GENETICALLY MODIFIED, LIVE ATTENUATED DENGUE VIRUS TYPE 3 VACCINE CANDIDATES

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Abstract. Three novel recombinant dengue type 3 (DEN3) virus vaccine candidates have been generated from a DEN3 virus isolated from a mild outbreak of dengue fever in the Sleman area of central Java in Indonesia in 1978. Antigenic chimeric viruses were prepared by replacing the membrane precursor and envelope (ME) proteins of recombinant DEN4 (rDEN4) virus with those from DEN3 Sleman/78 in the presence (rDEN3/4Δ30(ME)) and the absence (rDEN3/4(ME)) of the Δ30 mutation, a previously described 30-nucleotide deletion in the 3′ untranslated region. In addition, a full-length infectious cDNA clone was generated from the DEN3 isolate and used to produce rDEN3 virus and the vaccine candidate rDEN3Δ30. The chimeric viruses rDEN3/4(ME) and rDEN3/4Δ30(ME) appear to be acceptable vaccine candidates since they were restricted in replication in severe combined immune deficiency mice transplanted with human hepatoma cells, in rhesus monkeys, and in *Aedes* and *Toxorynchites* mosquitoes, and each was protective in rhesus monkeys against DEN3 virus challenge. The rDEN3/4(ME) and rDEN3/4Δ30(ME) viruses were comparable in all parameters evaluated, indicating that antigenic chimerization resulted in the observed high level of attenuation. Surprisingly, rDEN3Δ30 was not attenuated in any model tested when compared with wild-type rDEN3 and therefore, is not a vaccine candidate at present. Thus, the rDEN3/4(ME) and rDEN3/4Δ30(ME) antigenic chimeric viruses can be considered for evaluation in humans and for inclusion in a tetravalent dengue vaccine.

INTRODUCTION

The mosquito-borne dengue (DEN) viruses belong to the genus *Flavivirus* and contain a single-stranded positive-sense RNA genome.[1] A single polypeptide is co-translationally processed by viral and cellular proteases generating three structural proteins (C, M, and E) and at least seven non-structural proteins.[2] The genome organization of the DEN viruses is 5′-UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-UTR-3′ (UTR = untranslated region, C = capsid, prM = membrane precursor, E = envelope, NS = nonstructural). The DEN viruses (serotypes 1–4) cause disease ranging from a mild febrile illness to severe hemorrhagic fever and shock, and the disease burden associated with DEN virus infection continues to increase in tropical and semitropical countries inhabited by more than 2.5 billion people.[3] Annually, there are an estimated 50–100 million cases of dengue fever and 500,000 cases of the more severe and potentially lethal dengue hemorrhagic fever/shock syndrome (DHF/DSS).[4,5] Therefore, a vaccine effective against the four DEN virus serotypes is a public health priority, especially in many countries with very limited financial resources for health care.[6] A live attenuated virus vaccine is best suited to provide protection since it is economical to manufacture, and such vaccines can provide long-term immunity, with the live attenuated yellow fever virus vaccine serving as a successful model flavivirus vaccine.[7]

An effective live attenuated dengue virus vaccine must consist of a tetravalent formulation of components representing each serotype for two major reasons. First, multiple serotypes typically co-circulate in a region and the introduction of additional serotypes is common. Since each DEN virus serotype is capable of causing disease, prevention of disease requires protection against all four serotypes.[8,9] Second, the association of increased disease severity (DHF/DSS) in persons immune to one serotype undergoing an infection by a different serotype necessitates a vaccine that will confer long-term protection against all serotypes.[10–12] Development of a successful tetravalent vaccine has proven to be very complicated. Using

the conventional techniques of passage in tissue culture or mutagenesis, it has been difficult to develop monovalent vaccines against each of the four serotypes that exhibit a satisfactory balance between attenuation and immunogenicity.[13–15] In addition, it has been difficult to formulate a satisfactorily attenuated tetravalent vaccine that induces a broad neutralizing antibody response against each DEN virus serotype.[15–19]

We have previously described attenuated and immunogenic monovalent vaccine candidates for DEN1, DEN2, and DEN4 viruses that were generated by two distinct recombinant methodologies.[20–22] The first is the introduction of the Δ30 attenuating mutation into a wild-type DEN cDNA clone to generate a vaccine candidate. Specifically, the deletion of nucleotides 10478-10507 of the 3′-UTR (Δ30) of recombinant wild-type DEN4 virus yielded a vaccine candidate, rDEN4Δ30, which is safe, attenuated, and immunogenic in rhesus monkeys and humans.[20] Furthermore, incorporation of the Δ30 mutation into an infectious cDNA clone of DEN1 wild-type virus at a site homologous to that in DEN4 virus attenuated DEN1 virus for rhesus monkeys.[21] Using a second methodology, antigenic chimeric viruses have been generated by replacing M and E structural genes of DEN4 virus with those from DEN2 virus, and the resulting rDEN2/4 chimeric virus was attenuated and immunogenic in rhesus monkeys.[22] It was also possible to add the Δ30 mutation to the rDEN2/4 chimeric virus and maintain immunogenicity.[22] We have also described a set of point mutations that can attenuate wild-type rDEN4 virus for suckling mice,[23–25] for severe combined immune deficiency (SCID) mice transplanted with human liver cells (SCID-HuH-7),[26] for rhesus monkeys,[27] or for mosquitoes.[28] Since these mutations are in the non-structural gene regions of DEN4 virus, they can also be used to modify the attenuation phenotype of antigenic chimeric viruses with the DEN4 virus background. In addition, mutations identified in rDEN4 virus might be introduced into conserved sites of cDNA clones for other DEN viral serotypes in an attempt to transfer desired phenotypes. Thus, using the Δ30 mutation, the set of attenuating point mutations, and intertypic chimer-

ization, we have been able to generate attenuated DEN1, DEN2, and DEN4 virus vaccine candidates. To complete a set of four attenuated DEN virus monovalent components for a tetravalent DEN virus vaccine, a suitable DEN3 virus vaccine candidate is needed.

In the present study, a DEN3 wild-type virus (DEN3 Sleman/78) isolated in 1978 during a mild outbreak of dengue infection in the Sleman area of central Java in Indonesia was used as the parent to generate DEN3 virus vaccine candidates.[29] Studies by Gubler and others indicated that this DEN3 epidemic was characterized by lower viremia levels, milder illness, and less spread than previous DEN3 viral infections in the same area.[29] The possibility that the DEN3 Sleman/78 strain might represent a partially attenuated, naturally occurring variant of DEN3 virus made it an attractive virus to initiate the construction of live attenuated DEN3 virus vaccine candidates. Two antigenic chimeric viruses, rDEN3/4(ME) and rDEN3/4Δ30(ME), were constructed by replacing the ME structural proteins of rDEN4 virus (with and without the Δ30 mutation) with those from DEN3 Sleman/78. In addition, an infectious cDNA clone was generated from the DEN3 virus isolate and used to produce the first reported recombinant DEN3 virus and a third vaccine candidate, rDEN3Δ30. The three vaccine candidates, namely rDEN3/4(ME), rDEN3/4Δ30(ME), and rDEN3Δ30, were evaluated for replication in tissue culture, in SCID-HuH-7 mice, in rhesus monkeys, and in *Aedes* and *Toxorynchites* mosquitoes. Based on the results described here, a DEN3 virus vaccine candidate suitable for inclusion in a tetravalent vaccine formulation was identified.

