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Construction of Infectious cDNA Clones for Dengue 2 Virus: Strain 16681

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We identified nine nucleotide differences between the genomes of dengue-2 (DEN-2) 16681 virus and its vaccine derivative, strain PDK-53. These included a C-to-T (16681-to-PDK-53) mutation at nucleotide position 57 of the 5′-untranslated region, three silent mutations, and substitutions prM-29 Asp to Val, NS1-53 Gly to Asp, NS2A-181 Leu to Phe, NS3-250 Glu to Val, and NS4A-75 Gly to Ala. Unpassaged PDK-53 vaccine contained two genetic variants as a result of partial mutation at NS3-250. We constructed infectious cDNA clones for 16681 virus and each of the two PDK-53 variants. DEN-2 16681 clone-derived viruses were identical to the 16681 virus in plaque size and replication in LLC-MK_2 cells, replication in C6/36 cells, E and prM epitopes, and neurovirulence for suckling mice. PDK-53 virus and both clone-derived PDK-53 variants were attenuated in mice. However, the variant containing NS3-250-Glu was less temperature sensitive and replicated better in C6/36 cells than did PDK-53 virus. The variant containing NS3-250-Val had smaller, more diffuse plaques, decreased replication, and increased temperature sensitivity in LLC-MK_2 cells relative to PDK-53 virus. Both PDK-53 virus and the NS3-250-Val variant replicated poorly in C6/36 cells relative to 16681 virus. Unpassaged PDK-53 vaccine virus and the virus passaged once in LLC-MK_2 cells had genomes of identical sequence, including the mixed NS3-250-Glu/Val locus. Although the NS3-250-Val mutation clearly affected virus replication *in vitro,* it was not a major determinant of attenuation for PDK-53 virus in suckling mice.

INTRODUCTION

Dengue (DEN) viruses are mosquito-borne pathogens of the Flavivirus genus, family *Flaviviridae.* Flaviviruses are enveloped RNA viruses 45–50 nm in diameter that contain a single-stranded, positive-sense, capped RNA genome of approximately 11 kb. The gene organization is 5′-untranslated region (UTR)–capsid (C)–premembrane/membrane (prM/M)–envelope (E)–nonstructural protein 1 (NS1)–NS2A–NS2B–NS3–NS4A–NS4B–NS5–3′-UTR. The structural proteins C, M, and E and the nonstructural proteins NS1–NS5 are translated as a large precursor polyprotein molecule from a single, long open reading frame in the RNA genome. The individual mature viral proteins are processed from the polyprotein by both cellular and virus-specified proteases (Westaway *et al.,* 1985; Rice *et al.,* 1985; Coia *et al.,* 1988; Speight and Westaway, 1989). The virion consists of an icosahedral nucleocapsid that is surrounded by a cell-derived lipid membrane in which the E and M proteins are embedded.

DEN viruses of all four serotypes (DEN-1 to -4) are transmitted to humans primarily by *Aedes aegypti* mosquitoes in tropical and subtropical regions of the world and cause illness, ranging from dengue fever to the severe and often fatal dengue hemorrhagic fever/dengue shock syndrome. Investigators at Mahidol University in Bangkok, Thailand, have developed four live, attenuated candidate vaccine viruses, one for each of the four DEN serotypes, by serial passage of the parent viruses in cell culture (Yoksan *et al.,* 1986; Bhamarapravati *et al.,* 1987; Bhamarapravati and Yoksan, 1989, 1990; Vaughn *et al.,* 1996). These are currently the best live, attenuated vaccine virus candidates for dengue in terms of immunogenic efficacy and lack of reactogenicity in humans. The uncloned PDK-53 vaccine virus elicits neutralizing antibodies that last at least 1.5 years in adult human volunteers (Bhamarapravati *et al.,* 1987; Bhamarapravati and Yoksan, 1989; Vaughn *et al.,* 1996). We report here the genomic nucleotide sequences of the virulent 16681 strain of DEN-2 virus and its attenuated vaccine derivative, strain PDK-53. The PDK-53 vaccine contained two phenotypically different variants. We constructed full-genome-length, infectious cDNA clones for DEN-2 16681 virus and each variant in the PDK-53 vaccine.

[1] The nucleotide sequence data reported in this article have been deposited with the GenBank Data Library under Accession Nos. U87411 (16681) and U87412 (PDK-53).

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MATERIALS AND METHODS

Viruses

The DEN-2 parental 16681 virus was isolated in 1964 from the serum of a DHF/DSS patient in Bangkok, Thailand (Halstead and Simasthien, 1970). It was passaged several times in grivet monkey kidney BS-C-1 cells (Halstead and Simasthien, 1970), six times in rhesus monkey kidney LLC-MK_2 cells, once in a rhesus macaque monkey, twice in *Toxorhynchites amboinensis* mosquitoes, and then 53 times in primary dog kidney (PDK) cell culture to derive the PDK-53 vaccine virus (Halstead *et al.,* 1970; Halstead and Simasthien, 1970; Vaughn *et al.,* 1996). Except as noted, the PDK-53 virus was not passaged further in the present study; cDNA for the PDK-53 infectious clone was amplified directly from the PDK-53 vaccine, Lot 1, manufactured on April 8, 1983. Following the aforementioned mosquito passages, the 16681 virus seed used here was passaged once in primary green monkey kidney cells, twice in LLC-MK_2 cells, and four times in *Aedes albopictus* C6/36 mosquito cells. The 16681 and PDK-53 seeds contained 7.8×10^7 and 3.4×10^4 PFU/ml, respectively, as determined by plaque titration in Vero cell monolayers.

Cell culture

Viruses were grown in baby hamster kidney (BHK-21, clone 15), LLC-MK_2 (CCL-7; American Type Culture Collection), Vero, and C6/36 cells in Eagle's minimal essential medium containing antibiotic and 5% fetal bovine serum (FBS). Plaque titrations were performed in Vero or LLC-MK_2 cell monolayers grown in plastic six-well trays. To evaluate plaque size, virus-infected LLC-MK_2 monolayers were fixed with 2 ml of 3.7% (v/v) formaldehyde solution in phosphate-buffered saline for 2 hr. The agar overlay was removed, and the cells were stained with 1.25% (w/v) crystal violet for 2 min.

