JOURNAL OF VIROLOGY, June 1996, p. 3930–3937 Vol. 70, No. 6
0022-538X/96/$04.00+0

Dengue Type 4 Virus Mutants Containing Deletions in the 3′ Noncoding Region of the RNA Genome: Analysis of Growth Restriction in Cell Culture and Altered Viremia Pattern and Immunogenicity in Rhesus Monkeys

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Received 11 December 1995/Accepted 1 March 1996

The dengue type 4 virus (DEN4) genome contains a 384-nucleotide (nt) 3′ noncoding sequence in which the last 81 nt, predicted to form a secondary structure, are thought to be essential for virus replication. Immediately upstream of the secondary structure, short RNA sequences that are conserved among mosquito-borne flaviviruses have been identified. A series of deletions that range from 30 to 262 nt were introduced into this upstream region of full-length DEN4 cDNA to create viable deletion mutants, some of which might prove to be useful for inclusion in a live attenuated virus vaccine. When studied by an infectious-center assay, most full-length RNA transcripts of the deletion constructs exhibited reduced infectivity when transfected into simian LLC-MK_2 cells compared with the full-length RNA transcripts of wild-type parental virus. Deletion mutations that extended as far as the 5′ boundary of the 3′ noncoding region and whose 3′ boundary did not extend beyond the last 113 nt of the 3′ end were viable. With the exception of mutant 3′d 303-183, which contained a deletion of nt 303 to 183 from the 3′ terminus, deletion mutants produced plaques that appeared late on simian LLC-MK_2 cells or exhibited a small-plaque morphology on mosquito C6/36 cells compared with the wild-type virus. These mutants also replicated less efficiently and attained a lower titer in LLC-MK_2 cells than parental wild-type virus. Significantly, mutant 3′d 303-183 grew to a high titer and was least restricted in growth. Mutant 3′d 303-183 and four other moderately to severely restricted mutants were selected for evaluation of infectivity and immunogenicity in rhesus monkeys. There was a suggestion that occurrence and duration of viremia were reduced for some of the deletion mutants compared with the wild-type virus. However, more convincing evidence for attenuation of some of the mutants was provided by an analysis of antibody response to infection. Mutant 3′d 303-183 induced an antibody response equivalent to that stimulated by wild-type virus, whereas other mutants induced low to moderate levels of antibodies, as measured by radioimmunoprecipitation and virus neutralization. The immunogenicity of these 3′ DEN4 deletion mutants in monkeys appeared to correlate with their efficiency of growth in simian LLC-MK_2 cells. One or more mutants described in this paper may prove to be useful for immunization of humans against disease caused by dengue virus.

There are approximately 70 arthropod-borne viruses in the *Flavivirus* genus of the family *Flaviviridae*. The four dengue virus serotypes (DEN1 to DEN4) are most important in terms of human morbidity. Dengue outbreaks and epidemics continue to pose a public health problem in the tropical and subtropical regions, where its *Aedes aegypti* and *Aedes albopictus* mosquito vectors are abundant. Soon after DEN1 and DEN2 were isolated 50 years ago, serial passage in mouse brain was employed to attenuate these viruses for possible use in a live attenuated vaccine (15, 28, 29). Virus mutants selected by this procedure were shown to be partially attenuated for humans. This vaccine approach was not developed further because extensive virus purification would be required in order to remove contaminating mouse brain antigens. More recently, live candidate dengue vaccines were prepared by repeated passage in cultured cells of nonnatural hosts (9, 10, 22). Experience from different laboratories indicated that this new approach has not been consistently successful in producing attenuated vaccine strains. In an attempt to overcome these problems, we adapted a strategy of using genetic manipulation at the DNA level to construct stable attenuated mutants with defined mutations. This approach was made possible by our previous success in cloning a full-length DEN4 cDNA whose full-length RNA transcripts were infectious in cell culture (21).

The flavivirus positive-strand RNA genome contains approximately 11 kb. Sequence analysis indicated that 95% of the genome codes for a polyprotein with the three structural proteins in the order C–pre-M–E at the N terminus and nonstructural proteins NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 at the C terminus (8). The remaining sequences, which include approximately 100 nucleotides (nt) with an m_7G cap at the 5′ end and 400 to 600 nt at the 3′ end, are noncoding. Virion RNA is infectious when inoculated into an animal or transfected into cultured cells (1). During productive infection, the enveloped virus enters cells, presumably by receptor-mediated endocytosis, undergoes uncoating in acidified vesicles, and releases its virion RNA into the cytoplasm to initiate the viral replication cycle. In addition to serving as mRNA, virion RNA also directs the synthesis of genome-length minus-strand RNA that is

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present in the replicative intermediate, providing the template for transcription of mRNA and virion RNA by a semiconservative mechanism. It is thought that NS3 and NS5 are major components of the replicative machinery (4, 11, 23). Other viral proteins as well as cellular proteins may also play a role during viral replication.

The current model for the replication of positive-strand RNA viruses proposes that the 3′ noncoding (3′NC) region contains short sequences that serve as signals for the initiation of negative-strand RNA synthesis. Signal sequences for RNA packaging may also be present in this region. Attempts have been made to define such sequences. For example, thermodynamic analysis and biochemical probing of the 3′NC region of poliovirus RNA have provided evidence for the existence of a pseudoknot structure which may play a role in viral replication (17). In addition, the functional significance of the conserved 3′-terminal 19-nt sequence of the Sindbis alphavirus has been studied by mutational analysis (19). Interestingly, this study showed that with the exception of several deletions near the 3′ terminus, most short deletions produced viable mutants. These deletion mutants of Sindbis virus generally exhibited growth restriction and were found to be avirulent or less virulent than the wild-type virus for mice (18).

