Genetic Determinants of Dengue Type 4 Virus Neurovirulence for Mice

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Mouse-adapted dengue type 4 virus (DEN4) strain H241 is highly neurovirulent for mice, whereas its non-mouse-adapted parent is rarely neurovirulent. The genetic basis for the neurovirulence of the mouse-adapted mutant was studied by comparing intratypic chimeric viruses that contained the three structural protein genes from the parental virus or the neurovirulent mutant in the background sequence of nonneurovirulent DEN4 strain 814669. The chimera that contained the three structural protein genes from mouse neurovirulent DEN4 strain H241 proved to be highly neurovirulent in mice, whereas the chimera that contained the corresponding genes from its non-mouse-adapted parent was not neurovirulent. This finding indicates that most of the genetic loci for the neurovirulence of the DEN4 mutant lie within the structural protein genes. A comparison of the amino acid sequences of the parent and its mouse neurovirulent mutant proteins revealed that there were only five amino acid differences in the structural protein region, and three of these were located in the envelope (E) glycoprotein. Analysis of chimeras which contained one or two of the variant amino acids of the mutant E sequence substituting for the corresponding sequence of the parental virus identified two of these amino acid changes as important determinants of mouse neurovirulence. First, the single substitution of Ile for Thr-155 which ablated one of the two conserved glycosylation sites in parental E yielded a virus that was almost as neurovirulent as the mouse-adapted mutant. Thus, the loss of an E glycosylation site appears to play a role in DEN4 neurovirulence. Second, the substitution of Leu for Phe-401 also yielded a neurovirulent virus, but it was less neurovirulent than the glycosylation mutant. These findings indicate that at least two of the genetic loci responsible for DEN4 mouse neurovirulence map within the structural protein genes.

The four serotypes of dengue virus form a distinct antigenic subgroup of some 70 members of the arthropod-borne, serologically related flaviviruses. These flaviviruses are now classified within the *Flavivirus* genus of the family *Flaviviridae*, which contains two other genera, the human hepatitis C virus genus and the *Pestivirus* genus (9). Dengue viruses continue to cause major epidemics in most tropical and subtropical regions where the *Aedes* mosquito vector is abundant. Dengue virus infection usually causes fever, rash, and joint pain, but the disease is self-limited. However, in recent years, a more severe form of dengue, characterized by hemorrhagic fever and hemorrhagic shock, has occurred with increasing frequency, most notably in the dengue-epidemic region of southeast Asia (12). This form of dengue has a high fatality rate, especially in children and young adults. A safe and effective vaccine against dengue has not been developed.

Various strategies have been explored to produce an effective vaccine for prevention of dengue virus infection ever since the dengue virus was isolated more than half a century ago. During early studies, serial intracerebral passage of dengue type 1 or type 2 virus in mice was used to attenuate these viruses for development of candidate strains for inclusion in a vaccine to be used in humans (16, 24–27). Studies by Hotta showed that the Mochizuki strain (dengue type 1) virus lost its virulence for humans after 17 passages in mouse brain (16). Studies by Sabin revealed that dengue viruses at an intermediate mouse passage level, beginning at the 17th passage for type 1 virus and at the 18th passage for type 2 virus, were

satisfactorily attenuated and induced a lasting protective immunity against virus of the same serotype (24, 25). Sabin's studies further demonstrated that continued passage in mice selected highly neurovirulent mutants which produced paralytic disease in monkeys (25). Because of this increased neurovirulence, late-passage viruses were not considered safe for use as vaccines. However, these dengue viruses are valuable for use in studies designed to identify the genetic basis for loss of virulence for humans as well as the acquisition of neurovirulence for mice.

Following the construction of a full-length cDNA clone of dengue type 4 virus (DEN4) strain 814669, which can be used to produce infectious RNA transcripts (18), we recently constructed intertypic chimeric dengue viruses that contain the structural protein genes of DEN1 strain Western Pacific or DEN2 strain New Guinea C replacing the corresponding genes of DEN4 (3). A study with suckling mice demonstrated that the DEN2-DEN4 chimeric virus containing the three structural protein genes from the neurovirulent DEN2 strain retained much of the neurovirulence of the parental DEN2 strain (3). This suggested that most genetic determinants of mouse neurovirulence map within the three structural protein genes.

