



US 20100278773A1

(19) **United States**(12) **Patent Application Publication**
Chambers et al.(10) **Pub. No.: US 2010/0278773 A1**(43) **Pub. Date: Nov. 4, 2010**(54) **CHIMERIC FLAVIVIRUS VACCINES**(76) Inventors: **Thomas J. Chambers**, St. Louis,
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BOSTON, MA 02110 (US)(21) Appl. No.: **12/592,529**(22) Filed: **Nov. 25, 2009****Related U.S. Application Data**(63) Continuation of application No. 12/423,566, filed on
Apr. 14, 2009, now abandoned, which is a continuation
of application No. 10/701,122, filed on Nov. 4, 2003,
now abandoned, which is a continuation of application
No. 09/452,638, filed on Dec. 1, 1999, now Pat. No.
6,696,281, which is a continuation-in-part of applica-
tion No. 09/121,587, filed on Jul. 23, 1998, now Pat.
No. 6,962,708, which is a continuation-in-part of
application No. PCT/US98/03894, filed on Mar. 2,
1998, which is a continuation-in-part of applicationNo. 09/007,664, filed on Jan. 15, 1998, now aban-
doned, which is a continuation-in-part of application
No. 08/807,445, filed on Feb. 28, 1997, now aban-
doned.**Publication Classification**(51) **Int. Cl.**

<i>A61K 39/12</i>	(2006.01)
<i>A61K 39/29</i>	(2006.01)
<i>A61K 39/00</i>	(2006.01)
<i>A61K 38/21</i>	(2006.01)
<i>A61K 38/20</i>	(2006.01)
<i>A61P 35/00</i>	(2006.01)
<i>A61P 31/14</i>	(2006.01)
<i>C12N 7/01</i>	(2006.01)
<i>C07H 21/04</i>	(2006.01)

(52) **U.S. Cl.** **424/85.2**; 424/199.1; 424/85.7;
435/235.1; 536/23.72

(57)

ABSTRACT

A chimeric live, infectious, attenuated virus containing a yellow fever virus, in which the nucleotide sequence for a prM-E protein is either deleted, truncated, or mutated, so that functional prM-E protein is not expressed, and integrated into the genome of the yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed.

FIG. 1A

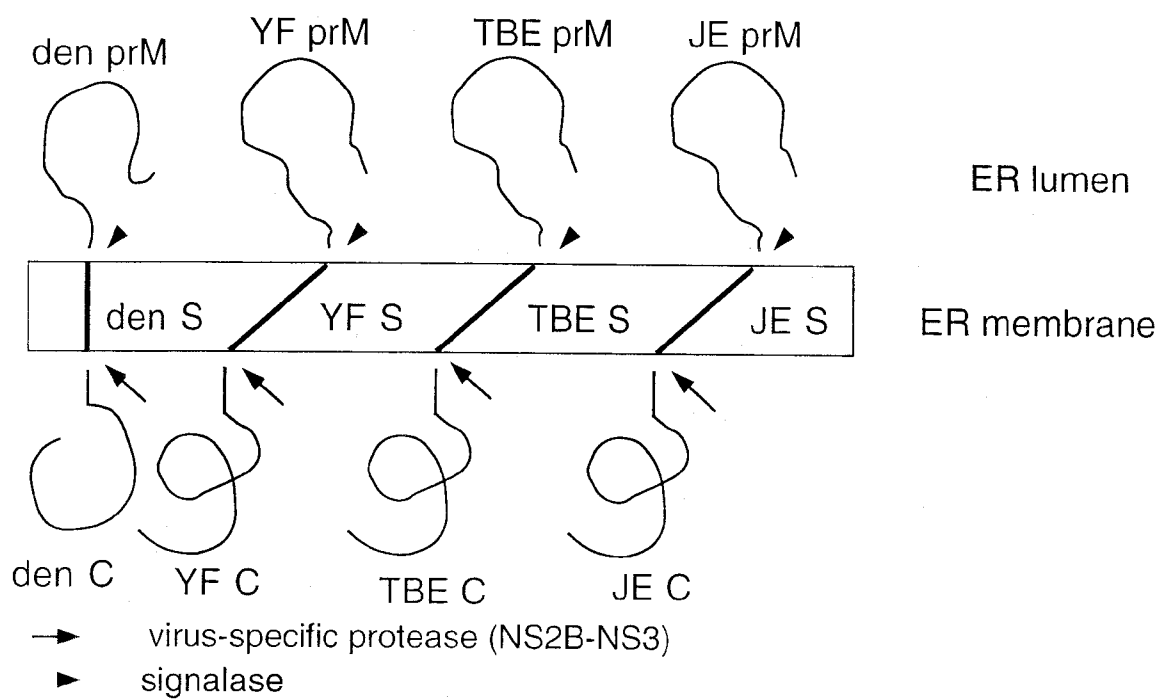
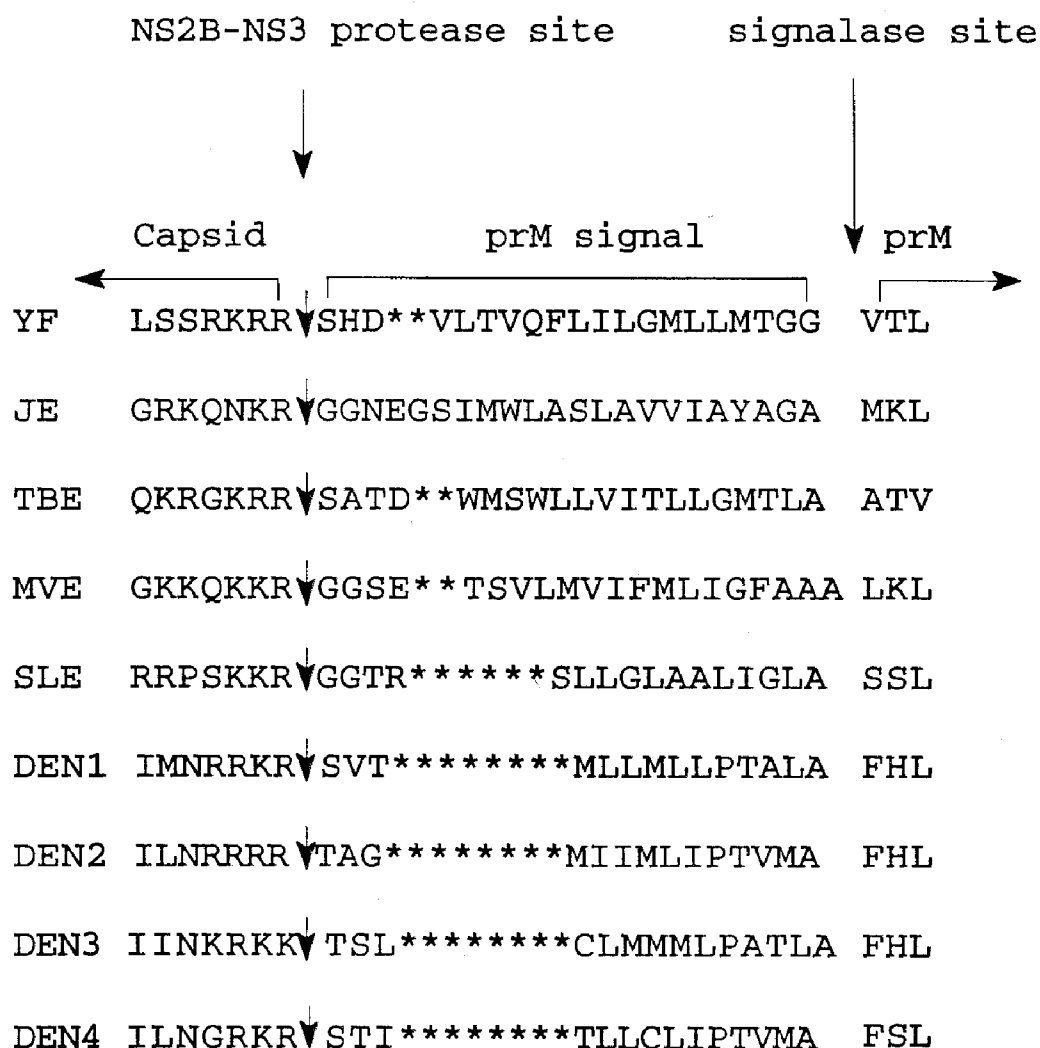