Cloning and sequencing of DEN-2 cDNA

Genomic RNA was extracted from virus seed as described by Lewis *et al.* (1992). Primer sequences were obtained from the published data of Blok *et al.* (1992). The reverse transcriptase/polymerase chain reaction (RT/PCR) was performed as described by Chang *et al.* (1995) and using the *Taq* Extender PCR additive as recommended (Stratagene). Six overlapping cDNA fragments containing genome nucleotide (nt) regions 1–1380 (*Sph*I site), 1380–2676 (*Hpa*I), 2676–4493 (*Kpn*I), 4493–6646 (*Nhe*I), 6646–9719 (*Bsp*HI), and 9719–10723 (*Xba*I) were amplified. Restriction sites *Sst*I and *Mlu*I and the promoter (5′-AAATTTAATACGACTCACTATA-3′) for T7 RNA polymerase (Milligan *et al.,* 1987) were engineered at the 5′ terminus of the cDNA. No extraneous 5′ G nt was inserted between the T7 promoter and the 5′-terminal A nt of the genomic cDNA. A unique *Xba*I site (beginning at nt 10723) was engineered at the 3′ terminus of the genomic cDNA to permit linearization prior to RNA transcription. Amplified cDNA was cloned into a TA vector (pGEM-T; Promega) by using electroporation-competent *Escherichia coli* XL1-Blue cells (Stratagene) prepared by the method of Dower *et al.* (1988). Cells were transformed by electroporation (Bio-Rad gene pulser set at 2.5 kV, 25 μF, and 200 ohms) and plated on YT agar (Sambrook *et al.,* 1989) containing 80 μg/ml ampicillin. Plasmid DNA was extracted from cell cultures by the alkaline-sodium dodecyl sulfate (SDS) procedure of Birnboim and Doly (1979).

Automated sequencing was performed as recommended (Applied Biosystems/Perkin–Elmer). Both strands of the cDNA were sequenced. The 5′- and 3′-terminal sequences of the genomes of 16681 and PDK-53 viruses were determined with the 5′ RACE kit (GIBCO BRL) and by tailing the genomic RNA with poly(A) using *E. coli* poly(A) polymerase as recommended (GIBCO BRL). RT/PCR amplification of the 3′ end was performed with virus-specific and oligo(dT) primers.

Construction of DEN-2 infectious cDNA clones

The 16681 clone was constructed first. Three intermediate clones containing nt regions 1–1380 (F1, *Sst*I/*Sph*I), 1380–4493 (F2, *Sph*I/*Kpn*I), and 4493–10723 (F3, *Kpn*I/*Xba*I) were constructed in the polylinker site of pUC19. The *Hin*dIII/*Ava*I region of pBR322 was replaced with the 57-bp *Hin*dIII/*Eco*RI polylinker site of pUC19 (*Ava*I and *Eco*RI sites were blunt-ended with Klenow). The *Sph*I site was removed by sequential treatment with *Sph*I, T4 DNA polymerase, and ligase to produce plasmid pBRUC-139S. DEN-2 16681 F3 cDNA was cloned into the *Kpn*I/*Xba*I sites of pBRUC-139S. The full-genome-length cDNA clone, pD2/IC-30P ("P" for parent virus), was constructed by ligating the *Sst*I–F1–*Sph*I + *Sph*I–F2–*Kpn*I + *Kpn*I–F3–pBRUC–139S–*Sst*I DNAs. PDK-53 virus-specific infectious clones, pD2/IC-130V ("V" for vaccine virus) were constructed by substituting regions of pD2/IC-30P with cognate cDNA derived from PDK-53 virus. All infectious clone plasmids were electroporated and replicated in *E. coli* XL1-Blue cells, except for pD2/IC-130V-F, which was derived in *E. coli* TB-1 cells. Plasmid pD2/IC-30P-A2 was derived by a single TB-1 passage of the original pD2/IC-30P-A clone.

DEN-2 genomic mRNA transcription and transfection

For RNA transcription, plasmids were linearized with *Xba*I, treated with 400 μg/ml proteinase K (Fisher Scientific) for 1 hr at 37°, extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1; AMRESCO) and once with chloroform, and then ethanol precipitated. *Xba*I linearization should result in an additional 4 nt at the 3′ terminus of the transcribed DEN-2 mRNA, since no effort was made to blunt-end the linearized DNA with mung bean

nuclease. Genomic mRNA was transcribed from 50–1000 ng of linearized cDNA template by using a commercial kit (AmpliScribe T7 kit; Epicentre Technologies). Reactions were incubated for 1.5 hr at 37° in the presence of 1.6–2.7 m*M* m^7-GpppA cap analog and then used without further treatment to transfect BHK-21 cells by electroporation as described by Liljeström *et al.* (1991). The transfected cultures were incubated for 6–8 days at 37°, at which time the culture medium was harvested, clarified by centrifugation, brought to 15% FBS, and stored in aliquots at −70°. Clone-derived viruses were passaged once in LLC-MK_2 cells before characterization.

Analysis of the structural proteins of clone-derived DEN-2 viruses

Virus structural proteins were visualized by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) of purified virus grown in LLC-MK_2 cells. The viruses were purified by sedimentation in glycerol/tartrate gradients (Obijeski *et al.,* 1976). Epitopes were mapped by indirect immunofluorescence assay (IFA; Riggs, 1979) by using a battery of well-defined murine monoclonal antibodies (MAbs) derived against DEN-2 virus (Henchal *et al.,* 1985). Virus-infected LLC-MK_2 cells were fixed in ice-cold acetone for 30 min. The MAb preparations were pretitrated and used at a 1:1000 dilution except for MAb 4E5, which was of lower titer and used at a final dilution of 1:40. The MAb binding was detected with a 1:100 dilution of fluorescein-labeled goat anti-mouse antibody (Jackson Immunoresearch Laboratories). Fluorescence intensity was scored on a 1+ to 4+ scale, with 4+ being the most intense. All cells were 100% infected, regardless of the infecting virus.

