

(19) **DANMARK**

(10) **DK/EP 2338508 T3**



Patent- og
Varemærkestyrelsen

(12) Oversættelse af
europæisk patentskrift

-
- (51) Int.Cl.: **A 61 K 39/00 (2006.01)** **A 61 K 39/12 (2006.01)** **A 61 K 39/42 (2006.01)**
C 07 H 21/04 (2006.01) **C 12 N 7/00 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2018-05-28**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2018-02-14**
- (86) Europæisk ansøgning nr.: **10177735.7**
- (86) Europæisk indleveringsdag: **2003-04-25**
- (87) Den europæiske ansøgnings publiceringsdag: **2011-06-29**
- (30) Prioritet: **2002-05-03 US 377860 P** **2002-12-23 US 436500 P**
- (62) Stamansøgningsnr: **03724319.3**
- (84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PT RO SE SI SK TR**
- (73) Patenthaver: **The Government of the United States of America, as represented by the Secretary Department of Health and Human Services, National Institutes of Health Office of, Technology Transfer , 6011 Executive Boulevard, Suite 325, Rockville, MD 20852-3804, USA**
- (72) Opfinder: **Whitehead, Stephen S., 9644 Duffer Way, Montgomery Village, MD 20886, USA**
Murphy, Brian R., 5410 Tuscarawus Road, Bethesda, MD 20816, USA
MARKOFF, Lewis, 6908 Nevis Road, Bethesda, MD 20817, USA
Falgout, Barry, 1400 Cove Lane, Apt. 103, Rockville, MD 20851, USA
Blaney, Joseph, 235 Montclair Road, Gettysburg, Pennsylvania 17325, USA
Hanley, Kathryn, 3860 Stoney Brooke Circle, Las Cruces, NM 88005, USA
LAI, Ching-Juh, 7353 Heatherhill Court, Bethesda, Maryland 20817, USA
- (74) Fuldmægtig i Danmark: **Plougmann Vingtoft A/S, Rued Langgaards Vej 8, 2300 København S, Danmark**
- (54) Benævnelse: **rDEN3/4delta 30(ME), rDEN2/4delta30(ME) eller rDEN1/4delta30(ME) rekombinant kimær Dengue-virus indeholdende en 30 nukleotiddeletion (delta30) i en sektion af den 3'-utranslaterede region af Dengue type 4 genom, hvor..**
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DESCRIPTION

Field of the Invention

[0001] The disclosure relates to a dengue virus vaccine containing chimeric dengue viruses of serotypes 1, 2, 3, and 4 that comprise a common 30 nucleotide deletion ($\Delta 30$) in the 3'-untranslated region of the genome of dengue virus serotypes 1, 2, 3, and 4.

Background of the Invention

[0002] Dengue virus is a positive-sense RNA virus belonging to the *Flavivirus* genus of the family *Flaviviridae*. Dengue virus is widely distributed throughout the tropical and semitropical regions of the world and is transmitted to humans by mosquito vectors. Dengue virus is a leading cause of hospitalization and death in children in at least eight tropical Asian countries (WHO 1997 *Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention, and Control* 2nd Edition, Geneva). There are four serotypes of dengue virus (DEN1, DEN2, DEN3, and DEN4) that annually cause an estimated 50-100 million cases of dengue fever and 500,000 cases of the more severe form of dengue virus infection known as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler, D.J. and Meltzer, M. 1999 *Adv Virus Res* 53:35-70). This latter disease is seen predominantly in children and adults experiencing a second dengue virus infection with a serotype different than that of their first dengue virus infection and in primary infection of infants who still have circulating dengue-specific maternal antibody (Burke, D.S. et al. 1988 *Am J Trop Med Hyg* 38:172-180; Halstead, S.B. et al. 1969 *Am J Trop Med Hyg* 18:997-1021; Thein, S. et al. 1997 *Am J Trop Med Hyg* 56:566-575). A dengue vaccine is needed to lessen disease burden caused by dengue virus, but none is licensed. Because of the association of more severe disease with secondary dengue virus infection, a successful vaccine must simultaneously induce immunity to all four serotypes. Immunity is primarily mediated by neutralizing antibody directed against the envelope (E) glycoprotein, a virion structural protein. Infection with one serotype induces long-lived homotypic immunity and a short-lived heterotypic immunity (Sabin, A. 1955 *Am J Trop Med Hyg* 4:198-207). Therefore, the goal of immunization is to induce a long-lived neutralizing antibody response against DEN1, DEN2, DEN3, and DEN4, which can best be achieved economically using live attenuated virus vaccines. This is a reasonable goal since a live attenuated vaccine has already been developed for the related yellow fever virus, another mosquito-borne flavivirus present in tropical and semitropical regions of the world (Monath, T.P. and Heinz, F.X. 1996 in: *Fields Virology*, Fields, D.M et al. eds. Philadelphia: Lippincott-Raven Publishers, pp. 961-1034).

[0003] Several live attenuated dengue vaccine candidates have been developed and evaluated in humans and non-human primates. The first live attenuated dengue vaccine candidates were host range mutants developed by serial passage of wild-type dengue viruses in the brains of mice and selection of mutants attenuated for humans (Kimura, R. and Hotta, S. 1944 *Jpn J Bacteriol* 1:96-99; Sabin, A.B. and Schlesinger, R.W. 1945 *Science* 101:640; Wisserman, C.L. et al. 1963 *Am J Trop Med Hyg* 12:620-623). Although these candidate vaccine viruses were immunogenic in humans, their poor growth in cell culture discouraged further development. Additional live attenuated DEN1, DEN2, DEN3, and DEN4 vaccine candidates have been developed by serial passage in non-human tissue culture (Angsubhakorn, S. et al. 1994 *Southeast Asian J Trop Med Public Health* 25:554-559; Bancroft, W.H. et al. 1981 *Infect Immun* 31:698-703; Bhamarapravati, N. et al. 1987 *Bull World Health Organ* 65:189-195; Eckels, K.H. et al. 1984 *Am J Trop Med Hyg* 33:684-698; Hoke, C.H. Jr. et al. 1990 *Am J Trop Med Hyg* 43:219-226; Kanesa-Thanan, N. et al. 2001 *Vaccine* 19:3179-3188) or by chemical mutagenesis (McKee, K.T. et al. 1987 *Am J Trop Med Hyg* 36:435-442). It has proven very difficult to achieve a satisfactory balance between attenuation and immunogenicity for each of the four serotypes of dengue virus using these approaches and to formulate a tetravalent vaccine that is safe and satisfactorily immunogenic against each of the four dengue viruses (Kanesa-Thanan, N. et al. 2001 *Vaccine* 19:3179-3188; Bhamarapravati, N. and Sutee, Y. 2000 *Vaccine* 18:44-47).

[0004] Two major advances using recombinant DNA technology have recently made it possible to develop additional promising live attenuated dengue virus vaccine candidates. First, methods have been developed to recover infectious dengue virus from cells transfected with RNA transcripts derived from a full-length cDNA clone of the dengue virus genome, thus making it possible to derive infectious viruses bearing attenuating mutations that have been introduced into the cDNA clone by site-directed mutagenesis (Lai, C.J. et al. 1991 *PNAS USA* 88:5139-5143). Second, it is possible to produce antigenic chimeric viruses in which the structural protein coding region of the full-length cDNA clone of dengue virus is replaced by that of a different dengue virus serotype or from a more divergent flavivirus (Bray, M. and Lai, C.J. 1991 *PNAS USA* 88:10342-10346; Chen, W. et al. 1995 *J Virol* 69:5186-5190; Huang, C.Y. et al. 2000 *J Virol* 74:3020-3028; Pletnev, A.G. and Men, R. 1998 *PNAS USA* 95:1746-1751). These techniques have been used to construct intertypic chimeric dengue viruses that have been shown to be effective in protecting monkeys against homologous dengue virus challenge (Bray, M. et al. 1996 *J Virol* 70:4162-4166). A similar strategy is also being used to develop attenuated antigenic chimeric dengue virus vaccines based on the attenuation of the yellow fever vaccine virus or the attenuation of the cell-culture passaged dengue viruses (Monath, T.P. et al. 1999 *Vaccine* 17:1869-1882; Huang, C.Y. et al. 2000 *J. Virol* 74:3020-3028).

[0005] Another study examined the level of attenuation for humans of a DEN4 mutant bearing a 30-nucleotide deletion ($\Delta 30$) introduced into its 3'-untranslated region by site-directed mutagenesis and that was found previously to be attenuated for rhesus monkeys (Men, R. et al. 1996 *J Virol* 70:3930-3937). Additional studies were carried out to examine whether this $\Delta 30$ mutation present in the DEN4 vaccine candidate was the major determinant of its attenuation for monkeys. It was found that the $\Delta 30$ mutation was indeed the major determinant of attenuation for monkeys, and that it specified a satisfactory balance between attenuation and immunogenicity for humans (Durbin, A.P. et al. 2001 *Am J Trop Med Hyg* 65:405-13).

[0006] Lai et al. (*Clinical and Diagnostic Virology* 10 (1998) 173-179) describe an evaluation of certain molecular strategies to develop a live dengue vaccine. They constructed intertypic chimeric cDNA in which the C-PreM-E or only the PreM-E structural protein genes of full-length DEN4 cDNA were replaced with the corresponding genes of DEN1, DEN2, or DEN3. This resulted in viable chimeric viruses that expressed the

protective structural proteins of DEN1, DEN2 or DEN3 against a background of DEN4 non-structural proteins. The chimeras were found to exhibit the predicted dengue type 1, 2 or 3 antigenicity, and were infectious for monkeys and capable of inducing protective immunity.

Summary of the Invention

[0007] The previously identified $\Delta 30$ attenuating mutation, created in dengue virus type 4 (DEN4) by the removal of 30 nucleotides from the 3' UTR, is also capable of attenuating a wild-type strain of dengue virus type 1 (DEN1). Removal of 30 nucleotides from the DEN1 3' UTR in a highly conserved region homologous to the DEN4 region encompassing the $\Delta 30$ mutation yielded a recombinant virus attenuated in rhesus monkeys to a level similar to recombinant virus DEN4 $\Delta 30$. This establishes the transportability of the $\Delta 30$ mutation and its attenuation phenotype to a dengue virus type other than DEN4. The effective transferability of the $\Delta 30$ mutation, described by this work, establishes the usefulness of the $\Delta 30$ mutation to attenuate and improve the safety of commercializable dengue virus vaccines of any serotype. We envision a tetravalent dengue virus vaccine containing dengue virus types 1, 2, 3, and 4 each attenuated by the $\Delta 30$ mutation. We also envision a tetravalent dengue virus vaccine containing recombinant antigenic chimeric viruses in which the structural genes of dengue virus types 1, 2, and 3 replace those of DEN4 $\Delta 30$; 1, 2, and 4 replace those of DEN3 $\Delta 30$; 1, 3, and 4 replace those of DEN2 $\Delta 30$; and 2, 3, and 4 replace those of DEN1 $\Delta 30$. In some instances, such chimeric dengue viruses are attenuated not only by the $\Delta 30$ mutation, but also by their chimeric nature. The presence of the $\Delta 30$ attenuating mutation in each virus component precludes the reversion to a wild-type virus by intertypic recombination. In addition, because of the inherent genetic stability of deletion mutations, the $\Delta 30$ mutation represents an excellent alternative for use as a common mutation shared among each component of a tetravalent vaccine.

Brief Description of the Drawings

[0008]

Figure 1. The live attenuated tetravalent dengue virus vaccine contains dengue viruses representing each of the 4 serotypes, with each serotype containing its full set of unaltered wild-type structural and non-structural proteins and a shared $\Delta 30$ attenuating mutation. The relative location of the $\Delta 30$ mutation in the 3' untranslated region (UTR) of each component is indicated by an arrow.

Figure 2. A. The $\Delta 30$ mutation removes 30 contiguous nucleotides (shaded) from the 3' UTR of DEN4. Nucleotides are numbered from the 3' terminus. B. Nucleotide sequence alignment of the TL2 region of DEN1, DEN2, DEN3, and DEN4 and their $\Delta 30$ derivatives. Also shown is the corresponding region for each of the four DEN serotypes. Upper case letters indicate sequence homology among all 4 serotypes, underlining indicates nucleotide pairing to form the stem structure. C. Predicted secondary structure of the TL2 region of each DEN serotype. Nucleotides that are removed by the $\Delta 30$ mutation are boxed (DEN1 - between nts 10562 - 10591, DEN2 Tonga/74 - between nts 10541 - 10570, DEN3 Sleman/78 - between nts 10535 - 10565, and DEN4 - between nts 10478 - 10507).

Figure 3. Viremia levels in rhesus monkeys inoculated with rDEN4 vaccine candidates bearing 5-FU derived mutations. Groups of four or two (rDEN4 and rDEN4 $\Delta 30$) monkeys were inoculated with $5.0 \log_{10}$ PFU virus subcutaneously. Serum was collected daily and virus titers were determined by plaque assay in Vero cells. The limit of virus detection was $0.7 \log_{10}$ PFU/ml. Mean virus titers are indicated for each group.

Figure 4. Viremia levels in rhesus monkeys inoculated with rDEN4 vaccine candidates bearing pairs of charge-to-alanine mutations in NSS. Groups of four or two (rDEN4 and rDEN4 $\Delta 30$) monkeys were inoculated with $5.0 \log_{10}$ PFU virus subcutaneously. Serum was collected daily and virus titers were determined by plaque assay in Vero cells. The limit of virus detection was $1.0 \log_{10}$ PFU/ml. Mean virus titers are indicated for each group. Viremia was not detected in any monkey after day 4.

Figure 5. The $\Delta 30$ mutation attenuates both DEN1 and DEN4 for rhesus monkeys. Groups of 4 monkeys were immunized subcutaneously with $5.0 \log_{10}$ PFU of the indicated virus. Serum was collected each day following immunization and virus titers were determined and are shown as mean $\log_{10}$ PFU/ml.

Figure 6. A. Diagram of the p2 (Tonga/74) full-length cDNA plasmid. Regions subcloned are indicated above the plasmid. Numbering begins at the 5' end of the viral sequence. B. The $\Delta 30$ mutation removes the indicated 30 nucleotides from the 3' UTR sequence to create p2A30.

Figure 7. Viremia levels in rhesus monkeys inoculated with DEN2 (Tonga/74), rDEN2, and rDEN2 $\Delta 30$ vaccine candidate. Groups of four monkeys were inoculated with $5.0 \log_{10}$ PFU virus subcutaneously. Serum was collected daily and virus titers were determined by plaque assay in Vero cells. The limit of virus detection was $0.7 \log_{10}$ PFU/ml. Mean virus titers are indicated for each group. Viremia was not detected in any monkey after day 8.

Figure 8. A. Diagram of the p3 (Sleman/78) full-length cDNA plasmid. Regions subcloned are indicated above the plasmid. Numbering begins at the 5' end of the viral sequence. The sequence and insertion location of the *SpeI* linker is shown. B. The $\Delta 30$ mutation removes the indicated 31 nucleotides from the 3' UTR sequence to create p3A30.

Figure 9. A. Recombinant chimeric dengue viruses were constructed by introducing either the CME or the ME regions of DEN2 (Tonga/74) into the DEN4 genetic background. The relative location of the $\Delta 30$ mutation in the 3' UTR is indicated by an arrow and intertypic junctions 1, 2, and 3 are indicated. B. Nucleotide and amino acid sequence of the intertypic junction regions. Restriction enzyme recognition sites used in assembly of each chimeric cDNA are indicated.

Figure 10. Growth kinetics in Vero cells of chimeric rDEN2/4 $\Delta 30$ viruses encoding single or combined Vero cell adaptation mutations. Vero cells were infected with the indicated viruses at an MOI of 0.01. At the indicated time points post-infection, 1 ml samples of tissue culture medium

were removed, clarified by centrifugation, and frozen at - 80°C. The level of virus replication was assayed by plaque titration in C6/36 cells. Lower limit of detection was 0.7 log₁₀PFU/ml. Replication levels on day 4 post-infection are indicated by the dashed line.

Figure 11. A. Recombinant chimeric dengue viruses were constructed by introducing either the CME or the ME regions of DEN3 (Sleman/78) into the DEN4 genetic background. The relative location of the A30 mutation in the 3' UTR is indicated by an arrow and intertypic junctions 1, 2, and 3 are indicated. Restriction enzyme recognition sites used in assembly of each chimeric cDNA are indicated. B. Nucleotide and amino acid sequence of the intertypic junction regions. Restriction enzyme recognition sites used in assembly of each chimeric cDNA are indicated.

Figure 12. A. Recombinant chimeric dengue viruses were constructed by introducing either the CME or the ME regions of DEN1 (Puerto Rico/94) into the DEN4 genetic background. The relative location of the A30 mutation in the 3' UTR is indicated by an arrow and intertypic junctions 1, 2, and 3 are indicated. Restriction enzyme recognition sites used in assembly of each chimeric cDNA are indicated. B. Nucleotide and amino acid sequence of the intertypic junction regions. Restriction enzyme recognition sites used in assembly of each chimeric cDNA are indicated.

Brief Description of the Sequences

[0009]

Serotype	GenBank Accession No. or description
DEN1	U88535
DEN2	Tonga/74
DEN3	Sleman/78
DEN4	AF326825

Brief Description of the SEQ ID NOs

[0010]

Identification	Figure, Table, or Appendix	SEQ ID NO.
TL2 region of DEN1	Figure 2	1
TL2 region of DEN2	Figure 2	2
TL2 region of DEN3	Figure 2	3
TL2 region of DEN 4	Figure 2	4
TL2 region of DEN1Δ30	Figure 2	5
TL2 region of DEN2Δ30	Figure 2	6
TL2 region of DEN3Δ30	Figure 2	7
TL2 region of DEN4Δ30	Figure 2	8
TL2 region of p2	Figure 6	9
TL2 region of p2Δ30	Figure 6	10
TL2 region of p3	Figure 8	11
TL2 region of p3Δ30	Figure 8	12
Spe1 linker in p3	Figure 8	13
rDEN2/4 junction 1	Figure 9	14-nt, 15-aa
rDEN2/4 junction 2	Figure 9	16-nt, 17-aa
rDEN2/4 junction 3	Figure 9	18-nt, 19-aa
rDEN3/4 junction 1	Figure 11	20-nt, 21-aa
rDEN3/4 junction 2	Figure 11	22-nt, 23-aa
rDEN3/4 junction 3	Figure 11	24-nt, 25-aa
rDEN1/4 junction 1	Figure 12	26-nt, 27-aa
rDEN1/4 junction 2	Figure 12	28-nt, 29-aa
rDEN1/4 junction 3	Figure 12	30-nt, 31-aa
D4 selected NS4B region	Table 15	32-nt, 33-aa
D1 selected NS4B region	Table 15	34-nt, 35-aa
D2 selected NS4B region	Table 15	36-nt, 37-aa
D3 selected NS4B region	Table 15	38-nt, 39-aa
CCACGGGCGCCGT	Table 26	40

Identification	Figure, Table, or Appendix	SEQ ID NO.
AAGGCCTGGA	Table 26	41
TATCCCCGGGAC	Table 26	42
AGAGCTCTCTC	Table 26	43
GAATCTCCACCCGGA	Table 26	44
CTGTCTGAATC	Table 26	45
DEN2 (Tonga/74) cDNA plasmid p2	Appendix 1	46-nt, 47-aa
DEN3 (Sleman/78) cDNA plasmid p3	Appendix 2	48-nt, 49-aa
DEN1 (Puerto Rico/94) CME chimeric region	Appendix 3	50-nt, 51-aa
DEN1 (Puerto Rico/94) ME chimeric region	Appendix 4	52-nt, 53-aa

Detailed Description of the Preferred Embodiment

Introduction

[0011] A molecular approach is herewith used to develop a genetically stable live attenuated tetravalent dengue virus vaccine. Each component of the tetravalent vaccine, namely, DEN1, DEN2, DEN3, and DEN4, must be attenuated, genetically stable, and immunogenic. A tetravalent vaccine is needed to ensure simultaneous protection against each of the four dengue viruses, thereby precluding the possibility of developing the more serious illnesses dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which occur in humans during secondary infection with a heterotypic wild-type dengue virus. Since dengue viruses can undergo genetic recombination in nature (Worobey, M. et al. 1999 PNAS USA 96:7352-7), the tetravalent vaccine should be genetically incapable of undergoing a recombination event between its four virus components that could lead to the generation of viruses lacking attenuating mutations. Previous approaches to develop a tetravalent dengue virus vaccine have been based on independently deriving each of the four virus components through separate mutagenic procedures, such as passage in tissue culture cells derived from a heterologous host. This strategy has yielded attenuated vaccine candidates (Bhamarapravati, N. and Sutee, Y. 2000 Vaccine 18:44-7). However, it is possible that gene exchanges among the four components of these independently derived tetravalent vaccines could occur in vaccinees, possibly creating a virulent recombinant virus. Virulent polioviruses derived from recombination have been generated in vaccinees following administration of a trivalent poliovirus vaccine (Guillot, S. et al. 2000 J Virol 74:8434-43).

[0012] The disclosure describes: (1) improvements to the previously described rDEN4Δ30 vaccine candidate, 2) attenuated rDEN1Δ30, rDEN2Δ30, and rDEN3Δ30 recombinant viruses containing a 30 nucleotide deletion (Δ30) in a section of the 3' untranslated region (UTR) that is homologous to that in the rDEN4Δ30 recombinant virus, (3) a method to generate a tetravalent dengue virus vaccine composed of rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, and rDEN4Δ30, 4) attenuated antigenic chimeric viruses, rDEN1/4Δ30, rDEN2/4Δ30, and rDEN3/4Δ30, for which the CME, ME, or E gene regions of rDEN4Δ30 have been replaced with those derived from DEN1, DEN2, or DEN3; alternatively rDEN1/3Δ30, rDEN2/3Δ30, and rDEN4/3Δ30 for which CME, ME, or E gene regions of rDEN3Δ30 have been replaced with those derived from DEN1, 2, or 4; alternatively rDEN1/2Δ30, rDEN3/2Δ30, and rDEN4/2Δ30 for which CME, ME, or E gene regions of rDEN2Δ30 have been replaced with those derived from DEN1, 3, or 4; and alternatively rDEN2/1Δ30, rDEN3/1Δ30, and rDEN4/1Δ30 for which CME, ME, or E gene regions of rDEN1Δ30 have been replaced with those derived from DEN2, 3, or 4, and 5) a method to generate a tetravalent dengue virus vaccine composed of rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, and rDEN4Δ30, alternatively rDEN1/3Δ30, rDEN2/3Δ30, rDEN4/3Δ30, and rDEN3Δ30, alternatively rDEN1/2Δ30, rDEN3/2Δ30, rDEN4/2Δ30, and rDEN2Δ30, and alternatively rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30, and rDEN1Δ30. These tetravalent vaccines are unique since they contain a common shared attenuating mutation which eliminates the possibility of generating a virulent wild-type virus in a vaccinee since each component of the vaccine possesses the same Δ30 attenuating deletion mutation. In addition, the rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30 tetravalent vaccine is the first to combine the stability of the Δ30 mutation with broad antigenicity. Since the A30 deletion mutation is in the 3' UTR of each virus, all of the proteins of the four component viruses are available to induce a protective immune response. Thus, the method provides a mechanism of attenuation that maintains each of the proteins of DEN1, DEN2, DEN3, and DEN4 viruses in a state that preserves the full capability of each of the proteins of the four viruses to induce humoral and cellular immune responses against all of the structural and non-structural proteins present in each dengue virus serotype.

[0013] As previously described, the DEN4 recombinant virus, rDEN4Δ30 (previously referred to as 2AΔ30), was engineered to contain a 30 nucleotide deletion in the 3' UTR of the viral genome (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13; Men, R. et al. 1996 J Virol 70:3930-7). Evaluation in rhesus monkeys showed the virus to be significantly attenuated relative to wild-type parental virus, yet highly immunogenic and completely protective. Also, a phase I clinical trial with adult human volunteers showed the rDEN4Δ30 recombinant virus to be safe and satisfactorily immunogenic (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13). To develop a tetravalent vaccine bearing a shared attenuating mutation in an untranslated region, we selected the A30 mutation to attenuate wild-type dengue viruses of serotypes 1, 2, and 3 since it attenuated wild-type DEN4 virus for rhesus monkeys and was safe in humans (Figure 1).

[0014] The Δ30 mutation was first described and characterized in the DEN4 virus (Men, R. et al. 1996 J Virol 70:3930-7). In DEN4, the mutation consists of the removal of 30 contiguous nucleotides comprising nucleotides 10478 - 10507 of the 3' UTR (Figure 2A) which form a putative stem-loop structure referred to as TL2 (Proutski, V. et al. 1997 Nucleic Acids Res 25:1194-202). Among the flaviviruses, large portions of the UTR form highly conserved secondary structures (Hahn, C.S. et al. 1987 J Mol Biol 198:33-41; Proutski, V. et al. 1997 Nucleic Acids Res 25:1194-202). Although the individual nucleotides are not necessarily conserved in these regions, appropriate base pairing preserves the stem-

loop structure in each serotype, a fact that is not readily apparent when only considering the primary sequence (Figure 2B, C).

Immunogenic Dengue Chimeras and Methods for Their Preparation

[0015] Immunogenic dengue chimeras and methods for preparing the dengue chimeras are provided herein. The immunogenic dengue chimeras are useful, alone or in combination, in a pharmaceutically acceptable carrier as immunogenic compositions to minimize, inhibit, or immunize individuals and animals against infection by dengue virus.

[0016] Chimeras of the disclosure comprise nucleotide sequences encoding the immunogenicity of a dengue virus of one serotype and further nucleotide sequences selected from the backbone of a dengue virus of a different serotype. These chimeras can be used to induce an immunogenic response against dengue virus.

[0017] In another aspect, the preferred chimera is a nucleic acid chimera comprising a first nucleotide sequence encoding at least one structural protein from a dengue virus of a first serotype, and a second nucleotide sequence encoding nonstructural proteins from a dengue virus of a second serotype different from the first. In the context of the invention, the dengue virus of the second serotype is DEN4. In another aspect, the structural protein can be the C protein of a dengue virus of the first serotype, the prM protein of a dengue virus of the first serotype, the E protein of a dengue virus of the first serotype, or any combination thereof.

[0018] The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a peptide by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (*i.e.*, amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

[0019] Furthermore, one of skill in the art will recognize that individual substitutions, deletions or additions in the amino acid sequence, or in the nucleotide sequence encoding for the amino acids, which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are conservatively modified variations, wherein the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1. 1) Alanine (A), Serine (S), Threonine (T);
2. 2) Aspartic acid (D), Glutamic acid (E);
3. 3) Asparagine (N), Glutamine (Q);
4. 4) Arginine (R), Lysine (K);
5. 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6. 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[0020] As used herein, the terms "virus chimera," "chimeric virus," "dengue chimera" and "chimeric dengue virus" means an infectious construct of the invention comprising nucleotide sequences encoding the immunogenicity of a dengue virus of one serotype and further nucleotide sequences derived from the backbone of a dengue virus of a different serotype.

[0021] As used herein, "infectious construct" indicates a virus, a viral construct, a viral chimera, a nucleic acid derived from a virus or any portion thereof which may be used to infect a cell.

[0022] As used herein, "nucleic acid chimera" means a construct of the invention comprising nucleic acid comprising nucleotide sequences encoding the immunogenicity of a dengue virus of one serotype and further nucleotide sequences derived from the backbone of a dengue virus of a different serotype. Correspondingly, any chimeric virus or virus chimera of the invention is to be recognized as an example of a nucleic acid chimera.

[0023] The structural and nonstructural proteins are to be understood to include any protein comprising or any gene encoding the sequence of the complete protein, an epitope of the protein, or any fragment comprising, for example, three or more amino acid residues thereof.

Dengue Chimeras

[0024] Dengue virus is a mosquito-borne flavivirus pathogen. The dengue virus genome contains a 5' untranslated region (5' UTR), followed by a capsid protein (C) encoding region, followed by a premembrane/membrane protein (prM) encoding region, followed by an envelope protein (E) encoding region, followed by the region encoding the nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) and finally a 3' untranslated region (3' UTR). The viral structural proteins are C, prM and E, and the nonstructural proteins are NS1-NS5. The structural and nonstructural proteins are translated as a single polyprotein and processed by cellular and viral proteases.

[0025] The dengue chimeras of the disclosure are constructs formed by fusing structural protein genes from a dengue virus of one serotype, *e.g.* DEN1, DEN2, DEN3, or DEN4, with non-structural protein genes from a dengue virus of a different serotype, *e.g.*, DEN1, DEN2, DEN3, or DEN4.

[0026] The attenuated, immunogenic dengue chimeras provided herein contain one or more of the structural protein genes, or antigenic portions thereof, of the dengue virus of one serotype against which immunogenicity is to be conferred, and the nonstructural protein genes of a dengue virus of a different serotype.

[0027] The chimera of the disclosure contains a dengue virus genome of one serotype as the backbone, in which the structural protein gene(s) encoding C, prM, or E protein(s) of the dengue genome, or combinations thereof, are replaced with the corresponding structural protein gene(s) from a dengue virus of a different serotype that is to be protected against. The resulting viral chimera has the properties, by virtue of being chimerized with a dengue virus of another serotype, of attenuation and is therefore reduced in virulence, but expresses antigenic epitopes of the structural gene products and is therefore immunogenic.

[0028] The genome of any dengue virus can be used as the backbone in the attenuated chimeras described herein. The backbone can contain mutations that contribute to the attenuation phenotype of the dengue virus or that facilitate replication in the cell substrate used for manufacture, e.g., Vero cells. The mutations can be in the nucleotide sequence encoding nonstructural proteins, the 5' untranslated region or the 3' untranslated region. The backbone can also contain further mutations to maintain the stability of the attenuation phenotype and to reduce the possibility that the attenuated virus or chimera might revert back to the virulent wild-type virus. For example, a first mutation in the 3' untranslated region and a second mutation in the 5' untranslated region will provide additional attenuation phenotype stability, if desired. In particular, a mutation that is a deletion of 30 nts from the 3' untranslated region of the DEN4 genome between nts 10478-10507 results in attenuation of the DEN4 virus (Men et al. 1996 J. Virology 70:3930-3933; Durbin et al. 2001 Am J Trop Med 65:405-413, 2001). Therefore, the genome of any dengue type 4 virus containing such a mutation at this locus can be used as the backbone in the attenuated chimeras described herein. Furthermore, other dengue virus genomes containing an analogous deletion mutation in the 3' untranslated region of the genomes of other dengue virus serotypes may also be used as the backbone structure. Such mutations may be achieved by site-directed mutagenesis using techniques known to those skilled in the art. It will be understood by those skilled in the art that the virulence screening assays, as described herein and as are well known in the art, can be used to distinguish between virulent and attenuated backbone structures.

Construction of Dengue Chimeras

[0029] The dengue virus chimeras described herein can be produced by substituting at least one of the structural protein genes of the dengue virus of one serotype against which immunity is desired into a dengue virus genome backbone of a different serotype, using recombinant engineering techniques well known to those skilled in the art, namely, removing a designated dengue virus gene of one serotype and replacing it with the desired corresponding gene of dengue virus of a different serotype. Alternatively, using the sequences provided in GenBank, the nucleic acid molecules encoding the dengue proteins may be synthesized using known nucleic acid synthesis techniques and inserted into an appropriate vector. Attenuated, immunogenic virus is therefore produced using recombinant engineering techniques known to those skilled in the art.

[0030] As mentioned above, the gene to be inserted into the backbone encodes a dengue structural protein of one serotype. Preferably the dengue gene of a different serotype to be inserted is a gene encoding a C protein, a prM protein and/or an E protein. The sequence inserted into the dengue virus backbone can encode both the prM and E structural proteins of the other serotype. The sequence inserted into the dengue virus backbone can encode the C, prM and E structural proteins of the other serotype. The dengue virus backbone is the DEN1, DEN2, DEN3, or DEN4 virus genome, or an attenuated dengue virus genome of any of these serotypes, and includes the substituted gene(s) that encode the C, prM and/or E structural protein(s) of a dengue virus of a different serotype, or the substituted gene(s) that encode the prM and/or E structural protein(s) of a dengue virus of a different serotype.

[0031] Suitable chimeric viruses or nucleic acid chimeras containing nucleotide sequences encoding structural proteins of dengue virus of any of the serotypes can be evaluated for usefulness as vaccines by screening them for phenotypic markers of attenuation that indicate reduction in virulence with retention of immunogenicity. Antigenicity and immunogenicity can be evaluated using *in vitro* or *in vivo* reactivity with dengue antibodies or immunoreactive serum using routine screening procedures known to those skilled in the art.

Dengue Vaccines

[0032] The preferred chimeric viruses and nucleic acid chimeras provide live, attenuated viruses useful as immunogens or vaccines. In a preferred embodiment, the chimeras exhibit high immunogenicity while at the same time not producing dangerous pathogenic or lethal effects.

[0033] The chimeric viruses or nucleic acid chimeras of this disclosure can comprise the structural genes of a dengue virus of one serotype in a wild-type or an attenuated dengue virus backbone of a different serotype. For example, the chimera may express the structural protein genes of a dengue virus of one serotype in either of a dengue virus or an attenuated dengue virus background of a different serotype.

[0034] The strategy described herein of using a genetic background that contains nonstructural regions of a dengue virus genome of one serotype, and, by chimerization, the properties of attenuation, to express the structural protein genes of a dengue virus of a different serotype has lead to the development of live, attenuated dengue vaccine candidates that express structural protein genes of desired immunogenicity. Thus, vaccine candidates for control of dengue pathogens can be designed.

[0035] Viruses used in the chimeras described herein are typically grown using techniques known in the art. Virus plaque or focus forming unit (FFU) titrations are then performed and plaques or FFU are counted in order to assess the viability, titer and phenotypic characteristics of the virus grown in cell culture. Wild type viruses are mutagenized to derive attenuated candidate starting materials.

[0036] Chimeric infectious clones are constructed from various dengue serotypes. The cloning of virus-specific cDNA fragments can also be accomplished, if desired. The cDNA fragments containing the structural protein or nonstructural protein genes are amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from dengue RNA with various primers. Amplified fragments are cloned into the cleavage sites of other intermediate clones. Intermediate, chimeric dengue clones are then sequenced to verify the sequence of the inserted dengue-specific cDNA.

