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Dengue 2 PDK-53 Virus as a Chimeric Carrier for Tetravalent Dengue Vaccine Development

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Attenuation markers of the candidate dengue 2 (D2) PDK-53 vaccine virus are encoded by mutations that reside outside of the structural gene region of the genome. We engineered nine dengue virus chimeras containing the premembrane (prM) and envelope (E) genes of wild-type D1 16007, D3 16562, or D4 1036 virus within the genetic backgrounds of wild-type D2 16681 virus and the two genetic variants (PDK53-E and PDK53-V) of the D2 PDK-53 vaccine virus. Expression of the heterologous prM-E genes in the genetic backgrounds of the two D2 PDK-53 variants, but not that of wild-type D2 16681 virus, resulted in chimeric viruses that retained PDK-53 characteristic phenotypic markers of attenuation, including small plaque size and temperature sensitivity in LLC-MK$_2$ cells, limited replication in C6/36 cells, and lack of neurovirulence in newborn ICR mice. Chimeric D2/1, D2/3, and D2/4 viruses replicated efficiently in Vero cells and were immunogenic in AG129 mice. Chimeric D2/1 viruses protected adult AG129 mice against lethal D1 virus challenge. Two tetravalent virus formulations, comprised of either PDK53-E- or PDK53-V-vectored viruses, elicited neutralizing antibody titers in mice against all four dengue serotypes. These antibody titers were similar to the titers elicited by monovalent immunizations, suggesting that viral interference did not occur in recipients of the tetravalent formulations. The results of this study demonstrate that the unique attenuation loci of D2 PDK-53 virus make it an attractive vector for the development of live attenuated flavivirus vaccines.

Dengue (DEN) viruses, which comprise four serotypes (D1 to D4), are members of the *Flavivirus* genus and contain a single-stranded positive-sense genomic RNA of approximately 11 kb. The RNA genome organization is 5′NCR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′NCR (where NCR is noncoding region, C is capsid, prM is premembrane, E is envelope, and NS is nonstructural protein). The structural proteins (C, prM, and E) and the NS proteins are translated as a single polyprotein precursor which is processed by cellular and viral proteases (37). DEN viruses are the leading cause of mosquito-transmitted viral disease in humans. DEN virus transmission results in an estimated 100 million cases of dengue fever and up to several hundred thousand cases of its more severe form, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), every year (16). Both viral virulence and host immune responses have been considered responsible for the pathogenesis of DHF/DSS (38). Immunopathological mechanisms, including antibody-dependent enhancement of virus replication and cell-mediated factors, have been regarded as important factors that contribute to DHF/DSS (31, 35, 39). It is a general concern that sequential infection with different serotypes of DEN virus will increase the risk of DHF/DSS through such immunopathological mechanisms (19, 31). Therefore, an efficacious tetravalent vaccine is needed to provide solid and long-term immunity against all four serotypes of DEN virus.

One of the most promising tetravalent DEN virus vaccine candidates consists of live attenuated DEN viruses that were derived by serial passage of wild-type viruses in primary dog kidney (PDK) cells or primary African green monkey kidney cells at Mahidol University, Bangkok, Thailand (45). Human phase I and II clinical trials have been conducted in Thailand and the United States, and the results indicate that these vaccine candidates are safe and immunogenic in humans (3, 5, 6, 14, 25, 40, 41, 44). However, some tetravalent formulations failed to induce neutralizing antibodies and/or equivalent T-cell responses against all four serotypes (3, 25, 40, 41). The Mahidol D2 vaccine virus, the PDK-53 strain, was derived by passage of the wild-type D2 16681 virus 53 times in PDK cells. It had the lowest 50% minimum infectious dose (5 PFU) among the four vaccine serotypes for humans (4). When tested alone, PDK-53 virus produced no untoward clinical symptoms, elicited neutralizing antibodies that lasted for at least 2 years, and induced significant memory T-cell responses in humans (4, 6, 14, 44).

The uncloned PDK-53 virus vaccine contains a mixture of two genotypic variants (29), designated PDK53-E and PDK53-V in this report. The PDK53-V variant contains all nine PDK-53 virus vaccine-specific nucleotide mutations, including the NS3-250 Glu-to-Val mutation. The PDK53-E variant contains eight of the nine mutations and the NS3-250 Glu of the wild-type 16681 virus. The phenotypic markers associated with the attenuation of PDK-53 virus, including small plaque size and temperature sensitivity in LLC-MK$_2$ cells, limited replication in C6/36 cells, and attenuation in newborn mice, are determined by mutations 5′-NCR-57 C-to-T, NS1-53 Gly-to-Asp,

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TABLE 1. Nucleotide and amino acid differences between recombinant and parental viruses

Recombinant virus	Parental virus		Nucleotide		Amino acid		Comment(s)
	Backbone	prM-E donor	Position[a]	Change	Position[b]	Change	
D2 16681-P48	D2 16681	D2 16681	453	A-G			Engineered *Mlu*I site for chimera, silent
			1531	C-T			Original clone marker from p30PA (29), silent
			2381–2382	TG-CC	E-482	Val-Ala	Engineered *Ngo*MIV cloning site for chimera
			2406	A-G			Original clone marker from p30PA (29), silent
D2 PDK53-E48	D2 PDK53-E	D2 PDK53-E	453	A-G			Engineered *Mlu*I site for chimera, silent
			900	T-C			Clone marker for PDK-53, silent
			2381–2382	TG-CC	E-482	Val-Ala	Engineered *Ngo*MIV cloning site for chimera
			5547	C-T			Silent mutation, T as 16681 strain
			8571	T-C			Silent mutation, C as 16681 strain
D2 PDK53-V48	D2 PDK53-V	D2 PDK53-V	453	A-G			Engineered *Mlu*I site for chimera, silent
			900	T-C			Clone marker for PDK-53, silent
			2381–2382	TG-CC	E-482	Val-Ala	Engineered *Ngo*MIV cloning site for chimera
			8571	T-C			Silent mutation, C as 16681 strain
D2/1-P	D2 16681-P48	D1 16007	1575	T-C			Engineered to remove internal *Xba*I, silent
D2/1-E	D2 PDK53-E48	D1 16007	1575	T-C			Engineered to remove internal *Xba*I, silent
D2/1-V	D2 PDK53-V48	D1 16007	1575	T-C			Engineered to remove internal *Xba*I, silent
D2/3-P	D2 16681-P48	D3 16562	550	C-T			Silent mutation in plasmids
			1968	A-T	E-345	His-Leu	Engineered for efficient growth in culture
D2/3-E	D2 PDK53-E48	D3 16562	550	C-T			Silent mutation in plasmids
			1968	A-T	E-345	His-Leu	Engineered for efficient growth in culture
D2/3-V	D2 PDK53-V48	D3 16562	550	C-T			Silent mutation in plasmids
			1968	A-T	E-345	His-Leu	Engineered for efficient growth in culture
D2/4-P	D2 16681-P48	D4 1036	396	A-C	C-100	Arg-Ser	Engineered for efficient growth in culture
			1403	A-G			Silent mutation in plasmid
			2029	C-T	E-364	Ala-Val	Engineered for efficient growth in culture
			2277	A-T	E-447	Met-Leu	Engineered for efficient growth in culture
D2/4-E	D2 PDK53-E48	D4 1036	396	A-C	C-100	Arg-Ser	Engineered for efficient growth in culture
			1403	A-G			Silent mutation in plasmid
			2029	C-T	E-364	Ala-Val	Engineered for efficient growth in culture
			2277	A-T	E-447	Met-Leu	Engineered for efficient growth in culture
D2/4-V	D2 PDK53-V48	D4 1036	396	A-C	C-100	Arg-Ser	Engineered for efficient growth in culture
			1403	A-G			Silent mutation in plasmid
			2029	C-T	E-364	Ala-Val	Engineered for efficient growth in culture
			2277	A-T	E-447	Met-Leu	Engineered for efficient growth in culture

[a] Position is given according to the backbone D2 virus or the wild-type prM-E donor virus.
[b] Only nonsilent mutations are shown.

and NS3-250 Glu-to-Val (9). Because these loci reside outside the structural region of the genome, chimeric viruses expressing structural genes of heterologous flaviviruses within the context of the PDK-53 background are expected to retain the attenuating phenotypic markers of the D2 PDK-53 virus. We previously showed that this held true for chimeric viruses containing the C-prM-E genes of the wild-type D1 16007 virus in the D2 PDK53-E and -V backbones (21). For the present study, we engineered chimeras containing the prM-E genes of the wild-type D1 16007, D3 16562, and D4 1036 viruses in the genetic background of D2 16681, PDK53-E, and PDK53-V viruses to explore the potential of the PDK-53 vaccine virus to serve as a vector for chimeric dengue vaccine development. Tetravalent DEN virus vaccine components developed with this strategy should have good replication efficiency in humans and consequently elicit effective immunity against all four serotypes, since each component would contain the same replication machinery of the candidate D2 PDK-53 vaccine virus.

