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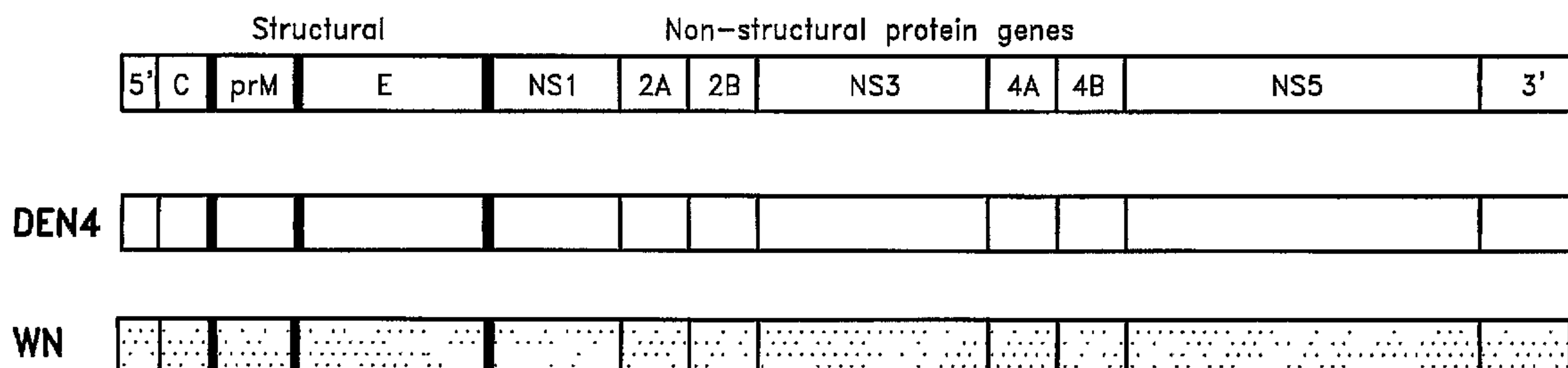
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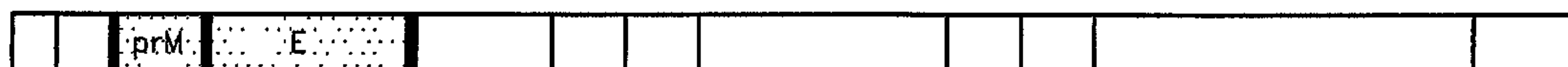
(54) Titre : PRODUCTION DE CHIMERES DU VIRUS DE LA DENGUE ET DU VIRUS DU NIL OCCIDENTAL EN VUE DE  
LEUR UTILISATION DANS UN VACCIN CONTRE LE VIRUS VIVANT A TITRE DE PREVENTION DE LA MALADIE  
PROVOQUEE PAR LE VIRUS DU NIL OCCIDENTAL

(54) Title: CONSTRUCTION OF WEST NILE VIRUS AND DENGUE VIRUS CHIMERAS FOR USE IN A LIVE VIRUS  
VACCINE TO PREVENT DISEASE CAUSED BY WEST NILE VIRUS

#### Positive-sense RNA virus:



#### New WN/DEN4 Chimeric Flavivirus:



- Infectious RNA transcribed in vitro from cDNA clones
- Infectious RNA transfected into cell culture (C6/36, Vero)
- Progeny virus recovered and evaluated in susceptible animals

#### (57) Abrégé/Abstract:

The present invention relates to attenuated, immunogenic West Nile virus chimeras built on a dengue virus backbone for the production of immunogenic, live, attenuated West Nile virus vaccines.

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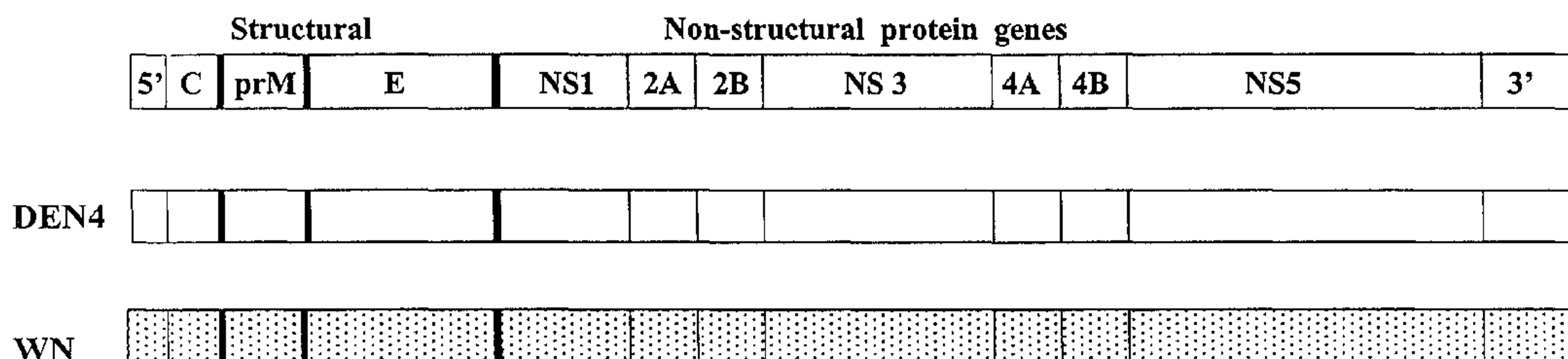
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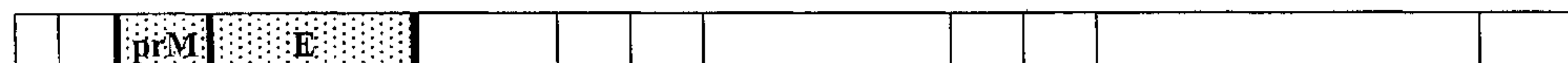
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(54) Title: CONSTRUCTION OF WEST NILE VIRUS AND DENGUE VIRUS CHIMERAS FOR USE IN A LIVE VIRUS VACCINE TO PREVENT DISEASE CAUSED BY WEST NILE VIRUS

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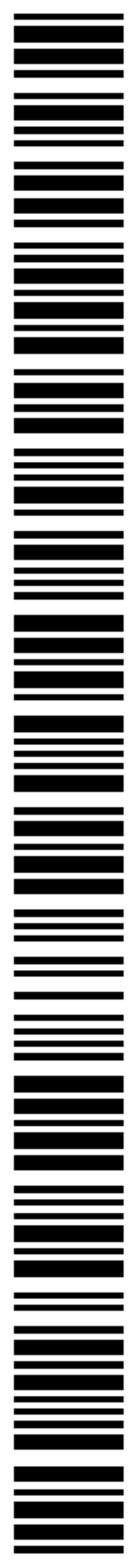


**New WN/DEN4 Chimeric Flavivirus:**



- Infectious RNA transcribed in vitro from cDNA clones
- Infectious RNA transfected into cell culture (C6/36, Vero)
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**CONSTRUCTION OF WEST NILE VIRUS AND DENGUE VIRUS CHIMERAS  
FOR USE IN A LIVE VIRUS VACCINE TO PREVENT DISEASE CAUSED BY  
WEST NILE VIRUS**

5

Field of the Invention

The present invention relates to attenuated, immunogenic West Nile virus chimeras built on a dengue virus backbone for the production of immunogenic, live, attenuated West Nile virus vaccines.

Background of the Invention

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Beginning with Fig. 1A, the flavivirus genome is a single-stranded, positive-sense RNA approximately 11 kb in length, containing a 5' untranslated region (5' UTR); a coding region encoding the three viral structural proteins; seven nonstructural proteins, designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5; and a 3' untranslated region (3' UTR). The viral structural proteins include the capsid (C), premembrane/membrane (prM) and envelope (E) proteins. The structural and nonstructural proteins are translated as a single polyprotein. The polyprotein is then processed by cellular and viral proteases.

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West Nile virus (WN) belongs to the family *Flaviviridae* that comprises more than 60 viruses, many of which are important human pathogens. WN is a member of the Japanese encephalitis virus (JE) serocomplex of mosquito-borne flaviviruses that includes St. Louis encephalitis, JE, and Murray Valley encephalitis viruses (Calisher, C.H. *et al.* 1989 *J Gen Virol* 70:27-43; Burke, D.S. & Monath, T.P. 2001 in: *Fields Virology*, eds. Knipe, D.M. & Howley, P.M. Lippincott Williams and Wilkins, Philadelphia, 4-th ed., pp. 1043-1125). Like other members of the JE antigenic complex, WN is maintained in a natural cycle that involves mosquito vectors and birds, while humans and equines are usually incidental hosts. For many years WN has been recognized as one of the most widely distributed flaviviruses with a geographic range including Africa, Australia, Europe, the Middle East and West Asia (Burke, D.S. & Monath, T.P. 2001 in: *Fields Virology*, eds. Knipe, D.M. & Howley, P.M. Lippincott Williams and Wilkins, Philadelphia, 4-th ed., pp. 1043-1125; Hayes, C.G. 1989 in: *The Arboviruses: Epidemiology and Ecology*, ed. Monath T.P. Boca Raton, FL CRC Press, Volume V, pp. 59-88). During 1999 WN first established itself in the USA in the Northeast and Mid-Atlantic States and more recently this virus extended its range to include the Southeastern and Western States (Anderson, J.F. *et al.*

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1999 *Science* **286**:2331-2333; Lanciotti, R.S. *et al.* 1999 *Science* **286**:2333-2337; Campbell, G. L. *et al.* 2002 *Lancet* **2**:519-529). In endemic regions, most human WN infections are asymptomatic or cause mild illness with symptoms of low-grade fever, headache, body aches, rash, myalgia, and polyarthropathy. However, human epidemics  
5 with severe disease have been reported in Israel, France, Romania, and Russia. In acute severe illness, the virus can cause hepatitis, meningitis and encephalitis leading to paralysis, and coma resulting in death. The neuropathologic lesions are similar to those of JE, with diffuse CNS inflammation and neuronal degeneration. Virus is also found in the spleen, liver, lymph nodes, and lungs of infected individuals. During the 1999 outbreak of WN in  
10 the USA, more than 60 people became ill and 7 died, while during 2002, morbidity was 3873 cases and there were 246 deaths (CDC Report: West Nile Update Current case Count, Jan. 2, 2003). Because of the recent and unexpected spread of WN from the Northeast to the Southeast and the West of the USA, this virus is considered a significant emerging disease threat that has embedded itself over a considerable region of the country.  
15 Currently, a licensed human vaccine is not available for prevention of WN disease. Mosquito control is the only practical strategy to combat the spread of disease, but effective spraying is difficult to perform in urban areas. Clearly, an effective vaccine is needed to protect at-risk populations.

Dengue viruses are mosquito-borne pathogens of the genus *Flavivirus* (family  
20 *Flaviviridae*). Four serotypes of dengue virus (DEN) have been identified, including dengue type 1 virus (DEN1), dengue type 2 virus (DEN2), dengue type 3 virus (DEN3) and dengue type 4 virus (DEN4). Live, attenuated dengue viruses of all four serotypes have been developed at Mahidol University in Thailand by passaging the wild-type viruses in primary dog kidney cell culture (Sabchareon, A. *et al.* 2002 *Am J Trop Med Hyg* **66**:264-  
25 272). These are currently the least promising live, attenuated vaccine candidates for immunization against dengue virus infection and/or disease because they are not well characterized as to the relative contributions of attenuation-associated mutations to the actual mechanism of attenuation nor as to the potential for reverse mutations to revert any of the vaccine candidates to the virulent biological phenotype of the wild-type dengue  
30 virus. These vaccine candidates have been designated by a combination of their dengue serotype, the cell line through which they were passaged and the number of times they were passaged. Thus, a dengue serotype 1 wild-type virus passaged in primary dog kidney

(PDK) cells 13 times is designated as DEN1 PDK13 virus. Other vaccine candidates are DEN2 PDK53, DEN3 PGMK30/FRhL3 (thirty passages in primary green monkey kidney cells, followed by three passages in fetal rhesus lung cells) and DEN4 PDK48. These four candidate vaccine viruses were derived by tissue culture passage of wild-  
 5 type parental DEN1 16007, DEN2 16681, DEN3 16562 and DEN4 1036 viruses, respectively.

Except for DEN2 PDK53 virus, the number and identity of the genetic mutations that accrued during multiple passages in cell culture and that are associated with the attenuation phenotype of the vaccine candidates are unknown. Neither the  
 10 relative contributions of such attenuation-associated mutations to the actual mechanism of attenuation, nor the potential for reverse mutations to revert any of the vaccine candidates to the virulent biological phenotype of the wild-type dengue virus are known for any of these four vaccine candidates. An understanding of the characteristics of a vaccine candidate is critical for the prediction of its stability and safety.

15 Accordingly, there is a need for attenuated, yet immunogenic flaviviruses to be used in the development of flavivirus vaccines to confer protection against flaviviruses. What would be ideal is a vaccine that would simultaneously protect an individual against flavivirus disease and be sufficiently characterized so that stability and safety are predictable.

## 20 Summary of the Invention

Chimeric flaviviruses that are attenuated and immunogenic are provided. Chimeric viruses containing the nonstructural protein genes of a dengue virus are used as a backbone into which the structural protein genes of a West Nile virus are substituted. These chimeric viruses exhibit pronounced immunogenicity in the absence  
 25 of the accompanying clinical symptoms of viral disease. The attenuated chimeric viruses are effective as immunogens or vaccines and may be combined in a pharmaceutical composition to confer immunity against West Nile virus.

In one aspect, the present invention provides a nucleic acid chimera comprising a first nucleotide sequence encoding a premembrane/ membrane (prM) protein and an  
 30 envelope (E) protein from a West Nile virus and a second nucleotide sequence

encoding a capsid (C) protein and nonstructural proteins from a wild-type strain of dengue type 4 virus, wherein the dengue type 4 virus is attenuated by a deletion of 30 nucleotides from the 3' untranslated region of the dengue type 4 genome corresponding to the TL2 stem-loop structure of nucleotides 10478-10507.

5           In another aspect, the invention provides a nucleic acid chimera comprising a first nucleotide sequence encoding a premembrane/ membrane (prM) protein and an envelope (E) protein from a West Nile virus and a second nucleotide sequence encoding a capsid (C) protein and nonstructural proteins from a wild-type strain of dengue type 4 virus, wherein the dengue type 4 virus is attenuated by a deletion of 30  
10   nucleotides from the 3' untranslated region of the dengue type 4 genome corresponding to the TL2 stem-loop structure of nucleotides 10478-10507, wherein a cleavage site is utilized for joining a dengue virus capsid protein and the West Nile virus prM protein, and wherein the West Nile virus prM protein contains aspartic acid (Asp) at a position 3 amino acids downstream of the cleavage site and contains threonine (Thr) at a  
15   position 6 amino acids downstream of the cleavage site.

          In another aspect, the invention provides a virus chimera comprising one or more than one nucleic acid chimera of the invention.

          In another aspect, the invention provides an immunogenic composition comprising one or more than one nucleic acid chimera of the invention or one or more  
20   than one virus chimera of the invention and a pharmaceutically acceptable carrier.

          In another aspect, the invention provides use of the composition of the invention for inducing an immune response in a subject.

          In another aspect, the invention provides use of the composition of the invention in the manufacture of a medicament for inducing an immune response in a subject.

25           In another aspect, the invention provides a vaccine composition comprising one or more than one nucleic acid chimera of the invention or one or more than one virus chimera of the invention and a pharmaceutically acceptable carrier.

          In another aspect, the invention provides use of the composition of the invention for preventing disease caused by West Nile virus in a subject.

