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(54) **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**

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 CPC **C12N 2770/24134**; **C12N 2770/24161**; **C12N 7/00**; **C12N 2770/24122**; **C12N 2770/24162**; **C12N 7/045**; **A61K 2039/5254**; **A61K 2039/5256**; **A61K 39/12**; **C07K 14/005**; **C07K 14/1825**
 USPC **424/202.1**, **199.1**, **205.1**, **218.1**; **435/235.1**, **236**, **320.1**

See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to a dengue virus tetravalent vaccine containing a common 30 nucleotide deletion ($\Delta 30$) in the 3'-untranslated region of the genome of dengue virus serotypes 1, 2, 3, and 4, or antigenic chimeric dengue viruses of serotypes 1, 2, 3, and 4.

42 Claims, 15 Drawing Sheets

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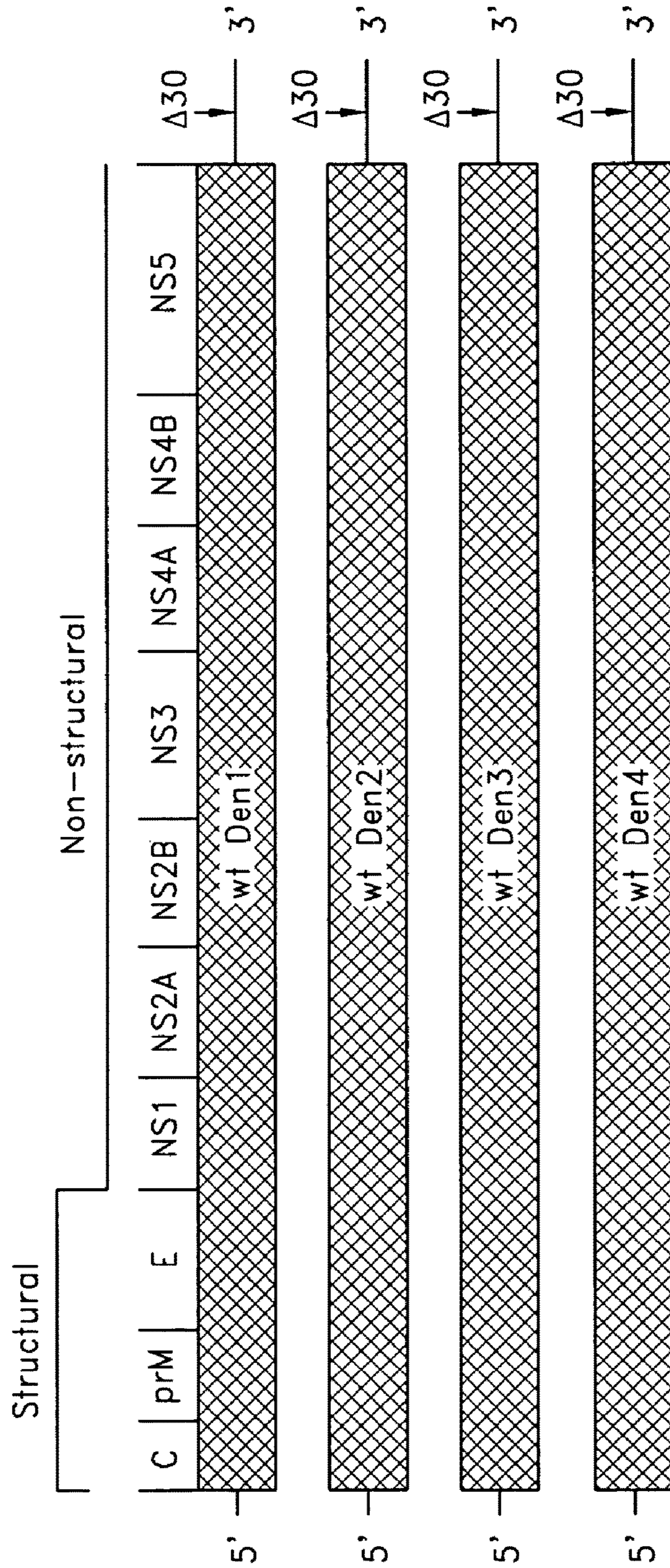


FIG. 1

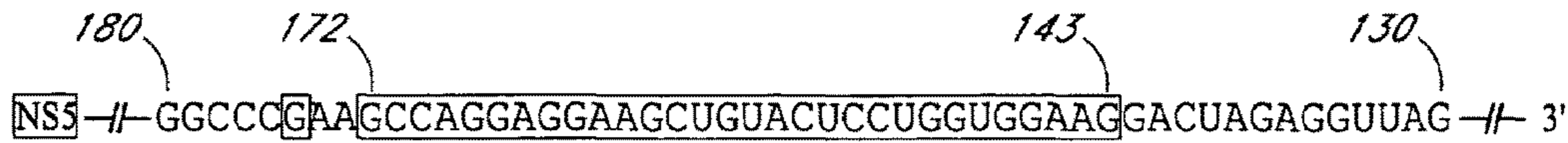


FIG. 2A

Δ30
 (172-143)

FIG. 2B

DEN1	GGGGCCC-AACACCAGGGGAAGCUGUACCCUGGUGGUAAGGACUAGA
DEN1Δ30	GGGGCCC-AA-----GACUAGA
DEN2	GGGGCCC-AAGGUGAGAUGAAGCUGUAGUCUCACUGGAAGGACUAGA
DEN2Δ30	GGGGCCC-AA-----GACUAGA
DEN3	GGGGCCCgAGCUCUGAGGGGAAGCUGUACCUCCUUGCAAAGGACUAGA
DEN3Δ30	GGGGCCCAA-----GACUAGA
DEN4	GGGGCCCgAAGCCAGGAGGAAGCUGUACUCCUGGUGGAAGGACUAGA
DEN4Δ30	GGGGCCC-AA-----GACUAGA

DEN1	GGGGCCC-AacaccagggGAAGCUGUAcccuggugguAAGGACUAGA
DEN2	GGGGCCC-AaggugagauGAAGCUGUAgucucacuggAAGGACUAGA
DEN3	GGGGCCCgAgcucugaggGAAGCUGUAccuccuugcaAAGGACUAGA
DEN4	GGGGCCCgAagccaggagGAAGCUGUAcuccugguggAAGGACUAGA

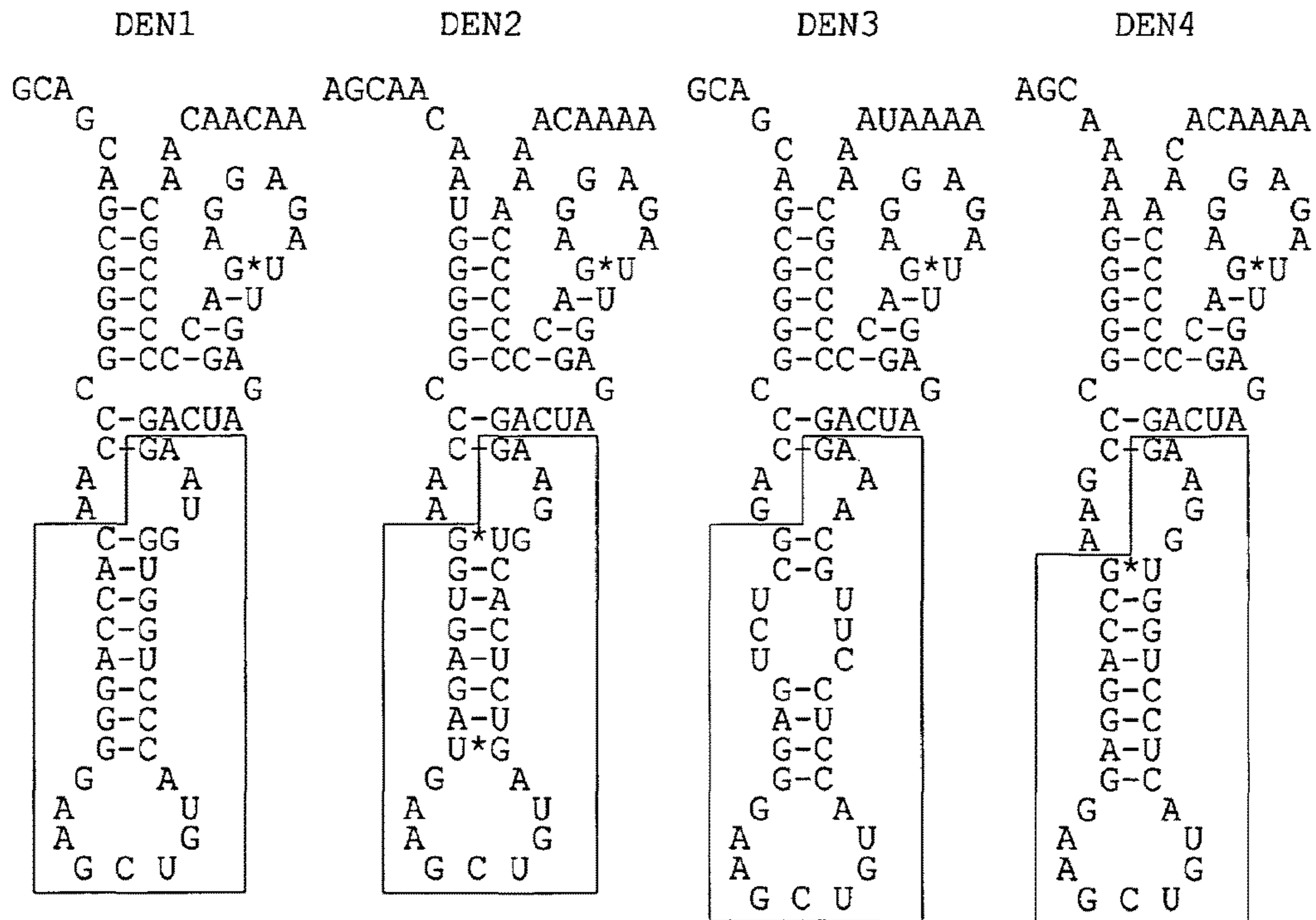


FIG. 2C

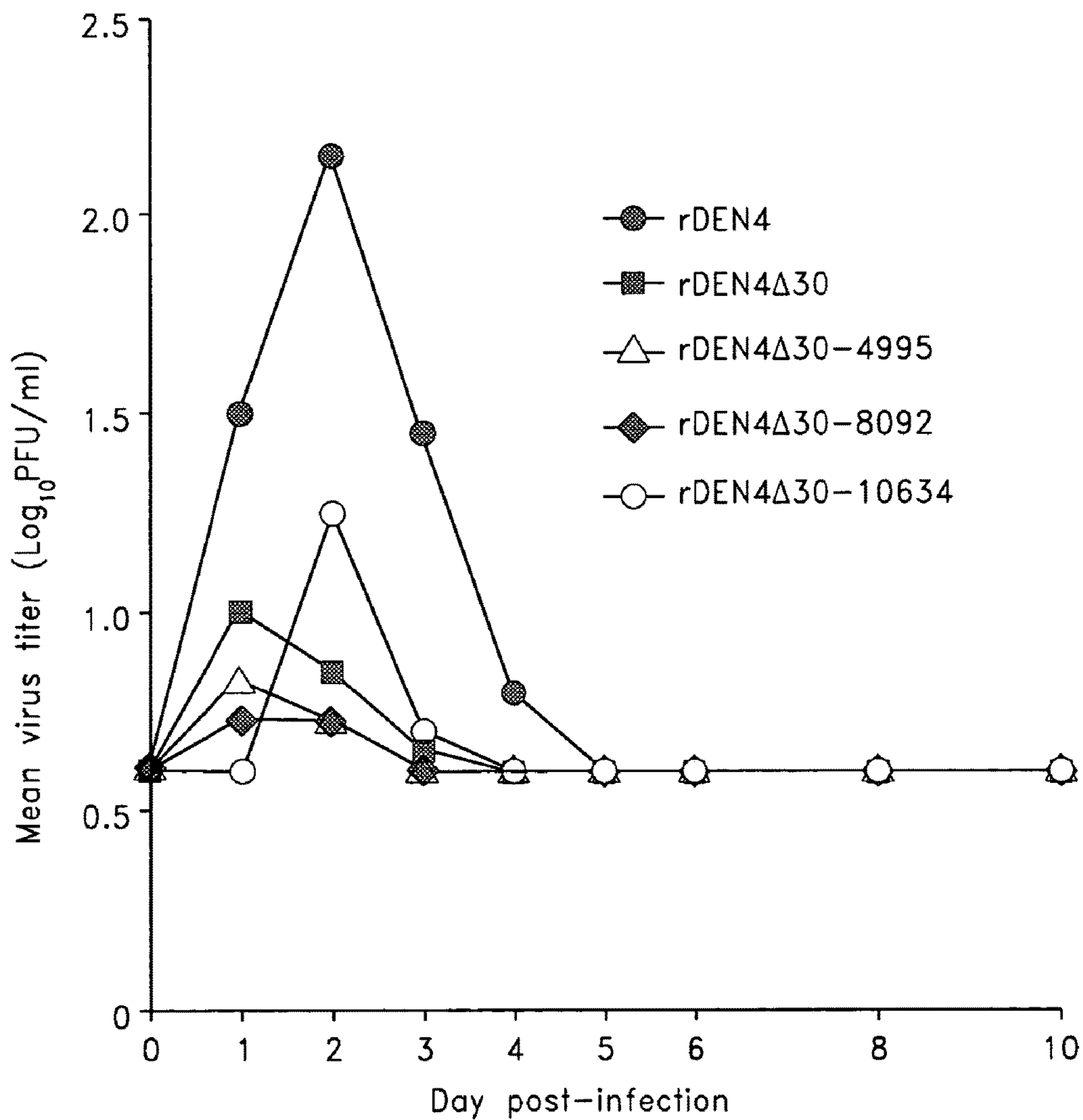


FIG. 3

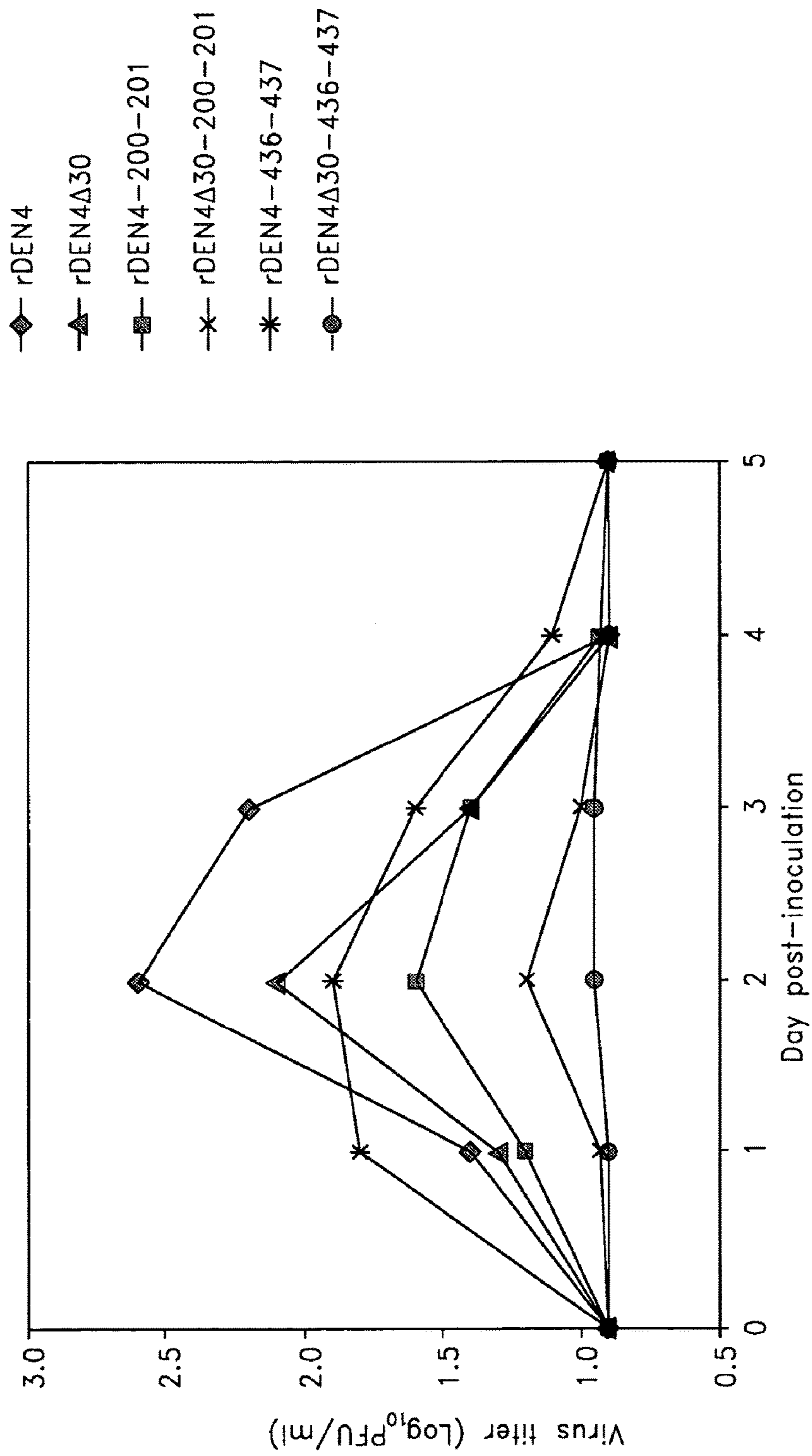


FIG. 4

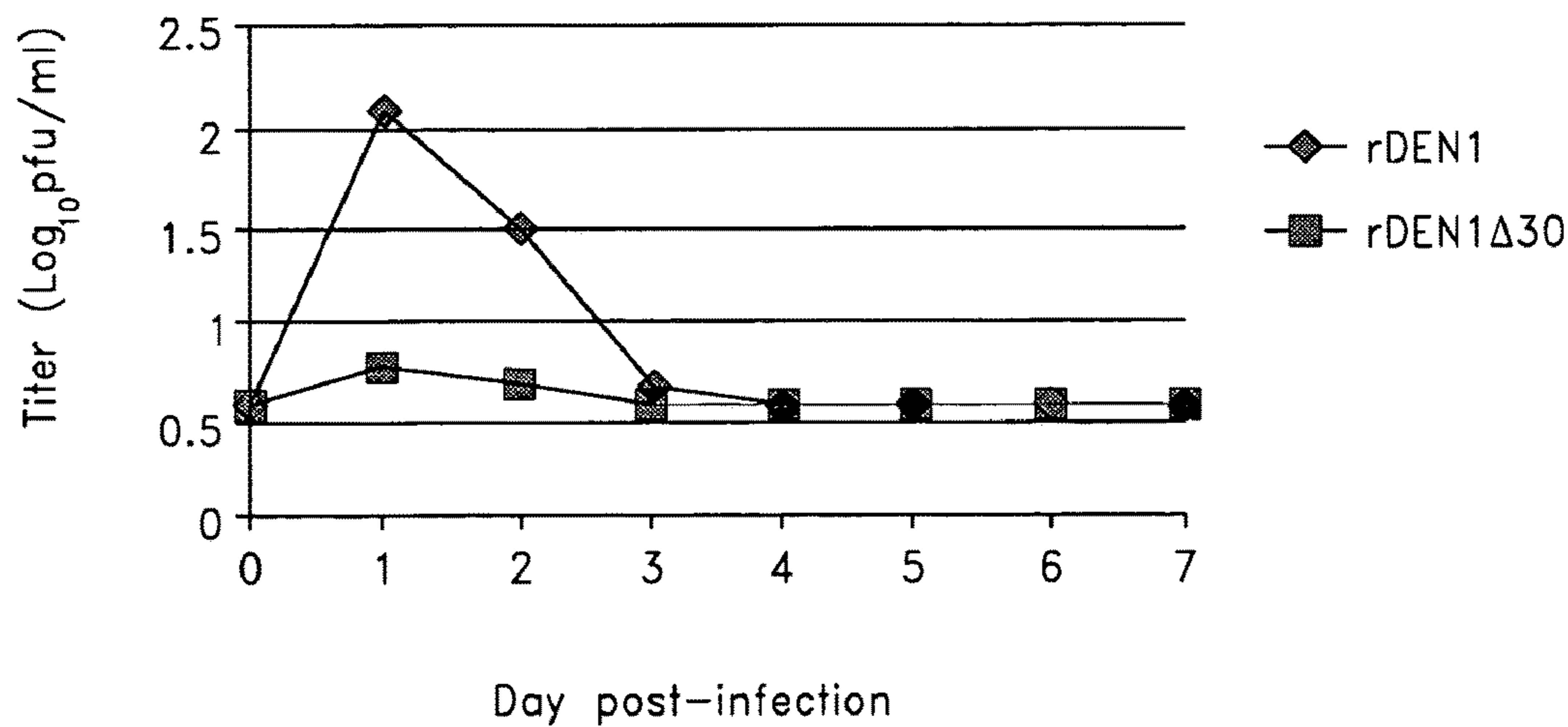
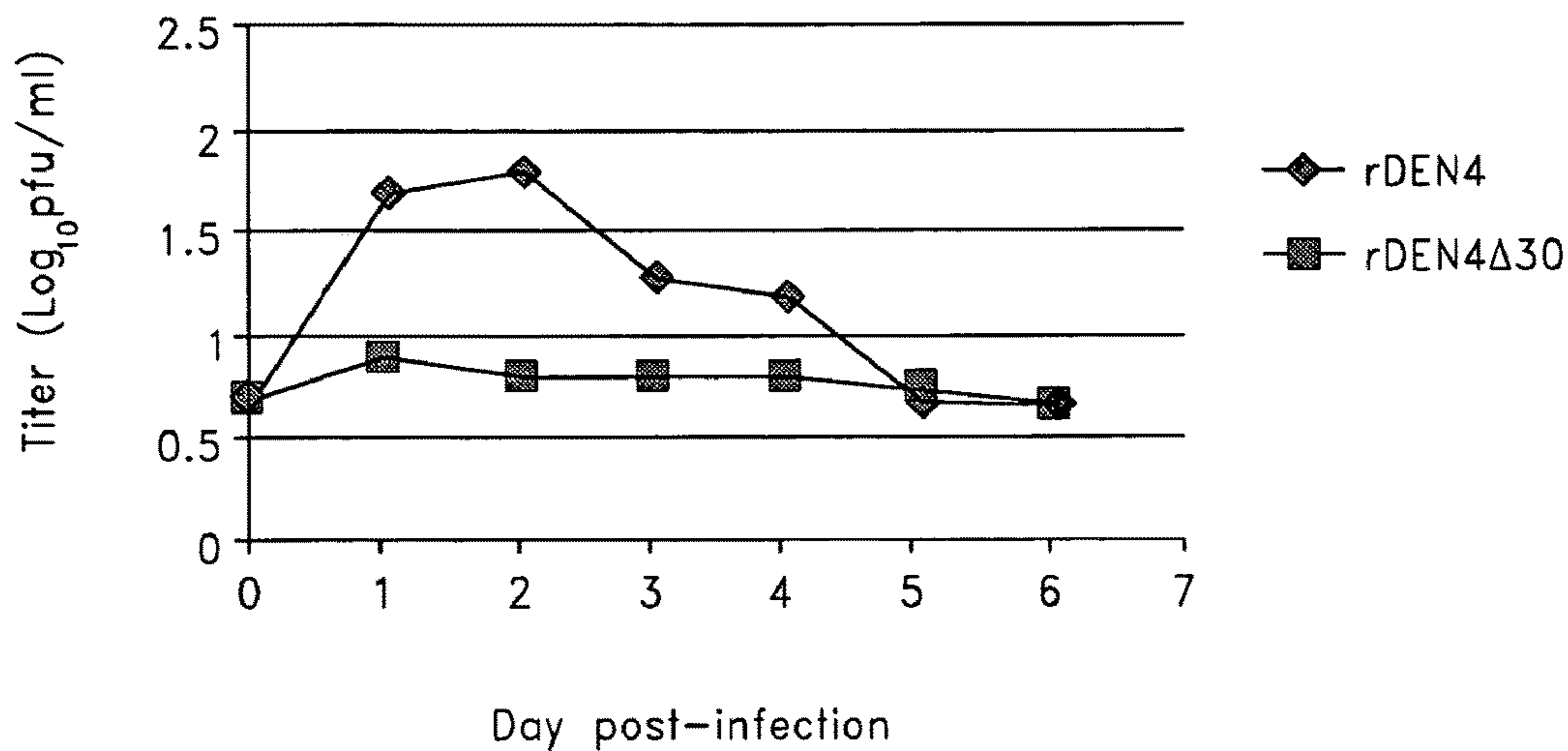


FIG. 5

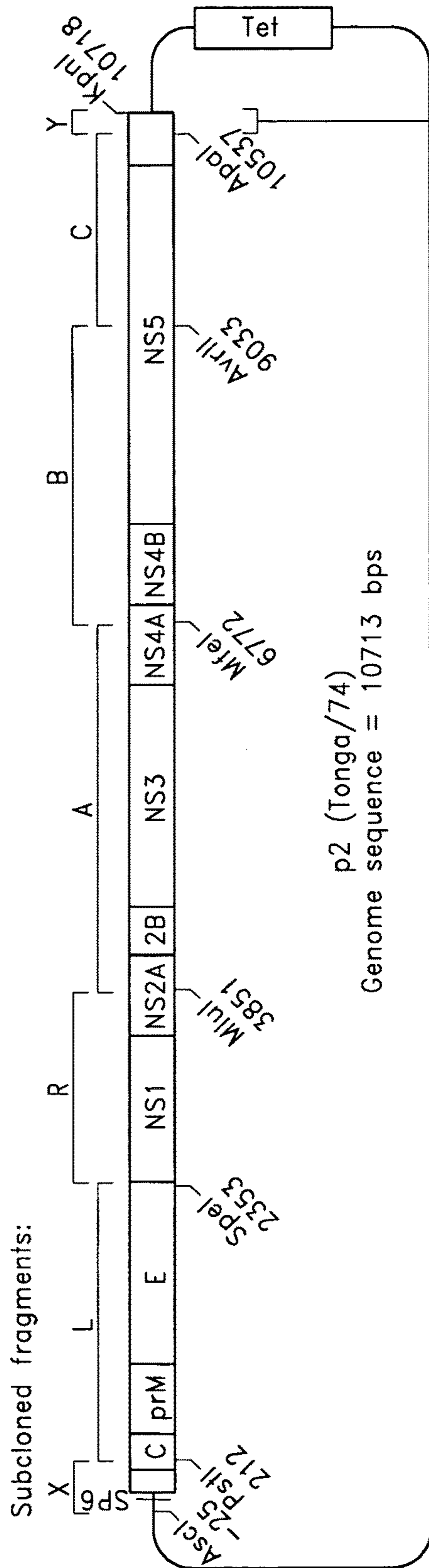


FIG. 6A

For p2Δ30:
substitute
fragment Y
with YΔ30

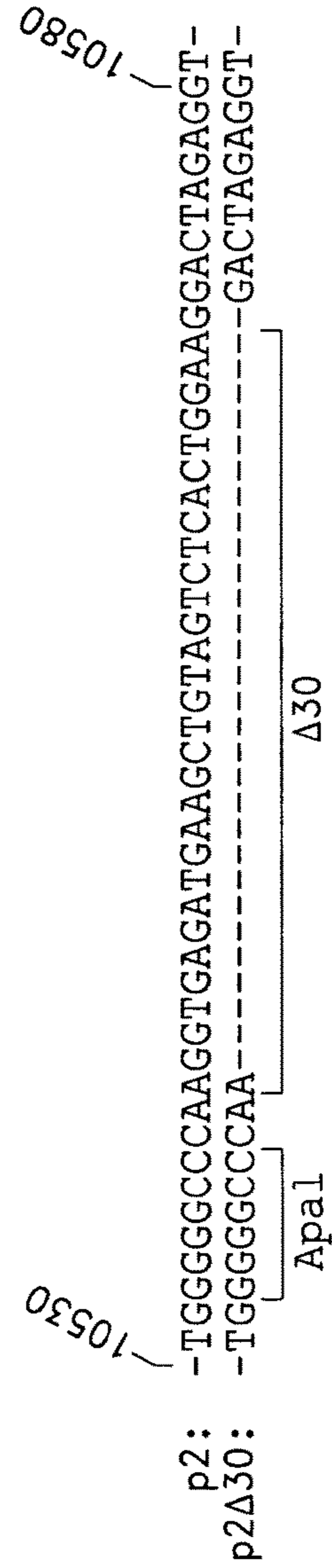


FIG. 6B

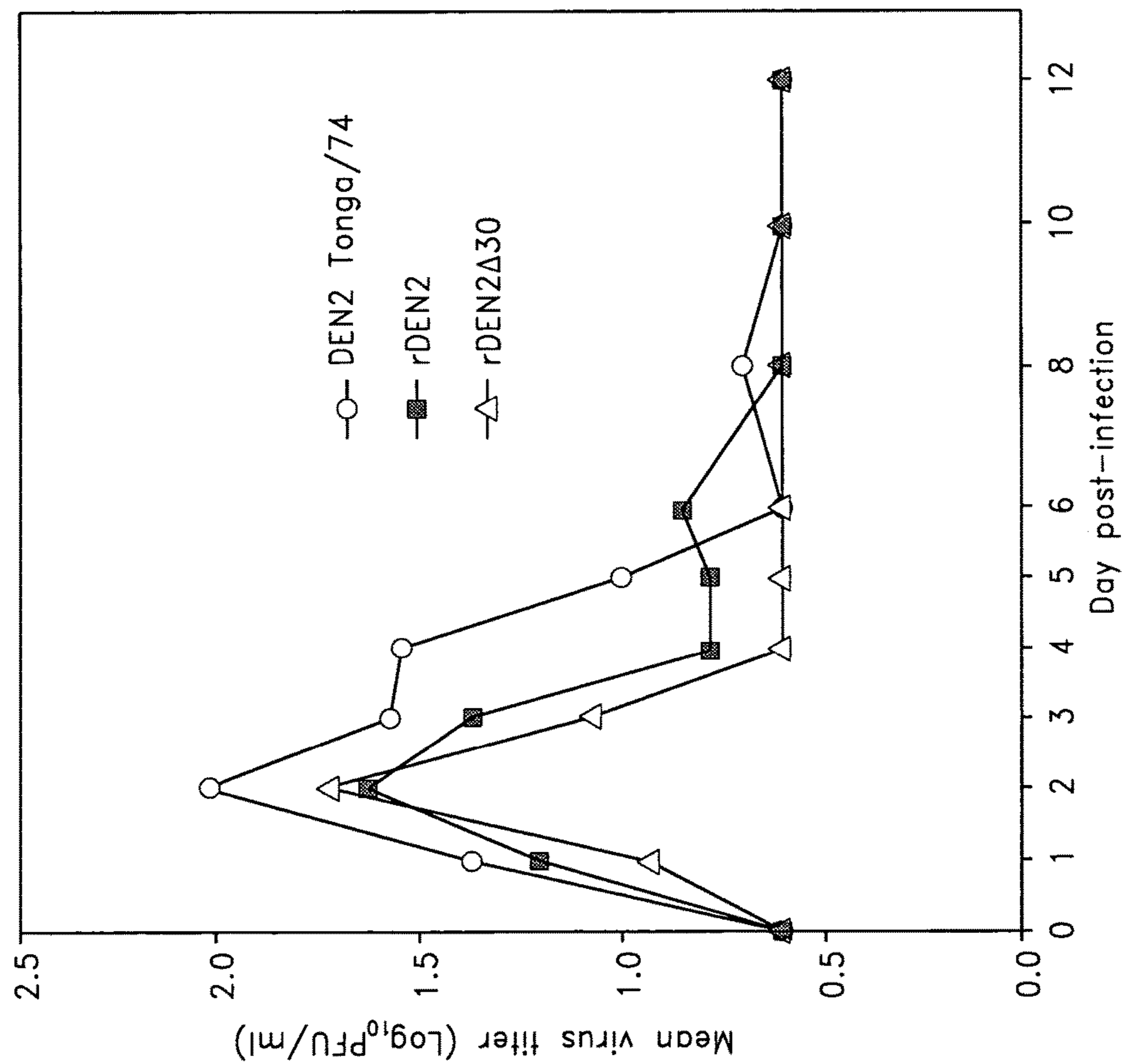


FIG. 7

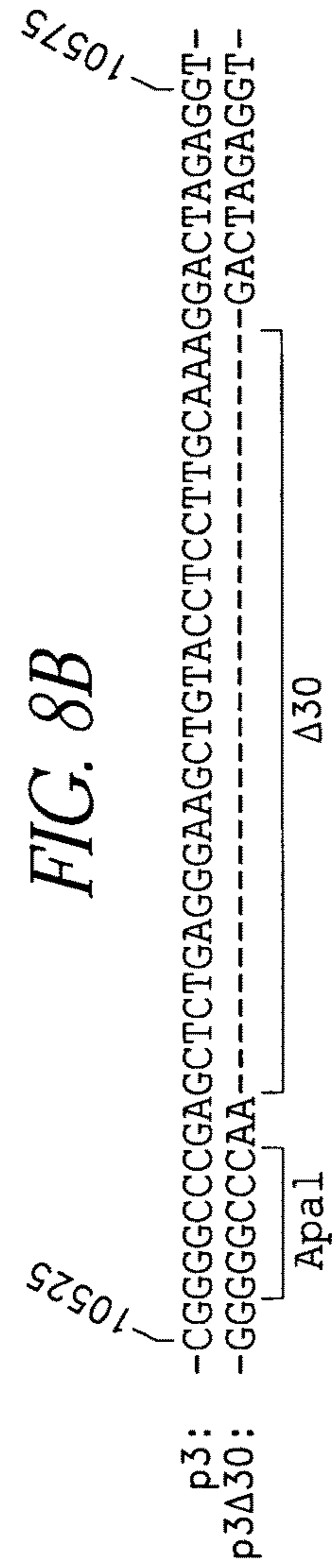
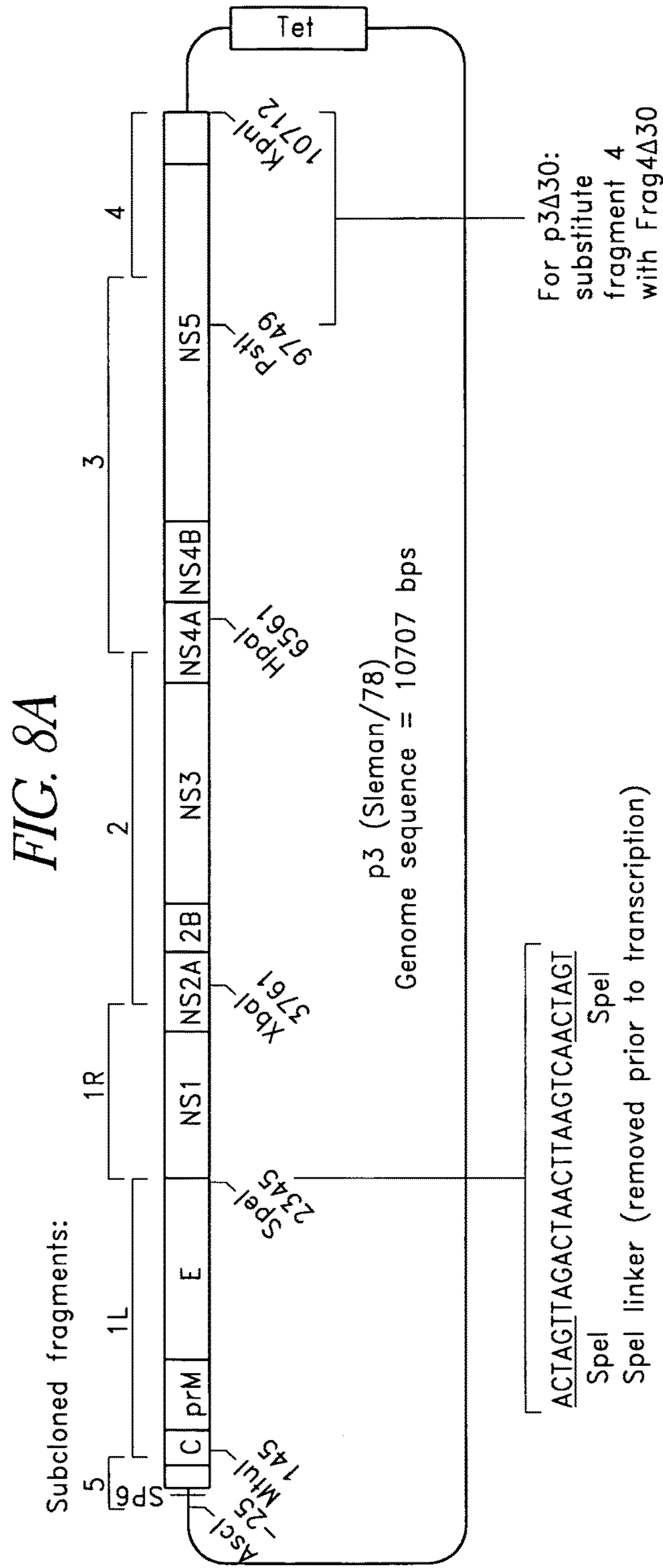


