Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10342–10346, November 1991
Medical Sciences

Construction of intertypic chimeric dengue viruses by substitution of structural protein genes

(chimera/viral proteins/neurovirulence)

MICHAEL BRAY AND CHING-JUH LAI

Molecular Viral Biology Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Communicated by Robert M. Chanock, August 28, 1991

ABSTRACT **Dengue virus contains an 11-kilobase positive-strand RNA genome that codes for, in one open reading frame, three structural proteins (capsid, premembrane, and envelope), followed by seven nonstructural proteins. The structural protein genes of a full-length cDNA clone of type 4 dengue virus were replaced with the corresponding genes of dengue 1 or dengue 2 to create intertypic chimeric cDNA. The RNA transcripts made from these templates were infectious when transfected into permissive cells in culture. Progeny of chimeric cDNA produced apparently authentic dengue 1 or dengue 2 structural proteins, together with dengue 4 nonstructural proteins, and as a consequence exhibited type 1 or type 2 serological specificity. Both of the chimeras ultimately grew to the same titer as their type 1 or type 2 parent, but the type 2/type 4 chimera grew very slowly. This chimera also produced small plaques; in contrast, the type 1/type 4 chimera produced normal size plaques. The type 2/type 4 chimera retained the mouse neurovirulence of the dengue 2 virus, which was the source of its structural protein genes. Each of the mice inoculated intracerebrally with the chimera died, but survival time was prolonged. The retardation of replication of the type 2/type 4 chimeric virus suggests that this virus and possibly other intertypic dengue virus chimeras with similar properties should be examined for attenuation in primates and possible usefulness in a live dengue virus vaccine for humans.**

Dengue is a mosquito-borne viral disease that occurs in tropical and subtropical regions throughout the world. The dengue virus subgroup causes more human disease than any other member of the flavivirus family. Dengue is characterized by fever, rash, severe headache, and joint pain. Its mortality rate is low. However, over the past few decades, a more severe form of dengue, characterized by hemorrhage and shock (dengue hemorrhagic fever/dengue shock syndrome, or DHF/DSS) has been observed with increasing frequency in children and young adults. DHF/DSS occurs most often during dengue virus infection of individuals previously infected with another dengue virus serotype, and this has led to the suggestion that immune enhancement of viral replication plays a role in pathogenesis of the more severe form of the disease (1).

Soon after their isolation in 1944, dengue viruses were passaged repeatedly in mouse brain, resulting in the selection of mouse neurovirulent mutants (2). Interestingly, studies performed in volunteers showed that mouse brain-adapted neurovirulent mutants of three strains of type 1 or type 2 virus were attenuated, but still immunogenic for humans (2–5). The mutants were not developed further as candidate vaccine strains because of concern for mouse brain antigens in the vaccine preparations. Since that time, virus mutants that (*i*) exhibited the small plaque size phenotype, and/or (*ii*) were temperature sensitive, and/or (*iii*) were adapted to cell cultures derived from an unnatural host (i.e., host range mutants) have been selected and evaluated as candidates for inclusion in a live attenuated virus vaccine (6–8). However, despite 25 years of such efforts, safe, effective dengue vaccines are still not available for general use.

There are four serotypes of dengue viruses (types 1–4), which are distinguishable by plaque reduction neutralization using serotype-specific monoclonal antibodies (mAbs) and by less specific tests using polyclonal sera (9, 10). The existence of serotypes was first discovered during early studies in human volunteers, which showed that infection with one dengue serotype induced durable homotypic immunity, whereas heterotypic immunity lasted only 3–6 months (2). It has been suggested that an effective dengue vaccine should contain all four serotypes in order to induce broad immunity that would preclude the occurrence of DHF/DSS.

The complete nucleotide sequences have been determined for dengue virus types 3 and 4 and several strains of type 2 virus, including the mouse-neurovirulent New Guinea C (NGC) strain; however, only the 5′ portion of the type 1 virus genome has been sequenced (11–16). The results of these studies indicate that the four dengue virus serotypes share a common genome organization. The genome of the dengue type 4 Caribbean strain 814669 was found to contain 10,646 nucleotides (11, 12). The first 101 nucleotides at the 5′ end and the last 384 at the 3′ end are noncoding. The remaining sequence codes for a 3386 amino acid polyprotein, which includes the three structural proteins—namely, capsid (C), pre-membrane (pre-M), and envelope (E), at its N terminus—followed by seven nonstructural proteins in the order NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The polyprotein is processed by cell signal peptidase(s) and by viral proteases to generate 11 or more viral proteins (17–20).