In another aspect, the invention provides use of the composition of the invention in the manufacture of a medicament for preventing disease caused by West Nile virus in a subject.

5 In another aspect, the invention provides an isolated nucleic acid probe or primer comprising 10-50 nucleotides that selectively binds to, and that is complementary to, either the nucleic acid encoding the cleavage site that separates the capsid protein and the premembrane protein of the nucleic acid chimera of the invention or the complementary strand thereto.

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#### Brief Description of the Drawings

**Fig. 1A** shows the translation and processing of the flavivirus polyprotein. At the top is depicted the viral genome with the structural and nonstructural protein coding regions, the 5'cap, and the 5' and 3' untranslated regions (UTRs) indicated. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Mature structural proteins are indicated by *shaded boxes* and the nonstructural

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proteins and structural protein precursors by *open boxes*. Contiguous stretches of uncharged amino acids are shown by *black bars*. *Asterisks* denote proteins with N-linked glycans but do not necessarily indicate the position or number of sites utilized. Cleavage sites for host signalase (♦), the viral serine protease (↓), furin or other Golgi-localized protease (♥), or unknown proteases (?) are indicated. Taken from Field's Virology, 2001 Fourth Edition, B.D. Lindenbach and C.M. Rice, page 998, Chapter 32.

**Fig. 1B** shows a strategy used to replace the genes for prM and E proteins of DEN4 with the corresponding genes of West Nile virus to produce WN/DEN4 chimeras that serve as candidate attenuated vaccine strains.

**Fig. 2** shows the structure of portions of chimeric WN/DEN4 cDNAs. The top bar depicts the chimeric virus cDNA genome from the 5' terminus of the genome to the 3' terminus of the NS1 gene. The solid black boxes represent hydrophobic domains in the polyprotein. The vertical solid arrow indicates the position of a potential NS2B-NS3 protease cleavage site in the polyprotein between the C and prM proteins (the first junction in chimeric genome). Cleavage sites for cellular signalase are indicated by open triangles (▽). A restriction enzyme-cleaved WN cDNA fragment bearing the sequence for the WN premembrane (prM) and envelope glycoprotein (E) structural protein genes was inserted into DEN4 cDNA at *Pst*I and *Xho*I sites, which are underlined. The second junction is located in the COOH-terminus of the WN E protein between the two hydrophobic domains.

The amino acid and nucleotide sequences of WN are presented in bold letters, and nucleotide numbering system is from GenBank accession No. AF196835. Infectivity of RNA transcripts from full-length cDNA constructs was tested by transfecting simian Vero or C6/36 mosquito cells and evaluating cell cultures for evidence of infection by immunofluorescence assay. The two clones in group 4 sustained a mutation of the amino acid +6 downstream from the cleavage site from I (isoleucine) to T (threonine) during cloning of cDNA (represented in the figure). Only these two clones were viable, yielding infectious virus following transfection of full length RNA transcripts.

\*Indicates amino acids in chimeric constructs that vary at the 3+ position downstream of protease cleavage site. \*\*Not applicable. †Two infectious chimeric WN/DEN4 viruses, namely clone 18 and 55 from group 4, were isolated.

Table of Sequences from Figure 2

| SEQUENCE                       | SEQ ID NO | SOURCE  |
|--------------------------------|-----------|---------|
| KKRGGRTGIA                     | 1         | WN      |
| AAGAAAAGAGGAGGAAAGACCGGAATTGCA | 2         | WN      |
| RKRSTITLLC                     | 3         | DEN4    |
| AGAAAAAGGTCAACGATAACATTGCTGTGC | 4         | DEN4    |
| RKRSAVTGIA                     | 5         | WN/DEN4 |
| AGAAAAAGGTCTGCAGTGACCGGAATTGCA | 6         | WN/DEN4 |
| RKRSAGTGIA                     | 7         | WN/DEN4 |
| AGAAAAAGGTCTGCAGGGACCGGAATTGCA | 8         | WN/DEN4 |
| RKRSADTGIA                     | 9         | WN/DEN4 |
| AGAAAAAGGTCTGCAGACACCGGAATTGCA | 10        | WN/DEN4 |
| RKRSADTGTA                     | 11        | WN/DEN4 |
| AGAAAAAGGTCTGCAGACACCGGAACTGCA | 12        | WN/DEN4 |
| INARD                          | 13        | WN      |
| ATCAATGCTCGTGAT                | 14        | WN      |
| LNSRN                          | 15        | DEN4    |
| CTGAACTCGAGGAAC                | 16        | DEN4    |
| INSRN                          | 17        | WN/DEN4 |
| ATCAACTCGAGGAAC                | 18        | WN/DEN4 |

**Fig. 3** shows the viremia of rhesus monkeys inoculated with parental WN or DEN4 virus or their WN/DEN4 chimera or its 3' deletion mutant WN/DEN4-3' $\Delta$ 30. Twenty rhesus monkeys (*Maccaca mulatta*) in groups of 4 were inoculated subcutaneously (SC) with WN, DEN4, WN/DEN4 clone 18 or WN/DEN4-3' $\Delta$ 30 clone 1. The quantity of virus in monkey serum was determined by direct titration in *Vero* cells using immunostaining focus-forming assay. Viremia was tested daily for 12 days post-inoculation for each monkey individually. Mean virus titer in serum of each monkey group shown; n is number of monkeys in group. The limit of detection of virus was  $10^{0.7}$  FFU/ml, and the WN/DEN4 and WN/DEN4-3' $\Delta$ 30 viruses were at or below the level of detection of virus in serum.

**Figure 4.** *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 DEN4 and DEN1 and their  $\Delta$ 30 derivatives. Also shown is the corresponding region for each of the four DEN serotypes, with upper case letters indicating sequence homology among all 4 serotypes, underlining indicating 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 for the already constructed DEN1 $\Delta$ 30, DEN4 $\Delta$ 30, DEN2 $\Delta$ 30 viruses are indicated (boxed) on the left and the proposed DEN3 $\Delta$ 30 virus is on the right (DEN1 – nts 10562 – 10591, DEN2

Tonga/74 – nts 10541 – 10570, DEN3 Sleman/78 – nts 10535 – 10565, and DEN4 – nts 10478 – 10507).

**Table of Sequences from Figure 4**

| SEQUENCE                                                                                     | SEQ ID NO | SOURCE  |
|----------------------------------------------------------------------------------------------|-----------|---------|
| GGCCCGAAGCCAGGAGGAAGCUGUACUCCUGGUGGAAG<br>GACUAGAGGUUAG                                      | 19        | DEN4    |
| GGGGCCCGAAGCCAGGAGGAAGCUGUACUCCUGGUGGA<br>AGGACUAGA                                          | 20        | DEN4    |
| GGGGCCCAAGACUAGA                                                                             | 21        | DEN4Δ30 |
| GGGGCCCAACACCAGGGGAAGCUGUACCCUGGUGGUAA<br>GGACUAGA                                           | 22        | DEN1    |
| GGGGCCCAAGACUAGA                                                                             | 23        | DEN1Δ30 |
| GGGGCCCAAGGUGAGAUGAAGCUGUAGUCUCACUGGAA<br>GGACUAGA                                           | 24        | DEN2    |
| GGGGCCCGAGCUCUGAGGGAAGCUGUACCUCCUUGCAAA<br>GGACUAGA                                          | 25        | DEN3    |
| GCAGCAGCGGGGGCCCAACACCAGGGGAAGCUGUAC<br>CCUGGUGGUAAAGGACUAGAGGUUAGAGGAGACCCC<br>CCGCAACAACAA | 26        | DEN1    |
| AGCAAAAGGGGGGCCCGAAGCCAGGAGGAAGCUGUA<br>CUCCUGGUGGAAGGACUAGAGGUUAGAGGAGACCC<br>CCCCAACACAAAA | 27        | DEN4    |
| AGCAACAAUGGGGGGCCCAAGGUGAGAUGAAGCUGU<br>AGUCUCACUGGAAGGACUAGAGGUUAGAGGAGACC<br>CCCCCAAACAAAA | 28        | DEN2    |
| GCAGCAGCGGGGGCCCGAGCUCUGAGGGAAGCUGUA<br>CCUCCUUGCAAAGGACUAGAGGUUAGAGGAGACCC<br>CCCGCAAUAAAA  | 29        | DEN3    |

**Brief Description of the Sequences**

|      | GenBank Accession No. or description |
|------|--------------------------------------|
| DEN1 | U88535                               |
| DEN2 | Tonga/74 (SEQ ID No: 30 and 31)*     |
| DEN3 | Sleman/78 (SEQ ID No: 32 and 33)**   |
| DEN4 | AF326825                             |

5 \* DEN2 (Tonga/74) cDNA plasmid p2

|                    |                         |
|--------------------|-------------------------|
| 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       |
| 5  | 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        |
| 10 | ** DEN3 (Sleman/78) cDNA plasmid p3 |                        |
|    | 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        |
| 15 | 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        |
| 20 | 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        |

#### Detailed Description of the Preferred Embodiment

25 Immunogenic WN/DEN flavivirus chimeras and methods for preparing the WN/DEN flavivirus chimeras are provided herein. The immunogenic WN/DEN flavivirus chimeras are useful, alone or in combination, in a pharmaceutically acceptable carrier as immunogenic compositions to immunize and protect individuals and animals against infection by West Nile virus.

30 Chimeras of the present invention comprise nucleotide sequences encoding the immunogenic structural proteins of a West Nile virus and further nucleotide sequences selected from the backbone of a dengue virus. Chimeric viruses derived from the nucleotide sequences can be used to induce an immunogenic response against West Nile virus.

35 In another embodiment, the preferred chimera is a nucleic acid chimera comprising a first nucleotide sequence encoding at least one structural protein from a West Nile virus, and a second nucleotide sequence encoding nonstructural proteins from a dengue virus. In another embodiment the dengue virus is attenuated. In another embodiment the dengue

virus is DEN4. In another embodiment, the structural protein can be the C protein of a West Nile virus, the prM protein of a West Nile virus, the E protein of a West Nile virus, or any combination thereof.

5 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.

10 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  
15 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);
- 20 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," "flavivirus chimera"  
25 and "chimeric flavivirus" means an infectious construct of the invention comprising nucleotide sequences encoding the immunogenicity of a West Nile virus and further nucleotide sequences derived from the backbone of a dengue virus.

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  
30 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 West Nile virus and further nucleotide sequences derived from the backbone of a dengue virus. Correspondingly, any chimeric flavivirus or flavivirus 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.

### **Flavivirus Chimeras**

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

The flavivirus chimeras of the invention are constructs formed by fusing structural protein genes from a West Nile virus with non-structural protein genes from a dengue virus, *e.g.*, DEN1, DEN2, DEN3, or DEN4.

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

The chimera of the invention contains a dengue virus genome 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 West Nile virus that is to be protected against. The resulting chimeric virus has the properties, by virtue of being chimerized with the dengue virus, of attenuation and is therefore reduced in virulence, but expresses antigenic epitopes of the WN 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 nonstructural proteins, the 5' untranslated region or the 3' untranslated region. The backbone can also contain further mutations to maintain the stability of the attenuation  
5 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  
10 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 Virol* **70**:3930-3933; Durbin *et al.* 2001 *Am J Trop Med* **65**:405-413). 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  
15 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.

## 20 **Construction of Flavivirus Chimeras**

The flavivirus chimeras described herein can be produced by substituting at least one of the structural protein genes of the West Nile virus against which immunity is desired into a dengue virus genome backbone, using recombinant engineering techniques well known to those skilled in the art, namely, removing a designated dengue virus gene and  
25 replacing it with the desired corresponding gene of West Nile virus. Alternatively, using the sequences provided in GenBank, the nucleic acid molecules encoding the flavivirus 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.

30 As mentioned above, the gene to be inserted into the backbone encodes a West Nile virus structural protein. Preferably the West Nile virus gene 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. The sequence inserted into the dengue virus backbone can encode the C, prM and E structural proteins. 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 West Nile virus or the substituted gene(s) that encode the prM and/or E structural protein(s) of a West Nile virus. In a particular embodiment of this invention, the substituted gene that encodes the structural protein of a West Nile virus directs the synthesis of a prM protein that contains Asp and Thr, respectively, at a position 3 and 6 amino acids downstream of the cleavage site that separates the capsid protein of DEN and the premembrane protein of West Nile virus.

Suitable chimeric viruses or nucleic acid chimeras containing nucleotide sequences encoding structural proteins of West Nile virus 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 West Nile antibodies or immunoreactive serum using routine screening procedures known to those skilled in the art.

### **Flavivirus 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 West Nile virus in a wild-type or an attenuated dengue virus backbone. For example, the chimera may express the structural protein genes of a West Nile virus in either of a dengue virus or an attenuated dengue virus background.

The strategy described herein of using a genetic background that contains nonstructural regions of a dengue virus genome, and, by chimerization, the properties of attenuation, to express the structural protein genes of a West Nile virus has lead to the development of live, attenuated flavivirus vaccine candidates that express structural protein genes of desired immunogenicity. Thus, vaccine candidates for control of West Nile virus 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  
5 derive attenuated candidate starting materials.

Chimeric infectious clones are constructed from various flavivirus strains. 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 flavivirus RNA with  
10 various primers. Amplified fragments are cloned into the cleavage sites of other intermediate clones. Intermediate, chimeric flavivirus clones are then sequenced to verify the sequence of the inserted flavivirus-specific cDNA.

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

#### **Method 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  
20 "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  
25 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  
30 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  
5 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  
10 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,  
15 rectal, aerosol, nasal, intramuscular, subcutaneous, intradermal, and topical. The composition may be administered in different forms, including, but not limited to, solutions, emulsions and suspensions, microspheres, particles, microparticles, nanoparticles and liposomes. It is expected that from about 1 to about 5 doses may be required per immunization schedule. Initial doses may range from about 100 to about 100,000 PFU or  
20 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  
25 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  
30 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 or domestic animals, such as horses or birds, especially individuals travelling to regions where West Nile 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 West Nile 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, copolymers such as polyoxyethylene-polyoxypropylene copolymers, including block copolymers, polymer p 1005, Freund's complete adjuvant (for animals), Freund's incomplete adjuvant; sorbitan monooleate, squalene, CRL-8300 adjuvant, alum, QS 21, muramyl dipeptide, CpG oligonucleotide motifs and combinations of CpG oligonucleotide motifs, trehalose, bacterial extracts, including mycobacterial extracts, detoxified endotoxins, membrane lipids, or combinations thereof.

### **Nucleic Acid Sequences**

Nucleic acid sequences of West Nile virus and dengue virus are useful for designing nucleic acid probes and primers for the detection of West Nile virus and dengue virus chimeras in a sample or specimen with high sensitivity and specificity. Probes or primers corresponding to West Nile virus and 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 West Nile virus and 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 West Nile virus sequence and 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”  
5 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.

10 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  
15 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  
20 stringent conditions and should have at least five nucleotides complementary to the sequence of interest as described in Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

If used as primers, the composition preferably includes at least two nucleic acid  
25 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 West Nile virus and dengue virus, the degree of complementarity between the hybridizing nucleic acid  
30 (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 West Nile virus and dengue virus can be inserted into a vector, such as a plasmid, and recombinantly expressed in a living organism to produce recombinant West Nile virus and dengue virus peptide and/or polypeptides.

The nucleic acid sequences of the invention include a diagnostic probe that serves to  
5 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  
10 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  
15 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.

