A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4

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Received 3 January 2003; returned to author for revision 12 February 2003; accepted 24 February 2003

Abstract

An acceptable live-attenuated dengue virus vaccine candidate should have low potential for transmission by mosquitoes. We have identified and characterized a mutation in dengue virus type 4 (DEN4) that decreases the ability of the virus to infect mosquitoes. A panel of 1248 mutagenized virus clones generated previously by chemical mutagenesis was screened for decreased replication in mosquito C6/36 cells but efficient replication in simian Vero cells. One virus met these criteria and contained a single coding mutation: a C-to-U mutation at nucleotide 7129 resulting in a Pro-to-Leu change in amino acid 101 of the nonstructural 4B gene (NS4B P101L). This mutation results in decreased replication in C6/36 cells relative to wild-type DEN4, decreased infectivity for mosquitoes, enhanced replication in Vero and human HuH-7 cells, and enhanced replication in SCID mice implanted with HuH-7 cells (SCID-HuH-7 mice). A recombinant DEN4 virus (rDEN4) bearing this mutation exhibited the same set of phenotypes. Addition of the NS4B P101L mutation to rDEN4 bearing a 30 nucleotide deletion (H9004 30) decreased the ability of the double-mutant virus to infect mosquitoes but increased its ability to replicate in SCID-HuH-7 mice. Although the NS4B P101L mutation decreases infectivity of DEN4 for mosquitoes, its ability to enhance replication in SCID-HuH-7 mice suggests that it might not be advantageous to include this specific mutation in an rDEN4 vaccine. The opposing effects of the NS4B P101L mutation in mosquito and vertebrate systems suggest that the NS4B protein is involved in maintaining the balance between efficient replication in the mosquito vector and the human host.

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Keywords: Flavivirus; Dengue virus; Vaccine; NS4B; Mutation; Aedes aegypti; Vero; HuH-7; C6/36; Toxorhynchites splendens

Introduction

The mosquito-borne dengue viruses (genus Flavivirus, family Flaviviridae, serotypes DEN1–DEN4) are the causative agents of dengue fever (DF) and its more severe manifestation, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Currently, dengue virus is the most significant arthropod-borne virus (arbovirus) in humans, causing an estimated 100 million cases of DF and 500,000 cases of DHF/DSS annually, resulting in enormous morbidity and thousands of deaths (Gubler, 1998). Moreover, the incidence of DHF is increasing dramatically in the developing world (Gubler, 2001). The most common risk factor associated with the emergence of DHF/DSS is the cocirculation of multiple dengue virus serotypes in a community (Gubler, 2001), since progression to DHF/DSS is more likely during second infection (Murgue et al., 2000; Vaughn et al., 2000). At present neither a licensed vaccine nor antiviral therapy is available for dengue virus.

Because of the association between second infection and DHF/DSS, an acceptable vaccine must induce immunity to all four serotypes concurrently and should have a low potential for transmission of the vaccine components by mosquitoes. To date, development of tetravalent live-attenuated vaccine candidates has resulted in uneven attenuation, and incomplete immunity (Bhamarapravati and Sutee, 2000;
Kanesa-thasan et al., 2001; Sabchareon et al., 2002), and, in some cases, wild-type levels of mosquito infectivity (Schoepf et al., 1991). Transmission of a live-attenuated tetravalent dengue vaccine by a mosquito has the potential to increase the level of DHF in a community for two reasons. First, transmission of dengue virus is not perfectly efficient, the four dengue serotypes differ in their infectivity for a particular strain of Aedes mosquito, and Aedes strains differ in their susceptibility to various serotypes (Rosen et al., 1985). Thus it is likely that at least some mosquitoes that fed on a vaccinated individual would transmit only a single serotype of a tetravalent vaccine. Individuals who were infected with a single vaccine serotype and subsequently infected with a different, wild-type serotype of dengue virus could be at increased risk for DHF. Second, multiple rounds of mosquito transmission could result in reversion of the attenuating mutations in vaccine viruses. The risks of uncontrolled transmission of a vaccine virus have been dramatically illustrated by the reversion to virulence of the oral poliovirus vaccine in Haiti and the Dominican Republic (Kew et al., 2002).