We recently succeeded in cloning a stable, full-length cDNA copy of type 4 dengue virus (designated p2A), which can serve as a template for *in vitro* transcription of infectious RNA (21). Thus, it is now possible to introduce specific mutations into a full-length dengue cDNA and recover viable dengue viruses bearing those mutations. Mutants produced in this manner should be useful for biologic studies and for construction of live virus vaccines. We now report the construction of two viable chimeric dengue viruses through replacement of the structural protein genes of the dengue type 4 cDNA clone with the corresponding genes of type 1 or type 2 dengue virus. These chimeric viruses exhibit the serologic specificity of type 1 or type 2 but retain the nonstructural proteins of type 4. The chimeric type 2/type 4

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; pfu, plaque-forming unit(s); E protein, envelope protein; C protein, capsid protein; pre-M protein, pre-membrane protein; IFA, immunofluorescence assay; HMAF, hyperimmune mouse ascitic fluid; WP strain, Western Pacific strain; NGC strain, New Guinea C strain; mAb, monoclonal antibody.

virus retains the neurovirulence phenotype of dengue 2 NGC strain, from which its structural genes were derived.

MATERIALS AND METHODS

Viruses. Dengue type 4 virus strain 814669 was used for construction of full-length cDNA that served as the source of full-length infectious RNA transcripts (11, 12). A preparation of dengue type 1 virus Western Pacific (WP) strain (D1 WP), fetal rhesus lung cell passage level 9, was kindly provided by K. Eckels (Walter Reed Army Institute of Research, Washington) (21). A mouse brain preparation of mouse neurovirulent dengue type 2 virus New Guinea C (NGC) strain (D2 NGC), mouse brain passage level 38, was kindly provided by D. Dubois (Walter Reed Army Institute of Research) (2). Dengue 1 and dengue 2 viruses were amplified by one passage in C6/36 mosquito cells. Each of these three viruses was then passaged once in LLC-MK_2 simian kidney cells, and the resulting virus suspension was used to study plaque morphology and mouse neurovirulence.

Cloning Vectors. Plasmid p5′-2, which contains the 5′ half of the dengue 4 genome cDNA, was modified to facilitate replacement of the three structural protein genes (22). First, a unique *Xho* I site was introduced through site-directed mutagenesis at nucleotide 2342 (A → G) of the dengue 4 sequence, near the site encoding the C terminus of E protein, creating p5′-2(Xho I). This nucleotide change did not alter the amino acid sequence. This vector was then digested at the unique *Bst*BI site within the dengue sequence and at the unique *Asp*718 site, which immediately follows the dengue sequence in p5′-2, and the fragment was ligated to the 3′ half of the dengue 4 genome, creating full-length p2A(Xho I). Second, the *Bgl* II site at nucleotide 88, which is conserved among flaviviruses, was made unique by removing three other *Bgl* II sites in p5′-2. The *Bgl* II site at the junction between pBR322 and the SP6 promoter was removed by inserting a *Not* I linker. Two other sites at 4128 and 4277 were removed by shortening the vector to the recently introduced *Pst* I site at 3473 (21). Plasmid p5′-2(Xho I, ΔPst I) was subsequently used for fragment exchange to create chimeric cDNA.

Chimeric cDNA. Dengue type 1 or type 2 virus grown in C6/36 mosquito cells was purified, and virion RNA was extracted (12). Dengue 1 first-strand cDNA was synthesized by reverse transcription using a negative-sense oligonucleotide that hybridized to nucleotides 2306–2338 of the dengue 1 sequence. This primer sequence contained a silent third base change (A → G) at nucleotide 2316 to create an *Xho* I site at the position corresponding to the site in the dengue 4 E gene in p5′-2(Xho I, ΔPst I). The dengue 1 cDNA was then used as the template to synthesize double-stranded DNA by PCR, using the negative-sense oligonucleotide and a positive-sense primer that hybridized to dengue 1 nucleotides 51–70 and contained the conserved *Bgl* II site. The PCR product was digested with *Bgl* II and *Xho* I and then cloned into p5′-2(Xho I, ΔPst I), replacing the corresponding dengue 4 sequence. The *Cla* I–*Xho* I fragment containing the dengue 1 sequence was then joined with the remaining dengue 4 cDNA from p2A(Xho I) to create full-length chimera p2A(D1 WP). Similarly, dengue 2 first-strand cDNA was synthesized by using a negative-sense primer that hybridized to dengue 2 nucleotides 2310–2364. This primer contains three base changes: T → C at 2333, A → G at 2336, and C → A at 2337. These changes create an *Xho* I site at the position corresponding to the *Xho* I site in the dengue 4 E gene described above. These changes did not alter the amino acid sequence. For double-strand DNA synthesis by PCR, the negative-sense primer was used, and the positive-sense primer was the same used for dengue 1. The PCR product was digested with *Bgl* II and *Xho* I and cloned into p5′-2(Xho I), and the *Cla* I–*Xho* I fragment was used to replace the corresponding fragment of p2A(Xho I) to create p2A(D2 NGC).