**West Nile virus/dengue type 4 virus chimeras that are reduced in neurovirulence and peripheral virulence without loss of immunogenicity or protective efficacy**

20 A candidate live attenuated vaccine strain was constructed for West Nile virus (WN), a neurotropic flavivirus that has recently emerged in the U.S. Considerable attenuation for mice was achieved by chimerization with dengue virus type 4 (DEN4). The genes for the structural premembrane (prM) and envelope (E) proteins of DEN4 present in a full-length infectious cDNA clone were replaced by the corresponding genes of WN  
25 strain NY99. Two of 18 full-length cDNA clones of a WN/DEN4 chimera yielded full-length RNA transcripts that were infectious when transfected into susceptible cells. The two infectious clones shared a motif in the transmembrane signal domain located immediately downstream of the NS2B-NS3 protease cleavage site that separates the DEN4 capsid protein and the WN premembrane protein of the chimera. This motif, Asp and Thr  
30 at a position 3 and 6 amino acids downstream of the cleavage site, respectively, was not present in the 16 non-infectious cDNA clones. The WN/DEN4 chimera was highly attenuated in mice compared to its WN parent; the chimera was at least 28,500 times less

neurovirulent in suckling mice inoculated intracerebrally and at least 10,000 times less virulent in adult mice inoculated intraperitoneally. Nonetheless, the WN/DEN4 chimera and a deletion mutant derived from it, were immunogenic and provided complete protection against lethal WN challenge. These observations provide the basis for pursuing the development of a live attenuated WN vaccine.

Recent advances in recombinant DNA technology have allowed us to develop a novel approach for constructing live attenuated flavivirus vaccines (Pletnev, A.G. *et al.* 1992 *PNAS USA* **89**:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS USA* **95**:1746-1751; Pletnev, A.G. *et al.* 2000 *Virology* **274**:26-31; Pletnev, A.G. *et al.* 2001 *J Virol* **75**:8259-8267). Our approach was made possible by the conservation among flaviviruses of genome organization, number of viral proteins, replicative strategy, gene expression, virion structure and morphogenesis (Lindenbach, B.D. & Rice, C.M. 2001 in: *Fields Virology*, eds. Knipe, D.M. & Howley, P.M. Lippincott Williams and Wilkins, Philadelphia, 4-th ed., pp. 1043-1125). All flaviviruses have a positive sense non-segmented RNA genome that encodes a single long polyprotein that is processed to yield capsid (C), premembrane (prM) and envelope glycoprotein (E) structural proteins followed by nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in that order. These shared properties suggested that viable chimeric viruses could be produced by replacing the genes for the viral structural proteins in a full-length infectious cDNA clone of a flavivirus with the corresponding viral genes (in cDNA form) of another flavivirus. When tested, this strategy was successful for chimeras that contained the sequence for viral structural proteins prM and E of tick-borne encephalitis virus (TBEV) or tick-borne Langat virus (LGT), while all other sequences were derived from the full-length infectious cDNA of mosquito-borne dengue type 4 virus (DEN4). This indicated that viral structural proteins of a disparate flavivirus, TBEV or LGT, could function in the context of cis-acting 5' and 3' sequences and nonstructural proteins of DEN4. Significantly, both chimeras proved to be highly attenuated in mice with respect to peripheral virulence, namely, the ability of a virus to spread to the CNS from a peripheral site of inoculation and cause encephalitis. Nonetheless, the chimeras proved to be immunogenic and able to induce resistance in mice against challenge with TBEV or LGT (Pletnev, A.G. *et al.* 1992 *PNAS USA* **89**:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS USA* **95**:1746-1751; Pletnev, A.G. *et al.* 2000 *Virology* **274**:26-31). It appeared that a favorable balance between reduction in virus

replication *in vivo* (attenuation) and induction of protective immunity had been achieved. We interpret this to mean that tick-borne flavivirus prM and E can interact in the context of DEN4 nonstructural proteins and cis-acting 5' and 3' sequences at a level sufficient for infectivity and induction of immunity but not sufficient for full expression of virulence that  
5 requires a high level of replication *in vivo* and ability to spread into the CNS.

Although a logical extension of this strategy was to construct WN/DEN4 chimeras, we realized that viability could not be predicted in advance because some flavivirus combinations such as some Langat virus(prM and E)/dengue virus chimeras, as well as dengue virus(prM and E)/Langat dengue virus chimeras, have not proven to be viable.  
10 Nevertheless, we were surprisingly successful in constructing viable WN/DEN4 chimeras in which the structural prM and E protein genes of the distantly related mosquito-borne WN were substituted for the corresponding genes of DEN4. We also generated a WN/DEN4 chimera with a 30 nucleotide deletion in the 3' untranslated region (3' UTR) that had previously been shown to render DEN4 safe but still immunogenic in adult volunteers  
15 (Durbin, A.P. *et al.* 2001 *Am J Trop Med Hyg* 65:405-413). Studies in mice were first performed to evaluate neurovirulence, peripheral virulence, immunogenicity, and protective efficacy of the newly constructed WN/DEN4 chimeric viruses.

### **Materials and Methods.**

#### **Cells and viruses**

20 Simian *Vero* cells (WHO seed passage 143) and mosquito C6/36 cells were obtained from Dr. L. Potash (Novavax Inc., Rockville, MD). These *Vero* cells are qualified for use in production of candidate human vaccines. Simian LLCMK<sub>2</sub> cells were purchased from the American Type Culture Collection (Manassas, VA). Starting with West Nile virus, the WN wild-type strain NY99-35262 used in this study was kindly provided by Dr.  
25 R. Lanciotti (Centers for Disease Control and Prevention, Fort Collins, CO). It was originally isolated from a Chilean flamingo at the Bronx Zoo (New York) in 1999 (Lanciotti, R.S. *et al.* 1999 *Science* 286:2333-2337). The sequence of WN NY99 genome is available as GenBank accession number AF196835, per Table 1, and other strains of WN may substitute for the sequence of WN NY99 genome. A virus suspension prepared in  
30 *Vero* cells had a titer of  $2.6 \times 10^7$  focus-forming units per milliliter (FFU/ml) as determined with *Vero* cells using an immunostaining focus-forming assay (Pletnev, A.G. 2001 *Virology* 282:288-300) and WN-specific mouse antibodies. Turning to dengue virus, wild-

type DEN4 Caribbean strain 814669 (GenBank accession number AF326573) was used, which replicated in *Vero* cells with a titer of  $1.1 \times 10^8$  FFU/ml. The sequence of recombinant DEN4 genome is available as GenBank accession number AF326825, per Table 1, and other strains of DEN4 may substitute for the sequence of DEN4 genome. The sequence of DEN1 genome is available as GenBank accession number U88536, the sequence of DEN2 genome is available as GenBank accession number M19197, and the sequence of DEN3 genome is available as GenBank accession number M93130, and any of these sequences may substitute for the sequence of DEN4 genome.

#### **Chimeric WN/DEN4 cDNA and recovery of infectious virus.**

Plasmid p2A(*Xho*I) (Bray, M. & Lai, C.-J. 1991 *PNAS USA* 88:10342-10346) containing the DEN4 full-length infectious cDNA, previously employed for recovery of chimeric TBEV/DEN4 and LGT/DEN4 viruses (Pletnev, A.G. *et al.* 1992 *PNAS USA* 89:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS. USA* 95:1746-1751), was used for construction of WN/DEN4 cDNA. This was achieved by substituting cDNA of the WN prM and E protein genes for those of the corresponding DEN4 genes (Fig. 1B). The source of WN cDNA was a PCR product that included nucleotides (nts) 233 to 2758 of the WN strain NY99 genome. This was also kindly provided by Dr. R. Lanciotti (CDC). The nucleotide sequence of the structural protein genes in this PCR fragment was determined and compared with the published sequence of WN NY99 (GenBank accession number AF196835). Three nucleotide differences ( $C_{1893} \rightarrow U$ ,  $C_{2370} \rightarrow U$  and  $C_{2385} \rightarrow A$ ) were identified in the E protein sequence, none of which resulted in an amino acid substitution.

Prior experience with construction and analysis of tick-borne/DEN4 chimeras indicated that we could not predict a priori the sequence of the DEN4 C protein/tick-borne flavivirus prM protein junction required for viability (Pletnev, A.G. *et al.* 1992 *PNAS USA* 89:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS. USA* 95:1746-1751). For this reason, we adopted an empirical approach and tested several different C/prM junction sequences (Fig. 2). This was not necessary for the downstream junction because it was located within the COOH-terminal region of WN E. Initially, 3 sets of C/prM junctions were tested but only one yielded a viable WN/DEN4 chimera (Fig. 2). The primers employed for construction of the chimeras by PCR used oligonucleotide 5'-TCAAAACAAAAGAAAAGATCTGCAGTGACCGGAATTGCAGTCATGATTGGC-3' (SEQ ID NO: 34), or 5'-

TCAAAACAAAAGAAAAGATCTGCAGGGACCGGAATTGCAGTCATGATTGGC-3'  
 (SEQ ID NO: 35), or 5'-  
 TCAAAACAAAAGAAAAGATCTGCAGACACCGGAATTGCAGTCATGATTGGC-3'  
 (SEQ ID NO: 36) as a forward primer and oligonucleotide 5'-  
 5 CCGCAAGAAACGTCATAGCAATTGACCTGTCACTCGAGTTGATTCCCATCCACA  
 ACAGAAGAGC-3' (SEQ ID NO: 37) as a reverse primer. Stable full-length WN/DEN4  
 cDNA clones were identified after transformation of *E. coli* BD 1528 with a ligation  
 mixture that contained the PCR product and the vector both of which were digested by *Pst*I  
 and *Xho*I (Fig. 2). Sequences at the junctions between WN and DEN4 genes in each  
 10 chimeric plasmid were verified.

Plasmid DNA containing full-length WN/DEN4 cDNA was linearized with *Asp*718.  
*In vitro* RNA synthesis and transfection of cells with its RNA transcripts were performed as  
 described previously (Pletnev, A.G. 2001 *Virology* **282**:288-300). Briefly, RNA transcripts  
 of full-length WN/DEN4 constructs listed in Fig. 2 were used to transfect simian LLCMK<sub>2</sub>,  
 15 simian *Vero* cells or mosquito C6/36 cells in the presence of LipofectAmine 2000 reagent  
 (GIBCO BRL, Gaithersburg, MD) in a BSL-3 laboratory generously provided by Dr. L.  
 Markoff (CBER, FDA). Transfected cells were examined by immunofluorescence assay  
 (IFA) for the presence of WN or DEN4 proteins using a WN- or DEN4-specific  
 hyperimmune mouse ascitic fluid (HMAF). Two infectious chimeric viruses containing  
 20 WN/DEN4 group 4 junctions (Fig. 2), namely, WN/DEN4 clone 18 and 55, were isolated.  
 The recovered chimeras were amplified once in simian *Vero* or mosquito C6/36 cells, viral  
 RNA was isolated and then reverse transcribed into cDNA that was used for sequence  
 analysis (Table 1). In a similar manner, the sequence of the *Vero* cell-derived WN/DEN4  
 clone 18 was determined after an additional purification by two rounds of terminal end-  
 25 point dilution and amplification in *Vero* cells infected at a multiplicity of 0.01. The  
 resulting virus suspension had a titer of 1.7x10<sup>6</sup> FFU/ml.

To introduce a deletion into the 3' untranslated region (UTR) of WN/DEN4  
 genome, the DNA fragment between the *Xho*I site (nt 2345 of DEN4 genome; GenBank  
 accession number AF326827) and the *Asp*718 site at the 3' end of plasmid WN/DEN4-18  
 30 DNA was replaced by the corresponding *Xho*I-*Asp*718-fragment derived from full-length  
 cDNA of a DEN4 mutant, clone p4Δ30 (Durbin *et al.* 2001 *Am. J Trop Med. Hyg* **65**:405-  
 413). This mutant had 30 nts deleted from the 3' untranslated region (UTR) of the genome

between nts 10478-10507. Full-length RNA generated by SP6 polymerase from 10 different plasmids was tested for infectivity by transfection of simian *Vero* cells. Two individual WN/DEN4-3' $\Delta$ 30 cDNA clones were infectious. The rescued deletion mutants, WN/DEN4-3' $\Delta$ 30 clone 1 and 78, were purified twice by terminal end-point dilution and  
5 amplified in *Vero* cells to a titer of  $1.4 \times 10^5$  and  $6 \times 10^4$  FFU/ml, respectively. Viral RNA was isolated, and complete sequence of the 3' deletion mutant genome was determined (Table 1).

#### Evaluation of parental and chimeric viruses in mice

Neurovirulence of *Vero* cell culture-propagated parental WN (strain NY99),  
10 parental DEN4 (strain 814669), chimeric WN/DEN4 (clone 18) and its deletion mutant (clone 1) was evaluated in a BSL-3 facility. Three-day-old Swiss Webster mice (Taconic Farms) in groups of 9 to 12 were inoculated by the intracerebral (IC) route with decimal dilutions ranging from 0.1 to  $10^5$  FFU of virus in 0.03 ml of MEM/0.25% human serum albumin. Mice were observed for 21 days for development of fatal encephalitis. The 50%  
15 lethal dose ( $LD_{50}$ ) of each virus was determined by the method of Reed and Muench (Reed, L.J. & Muench, H. 1938 *Am. J Hyg* **27**:493-497). Parental and chimeric viruses were also analyzed for peripheral virulence by intraperitoneal (IP) inoculation of 3-week-old Swiss female mice in groups of 10. Mice were inoculated with decimal dilutions of virus ranging from 0.1 to  $10^5$  FFU and observed for 28 days for fatal encephalitis. Moribund mice were  
20 humanely euthanized.

Mice that survived IP inoculation were bled on day 28 to evaluate the WN-specific neutralizing antibody response. Serum from mice in each group was pooled and the WN virus-neutralizing antibody titer of the serum pool was determined by FFU reduction assay in *Vero* cells as described previously (Pletnev, A.G. *et al.* 2001 *J Virol* **75**:8259-8267;  
25 Pletnev, A.G. 2001 *Virology* **282**:288-300). Briefly, a 1:10 dilution of pooled sera was prepared in MEM containing 2% fetal bovine serum (FBS) and then heat inactivated for 30 min at 56°C. Serial twofold dilutions of inactivated pooled sera were mixed with an equal volume of a virus suspension containing approximately 50 FFU of WN. The mixture was incubated for 30 min at 37°C, and 0.4 ml was then added to duplicate wells of *Vero* cells in  
30 a 6-well plate. After 1 h of absorption at 37°C, the inoculum was removed and cells were overlaid with MEM containing 2% FBS, 50  $\mu$ g/ml gentamycin, 0.25  $\mu$ g/ml fungizone, and 1% tragacanth gum. Antibody titer was determined after 2 days of incubation by an

immunostaining focus-forming assay (Pletnev, A.G. 2001 *Virology* **282**:288-300) that used WN-specific HMAF. Neutralizing antibody titer was the highest dilution of pooled sera that reduced focus formation 50% compared to sera collected from non-immunized mice.

The surviving mice were challenged IP on day 29 with 100 IP LD<sub>50</sub> (10<sup>3</sup> FFU) of parental WN virus and observed for fatal encephalitis for a period of 21 days. Moribund mice were humanely euthanized.