The 3′NC region of the RNA genome of mosquito-borne flaviviruses varies from 400 to 600 nt in length, of which the last 80 nt have the potential to form a stable secondary structure (6, 8, 26, 30, 31). Comparison of these 3′NC sequences has also identified short sequences that appear to be conserved among flaviviruses (8, 12). The functional role that these conserved sequences play during viral replication is still not clear. In the present study, we investigated the DEN4 3′NC sequence in an attempt to (i) delineate the sequence requirements for dengue virus replication and (ii) isolate stable, growth-impaired deletion mutants of DEN4 that could be evaluated as candidate vaccine strains. The infectivity of RNA transcripts containing a deletion in the 3′NC region was evaluated in cell culture, and evidence for the growth restriction of viable deletion mutants was sought. In addition, the infectivity and immunogenicity of several DEN4 deletion mutants were evaluated in monkeys.

MATERIALS AND METHODS

Construction of DEN4 deletion mutants. A full-length cDNA copy (clone 2A) of DEN4 strain 814669 was previously shown to produce infectious RNA transcripts (21). To introduce deletions into the 3′NC region, the DEN4 cDNA subfragment between the *Bam*HI site (nt 10104) and the *Asp*718 site (nt 10647) at the 3′ end was cloned in the $pGEM_3$ vector. The unique *Apa*I site (nt 10470) was used to engineer overlapping deletions upstream and downstream of the cleavage site in the 3′NC region (Fig. 1). Three deletions downstream of the *Apa*I site, 30, 60, or 90 nt in length, were introduced by site-directed mutagenesis. To accomplish this, the negative-strand primer for PCR was oligo 2955 (GAGCT GGTACCAGAACCTGTTGGATCAA), which contains the complementary sequence of the last 17 nt at the DEN4 3′ terminus and the *Asp*718 cleavage sequence (underlined). The positive-strand primer contains the targeted deletion sequence and the flanking *Apa*I cleavage sequence. Because the series of deletions is located near the 3′ terminus, a reverse-order numbering system, which assigns nt 1 to the last nucleotide (formerly DEN4 nt 10646), was adopted to designate the nucleotide positions of the deletion mutants. Accordingly, the three deletions constructed above were designated 3′d 172-143, 3′d 172-113, and 3′d 172-83, respectively. Four deletions that span 61, 121, 151, and 202 nt upstream of the *Apa*I site were constructed and designated 3′d 243-183, 3′d 303-183, 3′d 333-183, and 3′d 384-183, respectively (Fig. 1). Similarly, these upstream deletions, located between the *Bam*HI and *Apa*I sites, were generated by site-directed mutagenesis with positive-strand primer D122 (nt 9885 to 9902) and the negative-strand primer containing the targeted deletion and the flanking *Apa*I cleavage sequence. Each PCR DNA product was cleaved with *Apa*I plus *Bam*HI or *Apa*I plus *Asp*718, cloned, and sequenced to verify the presence of the deletion. Subsequently, the DNA fragments containing the deletions were each joined with the remaining DEN4 cDNA sequence and cloned in the pBR322 vector (21, 25).

Four additional constructs extending the deletion of 3′d 172-113 toward the 3′ end were also constructed. The size of the deletion was 66, 72, 78, or 84 nt, and these mutants were designated 3′d 172-107, 3′d 172-101, 3′d 172-95, and 3′d 172-89, respectively. To construct a mutant containing the combined deletions of 3′d 172-113 and 3′d 384-183, the DEN4 DNA fragments containing the respective deletions were joined at the *Apa*I site, and the resulting 262-nt deletion construct was designated 3′d 172-113/384-183.

RNA transcription, transfection, and recovery of viruses. The procedure for transcription of the mutant cDNA constructs and transfection of simian LLC-MK_2 cells or mosquito C6/36 cells with the RNA transcripts in the presence of lipofectin (Bethesda Research Laboratories) or DOTAP (Boehringer, Mannheim, Germany) was described earlier (21, 25). Ten days after transfection, cells in a 24-well plate were transferred to a T_{25} flask and a chamber slide. At 3 days and again 6 days later, cells on the slide were examined by an immunofluorescence assay (IFA) for the presence of DEN4 antigens. When evidence of DEN4 infection was detected in 70 to 100% of the cell population, the infected cells were lysed, and the virus was harvested in the form of a cell lysate. These virus samples contained 50% fetal calf serum (FCS). The titer was determined on C6/36 cells by plaque assay, and the samples were stored at −70°C.

Infectious-center assay. Subconfluent simian LLC-MK_2 cells in a 24-well plate were transfected with approximately 1 μg of the full-length DEN4 or deletion mutant RNA transcripts in the presence of DOTAP. After 16 to 18 h, approximately 3×10^4 transfected cells were suspended, transferred to a T_{25} flask, and allowed to settle. An excess of uninfected LLC-MK_2 cells was subsequently added to form a confluent monolayer, and an agarose overlay (SeaKem ME agarose; FMC Bioproducts, Rockland, Maine) was added 20 to 22 h after transfection. A second agarose overlay containing neutral red was added at 14 days, and the number of plaques was recorded the next day.

Sequencing deletion mutants. DEN4 deletion mutants recovered from LLC-MK_2 cells were amplified once in mosquito C6/36 cells. Viral RNA was extracted from a small aliquot of the amplified virus preparation. Reverse transcription of viral RNA was performed with oligo 2955 as the first-strand primer. The single-stranded cDNA product was used as the template for PCR with oligo 2955 and oligo D122 as primers. The PCR DNA product was cleaved with *Bam*HI and *Asp*718 for cloning in $pGEM_3$, and the cloned DNA insert was sequenced across the deletion junction to verify the deletion mutation.

Plaque morphology and growth rate analysis. The plaque assay of DEN4 deletion mutants was performed on LLC-MK_2 cells and on C6/36 cells (2, 14). Sea-Kem GTG agarose (FMC Bioproducts) was used in the overlay on C6/36 cells, whereas SeaKem ME agarose at 0.5% was used in the overlay on LLC-MK_2 cells. Diethyl aminoethyl dextran was not included in the agarose overlay for LLC-MK_2 cells. In each case, cell monolayers were stained with neutral red 6 days after infection, and plaque size was measured the next day. For one-step growth analysis, confluent LLC-MK_2 cells in a six-well plate were infected with the wild-type DEN4 or a deletion mutant at a multiplicity of infection (MOI) of 1 in medium 199 containing 2% FCS. After a 2-h adsorption period, the cells were rinsed once and refed with the same medium. At various times after infection, cells were scraped into 1 ml of medium 199 containing 50% FCS and lysed by freezing and thawing three times. The virus titer in the lysate was determined by a plaque assay on C6/36 cells. For growth rate analysis, confluent LLC-MK_2 cells in a T_{25} flask were infected with the wild-type DEN4 or a deletion mutant at an MOI of 0.01. Daily for 5 days, the infected cells were collected in 2 ml of medium 199 plus 50% FCS and lysed by freezing and thawing three times. The virus titer was determined by a plaque assay on C6/36 cells.