RNA Transcription, Transfection, and Recovery of Virus. Transfection of cells with full-length RNA transcripts was performed as described (22). Ten days after transfection, cells were trypsinized and transferred to a 6-well plate and to a chamber slide. Two days later, cells on the slide were tested by an immunofluorescence assay (IFA) for evidence of dengue viral antigens. If IFA showed that most cells were infected, the cells in the 6-well plate were trypsinized, mixed with a 6-fold excess of uninfected cells, and incubated until cytopathic effects (appearance of numerous dead cells in the medium) became evident, usually after 6 or 7 days. The infected cells were then harvested by removing the medium, scraping the cells, resuspending them in a standard volume of 50% Eagle's minimal essential medium/50% serum, and then freezing. If a small percentage of cells was positive, the cells were trypsinized, diluted 1:3 in fresh medium, and allowed to grow without addition of uninfected cells. The percentage of infected cells was then estimated on a weekly basis, and cell lysates were harvested and titered for virus at intervals.

Detection of Dengue Virus Antigens. Infected cells were analyzed by IFA using serotype-specific mAbs 1F1 (dengue 1), 3H5 (dengue 2), 5D4 (dengue 3), and 1H10 (dengue 4); nonstructural protein NS1-specific mAb 1G6 (dengue 4), originally produced by M. K. Gentry and E. Henchal (10); and fluorescein-conjugated anti-mouse antiserum (Kierkegaard & Perry Laboratories, Gaithersburg, MD). For each mAb, infected cells yielding a negative result were photographed for the same exposure time as positive cells. To analyze proteins of parental (wild-type) dengue viruses and of progeny virus v2A(Xho I), confluent LLC-MK_2 cells in a 6-well plate were infected with virus at an multiplicity of infection of 0.2. Six days after infection, cells were labeled with [^{35}S]methionine (50 μCi per well, specific activity of 600 Ci/mmol; 1 Ci = 37 GBq) in methionine-free medium for 6 hr, and cells were then lysed with RIPA (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) buffer. For chimeric virus v2A(D1 WP), labeling was performed when ≈100% of the cells were infected; for v2A(D2 NGC), labeling was performed when ≈30% were infected. Lysates were precipitated with the appropriate homotypic or heterotypic hyperimmune mouse ascitic fluid (HMAF) and analyzed by electrophoresis on SDS/12% polyacrylamide gels.

Plaque Morphology. Viruses were characterized for plaque morphology on LLC-MK_2 cells (9). Each parental virus was passaged once in LLC-MK_2 cells prior to analysis of plaque morphology. Neutral red overlay was added to cultures after 6–9 days of incubation.

Evaluation of Chimeric Viruses in Suckling Mice. Three-day-old Swiss mice were inoculated intracerebrally with 2000 plaque-forming units (pfu) of parental or chimeric dengue virus, in the form of a diluted lysate of virus-infected LLC-MK_2 cells. Negative control mice were injected with a lysate of uninfected cells. One litter was inoculated with parental dengue type 4 virus (814669), dengue type 1 virus WP, or dengue type 2 virus NGC. Two litters were inoculated with v2A(Xho I), v2A(D1 WP), or v2A(D2 NGC). Inoculated mice were monitored for 21 days for encephalitis and for death.

RESULTS

Stable Full-Length Chimeric cDNA. Transformation of *Escherichia coli* strain BD1528 bacteria with plasmid constructs p2A(Xho I), p2A(D1 WP), or p2A(D2 NGC) produced a stable plasmid population. The predicted structure of these plasmids is shown in Fig. 1. Their structure was verified by restriction enzyme mapping.

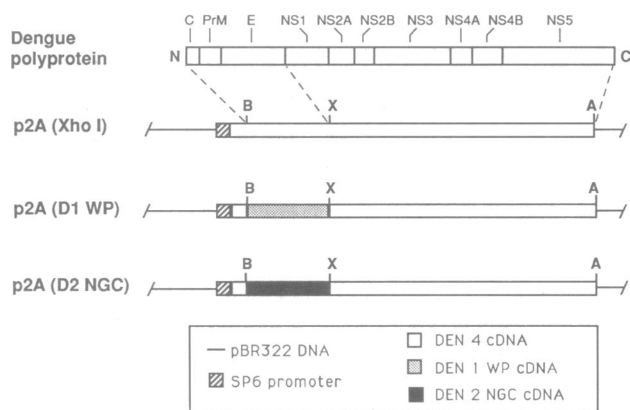

FIG. 1. Structure of full-length dengue virus cDNAs used for preparation of chimeric dengue viruses. The following plasmids containing full-length dengue cDNA were constructed: p2A(Xho I), a complete cDNA copy of the dengue 4 genome, in which a unique *Xho* I site was created near the 3′ end of the E gene; p2A(D1 WP), derived from p2A(Xho I) by replacing the sequence between the *Bgl* II site in the 5′ noncoding region and the unique *Xho* I site with the corresponding cDNA of dengue 1 WP strain; p2A(D2 NGC), prepared in the same manner by replacing the *Bgl* II–*Xho* I fragment with the corresponding cDNA from dengue 2 NGC strain. B, *Bgl* II; X, *Xho* I; A, *Asp* 718. DEN, dengue; PrM, pre-M.