The dengue viruses possess a 10.6-kb, single-stranded, positive-sense RNA genome that encodes three structural and at least seven nonstructural proteins. The genome, which codes for a single polypeptide, is organized as follows: 5'-UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-3'UTR (UTR: untranslated region; C: capsid; prM: membrane precursor; E: envelope; NS: nonstructural protein) (Rice, 1996). A deletion that removes nucleotides 10478–10507 of the 3' UTR (Δ30) was previously used to generate vaccine candidate rDEN4Δ30, which is attenuated for rhesus monkeys and humans (Durbin et al., 2001). In addition, the Δ30 mutation has been shown to decrease the ability of DEN4 to disseminate from the mosquito midgut to the head (Troyer et al., 2001). However, the Δ30 mutation does not affect the establishment of a midgut infection in mosquitoes nor does it altogether abolish dissemination when a high dose of virus is ingested (Troyer et al., 2001). Moreover, the effect of the Δ30 mutation on mosquito infection by the other three DEN serotypes is not yet known. To augment the attenuation conferred by Δ30 in the mosquito vector, we sought to identify and characterize mutations that specifically decrease the ability of DEN4 to infect the mosquito midgut while promoting efficient replication in Vero cells, the cell type to be used for vaccine manufacture.

To identify mosquito-attenuation mutations, we utilized a panel of virus clones generated by chemical mutagenesis (Blaney et al., 2001). Wild-type recombinant DEN4 virus rDEN4-2A was grown in the presence of 5-fluorouracil (5-FU) and 1248 clones were isolated. In the present study we screened each of these virus clones to identify mutant viruses that exhibit restricted replication in mosquito C6/36 cells but that retain efficient replication in simian Vero cells. This is a useful preliminary assay for decreased infectivity for intact mosquitoes, since reduced replication in cultured mosquito cells is often, though not always, associated with reduced infectivity for intact mosquitoes (Huang et al., 2000). Only one such virus clone was identified and this mutant exhibited both reduced infectivity for mosquitoes and significantly enhanced replication in Vero cells and HuH-7 (human hepatoma) cells. The genetic basis for this phenotype is a single amino acid change in NS4B.

**Results**

**Identification of small plaque mutant viruses in C6/36 cells**

A panel of 1248 virus clones was generated by 5-FU mutagenesis of rDEN4 in Vero cells (Blaney et al., 2001), and the efficiency of plaque formation (EOP) and plaque size of each of these virus clones were determined in C6/36 cells and compared to these phenotypes in Vero cells. While numerous mutant viruses replicated either well or poorly in both cell types, none showed decreased EOP in only C6/36 cells (arbitrarily defined as a 1000-fold decrease between EOP in Vero cells and C6/36 cells). However, one mutant virus (no. 5) produced plaques that were noticeably smaller than wild-type in C6/36 cells while maintaining large plaque size in Vero cells. Mutant virus no. 5 replicated to high titer (7.0 log10 PFU/ml) in both cell types. After three rounds of terminal dilution, this virus, designated rDEN4-2A-5, maintained the plaque size phenotype (Fig. 1) of the original mutant virus and was studied further. Analysis of the consensus sequence of the entire genome of rDEN4-2A-5 revealed that it contained only one coding mutation and two translationally silent mutations relative to its unmutagenized, wild-type, parental virus (Table 1). The single missense mutation, C to U, at nucleotide 7129 results in a Pro-to-Leu substitution in amino acid 101 of the NS4B protein (NS4B P101L), and it thus appeared that this mutation was responsible for the small plaque (sp) phenotype of rDEN4-2A-5 in C6/36 cells.

To confirm that the NS4B P101L mutation was indeed responsible for the sp phenotype in C6/36 cells, the mutation was introduced into a wt DEN4 cDNA clone to generate the rDEN4-7129 virus. Additionally, to investigate the interaction between the NS4B P101L mutation and the previously described Δ30 mutation (Durbin et al., 2001; Troyer et al., 2001), the NS4B P101L was introduced into a DEN4 cDNA carrying the Δ30 mutation to generate virus rDEN4Δ30-7129. Analysis of the consensus sequence demonstrated that both rDEN4-7129 and rDEN4Δ30-7129 contained the designated coding changes along with incidental, silent mutations (Table 1). Plaque size of these viruses was compared to rDEN4-C6/36, a recombinant wild-type DEN4 recovered in C6/36 cells that has a consensus sequence identical to that of the cDNA from which it was derived. Similar to rDEN4-2A-5, rDEN4-7129 showed reduced plaque size and normal titer in C6/36 cells relative to rDEN4-C6/36 (Fig. 1). The mean diameter of rDEN4-C6/36 plaques in C6/36 cells is twice that of rDEN4-7129, resulting in a fourfold difference in plaque area. Plaque diameter of rDEN4Δ30-7129 was significantly smaller than
that of rDEN4-7129 (Fig. 1). Moreover, in mammalian cells (Fig. 1), the plaque diameter of rDEN4-2A-5 and rDEN4-7129 was approximately twofold (Vero) and threefold (HuH-7) greater than that of rDEN4-C6/36, translating into a fourfold greater area in Vero cells and an eightfold greater area in HuH-7 cells. In Vero cells, rDEN4/C6/36 showed an intermediate plaque diameter between rDEN4-C6/36 and rDEN4-7129; in HuH-7 cells the plaque diameter of rDEN4Δ30-7129 was not different from that of rDEN4-7129.