DEN4 strain H241 was first isolated during a dengue epidemic in the Philippines in 1956. Subsequent isolation of DEN4 from hemorrhagic fever patients as well as patients with classical dengue fever elsewhere in Asia indicated that DEN4 was an important pathogen (13, 14). The first introduction of DEN4 into the Caribbean resulted in a large epidemic during 1980 to 1981 (15). Since that time, DEN4 has been isolated frequently along with other dengue virus serotypes during dengue epidemics in many regions of the Americas. Like

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DEN1 and DEN2, the prototype DEN4 strain H241 was successfully adapted by serial passage to replicate to high titer in mouse brain. Such a mouse-neurovirulent mutant has been used as a challenge virus in immunoprophylaxis studies in mice (4, 29).

This article describes the construction and characterization of intratypic DEN4 chimeric viruses containing the structural protein genes of the parent DEN4 strain H241 or its mouse-neurovirulent mutant that led to identification of the genetic basis for mouse neurovirulence.

MATERIALS AND METHODS

DEN4 strains. As described previously, DEN4 strain 814669, isolated in Dominica in 1981, was used to prepare full-length cDNA for sequence analysis (30). DEN4 parental strain H241, hereafter designated DEN4 H241-P, at passage 1 was recovered from the serum of a viremic monkey inoculated with the original human serum. The mouse-neurovirulent mutant of DEN4 H241, designated DEN4 H241-N, was identified after repeated mouse intracerebral passage (the exact passage number unknown) by J. McCowan at the Walter Reed Army Institute of Research, Washington, D.C., and was kindly supplied by D. Dubois and K. Eckels (Walter Reed Army Institute of Research). DEN4 H241-N, in the form of a mouse brain homogenate, was used previously as a challenge virus in experimental immunoprophylaxis studies in mice (4, 29). Both DEN4 H241-P and DEN4 H241-N were amplified by a single passage in mosquito C6/36 cells to yield seed virus. Each seed virus was passaged once in simian LLC-MK₂ cells and then in C6/36 cells. The resulting virus in the culture medium was titrated on LLC-MK₂ cell monolayers prior to evaluation of its mouse neurovirulence.

Cloning cDNA from DEN4 H241 strains. Seed stocks of the DEN4 H241 parent and the mouse-neurovirulent mutant were used to infect C6/36 cells, usually in two T₁₅₀ flasks, for preparation of virus from which virion RNA was to be extracted. Reverse transcription of virion RNA was performed with an oligonucleotide primer complementary to the DEN4 sequence at nucleotides (nt) 2369 to 2334, near the 3' end of the envelope protein gene. This oligonucleotide was used earlier to introduce the *Xho*I site at DEN4 nt 2342 for the construction of chimeric DEN4 cDNA (3).

To prepare double-stranded cDNA for the DEN4 H241 structural protein genes, the first cDNA strand was used as the template for a polymerase chain reaction (PCR) with the same first-strand primer and oligonucleotide D101, which is complementary to DEN4 nt 81 to 104, as the second primer. The PCR product, after cleavage with *Bgl*II and *Xho*I, was cloned in the p5'-2(*Xho*I) vector (3), replacing the corresponding sequence of DEN4 strain 814669. Several plasmids were isolated, and the sequence between the *Bgl*II and *Xho*I sites was determined by using the Sequenase 2.0 kit (United States Biochemical Corporation). In this manner, we were able to deduce the consensus sequence for clones of the parental virus and its neurovirulent mutant.

Site-directed mutagenesis. PCR-oligonucleotide-directed mutagenesis was performed to introduce mutations specifying amino acid substitutions in the parental DEN4 H241 envelope (E) glycoprotein sequence. Specifically, triplet ATA (Ile) was substituted for ACA (Thr) at amino acid position 155, and triplet CCC (Pro) was substituted for TCC (Ser) at position 156. Also, the Phe-401 to Leu substitution was achieved by replacing the *Bst*EII-*Xho*I fragment (nt 1438 to 2342) of the parental DEN4 H241 sequence with the corresponding neurovirulent DEN4 H241 sequence.