Recovery of Chimeric Viruses. Approximately one-half of the LLC-MK_2 cells transfected with RNA transcripts from p2A(Xho I) were positive by immunofluorescence staining for dengue virus antigens on day 12 (Fig. 2*A*). In a previous study, a similar proportion of antigen-positive cells, in the range of 20–50%, was observed 12 days after transfection with transcripts from p2A and p2A(Pst I), which in both instances yielded virus with wild-type phenotype (22). In contrast, on day 12 <1% of cells transfected with transcripts from p2A(D1 WP) or p2A(D2 NGC) were positive. The percentage of cells infected with v2A(D1 WP) increased to ≈5% by day 19, 30% by day 26, and 80% by day 33, at which time the titer of virus present in transfected cells was 5×10^6 pfu/ml (Fig. 2 *A* and *B*). In contrast, v2A(D2 NGC) grew more slowly: ≈1% of the cells were positive on day 19, but <33% of the cells had become infected by day 54. The virus titer was only 1.5×10^3 pfu/ml at 30 days and 2.5×10^3 pfu/ml at 44 days; it did not reach 10^4 pfu/ml until day 58. At 72 days after transfection, a majority of cells were infected, and the titer of the cell suspension was 10^5 pfu/ml.

The titer of virus produced by cells transfected with p2A(Xho I) RNA transcripts (10^6 pfu/ml) was the same as that observed when cell cultures were infected with type 4 virus at a multiplicity of infection (moi) of 0.1. In addition, the highest titer of virus produced by cultures transfected with the type 1/type 4 chimera (5×10^6 pfu/ml) was similar to that observed following infection of cell cultures with type 1 virus at an moi of 0.1 (1.5×10^7 pfu/ml). The highest titer of the type 2/type 4 chimera produced following transfection (10^5 pfu/ml) was similar to that produced by cell cultures infected with its type 2 parent at an moi of 0.1.

Characterization of Structural Proteins Produced by Chimeric Viruses. Indirect immunofluorescence was performed to characterize progeny viruses, as shown in Fig. 3. Cells infected with v2A(Xho I) were stained with dengue 4-specific mAb 1H10 by IFA but did not bind dengue 1-specific mAb 1F1 or dengue 2-specific mAb 3H5. As predicted, cells infected with v2A(D1 WP) reacted only with mAb 1F1, and those infected with v2A(D2 NGC) were stained only with mAb 3H5. mAb 5D4, specific for dengue 3 virus, did not react with any of the infected cells (data not shown). These results indicated that both chimeric viruses exhibited the antigenicity specified by their structural protein genes. As expected,

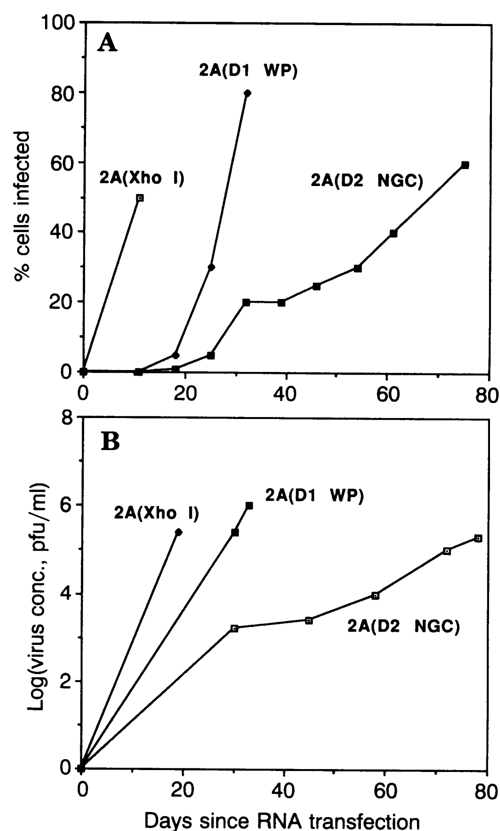

FIG. 2. Percentage of virus-infected cells and titer of virus following transfection with RNA transcripts. (*A*) LLC-MK_2 cells were transfected with the same concentration of RNA transcripts from p2A(Xho I), p2A(D1 WP), or p2A(D2 NGC). The percentage of virus-infected cells after transfection was estimated by IFA after replating in fresh medium. (*B*) Infected cells were harvested when a majority of cells was positive by IFA. Titration of virus was performed by plaque assay (9).

mAb 1G6, specific for dengue 4 nonstructural protein NS1, stained cells infected by progeny of the dengue type 4 cDNA [i.e., 2A(Xho I)], as well as either of the chimeric viruses, 2A(D1 WP) or 2A(D2 NGC), derived from the type 4 virus.