## **Results.**

### **Construction and recovery of chimeric WN/DEN4 viruses**

In total we constructed 18 plasmids that contained full-length chimeric WN/DEN4 cDNA which included the structural prM and E protein genes of the WN strain NY99 with all other sequences derived from DEN4 (Fig. 2). Full-length RNA generated by SP6 RNA polymerase from only 2 of the 18 chimeric cDNAs was infectious when transfected into mosquito C6/36 or simian *Vero* cells. Evidence for virus infectivity was detected by IFA. In the case of the 2 viable chimeric viruses, 80-100% of transfected cells were infected by day 5 as indicated by IFA using WN-specific HMAF. The 2 viable chimeric viruses (WN/DEN4 clones 18 and 55) had the C/prM intergenic junction sequence of group 4 chimera shown in Fig. 2, *i.e.*, +3 Asp and +6 Thr amino acids downstream of the cleavage site, respectively. The presence of this junction was confirmed by sequence analysis of the recovered chimeras. Also, the complete genomic sequence of the two chimeras rescued from cDNA in *Vero* cells was determined and compared with the consensus sequence of their parental WN NY99 and DEN4 viruses as well as the nucleotide sequence of the WN/DEN4 viral chimera insert in the plasmid DNA from which infectious RNA transcripts were derived (Table 1). Analysis of plasmid DNAs revealed 4 differences in nucleotide sequence from the consensus WN sequence determined by RT-PCR of a high titered suspension of WN strain NY99. Three of these differences produced amino acid substitutions in prM (Ile<sub>6</sub>→ Thr and Ile<sub>146</sub>→Val) and E (Thr<sub>282</sub>→ Ala). In addition, variability between (i) Glu<sub>92</sub> and Asp and (ii) Leu<sub>112</sub> and Ser was identified in the DEN4 NS3 and NS4B nonstructural proteins of the WN/DEN4 clone 55. Also, sequence of the *Vero* cell-grown WN/DEN4 clone 18 differed from its progenitor plasmid cDNA sequence in the DEN4 NS4B gene. A change U<sub>7162</sub>→C that caused the substitution Leu<sub>112</sub>→Ser was identified, which was observed previously (Blaney, J.E. *et al.* 2001 *J Virol* **75**:9731-9740).

Interestingly, a different substitution at this locus, Leu<sub>112</sub>→Phe, was also previously observed by Blaney *et al.* upon passage of wild-type DEN4 in *Vero* cells.

Following our success in constructing full-length infectious WN/DEN4 cDNAs, we constructed chimeric virus mutants with a 30 nucleotide deletion in their 3' untranslated region (UTR). Two mutants, WN/DEN4-3'Δ30 clone 1 and clone 78, were recovered from  
5 transfected *Vero* cells. The complete sequence of both these clones was analyzed (Table 1). Sequence of clone 78 differed from the sequence of plasmid DNA from which its infectious RNA transcripts were derived. A change of C<sub>7141</sub>→U produced an amino acid substitution Thr<sub>105</sub>→Ile in the NS4B protein. The WN/DEN4-3'Δ30 clone 1 also exhibited only one  
10 nucleotide difference from the plasmid cDNA sequence. This resulted in the same NS4B amino acid change (Leu<sub>112</sub>→Ser) that was observed in WN/DEN4 clone 18.

The WN/DEN4 chimera replicated more efficiently in *Vero* cells than did WN/DEN4-3'Δ30. The unmodified WN/DEN4 chimera reached a titer of 10<sup>6</sup> FFU/ml on day 6 in cells infected with a multiplicity of infection of 0.01; this was approximately 10-  
15 fold higher than the titer attained by the deletion mutant by day 6. The titer of the unmodified chimera was nearly the same as that attained by parental DEN4 under the same conditions.

#### Mouse neurovirulence.

Before evaluating chimeric viruses for virulence in mice, the *Vero* cell-rescued  
20 chimeric WN/DEN4 virus and its 3' deletion mutant were cloned biologically twice by terminal end-point dilution and then amplified in qualified *Vero* cells. The titer attained by the *Vero* cell-adapted WN/DEN4 clone 18 and WN/DEN4-3'Δ30 clone 1 was 1.7x10<sup>6</sup> FFU/ml and 1.4x10<sup>5</sup> FFU/ml, respectively.

Both chimeric WN/DEN4 virus and the deletion mutant WN/DEN4-3'Δ30 as well  
25 as parental WN strain NY99 and DEN4 strain 814669 viruses were evaluated in 3-day-old Swiss mice for neurovirulence by direct IC inoculation (Table 2). Wild-type WN NY99 grown in *Vero* cells was highly neurovirulent with an intracerebral LD<sub>50</sub> of 0.35 FFU in suckling Swiss mice. Wild-type DEN4 also grown in *Vero* cells was less neurovirulent with an IC LD<sub>50</sub> of 407 FFU. Both WN/DEN4 and WN/DEN4-3'Δ30 chimeric viruses  
30 exhibited a significant reduction in neurovirulence compared to their WN and DEN4 parents. All of the mice inoculated IC with 10<sup>3</sup> FFU of WN/DEN4 or its 3' deletion mutant survived during a 21 day observation period. At a higher dose of 10<sup>4</sup> FFU, only 4 of 11

mice inoculated with WN/DEN4 died. Thus, in suckling mice the WN/DEN4 chimera was more than 28,571 times less neurovirulent than its WN parent. The chimera with the 30 nt deletion was also significantly less neurovirulent than its WN parent. These observations are consistent with earlier observations that chimerization of TBEV or LGT with DEN4 significantly reduced their neurovirulence for mice (Pletnev, A.G. *et al.* 1992 *PNAS USA* 89:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS. USA* 95:1746-1751; Pletnev, A.G., Bray, M. & Lai, C.-J. 1993 *J Virol* 67:4956-4963).

#### Peripheral virulence in mice

Subsequently, we evaluated the chimeric viruses for peripheral virulence, *i.e.*, the ability of virus inoculated by the IP route to spread from a peripheral site to the central nervous system and cause encephalitis. Both chimeras were highly attenuated compared to their WN parent (Table 2 and 3). Notably, IP inoculation of  $10^4$  FFU of the deletion mutant chimera or  $10^5$  FFU of the unmodified chimera did not induce fatal encephalitis in any of the 3-week-old Swiss mice, whereas the IP  $LD_{50}$  for the WN parent was 10 FFU.

The chimeras were also evaluated in adult SCID mice because previous studies of tick-borne flaviviruses and their DEN4 chimeras indicated that SCID mice were a more sensitive detector of peripheral virulence than immunocompetent mice. Intraperitoneal inoculation of the maximum quantity of chimera,  $10^5$  FFU for WN/DEN4 and  $10^4$  FFU for WN/DEN4-3' $\Delta$ 30, did not produce encephalitis in any instance (Table 2). In contrast, the IP  $LD_{50}$  for parental WN was 6 FFU. These observations confirmed that the ablation of peripheral virulence of the WN chimeras had been achieved.

#### Immunogenicity and protective efficacy of chimeric viruses in mice

The two chimeras were immunogenic; a single IP inoculation of  $10^2$  FFU of the WN/DEN4 chimera induced a moderate level of serum WN neutralizing antibodies (1:93), while a 10-fold higher concentration ( $10^3$  FFU) induced a very high titer of WN neutralizing antibodies (1:1189) (Table 3). Also,  $10^3$  FFU of the chimeric WN/DEN4-3' $\Delta$ 30 deletion mutant stimulated a high level of such antibodies (1:292). Intraperitoneal challenge of the immunized mice on day 29 with 100 IP  $LD_{50}$  ( $10^3$  FFU) of parental WN indicated that the chimeras provided 90 to 100% protection against this high dose WN challenge (Table 3). There was a good correlation between the titer of serum WN neutralizing antibodies that developed in response to immunization and the degree of resistance induced. All unvaccinated control mice developed signs of CNS disease 7 to 13

days after challenge with 100 IP LD<sub>50</sub> of WN and these animals died shortly thereafter. To determine whether there was an age-related resistance of mice to WN, another group of 7-week-old mice also served as controls; they were the same age as immunized mice at time of challenge. This group of older control mice was challenged with one IP LD<sub>50</sub>,  
5 determined in 3-week-old mice. Seven of eight mice died during the 21 day observation period. This indicated that age-dependent resistance of mice to WN was not a factor in the observed protective effect of immunization.

**Table 1.** Mutations that were identified in genome of the WN/DEN4 or WN/DEN4-3'Δ30 chimera during cloning and rescue of chimera from cDNA in simian *Vero* cells

| Virus | Region of genome | NT (position)<br>* | WN/DEN4 |                   |          |                   | WN/DEN4-3' Δ30    |          |                          | Amino acid change |
|-------|------------------|--------------------|---------|-------------------|----------|-------------------|-------------------|----------|--------------------------|-------------------|
|       |                  |                    | pDNA**  | Recombinant virus |          | pDNA <sup>+</sup> | Recombinant virus |          |                          |                   |
|       |                  |                    |         | clone 55          | clone 18 |                   | clone 1           | clone 78 |                          |                   |
| WN    | prM              | U <sub>428</sub>   | C       | C                 | C        | C                 | C                 | C        | Ile <sub>6</sub> → Thr   |                   |
|       |                  | A <sub>847</sub>   | G       | G                 | G        | G                 | G                 | G        | Ile <sub>146</sub> → Val |                   |
|       | E                | A <sub>1566</sub>  | G       | G                 | G        | G                 | G                 | G        | silent                   |                   |
|       |                  | A <sub>1810</sub>  | G       | G                 | G        | G                 | G                 | G        | Thr <sub>282</sub> → Ala |                   |
| DEN4  | NS3              | A <sub>4799</sub>  | A       | C/a               | A        | A                 | A                 | A        | Glu <sub>92</sub> → Asp  |                   |
|       | NS4B             | C <sub>7141</sub>  | C       | C                 | C        | C                 | C                 | U        | Thr <sub>105</sub> → Ile |                   |
|       |                  | U <sub>7162</sub>  | U       | C/u               | C        | U                 | C                 | U        | Leu <sub>112</sub> → Ser |                   |

\*Numbering of nucleotide sequence of structural protein genes derived from the sequence of WN NY99 genome (GenBank accession number AF196835) and numbering of nucleotide sequence of nonstructural protein genes derived from the sequence of DEN4 genome (GenBank accession number AF326825).

\*\*Plasmid DNA.

<sup>+</sup> Comparison of the pDNA for the parental cDNA clones used to derive the chimeric viruses are indicated in Durbin, A. *et al.* 2001 *Am J Trop Med Hyg* 65:405-413

**Table 2.** Neurovirulence and peripheral virulence of parental West Nile virus (WN) or Dengue type 4 virus (DEN4) and their chimeric WN/DEN4 virus or its 3' deletion mutant WN/DEN4-3'Δ30 in mice as assayed by intracerebral (IC) or intraperitoneal (IP) inoculation

| Virus                           | Neurovirulence:                                                     |                          | Peripheral virulence:                                                |                                                                     |                          |
|---------------------------------|---------------------------------------------------------------------|--------------------------|----------------------------------------------------------------------|---------------------------------------------------------------------|--------------------------|
|                                 | LD <sub>50</sub> (FFU) after IC inoculation of 3-day-old Swiss mice | Reduction from WN parent | LD <sub>50</sub> (FFU) after IP inoculation of 3-week-old Swiss mice | LD <sub>50</sub> (FFU) after IP inoculation of 3-week-old SCID mice | Reduction from WN parent |
| DEN4                            | 407                                                                 | -                        | >100,000*                                                            | >100,000*                                                           | -                        |
| WN                              | 0.35                                                                | -                        | 10                                                                   | 6.0                                                                 | -                        |
| WN/DEN4 Chimera (clone 18)      | >10,000*                                                            | >28,571x                 | >100,000*                                                            | >100,000*                                                           | >10,000x                 |
| WN/DEN4-3'Δ30 Chimera (clone 1) | >1,000*                                                             | >2,857x                  | >10,000*                                                             | >10,000*                                                            | >1,000x                  |

Note: Each decimal dilution was tested in 9 to 12 mice in group.

\*Highest concentration tested.

**Table 3.** Peripheral virulence, antibody response and protective efficacy of parental (WN or DEN4) viruses and chimeric WN/DEN4 virus or its 3' deletion mutant WN/DEN4-3'Δ30 in 3-week-old Swiss mice

| Mice inoculated IP with         | Dose (FFU*) inoculated | Mortality after IP inoculation | Mean titer of WN neutralizing antibody in pooled sera on day 28 | Mortality after survivors inoculated IP with 100 IP LD <sub>50</sub> of WN on day 29 |
|---------------------------------|------------------------|--------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------------------------|
| WN                              | 0.1                    | 0/10                           | <1:10                                                           | 10/10 (100%)                                                                         |
|                                 | 1                      | 0/10                           | 1:24                                                            | 10/10 (100%)                                                                         |
|                                 | 10                     | 5/10                           | 1:40                                                            | 4/5 (80%)                                                                            |
|                                 | 100                    | 10/10                          |                                                                 |                                                                                      |
|                                 | 1,000                  | 9/10                           |                                                                 |                                                                                      |
|                                 | 10,000                 | 10/10                          |                                                                 |                                                                                      |
| WN/DEN4 Chimera (clone 18)      | 1                      | 0/10                           | 1:26                                                            | 10/10 (100%)                                                                         |
|                                 | 10                     | 0/10                           | 1:21                                                            | 9/10 (90%)                                                                           |
|                                 | 100                    | 0/10                           | 1:93                                                            | 7/10 (70%)                                                                           |
|                                 | 1,000                  | 0/10                           | 1:1189                                                          | 0/10 (0%)                                                                            |
|                                 | 10,000                 | 0/10                           | 1:585                                                           | 0/9** (0%)                                                                           |
|                                 | 100,000                | 0/10                           | 1:924                                                           | 0/10 (0%)                                                                            |
| WN/DEN4-3'Δ30 Chimera (clone 1) | 1                      | 0/10                           | 1:28                                                            | 9/10 (90%)                                                                           |
|                                 | 10                     | 0/10                           | <1:10                                                           | 9/10 (90%)                                                                           |
|                                 | 100                    | 0/10                           | 1:14                                                            | 8/10 (80%)                                                                           |
|                                 | 1,000                  | 0/10                           | 1:292                                                           | 1/10 (10%)                                                                           |
|                                 | 10,000                 | 0/10                           | 1:269                                                           | 0/10 (0%)                                                                            |
|                                 |                        |                                |                                                                 |                                                                                      |
| DEN4                            | 1,000                  | 0/10                           | <1:10                                                           | 10/10 (100%)                                                                         |
|                                 | 10,000                 | 0/10                           | 1:13                                                            | 8/10 (80%)                                                                           |
|                                 | 100,000                | 0/10                           | 1:22                                                            | 10/10 (100%)                                                                         |
| Control                         |                        |                                | <1:10                                                           | 10/10 (100%)                                                                         |

\*Focus forming unit.

\*\*One of the 10 mice inoculated died as a result of trauma; WN virus was not detected in the brain by tissue culture (*Vero* cell) assay.

Table 4. Chimeric WN/DEN4 and its 3' deletion mutant WN/DEN4-3'Δ30 are attenuated in rhesus monkeys

| Virus inoculated subcutaneously | Dose of virus (FFU) | No. of monkeys inoculated | Viremia     |                      |                                                                                        |
|---------------------------------|---------------------|---------------------------|-------------|----------------------|----------------------------------------------------------------------------------------|
|                                 |                     |                           | No. viremic | Mean duration (days) | Mean peak titer of viremia during 2 weeks post-inoculation log <sub>10</sub> (FFU/ml)* |
| WN/DEN4                         | 10 <sup>5</sup>     | 4                         | 3           | 1.5                  | 0.78                                                                                   |
|                                 | 10 <sup>6</sup>     | 4                         | 2           | 0.5                  | <0.7                                                                                   |
| WN/DEN4-3'Δ30                   | 10 <sup>5</sup>     | 4                         | 0           | 0                    | <0.7                                                                                   |
| WN                              | 10 <sup>5</sup>     | 2                         | 2           | 5.5                  | 2.63                                                                                   |
|                                 | 10 <sup>6</sup>     | 2                         | 2           | 5.5                  | 2.76                                                                                   |
| DEN4                            | 10 <sup>6</sup>     | 4                         | 4           | 3.8                  | 2.23                                                                                   |

\*Tested daily for 10 days.  
Note: 0.7 log<sub>10</sub>(FFU/ml) is a lowest level of detectable viremia in serum. 0.6 log10(FFU/ml) was used to calculate mean peak titer of viremia for animals that had no detectable viremia.