Intratypic DEN4 chimeras. The p5'-2 construct containing the structural protein genes from DEN4 H241-P or DEN4 H241-N was used to produce the respective chimeric full-length DEN4 cDNAs by addition of the remaining sequence from DEN4 strain 814669 (3, 18). Plasmids containing full-length intratypic DEN4 cDNA were designated p2A(H241-P) and p2A(H241-N), respectively. Transcription of the *Asp*718-linearized DEN4 cDNA template and transfection of LLC-MK₂ cells with the RNA transcripts in the presence of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl-ammonium methyl-sulfate (DOTAP) were performed as described before (21). Ten days after transfection, the cells in a 24-well plate were trypsinized and transferred to a 25-cm² culture flask and to a chamber slide. After 3 days of incubation, the cells on the chamber slide were stained for an immunofluorescence assay (IFA). If IFA showed positive evidence of infection, the cells in the 25-cm² flask were maintained until the cytopathic effect (appearance of numerous dead cells floating in the culture medium) was significant. Infected cells were then harvested together with 5 ml of fresh medium 199 containing 50% fetal bovine serum. The cell lysate was aliquoted and frozen at -70°C as the source of progeny virus.

Analysis of viral proteins. Confluent C6/36 cells in a 25-cm² culture flask were infected with DEN4 H241-P or its chimeric progeny 2A(H241-P) at a multiplicity of infection (MOI) of 0.01 for 5 days or with DEN4 H241-N or its chimeric progeny 2A(H241-N) at an MOI of 0.001 for 10 days. Infected cells were labeled with [³⁵S]methionine (150 μ Ci per flask; specific activity, 600 Ci/mmol; 1 Ci = 37 GBq) in 1 ml of methionine-free medium for 4 h. Cells were then lysed with RIPA buffer (1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 0.1 M Tris-hydrochloride [pH 7.5], 0.15 M NaCl), and the lysates were immunoprecipitated with DEN4-specific hyperimmune mouse ascitic fluid or a rabbit antiserum prepared against a 14-amino-acid peptide of E (amino acids 259 to 272 of the DEN4 E sequence) (20). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with and without prior digestion with endoglycosidase F (20).

Mouse neurovirulence. DEN4 strains and intratypic DEN4 chimeras were evaluated for mouse neurovirulence as described before (3, 21). For analysis of the 50% lethal dose (LD₅₀), 3-day-old outbred Swiss mice in groups of 6 to 10 were inoculated intracerebrally with serial dilutions of DEN4 or its derived chimera at a dose of 1 to 10³ PFU in 25 μ l of Eagle's basal medium with 0.25% human serum albumin. The survival rate analysis was also performed for the series of DEN4 parents and chimeric constructs at a fixed infectious dose. Briefly, 15 to 20 mice in each group were inoculated with a calculated dose of 125 PFU of DEN4 in the diluent mentioned above. Mice were then observed daily for signs of paralysis and death for 25 days postinoculation.

Titration of virus. The titers of the various DEN4 preparations were determined by a plaque assay on LLC-MK₂ cells (1). Briefly, monolayers of LLC-MK₂ cells were inoculated with serial dilutions of virus and overlaid with Seakem ME agarose (FMC BioProducts, Rockland, Maine) containing medium 199 supplemented with 10% fetal bovine serum, basal amino acids, and vitamins. Cells were stained with neutral red 8 days after infection, and the number of plaques was counted the following day.

RESULTS

Genetic variations of DEN4 strains. DEN4 H241-P and DEN4 strain 814669 were isolated 25 years apart from two

In the 2,232-nt sequence encompassing nearly all of the structural protein gene region, there were 121 nucleotide differences (94.6% homology) between the two DEN4 strains. These were mostly silent changes, occurring in the third position of a codon.

Comparison of amino acid sequences between the two DEN4 strains showed that there were two amino acid differ-

TABLE 1. Amino acid differences in the structural protein region between the parental DEN4 strain H241 and its neurovirulent mutant

Polyprotein amino acid no. ^a	Protein and position ^b	Amino acid	
		Parent	Neurovirulent mutant
81	C, 81	Thr	Ile
188	Pre-M, 78	Val	Ala
434	E, 155	Thr ^c	Ile
435	E, 156	Ser	Pro
680	E, 401	Phe	Leu

^a Amino acid residues are numbered according to their positions in the polyprotein.

^b The numbers indicate the amino acid positions in the named proteins and the numbering system that is used in this article.