Intracranial challenge of newborn mice

Newborn, 1-day-old, or 2-day-old outbred white ICR mice, maintained in a colony at the Centers for Disease Control and Prevention, were challenged intracranially with 10^4 PFU of virus diluted in 20 μl of diluent.

RESULTS

Nucleotide sequence analyses of DEN-2 16681 and PDK-53 viral genomes

At least two independent clones were sequenced for each amplified region of the 10,723-nt-long genomes of 16681 and PDK-53 (vaccine vial) viruses. Most of the amplicons contained errors, which were removed during construction of the full-genome-length cDNA clones. The genomes of 16681 and PDK-53 (passaged once in LLC-MK_2 cells to increase titer) viruses were also amplified as 10 overlapping fragments by RT/PCR, and both strands of the amplicons were sequenced directly without cloning. Except for position nt 5270, the sequences obtained by this analysis agreed exactly with the cDNA clone-derived sequences of both viral genomes. DEN-2 16681 virus contained A at nt 5270, whereas PDK-53 virus contained a mixture of both T and A at this position. To ensure that the mixed nt 5270 locus of PDK-53 virus was indicative of the vaccine itself, and not the result of the single LLC-MK_2 passage, the relevant vaccine vial-derived amplicon cDNA was sequenced directly and found to contain the same mixed A/T profile at nt 5270. Five of seven TA vector clones containing this amplicon had nt 5270-T, while two clones had nt 5270-A. These seven clones possessed the PDK-53 virus-specific nt 5547-C, which clearly indicated that all were derived from the PDK-53 vaccine virus.

The 9 nucleotide and five encoded amino acid differences between the genomes of 16681 virus and strain PDK-53 are summarized in Table 1. Three mutations in the E, NS3, and NS5 genes were silent. A C-to-T (16681-to-PDK-53) substitution occurred at nt 57 of the 5′-untranslated region. The five amino acid mutations included prM-29 Asp to Val, NS1-53 Gly to Asp, NS2A-181 Leu to Phe, NS3-250 Glu to Val, and NS4A-75 Gly to Ala. The PDK-53 vaccine virus contained two variants with respect to nt 5270; a significant part of the virus population retained the NS3-250-Glu of the parental 16681 virus. Our sequence data differed from those reported by Blok *et al.* (1992) at 85 nt positions (Fig. 1). They reported 53 16681/PDK-53 nucleotide differences (upward-pointing solid triangles in Fig. 1), whereas we identified only 9. Our data agreed with those of Blok *et al.* (1992) for 6 16681/PDK-53 nucleotide differences (downward-pointing triangles).

Derivation of viruses from DEN-2 infectious cDNA clones

Four viruses were derived from 16681-specific cDNA: (1) D2/IC-30P-A virus from the original infectious clone (pD2/IC-30P-A) of 16681 virus and a duplicate virus, D2/IC-30P-B, from plasmid that was extracted from an independent bacterial colony; (2) D2/IC-30P-A2 virus from the 30P-A plasmid that was passaged once in *E. coli* TB-1 cells; (3) D2/IC-30P-A5 virus from the 30P-A plasmid that was passaged four times in *E. coli* XL1-Blue cells; and (4) D2/IC-30P-A45 (plus duplicate 30P-A49) virus by engineering *Mlu*I (nt 451, silent A-to-G mutation at nt 453) and *Not*I (nt 2269, GA-to-CG [E-445 Gly-to-Ala] mutation at nt 2270–2271 and silent T-to-C mutation at nt 2274) sites into the 30P-A clone with mutagenic amplimers in PCR. The entire genomic cDNA sequence of pD2/IC-30P-A45 was identical to the expected nucleotide sequence of pD2/IC-30P-A, except for the engineered mutations. These mutations were introduced to permit future exchange of the prM and E genes of DEN-2 virus with those of heterologous flaviviruses.

Two variants were derived from PDK-53-specific cDNA: (1) D2/IC-130Vx-4 (plus duplicate 130Vx-7) virus,

TABLE 1

Summary of Nucleotide and Amino Acid Sequence Differences between the Genomes of DEN-2 16681 Virus and Its Vaccine Derivative, Strain PDK-53

Genome nucleotide position	Nucleotide		Amino acid		Amino acid position
	16681	PDK-53	16681	PDK-53	
57	C	T	—[a]	—	—
524	A	T	Asp	Val	prM-29
2055	C	T	Phe	Phe	E-373
2579	G	A	Gly	Asp	NS1-53
4018	C	T	Leu	Phe	NS2A-181
5270	A	A/T[b]	Glu	Glu/Val[b]	NS3-250
5547	T	C	Arg	Arg	NS3-342
6599	G	C	Gly	Ala	NS4A-75
8571	C	T	Val	Val	NS5-334

[a] —, 5′-untranslated region.
[b] The PDK-53 vaccine contained two genetic variants at genome nucleotide position 5270.

containing eight of the nine mutations in PDK-53 virus (Table 1) and encoding NS3-250-Glu, and (2) D2/IC-130Vc-K (plus duplicate 130Vc-C and 130Vc-L) virus, containing all nine PDK-53 virus-specific nucleotide mutations, including that encoding NS3-250-Val (Table 1). An additional virus, D2/IC-130V-F (plus duplicate 130V-J), containing the *Nhe*I (nt 6646) to *Xba*I (nt 10723) region of 16681 virus within the 130Vx-4 cDNA background, was also derived. This virus was essentially identical to the 130Vx-4 virus by the phenotypic analyses employed in this study (data not shown). Only a single silent mutation occurred in the *Nhe*I–*Xba*I region (Table 1).