[0037] Full genome-length chimeric plasmids constructed by inserting the structural or nonstructural protein gene region of dengue viruses into vectors are obtainable using recombinant techniques well known to those skilled in the art.

Methods of Administration

[0038] The viral chimeras described herein are individually or jointly combined with a pharmaceutically acceptable carrier or vehicle for administration as an immunogen or vaccine to humans or animals. The terms "pharmaceutically acceptable carrier" or "pharmaceutically acceptable vehicle" are used herein to mean any composition or compound including, but not limited to, water or saline, a gel, salve, solvent, diluent, fluid ointment base, liposome, micelle, giant micelle, and the like, which is suitable for use in contact with living animal or human tissue without causing adverse physiological responses, and which does not interact with the other components of the composition in a deleterious manner.

[0039] The immunogenic or vaccine formulations may be conveniently presented in viral plaque forming unit (PFU) unit or focus forming unit (FFU) dosage form and prepared by using conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets commonly used by one of ordinary skill in the art.

[0040] Preferred unit dosage formulations are those containing a dose or unit, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the present invention may include other agents commonly used by one of ordinary skill in the art.

[0041] The immunogenic or vaccine composition may be administered through different routes, such as oral or parenteral, including, but not limited to, buccal and sublingual, rectal, aerosol, nasal, intramuscular, subcutaneous, intradermal, and topical. The composition may be administered in different forms, including, but not limited to, solutions, emulsions and suspensions, microspheres, particles, microparticles, nanoparticles and liposomes. It is expected that from about 1 to about 5 doses may be required per immunization schedule. Initial doses may range from about 100 to about 100,000 PFU or FFU, with a preferred dosage range of about 500 to about 20,000 PFU or FFU, a more preferred dosage range of from about 1000 to about 12,000 PFU or FFU and a most preferred dosage range of about 1000 to about 4000 PFU or FFU. Booster injections may range in dosage from about 100 to about 20,000 PFU or FFU, with a preferred dosage range of about 500 to about 15,000, a more preferred dosage range of about 500 to about 10,000 PFU or FFU, and a most preferred dosage range of about 1000 to about 5000 PFU or FFU. For example, the volume of administration will vary depending on the route of administration. Intramuscular injections may range in volume from about 0.1 ml to 1.0 ml.

[0042] The composition may be stored at temperatures of from about -100°C to about 4°C. The composition may also be stored in a lyophilized state at different temperatures including room temperature. The composition may be sterilized through conventional means known to one of ordinary skill in the art. Such means include, but are not limited to, filtration. The composition may also be combined with bacteriostatic agents to inhibit bacterial growth.

Administration Schedule

[0043] The immunogenic or vaccine composition described herein may be administered to humans, especially individuals travelling to regions where dengue virus infection is present, and also to inhabitants of those regions. The optimal time for administration of the composition is about one to three months before the initial exposure to the dengue virus. However, the composition may also be administered after initial infection to ameliorate disease progression, or after initial infection to treat the disease.

Adjuvants

[0044] A variety of adjuvants known to one of ordinary skill in the art may be administered in conjunction with the chimeric virus in the immunogen or vaccine composition of this invention. Such adjuvants include, but are not limited to, the following: polymers, copolymers such as polyoxyethylene-polyoxypropylene copolymers, including block copolymers, polymer p 1005, Freund's complete adjuvant (for animals), Freund's incomplete adjuvant; sorbitan monooleate, squalene, CRL-8300 adjuvant, alum, QS 21, muramyl dipeptide, CpG oligonucleotide

motifs and combinations of CpG oligonucleotide motifs, trehalose, bacterial extracts, including mycobacterial extracts, detoxified endotoxins, membrane lipids, or combinations thereof.

Nucleic Acid Sequences

[0045] Nucleic acid sequences of dengue virus of one serotype and dengue virus of a different serotype are useful for designing nucleic acid probes and primers for the detection of dengue virus chimeras in a sample or specimen with high sensitivity and specificity. Probes or primers corresponding to dengue virus can be used to detect the presence of a vaccine virus. The nucleic acid and corresponding amino acid sequences are useful as laboratory tools to study the organisms and diseases and to develop therapies and treatments for the diseases.

[0046] Nucleic acid probes and primers selectively hybridize with nucleic acid molecules encoding dengue virus or complementary sequences thereof. By "selective" or "selectively" is meant a sequence which does not hybridize with other nucleic acids to prevent adequate detection of the dengue virus sequence. Therefore, in the design of hybridizing nucleic acids, selectivity will depend upon the other components present in the sample. The hybridizing nucleic acid should have at least 70% complementarity with the segment of the nucleic acid to which it hybridizes. As used herein to describe nucleic acids, the term "selectively hybridizes" excludes the occasional randomly hybridizing nucleic acids, and thus has the same meaning as "specifically hybridizing." The selectively hybridizing nucleic acid probes and primers can have at least 70%, 80%, 85%, 90%, 95%, 97%, 98% and 99% complementarity with the segment of the sequence to which it hybridizes, preferably 85% or more. Also contemplated herein are sequences, probes and primers that selectively hybridize to the encoding nucleic acid or the complementary, or opposite, strand of the nucleic acid. Specific hybridization with nucleic acid can occur with minor modifications or substitutions in the nucleic acid, so long as functional species-species hybridization capability is maintained. By "probe" or "primer" is meant nucleic acid sequences that can be used as probes or primers for selective hybridization with complementary nucleic acid sequences for their detection or amplification, which probes or primers can vary in length from about 5 to 100 nucleotides, or preferably from about 10 to 50 nucleotides, or most preferably about 18-24 nucleotides. Isolated nucleic acids are provided herein that selectively hybridize with the species-specific nucleic acids under stringent conditions and should have at least five nucleotides complementary to the sequence of interest as described in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

[0047] If used as primers, the composition preferably includes at least two nucleic acid molecules which hybridize to different regions of the target molecule so as to amplify a desired region. Depending on the length of the probe or primer, the target region can range between 70% complementary bases and full complementarity and still hybridize under stringent conditions. For example, for the purpose of detecting the presence of dengue virus, the degree of complementarity between the hybridizing nucleic acid (probe or primer) and the sequence to which it hybridizes is at least enough to distinguish hybridization with a nucleic acid from other organisms.

[0048] The nucleic acid sequences encoding dengue virus can be inserted into a vector, such as a plasmid, and recombinantly expressed in a living organism to produce recombinant dengue virus peptide and/or polypeptides.

[0049] The nucleic acid sequences disclosed herein include a diagnostic probe that serves to report the detection of a cDNA amplicon amplified from the viral genomic RNA template by using a reverse-transcription/polymerase chain reaction (RT/PCR), as well as forward and reverse amplimers that are designed to amplify the cDNA amplicon. In certain instances, one of the amplimers is designed to contain a vaccine virus-specific mutation at the 3'-terminal end of the amplimer, which effectively makes the test even more specific for the vaccine strain because extension of the primer at the target site, and consequently amplification, will occur only if the viral RNA template contains that specific mutation.

[0050] Automated PCR-based nucleic acid sequence detection systems have been recently developed. TaqMan assay (Applied Biosystems) is widely used. A more recently developed strategy for diagnostic genetic testing makes use of molecular beacons (Tyagi and Kramer, 1996 *Nature Biotechnology* 14:303-308). Molecular beacon assays employ quencher and reporter dyes that differ from those used in the TaqMan assay. These and other detection systems may be used by one skilled in the art.

Comparative EXAMPLE 1

Improvement of Dengue Virus Vaccine Candidate rDEN4Δ30

[0051] The safety of recombinant live-attenuated dengue-4 vaccine candidate rDEN4Δ30 was evaluated in twenty human volunteers who received a dose of 5.0 log₁₀ plaque forming units (PFU) (Durbin A.P. et al. 2001 *Am J Trop Med Hyg* 65:405-413). The vaccine candidate was found to be safe, well-tolerated and immunogenic in all of the vaccinees. However, five of the vaccinees experienced a transient elevation in alanine aminotransferase levels, three experienced neutropenia and ten vaccinees developed an asymptomatic macular rash, suggesting that it may be necessary to further attenuate this vaccine candidate.

[0052] Currently, a randomized, double-blind, placebo-controlled, dose de-escalation study is being conducted to determine the human infectious dose 50 (ID₅₀) of rDEN4Δ30. Each dose cohort consists of approximately twenty vaccinees and four placebo recipients. To date, complete data for doses of 3.0 log₁₀ PFU and 2.0 log₁₀ PFU has been collected. rDEN4Δ30 infected 100% of vaccinees when 3.0 log₁₀ PFU was administered and 95% of vaccinees when 2.0 log₁₀ PFU was administered (Table 1). The vaccine candidate caused no symptomatic illness at either dose (Table 1). One vaccinee who received 3.0 log₁₀ PFU experienced a transient elevation in alanine aminotransferase levels and approximately one fourth of the vaccinees experienced neutropenia at both doses (Table 1). Neutropenia was transient and mild. More than

half of the vaccinees developed a macular rash at both doses; the occurrence of rash was not correlated with vaccination dose or with viremia (Table 1 and Table 2). Neither peak titer nor onset of viremia differed between the 3.0 log₁₀ PFU and 2.0 log₁₀ PFU doses, though both measures of viremia were significantly lower for these doses than for a dose of 5.0 log₁₀ PFU (Table 3). The vaccine candidate was immunogenic in 95% of vaccinees at both doses and neutralizing antibody did not decline between days 28 and 42 post-vaccination (Table 4). Although the HID_{50} has not been determined yet, it is clearly less than 2.0 log₁₀ PFU. Interestingly, decreases in the dose of vaccine have had no consistent effect on immunogenicity, viremia, benign neutropenia or the occurrence of rash. Thus it will not necessarily be possible to further attenuate rDEN4Δ30 by decreasing the dose of virus administered, and other approaches must be developed.

Table 1. rDEN4Δ30 clinical summary

No. of subjects	Dose ^a	No. infected	No. with viremia	Mean peak titer ^b	No. volunteers with:			
					Fever	Rash	Neutropenia ^c	↑ALT
20	5.0	20	14	1.2(0.2)	1 ^d	10	3	5
20	3.0	20	7	0.4(0.0)	0	11	5	1 ^e
20	2.0	19	11	0.6(0.1)	1 ^d	16	4	0
8	0	0	0	0	0	0	0	0

^a Log₁₀ pfu

^b Log₁₀ pfu/mL

^c Neutropenia defined as ANC < 1500/dl

^d T Max in volunteer = 100.4°F

^e ALT day 0 = 78, ALT max = 91 (day 14)

Table 2. Pattern of rash in vaccinees

Dose ^a	No. with viremia	No. with rash	Viremia & rash	Viremia no rash	Mean day of onset ± SD	Mean duration (days ± SD)
5	14/20	10/20	9/20	5/20	8.1 ± 1.3 [A] ^a	3.6 ± 2.0 [A]
3	7/20	11/20	6/20	1/20	12.2 ± 1.4 [B]	5.0 ± 2.1 [A]
2	11/20	16/20	9/20	2/20	11.2 ± 1.4 [B]	6.9 ± 1.7 [B]

^alog₁₀ pfu

^bMeans in each column with different letters are significantly different ($\alpha = 0.05$)

Table 3. rDEN4Δ30 viremia summary

Dose ^a	# with viremia	Mean peak titer (log ₁₀ pfu/mL)	Mean onset of viremia (day ± SD)	Mean duration of viremia (day ± SD)
5	14	1.2 ± 0.2 [A]	5.8 ± 2.4 [A] ^b	4.4 ± 2.4 [A]
3	7	0.4 ± 0.0 [B]	9.1 ± 2.5 [B]	1.6 ± 1.0 [B]
2	11	0.6 ± 0.1 [B]	8.7 ± 2.4 [B]	2.6 ± 2.0 [A]

^alog₁₀ pfu

^bMeans in each column with different letters are significantly different ($\alpha = 0.05$)

Table 4. Immunogenicity of rDEN4Δ30

No. of subjects	Dose (log ₁₀)	No. infected	Geometric mean serum neutralizing antibody titer (range)		% seroconversion
			Day 28	Day 42	
20	5.0	20	567 (72 - 2455)	399 (45 - 1230)	100
20	3.0	20	156(5 - 2365)	158 (25 - 1222)	95
20	2.0	19	163 (5 - 943)	165 (5 - 764)	95
8	0	0	0	0	0

[0053] Two approaches have been taken to further attenuate rDEN4Δ30. This first is the generation and characterization of attenuating point mutations in rDEN4 using 5' fluorouracil mutagenesis (Blaney, J.E. Jr. et al. 2002 Virology 300: 125-139; Blaney, J.E. Jr. et al. 2001 J. Virol. 75: 9731-9740). This approach has identified a panel of point mutations that confer a range of temperature sensitivity (*ts*) and small plaque (*sp*) phenotypes in Vero and HuH-7 cells and attenuation (*att*) phenotypes in suckling mouse brain and SCID mice engrafted with HuH-7 cells (SCID)-HuH-7 mice). In this example, a subset of these mutations has been introduced to rDEN4Δ30 and the phenotypes of the resulting viruses evaluated.

[0054] A second approach was to create a series of paired charge-to-alanine mutations in contiguous pairs of charged amino acid residues in the rDEN4 NS5 gene. As demonstrated previously, mutation of 32 individual contiguous pairs of charged amino acid residues in rDEN4 NS5 conferred a range of *ts* phenotypes in Vero and HuH-7 cells and a range of *att* phenotypes in suckling mouse brain (Hanley, K.H. et al. 2002 J. Virol. 76 525-531). As demonstrated below, these mutations also confer an *att* phenotype in SCID-HuH-7 mice. These mutations have been introduced, either as single pairs or sets of two pairs, into rDEN4Δ30 to determine whether they are compatible with the Δ30 mutation and whether they enhance the *att* phenotypes of rDEN4Δ30.

[0055] A panel of rDEN4 viruses bearing individual point mutations have been characterized which possess temperature sensitive and/or small plaque phenotypes in tissue culture and varying levels of attenuated replication in suckling mouse brain when compared to wild type rDEN4 virus (Blaney, J. E. et al. 2002 Virology 300:125-139; Blaney, J. E. et al. 2001 J Virol. 75:9731-9740). Three mutations have been selected to combine with the $\Delta 30$ deletion mutation to evaluate their ability to further restrict replication of rDEN4 $\Delta 30$ in rhesus monkeys. First, the missense mutation in NS3 at nucleotide 4995 (Ser > Pro) which confers temperature sensitivity in *Vero* and HuH-7 cells and restricted replication in suckling mouse brain was previously combined with the $\Delta 30$ mutation (Blaney, J. E. et al. 2001 J Virol. 75:9731-9740). The resulting virus, rDEN4 $\Delta 30$ -4995, was found to be more restricted (1,000-fold) in mouse brain replication than rDEN4 $\Delta 30$ virus (<5-fold) when compared to wild type rDEN4 virus. Second, a missense mutation at nucleotide 8092 (Glu > Gly) which also confers temperature sensitivity in *Vero* and HuH-7 cells and 10,000-fold restricted replication in suckling mouse brain was combined with the $\Delta 30$ mutation here. Third, a substitution in the 3' UTR at nucleotide 10634 which confers temperature sensitivity in *Vero* and HuH-7 cells, small plaque size in HuH-7 cells, and approximately 1,000-fold restricted replication in suckling mouse brain and SCID mice transplanted with HuH-7 cells was combined with the $\Delta 30$ mutation here (Blaney, J. E. et al. 2002 Virology 300:125-139).

[0056] For the present investigation, subcloned fragments of p4 (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13) containing the above mutations were introduced into the p4A30 cDNA clone. For transcription and recovery of virus, cDNA was linearized with *Acc651* (isoschizomer of *KpnI* which cleaves leaving only a single 3' nucleotide) and used as template for transcription by SP6 RNA polymerase as previously described (Blaney, J. E. et al. 2002 Virology 300:125-139). C6/36 mosquito cells were transfected using liposome-mediated transfection and cell culture supernatants were harvested between days five and seven. Recovered virus was terminally diluted twice in *Vero* cells and passaged two (rDEN4 $\Delta 30$ -4995) or three (rDEN4 $\Delta 30$ -8092 and rDEN4 $\Delta 30$ -10634) times in *Vero* cells.

[0057] The complete genomic sequences of rDEN4 $\Delta 30$ -4995, rDEN4 $\Delta 30$ -8092, and rDEN4 $\Delta 30$ -10634 viruses were determined as previously described (Durbin et al. 2001 Am. J. Trop. Med. Hyg. 65:405-413). As expected, each rDEN4 $\Delta 30$ virus derivative contained the $\Delta 30$ mutation. Unexpectedly, in rDEN4 $\Delta 30$ -4995 virus, the nucleotide changes in the codon containing nucleotide 4995, resulted in a Ser > Leu amino acid change rather than a Ser > Pro change since the p4 $\Delta 30$ -4995 cDNA was designed to introduce the Ser > Pro change (Table 5). The p4 $\Delta 30$ -4995 cDNA clone was indeed found to encode a Ser > Pro change at nucleotide 4995, so it is unclear how the virus population acquired the Ser > Leu mutation. Nevertheless, this virus was evaluated to assess the phenotype specified by this missense mutation. rDEN4 $\Delta 30$ -4995 virus was also found to contain an incidental mutation at nucleotides 4725-6 which resulted in a single amino acid change (Ser > Asp). The rDEN4 $\Delta 30$ -8092 and rDEN4 $\Delta 30$ -10634 viruses contained the appropriate nucleotide substitutions as well as additional incidental mutations in E, NS4B and NS4B, respectively (Table 5).

Table 5. Missense and UTR mutations present in rDEN4 $\Delta 30$ virus derivatives bearing introduced point mutations.

Virus	Gene	Nucleotide position	Nucleotide substitution	Amino acid position ^a	Amino acid change
rDEN4 $\Delta 30$ -4995	NS3	4725	U > G	1542	Ser > Asp
	NS3	4726	C > A	1542	Ser > Asp
	NS3	4995 ^b	U > C	1632	Ser > Leu
rDEN4 $\Delta 30$ -8092	E	1612	A > C	504	Asp > Ala
	NS4B	7131	A > G	2344	Thr > Ala
	NS5	8092 ^b	A > G	2664	Glu > Gly
rDEN4 $\Delta 30$ -10634	NS4B	6969	A > U	2290	Met > Leu
	NS4B	7182	G > C	2361	Gly > Arg
	3'UTR	10634 ^b	U > C	none	none

^a Amino acid position in DEN4 polypeptide beginning with the methionine residue of the C protein (nucleotides 102-104) as position 1.

^b Mutation restricts replication in mouse models of DEN4 infection which were introduced by Kunkel mutagenesis.

[0058] Replication of the three modified rDEN4 $\Delta 30$ viruses were compared to rDEN4 $\Delta 30$ and wild type rDEN4 virus in the suckling mouse brain model and SCID mice transplanted with HuH-7 cells (SCID-HuH-7 mice). Experiments were conducted as previously described (Blaney, J. E. et al. 2002 Virology 300:125-139; Blaney, J. E. et al. 2001 J Virol. 75:9731-9740). Briefly, for infection of suckling mouse brain, groups of six seven-day-old mice were inoculated intracerebrally with 4.0 log₁₀ PFU of virus and the brain of each mouse was removed five days later. Clarified supernatants of 10% brain suspensions were then frozen at -70°C, and the virus titer was determined by plaque assay in *Vero* cells. For analysis of DEN4 virus replication in SCID-HuH-7 mice, four to six week-old SCID mice were injected intraperitoneally with 10⁷. HuH-7 cells. Five to six weeks after transplantation, mice were infected by direct inoculation into the tumor with 4.0 log₁₀ PFU of virus, and serum for virus titration was obtained by tail-nicking on day 7. The virus titer was determined by plaque assay in *Vero* cells.

[0059] Wild type rDEN4 virus replicated to 6.0 log₁₀PFU/g in suckling mouse brain, and rDEN4 $\Delta 30$ was restricted in replication by 0.7 log₁₀PFU/g, which is similar to previous observations (Table 6) (Blaney, J. E. et al. 2001 J Virol 75:9731-9740). rDEN4 $\Delta 30$ -4995, rDEN4 $\Delta 30$ -8092, and rDEN4 $\Delta 30$ -10634 viruses were found to have restricted replication in suckling mouse brain when compared to rDEN4 virus of 3.3, 2.8, and 2.4 log₁₀PFU/g, respectively. These results indicate that the additional attenuating mutations serve to further restrict replication of the rDEN4 $\Delta 30$ virus in mouse brain ranging from 50-fold (rDEN4 $\Delta 30$ -10634) to 400-fold (rDEN4 $\Delta 30$ -4995). In SCID-HuH-7 mice, virus titer of rDEN4 $\Delta 30$ virus was 0.4 log₁₀PFU/ml lower than rDEN4 virus, which is also similar to previous studies (Blaney, J. E. et al. 2002 Virology

300:125-139). Each modified rDEN4Δ30 virus was found to be further restricted in replication in SCID-HuH-7 mice (Table 6). rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634 viruses had restricted replication in SCID-HuH-7 mice when compared to rDEN4 virus of 2.9, 1.1, and 2.3 log₁₀PFU/g below the level of wild type rDEN4 virus, respectively. Two important observations were made: (1) The 4995, 8092 and 10634 mutations were compatible for viability with the Δ30 mutation, and (2) These three modified rDEN4Δ30 viruses had between a 10 and 1,000-fold reduction in replication in comparison to rDEN4 wild-type virus, which allows viruses with a wide range of attenuation in this model to be further evaluated in monkeys or humans.

Table 6. Addition of point mutations in NS3, NS5, or the 3' UTR to rDEN4Δ30 virus further attenuates the virus for suckling mouse brain and SCID-HuH-7 mice.

Virus	Replication in suckling mouse brain ^a			Replication in SCID-HuH-7 mice ^c		
	No. of mice	Virus titer ± SE log ₁₀ PFU/g brain	Mean log ₁₀ -unit reduction from wt ^b	No. of mice	Virus titer ± SE log ₁₀ PFU/ml serum	Mean log ₁₀ -unit reduction from wt ^b
rDEN4	12	6.0 ± 0.1	-	13	6.4 ± 0.2	-
rDEN4Δ30	12	5.3 ± 0.1	0.7	20	6.0 ± 0.2	0.4
rDEN4Δ30-4995	6	2.7 ± 0.4	3.3	5	3.5 ± 0.3	2.9
rDEN4Δ30-8092	6	3.2 ± 0.2	2.8	7	5.0 ± 0.4	1.1
rDEN4Δ30-10634	12	3.6 ± 0.1	2.4	5	4.4 ± 0.3	2.3

^a Groups of 6 suckling mice were inoculated i.c. with 10⁴ PFU of virus. Brains were removed 5 days later, homogenized, and titered in *Vero* cells.

^b Comparison of mean virus titers of mice inoculated with mutant virus and concurrent rDEN4 wt control.

^c Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10⁴ PFU virus. Serum was collected on day 6 and 7 and titered in *Vero* cells.

[0060] Based on the findings in the two mouse models of DEN4 virus infection, each of the rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634 viruses was evaluated in the rhesus macaque model of DEN4 infection which has been previously described (Durbin et al. 2001 Am. J. Trop. Med. Hyg. 65:405-413). Briefly, groups of four (rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634) or two (rDEN4, rDEN4Δ30, mock) monkeys were inoculated with 5.0 log₁₀PFU virus subcutaneously. Monkeys were observed daily and serum was collected on days 0 to 6, 8, 10, and 12, and virus titers were determined by plaque assay in *Vero* cells for measurement of viremia. On day 28, serum was drawn and the level of neutralizing antibodies was tested by plaque reduction assay in *Vero* cells as previously described (Durbin et al. 2001 Am. J. Trop. Med. Hyg. 65:405-413).

[0061] Viremia was detected beginning on day 1 post-infection and ended by day 4 in all monkeys (Table 7, Figure 3). Viremia was present in each monkey infected with rDEN4, rDEN4Δ30, or rDEN4Δ30-10634 virus, but only 2 out of 4 monkeys infected with rDEN4Δ30-4995 or rDEN4Δ30-8092 virus had detectable viremia. As expected, infection with rDEN4 virus resulted in the highest mean number of viremic days per monkey (3.0 days) as well as mean peak virus titer (2.2 log₁₀PFU/ml). Monkeys infected with rDEN4Δ30 virus had both a lower mean number of viremic days per monkey (2.0 days) and mean peak virus titer (1.1 log₁₀PFU/ml) compared to rDEN4 virus. Groups of monkeys infected with each of the modified rDEN4Δ30 viruses had a further restricted mean number of viremic days with those inoculated with rDEN4Δ30-8092 virus having the lowest value, 0.5 days, a 4-fold reduction compared to rDEN4Δ30 virus. The mean peak virus titer of monkeys infected with rDEN4Δ30-4995 (0.9 log₁₀PFU/ml) or rDEN4Δ30-8092 (0.7 log₁₀PFU/ml) was also lower than those infected with rDEN4Δ30 virus. However, the mean peak virus titer of monkeys infected with rDEN4Δ30-10634 (1.3 log₁₀PFU/ml) was slightly higher than those infected with rDEN4Δ30 particularly on day 2 (Figure 3).

Table 7. Addition of point mutations to rDEN4Δ30 further attenuates the virus for rhesus monkeys.

Virus ^a	No. of monkeys	No. of monkeys with viremia	Mean no. of viremic days per monkey ^b	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
					Day 0	Day 28
mock	2	0	0	<0.7	<10	<10
rDEN4	2	2	3.0	2.2 ± 0.6	<10	398
rDEN4Δ30	2	2	2.0	1.1 ± 0.4	<10	181
rDEN4Δ30-4995	4	2	0.8	0.9 ± 0.2	<10	78
rDEN4Δ30-8092	4	2	0.5	0.7 ± 0.1	<10	61
rDEN4Δ30-10634	4	4	1.3	1.3 ± 0.2	<10	107

^a Groups of rhesus monkeys were inoculated subcutaneously with 10⁵ PFU of the indicated virus in a 1 ml dose. Serum was collected on days 0 to 6, 8, 10, 12, and 28. Virus titer was determined by plaque assay in *Vero* cells.

^b Viremia was not detected in any monkey after day 4.

[0062] Serum collected on day 0 and 28 was tested for the level of neutralizing antibodies against rDEN4. No detectable neutralizing antibodies were found against DEN4 on day 0, as expected, since the monkeys were pre-screened to be negative for neutralizing antibodies against flaviviruses (Table 7). On day 28, monkeys infected with rDEN4 had a mean serum neutralizing antibody titer (reciprocal dilution) of 398 which was approximately two-fold higher than monkeys infected with rDEN4Δ30 virus (1:181). This result and the two-fold higher level of viremia in rDEN4 virus-infected monkeys are similar to results obtained previously (Durbin et al. 2001 Am. J. Trop. Med. Hyg. 65:405-413). Monkeys infected with rDEN4Δ30-4995 (1:78), rDEN4Δ30-8092 (1:61), and rDEN4Δ30-10634 (1:107) viruses each had a reduced mean serum neutralizing antibody titer compared to monkeys infected with rDEN4Δ30 virus. The four monkeys which had no detectable viremia did have serum neutralizing antibody titers indicating that they were indeed infected. Despite the slight increase in mean peak virus titer of rDEN4Δ30-10634 virus compared with rDEN4Δ30 virus, rDEN4Δ30-10634 virus had a lower mean serum neutralizing antibody titer compared to monkeys infected with rDEN4Δ30 virus. This and the lower mean number of viremic days per monkey suggests that the 10634 mutation can attenuate the replication of rDEN4Δ30 virus in monkeys.

[0063] On day 28 after inoculation, all monkeys were challenged with 5.0 log₁₀PFU wild type rDEN4 virus subcutaneously. Monkeys were observed daily and serum was collected on days 28 to 34, 36, and 38, and virus titers were determined by plaque assay in *Vero* cells for measurement of viremia after challenge. Twenty eight days after rDEN4 virus challenge, serum was drawn and the level of neutralizing antibodies was tested by plaque reduction assay in *Vero* cells. Mock-inoculated monkeys had a mean peak virus titer of 2.3 log₁₀PFU/ml after challenge with a mean number of viremic days of 3.5 (Table 8). However, monkeys inoculated with rDEN4, rDEN4Δ30, or each of the modified rDEN4Δ30 viruses had no detectable viremia, indicating that despite the decreased replication and immunogenicity of rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634 viruses, each was sufficiently immunogenic to induce protection against wild type rDEN4. Increases in mean neutralizing antibody titer were minimal (< 3-fold) following challenge in all inoculation groups except mock-infected providing further evidence that the monkeys were protected from the challenge.

Table 8. rDEN4Δ30 containing additional point mutations protects rhesus monkeys from wt DEN4 virus challenge

Virus ^a	No. of monkeys	Mean no. of viremic days per monkey after rDEN4 challenge	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
				Day 28	Day 56
Mock	2	3.5	2.3 ± 0.1	<10	358
rDEN4	2	0.0	<0.7	398	753
rDEN4Δ30	2	0.0	<0.7	181	202
rDEN4Δ30-4995	4	0.0	<0.7	78	170
rDEN4Δ30-8092	4	0.0	<0.7	61	131
rDEN4Δ30-10634	4	0.0	<0.7	107	177

^a 28 days after primary inoculation with the indicated viruses, rhesus monkeys were challenged subcutaneously with 10⁵ PFU rDEN4 virus in a 1 ml dose. Serum was collected on days 28 to 34, 36, 38, and 56. Virus titer was determined by plaque assay in *Vero* cells.

[0064] Taken together, these results indicate that the three point mutations, 4995, 8092, and 10634) described above do further attenuate the rDEN4Δ30 vaccine candidate in suckling mouse brain, SCID-HuH-7 mice, and rhesus monkeys. Because of additional incidental mutations (Table 4) present in each modified rDEN4Δ30 virus, the phenotypes cannot be directly attributed to the individual 4995, 8092, and 10634 point mutations. However, the presence of similar mouse-attenuation phenotypes in other rDEN4 viruses bearing one of these three mutations supports the contention that the 4995, 8092, and 10634 point mutations are responsible for the *att* phenotypes of the modified rDEN4Δ30 viruses. Since rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634 virus demonstrated decreased replication in rhesus monkeys while retaining sufficient immunogenicity to confer protective immunity, these viruses are contemplated as dengue vaccines for humans.

[0065] DEN4 viruses carrying both Δ30 and charge-to-alanine mutations were next generated. A subset of seven groups of charge-to-alanine mutations described above were identified that conferred between a 10-fold and 1,000-fold decrease in replication in SCID-HuH-7 mice and whose unmutated sequence was well-conserved across the four dengue serotypes. These mutations were introduced as single pairs and as two sets of pairs to rDEN4Δ30 using conventional cloning techniques. Transcription and recovery of virus and terminal dilution of viruses were conducted as described above. Assay of the level of temperature sensitivity of the charge-cluster-to-alanine mutant viruses in *Vero* and HuH-7 cells, level of replication in the brain of suckling mice and level of replication in SCID-HuH-7 mice was conducted as described above.

[0066] Introduction of one pair of charge-to-alanine mutations to rDEN4 produced recoverable virus in all cases (Table 9). Introduction of two pairs of charge-to-alanine mutations produced recoverable virus in two out of three cases (rDEN4Δ30-436-437-808-809 was not recoverable).

[0067] rDEN4Δ30 is not *ts* in *Vero* or HuH-7 cells. In contrast, seven of the seven sets of charge-to-alanine mutations used in this example conferred a *ts* phenotype in HuH-7 cells and five also conferred a *ts* phenotype in *Vero* cells. All six viruses carrying both Δ30 and charge-to-alanine mutations showed a *ts* phenotype in both *Vero* and HuH-7 cells (Table 9). rDEN4Δ30 is not attenuated in suckling mouse brain, whereas five of the seven sets of charge-to-alanine mutations conferred an *att* phenotype in suckling mouse brain (Table 10). Four of the viruses carrying both Δ30 and charge-to-alanine mutations were attenuated in suckling mouse brain (Table 10). In one case (rDEN4Δ30-23-24-396-397) combination of two mutations that did not attenuate alone resulted in an attenuated virus. Generally, viruses carrying both Δ30 and

charge-to-alanine mutations showed levels of replication in the suckling mouse brain more similar to their charge-to-alanine mutant parent virus than to rDEN4Δ30.

[0068] rDEN4Δ30 is attenuated in SCID-HuH-7 mice, as are six of the seven charge-to-alanine mutant viruses used in this example. Viruses carrying both Δ30 and charge-to-alanine mutations tended to show similar or slightly lower levels of replication in SCID-HuH-7 mice compared to their charge-to-alanine mutant parent virus (Table 10). In three cases, viruses carrying both A30 and charge-to-alanine mutations showed at least a fivefold greater reduction in SCID-HuH-7 mice than rDEN4Δ30.

[0069] The complete genomic sequence of five viruses (rDEN4-200-201, rDEN4Δ30-200-201, rDEN4-436-437 [clone 1], rDEN4A30-436-437, and rDEN4-23-24-200-201) that replicated to $> 10^5$ PFU/ml in Vero cells at 35°C and that showed a hundredfold or greater reduction in replication in SCID-HuH-7 mice was determined (Table 11). Each of the five contained one or more incidental mutations. In one virus, rDEN4Δ30-436-437, the one additional mutation has been previously associated with Vero cell adaptation (Blaney, J.E. Jr. et al. 2002 Virology 300:125-139). Each of the remaining viruses contained at least one incidental mutation whose phenotypic effect is unknown. Consequently, the phenotypes described cannot be directly attributed to the charge-to-alanine mutations. However, the fact that rDEN4 and rDEN4Δ30 viruses carrying the same charge-to-alanine mutations shared similar phenotypes provides strong support for the ability of charge-to-alanine mutations to enhance the attenuation of rDEN4Δ30. Because rDEN4-436-437 [clone 1] contained 4 incidental mutations, a second clone of this virus was prepared. rDEN4-436-437 [clone2] contained only one incidental mutation (Table 11), and showed the same phenotypes as rDEN4-436-437 in cell culture and SCID-HuH-7 mice. rDEN4-436-437 [clone 2] was used in the rhesus monkey study described below.

Table 9. Addition of charge-to-alanine mutations to rDEN4Δ30 confers a *ts* phenotype in both Vero and HuH-7 cells.