To test the relationship between plaque size and replication in tissue culture, we compared the kinetics and magnitude of replication of rDEN4-C6/36, rDEN4-7129, and rDEN4Δ30-7129 in C6/36, Vero, and HuH-7 cells (Fig. 2). Viruses carrying the NS4B P101L mutation showed a significant decrease in replication kinetics in C6/36 cells and a significant increase in replication kinetics in Vero and HuH-7 cells relative to rDEN4-C6/36. Replication of rDEN4Δ30-7129 was comparable to that of rDEN4-7129 in HuH-7 cells and lower than rDEN4-7129 in Vero and C6/36 cells. These findings reflect the plaque size differences in the specified cell types.

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**Fig. 1.** Plaque size of rDEN4-C6/36, rDEN4-2A-5, rDEN4-7129, and rDEN4Δ30-7129 in C6/36, Vero, and HuH-7 cells. Monolayers of confluent cells were infected with the indicated viruses and incubated for 5 days, and resulting plaques were visualized by immunoperoxidase staining. Values below each well represent the mean plaque diameter in mm ± standard error (n = 36 plaques per virus). For each cell type, plaque sizes were compared among the four viruses using a Tukey–Kramer post-hoc test; letters in parentheses next to mean plaque size indicate significantly different groups within each cell type, such that groups which share a letter are not significantly different and groups which do not share a letter are significantly different (P < 0.05).
Mosquito infectivity of small plaque mutant viruses

To determine whether reduced replication in C6/36 mosquito cells in tissue culture was an indication of reduced infectivity for intact mosquitoes, the percentage of Aedes aegypti infected after feeding on bloodmeals containing either mutant viruses bearing the NS4B P101L mutation or wild-type DEN4 viruses was determined (Fig. 3). Two wild-type viruses were used in this comparison: rDEN4-2A-13, derived from rDEN4-2A, is used for comparison with rDEN4-2A-5; rDEN4, derived from cDNA plasmids encoding identical virus polyproteins, was used in this comparison: rDEN4-2A-13, derived from rDEN4-2A-5 and rDEN4 were both derived from cDNA plasmids encoding identical virus polyproteins, yet each accumulated different incidental mutations during passage in cell culture (Blaney et al., 2001; Durbin et al., 2001). The data presented in Fig. 3 identify DEN4 viruses with three levels of infectivity. Wild-type DEN4 viruses (rDEN4-2A-13 and rDEN4) caused both a high percentage of total infections (infection of the midgut, irrespective of dissemination to the head) and a high percentage of disseminated infections of mosquitoes at doses of approximately 104.0 PFU. Virus carrying just the Δ30 mutation (rDEN4Δ30) caused a high percentage of total infections at a similar dose, but no disseminated infections, confirming previous observations (Troyer et al., 2001). Viruses carrying the NS4B P101L mutation caused a low percentage of total infections. Pairwise Fisher exact tests were used to compare the infectivity of each virus containing the NS4B P101L mutation to the comparable wild-type virus at the highest dose of each: rDEN4-2A-13 vs rDEN4-2A-5, n = 39, P < 0.001; rDEN4 vs rDEN4-7129, n = 68, P < 0.001; rDEN4Δ30 vs rDEN4Δ30-7129, n = 30, P < 0.001.

Since the NS4B P101L mutation prevented the virus from infecting the midgut of Ae. aegypti, it was not possible using this method to determine whether it also caused restriction in salivary gland infection. To assess this phenotype, we inoculated a subset of the viruses intrathoracically into Toxorhynchites splendens mosquitoes, thus bypassing the midgut. As shown in Table 2, both rDEN4-2A-5 and rDEN4-7129 infected a much lower percentage of mosquito heads (0 and 25%, respectively), following intrathoracic inoculations than their wild-type parent viruses (83 and 100%, respectively), and in the case of rDEN4-2A-5 this difference was significant.

Table 1
Nucleotide and amino acid differences among viruses utilized in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Nucleotide position</th>
<th>Gene/Region</th>
<th>Nucleotide change*</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>rDEN4-2A-5</td>
<td>7129</td>
<td>NS4B</td>
<td>C &gt; U</td>
<td>Pro &gt; Leu</td>
</tr>
<tr>
<td></td>
<td>7359</td>
<td>NS4B</td>
<td>C &gt; U</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>9047</td>
<td>NS5</td>
<td>U &gt; C</td>
<td>None</td>
</tr>
<tr>
<td>rDEN4-7129</td>
<td>7124</td>
<td>NS4B</td>
<td>C &gt; U</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>6521</td>
<td>NS4A</td>
<td>U &gt; C</td>
<td>None</td>
</tr>
<tr>
<td>rDEN4Δ30-7129</td>
<td>7129–7130</td>
<td>NS4B</td>
<td>CA &gt; UU</td>
<td>Pro &gt; Leu</td>
</tr>
<tr>
<td></td>
<td>10475</td>
<td>3' UTR</td>
<td>Deletionb</td>
<td>None</td>
</tr>
<tr>
<td>rDEN4-C6/36</td>
<td></td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

* The consensus sequence is compared to the cDNA from which each virus was derived. Translationally silent nucleotide changes incorporated into the mutagenesis primer in addition to the NS4B P101L mutation are not shown.