Unless specified otherwise, duplicate viruses were essentially identical to their primary clone-derived virus by

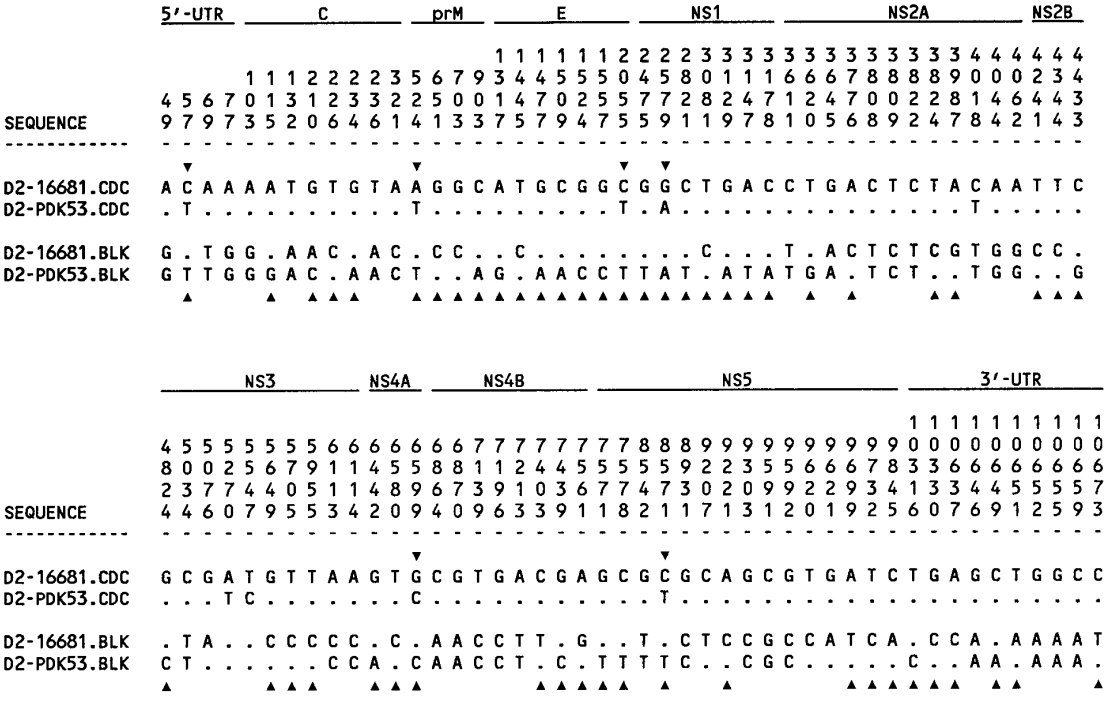

FIG. 1. Summary of the nucleotide sequence analyses of the genomes of DEN-2 16681 virus and its vaccine derivative, strain PDK-53. Our data agreed with those of Blok *et al.* (1992), except at the 85 nt positions shown. The genomic regions and positions of the relevant nucleotides are shown above the data determined in the present study (D2-16681.CDC and D2-PDK53.CDC) and by Blok *et al.* (D2-16681.BLK and D2-PDK53.BLK). Dots indicate positions of nucleotide identity with the D2-16681.CDC sequence. Upward-pointing solid triangles below the D2-PDK53.BLK sequence indicate the 53 16681/PDK-53 nucleotide differences reported by Blok *et al.* (1992). Downward-pointing triangles above the D2-16681.CDC sequence indicate those positions of 16681/PDK-53 difference at which our data agreed with those of Blok *et al.* Two variants in the PDK-53 vaccine contained A or T at nt 5270. The sequences of the PDK-53 virus present in the vaccine vial and after a single passage in LLC-MK_2 cells were identical.

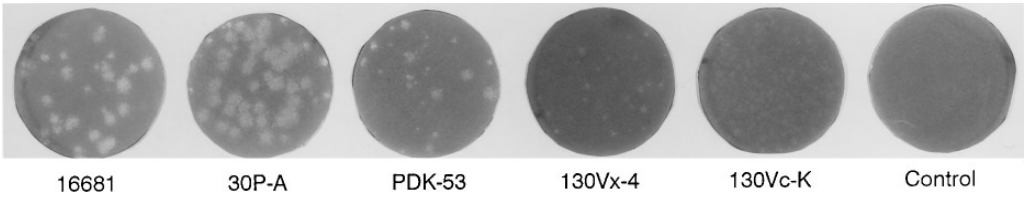

FIG. 2. Plaque phenotypes of DEN-2 16681, D2/IC-30P-A, PDK-53, D2/IC-130Vx-4 (NS3-250-Glu), and D2/IC-130Vc-K (NS3-250-Val) viruses at 9 days postinfection in LLC-MK_2 cells. Plaques were recorded by Polaroid photography. Control, uninfected cell monolayer.

the phenotypic analyses employed in this study (data not shown). All nine virus-specific genetic loci, as well as silent cDNA artifacts incorporated into the cDNA clones, were confirmed for each clone-derived virus by RT/PCR and sequencing of appropriate cDNA amplicons. Two silent cDNA artifacts were incorporated into the pD2/IC-30P clones at nt 1531 (C to T) and nt 2406 (A to G). A single silent artifact was incorporated into the pD2/IC-130Vx and pD2/IC-130Vc clones at nt 900 (T to C).

Analysis of the structural proteins of DEN-2 viruses

The SDS–PAGE profiles of the structural proteins of DEN-2 16681 and D2/IC-30P-A viruses were identical (data not shown). Authentic expression of the E and prM proteins in clone-derived viruses was demonstrated by MAb mapping of epitopes by IFA. E-specific MAbs (Henchal *et al.,* 1985) 3H5 (DEN-2 type-specific, neutralization [N] and hemagglutination-inhibition [HI] activities), 2H3 (type, HI), 4E5 (type, N, HI), 9D12 (subcomplex, N, HI), 13B7 (subcomplex), 1B7 (subcomplex, N, HI), and 4G2 (group, N, HI), as well as prM-specific MAb 2H2 (complex) reacted identically or nearly identically (scores of 2–4+) with LLC-MK_2 cells infected with 16681, 30P-A, PDK-53, 130V-F (130Vx-4 was not tested), or 130Vc-K virus. The flavivirus group-specific MAb 6B6C-1 (Roehrig *et al.,* 1983), elicited against St. Louis encephalitis virus, also reacted at 3–4+ with these viruses, whereas the DEN-1 virus-specific MAb 1F1 (Gubler, 1987) had no reactivity with these viruses (data not shown).