Table 5. Immunogenicity and protective efficacy of chimeric WN/DEN4 and its 3' deletion mutant WN/DEN4-3'Δ30 in rhesus monkeys

| Group of monkeys inoculated<br>SC with |                 | No. of monkeys | Geo. mean titer of WN serum<br>neutralizing antibody on post<br>immunization day 42 (range) | No. of monkeys viremic during 2 weeks<br>post challenge with 10 <sup>5</sup> FFU of WN<br>(Mean peak titer; log <sub>10</sub> FFU/ml)* |
|----------------------------------------|-----------------|----------------|---------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|
| Virus                                  | Dose (FFU)      |                |                                                                                             |                                                                                                                                        |
| WN/DEN4                                | 10 <sup>5</sup> | 4              | 1:661 (416-1126)                                                                            | 0                                                                                                                                      |
|                                        | 10 <sup>6</sup> | 4              | 1:501 (270-727)                                                                             | 0                                                                                                                                      |
| WN/DEN4-3'Δ30                          | 10 <sup>5</sup> | 4              | 1:186 (109-247)                                                                             | 0                                                                                                                                      |
| WN                                     | 10 <sup>5</sup> | 2              | 1:1318 (1305-1324)                                                                          | 0                                                                                                                                      |
|                                        | 10 <sup>6</sup> | 2              | 1:708 (599-842)                                                                             | 0                                                                                                                                      |
| DEN4                                   | 10 <sup>6</sup> | 4              | < 1:20                                                                                      | 4 (2.04**)                                                                                                                             |

\*Tested daily for 10 days.  
\*\*Mean duration of viremia was 3.75 days.

**Attenuation, immunogenicity and protective efficacy of West Nile/DEN4 chimeras in rhesus monkeys.**

It has been established that some non-human primates are readily infected with a number of flaviviruses by the peripheral route (Simmons *et al.* 1931 *Philipp J Sci* 44:1-247; 5 Rosen, 1958 *Am J Trop Med Hyg* 7:406-410). Thus, infection of monkeys represents the closest experimental system to flavivirus infection of humans. The response of monkeys to flavivirus infection is similar to that of humans in that there is a four to six day viremia, although lower primates do not usually develop clinical flavivirus symptoms. The objectives of flavivirus studies in monkeys are: (1) to evaluate the immunogenicity of 10 various candidate vaccines; (2) to evaluate the infectivity and virulence (attenuation phenotype) of candidate vaccines as measured by the duration of viremia in days and the peak virus titer in FFU/ml; and (3) to evaluate the protective efficacy of the candidate vaccines against challenge by wild-type flavivirus.

(1) Inoculation: Each monkey is inoculated with a total of  $10^5$  or  $10^6$  FFU of virus 15 diluted in L15 medium with SPG (Durbin, A. P. *et al.* 2001 *Am J Trop Med Hyg* 65:405-413). Normally, virus is inoculated by the subcutaneous route to anesthetized animals.

(2) Blood collection: Following inoculation of virus, blood samples of 3.0 ml are taken daily for two weeks and 5.0 ml at 3 weeks, 4 weeks, 5 weeks, and 6 weeks.

(3) Challenge with parental wild-type flavivirus: Where virus challenge is deemed 20 appropriate to evaluate the protective efficacy, monkeys are inoculated with wild-type virus at  $10^5$  FFU/dose in a 1.0 ml volume subcutaneously in the upper arm area.

(4) Laboratory assays: Serum samples are collected to be used to determine: (a) the duration and level of viremia by direct viral plaque or FFU assay; and (b) the titer of neutralizing antibodies induced as measured by FFU reduction neutralization test, all tests 25 well known to those skilled in the art of vaccine development.

Attenuation, immunogenicity, and protective efficacy of the West Nile/DEN4 chimeras were studied in 20 rhesus monkeys (Tables 4 and 5). Eight monkeys were inoculated subcutaneously (SC) with WN/DEN4 (clone 18); 4 animals received  $10^5$  FFU, while the other 4 received  $10^6$  FFU. Four monkeys were inoculated SC with  $10^5$  FFU of 30 WN/DEN4-3' $\Delta$ 30 (clone 1). A group of 4 monkeys was inoculated SC with parental West Nile virus; 2 animals received  $10^5$  FFU, while the other received  $10^6$  FFU. Finally, another group of 4 monkeys was inoculated SC with  $10^6$  of DEN4 (Table 4).

Each of the monkeys inoculated SC with  $10^5$  or  $10^6$  FFU of West Nile virus developed a viremia that lasted 5 to 6 days and attained a mean peak titer of 2.6 to 2.8  $\log_{10}$  (FFU/ml) (Fig. 3, Table 4). In contrast, WN/DEN4 induced viremia in only 5 of the 8 monkeys inoculated with  $10^5$  or  $10^6$  FFU. Viremia lasted only one to two days and attained a peak titer 100 fold lower than observed for WN infected monkeys. Significantly, each of the 4 monkeys inoculated SC with  $10^5$  FFU of the WN/DEN4-3' $\Delta$ 30 mutant failed to develop a detectable viremia.

Although the WN/DEN chimera and its deletion mutant were significantly attenuated for rhesus monkeys, these hybrid viruses induced a moderate to high level of serum WN neutralizing antibodies in each immunized animal (Table 5). The two chimeras also induced complete resistance to SC challenge with  $10^5$  FFU of West Nile virus on day 42 post immunization. Viremia of WN was not detected in any of the 12 monkeys immunized with WN/DEN4 or its deletion mutant. The West Nile challenge virus replicated efficiently in monkeys previously infected with DEN4 virus. This indicates that the high level of protection against WN challenge afforded by infection with WN/DEN4 chimeric viruses is specified by the WN protective antigens in the chimera and not by the DEN4 component of the chimera.

The  $\Delta$ 30 mutation was first described and characterized in the DEN4 virus (Men, R. *et al.* 1996 *J Virol* 70:3930-7). In DEN4, the mutation consists of the removal of 30 contiguous nucleotides comprising nucleotides 10478 – 10507 of the 3' UTR (Figure 4A) which form a putative stem-loop structure referred to as TL2 (Proutski, V. *et al.* 1997 *Nucleic Acids Res* 25:1194-202). Among the flaviviruses, large portions of the UTR form highly conserved secondary structures (Hahn, C.S. *et al.* 1987 *J Mol Biol* 198:33-41; Proutski, V. *et al.* 1997 *Nucleic Acids Res* 25:1194-202). Although the individual nucleotides are not necessarily conserved in these regions, appropriate base pairing preserves the stem-loop structure in each serotype, a fact that is not readily apparent when only considering the primary sequence (Figure 4B, C). We have demonstrated that the  $\Delta$ 30 mutation specifies an attenuation phenotype that is transportable to other DEN serotypes, DEN1 (Whitehead, S.S. *et al.* 2003 *J Virol* 77:1653-1657) and DEN2 (Tonga/74). This indicates that the  $\Delta$ 30 mutation is expected to have a corresponding effect on DEN3 wild-type virus. We envision constructing this remaining virus by deletion of the TL2 region of the virus, *e.g.*,

DEN3 (Sleman/78) (Figure 4C). These attenuated or wild type DEN1, DEN2, or DEN3 viruses could readily replace the DEN4 wild type or DEN4-3' $\Delta$ 30 viruses presented in these examples.

These findings specifically identify two candidate WN live attenuated virus vaccines. The first, WN/DEN4, is about 100-fold attenuated in comparison to WN wild-type virus as indicated by the greatly restricted level of viremia. The second virus, WN/DEN4-3' $\Delta$ 30, is more attenuated than WN/DEN4 as indicated by the absence of viremia in monkey serum and by the moderately decreased serum neutralizing antibody response. Thus, the methods and viruses taught provide live attenuated WN vaccines of differing levels of attenuation, each of which is highly protective against wild-type WN virus challenge. Similar attenuated WN/DEN chimeric viruses on a DEN1, DEN2, or DEN3 background are envisioned.

**Further attenuation of WN/DEN4 chimeras by introduction of additional mutations in the genes for the non-structural proteins of DEN4 that serve as a component of these vaccine candidates.**

We contemplate achieving an increase in the level of attenuation of the candidate vaccine WN/DEN4 or WN/DEN4-3' $\Delta$ 30 chimera if need be by adding one or more attenuation mutations to the DEN4 component of the chimeras. A large set of mutations that attenuate DEN4 in mice (Blaney, et al. 2001 *J Virol* **75**:9731-9740; Blaney, et al. 2002 *Virology* **300**:125-139; Hanley, et al. 2002 *J Virol* **76**:525-31) has been identified in the part of the DEN4 genome included in the WN/DEN4 chimeric viruses. Members from this set of attenuating mutations can be introduced in the WN/DEN4 chimeric virus to further attenuate these viruses. For example, it might be necessary to further attenuate the WN/DEN4 virus, which possesses some residual neurovirulence as indicated above. The feasibility of this approach to achieve further attenuation is exemplified by introducing a viable mutation that specifies a temperature sensitive phenotype as well as a phenotype of growth restriction in suckling mouse brain into the non-structural protein 3 (NS3) of the DEN4 component of the WN/DEN4 chimera. Mutation 4891 (isoleucine $\rightarrow$ threonine) had previously been identified at nucleotide 4891 of the NS3 gene of DEN4 (Blaney, et al. 2002 *Virology* **300**:125-139). Mutation 4891 specified two desirable phenotypes, i.e., temperature sensitivity and growth restriction in brain tissue. Similarly, mutation 4995

(serine→proline), also in NS3, specified the same two desirable phenotypes (Blaney, et al. 2001 *J Virology* 75:9731-9740, 2001). The 4891 and 4995 mutations also increase replication fitness of DEN4 in *Vero* cells, *i.e.*, they are *Vero* cell adaptation mutations. The wild type amino acid residue at DEN4 4891 (isoleucine) is conserved in DEN2 Tonga/74 and DEN3 Sleman/78, but not DEN1 West Pacific. The wild type amino acid residue at DEN4 4995 (serine) is conserved in DEN1 West Pacific, DEN2 Tonga/74, but not DEN3 Sleman. One or both of these mutations may also be included in a WN/DEN1, 2, or 3 chimera. Thus, their inclusion in WN/DEN4 virus is contemplated as achieving an increase in replication of the virus in *Vero* cells or the genetic stability of the mutation during manufacture in *Vero* cells.

### **Discussion.**

Initially, we demonstrated that although prM and E proteins of distantly related tick-borne and mosquito-borne flaviviruses are highly divergent, these proteins could be interchanged in some instances without loss of virus viability (Pletnev, A.G. *et al.* 1992 *PNAS USA* 89:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS USA* 95:1746-1751). This approach has been used to create new chimeric flaviviruses (Bray, M., Men, R. & Lai, C.-J. 1996 *J. Virol.* 70:4162-4166; Chambers, T.J. *et al.* 1999 *J Virol* 73:3095-3101; Guirakhoo, F. *et al.* 2000 *J Virol* 74:5477-5485; Huang, C.Y. *et al.* 2000 *J Virol* 74:3020-3028; Van Der Most, R.G. *et al.* 2000 *J Virol* 74:8094-8101; Caufour, P.S. *et al.* 2001 *Virus Res* 79:1-14).

Previously, we succeeded in constructing and recovering viable tick-borne/DEN4 chimeras (Pletnev, A.G. *et al.* 1992 *PNAS USA* 89:10532-10536; Pletnev, A.G. & Men, R. 1998 *PNAS USA* 95:1746-1751; Pletnev, A.G., Bray, M. & Lai, C.-J. 1993 *J Virol* 67:4956-4963). In these instances, the tick-borne flavivirus parent was tick-borne encephalitis virus, a highly virulent virus, or Langat virus, a naturally attenuated tick-borne virus. Thus, the two components of these chimeras had disparate vector hosts, namely ticks and in the case of DEN4, mosquitoes. Decreased efficiency of gene product interactions in the chimeras was thought to be the basis for the marked attenuation exhibited by these hybrid viruses. Nonetheless, although highly attenuated in mice, the TBEV/DEN4 and LGT/DEN4 chimeras were immunogenic and provided considerable protection against their parental tick-borne flavivirus. In the present instance, both virus parents of the WN/DEN4 chimeras are transmitted by mosquitoes. However, vector preference differs,

*Aedes* for DEN4 and *Culex* for WN (Burke, D.S. & Monath, T. P. 2001 in *Fields Virology*, eds. Knipe, D. M. & Howley, P. M. Lippincott Williams and Wilkins, Philadelphia, 4-th ed., pp. 1043-1125; Hayes, C.G. 1989 in *The Arboviruses: Epidemiology and Ecology*, ed. Monath T. P. Boca Raton, F. L.: CRC Press, Volume V, pp. 59-88).

5           In the present study, we constructed viable WN/DEN4 chimeras that contained a DEN4 genome whose genes for structural prM and E proteins were replaced by the corresponding genes of WN strain NY99. Among flaviviruses, the hydrophobic domain between C and prM ("transmembrane signal domain") varies in sequence and also varies in length from 14 to 20 amino acids (Stocks, C.E. & Lobigs, M. 1998 *J Virol* 72:2141-2149).  
 10       It acts as a signal sequence for translocation of prM protein into the endoplasmic reticulum lumen where post-translation maturation of this protein occurs (Lindenbach, B.D. & Rice, C.M. 2001 in *Fields Virology*, eds. Knipe, D. M. & Howley, P. M. Lippincott Williams and Wilkins, Philadelphia, 4-th ed., pp. 1043-1125). This signal peptide is flanked at its NH<sub>2</sub>-terminal region by the viral protease NS2B-NS3 cleavage site and at its COOH-terminal  
 15       region by a cellular signalase cleavage site. Four different junctions at the protease cleavage site between DEN4 C and WN prM protein were introduced separately in chimeric constructs (Fig. 2). The C/prM fusion sequence at the viral protease cleavage site (KR<sup>↓</sup>S) in the chimeras was constructed to be similar to that of the DEN4 parent, which provides its NS2B-NS3 protease for the processing of the chimeric polyprotein. However,  
 20       each of the chimeric constructs of group 1 and 2 chimeras contain a unique substitution in the transmembrane signal sequence at the third amino acid position downstream of the protease cleavage site, while another sequence is shared by group 3 and group 4 chimeras (Fig. 1A, Fig. 2). Thus, the transmembrane signal of the constructs is similar in length but exhibits polymorphism for group 1, group 2 and groups 3 and 4 together. This occurs at the  
 25       third amino acid position downstream of the protease cleavage site. Viable WN/DEN4 virus was recovered only when construct number 4 (Fig. 2) was employed to prepare RNA transcripts for transfection. Infectious virus was recovered from 2 of 5 separate clones that encoded Asp in the 3+ amino acid position. And only the 2 clones that also contained a second-site mutation at the 6+ amino acid position downstream of the protease cleavage  
 30       site that substituted Thr for Ile were infectious; this mutation occurred during cloning of cDNA in bacteria (Fig. 2, Table 1). In contrast, none of the 13 clones that encoded Gly or Val at the 3+ amino acid position produced infectious virus following transfection. This

suggests that the transmembrane signal sequence between C and prM is a determinant of viability in the context of a WN/DEN4 chimera. This is consistent with an earlier observation made with yellow fever virus that the transmembrane signal sequence between C and prM protein plays a role in viability and neurovirulence (Lee, E. *et al.* 2000 *J. Virol.* 5 74:24-32).

