358 (C→U; His_{130}→Tyr) in E. This unique amino acid substitution in E increased neuroinvasiveness of E5 in SCID mice as demonstrated previously (25). Of the eight recurring amino acid substitutions identified among the virus isolates, five (Asp_{135}→Asn, Leu_{267}→Ser, Gly_{291}→Glu, and Asp_{389}→Asn in E or Glu_{46}→Lys in NS3) restored the sequence of the wt LGT parent which is neuroinvasive in normal and immunodeficient mice (7, 27). A single amino acid substitution (Gly_{291}→Val) in the 651/E-Glu_{315} isolate occurred at the same site as one (Gly_{291}→Glu) that restored the sequence of wt LGT. Two recurring substitutions, one in C (Gln_{27}→Lys) and one in E (Val_{43}→Ile), are newly identified positions that could possibly contribute to neuroinvasiveness/neurovirulence.

DISCUSSION

In the present study, the major motivation to modify LGT was to produce a vaccine candidate that is free of detectable neuroinvasiveness in mice and hopefully would also show a decrease in its low level of residual neuroinvasiveness for humans (14, 37). A significant reduction of LGT neuroinvasiveness was achieved by the accumulation of amino acid changes, resulting in a stepwise decrease in neuroinvasiveness from wt LGT (highly neuroinvasive) to E5 (intermediate neuroinvasiveness) to E5-104 (not neuroinvasive). LGT E5 was initially selected from wt LGT by multiple passages in chicken embryo

TABLE 6. Nucleotide and amino acid differences between the sequences of the brain-adapted virus isolates and the sequence of virus used for i.p. inoculation of immunodeficient mice

Gene	Nucleotide		Amino acid change	Presence of specified mutation in virus isolate[a] from brain tissue of SCID mice inoculated i.p. with indicated virus[d]													
	Position (genome)	Change		E5-651		651/ E-Leu$_{119}$		651/ E-Asn$_{147}$		651/ E-Asn$_{205}$		651/ E-Glu$_{315}$		651/E$_{box}$		651/NS3-Glu$_{46}$	
				1	2	1	2	1	2	1	2	1	2	1	2	1	2
C	212	C→A	Gln$_{27}$→Lys											+	+		
prM	559	G→A	None			+											
E	1097	G→A	Val$_{43}$→Ile		+	+											
	1353	C→U	Thr$_{128}$→Ile					+									
	1358	C→U	His$_{130}$→Tyr[b]					+		+		+				+	
	1373	G→A	Asp$_{135}$→Asn[c]											+	+		
	1770	U→C	Leu$_{267}$→Ser[c]											+	+		
	1842	G→A	Gly$_{291}$→Glu[c]				+										
		G→U	Gly$_{291}$→Val										+				
	2135	G→A	Asp$_{389}$→Asn[c]				+						+				
	2274	G→A	Arg$_{435}$→Lys								+						
NS1	3247	G→A	None					+									
	3502	C→U	None		+												
NS2A	4031	U→C	None					+									
NS3	4733	G→A	Glu$_{46}$→Lys[c]													+	+
	5644	G→U	Gln$_{349}$→Asp	+													
NS4A	6594	G→U	Arg$_{45}$→Leu													+	
NS5	9308	G→A	Asp$_{549}$→Asn	+													
3′ NCR	10405	G→A	None	+													
	10419	C→U	None					+									
	10450	C→A	None		+												

[a] Virus isolates (1 and 2) were derived from brains of two separate moribund mice as indicated in Table 4.
[b] His$_{130}$→Tyr amino acid substitution was previously found to be responsible for the increased neuroinvasiveness of LGT virus in immunodeficient mice (25).
[c] A reversion to the sequence of parental E5 or wild-type LGT virus.
[d] Amino acid substitutions that emerged in two or more virus isolates are indicated by dashed boxes.