Plaque phenotypes of DEN-2 viruses in LLC-MK_2 cells

DEN-2 16681 and D2/IC-30P-A viruses (Fig. 2), as well as viruses 30P-A2, 30P-A5, and 30P-A45 (not shown), produced plaques of about 2.5-mm diameter at 9 days postinfection in LLC-MK_2 cells. PDK-53 virus from a vial of vaccine and 130Vx-4 virus (NS3-250-Glu) produced a

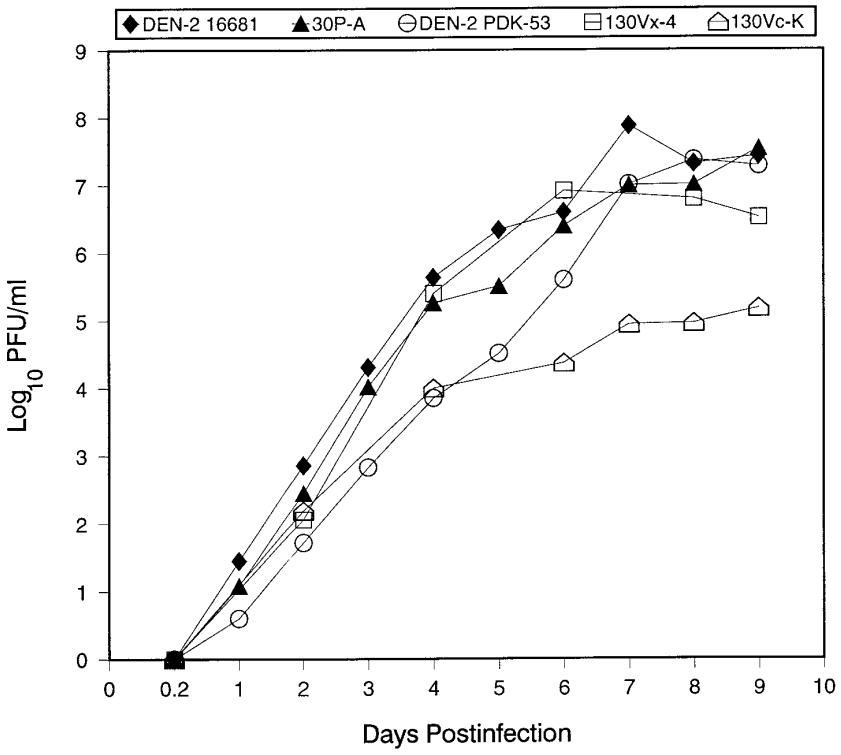

FIG. 3. Growth curves of DEN-2 16681, D2/IC-30P-A, PDK-53, D2/IC-130Vx-4 (NS3-250-Glu), and D2/IC-130Vc-K (NS3-250-Val) viruses in LLC-MK_2 cells grown in 75-cm^2 tissue culture flasks. The multiplicity of infection was approximately 0.004 PFU/cell.

mixed, smaller plaque phenotype. D2/IC-130Vc-K virus (NS3-250-Val) produced small, diffuse plaques. Longer incubation of 130Vc-K virus in Vero cell monolayers permitted better visualization of the virus plaques for purposes of titration (not shown).

Growth curves of DEN-2 viruses in LLC-MK_2 and C6/36 cells

DEN-2 16681 and D2/IC-30P-A viruses (Fig. 3), as well as 30P-A2 and 30P-A5 viruses (not shown), grew with nearly equal efficacy in LLC-MK_2 cells, reaching peak titers of 10^7–10^8 PFU/ml at 7–10 days postinfection. PDK-53 virus (open circles in Fig. 3) from a vial of vaccine, whose low titer set the low multiplicity of infection of 0.004 PFU/cell, and PDK-53 clone-derived 130Vx-4 virus (NS3-250-Glu) grew to peak titers of about 10^7 PFU/ml at 6–9 days postinfection. The somewhat delayed replication of PDK-53 virus was evident in a second experiment as well (not shown). D2/IC-130Vc-K virus (NS3-250-Val) replicated less efficiently, reaching a peak titer of $10^{5.0}$ PFU/ml at 9 days postinfection.

PDK-53 virus replicates with much lower efficiency than 16681 virus in *Ae. aegypti* mosquitoes (Khin *et al.,* 1994). We investigated virus replication in C6/36 cells (data not shown). DEN-2 16681 and D2/IC-30P-A viruses attained peak titers of $10^{8.3}$–$10^{8.7}$ PFU/ml at 7 days postinfection. PDK-53 clone-derived 130Vx-4 virus (NS3-250-Glu) replicated to a peak titer of $10^{6.2}$ PFU/ml. The 130Vc-K virus (NS3-250-Val) replicated poorly in C6/36 cells, achieving a peak titer of $10^{4.0}$ PFU/ml at 7 days postinfection. In the same experiment, the PDK-53 vaccine virus had a peak titer of $10^{3.0}$ PFU/ml. In a separate experiment, 16681, PDK-53, and 130Vc-L viruses attained peak titers of $10^{7.5}$, $10^{3.3}$, and $10^{2.3}$ PFU/ml, respectively, at 6–8 days postinfection in C6/36 cells (data not shown).

Temperature sensitivities of DEN-2 viruses in LLC-MK_2 cells

Viruses were tested for temperature sensitivity in LLC-MK_2 cells. DEN-2 16681 and D2/IC-30P-A virus titers were reduced by 89–90% at 39°, relative to the virus titer at 37°, at 8 days postinfection (Fig. 4). D2/IC-30P-A2 virus had 83% reduction in titer (not shown). As reported by Yoksan *et al.* (1986), virus from the PDK-53 vaccine vial was temperature sensitive, as indicated by a 99.4% reduction in titer at 39°. Clone-derived viruses representing the two genetic variants in the PDK-53 vaccine vial showed different temperature sensitivities. The 130Vx-4 virus (NS3-250-Glu) was more temperature-sensitive (97% reduction in titer) than 16681 virus, but less so than the PDK-53 vaccine virus. The NS3-250-Val variant, represented by D2/IC-130Vc-K virus, was highly temperature sensitive, with 99.98% reduction of virus titer at 39° relative to the virus titer at 37°.