Δ30 mutation (Durbin et al., 2001).

Δ30 mutation (Durbin et al., 2001).

Discussion

The goal of this study was to identify mutations that decreased infectivity of dengue virus for mosquitoes. Such mutations could be useful to restrict transmission of a dengue virus vaccine from vaccinees to mosquitoes. Of 1248 DEN4 virus clones screened, only one showed mosquito-specific attenuation in tissue culture, and this virus contained a single missense mutation in the NS4B gene. Small plaque size in mosquito cells in tissue culture was indicative of both decreased replication in mosquito cells in tissue...
culture and decreased infectivity for intact mosquitoes. Addition of the NS4B P101L mutation to DEN4 decreased replication of the rDEN4 derivative in C6/36 cells. Moreover, viruses carrying the NS4B P101L mutation were significantly restricted in their ability to infect the midgut of *Ae. aegypti* fed an infectious bloodmeal and to infect the head of *Tx. splendens* inoculated intrathoracically. Thus a single amino acid change in the NS4B gene strongly attenuated DEN4 for mosquitoes. The level of reduction in infectivity for intact mosquitoes appears far greater than the small effect on replication in C6/36 cells in tissue culture.

Studies of other flaviviruses have detected an association between mutations in NS4B and decreased mosquito infectivity. A yellow fever mutant virus generated by cell passage that was incapable of infecting mosquitoes was shown to carry multiple mutations in the NS4B gene, along with mutations in other genes (Dunster et al., 1999). Japanese encephalitis vaccine virus SA14-2-8, which shows reduced mosquito infectivity, was shown to carry one mutation in the NS4B gene, along with mutations in other genes (Ni et al., 1995). However, in neither case has the contribution of the NS4B mutations to the mosquito phenotype been defined. Conversely, several defined mutations outside of NS4B have been shown to decrease infectivity of dengue virus for mosquito cells in tissue culture. A small set of mutations (mutF) in the 3' UTR (Markoff et al., 2002; Zeng et al., 1998) and, independently, a combination of one mutation in the 5' UTR and one mutation in NS1 (Butrapet et al., 2000) are known to reduce replication in mosquito C6/36 cells. Among other arboviruses, mosquito infectivity has been studied most intensely in the alphaviruses (Strauss and Strauss, 1994). Mutations with host-specific effects have been documented for Sindbis virus, in which mutations in the viral polymerase (Lemm et al., 1990), 3' UTR (Kuhn et al., 1990), and glycoproteins (Boehme et al., 2000) result in loss of infectivity for mosquito cells in tissue culture. However, care must be taken in interpreting these results, since at least one study has shown that Sindbis viruses that replicate poorly in mosquito cells in cell culture nonetheless produce normal yields of virus in intact mosquitoes (Stollar and Hardy, 1984). Several evolutionary studies have investigated whether arboviruses passed exclusively in vertebrate cells tend to lose fitness in invertebrate cells (reviewed in Cooper and Scott, 2001). Only one of these studies has documented a loss of fitness in mosquito cells for virus passed in BHK (hamster) cells (Weaver et al., 1999).

In addition to its effect on mosquito infectivity, the NS4B P101L mutation also had pleiotropic effects on replication in mammalian cells. Addition of this mutation to DEN4 enhanced both plaque size and level of replication in both simian Vero and human HuH-7 cells relative to that of wild-type DEN4. Thus, the NS4B P101L mutation acts as a “trade-off” mutation that improves replication in mammalian systems at the cost of reduced replication in mosquitoes. Since little is known about the function of NS4B, it is impossible at this time to infer a mechanism by which the NS4B P101L mutation may effect this trade-off. The fact that the phenotype conferred by the mutation is host-specific suggests that the mutation may affect the interaction between NS4B and host cell factors unique to each host type. The attenuation in mosquitoes conferred by the NS4B P101L mutation may be useful in investigations of the mechanism of dengue virus infection of mosquitoes and specifically the role of NS4B in this process.