FIG. 1B



Virus names abbreviations

YF = yellow fever

JE = Japanese encephalitis

TBE = Tick-borne encephalitis

MVE = Murray Valley encephalitis

SLE = Saint Louis encephalitis

DEN1-4 = dengue serotypes 1-4

FIG. 2

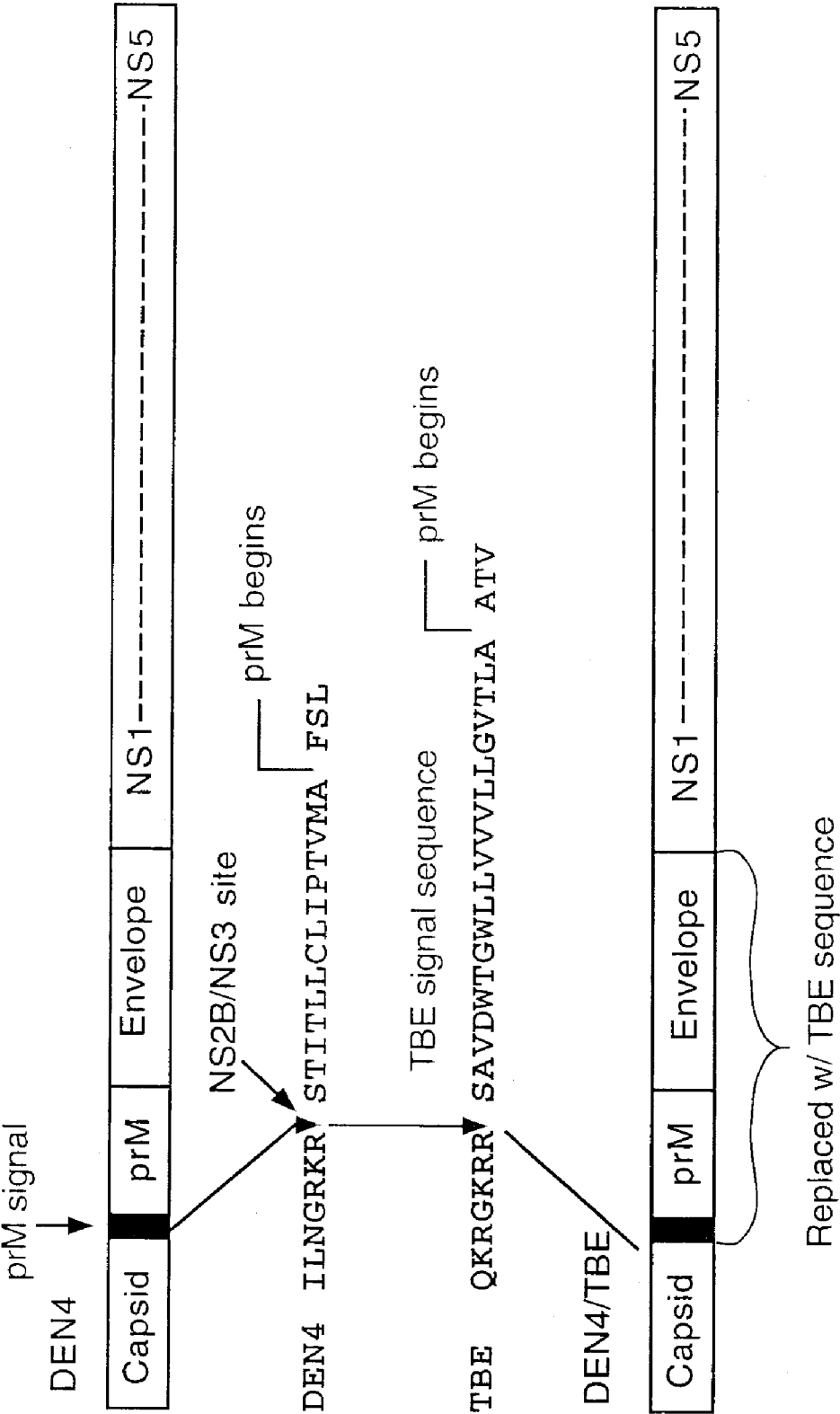


FIG. 3

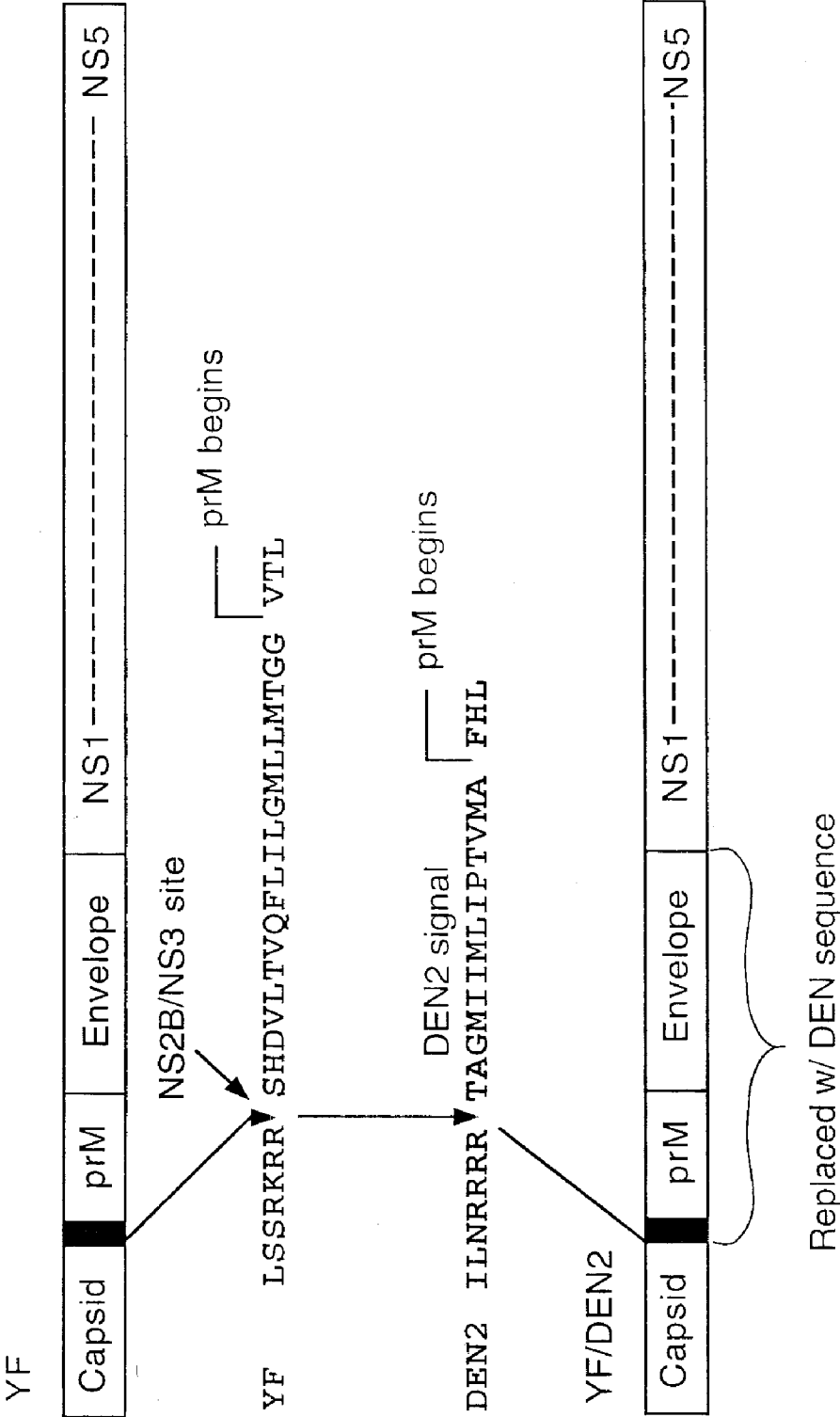


FIG. 4

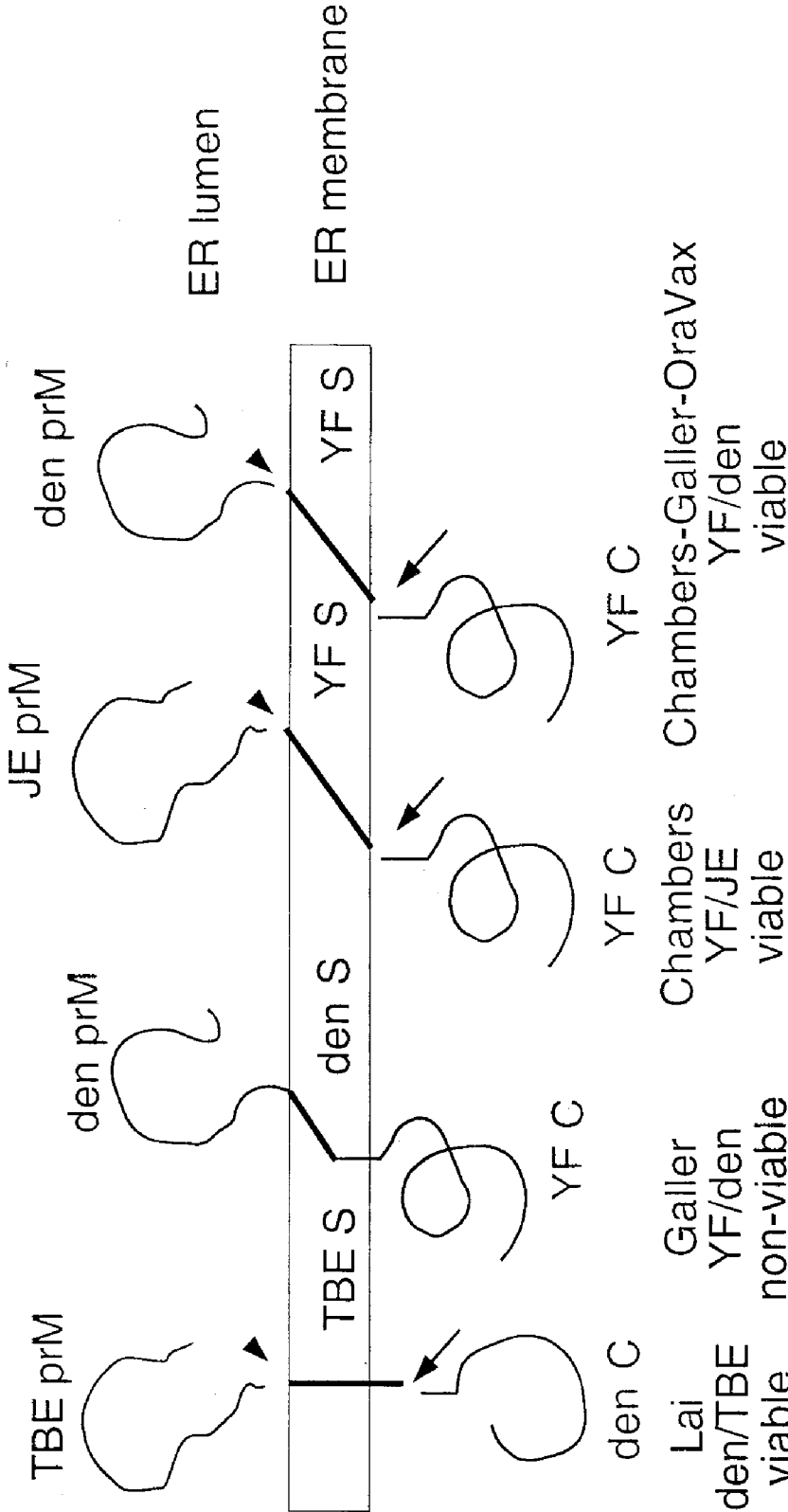


FIG. 5

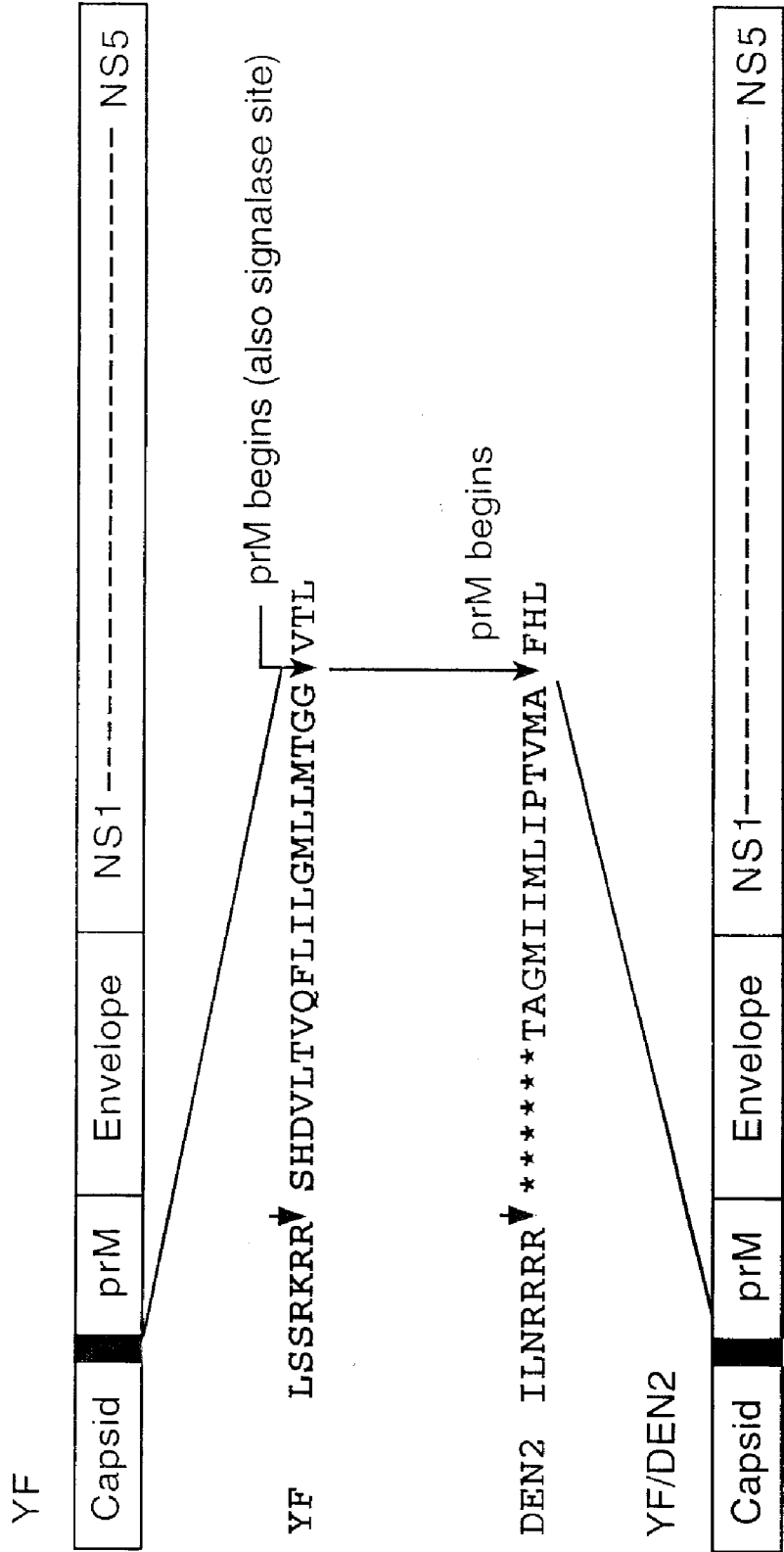
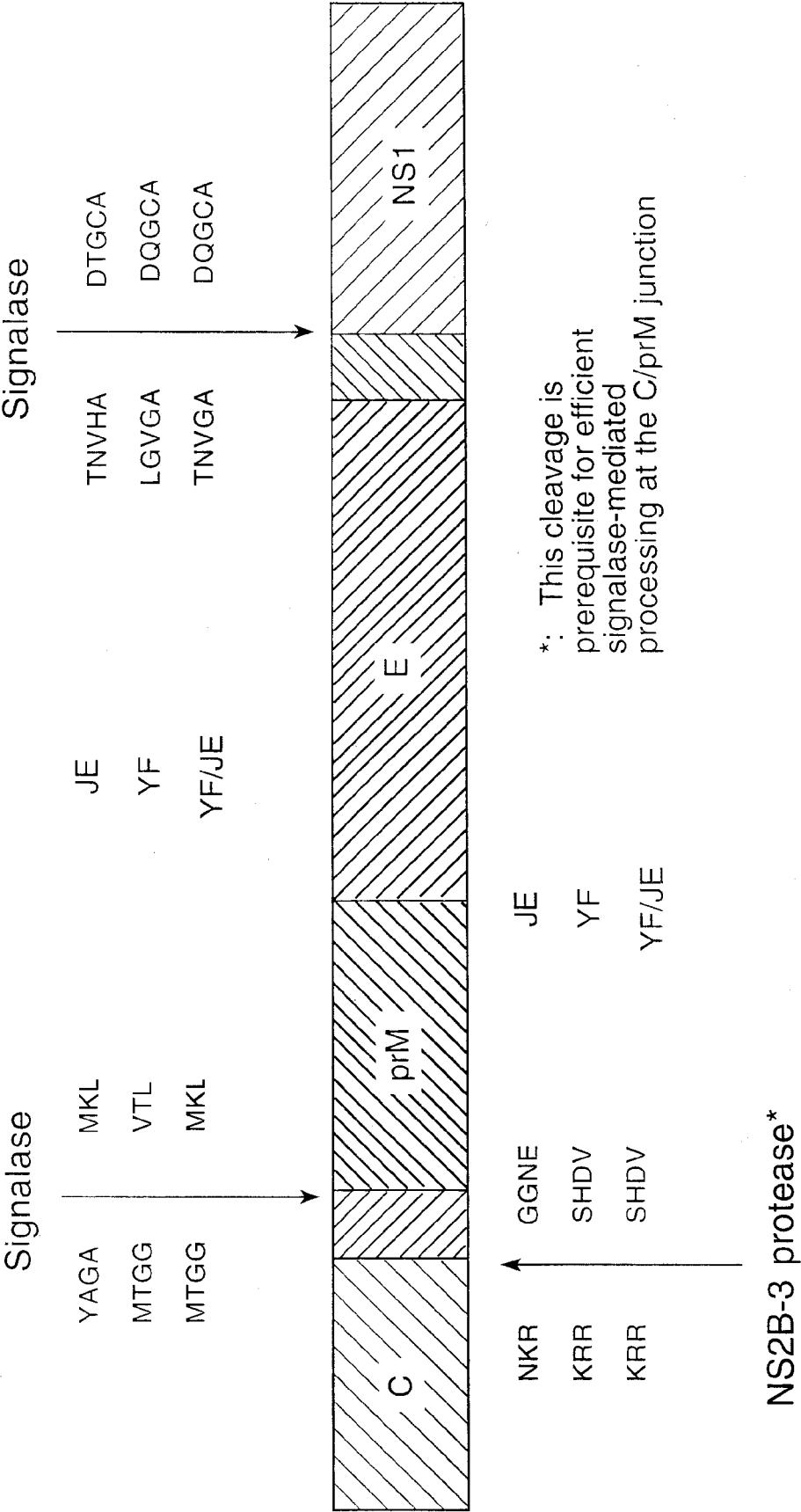