Neurovirulence of DEN-2 viruses in suckling mice

Groups of 16 newborn white ICR mice were inoculated intracranially with 10^4 PFU of original or clone-derived virus. DEN-2 16681 and 16681 clone-derived viruses, except for 30P-A2 virus, resulted in 50–81% mortality with an average survival time of 15–17 days (Table 2). Relative to control mice inoculated with diluent, significantly lower mean body weights ($P < 0.001$, Student's t test) were observed at 28 days postinfection in those mice that survived challenge with 30P-A or 30P-A45 virus (weights not shown).

In a separate experiment, intracranial challenge of 2-day-old mice (10 mice per group) with 10^4 PFU of 16681 or 30P-A virus resulted in 80% (average survival time of 15 days) or 90% (average survival time of 17 days) mortality, respectively, whereas challenge with 30P-A2 virus resulted in 10% mortality (data not shown). The reduced mortality caused by the 30P-A2 virus may have been due to a mutation of pD2/IC-30P-A2 during passage of pD2/IC-30P-A in *E. coli* TB-1 cells. However, the cDNA in pD2/IC-30P-A2 has not been sequenced to confirm this speculation.

Except for a single death (at 11 days postinfection) in the 130Vc-L group, all of the PDK-53 clone-derived viruses were attenuated for suckling mice by intracranial inoculation. Results for the 130Vx-4 and 130Vc-K viruses are shown in Table 2. Mice surviving challenge with 130V-F, 130V-J, 130Vx-4, and 130Vc-C viruses had lower ($P < 0.05$) mean body weights than the control group at 28 days postinfection. The 130Vc-L mouse group actually had a significantly greater ($P < 0.05$) mean body weight at 14 days postinfection. Because of low seed virus titer, the challenge dose for the 130Vc-L group was $10^{3.8}$ PFU. Mice challenged with the LLC-MK_2-1 passage of the PDK-53 vaccine virus all survived without significant weight loss (Table 2).

DISCUSSION

We report here the development of infectious cDNA clones for DEN-2 16681 virus and its vaccine derivative, strain PDK-53. The infectious clones of the 17D strain of yellow fever virus (Rice *et al.,* 1989) and DEN-2 PDK-53 virus (reported here) are currently the only flavivirus infectious clones that have been developed from vaccine viruses which have been tested in humans. DEN-2 16681 clone-derived D2/IC-30P viruses were identical to the 16681 virus in terms of the size of the structural proteins in the virion, expression of appropriate E and prM epitopes, plaque size in LLC-MK_2 cells, replication rate in LLC-MK_2 and C6/36 cells, relative lack of temperature sensitivity in LLC-MK_2 cells, and, except for the 30P-A2 virus, neurovirulence for suckling mice. Although the 30P-A2 virus was partially attenuated in mice, its replication phenotype *in vitro* was identical to that of the 16681 virus. We speculate that plasmid pD2/IC-30P-A2 may have ac-

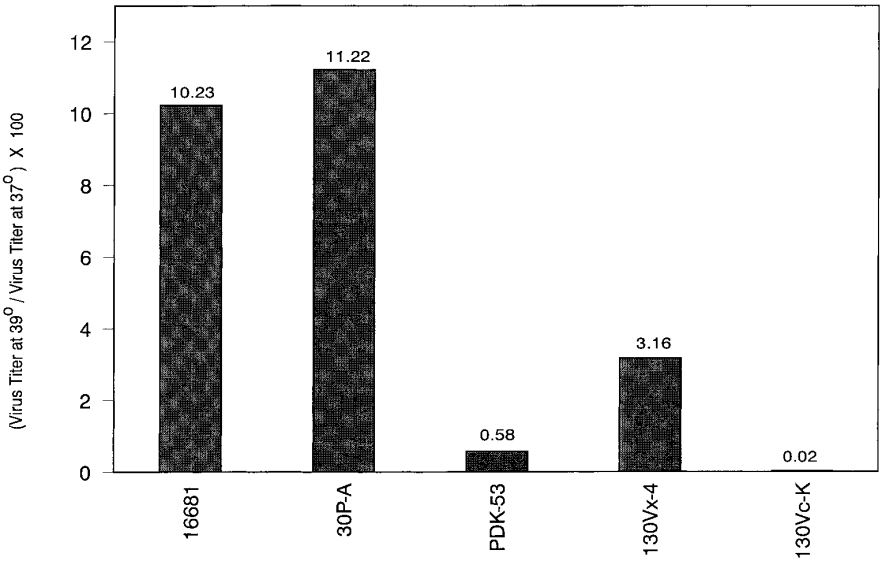

FIG. 4. Temperature sensitivities of DEN-2 16681, D2/IC-30P-A, PDK-53, D2/IC-130Vx-4 (NS3-250-Glu), and D2/IC-130Vc-K (NS3-250-Val) viruses in LLC-MK_2 cells grown in 75-cm^2 flasks at 37 or 39°. Cell culture media were harvested at 8 days postinfection and titrated in Vero cell monolayers. The bar heights and the numerical values over the bars indicate the percentage of virus replication at 39° relative to that at 37° ([virus titer at 39°/ virus titer at 37°] × 100). The multiplicity of infection was approximately 0.004 PFU/cell.

crued a mutation during passage of pD2/IC-30P-A in *E. coli* TB-1 cells.

We identified only 9 nucleotide mutations between the 16681 and the PDK-53 genomes. These data differed at 85 nt positions from the data of Blok *et al.* (1992), who reported 53 nucleotide differences for this virus pair. Our results showed that the PDK-53 vaccine vial contains a mixture of two phenotypically different variants as a result of partial mutation at NS3-250. Since both of the PDK-53 clone-derived variants were attenuated for newborn mice, the NS3-250 Glu-to-Val (16681-to-PDK-53) mutation is not a major determinant of attenuation for PDK-53 virus in mice. However, this mutation clearly affected virus replication *in vitro,* as demonstrated by the altered plaque phenotype and increased temperature sensitivity in LLC-MK_2 cells and the decreased replication rate in both LLC-MK_2 and C6/36 cells of the NS3-250-Val variant. The NS3-250 mutation may affect the proteinase or helicase activity of the conformationally mature NS3 protein of PDK-53 virus, although this mutation does not occur at the putative sequence motifs for these NS3 activities (Gorbalenya *et al.,* 1989a,b; Wengler *et al.,* 1991; Chambers *et al.,* 1991; Wengler and Wengler, 1991; Pause and Sonenberg, 1992; Arias *et al.,* 1993; Warrener *et al.,* 1993). The relative contribution of each of the two PDK-53 variants to the infectivity and immunogenic efficacy of the PDK-53 vaccine virus administered to humans is unknown.