cell culture for use as a live virus vaccine candidate to protect against illness caused by the members of TBEV complex (30, 38, 40, 41). There are 12 nt differences in the consensus sequence of the wt LGT and it E5 derivative (7), of which seven produced an amino acid substitution in E (Phe$_{119}$→Val and Asn$_{389}$→Asp), NS3 (Asn$_{22}$→Ser, Phe$_{248}$→Tyr, and Phe$_{317}$→Leu), and NS5 (Ser$_{422}$→Thr and Arg$_{542}$→Lys). These differences in the sequence make E5 virus significantly less neuroinvasive (i.p. LD$_{50}$ of $>10^7$ PFU) in Swiss mice compared to its wt LGT parent (i.p. LD$_{50}$ of $10^{3.7}$ PFU) (27). Nevertheless, wt LGT and E5 were highly neuroinvasive for immunodeficient mice (27). It appeared that the one or two amino acid mutations (Phe$_{119}$→Val or Asn$_{389}$→Asp) in the E protein of E5 contributed to its loss of neuroinvasiveness for adult Swiss mice since a recombinant LGT that differed from wt LGT at only these two amino acids was highly attenuated in immunocompetent mice inoculated i.p. (i.p. LD$_{50}$ of $>10^6$ PFU) (A. G. Pletnev, unpublished data). In addition, data from the present study indicate that a reverse mutation of Asp$_{389}$ to Asn in E independently arose in virus isolated from brains of SCID mice inoculated i.p. with either of two LGT E5 recombinant viruses (651/E-Leu$_{119}$ or 651/E-Glu$_{315}$) (Table 6), a finding that confirms the importance of the Asn$_{389}$→Asp substitution in the attenuation of LGT E5 for mice. The Asn$_{389}$→Asp mutation is located on the lateral surface of domain III of the E protein and corresponds to a site which is thought by others to attenuate TBEV or Murray Valley encephalitis virus for mice (reviewed in references 16 and 20). The contribution of the amino acid substitutions in NS3 and NS5 to the decreased neuroinvasiveness of E5 is unknown. Thus, at least one mutation in E5 (Asn$_{389}$→Asp in E) contributes to its decreased neuroinvasiveness.

Mutant virus E5-104 was generated by chemical mutagenesis of E5 and was found to lack neuroinvasiveness for the most susceptible host, the SCID mouse. E5-104 was at least $10^{6.2}$-fold or $10^{7.4}$-fold less neuroinvasive than E5 or wt LGT, respectively. This significant decrease in neuroinvasiveness of E5-104 compared to that of E5 was the result of the acquisition of 11 nt differences in sequence by E5-104, of which 7 produced an amino acid substitution in the structural envelope protein E or nonstructural protein NS3 or NS5 (Table 1). Only an amino acid change of Val$_{119}$→Leu in E of E5-104 occurred at the same site as a Phe$_{119}$→Val change that differentiated E5 from wt LGT. Thus, the seven coding mutations that developed in E5-104 are novel. The specific mutations that contributed to the decreased neurovirulence/neuroinvasiveness of E5-104 were next determined.

The first mutation identified in E5-104 that contributed to its attenuation was a Ser$_{267}$→Leu substitution in E. The contribution of this mutation to the attenuation of E5-104 for the brains of mice was revealed by an analysis of the genetic basis of the four-day delay in the onset of encephalitis in newborn mice that were inoculated i.c. with E5-104. Although E5-104 was nonneuroinvasive in immunodeficient mice, it remained neurovirulent in suckling Swiss mice when inoculated directly into the brain, indicating that the mutations in E5-104 had a much more limited effect on neurovirulence than on neuroinvasiveness. The altered neurovirulence was reflected in only a