MATERIALS AND METHODS

Viruses and cell cultures. Wild-type D1 16007, D2 16681, D3 16562, and D4 1036 viruses were available from the virus collection at the Centers for Disease Control and Prevention (CDC). D1 16007 and D2 16681 viruses were originally recovered from the sera of two patients with DHF/DSS in Thailand (20). D3 16562 virus was originally isolated from a DHF/DSS patient in the Philippines, and D4 1036 virus was obtained from a child with dengue fever in Indonesia (20). The D2 16681 virus was passaged several times in grivet monkey kidney BS-C-1 cells, six times in rhesus macaque monkeys, twice in *Toxorhynchites amboinensis* mosquitoes, and then 53 times in PDK cells at the Center for Vaccine Development, Mahidol University, to derive the PDK-53 vaccine virus (20, 45).

Viruses were grown in Vero, LLC-MK$_2$, and C6/36 cells as described previously (9, 21). Virus plaque titrations were performed under double agarose overlays in six-well plates of confluent Vero or LLC-MK$_2$ cells (9, 21). The second agarose overlay, containing neutral red vital stain, was added 7 days after infection, and plaques were counted 8 to 11 days after infection.

Construction of DEN virus intertypic chimeric plasmids. Three previously constructed D2 infectious clones, pD2-16681-P48, pD2-PDK53-E48, and pD2-PDK53-V48 (21) (Table 1), were used as vectors to construct the intertypic DEN virus chimeras.

(i) Chimeric D2/1 plasmids. We previously constructed six D2/1 chimeras containing the C-prM-E gene region of wild-type D1 16007 virus or its vaccine derivative, strain PDK-13, into the three D2 backbones (21). For this study, we constructed chimeras containing the prM-E gene region of D1 16007 virus in the D2 backbones. The prM-E cDNA fragment was amplified by PCR from the previously constructed D2/1 C-prM-E chimera with primers D1-435.Mlu (5′-GCGTTCCATCTGACGACGCGTGGGGGAGAGCCGCATA; the underlined sequence is the *Mlu*I site) and cD1-2394P.Ngo (5′-TGTGACCATGCCGGCTGCGATGCACATCATCGA; the underlined sequence is the *Ngo*MIV site) and was cloned into previously described intermediate D2 clones pD2I-P and

pD2I-E via engineered *Mlu*I-*Ngo*MIV sites (21). The intermediate clones were sequenced to verify the accuracy of the D1 virus-specific cDNA.

Fragments excised from the intermediate D2/1 clones with *Mlu*I and *Ngo*MIV were cloned into the full genome-length D2 vectors pD2-16681-P48, pD2-PDK53-E48, and pD2-PDK53-V48 to generate full-length D2/1 chimeric plasmids pD2/1-P, pD2/1-E, and pD2/1-V, respectively (Table 1). Plasmids were linearized by *Xba*I to generate the 3′ end of the cDNA for RNA transcription. An internal *Xba*I site at nucleotides (nt) 1575 to 1580 of D1 16007 virus was previously removed from the chimeric clones by site-directed mutagenesis (silent T-to-C mutation at nt 1575) (Table 1) (21).

(ii) Chimeric D2/3 plasmids. Multiple attempts to construct full-length chimeric D2/3 infectious clones failed in our laboratory. These plasmids, containing the prM-E genes of D3 16562 virus, were unstable in *Escherichia coli*. Therefore, we used in vitro ligation of two separate, subgenomic plasmids to generate the full-length chimeric D2/3 templates.

The cDNA fragment containing the prM-E genes of wild-type D3 16562 virus was amplified by reverse transcriptase (RT)-PCR from D3 viral RNA with primers D3-435.Mlu (5′-TGCTTTCCACTTAACTACGCGTGATGGAGAG CCGCGCA; the underlined sequence is the *Mlu*I site) and cD3-2394.Ngo (5′-TGTAATGATGCCGGCCGCGATGCATGAAAATGA; the underlined sequence is the *Ngo*MIV site). The amplified fragment was cloned into the *Mlu*I-*Ngo*MIV sites of the intermediate pD2I-P and pD2I-E clones. A restriction site for *Asc*I was introduced 16 nt downstream of the *Ngo*MIV site by site-directed mutagenesis to facilitate the in vitro ligation strategy. An additional mutation at nt 1968 (A to T), which changed amino acid E-345 from His to Leu (Table 1), was also introduced to permit derivation of viable chimeric viruses, as explained in Results. These intermediate chimeric D2/3 clones, pD2I/D3-P and pD2I/D3-E, were sequenced to verify the accuracy of the inserted D3 virus-specific cDNA. A silent C-to-T mutation was identified in both intermediate chimeric clones (Table 1).

Intermediate 3′-end D2 clones containing nt 2203 to 10723 of D2 16681, PDK53-E, or PDK53-V virus were obtained by deleting the 5′ end (including T7 promoter sequence and D2 nt 1 to 2202) of the virus-specific cDNA from the full-length clones pD2-16681-P48, pD2-PDK53-E48, and pD2-PDK53-V48, respectively. An *Asc*I site was also introduced 22 nt upstream of the *Ngo*MIV site to facilitate the in vitro ligation strategy.

The 5′-end pD2I/D3 and 3′-end D2 intermediate clones were digested with *Asc*I, treated with calf intestinal phosphatase, and then digested with *Ngo*MIV. The small excised *Asc*I-*Ngo*MIV fragments were removed by passing the digested DNA through spin columns (Qiagen, Valencia, Calif.). The 5′- and 3′-end linearized intermediate clones were then ligated together to obtain full genome-length chimeric D2/3-P, D2/3-E, and D2/3-V viral cDNAs. The *Asc*I and calf intestinal phosphatase treatments were designed to streamline the process (avoiding gel purification) and optimize the chance of obtaining correctly oriented full genome-length product. These ligated cDNAs were then cut with *Xba*I to produce the linearized 3′ end of the viral cDNA required for viral genomic RNA transcription.

(iii) Chimeric D2/4 plasmids. The cDNA fragment containing the prM-E genes of D4 1036 virus was amplified by RT-PCR from D4 viral RNA with primers D4-453.Mlu (5′-GGCGTTTCACTTGTCAACGCGTGATGGCGAAC CCCTCA; the underlined sequence is the *Mlu*I site) and cD4-2394.Ngo (5′-AG TGATTCCGCCGGCAGCTATGCACGTCATAGCCAT; the underlined sequence is the *Ngo*MIV site). Amplified fragments were cloned into the *Mlu*I-*Ngo*MIV sites of the intermediate pD2I-P and pD2I-E clones. Based on information described in Results, three mutations, C-100 Arg-to-Ser (in the D2 background), E-364 Ala-to-Val, and E-447 Met-to-Leu, were incorporated into the clones (Table 1). The chimeric intermediate D2/4 clones were sequenced before constructing the full genome-length chimeric D2/4 plasmids. A silent mutation resulting from RT-PCR was found at nt 1403 (A to G) in both intermediate clones (Table 1).

Fragments excised from the pD2-16681-P48, -PDK53-VE48, and -PDK53-V48 clones with *Ngo*MIV and *Sca*I (located in the pBR322-derived plasmid vector, downstream of the 3′ end of the D2 genomic cDNA) were cloned into *Ngo*MIV- and *Sca*I-cut chimeric D2/4 intermediate clones to obtain the full-length chimeric pD2/4-P, -E, and -V clones. Plasmids were linearized with *Xba*I to generate the 3′ terminus of the viral cDNA for RNA transcription.

Recovery of recombinant viruses. Recombinant plasmids were grown in *E. coli* XL1-Blue cells, and LLC-MK_2 or BHK-21 cells were transfected with transcribed recombinant viral RNA as described previously (21). Viral proteins expressed in the transfected cells were analyzed by indirect immunofluorescence assay (IFA). Virus-infected cells were fixed in cold acetone for 30 min. D1, D2, D3, and D4 virus-specific monoclonal antibodies 1F1, 3H5, 8A1, and 1H10, respectively, and a polyclonal mouse hyperimmune ascitic fluid against the D2 New Guinea C (NGC) virus were used in the assay. Binding was detected with fluorescein-labeled goat anti-mouse antibody. Viruses were harvested from transfected cell cultures when they were over 30% IFA positive with the polyclonal mouse ascitic fluid against D2 NGC virus, usually 6 to 11 days after transfection, and then were passaged once in LLC-MK_2 cells to obtain working seed stocks of virus.

Replication phenotypes of chimeric viruses in cell culture. For best comparison, all of the virus plaques, including those of the wild-type D1, D3, D4, and D2 backbone virus controls, were measured in the same test by use of the same batch of the LLC-MK_2 cells in six-well plates. Mean plaque diameters were calculated from 10 plaques for each virus 10 days after infection.