^c Thr-155 in the sequence Asn-Ile-Thr-155 constitutes a potential glycosylation site.

ences in the C region (98.2% homology) and four amino acid changes in pre-M (97.6% homology). In the 465-amino-acid E protein region, there were 16 amino acid differences (96.6% homology). Also, there was a change of a 6-amino-acid sequence, which appeared to be the result of a single nucleotide insertion and deletion; in DEN4 H241, 1494-GAA CCC AGG TCC GGA ATT GAT TTT-1517; in DEN4 814669, 1494-GAA CCA GGT CTG GAA TTG TAC TTT-1517. The degree of sequence relatedness between the two DEN4 strains appears to be similar to that between homotypic strains of DEN2 virus in the same region (2). The sequence homology between the two DEN4 strains is higher than that revealed by the fingerprint analysis (15).

Comparison of sequences between parental DEN4 H241 and its neurovirulent mutant. We then compared the nucleotide and amino acid sequences in the structural protein region between the parental DEN4 virus and its mouse-adapted neurovirulent mutant (Table 1). There were only 11 nucleotide changes, 6 of which were third-position silent mutations: G substituting for A-335 (A335G), A596G, T1355C, C1637T, T1739C, and C1799T. The other five mutations resulted in amino acid changes: C343T, T664C, C1402T, T1404C, and T2139C. In the C protein at position 81, Thr was changed to Ile, and in the pre-M protein at position 78, Val was mutated to Ala, which is a conservative change. In the E protein, there were three amino acid changes in the neurovirulent mutant located at positions 155, 156, and 401: Thr-155 to Ile, Ser-156 to Pro, and Phe-401 to Leu. Significantly, the Thr-155 to Ile substitution ablated one of the two potential glycosylation sites in E that are conserved among all four dengue virus serotypes. The other two substitutions were nonconservative amino acid changes.

Intratype DEN4 chimeric viruses. A series of intratype chimeric DEN4 cDNAs was constructed to analyze the genetic basis for mouse neurovirulence. Initially, we constructed two full-length chimeric DEN4 cDNA clones, designated p2A(H241-P) and p2A(H241-N), that contained the *Bgl*II-*Xho*I DNA fragment of DEN4 H241-P or DEN4 H241-N, respectively, substituting for the corresponding sequence of DEN4 814669. Because three changes are located in the E region, experiments were designed to determine the role of each of these changes in neurovirulence. For this purpose, three additional chimeric cDNAs that contained a single amino acid substitution and a fourth containing double substitutions in E were prepared by site-directed mutagenesis or by replacement of appropriate DNA fragments. The structures of

the chimeric constructs and their genotype designations are shown in Fig. 2. Transfections of LLC-MK₂ cells with RNA transcripts of these chimeric constructs all yielded progeny viruses, as detected by an IFA. This assay showed that approximately 75 to 100% of the cells were positive for dengue virus antigens on day 13 following transfection with RNA transcripts from p2A(H241-P), p2A(H241-P,I-155,P-156, p2A(H241-P,I-155), p2A(H241-P,P-156), or p2A(H241-P,L-401). The progeny viruses were harvested at day 20, and virus titers ranged from 4×10^4 to 2×10^5 PFU/ml. In contrast, only a few IFA-positive cells were observed on day 23 after transfection with RNA transcripts from p2A(H241-N). The percentage of infected cells increased to >80% by day 53, and the titer of progeny virus harvested at this time was 8×10^3 PFU/ml. The titer of progeny 2A(H241-N) was similar to that obtained following infection of the same cells with the parent DEN4 H241-N virus. This observation indicates that the mouse-neurovirulent DEN4 H241 replicated less efficiently than the prototype DEN4 H241 in LLC-MK₂ cells. The intratype DEN4 chimera containing the three structural protein genes from DEN4 H241-N also showed retarded growth in LLC-MK₂ cells.

Viral growth analysis. To further characterize the growth properties of both DEN4 H241-P and DEN4 H241-N and their chimeric progeny viruses, LLC-MK₂ cells on a chamber slide were inoculated with each virus at an MOI of 0.002. The number of infected cells was then monitored by IFA daily for 11 days. Infections with DEN4 H241-P or 2A(H241-P) spread rapidly from cell to cell. On day 1, about 20 foci, each containing a single IFA-positive cell, were observed in the chamber slide monolayer. The number of IFA-positive cells in each infected focus increased rapidly to about 20 on day 2 and to about 50 to 100 on day 3. The number of infected foci also increased. Approximately 50% of the cells became infected by day 4 and 80% by day 5, and most cells were positive for dengue virus antigens on day 6. In contrast, parental DEN4 H241-N and its derived chimera exhibited a slow virus spread: only a few foci, each consisting of two to three positive cells, were detected on day 3, and the number of positive cells in the infected focus was 10 to 20 on day 4 and increased to 100 to 200 on day 7. Approximately 70 to 80% of the cells were infected by day 11.