The +3 and +6 Asp and Thr motif at the capsid protein - prM protein cleavage site that was required for viability of the chimera could not be predicted from the sequence of either parent, i.e., DEN4 and West Nile virus, because neither parent had this +3 and +6 motif. Success was achieved by testing a number of disparate sequences at the cleavage 10 site and this led to the identification of the +3 and +6 Asp and Thr motif that was required for viability. For this reason, we advocate an empirical approach that embraces testing several different C/prM junction sequences for identification of other motifs that produce equally viable chimeric virus.

The WN strain NY99 exhibited considerable virulence in Swiss mice; its IC LD<sub>50</sub> 15 was 0.35 FFU for suckling mice and its IP LD<sub>50</sub> was 10 FFU for 3-week-old Swiss mice (Table 2). Nearly the same level of neurovirulence was observed for a wild-type strain of WN isolated in Israel that was evaluated in CD-1 (ICR) mice: IC LD<sub>50</sub> and IP LD<sub>50</sub> were estimated to be 1.1 and 4.3 PFU, respectively (Halevy, M. *et al.* 1994 *Arch Virol* 137:355-370). In addition, a high degree of genomic similarity (> 99.8%) between the WN NY99 20 and the WN Israel-1998 was recently confirmed by sequence analysis (Lanciotti, R.S. *et al.* 1999 *Science* 286:2333-2337) indicating that both highly pathogenic strains of WN, representing North American and Middle Eastern viruses, are closely related. Wild-type DEN4 Caribbean strain 814669 was moderately neurovirulent for suckling mice with an IC LD<sub>50</sub> of 407 FFU, and it was approximately 20 times more virulent than its cDNA cloned 25 virus (Pletnev, A.G. & Men, R. 1998 *PNAS USA* 95:1746-1751). In contrast, the WN/DEN4 chimera and its 3' deletion mutant were significantly less neurovirulent than their wild-type DEN4 or WN parent. Only at a high dose of 10<sup>4</sup> FFU did a minority of mice, inoculated IC with WN/DEN4 chimera, die. Also, the WN/DEN4 chimera inoculated IC at this dose caused death of suckling mice later than parental WN virus: 4-5 days post- 30 infection for wild-type WN compared to 9-13 days post-infection for the chimera. Additional methods and procedures are taught that allow further attenuation of the IC virulence of the WN/DEN4 chimeric virus by the introduction of mutations that are known

to attenuate DEN4 virus for the brain of mice. In addition, we also contemplate achieving further attenuation of WN/DEN4-3' $\Delta$ 30 by the incorporation of additional attenuating mutations.

Despite the high peripheral virulence of wild-type WN strain NY99 (IP LD<sub>50</sub> of 10  
5 FFU), chimerization of WN with DEN4 completely ablated this property of its WN parent. Thus, 3-week-old Swiss mice survived IP inoculation of 10<sup>4</sup> or 10<sup>5</sup> FFU of chimeric virus. Our observations are consistent with earlier findings that a similar large reduction of peripheral neurovirulence of TBEV or LGT occurs following chimerization with DEN4 (Pletnev, A.G. *et al.* 1992 *PNAS USA* **89**:10532-10536; Pletnev, A.G. & Men, R. 1998  
10 *PNAS. USA* **95**:1746-1751; Pletnev, A.G., Bray, M. & Lai, C.-J. 1993 *J Virol* **67**:4956-4963). Similar observations were made when the WN/DEN4 chimeras were tested in SCID mice for peripheral virulence (Table 2).

Although highly attenuated, the WN/DEN4 chimeras stimulated a moderate to high level of serum neutralizing antibodies against WN NY99 (Table 3). There was a strong  
15 correlation between the level of neutralizing antibodies to WN induced by immunization and resistance to subsequent lethal WN challenge. The immune response of mice inoculated with the chimeras was dose-dependent and indicated that the unmodified WN/DEN4 chimera was slightly more immunogenic than the corresponding 3' deletion mutant. However, 90 to 100% protection against WN challenge was achieved when a  
20 single 10<sup>3</sup> FFU dose of WN/DEN4 chimera or its 3' deletion mutant was used for immunization. A higher dose (10<sup>4</sup> FFU) of either chimera provided complete protection to WN challenge. The WN/DEN4 and WN/DEN4-3' $\Delta$ 30 were also highly attenuated, immunogenic, and protective against WN virus challenge in non-human primates (rhesus monkeys). Thus, the WN prM and E proteins of the chimeric viruses represent effective  
25 antigens able to induce complete protection to challenge with highly virulent WN in both mice and monkeys. Our observations concerning safety, immunogenicity, and protective efficacy of the chimeric WN/DEN4 vaccine candidates in mice and monkeys provide a basis for extending our evaluation of the vaccine candidates to humans and to domestic animals, such as horses or birds, which are at high risk. In this way, the use of the  
30 WN/DEN4 chimeras as vaccines is envisioned for humans and domestic animals, such as horses or birds.

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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.

## SEQUENCE LISTING

<110> The Government of the United States of America, as represented by the  
Secretary, Department of Health and Human Services;  
Walter Reed Army Institute Of Research

<120> CONSTRUCTION OF WEST NILE VIRUS AND DENGUE VIRUS CHIMERAS FOR USE IN  
A LIVE VIRUS VACCINE TO PREVENT DISEASE CAUSED BY WEST NILE VIRUS

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<141> 2003-01-09

<150> US 60/347,281

<151> 2002-01-10

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<212> PRT

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20          25          30
Phe Ser Leu Gly Met Leu Gln Gly Arg Gly Pro Leu Lys Leu Phe Met
35          40          45
Ala Leu Val Ala Phe Leu Arg Phe Leu Thr Ile Pro Pro Thr Ala Gly
50          55          60
Ile Leu Lys Arg Trp Gly Thr Ile Lys Lys Ser Lys Ala Ile Asn Val
65          70          75          80
Leu Arg Gly Phe Arg Lys Glu Ile Gly Arg Met Leu Asn Ile Leu Asn
85          90          95
Arg Arg Arg Arg Thr Val Gly Met Ile Ile Met Leu Thr Pro Thr Val
100         105         110
Met Ala Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val
115         120         125
Ser Arg Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Lys Asp Gly
130         135         140
Thr Asn Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu
145         150         155         160

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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 |     |
| 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  |  |  |
|      |      | 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 |  |  |

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 Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr Arg Gly Glu Asp Gly Cys  
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 Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu Lys Glu Glu Asn Leu  
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 Lys Gln Arg Ala Gly Val Leu Trp Asp Val Pro Ser Pro Pro Pro Val



|      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |  |  |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|--|--|
| 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  |  |  |
|      |      | 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                |
| 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                |
| 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                |
| 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                |
| Ser Ser Pro Asn Pro Thr Val | Glu Ala Gly Arg Thr Leu | Arg Val Leu         |
| 2645                        | 2650                    | 2655                |
| Asn Leu Val Glu Asn Trp Leu | Asn Asn Asn Thr Gln Phe | Cys Val Lys         |
| 2660                        | 2665                    | 2670                |
| Val Leu Asn Pro Tyr Met Pro | Ser Val Ile Glu Arg Met | Glu Thr Leu         |
| 2675                        | 2680                    | 2685                |
| Gln Arg Lys Tyr Gly Gly Ala | Leu Val Arg Asn Pro Leu | Ser Arg Asn         |
| 2690                        | 2695                    | 2700                |
| Ser Thr His Glu Met Tyr Trp | Val Ser Asn Ala Ser Gly | Asn Ile Val         |
| 2705                        | 2710                    | 2715                |
| 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  |  |  |
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| 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 |      |      |      |      |  |  |
| 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 |      |      |      |      |  |  |
| Trp  | Glu  | Pro  | Ser  | Arg  | Gly  | Trp  | Asn  | Asp  | Trp  | Thr  | Gln  | Val  | Pro  | Phe  | Cys  |  |  |
| 3185 |      |      |      |      | 3190 |      |      |      |      | 3195 |      |      |      |      | 3200 |  |  |
| Ser  | His  | His  | Phe  | His  | Glu  | Leu  | Ile  | Met  | Lys  | Asp  | Gly  | Arg  | Thr  | Leu  | Val  |  |  |
|      |      |      | 3205 |      |      |      |      |      | 3210 |      |      |      |      | 3215 |      |  |  |
| Val  | Pro  | Cys  | Arg  | Asn  | Gln  | Asp  | Glu  | Leu  | Ile  | Gly  | Arg  | Ala  | Arg  | Ile  | Ser  |  |  |
|      |      | 3220 |      |      |      |      | 3225 |      |      |      |      | 3230 |      |      |      |  |  |
| Gln  | Gly  | Ala  | Gly  | Trp  | Ser  | Leu  | Arg  | Glu  | Thr  | Ala  | Cys  | Leu  | Gly  | Lys  | Ser  |  |  |

|                                                                 |      |      |
|-----------------------------------------------------------------|------|------|
| 3235                                                            | 3240 | 3245 |
| Tyr Ala Gln Met Trp Ser Leu Met Tyr Phe His Arg Arg Asp Leu Arg |      |      |
| 3250                                                            | 3255 | 3260 |
| Leu Ala Ala Asn Ala Ile Cys Ser Ala Val Pro Ser His Trp Ile Pro |      |      |
| 3265                                                            | 3270 | 3275 |
| Thr Ser Arg Thr Thr Trp Ser Ile His Ala Ser His Glu Trp Met Thr |      | 3280 |
|                                                                 | 3285 | 3290 |
| Thr Glu Asp Met Leu Thr Val Trp Asn Arg Val Trp Ile Leu Glu Asn |      | 3295 |
|                                                                 | 3300 | 3305 |
| Pro Trp Met Glu Asp Lys Thr Pro Val Glu Ser Trp Glu Glu Ile Pro |      | 3310 |
|                                                                 | 3315 | 3320 |
| Tyr Leu Gly Lys Arg Glu Asp Gln Trp Cys Gly Ser Leu Ile Gly Leu |      | 3325 |
|                                                                 | 3330 | 3335 |
| Thr Ser Arg Ala Thr Trp Ala Lys Asn Ile Gln Thr Ala Ile Asn Gln |      | 3340 |
| 3345                                                            | 3350 | 3355 |
| Val Arg Ser Leu Ile Gly Asn Glu Glu Tyr Thr Asp Tyr Met Pro Ser |      | 3360 |
|                                                                 | 3365 | 3370 |
| Met Lys Arg Phe Arg Arg Glu Glu Glu Glu Ala Gly Val Leu Trp     |      | 3375 |
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| agtccttgga  | tcgcaagagg  | gagcaatgca  | cacagcactg  | acaggagcta  | cagagatcca  | 1740 |
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| attggaactc  | aaggggatga  | gctatgcaat  | gtgcttgaat  | gcctttgtgt  | tgaagaaaga  | 1860 |
| agtctccgaa  | acgcaacatg  | ggacaatact  | catcaagggt  | gagtacaaag  | gggaagatgc  | 1920 |
| accttgcaag  | attcctttct  | ccacggagga  | tggacaaggg  | aaagcccaca  | atggcagact  | 1980 |
| gatcacagct  | aacctcagtg  | tgaccaagaa  | ggaggagcct  | gtcaatattg  | aggcagaacc  | 2040 |
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| attaggaaaa  | atggtgcacc  | aaatattttg  | aagtgccttac | acagccctat  | ttagtggagt  | 2280 |
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<211> 3390
<212> PRT
<213> Dengue 3 (Sleman/78)
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| <400> 33 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Met      | Asn | Asn | Gln | Arg | Lys | Lys | Thr | Gly | Lys | Pro | Ser | Ile | Asn | Met | Leu |
| 1        |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |
| Lys      | Arg | Val | Arg | Asn | Arg | Val | Ser | Thr | Gly | Ser | Gln | Leu | Ala | Lys | Arg |
|          |     |     | 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 |     |     |     |
| 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 |     |     |     |
| Gly | Val | Leu | Asn | Ser | Leu | Gly | Lys | Met | Val | His | Gln | Ile | Phe | Gly | Ser |
| 705 |     |     |     |     | 710 |     |     |     |     | 715 |     |     |     |     | 720 |
| Ala | Tyr | Thr | Ala | Leu | Phe | Ser | Gly | Val | Ser | Trp | Ile | Met | Lys | Ile | Gly |
|     |     |     | 725 |     |     |     |     |     | 730 |     |     |     |     | 735 |     |
| Ile | Gly | Val | Leu | Leu | Thr | Trp | Ile | Gly | Leu | Asn | Ser | Lys | Asn | Thr | Ser |
|     |     |     | 740 |     |     |     |     | 745 |     |     |     |     | 750 |     |     |
| Met | Ser | Phe | Ser | Cys | Ile | Val | Ile | Gly | Ile | Ile | Thr | Leu | Tyr | Leu | Gly |