The action of the NS4B P101L mutation may also provide insight into the impact of host alternation on dengue virus evolution. Dengue virus, similar to many arthropod-borne RNA viruses (notably flaviviruses and alphaviruses), evolves at rates 10-fold slower than non-arthropod-borne viruses (Scott et al., 1994; Zanotto et al., 1996). One hypothesis for this
Fig. 3. Percentage of mosquitoes with detectable dengue virus in the midgut (Total Infections) or midgut and head (Disseminated Infections). Mosquitoes were assayed 21 days after being fed an infectious bloodmeal and dengue virus antigen in midgut and head tissues was identified by IFA. The virus dose was calculated by assuming that each mosquito takes a 2 μl bloodmeal. Sample sizes are listed above each bar; circles indicate that no mosquitoes were infected at a corresponding dose.
The NS4B P101L mutation identified in rDEN4-2A-5 and inserted into rDEN4-7129 causes a significant decrease in DEN4 infectivity for \textit{Toxorhynchites splendens} inoculated intrathoracically.

### Table 2

<table>
<thead>
<tr>
<th>Virus tested</th>
<th>Dose inoculated (log$_{10}$PFU$^a$)</th>
<th>No. mosquitoes tested</th>
<th>% Infected$^b,c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rDEN4</td>
<td>3.3</td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>7</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MID$_{50}$ = 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rDEN4-2A-13</td>
<td>4.0</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>9</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>MID$_{50}$ = 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rDEN4-7129</td>
<td>3.6</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MID$_{50}$ &gt; 4.3$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rDEN4-2A-5</td>
<td>3.4</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MID$_{50}$ &gt; 4.4$^e$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Amount of virus inoculated in a 0.22 $\mu$l dose.

$^b$ Percentage of mosquitoes with detectable dengue virus in head tissue; mosquitoes were assayed 14 days postinoculation, and dengue virus antigen was identified by IFA.

$^c$ Pairwise Fisher exact tests were used to compare each virus containing the NS4B P101L mutation with the appropriate wild-type virus. Because doses were not identical, the total number infected for each virus containing NS4B P101L was compared to the total number infected for the comparable wild-type at the next lower dose. Results for the two comparisons were as follows: rDEN4-2A-13 vs rDEN4-2A-5, $n = 18$, $P < 0.001$; rDEN4 vs rDEN4-7129, $n = 14$, $P = 0.1$.

$^d$ When infection was detected, but did not exceed a frequency of 50% at the highest dose of virus ingested, the MID$_{50}$ was estimated by assuming that a 10-fold more concentrated virus dose would infect 100% of the mosquitoes.

$^e$ When no infection was detected, the MID$_{50}$ was assumed to be greater than a 10-fold higher dose of virus than the one used.

The NS4B P101L mutation may be useful in one of the other three DEN serotypes or for the development of an pattern is that host alternation constrains evolution in arboviruses (Scott et al., 1994). The reasoning that underlies this idea is that the need to replicate in the very different environments of arthropod and vertebrate hosts causes arboviruses to evolve to a compromise genotype that allows them to replicate in each environment but does not maximize fitness in either. The NS4B P101L mutation appears to reveal such a compromise, since evolution to this genotype in DEN4 would probably improve fitness in the mammalian host, but would damage transmission by the mosquito vector.

The ability of the NS4B P101L mutation to act as a safeguard against mosquito transmission is clear, but its utility for an rDEN4 vaccine candidate depends upon its interaction with other attenuating mutations in the virus. Both the NS4B P101L mutation and $\Delta 30$ have similar effects on mosquito infectivity, $ts$ phenotypes in cell culture, and attenuation in suckling mouse brain; combining the two mutations in rDEN4$\Delta 30$-7129 did not change these phenotypic effects. In contrast, rDEN4$\Delta 30$ is attenuated in replication in SCID-HuH-7 mice, whereas rDEN4-7129 is enhanced relative to rDEN4. Combination of the two mutations resulted an intermediate phenotype and similar levels of replication to rDEN4. In human clinical trials, rDEN4$\Delta 30$ showed some residual hepatotoxicity when $10^5$ PFU virus was administered (Durbin et al., 2001). SCID-HuH-7 mice are a model for virus replication in the liver, and a decrease in attenuation for rDEN4$\Delta 30$ in this system would therefore be undesirable. At this time the NS4B P101L is not being incorporated into rDEN4$\Delta 30$ to modify the vaccine. Since the $\Delta 30$ mutation itself significantly restricts mosquito infectivity, this vaccine candidate is already limited in its potential for transmission (Troyer et al., 2001).