Analysis of viral proteins. Radioactive [³⁵S]methionine-labeled proteins prepared in C6/36 cells infected with DEN4 H241-P, DEN4 H241-N, or their derived chimeras were analyzed by immunoprecipitation with DEN4 hyperimmune mouse ascitic fluid and SDS-PAGE (Fig. 3). DEN4 H241-P and its derived chimera produced labeled bands that were identified as the pre-M, E, NS1, and NS3 proteins. Similar analysis showed that DEN4 H241-N and its derived chimera produced NS1 and NS3 proteins that migrated to the same locations as the counterpart proteins from the prototype parent. The pre-M proteins of DEN4 H241-N and 2A(H241-N) were detected at a reduced level and migrated slightly slower than the pre-M of DEN4 H241-P. The E glycoproteins of DEN4 H241-N and 2A(H241-N) migrated faster than the E protein of DEN4 H241-P or 2A(H241-P) (Fig. 3A). The calculated size difference between the two E proteins was about 3 kDa, a value equivalent to one carbohydrate moiety. The molecular size reduction observed for the E proteins of DEN4 H241-N and 2A(H241-N) was predicted, because the Thr-155→Ile substitution removes the potential N-glycosylation site at position 153 of E.

The E proteins were immunoprecipitated with an anti-DEN4 E peptide serum and further analyzed by digestion with endoglycosidase F (Fig. 3B). The result showed that the E

FIG. 2. DEN4 cDNAs used for preparation of chimeric viruses. Plasmid p2A(XhoI), which contains a complete cDNA copy of DEN4 strain 814669, was the basis for construction of all chimeric DEN4 cDNAs from the convenient *XhoI* site created near the 3' end of the E protein gene (3). p2A(H241-P) contains the structural protein gene defined by the sequence between the *BglII* site at nt 88 and the *XhoI* site of the DEN4 H241 parent; p2A(H241-N) contains the above-defined structural protein gene from the mouse-neurovirulent DEN4 H241 mutant. Plasmids p2A(H241-P,I-155,P-156), p2A(H241-P,I-155), p2A(H241-P,P-156), and p2A(H241-P,L-401) contain the same sequence as in p2A(H241-P) and the mutations specifying the indicated amino acid substitution in the E protein of the neurovirulent mutant. The mutations in the first three constructs were introduced by mutagenesis with an oligonucleotide-driven PCR. The mutation in the last construct, specifying a Leu substitution at position 401, was introduced by replacing the *BstEII-XhoI* fragment of DEN4 H241-P with the corresponding cDNA fragment of DEN4 H241-N. ▨, DEN4 (814669); □, DEN4 (H241 parent prototype); ▤, DEN4 (H241 neurovirulent mutant).

protein products of DEN4 H241-P and DEN4 H241-N were sensitive to endoglycosidase F digestion, and the sizes of both proteins appeared to be the same after digestion. This indicates that at least one of the two remaining potential N-glycosylation sites in DEN4 H241-N E protein is functional. On the other hand, the observed difference in the size of pre-M between the DEN4 H241-P and H241-N strains could not be explained by the conservative Val-78 to Ala substitution alone. Because DEN4 H241-N E glycosylation was altered, it was possible that pre-M could undergo differential posttranslational modification as a consequence of interaction with the E protein.

The viral proteins produced by other chimeric DEN4 viruses containing one or two amino acid substitutions in the E protein were also analyzed (Fig. 4). The results showed that 2A(H241-P) and two of its chimeric derivatives, 2A(H241-P,P-156) and 2A(H241-P,L-401), produced viral proteins of identical sizes. The E proteins produced by 2A(H241-P,I-155,P-156) and by 2A(H241-P,I-155) migrated faster than the E protein of 2A(H241-P), as predicted, because the glycosylation site sequence was removed.