Infectivity and immunogenicity in monkeys. Juvenile rhesus monkeys that weighed 3 to 6 kg and lacked immunoprecipitating antibodies for DEN4 antigens were inoculated with cDNA-derived wild-type DEN4 (clone 2A) or a deletion mutant. Wild-type DEN4 was grown in LLC-MK_2 cells, and each of the deletion mutants was similarly prepared. Each monkey was injected subcutaneously with 10^5 PFU (C6/36 cell plaquing titer) of virus diluted to a final volume of 1.0 ml with sterile phosphate-buffered saline, administered as two doses of 0.5×10^5 PFU of virus, one dose in each side of the upper shoulder area. Ketamine at 10 mg/kg was administered in order to anesthetize the animals prior to virus inoculation. Inoculated monkeys were bled daily for 13 days for viremia analysis. All serum specimens were handled and stored similarly. Blood samples were also collected at 2, 3, 4, 6, and 8 weeks for evaluation of antibody response. For viremia analysis, serum specimens were inoculated onto C6/36 cells to amplify any virus present (14). Briefly, an 80-μl serum sample, diluted with 220 μl of minimum essential medium plus 2% FCS, was added to C6/36 cells in a T_{25} flask for 1 h, and the cells were then fed with 5 ml of medium 199 plus 2% FCS. Following a medium change at day 7, the fluid medium at day 14 was collected for detection of any amplified virus. Detection of virus was performed by IFA or plaque assay on C6/36 cells as described above. Antibody response was analyzed by radioimmunoprecipitation (RIP) as well as by a plaque reduction-neutralization test (PRNT) as described previously (2, 20).

RESULTS

DEN4 cDNA constructs containing deletions in the 3′NC region. The last 81 nt of the 384-nt 3′NC region of the DEN4 RNA genome can potentially form a stable secondary structure which is thought to be essential for viral replication (32). Im-