Virus	AA changed b	No. nt changed	Mean virus titer (log ₁₀ PFU/ml) at indicated temperature (°C) ^a										
			Vero					HuH-7					
			35	37	38	39	AC	35	37	38	39	Δ	
rDEN4	none	0	7.4	7.1	7.7	7.2	0.2	7.7	7.5	7.5	7.4	0.3	
rDEN4Δ30	none	30	6.6	6.6	6.5	6.5	0.1	7.4	6.9	7.0	6.4	1.0	
rDEN4-23-24	KE	3	6.7	6.6	6.0	6.5	0.2	7.1	7.3	5.6	<u><1.7</u>	>5.4	
rDEN4Δ30-23-24			6.1	5.5	4.9	<u><1.7</u>	4.4	6.5	5.9	4.7	<u><1.7</u>	>4.2	
rDEN4-200-201	KH	4	5.3	4.8	4.8	4.3	1.0	5.7	5.4	2.7	<u><1.7</u>	>4.0	
rDEN4Δ30-200-201			6.0	5.3	5.6	<u><1.7</u>	>4.3	5.8	5.0	5.9	<u><1.7</u>	>4.1	
rDEN4-436-437	DK	4	5.2	4.2	3.4	<u>1.9</u>	3.3	5.9	4.9	3.2	<u><1.7</u>	>4.2	
rDEN4Δ30-436-437 [clone1]			6.3	5.7	5.5	<u><1.7</u>	>4.6	6.5	5.7	5.1	<u><1.7</u>	>4.8	
rDEN4-808-809	ED	3	4.6	4.1	<u><1.7</u>	<u><1.7</u>	>2.9	5.2	<u><1.7</u>	<u><1.7</u>	<u><1.7</u>	>3.5	
rDEN4Δ30-808-809			5.6	4.9	4.9	<u><1.7</u>	>3.9	5.9	4.8	5.1	<u><1.7</u>	>4.2	
rDEN4-23-24-200-201	KE, KH	7	6.0	5.2	4.2	<u><1.7</u>	>4.3	6.9	6.3	<u><1.7</u>	<u><1.7</u>	>5.2	
rDEN4Δ30-23-24-200-201			4.5	4.2	4.8	<u><1.7</u>	>2.8	4.9	4.5	2.9	<u><1.7</u>	>3.2	
rDEN4-23-24-396-397	KE, RE	7	6.5	5.8	5.5	<u><1.7</u>	>4.8	7.1	5.9	5.4	<u><1.7</u>	>5.4	
rDEN4Δ30-23-24-396-397			6.1	5.2	4.8	<u><1.7</u>	>4.4	6.9	5.4	4.9	<u><1.7</u>	>5.2	
rDEN-436-437-808-809	DK, ED	7	4.9	4.9	5.1	<u><1.7</u>	>3.2	5.5	3.2	<u><1.7</u>	<u><1.7</u>	>3.8	

^a Underlined values indicate a 2.5 or 3.5 log₁₀PFU/ml reduction in titer in Vero or HuH-7 cells, respectively, at the indicated temperature when compared to the permissive temperature (35°C).

^b Amino acid pair(s) changed to pair of Ala residues.

^c Reduction in titer (log₁₀pfu/ml) compared to the permissive temperature (35°C).

Table 10. Addition of charge-to-alanine mutations attenuates rDEN4Δ30 in suckling mouse brain and enhances attenuation in SCID-HuH-7 mice.

Virus	Replication in suckling mice ^a			Replication in SCID-HuH-7 mice ^c		
	n	Mean virus titer ± SE (log ₁₀ PFU/g brain)	Mean log reduction from wt ^b	n	Mean virus titer ± SE (log ₁₀ PFU/ml serum)	Mean log reduction from wt ^d
rDEN4	18	6.2 ± 0.4	--	33	5.4 ± 0.3	--
rDEN4Δ30	12	5.9 ± 0.8	0.2	8	3.4 ± 0.3	2.3
rDEN4-23-24	18	4.7 ± 0.1	1.6	19	4.7 ± 0.5	1.3
rDEN4A30-23-24	6	5.6 ± 0.3	0.7	7	4.6 ± 0.4	1.5
rDEN4-200-201	12	5.5 ± 0.5	0.6	12	3.7 ± 0.2	2.6
rDEN4Δ30-200-201	6	5.5 ± 0.6	0.1	4	3.3 ± 0.6	1.8
rDEN4-436-437	18	2.7 ± 0.4	3.5	10	2.9 ± 0.7	2.5

Virus	Replication in suckling mice ^a			Replication in SCID-HuH-7 mice ^c		
	n	Mean virus titer $\pm$ SE (log ₁₀ PFU/g brain)	Mean log reduction from wt ^b	n	Mean virus titer $\pm$ SE (log ₁₀ PFU/ml serum)	Mean log reduction from wt ^d
rDEN4Δ30-436-437 [clone1]	6	2.9 $\pm$ 0.3	3.4	4	2.3 $\pm$ 0.4	2.8
rDEN4-808-809	6	1.8 $\pm$ 0.1	3.1	8	3.2 $\pm$ 0.4	3.0
rDEN4Δ30-808-809	12	3.9 $\pm$ 0.7	2.1	4	3.7 $\pm$ 0.6	2.4
rDEN4-23-24-200-201		5.3 $\pm$ 0.5	0.7	13	3.4 $\pm$ 0.1	2.9
rDEN4Δ30-23-24-200-201	6	3.0 $\pm$ 0.2	2.6	5	1.8 $\pm$ 0.1	3.3
rDEN4-23-24-396-397	12	4.6 $\pm$ 0.9	1.5	8	3.6 $\pm$ 0.3	2.3
rDEN4Δ30-23-24-396-397	6	3.0 $\pm$ 0.2	2.6	5	2.2 $\pm$ 0.3	2.9
rDEN-436-437-808-809	6	<1.7 $\pm$ 0.0	3.6	8	2.1 $\pm$ 0.3	2.4

^a Groups of six suckling mice were inoculated i.c. with 10⁴ PFU virus in a 30 μ l inoculum. The brain was removed 5 days later, homogenized, and virus was quantitated by titration in *Vero* cells.

^b Determined by comparing the mean viral titers in mice inoculated with sample virus and concurrent wt controls (n = 6). The attenuation (*att*) phenotype is defined as a reduction of ≥ 1.5 log₁₀PFU/g compared to wt virus; reductions of ≥ 1.5 are listed in boldface.

^c Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10⁴ PFU virus.

^d Determined by comparing mean viral titers in mice inoculated with sample virus and concurrent wt controls. The attenuation phenotype is defined as a reduction of ≥ 1.5 log₁₀ PFU/g compared to wt virus; reductions of ≥ 1.5 are listed in boldface.

Table 11. Missense and UTR mutations present in rDEN4 virus derivatives bearing charge-to-alanine and the Δ30 mutation.

Virus	Gene ^{a,b}	Nucleotide position	Nucleotide substitution	Amino acid position ^c	Amino acid change ^b
rDEN4-200-201	prM	626	A>T	61	Glu > Asp
	NS4A	6659	C>T	93	Leu > Phe
	NS5	8160-8165	AAACA > GCAGC	200-201	LysHis > AlaAla
rDEN4Δ30-200-201	NS3	4830	G>A	102	Gly > Arg
	NS5	8106	G>A	181	Val > Ile
	NS5	8160-8165	AAACA>GCAGC	200-201	LysHis > AlaAla
	3' UTR	10478-10507	Δ30 deletion	None	None
rDEN4-436-437 [clone 1]	E	2331	T>G	464	Trp > Gly
	NS1	2845	C>T	140	Ser > Phe
	NS3*	4891	T>C	122	Ile > Thr
	NS5	8869-8873	GACAA>GCAGC	436-437	AspLys > AlaAla
	NS5	9659	A>G	699	Lys > Arg
rDEN4-436-437 [clone 2]	NS4B	7153	T>C	108	Val > Ala
	NS5	8869-8873	GACAA>GCAGC	436-437	AspLys > AlaAla
rDEN4Δ30-436-437	NS4B*	7163	A>C	111	Leu > Phe
	NS5	8869-73	GACAA>GCAGC	436-437	AspLys > AlaAla
	3' UTR	10478-10507	A30 deletion	None	None
rDEN4-23-24-200-201	NS3	6751	A>C	124	Lys > Thr
	NS5	7629-7633	AAAGA>GCAGC	23-24	LysGlu > AlaAla
	NS5	8160-8165	AAACA>GCAGC	200-201	LysHis > AlaAla

^a Asterisk indicates previously identified *Vero* cell adaptation mutation.

^b Bold values indicate mutations designed to occur in the designated virus.

^c Amino acid position in the protein product of the designated DEN4 gene; numbering starts with the amino terminus of the protein.

[0070] Based on the attenuation in the SCID-HuH7 mouse model, four of the charge-to-alanine mutant viruses (rDEN4-200-201, rDEN4Δ30-200-201, rDEN4-436-437 [clone 2], rDEN4Δ30-436-437) were evaluated in rhesus macaques as described above. As with the study of viruses carrying attenuating point mutations, viremia was detected on day 1 post-infection and ended by day 4 in all monkeys (Figure 4, Table 12). Viremia was detected in most of the monkeys infected; only one of the four monkeys infected with rDEN4A30-200-201 and one of the four monkeys infected with rDEN4Δ30-436-437 showed no detectable viremia. Monkeys infected with rDEN4 showed the highest mean peak virus titer; and in each case viruses carrying the Δ30 mutation showed an approximately 0.5 log decrease in mean peak virus titer relative to their parental viruses and a 0.5 to 2 day decrease in mean number of viremic days per monkey. Monkeys infected with viruses carrying both the Δ30 and charge-to-alanine mutations showed a two-fold reduction in mean peak viremia relative to those infected with rDEN4Δ30. This suggests

that addition of the charge-to-alanine mutations further attenuates rDEN4Δ30 for rhesus macaques.

[0071] As expected, none of the monkeys in this study showed detectable levels of neutralizing antibody on day 0. On day 28, every monkey infected with a virus showed a detectable levels of neutralizing antibody, indicating that all of the monkeys, even those that showed no detectable viremia, had indeed been infected. As in the study of attenuating point mutations, monkeys infected with rDEN4 had a mean serum neutralizing antibody titer (reciprocal dilution) which was approximately twice that of monkeys that had been infected with rDEN4Δ30. Monkeys infected with rDEN4-200-201 and rDEN4-436-437 [clone 2] had similar mean neutralizing antibody titers to rDEN4, and those infected with rDEN4Δ30-200-201 and rDEN4Δ30-436-437 had similar mean neutralizing antibody titers to rDEN4. In each case the addition of the Δ30 mutation to a virus resulted in a two-fold decrease in neutralizing antibody. Thus, although the addition of charge-to-alanine mutations to rDEN4Δ30 decreased mean peak viremia below that of rDEN4Δ30 alone, it did not affect levels of neutralizing antibody.

Table 12. Addition of paired charge-to-alanine mutations to rDEN4Δ30 further attenuates the virus for rhesus monkeys.

Virus ^a	No. of monkeys	No. of monkeys with viremia	Mean no. of viremic days per monkey ^b	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
					Day 0	Day 28
mock	2	0	0	<0.7	<5	<5
rDEN4	2	2	2.5	2.6 ± 0.3	<5	276
rDEN4Δ30	2	2	2.0	2.1 ± 0.1	<5	131
rDEN4-200,201	4	4	2.3	1.8 ± 0.3	<5	212
rDEN4Δ30-200,201	4	3	1.5	1.3 ± 0.2	<5	139
rDEN4-436,437 [c1 2]	4	4	3.3	1.8 ± 0.2	<5	273
rDEN4Δ30-436,437	4	3	1.3	1.0 ± 0.0	<5	143

^a Groups of rhesus monkeys were inoculated subcutaneously with 10⁵ PFU of the indicated virus in a 1 ml dose. Serum was collected on days 0 to 6, 8, 10 and 28. Virus titer was determined by plaque assay in *Vero* cells.

^b Viremia was not detected in any monkey after day 4.

[0072] After challenge with rDEN4 on day 28, mock-infected monkeys had a mean peak virus titer of 1.5 log₁₀PFU/ml and a mean number of viremic days of 3.0 (Table 13). However, none of the monkeys previously inoculated with rDEN4, rDEN4Δ30 or the charge-to-alanine mutant viruses showed detectable viremia. Additionally, none of the monkeys showed a greater than four-fold increase in serum neutralizing antibody titer. Together these data indicate that infection with any of the viruses, including those carrying both Δ30 and the charge-to-alanine mutations, protected rhesus macaques from challenge with rDEN4.

Table 13. rDEN4Δ30 containing charge-to-alanine mutations protects rhesus monkeys from wt DEN4 virus challenge

Virus ^a	No. of monkeys	Mean no. of viremic days per monkey after rDEN4 challenge	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
				Day 28	Day 56
mock	2	3.0	1.5 ± 0.7	<5	284
rDEN4	2	0.0	0.7	276	316
rDEN4Δ30	2	0.0	<0.7	131	96
rDEN4-200,201	4	0.0	<0.7	212	356
rDEN4Δ30-200,201	4	0.0	<0.7	139	132
rDEN4-436,437 [c1 2]	4	0.0	<0.7	273	401
rDEN4Δ30-436,437	4	0.0	<0.7	143	182

^a 28 days after primary inoculation with the indicated viruses, rhesus monkeys were challenged subcutaneously with 10⁵ PFU rDEN4 virus in a 1 ml dose. Serum was collected on days 28 to 34, 36, 10, and 56. Virus titer was determined by plaque assay in *Vero* cells.

[0073] Addition of charge-to-alanine mutations to rDEN4Δ30 can confer a range of *ts* phenotypes in both *Vero* and HuH-7 cells and *att* phenotypes in suckling mouse brain and can either enhance or leave unchanged attenuation in SCID-HuH-7 mice. Most importantly, addition of these mutations can decrease the viremia produced by rDEN4Δ30 in rhesus macaques without decreasing neutralizing antibody titer or protective efficacy. Thus addition of such mutations to rDEN4Δ30 is contemplated as enhancing attenuation in humans. Also, mutations are contemplated as being added that do not change the overall level of attenuation, but stabilize the attenuation phenotype because they themselves are independently attenuating even in the absence of the Δ30 mutation. Charge-to-alanine mutations are particularly useful because they occur outside of the structural gene regions, and so can be used to attenuate structural gene chimeric viruses. Moreover, they involve at least three nucleotide changes, making them unlikely to revert to wild type sequence.

[0074] A series of point mutations that enhance the replication of rDEN4 in *Vero* cells tissue culture have been identified; these are primarily located in the NS4B gene (Blaney, J. E. et. al. 2002 *Virology* 300:125-139; Blaney, J. E. et al. 2001 *J Virol* 75:9731-9740). *Vero* cell adaptation

mutations confer two desirable features upon a vaccine candidate. First, they enhance virus yield in Vero cells, the intended substrate for vaccine production, and thus render vaccine production more cost-effective. Second, although each of these Vero adaptation mutations are point mutations, they are likely to be extremely stable during vaccine manufacture, because they give a selective advantage in Vero cells. At least one Vero cell adaptation mutation, at position 7129, was also shown to decrease mosquito infectivity of rDEN4; poor mosquito infectivity is another desirable characteristic of a dengue vaccine candidate. To investigate the generality of this finding, we tested the effect of the remaining Vero cell adaptation mutations on the ability of rDEN4 to infect *Aedes aegypti* mosquitoes fed on an infectious bloodmeal. Table 14 shows the infectivity of each virus carrying a single Vero cell adaptation mutation at high titer. Of these, only one mutation, at position 7182, was associated with a large decrease in mosquito infectivity. Thus 7182 may be a particularly valuable mutation to include in an rDEN4 vaccine candidate, as it has opposite effects on replication in Vero cells and in mosquitoes.

Table 14. Effect of Vero cell adaptation mutations on rDEN4 mosquito infectivity

Virus	<i>Aedes aegypti</i> (oral infection)			
	Dose ^a (log ₁₀ pfu)	No. tested	% infected ^b	
			Midgut	Head
rDEN4	4.3	27	70	25
rDBN4-4891	4.4	23	74	13
rDEN4-4995	4.8	20	80	50
rDEN4-7153	4.8	20	80	30
rDEN4-7546	4.6	20	55	10
rDEN4-7162	5.0	20	55	25
rDEN4-7163	4.9	15	73	72
rDEN4-7182	5.0	20	20	0
rDEN4-7630	4.3	10	70	10

^aVirus titer ingested, assuming a 2 µl bloodmeal.

^bPercentage of mosquitoes with IFA detectable antigen in midgut or head tissue prepared 21 days after oral infection.

Comparative EXAMPLE 2

Generation and Characterization of a Recombinant DEN1 Virus Containing the Δ30 Mutation

[0075] We first sought to determine if the Δ30 mutation was able to satisfactorily attenuate a wild-type DEN virus other than the DEN4 serotype. To do this, the Δ30 mutation was introduced into the cDNA for DEN1 (Western Pacific). The pRS424DEN1WP cDNA clone (Puri, B. et al. 2000 Virus Genes 20:57-63) was digested with *Bam*HI and used as template in a PCR using Pfu polymerase with forward primer 30 (DEN1 nt 10515-10561 and 10592-10607) and the M13 reverse sequencing primer (101 nt beyond the 3' end of DEN1 genome sequence). The resulting PCR product was 292 bp and contained the A30 mutation. The pRS424DBN1WP cDNA was partially digested with *Apa*I, then digested to completion with *Sac*II and the vector was gel isolated, mixed with PCR product, and used to transform yeast strain YPH857 to yield growth on plates lacking tryptophan (Polo, S. et al. 1997 J Virol 71:5366-74). Positive yeast colonies were confirmed by PCR and restriction enzyme analysis. DNA isolated from two independent yeast colonies was used to transform *E. coli* strain STBL2. Plasmid DNA suitable for generating RNA transcripts was prepared and the presence of the Δ30 mutation was verified by sequence analysis.

[0076] For transcription and generation of virus, cDNA (designated pRS424DEN1Δ30) that was linearized with *Sac*II was used as template in a transcription reaction using SP6 RNA polymerase as described (Polo, S. et al. 1997 J Virol 71:5366-74). Transcription reactions were electroporated into LLC-MK2 cells and infection was confirmed by observation of CPE and immunofluorescence and harvested on day 14. Virus stocks were amplified on C6/36 mosquito cells and titered on LLC-MK2 cells. The genome of the resulting virus, rDEN1Δ30, was sequenced to confirm the presence of the Δ30 mutation. The Δ30 mutation removes nucleotides 10562-10591 of DEN1 (Figure 2B, C), which corresponds to the TL2 of DEN1. The virus replicates efficiently in Vero cell culture to titers of 6.5 log₁₀ PFU/ml, indicating that the Δ30 mutation is compatible with efficient growth of DEN1 in cell culture, a property essential for manufacture of the vaccine. Using similar techniques, parent virus rDEN1 was generated. Incidental mutations arising from virus passage in tissue culture were identified in both rDEN1 and rDEN1Δ30 using sequence analysis and are listed in Table 15. An additional rDBN1Δ30 virus was derived by transfection and amplification in Vero cells. Although this virus was not evaluated in the studies described below, its sequence analysis is included in Table 15. The properties of rDEN1Δ30 as a vaccine *in vivo* were next examined.

Table 15. Missense mutations present among the recombinant DEN1 viruses and correlation of NS4B region mutations with those found in DEN4

Virus	Transfection cell type	Gene	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change
wt rDEN1	LLC-MK2	prM	816	C > U	241	Ala > Val
		NS4B	7165 ^a	U > G	2357	Phe > Leu
		NS4B	7173 ^b	U > C	2360	Val > Ala
rDEN1Δ30	LLC-MK2	B	1748	A > U	552	Thr > Ser

Virus	Transfection cell type	Gene	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change
rDEN1Δ30	Vero	E	1545	A > G	484	Lys > Arg

^a Same nucleotide as 7154 in rDEN4.

^b Same nucleotide as 7162 in rDEN4

* Nucleotide and amino acid comparison of selected NS4B region:

	7	7	7	7	7	7
DEN4	1	1	1	1	1	1
base	3	4	5	6	7	8
Number:	8901234567890123456789012345678901234567					
	++	++	+++++	+	+	++
D4 7128-	CCACAAACCUUGACAGCATCCUUGAGUCATGCUUUAAGUCCAUUAUGCAUUAUAGGCCCA					
	P T T L T A S L V M L L V H T A I I G P					
D1 7139-	CGGCUAGCGUGACAGCGCGGUAUUUAUGCUAGUGGCUCAUUAUGCCAUUAUUGGACCC					
	P L T L T A A V P M L V A H T A I I G P					
D2 7135-	CCUAUAACCCUCACAGCGGCUUUCUUAUUGGUAACCAUUAUGCCAUUAUAGGACCG					
	P I T L T A A L L L L V A H T A I I G P					
D3 7130-	CCACUAACUCUCACAGCGCGGUAUUUAUGCUAGUGGCUCAUUAUGCCAUUAUAGGUCCA					
	P L T L T A A V L L L V T H T A I I G P					
	+	+	+	+	+	+

D4 = rDEN4

D1 = rDEN1(WP)

D2 = rDEN2(Tonga/74)

D3 = rDEN3(Sleman/78)

+Homology among all four serotypes

Nucleotides are underlined in even multiples of 10.

[0077] Evaluation of the replication, immunogenicity, and protective efficacy of rDEN1Δ30 and wild-type parental rDEN1 virus (derived from the pRS424DEN1WP cDNA) in juvenile rhesus monkeys was performed as follows. Dengue virus-seronegative monkeys were injected subcutaneously with 5.0 log₁₀ PFU of virus in a 1 ml dose divided between two injections in each side of the upper shoulder area. Monkeys were observed daily and blood was collected on days 0 - 10 and 28 and serum was stored at -70°C. Titer of virus in serum samples was determined by plaque assay in Vero cells as described previously (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13). Plaque reduction neutralization titers were determined for the day 28 serum samples as previously described (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13). All monkeys were challenged on day 28 with a single dose of 5.0 log₁₀ PFU of wild-type rDEN1 and blood was collected for 10 days. Virus titer in post-challenge sera was determined by plaque assay in Vero cells. Monkeys inoculated with full-length wild-type rDEN1 were viremic for 2 - 3 days with a mean peak titer of 2.1 log₁₀ PFU/ml (Table 16), and monkeys inoculated with rDEN1Δ30 were viremic for less than 1 day with a mean peak titer of 0.8 log₁₀ PFU/ml, indicating that the Δ30 mutation is capable of attenuating DEN1. As expected for an attenuated virus, the immune response, as measured by neutralizing antibody titer, was lower following inoculation with rDEN1Δ30 compared to inoculation with wild-type rDEN1 (Table 16), yet sufficiently high to protect the animals against wild-type DEN1 virus challenge. Wild-type rDEN1 virus was not detected in any serum sample collected following virus challenge, indicating that monkeys were completely protected following immunization with either full-length wild-type rDEN1 or recombinant virus rDEN1Δ30. The level of attenuation specified by the Δ30 mutation was comparable in both the DEN1 and DEN4 genetic backgrounds (Figure 5).

Table 16. The Δ30 mutation attenuates rDEN1 for rhesus monkeys

Virus*	n	Mean no. days with viremia	Mean peak titer (log ₁₀ pfu/ml)	Mean neutralization titer	Mean peak titer of challenge virus
rDEN1	4	2.8	2.1	1230	<0.7
rDBN1Δ30	4	0.5	0.8	780	<0.7

* Rhesus monkeys were inoculated subcutaneously with 5.0 log₁₀ PFU of virus. Serum samples were collected daily for 10 days. Serum for neutralization assay was collected on day 28. All monkeys were challenged on day 28 with 5.0 log₁₀ PFU of rDEN1.

[0078] As previously reported, rDEN4 virus replicated to greater than 6.0 log₁₀PFU/ml serum in SCID-HuH-7 mice, while the replication of rDEN4 virus bearing the A30 mutation was reduced by about 10-fold (Blaney, J.E. Jr. et al. 2002 Virology 300:125-139). The replication of rDEN1Δ30 was compared to that of wt rDEN1 in SCID-HuH-7 mice (Table 17). rDEN1Δ30 replicated to a level approximately 100-fold less than its wt rDEN1 parent. This result further validates the use of the SCID-HuH-7 mouse model for the evaluation of attenuated strains of DEN virus, with results correlating closely with those observed in rhesus monkeys.

Table 17. The A30 mutation attenuates rDEN1 for HuH-7-SCID mice

Virus	No. of Mice ⁵	Mean peak virus titer ⁶ (log ₁₀ pfu/ml ± SE)
wt rDEN1	9	7.3 ± 0.2
rDBN1Δ30	8	5.0 ± 0.3

⁵ Groups of HuH-7-SCID mice were inoculated directly into the tumor with 4.0 log₁₀pfu virus. Serum was collected on day 6 and 7, and virus titer was determined by plaque assay in Vero cells.

⁶ Significant difference was found between rDEN1 and rDEN1Δ30 viruses, Tukey-Kramer test ($P < 0.005$).

[0079] Finally, the infectivity of rDEN1 and rDEN1Δ30 for mosquitoes was assessed, using the methods described in detail in Example 5. Previously, the Δ30 mutation was shown to decrease the ability of rDEN4 to cross the mosquito midgut barrier and establish a salivary gland infection (Troyer, J.M. et al. 2001 Am J Trop Med Hyg 65:414-419). However neither rDEN1 nor rDEN1Δ30 was able to infect the midgut of *Aedes aegypti* mosquitoes efficiently via an artificial bloodmeal (Table 18), so it was not possible to determine whether Δ30 might further block salivary gland infection. A previous study also showed that the Δ30 had no effect on the infectivity of rDEN4 for *Toxorhynchites splendens* mosquitoes infected via intrathoracic inoculation (Troyer, J.M. et al. 2001 Am J Trop Med Hyg 65:414-419), and a similar pattern was seen for rDEN1 and rDEN1Δ30 (Table 18). The genetic basis for the inability of rDEN1 to infect the mosquito midgut has not been defined at this time. However, this important property of restricted infectivity for the mosquito midgut is highly desirable in a vaccine candidate since it would serve to greatly restrict transmission of the vaccine virus from a vaccinee to a mosquito vector.

Table 18. DEN1 and DEN1Δ30 viruses are both highly infectious for *Toxorhynchites splendens*, but do not infect *Aedes aegypti* efficiently.

Virus	<i>Toxorhynchites splendens</i> (intrathoracic inoculation)			<i>Aedes aegypti</i> (oral infection)			
	Dose ^a (log ₁₀ pfu)	No. tested	% infected ^b	Dose ^c (log ₁₀ (pfu)	No. tested	% infected ^d	
						Midgut	Head
rDEN1	3.5	7	100	4.0	26	11	0
	2.5	8	75				
	1.5	7	71				
	0.5	5	60				
			MID ₅₀ < 0.5			MID ₅₀ ≥ 4.4	
rDEN1 Δ30	2.7	8	100	3.2	20	10	0
	1.7	7	100				
	0.7	6	83				
			MID ₅₀ < 0.7			MID ₅₀ ≥ 3.6	

^a Amount of virus present in 0.22 μl inoculum.

^b Percentage of mosquitoes with IFA detectable antigen in head tissue prepared 14 days after inoculation.

^c Virus titer ingested, assuming a 2 μl bloodmeal.

^d Percentage of mosquitoes with IFA detectable antigen in midgut or head tissue prepared 21 days after oral infection. When virus infection was detected, but did not exceed a frequency of 50% at the highest dose of virus ingested, the MID₅₀ was estimated by assuming that a 10-fold more concentrated virus dose would infect 100% of the mosquitoes.

[0080] Thus, the Δ30 mutation, first described in DEN4, was successfully transferred to rDEN1. The resulting virus, rDEN1Δ30, was shown to be attenuated in monkeys and SCID-HuH-7 mice to levels similar to recombinant virus rDEN4Δ30, thereby establishing the conservation of the attenuation phenotype specified by the Δ30 mutation in a different DEN virus background. Based on the favorable results of rDEN4Δ30 in recent clinical trials (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13), it is predicted that rDEN1Δ30 will be suitably attenuated in humans. To complete the tetravalent vaccine, attenuated rDEN2 and rDEN3 recombinant viruses bearing the A30 mutation are contemplated as being prepared (See Examples 3 and 4 below). The demonstration that the Δ30 mutation specifies a phenotype that is transportable to another DEN serotype has important implications for development of the tetravalent vaccine. This indicates that the Δ30 mutation is expected to have a corresponding effect on DEN2 and DEN3 wild-type viruses.

Comparative EXAMPLE 3

Generation and Characterization of a Recombinant DEN2 Virus Containing the Δ30 Mutation

[0081] Evaluation of rDEN1Δ30 showed that it was satisfactorily attenuated. Based on this result, we sought to extend our technology to the creation of a DEN2 vaccine candidate. To do this, the Δ30 mutation was introduced into the cDNA of DEN2. A DEN2 virus isolate from a 1974 dengue epidemic in the Kingdom of Tonga (Tonga/74) (Gubler, D. J. et al. 1978 Am J Trop Med Hyg 27:581-589) was chosen to represent wt DEN2. The genome of DEN2 (Tonga/74) was sequenced in its entirety and served as consensus sequence for the construction of a full-length cDNA clone (Appendix 1). cDNA fragments of DEN2 (Tonga/74) were generated by reverse-transcription of the genome as indicated in Figure 6A. Each fragment was subcloned into a plasmid vector and sequenced to verify that it matched the consensus sequence as determined for the virus. This yielded seven cloned cDNA fragments spanning the genome. Cloned fragments were modified as follows: Fragment X, representing the 5' end of the genome was abutted to the SP6 promoter; Fragment L was modified to contain a translationally-silent *SpeI* restriction site at genomic nucleotide 2353; Fragment R was modified to contain a translationally-silent *SpeI* restriction site also at genomic nucleotide 2353, and to stabilize the eventual full-length clone, two additional translationally-silent mutations at nucleotides 2362 - 2364 and 2397 were created to ensure that translation stop codons were present in all reading frames other than that used to synthesize the virus polyprotein; Fragment A was modified at nucleotide 3582 to ablate a naturally occurring *SpeI* restriction site and at nucleotide 4497 to ablate a naturally occurring *KpnI* restriction site; Fragment C was modified at nucleotide 9374 to ablate a naturally occurring *KpnI* restriction site; and Fragment Y, representing the 3' end of the genome was abutted to a *KpnI* restriction site. Each fragment was added incrementally between the *AscI* and *KpnI* restriction

sites of DEN4 cDNA clone p4 (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13) to generate a full-length DEN2 cDNA clone (p2) with the same vector background successfully used to generate rDEN4 and rDEN4Δ30. cDNA clone p2 was sequenced to confirm that the virus genome region matched the DEN2 (Tonga/74) consensus sequence, with the exception of the translationally-silent modifications noted above. The Δ30 mutation was introduced into Fragment Y to generate Fragment YΔ30. To create p2A30, the Fragment Y region of p2 was replaced with Fragment YΔ30 (Figure 6A, B).

[0082] For transcription and generation of infectious virus, cDNA (p2 and p2A30) was linearized with *Acc65I* (isoschizomer of *KpnI* which cleaves leaving only a single 3' nucleotide) and used as template in a transcription reaction using SP6 RNA polymerase as previously described (Blaney, J. E. et. al. 2002 Virology 300:125-139). Transcripts were introduced into *Vero* cells or C6/36 mosquito cells using liposome-mediated transfection and cell culture supernatants were harvested on day 7.

[0083] rDEN2 virus was recovered from the p2 cDNA in both *Vero* and C6/36 cells, while rDEN2Δ30 was recovered from the p2A30 cDNA clone in only C6/36 cells (Table 19). The level of infectious virus recovered in C6/36 cells was comparable for the p2 and p2A30 cDNA clones when assayed by plaque titration and immunostaining in *Vero* or C6/36 cells. As previously observed, the efficiency of transfection in C6/36 cells was higher than that in *Vero* cells. Two rDEN2Δ30 viruses were recovered from independent cDNA clones, #2 and #10.

Table 19. rDEN2 virus is recovered in *Vero* and C6/36 cells, but rDEN2Δ30 virus is recovered only in C6/36 cells.

Transfection cell type	cDNA construct	Clone	Virus	Virus titer of transfection harvest (day 7) determined in the indicated cell type (log ₁₀ PFU/ml)	
				<i>Vero</i> cells	C6/36 cells
<i>Vero</i> cells	p2	#8A	rDEN2	3.1	4.3
	p2Δ30	#2	rDEN2Δ30	<0.7	<0.7
	p2Δ30	#10	rDEN2Δ30	<0.7	<0.7
C6/36 cells	p2	#8A	rDEN2	5.5	7.5
	p2Δ30	#2	rDEN2Δ30	4.8	7.6
	p2Δ30	#10	0	4.6	7.5

[0084] To produce working stocks of rDEN2 and rDEN2Δ30 viruses, transfection harvests were passaged and terminally diluted in *Vero* cells, and genomic sequences of the viruses were determined. The *Vero* cell transfection harvest of rDEN2 virus was terminally diluted once in *Vero* cells, and individual virus clones were passaged once in *Vero* cells. To assess whether any homologous *Vero* cell adaptation mutations identified in the rDEN4 NS4B 7100-7200 region were present in these virus clones, seven independent terminally diluted clones were sequenced over this region. Each of the seven rDEN2 viruses contained a single nucleotide substitution in this region at nucleotide 7169 (U > C) resulting in a Val > Ala amino acid change. This nucleotide corresponds to the 7162 mutation identified in rDEN4 (Blaney, J. E. et. al. 2002 Virology 300:125-139), which has a known *Vero* cell adaptation phenotype suggesting that this mutation may confer a replication enhancement phenotype in rDEN2 virus. One rDEN2 virus clone was completely sequenced and in addition to the 7169 mutation, a missense mutation (Glu > Ala) was found in NS5 at residue 3051 (Table 20).

Table 20. Missense mutations which accumulate in rDEN2 and rDEN2Δ30 viruses after transfection or passage in *Vero* cells.