Genetic loci determining DEN4 mouse neurovirulence. Three-day-old suckling mice were used to evaluate the neurovirulence of the 2A(XhoI), DEN4 H241-P, and H241-N strains and the derived chimeras 2A(H241-P) and 2A(H241-N). Two of 46 suckling mice inoculated intracerebrally with 1,000 PFU of 2A(XhoI) died of encephalitis on days 11 and 20. The mortality rate (4.3%) was similar to the result obtained previously (3). In the same experiment, 3 of 22 mice (14%) inoculated with 1,000 PFU of DEN4 H241-P died between

days 7 and 10, and 1 of 24 mice (4%) inoculated with the same dose of 2A(H241-P) died on day 19. The difference in mortality among the infected mouse groups was not significant ($P > 0.25$ by a χ^2 test) (Table 2). Fewer than 50% of the animals died even at the highest dose (1,000 PFU) tested. The LD₅₀ of DEN4 814669, H241-P, and its derived intratypic chimera was greater than 1,000 PFU. DEN4 H241-N proved to be highly neurovirulent; the calculated LD₅₀ was 0.6 PFU. Chimera 2A(H241-N) also proved to retain most of the mouse neurovirulence of its parent DEN4 H241-N; the calculated LD₅₀ of the virus was 23.3 PFU.

We also analyzed the mouse neurovirulence of chimeras engineered to contain single or double amino acid substitutions in the 2A(H241-P) genetic background. The results (Table 2) showed that chimera 2A(H241-P,P-156) was not neurovirulent, as the mortality rate of mice inoculated with 1,000 PFU of this virus did not differ from that of mice inoculated with its parent 2A(H241-P). Chimera 2A(H241-P,L-401) exhibited a moderate increase in neurovirulence, as the LD₅₀ of 91.7 PFU was much lower than that of nonneurovirulent 2A(H241-P) but higher than that of neurovirulent 2A(H241-N). Chimeras 2A(H241-P,I-155,P-156) and 2A(H241-P,I-155) were both neurovirulent, with an LD₅₀ of 23.1 and 2.6 PFU, respectively. These results provide evidence that both the Thr-155 to Ile and Phe-401 to Leu mutations in the DEN4 E sequence were determinants of DEN4 mouse neurovirulence. A comparison of neurovirulence suggests that Thr at position 155 is the most important determinant for the DEN4 neurovirulence phenotype in mice.

Animals were also inoculated intracerebrally with each virus

FIG. 3. Polyacrylamide gel analysis of viral proteins produced by parental and chimeric DEN4. (A) Aliquots of [35 S]methionine-labeled lysates of virus-infected C6/36 cells were immunoprecipitated with DEN4 hyperimmune mouse ascitic fluid, and the precipitates were analyzed by SDS-PAGE. The locations of the dengue virus E, pre-M, NS1, and NS3 proteins are indicated. Molecular size markers (in kilodaltons) are shown in lane M. (B) The same lysates were immunoprecipitated with a rabbit anti-E peptide serum, and the precipitates were digested with endoglycosidase F (+) or mock digested (-) prior to analysis by SDS-PAGE.

at a dose of 125 PFU, and the Kaplan-Meier survival curves were compared (Fig. 5). While most mice inoculated with the nonneurovirulent parent or its derived chimera survived for the 25-day observation period, all 15 mice inoculated with neurovirulent DEN4 H241-N died 7 to 11 days after inoculation. In the same experiment, 16 of 19 mice (84%) died between 10 and 15 days after inoculation with chimera 2A(H241-N). According to the Smirnov statistic, the difference between the survival distributions for DEN4 H241-N and its derived chimera 2A(H241-N) is highly significant ($P < 10^{-6}$), implying that the nonstructural region contributes to mouse neurovirulence. The groups infected with 2A(H241-P, L-401) ($P < 0.005$), 2A(H241-P, I-155, P-156) ($P < 0.001$), and 2A(H241-P, I-155) ($P < 10^{-6}$) were each significantly different from the group infected with 2A(H241-P). Similarly, the difference between the 2A(H241-P) and the 2A(H241-P, P-156) groups was highly significant ($P < 10^{-6}$). These data are consistent with the results of the LD₅₀ analysis described above. On the basis of this test, the survival curve for 2A(H241-P, I-155, P-156) is significantly different from that for 2A(H241-P, I-155) ($P < 0.05$). This observation suggests that the substitution of Pro for Ser-156 reduced the effect of the Ile-155 neurovirulence mutation.