FIG. 9A

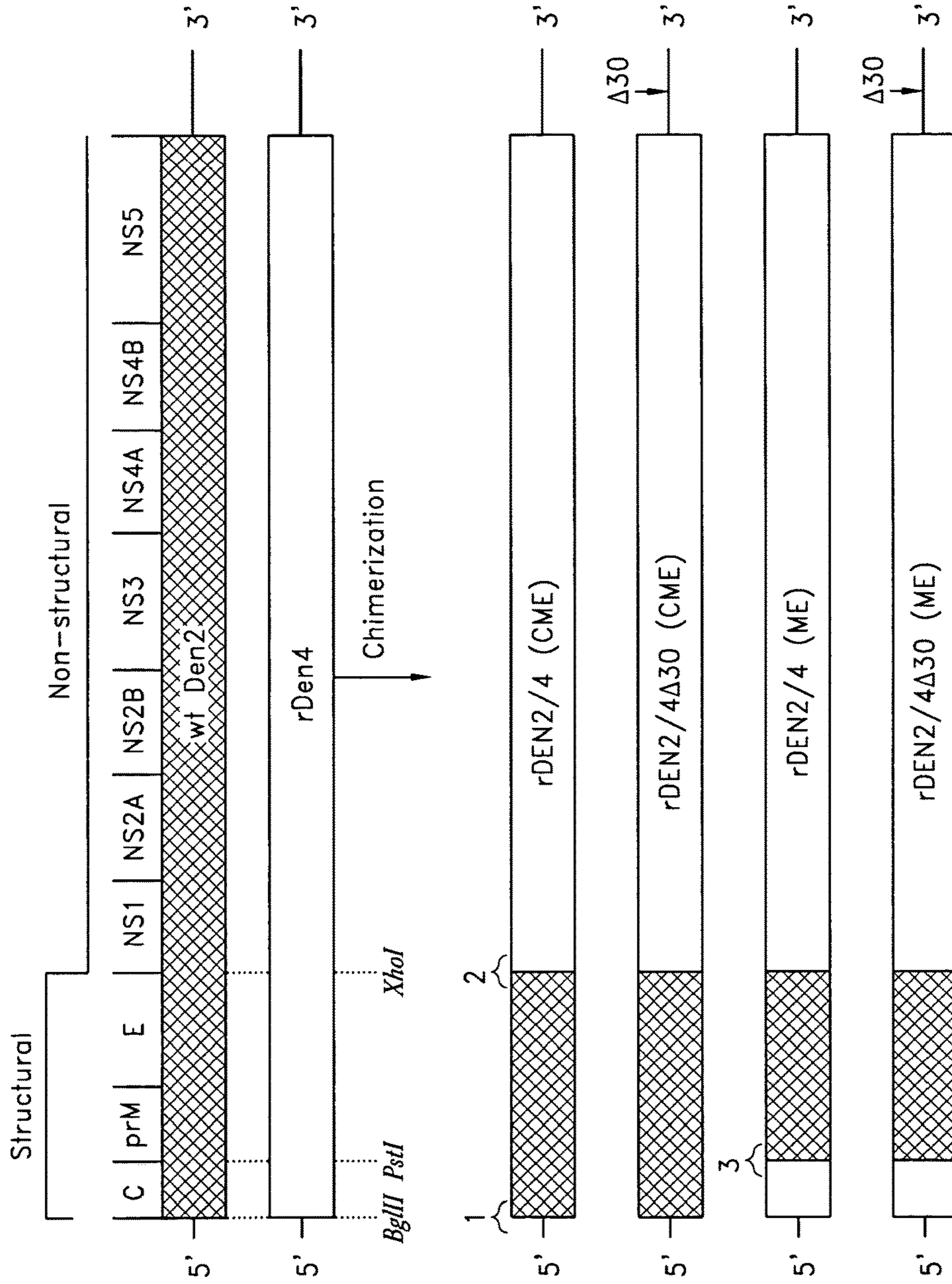
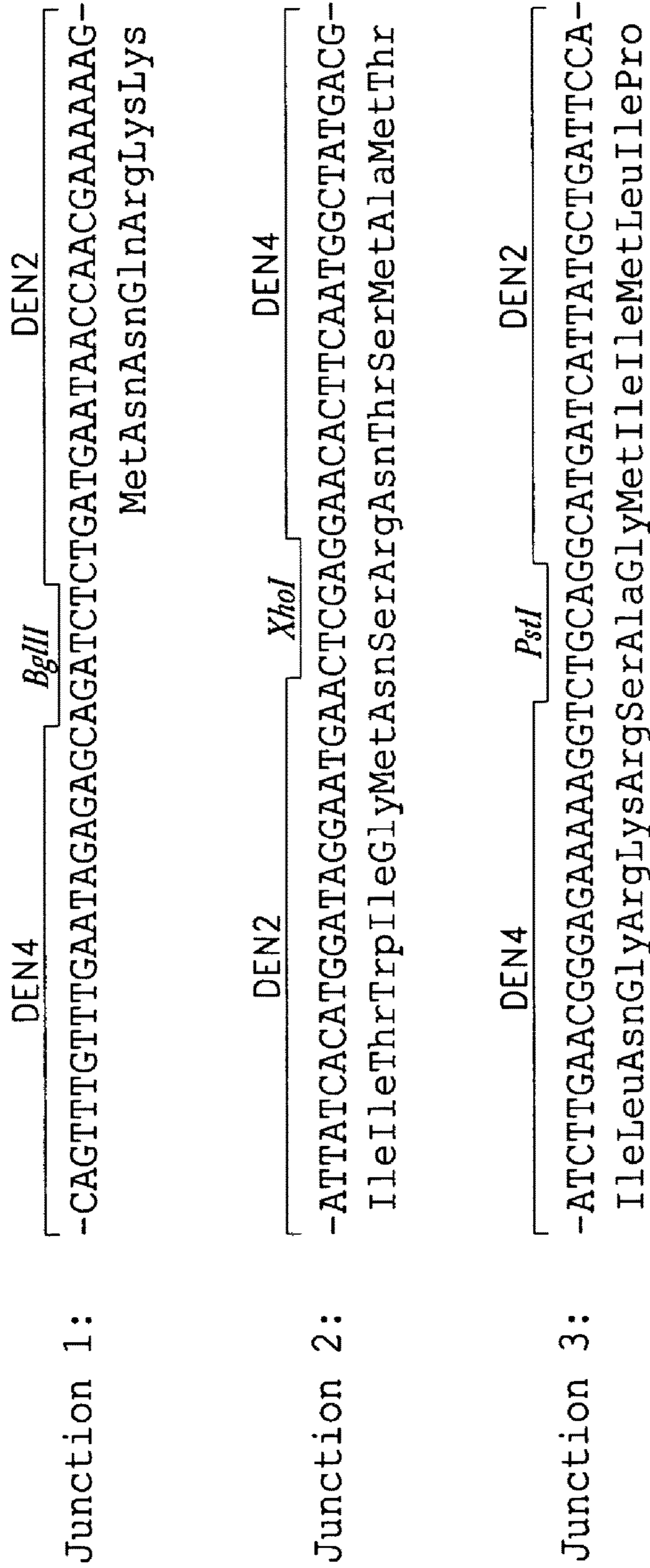


FIG. 9B



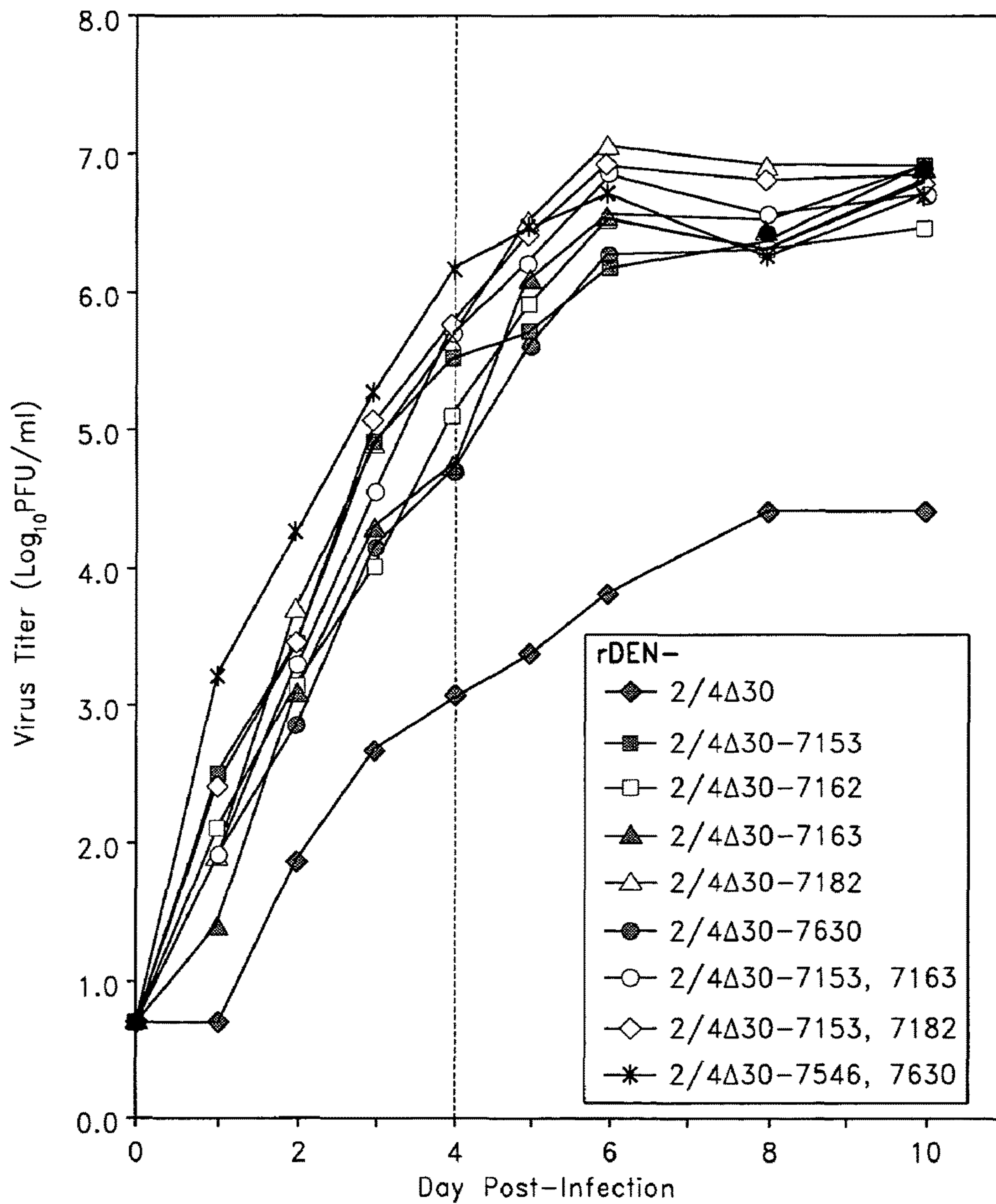


FIG. 10

FIG. 11A

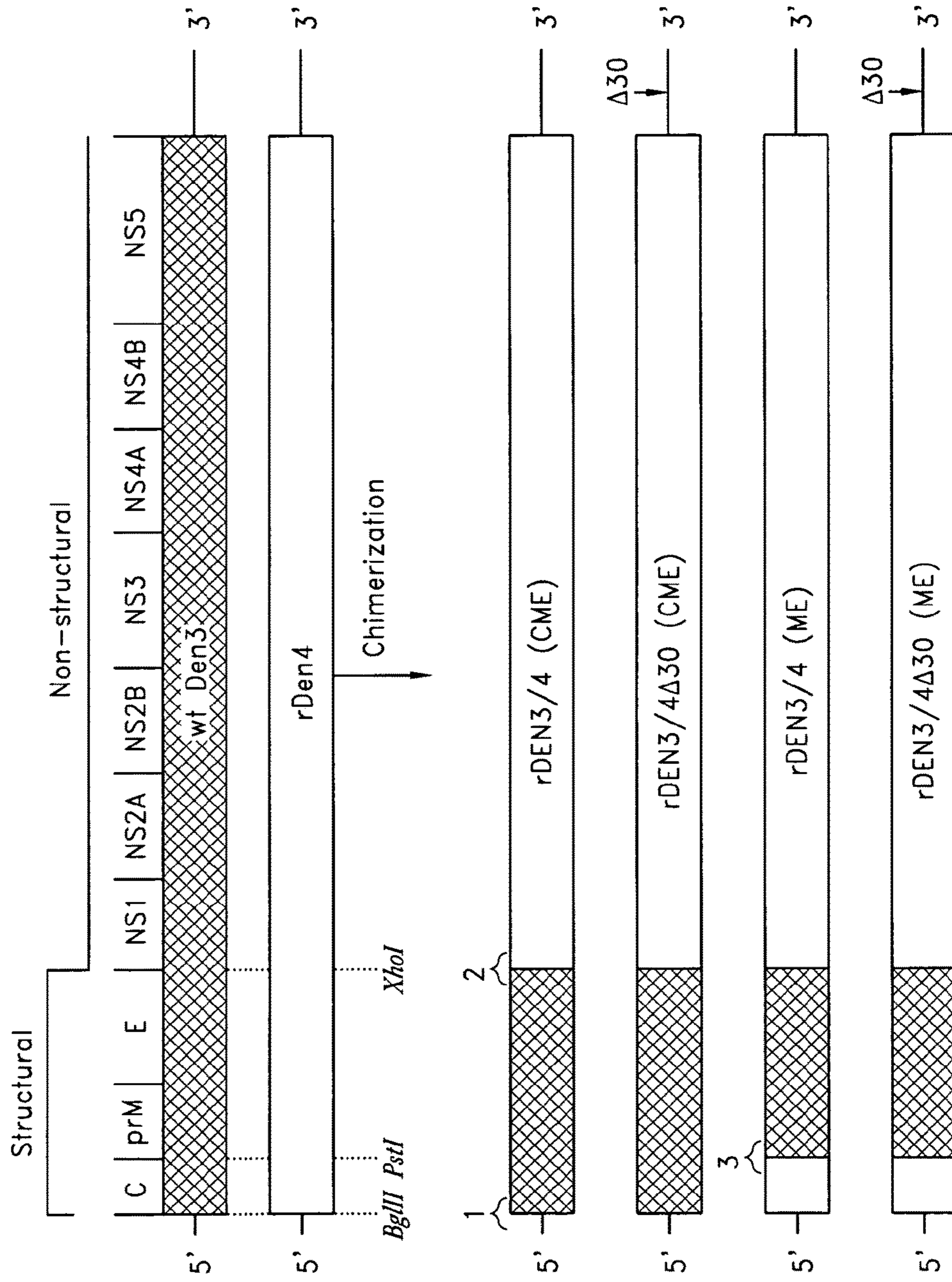


FIG. 12A

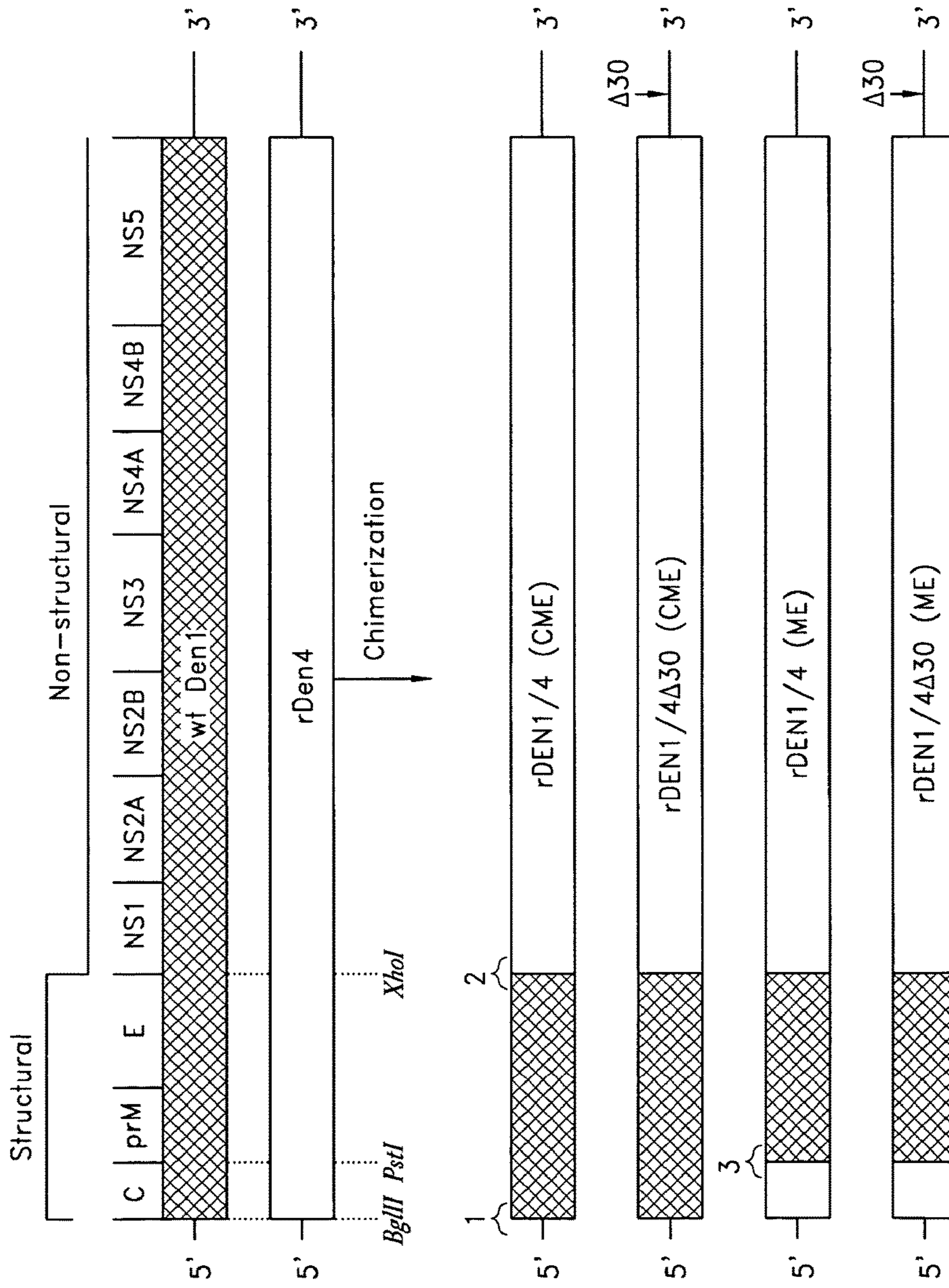
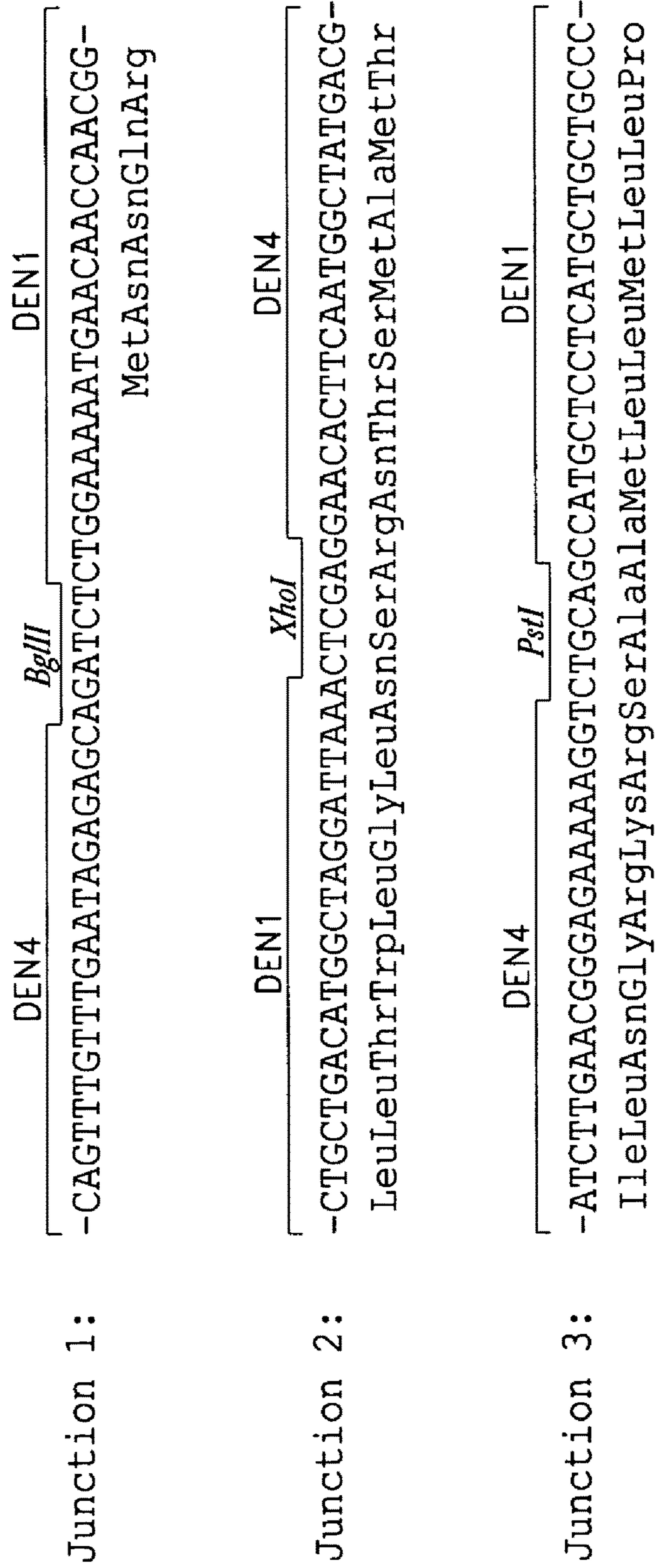


FIG. 12B



**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**

Matter enclosed in heavy brackets [] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue; a claim printed with strikethrough indicates that the claim was canceled, disclaimed, or held invalid by a prior post-patent action or proceeding.

RELATED APPLICATIONS

This application is a continuation and claims the benefit of priority of International Application No. PCT/US03/13279 filed Apr. 25, 2003, designating the United States of America and published in English on Nov. 13, 2003, as WO 03/092592, which claims the benefit of priority of U.S. Provisional Application No. 60/377,860 filed May 3, 2002 and U.S. Provisional Application No. 60/436,500 filed Dec. 23, 2002, all of which are hereby expressly incorporated by reference in their entireties.

FIELD OF THE INVENTION

The invention relates to a dengue virus tetravalent vaccine containing a common 30 nucleotide deletion (Δ 30) in the 3'-untranslated region of the genome of dengue virus serotypes 1, 2, 3, and 4, or antigenic chimeric dengue viruses of serotypes 1, 2, 3, and 4.

BACKGROUND OF THE INVENTION

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 semi-tropical 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).

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).

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).

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).

SUMMARY OF THE INVENTION

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

FIG. 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.

FIG. 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).

FIG. 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 log₁₀PFU 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 log₁₀PFU/ml. Mean virus titers are indicated for each group.

FIG. 4. Viremia levels in rhesus monkeys inoculated with rDEN4 vaccine candidates bearing pairs of charge-to-alanine mutations in NS5. Groups of four or two (rDEN4 and rDEN4 $\Delta 30$) monkeys were inoculated with 5.0 log₁₀PFU 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 log₁₀PFU/ml. Mean virus titers are indicated for each group. Viremia was not detected in any monkey after day 4.

FIG. 5. The $\Delta 30$ mutation attenuates both DEN1 and DEN4 for rhesus monkeys. Groups of 4 monkeys were immunized subcutaneously with 5.0 log₁₀PFU of the indicated virus. Serum was collected each day following immunization and virus titers were determined and are shown as mean log₁₀PFU/ml.

FIG. 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 p2 $\Delta 30$.

FIG. 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 log₁₀PFU 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 log₁₀PFU/ml. Mean virus titers are indicated for each group. Viremia was not detected in any monkey after day 8.

FIG. 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 p3 $\Delta 30$.

FIG. 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.

FIG. 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.

FIG. 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 $\Delta 30$ 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.

FIG. 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 $\Delta 30$ 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	
Serotype	GenBank Accession No. or description
DEN1	U88535
DEN2	Tonga/74
DEN3	Sleman/78
DEN4	AF326825

Brief Description of the SEQ ID NOs		
Identification	Figure, Table, or Appendix	SEQ ID NO.
TL2 region of DEN1	FIG. 2C	1
TL2 region of DEN2	FIG. 2C	2
TL2 region of DEN3	FIG. 2C	3
TL2 region of DEN4	FIG. 2C	4
TL2 region of DEN1 $\Delta 30$	FIG. 2B	5
TL2 region of DEN2 $\Delta 30$	FIG. 2B	6
TL2 region of DEN3 $\Delta 30$	FIG. 2B	7
TL2 region of DEN4 $\Delta 30$	FIG. 2B	8
TL2 region of p2	FIG. 6B	9
TL2 region of p2 $\Delta 30$	FIG. 6B	10
TL2 region of p3	FIG. 8B	11
TL2 region of p3 $\Delta 30$	FIG. 8B	12
SpeI linker in p3	FIG. 8A	13
rDEN2/4 junction 1	FIG. 9B	14-nt, 15-aa
rDEN2/4 junction 2	FIG. 9B	16-nt, 17-aa
rDEN2/4 junction 3	FIG. 9B	18-nt, 19-aa
rDEN3/4 junction 1	FIG. 11B	20-nt, 21-aa
rDEN3/4 junction 2	FIG. 11B	22-nt, 23-aa
rDEN3/4 junction 3	FIG. 11B	24-nt, 25-aa
rDEN1/4 junction 1	FIG. 12B	26-nt, 27-aa
rDEN1/4 junction 2	FIG. 12B	28-nt, 29-aa
rDEN1/4 junction 3	FIG. 12B	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
AAGGCCTGGA	Table 26	41
TATCCCCGGGAC	Table 26	42
AGAGCTCTCTC	Table 26	43
GAATCTCCACCCGGA	Table 26	44

-continued

Brief Description of the SEQ ID NOs		
Identification	Figure, Table, or Appendix	SEQ ID NO.
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

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).

The present invention describes: (1) improvements to the previously described rDEN4 $\Delta 30$ vaccine candidate, (2) attenuated rDEN1 $\Delta 30$, rDEN2 $\Delta 30$, and rDEN3 $\Delta 30$ recombinant viruses containing a 30 nucleotide deletion ($\Delta 30$) in a section of the 3' untranslated region (UTR) that is homologous to that in the rDEN4 $\Delta 30$ recombinant virus, (3) a method to generate a tetravalent dengue virus vaccine composed of rDEN1 $\Delta 30$, rDEN2 $\Delta 30$, rDEN3 $\Delta 30$, and rDEN4 $\Delta 30$, (4) attenuated antigenic chimeric viruses, rDEN1/4 $\Delta 30$, rDEN2/4 $\Delta 30$, and rDEN3/4 $\Delta 30$, for which the CME, ME, or E gene regions of rDEN4 $\Delta 30$ have been replaced with those derived from DEN1, DEN2, or DEN3; alternatively rDEN1/3 $\Delta 30$, rDEN2/3 $\Delta 30$, and rDEN4/3 $\Delta 30$ for which CME, ME, or E gene regions of rDEN3 $\Delta 30$ 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 Δ30 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.

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 a untranslated region, we selected the Δ30 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 (FIG. 1).

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 (FIG. 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 (FIG. 2B, C).

Immunogenic Dengue Chimeras and Methods for their Preparation

Immunogenic dengue chimeras and methods for preparing the dengue chimeras are provided herein. The immuno-

genic 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.

Chimeras of the present invention 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.

In another embodiment, 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 non-structural proteins from a dengue virus of a second serotype different from the first. In another embodiment the dengue virus of the second serotype is DEN4. In another embodiment, 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.

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.

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

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.

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.

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.

The structural and nonstructural proteins of the invention 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

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 non-structural 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.

The dengue chimeras of the invention 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.

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.

The chimera of the invention 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.

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 non-structural 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 of this invention.

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

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.

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.

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

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.

The chimeric viruses or nucleic acid chimeras of this invention 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.

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 proper-

ties 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.

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.

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.

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

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.

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.

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.

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, nano-particles 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.

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

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

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, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers, 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

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.

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 of this invention 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.

The present invention also contemplates 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, N.Y., 1989.

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.

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.

The nucleic acid sequences of the invention 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.

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.

EXAMPLE 1

Improvement of Dengue Virus Vaccine Candidate rDEN4Δ30

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.

Currently, a randomized, double-blind, placebo-controlled, dose de-escalation study is being conducted to determine the human infectious dose 50 (HID₅₀) of rDEN4Δ30. Each dose cohort consists of approximately twenty vaccinees and four placebo recipients. To date, complete data for doses of 3.0 log₁₀PFU and 2.0 log₁₀PFU has been collected. rDEN4Δ30 infected 100% of vaccinees when 3.0 log₁₀PFU was administered and 95% of vaccinees when 2.0 log₁₀PFU was administered (Table 1). The vaccine candidate caused no symptomatic illness at either dose (Table 1). One vaccinee who received 3.0 log₁₀PFU 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 log₁₀PFU and 2.0 log₁₀PFU doses, though both measures of viremia were significantly lower for these doses than for a dose of 5.0 log₁₀PFU (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 HID₅₀ has not been determined yet, it is clearly less than 2.0 log₁₀PFU. Interestingly, decreases in the dose of vaccine have had no consistent effect on immunogenicity, viremia, benign neutropenia or the occurrence of rash. Thus will not necessarily be possible to further attenuate rDEN4Δ30 by decreasing the dose of virus administered, and other approaches must be developed.

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TABLE 1

No. of subjects	Dose ^a	rDEN4Δ30 clinical summary						
		No. in- fected	No. with viremia	Mean peak titer ^b	No. volunteers with:			Neutro- penia ^c
					Fever	Rash		↑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

^aLog₁₀ pfu^bLog₁₀ pfu/mL^cNeutropenia defined as ANC < 1500/dl^dT Max in volunteer = 100.4° F.^eALT day 0 = 78, ALT max = 91 (day 14)

TABLE 2

Dose ^a	Pattern of rash in vaccinees					
	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 (α = 0.05)

TABLE 3

Dose ^a	rDEN4Δ30 viremia summary			
	# 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 (α = 0.05)

TABLE 4

No. of subjects	Dose (log ₁₀)	No. infected	Geometric mean serum neutralizing antibody titer (range)		% serocon- version
			Day 28		
			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

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

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mice). In this example, a subset of these mutations has been introduced to rDEN4Δ30 and the phenotypes of the resulting viruses evaluated.

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.

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 Δ30 deletion mutation to evaluate their ability to further restrict replication of rDEN4Δ30 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 Δ30 mutation (Blaney, J. E. et al. 2001 J Virol. 75:9731-9740). The resulting virus, rDEN4Δ30-4995, was found to be more restricted (1,000-fold) in mouse brain replication than rDEN4Δ30 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 Δ30 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 Δ30 mutation here (Blaney, J. E. et al. 2002 Virology 300:125-139).

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 p4Δ30 cDNA clone. For transcription and recovery of virus, cDNA was linearized with Acc65I (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 (rDEN4Δ30-4995) or three (rDEN4Δ30-8092 and rDEN4Δ30-10634) times in Vero cells.

The complete genomic sequences of rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634 viruses were deter-

mined as previously described (Durbin et al. 2001 Am. J. Trop. Med. Hyg. 65:405-413). As expected, each rDEN4Δ30 virus derivative contained the Δ30 mutation. Unexpectedly, in rDEN4Δ30-4995 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 p4Δ30-4995 cDNA was designed to introduce the Ser>Pro change (Table 5). The p4Δ30-4995 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. rDEN4Δ30-4995 virus was also found to contain an incidental mutation at nucleotides 4725-6 which resulted in a single amino acid change (Ser>Asp). The rDEN4Δ30-8092 and rDEN4Δ30-10634 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 rDEN4Δ30 virus derivatives bearing introduced point mutations.					
Virus	Gene	Nucleotide position	Nucleotide substitution	Amino acid position ^a	Amino acid change
rDEN4Δ30-4995	NS3	4725	U > G	1542	Ser > Asp
	NS3	4726	C > A	1542	Ser > Asp
	NS3	4995 ^b	U > C	1632	Ser > Leu
rDEN4Δ30-8092	E	1612	A > C	504	Asp > Ala
	NS4B	7131	A > G	2344	Thr > Ala
	NS5	8092 ^b	A > G	2664	Glu > Gly
rDEN4Δ30-10634	NS4B	6969	A > U	2290	Met > Leu
	NS4B	7182	G > C	2361	Gly > Arg
	3' UTR	10634 ^b	U > C	none	none

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

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

Replication of the three modified rDEN4Δ30 viruses were compared to rDEN4Δ30 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 log₁₀PFU 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 log₁₀PFU 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.

Wild type rDEN4 virus replicated to 6.0 log₁₀PFU/g in suckling mouse brain, and rDEN4Δ30 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). rDEN4Δ30-4995, rDEN4Δ30-8092, and rDEN4Δ30-10634 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 rDEN4Δ30 virus in mouse brain ranging from 50-fold (rDEN4Δ30-10634) to 400-fold (rDEN4Δ30-4995). In SCID-HuH-7 mice, virus titer of rDEN4Δ30 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

^aGroups 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.

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

^cGroups 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.

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).

Viremia was detected beginning on day 1 post-infection and ended by day 4 in all monkeys (Table 7, FIG. 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 (FIG. 3).

TABLE 7

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

^aGroups 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.

^bViremia was not detected in any monkey after day 4.

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.

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

^a28 days after primary inoculation with the indicated viruses, rhesus monkeys were challenged subcutaneously with 10⁷ 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.

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.

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.

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).

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.

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 Δ30 and charge-to-alanine mutations showed at least a fivefold greater reduction in SCID-HuH-7 mice than rDEN4Δ30.

The complete genomic sequence of five viruses (rDEN4-200-201, rDEN4Δ30-200-201, rDEN4-436-437 [clone 1], rDEN4Δ30-436-437, and rDEN4-23-24-200-201) that replicated to >10⁵ 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	Δ ^c	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	<1.7	>5.4
rDEN4Δ30-23-24			6.1	5.5	4.9	<1.7	4.4	6.5	5.9	4.7	<1.7	>4.2
rDEN4-200-201	KH	4	5.3	4.8	4.8	4.3	1.0	5.7	5.4	2.7	<1.7	>4.0
rDEN4Δ30-200-201			6.0	5.3	5.6	<1.7	>4.3	5.8	5.0	5.9	<1.7	>4.1
rDEN4-436-437	DK	4	5.2	4.2	3.4	1.9	3.3	5.9	4.9	3.2	<1.7	>4.2
rDEN4Δ30-436-437 [clone1]			6.3	5.7	5.5	<1.7	>4.6	6.5	5.7	5.1	<1.7	>4.8
rDEN4-808-809	ED	3	4.6	4.1	<1.7	<1.7	>2.9	5.2	<1.7	<1.7	<1.7	>3.5
rDEN4Δ30-808-809			5.6	4.9	4.9	<1.7	>3.9	5.9	4.8	5.1	<1.7	>4.2
rDEN4-23-24-200-201	KE, KH	7	6.0	5.2	4.2	<1.7	>4.3	6.9	6.3	<1.7	<1.7	>5.2
rDEN4Δ30-23-24-200-201			4.5	4.2	4.8	<1.7	>2.8	4.9	4.5	2.9	<1.7	>3.2
rDEN4-23-24-396-397	KE, RE	7	6.5	5.8	5.5	<1.7	>4.8	7.1	5.9	5.4	<1.7	>5.4
rDEN4Δ30-23-24-396-397			6.1	5.2	4.8	<1.7	>4.4	6.9	5.4	4.9	<1.7	>5.2
rDEN-436-437-808-809	DK, ED	7	4.9	4.9	5.1	<1.7	>3.2	5.5	3.2	<1.7	<1.7	>3.8

^aUnderlined values indicate a 2.5 or 3.5 log₁₀ PFU/ml reduction in titer in Vero or HuH-7 cells, respectively, at the indicated temperature when compared to the permissive temperature (35° C.).