TABLE 2

Neurovirulence of DEN-2 Viruses in Newborn White ICR Mice Following Intracranial Challenge with 10^4 PFU of Virus

Virus	Percentage mortality[a]	Average survival time Days (SD)
Diluent	0.0	—[b]
16681[c]	81.25	15.6 (2.1)
30P-A	50.0	16.8 (5.4)
30P-B[d]	75.0	15.3 (1.0)
30P-A2	25.0	14.0 (2.2)
30P-A5	68.75	15.5 (1.2)
30P-A45	68.75	15.0 (1.7)
30P-A49[d]	81.25	15.6 (1.0)
130Vx-4	0.0	—
130Vc-K	0.0	—
PDK-53[c]	0.0	—

[a] (Number of deaths/16 mice per group) × 100.

[b] —, not applicable.

[c] Wild-type DEN-2 16681 and PDK-53 viruses. The PDK-53 vaccine virus was passaged once in LLC-MK_2 cells to increase the virus titer for this experiment. All other viruses are clone-derived, D2/IC viruses.

[d] Duplicate of the preceding, primary clone-derived virus.

Although the E protein of flaviviruses often encodes determinants of attenuation and virulence (Barrett *et al.,* 1990), we identified no amino acid substitutions in the E protein of PDK-53 virus. The attenuation of PDK-53 virus is probably due to one or more of the mutations at 5′-UTR-57, prM-29, NS1-53, NS2A-181, and NS4A-75. Major determinants of attenuation have been shown to reside in the 5′-UTR of other positive-stranded viruses, including the Sabin poliovaccine viruses (Evans *et al.,* 1985; Macadam *et al.,* 1994) and the TC-83 vaccine strain of Venezuelan equine encephalitis virus (Kinney *et al.,* 1993). Cahour *et al.* (1995) demonstrated profound effects, in-

cluding decreased growth in LLC-MK_2 and C6/36 cells, of deletions engineered into the 5′-UTR of DEN-4 virus variants. Analyses of recombinant polio type I viruses obtained from infectious cDNA clones demonstrated that loci involved in attenuation were distributed in several regions of the genome and that alterations in antigenicity correlated poorly with virulence changes (Omata *et al.,* 1986).

Since no amino acid mutations occurred in the C, M, and E structural genes of PDK-53 virus, the infectious clones of the two PDK-53 variants might be useful genetic backgrounds for the expression of the structural genes of other DEN virus serotypes or other flaviviruses. Such chimeric viruses would be expected to retain the PDK-53 determinants of attenuation. The engineering of intertypic DEN chimeras within the genetic background of DEN-4 virus has been reported (Bray and Lai, 1991; Chen *et al.,* 1995; Bray *et al.,* 1996). The contribution of the prM-29 Asp-to-Val mutation to the attenuated phenotype of PDK-53 virus may be important. We plan to investigate chimeric viruses derived from genetic exchanges between DEN-2 16681 and PDK-53 viruses to define the genetic loci that encode the attenuated phenotype of the vaccine virus.

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REFERENCES

Arias, C. F., Preugschat, F., and Strauss, J. H. (1993). Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. *Virology* 193, 888–899.

Barrett, A. D. T., Dunster, L. M., Ledger, T. N., Gibson, C. A., Sil, B. K., Sanders, P. G., Gould, E. A., and Minor, P. D. (1990). Evidence that the envelope protein of flaviviruses encodes determinants of attenuation and virulence. *In* "Vaccines 90" (F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner, Eds.), pp. 101–104. Cold Spring Harbor Laboratory Press, New York.

Bhamarapravati, N., and Yoksan, S. (1989). Study of bivalent dengue vaccine in volunteers. *Lancet* 1, 1077.

Bhamarapravati, N., and Yoksan, S. (1990). The clinical trial of trivalent dengue vaccine. *Southeast Asian J. Trop. Med. Public Health* 21, 709.

Bhamarapravati, N., Yoksan, S., Chayaniyayothin, T., Angsubphakorn, S., and Bunyaratvej, A. (1987). Immunization with a live attenuated dengue-2-virus candidate vaccine (16681-PDK 53): Clinical, immunological and biological responses in adult volunteers. *Bull. WHO* 65, 189–195.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513–1523.

Blok, J., McWilliam, M., Butler, H. C., Gibbs, A. J., Weiller, G., Herring, B. L., Hemsley, A. C., Aaskov, J. G., Yoksan, S., and Bhamarapravati, N. (1992). Comparison of a dengue-2 virus and its candidate vaccine derivative: Sequence relationships with the flaviviruses and other viruses. *Virology* 187, 573–590.

Bray, M., and Lai, C.-J. (1991). Construction of intertypic chimeric dengue viruses by substitution of structural protein genes. *Proc. Natl. Acad. Sci. USA* 88, 10342–10346.

Bray, M., Men, R., and Lai, C.-J. (1996). Monkeys immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge. *J. Virol.* 70, 4162–4166.

Cahour, A., Pletnev, A., Vazeille-Falcoz, M., Rosen, L., and Lai, C.-J. (1995). Growth-restricted dengue virus mutants containing deletions in the 5′ noncoding region of the RNA genome. *Virology* 207, 68–76.

Chambers, T. J., Grakoui, A., and Rice, C. M. (1991). Processing of the yellow fever virus nonstructural polyprotein: A catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. *J. Virol.* 65, 6042–6050.

Chang, G.-J. J., Cropp, B. C., Kinney, R. M., Trent, D. W., and Gubler, D. J. (1995). Nucleotide sequence variation of the envelope protein gene identifies two distinct genotypes of yellow fever virus. *J. Virol.* 69, 5773–5780.

Chen, W., Kawano, H., Men, R., Clark, D., and Lai, C.-J. (1995). Construction of intertypic chimeric dengue viruses exhibiting type 3 antigenicity and neurovirulence for mice. *J. Virol.* 69, 5186–5190.