DISCUSSION

Previously, mouse-neurovirulent mutants of dengue type 1, 2, and 4 viruses were selected by serial intracerebral passage of wild-type human dengue viruses in mice. These neurovirulent

dengue virus mutants have been used primarily as challenge viruses in experimental mouse immunoprophylaxis studies. However, these mutants should also prove useful for mapping the genetic loci responsible for mouse neurovirulence. Previous studies of intertypic chimeric dengue viruses constructed by using DEN4 cDNA as a vector indicated that at least some genetic determinants for the mouse-neurovirulent phenotype of DEN2 strain NGC were located in its structural protein genes (3).

In the current study, we pursued this question in a more systematic manner by analyzing the genetic loci for mouse neurovirulence of DEN4 strain H241. This was achieved by constructing intratypic chimeric viruses that contained the three structural protein genes from this virus mutant or its nonneurovirulent parental virus. The chimera that contained the structural protein genes from the neurovirulent DEN4 H241 proved to be highly neurovirulent, whereas the chimera that contained the corresponding genes from the parent DEN4 H241 was not neurovirulent. This observation indicates that most of the genetic loci responsible for the neurovirulent phenotype of mouse-adapted DEN4 H241 map within the structural protein genes. The contribution of the noncoding and nonstructural protein gene regions to DEN4 mouse neurovirulence has not been examined. Analysis of mouse neurovirulence revealed that the LD₅₀ of the neurovirulent mutant H241-N was slightly higher than that of the chimera 2A(H241-N), which contains the structural genes of H241-N in the background of nonneurovirulent DEN4 strain 814669. In addition, the mean day of death was also significantly different between the two viruses. These data are consistent with the

FIG. 4. Analysis of viral proteins produced by DEN4 chimeras. The radiolabeled sample was prepared from C6/36 cells infected with the indicated chimera and then immunoprecipitated as described in the legend to Fig. 3A. The positions of dengue virus proteins and molecular weight markers (lane M) are indicated.

notion that genetic loci outside the structural protein genes may also contribute to DEN4 mouse neurovirulence.

Despite numerous passages in mouse brain, the neurovirulent DEN4 H241 mutant differed from the parental virus at only five codons in the structural protein region. This observation suggests that the genotype of the neurovirulent DEN4

TABLE 2. Mouse neurovirulence of parental DEN4 and intratypic DEN4 chimeras measured by LD₅₀ after intracerebral inoculation of suckling mice^a

Virus	LD ₅₀ (PFU)
2A(XhoI)	>1,000.0
H241-P	>1,000.0
2A(H241-P)	>1,000.0
H241-N ^b	0.6
2A(H241-N) ^b	23.3
2A(H241-P,I-155,P156) ^b	23.1
2A(H241-P,I-155) ^b	2.6
2A(H241-P,P-156)	>1,000.0
2A(H241-P,L-401) ^b	91.7

^a Three-day-old outbred Swiss mice in groups of 7 to 10 were inoculated with serial dilutions of nonneurovirulent, parental DEN4 or intratypic chimeras at doses of 5, 25, 125, and 1,000 PFU.

^b Tested at doses ranging from 0.04 to 1,000 PFU. For example, neurovirulent H241-N was tested at doses of 0.04, 0.2, 1, and 5 PFU; chimera 2A(H241-N) was tested at 0.2, 1, 5, 25, and 125 PFU; and chimera 2A(H241-P,I-155) was tested at 0.04 to 1,000 PFU. At least two independent experiments were performed.

FIG. 5. Mouse neurovirulence of DEN4 parents and various chimeras. Three-day-old outbred Swiss mice were inoculated intracerebrally with 125 PFU of parental or chimeric viruses. Infected mice were then observed daily for signs of paralysis and death for 25 days. Because many viruses were tested in this study, the results are shown in two separate panels for clarity.