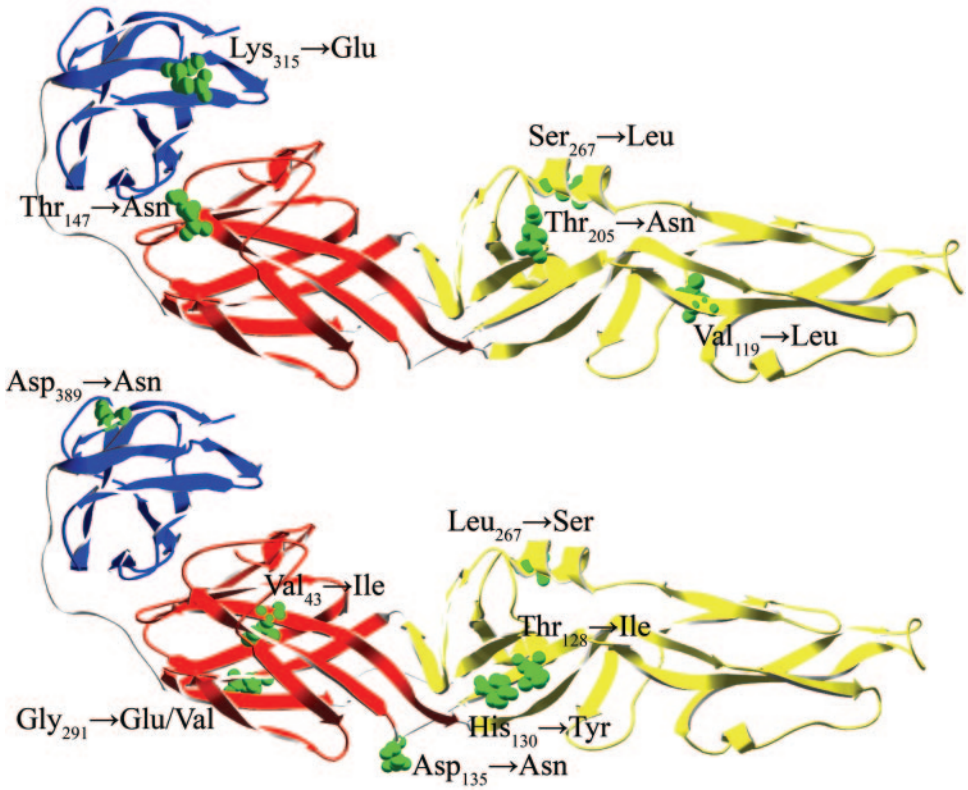

FIG. 3. Proposed three-dimensional structure of the E protein of LGT E5, based on the structure of the TBEV E protein ectodomain (31). The three structural domains I, II, and III are shown in red, yellow, and blue, respectively. Mutated residues identified in the E protein of the E5-104 mutant (Table 1) are shown in the upper panel. Mutated residues present in virus isolates from brain of mice are shown in the lower panel. The mutant residues are shown in green, and their position numbers and amino acid substitutions are indicated. The structure of the E protein ectodomain is displayed using the Swiss-Pdb Viewer version 3.7 (http://swissmodel.expasy.org/spdbv).

delay in the onset of death of the inoculated mice. It was assumed that the delay in the onset of encephalitis that was observed for E5-104 likely reflected the time that was needed for the virus to acquire mutations that enhanced its replicative capacity in the brain and led to encephalitis or death. The unique reverse mutation of Leu to Ser at position 267 in the E protein was identified in each of the two E5-104 isolates from moribund suckling mice, providing strong evidence that this mutation is important for replication of LGT in brain tissue and identifiying this site in E as one that contributes to neurovirulence. This same reverse mutation also arose in the brains of SCID mice inoculated i.p. with E5 derivatives containing Leu_{267}. The Ser_{267} residue is located in α-helical structure (αB) of domain II of E (Fig. 3) that is predicted to be involved in the interaction of two E protein monomers to form the dimer on the virion surface (31). A mutation at this position might disturb the structural and functional integrity of the E protein in the dimer and impair the biological properties of virus, including neurovirulence. It should be noted that an Ala residue at position 267 is present in the E proteins of most mosquito- and tick-borne flaviviruses, and only highly neurovirulent Far Eastern strains of TBEV and Omsk hemorrhagic fever virus as well as the less neurovirulent wt LGT have a Ser residue at this position (11).