Virus	Gene	Nucleotide position	Nucleotide substitution	Amino acid position ^a	Amino acid change
rDBN2 ^b (<i>Vero</i>)	NS4B	7169 ^c	U > C	2358	Val > Ala
	NS5	9248	A > C	3051	Glu > Ala
rDBN2Δ30 ^d (<i>Vero</i>)	NS3	4946	A > G	1617	Lys > Arg
	NS4B	7169 ^c	U > C	2358	Val > Ala

^a Amino acid position in DEN2 polypeptide beginning with the methionine residue of the C protein (nucleotides 97-99) as position 1.

^b Virus was recovered in *Vero* cells and terminally-diluted once in *Vero* cells. Virus stock was prepared in *Vero* cells.

^c Same nucleotide position as 7162 in rDEN4.

^d Virus was recovered in C6/36 cells and passaged three times in *Vero* cells. Virus was then terminally diluted and a stock was prepared in *Vero* cells.

[0085] Because both rDEN2 and rDEN2Δ30 viruses grown in *Vero* cells acquired the same mutation at nucleotide 7169, which corresponds to the *Vero* cell adaptation mutation previously identified in rDEN4 at nucleotide 7162, it was reasoned that this mutation is associated with growth adaptation of rDEN2 and rDEN2Δ30 in *Vero* cells. In anticipation that the 7169 mutation may allow rDEN2Δ30 to be recovered directly in *Vero* cells, the mutation was introduced into the rDEN2Δ30 cDNA plasmid to create p2A30-7169. Transcripts synthesized from p2A30-7169, as well as p2 and p2A30 were introduced into *Vero* cells or C6/36 mosquito cells using liposome-mediated transfection as described above. Virus rDEN2Δ30-7169 was recovered from the p2A30-7169 cDNA in both *Vero* and C6/36 cells, while rDEN2Δ30 was recovered from the p2A30 cDNA clone in only C6/36 cells (Table 21). The 7169 mutation is both necessary and sufficient for the recovery of rDEN2Δ30 in *Vero* cells.

Table 21. rDEN2Δ30-7169 virus containing the 7169 *Vero* cell adaptation mutation is recovered in both *Vero* and C6/36 cells

Transfection cell type	cDNA construct	Clone	Virus	Virus titer of transfection harvest (day 14) determined in C6/36 cells (log ₁₀ PFU/ml)
	p2	#8A	rDBN2	6.8

Transfection cell type	cDNA construct	Clone	Virus	Virus titer of transfection harvest (day 14) determined in C6/36 cells (log ₁₀ PFU/ml)
Vero cells	p2Δ30	#2	rDEN2Δ30	< 0.7
	p2Δ30-7169 ^a	#37	rDEN2Δ30-7169	5.1
C6136 cells	p2	#8A	rDEN2	6.9
	p2Δ30	#2	rDEN2Δ30	7.1
	p2Δ30-7169	#37	rDEN2Δ30-7169	7.2

^a Nucleotide 7169 in rDEN2 corresponds to nucleotide 7162 in rDEN4 which has been shown to be associated with growth adaptation in *Vero* cells.

[0086] To initially assess the ability of the Δ30 mutation to attenuate rDEN2 virus in an animal model, the replication of DEN2 (Tonga/74), rDEN2, and rDEN2Δ30 viruses was evaluated in SCID-HuH-7 mice. Previously, attenuation of vaccine candidates in SCID-HuH-7 mice has been demonstrated to be predictive of attenuation in the rhesus monkey model of infection (Examples 1 and 2). The recombinant viruses tested in this experiment were recovered in C6/36 cells. The DEN2 Tonga/74 virus isolate, rDEN2, and two independent rDEN2Δ30 viruses, (clones 20A and 21A) which were derived from two independent p2Δ30 cDNA clones, were terminally diluted twice in C6/36 cells prior to production of a working stock in C6/36 cells. These viruses should not contain any *Vero* cell adaptation mutations. DEN2 Tonga/74 virus replicated to a mean virus titer of 6.2 log₁₀PFU/ml in the serum of SCID-HuH-7 mice, and rDEN2 virus replicated to a similar level, 5.6 log₁₀PFU/ml (Table 22). Both rDEN2Δ30 viruses were greater than 100-fold restricted in replication compared to rDEN2 virus. These results indicate that the Δ30 mutation has an attenuating effect on replication of rDEN2 virus similar to that observed for rDEN4 and rDEN1 viruses.

Table 22. The Δ30 mutation restricts rDEN2 virus replication in SCID-HuH-7 mice.

Virus	No. of mice	Mean virus titer ± SE (log ₁₀ PFU/ml serum) ^a	Mean log ₁₀ -unit reduction from value for wt ^b
DEN2 (Tonga/74)	8	6.2 ± 0.3	-
rDEN2	9	5.6 ± 0.2	-
rDEN2Δ30 (clone 20A)	9	3.1 ± 0.2	2.5
rDEN2Δ30 (clone 21A)	9	2.9 ± 0.3	2.7

^a Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10⁴ PFU virus grown in C6/36 cells. Serum was collected on day 7 and titered in C6/36 cells.

^b Comparison of mean virus titers of mice inoculated with mutant virus and concurrent rDEN2 control.

[0087] DEN2 virus replication in SCID-HuH-7 mice was also determined using DEN2 (Tonga/74), rDEN2, and rDEN2Δ30 which were passaged in *Vero* cells (see Table 20, footnotes b and d). Both rDEN2 and rDEN2Δ30 had acquired a mutation in NS4B, nucleotide 7169, corresponding to the 7162 mutation identified in rDEN4 as *Vero* cell adaptation mutation. In the presence of the 7169 mutation, the Δ30 mutation reduced replication of rDEN2Δ30 by 1.0 log₁₀PFU/ml (Table 23). Previously, using virus grown in C6/36 cells and lacking the 7169 mutation, the Δ30 mutation reduced replication of 0 by about 2.5 log₁₀PFU/ml (Table 22). These results indicate that *Vero* cell growth adaptation in DEN2 may also confer a slight growth advantage in HuH-7 liver cells. Nevertheless, the attenuation conferred by the Δ30 mutation is still discernible in these *Vero* cell growth adapted viruses.

Table 23. The Δ30 mutation restricts *Vero* cell adapted rDEN2 virus replication in SCID-HuH-7 mice.

Virus	No. of mice	Mean virus titer ± SE (log ₁₀ PFU/ml serum) ^a	Mean log ₁₀ -unit reduction from value for wt ^b
DEN2 (Tonga/74)	6	5.9 ± 0.3	-
rDEN2	7	5.9 ± 0.2	-
rDEN2Δ30	9	4.9 ± 0.3	1.0

^a Groups of SCID-HuH-7 mice were inoculated directly into the tumor with 10⁴ PFU virus. Serum was collected on day 7 and titered in C6/36 cells.

^b Comparison of mean virus titers of mice inoculated with rDEN2Δ30 and rDEN2 control.

[0088] Evaluation of the replication, immunogenicity, and protective efficacy of rDEN2Δ30 and wild-type parental rDEN2 virus in juvenile rhesus monkeys was performed as follows. Dengue virus-seronegative monkeys were injected subcutaneously with 5.0 log₁₀ PFU of virus in a 1 ml dose divided between two injections in each side of the upper shoulder area. Monkeys were observed daily and blood was collected on days 0 - 10 and 28 and serum was stored at -70°C. Viruses used in this experiment were passaged in *Vero* cells, and recombinant viruses contained the mutations shown in Table 20 (See footnotes b and d). Titer of virus in serum samples was determined by plaque assay in *Vero* cells as described previously (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13). Plaque reduction neutralization titers were determined for the day 28 serum samples as previously described (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13). All monkeys were challenged on day

28 with a single dose of $5.0 \log_{10}$ PFU of wt DEN2 (Tonga/74) and blood was collected for 10 days. Virus titer in post-challenge sera was determined by plaque assay in Vero cells. Monkeys inoculated with wt DEN2 (Tonga/74) or rDEN2 were viremic for 4 - 5 days with a mean peak titer of 2.1 or 1.9 $\log_{10}$ PFU/ml, respectively.

[0089] Monkeys inoculated with rDEN2Δ30 were viremic for 2 - 3 days with a mean peak titer of 1.7 $\log_{10}$ PFU/ml (Table 24, Figure 7), indicating that the Δ30 mutation is capable of attenuating DEN2, although not to the same low level observed in rDEN1Δ30 (Table 16). As expected for an attenuated virus, the immune response, as measured by neutralizing antibody titer, was lower following inoculation with rDEN2Δ30 compared to inoculation with wt DEN2 (Tonga/74) or rDEN2 (Table 24), yet sufficiently high to protect the animals against wt DEN2 virus challenge (Table 25). Thus, the decreased number of days of viremia for rDEN2Δ30, the decreased mean peak titer, and the decreased serum antibody response indicate that the Δ30 mutation attenuates rDEN2 for rhesus monkeys.

Table 24. rDEN2Δ30 is slightly more attenuated for rhesus monkeys than rDEN2

Virus ^a	No. of monkeys	No. of monkeys with viremia	Mean no. of viremic days per monkey ^b	Mean peak virus titer ($\log_{10}$ PFU/ml $\pm$ SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
					Day 0	Day 28
mock	2	0	0	<0.7	<10	<10
DEN2 (Tonga/74)	4	4	4.5	2.1 $\pm$ 0.3	<10	311
rDEN2 (Vero)	4	4	4.0	1.9 $\pm$ 0.1	<10	173
rDEN2Δ30 (Vero)	4	4	2.8	1.7 $\pm$ 0.2	<10	91

^a Groups of rhesus monkeys were inoculated subcutaneously with 10^5 PFU of the indicated virus in a 1 ml dose. Serum was collected on days 0 to 6, 8, 10, 12, and 28. Virus titer was determined by plaque assay in Vero cells.

^b Viremia was not detected in any monkey after day 8.

Table 25. rDEN2Δ30 protects rhesus monkeys from wt DEN2 virus challenge

Virus ^a	No. of monkeys	Mean no. of viremic days per monkey after DEN2 challenge	Mean peak virus titer ($\log_{10}$ PFU/ml $\pm$ SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
				Day 28	Day 56
Mock	2	4.0	2.1 $\pm$ 0.1	<10	338
DEN2 (Tonga/74)	4	0	<0.7	311	334
rDEN2 (Vero)	4	0	<0.7	173	318
rDEN2Δ30 (Vero)	4	0	<0.7	91	267

^a 28 days after inoculation with the indicated viruses, monkeys were challenged subcutaneously with 10^5 PFU DEN2 (Tonga/74) in a 1 ml dose. Serum was collected on days 28 to 34, 36, 38, and 56. Virus titer was determined by plaque assay in Vero cells.

[0090] The infectivity of DEN2 (Tonga/74), rDEN2 and rDEN2Δ30 for *Aedes aegypti* mosquitoes via an artificial bloodmeal was evaluated using the methods described in detail in Example 5. However at doses of 3.3 to 3.5 $\log_{10}$ pfu ingested, none of these three viruses infected any mosquitoes, indicating that DEN2 (Tonga/74) is poorly infectious for *Aedes aegypti*. As with rDEN1, the genetic basis for this lack of infectivity remains to be defined. The important property of restricted infectivity for the mosquito midgut is highly desirable in a vaccine candidate because it would serve to greatly restrict transmission of the virus from a vaccinee to a mosquito vector.

[0091] Several missense mutation identified in rDEN4 have been demonstrated to confer attenuated replication in suckling mouse brain and/or SCID-HuH-7 mice (Blaney, J. E. et al 2002 Virology 300:125-139; Blaney, J. E. et al. 2001 J Virol 75:9731-9740). In addition, missense mutations that enhance replication of rDEN4 virus in Vero cells have been characterized. The significant sequence conservation among the DEN virus serotypes provides a strategy by which the mutations identified in rDEN4 viruses are contemplated as being used to confer similar phenotypes upon rDBN2 virus. Six mutations identified in rDEN4 virus that are at a site conserved in rDEN2 virus are being introduced into the p2 and p2Δ30 cDNA clones (Table 26). Specifically, two rDEN4 mutations, NS3 4891 and 4995, which confer Vero cell adaptation phenotypes and decreased replication in mouse brain, one mutation, NS4B 7182, which confers a Vero cell adaptation phenotype, and three mutations, NS1 2650, NS3 5097, and 3' UTR 10634 which confer decreased replication in mouse brain and SCID-HuH-7 mice are being evaluated. These mutations have been introduced into sub-cloned fragments of the p2 and p2Δ30 cDNA clones, and have been used to generate mutant full-length cDNA clones (Table 26), from which virus has been recovered in C6/36 cells (Table 27). The evaluation of these mutant rDEN2 viruses is contemplated as determining that such point mutations can be transported into a different DEN virus serotype and confer a similar useful phenotype, as has been demonstrated for the Δ30 deletion mutation.

Table 26. Introduction of conserved point mutations characterized in rDBN4 viruses into rDEN2 Tonga/74 virus.

Phenotype in rDEN4 virus				Mutation in rDEN4 virus			Mutation introduced into DEN2 virus			
Vero Adaptation ^a	Mouse brain att ^b	SCID-HuH-7 att ^c	Gene/region	Nucleotide position	Amino acid position ^d	Amino acid change	Nucleotide position	Amino acid position ^d	Amino add change	RE site / mutagenic region ^e
+	+	-	NS3	4891	1597	Ile > Thr	4889	1598	Ile > Thr	Nar I CCAcgGGcGCCGT
+	+	-	NS3	4995	1632	Ser > Pro	4993	1633	Ser > Pro	Stu I AAGGccTGGGA
+	-	-	NS4b	7182	2361	Gly > Ser	7189	2365	Gly > Ser	Xma I TATccCCGGGAC
-	+	+	NS1	2650	850	Asn > Ser	2648	851	Asn > Ser	Sac I AGAgcTctcTC
-	+	+	NS3	5097	1666	Asp > Asn	5095	1667	Asp > Asn	Xma I GaATCTCCACCCgGA
-	+	+	3'UTR	10634	n/a ^f	n/a	10698	n/a	n/a	none CTGTcGAATC

^a Presence of the indicated mutation increases plaque size in Vero cells two-fold or greater than rDEN4 virus.

^b Presence of the indicated mutation restricts replication in 7-day-old mouse brain greater than 100-fold compared to rDEN4 virus.

^c Presence of the indicated mutation restricts replication in SCID-HuH-7 mice greater than 100-fold compared to rDEN4 virus.

^d Amino acid position in DEN4 or DEN2 polyprotein beginning with the methionine residue of the C protein (nucleotides 102-104 or 97-99, respectively) as position 1.

^e Primers were engineered which introduced (underline) translationally-silent restriction enzyme (RE) sites. Lowercase letters indicate nt changes and bold letters indicate the site of the 5-FU mutation, which in some oligonucleotides differs from the original nucleotide substitution change in order to create a unique RE site. The change preserves the codon for the amino acid substitution.

^f Nucleotide substitution in the 3' UTR is U > C in DEN4 and DEN2 virus.

Table 27. rDEN2 viruses containing conserved 5-FU mutations are recovered in C6/36 cells.

Virus (nucleotide position in rDEN2)	Nucleotide position in rDEN4	Virus titer of transfection harvest (day 7) determined in C6/36 cells (log ₁₀ PFU/ml)
rDEN2-4889	4891	7.6
rDEN2-4993	4995	7.2
rDEN2-7189	7182	3.5
rDEN2-2648	2650	_a
rDEN2-5095	5097	_a
rDEN2-10698	10634	7.7

^a Transfection has not yet been attempted.

Comparative EXAMPLE 4

Generation and Characterization of a Recombinant DEN3 Virus Containing the Δ30 Mutation

[0092] Because rDEN1Δ30 was satisfactorily attenuated, we sought to extend our technology to the creation of a DEN3 vaccine candidate. To do this, the Δ30 mutation was introduced into the cDNA of DEN3, similar to the method used to create rDEN2Δ30. A DEN3 virus isolate from a 1978 dengue epidemic in rural Sleman, Central Indonesia (Sleman/78) (Gubler, D. J. et al. 1981 Am J Trop Med Hyg 30:1094-1099) was chosen to represent wt DEN3. The genome of DEN3 (Sleman/78) was sequenced in its entirety and served as consensus sequence for the construction of a full-length cDNA clone (Appendix 2). cDNA fragments of DEN3 (Sleman/78) were generated by reverse-transcription of the genome as indicated in Figure 8A. Each fragment was subcloned into a plasmid vector and sequenced to verify that it matched the consensus sequence as determined for the virus. This yielded six cloned cDNA fragments spanning the genome. Cloned fragments were modified as follows: Fragment 5, representing the 5' end of the genome was abutted to the SP6 promoter preceded by an *Ascl* restriction site; Fragment 1L was modified to contain a translationally-silent *SpeI* restriction site at genomic nucleotide 2345; Fragment 1R was modified to contain a translationally-silent *SpeI* restriction site also at genomic nucleotide 2345, and to stabilize the eventual full-length clone, three additional translationally-silent mutations at nucleotides 2354 - 2356, 2360 - 2362, and 2399 were created to ensure that translation stop codons were present in all reading frames other than that used to synthesize the virus polyprotein; Fragment 3 was modified at nucleotide 9007 to ablate a naturally occurring *KpnI* restriction site; and Fragment 4, representing the 3' end of the genome was abutted to a *KpnI* restriction site. Each fragment was added incrementally between the *Ascl* and *KpnI* restriction sites of DEN4 cDNA clone p4 (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13) to generate a full-length DEN3 cDNA clone with the same vector background successfully used to generate rDEN4 and rDEN2. However, a stable, full-length clone could not be recovered in *E. coli* when fragments 1L and 1R were combined into the same cDNA molecule. To overcome this instability, a synthetic DNA linker (Figure 8A) containing redundant termination codons in each of the forward and reverse open reading frames was introduced into the *SpeI* restriction site at the same time that fragment 1L was added to complete the full-length cDNA

construct. The resulting p3 clone containing the linker sequence was stable in *E. coli*, indicating that the linker sequence was sufficient to interrupt whatever deleterious element exists in this region. cDNA clone p3 was sequenced and the virus genome was found to match the DEN3 (Sleman/78) consensus sequence, with the exception of the linker sequence and translationally-silent modifications noted above (Appendix 2 - shown with the linker sequence removed). The $\Delta 30$ mutation was introduced into Fragment 4 to generate Fragment 4 $\Delta 30$. To create p3 $\Delta 30$, the Fragment 4 region of p3 was replaced with Fragment 4 $\Delta 30$ (Figure 8A, B).

[0093] For transcription and generation of infectious virus, cDNA plasmids p3 and p3 $\Delta 30$ were digested with *SpeI* and re-ligated to remove the linker sequence, linearized with *Acc65I* (isoschizomer of *KpnI* which cleaves leaving only a single 3' nucleotide), and used as templates in a transcription reaction using SP6 RNA polymerase as previously described (Blaney, J. E. et al. 2002 Virology 300:125-139). Transcripts were introduced into *Vero* cells or C6/36 mosquito cells using liposome-mediated transfection and cell culture supernatants were harvested on day 14.

[0094] rDEN3 virus was recovered from the p3 cDNA in both *Vero* and C6/36 cells, while rDEN3 $\Delta 30$ was recovered from the p3 $\Delta 30$ cDNA clone in only C6/36 cells (Table 28). The level of infectious virus recovered in C6/36 cells was comparable for the p3 and p3 $\Delta 30$ cDNA clones when assayed by plaque titration in *Vero* or C6/36 cells. As previously observed, the efficiency of transfection in C6/36 cells was higher than that in *Vero* cells. Two rDEN3 $\Delta 30$ viruses were recovered from independent cDNA clones, #22 and #41.

Table 28. rDEN3 virus is recovered in *Vero* and C6/36 cells, but rDEN3 $\Delta 30$ virus is recovered only in C6/36 cells.

Transfection cell type	cDNA construct	Clone	Virus	Virus titer of transfection harvest (day 14) determined in the indicated cell type (log ₁₀ PFU/ml)	
				<i>Vero</i> cells	C6/36 cells
<i>Vero</i> cells	p3	#50	rDEN3	5.2	6.3
	p3 $\Delta 30$	#22	rDEN3 $\Delta 30$	< 0.7	< 0.7
	p3 $\Delta 30$	#41	rDEN3 $\Delta 30$	< 0.7	< 0.7
C6/36 cells	p3	#50	rDEN3	5.2	6.0
	p3 $\Delta 30$	#22	rDEN3 $\Delta 30$	5.9	6.9
	p3 $\Delta 30$	#41	rDEN3 $\Delta 30$	5.1	7.2

[0095] To produce working stocks of viruses, transfection harvests will be passaged and terminally diluted in *Vero* cells, and genomic sequences of the viruses will be determined. To improve virus yield in *Vero* cells, the *Vero* cell adaptation mutation previously identified in rDEN4 at nucleotide 7162 was introduced into the homologous NS4B region of p3 and p3 $\Delta 30$ to create p3-7164 and p3 $\Delta 30$ -7164. This mutation creates a Val to Ala substitution at amino acid position 2357. As demonstrated for rDEN2 $\Delta 30$, this mutation allowed for the direct recovery of virus in *Vero* cells (Table 27) and is anticipated to have the same effect for rDEN3 $\Delta 30$.

[0096] To initially assess the ability of the $\Delta 30$ mutation to attenuate rDEN3 virus in an animal model, the replication of DEN3 (Sleman/78), rDEN3, and rDEN3 $\Delta 30$ viruses will be evaluated in SCID-HuH-7 mice and rhesus monkeys. Previously, attenuation of vaccine candidates in SCID-HuH-7 mice has been demonstrated to be predictive of attenuation in the rhesus monkey model of infection (Examples 1 and 2). The evaluation of these mutant rDEN3 viruses is contemplated as determining that the $\Delta 30$ deletion mutations can be transported into the DEN3 virus serotype and confer a similar useful phenotype, as has been demonstrated for DEN1, DEN2, and DEN4.

[0097] In summary, the strategy of introducing the $\Delta 30$ mutation into wild-type DEN viruses of each serotype to generate a suitably attenuated tetravalent vaccine formulation is a unique and attractive approach for several reasons. First, the mutation responsible for attenuation is a 30-nucleotide deletion in the 3' UTR, thus assuring that all of the structural and non-structural proteins expressed by each of the four components of the tetravalent vaccine are authentic wild-type proteins. Such wild-type proteins should elicit an antibody response that is broad based, rather than based solely on the M and E proteins that are present in chimeric dengue virus vaccine candidates (Guirakhoo, F. et al. 2001 J Virol 75:7290-304; Huang, C.Y. et al. 2000 J Virol 74:3020-8). The uniqueness of this approach derives from the fact that other live attenuated dengue virus vaccines have mutations in their structural or non-structural proteins (Butrapet, S. et al. 2000 J Virol 74:3011-9; Puri, B. et al. 1997 J Gen Virol 78:2287-91), therefore the immune response induced by these viruses will be to a mutant protein, rather than a wild-type protein. Second, deletion mutations are genetically more stable than point mutations, and reversion of the attenuation phenotype is unlikely. In humans, DEN4 $\Delta 30$ present in serum of vaccinees retained its $\Delta 30$ mutation, confirming its genetic stability *in vivo* (Durbin, A.P. et al. 2001 Am J Trop Med Hyg 65:405-13). The attenuating mutations in other existing dengue live attenuated vaccine candidates are based on less stable point mutations (Butrapet, S. et al. 2000 J Virol 74:3011-9; Puri, B. et al. 1997 J Gen Virol 78:2287-91). Third, since the $\Delta 30$ mutation is common to each of the four viruses of the tetravalent vaccine, recombination between any of the four vaccine serotypes would not lead to loss of the attenuating mutation or reversion to a wild-type phenotype. Recombination between components of the trivalent polio vaccine has been observed (Guillot, S. et al. 2000 J Virol 74:8434-43), and naturally occurring recombinant dengue viruses have been described (Worobey, M. et al. 1999 PNAS USA 96:7352-7) indicating the ability of this flavivirus to exchange genetic elements between two different viruses. Clearly, gene exchange is readily achieved between different DEN virus serotypes using recombinant cDNA techniques (Bray, M. and Lai, C.J. 1991 PNAS USA 88:10342-6). Fourth, viruses with wild-type structural proteins appear more infectious than viruses with altered structural proteins (Huang, C.Y. et al. 2000 J Virol 74:3020-80). This permits the use of a low quantity of each of the four virus components in the final vaccine, contributing to the low cost of manufacture. Low-cost manufacture is an essential element in defining the ultimate utility of a dengue virus vaccine.

EXAMPLE 5

Generation and Characterization of Intertypic Chimeric DEN2 Viruses Containing the $\Delta 30$ Mutation

[0098] The four serotypes of dengue virus are defined by antibody responses induced by the structural proteins of the virus, primarily by a neutralizing antibody response to the envelope (E) protein. These structural proteins include the E glycoprotein, a membrane protein (M), and a capsid (C) protein. The mature virus particle consists of a well-organized outer protein shell surrounding a lipid bilayer membrane and a less-well-defined inner nucleocapsid core (Kuhn, R.J. et al. 2002 Cell 108:717-25). The E glycoprotein is the major protective antigen and readily induces virus neutralizing antibodies that confer protection against dengue virus infection. An effective dengue vaccine must therefore minimally contain the E protein of all four serotypes, namely DEN1, DEN2, DEN3, and DEN4, thereby inducing broad immunity and precluding the possibility of developing the more serious illnesses DHF/DSS, which occur in humans during secondary infection with a heterotypic wild-type dengue virus. Based on a previously reported strategy (Bray, M. and Lai, C.J. 1991 PNAS USA 88:10342-6), a recombinant cDNA technology is being used to develop a live attenuated tetravalent dengue virus vaccine composed of a set of intertypic chimeric dengue viruses bearing the structural proteins of each serotype.

[0099] Following the identification of a suitably attenuated and immunogenic DEN4 recombinant virus, namely DEN4Δ30 (Durbin, A.P et al. 2001 Am J Trop Med Hyg 65:405-13), chimeric viruses based on the DEN4 cDNA have been generated in which the C-M-E (CME) or M-E (ME) genes have been replaced with the corresponding genes derived from the prototypic DEN2 New Guinea C (NGC) strain (Figure 9A). To create the CME chimeric viruses, the *Bgl*II / *Xho*I region of the cDNA for either rDEN4 or rDEN4Δ30 was replaced with a similar region derived from DEN2. Likewise, to create the ME chimeric viruses, the *Pst*I / *Xho*I region of the cDNA for either rDEN4 or rDEN4Δ30 was replaced with a homologous region derived from DEN2. The nucleotide and amino acid sequences of the resulting junctions are shown in Figure 9B. The GenBank accession number for the nucleotide sequence of rDEN4Δ30 is AF326837. The GenBank accession number for DEN2 NGC is M29095, which represents the mouse neurovirulent strain of DEN2 NGC and differs from the prototypic strain used here as previously documented (Bray, M. et al. 1998 J Virol 72:1647-51).

[0100] For transcription and generation of virus, chimeric cDNA clones were linearized and used as template in a transcription reaction using SP6 RNA polymerase as described (Durbin, A.P et al. 2001 Am J Trop Med Hyg 65:405-13). Transcripts were introduced into *Vero* cells using liposome-mediated transfection and recombinant dengue virus was harvested on day 7. The genomes of the resulting viruses were confirmed by sequence analysis of viral RNA isolated from recovered virus as previously described (Durbin, A.P et al. 2001 Am J Trop Med Hyg 65:405-13). Incidental mutations arising from virus passage in tissue culture were identified in all viruses and are listed in Table 29. Notably, each virus contained a missense mutation in NS4B corresponding to a previously identified mutation from rDEN4 and associated with adaptation to replication in *Vero* cells (See Table 30 for correlation of nucleotide positions between rDEN4 and chimeric viruses). All viruses replicated in *Vero* cells to titers in excess of 6.0 log₁₀ PFU/ml, indicating that the chimeric viruses, even those containing the A30 mutation, replicate efficiently in cell culture, a property essential for manufacture of the vaccine.

Table 29. Missense mutations observed among the *Vero* cell-grown chimeric DEN2/4 viruses

Virus	Gene	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change
rDEN2/4(CME)	NS4B	7161 ^a	A > U	2355	Leu > Phe
rDEN2/4Δ30(CME)	M	743	G > A	216	Gly > Glu
	E	1493	C > U	466	Ser > Phe
	NS4B	7544 ^b	C > T	2483	Ala > Val
rDEN2/4(ME)	E	1065	U > C	322	Phe > Leu
	NS4B	7163 ^a	A > U	2354	Leu > Phe
rDEN2/4Δ30(ME)	NS4B	7163 ^a	A > C	2354	Leu > Phe

^aSame nucleotide position as 7163 in rDEN4.
^bSame nucleotide position as 7546 in rDEN4.

Table 30. Nucleotide (nt) length differences for DEN chimeric viruses compared to rDEN4.

rDEN chimeric virus	nt difference from rDEN4 (following CME region)	ORF start (nt position)	Amino acid length		
			C	M	E
1/4 MB	0	102	113	166	495
1/4 CME	+3	102	114	166	495
2/4 ME	0	102	113	166	495
2/4 CME	-2	97	114	166	495
3/4 ME	-6	102	113	166	493
3/4 CME	-3	102	114	166	493
rDEN4	-	102	113	166	495

[0101] Results of a safety, immunogenicity, and efficacy study in monkeys are presented in Table 31. Monkeys inoculated with wild-type DEN2 were viremic for approximately 5 days with a mean peak titer of 2.1 log₁₀ PFU/ml, while monkeys inoculated with any of the chimeric DEN2 viruses were viremic for 1.2 days or less and had a mean peak titer of less than 1.0 log₁₀ PFU/ml. This reduction in the magnitude and duration of viremia clearly indicates that the chimeric viruses containing either the CME or ME proteins of DEN2 were more attenuated than the parental

DEN2 NGC virus. Neither the animals receiving the wild-type DEN2 nor the DEN2/4 chimeric viruses were ill. The decreased replication of the attenuated viruses in monkeys is accompanied by a reduction in the immune response of inoculated monkeys. This is indicated in Table 31 by approximately a 5-fold reduction in the level of neutralizing anti-body following inoculation with the chimeric viruses in comparison to titers achieved in animals inoculated with wild-type virus. Addition of the $\Delta 30$ mutation to the CME chimeric virus further attenuated the virus, such that rDEN2/4 $\Delta 30$ (CME) did not replicate in monkeys to a detectable level and did not induce a detectable immune response. This virus appeared over-attenuated, and if similar results were seen in humans, this virus would not be suitable for use as a vaccine. However, addition of the $\Delta 30$ mutation to the ME chimeric virus did not further attenuate this chimeric virus and the resulting rDEN2/4 $\Delta 30$ (ME) virus appears satisfactorily attenuated and immunogenic for use as a vaccine.

Table 31. Chimerization between dengue virus types 2 and 4 results in recombinant viruses which are attenuated for rhesus monkeys.

Group*	Virus	n	Mean no. days with viremia	Mean peak virus titer (log ₁₀ pfu/ml)	Geometric mean neutralizing antibody titer (reciprocal)
1	rDEN2/4 (CME)	6	1.2	0.9	50
2	rDEN2/4 $\Delta 30$ (CME)	8	0	<0.7	<5
3	rDEN2/4 (ME)	4	1.0	0.8	76
4	rDEN2/4 $\Delta 30$ (ME)	4	0.3	0.7	62
5	DEN2 NGC	6	5.5	2.1	312

* Rhesus monkeys were inoculated subcutaneously with 5.0 log₁₀ PFU of virus. Serum samples were collected daily for 10 days. Serum for neutralization assay was collected on day 28. Serum samples obtained before virus inoculation had a neutralizing antibody titer of <5.

[0102] As described in the previous examples, SCID mice transplanted with the HuH-7 cells are a sensitive model for the evaluation of dengue virus attenuation. Each chimeric DEN2/4 virus was inoculated into groups of SCID-HuH-7 mice and levels of virus in the serum were determined (Table 32). Chimeric viruses replicated to levels between 20- and 150-fold lower than either of the parental viruses (rDEN4 and DEN2-NGC). CME chimeric viruses were slightly more attenuated than the comparable ME chimeric viruses, with the $\Delta 30$ mutation providing a 0.5 log₁₀ reduction in replication. This level of attenuation exerted by the $\Delta 30$ mutation was similar to that observed previously for rDEN4 $\Delta 30$.

Table 32. Chimerization between dengue virus types 2 and 4 results in recombinant viruses which are attenuated for HuH-7-SCID mice.

Virus ^a	No. of mice	Mean peak virus titer (log ₁₀ pfu/ml $\pm$ SE)	Statistical group ^b
rDEN4	32	6.3 $\pm$ 0.2	A
DEN2-NGC	9	6.1 $\pm$ 0.2	A
rDEN2/4 (CME)	7	4.4 $\pm$ 0.3	B
rDEN2/4 $\Delta 30$ (CME)	7	3.9 $\pm$ 0.3	B
rDEN2/4 (ME)	6	4.8 $\pm$ 0.5	B
rDEN2/4 $\Delta 30$ (ME)	9	4.3 $\pm$ 0.2	B

^aGroups of HuH-7-SCID mice were inoculated into the tumor with 4.0 log₁₀ PFU of the indicated virus. Serum was collected on day 7 and virus titer was determined in *Vero* cells.

^bMean peak titers were assigned to statistical groups using the Tukey post-hoc test ($P < 0.05$). Groups with the same letter designation are not significantly different.

[0103] To evaluate the replication levels of each DEN2/4 chimeric virus in mosquitoes, two different genera of mosquitoes were experimentally infected. *Aedes aegypti* were infected by ingesting a virus-containing blood meal. By evaluating the presence of virus antigen in both the midgut and head tissue, infectivity could be determined for the local tissues (midgut), and the ability of virus to disseminate and replicate in tissues beyond the midgut barrier (head) could also be measured. The presence of virus in the head is limited by the ability of the ingested virus to replicate in the midgut and then disseminate to the salivary glands in the head, as well as the innate ability of the virus to replicate in the salivary glands. Intrathoracic inoculation of virus into *Toxorhynchites splendens* bypasses the mosquito midgut barrier. Parental viruses rDEN4 and DEN2-NGC readily infect *Ae. aegypti* and *T. splendens* (Table 33), with DEN2-NGC appearing to be much more infectious in *T. splendens*. Each of the rDEN2/4 chimeric viruses was also tested in both mosquito types. In many cases it was not possible to inoculate *Ae. aegypti* with an undiluted virus stock of sufficient titer to achieve a detectable infection due to the very low infectivity of several of the viruses. Nevertheless, it is clear that the rDEN2/4 chimeric viruses are less infectious for the midgut and head. Parental viruses rDEN4 and DEN2-NGC, administered at a maximum dose of approximately 4.0 log₁₀PFU, were detectable in 74% and 94% of midgut preparations, and 32% and 71% of head preparations, respectively. Among the chimeric viruses, the highest level of infectivity, as observed for rDBM2/4 $\Delta 30$ (CME), resulted in only 26% infected midgut samples and 6% head samples. In the more permissive *T. splendens*, the rDEN2/4 chimeric viruses were generally less infectious than either parental virus, with CME chimeric viruses being less infectious than ME viruses. It has previously been reported for DEN4 that the A30 mutation does not have a discernable effect on virus infectivity in *T. splendens* similar to that observed here for the rDEN2/4 chimeric viruses (Troyer, J.M. et al. 2001 Am J Trop Med Hyg 65:414-419).