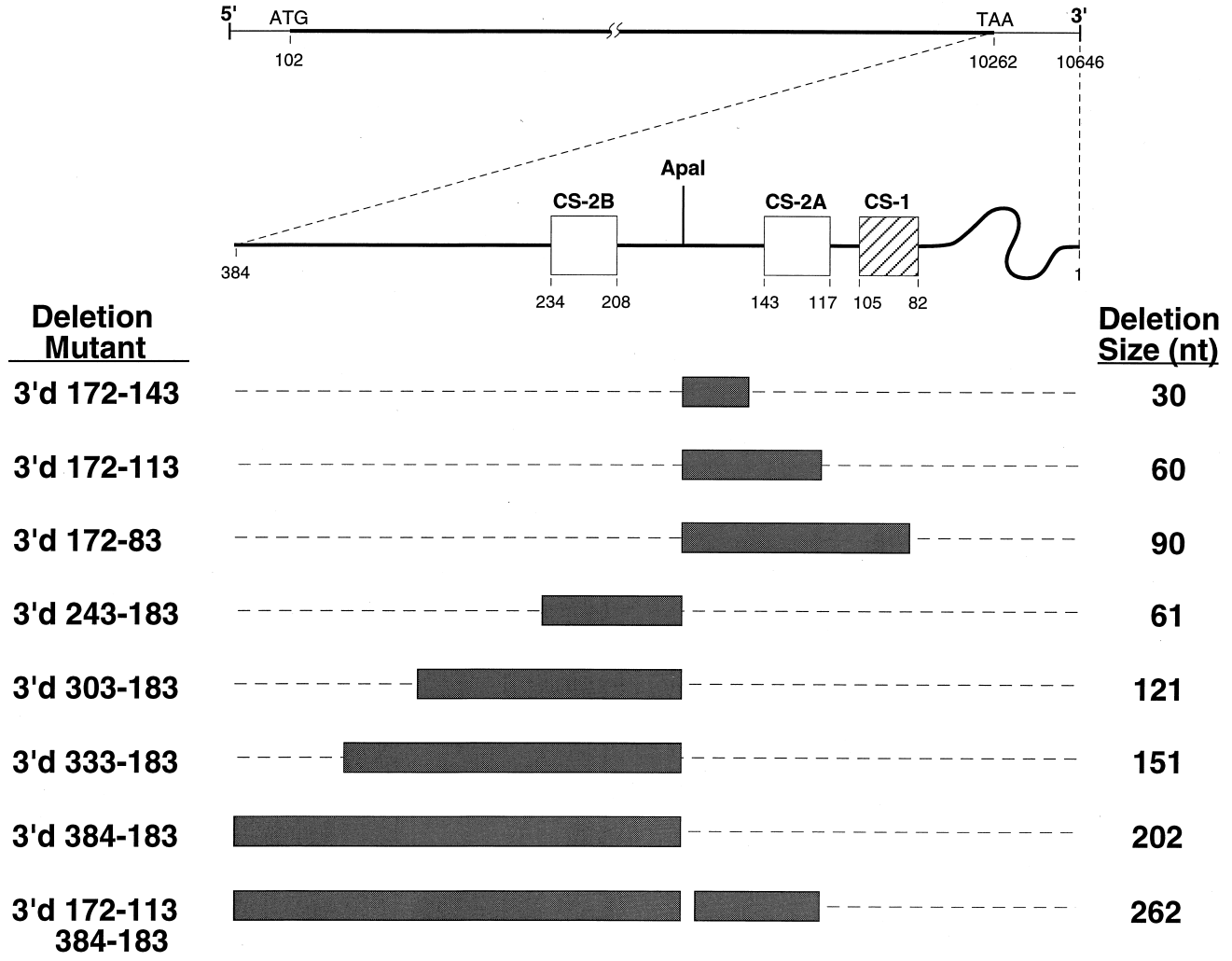

FIG. 1. Sequence of the DEN4 3′NC region and location of deletion mutations. The genome of DEN4 strain 814669 contains 10,646 nt, of which the last 384 nt at the 3′ terminus are noncoding. The locations of various sequence components in this region are designated with the reverse-order numbering system. These sequences include the 3′-distal secondary structure (nt 1 to 81) and flavivirus CS-1 (nt 82 to 105), CS-2A (nt 117 to 143), and CS-2B (nt 208 to 234). The *Apa*I site which was used to generate deletions is shown between CS-2A and CS-2B. The size of the deletion is shown on the right. Listed are eight deletion constructs, each of which is designated according to its deletion.

mediately preceding this region are two stretches of conserved sequences (CS) commonly present in mosquito-borne flaviviruses. One of these sequences, extending from nt 105 to nt 82 from the 3′ terminus, is designated CS-1; the other sequence is duplicated at nt 143 to 117 (CS-2A) and nt 234 to 208 (CS-2B), with one mismatch. We constructed DEN4 cDNA lacking one or more of these conserved sequences and attempted to recover viable viruses from the RNA transcripts by transfection of permissive simian LLC-MK_2 cells. The structures of these deletion constructs are shown in Fig. 1. Three overlapping deletions downstream of the *Apa*I site were constructed: 3′d 172-143, which retains CS-2A, CS-1, and the stem-loop structure; 3′d 172-113, which lacks the entire CS-2A sequence element; and 3′d 172-83, which lacks both CS-2A and CS-1 but retains the 3′ stem-loop secondary structure. Additionally, four overlapping deletions upstream of the *Apa*I site designated 3′d 243-183, 3′d 303-183, 3′d 333-183, and 3′d 384-183 were constructed. All four upstream deletion mutants lack the CS-2B element plus various lengths of the upstream sequence.

Infectivity of RNA transcripts. Initially, evidence for infectivity of the RNA transcripts prepared from the series of DEN4 DNA deletion constructs was sought by plaque formation in cell culture. Direct plaque assay by RNA transfection of permissive simian LLC-MK_2 cells or mosquito C6/36 cells was not successful, presumably because the cell monolayers were sensitive to the detergent in the transfection mixture. For this reason, an infectious-center assay was used to detect infectivity of full-length DEN4 and deletion mutant RNA transcripts. Transfection of full-length DEN4 or mutant 3′d 303-183 RNA produced approximately 20 to 30 plaques in the infectious-center assay, but the mutant plaques were faint (data not shown). Under the same conditions, RNA transcripts of 3′d 172-143 and 3′d 172-113 produced fewer plaques, i.e., 1 to 10. Also, these mutant plaques were faint compared with the large, clear wild-type virus plaques. These observations suggested that the viable deletion mutants were growth impaired. Deletion mutants 3′d 172-83, 3′d 333-183, and 3′d 384-183 failed to produce plaques in the infectious-center assay. However, production of progeny virus by cells transfected with transcripts of mutants 3′d 333-183 or 3′d 384-183 was detected when the

TABLE 1. Properties of DEN4 3′NC deletion mutants

Virus	Progeny virus recovered	Plaque formation on LLC-MK_2 cells		Plaque size (mm) on C6/36 cells[a]
		6 days	9 days	
Wild-type DEN4	+	+	+	11
3′d 172-83	−	ND[b]	ND	ND
3′d 172-113	+	−	+	10
3′d 172-143	+	−	+	9
3′d 243-183	+[c]	−	+	9
3′d 303-183	+	+	+	6
3′d 333-183	+	−	+	5
3′d 384-183	+	−	+	3
3′d 384-183/172-113	+	−	−	3

[a] Plaques developed by day 6.
[b] ND, not done.
[c] Progeny virus contained an additional deletion in the 3′NC region.

cells were assayed by IFA. Viable virus was not recovered from construct 3′d 172-83, whose deletion was apparently lethal.

Mapping the 3′NC sequence essential for infectivity. Mutant 3′d 172-83 repeatedly failed to produce plaques when tested by the infectious-center assay and did not yield progeny virus detectable by IFA following prolonged incubation of transfected cells. This deletion mutant lacked both the CS-2A and CS-1 sequences but retained the intact 3′ stem-loop structure. In contrast, mutant 3′d 172-113, which lacked the CS-2A but retained the CS-1 and the downstream stem-loop structure, was viable. This indicated that extension of the deletion from nt 172 to nt 83 to remove the CS-1 sequence abolished viability. The deletion limit for RNA infectivity in this region was assessed by analysis of four additional constructs, 3′d 172-89, 3′d 172-95, 3′d 172-101, and 3′d 172-107, each containing a deletion progressively shorter than that present in 3′d 172-83. Mutant 3′d 172-107 retained the entire CS-1 sequence, whereas the other mutants lacked various portions of it. When the infectivity of the RNA transcripts was assessed by transfection, none of the four deletion RNA constructs yielded progeny virus even after prolonged incubation. Thus, in the context of deletion 3′d 172-107, the presence of CS-1 and the downstream sequence was not sufficient for infectivity.

Stability of deletion mutants. Experiments were performed to determine if the progeny of deletion mutants retained the deletions originally introduced in the 3′NC region. Virion RNAs prepared from the progeny viruses were reverse transcribed, and the DNA fragment corresponding to the entire 3′NC region of each mutant was amplified by PCR or subsequently cloned in $pGEM_3$. The PCR DNA products of mutants 3′d 172-143, 3′d 172-113, 3′d 303-183, 3′d 333-183, and 3′d 384-183 all showed the predicted size. The presence of the deletion was verified by sequence analysis of the DNA clones. The PCR DNA product of 3′d 243-183 was smaller than that predicted for the deletion mutant. Sequence analysis of DNA clones revealed the presence of an additional deletion, possibly derived by genome rearrangement. For this reason, mutant 3′d 243-183 was not studied further.