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

^cReduction in titer (log₁₀ pfu/ml) compared to the permissive temperature (35° C.).

TABLE 10

Virus	Addition of charge-to-alanine mutations attenuates rDEN4Δ30 in suckling mouse brain and enhances attenuation in SCID-HuH-7 mice.					
	Replication in suckling mice ^a			Replication in SCID-HuH-7 mice ^b		
	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
rDEN4Δ30-23-24	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
rDEN4Δ30-436-437 [clone 1]	6	2.9 ± 0.3	3.4	4	2.3 ± 0.4	2.8
rDEN4-808-809	6	1.8 ± 0.1	3.1	8	3.2 ± 0.4	3.0
rDEN4Δ30-808-809	12	3.9 ± 0.7	2.1	4	3.7 ± 0.6	2.4
rDEN4-23-24-200-201	12	5.3 ± 0.5	0.7	13	3.4 ± 0.1	2.9
rDEN4Δ30-23-24-200-201	6	3.0 ± 0.2	2.6	5	1.8 ± 0.1	3.3
rDEN4-23-24-396-397	12	4.6 ± 0.9	1.5	8	3.6 ± 0.3	2.3
rDEN4Δ30-23-24-396-397	6	3.0 ± 0.2	2.6	5	2.2 ± 0.3	2.9
rDEN-436-437-808-809	6	<1.7 ± 0.0	3.6	8	2.1 ± 0.3	2.4

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

^bDetermined 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 log₁₀ PFU/g compared to wt virus; reductions of 1.5 are listed in boldface.

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

^dDetermined 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 log₁₀PFU/g compared to wt virus; reductions of ≥1.5 are listed in boldface. 55

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

TABLE 11-continued

Missense and UTR mutations present in rDEN4 virus derivatives bearing charge-to-alanine and the Δ 30 mutation.					
Virus	Gene ^{a,b}	Nucleotide position	Nucleotide substitution	Amino acid position ^c	Amino acid change ^b
rDEN4 Δ 30-200-201	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	Δ 30 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
rDEN4 Δ 30-436-437	NS4B*	7163	A > C	111	Leu > Phe
	NS5	8869-73	GACAA > GCAGC	436-437	AspLys > AlaAla
	3' UTR	10478-10507	Δ 30 deletion	None	None
	NS3	6751	A > C	124	Lys > Thr
rDEN4-23-24-200-201	NS5	7629-7633	AAAGA > GCAGC	23-24	LysGlu > AlaAla
	NS5	8160-8165	AAACA > GCAGC	200-201	LysHis > AlaAla

^aAsterisk indicates previously identified Vero cell adaptation mutation.

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

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

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Based on the attenuation in the SCID-HuH7 mouse model, four of the charge-to-alanine mutant viruses (rDEN4-200-201, rDEN4 Δ 30-200-201, rDEN4-436-437 [clone 2], rDEN4 Δ 30-436-437) 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 (FIG. 4, Table 12). Viremia was detected in most of the monkeys infected; only one of the four monkeys infected with rDEN4 Δ 30-200-201 and one of the four monkeys infected with rDEN4 Δ 30-436-437 showed no detectable viremia. Monkeys infected with rDEN4 showed the highest mean peak virus titer; and in each case viruses carrying the Δ 30 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 Δ 30 and charge-to-alanine mutations showed a two-fold reduction in mean peak viremia relative to those infected with rDEN4 Δ 30. This suggests that addition of the charge-to-alanine mutations further attenuates rDEN4 Δ 30 for rhesus macaques.

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 rDEN4 Δ 30. Monkeys infected with rDEN4-200-201 and rDEN4-436-437 [clone 2] had similar mean neutralizing antibody titers to rDEN4, and those infected with rDEN4 Δ 30-200-201 and rDEN4 Δ 30-436-437 had similar mean neutralizing antibody titers to rDEN4. In each case the addition of the Δ 30 mutation to a virus resulted in a two-fold decrease in neutralizing antibody. Thus, although the addition of charge-to-alanine mutations to rDEN4 Δ 30 decreased mean peak viremia below that of rDEN4 Δ 30 alone, it did not affect levels of neutralizing antibody.

TABLE 12

Addition of paired charge-to-alanine mutations to rDEN4 Δ 30 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 \pm 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 \pm 0.3	<5	276
rDEN4 Δ 30	2	2	2.0	2.1 \pm 0.1	<5	131
rDEN4-200, 201	4	4	2.3	1.8 \pm 0.3	<5	212
rDEN4 Δ 30-200, 201	4	3	1.5	1.3 \pm 0.2	<5	139

TABLE 12-continued

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
rDEN4-436, 437 [cl 2]	4	4	3.3	1.8 ± 0.2	<5	273
rDEN4Δ30-436, 437	4	3	1.3	1.0 ± 0.0	<5	143

^aGroups 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.

^bViremia was not detected in any monkey after day 4.

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.

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

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 [cl 2]	4	0.0	<0.7	273	401
rDEN4Δ30-436, 437	4	0.0	<0.7	143	182

^a28 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, 40, and 56. Virus titer was determined by plaque assay in Vero cells.

Addition of charge-to-alanine mutations to rDEN4Δ30 can confer a range of is 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.

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				
Aedes aegypti (oral infection)				
Virus	Dose ^a (log ₁₀ pfu)	No. tested	% infected ^b	
			Midgut	Head
rDEN4	4.3	27	70	25
rDEN4-4891	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.

EXAMPLE 2

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

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 BamHI 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 Δ30 mutation. The pRS424DEN1WP 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. Plas-

mid DNA suitable for generating RNA transcripts was prepared and the presence of the Δ30 mutation was verified by sequence analysis.

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 (FIG. 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 rDEN1Δ30 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	E	1748	A > U	552	Thr > Ser
rDEN1Δ30	Vero	E	1545	A > G	484	Lys > Arg

^aSame nucleotide as 7154 in rDEN4.

^bSame 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:	89012345678901234567890123456789012345678901234567					
	++	++	+ +++++ +	+ +	+ ++	+ +++++++ ++ ++ ++ ++
D4 7128-	CCACAACCUUGACAGCAUCCUAGUCAUGC	UUUAGUCCAUU	AUGCAAUA			
	UAGGCCCA					
	P	T	T	L	T	A
	S	L	V	M	L	L
	V	H	T	A	I	I
	G	P				
D1 7139-	CCGCUGACGCGACAGCGCGGUAUUU	AUGC	UAGUGGC	UCAUU	AUGCCAUA	
	AUUGGACCC					
	P	L	T	L	T	A
	A	V	P	M	L	V
	A	H	T	A	I	I
	G	P				
D2 7135-	CCUAUAACCCUCACAGCGGCUCUUCUUUU	AUUGGU	AGCACAUUAUGCCAUAUAG			
	GACCG					
	P	I	T	L	T	A
	A	L	L	L	L	V
	A	H	T	A	I	I
	G	P				

- continued

D3 7130- CCACUAACUCUCACAGCGGCAGUUCUCCUGCUAGUCACGCUUUAUGCUAU
UAUAGGUCCA
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.

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 (FIG. 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 peak titer of challenge virus |
| rDEN1 | 4 | 2.8 | 2.1 | 1230 |
| rDEN1Δ30 | 4 | 0.5 | 0.8 | 780 |

*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.

As previously reported, rDEN4 virus replicated to greater than 6.0 log₁₀ PFU/ml serum in SCID-HuH-7 mice, while the replication of rDEN4 virus bearing the Δ30 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 Δ30 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 |
| rDEN1Δ30 | 8 | 5.0 ± 0.3 |

⁵Groups of HuH-7-SCID mice were inoculated directly into the tumor with 4.0 log₁₀ pfu virus. Serum was collected on day 6 and 7, and virus titer was determined by plaque assay in Vero cells.

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

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 | 2.7 | 8 | 100 | 3.2 | 20 | 10 | 0 |
| Δ30 | 1.7 | 7 | 100 | | | | |
| | 0.7 | 6 | 83 | | | | |
| | | | MID ₅₀ < 0.7 | | | MID ₅₀ ≥ 3.6 | |

^aAmount of virus present in 0.22 μl inoculum.

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

^cVirus titer ingested, assuming a 2 μl bloodmeal.

^dPercentage 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.

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 Δ30 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.

EXAMPLE 3

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

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 FIG. 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 p2Δ30, the Fragment Y region of p2 was replaced with Fragment YΔ30 (FIG. 6A, B).

For transcription and generation of infectious virus, cDNA (p2 and p2Δ30) 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.

rDEN2 virus was recovered from the p2 cDNA in both Vero and C6/36 cells, while rDEN2Δ30 was recovered from the p2Δ30 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 p2Δ30 cDNA clones when

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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) | |
| | | | | Vero cells | C6/36 cells |
| Vero 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 | rDEN2Δ30 | 4.6 | 7.5 |

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 |
| rDEN2 ^b | NS4B | 7169 ^c | U > C | 2358 | Val > Ala |
| (Vero) | NS5 | 9248 | A > C | 3051 | Glu > Ala |
| rDEN2Δ30 ^d | NS3 | 4946 | A > G | 1617 | Lys > Arg |
| (Vero) | NS4B | 7169 ^c | U > C | 2358 | Val > Ala |

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

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

^cSame nucleotide position as 7162 in rDEN4.

^dVirus 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.

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

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into the rDEN2Δ30 cDNA plasmid to create p2Δ30-7169. Transcripts synthesized from p2Δ30-7169, as well as p2 and p2Δ30 were introduced into Vero cells or C6/36 mosquito cells using liposome-mediated transfection as described above. Virus rDEN2Δ30-7169 was recovered from the p2Δ30-7169 cDNA in both Vero and C6/36 cells, while rDEN2Δ30 was recovered from the p2Δ30 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) | |
| | | | | Vero cells | C6/36 cells |
| Vero cells | p2 | #8A | rDEN2 | 6.8 | |
| | p2Δ30 | #2 | rDEN2Δ30 | <0.7 | |
| | p2Δ30-7169 ^a | #37 | rDEN2Δ30-7169 | 5.1 | |
| C6/36 cells | p2 | #8A | rDEN2 | 6.9 | |
| | p2Δ30 | #2 | rDEN2Δ30 | 7.1 | |
| | p2Δ30-7169 | #37 | rDEN2Δ30-7169 | 7.2 | |

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

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 log₁₀ PFU/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 |

^aGroups 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.

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

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 rDEN2Δ30 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 |

^aGroups 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.

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

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 log₁₀PFU 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 log₁₀PFU 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 log₁₀PFU/ml, respectively.

Monkeys inoculated with rDEN2Δ30 were viremic for 2-3 days with a mean peak titer of 1.7 log₁₀PFU/ml (Table 24, FIG. 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 ± 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 ± 0.3 | <10 | 311 |
| rDEN2 (Vero) | 4 | 4 | 4.0 | 1.9 ± 0.1 | <10 | 173 |
| rDEN2Δ30 (Vero) | 4 | 4 | 2.8 | 1.7 ± 0.2 | <10 | 91 |

^aGroups 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.

^bViremia 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 (log ₁₀ PFU/ml ± SE) | Mean peak virus titer | Geometric mean serum neutralizing antibody titer (reciprocal dilution) | |
| | | | | Day 28 | Day 56 |
| Mock | 2 | 4.0 | 2.1 ± 0.1 | <10 | 338 |
| DEN2 (Tonga/74) | 4 | 0 | <0.7 | 311 | 334 |

TABLE 25-continued

| rDEN2Δ30 protects rhesus monkeys from wt DENT2 virus challenge | | | | | |
|--|----------------|--|-----------------------|--|--------|
| Virus ^a | No. of monkeys | Mean no. of viremic days per monkey after DEN2 challenge (log ₁₀ PFU/ml ± SE) | Mean peak virus titer | Geometric mean serum neutralizing antibody titer (reciprocal dilution) | |
| | | | | Day 28 | Day 56 |
| rDEN2 (Vero) | 4 | 0 | <0.7 | 173 | 318 |
| rDEN2Δ30 (Vero) | 4 | 0 | <0.7 | 91 | 267 |

^a28 days after inoculation with the indicated viruses, monkeys were challenged subcutaneously with 10⁵ 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.

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 log₁₀ 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.

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

15 mutations identified in rDEN4 viruses are contemplated as being used to confer similar phenotypes upon rDEN2 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 20 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 25 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 30 (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 rDEN4 viruses into rDEN2 Tonga/74 virus. | | | | | | | | | | |
|---|------------------------------|-----------------------------|-------------|-------------------------|------------------------------------|-------------------|-------------------------------------|------------------------------------|-------------------|---------------------------------------|
| Phenotype in rDEN4 virus | | | | Mutation in rDEN4 virus | | | Mutation introduced into DEN2 virus | | | |
| Vero Adap- tation ^a | Mouse brain att ^b | SCID-HuH-7 att ^c | Gene/region | Nucleo- tide position | Amino acid posi- tion ^d | Amino acid change | Nucleo- tide position | Amino acid posi- tion ^d | Amino acid change | RE site/mutagenic region ^e |
| + | + | - | NS3 | 4891 | 1597 | Ile > Thr | 4889 | 1598 | Ile > Thr | Nar I
CCA <u>cgGGcGCCGT</u> |
| + | + | - | NS3 | 4995 | 1632 | Ser > Pro | 4993 | 1633 | Ser > Pro | Stu I
AAGG <u>ccTGGA</u> |
| + | - | - | NS4b | 7182 | 2361 | Gly > Ser | 7189 | 2365 | Gly > Ser | Xma I
TAT <u>ccCCGGGAC</u> |
| - | + | + | NS1 | 2650 | 850 | Asn > Ser | 2648 | 851 | Asn > Ser | Sac I
AGAg <u>cTctcTC</u> |
| - | + | + | NS3 | 5097 | 1666 | Asp > Asn | 5095 | 1667 | Asp > Asn | Xma I
GaATCTCC <u>ACCCgGA</u> |

TABLE 26-continued

| Introduction of conserved point mutations characterized in rDEN4 viruses into rDEN2 Tonga/74 virus. | | | | | | | | | | |
|---|------------------|------------------|-------------|-------------------------|-------------------|--------|-------------------------------------|-------------------|--------|---------------------|
| Phenotype in rDEN4 virus | | | | Mutation in rDEN4 virus | | | Mutation introduced into DEN2 virus | | | |
| Vero | Mouse | SCID- | Gene/region | Nucleo- | Amino | Amino | Nucleo- | Amino | Amino | RE |
| tation ^a | att ^b | att ^c | | tide | acid | acid | tide | acid | acid | site/mutagenic |
| | | | | position | posi- | change | position | posi- | change | region ^e |
| | | | | | tion ^d | | | tion ^d | | |
| - | + | + | 3' UTR | 10634 | n/a ^f | n/a | 10698 | n/a | n/a | none
CTGTcGAATC |

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

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

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

^dAmino 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.

^ePrimers 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.

^fNucleotide 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 |

^aTransfection has not yet been attempted.

EXAMPLE 4

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

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 FIG. 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 AscI 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 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 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 (FIG. 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 Δ30 mutation was introduced into Fragment 4 to generate Fragment 4Δ30. To create p3Δ30, the Fragment 4 region of p3 was replaced with Fragment 4Δ30 (FIG. 8A, B).

For transcription and generation of infectious virus, cDNA plasmids p3 and p3Δ30 were digested with SpeI and religated 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.

rDEN3 virus was recovered from the p3 cDNA in both Vero and C6/36 cells, while rDEN3Δ30 was recovered from

the p3Δ30 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 p3Δ30 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 rDEN3Δ30 viruses were recovered from independent cDNA clones, #22 and #41.

TABLE 28

| rDEN3 virus is recovered in Vero and C6/36 cells,
but rDEN3Δ30 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) | |
| | | | | Vero cells | C6/36 cells |
| Vero cells | p3 | #50 | rDEN3 | 5.2 | 6.3 |
| | p3Δ30 | #22 | rDEN3Δ30 | <0.7 | <0.7 |
| | p3Δ30 | #41 | rDEN3Δ30 | <0.7 | <0.7 |
| C6/36 cells | p3 | #50 | rDEN3 | 5.2 | 6.0 |
| | p3Δ30 | #22 | rDEN3Δ30 | 5.9 | 6.9 |
| | p3Δ30 | #41 | rDEN3Δ30 | 5.1 | 7.2 |

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 p3Δ30 to create p3-7164 and p3Δ30-7164. This mutation creates a Val to Ala substitution at amino acid position 2357. As demonstrated for rDEN2Δ30, this mutation allowed for the direct recovery of virus in Vero cells (Table 27) and is anticipated to have the same effect for rDEN3Δ30.

To initially assess the ability of the Δ30 mutation to attenuate rDEN3 virus in an animal model, the replication of DEN3 (Sleman/78), rDEN3, and rDEN3Δ30 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 Δ30 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.

In summary, the strategy of introducing the Δ30 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, DEN4Δ30 present in serum of vaccinees retained its Δ30 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 Δ30 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 Δ30
Mutation

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.

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

(FIG. 9A). To create the CME chimeric viruses, the BglIII/XhoI 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 PstI/XhoI 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 FIG. 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).

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 log₁₀PFU/ml, indicating that the chimeric viruses, even those containing the Δ30 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 ME | 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 |

TABLE 30-continued

| 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 |
| 3/4 CME | -3 | 102 | 114 | 166 | 493 |
| rDEN4 | — | 102 | 113 | 166 | 495 |

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 log₁₀PFU/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 log₁₀PFU/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 antibody following inoculation with the chimeric viruses in comparison to titers achieved in animals inoculated with wild-type virus. Addition of the Δ30 mutation to the CME chimeric virus further attenuated the virus, such that rDEN2/4Δ30(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 Δ30 mutation to the ME chimeric virus did not further attenuate this chimeric virus and the resulting rDEN2/4Δ30(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 rDEN2/4Δ30 (CME) | 8 | 0 | <0.7 | <5 | |
| 3 rDEN2/4 (ME) | 4 | 1.0 | 0.8 | 76 | |
| 4 rDEN2/4Δ30 (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.

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 Δ30 mutation providing a 0.5 log₁₀ reduction in repli-

cation. This level of attenuation 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.

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

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 log₁₀ PFU, 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 rDEN2/4 $\Delta 30$ (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 $\Delta 30$ 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

| Virus | Dengue 2/4 chimeric viruses are less infectious compared to either parental virus strain in mosquitoes | | | | | | |
|---------------------------|--|-----------------------|------------------------------|--|--------|------------------------------|------------------------------|
| | <i>Toxorhynchites splendens</i>
(intrathoracic inoculation) | | | <i>Aedes aegypti</i>
(oral infection) | | | |
| | Dose ^a | No. | % | Dose ^c | No. | % infected ^d | |
| log ₁₀ pfu | tested | infected ^b | log ₁₀ pfu | tested | 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 |
| DEN2-NGC | | | MID ₅₀ = 2.2 | | | MID ₅₀ = 3.4 | MID ₅₀ \geq 4.1 |
| | 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 |
| rDEN2/4 (CME) | | | MID ₅₀ = 0.5 | | | MID ₅₀ = 3.2 | MID ₅₀ = 3.6 |
| | 3.9 | 9 | 11 | 4.4 | 11 | 9 | 0 |
| | 2.9 | 5 | 0 | 3.4 | 10 | 0 | 0 |
| rDEN2/4 $\Delta 30$ (CME) | | | MID ₅₀ \geq 4.3 | | | MID ₅₀ \geq 4.9 | Nc ^e |
| | 3.5 | 6 | 17 | 4.0 | 15 | 26 | 6 |
| rDEN2/4 (ME) | | | MID ₅₀ \geq 3.9 | | | MID ₅₀ \geq 4.3 | MID ₅₀ \geq 4.5 |
| | 2.5 | 6 | 17 | 3.0 | 10 | 0 | 0 |
| | 3.4 | 6 | 100 | 3.9 | 23 | 4 | 0 |
| rDEN2/4 $\Delta 30$ (ME) | | | MID ₅₀ = 2.8 | | | MID ₅₀ \geq 4.4 | Nc |
| | 2.4 | 5 | 20 | | | | |
| | 1.4 | 5 | 0 | | | | |
| rDEN2/4 $\Delta 30$ (ME) | | | MID ₅₀ = 2.8 | | | nc | Nc |
| | 2.6 | 11 | 9 | 3.1 | 30 | 0 | 0 |
| | | | MID ₅₀ \geq 3.0 | | | | |

^aAmount of virus present in 0.22 μ l inoculum.

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

^cVirus titer ingested, assuming a 2 μ l bloodmeal.

^dPercentage 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.

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

Chimerization of the DEN2 structural genes with rDEN4 $\Delta 30$ virus resulted in a virus, rDEN2/4 $\Delta 30$ (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.

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.

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.

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 (FIG. 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.

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 Δ30 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 Δ30, whereas the attenuation phenotype of chimeric viruses bearing just the ME region of DEN2 was unaltered by the addition of the Δ30 mutation.

This unexpected finding indicates that in a tetravalent vaccine comprised of individual component viruses bearing a shared attenuating mutation, such as the Δ30 mutation, only ME chimeric viruses can be utilized since CME chimeric viruses bearing the Δ30 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 Δ30 Mutation

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 BglII/XhoI region of the cDNA for either rDEN4 or rDEN4Δ30 with a similar region derived from DEN3 (Sleman/78) (FIG. 11A). Likewise, to create the ME chimeric viruses, the PstI/XhoI region of the cDNA for either rDEN4 or rDEN4Δ30 was replaced with a similar region derived from DEN3 (Sleman/78). The nucleotide and amino acid sequences of the resulting junctions are shown in FIG. 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 log₁₀PFU/ml, indicating that the chimeric viruses, even those containing the Δ30 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 |
|------------------|------|---------------------|-------------------|---------------------|-------------------|
| rDEN3/4Δ30 (CME) | M | 825 | T > C | 242 | Phe > Leu |
| | E | 1641 | C > T | 514 | Leu > Phe |
| | E | 2113 | A > G | 671 | Lys > Arg |
| rDEN3/4(ME) | NS4B | 7159 ^a | T > C | 2353 | Leu > Ser |
| | M | 460 | A > G | 120 | Asp > Gly |
| | NS4B | 7177 ^b | G > U | 2359 | Gly > Val |
| rDEN3/4Δ30 (ME) | NS5 | 7702 | C > U | 2534 | Ser > Phe |
| | E | 1432 | A > U | 444 | Gln > Leu |
| | NS4B | 7156 ^a | U > C | 2352 | Leu > Ser |
| | NS5 | 8692 | A > C | 2864 | Asn > His |

^aSame nucleotide position as 7162 in rDEN4.

^bSame nucleotide position as 7183 in rDEN4.

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

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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 \log_{10} 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 |
| DEN3-Sleman/78 | 23 | 6.4 \pm 0.2 | A |
| rDEN3/4 (CME) | 7 | 6.4 \pm 0.6 | A |
| rDEN3/4 $\Delta 30$ (CME) | 5 | 3.7 \pm 0.4 | B |
| rDEN3/4 (ME) | 6 | 4.2 \pm 0.7 | B |
| rDEN3/4 $\Delta 30$ (ME) | 7 | 4.7 \pm 0.4 | A, B |

^aGroups of HuH-7-SCID mice were inoculated into the tumor with 4.0 \log_{10} 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.

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 \log_{10} 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 wtDEN3 virus challenge (Table 37). It is clear that addition of the $\Delta 30$ mutation to rDEN3/4(CME) was capable of further attenuating the resulting virus rDEN3/4 $\Delta 30$ (CME).

TABLE 36

| The $\Delta 30$ 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 \pm SE) | Geometric mean serum neutralizing antibody titer (reciprocal dilution) | |
| | | | | Day 0 | Day 28 |
| DEN3 (Sleman/78) | 4 | 2.3 | 1.8 | <5 | 707 |

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TABLE 36-continued

| The $\Delta 30$ 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 \pm SE) | Geometric mean serum neutralizing antibody titer (reciprocal dilution) | |
| | | | | Day 0 | Day 28 |
| rDEN3/4 (CME) | 4 | 2.0 | 1.6 | <5 | 211 |
| rDEN3/4 $\Delta 30$ (CME) | 4 | 0 | <1.0 | <5 | 53 |
| rDEN3/4 (ME) | 4 | 0 | <1.0 | <5 | 70 |
| rDEN3/4 $\Delta 30$ (ME) | 4 | 0 | <1.0 | <5 | 58 |

^aGroups 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.

^bViremia 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 \pm SE) | Geometric mean serum neutralizing antibody titer (reciprocal dilution) | |
| | | | | Day 28 | Day 56 |
| Mock | 2 | 5.0 | 2.5 \pm 0.4 | <5 | 372 |
| DEN3 (Sleman/78) | 4 | 0 | <1.0 | 707 | 779 |
| rDEN3/4 (CME) | 4 | 0 | <1.0 | 211 | 695 |
| rDEN3/4 $\Delta 30$ (CME) | 4 | 0.8 | 1.1 \pm 0.2 | 53 | 364 |
| rDEN3/4 (ME) | 4 | 0 | <1.0 | 70 | 678 |
| rDEN3/4 $\Delta 30$ (ME) | 4 | 0 | <1.0 | 58 | 694 |

^a28 days after primary inoculation with the indicated viruses, rhesus monkeys were challenged subcutaneously with 10 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.

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 infectivity of several of the viruses. At a dose of approximately 2.8-2.9 \log_{10} PFU, 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 $\Delta 30$ (CME) is restricted in *Ae. aegypti*. By comparing infectivity of rDEN3/4(CME) and rDEN3/4 $\Delta 30$ (CME), it is clear that the $\Delta 30$ mutation is capable of further attenuating the chimeric virus for mosquitoes.

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TABLE 38

| Ability of DEN3/4 chimeric viruses to infect <i>Aedes aegypti</i> 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 ≥ 4.4 |
| DEN3 (Sleman) | 2.9 | 16 | 3 (18%) | 2 (12%) |
| | 1.9 | 10 | 1 (10%) | 0 |
| | | | MID ₅₀ ≥ 3.5 | MID ₅₀ +0 ≥ 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 ≥ 4.2 | MID ₅₀ ≥ 4.5 |
| DEN3/4Δ30 (CME) | 3.3 | 20 | 0 | 0 |
| | | | MID ₅₀ ≥ 4.3 | MID ₅₀ ≥ 4.3 |
| DEN3/4 (ME) | 3.4 | 15 | 0 | 0 |
| | | | MID ₅₀ ≥ 4.4 | MID ₅₀ ≥ 4.4 |

^aAmount of virus ingested, assuming a 2μ 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.

EXAMPLE 7

Generation and Characterization of Intertypic Chimeric DEN1 Viruses Containing the Δ30 Mutation

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 BglII/XhoI region of the cDNA for either rDEN4 or rDEN4Δ30 with a similar region derived from DEN1 (Puerto Rico/94) (FIG. 12A). Likewise, to create the ME chimeric viruses, the PstI/XhoI region of the cDNA for either rDEN4 or rDEN4Δ30 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 FIG. 12B.

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 log₁₀ PFU/ml, indicating that the chimeric viruses, even those containing the Δ30 mutation, replicate efficiently in cell culture. Genomic sequence analysis is currently underway to identify incidental mutations arising from virus passage in tissue culture.

To evaluate the replication levels of DEN1/4(CME) and rDEN1/4Δ30(CME) chimeric virus in mosquitoes, *Aedes aegypti* were infected by ingesting a virus-containing blood meal (Table 39). Parental virus rDEN4 infects *Ae. aegypti*

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with an MID₅₀ of 4.0 log₁₀ PFU. However, parental virus DEN1 (Puerto Rico/94), is unable to infect *Ae. aegypti* at a dose of up to 3.4 log₁₀ PFU. Thus CME chimeric viruses DEN1/4 and rDEN1/4Δ30 share this inability to infect *Ae. aegypti*. Therefore, it is unnecessary in *Ae. aegypti* to evaluate the effect of the Δ30 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 <i>Aedes aegypti</i> 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 |
| rDEN 1/4 (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 2μ 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.

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 | | | | | | | | | |
|---|--|------|------|------|------|------|------|------|------|
| | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 |
| 100 | AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACTGTTTTTTGATTAGAGAGCAGATCTCTGATGA
Met> | | | | | | | | |
| 200 | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 |
| ATAACCAACGGAAAAAGGCGAGAAAACACGCCTTTCAATATGCTGAAACGCGAGAGAAAACCGCGTGTCAACTGTACAACAGTTGACAAAGAGATTCTCACT
AsnAsnGlnArgLysLysAlaArgAsnThrProPheAsnMetLeuLysArgGluArgAsnArgValSerThrValGlnGlnLeuThrLysArgPheSerLeu> | | | | | | | | | |
| 300 | 210 | 220 | 230 | 240 | 250 | 260 | 270 | 280 | 290 |
| TGGAATGCTGCAGGGACGAGGACCACTAAAATTGTTTCATGGCCCTGGTGGCATTCTTCGTTTCCTAACAATCCCACCAACAGCAGGGATATTAAGA
GlyMetLeuGlnGlyArgGlyProLeuLysLeuPheMetAlaLeuValAlaPheLeuArgPheLeuThrIleProProThrAlaGlyIleLeuLysArg> | | | | | | | | | |
| 400 | 310 | 320 | 330 | 340 | 350 | 360 | 370 | 380 | 390 |
| TGGGAACAATTAATAAATCAAAGGCTATTAATGTTCTGAGAGGCTTCAGGAAGAGATTTGGAAGGATGCTGAATATCTTAAACAGGAGACGTAGAAGT
TrpGlyThrIleLysLysSerLysAlaIleAsnValLeuArgGlyPheArgLysGluIleGlyArgMetLeuAsnIleLeuAsnArgArgArgArgThr> | | | | | | | | | |
| 500 | 410 | 420 | 430 | 440 | 450 | 460 | 470 | 480 | 490 |
| TAGGCATGATCATCATGCTGACTCCAACAGTGTGGCGTTTCATCTGACCACACGCAACGGAGAACCACACATGATTGTGAGTACAGAAAAGGGAA
ValGlyMetIleIleMetLeuThrProThrValMetAlaPheHisLeuThrThrArgAsnGlyGluProHisMetIleValSerArgGlnGluLysGlyLys> | | | | | | | | | |
| 600 | 510 | 520 | 530 | 540 | 550 | 560 | 570 | 580 | 590 |
| AAGCTTCTGTTCAAGACAAAGGATGGCAGCAACATGTGTACCCTCATGGCCATGGACCTTGGTGTGAGTTGTGTAAGACACAATCACGTATAAATGTCCT
SerLeuLeuPheLysThrLysAspGlyThrAsnMetCysThrLeuMetAlaMetAspLeuGlyGluLeuCysGluAspThrIleThrTyrLysCysPro> | | | | | | | | | |
| 700 | 610 | 620 | 630 | 640 | 650 | 660 | 670 | 680 | 690 |
| TTTCTCAAGCAGAACGAACGAGACATAGATTGTTGGTGCAACTCCACGTCCACATGGGTAACCTATGGGACATGTACCACCACAGGAGACACAGAA
PheLeuLysGlnAsnGluProGluAspIleAspCysTrpCysAsnSerThrSerThrTrpValThrTyrGlyThrCysThrThrThrGlyGluHisArg> | | | | | | | | | |
| 800 | 710 | 720 | 730 | 740 | 750 | 760 | 770 | 780 | 790 |
| GAGAAAAAGATCAGTGGCGCTTGTTCACACGTGGGAATGGGATTGGAGACACGAACTGAAACATGGATCTCATCAGAAGGGGCCTGGAAACATGCCCA
ArgGluLysArgSerValAlaLeuValProHisValGlyMetGlyLeuGluThrArgThrGluThrTrpMetSerSerGluGlyAlaTrpLysHisAlaGln> | | | | | | | | | |
| 900 | 810 | 820 | 830 | 840 | 850 | 860 | 870 | 880 | 890 |
| GAGAATTGAACTTGGATTCTGAGACATCCAGGCTTTACCATAATGGCCGCAATCCTGGCATAACCCATAGGGACGACGATTTCCAAAGAGTCCTGATA
ArgIleGluThrTrpIleLeuArgHisProGlyPheThrIleMetAlaAlaIleLeuAlaTyrThrIleGlyThrThrHisPheGlnArgValLeuIle> | | | | | | | | | |
| 1000 | 910 | 920 | 930 | 940 | 950 | 960 | 970 | 980 | 990 |
| TTCATCTACTGACAGCCATCGCTCCTTCAATGACAATGCGCTGCATAGGAATATCAAATAGGGACTTTGTGGAAGGAGTGTGAGGAGGAGTTGGGTTG
PheIleLeuLeuThrAlaIleAlaProSerMetThrMetArgCysIleGlyIleSerAsnArgAspPheValGluGlyValSerGlyGlySerTrpVal> | | | | | | | | | |
| 1100 | 1010 | 1020 | 1030 | 1040 | 1050 | 1060 | 1070 | 1080 | 1090 |
| ACATAGTTTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAACAACCAACTGGACTTTGAACTGATAAAAAACAGAAGCCAAACAACCTGCCAC
AspIleValLeuGluHisGlySerCysValThrThrMetAlaLysAsnLysProThrLeuAspPheGluLeuIleLysThrGluAlaLysGlnProAlaThr> | | | | | | | | | |
| 1200 | 1110 | 1120 | 1130 | 1140 | 1150 | 1160 | 1170 | 1180 | 1190 |
| CTTAAGGAAGTACTGTATAGAGGCCAAACTGACCAACACGACAACAGACTCGCGCTGCCCAACACAAGGGGAACCCACCCTGAATGAAGAGCAGGACAAA
LeuArgLysTyrCysIleGluAlaLysLeuThrAsnThrThrThrAspSerArgCysProThrGlnGlyGluProThrLeuAsnGluGluGlnAspLys> | | | | | | | | | |
| 1300 | 1210 | 1220 | 1230 | 1240 | 1250 | 1260 | 1270 | 1280 | 1290 |
| AGGTTTGTCTGCAACATTCATGGTAGACAGAGGATGGGAAATGGATGTGGATTGTTTGGAAAAGGAGGCATCGTGACCTGTGCTATGTTACATGCA
ArgPheValCysLysHisSerMetValAspArgGlyTrpGlyAsnGlyCysGlyLeuPheGlyLysGlyGlyIleValThrCysAlaMetPheThrCys> | | | | | | | | | |
| 1400 | 1310 | 1320 | 1330 | 1340 | 1350 | 1360 | 1370 | 1380 | 1390 |
| AAAAGAACATGGAAGGAAAAATTGTTTCAGCCAGAAAACCTGGAATACACTGTCGTGATAACACCTCATTGAGGGAAGAATGCAGTGGGAAATGACAC
LysLysAsnMetGluGlyLysIleValGlnProGluAsnLeuGluTyrThrValValIleThrProHisSerGlyGluGluHisAlaValGlyAsnAspThr> | | | | | | | | | |
| 1500 | 1410 | 1420 | 1430 | 1440 | 1450 | 1460 | 1470 | 1480 | 1490 |
| AGGAAAACATGGTAAAGAAGTCAAGATAACACCACAGAGCTCCATCACAGAGGCGAACTGACAGGCTATGGCACTGTTACGATGGAGTGCTCTCCAAGA
GlyLysHisGlyLysGluValLysIleThrProGlnSerSerIleThrGluAlaGluLeuThrGlyTyrGlyThrValThrMetGluCysSerProArg> | | | | | | | | | |
| 1600 | 1510 | 1520 | 1530 | 1540 | 1550 | 1560 | 1570 | 1580 | 1590 |
| ACGGCCCTCGACTTCAATGAGATGGTGTGCTGCAAATGGAAGACAAAGCCTGGCTGGTGCACAGACAATGGTTCTAGACCTACCGTTGCCATGGCTGC | | | | | | | | | |

APPENDIX 1-continued

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

ThrGlyLeuAspPheAsnGluMetValLeuLeuGlnMetGluAspLysAlaTrpLeuValHisArgGlnTrpPheLeuAspLeuProLeuProTrpLeu>

