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Dengue virus type 3 vaccine candidates generated by introduction of deletions in the 3' untranslated region (3'-UTR) or by exchange of the DENV-3 3'-UTR with that of DENV-4

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Abstract

The dengue virus type 3 (DENV-3) vaccine candidate, rDEN3Δ30, was previously found to be under-attenuated in both SCID-HuH-7 mice and rhesus monkeys. Herein, two strategies have been employed to generate attenuated rDEN3 vaccine candidates which retain the full complement of structural and nonstructural proteins of DENV-3 and thus are able to induce humoral or cellular immunity to each of the DENV-3 proteins. First, using the predicted secondary structure of the 3' untranslated region (3'-UTR) of DENV-3 to design novel deletions, nine deletion mutant viruses were engineered and found to be viable. Four of nine deletion mutants replicated efficiently in Vero cells and were genetically stable. Second, chimeric rDENV-3 viruses were generated by replacement of the 3'-UTR of the rDENV-3 cDNA clone with that of rDENV-4 or rDEN4Δ30 yielding the rDEN3-3'D4 and rDEN3-3'D4Δ30 viruses, respectively. Immunization of rhesus monkeys with either of two deletion mutant viruses, rDEN3Δ30/31 and rDEN3Δ86, or with rDEN3-3'D4Δ30 resulted in infection without detectable viremia, with each virus inducing a strong neutralizing antibody response capable of conferring protection from DENV-3 challenge. The rDEN3Δ30/31 virus showed a strong host range restriction phenotype with complete loss of replication in C6/36 mosquito cells despite robust replication in Vero cells. In addition, rDEN3Δ30/31 had reduced replication in *Toxorynchites* mosquitoes following intrathoracic inoculation. The results are discussed in the context of vaccine development and the physical structure of the DENV 3'-UTR.

Introduction

The four dengue virus serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) circulate in tropical and subtropical regions of the world inhabited by more than 2.5 billion people [1]. The DENV are endemic in at least 100 countries and cause more disease in humans than any other arbovirus. Annually, an estimated 50–100 million dengue infections result in hundreds of thousands of cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), with children bearing the brunt of the disease burden [2,3]. DHF/DSS remains a leading cause of hospitalization and death of children in at least eight southeast Asian countries [2]. The

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dramatic increase in both the incidence and severity of disease caused by the four DENV serotypes over the past two decades is due in large part to the geographic expansion of the primary vectors, the peridomestic mosquito species *Aedes aegypti* and *Ae. albopictus*, and to the increased prevalence and co-circulation of the four DENV serotypes [1,4]. In urban settings, DENV are maintained in a cycle of transmission between *Aedes* species and humans, with no other apparent viral reservoir [5].

The DENV, members of the *Flaviviridae* family, have a spherical shape of approximately 40 to 60 nm diameter that contain a single-stranded positive-sense RNA genome [6]. A single viral polypeptide is co-translationally processed by viral and cellular proteases generating three structural proteins (capsid C, membrane M, and envelope E) and at least seven non-structural (NS) proteins. The genome organization of the DENV is 5'-UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-UTR-3' (UTR – untranslated region, prM – membrane precursor) [7].

In response to the increasing incidence and severity of DENV infection, an economical vaccine that prevents disease caused by the DENV has become a global public health priority. The cost-effectiveness, safety, and long-term efficacy associated with the live attenuated vaccine against yellow fever (YF) virus, another mosquito-borne flavivirus, serves as a model for the feasibility of developing a live attenuated DENV vaccine [8]. An effective DENV vaccine should confer protection for each serotype since all four serotypes commonly circulate in endemic regions, and secondary infection with a heterologous serotype is associated with increased disease severity [9]. Unfortunately, previous attempts to develop a tetravalent, live attenuated vaccine against the DENV have found that one or more vaccine components exhibited either under- or over-attenuation resulting in unacceptable reactogenicity or poor immunogenicity, respectively [10,11]. Modification of the concentration of one of the serotypes in a tetravalent vaccine has not been able to reliably correct problems of reactogenicity or over-attenuation [12–14]. Therefore, it appears that the path towards generation of an efficacious, live attenuated tetravalent DENV vaccine will include the development and evaluation of multiple vaccine candidates for each serotype to identify four individual viruses that can be combined into a formulation that successfully balances attenuation and immunogenicity. Access to a "menu" of vaccine candidates for each serotype will provide the flexibility to optimize a tetravalent formulation that is minimally reactogenic and that induces strong immunity to each serotype in humans.

Previously, we have employed two strategies for generating monovalent live attenuated vaccine candidates for each serotype that can then be combined into tetravalent formulations [15]. First, reverse genetics has been used to introduce an attenuating 30 nucleotide (nt) deletion (Δ30) mutation into the 3'-UTR of cDNA clones of each DENV serotype [16–21]. In initial studies, the rDEN4Δ30 vaccine candidate was found to be attenuated in rhesus monkeys and phase I/II clinical trials in humans have demonstrated that infection with vaccine virus results in low viremia, is strongly immunogenic, and exhibits minimal reactogenicity without serious adverse events [20,22]. Recently, the rDEN1Δ30 vaccine candidate, which was also attenuated in rhesus monkeys, has been found to share a similar set of properties in clinical trials as that observed for rDEN4Δ30: low viremia, strong immunogenicity, and minimal reactogenicity in 20 volunteers [21]. Unfortunately, the rDEN2Δ30 and rDEN3Δ30 vaccine candidates did not appear to be satisfactorily attenuated in rhesus monkeys during pre-clinical testing and will not be tested in humans [16,17]. Consequently, as an alternative strategy for vaccine development for DENV-2 and DENV-3, antigenic chimeric viruses have been generated by replacement of the structural proteins of the attenuated rDEN4Δ30 vaccine candidate with those from DENV-2 or DENV-3 yielding the rDEN2/4Δ30 and rDEN3/4Δ30 vaccine candidates, respectively [16,23]. The rDEN2/4Δ30 vaccine virus has been tested in humans and appears safe and strongly immunogenic [24], and clinical evaluation of the rDEN3/4Δ30 virus is currently

underway. However, the immune response to DENV-2 or DENV-3 would be directed predominantly against the M and E proteins or to cross-reactive determinants of DENV-4 proteins.

Here, we describe three additional vaccine candidates for the DENV-3 serotype generated by genetic modification of the 3'-UTR of the DENV-3 cDNA clone [16]. Development of these DENV-3 vaccine candidates, which possess the full complement of wild type DENV-3 proteins, is important for two reasons. First, the present vaccine candidate for DENV-3, rDEN3/4Δ30, may be found to be under- or over-attenuated in clinical trials as a monovalent vaccine or as a component of a tetravalent formulation. Second, an optimal vaccine for conferring protection from disease caused by DENV-3 may require induction of T cell responses against the entire set of DENV-3 proteins, rather than just the M and E which are the only DENV-3 sequences present in the rDEN3/4Δ30 chimeric virus. To generate additional DENV-3 vaccine candidates, nine novel deletions which encompass or supplement the original Δ30 deletion in the 3'-UTR were introduced into the rDENV-3 cDNA clone. Additionally, chimeric viruses were generated in which the 3'-UTR of the rDENV-3 cDNA clone was replaced with that of rDENV-4 or rDEN4Δ30. Viable viruses were analyzed for growth in Vero cells, attenuation in SCID mice transplanted with HuH-7 cells, and replication and immunogenicity in rhesus monkeys. Three mutant viruses (rDEN3Δ30/31, rDEN3Δ86, and rDEN3-3'D4Δ30) have preclinical phenotypes that suggest that they may be safe and immunogenic in humans.