FIG. 6



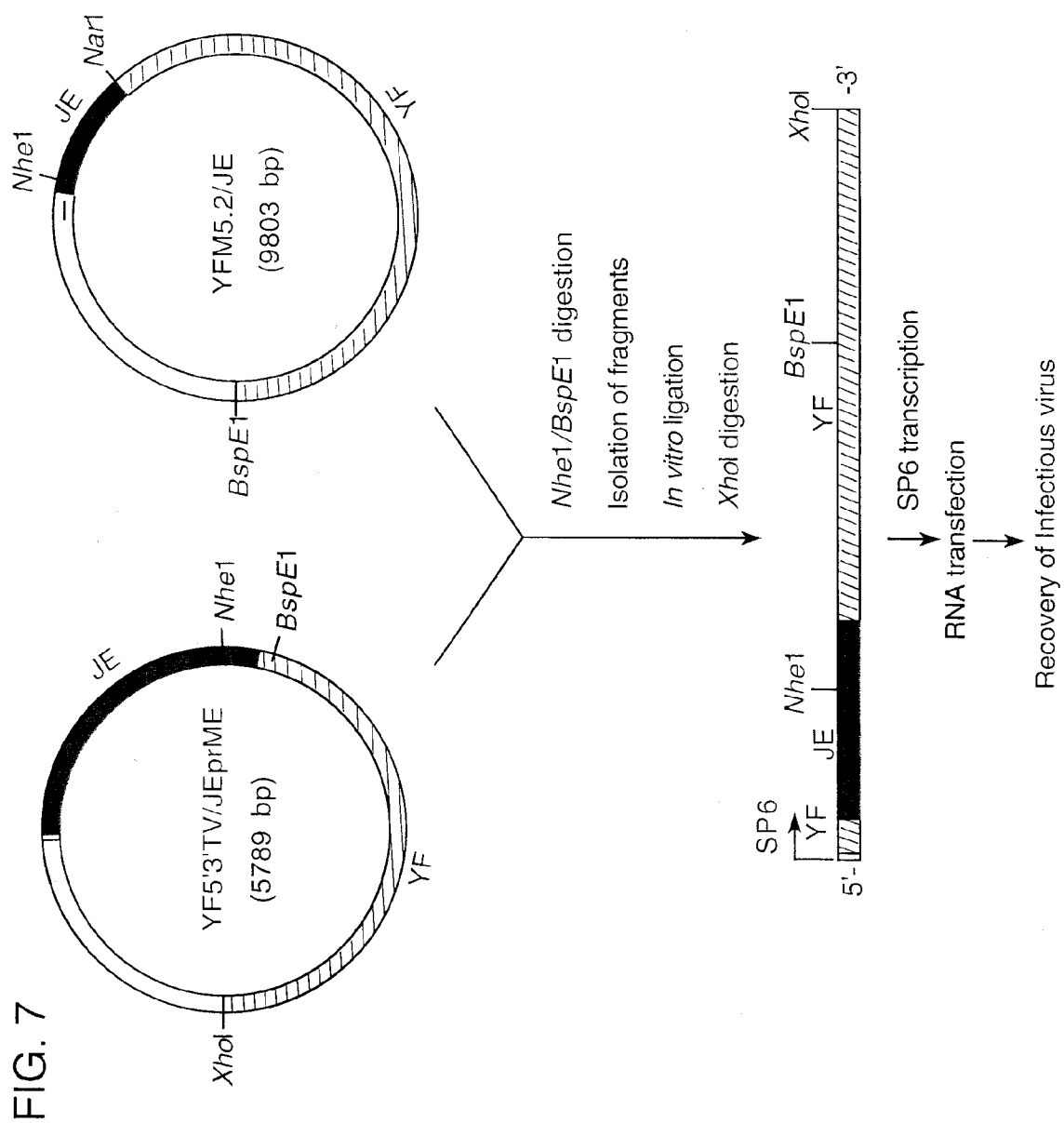


FIG. 8

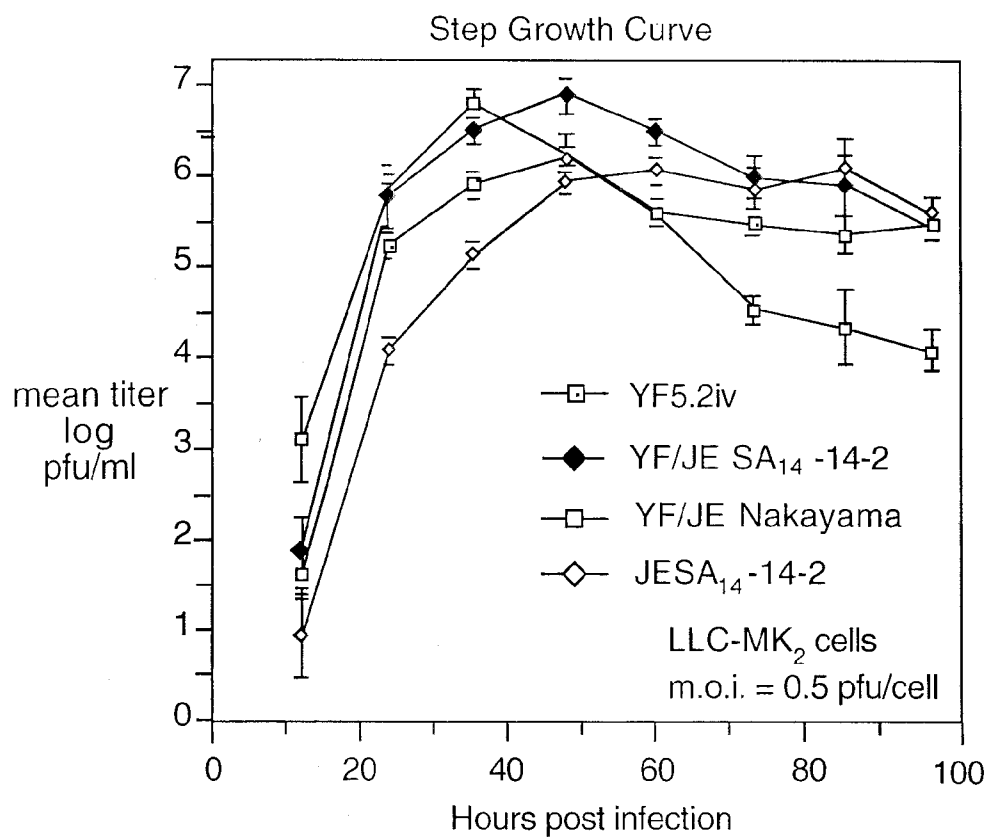
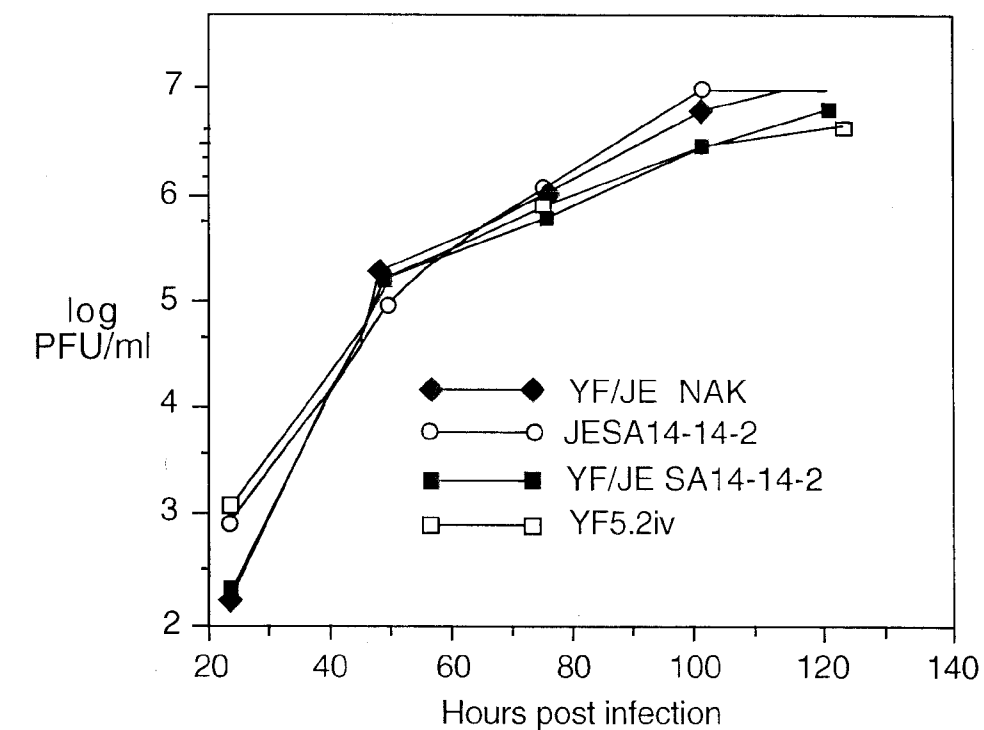