Growth for viral growth curves was measured in 75-cm^2 flasks of LLC-MK_2, Vero, or C6/36 cells at a multiplicity of infection (MOI) of approximately 0.001. After adsorption of virus for 2 h, 30 ml of Dulbecco's modified Eagle's medium (LLC-MK_2 and Vero cells) or overlay nutrient medium (C6/36 cells), each containing 5% fetal bovine serum and penicillin-streptomycin, was added, and the cultures were incubated in 5% CO_2 at 37°C (LLC-MK_2 and Vero cells) or 28°C (C6/36 cells). Aliquots of culture medium were harvested at 48-h intervals, adjusted to 12.5% fetal bovine serum, and stored at −80°C prior to plaque titration of virus.

Temperature sensitivity was tested in LLC-MK_2 cells. After virus adsorption for 2 h at 37°C, one set of cultures was incubated for 6 days at 37°C, and the other was incubated at 38.7°C. The virus was designated temperature sensitive if its titer at 38.7°C was reduced by 90% or more relative to its titer at 37°C 6 days after infection.

Sequencing of viral cDNA. The genomes of all of the working seed cultures of the chimeric viruses were fully sequenced, except for about 24 bases at the extreme 5′ and 3′ termini of the genome. Viral genomic RNA was extracted from the virus working seed with the QIAmp viral RNA kit (Qiagen). For each recombinant virus, seven to nine overlapping viral cDNA fragments which covered the entire viral genome were amplified by RT-PCR. They were sequenced by automated sequencing as described previously (21). Primers used for RT-PCR and sequencing will be provided upon request. They were based on the published sequences of D1 16007 (GenBank accession no. AF180817), D2 16681 (GenBank accession no. U87411), or unpublished prM-E sequences of D3 16562 and D4 1036 viruses.

Neurovirulence in suckling mice. Litters of newborn (less than 1 day old) outbred white ICR mice (colony maintained at CDC) were inoculated intracranially with 30 μl of diluent containing 10^4 PFU of virus. They were observed daily for 5 weeks, and a fatal end point was evidenced by moribund status, paralysis, or death.

Immunogenicity and protection in adult mice. Neutralizing antibody responses were tested in AG129 mice that were originally obtained from B & K Universal, Hull, United Kingdom, and are maintained in the animal facility at the Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, Colo. Mice were inoculated intraperitoneally (i.p.) with virus. Groups of mice (six mice per group) received 10^5 PFU of chimeric D2/3 virus, D2/4 virus, or chimeric E or V tetravalent formulations containing all four serotypes (D2 PDK-53 and chimeric D2/1, D2/3, and D2/4) of virus. Immunized mice were boosted with the same dose of the viruses 6 weeks later. Mice were bled 2 days prior to and 26 days after boosting. To study protection, groups of mice (six mice per group) received 10^4 PFU of D1 16007 or chimeric D2/1 virus. One group (11 mice) received phosphate-buffered saline (nonimmunized control). The mice were challenged i.p. with a lethal dose (10^7 PFU; over 20 50% lethal doses) of D1 Mochizuki virus 4 weeks after primary immunization. They were bled 1 day before challenge, and the surviving mice were bled again 30 days after challenge. Within each group, serum specimens were pooled for neutralization assays.

Neutralization assays. The plaque reduction neutralization test (PRNT) was performed in six-well plates of Vero cells as described previously (21). The mouse sera were heat inactivated (56°C for 30 min), and the tests were performed without addition of exogenous complement. Titrations of the input D1 16007, D2 16681, D3 16562, or D4 1036 virus were included in each assay. The neutralizing antibody titer was identified as the highest serum dilution, in serial twofold dilution series, that reduced the number of input virus plaques in the test by at least 50 or 70%.

To assess the specificity of the neutralizing epitopes present in the recombinant viruses, PRNT assays were also performed with hyperimmune mouse ascitic fluids made with D1 Hawaii, D2 NGC, D3 H-87, or D4 H-241 virus, as well as monoclonal anti-D2 3H5 antibody, all of which were obtained from the reference collection at CDC.

TABLE 2. Amino acid mutations found in preliminary D2/3 chimeras

Nucleotide[a]		Amino acid[a]		Mutation in D2/3 chimera[b]								
Position	Change	Position	Change	1P	1V	2V	1E	2E	3E[c]	4E[c]	5E[c]	6E[c]
1957	A-C	E-341	Q-H							H		
1968	A-T	E-345	H-L	L			L	L	L	L	L	L
2168	A-C	E-412	I-L		L							
2178	A-C	E-415	D-A			A						
3121	A-A/G	NS1-234	N-N/D		N/D							
6427	A-G	NS4A-18	T-A			A		A				
8192	A-C	NS5-208	N-T			T						

[a] Positions and changes are given according to the backbone D2 (16681 or PDK-53) virus or the D3 16562 prM-E donor.
[b] Preliminary D2/3 chimeras were derived from the chimeric plasmids containing authentic D2 and D3 sequences. These plasmids were sequenced to confirm the accuracy of the original sequences. Mutations indicated in this table were identified in the chimeric D2/3 viruses passaged once in LLC-MK_2 cells after recovery from transfected BHK-21 or LLC-MK_2 cells.
[c] Virus was sequenced only in the E gene region.

RESULTS

Construction and recovery of chimeric viruses. We successfully constructed chimeras containing the prM-E genes of D1, D3, and D4 viruses in the three D2 virus-specific genetic backbones of wild-type 16681 virus and both PDK53-E and PDK53-V variants of the candidate PDK-53 vaccine virus (Table 1). Viral titers after transfection of LLC-MK_2 or BHK-21 cells were 5 to 6 log_{10} PFU/ml. After one more passage in LLC-MK_2 cells, the working titers increased to 6 to 7 log_{10} PFU/ml. All of the D2/1 chimeras had the expected genomic nucleotide sequences (Table 1).

Originally, mutations occurred at several different positions in the genomes of nine independently derived D2/3 working seeds (Table 2). These mutations were not present in the corresponding plasmids. An A-to-T mutation at nt 1968 changed amino acid E-345 from His to Leu in seven of the nine working seeds (Table 2). This single mutation at E-345 was the only mutation that occurred in the genomes of D2/3-1P and -1E viruses, indicating that this mutation alone stabilized the viruses in culture. We introduced this mutation into all of the D2/3 cDNA clones, and the resulting recombinant viruses were stable and grew to high titers in LLC-MK_2 cells. The genomes of the final working seeds of the D2/3 chimeras all had the expected sequence, including a silent C-to-T mutation at nt 550 that occurred in all of the chimeric D2/3 plasmids (Table 1).

Originally, the chimeric D2/4 infectious clones containing the authentic sequences of D2 and D4 viruses only produced viable chimeric D2/4-P virus in transfected C6/36 cells. We were not able to recover infectious chimeric E or V virus from transfected C6/36 cells, and we were unable to recover any chimeric D2/4 virus from transfected LLC-MK_2 or BHK-21 cells. The D2/4-P chimera recovered from the transfected C6/36 cells produced pinpoint plaques in Vero cell monolayers, and viral titers were limited to 3 to 4.3 log_{10} PFU/ml in the transfected C6/36 cultures. After passage of the chimeric viruses in C6/36 cells one more time to obtain higher viral titers of 3.5 to 5.5 log_{10} PFU/ml, these viruses were passaged in LLC-MK_2 cells five times to obtain D2/4-P chimeras which would replicate efficiently in mammalian cells. In one case, the virus titer increased from 2.3 log_{10} PFU/ml at the first LLC-MK_2 cell passage to 6.5 log_{10} PFU/ml at the fifth LLC-MK_2 cell passage (Table 3). Four mutations, C-100 Arg-to-Ser, E-364 Ala-to-Ala/Val mix, E-447 Met-to-Leu, and NS4B-239 Ile-to-Val, were identified between the first passage in C6/36 cells and the fifth passage in LLC-MK_2 cells (Table 3). The three mutations shown in Table 3 that resided in the structural genes (C-100, E-364, and E-447) were incorporated into the final chimeric D2/4 infectious cDNA plasmids pD2/4-P, -E, and -V (Table 1). All these chimeric D2/4 clones produced viable high-titer (5 to 6 log_{10} PFU/ml) chimeric viruses in transfected LLC-MK_2 cells. The final working seeds of the D2/4 chimeras had the expected genomic sequence, including a silent A-to-G mutation at nt 1403 (Table 1).