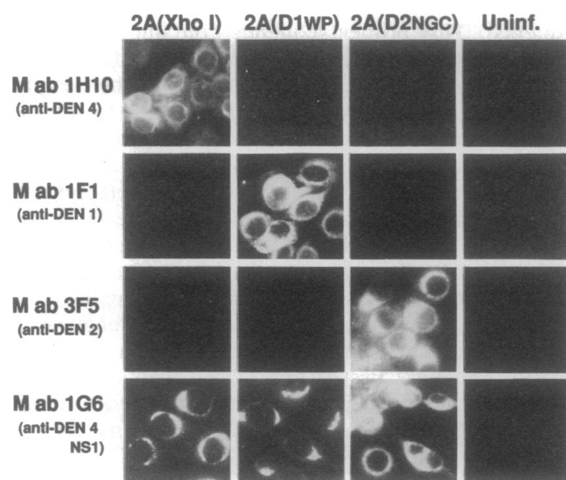

FIG. 3. Serotype analysis of chimeric dengue viruses. IFA was performed on LLC-MK_2 cells infected with v2A(Xho I), v2A(D1 WP), or v2A(D2 NGC) or on uninfected (Uninf.) cells. mAbs were used at dilutions of 1:50–1:300, whereas fluorescein-conjugated anti-mouse antibody was used at 1:100. The serotype specificity of the mAbs is shown at left.

The proteins of parental dengue type 4 virus and of its cDNA-derived progeny v2A(XhoI) appeared to be identical when analyzed by immunoprecipitation with homotypic HMAF followed by polyacrylamide gel electrophoresis (Fig. 4). The proteins of parental dengue type 1 virus (WP) and of dengue type 2 virus (NGC) were also analyzed. The E glycoproteins of dengue 1 and dengue 2 comigrated, but they migrated more slowly than the E protein of dengue 4. Similarly, the pre-M glycoproteins of dengue 1 and 2 comigrated, but they also migrated more slowly than the pre-M of dengue 4. The difference in relative molecular size of the E proteins was ≈4 kDa, whereas that of the pre-M proteins was about 1 kDa. These differences in molecular size of the E and pre-M proteins were probably due to variation in the extent of glycosylation or conformational differences. The immunoprecipitated proteins of viruses v2A(D1 WP) and v2A(D2 NGC) showed a hybrid pattern. Pre-M and E proteins of v2A(D1 WP), immunoprecipitated with dengue 1 HMAF, comigrated with pre-M and E proteins of parental dengue 1, but dengue 4 HMAF precipitated only bands that comigrated with dengue 4 nonstructural proteins. Similarly, pre-M and E of v2A(D2 NGC) comigrated with pre-M and E of parental dengue 2, whereas dengue 4 HMAF precipitated nonstructural proteins resembling those of type 4 virus.

Plaque Morphology of Parental and Progeny Viruses. Parental dengue type 4 virus and its cDNA-derived progeny v2A(Xho I) produced similar plaques on LLC-MK_2 cells (data not shown). The plaques of parental viruses were visible when stained with neutral red 6 days after infection, but chimera v2A(D2 NGC) failed to produce detectable plaques at that time. If staining was delayed until day 9, very small v2A(D2 NGC) plaques were detected; they were much smaller than those of v2A(Xho I) or parental dengue 2 NGC (Fig. 5). Plaques of dengue 4 814669 did not differ from those of progeny virus v2A(Xho I). Plaques of v2A(D1 WP) were similar to those of parental dengue 1 WP or v2A(Xho I).