Materials and Methods

Cells and Viruses

Vero cells (African green monkey kidney) were maintained in OptiPro SFM (Invitrogen, Grand Island, NY) supplemented with 4 mM L-glutamine (Invitrogen). HuH-7 cells (human hepatoma) were maintained in D-MEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine and 0.05 mg/ml gentamicin (Invitrogen). C6/36 cells (*Aedes albopictus* mosquito cells) were maintained at 32°C in Minimal Essential Medium (MEM) containing Earle's salts and 25 mM HEPES buffer (Invitrogen) and supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM non-essential amino acids (Invitrogen).

A recombinant DENV-3 (rDENV-3) virus strain was previously generated by reverse genetics from the biological isolate, Sleman/78, provided by Dr. Duane Gubler (John Burns School of Medicine, University of Hawaii) [16]. The virus was isolated during a 1978 dengue outbreak characterized by mild illness in the Sleman area of Central Java, Indonesia [25].

Genetic construction of rDEN3 deletion mutations

We sought to generate additional deletion mutations in the 3'-UTR that include the original Δ30 (nt 173–143, numbered from the 3' terminus) mutation previously introduced into rDENV-3 [16]. Figure 1B lists seven deletion mutations which encompass the original Δ30 mutation including Δ50, Δ61, Δ80, Δ86, Δ116A, Δ116B, and Δ146. In addition, the Δ30/31 mutation includes the original Δ30 mutation and an additional non-contiguous 31 nt deletion. The Δ31 mutation was also generated alone to discern the contribution of either Δ30 or Δ31 in the combined Δ30/31 deletion mutation. The positions in the predicted secondary structure of the DENV-3 3'-UTR of the nucleotides that border each deletion are indicated in Figure 1A.

PCR mutagenesis was used to introduce the nine new deletion mutations into the DENV-3 Sleman/78 cDNA plasmid, p3, which was previously used to generate the rDEN3Δ30 vaccine candidate [16]. The p3-frag.4 cDNA subclone (encompassing DENV-3 nucleotides 9749–

10,707) was used as the template for PCR reactions with mutagenic oligonucleotides. To generate full-length DENV-3 cDNA plasmids containing the deletion mutations, the *Pst*I-*Kpn*I fragments (963 nt) from the mutated p3-frag.4 cDNA subclones were introduced into the p3-7164 cDNA plasmid, and the presence of the appropriate deletion mutation was confirmed by sequence analysis. The p3-7164 plasmid encodes the 7164 Vero cell adaptation mutation (amino acid 115 in NS4B Val→Ala) which had previously been shown to enhance growth and transfection efficiency in Vero cells [16].

Genetic construction of rDENV-3 chimeric viruses with the 3'-UTR derived from rDENV-4 or rDEN4Δ30

Another strategy was used to generate novel rDENV-3 vaccine candidates that involved the replacement of the 3'-UTR of the rDENV-3 cDNA clone with that of rDENV-4 or rDEN4Δ30 (Figure 2). The p3-3'D4Δ30 plasmid was generated as follows. First, PCR mutagenesis was used to introduce a *Hpa*I restriction site into the p3-frag.4 cDNA subclone (Figure 2B). To introduce the rDEN4Δ30 3'-UTR into the p3-frag.4(*HpaI*) cDNA subclone, a 364 nt fragment encompassing the p4Δ30 3'-UTR was amplified by PCR using a forward primer (5'-AACAACAACAAACACCAAAGGCTATTG-3') and reverse primer (5'-CCTACCGGTACCAGAACCTGTTG-3'). To generate the p3-frag.4-3'D4Δ30 cDNA subclone, the *Hpa*I-*Kpn*I fragment was removed from p3-frag.4(*HpaI*) and replaced with the p4Δ30 3'-UTR PCR fragment which had been cleaved by *Kpn*I. The *Pst*I-*Kpn*I fragment of p3-frag.4-3'D4Δ30 was introduced into the p3 plasmid to make the full length cDNA clone, p3-3'D4Δ30. The sequence of the 3'-UTR and NS5 junction were confirmed to be correct.

To generate p3-3'D4, the 30 deleted nucleotides of the Δ30 deletion mutation were introduced into the p3-frag.4-3'D4Δ30 subclone. Briefly, the *Mlu*I – *Kpn*I fragment of p3-frag. 4-3'D4Δ30, which encompasses the Δ30 region, was replaced with the corresponding fragment of p4 to make the plasmid, p3-frag.4-3'D4. To generate a full-length chimeric genome, the *Pst*I – *Kpn*I fragment of p3 was replaced with the corresponding fragment of p3-frag.4-3'D4. The 3'-UTR sequence of the p3-3'D4 plasmid was determined to be correct as the missing 30 nt of the Δ30 mutation was replaced by wild type DENV-4 sequence.

Recovery and propagation of rDENV-3 viruses

For recovery of viruses, 5'-capped RNA transcripts were synthesized *in vitro* from cDNA plasmids and transfected into either Vero cells or C6/36 cells. Because of genetic instability in *E coli*, the p3 plasmid requires a linker with redundant stop codons flanked by *Spe*I restriction sites [16]. Prior to transcription and generation of infectious virus, these linker sequences were removed from mutant p3 cDNA plasmids by digestion with *Spe*I. Plasmids were then recircularized by ligation, linearized with *Acc*65I (isoschizomer of *Kpn*I which cleaves leaving only a single 3' nucleotide), and transcribed *in vitro* using SP6 polymerase. Purified transcripts were then transfected into Vero or C6/36 cells using DOTAP liposomes (Roche, Indianopolis, IN). After passage in Vero cells to reach a minimum virus titer of approximately $10^{6.0}$ PFU/ml, viruses were biologically cloned by two or three terminal dilutions before experimental stocks were prepared in Vero cells.

For analysis of replication in tissue culture, growth curves were performed in Vero cells and C6/36 cells. Tissue culture flasks (75 cm^2) of confluent cells were infected at a multiplicity of infection of 0.01. Aliquots of 0.5 ml were removed from flasks daily for seven days. After addition of SPG [22] to a concentration of 1X, samples were frozen on dry ice and stored at −80°C. Virus titer was determined by plaque assay on Vero cells for all samples. The limit of detection was $10^{1.0}$ PFU/ml.

Animal models of DEN virus infection

For analysis of virus replication in SCID-HuH-7 mice, four to six week-old SCID mice (Tac:Icr:Ha(ICR)-Prkdc^{scid}) (Taconic, Germantown, NY) were injected intraperitoneally with 0.1 mL of phosphate-buffered saline containing 10^7 HuH-7 cells which had been propagated in tissue culture [26]. Tumors were detected in the peritoneum five to six weeks after transplantation, and tumor-bearing mice were infected by direct inoculation into the tumor with 10^4 PFU of virus in 50 μ1 Opti-MEM I (Invitrogen). Serum was collected from infected mice on day 7 post-infection and frozen at −80°C. The virus titer was determined by plaque assay in Vero cells.