### Table 3

<table>
<thead>
<tr>
<th>Virus$^a$</th>
<th>Mean virus titer (log$_{10}$PFU/ml) at indicated temperature ($^\circ$C)</th>
<th>Replication in suckling mouse brain$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vero 35 37 38 39 $\Delta^b$</td>
<td>HuH-7 35 37 38 39 $\Delta$</td>
</tr>
<tr>
<td>rDEN4-2A-13</td>
<td>7.3 7.5 7.6 6.5 0.8</td>
<td>8.0 7.5 7.0 6.5 1.2</td>
</tr>
<tr>
<td>rDEN4-2A-5</td>
<td>6.7 7.3 7.3 7.3 0.5</td>
<td>7.5 7.0 6.5 6.5 0.7</td>
</tr>
<tr>
<td>rDEN4$^c$</td>
<td>7.3 7.5 7.6 7.0 0.3</td>
<td>7.4 7.3 7.3 7.3 0.7</td>
</tr>
<tr>
<td>rDEN4$\Delta 30$</td>
<td>6.6 6.6 6.5 6.5 0.1</td>
<td>7.6 7.3 7.3 7.3 0.7</td>
</tr>
<tr>
<td>rDEN4-7129</td>
<td>6.3 6.8 6.6 6.3 1.0</td>
<td>7.3 6.9 6.3 6.3 0.7</td>
</tr>
<tr>
<td>rDEN4$\Delta 30$-7129</td>
<td>6.3 6.8 6.6 6.3 1.0</td>
<td>7.3 6.9 6.3 6.3 0.7</td>
</tr>
</tbody>
</table>

$^a$ Each mutant virus was compared to the recombinant wild-type virus from which it was derived.

$^b$ Reduction in titer (log$_{10}$PFU/ml) at 39° Compared to permissive temperature (35°). Temperature sensitivity is defined in this study as a 2.5 or 3.5 log$_{10}$PFU/ml reduction in titer in Vero or HuH-7 cells, respectively, at the indicated temperatures when compared to permissive temperature (35°) (Hanley et al., 2002).

$^c$ Groups of six suckling mice were inoculated intracerebrally with $10^4$ PFU virus in a 30 $\mu$l inoculum. The brain was removed 5 days later and homogenized, and virus titers were determined in Vero cells.

$^d$ Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent wt controls ($n = 6$). The attenuation phenotype is defined as a $\geq$1 log$_{10}$PFU/g decrease in virus replication in suckling mouse brain. A minus (−) sign indicates an increase in replication relative to the wt control. A one-sample sign test was used to determine whether any of the mutant viruses showed a mean reduction or increase in titer significantly different from 0 ($df = 5$; $P > 0.3$ for each of the four comparisons).
inactivated tetravalent vaccine. DEN1, DEN2, and DEN3 all possess a Pro residue at the position corresponding to amino acid position 101 in DEN4 NS4B (Chang, 1997). Interestingly, Japanese encephalitis virus, Kunjin virus, and West Nile virus each possess a Leu at that position, the same amino acid encoded by the NS4B P101L mutation, yet are efficiently transmitted by mosquitoes. This may indicate that it is not the presence of Leu per se that damages mosquito infectivity but rather its effect on protein structure of NS4B. We are currently in the process of introducing a corresponding Pro-to-Leu mutation in the NS4B gene of the other three DEN serotypes to determine whether its effect on mosquito infection is transferable to each DEN serotype.

The NS4B P101L mutation falls into a cluster of mutations in the DEN4 NS4B gene that confer enhanced ability to replicate in Vero cells; these have been termed Vero cell adaptation mutations (Blaney et al., 2001, 2002). These Vero cell adaptation mutations are similar to the NS4B P101L mutation in that both confer enhanced replication in Vero cells in tissue culture. However, NS4B P101L is unique in both its ability to

![Graph](image-url)

Fig. 4. Mean difference (log_{10}PFU/ml serum) of peak level of replication of rDEN4-2A-5, rDEN4-7129, rDEN4Δ30-7129, and rDEN4Δ30 relative to a concurrent wild-type control virus (rDEN4) in SCID-HuH-7 mice. Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10^6 PFU of virus. Serum was collected on days 6 and 7 and titer determined in Vero cells. Values for rDEN4-2A-5 and rDEN4Δ30 represent results from a single experiment with one concurrent control. Values for rDEN4Δ30 and rDEN4-7129 represent data from two or three separate experiments, respectively. In these latter two cases, data were combined across experiments only after values were compared between experiments and found not to be significantly different (rDEN4Δ30, Student’s t-test, df = 1, P = 0.7; rDEN4-7129, ANOVA, df = 2, P = 0.9). Asterisks (*) indicate that the mean log_{10} difference is significantly different from 0 (one-sample t-test, P < 0.05).
HuH-7 cells (Nakabayashi et al., 1982) were maintained at 37°C supplemented with 10% FBS, 2 mM L-glutamine, 2 mM nonessential amino acids (Gibco), and 0.05 mg/ml gentamicin.