In order to determine the involvement of each mutation in the loss of neuroinvasiveness of E5-104, five recombinant viruses (651/E-Val_{119}, 651/E-Asn_{147}, 651/E-Asn_{205}, 651/E-Glu_{315}, and 651/NS3-Glu_{46}) that contained a single mutation in E (Val_{119}→Leu, Thr_{147}→Asn, Thr_{205}→Asn, or Lys_{315}→Glu) or in NS3 (Lys_{46}→Glu) were generated using the infectious cDNA clone E5-651 of LGT E5 (Table 2) and were evaluated in SCID mice (Table 4). Unfortunately, a single substitution of Ser_{267}→Leu in E or Asn_{17}→Asp in NS5 in the E5-651 cDNA did not yield a viable virus. For this reason, the recombinant 651/E_{box} virus that contains the five amino acid changes observed in E5-104 E protein, including the Ser_{267}→Leu substitution, was generated. Among the recombinant mutants, only three (651/NS3-Glu_{46}, 651/E-Glu_{315}, and 651/E_{box}) exhibited decreased neuroinvasiveness in SCID mice (Table 4 and 5). These three recombinants also were restricted in replication in murine neuroblastoma cells at the permissive temperature (32°C), as indicated by a 13-, 130-, or 1,890-fold decrease in virus titer in Neuro-2A cells compared to that in Vero cells, respectively. These findings indicate that a single mutation, Lys_{46}→Glu in NS3 or Lys_{315}→Glu in E, independently contributed to both *hr* and *att* phenotypes.

The novel attenuating mutation Lys_{315}→Glu in the E protein of E5-104 is located on the lateral surface of domain III (Fig. 3A), which has been proposed to be involved in cell receptor binding (31). A large set of the mutations affecting virulence in mice or cell tropism of both mosquito- and tick-borne flaviviruses was identified previously in this domain (re-

viewed in references 16 and 20). The replacement of a positively charged Lys_{315} residue with negatively charged Glu in β-sheet A of domain III could induce conformational alterations in this region of E that might affect the biological properties of E5 virus. In support of this proposal, we find that only viruses carrying this mutation (651/E-Glu_{315}, 651/E_{box}, and E5-104) had the *hr* phenotype in murine neuroblastoma cells and also had the *att* phenotype in SCID mice, providing strong evidence of the importance of Glu_{315} in cell tropism and neuroinvasiveness in mice.

The other independently attenuating mutation in E5-104 involved the exchange of the positively charged Lys residue by the negatively charged Glu residue at position 46 in NS3. This residue is located in the conserved N-terminal region (amino acids 46 to 60) of the trypsin-like serine protease domain in NS3 that contains the residues that are involved in the formation of the putative catalytic site (4, 13, 23; reviewed in reference 19). A substitution at position 47 or 54 in this conserved region of yellow fever virus has been shown to decrease both proteinase activity and virus replication (8), suggesting that the Lys_{46}→Glu mutation in NS3 of E5-104 may have a similar effect.

The information summarized above indicates that at least one mutation in the E protein of E5 (Asn_{389}→Asp) and three mutations in E5-104 (Ser_{267}→Leu and Lys_{315}→Glu in E and Lys_{46}→Glu in NS3) contribute to the restricted neuroinvasiveness of E5-104 in SCID mice. It is also possible that other E5- or E5-104-specific mutations play a role in the overall attenuation of E5-104, even though they were not specifically identified as attenuating mutations. For example, a negatively charged Asn_{17}→Asp mutation in E5-104 located in the N-terminal methyltransferase domain of NS5 protein may have an effect on RNA cap formation (17) since Asn_{17} is conserved among flaviviruses and its replacement by Ala in dengue-2 NS5 was found to affect GTP binding (12).

Thus, four or more mutations in E5-104 contribute to its greatly decreased neuroinvasiveness compared to that of wt LGT. The absence of death in the SCID mice inoculated i.p. with E5-104 indicates that phenotypic reversion to increased neuroinvasiveness appears rarely. The encouraging results obtained from analysis of the safety of E5-104 in immunodeficient mice constitute the basis for expanding the evaluation of its pathogenesis, immunogenicity, and protective efficacy in mice and nonhuman primates. The E5-104 mutant might be useful alone as a vaccine against disease caused by TBEV, but further studies will need to demonstrate its advantage compared to that of the other live attenuated TBEV vaccine candidates under study (27, 33). Alternatively, the attenuating mutations identified in E5-104 can be used to attenuate the TBEV strains that are responsible for causing most TBEV illness in Europe and Asia. As observed previously by others (2, 3, 5, 42), the attenuating mutations identified in one flavivirus can attenuate a related heterologous flavivirus if they are incorporated into the homologous sites.