To further investigate which mutations were involved in the fitness of the D2/4 chimeras in LLC-MK_2 cells, we engineered four more chimeric D2/4 clones in the D2 PDK53-E48 backbone to contain four different combinations (C-100-Ser, C-100-Ser plus E-447-Leu, E-364-Val plus E-447-Leu, and C-100-Ser plus E-364-Val plus E-447-Leu plus NS4b-239-Val) of the four mutations described in Table 3. C-100-Ser and E-447-Leu were the minimum mutations required for fitness of the D2/4 virus in LLC-MK_2 cells. Chimeras containing C-100-Ser alone or containing E-364-Val plus E-447-Leu were nonviable or unstable without acquiring more mutations after transfection and a single passage in LLC-MK_2 cells (data not shown). The two C-100-Ser and E-447-Leu mutations were also the mutations that occurred earliest in the passage lineage (Table 3). Except for the mutation at E-364, which developed a mixed Ala/Val locus at passage 5, the viruses were genetically stable between the second and fifth LLC-MK_2 passages (Table 3). These viruses were not passaged at the same MOI at each passage and may not have been harvested at peak titer. Therefore, the viral titers in Table 3 likely do not accurately reflect the relative fitness of the viruses at each LLC-MK_2 passage level. The reasons why these specific mutations enabled derivation of the chimeric D2/4 viruses in LLC-MK_2 are not clear at this point, but numerous repeated experiments resulted in the same conclusion, that these mutations increased the fitness of the D2/4 chimeras to replicate in mammalian cells.

Cells infected with chimeric viruses were tested by IFA with anti-E serotype-specific monoclonal antibodies 1F1, 3H5, 8A3, and 1H10. Each chimeric virus reacted only with the monoclo-

TABLE 3. Amino acid mutations found in a preliminary D2/4-P chimera after multiple passages in LLC-MK_2 cells

Cell type (passage no.)	Seed titer (log_{10} PFU/ml)	Presence of mutation			
		C-100[a] R to S	E-364[b] A to V	E-447[b] M to L	NS4B-239[a] I to V
C6/36 (0)[c]	4.3	R	A	M	I
C6/36 (1)	5.1	R	A	M	I
LLC-MK_2 (1)	2.3	S	A	M/L	I
LLC-MK_2 (2)	3.9	S	A	L	V
LLC-MK_2 (3)	5.4	S	A	L	V
LLC-MK_2 (4)	6.1	S	A	L	V
LLC-MK_2 (5)	6.5	S	A/V	L	V

[a] Amino acid positions and changes are based on parental D2 16681 backbone virus.
[b] Amino acid positions and changes are based on D4 1036 prM-E donor virus.
[c] C6/36-0 indicates virus seed recovered from C6/36 cells transfected with original chimeric D2/4 viral RNA.

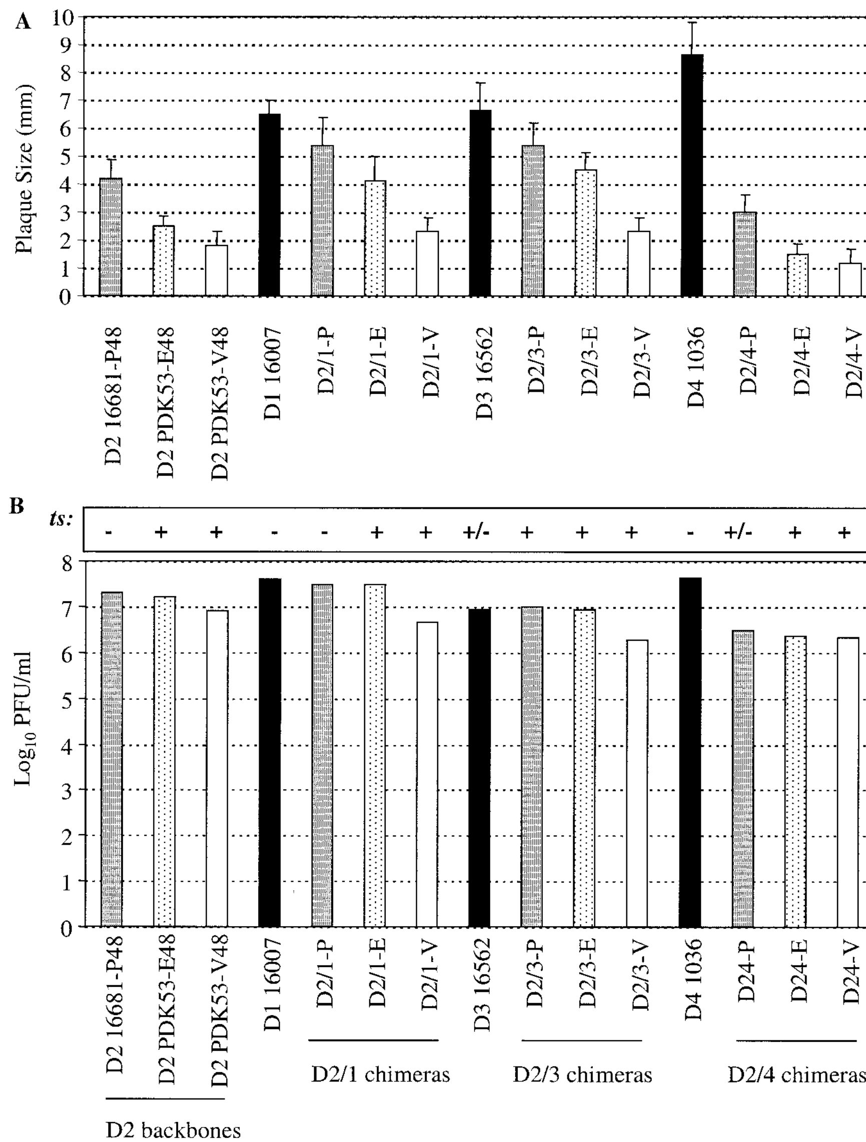

FIG. 1. Growth characteristics of chimeras in LLC-MK_2 cells. (A) Mean (± standard deviation) plaque diameters. Values were calculated from 10 individual plaques of each virus on day 10 after infection. (B) Temperature sensitivity (*ts*) and peak titers of viruses on day 6, 8, or 10 after infection. The *ts* scores were based on the reduction of the virus titers at 38.7°C versus the titers at 37°C (+ indicates titer reduction of 90% or greater at 38.7°C; +/− indicates reduction in the range that crosses the 90% cutting point from multiple experiments). The graph bar heights represent the log_{10} titers of the viruses at 37°C. The MOI was approximately 0.001 PFU/cell. Gray bars, D2 16681-P48 virus and chimeras containing that background; stippled bars, viruses containing the D2 PDK53-E48 background; white bars, D2 PDK53-V48 virus and chimeras with that background; black bars, wild-type D1, D3, and D4 viruses.

nal antibody that was specific for the E protein of the appropriate DEN virus serotype engineered in the chimera (data not shown), indicating that these chimeras successfully expressed and processed the serotype-specific prM-E proteins.

Plaque phenotypes of the chimeras. All of the chimeric D2/1, D2/3, and D2/4 viruses produced significantly smaller plaques than their prM-E donors, wild-type D1 16007, D3 16562, and D4 1036 viruses, respectively (Fig. 1A). The plaques of chimeric D2/1 and D2/3 viruses were larger than the plaques of their corresponding D2 backbone viruses (D2/1-P and D2/3-P versus D2 16681-P48 virus, D2/1-E and D2/3-E versus D2 PDK53-E48 virus, and D2/1-V and D2/3-V versus D2 PDK53-V48 virus). All three D2/4 chimeras produced smaller plaques than their backbone D2 viruses. The relative plaque sizes of the chimeric P, E, and V viruses for each prM-E serotype corresponded to the relative sizes of their backbone D2 viruses 16681-P48, PDK53-E48 and PDK53-V48, respectively. The chimeric P viruses produced the largest plaques in each serotype-specific prM-E group of chimeras, followed by the chimeric E viruses and then the chimeric V viruses.

Growth of the chimeras in LLC-MK_2, Vero, and C6/36 cell cultures. Temperature sensitivity was scored positive when viral growth was reduced by 90% or more at 38.7°C relative to the titer at 37°C (Fig. 1B). D1 16007, D2 16681-P48, and D4 1036 viruses were not temperature sensitive. D3 16562 virus

had an ambiguous (±) score. Wild-type D1 16007 virus was the least temperature-sensitive virus (titer reduced by 45 to 55%), and D2 16681-P48 virus showed 71 to 88% titer reduction at 38.7°C, similar to previously reported results (21). In multiple tests, D3 16562 and D4 1036 viral titers were reduced by 82 to 93% and 66 to 87%, respectively, at 38.7°C. Chimeras constructed in the D2 16681-P48 backbone showed different levels of temperature sensitivity. The D2/1-P chimera was not temperature sensitive (56 to 79% reduction), D2/4-P virus was somewhat sensitive with a ± score (87 to 95% reduction), and D2/3-P virus was temperature sensitive (92 to 98% reduction). Both D2 PDK53-E48 and -V48 viruses were temperature sensitive, with more than 90% reduction, and the chimeras (D2/1-E and -V, D2/3-E and-V, D2/4-E and -V) utilizing these two backbones reflected this level of temperature sensitivity. With 95 to 99.9% titer reduction at 38.7°C, the chimeric V viruses showed the greatest temperature sensitivity, while the chimeric E viruses demonstrated 90 to 98% reductions in titer.