Plaque morphology. Deletion mutants recovered from transfected simian LLC-MK_2 cells were amplified once by passage in these cells and then used for analysis of the plaque morphology on mosquito C6/36 cells. Each of the viable deletion mutants produced plaques 6 days after infection. The plaque size of deletion mutants varied, ranging from 3 to 10 mm, compared with the wild-type virus plaques, which averaged 11 mm (Table 1). The plaque size of mutants 3′d 172-143 and 3′d 172-113, which each contained a deletion located downstream of the *Apa*I site, was similar to that of the wild-type virus. In comparison, mutants with deletions located upstream of the *Apa*I site, except for 3′d 243-183, produced smaller plaques. In general, the larger the deletion, the smaller the plaque size. The plaque morphology of deletion mutants was also analyzed on simian LLC-MK_2 cells. With the exception of mutant 3′d 303-183, which produced plaques by 6 to 7 days, similar to the wild-type virus, the other deletion mutants did not produce plaques until 9 to 10 days after inoculation of cell culture (Table 1). Compared with the wild-type virus plaques, mutant plaques were faint (Fig. 2). Under these conditions, mutant 3′d 172-113/384-183, which sustained the largest deletion among these mutants, failed to produce plaques on simian LLC-MK_2 cells.

Growth analysis. The effect of deletion on viral replication in simian LLC-MK_2 cells was studied by one-step growth analysis. Mutants 3′d 172-113, 3′d 172-143, and 3′d 384-183 had a smaller burst size than did the wild-type virus after 24 h (Fig. 3A). When infection was initiated with a lower MOI (0.01) of virus, analysis of the growth yield showed that mutant 3′d 303-183 reached the wild-type virus level, whereas mutants 3′d 172-113 and 3′d 172-143, which were studied in a one-step

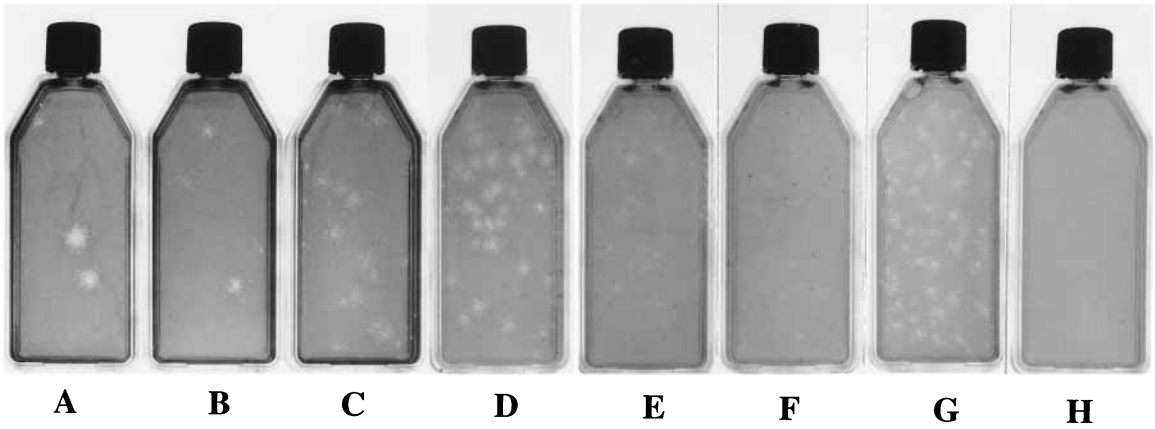

FIG. 2. Plaque morphology assay of wild-type DEN4 and derived deletion mutants. Monolayers of LLC-MK_2 cells were infected with progeny viruses recovered from RNA-transfected cells in a separate experiment. An agarose overlay was added to the infected LLC-MK_2 cells. Nine days after infection, cells in each flask were stained with neutral red. (A) Wild type; (B) 3′d 172-143; (C) 3′d 172-113; (D) 3′d 303-183; (E) 3′d 333-183; (F) 3′d 384-183; (G) 3′d 243-183; (H) 3′d 172-113/384-183.