MATERIALS AND METHODS

Cells and viruses. Vero cells (African green monkey kidney) were maintained in OptiPro SFM medium (Invitrogen, Carlsbad, CA) supplemented with 4 mM L-glutamine (Invitrogen). HuH-7 cells (human hepatoma)[30] were maintained in D-MEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and 0.05 mg/mL of 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).

The p4 cDNA clone (GenBank accession no. AY648301) derived from DEN4 virus strain 814669 (Dominica/81) was used to recover rDEN4 virus.[20,31] The rDEN4Δ30 virus was derived from plasmid p4Δ30, a cDNA clone containing a deletion of nucleotides 10,478-10,507 in the 3′ untranslated region (GenBank accession no. AY376438).[20,32]

Dengue virus type 3 biologic isolate, Sleman/78, was provided by Dr. Duane Gubler (Centers for Disease Control and Prevention, Fort Collins, CO). The virus was isolated during a 1978 dengue outbreak in the Sleman area of central Java in Indonesia.[29]

Sequence analysis. The genomic nucleotide sequence of the DEN3 Sleman/78 biologic isolate was determined on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using DEN3 virus-specific primers in BigDye terminator cycle sequencing reactions (Applied Biosystems). Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen,

Valencia, CA) from virus biologically cloned by three terminal dilutions in Vero cells. Reverse transcription was performed using random hexamer primers and the SuperScript First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen). Overlapping PCR fragments of approximately 2,000 basepairs were generated using DEN3 virus-specific primers and Advantage cDNA polymerase (ClonTech, Palo Alto, CA). Both strands of the resulting PCR fragments were sequenced directly using dye terminator reactions and the results were assembled into a consensus sequence. To determine the nucleotide sequence of the genomic 5′ and 3′ regions, the 5′ cap nucleoside of the viral genome was removed with tobacco acid pyrophosphatase (Epicentre Technologies, Madison, WI), followed by circularization of the genome using RNA ligase (Epicentre Technologies). An RT-PCR fragment spanning the ligation junction was generated and sequenced using DEN virus primers. For the DEN3 Sleman/78 consensus sequence, GenBank accession number AY648961 was assigned.

Genetic construction of chimeric DEN3 viruses. Chimeric viruses have been generated in which the ME genes of the p4 and p4Δ30 cDNA clones have been replaced with the corresponding genes derived from DEN3 Sleman/78. The genomic region within nucleotides 72-2334 was amplified by RT-PCR from the DEN3 virus Sleman/78 genome using DNA primers to introduce a translationally silent *Xho* I restriction enzyme recognition site at nucleotide 2336 (Figure 1A). The resulting fragment was cloned blunt-ended into vector pCR2.1 (Invitrogen) and site-directed mutagenesis was used to remove an unwanted *Xho* I site at nucleotide 1008 in a translationally silent manner. From the resulting vector, pCR3XRM, the *Bgl* II-*Xho* I fragment was cloned into a modified pUC118 vector containing the *Asc* I-*Bgl* II fragment (SP6 promoter and DEN4 5′-UTR) from plasmid p4 to generate plasmid pUC3Xinit (SP6 promoter, DEN4 virus 5′-UTR, and capsid-membrane-envelope [CME] region of DEN3 virus).

To generate ME chimeric cDNA plasmids, site-directed mutagenesis of pUC3Xinit was used to introduce a unique *Pst* I restriction enzyme recognition site at DEN3 virus nucleotide 400 immediately down stream of coding sequence for the trypsin cleavage site separating the C-protein from its anchor region. The *Asc* I-*Pst* I region of the resulting plasmid was replaced with that derived from p4 previously modified to contain a *Pst* I site in the same location. In the resulting plasmid, pUC-D3-ME, introduction of the *Pst* I cleavage site is translationally silent for the DEN4 virus portion of the chimeric molecule. However, DEN3 virus C protein amino acids 102-103 are changed from Ser-Leu to Ala-Val in the chimeric molecule (Figure 1B). Initially, ligation of the *Asc* I-*Xho* I fragment of pUC-D3-ME into p4 or p4Δ30 failed to yield genetically stable full-length cDNA plasmids. However, ligation in the presence of a synthetic DNA linker molecule containing termination codons in each of the forward and reverse coding frames and flanked by *Xho* I overhangs yielded the stable plasmids p4-D3L-ME and p4Δ30-D3L-ME. The nucleotide and amino acid sequences of the resulting junctions are shown in Figure 1B. The genomic region of each chimeric cDNA was sequenced as described in this report and GenBank accessions were assigned as follows (virus: accession numbers): p4-D3L-ME: AY656167 and p4Δ30-D3L-ME: AY656168.
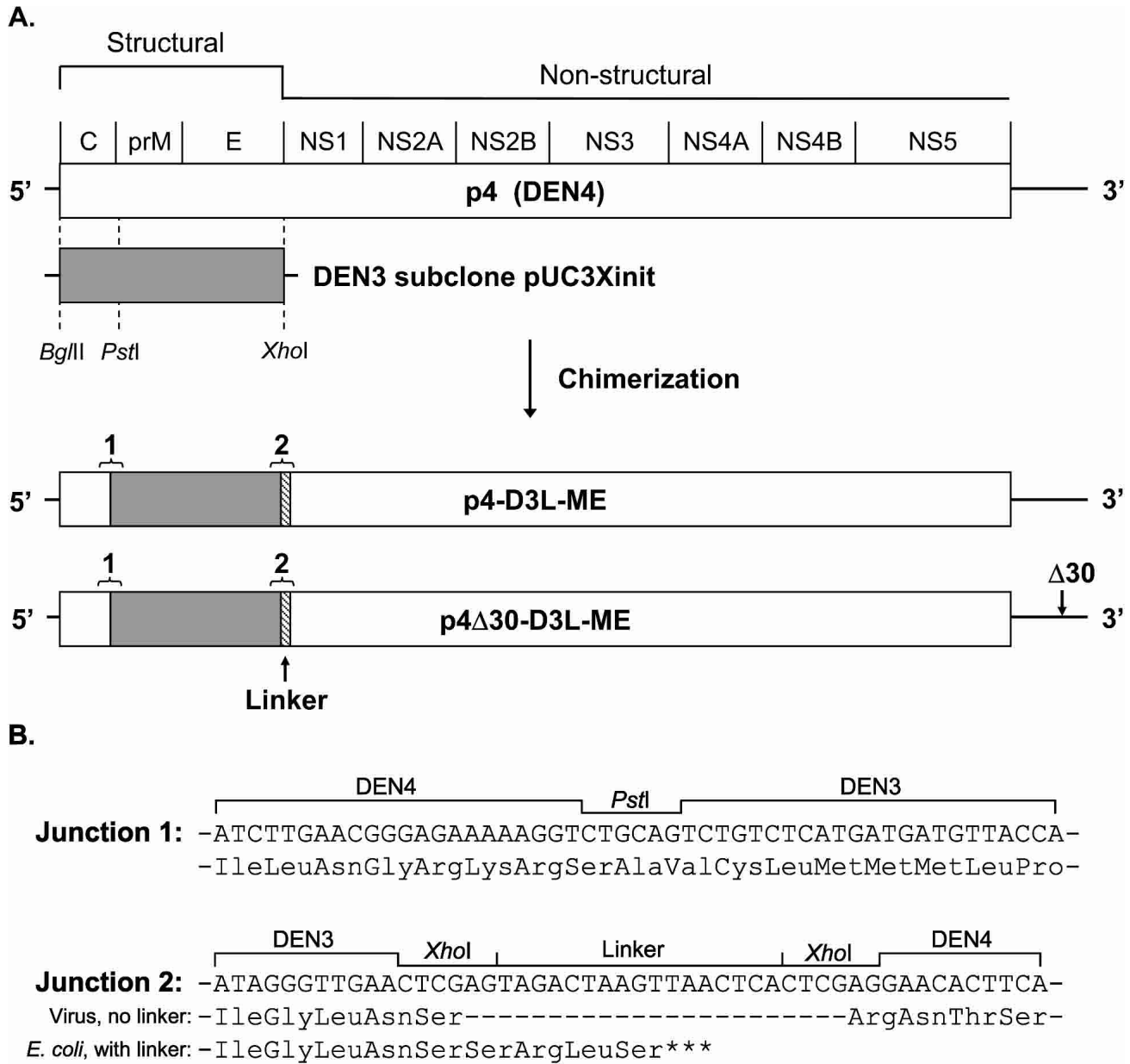
FIGURE 1. Molecular construction of the dengue type 3 (DEN3) virus chimeric cDNA plasmids. **A**, The membrane precursor and envelope (ME) structural protein coding region of the DEN4 cDNA plasmid p4 was replaced with the corresponding region from DEN3 (Sleman/78) capsid-membrane-envelope sub-clone pUC3Xinit. Restriction sites used to facilitate the construction are indicated. The virus genomic regions for each of the resulting full-length chimeric cDNA plasmids are shown and the presence of the Δ30 mutation is indicated in the 3′-untranslated region. The newly created cloning junctions are numbered 1–2 and the inserted linker sequence is indicated as a hatched box. The location of the linker is indicated. **B**, The nucleotide sequences surrounding the cloning junctions identified above are shown along with the predicted amino acid sequence corresponding to the virus polyprotein. For junction 2, the amino acid sequence is shown as predicted for the virus genome (with the linker sequence removed prior to transcription) and as predicted in *Escherichia coli* where cryptic expression of the virus polyprotein with the linker sequence intact would result in translational termination. The termination codon in the polyprotein open reading frame is indicated by * * *. Termination codons exist in the linker sequence for each of the additional forward and reverse open reading frames. C = capsid; prM = premembrane; NS = nonstructural.