Viruses were evaluated for replication and immunogenicity in rhesus macaques using established methods [22]. DENV-seronegative monkeys were injected subcutaneously with 10^5 PFU of virus diluted in L-15 medium (Invitrogen) or with a mock inoculum. Serum was collected on days 0–6, 8, 10 and 28 after inoculation and stored at −80°C. Virus titer in serum was determined for each day by plaque assay in Vero cells, and serum neutralizing antibody titer was determined for days 0 and 28 by plaque reduction neutralization test [22]. On day 35 post-infection, all monkeys were challenged by subcutaneous infection with 10^5 PFU of DENV-3 Sleman/78 wild type virus. Serum was collected on days 0–6, 8, and 10, frozen at −80°C, and the virus titer in serum samples was determined by plaque assay in Vero cells.

Replication in mosquitoes

Replication of rDENV-3 and rDEN3Δ30/31 was studied in *Toxorynchites amboinenesis* mosquitoes. Intrathoracic inoculation of serial ten-fold dilutions of test virus was performed as described previously [27]. After a 14 day incubation, heads were separated and homogenized in diluent. Virus titer in head homogenates was determined by plaque assay in Vero cells.

Results

Generation of rDENV-3 with 3’-UTR deletions

Transfections in Vero cells and C6/36 cells were performed with cDNA clones representing each of the nine deletion mutations listed in Figure 1B. Viruses with Δ30/31, Δ31, Δ50, Δ61, Δ80, Δ86, Δ116A, Δ116B, and Δ146 mutations were successfully recovered in C6/36 cells, whereas rDEN3Δ31 was the only virus that could also be recovered in Vero cells. The rDENV-3 deletion mutant viruses were then passaged in Vero cells before biological cloning by two terminal dilutions in Vero cells. Cloned viruses were then passaged two to seven times in Vero cells in an attempt to reach a titer of approximately $10^{6.0}$ PFU/ml which is considered sufficient to allow for cost-effective manufacture. Three recombinant viruses (rDEN3Δ50, rDEN3Δ116A, and rDEN3Δ146) were found to be excessively restricted for replication in Vero cells, despite being viable (Figure 1B), and were not studied further. The genetic sequence of the 3’-UTR was determined for the six remaining deletion mutant viruses that reached peak virus titers of approximately $10^{6.0}$ PFU/ml. The intended 3’-UTR sequence with the appropriate deletion was found for rDEN3Δ61, rDEN3Δ80, rDEN3Δ86 and rDEN3Δ30/31 (Figure 1B). However, two mutant viruses were found to contain additional deletions or mutations and were deemed to have potentially unstable genotypes. First, rDEN3Δ31 had the engineered 3’-UTR deletion of nt 258-228 but also contained a 25 nt deletion of nt 222–198. Second, rDEN3Δ116B had the engineered 3’-UTR deletion of nt 258-143 but also contained a 8 nt deletion of nt 430–423 and a single A->G substitution at nt 265. The observed genetic instability with these two viruses makes manufacturing a homogeneous suspension of virus a significant challenge so they were not studied further. For the nine original deletions constructed, four mutant viruses were found to replicate efficiently in Vero cells and to contain the engineered 3’-UTR sequence, and were studied further; rDEN3Δ61, rDEN3Δ80, rDEN3Δ86 and rDEN3Δ30/31 (Figure 1B).

Generation of rDENV-3 chimeric viruses with the 3'-UTR derived from rDENV-4 or rDEN4Δ30

The 3'-UTR chimeric virus, rDEN3-3'D4Δ30, was designed to be a vaccine candidate for inclusion in tetravalent formulations which share the Δ30 deletion mutation among all four serotypes (Figure 2). Sharing the Δ30 mutation amongst all serotypes is important since it precludes generation of a wild type recombinant virus during manufacture or use in vaccinees although recombination is viewed as an unlikely event [28]. The rDEN3-3'D4 virus was also generated to identify the contribution of the 3'-UTR chimerization and the Δ30 mutation to a phenotype observed for rDEN3-3'D4Δ30. rDEN3-3'D4 was recovered in C6/36 cells and Vero cells, whereas rDEN3-3'D4Δ30 was only recovered in Vero cells. Mutant viruses were then passaged once in Vero cells followed by biological cloning by two terminal dilutions in Vero cells. rDEN3-3'D4 and rDEN3-3'D4Δ30 were then passaged four or six times in Vero cells, respectively, and reached virus titers of greater than $10^{6.5}$ PFU/ml. The genetic sequence of the NS5 – 3'-UTR junction and the entire 3'-UTR was found to be as engineered for rDEN3-3'D4 and rDEN3-3'D4Δ30. Therefore, both viruses were studied further.

Replication of DENV-3 mutant viruses in SCID-HuH-7 mice

The four deletion mutant viruses (rDEN3Δ30/31, rDEN3Δ61, rDEN3Δ80, and rDEN3Δ86) and the rDEN3-3'D4 and rDEN3-3'D4Δ30 chimeric viruses were evaluated for level of replication in SCID-HuH-7 mice. This mouse model provided the original evidence that the rDEN3Δ30 virus was not attenuated compared to parent virus rDENV-3, while the antigenic chimeric virus, rDEN3/4Δ30, was approximately 100-fold restricted in replication in the mice when compared to wild type parent viruses [16].

As indicated in Table 1, wild type DENV-3 Sleman/78 replicated to a mean peak virus titer of $10^{6.9}$ PFU/ml. However, rDEN3Δ86 and rDEN3-3'D4Δ30 were more than 10-fold restricted in replication compared to wild type DENV-3 virus whereas the replication of rDEN3Δ30/31 was slightly less than 10-fold restricted. On the basis of this arbitrary cut-off, these three viruses were selected for further evaluation. It is important to note that the rDEN4Δ30 virus which has a well-characterized, non-reactogenic phenotype in humans was found to be only 6-fold restricted in replication in SCID-HuH-7 mice compared to wild type DENV-4[29].

Replication of DEN3 mutant viruses in tissue culture

The level of virus replication in both Vero cells and C6/36 mosquito cells was assessed for the rDEN3Δ30/31 and rDEN3Δ86 deletion mutant viruses and the rDEN3-3'D4 viruses with and without Δ30. Replication in Vero cells was analyzed because these cells are the substrate for manufacture, while growth in C6/36 cells was assessed because attenuation phenotypes in these mosquito cells may be associated with restricted replication in *Aedes* mosquitoes, which serve as the vector for DENV transmission [30].

The replication kinetics of each virus in both cell lines is shown in Figure 3. In Vero cells, rDEN3Δ30/31, rDEN3Δ86, and rDEN3-3'D4Δ30 replicated to a peak level that approximated that of wild type DENV-3 with similar kinetics. These three vaccine candidates reached peak virus titers of $10^{6.5}$ to $10^{6.7}$ PFU/ml which demonstrates the feasibility of manufacture for each of these viruses. In Vero cells, the rDEN3-3'D4 virus replicated to a peak titer of $10^{7.8}$ PFU/ml which is nearly 100-fold higher than that observed for wild type DENV-3 indicating that inclusion of the DENV-4 3'-UTR may augment replication in Vero cells. rDENV-4 replicates to a peak titer of approximately $10^{8.0}$ PFU/ml which indicates that the chimeric virus achieved a peak titer that does not exceed that of either of its parent viruses [18].