H241 had stabilized. Three of these amino acid changes in the neurovirulent mutant were located in the E protein sequence: Ile substituting for Thr-155, Pro for Ser-156, and Leu for Phe-401. Analysis of intratypic chimeras containing one or two of these changes constructed by site-directed mutagenesis allowed us to identify two specific changes that were responsible for neurovirulence. Specifically, the single substitution of Ile for Thr-155 which ablated one of the two conserved potential glycosylation sites in dengue virus E yielded progeny virus that was almost as neurovirulent as DEN4 H241-N. The available evidence did not permit distinction between the possibilities that the substitution of Ile itself or the loss of E glycosylation was responsible for neurovirulence. To answer to this question, we conducted a similar study which showed that a single substitution of Ala for Thr-155 at the corresponding glycosylation site of DEN4 strain 814669 also converted the nonneurovirulent parent to a neurovirulent virus (17a). Thus, loss of the glycosylation site in E appears to be an important contributor to DEN4 mouse neurovirulence.

The functional role that the carbohydrate moiety of flavivirus E glycoproteins plays during viral replication and pathogenesis is not known. Analysis of the amino acid sequences of the E glycoprotein from a variety of flaviviruses indicates that potential N-linked glycosylation sites vary in number and in position, although similarities are noted among members of the same subgroup. Kunjin and West Nile viruses do not contain an E glycosylation site sequence of the Asn-X-Thr/Ser type (5, 28). Japanese encephalitis virus and Murray Valley encephalitis virus contain only one such glycosylation site (5, 6). Host-specific as well as virus strain-specific differences in E glycosylation have been noted for yellow fever virus (8, 23).

The substitution of Leu for Phe-401 in DEN4 H241 was

identified as a second mutation that contributed to the acquisition of mouse neurovirulence. Our findings indicate that the chimera containing this substitution was less neurovirulent than the chimera bearing the glycosylation mutation discussed above. Sequence alignment analysis revealed that Phe occupied this position in the E sequence of all four prototype dengue viruses (7, 10, 11, 17, 19, 21, 30) as well as other flaviviruses. Leu substitution at position 401 of the neurovirulent DEN4 mutant resulted in a change from an aromatic amino acid to an aliphatic amino acid. It is of interest that an Ile substitution for Phe-401 occurred during the selection of a neurovirulent mutant from non-neurovirulent parental DEN2 New Guinea C during serial intracerebral passage in mice (3a).

On the basis of the Smirnov test, the difference in the survival distributions between the mouse groups infected with 2A(H241-P) and 2A(H241-P,P-156) had a *P* value of less than 10^{-6} . This indicates that the single substitution of Pro for Ser-156 in the parental DEN4 H241 E sequence had no apparent effect on mouse neurovirulence. Presumably, the Ile substitution for Thr-81 in C is also a silent mutation, because Ile also occupies this position in the C sequence of the nonneurovirulent DEN4 strain 814669. Similarly, the substitution of Ala for Val-78 observed in DEN4 H241 pre-M is a conservative change that did not alter neurovirulence.

Although highly neurovirulent in mice, DEN4 H241-N and the intratypic chimera containing all three structural genes of this virus in the background of DEN4 strain 814669 replicated slowly to a low titer in simian LLC-MK₂ cells. In contrast, the nonneurovirulent parental virus and its derived chimeras grew to a high titer in LLC-MK₂ cells. This indicates that the efficient growth of the dengue virus in simian cells is not linked to mouse neurovirulence. Neurovirulence is a complex pathological response to viral replication in the brain. It is likely that serial passage in mouse brain selected a virus mutant which replicated more efficiently in mouse brain, while at the same time the mutation responsible for neurovirulence caused restriction of replication in simian cells, yielding a neurovirulent mutant that also exhibited host range restriction. This dual phenotype is reminiscent of the highly monkey-neurovirulent poliovirus mutant that failed to replicate in cultures of non-neuronal cells (25a). We speculate that the mutations identified in E produce an alteration of the viral surface structure that facilitates binding of E to receptors on specialized cells in the brain. At present, the nature of such viral receptors remains unknown.

Although dengue virus infections in human do not manifest encephalitis symptoms, several other members of the *Flavivirus* genus cause acute encephalitis in humans, and there is significant homology (35 to 50%) among the E sequences of dengue viruses and other members of the *Flavivirus* genus. Although DEN4 from an intermediate mouse brain passage level has not been evaluated for utility as a vaccine, dengue virus type 1 and type 2 mutants selected in this manner were shown to be attenuated for humans (16, 24, 27). Analysis of amino acid changes in these viruses during serial mouse brain passage might help to identify other sequences that confer attenuation for humans and at the same time contribute to mouse neurovirulence.

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