Genetic construction of rDEN3 cDNA clone. The cDNA fragments of DEN3 Sleman/78 were generated by reverse transcription of the genome as indicated in Figure 2A. Each fragment (1L, 1R, 2, 3, 4, and 5) was subcloned into a plasmid vector and sequenced to verify that it matched the consensus sequence of the DEN3 Sleman/78 virus. This yielded six cloned cDNA fragments spanning the genome. Cloned fragments were modified as follows: Fragment 5, representing the

5′ end of the genome was abutted to the SP6 promoter preceded by an *Asc* I restriction endonuclease site; fragment 1L was modified to contain a translationally silent *Spe* I restriction endonuclease at genomic nucleotide 2345; fragment 1R was modified to contain a translationally silent *Spe* I restriction endonuclease site also at genomic nucleotide 2345, and to stabilize the eventual full-length clone, three additional translationally silent mutations at nucleotides 2354-2356, 2360-
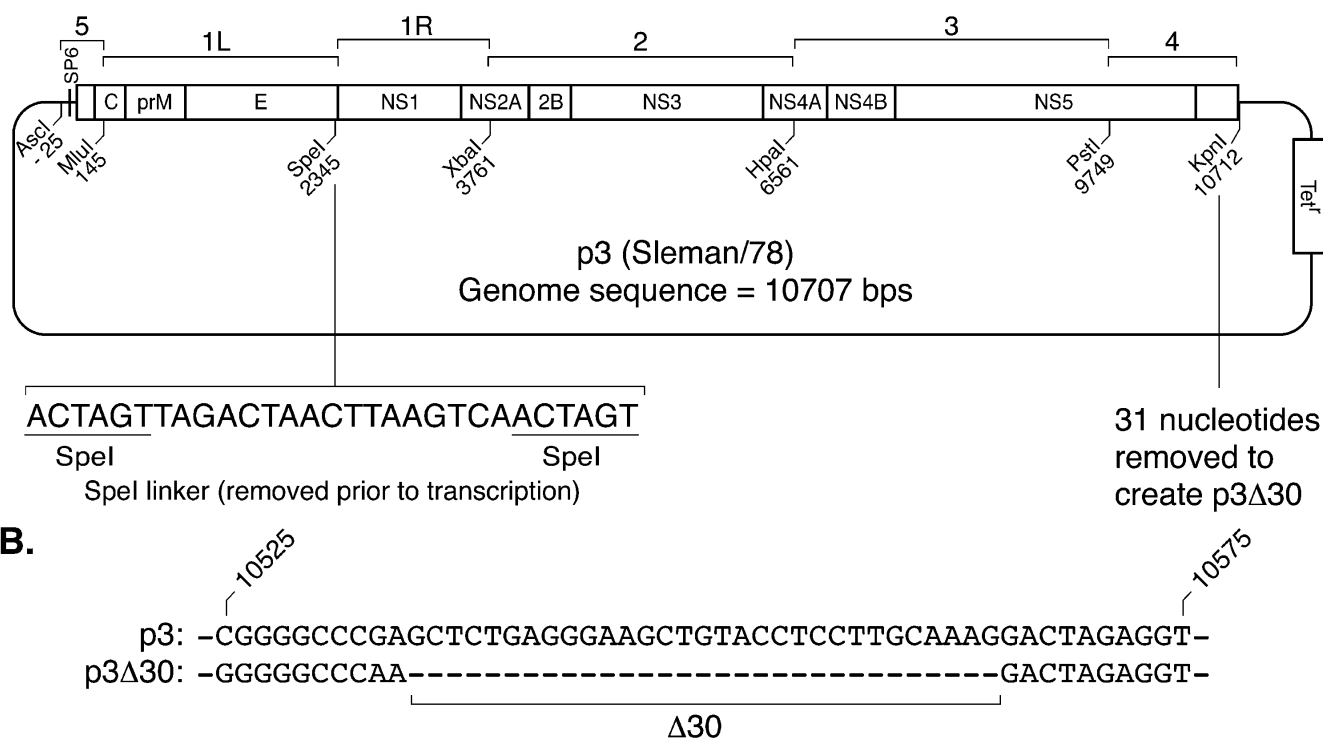
A. Subclone fragments:

ACTAGTTAGACTAACTTAAGTCAACTAGT
SpeI   SpeI
SpeI linker (removed prior to transcription)

31 nucleotides removed to create p3Δ30

B.

p3: –CGGGGCCCGAGCTCTGAGGGAAGCTGTACCTCCTTGCAAAGGACTAGAGGT–
p3Δ30: –GGGGGCCCAA––––––––––––––––––––––––––––––––GACTAGAGGT–

Δ30

FIGURE 2. Molecular construction of the dengue type 3 (DEN3) virus full-length cDNA plasmid p3. **A**, Diagram of the complete full-length DEN3 (Sleman/78) virus cDNA plasmid p3 is shown annotated with the restriction enzyme and corresponding cleavage site locations used to assemble the subcloned reverse transcriptase–polymerase chain reaction (RT-PCR) fragments. Restriction enzyme cleavage sites are numbered relative to nucleotide position in the virus genome. The corresponding genomic regions encoded by each subcloned RT-PCR fragment, 1L, 1R, 2, 3, 4, 5, are shown above the plasmid diagram. Relative positions of the SP6 promoter and tetracycline resistance gene (Tet^r) are indicated. The nucleotide sequence of the linker sequence inserted into the *Spe* I restriction site is shown. This linker sequence contains translational termination codons in each of the forward and reverse open reading frames and is necessary for stable replication of the plasmid in *Escherichia coli*. **B**, To generate plasmid p3Δ30, 31 nucleotides are removed from the 3′-untranslated region (UTR). The nucleotide sequence encompassing the Δ30 region is shown for the p3 parent cDNA and the resulting p3Δ30 cDNA. Nucleotide positions in the virus genome are indicated for p3. bps = basepairs. For definitions of other abbreviations, see Figure 1.