Analysis of virus replication in C6/36 cells demonstrated that rDEN3Δ86 and rDEN3-3'D4Δ30 reached peak titers approximately 10-fold lower than the peak virus titer of wild type DENV-3 virus, $10^{6.9}$ PFU/ml (Figure 3). The rDEN3-3'D4 virus replicated to a peak

titer similar to that observed for wild type DENV-3. The most striking result was the lack of replication of rDEN3Δ30/31 in C6/36 cells. After day 1, virus was not detected in culture medium from C6/36 cells infected with rDEN3Δ30/31 virus despite the efficient replication observed in Vero cells. These results were confirmed in a second independent growth curve experiment and indicate a host range attenuation phenotype in tissue culture.

Sequence analysis of rDENV-3 mutant viruses

The genomes of rDEN3Δ30/31, rDEN3Δ86, and rDEN3-3'D4Δ30 were fully sequenced to check for adventious mutations which may have arisen during passage in Vero cells and likely contribute to enhanced growth and adaptation in Vero cells (Table 2). Each virus was found to possess the 7164 Vero cell adaptation mutation that had been engineered into the cDNA plasmids. The rDEN3Δ30/31 virus was found to contain a single coding change in NS4B while rDEN3-3'D4Δ30 lacked coding changes but did contain a single nucleotide substitution in the 3'-UTR. Sequence analysis of rDEN3Δ86 revealed a coding change in M and a single nucleotide substitution in the 3'-UTR. Interestingly, a mixed population at nt 10267 (A→A/U) of rDEN3Δ86 was found that changes the stop codon (UAA) at the end of NS5 to UAU which encodes Tyr. This would serve to extend NS5 by only 2 amino acids (Tyr-Thr-End) since an inframe stop codon remains at nts 10271–10273.

Replication, immunogenicity, and protective efficacy of DENV-3 mutants in rhesus monkeys

Based on the attenuation in SCID-HuH-7 mice and efficient growth in Vero cells, rDEN3Δ30/31, rDEN3Δ86, and rDEN3-3'D4Δ30 were evaluated in rhesus monkeys. The mutant viruses were compared with wild type DENV-3 for level and duration of viremia, neutralizing antibody induction, and the ability to confer protection from wild type DENV-3 virus challenge. The rDEN3-3'D4 virus was also evaluated to identify a contribution of chimerization to attenuation with and without the Δ30 mutation.

Groups of four rhesus monkeys were inoculated subcutaneously with 10^5 PFU of the indicated virus (Table 3). Wild type DENV-3 Sleman/78 virus replicated in rhesus monkeys to a mean peak virus titer of $10^{1.8}$ PFU/ml serum with all monkeys developing viremia. These results parallel previous studies of DENV-3 in rhesus monkeys [16]. Viremia was not detected in any monkey infected with any of the three vaccine candidates, namely rDEN3Δ30/31, rDEN3Δ86, or rDEN3-3'D4Δ30, demonstrating a clear attenuation phenotype for each of these viruses in rhesus monkeys. Interestingly, the rDEN3-3'D4 virus was detected in 75% of monkeys with a mean peak virus titer of 1.3 $\log_{10}$PFU/ml serum suggesting that the presence of the Δ30 mutation is critical for attenuation of the rDEN3-3'D4Δ30 chimeric virus. Despite the lack of detectable viremia, mean neutralizing antibody levels in monkeys infected with rDEN3Δ30/31 and rDEN3Δ86 reached levels similar to that of wild type DENV-3, 1:253 (Table 3). In contrast, the rDEN3-3'D4Δ30 virus induced mean neutralizing antibody levels approximately three-fold lower than wild type DENV-3. However, 100% of monkeys immunized with each vaccine candidate seroconverted as defined by a four-fold or greater rise in serum neutralizing antibody levels after inoculation. Thus all monkeys were infected by each of the vaccine candidates despite the lack of detectable viremia. Determination of virus titer in serum after challenge with DENV-3 virus indicated that immunization with each of the vaccine candidates induced complete protection from detectable viremia as would be expected given the observed neutralizing antibody levels.

Replication of rDEN3Δ30/31 in *Toxorynchites* mosquitoes

Based on the attenuation of rDEN3Δ30/31 in rhesus monkeys and its restricted replication in C6/36 mosquito cells, rDENΔ30/31 was compared to wild type rDENV-3 for infectivity and level of replication in highly sensitive *Toxorynchites amboinensis* mosquitoes (Table 4). Ten-fold serial dilutions of virus were inoculated intrathoracically, and the ability to infect head

tissues was evaluated by performing a plaque assay on mosquito head homogenates after a 14 day incubation. The infectivity of rDENV-3 and rDENΔ30/31 was very similar as the 50% mosquito infectious dose was approximately $10^{1.3}$ PFU for both viruses (Table 4). However, the level of replication of rDENΔ30/31 in the heads of infected mosquitoes was approximately 5- to 30-fold reduced. This reduction was significant at the $10^{2.3}$ and $10^{1.3}$ PFU doses tested. This finding indicates that although rDENΔ30/31 has infectivity for *Toxorynchites* by intrathoracic infection similar to that of wild type rDENV-3, there is a statistically significant restriction in the level of replication in mosquitoes afforded by the Δ30/31 mutation.

Discussion

Reverse genetics has proven to be a very valuable tool to introduce attenuating mutations into dengue viruses to produce live attenuated DENV vaccine candidates [18,31]. Specifically, modification of the 3'-UTR as a means to attenuate DENV has resulted in the development of several promising live attenuated vaccine candidates [19,22,32,33]. The introduction of deletion mutations into untranslated regions is an attractive strategy for development of live attenuated DENV vaccines for two reasons. First, deletions have the potential for a high level of genetic stability and are unlikely to revert to wild type sequence. Second, attenuating deletion mutations in the 3'-UTR do not affect the sequence of translated gene products, and thus, the authentic wild type proteins encoded by the vaccine virus should induce the full spectrum of humoral and cell-mediated immune response in vaccinees. In contrast, chimeric vaccines viruses, such as the DEN3/4Δ30(ME) vaccine candidate [16], induce DENV-3-specific immunity to only the M and E proteins. The present study defines novel deletions and a DEN3/4 chimeric virus with a 3'-UTR swap and deletion that appear to be promising vaccine candidates for induction of protective immunity to the full set of DEN3 antigenic targets.

The DENV 3'-UTRs range in length from approximately 400–450 nucleotides and contain three defined regions, namely, the variable region which abuts the NS5 gene stop codon, the core region, and an essential 3' terminal stem loop structure which is well-conserved among the flaviviruses [34]. The DENV 3'-UTR is believed to play a key role in the regulation of viral replication by virtue of controlling RNA synthesis [35]. Both viral and cellular factors are known to bind to the 3'-UTR, and these interactions appear to regulate the efficiency of RNA replication [34]. There is some evidence suggesting that the attenuating effects of deletion mutations in the 3'-UTR are mediated by a disruption in secondary structure that results in a decrease in the efficiency of RNA replication [35]. Studies by Men et al. first characterized a panel of deletion mutations of varying length in the 3'-UTR of DENV-4 [33]. Deletions in the 3'-UTR from 30 to 262 nt in length were found to be viable and, with the exception of a single deletion (243–183), genetically stable. Various deletions including nt 172–143 (Δ30) were found to confer reduced replication in tissue culture and signs of attenuation in rhesus monkeys [33]. In our laboratory, the Δ30 deletion mutation that interrupts Structural Element 1 (SE-1) (Figure 1) in the 3'-UTR has been utilized for the development of vaccine candidates for each DENV serotype [16,17,19,22]. Although M-fold predictions suggest that elements of the secondary structure of the DENV 3'-UTRs are conserved, differences in the impact of Δ30 among the different DENV serotype suggests that such predictions fail to detect important structural variation [36,37]. RNAse mapping of the core region of the 3'-UTR of rDENV-4 has also pointed to important variation in secondary structure among the DEN serotypes [38]. For example, as initially predicted by Shurtleff et al. [36], RNAse mapping corroborates the prediction that the SE-1 (CS2) sequence of rDENV-4 folds into a single stem and loop "turret" rather than the double-branched dumbbell structure of rDENV-3. Moreover, because SE1 in rDENV-4 is composed of only a single branch, incorporation of Δ30 results in a decrease in length, rather than branching complexity, of SE1 in this serotype. Whether this putative variation in the structure of SE1 in rDENV-4 and rDENV-3 is borne out by RNAse mapping,

and whether it may explain the variation in the phenotypic impact of Δ30 among the viruses, is currently under investigation in our laboratories.