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

|      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |  |  |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|--|--|
| Ala  | Leu  | Ile  | Ala  | Thr  | Phe  | Lys  | Ile  | Gln  | Pro  | Phe  | Leu  | Ala  | Leu  | Gly  | Phe  |  |  |
|      |      |      |      | 1205 |      |      |      |      | 1210 |      |      |      |      |      | 1215 |  |  |
| Phe  | Leu  | Arg  | Lys  | Leu  | Thr  | Ser  | Arg  | Glu  | Asn  | Leu  | Leu  | Leu  | Gly  | Val  | Gly  |  |  |
|      |      |      | 1220 |      |      |      |      | 1225 |      |      |      |      |      | 1230 |      |  |  |
| Leu  | Ala  | Met  | Ala  | Thr  | Thr  | Leu  | Gln  | Leu  | Pro  | Glu  | Asp  | Ile  | Glu  | Gln  | Met  |  |  |
|      |      | 1235 |      |      |      |      | 1240 |      |      |      |      | 1245 |      |      |      |  |  |
| Ala  | Asn  | Gly  | Ile  | Ala  | Leu  | Gly  | Leu  | Met  | Ala  | Leu  | Lys  | Leu  | Ile  | Thr  | Gln  |  |  |
|      | 1250 |      |      |      |      | 1255 |      |      |      |      | 1260 |      |      |      |      |  |  |
| Phe  | Glu  | Thr  | Tyr  | Gln  | Leu  | Trp  | Thr  | Ala  | Leu  | Val  | Ser  | Leu  | Met  | Cys  | Ser  |  |  |
| 1265 |      |      |      |      | 1270 |      |      |      |      | 1275 |      |      |      |      | 1280 |  |  |
| Asn  | Thr  | Ile  | Phe  | Thr  | Leu  | Thr  | Val  | Ala  | Trp  | Arg  | Thr  | Ala  | Thr  | Leu  | Ile  |  |  |
|      |      |      | 1285 |      |      |      |      | 1290 |      |      |      |      |      | 1295 |      |  |  |
| Leu  | Ala  | Gly  | Ile  | Ser  | Leu  | Leu  | Pro  | Val  | Cys  | Gln  | Ser  | Ser  | Ser  | Met  | Arg  |  |  |
|      |      | 1300 |      |      |      |      |      | 1305 |      |      |      |      | 1310 |      |      |  |  |
| Lys  | Thr  | Asp  | Trp  | Leu  | Pro  | Met  | Ala  | Val  | Ala  | Ala  | Met  | Gly  | Val  | Pro  | Pro  |  |  |
|      | 1315 |      |      |      |      |      | 1320 |      |      |      |      | 1325 |      |      |      |  |  |
| Leu  | Pro  | Leu  | Phe  | Ile  | Phe  | Ser  | Leu  | Lys  | Asp  | Thr  | Leu  | Lys  | Arg  | Arg  | Ser  |  |  |
|      | 1330 |      |      |      |      | 1335 |      |      |      |      | 1340 |      |      |      |      |  |  |
| Trp  | Pro  | Leu  | Asn  | Glu  | Gly  | Val  | Met  | Ala  | Val  | Gly  | Leu  | Val  | Ser  | Ile  | Leu  |  |  |
| 1345 |      |      |      |      | 1350 |      |      |      |      | 1355 |      |      |      |      | 1360 |  |  |
| Ala  | Ser  | Ser  | Leu  | Leu  | Arg  | Asn  | Asp  | Val  | Pro  | Met  | Ala  | Gly  | Pro  | Leu  | Val  |  |  |
|      |      |      | 1365 |      |      |      |      | 1370 |      |      |      |      |      | 1375 |      |  |  |
| Ala  | Gly  | Gly  | Leu  | Leu  | Ile  | Ala  | Cys  | Tyr  | Val  | Ile  | Thr  | Gly  | Thr  | Ser  | Ala  |  |  |
|      |      | 1380 |      |      |      |      |      | 1385 |      |      |      |      | 1390 |      |      |  |  |
| Asp  | Leu  | Thr  | Val  | Glu  | Lys  | Ala  | Ala  | Asp  | Val  | Thr  | Trp  | Glu  | Glu  | Glu  | Ala  |  |  |
|      | 1395 |      |      |      |      |      | 1400 |      |      |      |      | 1405 |      |      |      |  |  |
| Glu  | Gln  | Thr  | Gly  | Val  | Ser  | His  | Asn  | Leu  | Met  | Ile  | Thr  | Val  | Asp  | Asp  | Asp  |  |  |
|      | 1410 |      |      |      |      | 1415 |      |      |      |      | 1420 |      |      |      |      |  |  |
| Gly  | Thr  | Met  | Arg  | Ile  | Lys  | Asp  | Asp  | Glu  | Thr  | Glu  | Asn  | Ile  | Leu  | Thr  | Val  |  |  |
| 1425 |      |      |      |      | 1430 |      |      |      |      | 1435 |      |      |      |      | 1440 |  |  |
| Leu  | Leu  | Lys  | Thr  | Ala  | Leu  | Leu  | Ile  | Val  | Ser  | Gly  | Ile  | Phe  | Pro  | Tyr  | Ser  |  |  |
|      |      |      | 1445 |      |      |      |      |      | 1450 |      |      |      |      | 1455 |      |  |  |
| Ile  | Pro  | Ala  | Thr  | Leu  | Leu  | Val  | Trp  | His  | Thr  | Trp  | Gln  | Lys  | Gln  | Thr  | Gln  |  |  |
|      |      | 1460 |      |      |      |      |      | 1465 |      |      |      |      | 1470 |      |      |  |  |
| Arg  | Ser  | Gly  | Val  | Leu  | Trp  | Asp  | Val  | Pro  | Ser  | Pro  | Pro  | Glu  | Thr  | Gln  | Lys  |  |  |
|      | 1475 |      |      |      |      |      | 1480 |      |      |      |      | 1485 |      |      |      |  |  |
| Ala  | Glu  | Leu  | Glu  | Glu  | Gly  | Val  | Tyr  | Arg  | Ile  | Lys  | Gln  | Gln  | Gly  | Ile  | Phe  |  |  |
|      | 1490 |      |      |      |      | 1495 |      |      |      |      | 1500 |      |      |      |      |  |  |
| Gly  | Lys  | Thr  | Gln  | Val  | Gly  | Val  | Gly  | Val  | Gln  | Lys  | Glu  | Gly  | Val  | Phe  | His  |  |  |
| 1505 |      |      |      |      | 1510 |      |      |      |      | 1515 |      |      |      |      | 1520 |  |  |
| Thr  | Met  | Trp  | His  | Val  | Thr  | Arg  | Gly  | Ala  | Val  | Leu  | Thr  | His  | Asn  | Gly  | Lys  |  |  |
|      |      |      | 1525 |      |      |      |      | 1530 |      |      |      |      | 1535 |      |      |  |  |
| Arg  | Leu  | Glu  | Pro  | Asn  | Trp  | Ala  | Ser  | Val  | Lys  | Lys  | Asp  | Leu  | Ile  | Ser  | Tyr  |  |  |
|      |      | 1540 |      |      |      |      |      | 1545 |      |      |      |      | 1550 |      |      |  |  |
| Gly  | Gly  | Gly  | Trp  | Lys  | Leu  | Ser  | Ala  | Gln  | Trp  | Gln  | Lys  | Gly  | Glu  | Glu  | Val  |  |  |
|      | 1555 |      |      |      |      |      | 1560 |      |      |      |      | 1565 |      |      |      |  |  |
| Gln  | Val  | Ile  | Ala  | Val  | Glu  | Pro  | Gly  | Lys  | Asn  | Pro  | Lys  | Asn  | Phe  | Gln  | Thr  |  |  |
|      | 1570 |      |      |      |      | 1575 |      |      |      |      | 1580 |      |      |      |      |  |  |
| Met  | Pro  | Gly  | Ile  | Phe  | Gln  | Thr  | Thr  | Thr  | Gly  | Glu  | Ile  | Gly  | Ala  | Ile  | Ala  |  |  |
| 1585 |      |      |      |      | 1590 |      |      |      |      | 1595 |      |      |      |      | 1600 |  |  |
| Leu  | Asp  | Phe  | Lys  | Pro  | Gly  | Thr  | Ser  | Gly  | Ser  | Pro  | Ile  | Ile  | Asn  | Arg  | Glu  |  |  |
|      |      |      | 1605 |      |      |      |      | 1610 |      |      |      |      |      | 1615 |      |  |  |
| Gly  | Lys  | Val  | Leu  | Gly  | Leu  | Tyr  | Gly  | Asn  | Gly  | Val  | Val  | Thr  | Lys  | Asn  | Gly  |  |  |
|      |      | 1620 |      |      |      |      |      | 1625 |      |      |      |      | 1630 |      |      |  |  |
| Gly  | Tyr  | Val  | Ser  | Gly  | Ile  | Ala  | Gln  | Thr  | Asn  | Ala  | Glu  | Pro  | Asp  | Gly  | Pro  |  |  |

|      |      |      |      |      |      |     |      |      |      |      |      |      |      |      |      |  |
|------|------|------|------|------|------|-----|------|------|------|------|------|------|------|------|------|--|
|      |      | 1635 |      |      |      |     | 1640 |      |      |      |      | 1645 |      |      |      |  |
| Thr  | Pro  | Glu  | Leu  | Glu  | Glu  | Glu | Met  | Phe  | Lys  | Lys  | Arg  | Asn  | Leu  | Thr  | Ile  |  |
|      | 1650 |      |      |      |      |     | 1655 |      |      |      |      | 1660 |      |      |      |  |
| Met  | Asp  | Leu  | His  | Pro  | Gly  | Ser | Gly  | Lys  | Thr  | Arg  | Lys  | Tyr  | Leu  | Pro  | Ala  |  |
| 1665 |      |      |      |      | 1670 |     |      |      |      | 1675 |      |      |      |      | 1680 |  |
| Ile  | Val  | Arg  | Glu  | Ala  | Ile  | Lys | Arg  | Arg  | Leu  | Arg  | Thr  | Leu  | Ile  | Leu  | Ala  |  |
|      |      |      |      | 1685 |      |     |      |      | 1690 |      |      |      |      |      | 1695 |  |
| Pro  | Thr  | Arg  | Val  | Val  | Ala  | Ala | Glu  | Met  | Glu  | Glu  | Ala  | Leu  | Lys  | Gly  | Leu  |  |
|      |      |      | 1700 |      |      |     |      | 1705 |      |      |      |      | 1710 |      |      |  |
| Pro  | Ile  | Arg  | Tyr  | Gln  | Thr  | Thr | Ala  | Thr  | Lys  | Ser  | Glu  | His  | Thr  | Gly  | Arg  |  |
|      | 1715 |      |      |      |      |     | 1720 |      |      |      |      | 1725 |      |      |      |  |
| Glu  | Ile  | Val  | Asp  | Leu  | Met  | Cys | His  | Ala  | Thr  | Phe  | Thr  | Met  | Arg  | Leu  | Leu  |  |
|      | 1730 |      |      |      | 1735 |     |      |      |      |      | 1740 |      |      |      |      |  |
| Ser  | Pro  | Val  | Arg  | Val  | Pro  | Asn | Tyr  | Asn  | Leu  | Ile  | Ile  | Met  | Asp  | Glu  | Ala  |  |
| 1745 |      |      |      |      | 1750 |     |      |      | 1755 |      |      |      |      |      | 1760 |  |
| His  | Phe  | Thr  | Asp  | Pro  | Ala  | Ser | Ile  | Ala  | Ala  | Arg  | Gly  | Tyr  | Ile  | Ser  | Thr  |  |
|      |      |      | 1765 |      |      |     |      |      | 1770 |      |      |      |      | 1775 |      |  |
| Arg  | Val  | Gly  | Met  | Gly  | Glu  | Ala | Ala  | Ala  | Ile  | Phe  | Met  | Thr  | Ala  | Thr  | Pro  |  |
|      |      |      | 1780 |      |      |     | 1785 |      |      |      |      |      | 1790 |      |      |  |
| Pro  | Gly  | Thr  | Ala  | Asp  | Ala  | Phe | Pro  | Gln  | Ser  | Asn  | Ala  | Pro  | Ile  | Gln  | Asp  |  |
|      | 1795 |      |      |      |      |     | 1800 |      |      |      |      | 1805 |      |      |      |  |
| Glu  | Glu  | Arg  | Asp  | Ile  | Pro  | Glu | Arg  | Ser  | Trp  | Asn  | Ser  | Gly  | Asn  | Glu  | Trp  |  |
|      | 1810 |      |      |      | 1815 |     |      |      |      | 1820 |      |      |      |      |      |  |
| Ile  | Thr  | Asp  | Phe  | Ala  | Gly  | Lys | Thr  | Val  | Trp  | Phe  | Val  | Pro  | Ser  | Ile  | Lys  |  |
| 1825 |      |      |      |      | 1830 |     |      |      |      | 1835 |      |      |      |      | 1840 |  |
| Ala  | Gly  | Asn  | Asp  | Ile  | Ala  | Asn | Cys  | Leu  | Arg  | Lys  | Asn  | Gly  | Lys  | Lys  | Val  |  |
|      |      |      | 1845 |      |      |     |      |      | 1850 |      |      |      |      | 1855 |      |  |
| Ile  | Gln  | Leu  | Ser  | Arg  | Lys  | Thr | Phe  | Asp  | Thr  | Glu  | Tyr  | Gln  | Lys  | Thr  | Lys  |  |
|      |      |      | 1860 |      |      |     |      | 1865 |      |      |      |      | 1870 |      |      |  |
| Leu  | Asn  | Asp  | Trp  | Asp  | Phe  | Val | Val  | Thr  | Thr  | Asp  | Ile  | Ser  | Glu  | Met  | Gly  |  |
|      | 1875 |      |      |      |      |     | 1880 |      |      |      |      | 1885 |      |      |      |  |
| Ala  | Asn  | Phe  | Lys  | Ala  | Asp  | Arg | Val  | Ile  | Asp  | Pro  | Arg  | Arg  | Cys  | Leu  | Lys  |  |
|      | 1890 |      |      |      | 1895 |     |      |      |      |      | 1900 |      |      |      |      |  |
| Pro  | Val  | Ile  | Leu  | Thr  | Asp  | Gly | Pro  | Glu  | Arg  | Val  | Ile  | Leu  | Ala  | Gly  | Pro  |  |
| 1905 |      |      |      |      | 1910 |     |      |      |      | 1915 |      |      |      |      | 1920 |  |
| Met  | Pro  | Val  | Thr  | Val  | Ala  | Ser | Ala  | Ala  | Gln  | Arg  | Arg  | Gly  | Arg  | Val  | Gly  |  |
|      |      |      | 1925 |      |      |     |      |      | 1930 |      |      |      |      | 1935 |      |  |
| Arg  | Asn  | Pro  | Gln  | Lys  | Glu  | Asn | Asp  | Gln  | Tyr  | Ile  | Phe  | Met  | Gly  | Gln  | Pro  |  |
|      |      |      | 1940 |      |      |     |      | 1945 |      |      |      |      | 1950 |      |      |  |
| Leu  | Asn  | Asn  | Asp  | Glu  | Asp  | His | Ala  | His  | Trp  | Thr  | Glu  | Ala  | Lys  | Met  | Leu  |  |
|      | 1955 |      |      |      |      |     | 1960 |      |      |      |      | 1965 |      |      |      |  |
| Leu  | Asp  | Asn  | Ile  | Asn  | Thr  | Pro | Glu  | Gly  | Ile  | Ile  | Pro  |      |      |      |      |  |

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

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 gagg 64

**WHAT IS CLAIMED IS:**

1. A nucleic acid chimera comprising a first nucleotide sequence encoding a premembrane/ membrane (prM) protein and an envelope (E) protein from a West Nile virus and a second nucleotide sequence encoding a capsid (C) protein and nonstructural proteins from a wild-type strain of dengue type 4 virus, wherein the dengue type 4 virus is attenuated by a deletion of 30 nucleotides from the 3' untranslated region of the dengue type 4 genome corresponding to the TL2 stem-loop structure of nucleotides 10478-10507.

2. A nucleic acid chimera comprising a first nucleotide sequence encoding a premembrane/ membrane (prM) protein and an envelope (E) protein from a West Nile virus and a second nucleotide sequence encoding a capsid (C) protein and nonstructural proteins from a wild-type strain of dengue type 4 virus, wherein the dengue type 4 virus is attenuated by a deletion of 30 nucleotides from the 3' untranslated region of the dengue type 4 genome corresponding to the TL2 stem-loop structure of nucleotides 10478-10507, wherein a cleavage site is utilized for joining a dengue virus capsid protein and the West Nile virus prM protein, and wherein the West Nile virus prM protein contains aspartic acid (Asp) at a position 3 amino acids downstream of the cleavage site and contains threonine (Thr) at a position 6 amino acids downstream of the cleavage site.

3. The nucleic acid chimera of Claim 1 or 2, wherein the dengue virus is adapted for increased growth in *Vero* cells.

4. A virus chimera comprising one or more than one nucleic acid chimera of any one of Claims 1-3.

5. An immunogenic composition comprising one or more than one nucleic acid chimera of any one of Claims 1-3 or one or more than one virus chimera of Claim 4 and a pharmaceutically acceptable carrier.

6. The composition of Claim 5 for use in the induction of an immune response.

7. Use of the composition of Claim 5 for inducing an immune response in a subject.

8. Use of the composition of Claim 5 in the manufacture of a medicament for inducing an immune response in a subject.
9. The use of Claim 7 or 8 wherein the subject is a non-human primate.
10. The use of Claim 7 or 8 wherein the subject is a human.
11. The use of Claim 7 or 8 wherein the subject is a horse or a bird.
12. A vaccine composition comprising one or more than one nucleic acid chimera of any one of Claims 1-3 or one or more than one virus chimera of Claim 4 and a pharmaceutically acceptable carrier.
13. The composition of Claim 12 for use in the prevention of disease caused by West Nile virus.
14. Use of the composition of Claim 12 for preventing disease caused by West Nile virus in a subject.
15. Use of the composition of Claim 12 in the manufacture of a medicament for preventing disease caused by West Nile virus in a subject.
16. The use of Claim 14 or 15 wherein the subject is a non-human primate.
17. The use of Claim 14 or 15 wherein the subject is a human.
18. The use of Claim 14 or 15 wherein the subject is a horse or a bird.