2362, and 2399 (see GenBank accession number AY656169 for specific sequences) were created to ensure that translation stop codons were present in all reading frames other than that used to synthesize the virus polyprotein; fragment 3 was modified at nucleotide 9007 to ablate a naturally occurring *Kpn* I restriction endonuclease site; and fragment 4, representing the 3′ end of the genome, was abutted to a *Kpn* I restriction endonuclease site. Fragment 2 was not modified.

Each fragment was added incrementally between the *Asc* I and *Kpn* I restriction endonuclease sites of the DEN4 virus cDNA clone p4 to generate a full-length DEN3 virus cDNA clone with the same vector background successfully used to generate rDEN4 virus. However, a stable, full-length clone could not be recovered in *Escherichia coli* when fragments 1L and 1R were combined into the same cDNA molecule. To overcome this instability, a synthetic DNA linker (Figure 2A) containing redundant termination codons in each of the forward and reverse open reading frames was introduced into the *Spe* I restriction endonuclease site at the same time that fragment 1L was added to complete the full-length cDNA construct. The resulting p3 clone containing the linker sequence was stable in *E. coli*, indicating that the linker sequence was sufficient to interrupt whatever deleterious element exists in this region. The cDNA clone p3 was sequenced and the virus genome region was found to match the DEN3

Sleman/78 virus consensus sequence, with the exception of the linker sequence and translationally silent modifications noted in this report. The Δ30 mutation (Figure 2B) was introduced into fragment 4 to generate fragment 4Δ30. To create p3Δ30, the fragment 4 region of p3 was replaced with Fragment 4Δ30 (Figure 2). The genomic region of each full-length cDNA was sequenced as described in this report and GenBank accessions were assigned as follows (cDNA clone accession numbers): p3: AY656169 and p3Δ30: AY656170).

Generation of recombinant dengue 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 as previously described.[26] Prior to transcription and generation of infectious virus, the linker sequences were removed from cDNA plasmids p4-D3L-ME and p4Δ30-D3L-ME by digestion with *Xho* I and removed from cDNA plasmids p3 and p3Δ30 by digestion with *Spe* I. Linker-less fragments were recircularized by ligation, linearized with *Acc* 65I (an isoschizomer of *Kpn* I that cleaves leaving only a single 3′ nucleotide), and transcribed *in vitro* using the AmpliCap SP6 Message Maker Kit (Epicentre Technologies). Purified transcripts were then transfected into Vero or C6/36 cells. Newly generated viruses were grown in Vero cells and biologically cloned by three rounds of terminal

dilution in Vero cells. Each virus used to infect rhesus monkeys was completely sequenced as described in this report to identify adventitious mutations that had accumulated during transfection and biologic cloning.

Replication in SCID-HuH-7 mice. The replication of viruses in SCID mice transplanted with HuH-7 cells (SCID-HuH-7 mice) has been described.[26] Four to six-week-old SCID mice (Tac:Icr:Ha[ICR]-*Prkdc*[scid]; Taconic, Germantown, NY) were injected intraperitoneally with 10^7 HuH-7 cells suspended in 0.2 mL of phosphate-buffered saline. Tumors were detected in the peritoneum, and mice were infected by direct inoculation into the tumor with 10^4 plaque-forming units (PFU) of virus in 0.05 mL of Opti-MEM (Invitrogen). On day 7 postinfection, serum was obtained from cardiac blood and stored at $-70°$C. Virus titer in serum samples was determined by plaque assay in Vero cells.[20]

Replication, immunogenicity, and efficacy in rhesus monkeys. The chimeric viruses and rDEN3 viruses were evaluated in rhesus macaques in two separate experiments using established methods.[20] Dengue virus seronegative monkeys were injected subcutaneously with 10^5 PFU virus diluted in L-15 medium (Invitrogen) or with a mock inoculum. Serum was collected on days 0–6, 8 and 28 after inoculation and stored at $-70°$C. Virus titer was determined for each day by plaque assay in Vero cells and serum neutralizing antibody titer was determined for days 0 and 28 by a plaque-reduction neutralization test.[20] On day 28, monkeys were challenged with 10^5 PFU of DEN3 Sleman/78, and serum was collected on days 29–34 and 56. Virus titer was determined for days 28–34 and serum neutralizing antibody titer was determined for day 56.

Replication in mosquitoes. Virus replication in *Ae. aegypti* and *Toxorynchites amboinensis* was evaluated as previously described.[33] Briefly, *Ae. aegypti* were administered blood meals containing serial 10-fold dilutions of virus. After 21 days, antigen was detected in head and midgut preparations by immunoflourescence assay using DEN3 or DEN4 virus-specific hyperimmune mouse ascitic fluid and fluorescein isothiocyanate conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories. Gaithersburg, MD). The 50% mosquito infectious dose (MID_{50}) was determined by the method of Reed and Muench.[34] *Toxorynchites amboinensis* were inoculated intrathoracically with a 0.22-μL dose containing serial 10-fold dilutions of virus and held for 14 days. Head preparations were made and antigen visualized as described in this report.

RESULTS

Recovery and sequence analysis of rDEN3/4(ME) and rDEN3/4(ME)Δ30 viruses. The first method used to generate an attenuated DEN3 virus vaccine candidate involved the construction of an antigenic chimeric virus bearing the ME gene region of DEN3 virus in a wild-type DEN4 virus background (Figure 1A). Since the E protein induces neutralizing antibodies and is the major protective antigen for dengue viruses, the rDEN3/4 antigenic chimeric viruses were designed to induce immunity to DEN3 virus. The attenuation of the chimeric virus would result from chimerization *per se*, a process that is known to attenuate antigenic chimeric flaviviruses *in vivo*.[22,35,36] A derivative of the rDEN3/4(ME) virus was constructed to contain the Δ30 mutation, which attenu-

ates DEN1 and DEN4 viruses for mice, monkeys, mosquitoes, or humans.[20,21]