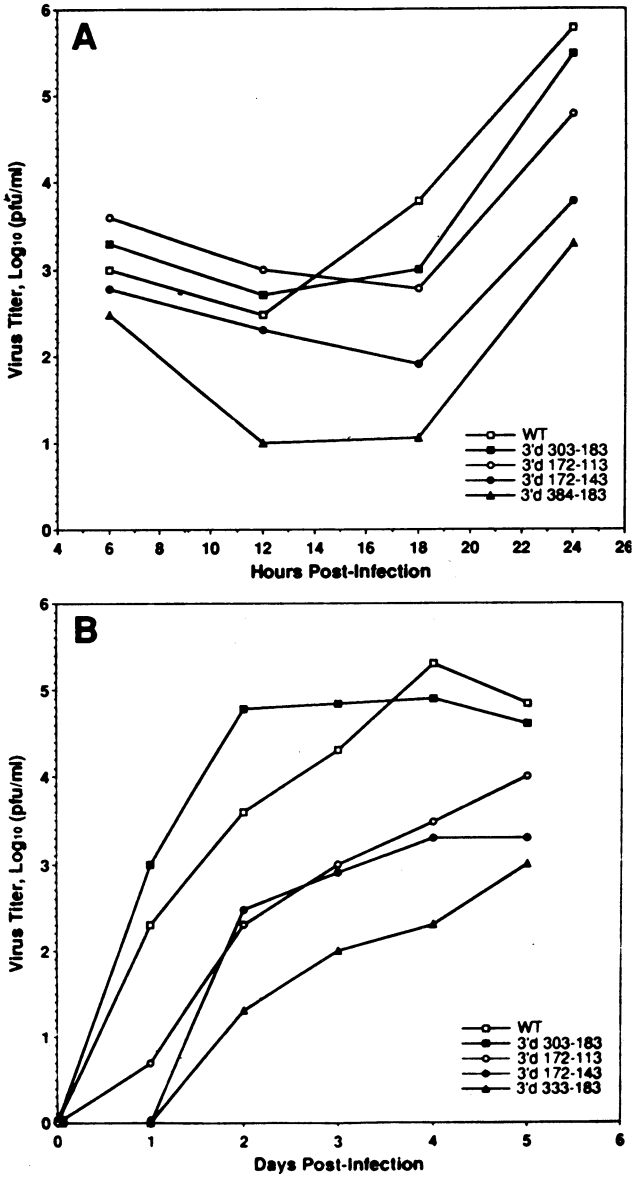

FIG. 3. Growth analysis of deletion mutants in primate LLC-MK_2 cells. (A) For analysis of the one-step growth curve, LLC-MK_2 cells were infected with the indicated virus at an MOI of 1. Following virus adsorption for 1 h, cells were rinsed, fresh medium was added, and cells were incubated for various times prior to lysis. The virus titer in the cell lysate was measured on C6/36 cells. (B) Growth analysis was performed as above except that an MOI of 0.01 was used for infection. WT, wild type.

growth analysis, and another mutant, 3′d 333-183, grew to a lower titer (Fig. 3B). From the small-plaque morphology, delayed appearance of plaques on simian LLC-MK_2 cells, and/or reduced growth, four of the deletion mutants appeared to be restricted in cell culture. Significantly, mutant 3′d 303-183, which sustained a 121-nt deletion, was the least growth restricted and able to grow as efficiently as wild-type virus.

Response of monkeys to infection with wild-type DEN4 and its derived deletion mutants. Of the various experimental animals, monkeys have proved to be the surrogates that most closely resemble humans in their response to dengue virus infection (20, 27). The response of these primates to infection with dengue virus is similar to that of humans in that there is a viremia that lasts several days; however, infected monkeys do not develop symptoms of dengue disease. It has been suggested that the occurrence and duration of viremia in monkeys might provide useful information predictive of infectivity of dengue virus for humans and possibly virulence as well (16). For this reason, some of the DEN4 3′ deletion mutants were studied in monkeys. In the first study, six monkeys were inoculated with 10^5 PFU of DEN4 (clone 2A) (Table 2). Five of these six monkeys sustained a viremia that lasted 1 to 5 days (mean, 3.4 days). The amount of virus present in serum was very low ($\leq$1 PFU per 80 μl of serum), because it could not be detected by direct plaque assay. This meant that our assay for viremia, which entailed amplification of virus in cell culture before detection was attempted, was qualitative at best. Two additional studies which included deletion mutants were subsequently performed.

In study 2 (Table 2), groups of four rhesus monkeys were inoculated subcutaneously with 10^5 PFU of deletion mutant 3′d 333-183, 3′d 303-183, or 3′d 172-143. Two additional monkeys were inoculated with the same dose of wild-type virus and served as positive controls. Interestingly, viremia was not detected in monkeys inoculated with mutant 3′d 333-183 or 3′d 172-143. Only one monkey of four inoculated with 3′d 303-183 developed a brief 1-day viremia. Each of the two monkeys infected with wild-type DEN4 also had 1 day of viremia. The significance of our failure to detect viremia with two deletion mutants and the short duration of viremia in response to the third mutant is not clear, because the level of viremia achieved by wild-type virus was so low.

A third study in which monkeys in groups of four were inoculated with mutant 3′d 172-113 or 3′d 384-183 or wild-type virus was performed (Table 2). Each of the four monkeys infected with wild-type DEN4 showed 3 to 4 days of viremia. Two of four monkeys infected with mutant 3′d 172-113 showed 3 to 4 days of viremia, and two of four monkeys infected with mutant 3′d 384-183 showed a brief 1-day viremia. In study three and to a lesser extent in the second study, there was a suggestion that the deletion mutants induced less viremia than their wild-type parental virus. Thus, in the aggregate, 11 of 12 monkeys inoculated with wild-type parental virus developed viremia, which in eight instances lasted 3 to 4 days. In contrast, two of the five deletion mutants tested did not cause detectable viremia, and the other three deletion mutants induced viremia in only one or two of four inoculated animals. It should be noted that these differences should not be overinterpreted because of the very low level of viremia caused by DEN4. It is clear, however, that the mutants did not exhibit an enhancement of viremia.

Immunogenicity of wild-type DEN4 and deletion mutants in monkeys. It appeared that a more quantitative assessment of attenuation of the 3′ deletion mutants was achieved when the antibody response of the inoculated monkeys was analyzed. Antibody response to infection with wild-type DEN4 or a deletion mutant in monkeys was initially analyzed by RIP. As early as 2 weeks after infection, DEN4 E and NS1 antibodies were detected in sera from monkeys inoculated with wild-type DEN4 or mutant 3′d 303-183, whereas detection of these antibodies in monkeys infected with other deletion mutants was delayed (data not shown). As estimated by the intensity of the precipitated radiolabeled E or NS1 protein, antibody response in infected monkeys reached its highest level at 6 to 8 weeks. Figure 4 shows the results of RIP analysis of sera collected 8 weeks after infection. Each of six monkeys infected with wild-type DEN4 as well as each of the four monkeys infected with mutant 3′d 303-183 or 3′d 172-113 developed a high level of E antibodies. In comparison, the level of antibody response to

TABLE 2. Viremia and antibody response of monkeys following inoculation with 10^5 PFU of DEN4 or derived deletion mutants