1610 1620 1630 1640 1650 1660 1670 1680 1690

1700
CCGGAGCAGACACACAAGGATCAAATTGGATACAGAAAGAAACACTGGTCACCTTCAAAAATCCCCATGCGAAAAACAGGATGTTGTTGTCTTAGGATC
ProGlyAlaAspThrGlnGlySerAsnTrpIleGlnLysGluThrLeuValThrPheLysAsnProHisAlaLysLysGlnAspValValValLeuGlySer>

1710 1720 1730 1740 1750 1760 1770 1780 1790

1800
CCAAGAGGGGGCCATGCATACAGCACTCACAGGGGCTACGGAAATCCAGATGTCATCAGGAAACCTGCTGTTACAGGACATCTCAAGTGCAGGCTGAGA
GlnGluGlyAlaMetHisThrAlaLeuThrGlyAlaThrGluIleGlnMetSerSerGlyAsnLeuLeuPheThrGlyHisLeuLysCysArgLeuArg>

1810 1820 1830 1840 1850 1860 1870 1880 1890

1900
ATGGACAAATTACAACCTAAAGGGATGTCATACTCCATGTGCACAGGAAAGTTTAAAATTGTGAAGGAAATAGCAGAAACACAACATGGAACAATAGTCA
MetAspLysLeuGlnLeuLysGlyMetSerTyrSerMetCysThrGlyLysPheLysIleValLysGluIleAlaGluThrGlnHisGlyThrIleVal>

1910 1920 1930 1940 1950 1960 1970 1980 1990

2000
TTAGAGTACAATATGAAGGAGACGGCTCTCCATGCAAGATCCCCTTTGAGATAATGGATCTGGAAAAAGACATGTTTTGGGCCGCTGATCACAGTCAA
IleArgValGlnTyrGluGlyAspGlySerProCysLysIleProPheGluIleMetAspLeuGluLysArgHisValLeuGlyArgLeuIleThrValAsn>

2010 2020 2030 2040 2050 2060 2070 2080 2090

2100
CCCAATTGTAACAGAAAAGGACAGTCCAGTCAACATAGAAGCAGAACCTCCATTCGGAGACAGCTACATCATAGGAGTGAACCCAGGACAATTGAAG
ProIleValThrGluLysAspSerProValAsnIleGluAlaGluProProPheGlyAspSerTyrIleIleIleGlyValGluProGlyGlnLeuLys>

2110 2120 2130 2140 2150 2160 2170 2180 2190

2200
CTGGACTGGTTCAAGAAAGGAAGTTCCATCGGCCAAATGTTTGGAGACAACAATGAGGGGAGCGAAAAGAATGGCCATTTTGGGTGACACAGCCTGGGATT
LeuAspTrpPheLysLysGlySerSerIleGlyGlnMetPheGluThrThrMetArgGlyAlaLysArgMetAlaIleLeuGlyAspThrAlaTrpAsp>

2210 2220 2230 2240 2250 2260 2270 2280 2290

2300
TTGGATCTCTGGGAGGAGTGTTCACATCAATAGGAAAGGCTCTCCACCAGGTTTTTGGAGCAATCTACGGGGCTGCTTTTCAGTGGGGTCTCATGGACTAT
PheGlySerLeuGlyGlyValPheThrSerIleGlyLysAlaLeuHisGlnValPheGlyAlaIleTyrGlyAlaAlaPheSerGlyValSerTrpThrMet>

2310 2320 2330 2340 2350 2360 2370 2380 2390

2400
GAAGATCCTCATAGGAGTTATCATCACATGGATAGGAATGAACTCACGTAGCACTAGTCTGAGCGTGTCACTGGTGTAGTGGGAATCGTGACACTTTAC
LysIleLeuIleGlyValIleIleThrTrpIleGlyMetAsnSerArgSerThrSerLeuSerValSerLeuValLeuValGlyIleValThrLeuTyr>

2410 2420 2430 2440 2450 2460 2470 2480 2490

2500
TTGGGAGTTATGGTGCAGGCCGATAGTGGTTGCGTTGTGAGCTGGAAGAACAAGAATAAAATGTGGCAGTGAATATTCGTACAGATAACGTGCATA
LeuGlyValMetValGlnAlaAspSerGlyCysValValSerTrpLysAsnLysGluLeuLysCysGlySerGlyIlePheValThrAspAsnValHis>

2510 2520 2530 2540 2550 2560 2570 2580 2590

2600
CATGGACAGAACAATAACAAGTTCCAACCAGAATCCCCTTCAAACTGGCCTCAGCCATCCAGAAAGCGCATGAAGAGGGCATCTGTGGAATCCGCTCAGT
ThrTrpThrGluGlnTyrLysPheGlnProGluSerProSerLysLeuAlaSerAlaIleGlnLysAlaHisGluGluGlyIleCysGlyIleArgSerVal>

2610 2620 2630 2640 2650 2660 2670 2680 2690

2700
AACAGACTGGAAAATCTTATGTGGAACAGATAACATCAGAATTGAATCATATCTATCAGAAAATGAAGTGAACCTGACCATCATGACAGGAGACATC
ThrArgLeuGluAsnLeuMetTrpLysGlnIleThrSerGluLeuAsnHisIleLeuSerGluAsnGluValLysLeuThrIleMetThrGlyAspIle>

2710 2720 2730 2740 2750 2760 2770 2780 2790

2800
AAAGGAATCATGCAGGTAGGAAAACGATCTTTGCGGCCCTCAACCCACTGAGTTGAGGTATTCATGGAACATGGGGTAAAGCGAAAAATGCTCTCCACAG
LysGlyIleMetGlnValGlyLysArgSerLeuArgProGlnProThrGluLeuArgTyrserTrpLysThrTrpGlyLysAlaLysMetLeuSerThr>

2810 2820 2830 2840 2850 2860 2870 2880 2890

2900
AACTCCACAATCAGACCTCCTCATTGATGGTCCCGAAACAGCAGAATGCCCAACACAAACAGAGCTTGGAAATCACTGGAAGTTGAGGACTACGGCTT
GluLeuHisAsnGlnThrPheLeuIleAspGlyProGluThrAlaGluCysProAsnThrAsnArgAlaTrpAsnSerLeuGluValGluAspTyrGlyPhe>

2910 2920 2930 2940 2950 2960 2970 2980 2990

3000
TGGAGTATTCACCTACCAATATATGGCTAAGATTGAGAGAAAAGCAGGATGTATTTGTGACTCAAATCATGTGCAGCGGCCATAAAGGACAACAGAGCC
GlyValPheThrThrAsnIleTrpLeuArgLeuArgGluLysGlnAspValPheCysAspSerLysLeuMetSreAlaAlaIleLysAspAsnArgAla>

3010 3020 3030 3040 3050 3060 3070 3080 3090

3100
GTCCATGCTGATATGGGTTATTTGGATAGAAAGCGCACTCAATGATACATGGAAGATAGAGAAAGCTTCTTTTCAATTGAAGTCAAAGTTGCCACTGGCCAA
ValHisAlaAspMetGlyTryTrpIleGluSerAlaLeuAsnAspThrTrpLysIleGluLysAlaSerPheIleGluValLysSerCysHisTrpPro>

3110 3120 3130 3140 3150 3160 3170 3180 3190

APPENDIX 1-continued

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

3200
AGTCACACACCCTATGGAGTAATGGAGTGTCTAGAAAGCGAGATGGTCATTCCAAGAATTTTCGCTGGACCAGTGTCAACATAATAACAGACCAGGCTA
LysSerHisThrLeuTrpSerAsnGlyValLeuGluSerGluMetValIleProLysAsnPheAlaGlyProValSerGlnHisAsnAsnArgProGlyTyr>

3210 3220 3230 3240 3250 3260 3270 3280 3290

3300
TTACACAAACAGCAGGACCTTGGCATCTAGGCAAGCTTGAGATGGACTTTGATTTCTGCGAAGGGACTACAGTGGTGGTAACCGAGAACTGTGGAAAC
TyrThrGlnThrAlaGlyProTrpHisLeuGlyLysLeuGluMetAspPheAspPheCysGluGlyThrThrValValValThrGluAsnCysGlyAsn>

3310 3320 3330 3340 3350 3360 3370 3380 3390

3400
AGAGGGCCCTCTTTAAGAACAACCACTGCCTCAGGAAAACCTATAACGGAATGGTGTGTGCGATCTTGCACACTACCACCCTAAGATACAGAGGTGAGG
ArgGlyProSerLeuArgThrThrThrAlaSerGlyLysLeuIleThrGluTrpCysCysArgSerCysThrLeuProProLeuArgTyrArgGlyGlu>

3410 3420 3430 3440 3450 3460 3470 3480 3490

3500
ATGGATGTTGGTACGGGATGGAAATCAGACCATTGAAAGAGAAAGAAGAAAATCTGGTCAGTTCTCTGGTTACAGCCGGACATGGGCAGATTGACAATTT
AspGlyCysTrpTyrGlyMetGluIleArgProLeuLysGluLysGluGluAsnLeuValSerSerLeuValThrAlaGlyHisGlyGlnIleAspAsnPhe>

3510 3520 3530 3540 3550 3560 3570 3580 3590

3600
CTCATTAGGAATCTTGGGAATGGCACTGTTCTTGAAGAAATGCTCAGGACTCGAGTAGGAACAAAACATGCAATATTACTCGTCGCAGTTTCTTTCTG
SerLeuGlyIleLeuGlyMetAlaLeuPheLeuGluGluMetLeuArgThrArgValGlyThrLysHisAlaIleLeuLeuValAlaValSerPheVal>

3610 3620 3630 3640 3650 3660 3670 3680 3690

3700
ACGCTAATCACAGGAACATGTCTTTTAGAGACCTGGGAAGAGTGTGGTTATGGTGGGTGCCACCATGACAGATGACATAGGCATGGGTGTGACTTATC
ThrLeuIleThrGlyAsnMetSerPheArgAspLeuGlyArgValMetValMetValGlyAlaThrMetThrAspAspIleGlyMetGlyValThrTyr>

3710 3720 3730 3740 3750 3760 3770 3780 3790

3800
TCGCTCTACTAGCAGCTTTTAGAGTCAGACCAACCTTTCAGCTGGACTGCTCTTGGAGAAAACCTGACCTCAAGGAATTAATGATGACTACCATAGGAAT
LeuAlaLeuLeuAlaAlaPheArgValArgProThrPheAlaAlaGlyLeuLeuLeuArgLysLeuThrSerLysGluLeuMetMetThrThrIleGlyIle>

3810 3820 3830 3840 3850 3860 3870 3880 3890

3900
CGTTCTTCTCTCCAGAGTAGCATAACAGAGACCATTCTTGAAGTACCGACGCGTTAGCTCTAGGCATGATGGTCTCAAGATGGTGAGAAACATGGAA
ValLeuLeuSerGlnSerSerIleProGluThrIleLeuGluLeuThrAspAlaLeuAlaLeuGlyMetMetValLeuLysMetValArgAsnMetGlu>

3910 3920 3930 3940 3950 3960 3970 3980 3990

4000
AAATATCAGCTGGCAGTGACCATCATGGCTATTTTGTGCGTCCCAAATGCTGTGATATTACAGAACGCATGGAAAGTGGAGTTGCACAATATTGGCAGTGG
LysTyrGlnLeuAlaValThrIleMetAlaIleLeuCysValProAsnAlaValIleLeuGlnAsnAlaTrpLysValSerCysThrIleLeuAlaVal>

4010 4020 4030 4040 4050 4060 4070 4080 4090

4100
TGCTGTTTTCCCCCTGCTCTTAACATCCTCAACAGAAAGCGGACTGGATAACCATTAGCGTTGACGATCAAAGGTCTTAATCCAACAGCCATTTTTCT
ValSerValSerProLeuLeuLeuThrSerSerGlnGlnLysAlaAspTrpIleProLeuAlaLeuThrIleLysGlyLeuAsnProThrAlaIlePheLeu>

4110 4120 4130 4140 4150 4160 4170 4180 4190

4200
AACAAACCTCTCAAGAACCAACAAGAAAAGGAGCTGGCCTTTAAATGAGGCCATCATGGCGGTTGGGATGGTGAGTATCTTGGCCAGCTCTCTTTAAAG
ThrThrLeuSerArgThrAsnLysLysArgSerTrpProLeuAsnGluAlaIleMetAlaValGlyMetValSerIleLeuAlaSerSerLeuLeuLys>

4210 4220 4230 4240 4250 4260 4270 4280 4290

4300
AATGACATCCCCATGACAGGACCATTAGTGGCTGGAGGGCTCCTTACTGTGTGCTACGTGCTAACTGGGCGGTCAGCCGATCTGGAATTAGAGAGAGCTA
AsnAspIleProMetThrGlyProLeuValAlaGlyGlyLeuLeuThrValCysTyrValLeuThrGlyArgSerAlaAspLeuGluLeuGluArgAla>

4310 4320 4330 4340 4350 4360 4370 4380 4390

4400
CCGATGTCAAATGGGATGACCAGGCAGAGATATCAGGTAGCAGTCCAATCCTGTCAAGAACAATATCAGAAGATGGCAGCATGTCAATAAAGAATGAAGA
ThrAspValLysTrpAspAspGlnAlaGluIleSerGlySerSerProIleLeuSerIleThrIleSerGluAspGlySerMetSerIleLysAsnGluGlu>

4410 4420 4430 4440 4450 4460 4470 4480 4490

4500
GGAAGAGCAAACACTGACTATACTCATTAGAACAGGATTGCTTGTGATCTCAGGACTCTTTCCGGTATCAATACCAATTACAGCAGCAGCATGGTATCTG
GluGluGlnThrLeuTheIleLeuIleArgThrGlyLeuLeuValIleSerGlyLeuPheProValSerIleProIleThrAlaAlaAlaTrpTyrLeu>

4510 4520 4530 4540 4550 4560 4570 4580 4590

4600
TGGGAAGTAAAGAAACAACGGGCTGGAGTGTGGGATGTCCCTCACCACCACCGTGGGAAAAGCTGAATTGGAAGATGGAGCCTACAGAATCAAGC
TrpGluValLysLysGlnArgAlaGlyValLeuTrpAspValProSerProProProValGlyLysAlaGluLeuGluAspGlyAlaTyrArgIleLys>

4610 4620 4630 4640 4650 4660 4670 4680 4690

APPENDIX 1-continued

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

4700
 AAAAAGGAATCCTTGGATATTTCCAGATCGGAGCTGGAGTTTACAAAGAAGGAACATTTACACAATGTGGCACGTCACACGTGGCGCTGCCTAATGCA
 GlnLysGlyIleLeuGlyTyrSerGlnIleGlyAlaGlyValTyrLysGluGlyThrPheHisThrMetTrpHisValThrArgGlyAlaValLeuMetHis>

4710 4720 4730 4740 4750 4760 4770 4780 4790

4800
 TAAGGGGAAGAGGATTGAACCATCATGGGCGGACGTCAGAAAGACTTAATATCATATGGAGGAGGTTGGAAGCTAGAAGGAGAATGGAAAGAAGGAGAA
 LysGlyLysArgIleGluProSerTrpAlaAspValLysLysAspLeuIleSerTyrGlyGlyGlyTrpLysLeuGluGlyGluTrpLysGluGlyGlu>

4810 4820 4830 4840 4850 4860 4870 4880 4890

4900
 GAAGTCCAGGTCTTGGCATTGGAGCCAGGGAAAAATCCAAGAGCCGTCCAAACAAAGCCTGGCCTTTTTAGAACCAACACTGGAACCATAGGTGCCGTAT
 GluValGlnValLeuAlaLeuGluProGlyLysAsnProArgAlaValGlnThrLysProGlyLeuPheArgThrAsnThrGlyThrIleGlyAlaVal>

4910 4920 4930 4940 4950 4960 4970 4980 4990

5000
 CTCTGGACTTTTCCCCTGGGACGTCAGGATCTCCAATCGTCGACAAAAAGGAAAAGTTGTAGGTCTCTATGGCAATGGTGTGTTACAAGGAGTGGAGC
 SerLeuAspPheSerProGlyThrSerGlySerProIleValAspLysLysGlyLysValValGlyLeuTyrGlyAsnGlyValValThrArgSerGlyAla>

5010 5020 5030 5040 5050 5060 5070 5080 5090

5100
 ATATGTGAGTGCCATAGCTCAGACTGAAAAAGCATTGAAGACAATCCAGAGATTGAAGATGACATCTTTCGAAAGAGAAGATTGACTATCATGGATCTC
 TyrValSerAlaIleAlaGlnThrGluLysSerIleGluAspAsnProGluIleGluAspAspIlePheArgLysArgArgLeuThrIleMetAspLeu>

5110 5120 5130 5140 5150 5160 5170 5180 5190

5200
 CACCCAGGAGCAGGAAAGACAAAGAGATACCTCCCGCCATAGTCAGAGAGGCCATAAAAAGAGGCTTGAGAACACTAATCCTAGCCCCACTAGAGTCG
 HisProGlyAlaGlyLysThrLysArgTyrLeuProAlaIleValArgGluAlaIleLysArgGlyLeuArgThrLeuIleLeuAlaProThrArgVal>

5210 5220 5230 5240 5250 5260 5270 5280 5290

5300
 TGGCAGCTGAAATGGAGGAAGCCCTTAGAGGACTTCCAATAAGATACAAACTCCAGCTATCAGGGCTGAGCACACCGGGCGGGAGATTGTAGACTTAAT
 ValAlaAlaGluMetGluGluAlaLeuArgGlyLeuProIleArgTyrGlnThrProAlaIleArgAlaGluHisThrGlyArgGluIleValAspLeuMet>

5310 5320 5330 5340 5350 5360 5370 5380 5390

5400
 GTGTCATGCCACATTTACCATGAGGCTGCTATCACCAATCAGGGTGCCAAATTACAACCTGATCATCATGGACGAAGCCATTTTACAGATCCAGCAAGC
 CysHisAlaThrPheThrMetArgLeuLeuSerProIleArgValProAsnTyrAsnLeuIleIleMetAspGluAlaHisPheThrAspProAlaSer>

5410 5420 5430 5440 5450 5460 5470 5480 5490

5500
 ATAGCAGCTAGGGGATACATCTCAACTCGAGTGGAGATGGGGGAGGCAGCTGGAATTTTTATGACAGCCACTCCTCCGGGTAGTAGAGATCCATTTCTC
 IleAlaAlaArgGlyTyrIleSerThrArgValGluMetGlyGluAlaAlaGlyIlePheMetThrAlaThrProProGlySerArgAspProPhePro>

5510 5520 5530 5540 5550 5560 5570 5580 5590

5600
 AGAGCAATGCACCAATTATGGACGAAGAAAGAGAAATTCGGAACGTTTCATGGAACCTGCGGCACGAGTGGGTACGGATTTTAAAGGAAAGACTGTCTG
 GlnSerAsnAlaProIleMetAspGluGluArgGluIleProGluArgSerTrpAsnSerGlyHisGluTrpValThrAspPheLysGlyLysThrValTrp>

5610 5620 5630 5640 5650 5660 5670 5680 5690

5700
 GTTGTTCACCAAGCATAAAAACCGAAATGACATAGCAGCCTGCCTGAGAAAGAAATGGAAAGAGGGTGATACAACCTCAGTAGGAAGACCTTTGATTCTGAA
 PheValProSerIleLysThrGlyAsnAspIleAlaAlaCysLeuArgLysAsnGlyLysArgValIleGlnLeuSerArgLysThrPheAspSerGlu>

5710 5720 5730 5740 5750 5760 5770 5780 5790

5800
 TATGTCAAGACTAGAACCAATGACTGGGATTTCTGTTTACAACCTGACATCTCGGAAATGGGCGCCAACTTTAAAGCTGAGAGGGTCATAGACCCAGAC
 TyrValLysThrArgThrAsnAspTrpAspPheValValThrThrAspIleSerGluMetGlyAlaAsnPheLysAlaGluArgValIleAspProArg>

5810 5820 5830 5840 5850 5860 5870 5880 5890

5900
 GCTGCATGAAACAGTTATATTGACAGACGGCGAAGAGCGGGTGATTCTGGCAGGACCCATGCCAGTGACCCACTCTAGTGCAGCACAAAGAAGAGGGAG
 ArgCysMetLysProValIleLeuThrAspGlyGluGluArgValIleLeuAlaGluProMetProValThrHisSerSerAlaAlaGlnArgArgGlyArg>

5910 5920 5930 5940 5950 5960 5970 5980 5990

6000
 AATAGGAAGGAATCCAAGGAATGAAAATGATCAATATATATATATATGGGGGAACCACTGGAAAATGATGAAGACTGTGCGCACCTGGAAGGAAGCTAAGATG
 IleGlyArgAsnProArgAsnGluAsnAspGlnTyrIleTyrMetGlyGluProLeuGluAsnAspGluAspCysAlaHisTrpLysGluAlaLysMet>

6010 6020 6030 6040 6050 6060 6070 6080 6090

6100
 CTCTAGATAATATCAACACACCTGAAGGAATCATTCCAGCTTGTTTCGAGCCAGAGCGTGAAAAGGTGGATGCCATTGACGGTGAATATCGCTTGAGAG
 LeuLeuAspAsnIleAsnThrProGluGlyIleIleProSerLeuPheGluProGluArgGluLysValAspAlaIleAspGlyGluTyrArgLeuArg>

6110 6120 6130 6140 6150 6160 6170 6180 6190

APPENDIX 1-continued

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

6200
GAGAAGCACGGAAAACCTTTGTGGACCTAATGAGAAGAGGAGACCTACCAGTCTGGTTGGCTTATAAAGTGGCAGCTGAAGGTATCAACTACGCAGACAG
GlyGluAlaArgLysThrPheValAspLeuMetArgArgGlyAspLeuProValTrpLeuAlaTyrLysValAlaAlaGluGlyIleAsnTyrAlaAspArg>

6210 6220 6230 6240 6250 6260 6270 6280 6290

6300
AAGATGGTGTGGTGGACCGAACCAGAAACAATCAAATCTTGGGAAGAAAATGTGGAAGTGGAAATCTGGACAAAGGAAGGGAAAGGAAAAATTGAAACCT
ArgTrpCysPheAspGlyThrArgAsnAsnGlnIleLeuGluGluAsnValGluValGluIleTrpThrLysGluGlyGluArgLysLysLeuLysPro>

6310 6320 6330 6340 6350 6360 6370 6380 6390

6400
AGATGGTTAGATGCTAGGATCTACTCCGACCCACTGGCGCTAAAAGAGTTCAAGGAATTTGCAGCCGGAAGAAAGTCCCTAACCTGAACCTAATTACAG
ArgTrpLeuAspAlaArgIleTyrSerAspProLeuAlaLeuLysGluPheLysGluPheAlaAlaGlyArgLysSerLeuThrLeuAsnLeuIleThr>

6410 6420 6430 6440 6450 6460 6470 6480 6490

6500
AGATGGGCAGACTCCCAACTTTTATGACTCAGAAGGCCAGAGATGCACTAGACAACCTGGCGGTGCTGCACACGGCTGAAGCGGGTGGAAAGGCATACAA
GluMetGlyArgLeuProThrPheMetThrGlnLysAlaArgAspAlaLeuAspAsnLeuAlaValLeuHisThrAlaGluAlaGlyGlyLysAlaTyrAsn>

6510 6520 6530 6540 6550 6560 6570 6580 6590

6600
TCATGCTCTCAGTGAATTACCGGAGACCCTGGAGACATTGCTTTTGTGACACTGTTGGCCACAGTCACGGGAGGAATCTTCCTATTCCTGATGAGCGGA
HisAlaLeuSerGluLeuProGluThrLeuGluThrLeuLeuLeuLeuThrLeuLeuAlaThrValThrGlyGlyIlePheLeuPheLeuMetSerGly>

6610 6620 6630 6640 6650 6660 6670 6680 6690

6700
AGGGGTATGGGGAAGATGACCTGGGAATGTGCTGCATAATCACGGCCAGCATCCTCTTATGGTATGCACAAATACAGCCACATTGGATAGCAGCCTCAA
ArgGlyMetGlyLysMetThrLeuGlyMetCysCysIleIleThrAlaSerIleLeuLeuTryTyrAlaGlnIleGlnProHisTrpIleAlaAlaSer>

6710 6720 6730 6740 6750 6760 6770 6780 6790

6800
TAATATTGGAGTTCTTTCTCATAGTCTTGCTCATTCCAGAACCAGAAAAGCAGAGGACACCTCAGGATAATCAATTGACTTATGTCATCATAGCCATCCT
IleIleLeuGluPhePheLeuIleValLeuLeuIleProGluProGluLysGlnArgThrProGlnAspAsnGlnLeuThrTyrValIleIleAlaIleLeu>

6810 6820 6830 6840 6850 6860 6870 6880 6890

6900
CACAGTGGTGGCCGCAACCATGGCAAACGAAATGGGTTTTCTGGAAAAACAAGAAAGACCTCGGACTGGGAAACATTGCAACTCAGCAACCTGAGAGC
ThrValValAlaAlaThrMetAlaAsnGluMetGlyPheLeuGluLysThrLysLysAspLeuGlyLeuGlyAsnIleAlaThrGlnGlnProGluSer>

6910 6920 6930 6940 6950 6960 6970 6980 6990

7000
AACATTCTGGACATAGATCTACGTCCTGCATCAGCATGGACGTTGTATGCCGTGGCTACAACATTTATCACACCAATGTTGAGACATAGCATTGAAAATT
AsnIleLeuAspIleAspLeuArgProAlaSerAlaTrpThrLeuTyrAlaValAlaThrThrPheIleThrProMetLeuArgHisSerIleGluAsn>

7010 7020 7030 7040 7050 7060 7070 7080 7090

7100
CCTCAGTAAATGTGTCCCTAACAGCCATAGCTAACCAAGCCACAGTGCTAATGGGTCTCGGAAAAGGATGGCCATTGTCAAAGATGGACATTGGAGTTCC
SerSerValAsnValSerLeuThrAlaIleAlaAsnGlnAlaThrValLeuMetGlyLeuGlyLysGlyTrpProLeuSerLysMetAspIleGlyValPro>

7110 7120 7130 7140 7150 7160 7170 7180 7190

7200
CCTCCTTGCTATTGGGTGTTACTCACAAGTCAACCCTATAACCCTCACAGCGGCTCTTCTTTTATTGGTAGCACATTATGCCATCATAGGACCGGGACTT
LeuLeuAlaIleGlyCysTyrSerGlnValAsnProIleThrLeuThrAlaAlaLeuLeuLeuLeuValAlaHisTyrAlaIleIleGlyProGlyLeu>

7210 7220 7230 7240 7250 7260 7270 7280 7290

7300
CAAGCCAAAGCAACTAGAGAAGCTCAGAAAAGAGCAGCAGCGGGCATCATGAAAAACCAACTGTGGATGGAATAACAGTGATAGATCTAGATCCAATAC
GlnAlaLysAlaThrArgGluAlaGlnLysArgAlaAlaAlaGlyIleMetLysAsnProThrValAspGlyIleThrValIleAspLeuAspProIle>

7310 7320 7330 7340 7350 7360 7370 7380 7390

7400
CCTATGATCCAAAGTTTGAAGCAGTTGGGACAAGTAATGCTCCTAGTCTCTGCGTGACCAAGTGCTGATGATGAGGACTACGTGGGCTTTGTGTGA
ProTyrAspProLysPheGluLysGlnLeuGlyGlnValMetLeuLeuValLeuCysValThrGlnValLeuMetMetArgThrThrTrpAlaLeuCysGlu>

7410 7420 7430 7440 7450 7460 7470 7480 7490

7500
AGCCTTAACCTAGCAACTGGACCCGTGTCCACATTGTGGGAAGGAAATCCAGGGAGATTCTGGAACACAACCATTGCAGTGTCAATGGCAAACATCTTT
AlaLeuThrLeuAlaThrGlyProValSerThrLeuTrpGluGlyAsnProGlyArgPheTrpAsnThrThrIleAlaValSerMetAlaAsnIlePhe>

7510 7520 7530 7540 7550 7560 7570 7580 7590

7600
AGAGGGAGTTACCTGGCTGGAGCTGGACTTCTCTTTTCTATCATGAAGAACAACCAAGCAGCAGAGAAGAGGAACTGGCAATATAGGAGAAACGTTAGGAG
ArgGlySerTyrLeuAlaGlyAlaGlyLeuLeuPheSerIleMetLysAsnThrThrSerThrArgArgGlyThrGlyAsnIleGlyGluThrLeuGly>

7610 7620 7630 7640 7650 7660 7670 7680 7690

APPENDIX 1-continued

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

7700
AGAAATGGAAAAGCAGACTGAACGCATTGGGGAAAAGTGAATTCCAGATCTACAAAAAAGTGAATTCAAGAAGTGGACAGAACCTTAGCAAAAGAAGG
GluLysTrpLysSerArgLeuAsnAlaLeuGlyLysSerGluPheGlnIleTyrLysLysSerGlyIleGlnGluValAspArgThrLeuAlaLysGluGly>

7710 7720 7730 7740 7750 7760 7770 7780 7790

7800
CATTAAGAGAGAGAAACGGATCATCACGCTGTGTCGCGAGGCTCAGCAAACTGAGATGGTTTCGTTGAAAGGAATTTGGTCACACCAGAAGGGAAAGTA
IleLysArgGlyGluThrAspHisHisAlaValSerArgGlySerAlaLysLeuArgTrpPheValGluArgAsnLeuValThrProGluGlyLysVal>

7810 7820 7830 7840 7850 7860 7870 7880 7890

7900
GTGACCTTGGTTGTGGCAGAGGGGGCTGGTCATACTATTGTGGAGGATTAAGAATGTAAGAGAAGTTAAAGGCTTAACAAAAGGAGGACCAGGACACG
ValAspLeuGlyCysGlyArgGlyGlyTrpSerTyrTyrCysGlyGlyLeuLysAsnValArgGluValLysGlyLeuThrLysGlyGlyProGlyHis>

7910 7920 7930 7940 7950 7960 7970 7980 7990

8000
AAGAACCTATCCCTATGTCAACATATGGGTGGAATCTAGTACGCTTACAGAGCGGAGTTGATGTTTTTTTTTGTCCACCAGAGAAGTGTGACACATTGTT
GluGluProIleProMetSerThrTyrGlyTrpAsnLeuValArgLeuGlnSerGlyValAspValPhePheValProProGluLysCysAspThrLeuLeu>

8010 8020 8030 8040 8050 8060 8070 8080 8090

8100
GTGTGACATAGGGGAATCATCAACAAATCCACGGTAGAAGCGGGACGAACACTCAGAGTCTCAACCTAGTGGAAAATTGGCTGAACAATAACACCCAA
CysAspIleGlyGluSerSerProAsnProThrValGluAlaGlyArgThrLeuArgValLeuAsnLeuValGluAsnTrpLeuAsnAsnAsnThrGln>

8110 8120 8130 8140 8150 8160 8170 8180 8190

8200
TTTTGCGTAAAGGTTCTTAACCGTACATGCCCTCAGTCATTGAAAGAATGGAAACCTTACAACGGAAATACGGAGGAGCCTTGGTGAGAAATCCACTCT
PheCysValLysValLeuAsnProTyrMetProSerValIleGluArgMetGluThrLeuGlnArgLysTyrGlyGlyAlaLeuValArgAsnProLeu>

8210 8220 8230 8240 8250 8260 8270 8280 8290

8300
CACGGAATCCACACATGAGATGTACTGGGTGTCCAATGCTTCCGGAACATAGTGTGCATCAGTGAACATGATTTCAAGAATGCTGATCAACAGATTAC
SerArgAsnSerThrHisGluMetTyrTrpValSerAsnAlaSerGlyAsnIleValSerSerValAsnMetIleSerArgMetLeuIleAsnArgPheThr>

8310 8320 8330 8340 8350 8360 8370 8380 8390

8400
TATGAGACACAAGAAGGCCACCTATGAGCCAGATGTCGACCTCGGAAGCGGAACCCGCAATATTGGAATTGAAAGTGAGACACCGAACCTAGACATAATT
MetArgHisLysLysAlaThrTyrGluProAspValAspLeuGlySerGlyThrArgAsnIleGlyIleGluSerGluThrProAsnLeuAspIleIle>

8410 8420 8430 8440 8450 8460 8470 8480 8490

8500
GGGAAAAGAAATAGAAAAATAAAACAAGAGCATGAAACGTCATGGCACTATGATCAAGACCACCCATACAAAACATGGGCTTACCATGGCAGCTATGAAA
GlyLysArgIleGluLysIleLysGlnGluHisGluThrSerTrpHisTyrAspGlnAspHisProTyrLysThrTrpAlaTyrHisGlySerTyrGlu>

8510 8520 8530 8540 8550 8560 8570 8580 8590

8600
CAAAACAGACTGGATCAGCATCATCCATGGTGAACGGAGTAGTCAGATTGCTGACAAAACCTGGGACGTTGTTCCAATGGTGACACAGATGGCAATGAC
ThrLysGlnThrGlySerAlaSerSerMetValAsnGlyValValArgLeuLeuThrLysProTrpAspValValProMetValThrGlnMetAlaMetThr>

8610 8620 8630 8640 8650 8660 8670 8680 8690

8700
AGACACAACCTCTTTTGGACAACAGCGCTCTTCAAAGAGAAGGTGGATACGAGAACCACCAAGAACCAAAAGAGGACAAAAAACTAATGAAAATCACG
AspThrThrPropheGlyGlnGlnArgValPheLysGluLysValAspThrArgThrGlnGluProLysGluGlyThrLysLysLeuMetLysIleThr>

8710 8720 8730 8740 8750 8760 8770 8780 8790

8800
GCAGAGTGGCTCTGAAAGAACTAGGAAAGAAAAGACACCTAGAATGTGTACCAGAGAAGAATTCACAAAAAAGGTGAGAAGCAATGCAGCCTTGGGGG
AlaGluTrpLeuTrpLysGluLeuGlyLysLysLysThrProArgMetCysThrArgGluGluPheThrLysLysValArgSerAsnAlaAlaLeuGly>

8810 8820 8830 8840 8850 8860 8870 8880 8890

8900
CCATATTCACCGATGAGAACAAGTGGAAATCGGCGCTGAAGCCGTTGAAGATAGTAGGTTTTGGGAGCTGGTTGACAAGGAAAGGAACTCCATCTTGA
AlaIlePheThrAspGluAsnLysTrpLysSerAlaArgGluAlaValGluAspSerArgPheTrpGluLeuValAspLysGluArgAsnLeuHisLeuGlu>

8910 8920 8930 8940 8950 8960 8970 8980 8990

9000
AGGGAAATGTGAAACATGTGTATACAACATGATGGGGAAAAGAGAGAAAAAAGTAGGAGAGTTTGGTAAAGCAAAGGCAGCAGAGCCATATGGTACATG
GlyLysCysGluThrCysValTyrAsnMetMetGlyLysArgGluLysLysLeuGlyGluPheGlyLysAlaLysGlySerArgAlaIleTrpTyrMet>

9010 9020 9030 9040 9050 9060 9070 9080 9090

9100
TGGCTCGGAGCACGCTTCTTAGAGTTTGAAGCCCTAGGATTTTTGAATGAAGACCATTTGGTTCTCCAGAGAGAACTCCCTGAGTGGAGTGAAGGAGAAG
TrpLeuGlyAlaArgPheLeuGluPheGluAlaLeuGlyPheLeuAsnGluAspHisTrpPheSerArgGluAsnSerLeuSerGlyValGluGlyGlu>

9110 9120 9130 9140 9150 9160 9170 9180 9190

9200
GGCTGCATAAGCTAGGTTACATCTTAAGAGAGGTGAGCAAGAAAGAGGAGGCAATGTATGCCGATGACACCGCAGGCTGGGACACAAGAATCACAAAT
GlyLeuHisLysLeuGlyTyrIleLeuArgGluValSerLysLysGluGlyGlyAlaMetTyrAlaAspAspThrAlaGlyTrpAspThrArgIleThrIle>

APPENDIX 1-continued

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

9210 9220 9230 9240 9250 9260 9270 9280 9290
9300
AGAGGATTTGAAAAATGAAGAAATGATAACGAACCACATGGCAGGAGAACACAAGAACTTGCCGAGGCCATTTTTAAATTGACGTACCAAAACAAGGTG
GluAspLeuLysAsnGluGluMetIleThrAsnHisMetAlaGlyGluHisLysLysLeuAlaGluAlaIlePheLysLeuThrTyrGlnAsnLysVal>