Construction of a stable, full-length DEN3/4(ME) chimeric cDNA clone was possible in *E. coli* only after the inclusion of a short DNA linker molecule near the E-NS1 junction at the *Xho* I restriction site (Figure 1). Although the subclones pUC3Xinit and pUC-D3-ME, each containing the structural genes of DEN3 virus, and the full-length DEN4 plasmids p4 and p4Δ30 were stable in *E. coli*, recombinant DEN3/4(ME) chimeric plasmid derivatives constructed using the *Xho* I restriction site were uniformly unstable. Most often, the resulting plasmids contained large deletions encompassing the E and NS1 regions. Alternatively, a translational stop codon was detected in the polyprotein open reading frame (ORF) at codon 147 of NS1. At this same time, efforts to construct DEN1 full-length chimeric plasmids using similar methods were also confounded by genetic instability, and a 4,000-basepair insertion derived from the *E. coli* genome was detected at the *Xho* I cloning site of one of the resulting plasmids. Taken together, these observations lead to the hypothesis that a gene product deleterious for growth in *E. coli* was being synthesized after the DEN3 virus structural genes were chimerized with the DEN4 virus nonstructural genes and that only plasmids with modifications in this region were stable in *E. coli*. Therefore, an *Xho* I linker sequence was designed to contain termination codons in each of the forward and reverse coding frames (Figure 1). Ligation of the *Asc* I-*Xho* I fragment containing the DEN3 virus structural genes into p4 and p4Δ30 in the presence of this *Xho* I linker interrupted each ORF in this region and consistently yielded genetically stable full-length chimeric plasmids. Following removal of the linker by *Xho* I digestion, recircularization of the cDNA, and linearization at the 3′ end of the genomic region, infectious RNA transcripts were synthesized *in vitro* from the SP6 promoter.

The chimeric viruses could be recovered from transfected RNA in C6/36 cells but not in Vero cells. However, the recovered viruses adapted to replicate efficiently in Vero cells and were terminally diluted in this cell line. The rDEN3/4(ME) virus reached a titer of $10^{6.8}$ PFU/mL in Vero cells, while rDEN3/4Δ30(ME) replicated to $10^{6.4}$ PFU/mL and had a smaller plaque size. The rDEN3/4(ME) and rDEN3/4Δ30(ME) chimeric viruses that were biologically cloned in Vero cells accumulated incidental mutations, some of which were previously identified in DEN4 virus NS4B and were associated with enhanced replication of rDEN4 virus in Vero cells (Table 1).[24,37]

Recovery and sequence analysis of rDEN3Δ30 virus. In a second approach to generating a DEN3 virus vaccine candidate, a full-length cDNA clone for the DEN3 Sleman/78 virus was generated with or without the Δ30 mutation in the 3′-UTR (Figure 2). As observed for the chimeric cDNAs, amplification of the plasmid containing the full-length DEN3 virus cDNA in *E. coli* was problematic. To produce a stable cDNA clone, an *Spe* I linker containing redundant termination codons was incorporated between the 1L and 1R fragments (Figure 2). The rDEN3 virus could be recovered in Vero and C6/36 cells following RNA transfection. However, the addition of the Δ30 mutation to rDEN3 virus yielded the rDEN3Δ30 virus vaccine candidate that could only be recovered in C6/36 cells. After passage in Vero cells, rDEN3 and rDEN3Δ30 viruses both accumulated a single nucleotide

TABLE 1

Incidental missense mutations identified in the chimeric rDEN3/4 and rDEN3 viruses that were biologically cloned and passaged in Vero cells*

Virus	Recovery cell type	Gene	Nucleotide position	Nucleotide substitution	Amino acid position	Amino acid change
rDEN3/4(ME)	C6/36	M	460	A > G	7	Asp > Gly
		NS4B	7177†‡	G > U	119	Gly > Val
		NS5	7702	C > U	49	Ser > Phe
rDEN3/4Δ30(ME)	C6/36	E	1432	A > U	165	Gln > Leu
		NS4B	7156‡§	U > C	112	Leu > Ser
		NS5	8692	A > C	379	Asn > His
rDEN3	Vero	NS4B	7164¶	U > C	115	Val > Ala
rDEN3Δ30	Vero	NS4B	7164¶	U > C	115	Val > Ala

* rDen = recombinant dengue; M = membrane; NS = nonstructural; E = envelope.
† Same nucleotide position as 7183 in rDEN4 virus in same codon as 7182 mutation identified as Gly > Ser change.[37]
‡ Previously identified as a Vero cell adaptation mutation in rDEN4 virus.[37]
§ Same mutation identified in rDEN4 virus at nucleotide position 7162. Amino acid change: Leu > Ser.[37]
¶ Nucleotide position 7164 in rDEN3 virus corresponds to nucleotide 7162 in rDEN4 virus, but wild-type amino acids are Val and Leu, respectively.[37]

change in NS4B at nucleotide 7164 specifying an amino acid substitution that had been previously reported to occur at the homologous site following passage of DEN4 virus in Vero cells (Table 1).[37] Inclusion of the Vero cell adaptation mutation at position 7164 in the rDEN3Δ30 virus cDNA clone permitted direct recovery of the virus in Vero cells, and the rDEN3Δ30 virus replicated to a mean virus titer of 10^7 PFU/mL in Vero cells. Thus, three DEN3 vaccine candidates, rDEN3Δ30 and the rDEN3/4(ME) and rDEN3/4Δ30(ME) antigenic chimeric viruses, were generated and next evaluated in SCID-HuH-7 mice, rhesus monkeys, and mosquitoes.

Replication of rDEN3 viruses in SCID-HuH-7 mice. The SCID mice transplanted with HuH-7 human hepatoma cells (SCID-HuH-7 mice) were used to investigate the growth properties of the chimeric rDEN3 vaccine candidate viruses. Previously, an *att* phenotype in SCID-HuH-7 mice has been shown to be a predictor of reduced replication in rhesus monkeys.[21,22] Wild-type DEN3 Sleman/78 virus, similar to wild-type DEN1, DEN2, and DEN4 viruses, replicated efficiently in SCID-HuH-7 mice and reached a mean titer in serum of greater than 6.0 $\log_{10}$ PFU/mL (Table 2).[21,22,26] Chimerization of the DEN3 ME virus genes with rDEN4 virus had a significant attenuating effect on replication, with the rDEN3/4(ME) virus being approximately 100-fold restricted when compared with the replication of either the DEN3 or DEN4 parent viruses. Introduction of the Δ30 mutation into rDEN3/4(ME) virus did not appear to confer further attenuation. Thus, the major determinant of attenuation of the two versions of the chimeric viruses in SCID-HuH-7 mice was chimerization and not the introduction of the Δ30 mutation.

TABLE 2

Effect of chimerization between rDEN3 and rDEN4 viruses on attenuation in SCID-HuH-7 mice*

Virus†	No. of mice	Mean peak virus titer ($\log_{10}$ PFU/mL ± SE)	Statistical group‡
rDEN4	40	6.4 ± 0.1	A
DEN3-Sleman/78	31	6.4 ± 0.2	A
rDEN3/4(ME)	6	4.2 ± 0.8	B
rDEN3/4Δ30(ME)	7	4.7 ± 0.4	B
rDEN3	9	6.1 ± 0.2	–
rDEN3Δ30	8	5.8 ± 0.3	–

* rDEN = recombinant dengue; PFU = plaque-forming units; M = membrane; E = envelope.
† Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10^4 PFU of virus. Serum was collected on day 7 and titered in Vero cells.
‡ Mean peak titers were assigned to statistical groups using the Tukey post-hoc test ($P < 0.05$). Groups with a different letter designation are significantly different.