Due to the wild type phenotype of rDEN3Δ30, which contains a deletion in SE-1, it was hypothesized that the presence of the unaltered reciprocal structure, SE-2, might be sufficient for efficient structure and function of the overall DENV-3 3'-UTR [16]. Therefore, the additional deletions described here were generated that extend into SE-2. In addition, the Δ30/31 deletion mutation was generated to disrupt both of the reciprocal structures, SE-1 and SE-2. All of the deletions were found to be viable and therefore, may be useful for basic studies of the structure and function of the DENV-3 3'-UTR. However, only deletion viruses that had adequate replication in Vero cells and that maintained the introduced deletion without major modification were considered in this study since these are characteristics required for vaccine candidates. Nonetheless, it is intriguing that while Δ30 in SE1 is highly stable in all four DENV serotypes [16,17,19,22], Δ31 in SE2 triggered an additional large deletion within the same structural element. Although previous studies have found the structure of these two elements to be almost identical [37], this discrepancy in their tolerance of a homologous deletion suggests differences in either secondary or tertiary structure.

Of the nine DENV-3 viruses with 3'-UTR deletions recovered and evaluated in the present study, two viruses, rDEN3Δ86 and rDEN3Δ30/31, appear to be suitable vaccine candidates. Both rDEN3Δ86 and rDEN3Δ30/31 were mildly attenuated in SCID-HuH-7 mice, but in rhesus monkeys, the viruses were highly attenuated as indicated by the absence of viremia in immunized animals. These results suggest that the disruption of both SE-2 and SE-1 is necessary for attenuation of DENV-3. These findings further demonstrate that while the general secondary structure of the DENV 3'-UTRs is believed to be conserved, there are significant differences between the serotypes that are not currently understood. Despite the absence of viremia in rhesus monkeys immunized with rDEN3Δ86 and rDEN3Δ30/31, a robust neutralizing antibody response developed suggesting that these deletions confer strong attenuation while still allowing a level of replication sufficient for development of an antibody response. The antibody response in monkeys immunized with rDEN3Δ86 and rDEN3Δ30/31 reached levels similar to that of monkeys infected with wild type DEN3 which makes these vaccine candidates particularly attractive for study in humans.

In addition to a balanced level of attenuation and immunogenicity, a particular concern for live attenuated vaccines for arboviruses to be used in humans is the potential for unwanted replication and transmission in mosquito vectors. Ideally, a live attenuated DENV vaccine candidate should have two characteristics to block transmission. First, vaccine candidates should replicate to low levels in humans, which serves to decrease the likelihood of a feeding mosquito becoming infected. In the case of rDEN3Δ86 and rDEN3Δ30/31, the lack of detectable viremia in rhesus monkeys suggests that they would indeed replicate to low levels in humans similar to other DENV vaccine candidates studied in our laboratory [18]. Second, live attenuated DENV vaccine candidates should have an inherent restriction for growth in mosquitoes, as observed for the rDEN4Δ30 virus [27]. Therefore, the DENV-3 described here were assayed for replication in C6/36 cells which are derived from *Aedes albopictus.* A single virus, rDEN3Δ30/31, was found to be defective for replication in C6/36 cells while capable of efficient replication in Vero cells. Since previous studies have demonstrated that wild type DENV-3 Sleman/78 has low infectivity for orally fed *Aedes aegyptii* mosquitoes [16], rDEN3Δ30/31 was tested for infectivity and replication in intrathoracically-infected *Toxorynchites* mosquitoes. In this highly sensitive mosquito, rDEN3Δ30/31 was found to have a small but statistically significant decrease in replication. Therefore, the rDEN3Δ30/31 vaccine candidate appears to have several factors which would prevent transmission of the virus from vaccinee to mosquito: (1) development of low or no viremia in vaccinees, (2) the

natural low infectivity of DENV-3 Sleman/78 for *Aedes* [16], and (3) attenuation conferred by the Δ30/31 mutation.

An alternative method for developing a DENV-3 vaccine candidate was also pursued by swapping the 3'-UTR of DENV-3 with that of rDENV-4 or rDEN4Δ30. This is a new strategy for attenuation of a flavivirus, although chimeric live oral poliovirus vaccines have been generated by swapping the 5' UTR of a modified Sabin type 3 strain with that of type 1 and 2 in an effort to increase genetic stability [39]. In addition, Markoff and colleagues previously constructed chimeric West Nile virus/DENV-2 viruses with swaps confined to the 3' terminal stem loop structure that were found to be attenuated for replication in tissue culture [40]. Alignment of DENV-3 Sleman/78 and rDENV-4 reveals that the DENV-3 3'-UTR contains an approximately 60 nt extension in the variable region near the beginning of the 3'-UTR. Excluding this additional sequence in the DENV-3 3'-UTR, the nucleotide homology of the DENV-3 and DENV-4 3'-UTR is only 75%. However, the linear order of specific structural elements of the 3'-UTR appears to be conserved among the four serotypes [36,37,41] even though as discussed above, the configuration of individual elements may differ among the serotypes. Therefore, the ability of the DENV-4 3'-UTR to function normally in the context of the rest of the DENV-3 genome was not difficult to envision. In fact, the rDEN3-3'D4 virus showed no signs of attenuation when compared to wild type DENV-3 in tissue culture, SCID-HuH-7 mice, or rhesus monkeys. Surprisingly, the rDEN3-3'D4 virus appeared to replicate better than wild type DENV-3 in Vero cells, and this virus might be useful to achieve the high virus yields required for manufacture of an inactivated DENV-3 vaccine. When the Δ30 mutation was introduced into the DENV-4 3'-UTR (rDEN3-3'D4Δ30), significant attenuation was observed in C6/36 cells, Vero cells, SCID-HuH-7 mice, and rhesus monkeys. The impact of Δ30 in rDEN3-3'D4 is generally consistent with its impact in rDEN4, save that in the latter case the addition of Δ30 does not cause attenuation in Vero cells [27]. This finding further indicates that the context of the Δ30 deletion dictates whether the mutation will confer attenuation upon a given virus. The present studies do not elucidate why the Δ30 mutation fails to attenuate DENV-3 in the context of the DENV-3 3'-UTR. The only insight into the molecular mechanism by which the Δ30 mutation confers attenuation upon the DENV comes from a study of DENV replicons. Alvarez et al. found that a replicon containing the 3'-UTR with the Δ30 deletion had 50- to 100-fold decreased RNA replication in BHK or C6/36 cells [35]. Further studies are needed to discern the structural basis for the divergent effects observed for deletion mutations in DENV-3 versus DENV-4.