Coia, G., Parker, M. D., Speight, G., Byrne, M. E., and Westaway, E. G. (1988). Nucleotide and complete amino acid sequences of Kunjin virus: Definitive gene order and characteristics of the virus-specified proteins. *J. Gen. Virol.* 69, 1–21.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16, 6127–6145.

Evans, D. M. A., Dunn, G., Minor, P. D., Schild, G. C., Cann, A. J., Stanway, G., Almond, J. W., Currey, K., and Maizel, J. V., Jr. (1985). Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature* 314, 548–550.

Gorbalenya, A. E., Donchenko, A. P., Koonin, E. V., and Blinov, V. M. (1989a). N-terminal domains of putative helicases of flavi and pestiviruses may be serine proteases. *Nucleic Acids Res.* 17, 3889–3897.

Gorbalenya, A., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989b). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* 17, 4713–4730.

Gubler, D. J. (1987). Application of serotype-specific monoclonal antibodies for identification of dengue viruses. *In* "Arboviruses in Arthropod Cells *in Vitro*" (C. Yunker, Ed.), Vol. 2, pp. 3–14. CRC Press, Boca Raton, FL.

Halstead, S. B., and Simasthien, P. (1970). Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. *Yale J. Biol. Med.* 42, 276–292.

Halstead, S. B., Udomsakdi, S., Simasthien, P., Singharaj, P., Sukhavachana, P., and Nisalak, A. (1970). Observations related to pathogenesis of dengue hemorrhagic fever. I. Experience with classification of dengue viruses. *Yale J. Biol. Med.* 42, 261–275.

Henchal, E. A., McCown, J. M., Burke, D. S., Seguin, M. C., and Brandt, W. E. (1985). Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. *Am. J. Trop. Med. Hyg.* 34, 162–169.

Khin, M. M., Jirakanjanakit, N., Yoksan, S., and Bhamarapravati, N. (1994). Infection, dissemination, transmission, and biological attributes of dengue-2 PDK53 candidate vaccine virus after oral infection in *Aedes aegypti. Am. J. Trop. Med. Hyg.* 51, 864–869.

Kinney, R. M., Chang, G.-J., Tsuchiya, K. R., Sneider, J. M., Roehrig, J. T., Woodward, T. M., and Trent, D. W. (1993). Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5′-noncoding region and the E2 envelope glycoprotein. *J. Virol.* 67, 1269–1277.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Lewis, J. A., Chang, G.-J., Lanciotti, R. S., and Trent, D. W. (1992). Direct sequencing of large flavivirus PCR products for analysis of genome variation and molecular epidemiological investigations. *J. Virol. Methods* 38, 11–24.

Liljeström, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991). *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: The small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* 65, 4107–4113.

Macadam, A. J., Stone, D. M., Almond, J. W., and Minor, P. D. (1994). The 5′ noncoding region and virulence of poliovirus vaccine strains. *Trends Microbiol.* 2, 449–454.

Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987). Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* 15, 8783–8798.

Obijeski, J. F., Bishop, D. H. L., Murphy, F. A., and Palmer, E. L. (1976). Structural proteins of La Crosse virus. *J. Virol.* 19, 985–997.

Omata, T., Kohara, M., Kuge, S., Komatsu, T., Abe, S., Semler, B. L., Kameda, A., Itoh, H., Arita, M., Wimmer, E., and Nomoto, A. (1986). Genetic analysis of the attenuation phenotype of poliovirus type 1. *J. Virol.* 58, 348–358.

Pause, A., and Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: The mammalian translation initiation factor eIF-4A. *EMBO J.* 11, 2643–2654.

Rice, C. M., Grakoui, A., Galler, R., and Chambers, T. J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by *in vitro* ligation. *New Biol.* 1, 285–296.

Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L., and Strauss, J. H. (1985). Nucleotide sequence of yellow fever virus: Implications for flavivirus gene expression and evolution. *Science* 229, 726–735.

Riggs, J. L. (1979). Immunofluorescent staining. *In* "Diagnostic Procedures for Viral, Rickettsia, and Chlamydial Infections," 5th ed. (E. H. Lennette and N. J. Schmidt, Eds.). Am. Public Health Assoc., Washington, D.C.

Roehrig, J. T., Mathews, J. H., and Trent, D. W. (1983). Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. *Virology* 128, 118–126.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Speight, G., and Westaway, G. (1989). Positive identification of NS4A, the last of the hypothetical nonstructural proteins of flaviviruses. *Virology* 170, 299–301.

Vaughn, D. W., Hoke, C. H., Jr., Yoksan, S., LaChances, R., Innis, B. L., Rice, R. M., and Bhamarapravati, N. (1996). Testing of a dengue 2 live-attenuated vaccine (strain 16681 PDK 53) in ten American volunteers. *Vaccine* 14, 329–336.

Warrener, P., Tamura, J. K., and Collett, M. S. (1993). RNA-stimulated NTPase activity associated with yellow fever virus NS3 protein expressed in bacteria. *J. Virol.* 67, 989–996.

Wengler, G., and Wengler, G. (1991). The carboxy-terminal part of the NS3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. *Virology* 184, 707–715.

Wengler, G., Czaya, G., Färber, P. M., and Hegemann, J. H. (1991). *In vitro* synthesis of West Nile virus proteins indicates that the amino-terminal segment of the NS3 protein contains the active centre of the protease which cleaves the viral polyprotein after multiple basic amino acids. *J. Gen. Virol.* 72, 851–858.

Westaway, E. G., Brinton, M. A., Gaidamovich, S. Ya., Horzinek, M. C., Igarashi, A., Kääriänen, L., Lvov, D. K., Porterfield, J. S., Russell, P. K., and Trent, D. W. (1985). Flaviviridae. *Intervirology* 74, 183–192.

Yoksan, S., Bhamarapravati, N., and Halstead, S. B. (1986). Dengue virus vaccine development: Study on biological markers of uncloned dengue 1–4 viruses serially passaged in primary kidney cells. *In* "Arbovirus Research in Australia, Proceedings of the Fourth Symposium" (T. D. St. George, B. H. Kay, and J. Blok, Eds.), pp. 35–38. CSIRO/QIMR, Brisbane.