Table 33. Dengue 2/4 chimeric viruses are less infectious compared to either parental virus strain in mosquitoes

Virus	<i>Toxorhynchites splendens</i> (intrathoracic inoculation)			<i>Aedes aegypti</i> (oral infection)			
	Dose ^a log ₁₀ pfu	No. tested	% infected ^b	Dose ^c log ₁₀ pfu	No. tested	% infected ^d	
						Midgut	Head
rDEN4	3.3	6	83	3.8	38	74	32
	2.3	7	57	2.8	15	26	6
	1.3	6	0	1.8	20	10	5
			MID ₅₀ =2.2			MID ₅₀ =3.4	MID ₅₀ ≥ 4.1
DEN2-NGC	2.5	5	100	4.0	17	94	71
	1.2	15	93	3.0	25	36	16
	0.2	4	75	2.0	30	0	0
	0.02	8	38				
			MID ₅₀ =0.5			MID ₅₀ =3.2	MID ₅₀ =3.6
rDEN2/4 (CME)	3.9	9	11	4.4	11	9	0
	2.9	5	0	3.4	10	0	0
			MID ₅₀ ≥4.3			MID ₅₀ ≥4.9	Nc ^e
rDEN2/4Δ30 (CME)	3.5	6	17	4.0	15	26	6
	2.5	6	17	3.0	10	0	0
			MID ₅₀ ≥3.9			MID ₅₀ ≥4.3	MID ₅₀ ≥4.5
rDEN2/4 (MB)	3.4	6	100	3.9	23	4	0
	2.4	5	20				
	1.4	5	0				
			MID ₅₀ =2.8			MID ₅₀ ≥4.4	Ne
rDEN2/4Δ30 (ME)	2.6	11	9	3.1	30	0	0
			MID ₅₀ ≥3.0			nc	Nc

^a Amount of virus present in 0.22 µl inoculum.

^b Percentage of mosquitoes with IFA detectable antigen in head tissue prepared 14 days after inoculation.

^c Virus titer ingested, assuming a 2 µl bloodmeal.

^d Percentage of mosquitoes with IFA detectable antigen in midgut or head tissue prepared 21 days after oral infection. When virus infection was detected, but did not exceed a frequency of 50% at the highest dose of virus ingested, the MID₅₀ was estimated by assuming that a 10-fold more concentrated virus dose would infect 100% of the mosquitoes.

^enc = not calculated, since virus antigen was not detected.

[0104] Chimerization of the DEN2 structural genes with rDEN4Δ30 virus resulted in a virus, rDEN2/4Δ30(CME), that had decreased replication in *Vero* cells compared to either parent virus. To evaluate *Vero* cell adaptation mutations (Blaney, J. E. et al. 2002 Virology 300:125-139) as a means of increasing the virus yield of a DEN vaccine candidate in *Vero* cells, selected mutations were introduced into this chimeric virus. Accordingly, rDEN2/4Δ30(CME) viruses bearing adaptation mutations were recovered, terminally diluted, and propagated in C6/36 cells to determine if the virus yield in *Vero* cells could be increased.

[0105] rDEN2/4Δ30(CME) viruses bearing *Vero* cell adaptation mutations were generated as follows. DNA fragments were excised from rDEN4 cDNA constructs encompassing single or double DEN4 *Vero* cell adaptation mutations and introduced into the cDNA clone of rDEN2/4Δ30(CME). The presence of the *Vero* cell adaptation mutation was confirmed by sequence analysis, and RNA transcripts derived from the mutant cDNA clones were transfected, terminally diluted, and propagated in C6/36 cells.

[0106] For evaluation of growth kinetics, *Vero* cells were infected with the indicated viruses at a multiplicity of infection (MOI) of 0.01. Confluent cell monolayers in duplicate 25-cm² tissue culture flasks were washed and overlaid with a 1 ml inoculum containing the indicated virus. After a two hour incubation at 37°C, cells were washed three times in MEM and 5 ml of MEM supplemented with 2% FBS was added. A 1 ml aliquot of tissue culture medium was removed, replaced with fresh medium, and designated the day 0 time-point. At the indicated time points post-infection, 1 ml samples of tissue culture medium were removed, clarified by centrifugation, and frozen at -80°C. The level of virus replication was assayed by plaque titration in C6/36 cells and visualized by immunoperoxidase staining. The limit of detection was < 0.7 log₁₀PFU/mL.

[0107] The growth properties of rDEN2/4Δ30(CME) viruses bearing single *Vero* cell adaptation mutations at NS4B -7153, -7162, -7163, -7182, NS5 -7630 or three combinations of mutations were compared in *Vero* cells with rDEN2/4Δ30(CME) virus (Figure 10). Without an introduced *Vero* cell adaptation mutation, rDEN2/4Δ30(CME) virus yield peaked at 4.4 log₁₀PFU/ml. Each individual adaptation mutation and the combined mutations conferred a substantial increase in replication. Specifically, rDEN2/4Δ30(CME)-7182 grew to the highest titer of 7.1 log₁₀PFU/ml, which was a 500-fold increase in yield. rDEN2/4Δ30(CME)-7162 had the lowest yield but still was increased 125-fold over the level of replication by rDEN2/4Δ30(CME) virus. Introduction of two adaptation mutations into rDEN2/4Δ30(CME) virus did not significantly increase virus yield over that of viruses bearing single *Vero* cell adaptation mutations. The observed increase of up to 500-fold in virus yield by the introduction of a *Vero*

cell adaptation mutation into this chimeric vaccine candidate demonstrates the value of identifying and characterizing specific replication-promoting sequences in DEN viruses.

[0108] These results have particular significance for the development of a live attenuated dengue virus vaccine. First, it is clear that chimerization leads to attenuation of the resulting virus, as indicated by studies in rhesus monkeys, HuH7-SCID mice and mosquitoes. Although this conclusion was not made in the previous study with DEN2/DEN4 or DEN1/DEN4 chimeric viruses (Bray, M. et al. 1996 J Virol 70:4162-6), careful examination of the data would suggest that the chimeric viruses are more attenuated in monkeys compared to the wild-type parent viruses. Second, the A30 mutation can further augment this attenuation in a chimeric-dependent manner. Specifically, in this example, chimeric viruses bearing the CME region of DEN2 were over-attenuated by the addition of $\Delta 30$, whereas the attenuation phenotype of chimeric viruses bearing just the ME region of DEN2 was unaltered by the addition of the $\Delta 30$ mutation. This unexpected finding indicates that in a tetravalent vaccine comprised of individual component viruses bearing a shared attenuating mutation, such as the A30 mutation, only ME chimeric viruses can be utilized since CME chimeric viruses bearing the $\Delta 30$ mutation can be over-attenuated in rhesus monkeys and might provide only limited immunogenicity in humans.

EXAMPLE 6

Generation and Characterization of Intertypic Chimeric DEN3 Viruses Containing the $\Delta 30$ Mutation

[0109] Chimeric viruses based on the DEN4 cDNA have been generated in which the CME or ME genes have been replaced with the corresponding genes derived from DEN3 (Sleman/78), a virus isolate from the 1978 dengue outbreak in the Sleman region of Indonesia (Gubler, D.J. et al. 1981 Am J Trop Med Hyg 30:1094-1099) (Appendix 2). As described in Example 5 for the DEN2 chimeric viruses, CME chimeric viruses for DEN3 were generated by replacing the *Bgl*II / *Xho*I region of the cDNA for either rDEN4 or rDEN4 $\Delta 30$ with a similar region derived from DEN3 (Sleman/78) (Figure 11A). Likewise, to create the ME chimeric viruses, the *Pst*I / *Xho*I region of the cDNA for either rDEN4 or rDEN4 $\Delta 30$ was replaced with a similar region derived from DEN3 (Sleman/78). The nucleotide and amino acid sequences of the resulting junctions are shown in Figure 11B. The genomes of the resulting viruses were confirmed by sequence analysis of viral RNA isolated from recovered virus as previously described (Durbin, A.P et al. 2001 Am J Trop Med Hyg 65:405-13). Incidental mutations arising from virus passage in tissue culture were identified in all viruses and are listed in Table 34. Notably, each virus contained a missense mutation in NS4B corresponding to a previously identified mutation from rDEN4 and associated with adaptation to growth in Vero cells (See Table 30 for correlation of nucleotide positions between rDEN4 and chimeric viruses). All viruses replicated in Vero cells to titers in excess of 5.7 log₁₀ FFU/ml, indicating that the chimeric viruses, even those containing the $\Delta 30$ mutation, replicate efficiently in cell culture, a property essential for manufacture of the vaccine.

Table 34. Missense mutations observed among Vero cell-grown chimeric DEN3/4 viruses

Virus	Gene	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change
rDEN3/4 $\Delta 30$ (CME)	M	825	T > C	242	Phe > Leu
	E	1641	C > T	514	Leu > Phe
	E	2113	A > G	671	Lys > Arg
	NS4B	7159 ^a	T > C	2353	Leu > Ser
rDEN3/4(ME)	M	460	A > G	120	Asp > Gly
	NS4B	7177 ^b	G > U	2359	Gly > Val
	NS5	7702	C > U	2534	Ser > Phe
rDEN3/4 $\Delta 30$ (MB)	E	1432	A > U	444	Gln > Leu
	NS4B	7156 ^a	U > C	2352	Leu > Ser
	NS5	8692	A > C	2864	Asn > His
^a Same nucleotide position as 7162 in rDEN4.					
^b Same nucleotide position as 7183 in rDEN4.					

[0110] As described in the previous examples, SCID mice transplanted with HuH-7 cells are a sensitive model for the evaluation of dengue virus attenuation. Each chimeric DEN3/4 virus was inoculated into groups of SCID-HuH-7 mice and levels of virus in the serum were determined (Table 35). While chimeric virus rDEN3/4 (CME) was not attenuated, the remaining chimeric viruses replicated to levels between 40- and 400-fold lower than either of the parental viruses (rDEN4 and DEN3-Sleman/78). In the CME chimeric virus, the $\Delta 30$ mutation providing a remarkable 2.7 log₁₀ reduction in replication. This level of attenuation conferred by the $\Delta 30$ mutation in the CME chimeric virus was much greater than that observed previously for rDEN4 $\Delta 30$. The rDEN3/4 (ME) virus was 100-fold reduced in replication compared to either parent virus indicating that the ME chimerization was attenuating per se. Addition of the $\Delta 30$ mutation to rDEN3/4 (ME) did not result in additional attenuation.

Table 35. Chimerization between dengue virus types 3 and 4 results in recombinant viruses which are attenuated for HuH-7-SCID mice.

Virus ^a	No. of mice	Mean peak virus titer (log ₁₀ pfu/ml $\pm$ SE)	Statistical group ^b
rDEN4	32	6.3 $\pm$ 0.2	A

Virus ^a	No. of mice	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Statistical group ^b
DEN3-Sleman/78	23	6.4 ± 0.2	A
rDEN3/4 (CME)	7	6.4 ± 0.6	A
rDEN3/4Δ30 (CME)	5	3.7 ± 0.4	B
rDEN3/4 (ME)	6	4.2 ± 0.7	B
rDEN3/4Δ30 (ME)	7	4.7 ± 0.4	A,B

^aGroups of HuH-7-SCID mice were inoculated into the tumor with 4.0 log₁₀ PFU of the indicated virus. Serum was collected on day 7 and virus titer was determined in *Vero* cells.

^bMean peak titers were assigned to statistical groups using the Tukey post-hoc test ($P < 0.05$). Groups with the same letter designation are not significantly different.

[0111] Evaluation of the replication and immunogenicity of the DEN3 chimeric recombinant viruses and wild-type DEN3 virus in monkeys was performed as described in Example 5. Results of this safety and immunogenicity study in monkeys are presented in Table 36. Monkeys inoculated with rDEN3/4(CME) and wild-type DEN (Sleman/78) were viremic for approximately 2 days with a mean peak titer of between 1.6 and 1.8 log₁₀ PFU/ml, respectively, indicating that chimerization of the CME structural genes of DEN3 did not lead to attenuation of virus replication, a different pattern than that observed for DEN2 chimerization (Table 31). However, chimerization of the ME structural genes resulted in attenuated viruses with undetectable viremia in monkeys, although all monkeys seroconverted with a greater than 10-fold increase in serum antibody levels. As expected for an attenuated virus, the immune response, as measured by neutralizing antibody titer, was lower following inoculation with any of the chimeric viruses compared to inoculation with wt DEN3 (Sleman/78), yet sufficiently high to protect the animals against wt DEN3 virus challenge (Table 37). It is clear that addition of the Δ30 mutation to rDEN3/4(CME) was capable of further attenuating the resulting virus rDEN3/4Δ30(CME).

Table 36. The Δ30 mutation further attenuates rDEN3/4(CME) for rhesus monkeys

Virus ^a	No. of monkeys	Mean no. of viremic days per monkey ^b	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
				Day 0	Day 28
DEN3 (Sleman/78)	4	2.3	1.8	<5	707
rDEN3/4 (CME)	4	2.0	1.6	<5	211
rDEN3/4Δ30 (CME)	4	0	<1.0	<5	53
rDEN3/4 (ME)	4	0	<1.0	<5	70
rDEN3/4Δ30 (ME)	4	0	<1.0	<5	58

^a Groups of rhesus monkeys were inoculated subcutaneously with 10⁵ PFU of the indicated virus in a 1 ml dose. Serum was collected on days 0 to 6, 8, 10, 12, and 28. Virus titer was determined by plaque assay in *Vero* cells.

^b Viremia was not detected in any monkey after day 4.

Table 37. rDEN3/4 chimeric viruses protect rhesus monkeys from wt DEN3 virus challenge

Virus ^a	No. of monkeys	Mean no. of viremic days per monkey after rDEN3 challenge	Mean peak virus titer (log ₁₀ PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
				Day 28	Day 56
Mock	2	5.0	2.5 ± 0.4	<5	372
DEN3 (Sleman/78)	4	0	<1.0	707	779
rDEN3/4 (CME)	4	0	<1.0	211	695
rDEN2/4Δ30 (CME)	4	0.8	1.1 ± 0.2	53	364
rDEN3/4 (ME)	4	0	<1.0	70	678
rDEN3/4Δ30 (ME)	4	0	<1.0	58	694

^a 28 days after primary inoculation with the indicated viruses, rhesus monkeys were challenged subcutaneously with 10⁵ PFU DEN3 (Sleman/78) virus in a 1 ml dose. Serum was collected on days 28 to 34, 36, 38, and 56. Virus titer was determined by plaque assay in *Vero* cells.

[0112] To evaluate the replication levels of each DEN3/4 chimeric virus in mosquitoes, *Aedes aegypti* were infected by ingesting a virus-containing blood meal (Table 38). Parental viruses rDEN4 and DEN3 (Sleman/78) readily infect *Ae. aegypti*. Each of the rDEN3/4 chimeric viruses was also tested. In many cases it was not possible to infect *Ae. aegypti* with an undiluted virus stock of sufficient titer to achieve a detectable infection due to the very low inactivity of several of the viruses. At a dose of approximately 2.8 - 2.9 log₁₀PFU, rDEN4, DEN3

(Sleman/78), and rDEN3/4(CME) were equally infectious and disseminated to the head with equal efficiency. For the remaining chimeric viruses, infection was not detectable even at a dose of $3.4 \log_{10}$ PFU, indicating that replication of rDEN3/4(ME) and rDEN3/4 Δ 30(CME) is restricted in *Ae. aegypti*. By comparing infectivity of rDEN3/4(CME) and rDEN3/4 Δ 30(CME), it is clear that the Δ 30 mutation is capable of further attenuating the chimeric virus for mosquitoes.

Table 38. Ability of DEN3/4 chimeric viruses to infect *Aedes aegypti* fed an infectious bloodmeal.

Virus Tested	Dose Ingested ($\log_{10}$ pfu) ^a	No. Mosquitoes Tested	No. (%) Midgut Infections ^{b,c,d}	No. (%) Disseminated Infections ^e
rDEN4	3.8	18	14 (77%)	2 (14%)
	2.8	20	7 (34%)	2 (10%)
	1.8	18	0	0
			MID ₅₀ = 3.4	MID ₅₀ $\geq$ 4.4
DEN3 (Sleman)	2.9	16	3 (18%)	2 (12%)
	1.9	10	1 (10%)	0
			MID ₅₀ $\geq$ 3.5	MID ₅₀ $\geq$ 3.5
rDEN3/4 (CME)	3.9	20	6 (30%)	2 (10%)
	2.9	18	4 (22%)	0
	1.9	13	1 (7%)	0
			MID ₅₀ $\geq$ 4.2	MID ₅₀ $\geq$ 4.5
DEN3/4 Δ 30 (CME)	3.3	20	0	0
			MID ₅₀ $\geq$ 4.3	MID ₅₀ $\geq$ 4.3
DEN3/4 (ME)	3.4	15	0	0
			MID ₅₀ $\geq$ 4.4	MID ₅₀ $\geq$ 4.4

^a Amount of virus ingested, assuming a 2 μ bloodmeal.

^b Number (percentage) of mosquitoes with detectable dengue virus in midgut tissue; mosquitoes were assayed 21 days post feed, and dengue virus antigen was identified by IFA.

^c When infection was detected, but did not exceed a frequency of 50% at the highest dose of virus ingested, the MID₅₀ was estimated by assuming that a 10-fold more concentrated virus dose would infect 100% of the mosquitoes.

^d When no infection was detected, the MID₅₀ was assumed to be greater than a 10-fold higher dose of virus than the one used.

^e Number (percentage) of mosquitoes with detectable dengue virus antigen in both midgut and head tissue.

EXAMPLE 7

Generation and Characterization of Intertypic Chimeric DEN1 Viruses Containing the Δ 30 Mutation

[0113] Chimeric viruses based on the DEN4 cDNA have been generated in which the CME or ME genes have been replaced with the corresponding genes derived from DEN1 (Puerto Rico/94), a virus isolate from a 1994 dengue outbreak in Puerto Rico (Appendices 3 and 4). As described in Example 4 for the DEN2 chimeric viruses, CME chimeric viruses for **DEN1** were generated by replacing the *Bgl*II / *Xho*I region of the cDNA for either rDEN4 or rDEK4 Δ 30 with a similar region derived from DEN1 (Puerto Rico/94) (Figure 12A). Likewise, to create the ME chimeric viruses, the *Pst*I / *Xho*I region of the cDNA for either rDEN4 or rDEN4 Δ 30 was replaced with a similar region derived from DEN1 (Puerto Rico/94). The nucleotide and amino acid sequences of the resulting junctions are shown in Figure 12B.

[0114] For transcription and generation of virus, chimeric cDNA clones were linearized and used as template in a transcription reaction using SP6 RNA polymerase as described. Transcripts were introduced into C6/36 mosquito cells using liposome-mediated transfection and recombinant dengue virus was harvested between day 7 and 14. Viruses were subsequently grown in *Vero* cells and biologically cloned by terminal dilution in *Vero* cells. All viruses replicated in *Vero* cells to titers in excess of $6.0 \log_{10}$ PFU/ml, indicating that the chimeric viruses, even those containing the Δ 30 mutation, replicate efficiently in cell culture. Genomic sequence analysis is currently underway to identify incidental mutations arising from virus passage in tissue culture.

[0115] To evaluate the replication levels of DEN1/4(CME) and rDEN1/4 Δ 30(CME) chimeric virus in mosquitoes, *Aedes aegypti* were infected by ingesting a virus-containing blood meal (Table 39). Parental virus rDEN4 infects *Ae. aegypti* with an MID₅₀ of $4.0 \log_{10}$ PFU. However, parental virus DEN1(Puerto Rico/94), is unable to infect *Ae. aegypti* at a dose of up to $3.4 \log_{10}$ PFU. Thus CME chimeric viruses DEN1/4 and rDEN1/4 Δ 30 share this inability to infect *Ae. aegypti*. Therefore, it is unnecessary in *Ae. aegypti* to evaluate the effect of the Δ 30 mutation on the infectivity of the DEN1/4 chimeric viruses, in a manner similar to that used for the DEN2/4 and DEN3/4 chimeric viruses.

Table 39. Inability of DEN1/4 chimeric viruses to infect *Aedes aegypti* fed an infectious bloodmeal.

Virus tested	Dose ingested (log ₁₀ pfu) ^a	No. Mosquitoes Tested	No. (%) Midgut Infections ^{b,c,d}	No. (%) Disseminated Infections ^e
rDEN4	4.3	21	18 (85%)	8 (44%)
	3.3	15	3 (20%)	0
	2.3	20	0	0
			MID ₅₀ = 4.0	MID ₅₀ ≥ 4.3
DEN1 (Puerto Rico/94)	3.4	21	0	0
			MID ₅₀ ≥ 4.4	MID ₅₀ ≥ 4.4
rDEN1/4 (CME)	3.8	20	0	0
			MID ₅₀ ≥ 4.8	MID ₅₀ ≥ 4.8
rDEN1/4Δ30 (CME)	2.8	20	0	0
			MID ₅₀ ≥ 3.8	MID ₅₀ ≥ 3.8

^aAmount of virus ingested, assuming a 2μl bloodmeal.

^bNumber (percentage) of mosquitoes with detectable dengue virus in midgut tissue; mosquitoes were assayed 21 days post feed, and dengue virus antigen was identified by IFA.

^cWhen infection was detected, but did not exceed a frequency of 50% at the highest dose of virus ingested, the MID₅₀ was estimated by assuming that a 10-fold more concentrated virus dose would infect 100% of the mosquitoes.

^dWhen no infection was detected, the MID₅₀ was assumed to be greater than a 10-fold higher dose of virus than the one used.

^eNumber (percentage) of mosquitoes with detectable dengue virus antigen in both midgut and head tissue.

[0116] As described in the previous examples, SCID mice transplanted with the HuH-7 cells are a sensitive model for the evaluation of dengue virus attenuation. Each chimeric DEN1/4 virus was inoculated into groups of SCID-HuH-7 mice and levels of virus in the serum were determined (Table 40). Chimeric viruses replicated to levels between 15- and 250-fold lower than either of the parental viruses, rDEN4 and DEN1 (Puerto Rico/94). CME chimeric viruses were more attenuated than the comparable ME chimeric viruses, with the Δ30 mutation providing a 0.8 log₁₀ reduction in replication. This level of attenuation exerted by the Δ30 mutation in the CME chimeric viruses was similar to that observed previously for rDEN4Δ30. However, the attenuating effect of the Δ30 mutation in the ME chimeric viruses is indiscernible.

Table 40. Chimerization between dengue virus types 1 and 4 results in recombinant viruses which are attenuated for HuH-7-SCID mice.

Virus ^a	No. of mice	Mean peak virus titer (log ₁₀ pfu/ml ± SE)	Statistical group ^b
rDEN4	32	6.3 ± 0.2	A
DEN1 (Puerto Rico/94)	4	6.4 ± 0.2	A
rDEN1/4 (CME)	8	4.7 ± 0.2	B,C
rDEN1/4Δ30 (CME)	6	3.9 ± 0.4	C
rDEN1/4 (ME)	6	5.0 ± 0.2	B
rDEN1/4Δ30 (ME)	6	5.1 ± 0.3	B

^aGroups of HuH-7-SCID mice were inoculated into the tumor with 4.0 log₁₀ PFU of the indicated virus. Serum was collected on day 7 and virus titer was determined in Vero cells.

^bMean peak titers were assigned to statistical groups using the Tukey post-hoc test ($P < 0.05$). Groups with the same letter designation are not significantly different

APPENDIX 1

Nucleotide and amino acid sequence of DEN2 (Tonga/74) cDNA plasmid p2

[0117]

Bases 1 to 10713: DEN2 virus genome cDNA

Bases 97 to 10269: DEN2 polyprotein ORF

Bases 97 to 438: C protein ORF

Bases 439 to 936: prM protein ORF

Bases 937 to 2421: B protein ORF

Bases 2422 to 3477: NS1 protein ORF

Bases 3478 to 4131: NS2A protein ORF

Bases 4132 to 4521: NS2B protein ORF

Bases 4522 to 6375: NS3 protein ORF

Bases 6376 to 6756: NS4A protein ORF

Bases 6757 to 6825: 2K protein ORF

Bases 6826 to 7569: NS4B protein ORF

Bases 7570 to 10269: NS5 protein ORF

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10      20      30      40      50      60      70      80      90     100
AGCTGTAGCTACGTCGAGTACACAAAGACAGATCTCTTGGGGAGCTAAGCTCAACGCTGTTCTAACGTTTCTTGATTAGAGAGCGAGATCTTGATGA
Met>

110     120     130     140     150     160     170     180     190     200
ATAACCAACAAAARAGCGAGAAACACCCCTTTTCAATATGCTTAAACGGAGAGAAACCGCTGTCACAGTCAACAACAGTTGACAAAGGATCTCTCACT
AenAesGluArgLysLysAlaArgAnThrProPheAesMetLeuLysArgGluArgAenArgValSerThrValGlnGlnLeuThrLysArgPheSerLeu>

210     220     230     240     250     260     270     280     290     300
TGGAAATGCTGAGAGAGAGACCACTAAATATTTTCATGSCCTGCTGGCAATCTCTTGGTTCTACAAATCCACAGACAGAGGATATTAAGAAAGA
GlyMetLeuGlnGlyArgGlyProLeuLysLeuPheMetAlaLeuValAlaLeuAenArgPheLeuThrIleProProThrAlaGlyIleLeuLysArg>

310     320     330     340     350     360     370     380     390     400
TGGGAGACATTAAGAAATCAAAAGGCTATTAATGTTCTGGAGAGGCTTCAGGAAACAGATTGAGAGGATGCTGAATATCTTAACAGAGAGCTAGAACRG
TrpGlyThrIleLysLysSerLysAlaIleAenValLeuAenGlyPheArgLysGlnIleGlyArgMetLeuAenIleLeuAenArgArgArgArgThr>

410     420     430     440     450     460     470     480     490     500
TAGGCAAGCTCATCTGAGACTCGAAGAGGATGAGGCTTCTATCTGACACACGCGACAGAGAGAGAGCTCTCATGATTTCCTGTAACAGAAAGAGAGAA
ValGlyMetCileIleMetLeuThrProThrValMetAlaPheHisLeuThrThrArgAenGlyGluProHisMetCileValSerArgGlnGlnLysGlyLys>

510     520     530     540     550     560     570     580     590     600
AAGCCCTCTCTTCAGAGACAAAGATGCGACAGACATCTGACCTCTATGSCCATGACCTTGGTGGTGTGGTGAAGACACAAATCACATTAATATCTCT
SerLeuLeuPheLysThrLysAspGlyThrAenMetCysThrLeuMetAlaMetAspLeuGlyGluLeuLysGlnLysPheIleThrCysLysCysPro>

610     620     630     640     650     660     670     680     690     700
TTTCTCAAGAGAAACGAGAACAGACATGATTTGAGTGCACCTCCAGTCCACATGCGTAACCTTAAGGACATGTACCCACACAGAGAGACAGAA
PheLeuLysGlnAenGluProGluArgLysLeuAspCysThrCysAenSerThrSerThrTrpValThrThrGlyThrCysThrThrThrGlyGluHisArg>

710     720     730     740     750     760     770     780     790     800
AGAGAAAAAGATCAATGCTGCTTGTCCACACCTGGGAAAGGATGAGACACAGAACCTGAACATGGATGTCATCAGAGAGGAGGCTGGAAACATGCCCA
ArgGlyLysArgSerValAlaLeuValProHisValGlyMetGlyLeuGlnThrArgThrGlnThrTrpMetSerSerGlnGlyAlaTrpLysHisAlaGln>

810     820     830     840     850     860     870     880     890     900
GAGATATGAACATTAATCTGAGAGACGACAGGCTTTCACCATATGCGCGCAATCTCGCATACACCATAGGAGACACATTTCCAAAGAGCTCTGATA
ArgIleGlnThrTrpIleLeuArgHisIleProGlyPheThrIleMetAlaAlaIleLeuAlaLysThrIleGlyThrThrHisPheGlnArgValLeuIle>

910     920     930     940     950     960     970     980     990     1000
TTCAGCTCTTCTGACACCATCTCCCTCTCATGACAAAGGCTCCAGAGAAATCAAGATGAGAACTCTGAGAGAGAGATGTCAGAGAGAGATGAGATG
PheIleLeuThrAlaIleAlaProMetThrMetArgCysIleGlyIleSerAenArgAenPheValGlnGlyValSerGlyGlySerTrpVal>

1010    1020    1030    1040    1050    1060    1070    1080    1090    1100
ACATAGTTTGAAGACAGAGATTTGGAGACGAGATGGCAAAAGACAGACCACTGAGCTGTGAAGTGAATAAGACAGAGGCTAAACAGACCTGCCAC
AspIleValLeuGlnGlnGlySerCysValThrThrMetAlaLysAenLysProThrLeuAenPheGlnLysThrGlnAlaLysGlnProAlaThr>

1110    1120    1130    1140    1150    1160    1170    1180    1190    1200
CTTAGAGAGATCTGATAGAGGCGCAACTGACCAACACACAGACAGACTGCGCTGCCACACAGAGGAGACCCACCTGAATAGAGAGCGAGACAGAA
LeuArgGlyTyrCysIleGlnAlaLysLeuThrAenThrThrThrAspSerArgCysProThrGlnGlyGluProThrLeuAenGlnGlnAspLys>

1210    1220    1230    1240    1250    1260    1270    1280    1290    1300
AGTTTCTGTCGAACATCTCATGACAGAGAGATGAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
ArgPheValCysLysHisAenMetValAspArgGlyTrpGlyAenGlyCysGlyLeuPheGlyIleGlyIleValThrCysAlaMetPheThrCys>

1310    1320    1330    1340    1350    1360    1370    1380    1390    1400
AAGAGACATGAGAGAGAAATTTGGAGACGAGAACTGGAAATACACTGTGCTGATACACCTCATTCAGGAGAGAAACATGAGTGGAGAAATGACAC
LysLysAenMetGlnLysIleValGlnProGluAenLeuGlnThrThrValValIleThrProHisSerGlyGlnGlnHisAlaValGlyAenAspThr>

1410    1420    1430    1440    1450    1460    1470    1480    1490    1500
AGGAAACATGCTGAAGAGATCAAGATTAACACACAGAGGCTCCATCAGAGAGGAGACATGACAGGCTATGCGCATGTTACAGATGAGATGCTCTCCAGAA
GlyLysHisGlyLysGlnValIleThrProGlnSerSerIleThrGlnAlaGlnLeuThrGlyThrGlyThrValThrMetGlnCysSerProArg>

1510    1520    1530    1540    1550    1560    1570    1580    1590    1600
ACGGGCTCTGACTTCATGAGATGATGTGTGCTGCAATAGAGACAAAGGCTGGCTGGTGGACAGACATGTTCTTACACTACCGTTGCCATGGCTGC
ThrLysLeuAspPheAenGlnMetValLeuLeuGlnMetGluAspLysAlaTrpLeuValHisArgGlnTrpPheLeuAenPheProLeuProThrLeu>

1610    1620    1630    1640    1650    1660    1670    1680    1690    1700
CTGAGAGACACACACAGAGATCAATAGATACAGAGACAGACACTGGTCACTTCAAAATGCCCATGCCAGAAACAGATGTTGTTGCTCTAGAGTC
ProGlyAlaAspTrpGlnGlnLysSerAenThrIleGlnLysGlnThrLeuValThrPheLysAenProHisAlaLysLysAlaAspValValLeuGlnDer>

1710    1720    1730    1740    1750    1760    1770    1780    1790    1800
CCAGAGAGGAGAGACATGACAGACTCATGAGAGGCTACGAAATCCAGATGATCATGACAGAACTGCTGTGACAGAGACATCTCAAGTCCAGGCTGAC
GlnGlnGlyAlaMetCileThrAlaLeuThrGlyAlaThrGlnIleGlnMetSerSerGlyAenLeuLeuPheThrGlyHisLeuLysCysArgLeuArg>

1810    1820    1830    1840    1850    1860    1870    1880    1890    1900
ATGACAAATTAACACTTAAGAGATCTCATACTCCATGTCACAGAAAGTTTAAATTTGTGAAGAGAAATAGCAGAAACACACATGAGACAAATGATCA
MetAspLysLeuGlnLysGlyMetSerThrSerMetCysThrGlyLysPheLysIleValLysGlnIleAlaGlnThrGlnHisGlyThrIleVal>

1910    1920    1930    1940    1950    1960    1970    1980    1990    2000
TTAGAGTCAATATGAGAGAGAGAGGCTCTCCATGCAAAATGCCCTCTGAGATATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGAT
IleArgValGlnTrpGlnGlyAspLysSerProCysLysIleProPheGlnIleMetAspLeuGlnLysArgHisValLeuGlyArgLeuIleThrValAen>

2010    2020    2030    2040    2050    2060    2070    2080    2090    2100
CCCAATGTCAGACATGACAGACTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
ProIleValThrGlnLysAspSerProValAenIleGlnAlaGlnProProPheGlyAspSerTyrIleIleIleGlyValGlnProGlyGlnLys>

2110    2120    2130    2140    2150    2160    2170    2180    2190    2200
CTGAGCTGCTGAGAGAGAGAGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
LeuAspTrpPheLysLysGlySerSerIleGlyGlnMetPheGlnThrThrMetArgGlyAlaLysArgMetAlaIleLeuGlyAspThrAlaTrpAsp>