FIG. 9

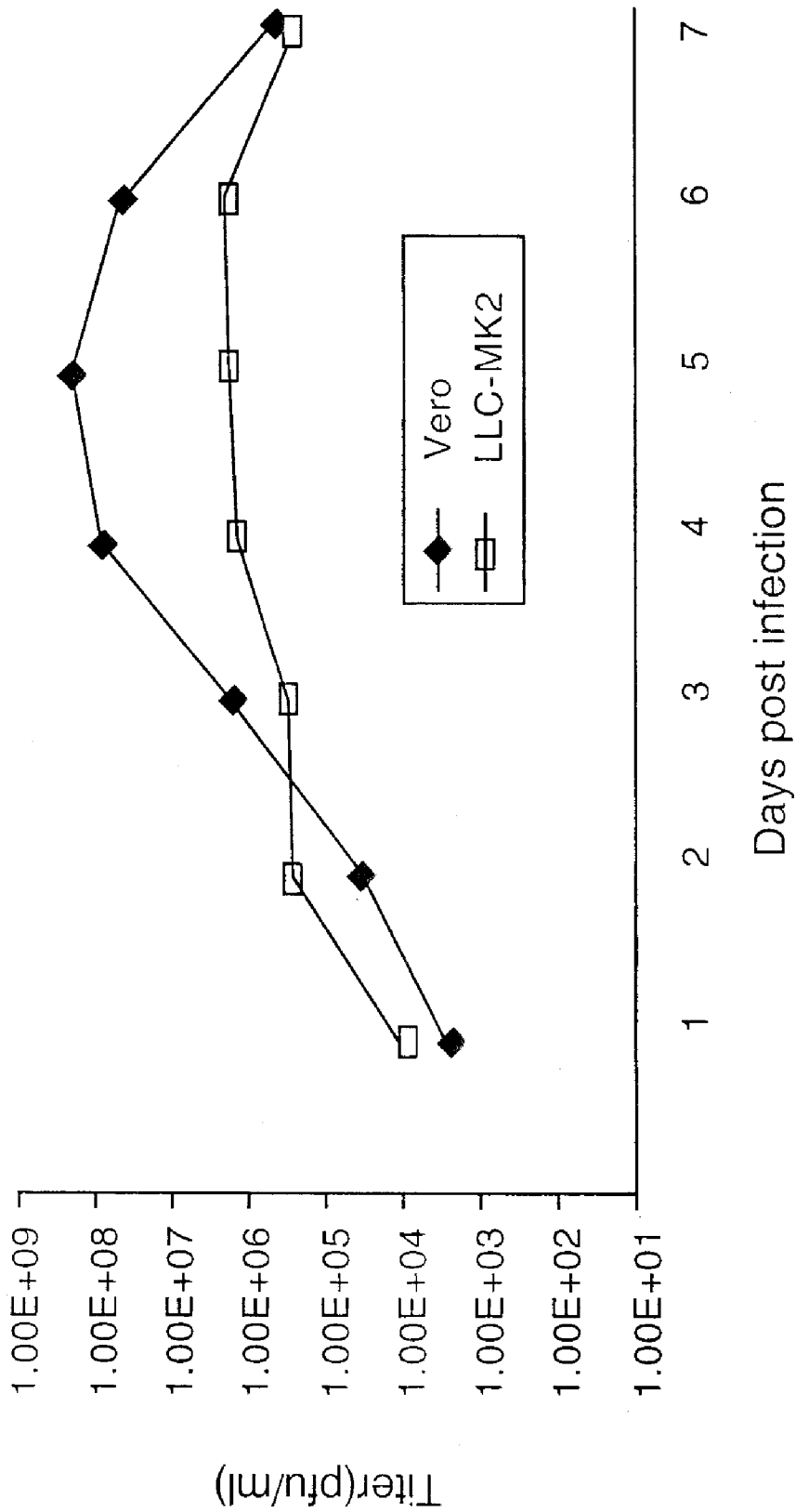


FIG. 10

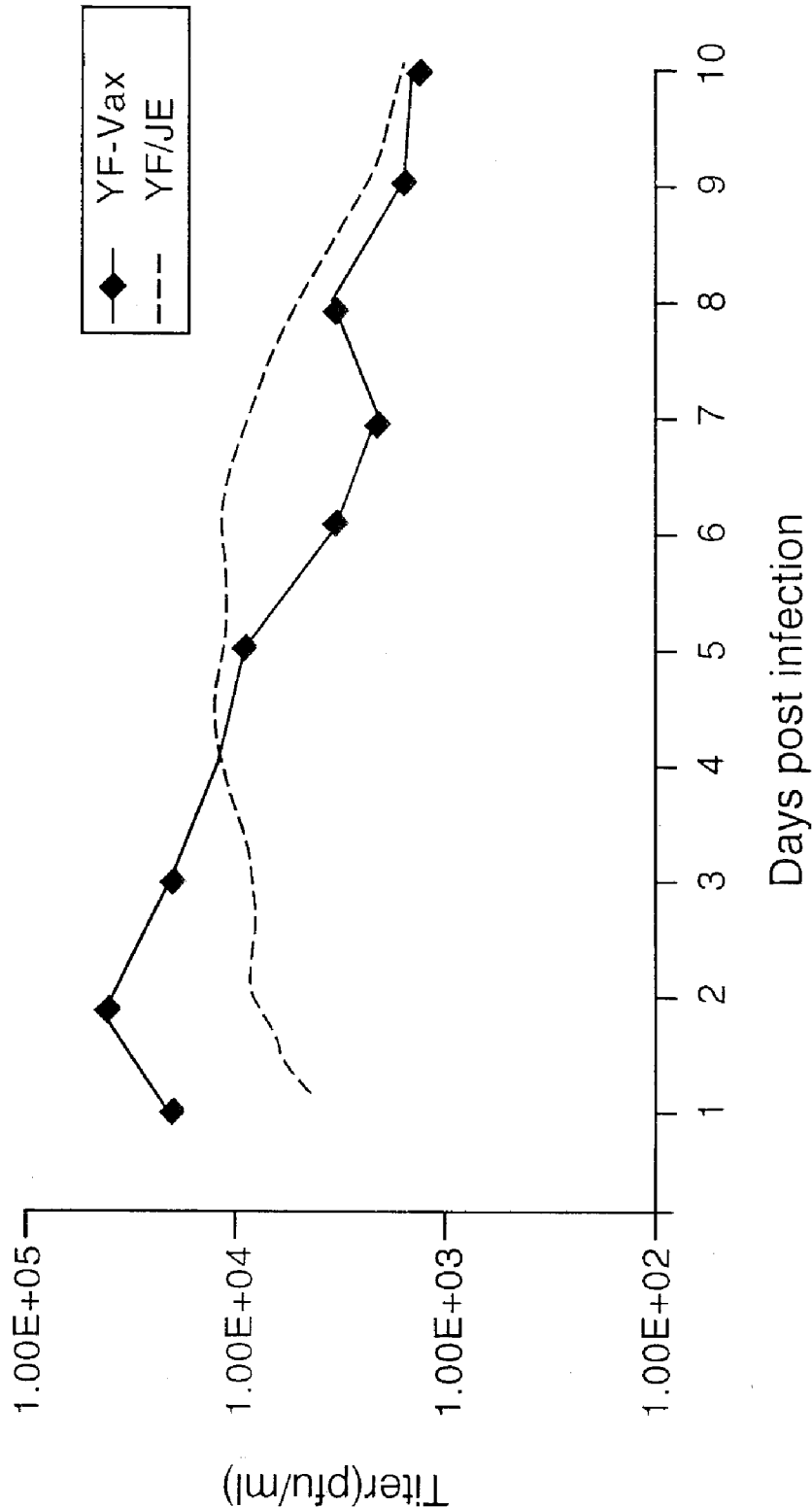


FIG. 11A

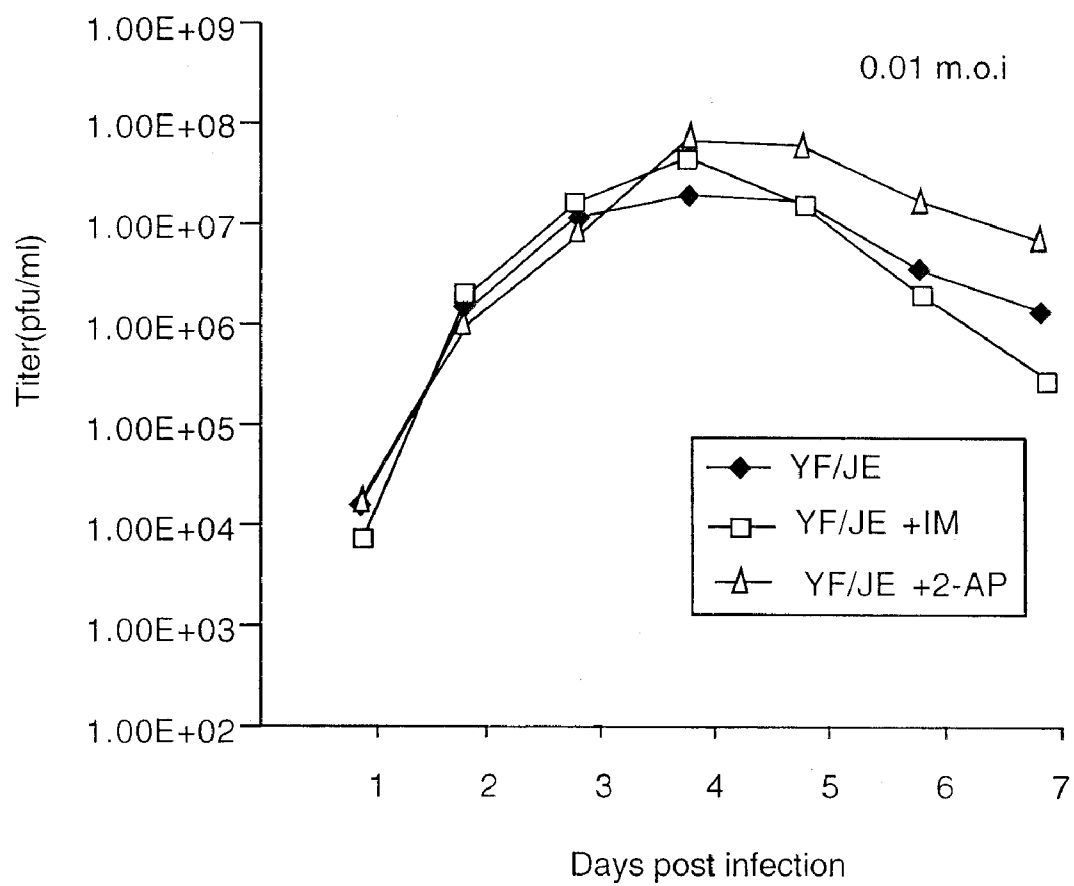


FIG. 11B

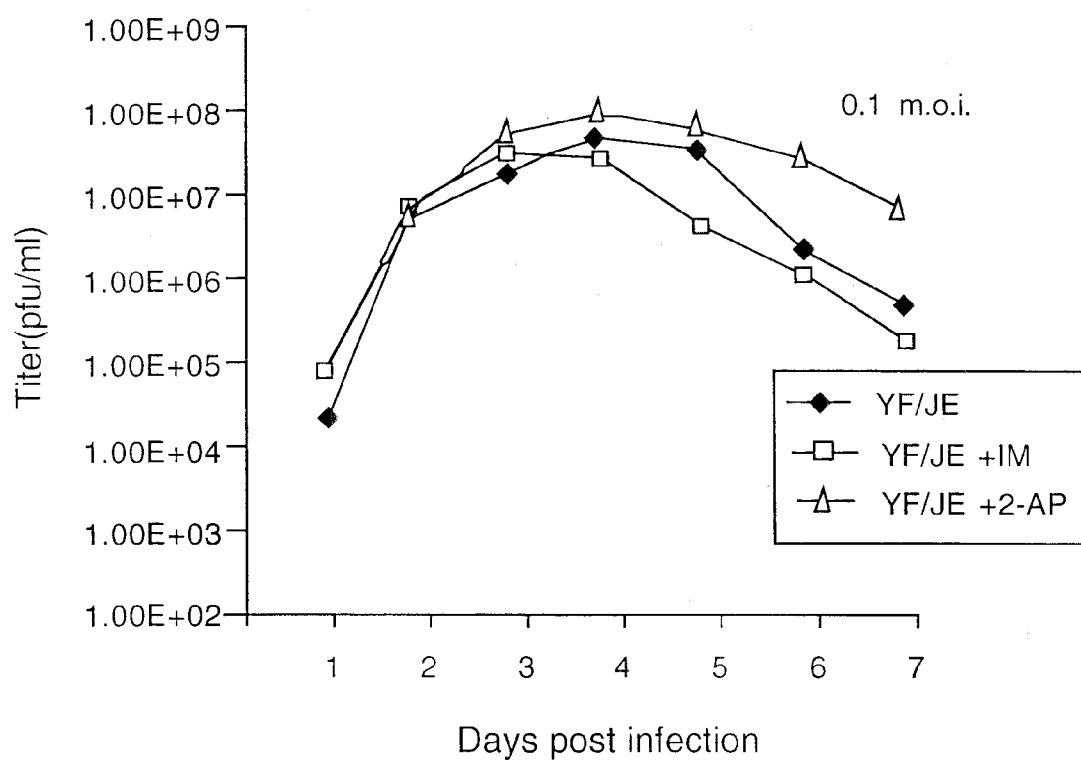
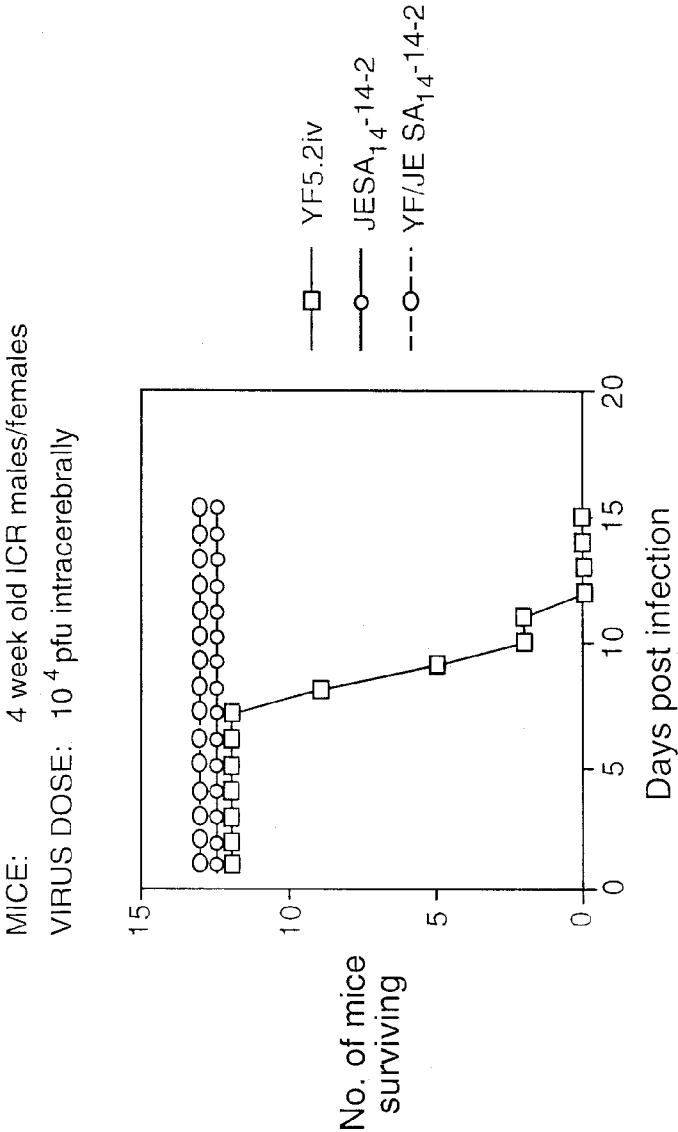