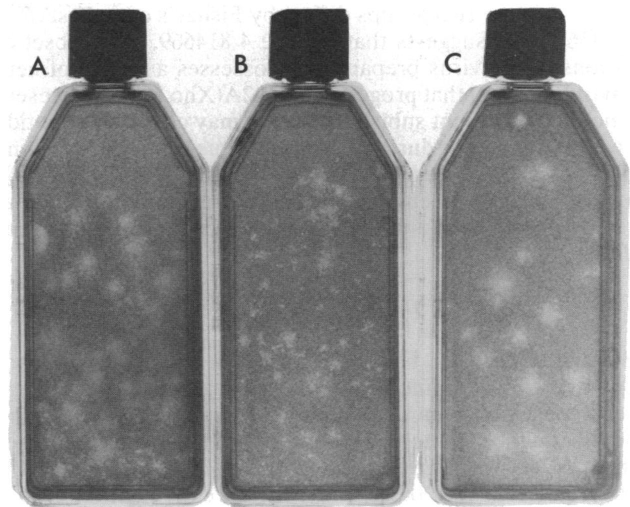

FIG. 5. Morphology of viral plaques on LLC-MK_2 cells. Monolayers of LLC-MK_2 cells were infected with dengue virus, followed by an agarose overlay (9). Neutral red overlay was applied 9 days after infection of the cell monolayer, and plaques were photographed the next day. (*A*) 2A(Xho I). (*B*) 2A(D2 NGC). (*C*) Wild-type NGC.

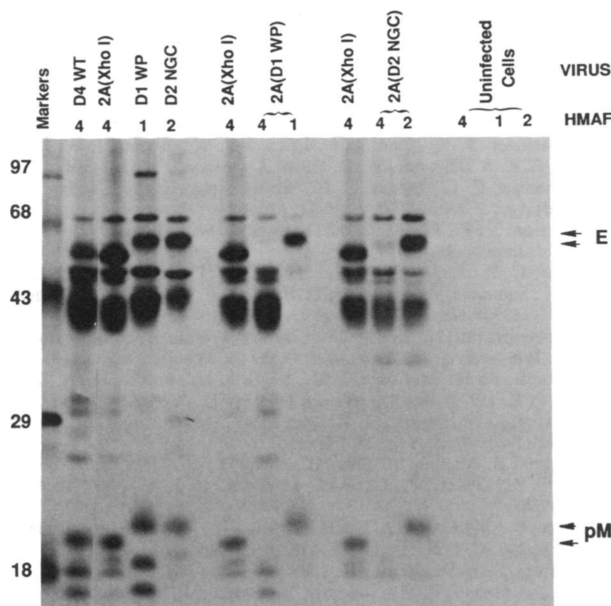

FIG. 4. Polyacrylamide gel analysis of dengue types 1, 2, and 4 viral proteins produced by parental and chimeric dengue viruses. [^{35}S]Methionine-labeled lysates of virus-infected LLC-MK_2 cells or uninfected control cells were immunoprecipitated with HMAF raised against dengue 1, 2, or 4 and analyzed by electrophoresis on an SDS/12% polyacrylamide gel (acrylamide/*N,N'*-methylenebisacrylamide = 60:1.6). At the top of each lane, virus is indicated vertically, and the HMAF with which the lysate was immunoprecipitated is indicated horizontally. Markers, protein molecular size markers in kilodaltons. The locations of the E and pre-M (pM) glycoproteins of dengue types 1, 2, and 4 are indicated. WT, wild type.

Virulence of Viruses for Suckling Mice. Chimera v2A(D2 NGC) was compared with its parental viruses, mouse brain-adapted dengue 2 NGC and v2A(Xho I), for mouse neurovirulence. Dengue 2 NGC proved to be the most rapidly lethal: each of 10 mice died of encephalitis on day 5 or day 6 postinoculation, with a mean survival time of 5.1 days, while each of the 15 mice inoculated with chimera v2A(D2 NGC) survived 8–11 days before dying, with a mean survival time of 9.1 days (Fig. 6). This difference in survival distributions is significant ($P = 0.00001$, Smirnov two-point statistic D_{mn}). We also compared parental virus dengue 4 814669 to its progeny v2A(Xho I). Five of 12 mice inoculated with the parental virus died of encephalitis; the first death occurred on day 11, while only 1 of 18 mice inoculated with v2A(Xho I) died 16 days after inoculation; the rest remained healthy. Although the survival distributions do not differ significantly according to the Smirnov statistic ($P > 0.14$), the percent

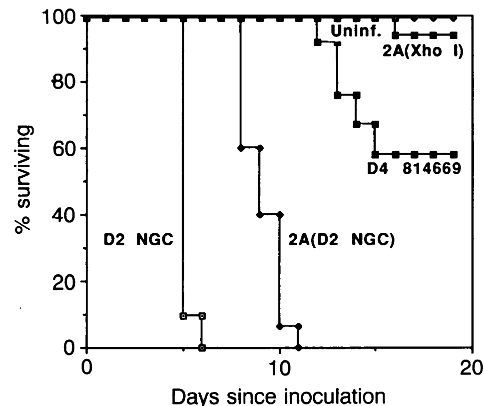

FIG. 6. Mouse neurovirulence of parental dengue type 2 NGC or dengue type 4 virus or type 2/type 4 chimeric virus. Three-day-old Swiss mice were inoculated intracerebrally with 2000 pfu of parental or chimeric dengue virus, in the form of a diluted lysate of infected LLC-MK_2 cells. The type of virus and the number of mice injected were dengue 2 (NGC), 10 mice; v2A(D2 NGC), 15 mice; dengue 4 814669, 12 mice; v2A(Xho I), 18 mice. Negative controls (11 mice) were injected with a diluted lysate of uninfected (Uninf.) cells. Mice were then monitored daily for signs of encephalitis and for death.