Additional information regarding the genetic determinants of neuroinvasiveness and neurovirulence of the LGT was obtained following complete genome sequence analysis of the isolates of recombinant viruses from the brains of SCID mice (Table 6). Immunodeficient SCID mice provide the opportunity for prolonged replication of virus, leading to the emergence of mutations that increase the fitness of a virus to spread from a peripheral inoculation site to the CNS or that increase its ability to replicate in the brain and cause encephalitis. Sequence analysis of the virus that becomes the predominant species in the brain identifies such facilitating mutations, and their presence in the brain of two or more animals is interpreted to indicate their contribution to increased fitness. Mutations observed in viruses isolated from brains did not occur randomly across the genomes and resided mainly on the envelope proteins, confirming the important role that E plays in neuroinvasiveness/neurovirulence.

Eight such mutations were identified in virus isolates from the brains of mice inoculated with recombinant viruses (Table 6, boxes) and can be divided into two groups: mutations (Asp_{135}→Asn, Leu_{267}→Ser, Gly_{291}→Glu, or Asp_{389}→Asn in E or Glu_{46}→Lys in NS3) that restored the sequence of wt LGT (group 1) and mutations (Gln_{27}→Lys in C and Val_{43}→Ile, His_{130}→Tyr, or Gly_{291}→Val in E) at sites that are conserved in TBE viruses (group 2). Of the five substitutions observed in group 1, the occurrence of reverse mutations at three positions (Leu_{267}→Ser and Asp_{389}→Asn in E and Glu_{46}→Lys in NS3) confirms our previous findings of the importance of these sites in the attenuation of E5 or E5-104. The other two mutations in group 1 (Asp_{135}→Asn and Gly_{291}→Glu) are newly identified sites that could also contribute to neuroinvasiveness/neurovirulence of TBE viruses for mice.

The mutations in group 2 (Gln_{27}→Lys in C and Val_{43}→Ile, His_{130}→Tyr, or Gly_{291}→Val in E) are interesting in that they are counterintuitive, i.e., they are substitutions at newly identified sites that are conserved in virulent TBE viruses to amino acid residues that are not conserved among flaviviruses at these positions, yet the mutations appear to increase the neuroinvasiveness/neurovirulence of the specific recombinant virus for mice. The exact mechanism by which these mutations are able to enhance the replicative fitness or neuroinvasiveness of recombinant viruses in SCID mice remains to be determined. The His_{130}→Tyr mutation in E (group 2) independently emerged by itself in three brain isolates (651/E-Asn_{147}, 651/E-Asn_{205}, and 651/E-Glu_{315}) or was identified together with two additional mutations in the NS3 protein in the 651/NS3-Glu_{46} isolate (Table 6). A mutation at position 130 is located within a hinge region connecting domains I and II of E protein (Fig. 3B), which is predicted to be involved in acid-induced conformational change of the E protein and is important in viral virulence (31; reviewed in reference 16). As we demonstrated previously (25), this single amino acid change of His_{130} to Tyr in E protein is directly associated with an increase in neuroinvasiveness of the E5 virus in SCID mice. Our findings are consistent with previous observations that mutations in the hinge regions that are linking the E protein domains of Japanese encephalitis, Murray Valley encephalitis, yellow fever, or TBE viruses can affect either neurovirulence or neuroinvasiveness in normal mice (reviewed in references 16 and 20). It is possible that each of the four mutations in group 2 (Gln_{27}→Lys in C and Val_{43}→Ile, His_{130}→Tyr, and Gly_{291}→Val in E) promotes increased neuroinvasiveness/neurovirulence in mammals but is actively selected against in nature, and thus they are not present in circulating TBEV strains. Such mutations might identify sequences in C or E that are

required for efficient replication in the tick vector. This possibility could be studied further.

The identification of residues that are potentially involved in neurotropism, affect neuroinvasiveness, and promote attenuation of flaviviruses is particularly important for the development and quality control of a live attenuated virus vaccine during its manufacture and use in humans. Neuroinvasiveness-enhancing mutations can be monitored during all phases of vaccine manufacture and evaluation in humans. Also, our findings in the present study with E5-104 and those in previous studies with chimeric flaviviruses (33) associate the restriction of replication in cells of neural origin and attenuation in mice and monkeys, and this correlation might be more generally applicable and prove to be useful in the development of attenuated derivatives of other neurotropic viruses.

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