FIG. 12



Virus	Survival	P
YF5.2iv	0/12 (0%)	-
JESA ₁₄ -14-2	12/12 (100%)	<0.001
YF/JE SA ₁₄ -14-2	13/13 (100%)	<0.001

FIG. 13

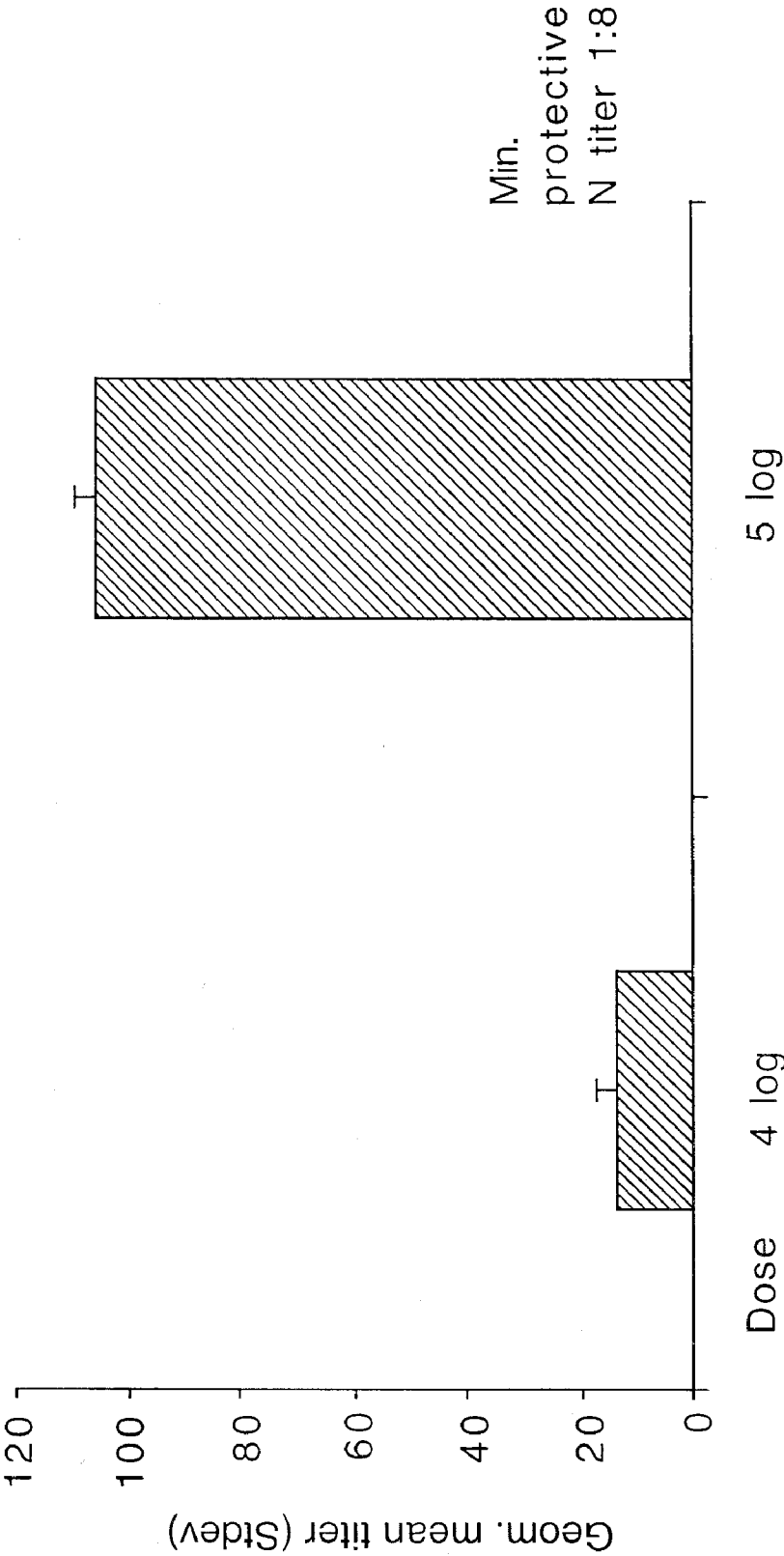


FIG. 14A

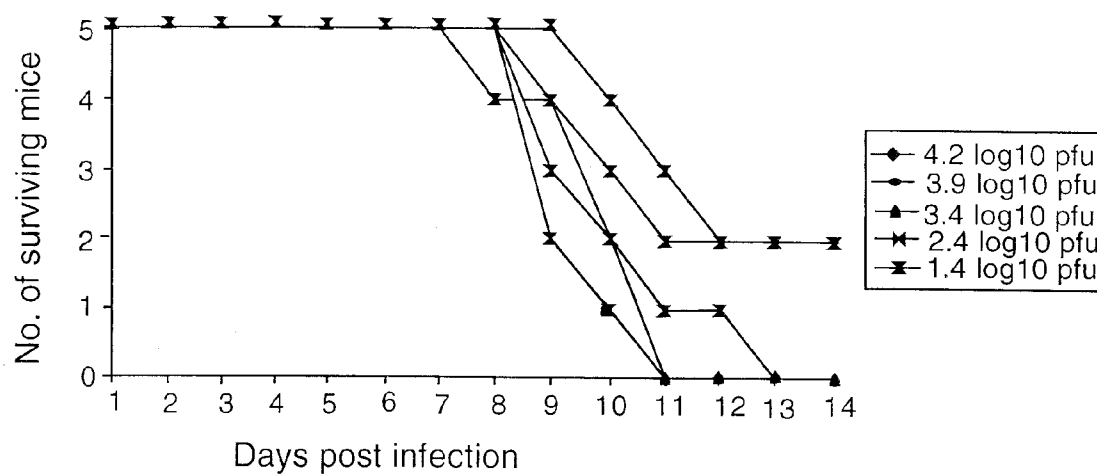


FIG. 14B

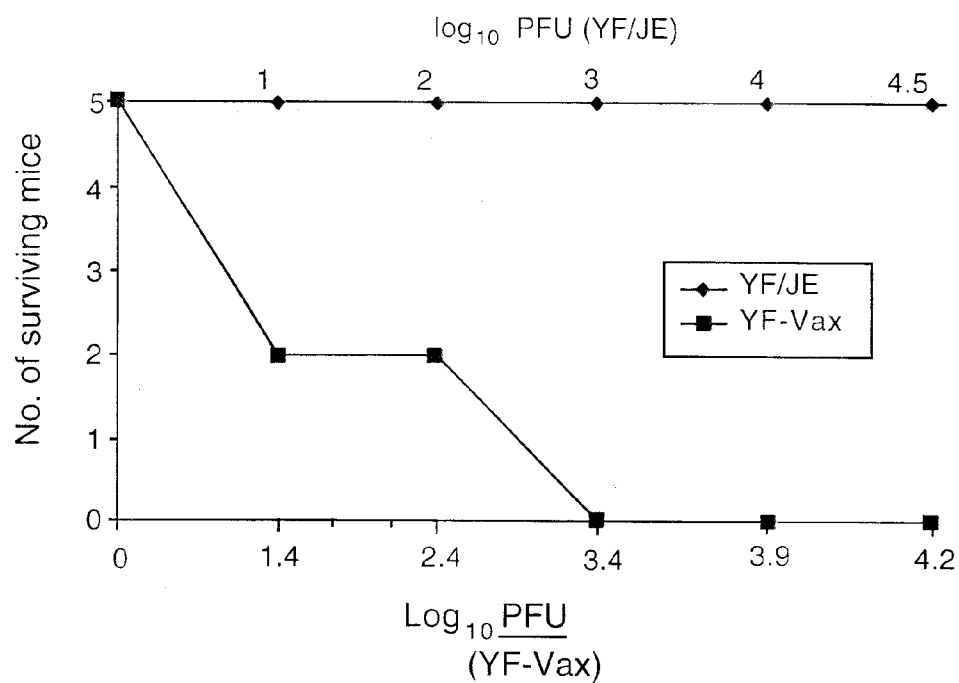


FIG. 15

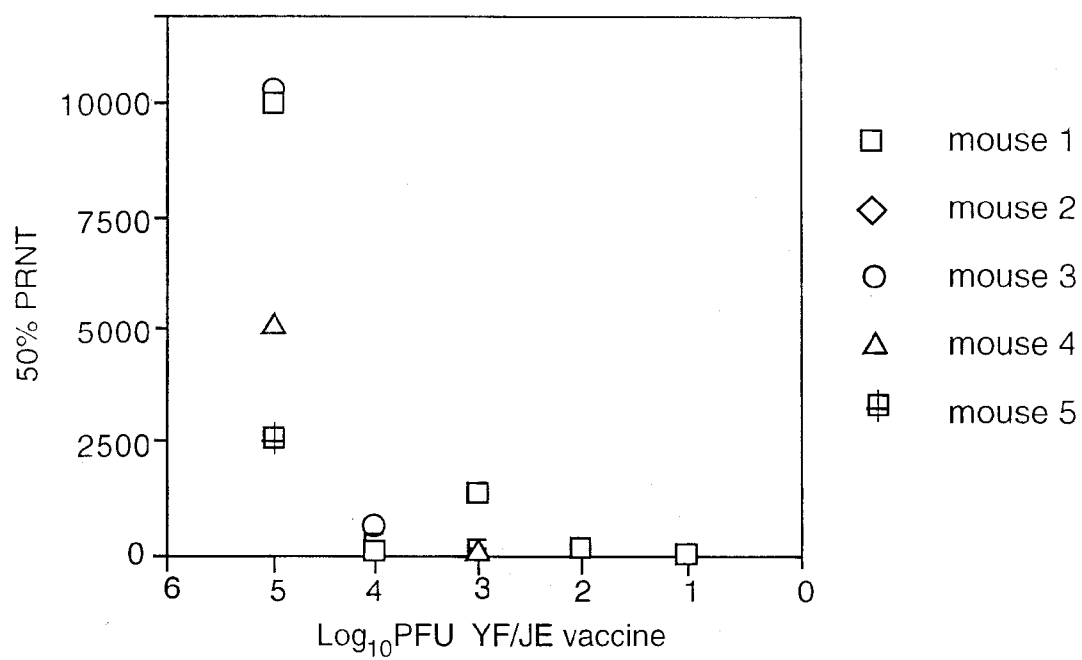
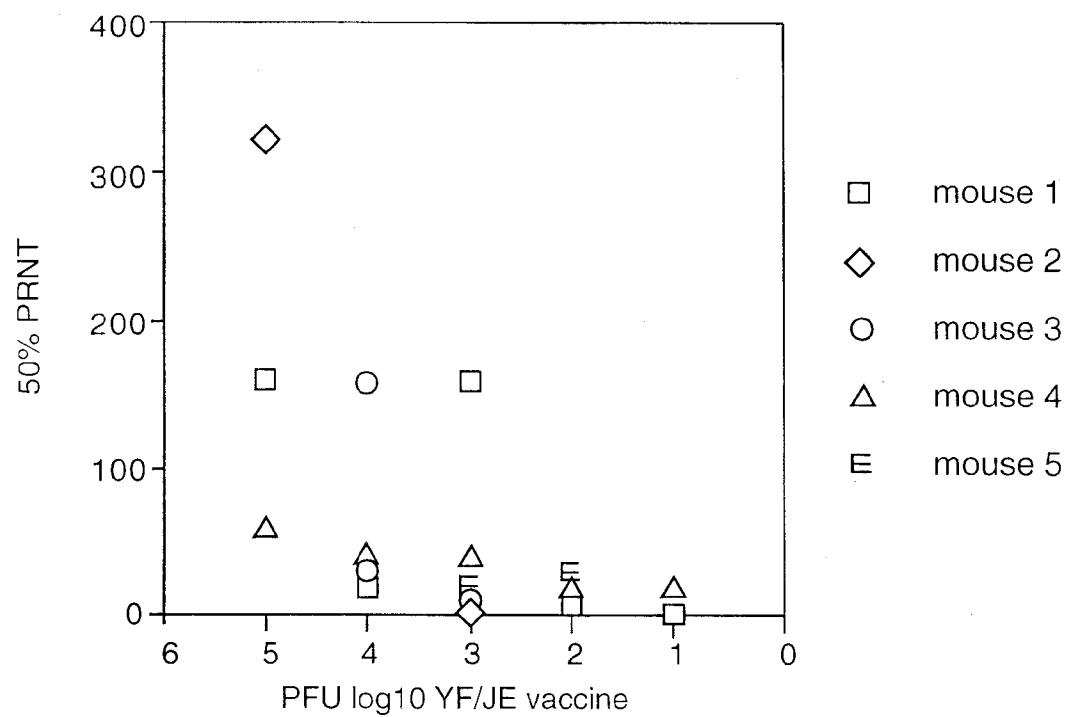