survival of the two groups differs by Fisher's exact test ($P = 0.0256$). This suggests that dengue 4 814669, or a subset of virions in the virus preparation, possesses a degree of neurovirulence and that progeny virus v2A(Xho I) may represent a nonneurovirulent subpopulation or may contain nucleotide changes that arose during cloning or virus propagation. None of 12 mice inoculated with parental dengue type 1 virus (WP), 18 injected with its chimeric progeny v2A(D1 WP), or 11 that received a lysate of uninfected cells developed encephalitis or died.

DISCUSSION

Sequence analysis has shown that there is a significant conservation of amino acid sequence among the four dengue serotypes. With regard to the three structural proteins, amino acid sequence homology between dengue 4 and the other three serotypes ranges from 62% to 74% for C, pre-M, or E (13–16). Because of these similarities, we used our full-length dengue 4 cDNA clone to engineer chimeric cDNA that contained the three structural protein genes from the dengue type 1 virus WP strain or a mouse neurovirulent mutant of dengue type 2 virus, NGC strain, plus the nonstructural protein genes and the 5′ and 3′ noncoding sequences of dengue type 4 virus. RNA transcripts derived from each of the cDNA templates were used to transfect permissive LLC-MK_2 cells, and infectious progeny virus was recovered. Serologic analysis and identification of viral proteins confirmed that the chimeric viruses produced the corresponding structural proteins and exhibited type 1 or type 2 antigenicity.

Although the two chimeric dengue viruses and the parental type 4 virus had the same genomic structure and shared the 5′ and 3′ noncoding regions as well as the nonstructural protein genes of type 4 virus, the growth phenotype of these viruses on cultured cells varied. After an initial delay, the growth rate of chimera v2A(D1 WP) appeared to be similar to that of parental v2A(Xho I). On the other hand, the growth rate of chimera v2A(D2 NGC) was markedly reduced, but this virus ultimately attained the titer of its parental dengue type 2 virus. We speculate that the slow growth phenotype of v2A(D2 NGC) resulted from suboptimal viral gene expression, assembly, and/or maturation. This may reflect relative incompatibility of type 2 structural proteins and type 4 viral nonstructural proteins such as viral proteases. It is also possible that viral RNA packaging requires specific interaction between the C protein and primary sequence or secondary structure of viral RNA. A mismatch between the dengue 2 C protein and dengue 4 RNA sequences, possibly in the noncoding regions, could also be responsible for slow replication of this chimeric virus. Accordingly, it will be interesting to determine the effect of replacing the C protein of v2A(D2 NGC) with the C protein of dengue 4 or replacing the dengue 4 sequence of the 5′ noncoding region of the chimera with the 5′ noncoding sequence of dengue 2. Another possible explanation for the slow growth rate of v2A(D2 NGC) is alteration in the dengue 2 or dengue 4 sequence that arose during cloning, as mentioned above.

Study of chimera v2A(D2 NGC) in suckling mice demonstrated that the virus retained much of the neurovirulence of parental dengue 2 NGC. This suggests that most of the genetic determinants of neurovirulence map within the three structural protein genes. The delay in mortality caused by the chimeric virus could also reflect the type 2/type 4 incompatibilities discussed above or a degree of attenuation of v2A(Xho I), from which v2A(D2 NGC) is partially derived. Alternatively, specific sequences in dengue 2 noncoding regions or the nonstructural protein genes may be needed for rapid growth of virus in mouse brain. Previous clinical studies showed that three independently derived, mouse-adapted, highly neurovirulent mutant strains of dengue type 1 or type 2 virus were attenuated for humans (3–5). Therefore, it is possible that identification of the site or sites responsible for neurovirulence may help to identify mutations conferring attenuation for humans. These questions can be addressed by genetic mapping and phenotype analysis of the appropriate dengue virus chimeras.