9310 9320 9330 9340 9350 9360 9370 9380 9390
9400
GTGCGTGTGCAAAGACCAACACCAAGAGGCACAGTAATGGACATCATATCGAGAAGAGACCAAGGGGTAGTGGACAAGTTGGCACCTATGGCCTCAACA
ValArgValGlnArgProThrProArgGlyThrValMetAspIleIleSerArgArgAspGlnArgGlySerGlyGlnValGlyThrTyrGlyLeuAsn>

9410 9420 9430 9440 9450 9460 9470 9480 9490
9500
CTTTCACCAACATGGAAGCACAATAATTAGGCAAATGGAGGGGAAGGAATCTTCAAAGCATCCAGCACTTGACAGCCTCAGAAGAAATCGCTGTGCA
ThrPheThrAsnMetGluAlaGlnLeuIleArgGlnMetGluGlyGluGlyIlePheLysSerIleGlnHisLeuThrAlaSerGluGluIleAlaValGln>

9510 9520 9530 9540 9550 9560 9570 9580 9590
9600
AGATTGGCTAGTAAGAGTAGGGCGTGAAAGGTTGTCAAGAATGGCCATCAGTGGAGATGATTGTGTGTGAAACCTTTAGATGATAGATTTGCAAGAGCT
AspTrpLeuValArgValGlyArgGluArgLeuSerArgMetAlaIleSerGlyAspAspCysValValLysProLeuAspAspArgPheAlaArgAla>

9610 9620 9630 9640 9650 9660 9670 9680 9690
9700
CTAACAGCTCTAAATGACATGGGAAAGGTTAGGAAGGACATACAGCAATGGGAGCCCTCAAGAGGATGGAACGACTGGACGCAGGTGCCCTTCTGTTCAC
LeuThrAlaLeuAsnAspMetGlyLysValArgLysAspIleGlnGlnTrpGluProSerArgGlyTrpAsnAspTrpThrGlnValProPheCysSer>

9710 9720 9730 9740 9750 9760 9770 9780 9790
9800
ACCATTTTACGAGTTAATTATGAAAGATGGTCGCACACTCGTAGTTCCATGCAGAAACCAAGATGAATTGATCGGCAGAGCCGAATTTCCAGGGAGC
HisHisPheHisGluLeuIleMetLysAspGlyArgThrLeuValValProCysArgAsnGlnAspGluLeuIleGlyArgAlaArgIleSerGlnGlyAla>

9810 9820 9830 9840 9850 9860 9870 9880 9890
9900
TGGGTGGTCTTTACGGGAGACGGCCTGTTTGGGGAAGTCTTACGCCCAAATGTGGAGCTTGATGTACTTCCACAGACGTGATCTCAGGCTAGCGGCAAAT
GlyTrpSerLeuArgGluThrAlaCysLeuGlyLysSerTyrAlaGlnMetTrpSerLeuMetTyrPheHisArgArgAspLeuArgLeuAlaAlaAsn>

9910 9920 9930 9940 9950 9960 9970 9980 9990
10000
GCCATCTGCTCGGCAGTCCCATCACACTGGATTCCAACAAGCCGGACAACCTGGTCCATACACGCCAGCCATGAATGGATGACGACGGAAGACATGTTGA
AlaIleCysSerAlaValProSerHisTrpIleProThrSerArgThrThrTrpSerIleHisAlaSerHisGluTrpMetThrThrGluAspMetLeu>

10010 10020 10030 10040 10050 10060 10070 10080 10090
10100
CAGTTTGGAAACAGAGTGTGGATCCTAGAAAATCCATGGATGGAAGACAAAACCTCCAGTGGAAATCATGGGAGGAAATCCCATACCTGGGAAAAAGAGAAGA
ThrValTrpAsnArgValTrpIleLeuGluAsnProTrpMetGluAspLysThrProValGluSerTrpGluGluIleProTyrLeuGlyLysArgGluAsp>

10110 10120 10130 10140 10150 10160 10170 10180 10190
10200
CCAATGGTGCAGCTCGCTGATTGGGCTGACAAGCAGAGCCACCTGGGCGAAGAATATCCAGACAGCAATAAACCAAGTCAGATCCCTCATTTGGCAATGAG
GlnTrpCysGlySerLeuIleGlyLeuThrSerArgAlaThrTrpAlaLysAsnIleGlnThrAlaIleAsnGlnValArgSerLeuIleGlyAsnGlu>

10210 10220 10230 10240 10250 10260 10270 10280 10290
10300
GAATACACAGATTACATGCCATCCATGAAAAGATTGAGAAGAGAAGAGGAAGAGGCAGGAGTTTTGTGGTAGAAAAACATGAAACAAAACAGAAGTCAGG
GluTyrThrAspTyrMetProSerMetLysArgPheArgArgGluGluGluGluAlaGlyValLeuTrp***>

10310 10320 10330 10340 10350 10360 10370 10380 10390
10400
TCGGATTAAGCCATAGTACGGGAAAAACTATGCTACCTGTGAGCCCCGTCAAGGACGTTAAAAGAAGTCAGGCCATTTTGTGATGCCATAGCTTGAGCAAA

10410 10420 10430 10440 10450 10460 10470 10480 10490
10500
CTGTGCAGCCTGTAGCTCCACCTGAGAAGGTGTAATAAATCCGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAGGA

10510 10520 10530 10540 10550 10560 10570 10580 10590
10600
GACCCCTCCCTTACAGATCGCAGCAACAATGGGGGCCAAGGTGAGATGAAGCTGTAGTCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCAAAA

10610 10620 10630 10640 10650 10660 10670 10680 10690
10700
CAAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGGACGCCAGAAAATGGAATGGTGTCTGTTGA

10710 10720 10730 10740 10750 10760 10770 10780 10790
10800
ATCAACAGGTTCTGGTACCGGTAGGCATCGTGGTGTACGCTCGTCTTGGTATGGCTTCATTGAGCTCCGGTCCCAACGATCAAGGCGAGTTACATG

10810 10820 10830 10840 10850 10860 10870 10880 10890
10900

APPENDIX 1-continued

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

ATCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTATCACTCATGGTTATGGCAGCA

10910 10920 10930 10940 10950 10960 10970 10980 10990

11000
CTGCATAATTCTTACTGTGCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATCTGAGAATAGTGTATGCGGCGACCGA

11010 11020 11030 11040 11050 11060 11070 11080 11090

11100
GTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTC

11110 11120 11130 11140 11150 11160 11170 11180 11190

11200
AAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTACTTTTACCAGCGTTTCTGGGTGAGCA

11210 11220 11230 11240 11250 11260 11270 11280 11290

11300
AAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTTCTCTTTTCAATATTATTGAAGCATTATC

11310 11320 11330 11340 11350 11360 11370 11380 11390

11400
AGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGGGTTCCGCGCACATTTCCCGAAAAGTGCCACCTGACGT

11410 11420 11430 11440 11450 11460 11470 11480 11490

11500
CTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCGA

11510 11520 11530 11540 11550 11560 11570 11580 11590

11600
TAAGCTTTAATGCGGTAGTTTATCACAGTTAAATGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAAATGCGCTCATCGTCATCCTCGGCACCGTC

11610 11620 11630 11640 11650 11660 11670 11680 11690

11700
ACCCTGGATGCTGTAGGCATAGGCTTGTTATGCCGGTACTGCCGGGCCCTTTCGGGATATCGTCCATTCGACAGCATCGCCAGTCACTATGGCGTGC

11710 11720 11730 11740 11750 11760 11770 11780 11790

11800
TGCTGGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTGGCCGCCGCCAGTCTGCTCGCTTCTGCTACT

11810 11820 11830 11840 11850 11860 11870 11880 11890

11900
TGGAGCCACTATCGACTACGCGATCATGGCGACCACCCGTCCTGTGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCCACAGGTGCG

11910 11920 11930 11940 11950 11960 11970 11980 11990

12000
GTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTCGGCGTGGGTATGGTGGCAGGCC

12010 12020 12030 12040 12050 12060 12070 12080 12090

12100
CCGTGGCCGGGGACTGTTGGGCGCCATCTCCTTGATGCACCATTCCTTGCGGCGGGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCTAAT

12110 12120 12130 12140 12150 12160 12170 12180 12190

12200
GCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGGGGCATGACTATCGTCGCCGCA

12210 12220 12230 12240 12250 12260 12270 12280 12290

12300
CTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCAATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGA

12310 12320 12330 12340 12350 12360 12370 12380 12390

12400
TCGGCCTGTCGCTTTCGGTATTCGGAATCTTGACGCCCTCGTCAAGCCTTCGTCCTACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTAT

12410 12420 12430 12440 12450 12460 12470 12480 12490

12500
CGCCGGCATGGCGCCGACGCGCTGGGCTACGCTTGTGCTGGCGTTTCGCGACGCGAGGCTGGATGGCCTTCCCATTATGATTCTTCTCGCTTCCGGCGGC

12510 12520 12530 12540 12550 12560 12570 12580 12590

12600
ATCGGGATGCCCGCTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCTTACCAGCCTAACTT

12610 12620 12630 12640 12650 12660 12670 12680 12690

12700
CGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTG

12710 12720 12730 12740 12750 12760 12770 12780 12790

12800
CCTCCC CGGTTGCGTTCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTACCACCTCAAGAATTGGAG

APPENDIX 1-continued

| Nucleotide and amino acid sequence of DEN2 (Tonga/74) cDNA plasmid p2 | | | | | | | | | |
|---|--|-------|-------|-------|-------|-------|-------|-------|--|
| 12810 | 12820 | 12830 | 12840 | 12850 | 12860 | 12870 | 12880 | 12890 | |
| 12900 | CCAATCAATTCTTGC GGAGAACTGTGAATGCGCAAACCAACCCCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTC | | | | | | | | |
| 12910 | 12920 | 12930 | 12940 | 12950 | 12960 | 12970 | 12980 | 12990 | |
| 13000 | GGGCAGCGTTGGGTCTTGGCCACGGGTGCGCATGATCGTGCTCCTGTCTGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTTACTGGTTAGCAGAATGAA | | | | | | | | |
| 13010 | 13020 | 13030 | 13040 | 13050 | 13060 | 13070 | 13080 | 13090 | |
| 13100 | TCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTC | | | | | | | | |
| 13110 | 13120 | 13130 | 13140 | 13150 | 13160 | 13170 | 13180 | 13190 | |
| 13200 | TGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACGAA | | | | | | | | |
| 13210 | 13220 | 13230 | 13240 | 13250 | 13260 | 13270 | 13280 | 13290 | |
| 13300 | GCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCATAACGCCAGTTGTTTACCCTCACACGTTCCAGTAACCGGCATGTTTCATCA | | | | | | | | |
| 13310 | 13320 | 13330 | 13340 | 13350 | 13360 | 13370 | 13380 | 13390 | |
| 13400 | TCAGTAACCCGTATCGTGAGCATCCTCTCTCGTTTCATCGGTATCATTACCCCATGAACAGAAATCCCCCTTACACGGAGGCATCAGTGACCAAACAGG | | | | | | | | |
| 13410 | 13420 | 13430 | 13440 | 13450 | 13460 | 13470 | 13480 | 13490 | |
| 13500 | AAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTG | | | | | | | | |
| 13510 | 13520 | 13530 | 13540 | 13550 | 13560 | 13570 | 13580 | 13590 | |
| 13600 | TGAATCGCTTACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACG | | | | | | | | |
| 13610 | 13620 | 13630 | 13640 | 13650 | 13660 | 13670 | 13680 | 13690 | |
| 13700 | GTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGGGGCGCAGCCATGACCCAGTCAC | | | | | | | | |
| 13710 | 13720 | 13730 | 13740 | 13750 | 13760 | 13770 | 13780 | 13790 | |
| 13800 | GTAGCGATAGCGGAGTGATACTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGACCATATGCGGTGTGAAATACCGCACAGATGCGTA | | | | | | | | |
| 13810 | 13820 | 13830 | 13840 | 13850 | 13860 | 13870 | 13880 | 13890 | |
| 13900 | AGGAGAAAATACCGCATCAGGCGCTTCCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTTCGGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGG | | | | | | | | |
| 13910 | 13920 | 13930 | 13940 | 13950 | 13960 | 13970 | 13980 | 13990 | |
| 14000 | CGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGCCCGCTTGCT | | | | | | | | |
| 14010 | 14020 | 14030 | 14040 | 14050 | 14060 | 14070 | 14080 | 14090 | |
| 14100 | GGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCG | | | | | | | | |
| 14110 | 14120 | 14130 | 14140 | 14150 | 14160 | 14170 | 14180 | 14190 | |
| 14200 | TTTCCCCCTGGAAGCTCCCTCGTGCCTCTCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTCTCCCTTCGGAAGCGTGCGCTTTCTC | | | | | | | | |
| 14210 | 14220 | 14230 | 14240 | 14250 | 14260 | 14270 | 14280 | 14290 | |
| 14300 | ATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCCGCTCCAAGCTGGGCTGTGTGCACGAACCCCGTTCAGCCCGACCGCTGCGCCTTATC | | | | | | | | |
| 14310 | 14320 | 14330 | 14340 | 14350 | 14360 | 14370 | 14380 | 14390 | |
| 14400 | CGGTAACATATCGTCTTGTAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGT | | | | | | | | |
| 14410 | 14420 | 14430 | 14440 | 14450 | 14460 | 14470 | 14480 | 14490 | |
| 14500 | GCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAATCCAGTTACCTTCGGAAGGAG | | | | | | | | |
| 14510 | 14520 | 14530 | 14540 | 14550 | 14560 | 14570 | 14580 | 14590 | |
| 14600 | TTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACCGCGAGAAAAAAGGATCTCAAGAAGA | | | | | | | | |
| 14610 | 14620 | 14630 | 14640 | 14650 | 14660 | 14670 | 14680 | 14690 | |
| 14700 | TCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC | | | | | | | | |
| 14710 | 14720 | 14730 | 14740 | 14750 | 14760 | 14770 | 14780 | 14790 | |
| 14800 | | | | | | | | | |

APPENDIX 1-continued

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

CTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTATATATAGATAAACTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG

14810 14820 14830 14840 14850 14860 14870 14880 14890

14900
CGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGAT

14910 14920 14930 14940 14950 14960 14970 14980 14990

15000
ACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCCTCC

15010 15020 15030 15040 15050 15060 15070 15080 15090

15100
ATCCAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTAGTTCCGAGTTAATAGTTTGGCGAACGTTGTTGCCATTGCTGCAAGATCTGGCTAGCGAT

15110 15120 15130 15140 15150
GACCCGCTGATTGGTTCGCTGACCAATTTCCGGGCGCGCGATTAGGTGACACTATAG

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: E 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

APPENDIX 2

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

10 20 30 40 50 60 70 80 90 100
AGTTGTTAGTCTACGTGGACCGACAAGAACAGTTTCGACTCGGAAGCTTGCTTAACGTAGTACTGACAGTTTTTTTATTAGAGAGCAGATCTCTGATGAACMet
Asn>

110 120 130 140 150 160 170 180 190 200
AACCAACGGAAAAGACGGGAAAACCGTCTATCAATATGCTGAAACGCGTGAGAAACCGTGTGTCAACTGGATCACAGTTGGCGAAGAGATTCTCAAGAG
AsnGlnArgLysLysThrGlyLysProSerIleAsnMetLeuLysArgValArgAsnArgValSerThrGlySerGlnLeuAlaLysArgPheSerArg>

210 220 230 240 250 260 270 280 290 300
GACTGCTGAACGGCCAAGGACCAATGAAATTGGTTATGGCGTTCATAGCTTTCCTCAGATTTCTAGCCATTCCACCGACAGCAGGAGTCTGGCTAGATG
GlyLeuLeuAsnGlyGlnGlyProMetLysLeuValMetAlaPheIleAlaPheLeuArgPheLeuAlaIleProProThrAlaGlyValLeuAlaArgTrp>

310 320 330 340 350 360 370 380 390 400
GGGAACCTTTAAGAAGTCGGGGCTATTAAGGTCCTGAGAGGCTTCAAGAAGGAGATCTCAAACATGCTGAGCATTATCAACAGACGGAAAAGACATCG
GlyThrPheLysLysSerGlyAlaIleLysValLeuArgGlyPheLysLysGluIleSerAsnMetLeuSerIleIleAsnArgArgLysLysThrSer>

410 420 430 440 450 460 470 480 490 500
CTCTGTCTCATGATGATGTTACCAGCAACACTTGCTTCCACTTGACTTCACGAGATGGAAGCCGCGCATGATGTGGGGAAGAATGAAAGAGGAAAAT
LeuCysLeuMetMetLeuProAlaThrLeuAlaPheHisLeuThrSerArgAspGlyGluProArgMetIleValGlyLysAsnGluArgGlyLys>

510 520 530 540 550 560 570 580 590 600
CCCTACTTTTTAAGACAGCCTCTGGAATCAACATGTGCACACTCATAGCCATGGATTTGGGAGAGATGTGTGATGACACGGTCACCTACAAATGCCCCCT
SerLeuLeuPheLysThrAlaSerGlyIleAsnMetCysThrLeuIleAlaMetAspLeuGlyGluMetCysAspAspThrValThrTyrLysCysProLeu>

610 620 630 640 650 660 670 680 690 700
CATTACTGAAGTGGAGCCTGAAGACATTGACTGCTGGTGCAACCTTACATCGACATGGGTGACCTACGGAACGTGCAATCAAGCTGGAGAGCACAGACGC
IleThrGluValGluProGluAspIleAspCysTrpCysAsnLeuThrSerThrTrpValThrTyrGlyThrCysAsnGlnAlaGlyGluHisArgArg>

710 720 730 740 750 760 770 780 790 800
GACAAAAGATCGGTGGCGTTAGCTCCCATGTCGGCATGGGACTGGACACACGCACCCAAACCTGGATGTCGGCTGAAGGAGCTTGGAGACAGGTTCGAGA
AspLysArgSerValAlaLeuAlaProHisValGlyMetGlyLeuAspThrArgThrGlnThrTrpMetSerAlaGluGlyAlaTrpArgGlnValGlu>

810 820 830 840 850 860 870 880 890 900
AGGTAGAGACATGGGCCCTTAGGCACCCAGGGTTCACAATACTAGCCCTATTTCTTGGCCATTACATAGGCACTTCTTGGACCCAGAAAGTGGTTATTTT
LysValGluThrTrpAlaPheArgHisProGlyPheThrIleLeuAlaLeuPheLeuAlaHisTyrIleGlyThrSerLeuThrGlnLysValValIlePhe>

910 920 930 940 950 960 970 980 990 1000
CATACTACTAATGCTGGTCAACCCATCCATGACAATGAGATGCGTGGGAGTAGGAAACAGAGATTTTGTGGAAAGCCTATCAGGAGCTACGTGGGTTGAC
IleLeuLeuMetLeuValThrProSerMetThrMetArgCysValGlyValGlyAsnArgAspPheValGluGlyLeuSerGlyAlaThrTrpValAsp>

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100

APPENDIX 2-continued

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

GTGGTCTCGAGCAGGTTGGTGTGTGACTACCATGGCTAAGAACAAGCCCAGCTGGATATAGAGCTCCAGAAGACCGAGGCCACCAACTGGCGACCC
 ValValLeuGluHisGlyGlyCysValThrThrMetAlaLysAsnLysProThrLeuAspIleGluLeuGlnLysThrGluAlaThrGlnLeuAlaThr>

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 TAAGGAACTATGTATTGAGGAAAAATTACCAACGTAACAACCGACTCAAGGTGCCCCACCAAGGGGAAGCGATTTTACCTGAGGAGCAGGACCAGAA
 LeuArgLysLeuCysIleGluGlyLysIleThrAsnValThrThrAspSerArgCysProThrGlnGlyGluAlaIleLeuProGluGluGlnAspGlnAsn>

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
 CCACGTGTGCAAGCACACATACGTGGACAGAGGCTGGGGAAACGGTTGTGGTTTGTGGCAAGGGGAAGCCTGGTAACATGCGCGAAATTTCAATGTTTG
 HisValCysLysHisThrTyrValAspArgGlyTrpGlyAsnGlyCysGlyLeuPheGlyLysGlySerLeuValThrCysAlaLysPheGlnCysLeu>

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
 GAATCAATAGAGGGAAAAGTGGTGCAGCATGAGAACCCTCAAATACACCGTCATCATCACAGTGCACACAGGAGATCAACACCAGGTGGGAAATGAAACGC
 GluSerIleGluGlyLysValValGlnHisGluAsnLeuLysTyrThrValIleIleThrValHisThrGlyAspGlnHisGlnValGlyAsnGluThr>

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
 AGGGAGTCACGGCTGAGATAACACCCAGGCATCAACCGTTGAAGCCATCTTACCTGAATATGGAACCCCTGGGCTAGAATGCTCACCCACGGACAGGTTT
 GlnGlyValThrAlaGluIleThrProGlnAlaSerThrValGluAlaIleLeuProGluTyrGlyThrLeuGlyLeuGluCysSerProArgThrGlyLeu>

1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
 AGATTTCAATGAAATGATTTTGTGACAATGAAGAACAAGCATGGATGGTACATAGACAATGGTTTTTTGACCTACCTTTACCATGGACATCAGGAGCT
 AspPheAsnGluMetIleLeuLeuThrMetLysAsnLysAlaTrpMetValHisArgGlnTrpPhePheAspLeuProLeuProTrpThrSerGlyAla>

1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
 ACAACAGAAACACCAACCTGGAATAAGAAAGAGCTTCTGTGACATTCAAAAACGCACATGCAAAAAAGCAAGAAGTAGTAGTCCTTGGATCGCAAGAGG
 ThrThrGluThrProThrTrpAsnLysLysGluLeuLeuValThrPheLysAsnAlaHisAlaLysLysGlnGluValValValLeuGlySerGlnGlu>

1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
 GAGCAATGCACACAGCACTGACAGGAGCTACAGAGATCCAAACCTCAGGAGGCACAAGTATTTTTGCGGGCACTTAAAATGTAGACTCAAGATGGACAA
 GlyAlaMetHisThrAlaLeuThrGlyAlaThrGluIleGlnThrSerGlyGlyThrSerIlePheAlaGlyHisLeuLysCysArgLeuLysMetAspLys>

1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
 ATTGGAATCAAGGGGATGAGCTATGCAATGTGCTTGAATGCCTTTGTGTTGAAGAAAGAAGTCTCCGAAACGCAACATGGGACAATACTCATCAAGGTT
 LeuGluLeuLysGlyMetSerTyrAlaMetCysLeuAsnAlaPheValLeuLysLysGluValSerGluThrGlnHisGlyThrIleLeuIleLysVal>

1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
 GAGTACAAAGGGGAAGATGCACCTTGCAAGATTCCTTCTCCACGGAGGATGGACAAGGGAAAGCCACAATGGCAGACTGATCAGCTAACCCAGTGG
 GluTyrLysGlyGluAspAlaProCysLysIleProPheSerThrGluAspGlyGlnGlyLysAlaHisAsnGlyArgLeuIleThrAlaAsnProVal>

2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
 TGACCAAGAAGGAGGAGCCTGTCAATATTGAGGCAGAACCTCCTTTTGGGGAAAGCAATATAGTAATTGGAATTGGAGACAAAGCCTTAAAATCAACTG
 ValThrLysLysGluGluProValAsnIleGluAlaGluProProPheGlyGluSerAsnIleValIleGlyIleGlyAspLysAlaLeuLysIleAsnTrp>

2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
 GTACAAGAAGGGGAAGCTCGATTGGGAAGATGTTTCGAGGCCACTGCCAGAGGTGCAAGGCGCATGGCCATCTGGGAGACACAGCCTGGGACTTTGGATCA
 TyrLysLysGlySerSerIleGlyLysMetPheGluAlaThrAlaArgGlyAlaArgArgMetAlaIleLeuGlyAspThrAlaTrpAspPheGlySer>

2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
 GTAGGTGGTGTTTTAAATTCATTAGGAAAAATGGTGCACCAATATTTGGAAGTGCTTACACAGCCCTATTTAGTGGAGTCTCCTGGATAATGAAAATTG
 ValGlyGlyValLeuAsnSerLeuGlyLysMetValHisGlnIlePheGlySerAlaTyrThrAlaLeuPheSerGlyValSerTrpIleMetLysIle>

2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
 GAATAGGTGTCCTTTTAACTGGATAGGGTTGAATTCAAAAACACTAGTATGAGCTTTAGCTGCATTGTGATAGGAATCATTACACTCTATCTGGGAGC
 GlyIleGlyValLeuLeuThrTrpIleGlyLeuAsnSerLysAsnThrSerMetSerPheSerCysIleValIleGlyIleIleThrLeuTyrLeuGlyAla>

2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
 CGTGGTGAAGCTGACATGGGGTGTGTCATAAAGTGGAAAGGCAAGAACTCAAATGTGGAAGTGAATTTTCGTCACTAATGAGGTCCACACCTGGACA
 ValValGlnAlaAspMetGlyCysValIleAsnTrpLysGlyLysGluLeuLysCysGlySerGlyIlePheValThrAsnGluValHisThrTrpThr>

2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
 GAGCAATACAAATTTCAAGCAGACTCCCCAAAAGACTGGCGACAGCCATTGCAGGCGCTTGGGAGAATGGAGTGTGCGGAATCAGGTCGACAACAGAA
 GluGlnTyrLysPheGlnAlaAspSerProLysArgLeuAlaThrAlaIleAlaGlyAlaTrpGluAsnGlyValCysGlyIleArgSerThrThrArg>

2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
 TGGAGAACCTCTTGTGGAAGCAATAGCCAATGAACGAACTACATATTATGGGAAAACAACATCAAATTAACGGTAGTTGTGGGTGATATAATTGGGGT
 MetGluAsnLeuLeuTrpLysGlnIleAlaAsnGluLeuAsnTyrIleLeuTrpGluAsnAsnIleLysLeuThrValValValGlyAspIleIleGlyVal>

2710 2720 2730 2740 2750 2760 2770 2780 2790 2800
 CTTAGAGCAAGGGAAAAGAACACTAACACCACAACCCATGGAACATAAATATTCATGGAAAACATGGGGAAAGGCGAAGATAGTGACAGCTGAAACACAA
 LeuGluGlnGlyLysArgThrLeuThrProGlnProMetGluLeuLysTyrSerTrpLysThrTrpGlyLysAlaLysIleValThrAlaGluThrGln>

2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
 AATTCCTCTTTTATAATAGATGGGCCAACACACCCAGAGTGTCCAAGTGCCTCAAGAGCATGGAATGTGTGGGAGGTGGAAGATTACGGGTTCCGAGTCT
 AsnSerSerPheIleIleAspGlyProAsnThrProGluCysProSerAlaSerArgAlaTrpAsnValTrpGluValGluAspTyrGlyPheGlyVal>

2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
 TCACAATAACATATGGCTGAAACTCCGAGAGATGTACACCAACTATGTGACCACAGGCTAATGTCCGCGAGCCGTTAAGGATGAGAGGGCCGTACACGC
 PheThrThrAsnIleTrpLeuLysLeuArgGluMetTyrThrGlnLeuCysAspHisArgLeuMetSerAlaAlaValLysAspGluArgAlaValHisAla>

APPENDIX 2-continued

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

3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
 CGACATGGGCTATTGGATAGAAAGCCAAAAGAATGGAAGTTGGAAGCTAGAAAAGGCATCCCTCATAGAGGTAAAAACCTGCACATGGCCAAAATCACAC
 AspMetGlyTyrTrpIleGluSerGlnLysAsnGlySerTrpLysLeuGluLysAlaSerLeuIleGluValLysThrCysThrTrpProLysSerHis>

3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
 ACTCTTTGGAGCAATGGTGTGCTAGAGAGTGACATGATCATCCCAAAGAGTCTGGCTGGTCCCATTTTCGCAACACAACACTACAGGCCCGGATACCACACC
 ThrLeuTrpSerAsnGlyValLeuGluSerAspMetIleIleProLysSerLeuAlaGlyProIleSerGlnHisAsnTyrArgProGlyTyrHisThr>

3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
 AACGGCAGGACCCCTGGCACTTAGGAAAATTGGAGCTGGACTTCAACTATTGTGAAGGAACAACAGTTGTTCATCACAGAAAATTGTGGGACAAGAGGCC
 GlnThrAlaGlyProTrpHisLeuGlyLysLeuGluLeuAspPheAsnTyrCysGluGlyThrThrValValIleThrGluAsnCysGlyThrArgGlyPro>

3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
 ATCACTGAGAACAACAACAGTGTGAGGAAGTTGATACCGAATGGTGTGCGCTCGTGTACACTTCCCTCCCTGCGATACATGGGAGAAGACGGCTGC
 SerLeuArgThrThrThrValSerGlyLysLeuIleHisGluTrpCysCysArgSerCysThrLeuProProLeuArgTyrMetGlyGluAspGlyCys>

3410 3420 3430 3440 3450 3460 3470 3480 3490 3500
 TrpTyrGlyMetGluIleArgProIleAsnGluLysGluGluAsnMetValLysSerLeuValSerAlaGlySerGlyLysValAspAsnPheThrMet>
 TGGTATGGCATGGAATTAGACCCATTAATGAGAAAGAAGAGAACATGGTAAAGTCTTTAGTCTCAGCAGGAGTGGAAAGGTGGATAACTTCACAATGG

3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
 GTGTCTTGTGTTTGGCAATCCTTTTTGAAGAGGTGATGAGAGGAPAATTTGGGAPAAAGCACATGATTGCGAGGGTCTCTTACGTTTGTACTCCTCT
 GlyValLeuCysLeuAlaIleLeuPheGluGluValMetArgGlyLysPheGlyLysLysHisMetIleAlaGlyValLeuPheThrPheValLeuLeu>

3610 3620 3630 3640 3650 3660 3670 3680 3690 3700
 CTCAGGGCAAATAACATGGAGAGACATGGCGCACACTCATAATGATTGGGTCCAACGCCTCTGACAGAAATGGGAATGGGCGTCACTTACCTAGCATTG
 SerGlyGlnIleThrTrpArgAspMetAlaHisThrLeuIleMetIleGlySerAsnAlaSerAspArgMetGlyMetGlyValThrTyrLeuAlaLeu>

3710 3720 3730 3740 3750 3760 3770 3780 3790 3800
 ATTGCAACATTTAAAATTCAGCCATTTTGGCTTTGGGATCTTCTCCTGAGGAACTGACATCTAGAGAAAATTTATTGTTGGGAGTTGGGTTGGCCATGG
 IleAlaThrPheLysIleGlnProPheLeuAlaLeuGlyPhePheLeuArgLysLeuThrSerArgGluAsnLeuLeuLeuGlyValGlyLeuAlaMet>

3810 3820 3830 3840 3850 3860 3870 3880 3890 3900
 CAACAACGTTACAACAGCCAGAGGACATTGAACAAATGGCGAATGGAATAGCTTTAGGGCTCATGGCTCTTAAATTAATAACACAATTTGAAACATACCA
 AlaThrThrLeuGlnLeuProGluAspIleGluGlnMetAlaAsnGlyIleAlaLeuGlyLeuMetAlaLeuLysLeuIleThrGlnPheGluThrTyrGln>

3910 3920 3930 3940 3950 3960 3970 3980 3990 4000
 ACATGGACGGCATTAGTCTCCCTAATGTGTTCAAATACAATTTTACGTTGACTGTTGCTGGGAGAACAGCCACCCTGATTTTGGCCGGAATTTCTCTT
 LeuTrpThrAlaLeuValSerLeuMetCysSerAsnThrIlePheThrLeuThrValAlaTrpArgThrAlaThrLeuIleLeuAlaGlyIleSerLeu>

4010 4020 4030 4040 4050 4060 4070 4080 4090 4100
 TTGCCAGTGTGCCAGTCTTCGAGCATGAGGAAAACAGATTGGCTCCCAATGGCTGTGGCAGCTATGGGAGTTCCACCCTACCCTTTTATTTTTCAGTT
 LeuProValCysGlnSerSerSerMetArgLysThrAspTrpLeuProMetAlaValAlaAlaMetGlyValProProLeuProLeuPheIlePheSer>

4110 4120 4130 4140 4150 4160 4170 4180 4190 4200
 TGAAAGATACGCTCAAAGGAGAAGCTGGCCACTGAATGAGGGGGTGATGGCTGTTGGACTTGTGAGTATCTAGCTAGTTCTCTCTTAGGAATGACGT
 LeuLysAspThrLeuLysArgArgSerTrpProLeuAsnGluGlyValMetAlaValGlyLeuValSerIleLeuAlaSerSerLeuLeuArgAsnAspVal>

4210 4220 4230 4240 4250 4260 4270 4280 4290 4300
 GCCATGGCTGGACCATTAGTGGCTGGGGGCTTGCTGATAGCGTGCTACGTCATAACTGGCAGTCAGCAGACCTCACTGTAGAAAAAGCAGCAGATGTC
 ProMetAlaGlyProLeuValAlaGlyGlyLeuLeuIleAlaCysTyrValIleThrGlyThrSerAlaAspLeuThrValGluLysAlaAlaAspVal>

4310 4320 4330 4340 4350 4360 4370 4380 4390 4400
 ACATGGGAGGAAGAGGCTGAGCAAACAGGAGTGTCCACAATTTAATGATCACAGTTGATGACGATGGAACAATGAGAATAAAAGATGATGAGACTGAGA
 ThrTrpGluGluGluAlaGluGlnThrGlyValSerHisAsnLeuMetIleThrValAspAspAspGlyThrMetArgIleLysAspAspGluThrGlu>

4410 4420 4430 4440 4450 4460 4470 4480 4490 4500
 ACATCTTAACAGTGCCTTTGAAAACAGCATTACTAATAGTGTGAGGCATTTTCCATACTCCATAACCGCAACACTGTTGGTCTGGCACACTTGGCAAAA
 AsnIleLeuThrValLeuLeuLysThrAlaLeuLeuIleValSerGlyIlePheProTyrSerIleProAlaThrLeuLeuValTrpHisThrTrpGlnLys>

4510 4520 4530 4540 4550 4560 4570 4580 4590 4600
 GCAAACCCAAAGATCCGGTGTCTATGGGACGTTCCAGCCCCCAGAGACACAGAAAGCAGAAGTGGAAAGGGGTTTATAGGATCAAGCAGCAAGGA
 GlnThrGlnArgSerGlyValLeuTrpAspValProSerProProGluThrGlnLysAlaGluLeuGluGluGlyValTyrArgIleLysGlnGlnGly>

4610 4620 4630 4640 4650 4660 4670 4680 4690 4700
 ATTTTGGGAAAACCAAGTGGGGTGGAGTACAAAAGAAGGAGTTTCCACACCATGTGGCACGTCACAAGAGGAGCAGTGTGACACACAATGGGA
 IlePheGlyLysThrGlnValGlyValGlyValGlnLysGluGlyValPheHisThrMetTrpHisValThrArgGlyAlaValLeuThrHisAsnGly>

4710 4720 4730 4740 4750 4760 4770 4780 4790 4800
 AAAGACTGGAACCAAACCTGGGCTAGCGTGAAAAAGATCTGATTTTCATACGGAGGAGGATGGAAATGAGTGCACAATGGCAAAAAGGAGAGGAGGTGCA
 LysArgLeuGluProAsnTrpAlaSerValLysLysAspLeuIleSerTyrGlyGlyGlyTrpLysLeuSerAlaGlnTrpGlnLysGlyGluGluValGln>

4810 4820 4830 4840 4850 4860 4870 4880 4890 4900
 GGTTATTGCCGTAGAGCCTGGGAAGAACCAAGAATTTCAAACCATGCCAGGCATTTTCCAGACAACAACAGGGGGAGATAGGAGCGATTGCACTGGAC
 ValIleAlaValGluProGlyLysAsnProLysAsnPheGlnThrMetProGlyIlePheGlnThrThrThrGlyGluIleGlyAlaIleAlaLeuAsp>

4910 4920 4930 4940 4950 4960 4970 4980 4990 5000

APPENDIX 2-continued

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

TTCAAGCCTGGAACCTCAGGATCTCCATCATAAACAGAGAGGGAAAGGTACTGGGATTGTATGGCAATGGAGTGGTCACAAAGAATGGTGGCTATGTCA
 PheLysProGlyThrSerGlySrProIleIleAsnArgGluGlyLysValLeuGlyLeuTyrGlyAsnGlyValValThrLysAsnGlyGlyTyrVal>

5010 5020 5030 5040 5050 5060 5070 5080 5090 5100
 GTGGAATAGCACAAACAAATGCAGAACCAGACGGACCGACACCAGAGTTGGAAGAAGAGATGTTCAAAAAGCGAAATCTAACATAATGGATCTCCATCC
 SerGlyIleAlaGlnThrAsnAlaGluProAspGlyProThrProGluLeuGluGluGluMetPheLysLysArgAsnLeuThrIleMetAspLeuHisPro>