FIG. 16

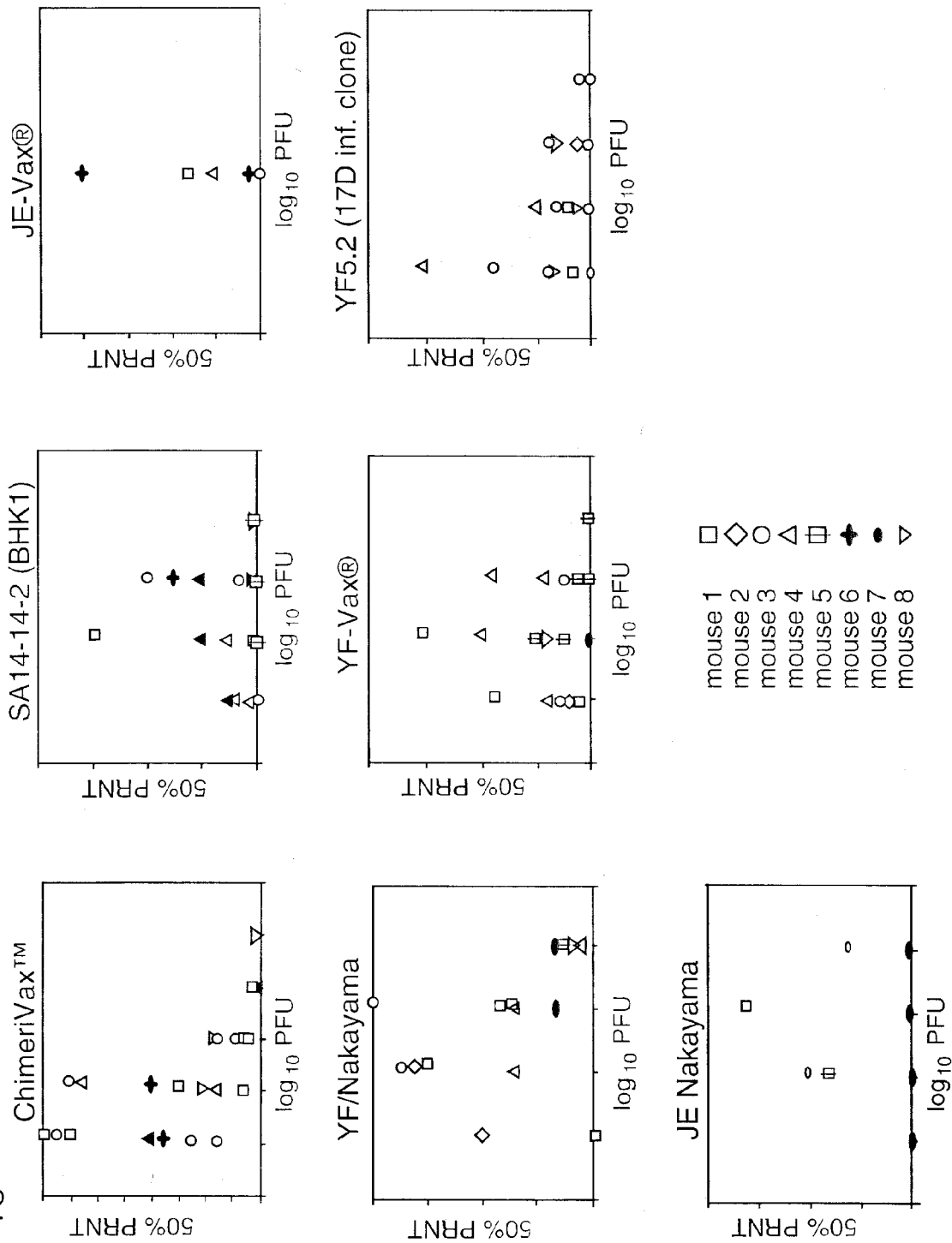


FIG. 17

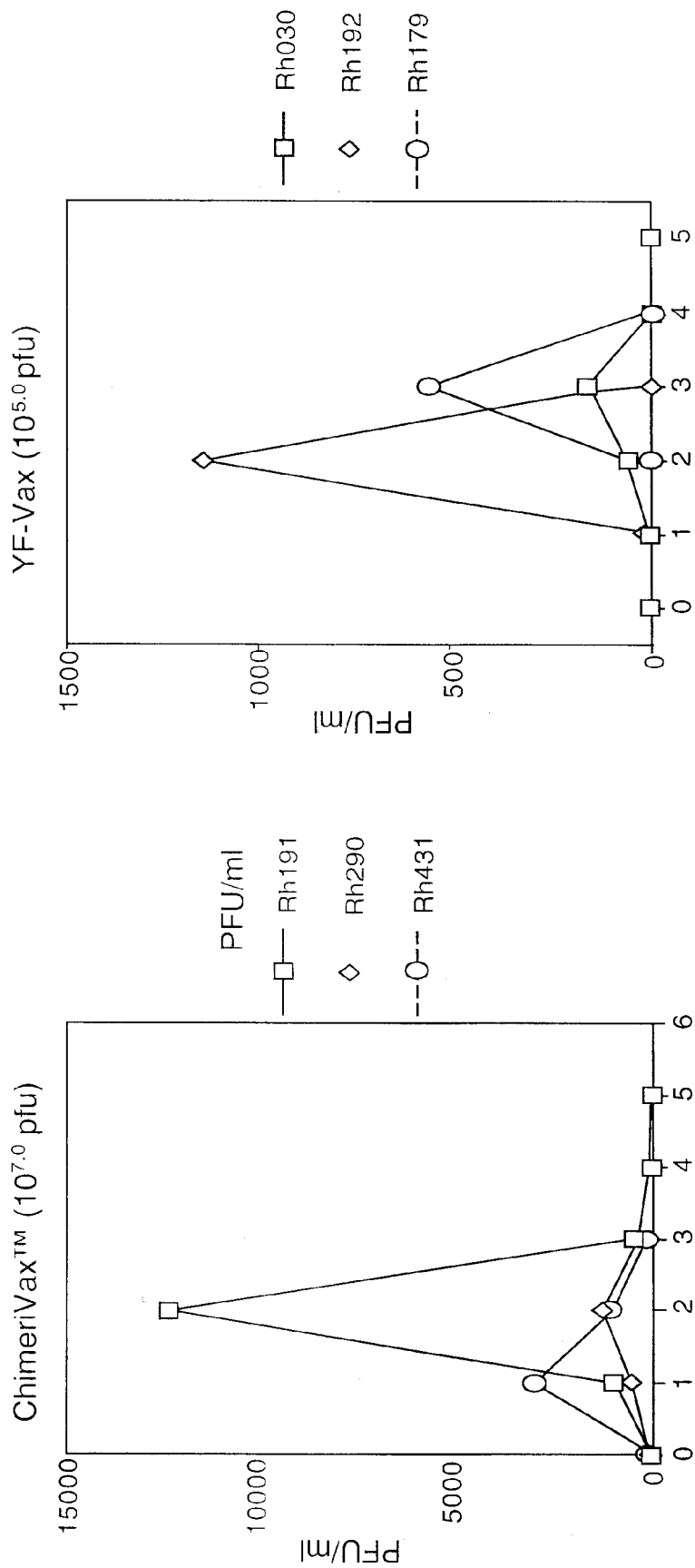


FIG. 18

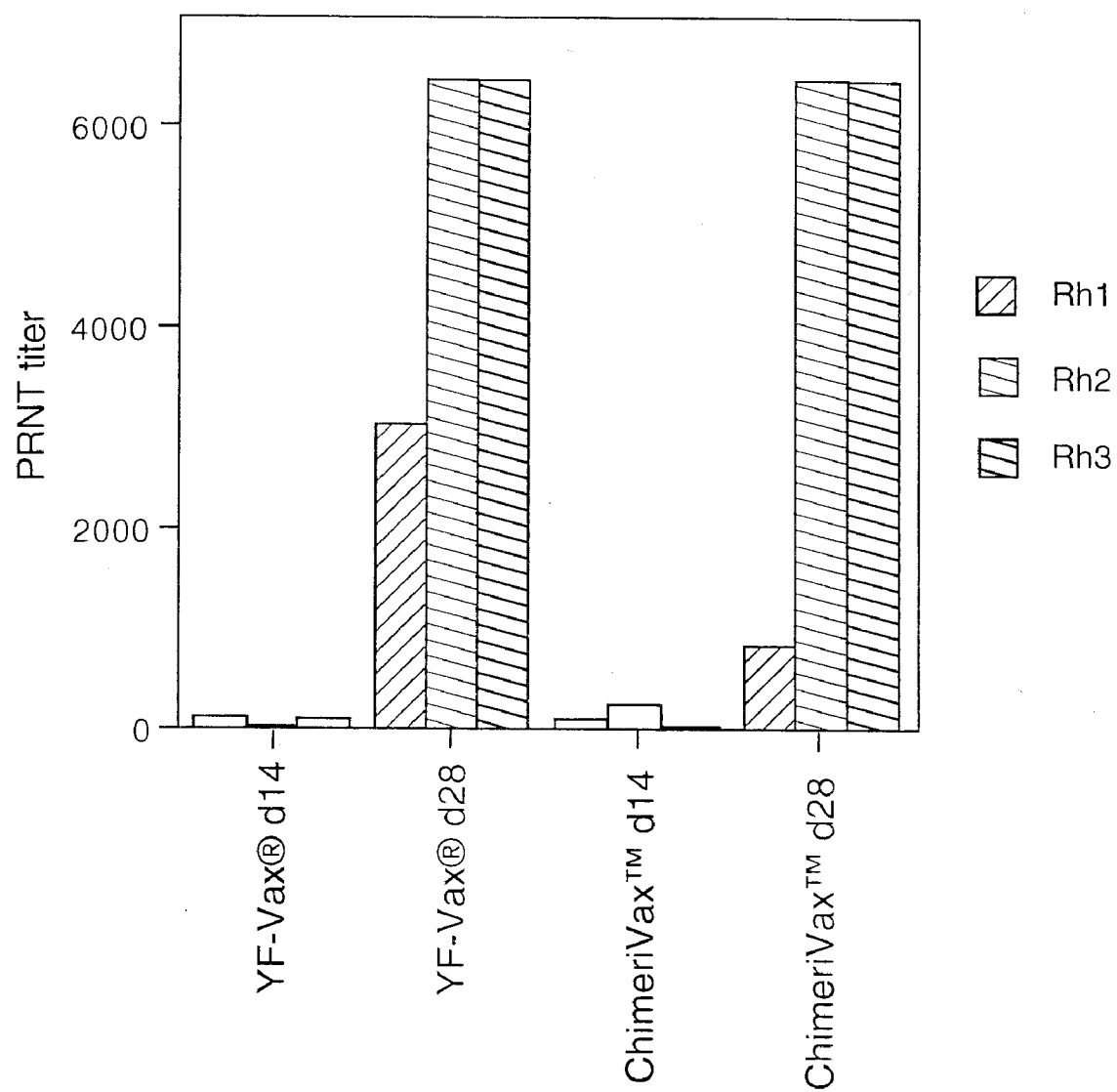