The current strategy for immunization against dengue favors the use of a vaccine preparation containing all four dengue serotypes. This would protect individuals in endemic areas from the risk of severe dengue resulting from reinfection with a different dengue serotype. Our success in constructing viable chimeric dengue viruses offers the possibility of producing viruses representing each of the four dengue serotypes on a single genetic background of shared 5′ and 3′ noncoding sequences and nonstructural protein sequences. If mutations conferring attenuation can be engineered within these shared regions, it may be possible to construct a set of chimeric viruses that include all four dengue serotype specificities. However, intertypic incompatibility such as that exhibited by the type 4/type 2 chimera may be sufficient to produce a satisfactory level of attenuation, without loss of immunogenicity, thus yielding promising candidate live virus vaccine strains. It may also be possible to develop chimeric virus vaccines against other important flavivirus pathogens, such as Japanese encephalitis virus or tick-borne encephalitis virus, by substituting their structural protein genes into the full-length dengue type 4 virus cDNA. Since these novel viruses may show altered pathogenicity, such investigations must be carried out with caution, under appropriate biological containment.

We are grateful to Dr. Robert Chanock for encouragement of this work, to Drs. K. Eckels and D. Dubois for providing viruses and cells, to Dr. David Alling for statistical analysis, to Drs. B. Falgout and L. Markoff and other staff members in the Molecular Viral Biology Section for helpful discussions, to Ms. M. Pethel for providing plasmid p5′-2(Pst I), and to Mr. T. Popkin for expert photographic work.

1. Halstead, S. B. (1988) *Science* **239**, 476–481.
2. Sabin, A. B. (1952) *Am. J. Trop. Med. Hyg.* **1**, 30–50.
3. Sabin, A. B. (1955) *Am. J. Trop. Med. Hyg.* **4**, 198–207.
4. Schlesinger, R. W., Gordon, I., Frankel, J. W., Winter, J. W., Patterson, P. R. & Dorrance, W. R. (1956) *J. Immunol.* **77**, 352–364.
5. Wisseman, C. L., Sweet, B. H., Rosenzweig, E. C. & Eylar, O. R. (1963) *Am. J. Trop. Med. Hyg.* **12**, 620–623.
6. Harrison, V. R., Eckels, K. H., Sagartz, J. W. & Russell, P. K. (1977) *Infect. Immun.* **18**, 151–156.
7. Hoke, C. H., Malinoski, F. J., Eckels, K. H., Scott, R. M., Dubois, D. R., Summers, P. L., Simms, T., Burrous, J., Hasty, S. E. & Bancroft, W. H. (1990) *Am. J. Trop. Med. Hyg.* **43**, 219–226.
8. Bhamarapravati, N., Yoksan, S., Chayaniyayothin, T., Angsubphakorn, S. & Bunyaratvej, A. (1987) *Bull. WHO* **65**, 189–195.
9. Bancroft, W. H., McCown, J. M., Lago, P. M., Brandt, W. E. & Russell, P. K. (1979) *Pan Am. Health Organ. Sci. Publ.* **375**, 175–178.
10. Henchal, E. A., Gentry, M. K., McCown, J. M. & Brandt, W. E. (1982) *Am. J. Trop. Med. Hyg.* **31**, 548–555.
11. Mackow, E., Makino, Y., Zhao, B., Zhang, Y. M., Markoff, L., Buckler-White, A., Guiler, M., Chanock, R. & Lai, C. J. (1987) *Virology* **159**, 217–228.
12. Zhao, B., Mackow, E., Buckler-White, A., Markoff, L., Chanock, R. M., Lai, C. J. & Makino, Y. (1986) *Virology* **155**, 77–88.
13. Osatomi, K. & Sumiyoshi, H. (1990) *Virology* **176**, 643–647.
14. Irie, A., Mohan, P. M., Sasaguri, Y., Putnak, R. & Padmanabhan, R. (1989) *Gene* **75**, 197–211.
15. Mason, P. W., McAda, P. C., Mason, T. L. & Fournier, M. J. (1987) *Virology* **161**, 262–267.
16. Hahn, Y. S., Galler, R., Hunkapiller, T., Dalrymple, J., Strauss, J. & Strauss, E. (1988) *Virology* **162**, 167–180.
17. Markoff, L. (1989) *J. Virol.* **63**, 3345–3352.
18. Falgout, B., Chanock, R. M. & Lai, C. J. (1989) *J. Virol.* **63**, 1852–1860.
19. Falgout, B., Pethel, M., Zhang, Y. M. & Lai, C. J. (1991) *J. Virol.* **65**, 2467–2475.
20. Hori, H. & Lai, C. J. (1990) *J. Virol.* **64**, 4573–4577.
21. McKee, K. T., Bancroft, W. H., Eckels, K. H., Redfield, R. R., Summers, P. L. & Russell, P. K. (1987) *Am. J. Trop. Med. Hyg.* **36**, 435–442.
22. Lai, C. J., Zhao, B., Hori, H. & Bray, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5139–5143.