All of the chimeric viruses reached peak titers of 6.3 to 7.7 $\log_{10}$ PFU/ml between 6 and 10 days after infection in LLC-MK_2 cells (Fig. 1B). PDK53-V48 virus and the three chimeric V viruses had the lowest peak titers within each serotype-specific group. The chimeric D2/1-P and -E viruses attained peak titers that were similar to the peak titer of the wild-type D1 16007 virus and greater than the peak titers of their backbone 16681-P48 and PDK53-E48 viruses, respectively. The D2/3-P and -E chimeras had peak titers that were close to those of wild-type D3 16562 and their backbone viruses. Chimeric D2/4-P and -E viruses, however, had peak titers that were 14.5- and 18.8-fold lower than that of wild-type D4 1036 virus and 6.5- and 6.4-fold lower than the peak titers of the backbone 16681-P48 and PDK53-E48 viruses, respectively. Although all three D2/4 chimeras reached similar peak titers in LLC-MK_2 cells, the D2/4-E and -V chimeras replicated somewhat slower than D2/4-P and other chimeras. D2/4-E and -V chimeras reached peak titers at approximately 8 to 10 days postinfection, while other chimeras reached peak titers between 6 and 8 days postinfection.

Vero cells were also used to assess the replication efficiency of the chimeric viruses that were constructed within the D2 PDK-53 vaccine backbones (E48 and V48). All of the viruses reached peak titers of 6.7 to 7.2 $\log_{10}$ PFU/ml 10 days after infection (Fig. 2A). The D2/1 and D2/3 chimeras reached peak titers that were comparable to the peak titers of their backbone PDK53-E48 (7.6 $\log_{10}$ PFU/ml) and PDK53-V48 (7.3 $\log_{10}$ PFU/ml) viruses, respectively. Chimeric D2/4 viral peak titers were again slightly lower (Fig. 2A).

In C6/36 cells, the backbone 16681-P48 virus replicated much more efficiently (peak titer of 8.4 $\log_{10}$ PFU/ml) than did the D2PDK53-E48 and -V48 viruses (5.5 and 4.5 $\log_{10}$ PFU/ml, respectively) within the 12 days of incubation (Fig. 2B). The chimeras constructed in the 16681-P48 backbone (chimeric P viruses) grew better than the chimeras with the PDK53-E48 backbone (chimeric E viruses), which in turn grew somewhat more efficiently than chimeras with the PDK53-V48 backbone (chimeric V viruses). The chimeric P viruses had lower peak titers than their backbone 16681-P48 and parental D1 16007, D3 16562, and D4 1036 viruses, respectively. The chimeric D2/3-E and D2/4-E viruses reached peak titers that were somewhat lower than that of their backbone PDK53-E48 virus. Titers of the D2/3-V and D2/4-V viruses were close to that of PDK53-V48 backbone virus. The D2/1-E and -V chimeras replicated to peak titers that were more than 10-fold lower than the peak titers of the backbone PDK53-E48 and -V48 viruses, respectively. Like the relative peak titers of D2 PDK53-E48 and PDK53-V48 viruses, the peak titer of D2/1-V virus was 10-fold lower than that of D2/1-E virus.

Neutralization of chimeric viruses. To assess the specificity of the neutralization epitopes of the chimeric viruses, we tested the chimeric P viruses against anti-DEN virus mouse ascitic fluids and a monoclonal anti-D2 antibody in a PRNT (70% endpoints shown in Table 4). Anti-D1, -D3, and -D4 ascitic fluids neutralized the appropriate serotype-specific wild-type and chimeric P viruses to equivalent reciprocal titers. Although D2 polyclonal ascitic fluid showed variable cross-reactivity against the heterologous DEN viruses, the monoclonal antibody 3H5, which neutralized D2 16681 virus to a high titer, cross-reacted minimally or not at all with viruses containing the E protein of D1, D3, or D4 virus. All three chimeric P viruses expressed serotype-specific neutralizing epitopes similar to their parental, wild-type prM-E donor viruses.

Neurovirulence in suckling mice. We expected that the D2/1 chimeras in the PDK53-E or -V backbone in this study would be non-neurovirulent for newborn ICR mice, because the wild-type D1 16007 virus itself was only slightly neurovirulent, causing some weight loss in newborn mice without mortality (21). Our previous D2/1 chimeras, constructed with C-prM-E of D1 16007 virus in PDK-53 backgrounds, were avirulent for newborn ICR mice (21). Therefore, for this study we focused on the D2/3 and D2/4 chimeras. The D2/3-P and D2/4-P chimeras were not as lethal as their backbone 16681-P48 virus or their prM-E donor viruses, D3 16562 and D4 1036 (Fig. 3). However, they still killed 32.5 and 62.5% of the mice, respectively, with longer average surviving times. The backbone PDK53-E48 and -V48 viruses as well as all of the D2/3 and D2/4 chimeric E and V viruses constructed in these backbones were non-neurovirulent for newborn ICR mice.

Immunogenicity and protective efficacies of chimeras in mice. AG129 mice have been used previously as a mouse challenge model for D2 viruses, including the D2 PDK-53 strain (23). A D1 virus, strain Mochizuki, which was passaged twice in mouse brain and once in C6/36 cells, was lethal for adult AG129 mice when administered by i.p. inoculation (A. J. Johnson and J. T. Roehrig, unpublished data). Table 5 shows the protective effect of our D2/1 chimeras against lethal D1 Mochizuki viral challenge in AG129 mice. Mice immunized with D1 16007 or D2/1 chimeras all survived virus challenge. We included our previous D2/1 chimeras, D2/1-EP and -VP, constructed with the C-prM-E gene region from D1 16007 virus (21), for comparison with the D2/1 chimeras containing the prM-E gene of D1 16007 virus. The chimeras expressing the prM-E genes of D1 virus elicited higher primary neutralizing titers (pooled reciprocal titers, which neutralized at least 70% of the input virus [$PRNT_{70}$ titers], of 320 to 1,280) than the chimeras with C-prM-E genes of D1 virus (pooled reciprocal $PRNT_{70}$ titer of 80). The PRNT titers elicited by D2/1-P, -E, and -V chimeras in the AG129 mice were equivalent to the titers elicited by the wild-type D1 16007 virus. All of the surviving mice had high neutralizing antibody titers against D1 16007 virus after challenge. All of the mice inoculated with