2210    2220    2230    2240    2250    2260    2270    2280    2290    2300
TGGATCTCTGAGAGAGAGAGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
PheGlySerLeuGlyValPheThrSerIleGlyLysAlaLeuHisGlnValPheGlyAlaIleLysGlyAlaAlaPheSerGlyValSerTrpThrMet>

2310    2320    2330    2340    2350    2360    2370    2380    2390    2400
GAGATCTCTGAGAGAGAGAGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
ArgAlaGlnTrpPheLysLysGlySerSerIleGlyGlnMetPheGlnThrThrMetArgGlyAlaLysArgMetAlaIleLeuGlyAspThrAlaTrpAsp>

2410    2420    2430    2440    2450    2460    2470    2480    2490    2500
TTGAGATCTGAGAGAGAGAGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
LeuGlyValMetValGlnAlaAspSerGlyCysValValSerTrpLysAenLysGlnLysCysGlySerGlyIlePheValThrAspAenValHis>

2510    2520    2530    2540    2550    2560    2570    2580    2590    2600
CTGAGATCTGAGAGAGAGAGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
ThrThrThrGlnGlnLysLysGlnGlnGlnSerProSerLysLeuAlaSerAlaIleGlnLysAlaHisGlnGlnGlyIleCysGlyIleArgSerVal>

2610    2620    2630    2640    2650    2660    2670    2680    2690    2700
AAGAGACTTGAGAAATTTTATGAGAGAGAGATACATCAAGAAATGATCATCATCTCTGAGAAATGAGATGAGATGAGATGAGATGAGATGAGATGAGAT
ThrArgIleGlnAenMetTrpLysGlnIleThrSerGlnLeuAenHisIleLeuSerGlnAenGlnValLysLeuThrIleMetThrGlyAspIle>

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131110 131210 131310 131410 131510 131610 131710 131810 131910 132010
TGGAAAGCCGGGAATCGACGCCCTCGACCAATTGATTTCCGGATCTGCACTCCAGAGATGCTCTGGCATACCTCGCTTGGGAACCACTCATCTCTGATTATACAGG

132110 132210 132310 132410 132510 132610 132710 132810 132910 133010
GCGCTGGCATTCAGACCTGATGATGTTCTCTCTGCTGCTCGCGCGATGCTATACCCGCAATGTGATTACCGTACCAAGCTTTCAGTAAACGCGATCTCATATG

133110 133210 133310 133410 133510 133610 133710 133810 133910 134010
TCGTAAAGCCGTATGTGTAAAGTCTCTCTCTCTGTTTCTATGCTGTATCATTCACCCATGAACGAATAATCCCTCTACACGAGAGCTTCAGTACGAACAAACAGG

134110 134210 134310 134410 134510 134610 134710 134810 134910 135010
AAAAAGAGCGCCCTTAACTAGCCGCGCTTTATCAAGAGCCAGACATTTACCGCTTCTGAGGAAGCAACTCGAGAGCTGGAGTCGGATGAAACGAGCGACAGCATCT

135110 135210 135310 135410 135510 135610 135710 135810 135910 136010
TGAAAGCTTTACAGAACCGGTATGATGCTTTACCAAGACCTGCTCTGAGGCTTTGGTATGAAGAGCTTGAAACCTCTACACATGCACTGCACTCCCGAGAGCT

136110 136210 136310 136410 136510 136610 136710 136810 136910 137010
GTCTACGCTTTGCTGTAAACGGATGCTCGCGAGCGAGACGACCCCTCTGAGCGGCTCTAGCGGCTTTTCGCGGTTCTCGAGGCGACGCCATGACCCATCTCAC

137110 137210 137310 137410 137510 137610 137710 137810 137910 138010
GTACGGATCGAGAGTGTATGCTGTACTACTTACGTCAGACGAGAGATGTACTACGATGAGTCAAGCATATGCGGTGTGAATACCCGACGAGCTGATAGCTGATA

138110 138210 138310 138410 138510 138610 138710 138810 138910 139010
AGAGGAAATACCCGATCACTAGGCGCTCTTCCCTCTGCTGCTCACTACTGCTCGCGAGCTGCTCTGCTCGGCTCGCGACCGGTTACAGCTACTCTCAAAAGC

139110 139210 139310 139410 139510 139610 139710 139810 139910 140010
CGTATATACGCTTATCGACAGATTCAGGAGATACGCGACAGAAATACATCTGTACCAAAAGCCGACGAAAGAGCCGAGAACCGCTAAAGAGACCGGCTTTCCT

140110 140210 140310 140410 140510 140610 140710 140810 140910 141010
GCGCTTTTTCATAGAGCTCGCGCCCTCGACGAGACATCAAAAATCGAGCGCTCAAGCTCAGAGCGTGAGTAACCCGACGAGACTATAAAGATACAGAGCG

141110 141210 141310 141410 141510 141610 141710 141810 141910 142010
TTTTCCTCTCGAAGCTCCCTCTGTCGCTCTCTCTTTGAGCCCTCGCGCTTACCGAGATCTCTCGCGCTTTCTCCCTCTCGGGAAGCGTACGCTTTCTCT

142110 142210 142310 142410 142510 142610 142710 142810 142910 143010
ATAGCTCGAGCGTGTATGCTATCTGAGTTGGTGTATGCTGCTCTGCTCGCTGAGGCTGTGGTGGAGCAAGCCCGCTGAGCGGCGATCGCTGCTGTATCG

143110 143210 143310 143410 143510 143610 143710 143810 143910 144010
CGGTAACTATCGCTCTGAGTCCCAACCGCTTAAAGACAGACATTTGCCACCTGGAGCAGCAGCATGTGTAACAGGATATAGCAGACGAGCGTATGTAGGCGGCT

144110 144210 144310 144410 144510 144610 144710 144810 144910 145010
GTCACAGAGCTCTTGAAGTGTGAGCTCTACACGCTACCTAGACAGAGCAGATATGTGATCTAGCGCTCTGCGAGCGAGTATACTCTGGAAAGAGCG

145110 145210 145310 145410 145510 145610 145710 145810 145910 146010
TTGCTGATCTTGATCTGATCTCGCAAAAGACAGCACCGCTGTATCGCTGTTTCTTTCTGACACAGAGATTACGCGCAGAAAGAGAGCTCTCAAGAGAGC

146110 146210 146310 146410 146510 146610 146710 146810 146910 147010
TCTCTTGATCTTTTCTACAGGGGTGTGACGCTCAGTGTGACAGAAATCTCAGCTTAAAGGATTTTGGTGTACAGAGATTAACAAAGAGATTTCTACCTAGATCT

147110 147210 147310 147410 147510 147610 147710 147810 147910 148010
CTTTTAATTAAGAAATGTTTAAACATCTAAAGATATATATGTGATTAATCTGTCTACAGCTTACCAATGCTTAATCACTAGTACAGCATCTATCTAGCG

148110 148210 148310 148410 148510 148610 148710 148810 148910 149010
CGATCTGCTGATTTGCTGTGATGATGTGCTTGTCTGCTCTCCGTGCTGTATGATTAAGTCTACGACAGCGAGGCTTACCATCTGGCCCCAGTGTGCAATGAT

149110 149210 149310 149410 149510 149610 149710 149810 149910 150010
ACCGGAGAGCCGACGCTACCCGCTCTCGAGATTATCTAGCATATAAACGAGCAGCGAGCGAGAGCGGCGAGCGGCGAGAGTGTCTCGCATCTTATCCGCTCTCT

150110 150210 150310 150410 150510 150610 150710 150810 150910 151010
ATCTAGCTATTATGTTTCTCGCGAGCGTAGATATATATATTTCTCCGCACTATATGTTTGTGGCAAGTTGTGTGTCAGATTCGCAAGCTCTGGCTAGCAGCG

151110 151210 151310 151410 151510
GACCTGCTGATTTGGTCTGCTACCATTTCCGCGGCGCCGATTTAGAGTACGACTATAG

APPENDIX 2

Nucleotide and amino acid sequence of DEN3 (Sleman/78) cDNA plasmid p3

[0118]

Bases 1 to 10707: DEN3 virus genome cDNA

Bases 95 to 10264: DEN3 polyprotein ORF

Bases 95 to 436: C protein ORF

Bases 437 to 934: prM protein ORF

Bases 935 to 2413: E protein ORF

Bases 2414 to 3469: NS1 protein ORF

Bases 3470 to 4123: NS2A protein ORF

Bases 4124 to 4513: NS2B protein ORF

Bases 4514 to 6370: NS3 protein ORF

Bases 6371 to 6751: NS4A protein ORF

Bases 6752 to 6820: 2K protein ORF

Bases 6821 to 7564: NS4B protein ORF

Bases 7575 to 10264: NS5 protein ORF

10 20 30 40 50 60 70 80 90 100
AGTGTGTGTTCTACGTGGACCGACAGAACAGCTTTGACCTCGGAAGTTGCTTACGGTACTGACAGTTTTTTTATGAGAGCAGATCTCTGTGATGAC
MeLae2>

[illegible]

[illegible]

APPENDIX 3

Nucleotide and amino acid sequence of DEN1 (Puerto Rico/94) CME chimeric region

[0119]

Bases 1 to 88 (BgIII): DEN4

Bases 89 (BglIII) to 2348 (XhoI): DEN1

Bases 2349 (XhoI) to 2426: DEN4

Bases 102 to 443: C protein ORF

Bases 444 to 941: prM protein ORF

Bases 942 to 2426: E protein ORF

130 140 150 160 170 180 190 200
ASTTGTGTCCTCTGTGACACCGACCAAGCAATCTTCBAATCTCGAAGCTTGCCTAACCAACCTCTCAACACTTGTGTTGATGATGAGCAATCTCTGGAAA

210 220 230 240 250 260 270 280 290 300
AATCAACCAACCAACCAAGGAAAGAAGCGGTGTGACGCTCTCAATATATCTCGAAACCGCAAGAACCTCTCAACTGGTCTCACAGTCTGGGGAAGGAGCT
MetAdeAanGlnArgLysIlySerIleGlyArgProSerPheAanMetLeuIleArgAlaAargAanValSerThrIleGlyGlnAlaIleArgPhe

310 320 330 340 350 360 370 380 390 400
TCGAAAGAGCTCTTTCACCGCAAGCAACCAATGAATCTGAGCGCTCTCATGACATCTCTAGACTCTTAGCCATCAACCCCAACCAAGCAATCTGAAATCT
SerIlyGlyLeuLeuSerGlyGlnIlyProMetIleGlnValMetAlaIlePheIleAlaPheLeuArgPheLeuAlaIleProProThrAlaGlyIleLeu

410 420 430 440 450 460 470 480 490 500
CTGATAGGAGCTCTCTCAGAGAGAAAGGAGGATCAAGACTTACAGCGCTTCAAAAGAGAGATCTCAAGCATGTGAAACTATTATGACAGCAAGGAAAGAA
AlaArgTrpSerIleIlePheIlyGlnIlyAlaIleIleValLeuArgLysPheIleGlnIleSerSerMetLeuAanIleIleAanArgLysIleLeu

510 520 530 540 550 560 570 580 590 600
AGCTGTGACCTGCTCTCTATCTGTGCGCCACCAAGCTTGGCTCTCTCTTGTACCAACCAAGGGGAGAGGACCCACCATATATGTTTASTAGCAAGAAAG
SerValThrMetLeuLeuLeuLeuProThrAlaLeuAlaPheIleIleThrThrArgGlyIlyIleProIleMetIleValSerIleGlnIleArg

610 620 630 640 650 660 670 680 690 700
GGAAGATCTGTGTTGTTAAGACTTCAAGCGGCATCATCATCTGACACTCTCTCTGCAATGGAATTGGAGAGCAATTAGCGAGAGCAACAATGACTCAAT
GlyIysSerLeuLeuPheIlyThrSerAlaGlyIleAanMetCysThrLeuIleAlaMetAlaPheLeuGlyIleLeuCysIleAspThrMetThrThrIleGly

710 720 730 740 750 760 770 780 790 800
GCCCGCCGACCTACAGAGCGCAAGCAACAAGTGTGCTGTGCTGTGCTGTCACCAAGCAATGGTGTGCTATGAGCTCTCTGAAAGCGCGCAAGCT
CysProArgIleThrGluAlaIleProArgAspValAspCysProThrCysAlaIleThrAspThrThrIlePheIleThrIleCysSerIleThrIleGlyIleAla

810 820 830 840 850 860 870 880 890 900
CCGAGAGCAACAAGCTCTGCTGACACTGCGCCCAACAGAGCTGGCTGGCTTAGAGAAAGCAACAACCAATGAGTGTCTCTTAAACTGCTGTGGAACGA
ArgArgGlyPheLeuLeuIleAlaIleProIleIleValGlyIleGlyIleGlyIleIleArgThrIleThrIlePheIleGlyIleAlaIleThrIleGlyIle

910 920 930 940 950 960 970 980 990 1000
GTACAAAGAAATGAAATGCTTGTGAGCAAGCTTCAAGCTCTGACAGCCCTTTTATGACCAACCTCATGCAAGACCATCTATTATCAGAAAGGCA
ValGlnIlyValGlnIleThrThrAlaLeuArgGlyIleIleIlePheThrValThrAlaIlePheLeuAlaAlaIleIleIleThrIleIleThrIleGlyIle

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
TCATCTCTCTCTCTCTGCTCACTGATCAACCTCAAGTCGATCGATGTGTGGAGATGAAACAGAAAGACTCTCTGTAGAGACTCTCTACAGACACTGT
IleIlePheIleLeuLeuLeuValThrProSerMetAlaMetIleGlyCysValIleIleIlyAanArgPhePheValGlnIlyLeuSerGlyIleThrThr

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
GGTGGCTGTGATCTGAGCACTGGAGCTGAGCTCAACCACTCAACCTCAACCACTCAACCACTCAACCACTCAACCACTCAACCACTCAACCACTCAACCACT
ValAspValValLeuIleGlyIleGlyIleGlyIleValThrThrMetAlaIleAspIleAspIleProThrIleAspIleLeuIleIleIleIleValThrIleThrPro

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
ACTCGAAGCTTGTGCTGCAACAGCTTGTGAGCAAGCTCTGAGAGCTGTGAGCTCGCACTTATGCGAAGAAAGCAAGCTATGCTGTGTGCTATGCA
AspSerAanPheValCysArgArgIlePheValAspArgIleGlyIleGlyIleCysIleIlePheIleIleGlyIleSerLeuLeuIleThrCysAlaIlePheIly

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
GTGTGTGCAAGAAATGAGAGAGAGATGATCTATATTAAGAACTTAAAACTTCACTGATATGTCATCTCCACACTGGGAGACAGCAAGTGTGAAAC
CysValThrIleGlnIlyIleValGlnIlyValGlnIlyValGlnIlyValGlnIlyValGlnIlyValGlnIlyValGlnIlyValGlnIlyValGlnIlyValGlnIly

1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
GAGCATCTAGACACACTTGGACATAATACCACTTCAAGCTCTTACCTGGAAATACAGCTCAGCTACGAGCCTCAGATCTGGAGCTCTCCG									
CTGCThThrGluGluGluGlyThrLeuAlaThrLeuThrProGluAlaProThrThrSerGluGluGlnGlnLeuThrAspTyrGlyAlaLeuThrLeuAspCysSer>									
1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
CTGAGACAGTCACTGCTTAAATGCTTCTCTTCTTCACTGATCAAGAAATCAATGCTTGGCTGACACAGACAGCTTCTGACATCTACAGCTCTCT									
ProGlyThrGlyLeuSerProGluGlnMetValLeuLeuThrMetTyrGlyGlySerTyrLeuValHisGlyGlnThrPheThrAspLeuProLeuProTyr>									
1610	1620	1630	1640	1650	1660	1670	1680	1690	1700
GACTCTCAGAGAGCTTCAACATCTCAGAGAGCTTGGACACAGACAGATATTTGCTGCTCACTTCAAGACAGAGCTCATGCAAGAAACACAGAGATGATCTACTG									
ThrSerGlyPheSerThrSerGlnGlyThrThrPheAsnArgGlnAspLeuLeuValThrPheTyrThrThrAlaHisAlaTyrTyrGlnGlnGluValValLeu>									
1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
GGATCACTGAGAGAGGAGGAGTCACTGCTCTGCTCTGCTCTGCTCTGACAGACAGAGACACAGACATCTTTCTAGACATCTCAAGATCAAGAACTCACTG									
GlySerGlnGlyGlyAlaMetHisThrAlaLeuThrGlyAlaThrGluAlaGlnThrSerGlyThrThrThrIleProGluAlaGlyHisLeuGlyCysArg>									
1810	1820	1830	1840	1850	1860	1870	1880	1890	1900
TAAAGATGATTAAGTCACTTAAAGAGAGGATCATCTGATATGCTCAGACAGCTCATTTAAAGCTAGAGAGAGAGATCTGAGACACAGCATCTGAACTCT									
LeuIleSerMetAspTyrLeuThrLeuTyrGlyMetSerTyrValMetTyrThrGlySerPheTyrLeuGlyGlyGluValAlaGlnThrIleGlnAlaGlyThrVal>									
1910	1920	1930	1940	1950	1960	1970	1980	1990	2000
TTCATCTCTGGCTTAAATTCAGAGAGACACATATGGCATCGCAATTCCTTTCTTGGCCCAACATCAGAGAAAGAGATGACCCACCAATATGGCAATATGATGAC									
LeuValGlnValTyrGlyGluThrThrAspAlaProCysTyrIleProPheSerAlaGlnAspGlyGlyValThrIleAsnGlnArgLeuIleThr>									
2010	2020	2030	2040	2050	2060	2070	2080	2090	2100
GGCACCCCATGCTGCTCTCAACAGAAACAAACCATCTCAGATCTCAGACAGACACCACTTTTCTGTGACATCTACATCTCTGTGTGAGGCGCATGAAAGAGCT									
AlaAsnProValThrThrAspTyrGlyProValAlaGlnIleGluThrLeuProPheGlyGlySerTyrIleValAlaGlyAlaGlyGlyAlaGlyAlaLeu>									
2110	2120	2130	2140	2150	2160	2170	2180	2190	2200
TGAACATCTGCTGTTCAGAGAGAGACAGATCAAGAAATTTTCAAGCACTCTGCTCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG									
LeuLysLeuSerTyrTrpPheTyrGlyGlySerSerLeuGlyTyrMetPheGluAlaThrAlaArgGlyAlaArgArgMetAlaAlaLeuGlyTyrPheThrAlaTrp>									
2210	2220	2230	2240	2250	2260	2270	2280	2290	2300
GGACTTCTCTTATAGAGAGAGAGCTTTCACATCAATGGAGAAATATGTTACACCAAGCTTTTGGAGCCGCATATGGGCTTCTTCTGAGGAGTCTTCTTGG									
AspPheGlySerLeuGlyGlyValThrThrSerValGlyTyrLeuValGlnValPheGlyAlaAlaTyrGlyValLeuPheSerSerGlyValSerTyr>									
2310	2320	2330	2340	2350	2360	2370	2380	2390	2400
ACCACTGAAATAGAGATAGCGATCTCTCTGACAGAGGCTGAGTGTAACTGAGAGAGACACTTCAATGGCTGACAGCTGAGCTGAGCTTCTGGAGGAATCACT									
ThrMetTyrGlyLeuGlyGlyLeuLeuThrThrProGlyLeuAsnSerArgAsnThrSerMetAlaPheThrTyrGlnLeuAlaValGlyGlyTyrThr>									
2410	2420								
TTCTTCTGGGCTTCACTGTTCAAGCA									
LeuPheLeuGlyPheThrValGlnAla>									

APPENDIX 4

Nucleotide and amino acid sequence of DEN1 (Puerto Rico/94) ME chimeric region

[0120]

Bases 1 to 404 (PstI): DEN4

Bases 405 (PstI) to 2345 (XhoI): DEN1

Bases 2346 (XhoI) to 2423: DEN4

Bases 102 to 440: C protein ORF

Bases 441 to 938: prM protein ORF

Bases 939 to 2423: E protein ORF

[illegible]

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1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
CBAACCTTGTCTGAGAGACGTTTCTGAGACAGAGACCTGGGGTAAATGACACGCACTATTGGAAAAGGAGCCTACACGCTGTCTAAAGTTCAAGTGG
SerAanPheValCysArgArgThrPheValAspArgGlyTyrGlyAsnGlyCysGlyLeuPheGlyLysGlySerLeuLeuThrCysAlaLysPheLysCys>

1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
TGTGACAAATCTGAGAGAGATGTTCTCAATATGAAAGACTTAAATATTCAGTGATAGTCACCTGTCCACACTGGAGACCAACACAGTCTGGAAACAG
ValTrLysLeuGlyGlyValLeuValLeuTyrGlyAsnLeuLysTyrSerValLeuValThrValHisThrGlyAspGlnHisGlnValGlyAsnGlu>

1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
ACTACAGAAATGGAACCAATGCAACCAACACCTCAAGCTCCTACGTCGGAATACAGCTGACTGACTACCGAGCCCTACATTTGAGACTGCTCCGCTTA
ThrThrGlnHisGlyThrIleAlaThrIleThrProGlnAlaProThrSerGlnIleGlnLeuThrAspTyrGlyAlaLeuThrLeuAspCysSerPro>

1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
GRACAGGCTGGACCTTAAATGAGATGCTTCTATTGACAAATGAAAGAAATCATGGCTGTGTCGCAACCAATGTTTCTAGACTTACCACCTGCTTGAGAC
ArgThrGlyLysAspPheAsnGlnMetValLeuLeuTyrMetLysGlyLysSerTrpLeuValHisLysGlnTrpPheLeuAspLeuProLeuProTrpThr>

1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
TTCAGGAGCTTCAACTCTCAAGGACTTGGAGCAGACAGAGATTTCCTGTCACATTCAAGACAGCTCATGCAAGAAACAGGAAGTAGTCTACTGAGGA
SerGlyAlaSerThrSerGlnGluThrTrpPheArgGlnAspLeuValThrPheLysThrAlaHisAlaLysLysGlnGluValValValLeuGly>

1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
TCMCAAGAGAGACCAATGCACTGCTGCTGACTGGGCGACAGAAATGCGAGACCTTCGAGGACCTGAAATGCGAGACTRA
SerGlnGlyLysAlaMetHisThrAlaLeuThrGlyAlaThrGlnIleGlnThrSerGlyThrThrThrIlePheAlaGlyHisLeuLysCysArgLeu>

1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
AATCGGATTAACCTGACTTTAAAGAGGACCTCATATGTAATGTCACAGGCTCATTTAAAGCTAGAGAAAGAGATGCTGTGAGACCCAGCATGGAACCTTTT
LysMetAspLysLeuThrLeuLysGlyMetSerTyrValMetCysThrGlySerIleLysLeuGlyLysGluValAlaGluThrGlnHisGlyThrValLeu>

1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
AGTCTGGTTPAATACCGAAGAGACAGATCCGCCACAGATCCCTTTTCGCCCGAAGATGCAAAAGAGATGACCCGAAATGGAGATTTGATACAGCC
ValGlnValLysTyrGlyLysThrAspAlaProCysLysIleProPheSerAlaGlnAspGlnLysGlyValThrGlnAsnGlyArgLeuIleThrAla>

2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
AADCCTATATCTCATGACAGAGAAATACCACTGACATTTGAGACAGAACCACTTTTGTGAGAGCTACATCTGCTGAGGGCAGGTGAAAGAACTTTGA
AsnProIleValThrAspLysGlyLysProValAsnIleGlnThrGluProProPheGlyLysSerTyrIleValValGlyLysGlyLysAlaLeu>

2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
AACTGAGCTGCTTCAAGAGAGGAGACAGCATAGAGAAATGTCGACCAACTGCCCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
LysLeuSerTrpPheLysLysGlySerSerIleGlyLysMetPheGluAlaThrAlaArgGlyAlaArgArgMetAlaIleLeuGlyAspThrAlaTrpAsp>

2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
CTTTCGCTCTATAGAGAGATGTTTCACTACACTGAGAGAAATTCATACACAGAGTTTTCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
PheGlySerIleGlyLysValPheThrSerValGlyLysLeuValHisGlnValPheSerYalaAlaTyrGlyValLeuPheSerGlyValSerTrpThr>

2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
ATGAAAAACGAAATAGAGATTTCTGATGACGGCTAATTAATCTGACCAACCACTTCAATGGCTATGACGTCATAGCTGTTGGAGAAATCACTCTGT
MetLysLleGlyLleGlyLleLeuLeuThrTrpLeuGlyLeuAsnSerArgAsnThrSerMetAlaMetThrCysIleAlaValGlyGlyIleThrLeu>

2410      2420
TTCAGGACTTCAACATTTCAGCA
PheLeuGlyPheThrValGlnAla

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[0121] While the present invention has been described in detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention as defined in the claims. The following items are also disclosed:

1. An immunogenic composition being tetravalent and containing a common 30 nucleotide deletion in the 3' untranslated region of dengue types 1, 2, 3, and 4 comprising a nucleic acid comprising a first nucleotide sequence encoding at least one structural protein from a first dengue virus and a second nucleotide sequence encoding nonstructural proteins from a second dengue virus, wherein the second dengue virus is attenuated by a deletion of about 30 nucleotides from the 3' untranslated region of the dengue genome corresponding to the TL2 stem-loop structure.
2. The composition of item 1, wherein the nucleic acid further comprises a mutation generating a mutant having a phenotype wherein the phenotype is temperature sensitivity in *Vero* cells or the human liver cell line HuH-7, host-cell restriction in mosquito cells or the human liver cell line HuH-7, host-cell adaptation for improved replication in *Vero* cells, or attenuation in mice or monkeys.
3. The composition of item 1 or 2, wherein the serotype of the first dengue virus is the same as the serotype of the second dengue virus.
4. The composition of item 3, wherein the serotype is type 1.
5. The composition of item 4, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 1 genome corresponding to the TL2 stem-loop structure between about nucleotides 10562-10591.
6. The composition of item 3, wherein the serotype is type 2.
7. The composition of item 6, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 2 genome corresponding to the TL2 stem-loop structure between about nucleotides 10541-10570.
8. The composition of item 3, wherein the serotype is type 3.
9. The composition of item 8, wherein the deletion is by a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 3 genome corresponding to the TL2 stem-loop structure between about nucleotides 10535-10565.
10. The composition of item 3, wherein the serotype is type 4.
11. The composition of item 10, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 4 genome corresponding to the TL2 stem-loop structure between about nucleotides 10478-10507.
12. The composition of item 1 or 2, wherein the serotype of the first dengue virus is different from the serotype of the second dengue virus and wherein nucleic acid is a nucleic acid chimera.
13. The composition of item 12, wherein the serotype of the second dengue virus having the deletion is type 1.
14. The composition of item 13, wherein the serotype of the first dengue virus is type 2.
15. The composition of item 13, wherein the serotype of the first dengue virus is type 3.
16. The composition of item 13, wherein the serotype of the first dengue virus is type 4.
17. The composition of any of items 13-16, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.
18. The composition of item 17, wherein the structural proteins are prM and E proteins.
19. The composition of any of items 13-18, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 1 genome corresponding to the TL2 stem-loop structure between about nucleotides 10562 and 10591.
20. The composition of item 12, wherein the serotype of the second dengue virus having the deletion is type 2.
21. The composition of item 20, wherein the serotype of the first dengue virus is type 1.

22. 22. The composition of item 20, wherein the serotype of the first dengue virus is type 3.
23. 23. The composition of item 20, wherein the serotype of the first dengue virus is type 4.
24. 24. The composition of any of items 20-23, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.
25. 25. The composition of item 24, wherein the structural proteins are prM and E proteins.
26. 26. The composition of any of items 20-25, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 2 genome corresponding to the TL2 stem-loop structure between about nucleotides 10541 and 10570.
27. 27. The composition of item 12, wherein the serotype of the second dengue virus having the deletion is type 3.
28. 28. The composition of item 27, wherein the serotype of the first dengue virus is type 1.
29. 29. The composition of item 27, wherein the serotype of the first dengue virus is type 2.
30. 30. The composition of item 27, wherein the serotype of the first dengue virus is type 4.
31. 31. The composition of any of items 27-30, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.
32. 32. The composition of item 31, wherein the structural proteins are prM and B proteins.
33. 33. The composition of any of items 27-32, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 3 genome corresponding to the TL2 stem-loop structure between about nucleotides 10535 and 10565.
34. 34. The composition of item 12, wherein the serotype of the second dengue virus having the deletion is type 4.
35. 35. The composition of item 34, wherein the serotype of the first dengue virus is type 1.
36. 36. The composition of item 34, wherein the serotype of the first dengue virus is type 2.
37. 37. The composition of item 34, wherein the serotype of the first dengue virus is type 3.
38. 38. The composition of any of items 34-37, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.
39. 39. The composition of item 38, wherein the structural proteins are prM and E proteins.
40. 40. The composition of any of items 34-39, wherein the deletion is a deletion of about 30 nucleotides from the 3' untranslated region of the dengue type 4 genome corresponding to the TL2 stem-loop structure between about nucleotides 10478 and 10507.
41. 41. The composition of any of items 1-40 wherein the one or more nucleic acid or nucleic acid chimera comprises a virus or virus chimera.
42. 42. The composition of any of items 1-41 for use in the induction of an immune response.
43. 43. A method of inducing an immune response in a subject comprising administering an effective amount of the composition of any of items 1-41 to the subject.
44. 44. The method of item 43 wherein the subject is a human.
45. 45. A tetravalent vaccine comprising the composition of any of items 1-41.
46. 46. The vaccine of item 45 for use in the prevention of disease caused by dengue virus.
47. 47. A method of preventing disease caused by dengue virus in a subject comprising administering an effective amount of the vaccine of item 45 to the subject.
48. 48. The method of item 47 wherein the subject is a human.
49. 49. An isolated nucleic acid probe or primer that selectively hybridizes with and possesses at least five nucleotides complementary to the nucleic acid or the complementary strand of the nucleic acid encoding the cleavage site that separates the capsid protein and the premembrane protein of the nucleic acid chimera comprising the composition of item 18, 25, 32, or 39.
50. 50. A composition, optionally comprising a mutation selected from the group consisting of temperature sensitivity in *Vero* cells or the human liver cell line HuH-7, host-cell restriction in mosquito cells or the human liver cell line HuH-7, host-cell adaptation for improved replication in *Vero* cells, or attenuation in mice or monkeys, the composition comprising a member selected from the group consisting of:
 1. (1) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30,
 2. (2) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30,
 3. (3) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30,
 4. (4) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30,
 5. (5) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30,
 6. (6) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
 7. (7) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
 8. (8) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
 9. (9) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30,
 10. (10) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
 11. (11) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
 12. (12) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30,
 13. (13) rDBN1Δ30, rDBN2Δ30, rDEN3/4Δ30, rDEN4Δ30,
 14. (14) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
 15. (15) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
 16. (16) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
 17. (17) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30,
 18. (18) rDEN1Δ30, rDEN2/1Δ30, rDEN3A30, rDEN4/1Δ30,
 19. (19) rDEN1Δ30, rDEN2/1Δ30, rDEN3A30, rDEN4/2Δ30,
 20. (20) rDEN1Δ30, rDEN2/1Δ30, rDEN3A30, rDEN4/3Δ30,
 21. (21) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30,
 22. (22) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
 23. (23) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
 24. (24) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
 25. (25) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4Δ30,

26. (26) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
27. (27) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
28. (28) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2A30, rDEN4/3Δ30,
29. (29) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4Δ30,
30. (30) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
31. (31) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
32. (32) rDEN1Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
33. (33) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4Δ30,
34. (34) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/1Δ30,
35. (35) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/2Δ30,
36. (36) rDEN1Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30,
37. (37) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4Δ30,
38. (38) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
39. (39) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
40. (40) rDEN1Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
41. (41) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4Δ30,
42. (42) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
43. (43) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
44. (44) rDEN1Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/3Δ30,
45. (45) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4Δ30,
46. (46) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
47. (47) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
48. (48) rDEN1Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
49. (49) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4Δ30,
50. (50) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/1Δ30,
51. (51) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/2Δ30,
52. (52) rDEN1Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/3Δ30,
53. (53) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4Δ30,
54. (54) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
55. (55) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
56. (56) rDEN1Δ30, rDEN2/4Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
57. (57) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4Δ30,
58. (58) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
59. (59) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
60. (60) rDEN1Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/3Δ30,
61. (61) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4Δ30,
62. (62) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
63. (63) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
64. (64) rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
65. (65) rDEN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30,
66. (66) rDBN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30,
67. (67) rDBN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30,
68. (68) rDBN1/2Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30,
69. (69) rDBN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30,
70. (70) rDBN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
71. (71) rDBN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
72. (72) rDBN1/2Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
73. (73) rDBN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30,
74. (74) rDBN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
75. (75) rDBN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
76. (76) rDBN1/2Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30,
77. (77) rDBN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4Δ30,
78. (78) rDBN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
79. (79) rDBN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
80. (80) rDBN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
81. (81) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30,
82. (82) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/1Δ30,
83. (83) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/2Δ30,
84. (84) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/3Δ30,
85. (85) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30,
86. (86) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
87. (87) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
88. (88) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
89. (89) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4Δ30,
90. (90) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
91. (91) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
92. (92) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/3Δ30,

93. (93) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4Δ30,
94. (94) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
95. (95) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
96. (96) rDBN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
97. (97) rDBN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4Δ30,
98. (98) rDBN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/1Δ30,
99. (99) rDBN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/2Δ30,
100. (100) rDBN1/2Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30,
101. (101) rDBN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4Δ30,
102. (102) rDBN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
103. (103) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
104. (104) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
105. (105) rDEN1/2Δ30, rDEN2/3A30, rDEN3/2A30, rDEN4Δ30,
106. (106) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
107. (107) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
108. (108) rDEM1/2Δ, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/3Δ
109. (109) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4Δ30,
110. (110) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
111. (111) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4A30, rDEN4/2Δ30,
112. (112) rDEN1/2Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
113. (113) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3A30, rDEN4Δ30,
114. (114) rDEN1/2Δ30, rDEN2/4Δ30, rDEN3Δ30, rDEN4/1Δ30,
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SEQUENCE LISTING

[0122]

<110> The Government of the United States of America, as represented by the Secretary, Health and Human Services

<120> DENGUE TETRAVALENT VACCINE CONTAINING A COMMON 30 NUCLEOTIDE DELETION IN THE 3' -UTR OF DENGUE TYPES 1, 2, 3, AND 4, OR ANTIGENIC CHIMERIC DENGUE VIRUSES 1, 2, 3, AND 4