5110 5120 5130 5140 5150 5160 5170 5180 5190 5200
 CGGGTCAGGAAAGACGCGGAAATATCTTCCAGCTATTGTTAGAGAGGCAATCAAGAGACGCTTAAGGACTCTAATTTTGGCACCAACAAGGGTAGTTGCA
 GlySerGlyLysThrArgLysTyrLeuProAlaIleValArgGluAlaIleLysArgArgLeuArgThrLeuIleLeuAlaProThrArgValValAla>

5210 5220 5230 5240 5250 5260 5270 5280 5290 5300
 GCTGAGATGGAAGAAGCATTGAAAGGGCTCCCAATAAGGTATCAAACAACCTGCAACAAAATCTGAACACACAGGGAGAGAGATTGTTGATCTAATGTGCC
 AlaGluMetGluGluAlaLeuLysGlyLeuProIleArgTyrGlnThrThrAlaThrLysSerGluHisThrGlyArgGluIleValAspLeuMetCys>

5310 5320 5330 5340 5350 5360 5370 5380 5390 5400
 ACGCAACGTTTACAATGCGTTTGTGTGTCACCAGTCAGGGTTCCAAACTACAACCTTGATAATAATGGATGAGGCTCATTTTACAGACCCAGCCAGTATAGC
 HisAlaThrPheThrMetArgLeuLeuSerProValArgValProAsnTyrAsnLeuIleIleMetAspGluAlaHisPheThrAspProAlaSerIleAla>

5410 5420 5430 5440 5450 5460 5470 5480 5490 5500
 GGCTAGAGGGTACATATCAACTCGTGTAGGAATGGGAGAGGCGCAATTTTCATGACAGCCACACCCCTGGAACAGCTGATGCCTTTTCTCAGAGC
 AlaArgGlyTyrIleSerThrArgValGlyMetGlyGluAlaAlaAlaIlePheMetThrAlaThrProProGlyThrAlaAspAlaPheProGlnSer>

5510 5520 5530 5540 5550 5560 5570 5580 5590 5600
 AACGCTCCAATTCAAGATGAAGAAAGAGACATACCAGAACGCTCATGGAATTCAGGCAATGAATGGATTACCGACTTTGCCGGAAGACGGTGTGGTTTG
 AsnAlaProIleGlnAspGluGluArgAspIleProGluArgSerTrpAsnSerGlyAsnGluTrpIleThrAspPheAlaGlyLysThrValTrpPhe>

5610 5620 5630 5640 5650 5660 5670 5680 5690 5700
 TCCCTAGCATCAAAGCTGGAATGACATAGCAAACCTGCTGCGGAAAATGGAAAAAGGTCATTCAACTTAGTAGGAAGACTTTTGGACACAGAATATCA
 ValProSerIleLysAlaGlyAsnAspIleAlaAsnCysLeuArgLysAsnGlyLysLysValIleGlnLeuSerArgLysThrPheAspThrGluTyrGln>

5710 5720 5730 5740 5750 5760 5770 5780 5790 5800
 AAAGACTAAACTAAATGATTGGGACTTTGTGGTGACAACAGACATTTTCAGAAATGGGAGCCAATTTCAAAGCAGACAGAGTGATCGACCCAAGAAGATGT
 LysThrLysLeuAsnAspTrpAspPheValValThrThrAspIleSerGluMetGlyAlaAsnPheLysAlaAspArgValIleAspProArgArgCys>

5810 5820 5830 5840 5850 5860 5870 5880 5890 5900
 CTCAGCCAGTGATTTTACAGACGGACCCGAGCGCTGATCCTGGCGGGACCAATGCCAGTCACCGTAGCGCGCTGCGCAAAGGAGAGGGAGAGTTG
 LeuLysProValIleLeuThrAspGlyProGluArgValIleLeuAlaGlyProMetProValThrValAlaSerAlaAlaGlnArgArgGlyArgVal>

5910 5920 5930 5940 5950 5960 5970 5980 5990 6000
 GCAGGAACCCACAAAAGAAAATGACCAATACATATTCATGGGCCAGCCCTCAATAATGATGAAGACCATGCTCACTGGACAGAAGCAAAAATGCTGCT
 GlyArgAsnProGlnLysGluAsnAspGlnTyrIlePheMetGlyGlnProLeuAsnAsnAspGluAspHisAlaHisTrpThrGluAlaLysMetLeuLeu>

6010 6020 6030 6040 6050 6060 6070 6080 6090 6100
 AGACAACATCAACACACCAGAAGGGATCATAACCAGCTCTTTTGAACCAGAAAGGAGAAGTCAGCCGCCATAGACGGCGAATACCGCTGAAGGGTGAG
 AspAsnIleAsnThrProGluGlyIleIleProAlaLeuPheGluProGluArgGluLysSerAlaAlaIleAspGlyGluTyrArgLeuLysGlyGlu>

6110 6120 6130 6140 6150 6160 6170 6180 6190 6200
 TCCAGGAAGACCTTCGTGGAACCTCATGAGGAGGGGTGACCTCCAGTTTGGCTAGCCATAAAGTAGCATCAGAAGGGATCAAATATACAGATAGAAAGT
 SerArgLysThrPheValGluLeuMetArgArgGlyAspLeuProValTrpLeuAlaHisLysValAlaSerGluGlyIleLysTyrThrAspArgLys>

6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
 GGTGTTTTGATGGAGAACGCAACAATCAAATTTTAGAGGAGAATATGGATGTGGAAATCTGGACAAAGGAAGGAGAAAAGAAAAATTGAGACCTAGGTG
 TrpCysPheAspGlyGluArgAsnAsnGlnIleLeuGluGluAsnMetAspValGluIleTrpThrLysGluGlyGluLysLysLysLeuArgProArgTrp>

6310 6320 6330 6340 6350 6360 6370 6380 6390 6400
 GCTTGATGCCCGCACTTATTCAGATCCCTTAGCGCTCAAGGAATTCAGGACTTTGCGGCTGGTAGAAAGTCAATTGCCCTTGATCTTGTGACAGAAATA
 LeuAspAlaArgThrTyrSerAspProLeuAlaLeuLysGluPheLysAspPheAlaAlaGlyArgLysSerIleAlaLeuAspLeuValThrGluIle>

6410 6420 6430 6440 6450 6460 6470 6480 6490 6500
 GGAAGAGTGCCTTCACACTTAGCTCACAGAACGAGAAACGCCCTGGACAATCTGGTGTGTTGCACACGTCAGAACATGGCGGGAGGGCTACAGGCATG
 GlyArgValProSerHisLeuAlaHisArgThrArgAsnAlaLeuAspAsnLeuValMetLeuHisThrSerGluHisGlyGlyArgAlaTyrArgHis>

6510 6520 6530 6540 6550 6560 6570 6580 6590 6600
 CAGTGGAGGAACCTACCAGAAACAATGGAAACACTCTTACTCCTGGGACTCATGATCCTGTTAACAGGTGGGCAATGCTTTTCTTGATATCAGGTAAAGG
 AlaValGluGluLeuProGluThrMetGluThrLeuLeuLeuLeuGlyLeuMetIleLeuLeuThrGlyGlyAlaMetLeuPheLeuIleSerGlyLysGly>

6610 6620 6630 6640 6650 6660 6670 6680 6690 6700
 GATTGGAAAGACTTCAATAGGACTCATTTGTGTAGCTGCTTCCAGCGGTATGTTATGGATGGCTGATGTCCCACTCCAATGGATCGCGTCTGCCATAGTC
 IleGlyLysThrSerIleGlyLeuIleCysValAlaAlaSerSerGlyMetLeuTrpMetAlaAspValProLeuGlnTrpIleAlaSerAlaIleVal>

6710 6720 6730 6740 6750 6760 6770 6780 6790 6800
 CTGGAGTTTTTTATGATGGTGTACTTATACCAGAACCAGAAAAGCAGAGAATCCCAAGACAATCAACTCGCATATGTCGTGATAGGCATACTCACAC
 LeuGluPhePheMetMetValLeuLeuIleProGluProGluLysGlnArgThrProGlnAspAsnGlnLeuAlaTyrValValIleGlyIleLeuThr>

6810 6820 6830 6840 6850 6860 6870 6880 6890 6900
 TGGCTGCAATAGTAGCAGCCAATGAAATGGGACTGTTGGAAACCACAAAGAGAGATTTAGGAATGTCCAAGAACCAGGTGTTGTTTCTCAACCAGCTA
 LeuAlaAlaIleValAlaAlaAsnGluMetGlyLeuLeuGluThrThrLysArgAspLeuGlyMetSerLysGluProGlyValValSerProThrSerTyr>

APPENDIX 2-continued

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

6910 6920 6930 6940 6950 6960 6970 6980 6990 7000
 TTTGGATGTGGACTTGCACCCAGCATCAGCCTGGACATTTGTACGCTGTGGCCACAACAGTAATAACACCAATGTTGAGACATACCATAGAGAATTCACA
 LeuAspValAspLeuHisProAlaSerAlaTrpThrLeuTyrAlaValAlaThrThrValIleThrProMetLeuArgHisThrIleGluAsnSerThr>

7010 7020 7030 7040 7050 7060 7070 7080 7090 7100
 GCAAATGTGTCCCTGGCAGCTATAGCCAACCAGGCAGTGGTCTGATGGGTTTAGACAAAGGATGGCCGATATCGAAAATGGACTTAGGCGTGCCACTAT
 AlaAsnValSerLeuAlaAlaIleAlaAsnGlnAlaValValLeuMetGlyLeuAspLysGlyTrpProIleSerLysMetAspLeuGlyValProLeu>

7110 7120 7130 7140 7150 7160 7170 7180 7190 7200
 TGGCACTGGGTTGTTATTACAAGTGAACCCACTAACTCTCACAGCGGCAGTTCCTCTGCTAGTCACGCATATGCTATTATAGGTCCAGGATTGCAGGC
 LeuAlaLeuGlyCysTyrSerGlnValAsnProLeuThrLeuThrAlaAlaValLeuLeuLeuValThrHisTyrAlaIleIleGlyProGlyLeuGlnAla>

7210 7220 7230 7240 7250 7260 7270 7280 7290 7300
 AAAAGCCACTCGTGAAGCTCAAAAAGGACAGCTGCTGGAATAATGAAGAATCCAACGGTGGATGGGATAATGACAATAGACCTAGATCCTGTAATATAC
 LysAlaThrArgGluAlaGlnLysArgThrAlaAlaGlyIleMetLysAsnProThrValAspGlyIleMetThrIleAspLeuAspProValIleTyr>

7310 7320 7330 7340 7350 7360 7370 7380 7390 7400
 GATTCAAAATTTGAAAAGCAACTAGGACAGGTTATGCTCCTGGTCTGTGTGCAGTCAACTTTTGTAAATGAGAACATCATGGGCTTTTGTGAAGCTC
 AspSerLysPheGluLysGlnLeuGlyGlnValMetLeuLeuValLeuCysAlaValGlnLeuLeuLeuMetArgThrSerTrpAlaPheCysGluAla>

7410 7420 7430 7440 7450 7460 7470 7480 7490 7500
 TAACCCTAGCCACAGGACCAATAACAACACTCTGGGAAGGATCACCTGGGAAGTCTGGAACACCACGATAGCTGTTCCATGGCGAACATCTTTAGAGG
 LeuThrLeuAlaThrGlyProIleThrThrLeuTrpGluGlySerProGlyLysPheTrpAsnThrThrIleAlaValSerMetAlaAsnIlePheArgGly>

7510 7520 7530 7540 7550 7560 7570 7580 7590 7600
 GAGCTATTTAGCAGGAGCTGGGCTTGCTTTTTCTATCATGAAATCAGTTGGAACAGGAAAGAGAGGGACAGGGTCACAGGGTGAACCTTGGGAGAAAAG
 SerTyrLeuAlaGlyAlaGlyLeuAlaPheSerIleMetLysSerValGlyThrGlyLysArgGlyThrGlySerGlnGlyGluThrLeuGlyGluLys>

7610 7620 7630 7640 7650 7660 7670 7680 7690 7700
 TGGAAAAGAAATTGAATCAATTACCCCGAAAGAGTTTACCTTTTACAAGAAATCCGGAATCACTGAAGTGGATAGAACAGAAGCCAAAGAAGGGTTGA
 TrpLysLysLysLeuAsnGlnLeuProArgLysGluPheAspLeuTyrLysLysSerGlyIleThrGluValAspArgThrGluAlaLysGluGlyLeu>

7710 7720 7730 7740 7750 7760 7770 7780 7790 7800
 AAAGAGGAGAAATAACACACCATGCCGTGTCCAGAGGCAGCGCAAACTTCAATGGTTCGTGGAGAGAAACATGGTCATCCCCGAAGGAAGATCATAGA
 LysArgGlyGluIleThrHisHisAlaValSerArgGlySerAlaLysLeuGlnTrpPheValGluArgAsnMetValIleProGluGlyArgValIleAsp>

7810 7820 7830 7840 7850 7860 7870 7880 7890 7900
 CTTAGGCTGTGGAAGAGGAGGCTGGTCATATTATTGTGCAGGACTGAAAAAGTTACAGAAGTGCAGGATACACAAAAGGCGGCCAGGACATGAAGAA
 LeuGlyCysGlyArgGlyGlyTrpSerTyrTyrCysAlaGlyLeuLysLysValThrGluValArgGlyTyrThrLysGlyGlyProGlyHisGluGlu>

7910 7920 7930 7940 7950 7960 7970 7980 7990 8000
 CCACTACCTATGTCTACATACGGATGGAACATAGTCAAGTTAATGAGTGGAAAGGATGTGTTTTATCTTCCACCTGAAAAGTGTGATACTCTATTGTGTG
 ProValProMetSerThrTyrGlyTrpAsnIleValLysLeuMetSerGlyLysAspValPheTyrLeuProProGluLysCysAspThrLeuLeuCys>

8010 8020 8030 8040 8050 8060 8070 8080 8090 8100
 ACATTGGAGAATCTTCACCAAGCCCAACAGTGGAAAGAAAGCAGAACCATAAGAGTCTTGAAGATGGTTGAACCATGGCTAAAAATAACCAGTTTTGCAT
 AspIleGlyGluSerSerProSerProThrValGluGluSerArgThrIleArgValLeuLysMetValGluProTrpLeuLysAsnAsnGlnPheCysIle>

8110 8120 8130 8140 8150 8160 8170 8180 8190 8200
 TAAAGTATTGAACCTTACATGCCAACTGTGATTGAGCACCTAGAAAGACTACAAGGAAACATGGAGGAATGCTTGTGAGAAATCCACTCTCACGAAAC
 LysValLeuAsnProTyrMetProThrValIleGluHisLeuGluArgLeuGlnArgLysHisGlyGlyMetLeuValArgAsnProLeuSerArgAsn>

8210 8220 8230 8240 8250 8260 8270 8280 8290 8300
 TCCACGCACGAAATGTACTGGATATCTAATGGCACAGGCAATATCGTTTTCTTCAGTCAACATGGTATCCAGATTGCTACTTAACAGATTCACAATGACAC
 SerThrHisGluMetTyrTrpIleSerAsnGlyThrGlyAsnIleValSerSerValAsnMetValSerArgLeuLeuLeuAsnArgPheThrMetThr>

8310 8320 8330 8340 8350 8360 8370 8380 8390 8400
 ATAGGAGACCCACCATAGAGAAAGATGTGGATTTAGGAGCGGGACCCGACATGTCATGCGGAACAGAAACACCCAACATGGATGTCAATGGGGAAAG
 HisArgArgProThrIleGluLysAspValAspLeuGlyAlaGlyThrArgHisValAsnAlaGluProGluThrProAsnMetAspValIleGlyGluArg>

8410 8420 8430 8440 8450 8460 8470 8480 8490 8500
 AATAAGAAGGATCAAGGAGGAGCATAGTTCAACATGGCACTATGATGATGAAAATCCTTATAAAACGTGGGCTTACCATGGATCCTATGAAGTTAAGGCC
 IleArgArgIleLysGluGluHisSerSerThrTrpHisTyrAspAspGluAsnProTyrLysThrTrpAlaTyrHisGlySerTyrGluValLysAla>

8510 8520 8530 8540 8550 8560 8570 8580 8590 8600
 ACAGGCTCAGCCTCCTCCATGATAAATGGAGTCGTGAACTCCTCACGAAACCATGGGATGTGGTGGCCATGGTGACACAGATGGCAATGACGGATACAA
 ThrGlySerAlaSerSerMetIleAsnGlyValValLysLeuLeuThrLysProTrpAspValValProMetValThrGlnMetAlaMetThrAspThr>

8610 8620 8630 8640 8650 8660 8670 8680 8690 8700
 CCCCATTGCGCCAGCAAAGGTTTTTAAAGAGAAAGTGGACACCAGGACACCCAGACCTATGCCAGGAACAAGAAAGGTTATGGAGATCACAGCGGAATG
 ThrProPheGlyGlnGlnArgValPheLysGluLysValAspThrArgThrProArgProMetProGlyThrArgLysValMetGluIleThrAlaGluTrp>

8710 8720 8730 8740 8750 8760 8770 8780 8790 8800
 GCTTTGGAGAACCCTGGGAAGGAACAAAAGACCCAGATTATGTACGAGAGAGGAGTTACAAAAAGGTCAGAACCAACGCAGCTATGGGCGCCGTTTTT
 LeuTrpArgThrLeuGlyArgAsnLysArgProArgLeuCysThrArgGluGluPheThrLysLysValArgThrAsnAlaAlaMetGlyAlaValPhe>

8810 8820 8830 8840 8850 8860 8870 8880 8890 8900

APPENDIX 2-continued

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

AATTCTCTTACTGTTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCT
 11010 11020 11030 11040 11050 11060 11070 11080 11090 11100
 CTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCAAGGAT
 11110 11120 11130 11140 11150 11160 11170 11180 11190 11200
 CTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACCTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACA
 11210 11220 11230 11240 11250 11260 11270 11280 11290 11300
 GGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCATTATCAGGGTT
 11310 11320 11330 11340 11350 11360 11370 11380 11390 11400
 ATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGGGTTCCGCGCACATTTCCCGAAAAGTGCCACCTGACGTCTAAGA
 11410 11420 11430 11440 11450 11460 11470 11480 11490 11500
 AACCATATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCGATAAGCT
 11510 11520 11530 11540 11550 11560 11570 11580 11590 11600
 TTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCTCGGCACCGTCACCCCTG
 11610 11620 11630 11640 11650 11660 11670 11680 11690 11700
 GATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCTCTTGGGGATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTGG
 11710 11720 11730 11740 11750 11760 11770 11780 11790 11800
 CGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCCAGTCCTGCTCGCTTCGCTACTTGGAGC
 11810 11820 11830 11840 11850 11860 11870 11880 11890 11900
 CACTATCGACTACGCGATCATGGCGACCACCCGCTCTGTGGATCCTCTACGCCGACGCATCGTGGCCGCATCACCGGCGCCACAGGTGCGGTTGCT
 11910 11920 11930 11940 11950 11960 11970 11980 11990 12000
 GCGCCTATATCGCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTCGGCGTGGGTATGGTTGGCAGGCCCGTGG
 12010 12020 12030 12040 12050 12060 12070 12080 12090 12100
 CCGGGGACTGTTGGGCGCATCTCCTTGCATGCACCATTCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCTAATGCAGGA
 12110 12120 12130 12140 12150 12160 12170 12180 12190 12200
 GTCGATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGTCTCCTTCCGGTGGGCGGGGCATGACTATCGTCGCCGCACTTATG
 12210 12220 12230 12240 12250 12260 12270 12280 12290 12300
 ACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCAATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCC
 12310 12320 12330 12340 12350 12360 12370 12380 12390 12400
 TGTGCTTGCAGTATTCGGAATCTTGCACGCCCTCGTCAAGCCTTCGTCACTGGTCCCGCCACCAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGG
 12410 12420 12430 12440 12450 12460 12470 12480 12490 12500
 CATGGCGGCCGACGCGTGGGCTACGTCTTGTGCGGTTTCGCGACGCGAGGCTGGATGGCCTTCCCATTATGATTCTTCTCGCTTCCGGCGGCATCGGG
 12510 12520 12530 12540 12550 12560 12570 12580 12590 12600
 ATGCCCGGCTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCTTACCAGCCTAACTTCGATCA
 12610 12620 12630 12640 12650 12660 12670 12680 12690 12700
 CTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCC
 12710 12720 12730 12740 12750 12760 12770 12780 12790 12800
 CGCGTTGCGTGCAGGTCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTACCACTCAAGAATTGGAGCCAATC
 12810 12820 12830 12840 12850 12860 12870 12880 12890 12900
 AATTCTTGGGAGAACTGTGAATGCGCAAACCAACCCCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGACGCGGCGCATCTCGGGCAG
 12910 12920 12930 12940 12950 12960 12970 12980 12990 13000
 CGTTGGGTCCTGGCCACGGGTGCGCATGATCGTGTCTCTGTGCTTGGAGACCCGGCTAGGCTGGCGGGGTTGCCTTACTGGTTAGCAGAAATGAATCACCC
 13010 13020 13030 13040 13050 13060 13070 13080 13090 13100
 ATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAA
 13110 13120 13130 13140 13150 13160 13170 13180 13190 13200
 CGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACGAAGCGCTG
 13210 13220 13230 13240 13250 13260 13270 13280 13290 13300
 GCATTGACCCTGAGTGATTTTCTCTGGTCCC GCCGATCCATAACCGCCAGTTGTTTACCCTACAACGTTCCAGTAACCGGGCATGTTTCATCATCAGTA
 13310 13320 13330 13340 13350 13360 13370 13380 13390 13400
 ACCCGTATCGTGAGCATCTCTCTCGTTTCATCGGTATCATTACCCCATGAACAGAAATCCCCCTTACACGAGGCATCAGTGACCAAACAGGAAAAA
 13410 13420 13430 13440 13450 13460 13470 13480 13490 13500
 CCGCCCTAACATGGCCCGCTTATCAGAAGCCAGACATTAACGCTTCTGGAGAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATC
 13510 13520 13530 13540 13550 13560 13570 13580 13590 13600

APPENDIX 2-continued

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

GCTTCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACA
 13610 13620 13630 13640 13650 13660 13670 13680 13690 13700
 GCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGGGGCGCAGCCATGACCCAGTCACGTAGCG
 13710 13720 13730 13740 13750 13760 13770 13780 13790 13800
 ATAGCGGAGTGTATACTGGCTTAAGTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGA
 13810 13820 13830 13840 13850 13860 13870 13880 13890 13900
 AAATACCGCATCAGGCGCTCTCCGCTTCCCTCGCTCACTGACTCGCTGCGCTCGGTCTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAA
 13910 13920 13930 13940 13950 13960 13970 13980 13990 14000
 TACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT
 14010 14020 14030 14040 14050 14060 14070 14080 14090 14100
 TTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCC
 14110 14120 14130 14140 14150 14160 14170 14180 14190 14200
 CCTGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCTGCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTCTCATAGCT
 14210 14220 14230 14240 14250 14260 14270 14280 14290 14300
 CACGCTGTAGGTATCTCAGTTCGGTGTAGGTCTGCTCAAGCTGGGCTGTGTGCACGAACCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGGTAA
 14310 14320 14330 14340 14350 14360 14370 14380 14390 14400
 CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA
 14410 14420 14430 14440 14450 14460 14470 14480 14490 14500
 GAGTCTTGAAGTGGTGGCTAACTACGGCTACACTAGAAGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
 14510 14520 14530 14540 14550 14560 14570 14580 14590 14600
 GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTT
 14610 14620 14630 14640 14650 14660 14670 14680 14690 14700
 GATCTTTTCTACGGGTCTGACGCTCATTGGAACGAAACTCACGTTAAGGGATTTGGTTCATGAGATTATCAAAAAGGATCTTACCTAGATCCTTTTA
 14710 14720 14730 14740 14750 14760 14770 14780 14790 14800
 AATTAATAATGAAGTTTTAAATCAATCTAAAGTATATAGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCT
 14810 14820 14830 14840 14850 14860 14870 14880 14890 14900
 GTCTATTTGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCG
 14910 14920 14930 14940 14950 14960 14970 14980 14990 15000
 AGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTTGCAACTTTATCCGCCTCCATCCAG
 15010 15020 15030 15040 15050 15060 15070 15080 15090 15100
 TCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCACAACGTTGTTGCCATTGCTGCAAGATCTGGCTAGCGATGACCCCT
 15110 15120 15130 15140 15150
 GCTGATTGGTTCGCTGACCATTTCCGGGCGCGCCGATTTAGGTGACACTATAG

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

APPENDIX 3

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

AGTTGTTAGTCTGTGTGGACCGACAAGGACAGTTCCAAAATCGGAAGCTTGCTTAACACAGTTCTAACAGTTTGTGTTGAATAGAGAGCAGATCTCTGGAAA
 10 20 30 40 50 60 70 80 90 100
 AATGAACAACCAACGGAAAAAGACGGGTCGACCGTCTTTCAATATGCTGAAACGCGGAGAAACCGCGTGTCAACTGGTTTCACAGTTGGCGAAGAGATT
 110 120 130 140 150 160 170 180 190 200
 MetAsnAsnGlnArgLysLysThrGlyArgProSerPheAsnMetLeuLysArgAlaArgAsnArgValSerThrGlySerGlnLeuAlaLysArgPhe>
 210 220 230 240 250 260 270 280 290 300

APPENDIX 3-continued

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

TCAAAAGGATTGCTTTCAGGCCAAGGACCCATGAAATTGGTGTGGCTTTCATAGCATTTCTAAGATTTCTAGCCATACCCCAACAGCAGGAATTTTGG
 SerLysGlyLeuLeuSerGlyGlnGlyProMetLysLeuValMetAlaPheIleAlaPheLeuArgPheLeuAlaIleProProThrAlaGlyIleLeu>

310 320 330 340 350 360 370 380 390 400
 CTAGATGGAGCTCATTCAAGAAGAATGGAGCGATCAAAGTGTACGGGGTTTCAAAAAGAGATCTCAAGCATGTTGAACATTATGAACAGGAGGAAAA
 AlaArgTrpSerSerPheLysLysAsnGlyAlaIleLysValLeuArgGlyPheLysLysGluIleSerSerMetLeuAsnIleMetAsnArgArgLysLys>

410 420 430 440 450 460 470 480 490 500
 ATCTGTGACCATGCTCCTCATGCTGCTGCCCCACAGCCCTGGCGTTCATTTGACCACACGAGGGGAGAGCCACACATGATAGTTAGTAAGCAGGAAAGA
 SerValThrMetLeuLeuMetLeuLeuProThrAlaLeuAlaPheHisLeuThrThrArgGlyGlyGluProHisMetIleValSerLysGlnGluArg>

510 520 530 540 550 560 570 580 590 600
 GGAAAGTCACTGTTGTTTAAAGACCTCTGCAGGCATCAATATGTGCACTCTCATTGCGATGGATTTGGGAGAGTTATGCGAGGACACAATGACCTACAAAT
 GlyLysSerLeuLeuPheLysThrSerAlaGlyIleAsnMetCysThrLeuIleAlaMetAspLeuGlyGluLeuCysGluAspThrMetThrTyrLys>

610 620 630 640 650 660 670 680 690 700
 GCCCCCGGATCACTGAGGCGGAACCAGATGACGTTGACTGCTGGTGAATGCCACAGACACATGGGTGACCTATGGGACGTGTTCTCAAACCGGCGAACA
 CysProArgIleThrGluAlaGluProAspAspValAspCysTrpCysAsnAlaThrAspThrTrpValThrTyrGlyThrCysSerGlnThrGlyGluHis>

710 720 730 740 750 760 770 780 790 800
 CCGACGAGACAAACGTTCCGTGGCACTGGCCCCACACGTGGGACTTGGTCTAGAAACAAGAACCAGAAACATGGATGTCTCTGAAGGTGCCTGGAAACAA
 ArgArgAspLysArgSerValAlaLeuAlaProHisValGlyLeuGlyLeuGluThrArgThrGluThrTrpMetSerSerGluGlyAlaTrpLysGln>

810 820 830 840 850 860 870 880 890 900
 GTACAAAAGTGGAGACTTGGGCTTTGAGACACCCAGGATTCACGGTGACAGCCCTTTTTTTAGCACATGCCATAGGAACATCCATTACTCAGAAAGGGA
 ValGlnLysValGluThrTrpAlaLeuArgHisProGlyPheThrValThrAlaLeuPheLeuAlaHisAlaIleGlyThrSerIleThrGlnLysGly>

910 920 930 940 950 960 970 980 990 1000
 TCATTTTCATTCTGCTGATGCTAGTAACACCATCAATGGCCATGCGATGTGTGGGAATAGGCAACAGAGACTTCGTTGAAGGACTGTCAGGAGCAACGTG
 IleIlePheIleLeuLeuMetLeuValThrProSerMetAlaMetArgCysValGlyIleGlyAsnArgAspPheValGluGlyLeuSerGlyAlaThrTrp>

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
 GGTGGACGTGGTATTGGAGCATGGAAGCTGCGTACCACCATGGCAAAAGATAAACCAACATTGGACATTGAACTCTGAAGACGGAGTCAAAACCCCT
 ValAspValValLeuGluHisGlySerCysValThrThrMetAlaLysAspLysProThrLeuAspIleGluLeuLysThrGluValThrAsnPro>

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 GCCGCTTTCGCAAACTGTGCATTGAAGCTAAAATATCAAACACCACCACCGATTCAAGGTGTCCAACACAAGGAGAGGCTACACTGGTGAAGAACAGG
 AlaValLeuArgLysLeuCysIleGluAlaLysIleSerAsnThrThrThrAspSerArgCysProThrGlnGlyGluAlaThrLeuValGluGluGln>

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
 ACTCGAACTTTGTGTGTCGACGAACGTTTGTGGACAGAGGCTGGGGTAATGGCTGCGGACTATTTGGAAAAGGAAGCCTACTGACGTGTGCTAAGTTCAA
 AspSerAsnPheValCysArgArgThrPheValAspArgGlyTrpGlyAsnGlyCysGlyLeuPheGlyLysGlySerLeuLeuThrCysAlaLysPheLys>

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
 GTGTGTGACAAAAGTAAAGGAAAGATAGTTCAATATGAAAACCTAAAATATTCAGTGATAGTCACTGTCCACACTGGGGACCAGCACCAGGTGGGAAAC
 CysValThrLysLeuGluGlyLysIleValGlnTyrGluAsnLeuLysTyrSerValIleValThrValHisThrGlyAspGlnHisGlnValGlyAsn>

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
 GAGACTACAGAACATGGAACAATTGCAACCATAACACCTCAAGCTCCTACGTCGGAAATACAGCTGACTGACTACGGAGCCCTCACATTGGACTGCTCGC
 GluThrThrGluHisGlyThrIleAlaThrIleThrProGlnAlaProThrSerGluIleGlnLeuThrAspTyrGlyAlaLeuThrLeuAspCysSer>

1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
 CTAGAACAGGGCTGGACTTTAATGAGATGGTCTATTGACAATGAAAGAAAAATCATGGCTTGTCCACAAACAATGGTTTCTAGACTTACCCTGCCTTG
 ProArgThrGlyLeuAspPheAsnGluMetValLeuLeuThrMetLysGluLysSerTrpLeuValHisLysGlnTrpPheLeuAspLeuProLeuProTrp>

1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
 GACTTCAGGAGCTTCAACATCTCAAGAGACTTGAACAGACAAGATTTGCTGGTCAATTCAAGACAGCTCATGCAAAGAAACAGGAAGTAGTCTGACTG
 ThrSerGlyAlaSerThrSerGlnGluThrTrpAsnArgGlnAspLeuLeuValThrPheLysThrAlaHisAlaLysLysGlnGluValValValLeu>

1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
 GGATCACAGGAAGGAGCAATGCACACTGCGTTGACTGGGGCGACAGAAATCCAGACGTGAGGAACGACAACAATCTTTGAGGACACCTGAAATGCAGAC
 GlySerGlnGluGlyAlaMetHisThrAlaLeuThrGlyAlaThrGluIleGlnThrSerGlyThrThrThrIlePheAlaGlyHisLeuLysCysArg>

1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
 TAAAAATGGATAAACTGACTTTAAAAGGGAGTCATATGTAATGTGCACAGGCTCATTTAAGCTAGAGAAGGAAGTGGCTGAGACCCAGCATGGAAGTGT
 LeuLysMetAspLysLeuThrLeuLysGlyMetSerTyrValMetCysThrGlySerPheLysLeuGluLysGluValAlaGluThrGlnHisGlyThrVal>

1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
 TTTAGTGCAGGTTAAATACGAAGGAACAGATGCGCCATGCAAGATCCCTTTTTCGGCCAAGATGAGAAAGGAGTGACCCAGAATGGGAGATTGATAACA
 LeuValGlnValLysTyrGluGlyThrAspAlaProCysLysIleProPheSerAlaGlnAspGluLysGlyValThrGlnAsnGlyArgLeuIleThr>

2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
 GCCAACCCCATAGTCACTGACAAAGAAAAACCAGTCAACATTGAGACAGAACCCTTTTTGGTGAGAGCTACATCGTGGTAGGGCAGGTGAAAAAGCTT
 AlaAsnProIleValThrAspLysGluLysProValAsnIleGluThrGluProProPheGlyGluSerTyrIleValValGlyAlaGlyGluLysAla>

2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
 TGAACCTGAGCTGGTTCAAGAAAGGGAGCAGCATAGGAAAATGTTTCAAGCAACTGCCCCGAGGAGCGCAAGGATGGCTATCCTGGGAGACACCCGATG
 LeuLysLeuSerTrpPheLysLysGlySerSerIleGlyLysMetPheGluAlaThrAlaArgGlyAlaArgArgMetAlaIleLeuGlyAspThrAlaTrp>

APPENDIX 3-continued

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

2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
 GGACTTTGGCTCTATAGGAGGAGTGTTCACATCAGTGGGAAAATTGGTACACCAGGTTTTTGGAGCCGCATATGGGGTTCTGTTTCAGCGGTGTTTCTTGG
 AspPheGlySerIleGlyGlyValPheThrSerValGlyLysLeuValHisGlnValPheGlyAlaAlaTyrGlyValLeuPheSerGlyValSerTyr>

2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
 ACCATGAAAATAGGAATAGGGATTCTGCTGACATGGCTAGGATTAACCTCGAGGAACACTTCAATGGCTATGACGTGCATAGCTGTTGGAGGAATCACTC
 ThrMetLysIleGlyIleGlyIleLeuLeuThrTrpLeuGlyLeuAsnSerArgAsnThrSerMetAlaMetThrCysIleAlaValGlyGlyIleThr>

2410 2420
 TGTTTCTGGGCTTCACAGTTCAAGCA
 LeuPheLeuGlyPheThrValGlnAla>

Bases 1 to 88 (BglIII): 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

APPENDIX 4

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

10 20 30 40 50 60 70 80 90 100
 AGTTGTTAGTCTGTGTGGACCGACAAGGACAGTTCCAAATCGGAAGCTTGCTTAACACAGTTCCTAACAGTTGTTTGAATAGAGAGCAGATCTCTGGAAA

110 120 130 140 150 160 170 180 190 200
 AATGAACCAACGAAAAAGGTGGTTAGACCACCTTTCATATGCTGAAACGCGAGAGAAACCGCGTATCAACCCCTCAAGGGTTGGTGAAGAGATTCTCA
 MetAsnGlnArgLysLysValValArgProProPheAsnMetLeuLysArgGluArgAsnArgValSerThrProGlnGlyLeuValLysArgPheSer>

210 220 230 240 250 260 270 280 290 300
 ACCGGACTTTTTCTGGGAAAGGACCCTTACGGATGGTCTAGCATTATCAGCTTTTGGCGAGTCCCTTCCATCCCACCAACAGCAGGGATTCTGAAGA
 ThrGlyLeuPheSerGlyLysGlyProLeuArgMetValLeuAlaPheIleThrPheLeuArgValLeuSerIleProProThrAlaGlyIleLeuLys>

310 320 330 340 350 360 370 380 390 400
 GATGGGGACAGTTGAAGAAAATAAGGCCATCAAGATACTGATTGGATTTCAGGAAGGAGATAGGCCCGCATGCTGAACATCTTGAACGGGAGAAAAAGGTC
 ArgTrpGlyGlnLeuLysLysAsnLysAlaIleLysIleLeuIleGlyPheArgLysGluIleGlyArgMetLeuAsnIleLeuAsnGlyArgLysArgSer>