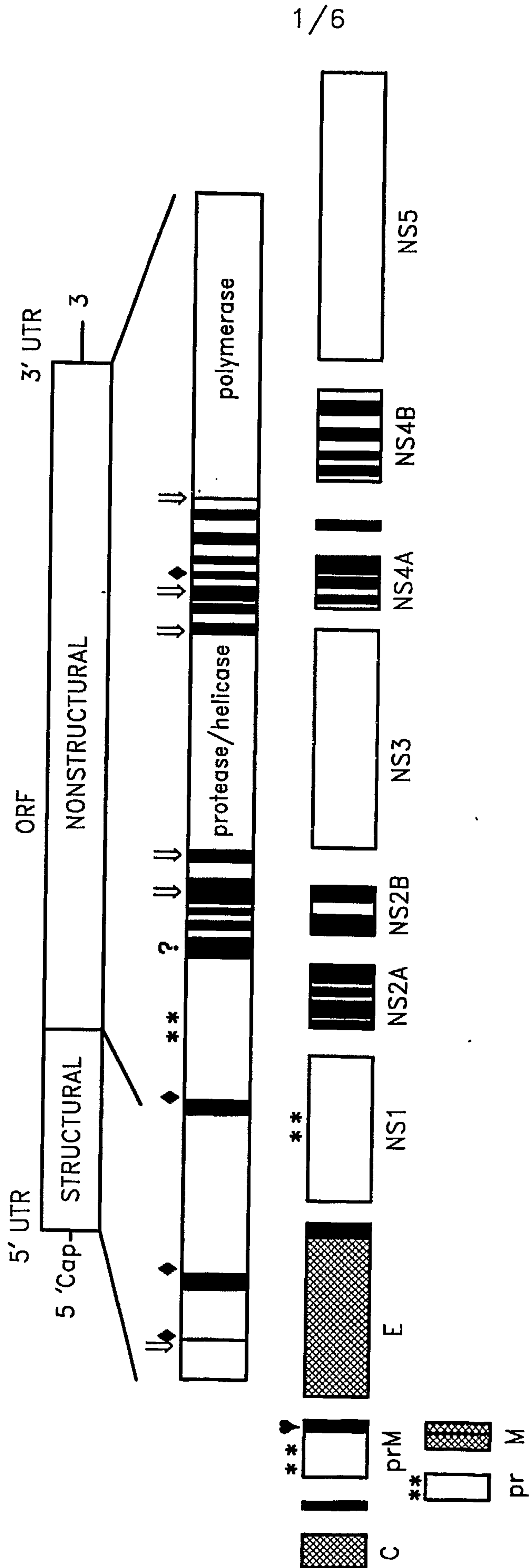
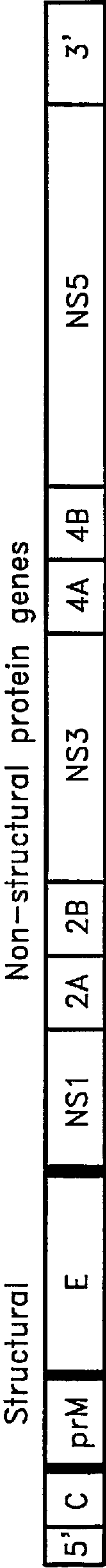


FIG. 1A

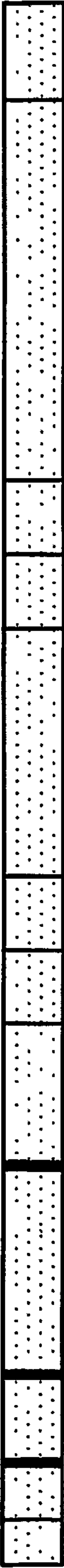
Positive-sense RNA virus:



DEN4



WN



New WN/DEN4 Chimeric Flavivirus:



- Infectious RNA transcribed in vitro from cDNA clones
- Infectious RNA transfected into cell culture (C6/36, Vero)
- Progeny virus recovered and evaluated in susceptible animals

FIG. 1B



4/6

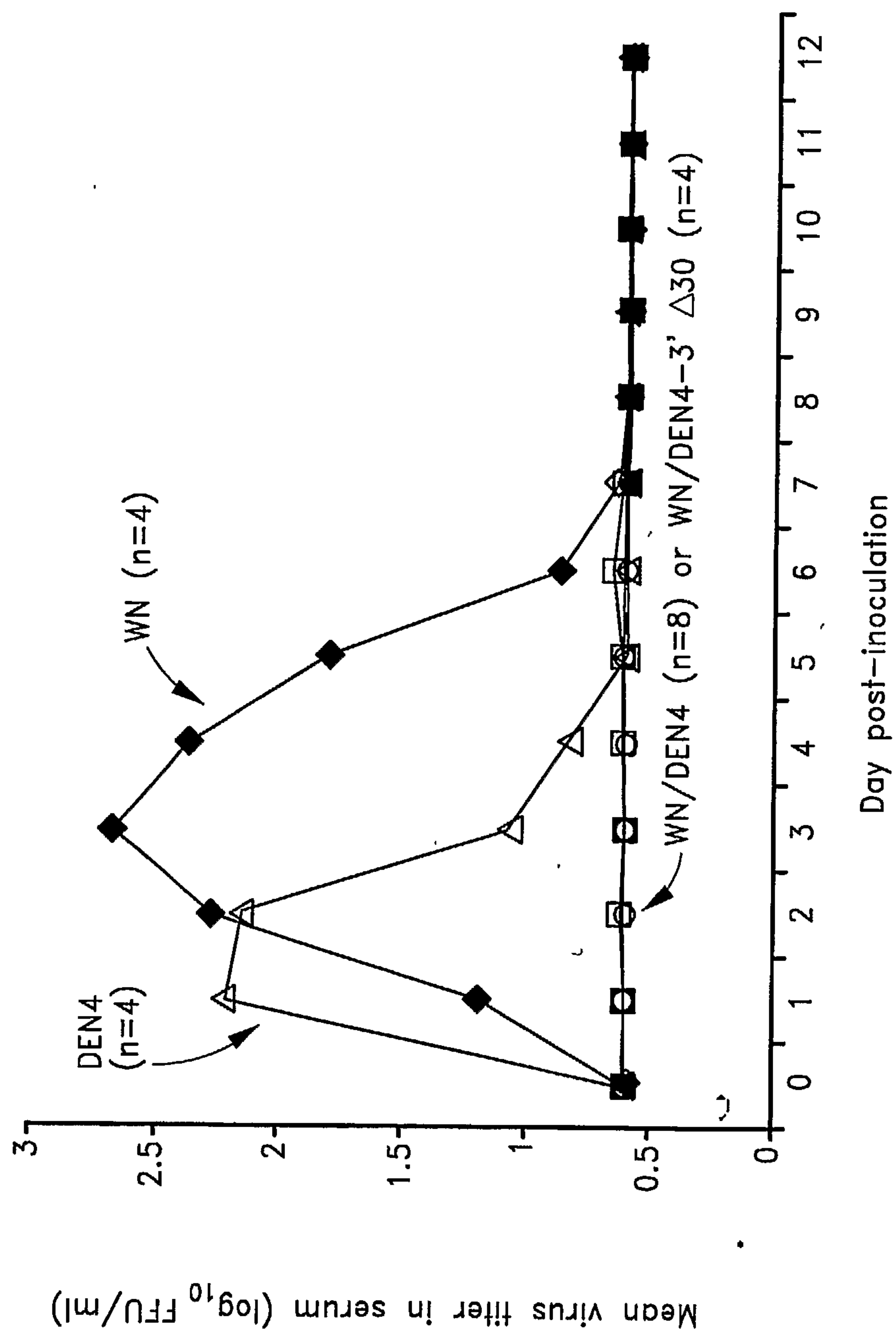


FIG. 3

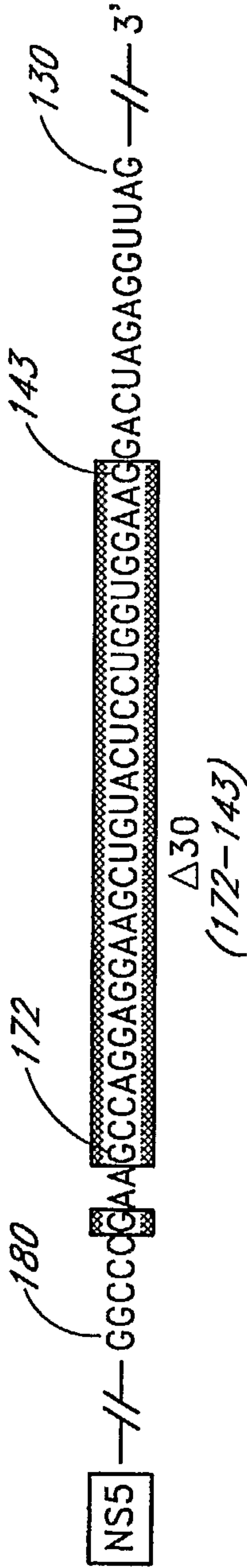


FIG. 4A

|         |                                                     |
|---------|-----------------------------------------------------|
| DEN4    | GGGGCCCGAAGCCAGGAGGAGCUGUACUCCUGGUGGAAGGACUAGA      |
| DEN4Δ30 | GGGGCCC-AA-----GACUAGA                              |
| DEN1    | GGGGCCC-AACACCAGGGAAGCUGUACCCUGGUGGUAAGGACUAGA      |
| DEN1Δ30 | GGGGCCC-AA-----GACUAGA                              |
| DEN1    | GGGGCCC-Aa caccagggGAAGCUGUA cccuggugguAAGGACUAGA   |
| DEN2    | GGGGCCC-Aag gugagauGAAGCUGUAgucucacucuggAAGGACUAGA  |
| DEN3    | GGGGCCCgA gcucugaggGAAGCUGUA ccuc cuu gcaAAGGACUAGA |
| DEN4    | GGGGCCCgAagccaggagGAAGCUGUA cuccuggu gAAGGACUAGA    |

FIG. 4B

Completed Δ30 viruses

Proposed

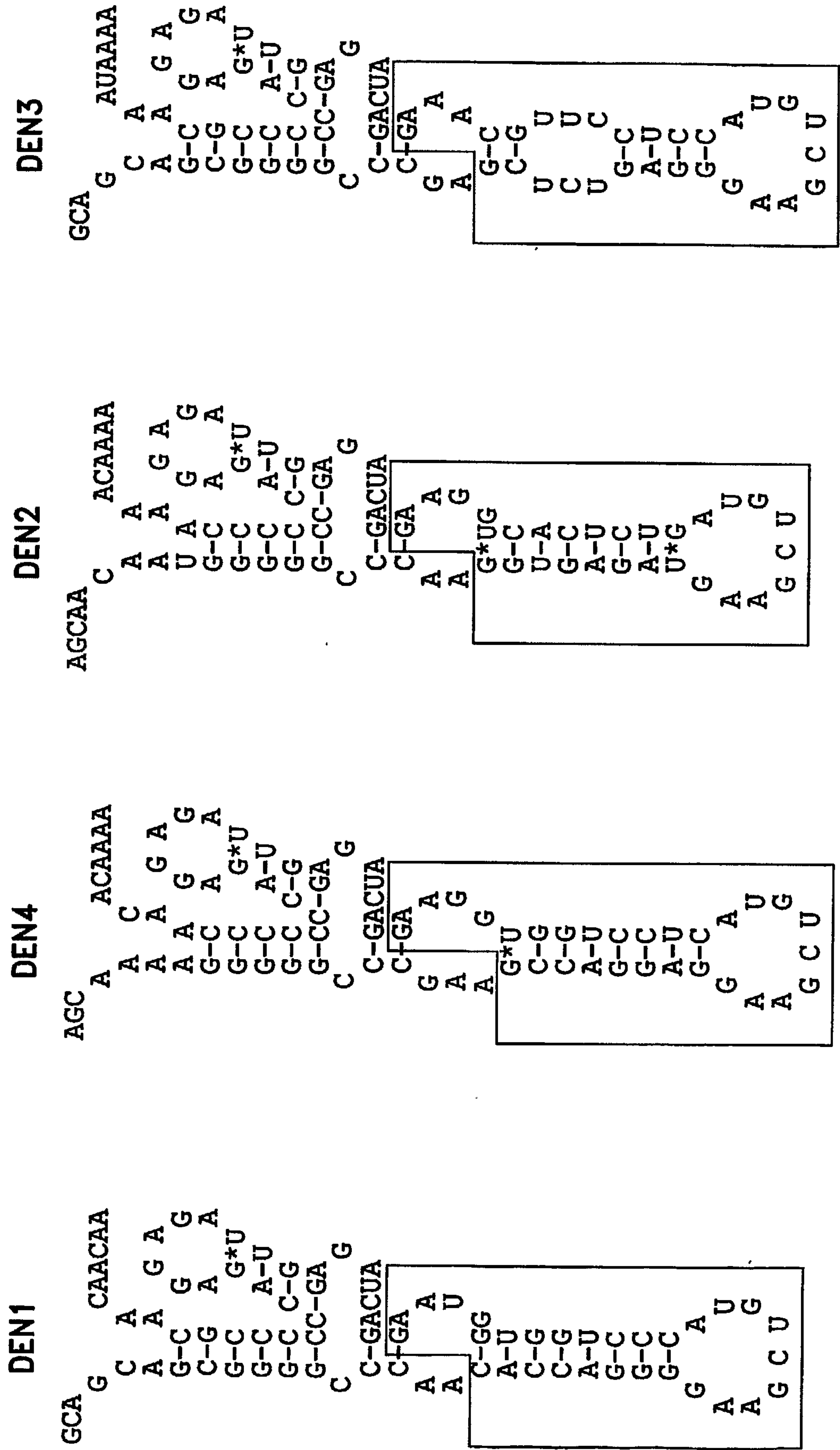
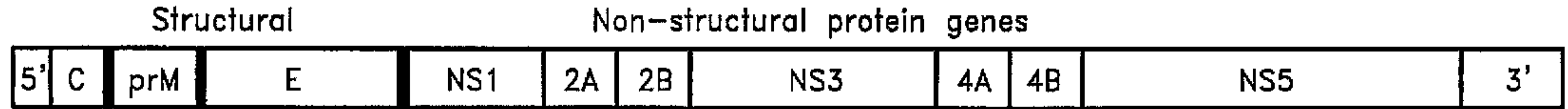


FIG. 4C

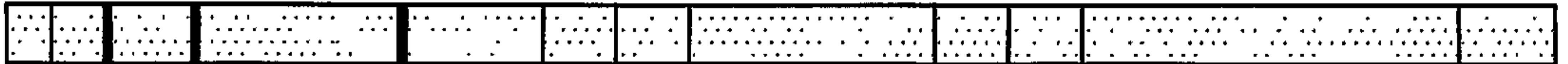
## Positive-sense RNA virus:



**DEN4**



**WN**



## New WN/DEN4 Chimeric Flavivirus:



- Infectious RNA transcribed in vitro from cDNA clones
- Infectious RNA transfected into cell culture (C6/36, Vero)
- Progeny virus recovered and evaluated in susceptible animals