Based on the restricted replication and strong immunogenicity in rhesus monkeys observed for rDEN3Δ30/31, rDEN3Δ86, and rDEN3-3'D4Δ30 as well as the mosquito restriction of rDEN3Δ30/31, the viruses were re-derived to generate Clinical Lots suitable for administration in humans. Seed stocks of rDEN3Δ30/31 and rDEN3-3'D4Δ30 were successfully generated and found to retain the correct modified 3' UTR sequences along with differing adventitious mutations (data not shown). However, the rederived rDEN3Δ86 virus was found to contain an expanded deletion mutation (346–108) and this genetic instability will preclude further evaluation of this virus as a vaccine candidate. Therefore, only Clinical Lots of rDEN3Δ30/31 and rDEN3-3'D4Δ30 are currently being manufactured and will be tested in humans. At present, the rDEN3/4Δ30 antigenic chimeric virus is currently being evaluated in a clinical trial and will most likely be the DENV-3 component of the first tetravalent formulation that we will test in humans [16]. Should the rDEN3/4Δ30 virus be over- or under-attenuated in humans, then rDEN3Δ30/31 or rDEN3-3'D4Δ30 may be a suitable reserve vaccine candidate. The fact that rDEN3Δ30/31 and rDEN3-3'D4Δ30 possess the full complement of DENV-3 antigenic targets instead of only the M and E genes (such as rDEN3/4Δ30) suggests that they might be preferable to the antigenic chimeric virus.

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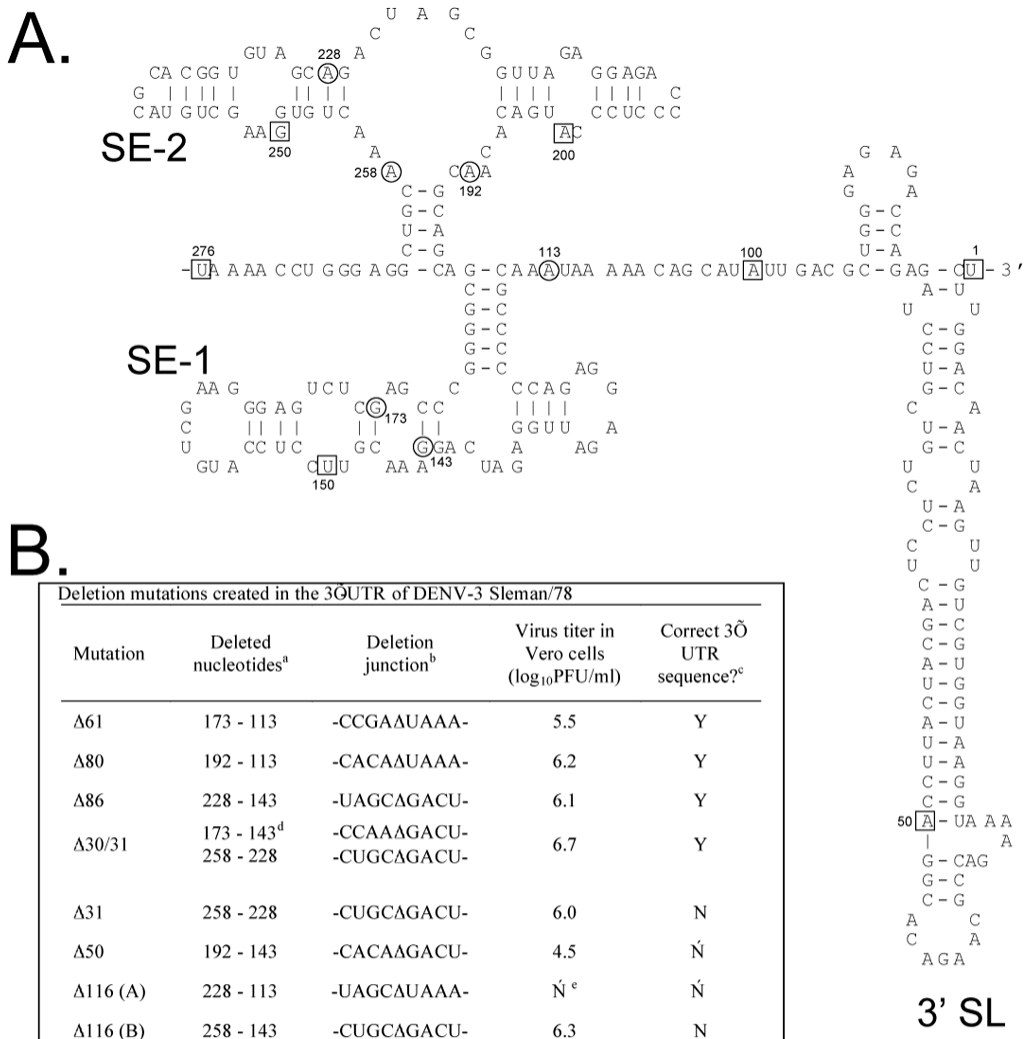
A.



B.

Deletion mutations created in the 3ÕUTR of DENV-3 Sleman/78

Mutation	Deleted nucleotides[a]	Deletion junction[b]	Virus titer in Vero cells (log_{10}PFU/ml)	Correct 3Õ UTR sequence?[c]
Δ61	173 - 113	-CCGAΔUAAA-	5.5	Y
Δ80	192 - 113	-CACAΔUAAA-	6.2	Y
Δ86	228 - 143	-UAGCΔGACU-	6.1	Y
Δ30/31	173 - 143[d] 258 - 228	-CCAAΔGACU- -CUGCΔGACU-	6.7	Y
Δ31	258 - 228	-CUGCΔGACU-	6.0	N
Δ50	192 - 143	-CACAΔGACU-	4.5	Ń
Δ116 (A)	228 - 113	-UAGCΔUAAA-	Ń [e]	Ń
Δ116 (B)	258 - 143	-CUGCΔGACU-	6.3	N
Δ146	258 - 113	-CUGCΔUAAA-	Ń [e]	Ń

[a] Numbering is from the 3Õend of viral genome.
[b] Four bordering nucleotides on either side of the deletion (Ę) are indicated.
[c] The 3ÕUTR of virus genomes was sequenced. Y indicates that deletion was intact and no other changes were found in the 3ÕUTR (viruses were further studied). N indicates that additional deletion/changes were found as described in text. Dash indicates that virus was not sequenced because of low titer.
[d] The Ę30 designation was used for consistency with DEN4Ę30, however, the Ę30 deletion of DENV-3 actually contains 31 deleted nt and a substitution (G->A) at nt 175 (Blaney et al., Am. J. Trop. Med. Hyg., 2004, 71:811-821).
[e] Virus could not be adapted to growth in Vero cells although it replicated in C6/36 cells.

Figure 1.

A. The predicted secondary structure for the 3'-UTR of wt DENV-3 Sleman/78. The sequence used for construction of the secondary structure model is the last 276 nucleotides of DENV-3 (nucleotides 10432 – 10707 from GenBank accession number AY648961). The M-fold program [42,43] was used and nucleotides 267–276 and 95–104 were constrained to be single stranded in order to prevent circularization of the structure and loss of the conserved terminal 3' hairpin stem loop (designated 3' SL). Nucleotides are boxed at every 50 bases for reference to indicate position relative to the 3' end. Circled nucleotides represent borders of the described deletions. The two stem-loop structural elements, designated SE-1 and SE-2, correspond approximately to the previously described TL2 and TL1, DB1 and DB2, and A3 and A2

structural elements [36,37,41]. **B.** Summary of the recovery and properties of the nine mutant viruses with deletions introduced into the 3'-UTR.