The rDEN3 and rDEN3Δ30 viruses were also evaluated in SCID-HuH-7 mice (Table 2). Both viruses achieved a titer of approximately 6.0 $\log_{10}$ PFU/mL in serum. Thus, the Δ30 mutation did not attenuate DEN3 wild-type virus for SCID-HuH-7 mice whereas this mutation readily attenuated both rDEN1 and rDEN4 viruses for these mice.[21,26]

Replication, immunogenicity, and protective efficacy of rDEN3 viruses in rhesus monkeys. The replication (viremia), immunogenicity, and protective efficacy of the rDEN3 vaccine candidates and wild-type viruses in monkeys were studied. Monkeys inoculated with wild-type DEN3 Sleman/78 virus were viremic for 2.3 days with a mean peak titer of 1.8 $\log_{10}$ PFU/mL (Table 3). Neither rDEN3/4(ME) nor rDEN3/4Δ30(ME) virus produced detectable viremia in any monkey, but all monkeys seroconverted with a geometric mean neutralizing antibody titer of 1:70 and 1:58, respectively. The level of neutralizing antibodies induced by the chimeric virus vaccine candidates was approximately 10-fold less than that induced by DEN3 wild-type virus, a finding consistent with the decreased replication exhibited by the DEN3/4 chimeric viruses. Thus, chimerization attenuated the rDEN3/4(ME) viruses for monkeys as it did for the SCID-HuH-7 mice. However, an independent attenuating effect of the Δ30 mutation could not be identified in monkeys. When vaccinated monkeys were challenged with DEN3 Sleman/78 virus, all monkeys were protected, as indicated by the lack of viremia (Table 3).

In contrast to the chimeric viruses, rDEN3Δ30 virus does not appear to be attenuated in rhesus monkeys (Table 4). There was no difference in the mean number of viremic days or mean peak virus titer between rDEN3 virus (2.3 days; 1.4 $\log_{10}$ PFU/mL) and rDEN3Δ30 virus (2.0 days; 1.5 $\log_{10}$ PFU/mL). All monkeys inoculated with rDEN3Δ30 virus seroconverted with neutralizing antibody titers equivalent to rDEN3 virus and, not surprisingly, all monkeys were protected upon challenge with DEN3 Sleman/78 virus. The mean neutralizing antibody titers for rDEN3Δ30 virus were approximately five-fold greater than the more attenuated rDEN3/4(ME) and rDEN3/4Δ30(ME) chimeric viruses. Thus, the Δ30 mutation failed to attenuate DEN3 virus for both SCID-HuH-7 mice and monkeys.

Replication of rDEN3 viruses in mosquitoes. The rDEN3/4(ME) chimeric viruses were compared with the parent rDEN4 and DEN3 Sleman/78 viruses for infectivity for *Ae. aegypti* fed on an infectious blood meal and for *T. amboinensis* inoculated intrathoracically (Table 5). The DEN3 Sle-

TABLE 3
Effect of chimerization between rDEN3 and rDEN4 viruses on attenuation in rhesus monkeys*

Virus†	No. of monkeys	% of Monkeys with viremia	Mean no. of viremic days per monkey‡	Mean peak virus titer ($\log_{10}$ PFU/mL ± SE)§	Geometric mean serum neutralizing antibody titer (reciprocal dilution)¶			Virus replication after challenge#	
					Day 0	Day 28	% With four-fold increase	% With viremia	Mean peak virus titer ($\log_{10}$ PFU/mL ± SE)§
Mock	2	0	0	< 0.7	< 5	< 5	0	100	2.5 ± 0.4
rDEN4**	4	100	3.8	1.5 ± 0.2	< 10	446	100	ND	ND
DEN3-Sleman/78	4	100	2.3	1.8 ± 0.1	< 5	707	100	0	< 1.0
rDEN3/4(ME)	4	0	0	< 0.7	< 5	70	100	0	< 1.0
rDEN3/4Δ30(ME)	4	0	0	< 0.7	< 5	58	100	0	< 1.0

* rDEN = recombinant dengue; PFU = plaque-forming units; M = membrane; E = envelope.
† Groups of rhesus monkeys were inoculated subcutaneously with 10^5 PFU of the indicated virus in a 1-ml dose. Serum was collected daily on days 0–6 and on days 8, 10, and 28.
‡ Viremia was not detected in any monkey after day 5.
§ Virus titer in serum was determined by plaque assay in Vero cells. The lower limit of detection was 0.7 $\log_{10}$ PFU/mL.
¶ Plaque reduction (60%) neutralizing antibody titers were determined using DEN3-Sleman/78 virus.
# Vaccinated rhesus monkeys were inoculated subcutaneously with 10^5 PFU of DEN3 virus in a 1-ml dose. Serum was collected daily on days 0–8. ND = not done (monkeys not challenged).
** Historical data as reported by Durbin and others[20] and included here for comparison.

man/78 wild-type virus was found to be less infectious than wild-type rDEN4 virus for the midgut of *Ae. aegypti* ($P < 0.001$, by Fisher's exact test). The infectivity of rDEN3/4(ME) and rDEN3/4Δ30(ME) viruses for the midgut of *Ae. aegypti* was similar to that of their DEN3 virus parent. Although neither chimerization nor the Δ30 mutation had a discernable effect on infectivity of the rDEN3/4(ME) virus chimeras for the midgut of *Ae. aegypti*, chimerization affected the ability of the virus to spread from the midgut to the head.

The percentage of rDEN4 and DEN3 virus disseminated infections (head infections in mosquitoes with virus present in the midgut) was 43% and 50%, respectively, at the highest dose tested (Table 5 and Figure 3). However, disseminated infection was not observed at the highest dose tested for rDEN3/4(ME) virus (0/13 midgut infections spreading to the head) or for rDEN3/4Δ30(ME) virus (0/2). The number of observed rDEN3/4(ME) virus disseminated infections (zero) is significantly lower than the number expected (six) when compared with rDEN4 virus ($n = 13$; $P = 0.004$, by chi-square test). Thus, chimerization between DEN3 and DEN4 viruses lead to a decrease in dissemination.

This defect in dissemination was further investigated by directly inoculating virus intrathoracically into *T. amboinensis* and measuring the ability of the virus to infect the head tissues. If the block in dissemination resulted solely from an inability of the chimeric virus to overcome the midgut escape barrier, the virus would readily infect the head of intrathoracically inoculated *T. amboinensis*. Conversely, loss of infectivity for the head of *T. amboinensis* inoculated by the intrathoracic route would identify a defect in the ability of the

chimeric virus to replicate in tissues outside of the midgut. Both rDEN3/4(ME) and rDEN3/4Δ30(ME) viruses were found to be significantly less infectious for *T. amboinensis* when compared with either rDEN4 or DEN3 virus (Table 5). The MID_{50} of rDEN4 and DEN3 viruses were < 0.6 and < 0.3 PFU, respectively, whereas each chimeric virus was at least 100-fold less infectious (MID_{50} = 70 or 76 PFU). Thus, the inability of the chimeric viruses to spread to the head of the orally fed mosquitoes at least partially reflects a decreased ability of the virus to replicate in the tissues of the mosquito beyond the midgut.

The rDEN3 and rDEN3Δ30 viruses were also compared for infectivity in both mosquito models to determine the effect of the Δ30 mutation upon replication. As observed in the SCID-HuH-7 mice and rhesus monkeys, rDEN3Δ30 virus did not demonstrate decreased replication compared with rDEN3 virus in *Ae. aegypti* or *T. amboinensis*.