410 420 430 440 450 460 470 480 490 500
 TGCAGCCATGCTCCTCATGCTGCTGCCACAGCCCTGGCGTTCATTTGACCACACGAGGGGGAGAGCCACACATGATAGTTAGTAAGCAGGAAAGAGGA
 AlaAlaMetLeuLeuMetLeuLeuProThrAlaLeuAlaPheHisLeuThrThrArgGlyGlyGluProHisMetIleValSerLysGlnGluArgGly>

510 520 530 540 550 560 570 580 590 600
 AAGTCACTGTTGTTTAAAGACCTCTGCAGGCATCAATATGTGCACTCTCATTGCGATGGATTTGGGAGAGTTATGCGAGGACACAATGACCTACAAATGCC
 LysSerLeuLeuPheLysThrSerAlaGlyIleAsnMetCysThrLeuIleAlaMetAspLeuGlyGluLeuCysGluAspThrMetThrTyrLysCys>

610 620 630 640 650 660 670 680 690 700
 CCCGGATCACTGAGGCGGAACCAGATGACGTTGACTGCTGGTGCAATGCCACAGACACATGGGTGACCTATGGGACGTGTTCTCAAACCGGCGAACACCG
 ProArgIleThrGluAlaGluProAspAspValAspCysTrpCysAsnAlaThrAspThrTrpValThrTyrGlyThrCysSerGlnThrGlyGluHisArg>

710 720 730 740 750 760 770 780 790 800
 ACGAGACAAACGTTCCGTGGCACTGGCCCCACAGTGGGACTTGGTCTAGAAACAAGAACCGAAACATGGATGTCCTCTGAAGGTGCTGAAACAAGTA
 ArgAspLysArgSerValAlaLeuAlaProHisValGlyLeuGlyLeuGluThrArgThrGluThrTrpMetSerSerGluGlyAlaTrpLysGlnVal>

810 820 830 840 850 860 870 880 890 900
 CAAAAGTGGAGACTTGGGCTTTGAGACACCCAGGATTCACGGTGACAGCCCTTTTTTAGCACATGCCATAGGAACATCCATTACTCAGAAAGGGATCA
 GlnLysValGluThrTrpAlaLeuArgHisProGlyPheThrValThrAlaLeuPheLeuAlaHisAlaIleGlyThrSerIleThrGlnLysGlyIle>

910 920 930 940 950 960 970 980 990 1000
 TTTTCATTCTGCTGATGCTAGTAACACCATCAATGGCCATGCGATGTGTGGGAATAGGCAACAGAGACTTCGTTGAAGGACTGTCAGGAGCAACGTGGGT
 IlePheIleLeuLeuMetLeuValThrProSerMetAlaMetArgCysValGlyIleGlyAsnArgAspPheValGluGlyLeuSerGlyAlaThrTrpVal>

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
 GGACGTGGTATTGGAGCATGGAAGCTGCGTCACCACCATGGCAAAAGATAAACAACATTGGACATTGAACTCTTGAAGACGGAGGTCACAAACCTGCC
 AspValValLeuGluHisGlySerCysValThrThrMetAlaLysAspLysProThrLeuAspIleGluLeuLeuLysThrGluValThrAsnProAla>

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 GTCTTGCCAAACCTGTGCATTGAAGCTAAAATATCAAACACCACCACCGATTCAAGGTGTCCAACACAAGGAGAGGCTACACTGGTGAAGAACAGGACT
 ValLeuArgLysLeuCysIleGluAlaLysIleSerAsnThrThrThrAspSerArgCysProThrGlnGlyGluAlaThrLeuValGluGluGlnAsp>

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
 CGAACTTTGTGTGTCGACGAACGTTTGTGGACAGAGGCTGGGGTAATGGCTGCGGACTATTTGAAAAGGAAGCCTACTGACGTGTGCTAAGTTCAAGTG
 SerAsnPheValCysArgArgThrPheValAspArgGlyTrpGlyAsnGlyCysGlyLeuPheGlyLysGlySerLeuLeuThrCysAlaLysPheLysCys>

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400

APPENDIX 4-continued

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

TGTGACAAAAC TAGAAGGAAAG ATAGTTCAATATGAAAAC TAAAATATT CAGTGATAGTCACTGTCCACACTGGGGACCAGCACCAGGTGGGAAACGAG
ValThrLysLeuGluGlyLysIleValGlnTyrGluAsnLeuLysTyrSerValIleValThrValHisThrGlyAspGlnHisGlnValGlyAsnGlu>

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
ACTACAGAACATGGAACAATTGCAACCATAACACCTCAAGCTCCTACGTCGGAAATACAGCTGACTGACTACGGAGCCCTCACATTGGACTGCTCGCCTA
ThrThrGluHisGlyThrIleAlaThrIleThrProGlnAlaProThrSerGluIleGlnLeuThrAspTyrGlyAlaLeuThrLeuAspCysSerPro>

1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
GAACAGGGCTGGACTTTAATGAGATGGTTCTATTGACAAATGAAAGAAAATCATGGCTTGCCACAAACAATGGTTTCTAGACTTACCACTGCCTTGGAC
ArgThrGlyLeuAspPheAsnGluMetValLeuLeuThrMetLysGluLysSerTrpLeuValHisLysGlnTrpPheLeuAspLeuProLeuProTrpThr>

1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
TTCAGGAGCTTCAACATCTCAAGAGACTTGAACAGACAAAGATTTGCTGGTCACATTCAAGACAGCTCATGCAAAGAAACAGGAAGTAGTCGTACTGGGA
SerGlyAlaSerThrSerGlnGluThrTrpAsnArgGlnAspLeuLeuValThrPheLysThrAlaHisAlaLysLysGlnGluValValValLeuGly>

1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
TCACAGGAAGGAGCAATGCACACTGCGTTGACTGGGGCGACAGAAATCCAGACGTCAGGAACGACAACAATCTTTGACAGACACCTGAAATGCAGACTAA
SerGlnGluGlyAlaMetHisThrAlaLeuThrGlyAlaThrGluIleGlnThrSerGlyThrThrThrIlePheAlaGlyHisLeuLysCysArgLeu>

1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
AAATGGATAAACTGACTTTAAAAGGGATGTCATATGTAATGTGCACAGGCTCATTTAAGCTAGAGAAGGAGTGGCTGAGACCCAGCATGGAACTGTTTT
LysMetAspLysLeuThrLeuLysGlyMetSerTyrValMetCysThrGlySerPheLysLeuGluLysGluValAlaGluThrGlnHisGlyThrValLeu>

1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
AGTGCAGGTTAAATACGAAGGAACAGATGCGCCATGCAAGATCCCTTTTTCGGCCCAAGATGAGAAAGGAGTGACCCAGAATGGGAGATTGATAACAGCC
ValGlnValLysTyrGluGlyThrAspAlaProCysLysIleProPheSerAlaGlnAspGluLysGlyValThrGlnAsnGlyArgLeuIleThrAla>

2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
AACCCCATAGTCACTGACAAAGAAAACAGTCAACATTTGAGACAGAACCACCTTTTGGTGAGAGCTACATCGTGGTAGGGGCAGGTGAAAAAGCTTTGA
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2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
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2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
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PheGlySerIleGlyGlyValPheThrSerValGlyLysLeuValHisGlnValPheGlyAlaAlaTyrGlyValLeuPheSerGlyValSerTrpThr>

2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
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2410 2420
TTCTGGGCTTACAGTTCAAGCA
PheLeuGlyPheThrValGlnAla

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

While the present invention has been described in some 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. All figures, tables, appendices, patents, patent applications and publications, referred to above, are hereby incorporated by reference.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 53

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<400> SEQUENCE: 1

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aggagacccc ccgcaacaac aa 82

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 <213> ORGANISM: Dengue 2 virus

<400> SEQUENCE: 2

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agaggagacc cccccaaaac aaaa 84

<210> SEQ ID NO 3
 <211> LENGTH: 83
 <212> TYPE: RNA
 <213> ORGANISM: Dengue 3 virus

<400> SEQUENCE: 3

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gaggagaccc cccgcaaaaua aaa 83

<210> SEQ ID NO 4
 <211> LENGTH: 83
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<400> SEQUENCE: 4

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gaggagaccc cccaacaca aaa 83

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 <223> OTHER INFORMATION: Dengue 1 delta 30

<400> SEQUENCE: 5

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<210> SEQ ID NO 6
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<210> SEQ ID NO 7
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<400> SEQUENCE: 7

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<210> SEQ ID NO 8
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<212> TYPE: RNA
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 <223> OTHER INFORMATION: Dengue 4 delta 30

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 <210> SEQ ID NO 9
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 <400> SEQUENCE: 11

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 <400> SEQUENCE: 12

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 <210> SEQ ID NO 13
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 <220> FEATURE:
 <223> OTHER INFORMATION: Spe1 linker in p3

 <400> SEQUENCE: 13

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 <210> SEQ ID NO 14
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 <212> TYPE: DNA
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<400> SEQUENCE: 15

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<210> SEQ ID NO 16
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 <212> TYPE: DNA
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 <212> TYPE: PRT
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<400> SEQUENCE: 17

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Thr

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 <212> TYPE: DNA
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 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN2/4 junction 3

<400> SEQUENCE: 18

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<210> SEQ ID NO 19
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 <212> TYPE: PRT
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 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN2/4 junction 3

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Pro

<210> SEQ ID NO 20
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 <212> TYPE: DNA
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 <220> FEATURE:
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<400> SEQUENCE: 21

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<210> SEQ ID NO 22
 <211> LENGTH: 51
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN3/4 junction 2

<400> SEQUENCE: 22

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<210> SEQ ID NO 23
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 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
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 <223> OTHER INFORMATION: rDEN3/4 junction 2

<400> SEQUENCE: 23

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Thr

<210> SEQ ID NO 24
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 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN3/4 junction 3

<400> SEQUENCE: 24

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<210> SEQ ID NO 25
 <211> LENGTH: 17
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN3/4 junction 3

<400> SEQUENCE: 25

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Pro

<210> SEQ ID NO 26
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 <212> TYPE: DNA
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<400> SEQUENCE: 26

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 <212> TYPE: PRT
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 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN1/4 junction 1

<400> SEQUENCE: 27

Met Asn Asn Gln Arg
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<210> SEQ ID NO 28
 <211> LENGTH: 51
 <212> TYPE: DNA
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 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN1/4 junction 2

<400> SEQUENCE: 28

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<210> SEQ ID NO 29
 <211> LENGTH: 17
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN1/4 junction 2

<400> SEQUENCE: 29

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Thr

<210> SEQ ID NO 30
 <211> LENGTH: 51
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN1/4 junction 3

<400> SEQUENCE: 30

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<210> SEQ ID NO 31
 <211> LENGTH: 17
 <212> TYPE: PRT
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 <220> FEATURE:
 <223> OTHER INFORMATION: rDEN1/4 junction 3

<400> SEQUENCE: 31

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Pro

<210> SEQ ID NO 32
 <211> LENGTH: 60
 <212> TYPE: RNA
 <213> ORGANISM: Dengue 4 virus

<400> SEQUENCE: 32

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<210> SEQ ID NO 33
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Dengue 4 virus

<400> SEQUENCE: 33

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 Ile Ile Gly Pro
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<210> SEQ ID NO 34
 <211> LENGTH: 60
 <212> TYPE: RNA
 <213> ORGANISM: Dengue 1 virus

<400> SEQUENCE: 34

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<210> SEQ ID NO 35
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Dengue 1 virus

<400> SEQUENCE: 35

Pro Leu Thr Leu Thr Ala Ala Val Pro Met Leu Val Ala His Thr Ala
 1 5 10 15
 Ile Ile Gly Pro
 20

<210> SEQ ID NO 36
 <211> LENGTH: 60
 <212> TYPE: RNA
 <213> ORGANISM: Dengue 2 virus

<400> SEQUENCE: 36

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<210> SEQ ID NO 37
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 <212> TYPE: PRT
 <213> ORGANISM: Dengue 2 virus

<400> SEQUENCE: 37

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 1 5 10 15
 Ile Ile Gly Pro
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<210> SEQ ID NO 38
 <211> LENGTH: 60
 <212> TYPE: RNA
 <213> ORGANISM: Dengue 3 virus

<400> SEQUENCE: 38

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<210> SEQ ID NO 39
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Dengue 3 virus

<400> SEQUENCE: 39

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Pro Leu Thr Leu Thr Ala Ala Val Leu Leu Leu Val Thr His Thr Ala
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Ile Ile Gly Pro
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<210> SEQ ID NO 40
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 <220> FEATURE:
 <223> OTHER INFORMATION: primer

<400> SEQUENCE: 40

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<210> SEQ ID NO 41
 <211> LENGTH: 10
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: primer

<400> SEQUENCE: 41

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<210> SEQ ID NO 42
 <211> LENGTH: 12
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: primer

<400> SEQUENCE: 42

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<210> SEQ ID NO 43
 <211> LENGTH: 11
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: primer

<400> SEQUENCE: 43

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<210> SEQ ID NO 44
 <211> LENGTH: 15
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: primer

<400> SEQUENCE: 44

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<210> SEQ ID NO 45
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 <400> SEQUENCE: 46

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<212> TYPE: PRT

<213> ORGANISM: Dengue 2 virus (Tonga/74)

<400> SEQUENCE: 47

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Phe Ser Leu Gly Met Leu Gln Gly Arg Gly Pro Leu Lys Leu Phe Met
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Ile Leu Lys Arg Trp Gly Thr Ile Lys Lys Ser Lys Ala Ile Asn Val
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Arg Arg Arg Arg Thr Val Gly Met Ile Ile Met Leu Thr Pro Thr Val
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Met Ala Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val
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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
 245 250 255

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
 290 295 300

Leu Glu His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr
 305 310 315 320

Leu Asp Phe Glu Leu Ile Lys Thr Glu Ala Lys Gln Pro Ala Thr Leu
 325 330 335

Arg Lys Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Thr Asp Ser
 340 345 350

Arg Cys Pro Thr Gln Gly Glu Pro Thr Leu Asn Glu Glu Gln Asp Lys
 355 360 365

Arg Phe Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly
 370 375 380

Cys Gly Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr
 385 390 395 400

Cys Lys Lys Asn Met Glu Gly Lys Ile Val Gln Pro Glu Asn Leu Glu
 405 410 415

Tyr Thr Val Val Ile Thr Pro His Ser Gly Glu Glu His Ala Val Gly
 420 425 430

Asn Asp Thr Gly Lys His Gly Lys Glu Val Lys Ile Thr Pro Gln Ser
 435 440 445

Ser Ile Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu
 450 455 460

Cys Ser Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln
 465 470 475 480

Met Glu Asp Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu
 485 490 495

Pro Leu Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile
 500 505 510

Gln Lys Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln
 515 520 525

Asp Val Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu
 530 535 540

Thr Gly Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr
 545 550 555 560

Gly His Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly
 565 570 575

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| | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Tyr | Ser | Met | Cys | Thr | Gly | Lys | Phe | Lys | Ile | Val | Lys | Glu | Ile |
| | | | 580 | | | | | 585 | | | | | 590 | | |
| 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 | |
| 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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| 995 | | | 1000 | | | 1005 | | |
|-------------|-------------|-------------|-------------|-------------|---------|------|--|--|
| Asn Gly Val | Leu Glu Ser | Glu Met Val | Ile Pro Lys | Asn Phe Ala | Gly | | | |
| 1010 | | 1015 | | 1020 | | | | |
| Pro Val Ser | Gln His Asn | Asn Arg Pro | Gly Tyr Tyr | Thr Gln Thr | Ala | | | |
| 1025 | | 1030 | | 1035 | 1040 | | | |
| Gly Pro Trp | His Leu Gly | Lys Leu Glu | Met Asp Phe | Asp Phe Cys | Glu | | | |
| | 1045 | | 1050 | | 1055 | | | |
| Gly Thr Thr | Val Val Val | Thr Glu Asn | Cys Gly Asn | Arg Gly Pro | Ser | | | |
| | 1060 | | 1065 | | 1070 | | | |
| Leu Arg Thr | Thr Thr Thr | Ala Ser Gly | Lys Leu Ile | Thr Glu Trp | Cys Cys | | | |
| | 1075 | | 1080 | | 1085 | | | |
| Arg Ser Cys | Thr Leu Pro | Pro Leu Arg | Tyr Arg Gly | Glu Asp Gly | Cys | | | |
| 1090 | | 1095 | | 1100 | | | | |
| Trp Tyr Gly | Met Glu Ile | Arg Pro Leu | Lys Glu Lys | Glu Glu Asn | Leu | | | |
| 1105 | | 1110 | | 1115 | 1120 | | | |
| Val Ser Ser | Leu Val Thr | Ala Gly His | Gly Gln Ile | Asp Asn Phe | Ser | | | |
| | 1125 | | 1130 | | 1135 | | | |
| Leu Gly Ile | Leu Gly Met | Ala Leu Phe | Leu Glu Glu | Met Leu Arg | Thr | | | |
| | 1140 | | 1145 | | 1150 | | | |
| Arg Val Gly | Thr Lys His | Ala Ile Leu | Leu Val Ala | Val Ser Phe | Val | | | |
| | 1155 | | 1160 | | 1165 | | | |
| Thr Leu Ile | Thr Gly Asn | Met Ser Phe | Arg Asp Leu | Gly Arg Val | Met | | | |
| 1170 | | 1175 | | 1180 | | | | |
| Val Met Val | Gly Ala Thr | Met Thr Asp | Asp Ile Gly | Met Gly Val | Thr | | | |
| 1185 | | 1190 | | 1195 | 1200 | | | |
| Tyr Leu Ala | Leu Leu Ala | Ala Phe Arg | Val Arg Pro | Thr Phe Ala | Ala | | | |
| | 1205 | | 1210 | | 1215 | | | |
| Gly Leu Leu | Leu Arg Lys | Leu Thr Ser | Lys Glu Leu | Met Met Thr | Thr | | | |
| | 1220 | | 1225 | | 1230 | | | |
| Ile Gly Ile | Val Leu Leu | Ser Gln Ser | Ser Ile Pro | Glu Thr Ile | Leu | | | |
| 1235 | | 1240 | | 1245 | | | | |
| Glu Leu Thr | Asp Ala Leu | Ala Leu Gly | Met Met Val | Leu Lys Met | Val | | | |
| 1250 | | 1255 | | 1260 | | | | |
| Arg Asn Met | Glu Lys Tyr | Gln Leu Ala | Val Thr Ile | Met Ala Ile | Leu | | | |
| 1265 | | 1270 | | 1275 | 1280 | | | |
| Cys Val Pro | Asn Ala Val | Ile Leu Gln | Asn Ala Trp | Lys Val Ser | Cys | | | |
| | 1285 | | 1290 | | 1295 | | | |
| Thr Ile Leu | Ala Val Val | Ser Val Ser | Pro Leu Leu | Leu Thr Ser | Ser | | | |
| | 1300 | | 1305 | | 1310 | | | |
| Gln Gln Lys | Ala Asp Trp | Ile Pro Leu | Ala Leu Thr | Ile Lys Gly | Leu | | | |
| | 1315 | | 1320 | | 1325 | | | |
| Asn Pro Thr | Ala Ile Phe | Leu Thr Thr | Leu Ser Arg | Thr Asn Lys | Lys | | | |
| 1330 | | 1335 | | 1340 | | | | |
| Arg Ser Trp | Pro Leu Asn | Glu Ala Ile | Met Ala Val | Gly Met Val | Ser | | | |
| 1345 | | 1350 | | 1355 | 1360 | | | |
| Ile Leu Ala | Ser Ser Leu | Leu Lys Asn | Asp Ile Pro | Met Thr Gly | Pro | | | |
| | 1365 | | 1370 | | 1375 | | | |
| Leu Val Ala | Gly Gly Leu | Leu Thr Val | Cys Tyr Val | Leu Thr Gly | Arg | | | |
| | 1380 | | 1385 | | 1390 | | | |
| Ser Ala Asp | Leu Glu Leu | Glu Arg Ala | Thr Asp Val | Lys Trp Asp | Asp | | | |
| 1395 | | 1400 | | 1405 | | | | |
| Gln Ala Glu | Ile Ser Gly | Ser Ser Pro | Ile Leu Ser | Ile Thr Ile | Ser | | | |
| 1410 | | 1415 | | 1420 | | | | |

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Glu Asp Gly Ser Met Ser Ile Lys Asn Glu Glu Glu Glu Gln Thr Leu
 1425 1430 1435 1440
 Thr Ile Leu Ile Arg Thr Gly Leu Leu Val Ile Ser Gly Leu Phe Pro
 1445 1450 1455
 Val Ser Ile Pro Ile Thr Ala Ala Ala Trp Tyr Leu Trp Glu Val Lys
 1460 1465 1470
 Lys Gln Arg Ala Gly Val Leu Trp Asp Val Pro Ser Pro Pro Pro Val
 1475 1480 1485
 Gly Lys Ala Glu Leu Glu Asp Gly Ala Tyr Arg Ile Lys Gln Lys Gly
 1490 1495 1500
 Ile Leu Gly Tyr Ser Gln Ile Gly Ala Gly Val Tyr Lys Glu Gly Thr
 1505 1510 1515 1520
 Phe His Thr Met Trp His Val Thr Arg Gly Ala Val Leu Met His Lys
 1525 1530 1535
 Gly Lys Arg Ile Glu Pro Ser Trp Ala Asp Val Lys Lys Asp Leu Ile
 1540 1545 1550
 Ser Tyr Gly Gly Gly Trp Lys Leu Glu Gly Glu Trp Lys Glu Gly Glu
 1555 1560 1565
 Glu Val Gln Val Leu Ala Leu Glu Pro Gly Lys Asn Pro Arg Ala Val
 1570 1575 1580
 Gln Thr Lys Pro Gly Leu Phe Arg Thr Asn Thr Gly Thr Ile Gly Ala
 1585 1590 1595 1600
 Val Ser Leu Asp Phe Ser Pro Gly Thr Ser Gly Ser Pro Ile Val Asp
 1605 1610 1615
 Lys Lys Gly Lys Val Val Gly Leu Tyr Gly Asn Gly Val Val Thr Arg
 1620 1625 1630
 Ser Gly Ala Tyr Val Ser Ala Ile Ala Gln Thr Glu Lys Ser Ile Glu
 1635 1640 1645
 Asp Asn Pro Glu Ile Glu Asp Asp Ile Phe Arg Lys Arg Arg Leu Thr
 1650 1655 1660
 Ile Met Asp Leu His Pro Gly Ala Gly Lys Thr Lys Arg Tyr Leu Pro
 1665 1670 1675 1680
 Ala Ile Val Arg Glu Ala Ile Lys Arg Gly Leu Arg Thr Leu Ile Leu
 1685 1690 1695
 Ala Pro Thr Arg Val Val Ala Ala Glu Met Glu Glu Ala Leu Arg Gly
 1700 1705 1710
 Leu Pro Ile Arg Tyr Gln Thr Pro Ala Ile Arg Ala Glu His Thr Gly
 1715 1720 1725
 Arg Glu Ile Val Asp Leu Met Cys His Ala Thr Phe Thr Met Arg Leu
 1730 1735 1740
 Leu Ser Pro Ile Arg Val Pro Asn Tyr Asn Leu Ile Ile Met Asp Glu
 1745 1750 1755 1760
 Ala His Phe Thr Asp Pro Ala Ser Ile Ala Ala Arg Gly Tyr Ile Ser
 1765 1770 1775
 Thr Arg Val Glu Met Gly Glu Ala Ala Gly Ile Phe Met Thr Ala Thr
 1780 1785 1790
 Pro Pro Gly Ser Arg Asp Pro Phe Pro Gln Ser Asn Ala Pro Ile Met
 1795 1800 1805
 Asp Glu Glu Arg Glu Ile Pro Glu Arg Ser Trp Asn Ser Gly His Glu
 1810 1815 1820
 Trp Val Thr Asp Phe Lys Gly Lys Thr Val Trp Phe Val Pro Ser Ile
 1825 1830 1835 1840

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Lys Thr Gly Asn Asp Ile Ala Ala Cys Leu Arg Lys Asn Gly Lys Arg
 1845 1850 1855
 Val Ile Gln Leu Ser Arg Lys Thr Phe Asp Ser Glu Tyr Val Lys Thr
 1860 1865 1870
 Arg Thr Asn Asp Trp Asp Phe Val Val Thr Thr Asp Ile Ser Glu Met
 1875 1880 1885
 Gly Ala Asn Phe Lys Ala Glu Arg Val Ile Asp Pro Arg Arg Cys Met
 1890 1895 1900
 Lys Pro Val Ile Leu Thr Asp Gly Glu Glu Arg Val Ile Leu Ala Gly
 1905 1910 1915 1920
 Pro Met Pro Val Thr His Ser Ser Ala Ala Gln Arg Arg Gly Arg Ile
 1925 1930 1935
 Gly Arg Asn Pro Arg Asn Glu Asn Asp Gln Tyr Ile Tyr Met Gly Glu
 1940 1945 1950
 Pro Leu Glu Asn Asp Glu Asp Cys Ala His Trp Lys Glu Ala Lys Met
 1955 1960 1965
 Leu Leu Asp Asn Ile Asn Thr Pro Glu Gly Ile Ile Pro Ser Leu Phe
 1970 1975 1980
 Glu Pro Glu Arg Glu Lys Val Asp Ala Ile Asp Gly Glu Tyr Arg Leu
 1985 1990 1995 2000
 Arg Gly Glu Ala Arg Lys Thr Phe Val Asp Leu Met Arg Arg Gly Asp
 2005 2010 2015
 Leu Pro Val Trp Leu Ala Tyr Lys Val Ala Ala Glu Gly Ile Asn Tyr
 2020 2025 2030
 Ala Asp Arg Arg Trp Cys Phe Asp Gly Thr Arg Asn Asn Gln Ile Leu
 2035 2040 2045
 Glu Glu Asn Val Glu Val Glu Ile Trp Thr Lys Glu Gly Glu Arg Lys
 2050 2055 2060
 Lys Leu Lys Pro Arg Trp Leu Asp Ala Arg Ile Tyr Ser Asp Pro Leu
 2065 2070 2075 2080
 Ala Leu Lys Glu Phe Lys Glu Phe Ala Ala Gly Arg Lys Ser Leu Thr
 2085 2090 2095
 Leu Asn Leu Ile Thr Glu Met Gly Arg Leu Pro Thr Phe Met Thr Gln
 2100 2105 2110
 Lys Ala Arg Asp Ala Leu Asp Asn Leu Ala Val Leu His Thr Ala Glu
 2115 2120 2125
 Ala Gly Gly Lys Ala Tyr Asn His Ala Leu Ser Glu Leu Pro Glu Thr
 2130 2135 2140
 Leu Glu Thr Leu Leu Leu Leu Thr Leu Leu Ala Thr Val Thr Gly Gly
 2145 2150 2155 2160
 Ile Phe Leu Phe Leu Met Ser Gly Arg Gly Met Gly Lys Met Thr Leu
 2165 2170 2175
 Gly Met Cys Cys Ile Ile Thr Ala Ser Ile Leu Leu Trp Tyr Ala Gln
 2180 2185 2190
 Ile Gln Pro His Trp Ile Ala Ala Ser Ile Ile Leu Glu Phe Phe Leu
 2195 2200 2205
 Ile Val Leu Leu Ile Pro Glu Pro Glu Lys Gln Arg Thr Pro Gln Asp
 2210 2215 2220
 Asn Gln Leu Thr Tyr Val Ile Ile Ala Ile Leu Thr Val Val Ala Ala
 2225 2230 2235 2240
 Thr Met Ala Asn Glu Met Gly Phe Leu Glu Lys Thr Lys Lys Asp Leu
 2245 2250 2255
 Gly Leu Gly Asn Ile Ala Thr Gln Gln Pro Glu Ser Asn Ile Leu Asp

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| 2260 | | | | | 2265 | | | | | 2270 | | | | | |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|-----|
| Ile | Asp | Leu | Arg | Pro | Ala | Ser | Ala | Trp | Thr | Leu | Tyr | Ala | Val | Ala | Thr |
| | 2275 | | | | | | 2280 | | | | | 2285 | | | |
| Thr | Phe | Ile | Thr | Pro | Met | Leu | Arg | His | Ser | Ile | Glu | Asn | Ser | Ser | Val |
| | 2290 | | | | | 2295 | | | | 2300 | | | | | |
| Asn | Val | Ser | Leu | Thr | Ala | Ile | Ala | Asn | Gln | Ala | Thr | Val | Leu | Met | Gly |
| 2305 | | | | | 2310 | | | | 2315 | | | | | 2320 | |
| Leu | Gly | Lys | Gly | Trp | Pro | Leu | Ser | Lys | Met | Asp | Ile | Gly | Val | Pro | Leu |
| | | | | 2325 | | | | | 2330 | | | | 2335 | | |
| Leu | Ala | Ile | Gly | Cys | Tyr | Ser | Gln | Val | Asn | Pro | Ile | Thr | Leu | Thr | Ala |
| | | | 2340 | | | | | | 2345 | | | 2350 | | | |
| Ala | Leu | Leu | Leu | Leu | Val | Ala | His | Tyr | Ala | Ile | Ile | Gly | Pro | Gly | Leu |
| | | | 2355 | | | | 2360 | | | | | 2365 | | | |
| Gln | Ala | Lys | Ala | Thr | Arg | Glu | Ala | Gln | Lys | Arg | Ala | Ala | Ala | Gly | Ile |
| | 2370 | | | | | 2375 | | | | | 2380 | | | | |
| Met | Lys | Asn | Pro | Thr | Val | Asp | Gly | Ile | Thr | Val | Ile | Asp | Leu | Asp | Pro |
| 2385 | | | | | 2390 | | | | 2395 | | | | 2400 | | |
| Ile | Pro | Tyr | Asp | Pro | Lys | Phe | Glu | Lys | Gln | Leu | Gly | Gln | Val | Met | Leu |
| | | | 2405 | | | | | | 2410 | | | 2415 | | | |
| Leu | Val | Leu | Cys | Val | Thr | Gln | Val | Leu | Met | Met | Arg | Thr | Thr | Trp | Ala |
| | | | 2420 | | | | | 2425 | | | | | 2430 | | |
| Leu | Cys | Glu | Ala | Leu | Thr | Leu | Ala | Thr | Gly | Pro | Val | Ser | Thr | Leu | Trp |
| | | 2435 | | | | | 2440 | | | | | 2445 | | | |
| Glu | Gly | Asn | Pro | Gly | Arg | Phe | Trp | Asn | Thr | Thr | Ile | Ala | Val | Ser | Met |
| | 2450 | | | | | 2455 | | | | | 2460 | | | | |
| Ala | Asn | Ile | Phe | Arg | Gly | Ser | Tyr | Leu | Ala | Gly | Ala | Gly | Leu | Leu | Phe |
| 2465 | | | | | 2470 | | | | 2475 | | | | | 2480 | |
| Ser | Ile | Met | Lys | Asn | Thr | Thr | Ser | Thr | Arg | Arg | Gly | Thr | Gly | Asn | Ile |
| | | | 2485 | | | | | | 2490 | | | 2495 | | | |
| Gly | Glu | Thr | Leu | Gly | Glu | Lys | Trp | Lys | Ser | Arg | Leu | Asn | Ala | Leu | Gly |
| | | 2500 | | | | | | 2505 | | | | | 2510 | | |
| Lys | Ser | Glu | Phe | Gln | Ile | Tyr | Lys | Lys | Ser | Gly | Ile | Gln | Glu | Val | Asp |
| | | 2515 | | | | | 2520 | | | | 2525 | | | | |
| Arg | Thr | Leu | Ala | Lys | Glu | Gly | Ile | Lys | Arg | Gly | Glu | Thr | Asp | His | His |
| | 2530 | | | | | 2535 | | | | | 2540 | | | | |
| Ala | Val | Ser | Arg | Gly | Ser | Ala | Lys | Leu | Arg | Trp | Phe | Val | Glu | Arg | Asn |
| 2545 | | | | | 2550 | | | | 2555 | | | | | 2560 | |
| Leu | Val | Thr | Pro | Glu | Gly | Lys | Val | Val | Asp | Leu | Gly | Cys | Gly | Arg | Gly |
| | | | | 2565 | | | | | 2570 | | | 2575 | | | |
| Gly | Trp | Ser | Tyr | Tyr | Cys | Gly | Gly | Leu | Lys | Asn | Val | Arg | Glu | Val | Lys |
| | | 2580 | | | | | | 2585 | | | | | 2590 | | |
| Gly | Leu | Thr | Lys | Gly | Gly | Pro | Gly | His | Glu | Glu | Pro | Ile | Pro | Met | Ser |
| | | 2595 | | | | | 2600 | | | | | 2605 | | | |
| Thr | Tyr | Gly | Trp | Asn | Leu | Val | Arg | Leu | Gln | Ser | Gly | Val | Asp | Val | Phe |
| | 2610 | | | | | 2615 | | | | | 2620 | | | | |
| 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 | | | | |

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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
 2740 2745 2750

Thr Arg Asn Ile Gly Ile Glu Ser Glu Thr Pro Asn Leu Asp Ile Ile
 2755 2760 2765

Gly Lys Arg Ile Glu Lys Ile Lys Gln Glu His Glu Thr Ser Trp His
 2770 2775 2780

Tyr Asp Gln Asp His Pro Tyr Lys Thr Trp Ala Tyr His Gly Ser Tyr
 2785 2790 2795 2800

Glu Thr Lys Gln Thr Gly Ser Ala Ser Ser Met Val Asn Gly Val Val
 2805 2810 2815

Arg Leu Leu Thr Lys Pro Trp Asp Val Val Pro Met Val Thr Gln Met
 2820 2825 2830

Ala Met Thr Asp Thr Thr Pro Phe Gly Gln Gln Arg Val Phe Lys Glu
 2835 2840 2845

Lys Val Asp Thr Arg Thr Gln Glu Pro Lys Glu Gly Thr Lys Lys Leu
 2850 2855 2860

Met Lys Ile Thr Ala Glu Trp Leu Trp Lys Glu Leu Gly Lys Lys Lys
 2865 2870 2875 2880

Thr Pro Arg Met Cys Thr Arg Glu Glu Phe Thr Lys Lys Val Arg Ser
 2885 2890 2895

Asn Ala Ala Leu Gly Ala Ile Phe Thr Asp Glu Asn Lys Trp Lys Ser
 2900 2905 2910

Ala Arg Glu Ala Val Glu Asp Ser Arg Phe Trp Glu Leu Val Asp Lys
 2915 2920 2925

Glu Arg Asn Leu His Leu Glu Gly Lys Cys Glu Thr Cys Val Tyr Asn
 2930 2935 2940

Met Met Gly Lys Arg Glu Lys Lys Leu Gly Glu Phe Gly Lys Ala Lys
 2945 2950 2955 2960

Gly Ser Arg Ala Ile Trp Tyr Met Trp Leu Gly Ala Arg Phe Leu Glu
 2965 2970 2975

Phe Glu Ala Leu Gly Phe Leu Asn Glu Asp His Trp Phe Ser Arg Glu
 2980 2985 2990

Asn Ser Leu Ser Gly Val Glu Gly Glu Gly Leu His Lys Leu Gly Tyr
 2995 3000 3005

Ile Leu Arg Glu Val Ser Lys Lys Glu Gly Gly Ala Met Tyr Ala Asp
 3010 3015 3020

Asp Thr Ala Gly Trp Asp Thr Arg Ile Thr Ile Glu Asp Leu Lys Asn
 3025 3030 3035 3040

Glu Glu Met Ile Thr Asn His Met Ala Gly Glu His Lys Lys Leu Ala
 3045 3050 3055

Glu Ala Ile Phe Lys Leu Thr Tyr Gln Asn Lys Val Val Arg Val Gln
 3060 3065 3070

Arg Pro Thr Pro Arg Gly Thr Val Met Asp Ile Ile Ser Arg Arg Asp
 3075 3080 3085

Gln Arg Gly Ser Gly Gln Val Gly Thr Tyr Gly Leu Asn Thr Phe Thr
 3090 3095 3100

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Asn Met Glu Ala Gln Leu Ile Arg Gln Met Glu Gly Glu Gly Ile Phe
 3105 3110 3115 3120
 Lys Ser Ile Gln His Leu Thr Ala Ser Glu Glu Ile Ala Val Gln Asp
 3125 3130 3135
 Trp Leu Val Arg Val Gly Arg Glu Arg Leu Ser Arg Met Ala Ile Ser
 3140 3145 3150
 Gly Asp Asp Cys Val Val Lys Pro Leu Asp Asp Arg Phe Ala Arg Ala
 3155 3160 3165
 Leu Thr Ala Leu Asn Asp Met Gly Lys Val Arg Lys Asp Ile Gln Gln
 3170 3175 3180
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<210> SEQ ID NO 49