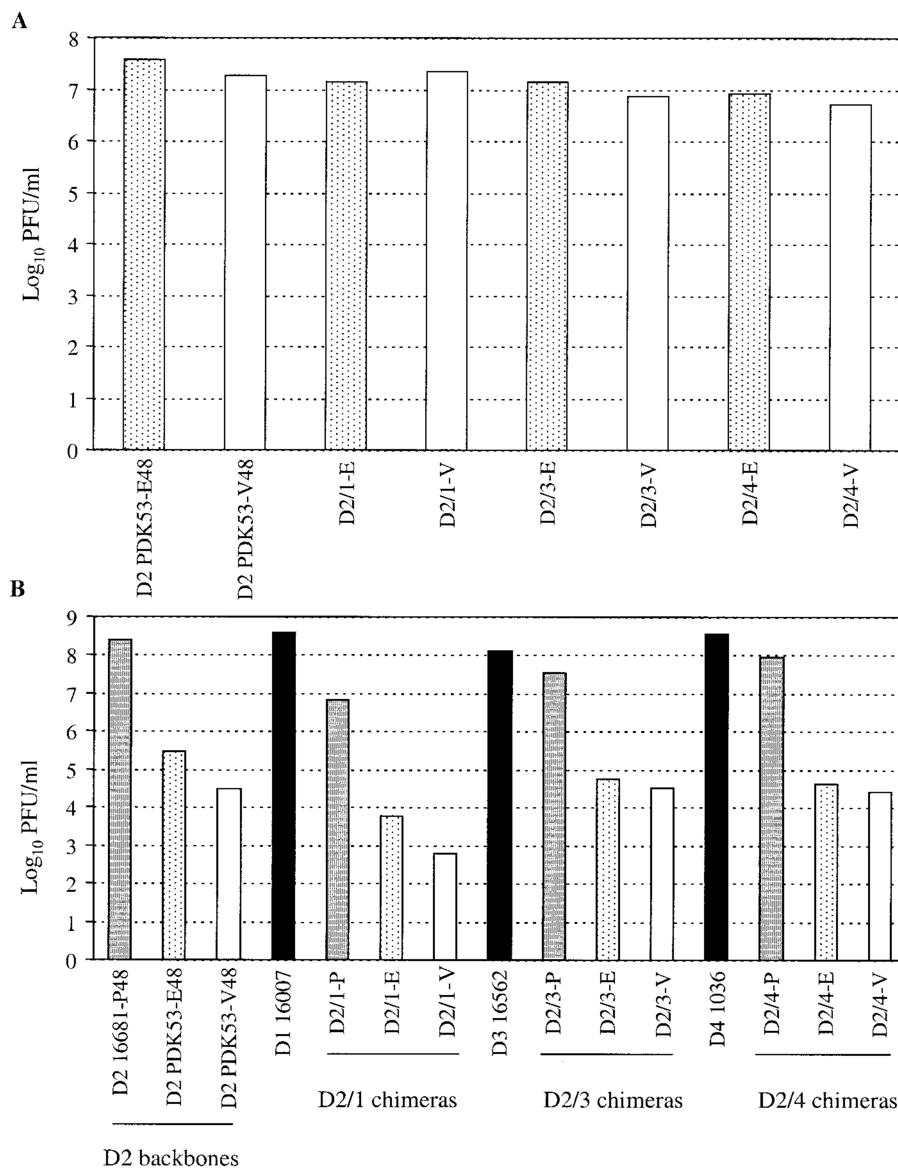

FIG. 2. Growth characteristics of chimeras in Vero or C6/36 cells. Cells were infected at an approximate MOI of 0.001 PFU/ml. (A) Peak titers of chimeric E and V viruses in Vero cells at day 10 postinfection. (B) Peak titers of viruses in C6/36 cells within 12-day cultures. Gray bars, D2 16681-P48 virus and the chimeras within that background; stipple bars, D2 PDK53-E48 virus and the chimeras within that carrier; white bars, D2 PDK53-V48 virus and chimeras with that backbone; black bars, wild-type D1, D3, and D4 viruses.

phosphate-buffered saline (mock immunization) died between 6 and 15 days (average survival time of 11.5 ± 4.2 days) after challenge.

We tested the immunogenicity of the D2/3 and D2/4 chimeras in AG129 mice, but we did not perform virus challenges due to the lack of D3 and D4 strains that are lethal for AG129 mice. The neutralizing antibody titers shown in Table 6 were against the homologous wild-type virus (D3 16562 virus for D2/3 chimeras and D4 1036 virus for D2/4 chimeras). All of the D2/3 and D2/4 chimeric viruses were immunogenic in these mice. Primary titers elicited by the D2/3 chimeras were slightly lower or equivalent to the titer induced by wild-type D3 16562 virus. Chimeric D2/3 boosted titers were similar to that of D3 16562 virus. The D2/4-P chimera was as immunogenic as the wild-type D4 1036 virus in mice. However, the D2/4-E and -V chimeras containing the same structural proteins as the D2/4-P virus elicited lower primary and boosted titers than did the D2/4-P chimera.

We also immunized AG129 mice with two tetravalent virus formulations. The tetra-E formulation contained D2/1-E, D2 PDK53-E48, D2/3-E, and D2/4-E viruses (all with the PDK53-E48 backbone), while the tetra-V formulation contained D2/1-V, D2 PDK53-V48, D2/3-V, and D2/4-V viruses (with the PDK53-V backbone). Both the tetra-E and tetra-V formulations elicited neutralizing antibody titers against all four DEN virus serotypes after primary immunization (Table 7). Similar titers were elicited by both tetra-E and tetra-V formulations. Titers were highest versus the D2 16681 virus and lowest versus the D4 1036 virus. The chimeric E and V tetravalent formulations elicited primary $PRNT_{50}$ titers against D1, D3, and D4

TABLE 4. Neutralization titers of mouse anti-DEN ascitic fluids (AF) and monoclonal anti-D2-3H5 antibody against chimeric P and parental viruses

Virus	PRNT titer[a]				
	D1-AF	D2-AF	D2-3H5	D3-AF	D4-AF
D2 16681	20	1,280	>40,960	20	<20
D1 16007	320	20	20	20	<20
D2/1-P	320	40	<20	20	20
D3 16562	40	80	<20	1,280	20
D2/3-P	80	320	20	1,280	80
D4 1036	<20	20	<20	<20	320
D2/4-P	<20	40	<20	20	640

[a] Reciprocal antibody dilution that reduced input D1 16007, D2 16681, D3 16562, or D4 1036 virus 70% or more. D1-, D2-, D3-, and D4-AF are hyperimmune ascitic fluids against D1, D2, D3, and D4 viruses, respectively.

TABLE 5. Immunogenicity and protective efficacy of D2/1 chimeras in AG129 mice[a]

Virus	PRNT titer before challenge[b]	After challenge	
		No. of survivors/N[c]	PRNT titer[b]
PBS control	<10 (<10)	0/11	NA[d]
D1 16007	640 (2,560)	6/6	2,560 (10,240)
D2/1-P	1,280 (2,560)	6/6	2,560 (2,560)
D2/1-E	320 (1,280)	6/6	2,560 (2,560)
D2/1-V	640 (640)	6/6	2,560 (2,560)
D2/1-EP	80 (160)	6/6	1,280 (2,560)
D2/1-VP	80 (160)	6/6	1,280 (1,280)

[a] Three- to five-week-old AG129 mice were immunized with 10^4 PFU of virus and challenged with 10^7 PFU of D1 Mochizuki virus 4 weeks after immunization.
[b] Reciprocal dilution of the pooled sera from each group that neutralized at least 70% (or 50%, in parentheses) of the input D1 16007 PFU.
[c] N, total number of mice per group.
[d] NA, not applicable.

viruses (Table 7) that were similar to those elicited by monovalent immunization with chimeric D2/1, D2/3, or D2/4 virus (Tables 5 and 6). High boosted titers, including those against D4 virus, were induced by these tetravalent formulations.

DISCUSSION

Three major genetic determinants, including the mutations 5′ NC-57 C-to-T, NS1-53 Gly-to-Asp, and NS3-250 Glu-to-Val, contribute to the phenotypic attenuation markers of the candidate D2 PDK-53 vaccine virus (9). The only amino acid mutation that occurs in the structural protein region of the PDK-53 viral genome, prM-29 Asp-to-Val, has little or no effect on the viral phenotype (9). Restriction of its attenuation loci outside of the structural gene region makes the PDK-53 viral genetic background ideal for development of chimeric DEN virus or other flavivirus vaccine candidates. We previously showed that chimeric D2/1 viruses containing the C-prM-E region of wild-type D1 16007 virus or the C-prM-E region of the Mahidol candidate D1 PDK-13 vaccine virus retained the D2 PDK-53 characteristic attenuation markers (21). For this report, we constructed chimeras that expressed the prM-E proteins of wild-type D1, D3, or D4 virus in the D2 16681-P48, PDK53-E48, and PDK53-V48 backbones to further investigate the potential application of the attenuated genetic background of PDK-53 virus to chimeric DEN virus vaccine development. Relative to the appropriate wild-type virus, all of the chimeras that we constructed within the PDK53-E48 or -V48 backbone retained the D2 PDK-53 attenuation markers, including temperature sensitivity and reduced plaque size in mammalian LLC-MK_2 cells, decreased ability to replicate in mosquito cell culture, and lack of neurovirulence in newborn mice. We demonstrated the immunogenicity and, in the case of D2/1 chimeras, protective efficacy of the chimeras as experimental vaccines against DEN viral challenge in mice.

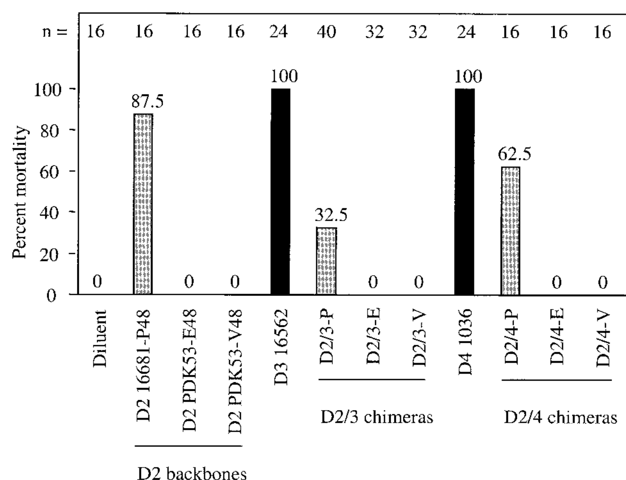

FIG. 3. Neurovirulence of chimeric D2/3 and D2/4 viruses in newborn mice. Newborn ICR mice were inoculated with 10^4 PFU of virus by the intracranial route. Percent mortality is indicated directly over each graph bar. Gray bars, D2 16681-P48 virus and the chimeras within that background; black bars, wild-type D3 and D4 viruses. n, number of mice per group, indicated at the top of the figure. Average survival times of D2 16681-P48, D3 16562, D2/3-P, D4 1036, and D2/4-P viruses were 15.6 ± 2.6, 14.1 ± 2.1, 19 ± 2.1, 8.6 ± 0.6, and 17.8 ± 2.8 days, respectively.