DISCUSSION

Efforts to develop a DEN3 component for a live attenuated tetravalent DEN virus vaccine have proven challenging and exemplify the difficulty of DEN vaccine development.[14–16] In the Mahidol University/Aventis Pasteur vaccine, the DEN3 component was the most reactogenic of the four serotypes when evaluated as a monovalent vaccine and was the dominant component in a tetravalent vaccine since it achieved higher levels of viremia and induced more neutralizing antibody than any of the other three components. The DEN3 virus strain attained the highest levels of viremia and induced

TABLE 4
Effect of the addition of the Δ30 deletion mutation to rDEN3 virus on attenuation for rhesus monkeys*

Virus†	No. of Monkeys	% of monkeys with viremia	Mean no. of viremic days per monkey‡	Mean peak virus titer ($\log_{10}$ PFU/mL ± SE)§	Geometric mean serum neutralizing antibody titer (reciprocal dilution)¶			Virus replication after challenge#	
					Day 0	Day 28	% With four-fold increase	% With viremia	Mean peak virus titer ($\log_{10}$ PFU/mL ± SE)§
Mock	2	0	0	< 1.0	< 10	< 10	0	100	2.6 ± 0.1
DEN3-Sleman/78	4	75	2.5	2.0 ± 0.4	< 10	325	100	0	< 1.0
rDEN3	4	100	2.3	1.4 ± 0.2	< 10	363	100	0	< 1.0
rDEN3 Δ30	4	100	2.0	1.5 ± 0.2	< 10	265	100	0	< 1.0

* rDEN = recombinant dengue; PFU = plaque-forming units.
† Groups of rhesus monkeys were inoculated subcutaneously with 10^5 PFU of the indicated virus in a 1-ml dose. Serum was collected daily on days 0–6 and on days 8, 10, and 28.
‡ Viremia was not detected in any monkey after day 6.
§ Virus titer in serum was determined by plaque assay in Vero cells. The lower limit of detection was 1.0 $\log_{10}$ PFU/mL.
¶ Plaque reduction (60%) neutralizing antibody titers were determined using DEN3-Sleman/78 virus.
# Vaccinated rhesus monkeys were inoculated subcutaneously with 10^5 PFU of DEN3 virus in a 1-ml dose. Serum was collected daily on days 0–8.

TABLE 5
Effect of chimerization between rDEN3 and rDEN4 viruses on infectivity for *Aedes aegypti* and *Toxorynchites amboinensis**

	Ae. aegypti (oral infection)				*T. amboinensis* (intrathoracic inoculation)		
			No. infected (%)‡				
Virus	Dose (log$_{10}$ PFU)†	No. tested	Midgut	Head	Dose (log$_{10}$ PFU)§	No. tested	% Infected¶
rDEN4	3.8	38	28 (74)	12 (32)	1.8	6	100
	2.8	15	4 (26)	1 (6)	0.8	7	71
	1.8	20	2 (10)	1 (5)	−0.2	5	60
							MID$_{50}$ < 0.6
DEN3	4.1	28	4 (14)	2 (7)	1.6	6	100
	3.1	10	2 (20)	0 (0)	0.6	6	100
					−0.5	6	100
							MID$_{50}$ < 0.3
rDEN3/4(ME)	3.8	40	13 (33)	0 (0)	1.8	8	50
	2.8	12	0 (0)	0 (0)	0.8	5	40
					−0.2	5	0
							MID$_{50}$ = 70
rDEN3/4Δ30(ME)	3.5	20	2 (10)	0 (0)	2.0	5	60
	2.5	9	1 (11)	0 (0)	1.0	5	40
					0.1	5	20
							MID$_{50}$ = 76

* rDEN = recombinant dengue; PFU = plaque-forming units; MID$_{50}$ = 50% mosquito infectious dose; M = membrane; E = envelope.
† Virus titer ingested, assuming a 2-μL blood meal.
‡ Number and percentage of mosquitoes with antigen detectable by immunofluorescence assay (IFA) in midgut or head tissue prepared 21 days after oral infection.
§ Amount of virus present in a 0.22-μL inoculum.
¶ Percentage of mosquitoes with antigen detectable by IFA in head tissue prepared 14 days post-inoculation.

the highest level of antibody even when tested at a dose of 1 log$_{10}$ 50% tissue culture infectious doses, 100-fold lower than that of the DEN1, DEN2, or DEN4 virus components.[38] However, decreasing the DEN3 virus component dose did appear to lower the reactogenicity of the tetravalent vaccine.[16] For the DEN3 virus candidates developed at Walter Reed Army Institute of Research, DEN3 virus strain CH53489 passaged 10 times in primary dog kidney cells (PDK-10) was immunogenic, yet too reactogenic for further use in humans. At passage level PDK-20, it was found to be acceptably attenuated, but seroconversion rates to this strain in monovalent or tetravalent formulations are only approximately 50% after a single dose.[14,19,39]
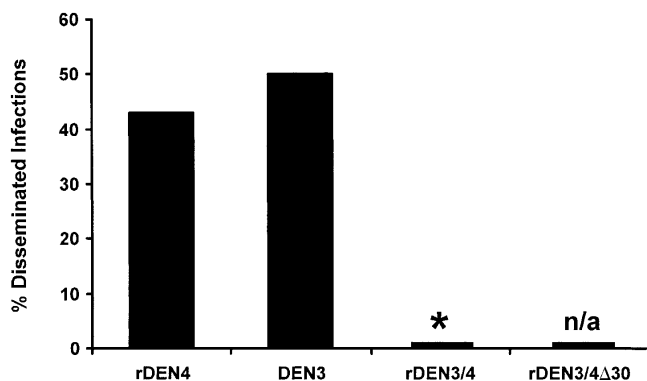

FIGURE 3. Decreased dissemination of recombinant dengue type 3 rDEN3/4(ME) virus and rDEN3/4Δ30(ME) virus in *Aedes aegypti*. The percentage of midgut infections that progressed to head infections is presented for the highest dose tested for each virus. The asterisk indicates that the number of observed rDEN3/4(ME) disseminated infections (zero) is significantly different than the number expected (6) when compared with rDEN4 (n = 13; P = 0.004, by a one-group chi-square test). n/a indicates that the number of expected rDEN3/4Δ30(ME) disseminated infections (1) was too few to analyze. M = membrane; E = envelope.

The antigenic chimeric DEN3/4 viruses described in the present study have many properties desirable for a DEN3 virus vaccine candidate, and may serve as an alternative DEN3 virus vaccine candidate. Chimerization of the ME genes of the DEN3 Sleman/78 virus with rDEN4 virus resulted in a virus that was 100-fold restricted in replication in SCID-HuH-7 mice (Table 2) compared with either parent virus. The rDEN3/4(ME) and rDEN3/4Δ30(ME) virus vaccine candidates were also both attenuated in rhesus monkeys. Whereas wild-type DEN3 and DEN4 parent viruses were readily recovered from the blood of monkeys, there was no detectable viremia in monkeys infected with the chimeras. Monkeys infected with the chimeras developed a geometric mean titer of serum neutralizing antibody of approximately 1:70 (Table 3), a level approximately 10-fold lower than that of monkeys infected with wild-type virus. The lower level of serum neutralizing antibody response of monkeys infected with the rDEN3/4 chimeric viruses compared with that of monkeys infected with wild-type virus is consistent with their decreased replication. Decreased immunogenicity of dengue virus and other flavivirus vaccines in monkeys is commonly observed.[18,20,40] Despite the restricted replication and decreased serum antibody response, monkeys immunized with either rDEN3/4 virus vaccine candidate were resistant to replication of DEN3 challenge virus.