<211> LENGTH: 3390

<212> TYPE: PRT

<213> ORGANISM: Dengue 3 (Sleman/78) virus

<400> SEQUENCE: 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
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

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 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 | Ser | 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 |
| | | 675 | | | | | 680 | | | | | 685 | | | |
| Arg | Met | Ala | Ile | Leu | Gly | Asp | Thr | Ala | Trp | Asp | Phe | Gly | Ser | Val | Gly |
| | 690 | | | | | 695 | | | | | 700 | | | | |
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| 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 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 |
| 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 |
| Ser Leu Val | Ser Ala Gly Ser Gly Lys Val | Asp Asn Phe Thr Met Gly | |
| | 1125 | 1130 | 1135 |

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 Arg Leu Glu Pro Asn Trp Ala Ser Val Lys Lys Asp Leu Ile Ser Tyr
 1540 1545 1550

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| | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|
| 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 | | | | |

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| 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 |
| Thr Arg Asn Ala Leu Asp Asn Leu Val Met Leu His Thr Ser Glu His
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
2145 | 2150 | 2155 2160 |
| Met Leu Phe Leu Ile Ser Gly Lys Gly Ile Gly Lys Thr Ser Ile Gly
2165 | 2170 | 2175 |
| Leu Ile Cys Val Ala Ala Ser Ser Gly Met Leu Trp Met Ala Asp Val
2180 | 2185 | 2190 |
| Pro Leu Gln Trp Ile Ala Ser Ala Ile Val Leu Glu Phe Phe Met Met
2195 | 2200 | 2205 |
| Val Leu Leu Ile Pro Glu Pro Glu Lys Gln Arg Thr Pro Gln Asp Asn
2210 | 2215 | 2220 |
| Gln Leu Ala Tyr Val Val Ile Gly Ile Leu Thr Leu Ala Ala Ile Val
2225 | 2230 | 2235 2240 |
| Ala Ala Asn Glu Met Gly Leu Leu Glu Thr Thr Lys Arg Asp Leu Gly
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 |
| Val Ile Thr Pro Met Leu Arg His Thr Ile Glu Asn Ser Thr Ala Asn
2290 | 2295 | 2300 |
| Val Ser Leu Ala Ala Ile Ala Asn Gln Ala Val Val Leu Met Gly Leu
2305 | 2310 | 2315 2320 |
| Asp Lys Gly Trp Pro Ile Ser Lys Met Asp Leu Gly Val Pro Leu Leu
2325 | 2330 | 2335 |
| Ala Leu Gly Cys Tyr Ser Gln Val Asn Pro Leu Thr Leu Thr Ala Ala
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 |

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Ile Tyr Asp Ser Lys Phe Glu Lys Gln Leu Gly Gln Val Met Leu Leu
2405 2410 2415

Val Leu Cys Ala Val Gln Leu Leu Leu Met Arg Thr Ser Trp Ala Phe
2420 2425 2430

Cys Glu Ala Leu Thr Leu Ala Thr Gly Pro Ile Thr Thr Leu Trp Glu
2435 2440 2445

Gly Ser Pro Gly Lys Phe Trp Asn Thr Thr Ile Ala Val Ser Met Ala
2450 2455 2460

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

Lys Glu Phe Asp Leu Tyr Lys Lys Ser Gly Ile Thr Glu Val Asp Arg
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
2725 2730 2735

Arg Arg Pro Thr Ile Glu Lys Asp Val Asp Leu Gly Ala Gly Thr Arg
2740 2745 2750

His Val Asn Ala Glu Pro Glu Thr Pro Asn Met Asp Val Ile Gly Glu
2755 2760 2765

Arg Ile Arg Arg Ile Lys Glu Glu His Ser Ser Thr Trp His Tyr Asp
2770 2775 2780

Asp Glu Asn Pro Tyr Lys Thr Trp Ala Tyr His Gly Ser Tyr Glu Val
2785 2790 2795 2800

Lys Ala Thr Gly Ser Ala Ser Ser Met Ile Asn Gly Val Val Lys Leu
2805 2810 2815

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Leu Thr Lys Pro Trp Asp Val Val Pro Met Val Thr Gln Met Ala Met
 2820 2825 2830

Thr Asp Thr Thr Pro Phe Gly Gln Gln Arg Val Phe Lys Glu Lys Val
 2835 2840 2845

Asp Thr Arg Thr Pro Arg Pro Met Pro Gly Thr Arg Lys Val Met Glu
 2850 2855 2860

Ile Thr Ala Glu Trp Leu Trp Arg Thr Leu Gly Arg Asn Lys Arg Pro
 2865 2870 2875 2880

Arg Leu Cys Thr Arg Glu Glu Phe Thr Lys Lys Val Arg Thr Asn Ala
 2885 2890 2895

Ala Met Gly Ala Val Phe Thr Glu Glu Asn Gln Trp Asp Ser Ala Arg
 2900 2905 2910

Ala Ala Val Glu Asp Glu Glu Phe Trp Lys Leu Val Asp Arg Glu Arg
 2915 2920 2925

Glu Leu His Lys Leu Gly Lys Cys Gly Ser Cys Val Tyr Asn Met Met
 2930 2935 2940

Gly Lys Arg Glu Lys Lys Leu Gly Glu Phe Gly Lys Ala Lys Gly Ser
 2945 2950 2955 2960

Arg Ala Ile Trp Tyr Met Trp Leu Gly Ala Arg Tyr Leu Glu Phe Glu
 2965 2970 2975

Ala Leu Gly Phe Leu Asn Glu Asp His Trp Phe Ser Arg Glu Asn Ser
 2980 2985 2990

Tyr Ser Gly Val Glu Gly Glu Gly Leu His Lys Leu Gly Tyr Ile Leu
 2995 3000 3005

Arg Asp Ile Ser Lys Ile Pro Gly Gly Ala Met Tyr Ala Asp Asp Thr
 3010 3015 3020

Ala Gly Trp Asp Thr Arg Ile Thr Glu Asp Asp Leu His Asn Glu Glu
 3025 3030 3035 3040

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

Thr Pro Lys Gly Thr Val Met Asp Ile Ile Ser Arg Lys Asp Gln Arg
 3075 3080 3085

Gly Ser Gly Gln Val Gly Thr Tyr Gly Leu Asn Thr Phe Thr Asn Met
 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 Glu Asn Pro His Leu Leu Glu Lys Lys Val Thr Gln Trp
 3125 3130 3135

Leu Glu Thr 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
 3170 3175 3180

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

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| 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
3315 | 3320 | 3325 |
| Leu Gly Lys Arg Glu Asp Gln Trp Cys Gly Ser Leu Ile Gly Leu Thr
3330 | 3335 | 3340 |
| 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
3365 | 3370 | 3375 |
| Lys Arg Phe Arg Lys Glu Glu Glu Ser Glu Gly Ala Ile Trp
3380 | 3385 | 3390 |

<210> SEQ ID NO 50
 <211> LENGTH: 2426
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Dengue 1 CME chimeric region

<400> SEQUENCE: 50

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agttgttagt ctgtgtggac cgacaaggac agttccaaat cggaagcttg cttaacacag      60
ttctaacagt ttgtttgaat agagagcaga tctctggaaa aatgaacaac caacggaaaa      120
agacgggtcg accgtctttc aatatgctga aacgcgcgag aaaccgcgtg tcaactggtt      180
cacagttggc gaagagattc tcaaaaggat tgctttcagg ccaaggaccc atgaaattgg      240
tgatggcttt catagcattt ctaagatttc tagccatacc cccaacagca ggaatthttg      300
ctagatggag ctcatcaag aagaatggag cgatcaaagt gttacggggt ttcaaaaaag      360
agatctcaag catggtgaac attatgaaca ggaggaaaaa atctgtgacc atgctcctca      420
tgctgctgcc cacagccctg gcgttccatt tgaccacacg aggggggagag ccacacatga      480
tagttagtaa gcaggaaaga ggaaagtcac tgttgtttaa gacctctgca ggcacatcaata      540
tgtgcaactc cattgcatg gatttgggag agttatgcca ggacacaatg acctacaaat      600
gccccggat cactgaggcg gaaccagatg acgttgactg ctggtgcaat gccacagaca      660
catgggtgac ctatgggacg tgttctcaaa ccggcgaaca ccgacgagac aaacgttccg      720
tggcactggc cccacacgtg ggacttggtc tagaaacaag aaccgaaaca tggatgtcct      780
ctgaagggtc ctggaacaaa gtacaaaaag tggagacttg ggctttgaga caccaggat      840
tcacgggtgac agcccttttt ttagcacatg ccataggaac atccattact cagaaagga      900
tcattttcat tctgctgatg ctagtaacac catcaatggc catgcatgt gtgggaatag      960
gcaacagaga cttcgttgaa ggactgtcag gagcaacgtg ggtggacgtg gtattggagc     1020
atggaagctg cgtcaccacc atggcaaaag ataaaccaac attggacatt gaactcttga     1080
agacggaggt cacaaacct gccgtcttgc gcaaactgtg cattgaagct aaaatatcaa     1140
acaccaccac cgattcaagg tgtccaacac aaggagaggg tacactggtg gaagaacagg     1200

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actcgaactt tgtgtgtcga cgaacgtttg tggacagagg ctggggtaat ggctgctggac 1260
tatttggaag aggaagccta ctgacgtgtg ctaagttcaa gtgtgtgaca aaactagaag 1320
gaaagatagt tcaatatgaa aacttaaaat attcagtgat agtcactgtc cacactgggg 1380
accagcacca ggtgggaaac gagactacag aacatggaac aattgcaacc ataacacctc 1440
aagctcctac gtcggaaata cagctgactg actacggagc cctcacattg gactgctcgc 1500
ctagaacagg gctggacttt aatgagatgg ttctattgac aatgaaagaa aaatcatggc 1560
ttgtccacaa acaatggttt ctgacttac cactgccttg gacttcagga gcttcaacat 1620
ctcaagagac ttggaacaga caagatttgc tggtcacatt caagacagct catgcaaaga 1680
aacaggaagt agtcgtactg ggatcacagg aaggagcaat gcacactgcg ttgactgggg 1740
cgacagaaat ccagacgtca ggaacgacaa caatctttgc aggacacctg aaatgcagac 1800
taaaaatgga taaactgact ttaaaaggga tgtcatatgt aatgtgcaca ggctcattta 1860
agctagagaa ggaagtggct gagacccagc atggaactgt tttagtgcag gttaaatacg 1920
aaggaacaga tgcgccatgc aagatccctt tttcggcca agatgagaaa ggagtgacct 1980
agaatgggag attgataaca gccaacccca tagtcactga caaagaaaaa ccagtcaaca 2040
ttgagacaga accacctttt ggtgagagct acatcgtggg aggggcaggt gaaaaagctt 2100
tgaaaactgag ctggttcaag aaaggagca gcatagggaa aatgttcgaa gcaactgccc 2160
gaggagcgcg aaggatggct atcctgggag acaccgcatg ggactttggc tctataggag 2220
gagtgttcac atcagtggga aaattggtac accaggtttt tggagccgca tatggggttc 2280
tgttcagcgg tgtttcttgg accatgaaaa taggaatagg gattctgctg acatggctag 2340
gattaaactc gaggaacact tcaatggcta tgacgtgcat agctgttggg ggaatcactc 2400
tgtttctggg cttcacagtt caagca 2426

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<210> SEQ ID NO 51

<211> LENGTH: 775

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Dengue 1 CME chimeric region

<400> SEQUENCE: 51

```

Met Asn Asn Gln Arg Lys Lys Thr Gly Arg Pro Ser Phe Asn Met Leu
 1             5             10             15

Lys Arg Ala Arg Asn Arg Val Ser Thr Gly Ser Gln Leu Ala Lys Arg
          20             25             30

Phe Ser Lys Gly Leu Leu Ser 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

Ile Leu Ala Arg Trp Ser Ser Phe Lys Lys Asn Gly Ala Ile Lys Val
65             70             75             80

Leu Arg Gly Phe Lys Lys Glu Ile Ser Ser Met Leu Asn Ile Met Asn
          85             90             95

Arg Arg Lys Lys Ser Val Thr Met Leu Leu Met Leu Leu Pro Thr Ala
          100            105            110

Leu Ala Phe His Leu Thr Thr Arg Gly Gly Glu Pro His Met Ile Val
          115            120            125

Ser Lys Gln Glu Arg Gly Lys Ser Leu Leu Phe Lys Thr Ser Ala Gly
          130            135            140

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ile | Asn | Met | Cys | Thr | Leu | Ile | Ala | Met | Asp | Leu | Gly | Glu | Leu | Cys | Glu | 145 | 150 | 155 | 160 |
| Asp | Thr | Met | Thr | Tyr | Lys | Cys | Pro | Arg | Ile | Thr | Glu | Ala | Glu | Pro | Asp | 165 | 170 | 175 | |
| Asp | Val | Asp | Cys | Trp | Cys | Asn | Ala | Thr | Asp | Thr | Trp | Val | Thr | Tyr | Gly | 180 | 185 | 190 | |
| Thr | Cys | Ser | Gln | Thr | Gly | Glu | His | Arg | Arg | Asp | Lys | Arg | Ser | Val | Ala | 195 | 200 | 205 | |
| Leu | Ala | Pro | His | Val | Gly | Leu | Gly | Leu | Glu | Thr | Arg | Thr | Glu | Thr | Trp | 210 | 215 | 220 | |
| Met | Ser | Ser | Glu | Gly | Ala | Trp | Lys | Gln | Val | Gln | Lys | Val | Glu | Thr | Trp | 225 | 230 | 235 | 240 |
| Ala | Leu | Arg | His | Pro | Gly | Phe | Thr | Val | Thr | Ala | Leu | Phe | Leu | Ala | His | 245 | 250 | 255 | |
| Ala | Ile | Gly | Thr | Ser | Ile | Thr | Gln | Lys | Gly | Ile | Ile | Phe | Ile | Leu | Leu | 260 | 265 | 270 | |
| Met | Leu | Val | Thr | Pro | Ser | Met | Ala | Met | Arg | Cys | Val | Gly | Ile | 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 | Ser | Cys | Val | Thr | Thr | Met | Ala | Lys | Asp | Lys | Pro | Thr | 305 | 310 | 315 | 320 |
| Leu | Asp | Ile | Glu | Leu | Leu | Lys | Thr | Glu | Val | Thr | Asn | Pro | Ala | Val | Leu | 325 | 330 | 335 | |
| Arg | Lys | Leu | Cys | Ile | Glu | Ala | Lys | Ile | Ser | Asn | Thr | Thr | Thr | Asp | Ser | 340 | 345 | 350 | |
| Arg | Cys | Pro | Thr | Gln | Gly | Glu | Ala | Thr | Leu | Val | Glu | Glu | Gln | Asp | Ser | 355 | 360 | 365 | |
| Asn | Phe | Val | Cys | Arg | Arg | Thr | Phe | Val | Asp | Arg | Gly | Trp | Gly | Asn | Gly | 370 | 375 | 380 | |
| Cys | Gly | Leu | Phe | Gly | Lys | Gly | Ser | Leu | Leu | Thr | Cys | Ala | Lys | Phe | Lys | 385 | 390 | 395 | 400 |
| Cys | Val | Thr | Lys | Leu | Glu | Gly | Lys | Ile | Val | Gln | Tyr | Glu | Asn | Leu | Lys | 405 | 410 | 415 | |
| Tyr | Ser | Val | Ile | Val | Thr | Val | His | Thr | Gly | Asp | Gln | His | Gln | Val | Gly | 420 | 425 | 430 | |
| Asn | Glu | Thr | Thr | Glu | His | Gly | Thr | Ile | Ala | Thr | Ile | Thr | Pro | Gln | Ala | 435 | 440 | 445 | |
| Pro | Thr | Ser | Glu | Ile | Gln | Leu | Thr | Asp | Tyr | Gly | Ala | Leu | Thr | Leu | Asp | 450 | 455 | 460 | |
| Cys | Ser | Pro | Arg | Thr | Gly | Leu | Asp | Phe | Asn | Glu | Met | Val | Leu | Leu | Thr | 465 | 470 | 475 | 480 |
| Met | Lys | Glu | Lys | Ser | Trp | Leu | Val | His | Lys | Gln | Trp | Phe | Leu | Asp | Leu | 485 | 490 | 495 | |
| Pro | Leu | Pro | Trp | Thr | Ser | Gly | Ala | Ser | Thr | Ser | Gln | Glu | Thr | Trp | Asn | 500 | 505 | 510 | |
| Arg | Gln | Asp | Leu | Leu | Val | Thr | Phe | Lys | Thr | Ala | His | Ala | Lys | Lys | Gln | 515 | 520 | 525 | |
| Glu | Val | Val | Val | Leu | Gly | Ser | Gln | Glu | Gly | Ala | Met | His | Thr | Ala | Leu | 530 | 535 | 540 | |
| Thr | Gly | Ala | Thr | Glu | Ile | Gln | Thr | Ser | Gly | Thr | Thr | Thr | Ile | Phe | Ala | 545 | 550 | 555 | 560 |
| Gly | His | Leu | Lys | Cys | Arg | Leu | Lys | Met | Asp | Lys | Leu | Thr | Leu | Lys | Gly | | | | |

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| 565 | | | | 570 | | | | 575 | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Tyr | Val | Met | Cys | Thr | Gly | Ser | Phe | Lys | Leu | Glu | Lys | Glu | Val |
| | | | 580 | | | | | 585 | | | | 590 | | | |
| Ala | Glu | Thr | Gln | His | Gly | Thr | Val | Leu | Val | Gln | Val | Lys | Tyr | Glu | Gly |
| | | | 595 | | | | 600 | | | | 605 | | | | |
| Thr | Asp | Ala | Pro | Cys | Lys | Ile | Pro | Phe | Ser | Ala | Gln | Asp | Glu | Lys | Gly |
| | | | 610 | | | | 615 | | | | 620 | | | | |
| Val | Thr | Gln | Asn | Gly | Arg | Leu | Ile | Thr | Ala | Asn | Pro | Ile | Val | Thr | Asp |
| | | | 625 | | | 630 | | | | 635 | | | | | 640 |
| Lys | Glu | Lys | Pro | Val | Asn | Ile | Glu | Thr | Glu | Pro | Pro | Phe | Gly | Glu | Ser |
| | | | 645 | | | | | | | 650 | | | | 655 | |
| Tyr | Ile | Val | Val | Gly | Ala | Gly | Glu | Lys | Ala | Leu | Lys | Leu | Ser | Trp | Phe |
| | | | 660 | | | | | | | 665 | | | | 670 | |
| Lys | Lys | Gly | Ser | Ser | Ile | Gly | Lys | Met | Phe | Glu | Ala | Thr | Ala | Arg | Gly |
| | | | 675 | | | | | | | | | | | 685 | |
| Ala | Arg | Arg | Met | Ala | Ile | Leu | Gly | Asp | Thr | Ala | Trp | Asp | Phe | Gly | Ser |
| | | | 690 | | | | 695 | | | | 700 | | | | |
| Ile | Gly | Gly | Val | Phe | Thr | Ser | Val | Gly | Lys | Leu | Val | His | Gln | Val | Phe |
| | | | 705 | | | | 710 | | | 715 | | | | | 720 |
| Gly | Ala | Ala | Tyr | Gly | Val | Leu | Phe | Ser | Gly | Val | Ser | Trp | Thr | Met | Lys |
| | | | 725 | | | | | | | 730 | | | | 735 | |
| Ile | Gly | Ile | Gly | Ile | Leu | Leu | Thr | Trp | Leu | Gly | Leu | Asn | Ser | Arg | Asn |
| | | | 740 | | | | | | | 745 | | | | 750 | |
| Thr | Ser | Met | Ala | Met | Thr | Cys | Ile | Ala | Val | Gly | Gly | Ile | Thr | Leu | Phe |
| | | | 755 | | | | 760 | | | | | | | 765 | |
| Leu | Gly | Phe | Thr | Val | Gln | Ala | | | | | | | | | |
| | | | 770 | | | 775 | | | | | | | | | |

<210> SEQ ID NO 52

<211> LENGTH: 2423

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Dengue 1 ME chimeric region

<400> SEQUENCE: 52

```

agttgtagt ctgtgtggac cgacaaggac agttccaaat cggaagcttg cttaacacag      60
ttctaacagt ttgtttgaat agagagcaga tctctggaaa aatgaaccaa cgaaaaaagg      120
tggttagacc acctttcaat atgctgaaac gcgagagaaa ccgcgtatca acccctcaag      180
ggttggtgaa gagattctca accggacttt tttctgggaa aggaccctta cggatggtgc      240
tagcattcat cacgtttttg cgagtccttt ccatcccacc aacagcaggg attctgaaga      300
gatggggaca gttgaagaaa aataaggcca tcaagatact gattggattc aggaaggaga      360
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<220> FEATURE:

<223> OTHER INFORMATION: Dengue 1 ME chimeric region

<400> SEQUENCE: 53

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

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| Arg | Lys | Arg | Ser | Ala | Ala | Met | Leu | Leu | Met | Leu | Leu | Pro | Thr | Ala | Leu | 100 | 105 | 110 | |
| Ala | Phe | His | Leu | Thr | Thr | Arg | Gly | Gly | Glu | Pro | His | Met | Ile | Val | Ser | 115 | 120 | 125 | |
| Lys | Gln | Glu | Arg | Gly | Lys | Ser | Leu | Leu | Phe | Lys | Thr | Ser | Ala | Gly | Ile | 130 | 135 | 140 | |
| Asn | Met | Cys | Thr | Leu | Ile | Ala | Met | Asp | Leu | Gly | Glu | Leu | Cys | Glu | Asp | 145 | 150 | 155 | 160 |
| Thr | Met | Thr | Tyr | Lys | Cys | Pro | Arg | Ile | Thr | Glu | Ala | Glu | Pro | Asp | Asp | 165 | 170 | 175 | |
| Val | Asp | Cys | Trp | Cys | Asn | Ala | Thr | Asp | Thr | Trp | Val | Thr | Tyr | Gly | Thr | 180 | 185 | 190 | |
| Cys | Ser | Gln | Thr | Gly | Glu | His | Arg | Arg | Asp | Lys | Arg | Ser | Val | Ala | Leu | 195 | 200 | 205 | |
| Ala | Pro | His | Val | Gly | Leu | Gly | Leu | Glu | Thr | Arg | Thr | Glu | Thr | Trp | Met | 210 | 215 | 220 | |
| Ser | Ser | Glu | Gly | Ala | Trp | Lys | Gln | Val | Gln | Lys | Val | Glu | Thr | Trp | Ala | 225 | 230 | 235 | 240 |
| Leu | Arg | His | Pro | Gly | Phe | Thr | Val | Thr | Ala | Leu | Phe | Leu | Ala | His | Ala | 245 | 250 | 255 | |
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| Leu | Val | Thr | Pro | Ser | Met | Ala | Met | Arg | Cys | Val | Gly | Ile | Gly | Asn | Arg | 275 | 280 | 285 | |
| Asp | Phe | Val | Glu | Gly | Leu | Ser | Gly | Ala | Thr | Trp | Val | Asp | Val | Val | Leu | 290 | 295 | 300 | |
| Glu | His | Gly | Ser | Cys | Val | Thr | Thr | Met | Ala | Lys | Asp | Lys | Pro | Thr | Leu | 305 | 310 | 315 | 320 |
| Asp | Ile | Glu | Leu | Leu | Lys | Thr | Glu | Val | Thr | Asn | Pro | Ala | Val | Leu | Arg | 325 | 330 | 335 | |
| Lys | Leu | Cys | Ile | Glu | Ala | Lys | Ile | Ser | Asn | Thr | Thr | Thr | Asp | Ser | Arg | 340 | 345 | 350 | |
| Cys | Pro | Thr | Gln | Gly | Glu | Ala | Thr | Leu | Val | Glu | Glu | Gln | Asp | Ser | Asn | 355 | 360 | 365 | |
| Phe | Val | Cys | Arg | Arg | Thr | Phe | Val | Asp | Arg | Gly | Trp | Gly | Asn | Gly | Cys | 370 | 375 | 380 | |
| Gly | Leu | Phe | Gly | Lys | Gly | Ser | Leu | Leu | Thr | Cys | Ala | Lys | Phe | Lys | Cys | 385 | 390 | 395 | 400 |
| Val | Thr | Lys | Leu | Glu | Gly | Lys | Ile | Val | Gln | Tyr | Glu | Asn | Leu | Lys | Tyr | 405 | 410 | 415 | |
| Ser | Val | Ile | Val | Thr | Val | His | Thr | Gly | Asp | Gln | His | Gln | Val | Gly | Asn | 420 | 425 | 430 | |
| Glu | Thr | Thr | Glu | His | Gly | Thr | Ile | Ala | Thr | Ile | Thr | Pro | Gln | Ala | Pro | 435 | 440 | 445 | |
| Thr | Ser | Glu | Ile | Gln | Leu | Thr | Asp | Tyr | Gly | Ala | Leu | Thr | Leu | Asp | Cys | 450 | 455 | 460 | |
| Ser | Pro | Arg | Thr | Gly | Leu | Asp | Phe | Asn | Glu | Met | Val | Leu | Leu | Thr | Met | 465 | 470 | 475 | 480 |
| Lys | Glu | Lys | Ser | Trp | Leu | Val | His | Lys | Gln | Trp | Phe | Leu | Asp | Leu | Pro | 485 | 490 | 495 | |
| Leu | Pro | Trp | Thr | Ser | Gly | Ala | Ser | Thr | Ser | Gln | Glu | Thr | Trp | Asn | Arg | | | | |

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
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| | 515 | | | | | | 520 | | | | | 525 | | | |
| Val | Val | Val | Leu | Gly | Ser | Gln | Glu | Gly | Ala | Met | His | Thr | Ala | Leu | Thr |
| | 530 | | | | | 535 | | | | | 540 | | | | |
| Gly | Ala | Thr | Glu | Ile | Gln | Thr | Ser | Gly | Thr | Thr | Thr | Ile | Phe | Ala | Gly |
| 545 | | | | | 550 | | | | | 555 | | | | | 560 |
| His | Leu | Lys | Cys | Arg | Leu | Lys | Met | Asp | Lys | Leu | Thr | Leu | Lys | Gly | Met |
| | | | 565 | | | | | | 570 | | | | | 575 | |
| Ser | Tyr | Val | Met | Cys | Thr | Gly | Ser | Phe | Lys | Leu | Glu | Lys | Glu | Val | Ala |
| | | 580 | | | | | | 585 | | | | | 590 | | |
| Glu | Thr | Gln | His | Gly | Thr | Val | Leu | Val | Gln | Val | Lys | Tyr | Glu | Gly | Thr |
| | | 595 | | | | | 600 | | | | | 605 | | | |
| Asp | Ala | Pro | Cys | Lys | Ile | Pro | Phe | Ser | Ala | Gln | Asp | Glu | Lys | Gly | Val |
| 610 | | | | | | 615 | | | | | 620 | | | | |
| Thr | Gln | Asn | Gly | Arg | Leu | Ile | Thr | Ala | Asn | Pro | Ile | Val | Thr | Asp | Lys |
| 625 | | | | | | 630 | | | | | 635 | | | | 640 |
| Glu | Lys | Pro | Val | Asn | Ile | Glu | Thr | Glu | Pro | Pro | Phe | Gly | Glu | Ser | Tyr |
| | | | | 645 | | | | | 650 | | | | | 655 | |
| Ile | Val | Val | Gly | Ala | Gly | Glu | Lys | Ala | Leu | Lys | Leu | Ser | Trp | Phe | Lys |
| | | | 660 | | | | | 665 | | | | | 670 | | |
| Lys | Gly | Ser | Ser | Ile | Gly | Lys | Met | Phe | Glu | Ala | Thr | Ala | Arg | Gly | Ala |
| | | 675 | | | | | 680 | | | | | 685 | | | |
| Arg | Arg | Met | Ala | Ile | Leu | Gly | Asp | Thr | Ala | Trp | Asp | Phe | Gly | Ser | Ile |
| | 690 | | | | | 695 | | | | | 700 | | | | |
| Gly | Gly | Val | Phe | Thr | Ser | Val | Gly | Lys | Leu | Val | His | Gln | Val | Phe | Gly |
| 705 | | | | | | 710 | | | | | 715 | | | | 720 |
| Ala | Ala | Tyr | Gly | Val | Leu | Phe | Ser | Gly | Val | Ser | Trp | Thr | Met | Lys | Ile |
| | | | | 725 | | | | | 730 | | | | | 735 | |
| Gly | Ile | Gly | Ile | Leu | Leu | Thr | Trp | Leu | Gly | Leu | Asn | Ser | Arg | Asn | Thr |
| | | | 740 | | | | | 745 | | | | | 750 | | |
| Ser | Met | Ala | Met | Thr | Cys | Ile | Ala | Val | Gly | Gly | Ile | Thr | Leu | Phe | Leu |
| | | 755 | | | | | 760 | | | | | 765 | | | |
| Gly | Phe | Thr | Val | Gln | Ala | | | | | | | | | | |
| | | | | 770 | | | | | | | | | | | |

What is claimed is:

1. A tetravalent immunogenic composition comprising
 - a) a first attenuated virus that is immunogenic against dengue serotype 1,
 - b) a second attenuated virus that is immunogenic against dengue serotype 2,
 - c) a third attenuated virus that is immunogenic against dengue serotype 3, and
 - d) a fourth attenuated virus that is immunogenic against dengue serotype 4,
 wherein each of a), b), c) and d) comprises a nucleic acid comprising
 - i) a first nucleotide sequence encoding at least one structural protein from a first dengue virus,
 - ii) a second nucleotide sequence encoding nonstructural proteins from the first dengue virus or a second dengue virus, and
 - iii) a 3' untranslated region, wherein the 3' untranslated region contains a deletion of about 30 nucleotides corresponding to the TL2 stem-loop structure, and wherein both the 3' untranslated region and the

second nucleotide sequence encoding nonstructural proteins are from either the first dengue virus or the second dengue virus;

wherein the tetravalent immunogenic composition is not rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4Δ30.

2. The tetravalent immunogenic composition of claim 1, wherein the nucleic acid of at least one of a), b), c) or d) further comprises a mutation that confers 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 claim 1, wherein the first nucleotide sequence encoding at least one structural protein and the second nucleotide sequence encoding nonstructural proteins of at least one of a), b), c) and d) are from the first dengue virus.

4. The composition of claim 3, wherein the deletion of a) 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.

5 **5.** The composition of claim **3**, wherein the deletion of b) 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.

10 **6.** The composition of claim **3**, wherein the deletion of c) 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.

15 **7.** The composition of claim **3**, herein the deletion of d) 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.

20 **8.** The composition of claim **1**, wherein the first nucleotide sequence encoding at least one structural protein of at least one of a), b), c), and d) is from the first dengue virus and the second nucleotide sequence encoding nonstructural proteins of at least one of a), b), c), and d) is from the second dengue virus, wherein the serotype of the first dengue virus and the second dengue virus are different.

9. The composition of claim **8**, wherein the serotype of the second dengue virus having the deletion is type 1.

10. The composition of claim **9**, wherein the serotype of the first dengue virus of b) is type 2.

30 **11.** The composition of claim **9**, wherein the serotype of the first dengue virus of c) is type 3.

12. The composition of claim **9**, wherein the serotype of the first dengue virus of d) is type 4.

35 **13.** The composition of claim **9**, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.

14. The composition of claim **13**, wherein the structural proteins are prM and E proteins.

40 **15.** The composition of claim **9**, wherein the deletion of a) 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.

45 **16.** The composition of claim **8**, wherein the serotype of the second dengue virus having the deletion is type 2.

17. The composition of claim **16**, wherein the serotype of the first dengue virus of a) is type 1.

18. The composition of claim **16**, wherein the serotype of the first dengue virus of c) is type 3.

50 **19.** The composition of claim **16**, wherein the serotype of the first dengue of d) virus is type 4.

20. The composition of claim **16**, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.

55 **21.** The composition of claim **20**, wherein the structural proteins are prM and E proteins.

60 **22.** The composition of claim **16**, 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.

23. The composition of claim **8**, wherein the serotype of the second dengue virus having the deletion is type 3.

24. The composition of claim **23**, wherein the serotype of the first dengue virus of a) is type 1.

25. The composition of claim **23**, wherein the serotype of the first dengue virus of b) is type 2.

26. The composition of claim **23**, wherein the serotype of the first dengue virus of d) is type 4.

27. The composition of claim **23**, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.

28. The composition of claim **27**, wherein the structural proteins are prM and E proteins.

29. The composition of claim **23**, 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.

30. The composition of claim **8**, wherein the serotype of the second dengue virus having the deletion is type 4.

31. The composition of claim **30**, wherein the serotype of the first dengue virus of a) is type 1.

32. The composition of claim **30**, wherein the serotype of the first dengue virus of b) is type 2.

33. The composition of claim **30**, wherein the serotype of the first dengue virus of c) is type 3.

34. The composition of claim **30**, wherein the first nucleotide sequence encodes at least two structural proteins of the first dengue virus.

25 **35.** The composition of claim **34**, wherein the structural proteins are prM and E proteins.

36. The composition of claim **30**, 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.

37. A method of inducing an immune response in a subject comprising administering an effective amount of the composition of claim **1** to the subject.

35 **38.** The method of claim **37** wherein the subject is a human.

39. A tetravalent vaccine comprising the composition of claim **1**.

40 **40.** A method of preventing disease caused by dengue virus in a subject comprising administering an effective amount of the vaccine of claim **39** to the subject.

41. The method of claim **40** wherein the subject is a human.

42. The tetravalent immunogenic composition defined in claim **1**, selected from the group consisting of:

- (1) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4Δ30,
- (2) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/1Δ30,
- (3) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/2Δ30,
- (4) rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, rDEN4/3Δ30,
- (5) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4Δ30,
- (6) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/1Δ30,
- (7) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/2Δ30,
- (8) rDEN1Δ30, rDEN2Δ30, rDEN3/1Δ30, rDEN4/3Δ30,
- (9) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4Δ30,
- (10) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
- (11) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/2Δ30,
- (12) rDEN1Δ30, rDEN2Δ30, rDEN3/2Δ30, rDEN4/3Δ30,
- (13) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4Δ30,
- (14) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
- (15) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/2Δ30,
- (16) rDEN1Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
- (17) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4Δ30,

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- (18) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/1Δ30,
 (19) rDEN1Δ30, rDEN2/1Δ30, rDEN3Δ30, rDEN4/2Δ30,
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 (21) rDEN1Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30,
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 (28) rDEN1Δ30, rDEN2/1Δ30, rDEN3/2Δ30, rDEN4/3Δ30,
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 (78) rDEN1/2Δ30, rDEN2Δ30, rDEN3/4Δ30, rDEN4/1Δ30,
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 (85) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/1Δ30, rDEN4Δ30,

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 (95) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/2Δ30, 20
 (96) rDEN1/2Δ30, rDEN2/1Δ30, rDEN3/4Δ30, rDEN4/3Δ30,
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 (164) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3Δ30, rDEN4/3Δ30, 25
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 (167) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/1Δ30, rDEN4/2Δ30, 30
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 (169) rDEN1/3Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4Δ30, 35
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188

- (186) rDEN1/3Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/1Δ30,
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1Δ30,
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2Δ30,
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2Δ30,
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3Δ30,
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 (234) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/ 30
1Δ30,
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 (236) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/2Δ30, rDEN4/
3Δ30,
 (237) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, 35
rDEN4Δ30,

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- (238) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/
1Δ30,
 (239) rDEN1/4Δ30, rDEN2/3Δ30, rDEN3/4Δ30, rDEN4/
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3Δ30,
 (241) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3Δ30,
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1Δ30,
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2Δ30,
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3Δ30,
 (249) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/2Δ30,
rDEN4Δ30,
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1Δ30,
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2Δ30,
 (252) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/2Δ30, rDEN4/
3Δ30,
 (254) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/
1Δ30,
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2Δ30, and
 (256) rDEN1/4Δ30, rDEN2/4Δ30, rDEN3/4Δ30, rDEN4/
3Δ30.

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