TABLE 6. Immunogenicity of D2/3 and D2/4 chimeras in AG129 mice[a]

Virus	PRNT titer[b]	
	Primary	Boosted
D3 16562	160 (320)	320 (640)
D2/3-P	160 (320)	320 (640)
D2/3-E	40 (160)	160 (640)
D2/3-V	80 (160)	320 (640)
D4 1036	320 (640)	640 (1,280)
D2/4-P	160 (320)	320 (640)
D2/4-E	20 (80)	160 (320)
D2/4-V	20 (40)	80 (320)

[a] Six- to eight-week-old AG129 mice were immunized with 10^5 PFU of virus, bled on day 40, and boosted with the same dose of virus on day 42. Mice were bled again 26 days after boosting.
[b] Reciprocal dilution of pooled sera in each group yielding at least 70% (or 50%, in parentheses) plaque reduction of the D3 16562 (for D3 and D2/3 groups) or D4 1036 (for D4 and D2/4 groups) virus.

TABLE 7. Immunogenicity of tetravalent chimeric DEN viruses in AG129 mice[a]

Viruses	PRNT titer[b]							
	D1 16007		D2 16681		D3 16562		D4 1036	
	Primary	Boosted	Primary	Boosted	Primary	Boosted	Primary	Boosted
Tetra-E	160 (640)	640 (1,280)	640 (1,280)	1,280 (1,280)	80 (160)	320 (640)	40 (80)	160 (320)
Tetra-V	160 (640)	640 (2,560)	640 (1,280)	1,280 (2,560)	80 (160)	640 (1,280)	40 (80)	160 (320)

[a] Six- to eight-week-old AG129 mice were immunized with tetravalent virus containing 10^5 PFU each of D2/1-E, D2 PDK53-E, D2/3-E, and D2/4-E viruses (Tetra-E) or D2/1-V, D2 PDK53-V, D2/3-V, and D2/4-V viruses (Tetra-V), bled on day 40, and boosted with the same tetravalent dose of viruses on day 42. Mice were bled again 30 days after boosting.

[b] Reciprocal dilution of the pooled sera in each group yielding at least 70% (or 50%, in parentheses) plaque reduction of D1 16007, D2 16681, D3 16562, and D4 1036 viruses.

Genome sequence and fitness-enhancing amino acid substitutions of the chimeric viruses. Genome sequencing of our chimeric D2/1 viruses revealed no unexpected mutations at LLC-MK_2 passage 2 or 3 posttransfection. However, our original chimeric D2/3 and D2/4 cDNA clones produced either no infectious chimeric virus (D2/4) or chimeric virus with low replication efficiency (D2/3) following transfection of mammalian cells. We identified and incorporated into our cDNA clones mutations that imparted to chimeric D2/3 and D2/4 viruses fitness for replication in LLC-MK_2 and Vero cells. A single E-345 His-to-Leu mutation was engineered into the D2/3 chimeras. The permissive mechanism of this mutation is unclear. However, the D3 E-345 locus aligns with the E-352 locus of tick-borne encephalitis (TBE) virus. This locus resides on the lateral surface, just upstream of the D strand, of domain III in the E protein of TBE virus (36). Mutations in the lateral surface of domain III have been associated with changes in host range, tropism, and virulence or attenuation (36).

To enhance genetic stability and replication efficiency in mammalian cell culture, we engineered three mutations, C-100 Arg-to-Ser, E-364 Ala-to-Val, and E-447 Met-to-Leu, into the D2/4 chimeras. We later determined that the C-100 and E-447 mutations were sufficient to stabilize the D2/4 virus in mammalian cells. According to sequence alignment with TBE virus, the D2/4 E-447 Met-to-Leu mutation is located in a predicted α-helical (H2) element in the stem region of the flaviviral E protein. This H2 element was found to be important for the stability of the prM-E dimer (1). Most other sequenced flaviviruses, including D1 virus, D3 virus, viruses of the Japanese encephalitis complex, and yellow fever virus, contain Leu at this position after sequence alignment. D2 viruses contain Ala at this position and TBE viruses possess Leu or Ile. The engineered C-100 Arg-to-Ser substitution in the D2/4 chimeras occurs at the flaviviral polyprotein site R/KR/K ↓ S/T/G, which is cleaved by the viral NS2B-NS3 protease complex to convert the intracellular form of the C protein to virion C protein. The chimeric D2/4 C-100 Arg-to-Ser change may shift the cleavage site from the original D2 virus-specific $RRRR_{C\text{-}100}$ ↓ SA to RRR ↓ $S_{C\text{-}100}$SA of the D2/4 chimeras, which would mean that the C-100-Ser of the D2/4 chimeras was cleaved from the mature virions. Wild-type D4 virus contains only three basic amino acids preceding this polyprotein cleavage site (GRRR ↓ S), while D1, D2, and D3 viruses all have four basic amino acids (K or R). The C-100 Arg-to-Ser substitution in the D2/4 chimeras may present a more appropriate polyprotein sequence motif for processing and maturation of the proteins expressed by the chimeric D2/4 viral genome in mammalian cells.

The fitness-enhancing amino acid substitutions that we incorporated into our D2/3 and D2/4 chimeras had no apparent effect on the attenuation markers or immunogenicity of these viruses. The prM and E proteins of the chimeras appeared to possess the appropriate antigenic properties, as suggested by their serotype-specific reactivity against monoclonal antibodies in IFA and by their serotype-specific neutralizing epitopes (Table 4). Amino acid substitutions in structural proteins have been identified in other flaviviral chimeras after transfection and multiple passages in cell cultures (17). Until we have a better understanding of flaviviral protein sequences and interactions during intracellular viral maturation and in mature virions, developing certain chimeric flavivirus vaccine candidates may involve empirical identification of fitness-enhancing genomic mutations that permit successful genetic engineering of replication-competent, immunogenic chimeric viruses.

Replication phenotypes of chimeric viruses in cell cultures. Reduction of plaque size in LLC-MK_2 cells relative to the plaque sizes of their wild-type prM-E donor viruses was observed for all of the chimeras. These results were consistent with the previous demonstration that the 5′NC-57-T, NS1-53-Asp, and NS3-250-Val mutations all contribute to the small-plaque phenotype of the candidate PDK-53 vaccine virus (9). The results also indicated that the plaque phenotype was determined by both the donor prM-E genes and the D2 carrier background. However, the D2/4 chimeras produced smaller plaques than the respective carrier D2 viruses, even though the D4 1036 virus produced the largest plaques among all of the viruses tested in this report. The D2/4 chimeras also generally grew to somewhat lower peak titers than other chimeras and the D2 backbone viruses in LLC-MK_2 and Vero cells. These results indicated that some incompatibility still existed between the D4 prM-E genes and the carrier D2 background in our genetically modified D2/4 chimeras. Flavivirus replication involves *cis*-acting interactions between viral structural proteins, nonstructural proteins, and RNA structures, and these interactions may not have been fully compatible in the D2/4 chimeras. Nevertheless, all of the D2/1, D2/3, and D2/4 chimeric E and chimeric V viruses replicated to peak titers of 6.3 to 7.4 $\log_{10}$ PFU/ml in LLC-MK_2 and Vero cells. The efficient growth of these chimeras in Vero cells is encouraging, because this is one of the cell lines that are licensed for manufacturing viral vaccines.

The temperature-sensitive phenotype of D2 PDK-53 virus has been attributed to a synergism between the NS1-53-Asp and NS3-250-Val loci (9). The PDK53-V variant, containing both the NS1-53 and NS3-250 loci, was more temperature sensitive than the PDK53-E variant with only the NS1-53 mutation of PDK-53 virus (9, 21). In the present study, all of the chimeric E and V viruses retained the temperature-sensitive phenotype of their respective D2 PDK-53 carriers. Although we observed that the chimeric V viruses usually were slightly more temperature sensitive than the chimeric E viruses within the same serotype-specific prM-E chimeric group, the differences were not as obvious as the difference between PDK53-V and PDK53-E viruses.