The phenotypes of reduced replication in both SCID-HuH-7 mice and monkeys of the DEN3/4 virus is shared with that of similarly constructed rDEN2/4 chimeric viruses.[22] However, introduction of the Δ30 mutation into either the rDEN3/4(ME) or the rDEN2/4(ME) chimeric viruses did not further attenuate the chimeras for SCID-HuH-7 mice or monkeys.[22] These findings indicate that chimerization of DEN4 virus with either DEN2 or DEN3 virus was the primary determinant of attenuation and that this attenuation was not discernibly augmented by the addition of the Δ30 mutation. However, the presence of the Δ30 mutation, which is known to attenuate the DEN4 virus and which is in the DEN4 component of the

chimeric viruses, could potentially serve to enhance the phenotypic stability of the rDEN3/4Δ30(ME) virus vaccine candidate.

The rDEN3/4(ME) and rDEN3/4Δ30(ME) chimeric viruses were also restricted in their replication in mosquitoes, specifically in their ability to disseminate from the midgut to the head of orally infected mosquitoes. Since both viruses were at least 100-fold less infectious than their wild-type parent viruses following intrathoracic inoculation of *Toxorynchites*, the restriction in dissemination was partially ascribed to a decreased ability to initiate an infection in tissues outside the midgut. The phenotypes of restricted dissemination and decreased infectivity of the DEN3/4 chimeric viruses following intrathoracic inoculation of *Toxorynchites* is shared with that of similarly constructed rDEN2/4 chimeric viruses.[22] For both DEN3/4 and DEN2/4 chimeric viruses, the addition of the Δ30 mutation did not modify the infectivity of the virus for orally fed or intrathoracically inoculated mosquitoes. This observation indicates that chimerization was the major factor responsible for the restricted dissemination of the virus in mosquitoes.

There is very little information regarding the generation of DEN3 intertypic chimeric viruses and the effect this chimerization has on the biologic properties of the virus. Previously, the feasibility of generating a chimeric virus in which the CME genes of DEN4 were replaced by those from a DEN3 parent virus was established, but the biologic behavior of the chimeric virus *in vivo* was not studied in detail.[41] A second DEN3 virus chimera in which the ME genes of DEN2 virus were replaced with those from DEN3 virus exhibited decreased mouse neurovirulence compared with that of its DEN3 virus parent.[42] The residual neurovirulence of this DEN3/2 chimeric virus was eliminated by the addition of attenuating mutations into its NS genes.[42] Evaluation of these chimeric viruses in non-human primates has not been reported, but the attenuation phenotype in mice suggests that similar to the DEN3/4 chimeric viruses described here, they might also be attenuated.

In a second approach to create a DEN3 virus vaccine candidate, we generated an infectious cDNA clone of DEN3 virus and introduced the Δ30 mutation into its 3′-UTR in a region homologous to its location in DEN4Δ30 virus. The Sleman/78 DEN3 virus was chosen as parent for generating DEN3 virus vaccine candidates since the DEN3 epidemic from which this virus was isolated in Sleman in central Java was characterized by lower viremia levels, milder illness, and weaker spread than previous DEN3 virus infections in neighboring Bantul in central Java.[29] In the past, naturally attenuated viruses have been used as live virus vaccines.[43] The poliovirus type 2 component in the trivalent Sabin live-attenuated vaccine was selected because it was found to be highly attenuated in primate brain and spinal cord prior to any attempt to attenuate it.[44] In the DEN3 epidemic in Sleman, virus isolation rates from patients with primary or secondary DEN3 virus infection were at least two-fold lower than that from Bantul. In addition, mean levels of virus detected by mosquito inoculation of serum from Sleman patients was 100-fold lower than those reported from Bantul. Severity of illness was also reduced in the Sleman epidemic. Thus, the clinical and virologic evidence suggested that the Sleman epidemic was caused by a partially attenuated variant of DEN3 virus. The less virulent nature of the Sleman/78 virus isolate suggested it would be a safe parent virus to use and would be easier to attenuate than more virulent DEN3 virus strains. However, it is clear that the Sleman/78 virus isolate, in contrast to the Sabin poliovirus type 2 vaccine strain, would require further attenuation for use as a vaccine. In the present study, we found that the introduction of the Δ30 mutation into the DEN3 Sleman/78 virus failed to further attenuate its replication in SCID-HuH-7 mice, monkeys, or mosquitoes. This finding was both surprising and disappointing based on the previous results obtained with rDEN4Δ30 and rDEN1Δ30 viruses, which are both clearly attenuated in mice, rhesus monkeys, or mosquitoes.[20,21] The Δ30 mutation, generated first in DEN4 virus, removes a stem-loop structure (TL2) from the 3′-UTR predicted secondary structure.[32,45] Although there is significant sequence heterogeneity in this region among the four serotypes, the base-pairing indicated in the predicted secondary structure preserves the stem-loop for each of the four serotypes and this region has been implicated in virus pathogenicity.[21,46] Inspection of the TL2 stem-loop structure in the 3′-UTR of DEN3 Sleman/78 virus does not provide a clue as to why the Δ30 mutation did not confer an attenuation phenotype on the Sleman/78 virus. Despite the fact that sufficient replication of the DEN3 Sleman/78 parent virus was seen in monkeys to enable measurement of attenuation due to the introduction of the Δ30 mutation, attenuation was not observed. We therefore favor the interpretation that the specific configuration of the DEN3 virus 3′-UTR tolerates the introduction of the Δ30 mutation. In each of the four DEN virus serotypes, the TL2 structure is present in addition to a reciprocal stem loop structure (TL1) similar in size and context. The findings from the present study suggest that DEN3 virus, in contrast to DEN1 and DEN4 viruses, can function well with the presence of only a single TL structure. This hypothesis is currently being experimentally examined.

The most promising vaccine candidate from the present study would appear to be rDEN3/4Δ30(ME) virus, based on the greater than 10-fold reduced replication in SCID-HuH-7 mice, lack of viremia in rhesus monkeys, satisfactory immunogenicity and efficacy, and reduced infectivity for *Aedes* and *Toxorynchites* mosquitoes. The rDEN3/4Δ30(ME) virus vaccine candidate effectively has two genetically stable attenuating factors, the Δ30 deletion and the attenuating effect of chimerization. Furthermore, the rDEN3/4Δ30(ME) virus vaccine candidate would be the preferred vaccine candidate to use in a tetravalent vaccine since the presence of the Δ30 mutation in each component of the tetravalent vaccine would preclude the generation of a wild-type DEN4 virus by recombination.[47] Currently, this virus is being prepared as a clinical lot for evaluation in humans. Unlike rDEN4Δ30 and rDEN1Δ30 viruses, which are being evaluated in humans, the rDEN3Δ30 virus did not possess any indicators of attenuation as measured here.[20,21] Therefore, despite the potential partial, natural attenuation of the DEN3 Sleman/78 virus strain, the rDEN3Δ30 vaccine candidate will need to be further attenuated by addition of mutations before this virus can be evaluated in humans, and such efforts are ongoing.

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