Study no.	Virus inoculated[a]	Monkey no.	Detection of viremia[b] on day postinoculation:								Total days of viremia	Antibody titer[c] measured by 50% plaque reduction	
			3	4	5	6	7	8	9	10		Individual	Geometric mean
1	Wild-type DEN4 (2A)	H-151	−	+	+	+	+	−	−	−	4	340	370
		H-153	−	+	+	−	+	−	−	−	3	415	
		H-154	−	−	−	−	−	−	−	−	0	400	
		H-157	−	+	+	+	+	−	−	−	4	425	
		H-052	+	+	+	+	+	−	−	−	5	475	
		H-887	−	−	+	−	−	−	−	−	1	225	
2	Wild-type DEN4 (2A)	H-228	−	−	+	−	−	−	−	−	1	290	300
		H-229	−	−	+	−	−	−	−	−	1	310	
	3′d 333-183	H-231	−	−	−	−	−	−	−	−	0	<40	52
		H-232	−	−	−	−	−	−	−	−	0	70	
		H-233		−	−	−	−	−	−	−			
		H-234	−	−	−	−	−	−	−	−	0	<40	
	3′d 303-183	H-235	−	−	−	−	−	−	−	−	0	370	334
		H-236	−	−	−	−	−	+	−	−	1	410	
		H-237	−	−	−	−	−	−	−	−	0	230	
		H-238	−	−	−	−	−	−	−	−	0	360	
	3′d 172-143	H-239	−	−	−	−	−	−	−	−	0	55	83
		H-240	−	−	−	−	−	−	−	−	0	150	
		H-241	−	−	−	−	−	−	−	−	0	30	
		H-244	−	−	−	−	−	−	−	−	0	195	
3	Wild-type DEN4 (2A)	H-141	−	+	−	+	+	+	−	−	4	380	307
		H-146	−	−	+	+	+	+	−	−	4	410	
		H-184	−	−	+	+	+	−	−	−	3	300	
		H-185	−	−	−	+	+	+	−	−	3	190	
	3′d 172-113	H-012	−	−	−	−	−	−	−	−	0	120	115
		H-175	−	−	−	−	+	+	+	+	4	200	
		H-176	−	−	+	+	+	−	−	−	3	180	
		H-191	−	−	−	−	−	−	−	−	0	120	
	3′d 384-183	H-047	−	−	−	−	−	−	−	−	0	40	28
		H-142	−	−	−	−	+	−	−	−	1	40	
		H-279	−	−	−	−	−	+	−	−	1	20	
		H-282	−	−	−	−	−	−	−	−	0	20	

[a] Viruses were derived by transfection of simian LLC-MK_2 cells with transcripts of cDNA and subsequent passage in these cells.
[b] Serum samples from days 3 to 10 were analyzed. Day 1 and 2 samples were not analyzed because they might contain residual virus from the inoculum.
[c] Reciprocal of antibody concentration.

infection with 3′d 172-143 was reduced. With one exception (monkey H-232), monkeys responded poorly or not at all to inoculation with mutant 3′d 333-183 or 3′d 384-183. The level of NS1 antibodies was highest in monkeys infected with the wild-type virus or with mutant 3′d 303-183. Other mutants induced a low level of NS1 antibodies.

DEN4-neutralizing antibodies in the monkey sera were also measured by PRNT. Table 2 shows that the monkeys inoculated with the wild-type virus developed a high neutralizing antibody titer (geometric mean titer ranging from 1:300 to 1:370). Monkeys infected with mutant 3′d 303-183 also achieved a high antibody titer (geometric mean titer, 1:334). Mutants 3′d 172-113 and 3′d 173-143 induced a moderate antibody response in monkeys (geometric mean titer, 1:115 and 1:83, respectively). In contrast, mutants 3′d 333-183 and 3′d 384-183 elicited a low titer of neutralizing antibodies (geometric mean titer, 1:52 and 1:28, respectively). There was an excellent correlation between the level of E antibodies measured by RIP and the level of neutralizing antibodies measured by PRNT.

DISCUSSION

The successful construction and cloning of full-length DEN4 cDNA which yields infectious RNA transcripts has afforded us opportunities for detailed molecular analysis of the RNA genome and for the development of new dengue vaccine strategies. Previously, we employed DEN4 cDNA to construct mutants containing amino acid substitutions at the NS1-NS2A cleavage junction that were later shown to reduce cleavage of the NS1-NS2A region of the viral polyprotein. Such DEN4 mutants appeared to be growth restricted, as indicated by reduced size of plaques in cell culture (24). Because mutants containing a single amino acid substitution often exhibit reversion to the wild-type phenotype, we turned to construction of deletion mutants which should be more stable and thus more suitable for use as candidate vaccine strains. For this purpose, we initially constructed DEN4 deletion mutants lacking sequences in the 5′NC region and showed that some of these deletion mutants replicated poorly in cell culture (7). In this study, the 3′NC sequence was chosen as the target site for

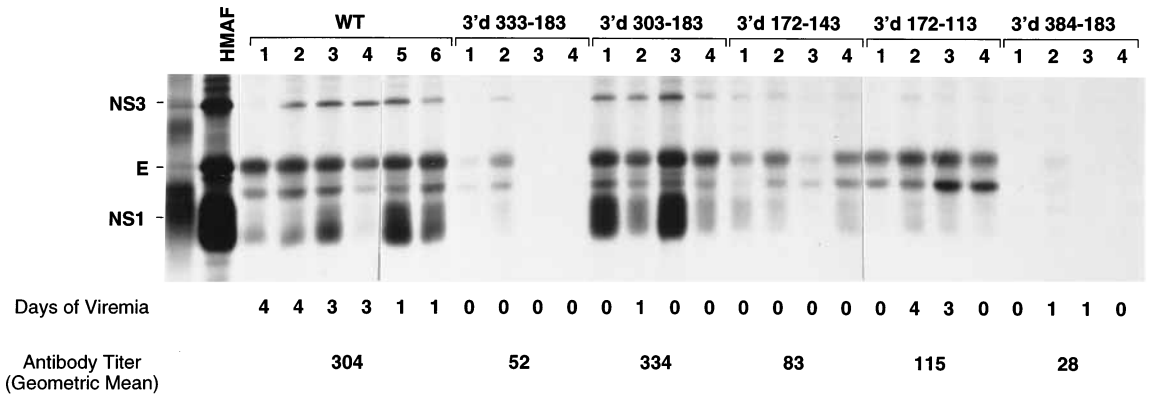

FIG. 4. RIP analysis of seroresponse to infection with wild-type DEN4 and deletion mutants in monkeys. Serum samples collected 8 weeks postinfection were analyzed by RIP. The [^{35}S]methionine-labeled lysate was prepared from cells infected with wild-type (WT) DEN4. An excess of the labeled antigens was used in 200 μl of the reaction mixture, to which 5 μl of each serum sample was added. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Within each virus group, the serum samples were from the indicated monkeys (from left to right): WT, H-141, H-146, H-184, H-185, H-228, and H-229; mutant 3′d 333-183, H-231, H-232, H-233, and H-234; mutant 3′d 303-183, H-235, H-236, H-237, and H-238; mutant 3′d 172-143, H-239, H-240, H-241, and H-244; mutant 3′d 172-113, H-012, H-175, H-176, and H-191; and mutant 3′d 384-183, H-047, H-142, H-279, and H-282. The unmarked lane contains molecular size markers; lane HMAF shows dengue virus proteins precipitated by HMAF.