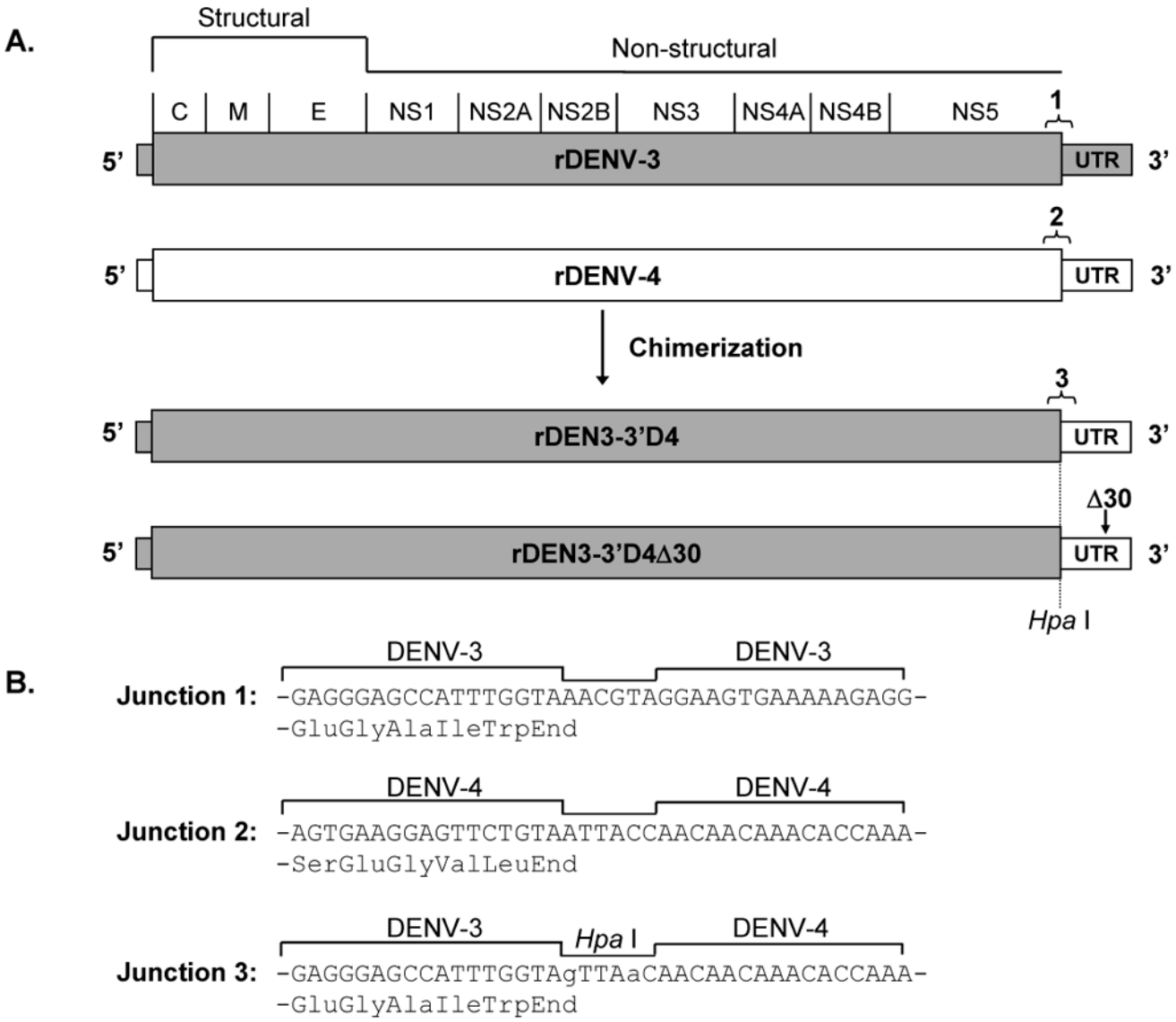


Figure 2.

Chimerization of rDEN3 with the 3'-UTR of rDENV-4 or rDEN4Δ30. **A.** Recombinant 3'-UTR chimeric dengue viruses were constructed by replacing the 3'-UTR of rDENV-3 with regions derived from either rDENV-4 or rDEN4Δ30. The relative location of the Δ30 mutation in the 3'-UTR is indicated by an arrow. The junctions between the ORF and UTR for rDENV-3 and rDENV-4 are indicated as junctions 1 and 2, respectively. Intertypic junction 3 is also indicated for the resulting chimeric viruses. **B.** Nucleotide and amino acid sequence of the junction regions are shown. For junction 3, nucleotide substitutions used to introduce a unique *Hpa*I restriction enzyme recognition site are shown in lower case.