<130> K2825 EP/1 S3

<150> US 60/377,860

<151> 2002-05-03

<150> US 60/436,500

<151> 2002-12-23

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<211> 3391

<212> PRT

<213> Dengue 2 virus (Tonga/74)

<400> 47

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Lys Arg Glu Arg Asn Arg Val Ser Thr Val Gln Gln Leu Thr Lys Arg
20     25     30
Phe Ser Leu Gly Met Leu Gln Gly Arg Gly Pro Leu Lys Leu Phe Met
35     40     45
Ala Leu Val Ala Phe Leu Arg Phe Leu Thr Ile Pro Pro Thr Ala Gly
50     55     60
Ile Leu Lys Arg Trp Gly Thr Ile Lys Lys Ser Lys Ala Ile Asn Val
65     70     75     80
Leu Arg Gly Phe Arg Lys Glu Ile Gly Arg Met Leu Asn Ile Leu Asn
85     90     95

Arg Arg Arg Arg Thr Val Gly Met Ile Ile Met Leu Thr Pro Thr Val
100    105    110
Met Ala Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val
115    120    125
Ser Arg Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Lys Asp Gly
130    135    140
Thr Asn Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu
145    150    155    160
Asp Thr Ile Thr Tyr Lys Cys Pro Phe Leu Lys Gln Asn Glu Pro Glu
165    170    175
Asp Ile Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly
180    185    190
Thr Cys Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala
195    200    205
Leu Val Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp
210    215    220
Met Ser Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Thr Trp
225    230    235    240
Ile Leu Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr
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Thr Ile Gly Thr Thr His Phe Gln Arg Val Leu Ile Phe Ile Leu Leu
260    265    270
Thr Ala Ile Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn
275    280    285
Arg Asp Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val
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Leu Glu His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr
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Cys Lys Lys Asn Met Glu Gly Lys Ile Val Gln Pro Glu Asn Leu Glu
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Tyr Thr Val Val Ile Thr Pro His Ser Gly Glu Glu His Ala Val Gly
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Cys Ser Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln
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Gln Lys Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln
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Thr Gly Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr
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Ala Glu Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly
595    600    605
Asp Gly Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys
610    615    620
Arg His Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu
625    630    635    640
Lys Asp Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser
645    650    655
Tyr Ile Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asp Trp Phe
660    665    670
Lys Lys Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly
675    680    685
Ala Lys Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser
690    695    700
Leu Gly Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe
705    710    715    720
Gly Ala Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys
725    730    735

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Ile Leu Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser
 740 745 750
 Thr Ser Leu Ser Val Ser Leu Val Leu Val Gly Ile Val Thr Leu Tyr
 755 760 765
 Leu Gly Val Met Val Gln Ala Asp Ser Gly Cys Val Val Ser Trp Lys
 770 775 780
 Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Phe Val Thr Asp Asn Val
 785 790 795 800
 His Thr Trp Thr Glu Gln Tyr Lys Phe Gln Pro Glu Ser Pro Ser Lys
 805 810 815
 Leu Ala Ser Ala Ile Gln Lys Ala His Glu Glu Gly Ile Cys Gly Ile
 820 825 830
 Arg Ser Val Thr Arg Leu Glu Asn Leu Met Trp Lys Gln Ile Thr Ser
 835 840 845
 Glu Leu Asn His Ile Leu Ser Glu Asn Glu Val Lys Leu Thr Ile Met
 850 855 860
 Thr Gly Asp Ile Lys Gly Ile Met Gln Val Gly Lys Arg Ser Leu Arg
 865 870 875 880
 Pro Gln Pro Thr Glu Leu Arg Tyr Ser Trp Lys Thr Trp Gly Lys Ala
 885 890 895
 Lys Met Leu Ser Thr Glu Leu His Asn Gln Thr Phe Leu Ile Asp Gly
 900 905 910
 Pro Glu Thr Ala Glu Cys Pro Asn Thr Asn Arg Ala Trp Asn Ser Leu
 915 920 925
 Glu Val Glu Asp Tyr Gly Phe Gly Val Phe Thr Thr Asn Ile Trp Leu
 930 935 940
 Arg Leu Arg Glu Lys Gln Asp Val Phe Cys Asp Ser Lys Leu Met Ser
 945 950 955 960
 Ala Ala Ile Lys Asp Asn Arg Ala Val His Ala Asp Met Gly Tyr Trp
 965 970 975

 Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Ile Glu Lys Ala Ser Phe
 980 985 990
 Ile Glu Val Lys Ser Cys His Trp Pro Lys Ser His Thr Leu Trp Ser
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 Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu Lys Glu Glu Asn Leu
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 1845 1850 1855

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 Phe Val Pro Pro Glu Lys Cys Asp Thr Leu Leu Cys Asp Ile Gly Glu
 2625 2630 2635 2640
 Ser Ser Pro Asn Pro Thr Val Glu Ala Gly Arg Thr Leu Arg Val Leu
 2645 2650 2655
 Asn Leu Val Glu Asn Trp Leu Asn Asn Asn Thr Gln Phe Cys Val Lys
 2660 2665 2670
 Val Leu Asn Pro Tyr Met Pro Ser Val Ile Glu Arg Met Glu Thr Leu
 2675 2680 2685
 Gln Arg Lys Tyr Gly Gly Ala Leu Val Arg Asn Pro Leu Ser Arg Asn
 2690 2695 2700
 Ser Thr His Glu Met Tyr Trp Val Ser Asn Ala Ser Gly Asn Ile Val
 2705 2710 2715 2720
 Ser Ser Val Asn Met Ile Ser Arg Met Leu Ile Asn Arg Phe Thr Met
 2725 2730 2735

 Arg His Lys Lys Ala Thr Tyr Glu Pro Asp Val Asp Leu Gly Ser Gly
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 Thr Arg Asn Ile Gly Ile Glu Ser Glu Thr Pro Asn Leu Asp Ile Ile
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cagccagccg gaagggccga ggcagagaagt ggtccctgcaa ctttattccgc cttccatccag 15000
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gttggtgcca ttgctgcaag atctggcctag cgatgacct gctgattggt tcgctgacca 15120
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<210> 49

<211> 3390

<212> PRT

<213> Dengue 3 (Sleman/78) virus

<400> 49

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20     25     30
Phe Ser Arg Gly Leu Leu Asn Gly Gln Gly Pro Met Lys Leu Val Met
35     40     45
Ala Phe Ile Ala Phe Leu Arg Phe Leu Ala Ile Pro Pro Thr Ala Gly
50     55     60
Val Leu Ala Arg Trp Gly Thr Phe Lys Lys Ser Gly Ala Ile Lys Val
65     70     75     80
Leu Arg Gly Phe Lys Lys Glu Ile Ser Asn Met Leu Ser Ile Ile Asn
85     90     95
Arg Arg Lys Lys Thr Ser Leu Cys Leu Met Met Met Leu Pro Ala Thr
100    105    110
Leu Ala Phe His Leu Thr Ser Arg Asp Gly Glu Pro Arg Met Ile Val
115    120    125
Gly Lys Asn Glu Arg Gly Lys Ser Leu Leu Phe Lys Thr Ala Ser Gly
130    135    140
Ile Asn Met Cys Thr Leu Ile Ala Met Asp Leu Gly Glu Met Cys Asp
145    150    155    160
Asp Thr Val Thr Tyr Lys Cys Pro Leu Ile Thr Glu Val Glu Pro Glu
165    170    175
Asp Ile Asp Cys Trp Cys Asn Leu Thr Ser Thr Trp Val Thr Tyr Gly
180    185    190
Thr Cys Asn Gln Ala Gly Glu His Arg Arg Asp Lys Arg Ser Val Ala
195    200    205
Leu Ala Pro His Val Gly Met Gly Leu Asp Thr Arg Thr Gln Thr Trp
210    215    220
Met Ser Ala Glu Gly Ala Trp Arg Gln Val Glu Lys Val Glu Thr Trp
225    230    235    240

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Ala Phe Arg His Pro Gly Phe Thr Ile Leu Ala Leu Phe Leu Ala His
245    250    255
Tyr Ile Gly Thr Ser Leu Thr Gln Lys Val Val Ile Phe Ile Leu Leu
260    265    270
Met Leu Val Thr Pro Ser Met Thr Met Arg Cys Val Gly Val Gly Asn
275    280    285
Arg Asp Phe Val Glu Gly Leu Ser Gly Ala Thr Trp Val Asp Val Val
290    295    300
Leu Glu His Gly Gly Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr
305    310    315    320
Leu Asp Ile Glu Leu Gln Lys Thr Glu Ala Thr Gln Leu Ala Thr Leu
325    330    335
Arg Lys Leu Cys Ile Glu Gly Lys Ile Thr Asn Val Thr Thr Asp Ser
340    345    350
Arg Cys Pro Thr Gln Gly Glu Ala Ile Leu Pro Glu Glu Gln Asp Gln
355    360    365
Asn His Val Cys Lys His Thr Tyr Val Asp Arg Gly Trp Gly Asn Gly
370    375    380
Cys Gly Leu Phe Gly Lys Gly Ser Leu Val Thr Cys Ala Lys Phe Gln
385    390    395    400
Cys Leu Glu Ser Ile Glu Gly Lys Val Val Gln His Glu Asn Leu Lys
405    410    415
Tyr Thr Val Ile Ile Thr Val His Thr Gly Asp Gln His Gln Val Gly
420    425    430
Asn Glu Thr Gln Gly Val Thr Ala Glu Ile Thr Pro Gln Ala Ser Thr
435    440    445
Val Glu Ala Ile Leu Pro Glu Tyr Gly Thr Leu Gly Leu Glu Cys Ser
450    455    460
Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Ile Leu Leu Thr Met Lys
465    470    475    480
Asn Lys Ala Trp Met Val His Arg Gln Trp Phe Phe Asp Leu Pro Leu
485    490    495
Pro Trp Thr Ser Gly Ala Thr Thr Glu Thr Pro Thr Trp Asn Lys Lys
500    505    510
Glu Leu Leu Val Thr Phe Lys Asn Ala His Ala Lys Lys Gln Glu Val
515    520    525
Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly
530    535    540
Ala Thr Glu Ile Gln Thr Ser Gly Gly Thr Ser Ile Phe Ala Gly His
545    550    555    560
Leu Lys Cys Arg Leu Lys Met Asp Lys Leu Glu Leu Lys Gly Met Ser
565    570    575
Tyr Ala Met Cys Leu Asn Ala Phe Val Leu Lys Lys Glu Val Scr Glu
580    585    590
Thr Gln His Gly Thr Ile Leu Ile Lys Val Glu Tyr Lys Gly Glu Asp
595    600    605
Ala Pro Cys Lys Ile Pro Phe Ser Thr Glu Asp Gly Gln Gly Lys Ala
610    615    620
His Asn Gly Arg Leu Ile Thr Ala Asn Pro Val Val Thr Lys Lys Glu
625    630    635    640
Glu Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Glu Ser Asn Ile
645    650    655
Val Ile Gly Ile Gly Asp Lys Ala Leu Lys Ile Asn Trp Tyr Lys Lys
660    665    670
Gly Ser Ser Ile Gly Lys Met Phe Glu Ala Thr Ala Arg Gly Ala Arg

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Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Val Gly
690      695      700
Gly Val Leu Asn Ser Leu Gly Lys Met Val His Gln Ile Phe Gly Ser
705      710      715      720
Ala Tyr Thr Ala Leu Phe Ser Gly Val Ser Trp Ile Met Lys Ile Gly
725      730      735
Ile Gly Val Leu Thr Trp Ile Gly Leu Asn Ser Lys Asn Thr Ser
740      745      750
Met Ser Phe Ser Cys Ile Val Ile Gly Ile Ile Thr Leu Tyr Leu Gly
755      760      765
Ala Val Val Gln Ala Asp Met Gly Cys Val Ile Asn Trp Lys Gly Lys
770      775      780
Glu Leu Lys Cys Gly Ser Gly Ile Phe Val Thr Asn Glu Val His Thr
785      790      795      800
Trp Thr Glu Gln Tyr Lys Phe Gln Ala Asp Ser Pro Lys Arg Leu Ala
805      810      815
Thr Ala Ile Ala Gly Ala Trp Glu Asn Gly Val Cys Gly Ile Arg Ser
820      825      830
Thr Thr Arg Met Glu Asn Leu Leu Trp Lys Gln Ile Ala Asn Glu Leu
835      840      845
Asn Tyr Ile Leu Trp Glu Asn Asn Ile Lys Leu Thr Val Val Val Gly
850      855      860
Asp Ile Ile Gly Val Leu Glu Gln Gly Lys Arg Thr Leu Thr Pro Gln
865      870      875      880
Pro Met Glu Leu Lys Tyr Ser Trp Lys Thr Trp Gly Lys Ala Lys Ile
885      890      895
Val Thr Ala Glu Thr Gln Asn Ser Ser Phe Ile Ile Asp Gly Pro Asn
900      905      910
Thr Pro Glu Cys Pro Ser Ala Ser Arg Ala Trp Asn Val Trp Glu Val
915      920      925
Glu Asp Tyr Gly Phe Gly Val Phe Thr Thr Asn Ile Trp Leu Lys Leu
930      935      940
Arg Glu Met Tyr Thr Gln Leu Cys Asp His Arg Leu Met Ser Ala Ala
945      950      955      960
Val Lys Asp Glu Arg Ala Val His Ala Asp Met Gly Tyr Trp Ile Glu
965      970      975
Ser Gln Lys Asn Gly Ser Trp Lys Leu Glu Lys Ala Ser Leu Ile Glu
980      985      990
Val Lys Thr Cys Thr Trp Pro Lys Ser His Thr Leu Trp Ser Asn Gly
995      1000      1005
Val Leu Glu Ser Asp Met Ile Ile Pro Lys Ser Leu Ala Gly Pro Ile
1010      1015      1020
Ser Gln His Asn Tyr Arg Pro Gly Tyr His Thr Gln Thr Ala Gly Pro
1025      1030      1035      1040
Trp His Leu Gly Lys Leu Glu Leu Asp Phe Asn Tyr Cys Glu Gly Thr
1045      1050      1055
Thr Val Val Ile Thr Glu Asn Cys Gly Thr Arg Gly Pro Ser Leu Arg
1060      1065      1070
Thr Thr Thr Val Ser Gly Lys Leu Ile His Glu Trp Cys Cys Arg Ser
1075      1080      1085
Cys Thr Leu Pro Pro Leu Arg Tyr Met Gly Glu Asp Gly Cys Trp Tyr
1090      1095      1100
Gly Met Glu Ile Arg Pro Ile Asn Glu Lys Glu Glu Asn Met Val Lys
1105      1110      1115      1120

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Ser Leu Val Ser Ala Gly Ser Gly Lys Val Asp Asn Phe Thr Met Gly
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Val Leu Cys Leu Ala Ile Leu Phe Glu Glu Val Met Arg Gly Lys Phe
1140      1145      1150
Gly Lys Lys His Met Ile Ala Gly Val Leu Phe Thr Phe Val Leu Leu
1155      1160      1165
Leu Ser Gly Gln Ile Thr Trp Arg Asp Met Ala His Thr Leu Ile Met
1170      1175      1180
Ile Gly Ser Asn Ala Ser Asp Arg Met Gly Met Gly Val Thr Tyr Leu
1185      1190      1195      1200
Ala Leu Ile Ala Thr Phe Lys Ile Gln Pro Phe Leu Ala Leu Gly Phe
1205      1210      1215
Phe Leu Arg Lys Leu Thr Ser Arg Glu Asn Leu Leu Leu Gly Val Gly
1220      1225      1230
Leu Ala Met Ala Thr Thr Leu Gln Leu Pro Glu Asp Ile Glu Gln Met
1235      1240      1245
Ala Asn Gly Ile Ala Leu Gly Leu Met Ala Leu Lys Leu Ile Thr Gln
1250      1255      1260
Phe Glu Thr Tyr Gln Leu Trp Thr Ala Leu Val Ser Leu Met Cys Ser
1265      1270      1275      1280
Asn Thr Ile Phe Thr Leu Thr Val Ala Trp Arg Thr Ala Thr Leu Ile
1285      1290      1295
Leu Ala Gly Ile Ser Leu Leu Pro Val Cys Gln Ser Ser Ser Met Arg
1300      1305      1310
Lys Thr Asp Trp Leu Pro Met Ala Val Ala Ala Met Gly Val Pro Pro
1315      1320      1325
Leu Pro Leu Phe Ile Phe Ser Ser Leu Lys Asp Thr Leu Lys Arg Arg Ser
1330      1335      1340
Trp Pro Leu Asn Glu Gly Val Met Ala Val Gly Leu Val Ser Ile Leu
1345      1350      1355      1360
Ala Ser Ser Leu Leu Arg Asn Asp Val Pro Met Ala Gly Pro Leu Val
1365      1370      1375
Ala Gly Gly Leu Leu Ile Ala Cys Tyr Val Ile Thr Gly Thr Ser Ala
1380      1385      1390
Asp Leu Thr Val Glu Lys Ala Ala Asp Val Thr Trp Glu Glu Glu Ala
1395      1400      1405
Glu Gln Thr Gly Val Ser His Asn Leu Met Ile Thr Val Asp Asp Asp
1410      1415      1420
Gly Thr Met Arg Ile Lys Asp Asp Glu Thr Glu Asn Ile Leu Thr Val
1425      1430      1435      1440
Leu Leu Lys Thr Ala Leu Leu Ile Val Ser Gly Ile Phe Pro Tyr Ser
1445      1450      1455
Ile Pro Ala Thr Leu Leu Val Trp His Thr Trp Gln Lys Gln Thr Gln
1460      1465      1470
Arg Ser Gly Val Leu Trp Asp Val Pro Ser Pro Pro Glu Thr Gln Lys
1475      1480      1485

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Ala Glu Leu Glu Glu Gly Val Tyr Arg Ile Lys Gln Gln Gly Ile Phe
 1490 1495 1500
 Gly Lys Thr Gln Val Gly Val Gly Val Gln Lys Glu Gly Val Phe His
 1505 1510 1515 1520
 Thr Met Trp His Val Thr Arg Gly Ala Val Leu Thr His Asn Gly Lys
 1525 1530 1535
 Arg Leu Glu Pro Asn Trp Ala Ser Val Lys Lys Asp Leu Ile Ser Tyr
 1540 1545 1550
 Gly Gly Gly Trp Lys Leu Ser Ala Gln Trp Gln Lys Gly Glu Glu Val
 1555 1560 1565
 Gln Val Ile Ala Val Glu Pro Gly Lys Asn Pro Lys Asn Phe Gln Thr
 1570 1575 1580
 Met Pro Gly Ile Phe Gln Thr Thr Thr Gly Glu Ile Gly Ala Ile Ala
 1585 1590 1595 1600
 Leu Asp Phe Lys Pro Gly Thr Ser Gly Ser Pro Ile Ile Asn Arg Glu
 1605 1610 1615
 Gly Lys Val Leu Gly Leu Tyr Gly Asn Gly Val Val Thr Lys Asn Gly
 1620 1625 1630
 Gly Tyr Val Ser Gly Ile Ala Gln Thr Asn Ala Glu Pro Asp Gly Pro
 1635 1640 1645
 Thr Pro Glu Leu Glu Glu Glu Met Phe Lys Lys Arg Asn Leu Thr Ile
 1650 1655 1660
 Met Asp Leu His Pro Gly Ser Gly Lys Thr Arg Lys Tyr Leu Pro Ala
 1665 1670 1675 1680
 Ile Val Arg Glu Ala Ile Lys Arg Arg Leu Arg Thr Leu Ile Leu Ala
 1685 1690 1695
 Pro Thr Arg Val Val Ala Ala Glu Met Glu Glu Ala Leu Lys Gly Leu
 1700 1705 1710
 Pro Ile Arg Tyr Gln Thr Thr Ala Thr Lys Ser Glu His Thr Gly Arg
 1715 1720 1725
 Glu Ile Val Asp Leu Met Cys His Ala Thr Phe Thr Met Arg Leu Leu
 1730 1735 1740
 Ser Pro Val Arg Val Pro Asn Tyr Asn Leu Ile Ile Met Asp Glu Ala
 1745 1750 1755 1760
 His Phe Thr Asp Pro Ala Ser Ile Ala Ala Arg Gly Tyr Ile Ser Thr
 1765 1770 1775
 Arg Val Gly Met Gly Glu Ala Ala Ala Ile Phe Met Thr Ala Thr Pro
 1780 1785 1790
 Pro Gly Thr Ala Asp Ala Phe Pro Gln Ser Asn Ala Pro Ile Gln Asp
 1795 1800 1805
 Glu Glu Arg Asp Ile Pro Glu Arg Ser Trp Asn Ser Gly Asn Glu Trp
 1810 1815 1820
 Ile Thr Asp Phe Ala Gly Lys Thr Val Trp Phe Val Pro Ser Ile Lys
 1825 1830 1835 1840
 Ala Gly Asn Asp Ile Ala Asn Cys Leu Arg Lys Asn Gly Lys Lys Val
 1845 1850 1855
 Ile Gln Leu Ser Arg Lys Thr Phe Asp Thr Glu Tyr Gln Lys Thr Lys
 1860 1865 1870
 Leu Asn Asp Trp Asp Phe Val Val Thr Thr Asp Ile Ser Glu Met Gly
 1875 1880 1885
 Ala Asn Phe Lys Ala Asp Arg Val Ile Asp Pro Arg Arg Cys Leu Lys
 1890 1895 1900
 Pro Val Ile Leu Thr Asp Gly Pro Glu Arg Val Ile Leu Ala Gly Pro
 1905 1910 1915 1920
 Met Pro Val Thr Val Ala Ser Ala Ala Gln Arg Arg Gly Arg Val Gly
 1925 1930 1935
 Arg Asn Pro Gln Lys Glu Asn Asp Gln Tyr Ile Phe Met Gly Gln Pro
 1940 1945 1950
 Leu Asn Asn Asp Glu Asp His Ala His Trp Thr Glu Ala Lys Met Leu
 1955 1960 1965
 Leu Asp Asn Ile Asn Thr Pro Glu Gly Ile Ile Pro Ala Leu Phe Glu
 1970 1975 1980
 Pro Glu Arg Glu Lys Ser Ala Ala Ile Asp Gly Glu Tyr Arg Leu Lys
 1985 1990 1995 2000
 Gly Glu Ser Arg Lys Thr Phe Val Glu Leu Met Arg Arg Gly Asp Leu
 2005 2010 2015
 Pro Val Trp Leu Ala His Lys Val Ala Ser Glu Gly Ile Lys Tyr Thr
 2020 2025 2030
 Asp Arg Lys Trp Cys Phe Asp Gly Glu Arg Asn Asn Gln Ile Leu Glu
 2035 2040 2045
 Glu Asn Met Asp Val Glu Ile Trp Thr Lys Glu Gly Glu Lys Lys Lys
 2050 2055 2060
 Leu Arg Pro Arg Trp Leu Asp Ala Arg Thr Tyr Ser Asp Pro Leu Ala
 2065 2070 2075 2080
 Leu Lys Glu Phe Lys Asp Phe Ala Ala Gly Arg Lys Ser Ile Ala Leu
 2085 2090 2095
 Asp Leu Val Thr Glu Ile Gly Arg Val Pro Ser His Leu Ala His Arg
 2100 2105 2110
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 2115 2120 2125
 Gly Gly Arg Ala Tyr Arg His Ala Val Glu Glu Leu Pro Glu Thr Met
 2130 2135 2140
 Glu Thr Leu Leu Leu Leu Gly Leu Met Ile Leu Leu Thr Gly Gly Ala
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 Met Leu Phe Leu Ile Ser Gly Lys Gly Ile Gly Lys Thr Ser Ile Gly
 2165 2170 2175
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 Pro Leu Gln Trp Ile Ala Ser Ala Ile Val Leu Glu Phe Phe Met Met
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 2245 2250 2255
 Met Ser Lys Glu Pro Gly Val Val Ser Pro Thr Ser Tyr Leu Asp Val
 2260 2265 2270
 Asp Leu His Pro Ala Ser Ala Trp Thr Leu Tyr Ala Val Ala Thr Thr

2275 2280 2285
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 2290 2295 2300
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 Asp Lys Gly Trp Pro Ile Ser Lys Met Asp Leu Gly Val Pro Leu Leu
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 2340 2345 2350
 Val Leu Leu Leu Val Thr His Tyr Ala Ile Ile Gly Pro Gly Leu Gln
 2355 2360 2365
 Ala Lys Ala Thr Arg Glu Ala Gln Lys Arg Thr Ala Ala Gly Ile Met
 2370 2375 2380
 Lys Asn Pro Thr Val Asp Gly Ile Met Thr Ile Asp Leu Asp Pro Val
 2385 2390 2395 2400
 Ile Tyr Asp Ser Lys Phe Glu Lys Gln Leu Gly Gln Val Met Leu Leu
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 Cys Glu Ala Leu Thr Leu Ala Thr Gly Pro Ile Thr Thr Leu Trp Glu

 2435 2440 2445
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 Asn Ile Phe Arg Gly Ser Tyr Leu Ala Gly Ala Gly Leu Ala Phe Ser
 2465 2470 2475 2480
 Ile Met Lys Ser Val Gly Thr Gly Lys Arg Gly Thr Gly Ser Gln Gly
 2485 2490 2495
 Glu Thr Leu Gly Glu Lys Trp Lys Lys Lys Leu Asn Gln Leu Pro Arg
 2500 2505 2510
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 2515 2520 2525
 Thr Glu Ala Lys Glu Gly Leu Lys Arg Gly Glu Ile Thr His His Ala
 2530 2535 2540
 Val Ser Arg Gly Ser Ala Lys Leu Gln Trp Phe Val Glu Arg Asn Met
 2545 2550 2555 2560
 Val Ile Pro Glu Gly Arg Val Ile Asp Leu Gly Cys Gly Arg Gly Gly
 2565 2570 2575
 Trp Ser Tyr Tyr Cys Ala Gly Leu Lys Lys Val Thr Glu Val Arg Gly
 2580 2585 2590
 Tyr Thr Lys Gly Gly Pro Gly His Glu Glu Pro Val Pro Met Ser Thr
 2595 2600 2605
 Tyr Gly Trp Asn Ile Val Lys Leu Met Ser Gly Lys Asp Val Phe Tyr
 2610 2615 2620
 Leu Pro Pro Glu Lys Cys Asp Thr Leu Leu Cys Asp Ile Gly Glu Ser
 2625 2630 2635 2640
 Ser Pro Ser Pro Thr Val Glu Glu Ser Arg Thr Ile Arg Val Leu Lys
 2645 2650 2655
 Met Val Glu Pro Trp Leu Lys Asn Asn Gln Phe Cys Ile Lys Val Leu
 2660 2665 2670
 Asn Pro Tyr Met Pro Thr Val Ile Glu His Leu Glu Arg Leu Gln Arg
 2675 2680 2685
 Lys His Gly Gly Met Leu Val Arg Asn Pro Leu Ser Arg Asn Ser Thr
 2690 2695 2700
 His Glu Met Tyr Trp Ile Ser Asn Gly Thr Gly Asn Ile Val Ser Ser
 2705 2710 2715 2720
 Val Asn Met Val Ser Arg Leu Leu Leu Asn Arg Phe Thr Met Thr His
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 Arg Arg Pro Thr Ile Glu Lys Asp Val Asp Leu Gly Ala Gly Thr Arg
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 His Val Asn Ala Glu Pro Glu Thr Pro Asn Met Asp Val Ile Gly Glu
 2755 2760 2765
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 2770 2775 2780
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 Gly Lys Arg Glu Lys Lys Leu Gly Glu Phe Gly Lys Ala Lys Gly Ser
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 Lys Ile Thr Gln Gln Met Asp Pro Glu His Arg Gln Leu Ala Asn Ala
 3045 3050 3055
 Ile Phe Lys Leu Thr Tyr Gln Asn Lys Val Val Lys Val Gln Arg Pro
 3060 3065 3070

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Thr Pro Lys Gly Thr Val Met Asp Ile Ile Ser Arg Lys Asp Gln Arg
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3090          3095          3100
Glu Ala Gln Leu Ile Arg Gln Met Glu Gly Glu Gly Val Leu Ser Lys
3105          3110          3115          3120
Ala Asp Leu Gln Asn Pro His Leu Leu Glu Lys Lys Val Thr Gln Trp
3125          3130          3135
Leu Glu Thr Lys Lys Gly Val Glu Arg Leu Lys Arg Met Ala Ile Ser Gly
3140          3145          3150
Asp Asp Cys Val Val Lys Pro Ile Asp Asp Arg Phe Ala Asn Ala Leu
3155          3160          3165
Leu Ala Leu Asn Asp Met Gly Lys Val Arg Lys Asp Ile Pro Gln Trp
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Gln Pro Ser Lys Gly Trp His Asp Trp Gln Gln Val Pro Phe Cys Ser
3185          3190          3195          3200
His His Phe His Glu Leu Ile Met Lys Asp Gly Arg Lys Leu Val Val
3205          3210          3215
Pro Cys Arg Pro Gln Asp Glu Leu Ile Gly Arg Ala Arg Ile Ser Gln
3220          3225          3230
Gly Ala Gly Trp Ser Leu Arg Glu Thr Ala Cys Leu Gly Lys Ala Tyr
3235          3240          3245
Ala Gln Met Trp Thr Leu Met Tyr Phe His Arg Arg Asp Leu Arg Leu
3250          3255          3260
Ala Ser Asn Ala Ile Cys Ser Ala Val Pro Val His Trp Val Pro Thr
3265          3270          3275          3280
Ser Arg Thr Thr Trp Ser Ile His Ala His His Gln Trp Met Thr Thr
3285          3290          3295
Glu Asp Met Leu Thr Val Trp Asn Arg Val Trp Ile Glu Asp Asn Pro
3300          3305          3310
Trp Met Glu Asp Lys Thr Pro Val Lys Thr Trp Glu Asp Val Pro Tyr

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3315          3320          3325
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Ser Arg Ala Thr Trp Ala Gln Asn Ile Leu Thr Ala Ile Gln Gln Val
3345          3350          3355          3360
Arg Ser Leu Ile Gly Asn Glu Glu Phe Leu Asp Tyr Met Pro Ser Met
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Lys Arg Phe Arg Lys Glu Glu Glu Ser Glu Gly Ala Ile Trp
3380          3385          3390

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<211> 2426

<212> DNA

<213> Artificial Sequence

<220>

<223> Dengue 1 CME chimeric region

<400> 50

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tgatgggttt catagcattt ctaagatttc tagccatacc cccaacagca ggaatttttg 300
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gccccgggat cactgagcgc gaaccagatg acgttgaatg ctggtgcaat gccacagaca 660
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65     70     75     80
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85     90     95
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50          55          60
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65          70          75          80
Ile Gly Phe Arg Lys Glu Ile Gly Arg Met Leu Asn Ile Leu Asn Gly
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REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

- 1.** Rekombinant kimær Denguevirus omfattende en nukleinsyre-kimære omfattende en første nukleotidsekvens der koder for strukturproteinet C fra Dengue type 4 virus og strukturproteinerne M og E fra Denguevirus type 1, Denguevirus type 2 eller Denguevirus type 3, hvor regionen af Dengue type 4 virus, der koder for strukturproteinerne M og E, er erstattet af regionen, der koder for strukturproteinerne M og E fra Denguevirus type 1, Denguevirus type 2, eller Denguevirus type 3, og en anden nukleotidsekvens der koder for ikke-strukturproteiner fra Dengue type 4 virus og den 3'-utranslaterede region af Dengue type 4-genomet indeholdende en 30 nukleotiddeletion, der svarer til TL2-hårnålestrukturen mellem nukleotiderne 10478-10507 af Dengue type 4-genomet.
- 2.** Rekombinant kimær Denguevirus ifølge krav 1, yderligere omfattende en mutation der tilvejebringer en fænotype, hvor fænotypen er temperaturfølsomhed i *Vero*-celler eller den humane levercellelinje HuH-7, værts-cellerestriktion i moskitoceller eller den humane levercellelinje HuH-7, værts-celleteilpasning til forbedret replikation i *Vero*-celler, eller svækkelse i mus eller aber.
- 3.** Rekombinant kimær Denguevirus ifølge krav 1 eller 2 til anvendelse i induktionen af et beskyttende immunrespons.
- 4.** Rekombinant kimær Denguevirus til anvendelse i inducering af et beskyttende immunrespons ifølge krav 3, hvor den rekombinante kimære Denguevirus er omfattet i en vaccine, der skal administreres til et individ i en effektiv mængde.
- 5.** Rekombinant kimær Denguevirus ifølge krav 1 eller 2 til anvendelse i forebyggelsen af en sygdom forårsaget af Denguevirus.
- 6.** Rekombinant kimær Denguevirus til anvendelse i forebyggelse af en sygdom forårsaget af Denguevirus ifølge krav 5, hvor den rekombinante kimære Denguevirus er omfattet i en vaccine, der skal administreres til et individ i en effektiv mængde.

7. Vaccine eller immunogen sammensætning omfattende mindst én rekombinant kimær Denguevirus ifølge krav 1 eller 2.

8. Vaccinen eller den immunogene sammensætning ifølge krav 7 der er en
5 Denguevirus vaccine eller en Denguevirus-immunogen sammensætning.

9. Vaccinen eller den immunogene sammensætning ifølge krav 7 eller 8 der er en tetravalent vaccine eller en tetravalent immunogen sammensætning.