In *Aedes albopictus* C6/36 cells, peak titers of the chimeric P viruses were lower than those of their respective wild-type prM-E donor viruses (Fig. 2B), suggesting a certain level of incompatibility between the replication machinery of the D2 backbone and the heterologous structural gene region. Reduced replication of D2 PDK-53 virus in *Aedes aegypti* (27) and C6/36 cells (9, 29) may constitute a biological attenuation marker of PDK-53 virus. Low oral infection and dissemination rates in mosquitoes have been suggested to represent attenuation markers for flaviviruses (7, 13, 22, 24, 33, 34, 42). The PDK-53 virus is not transmitted by *A. aegypti* mosquitoes (27). This restriction reduces the potential for secondary transmission of the vaccine virus, thereby providing an important safety factor. The C6/36 replication phenotype of PDK-53 virus may reflect its restricted replication in mosquitoes (29). Similar low-replication phenotypes have been observed in C6/36 cells for a D4 vaccine candidate, 2AΔ30 (43), and a D4 mutant containing a deletion in the 5′ NCR (11). The crippled C6/36 replication phenotype of D2 PDK-53 virus is encoded predominantly, independently and synergistically, by the 5′NC-57 C-to-T and NS1-53 Gly-to-Asp mutations (9). In our present study, all of the chimeric E and V viruses showed decreased peak replication titers that were approximately 10^3- to 10^4-fold lower than the peak titers of the chimeric P viruses and approximately 10^3- to 10^6-fold lower than the wild-type D1, D3, and D4 viruses in C6/36 cells. Based on the previous analysis of D2 PDK-53 viral replication in mosquitoes (27), we predict that the limited replication phenotype of the D2/1-, D2/3-, and D2/4-E and -V chimeras in C6/36 cells is an accurate indicator of their relative incompetence for replication, dissemination, and transmission in mosquitoes.

Attenuation, immunogenicity, and protective efficacy of the chimeric viruses in mice. A unique characteristic of the D2 PDK-53 virus is that the determinants of its attenuated phenotype in mice reside at the 5′ NCR-57 and NS1-53 loci (9). Most studies have identified major mouse virulence determinants in the E protein of the investigated flaviviruses (2, 12, 26). Previously, we showed that attenuation markers were retained in chimeric D2/1 viruses (21). In this study, we demonstrated that the D2 PDK53-E48 and -V48 backbones were sufficient to attenuate the D2/3-E, D2/3-V, D2/4-E, and D2/4-V chimeras expressing the prM-E proteins from the more neurovirulent (in mice) wild-type D3 16562 and D4 1036 viruses. Chimeric D2/3-P and D2/4-P viruses constructed in the wild-type D2 16681-P48 background retained neurovirulence for newborn ICR mice. However, the D2/3-P and D2/4-P viruses were less virulent than their wild-type D3 and D4 prM-E donor viruses, as well as the D2 16681-P48 carrier virus, indicating that the juxtaposition of heterologous genes in the chimeric DEN viruses further contributed to attenuation in mice.

AG129 mice, which lack alpha/beta interferon and gamma interferon receptor genes, have been successfully used as a mouse model for D2 vaccine testing (23). In this study, single-dose immunization of AG129 mice with chimeric D2/1 viruses induced high-titer neutralizing antibodies and protected all immunized mice from lethal challenge with the virulent D1 Mochizuki virus. $PRNT_{70}$ titers against D1 16007 virus elicited by chimeric D2/1-P, -E, and -V viruses were equal to or within twofold of the titers elicited by wild-type D1 16007 virus. This suggests that the prM-E proteins expressed by these chimeras provided optimal immunogenicity in these mice. Previous chimeras D2/1-EP and -VP, constructed with the C-prM-E proteins from D1 16007 virus, elicited lower titers than those elicited by current D2/1-E and -V chimeras, constructed with prM-E proteins of the same D1 16007 virus, suggesting that the D2/1-EP and -VP chimeras may not replicate as well as D2/1-E and -P chimeras in AG129 mice. The flaviviral C protein is not only a structural component of mature virions; it also appears to be involved in viral replication. Studies with subgenomic replicons of Kunjin virus suggest that the first 20 amino acids of C or the RNA sequence itself plays a role in virus replication (28). Therefore, DEN virus chimeras maintaining the D2 virus-specific C gene in the D2 backbones might replicate with efficiencies more similar to that of the D2 virus itself.

All of the D2/3 and D2/4 chimeras were immunogenic in AG129 mice, and one or two immunizations were sufficient to induce significant neutralizing antibodies. The D2/3 chimeras elicited primary and boosted $PRNT_{50}$ titers that were similar to those induced by wild-type D3 16562 virus, suggesting that optimal immunogenicity of the D2/3 chimeras in AG129 mice was achieved and that the single engineered mutation at the E-345 locus did not affect this immunogenicity. The D2/4-P chimera appeared to be as immunogenic as the wild-type D4 1036 virus in AG129 mice, again indicating that the three mutations engineered into the D2/4 chimeras did not adversely affect their immunogenicity. Although the D2/4-E and -V chimeras expressed the same prM-E structural proteins as the D2/4-P chimera, neutralizing titers elicited by these two chimeras were somewhat lower than those elicited by the D2/4-P and D4 1036 viruses. Though the peak titers among D2/4 chimeras were similar in LLC-MK_2 cells, we observed that the D2/4-E and -V chimeras were slower to reach their peak titers (8 to 10 days postinfection) than was the D2/4-P chimera (6 to 8 days postinfection). This lower replication rate in cell culture suggested a possible slower growth of these chimeras in mice, which might affect their immunogenicity. Boosted reciprocal neutralizing antibody titers elicited by D2/4-E and -V viruses in the mice were high, only fourfold lower than the boosted $PRNT_{50}$ titers elicited by wild-type D4 1036 virus. We were unable to determine protective efficacies of the D2/3 and D2/4 chimeras in AG129 mice. However, these chimeras should be protective based on the primary or boosted neutralizing titers, which were close to the protective primary neutralizing titers elicited by D2/1 chimeras in mice. Tetravalent formulations of chimeric E or V viruses successfully induced neutralizing antibodies against all four DEN virus serotypes after primary immunization. The reciprocal $PRNT_{50}$ titers were very similar

to the homologous titers elicited by each monovalent virus, indicating that no significant interference occurred among the four serotype viruses in the tetravalent formulations. In mice, there was no difference between the levels of immunogenicity of the E chimeras and those of the V chimeras within each serotype-specific experimental group.

Neurovirulence and immunogenicity results in mice may not accurately reflect the human response to DEN virus infection. Nevertheless, D2/1-, D2/3-, and D2/4-E and -V chimeras retained all of the investigated phenotypic attenuation markers that are associated with the D2 PDK-53 vaccine candidate, and they induced neutralizing antibodies following primary immunization in mice. Comparison of the chimeric E and V viruses with chimeric P viruses clearly demonstrated the specific attenuating effects of the PDK53-E and PDK53-V genetic backgrounds. Reversion of the D2 PDK-53 vaccine virus to the virulent 16681 wild-type viral attributes for these phenotypic markers required at least simultaneous back mutations at the two dominant attenuating loci, 5′-NCR-57 and NS1-53 (9). Furthermore, our previous chimeric D2/1 constructs in the two PDK-53 backbones have been shown to be safe and immunogenic in monkeys (10).

Applications of D2 PDK-53 virus as carrier for DEN virus vaccine development. Engineering of chimeric flaviviruses for vaccine development has been intensely investigated in recent years (recently reviewed in reference 30). Heterologous flaviviral structural proteins have been expressed in chimeras based on several flaviviral vectors, including D4 814669 (8, 15, 32), yellow fever virus 17D (17, 18), and D2 PDK-53 (21). The D2 PDK-53 vaccine virus is unique, because the genomic determinants of its characteristic phenotypic markers of attenuation have been identified, and these determinants reside outside of the structural gene region. The attenuated PDK-53 vectors may permit straightforward development of chimeric flaviviral vaccine candidates, because introducing additional determinants of attenuation into the donor prM-E genes of the heterologous flavivirus may not be necessary to preserve the attenuated phenotype of the resulting chimera. This uniform strategy affords the possibility of expressing prM-E proteins of optimal immunogenicity in possible situations where immunization with region-specific strains of virus may be desirable. It is likely that the fitness-enhancing amino acid mutations incorporated into our D2/3 and D2/4 viruses would also be effective in expressing the prM-E genes of other D3 and D4 strains. If these engineered mutations have no deleterious effect on chimeric viral immunogenicity, as seemed to be the case for this report, then this strategy may permit the engineering of chimeric flaviviral vaccines possessing optimal, wild-type immunogenicity. Because all four components of a tetravalent DEN virus vaccine utilizing the D2 PDK-53-E or -V vector would possess identical loci of attenuation outside of the structural gene region, any potential recombination events in the vaccinee would result in either no change in the resulting recombinants, in the case of recombination outside of the prM-E gene region, or preservation of the PDK-53 characteristic attenuation markers in genomes resulting from recombination in the prM-E gene region. Uniform genetic loci of attenuation should permit more straightforward design of rapid genetic assays to verify retention of these loci during vaccine manufacturing and during follow-up surveys of virus isolation during field testing of the tetravalent vaccine. Finally, a tetravalent DEN virus vaccine whose individual viral components possess the uniform replicative machinery of the PDK-53 virus may replicate more efficiently and uniformly in human vaccinees to induce efficient immunity against all four DEN virus serotypes.

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