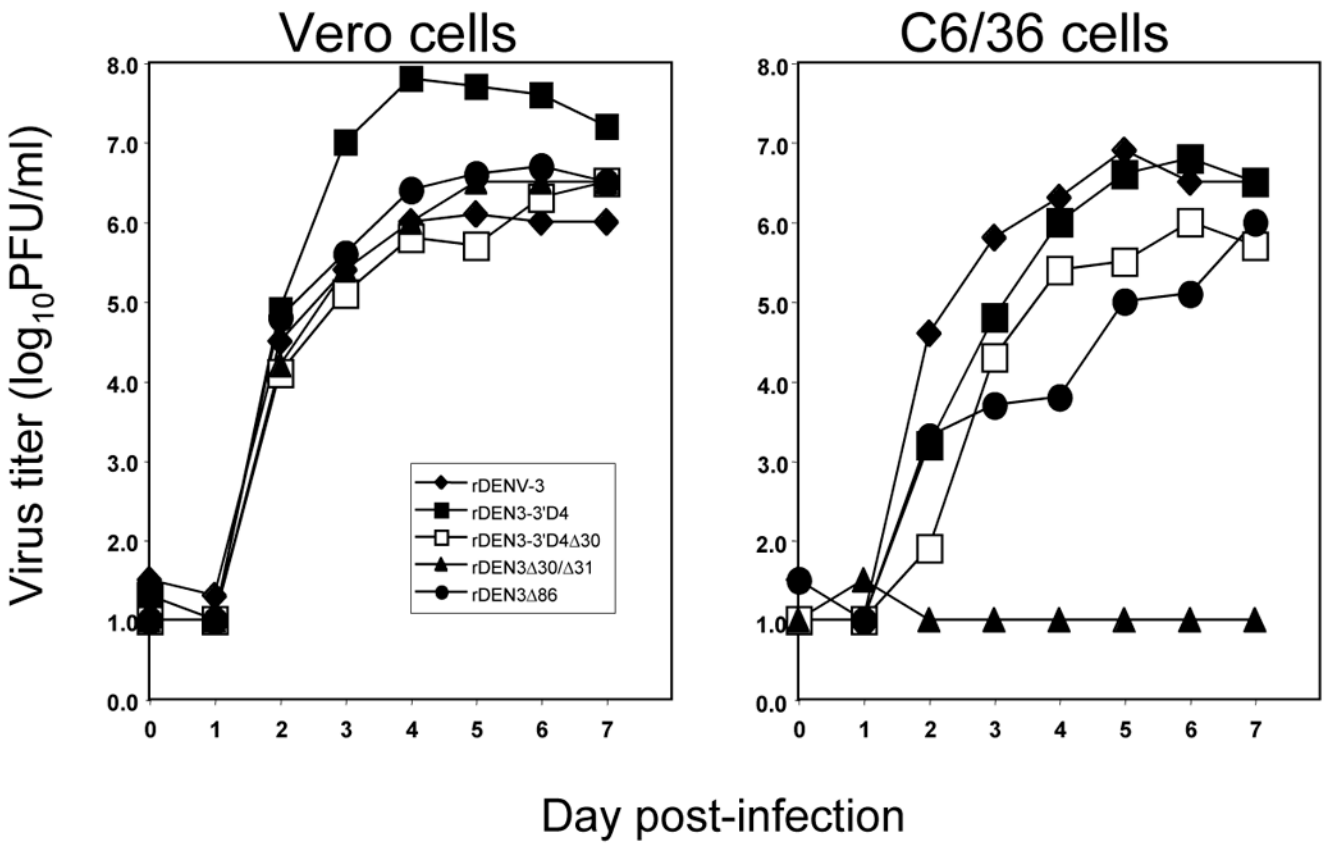


Figure 3.
Replication of rDENV-3 in Vero cells and C6/36 cells. Cells were infected at an MOI of 0.01. Virus titer of supernatants was determined by plaque assay in Vero cells for all samples. The limit of detection is 10^1 PFU/ml.

Table 1

Replication of mutant DENV-3 viruses in SCID-HuH-7 mice.

Virus[a]	Analysis group	No. of mice	Mean peak virus titer (log_{10}PFU/ml ± SE)	Mean log_{10} reduction from DEN3 virus titer
DENV-3 (Sleman/78)		8	6.9 ± 0.1	
rDEN3Δ30/31	1	8	6.0 ± 0.3	0.9
rDEN3Δ61	1	9	6.3 ± 0.2	0.6
rDEN3Δ80	1	9	6.3 ± 0.3	0.6
rDEN3Δ86	1	10	5.6 ± 0.4[b]	1.3
rDEN3-3'D4	2	11	6.5 ± 0.4	0.4
rDEN3-3'D4Δ30	2	9	5.7 ± 0.2[b]	1.2

[a] Groups of SCID-HuH-7 mice were inoculated into the tumor with 10^4 PFU of the indicated virus. Serum was collected on day 7 and virus titer was determined in Vero cells.

[b] Analysis groups 1 and 2 were independently compared to wild type DENV-3. Mean virus titers are significantly different from DENV-3 virus titer as determined by Tukey-Kramer post-hoc test ($P < 0.05$).

Table 2

Adventitious mutations that were identified in rDEN3Δ30/31, rDEN3Δ86, and rDEN3-3'D4Δ30

Virus[a]	Gene	Nucleotide position	Nucleotide substitution	Amino acid position	Amino acid change
rDEN3Δ30/31	NS4B	7398	C → U	193	Ala → Val
rDEN3Δ86	M	512	A → G	26	Lys → Glu
	NS3	6076	C → U	521	*silent*
	NS5	8623	U → C	353	*silent*
	NS5	10267[b]	A → U	END	end → Tyr
	3' UTR	10455	G → C	—	—
rDEN3-3'D4Δ30	C	250	U → C	52	*silent*
	NS3	5899	U → C	462	*silent*
	3' UTR	10534	A →G	—	—

[a] rDEN3Δ30/31 and rDEN3Δ86 were compared to the DENV-3 p3 plasmid cDNA (Genbank # AY656169). rDEN3-3'D4Δ30 was compared to the DENV-3 p3 plasmid cDNA clone (5' UTR and genes) and the DEN4 p4 cDNA clone (Genbank # AY648301) for the 3' UTR.

[b] There is a mixed population at this nt position (A→A/U) that changes the stop codon (UAA) at the end of NS5 to UAU which encodes Tyr. This would serve to extend NS5 by only 2 amino acids (Tyr-Thr-End) since an inframe stop codon remains at nts 10271–10273.

Table 3

Replication and immunogenicity of rDENV-3 mutant viruses in rhesus monkeys.

Virus[a]	No. of monkeys	% of monkeys with viremia	Mean no. of viremic days per monkey	Mean peak virus titer[b] (log_{10}PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)[c]		Post-challenged[d]	
					Day 0	Day 28	% of monkeys with viremia	Mean peak virus titer[b] (log_{10}PFU/ml ± SE)
DENV-3 (Sleman/78)	4	100	3.5	1.8 ± 0.1	< 5	253	0	< 1.0
rDENΔ330/31	4	0	0	< 1.0	< 5	304	0	< 1.0
rDEN3Δ86	4	0	0	< 1.0	< 5	224	0	< 1.0
rDEN3-3'D4	4	75	1.5	1.3 ± 0.2	< 5	229	0	<1.0
rDEN3-3'D4Δ30	4	0	0	< 1.0	< 5	77	0	< 1.0
mock infected	2	0	0	< 1.0	< 5	< 5	100	1.8 ± 0.2

[a] Groups of rhesus monkeys were inoculated subcutaneously on day 0 with 10^5 PFU of the indicated virus in a 1 ml dose. Serum was collected daily for 10 days and on day 28.

[b] Virus titer in serum was determined by plaque assay in Vero cells. Mean peak virus titer of DENV-3-infected rhesus monkeys was significantly different from that of other groups as determined by Tukey-Kramer post-hoc test ($P < 0.05$).

[c] Plaque reduction (60%) neutralizing antibody titers were determined using DENV-3 (Sleman/78) virus on Vero cells.

[d] Monkeys were challenged after 35 days with DENV-3 (Sleman/78) administered subcutaneously in a 1 ml dose containing 10^5 PFU. Serum was collected daily for 10 days.

Table 4

Replication of rDENV-3 and rDEN3Δ30/31 in *Toxorynchites amboinensis*

Virus	Dose[a] (log_{10}PFU)	No tested	% infected[b]	Mean virus titer[c] (log_{10}PFU/head)	Reduction (log_{10}) compared to same dose of wt virus
rDENV-3 wt	2.3	20	90	4.2 ± 0.1[d]	
	1.3	19	53	4.2 ± 0.1[e]	
	0.3	17	18	4.3 ± 0.3	
rDEN3Δ30/31	2.3	12	83	2.7 ± 0.3[d]	1.5
	1.3	16	44	3.1 ± 0.3[e]	1.1
	0.3	8	13	3.6 ± 0.0	0.7

[a] Virus titer administered intrathoracically in a 0.2 ul inoculum.

[b] Percentage of mosquitoes with detectable virus at day 14 post-inoculation was determined by plaque assay on mosquito head homogenates in Vero cells.

[c] Calculated using only values of virus-positive heads.

[d] For $10^{2.3}$ PFU dose of rDENV-3 and rDEN3Δ30/31, mean virus titers were significantly different as determined by a Tukey-Kramer post-hoc test ($P < 0.001$).

[e] For $10^{1.3}$ PFU dose of rDENV-3 and rDEN3Δ30/31, mean virus titers were significantly different as determined by a Tukey-Kramer post-hoc test ($P < 0.005$).