10 **10.** Vaccinen eller den immunogene sammensætning ifølge et hvilket som helst af kravene 7 til 9 omfattende rDEN1/4Δ30 (ME), rDEN2/4Δ30 (ME) og rDEN3/4Δ30 (ME), hvor:

15 rDEN1/4Δ30 (ME) er en kimær Denguevirus ifølge krav 1 omfattende en nukleinsyre-kimære omfattende en første nukleotidsekvens der koder for strukturproteinet C fra Dengue type 4 virus og strukturproteinerne M og E fra Denguevirus type 1, hvor regionen af Dengue type 4 virusen, der koder for strukturproteinerne M og E, er erstattet af regionen, der koder for strukturproteinerne M og E fra Denguevirus type 1, og en anden nukleotidsekvens der koder for ikke-strukturproteiner fra Dengue type 4
20 virus, og den 3'-utranslaterede region af Dengue type 4-genomet indeholdende 30 nukleotiddeletionen;

25 rDEN2/4Δ30 (ME) er en kimær Denguevirus ifølge krav 1 omfattende en nukleinsyre-kimære omfattende en første nukleotidsekvens der koder for strukturproteinet C fra Dengue type 4 virus og strukturproteinerne M og E fra Denguevirus type 2, hvor regionen af Dengue type 4 virusen, der koder for strukturproteinerne M og E, er erstattet af regionen, der koder for strukturproteinerne M og E fra Denguevirus type 2, og en anden nukleotidsekvens der koder for ikke-strukturproteiner fra Dengue type 4 virus, og den 3'-utranslaterede region af Dengue type 4-genomet
30 indeholdende 30 nukleotiddeletionen; og

rDEN3/4Δ30 (ME) er en kimær Dengue-virus ifølge krav 1 omfattende en nukleinsyre-kimære omfattende en første nukleotidsekvens der koder for strukturproteinet C fra Dengue type 4 virus og strukturproteinerne M og E

5 fra Denguevirus type 3, hvor regionen af Dengue type 4 virusen, der koder for strukturproteinerne M og E, er erstattet af regionen, der koder for strukturproteinerne M og E fra Denguevirus type 3, og en anden nukleotidsekvens der koder for ikke-strukturproteiner fra Dengue type 4 virus, og den 3'-utranslaterede region af Dengue type 4-genomet indeholdende 30 nukleotiddeletionen.

10 **11.** Rekombinant kimær Denguevirus til anvendelse i induktionen af en beskyttende immunrespons ifølge krav 3 eller 4, der skal administreres til et humant individ.

15 **12.** Rekombinant kimær Denguevirus til anvendelse i forebyggelse af en sygdom forårsaget af Denguevirus ifølge krav 5 eller 6, der skal administreres til et humant individ.

DRAWINGS

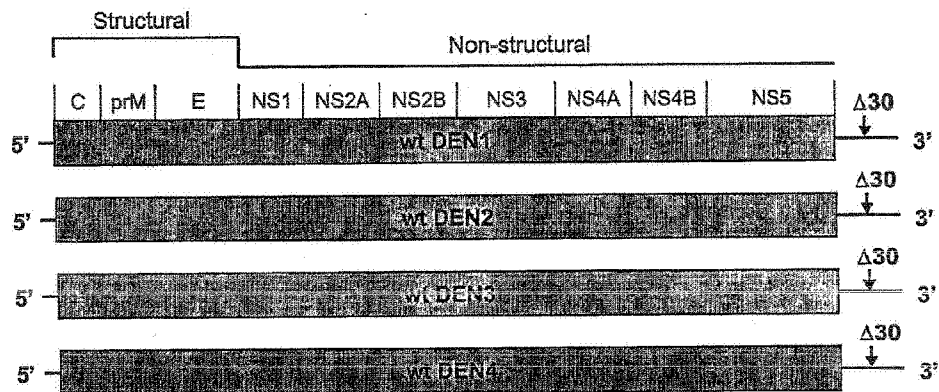
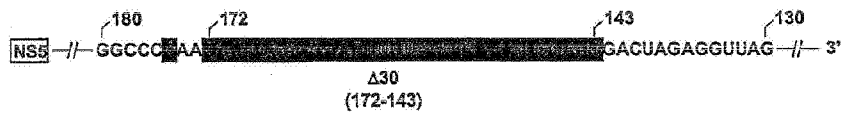


Figure 1

A.



B.

DEN1 GGGGCCC-AACACCAGGGGAAGCUGUACCCUGGUGGUAAGGACUAGA
 DEN1Δ30 GGGGCCC-AA-----GACUAGA
 DEN2 GGGGCCC-AAGGUGAGAUGAAGCUGUAGUCUCACUGGAAGGACUAGA
 DEN2Δ30 GGGGCCC-AA-----GACUAGA
 DEN3 GGGGCCCCGAGCUCUGAGGGGAAGCUGUACCUCUUGCAAAGGACUAGA
 DEN3Δ30 GGGGCCCCAA-----GACUAGA
 DEN4 GGGGCCCCGAAGCCAGGAGGAAGCUGUACUCCUGGUGGAAGGACUAGA
 DEN4Δ30 GGGGCCC-AA-----GACUAGA

DEN1 GGGGCCC-AacaccagggaAGCUGUAcccugguugguAAGGACUAGA
 DEN2 GGGGCCC-AagguagagauGAAGCUGUAgucucacuggAAGGACUAGA
 DEN3 GGGGCCCCgAgucugagggaAGCUGUAgcuccuuggaAAGGACUAGA
 DEN4 GGGGCCCCgAagccaggagGAAGCUGUAcuccugguuggAAGGACUAGA

C.

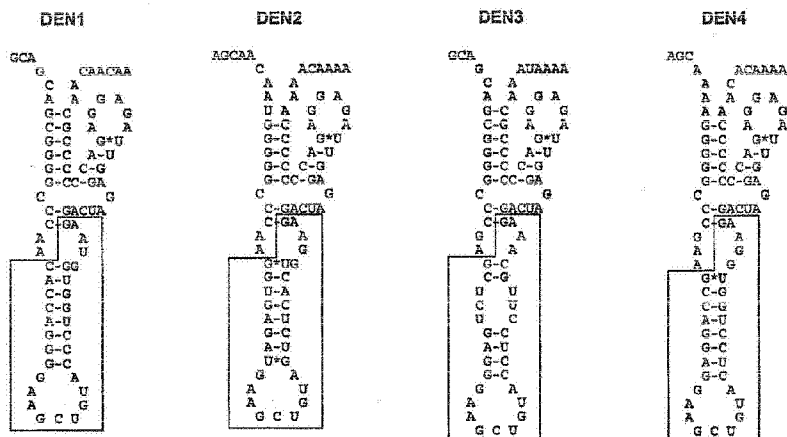


Figure 2

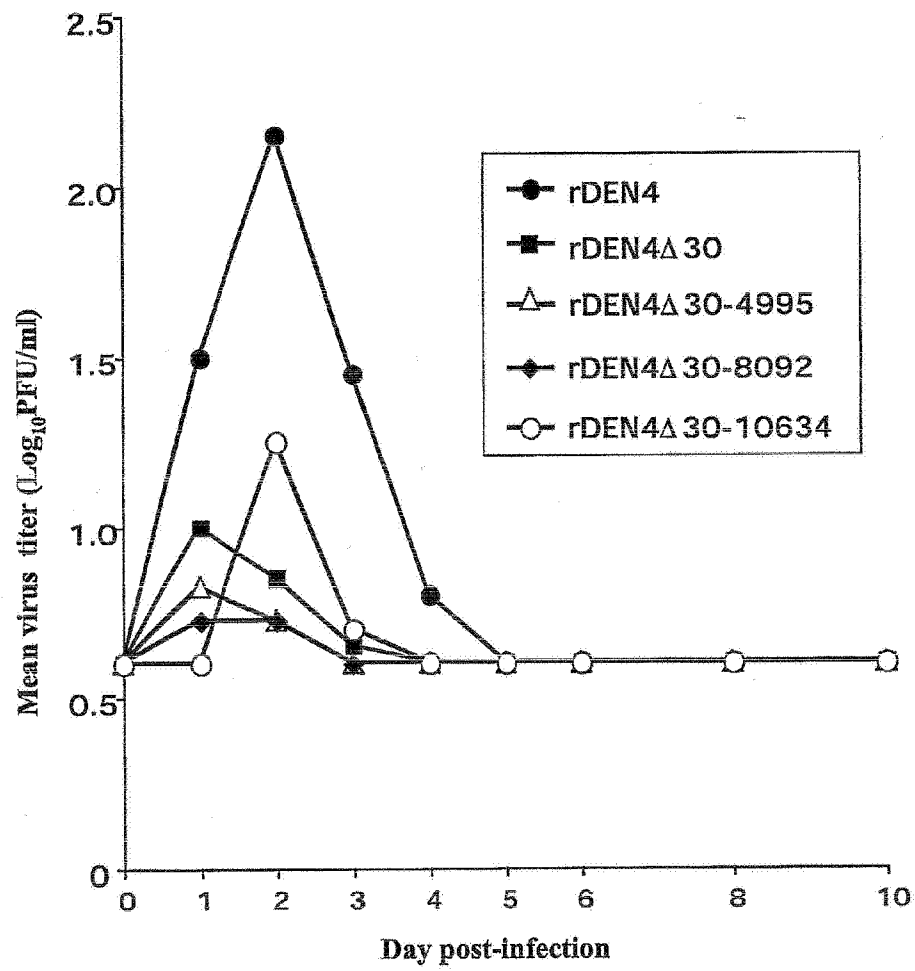


Figure 3

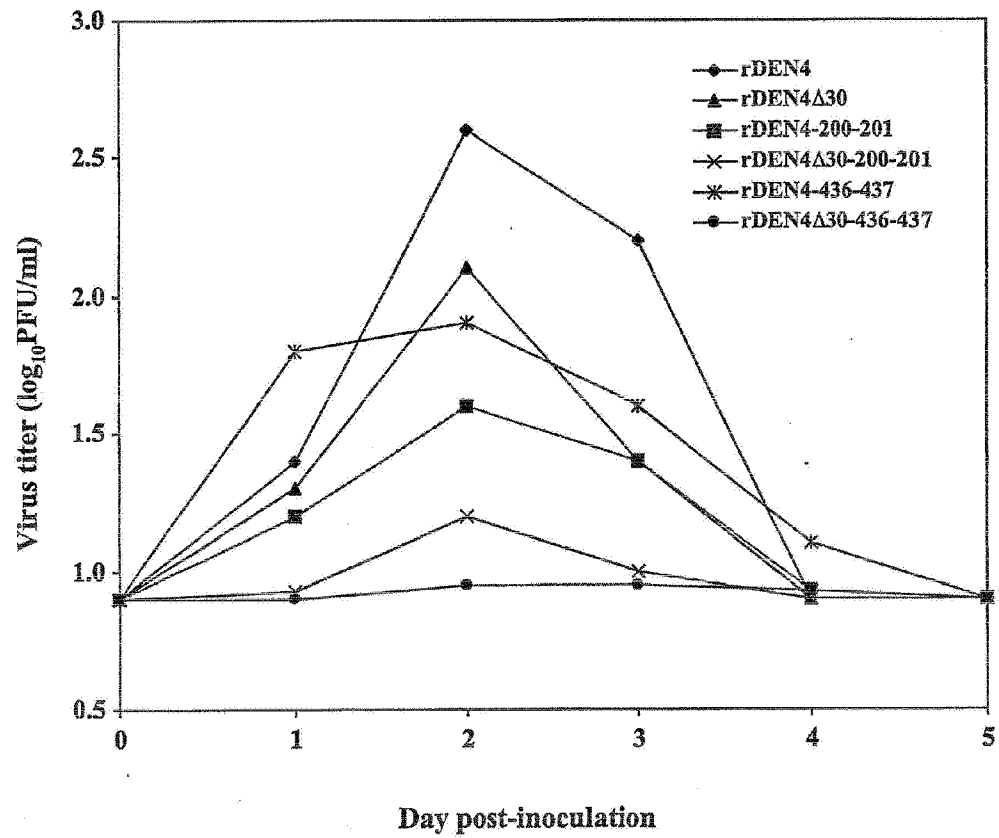


Figure 4

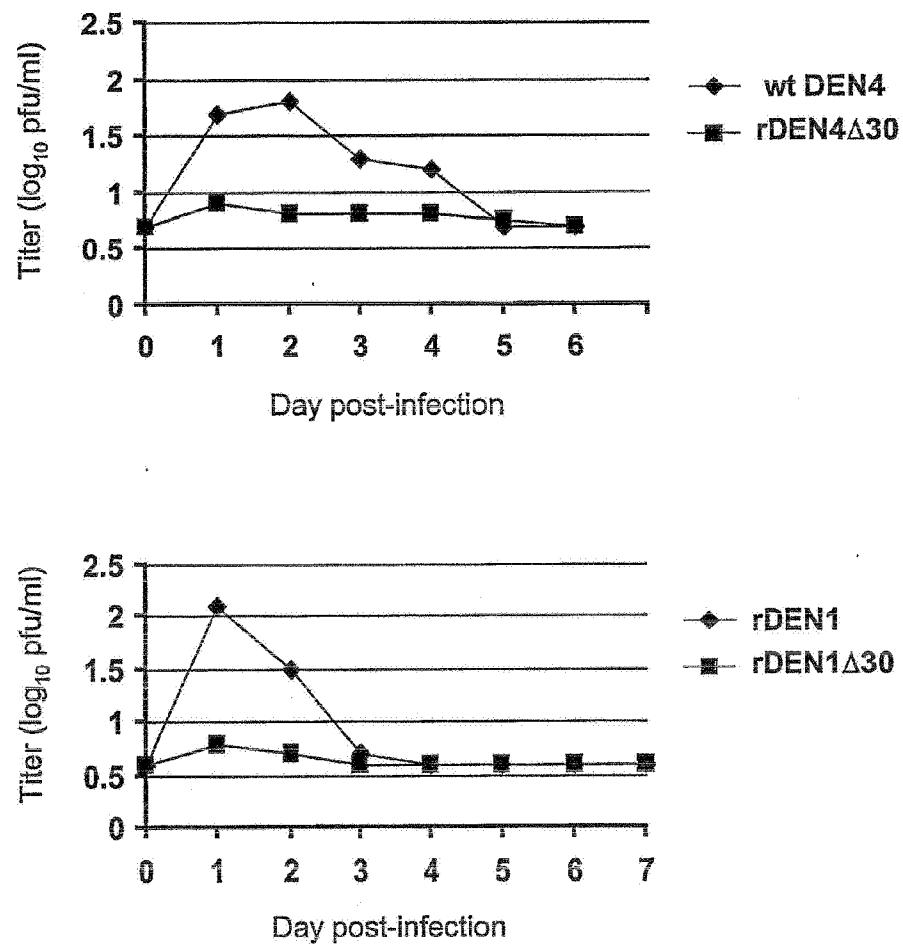
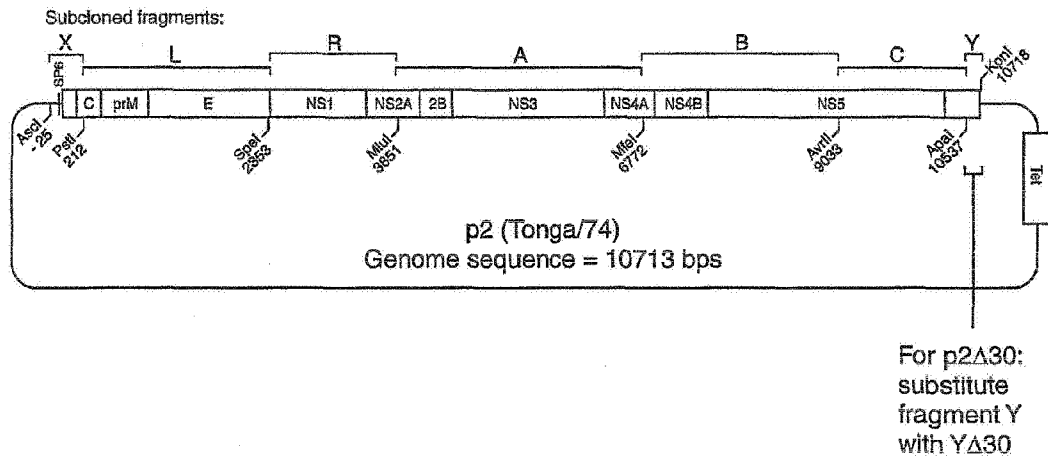


Figure 5

A.



B.

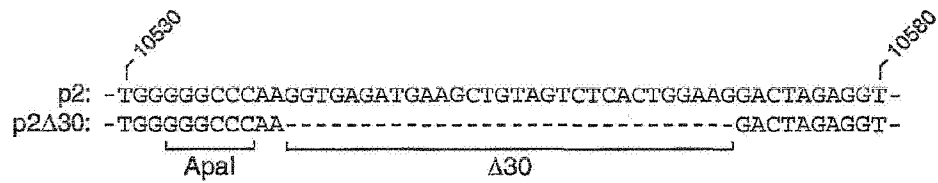


Figure 6

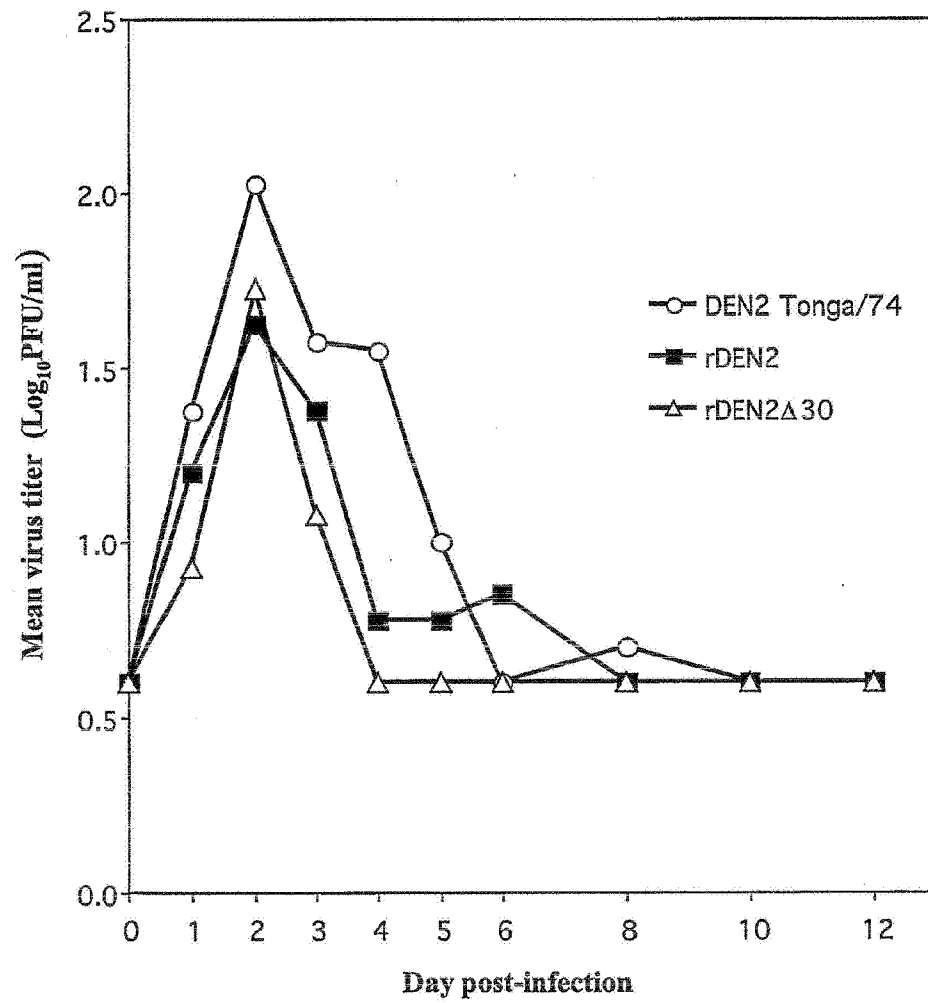
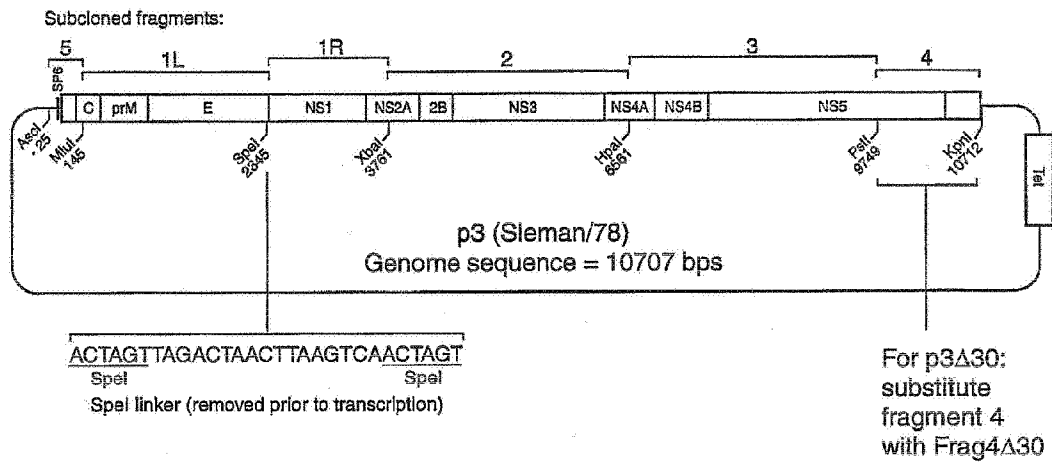


Figure 7

A.



B.

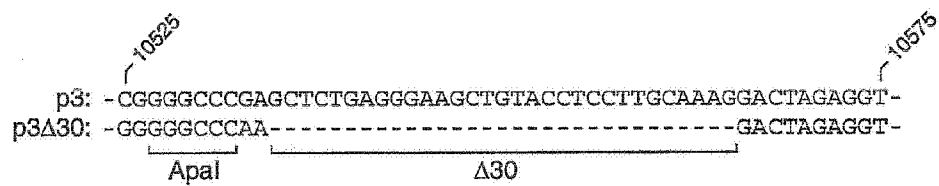


Figure 8

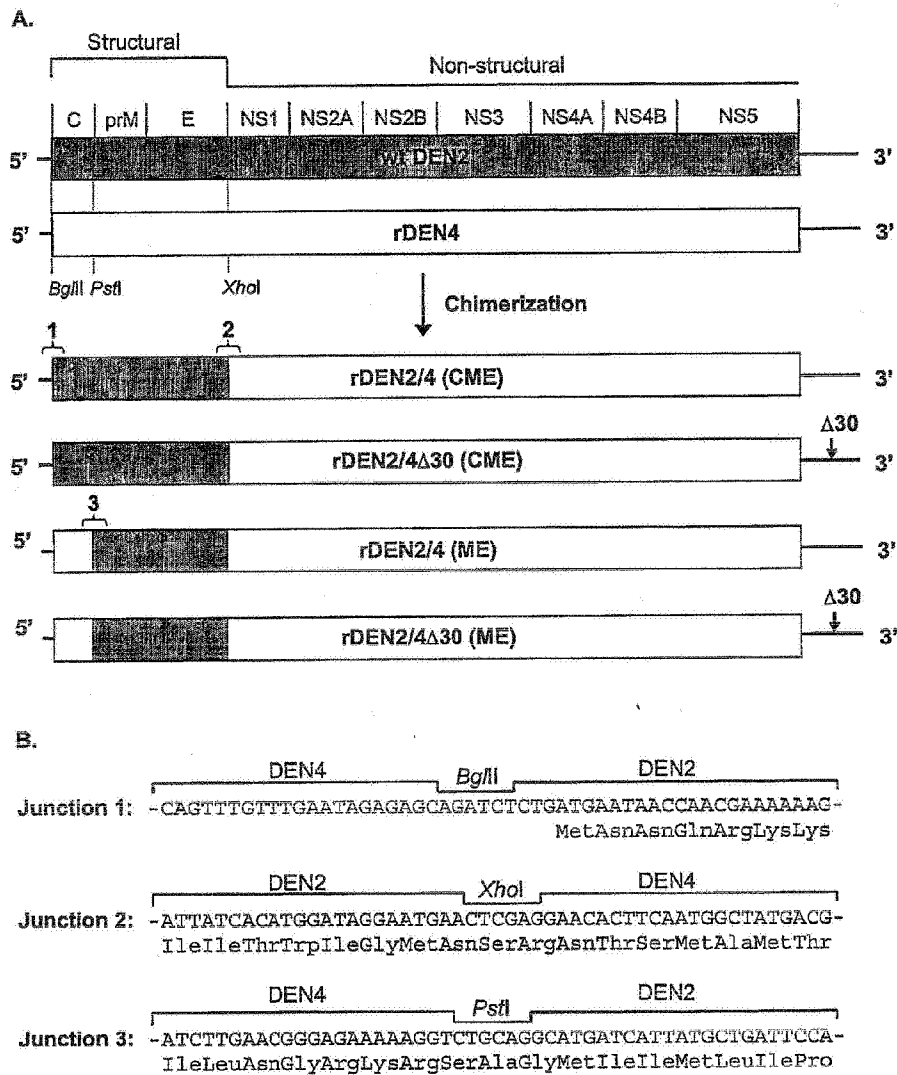


Figure 9

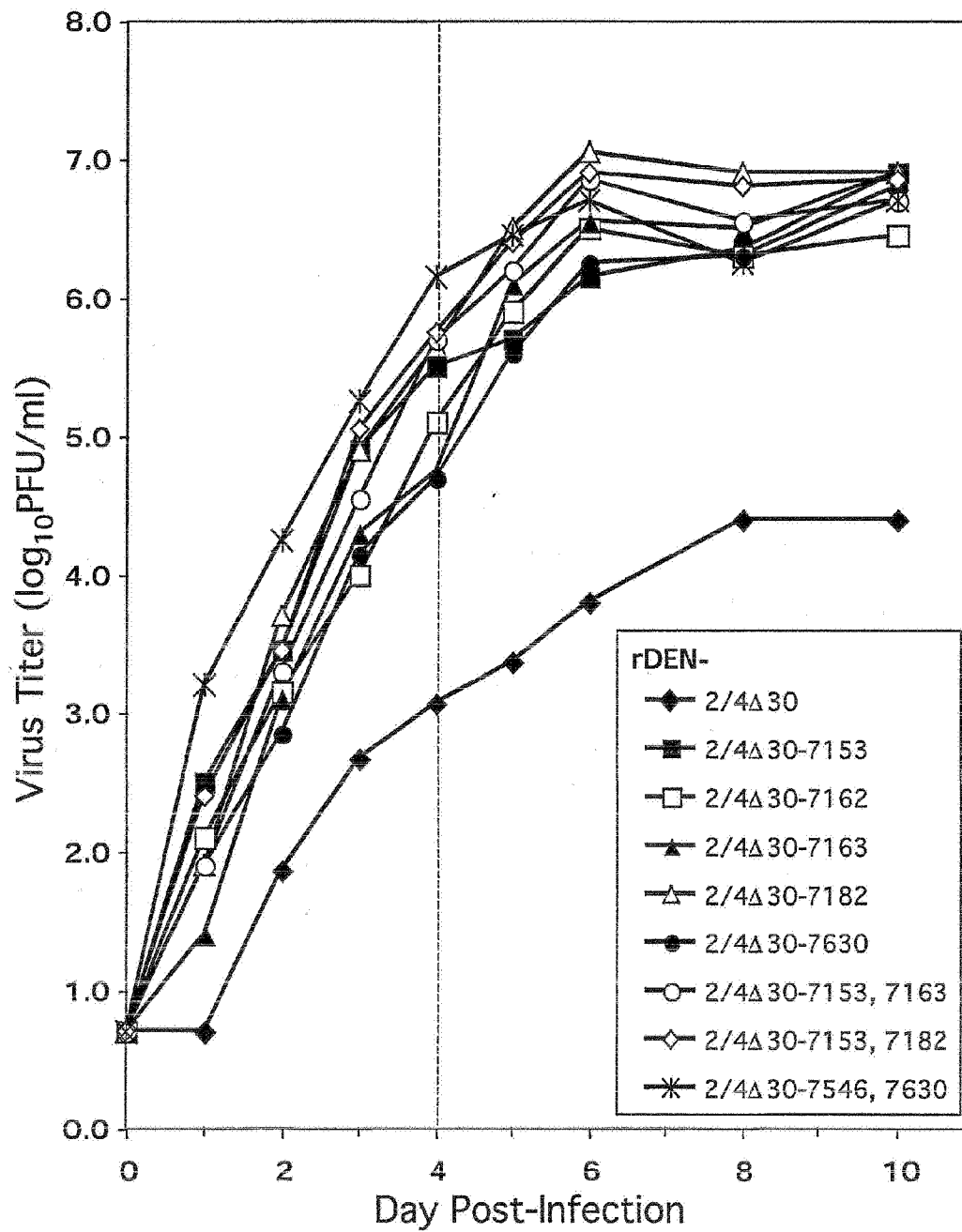


Figure 10

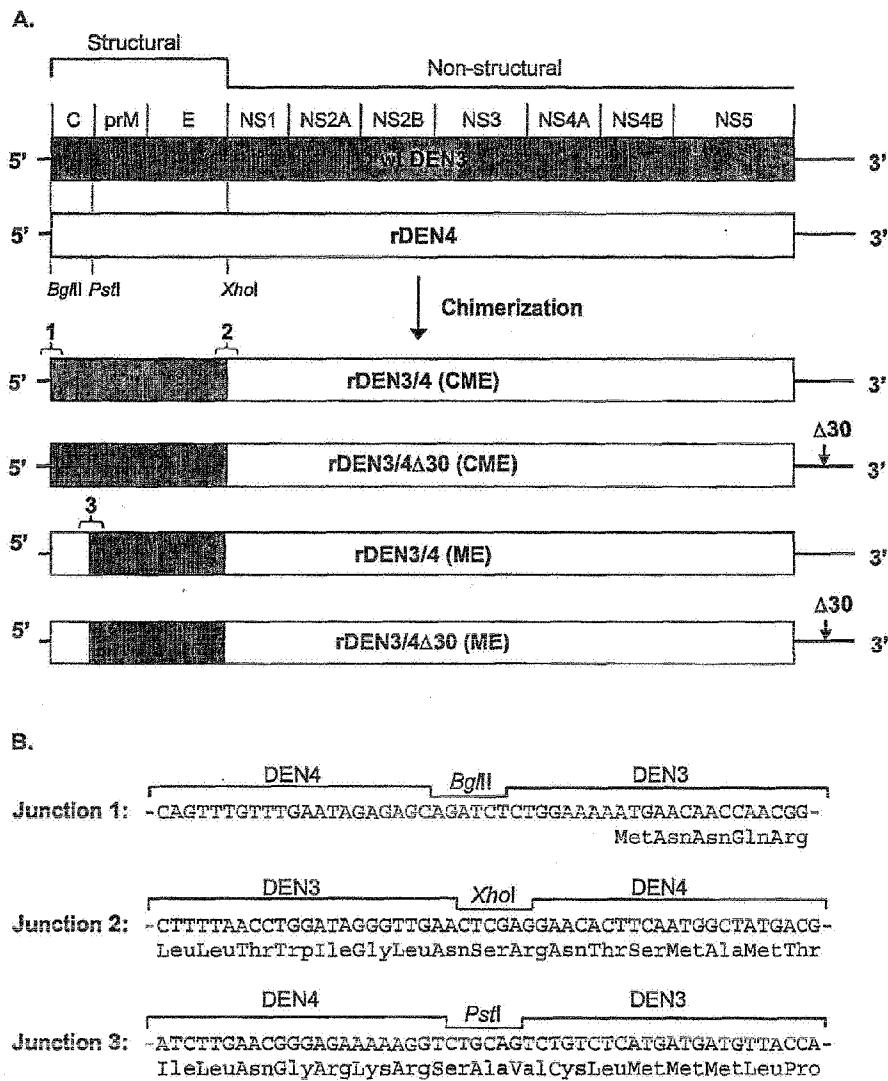


Figure 11

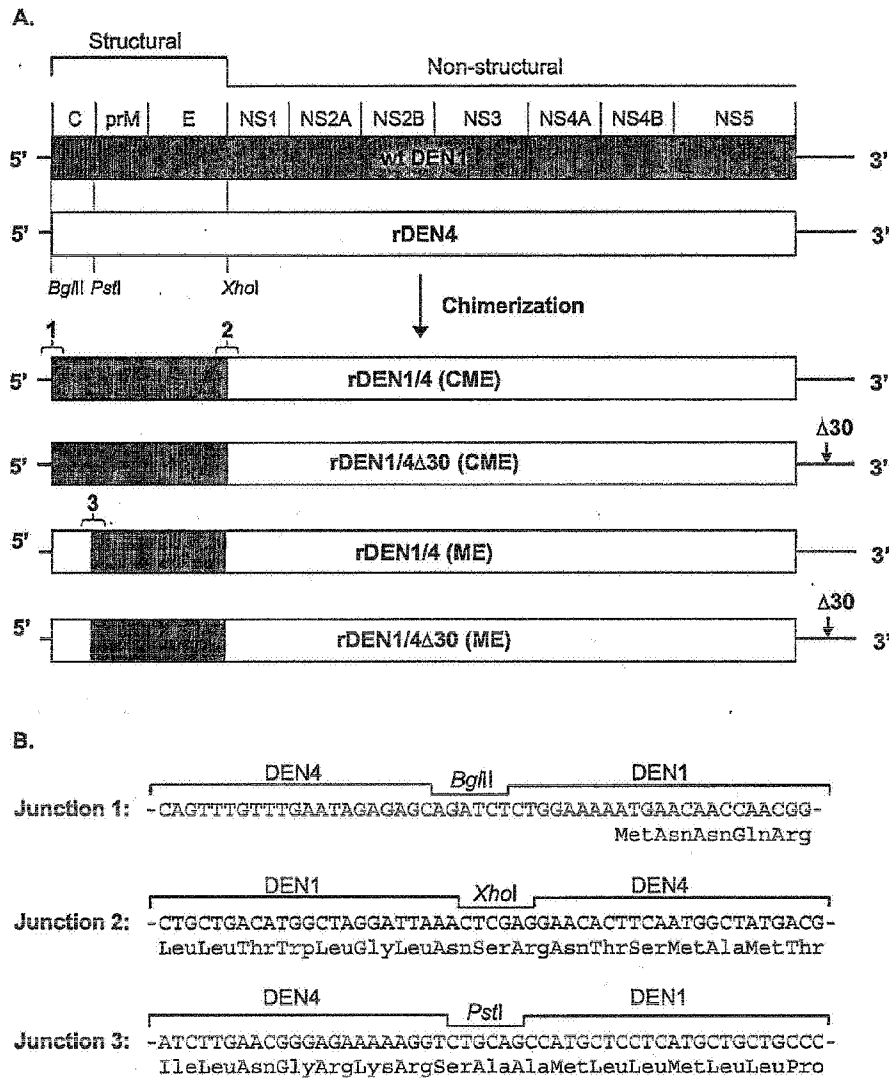


Figure 12