creating deletions. The 3′NC is thought to play an important role in the initiation and regulation of viral RNA replication. The 3′NC region includes at its terminus a potentially stable secondary RNA structure, and upstream there are several domains that are conserved among flaviviruses. Also, the long length of the 3′NC region (approximately 380 to 600 nt) suggested that viable mutants containing sizable deletions could be isolated.

The 3′-distal sequence of approximately 80 nt which can potentially form a stem-loop RNA structure is thought to be essential for viral replication (6, 32). Deletions in this region would most likely lead to disruption of the secondary structure and loss of viability. Instead, we introduced deletions into the region that contains conserved flavivirus CS-1, CS-2A, and CS-2B domains. Because these domains are so highly conserved, it was considered likely that they participated in essential interactions with cellular or viral proteins during viral replication. Deletion of part or all of these conserved sequences might therefore reduce the efficiency of virus replication. To our surprise, sizable regions of the 3′NC sequence that were located at 113 nt or more upstream of the 3′ terminus of the noncoding sequence could be deleted without loss of viability. These deletions removed either CS-2A or CS-2B or both. Interestingly, deletion 3′d 172-113 yielded viable virus, but 3′d 172-107 did not. It appears that the sequence between nt 112 and 107 plays a critical role in virus replication. This sequence is located upstream of the region between nt 96 and 106 that is proposed to be potentially capable of pairing with a complementary sequence near the 5′ end and thus forming a "panhandle" RNA structure (12). Deletion of this sequence might disrupt the RNA structure and prevent its possible interactions with viral or host cell proteins. The consequences of these deletions were variable. Thus, the viable mutants exhibited a spectrum of growth restriction in cell culture, as indicated by plaque development and size as well as one-step growth analysis.

Analysis of plaque morphology and growth yield showed that the viable deletion mutants exhibited a range of growth restriction in cultured cells. Mutant 3′d 303-183 grew to the wild-type virus level but produced plaques that were faint compared with the wild-type plaques. It is likely that the entire 3′NC sequence is required for efficient viral replication. In general, growth restriction was more profound in primate LLC-MK_2 cells than in mosquito C6/36 cells. This finding suggests that host factors play an important role in efficient viral replication.

DEN4 mutants restricted in growth capacity might prove to be attenuated, because almost every other licensed or well-studied live vaccine strain is restricted in its growth in humans as well as in appropriate experimental animal systems. However, in terms of vaccine production, it is necessary that the virus mutants be competent as regards efficient viral replication in cell culture in order to yield a satisfactory titer. In this context, it should be noted that mutant 3′d 303-183 grew to a titer of approximately 10^6 PFU/ml in primate-derived LLC-MK_2 cells and in mosquito C6/36 cells. This level of growth is similar to that of the wild-type DEN4 as well as many candidate dengue vaccine strains produced and evaluated by other investigators (16, 22).

The immunogenicity of cDNA-derived DEN4 and its deletion mutants was evaluated in monkeys because these primates represent the most relevant experimental surrogate for the study of dengue virus infection. DEN4 recovered from full-length cDNA proved to be infectious for monkeys. Monkeys infected with wild-type virus developed a high titer of neutralizing antibodies, and 11 of 12 such infected monkeys developed a viremia that lasted 1 to 5 days. In contrast, viremia was detected less often in monkeys infected with the deletion mutants: only 5 of 20 such monkeys developed viremia. Thus, overall, there appeared to be a reduction in viremia response to each of these deletion mutants compared with that observed with the wild-type virus. However, this interpretation should be considered with some caution, because the level of viremia produced by wild-type virus was very low.

Antibody response to dengue virus infection in monkeys exhibited a pattern that correlated with the level of virus growth in cell culture. Thus, (i) monkeys infected with the wild-type virus developed a high titer of neutralizing antibodies, regardless of duration of viremia; (ii) mutants 3′d 384-183 and 3′d 333-183, which were most severely restricted for growth in cultured cells, induced a low level of antibodies; (iii) mutants 3′d 172-113 and 3′d 172-143, which were less restricted in growth in vitro, induced a moderate antibody response; and (iv) mutant 3′d 303-183, which replicated almost as well as wild-type virus in vitro, elicited the same level of antibody response as the wild-type parental virus.

From the observations just described, mutant 3′d 303-183, which sustained a 121-nt deletion but remained highly immunogenic, is of particular interest as a candidate for further evaluation in vivo. Mutants 3′d 172-113 and 3′d 172-143, which induced a moderate level of antibodies, might also prove to be interesting vaccine strain candidates.

If we are successful in identifying one or more deletion mutations in the 3′NC region of DEN4 that specify a satisfactory level of attenuation of this virus without significant loss of immunogenicity, such a deletion could also be used to attenuate chimeric DEN1, DEN2, and DEN3 viruses that are constructed by substituting the structural protein genes of these viruses for the corresponding genes of DEN4.

ACKNOWLEDGMENTS

We thank W. London and the staff of Bioqual Inc. (Rockville, Md.) and C. Malaga and his staff at the University of Puerto Rico, San Juan, for their work on the series of primate studies and P. Russell, K. Eckels, and C. Hoke, Jr., for useful advice.

This research project received partial financial support from the WHO Global Programme for Vaccines.

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