Each of 1248 virus clones generated using 5'-fluorouracil mutagenesis of rDEN4-2A (Blaney et al., 2001) was titered in C6/36 cells. Confluent C6/36 monolayers on 24-well plates were inoculated in duplicate with serial 10-fold dilutions of virus, incubated under the conditions described above for 2 h, and overlaid with OptiMEM (Gibco) containing 0.8% methylcellulose, and supplemented with 2% FBS, 2 mM glutamine, and 0.05 mg/ml gentamycin. Plates were further incubated for 5 days and then immunostained as previously described (Durbin et al., 2001). The titer of each virus clone in C6/36 cells (log_{10}PFU/ml) and in Vero cells (TCID_{50}/ml) was compared. Mutant virus no. 5, which showed small plaque size in C6/36 cells, was biologically cloned by three rounds of terminal dilution in Vero cells followed by an additional amplification in Vero cells using methods previously described (Hanley et al., 2002). The resulting mutant virus was designated rDEN4-2A-5.

The wild-type DEN4 viruses used in this study were generated from either the 2A cDNA or the p4 cDNA, a modified version of 2A in which selected restriction enzyme sites have been either ablated or introduced through translationally silent nucleotide substitutions (Durbin et al., 2001; Lai et al., 1991). Therefore 2A, and consequently p4, both encode the same polyprotein of the parent virus DEN4 strain 814669 (Dominica 1981). One wild-type virus, rDEN4-2A-13, was generated by passaging virus rDEN4-2A, derived from cDNA 2A, in Vero cells in parallel with the 5'-fluorouracil-treated virus clones (Blaney et al., 2001). Therefore rDEN4-2A-13 is used for comparison with rDEN4-2A-5, a 5'-fluorouracil-treated virus clone. rDEN4 was derived from the cDNA p4 (Durbin et al., 2001; Lai et al., 1991) and amplified via one additional passage in Vero cells. rDEN4 is used for comparison with rDEN4Δ30 (Durbin et al., 2001), rDEN4Δ7129, and rDEN4Δ30-7129.

Both rDEN4-2A-13 and rDEN4 accumulated different sets of incidental mutations during passage. To generate a wild-type DEN4 that did not contain any mutations and could be used in plaque size and multicycle replication assays, rDEN4-C6/36 was derived from cDNA p4 and cloned in C6/36 cells (J.E. Blaney et al., unpublished results). Sequence analysis verified that the whole genomic consensus sequence rDEN4-C6/36 exactly matches its predicted sequence derived from p4 (Table 1).

Vaccine candidate rDEN4Δ30 was derived from plasmid p4Δ30, a cDNA clone containing a deletion of nucleotides 10,478–10,507 in the 3' UTR, (Durbin et al., 2001; Men et al., 1996). GenBank Accession Nos. (virus: accession number) were previously assigned as follows: 814669: AF326573, rDEN4: AF326825, rDEN4Δ30: AF326827.

rDEN4-7129 and rDEN4Δ30-7129 were generated via site-directed mutagenesis (Kunkel, 1985) as previously described (Hanley et al., 2002). Briefly, a subclone suitable for mutagenesis was derived from full-length DEN4 plasmid p4 by digestion with restriction enzymes PmlI and XmaI at nucleotide

**Methods and materials**

**Cells and viruses**

Vero cells (African green monkey kidney) were maintained at 37°C in an atmosphere of 5% CO₂ in MEM (Gibco) containing 10% FBS, 2 mM L-glutamine, 0.05 mg/ml gentamicin (Gibco). HuH-7 cells (Nakabayashi et al., 1982) were maintained at 37°C in an atmosphere of 5% CO₂ in D-MEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and 0.05 mg/ml gentamicin. C6/36 cells (Aedes albopictus mosquito cells) were maintained at 23°C in an atmosphere of 5% CO₂ in MEM (Gibco) containing 10% FBS, 2 mM L-glutamine, 2 mM nonessential amino acids (Gibco), and 0.05 mg/ml gentamicin.

HuH-7 cells (Nakabayashi et al., 1982) were maintained at glutamine (Gibco) and 0.05 mg/ml gentamicin (Gibco).
positions 6318 and 7444, respectively. This fragment was then subcloned into a modified pUC vector and site-directed mutagenesis was performed. The primer used to create the NS4B P101L mutation was 7112-CTATTCTGAAGTtAACttAA-CAACCTTGAC-7141 with lower case indicating changes from wild-type sequence. The T substitution at nucleotide 7124 creates a translationally silent HpaI restriction site useful for screening mutant plasmids. The TT pair of substitutions at nucleotides 7128–7129 create the Pro-to-Leu codon change. Two nucleotides in this codon were altered to make the mutation more resistant to reversion. Restriction enzyme fragments bearing the NS4B P101L mutation were cloned back into the full-length p4 and p4Δ30 plasmids, each of which was sequenced to confirm the presence of the desired mutation. 5′-Capped RNA transcripts were synthesized in vitro from the mutagenized cDNA templates, transfected in Vero cells, and biologically cloned by two rounds of terminal dilution in Vero cells (Blaney et al., 2002). To avoid selection against the intended small plaque phenotype of the recombinant viruses, transfection and terminal dilution were performed in Vero cells rather than C6/36 cells. The resulting viruses were designated rDEN4-7129 and rDEN4Δ30-7129. Each virus was completely sequenced to ascertain whether any incidental mutations had occurred during transfection and cloning.

**Plaque size and multicycle replication kinetics**

The plaque size and multicycle replication kinetics of rDEN4-7129 and rDEN4Δ30-7129 were compared to that of rDEN4-C6/36 in C6/36, Vero, and HuH-7 cells. To measure plaque size, serial threefold dilutions of the indicated viruses were inoculated in triplicate onto confluent Vero, HuH-7, and C6/36 cell monolayers in 24-well plates. After incubation at 37°C (Vero) or 32°C (C6/36) for 2 h, monolayers were overlayed with medium containing 0.8% methylcellulose. Following incubation for 5 days, plaques were visualized by immunoperoxidase staining and digitally photographed. For each of the virus-infected wells, the diameter of 36 well-isolated plaques were measured on the digital image using Adobe Photoshop.

To determine replication kinetics, confluent cell monolayers in 25-cm² tissue-culture flasks were infected with virus at a multiplicity of infection (M.O.I.) of 0.01 in a 1.5-ml inoculum. After a 2-h incubation at 37°C, cell monolayers were washed three times in PBS and 5 ml of culture medium supplemented with 2% FBS was added. A 1-ml aliquot of tissue culture medium was removed (0 h time point) and replaced with 1 ml of fresh medium. At designated time points, additional samples of tissue culture medium were removed (with fresh medium replacement), clarified by centrifugation, and frozen at −70°C. Virus titer at each time point was determined by plaque titration in Vero cells.

**Mosquito maintenance and infection**

Methods for the maintenance of *Ae. aegypti* and *Tx. splendens* mosquitoes, the infection of *Ae. aegypti* via an infectious blood-meal, and the infection of *Tx. splendens* via intrathoracic inoculation have been previously described (Troyer et al., 2001). Head and midgut preparations of *Ae. aegypti* and head preparations of *Tx. splendens* were made on glass slides as previously described (Sumanachiraporn et al., 1998; Troyer et al., 2001). Immunofluorescence assay (IFA) was conducted as described previously (Troyer et al., 2001) except that hyperimmune mouse ascites fluid specific for DEN-4 diluted 1/100 in PBS-Tween 20 (0.05%) was used as the primary antibody. The infectious dose required to infect 50% of mosquitoes (MID₅₀) was determined by the method of Reed and Muench (1938).

**Characterization of attenuation phenotypes**

Viruses were screened for *ts* phenotype by assessing plaque formation at permissive (35°C) and various restrictive temperatures (37, 38, and 39°C) in Vero and HuH-7 cell monolayers in 24-well plates as described (Hanley et al., 2002). The replication of viruses was evaluated in Swiss Webster suckling mice (Taconic, Germantown, NY) using groups of six 1-week-old mice that were inoculated intracerebrally with a 30 μl inoculum containing 10⁴ PFU of virus as previously described (Blaney et al., 2001; Hanley et al., 2002). The replication of viruses in SCID mice transplanted with HuH-7 cells (SCID-HuH-7 mice) was determined as described by Blaney et al., (2002). Briefly, four to six-week-old SCID mice (Tac:ICr:Ha(ICR)-Prkdc(scid)) (Taconic) were injected intraperitoneally with 10⁷ HuH-7 cells suspended in 200 μl phosphate-buffered saline. Five to six weeks after transplantation, tumors were detected in the peritoneum and mice were infected by direct inoculation into the tumor with 10⁷ PFU of virus in 50 μl Opti-MEM. Serum for virus titration was obtained by tail-nicking on days 6 and 7 postinfection, and virus titer was determined by plaque assay in Vero cells. Tumors were excised and weighed to confirm uniformity of the experimental groups.

**Acknowledgments**

We are grateful for the contributions of Cai Yen Firestone to this research. We thank Andre Laughinghouse and Kevin Lee of NIAID for assistance in the maintenance and inoculation of *Tx. splendens*. Timothy Endy and Russ Coleman of AFRIMS (Thailand) generously provided us with *Tx. splendens* as well as expert advice on their rearing and on inoculations; Gary Clark of CDC (Puerto Rico) generously provided us with *Ae. aegypti*. We thank Dr. Robert Chanock for reviewing the manuscript.

**References**


yields mutant viruses which are temperature sensitive in vero cells or human liver cells and attenuated in mice. J. Virol. 75 (20), 9731–9740.