FIG. 19

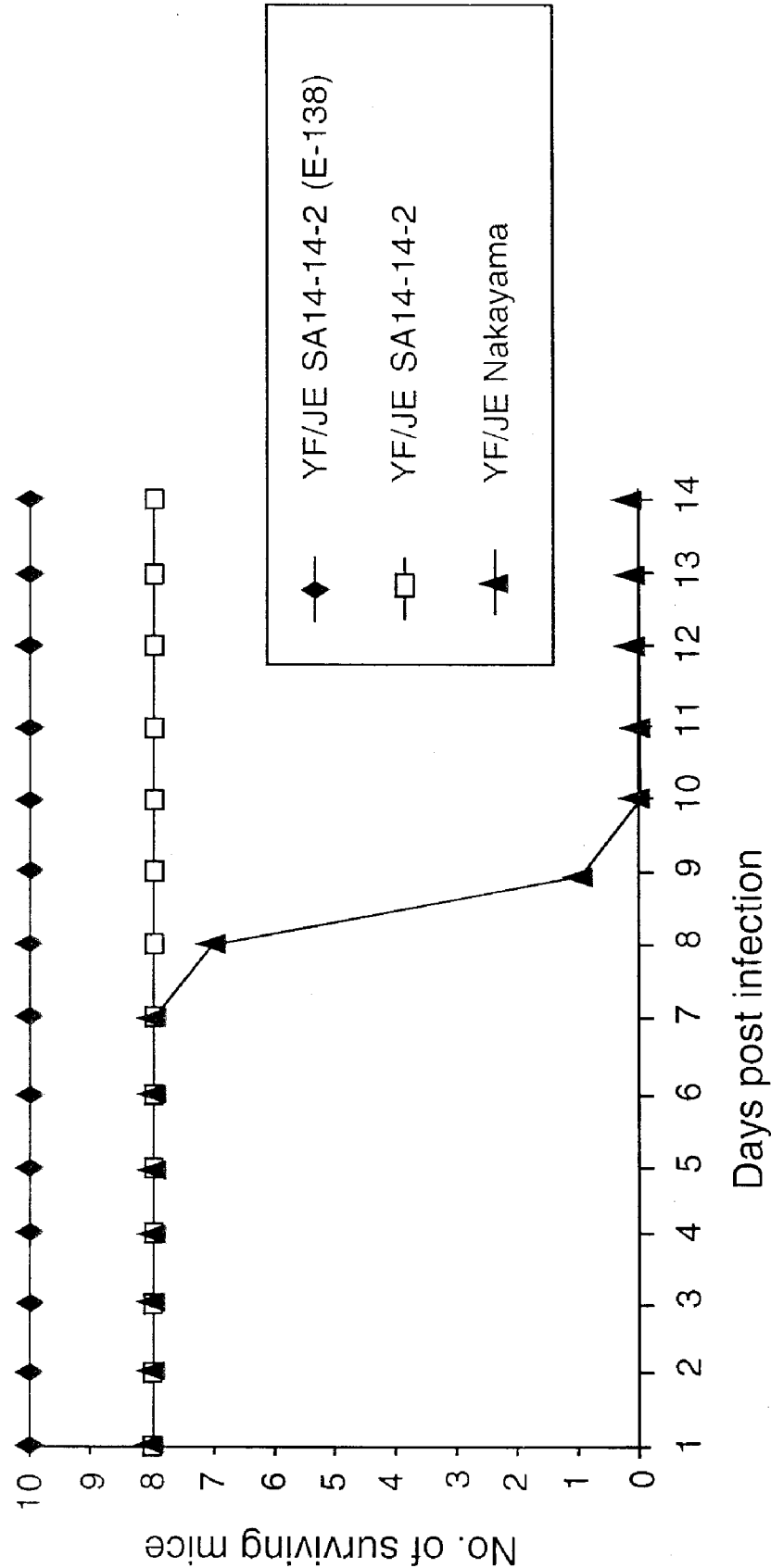


FIG. 20

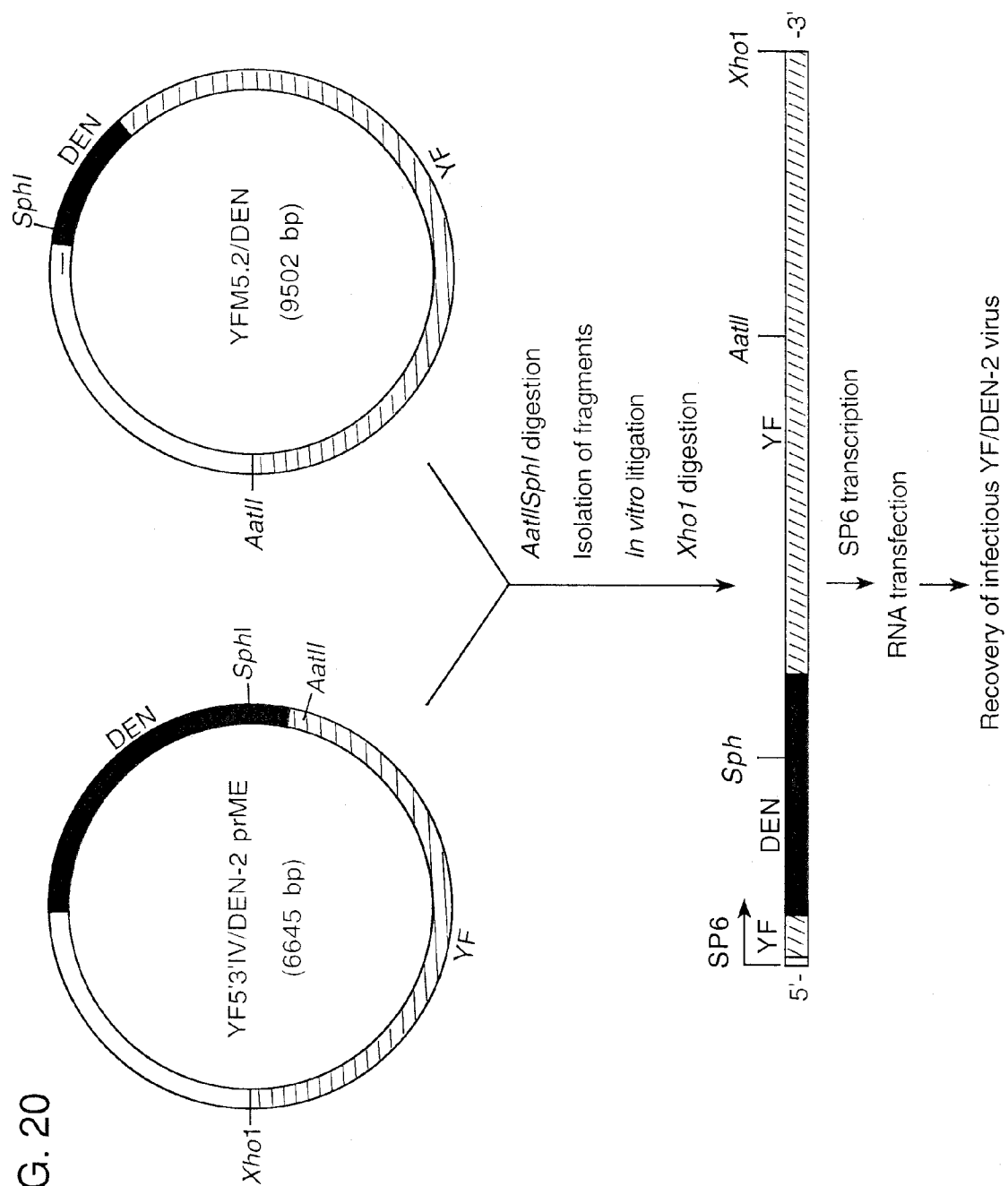


FIG. 21

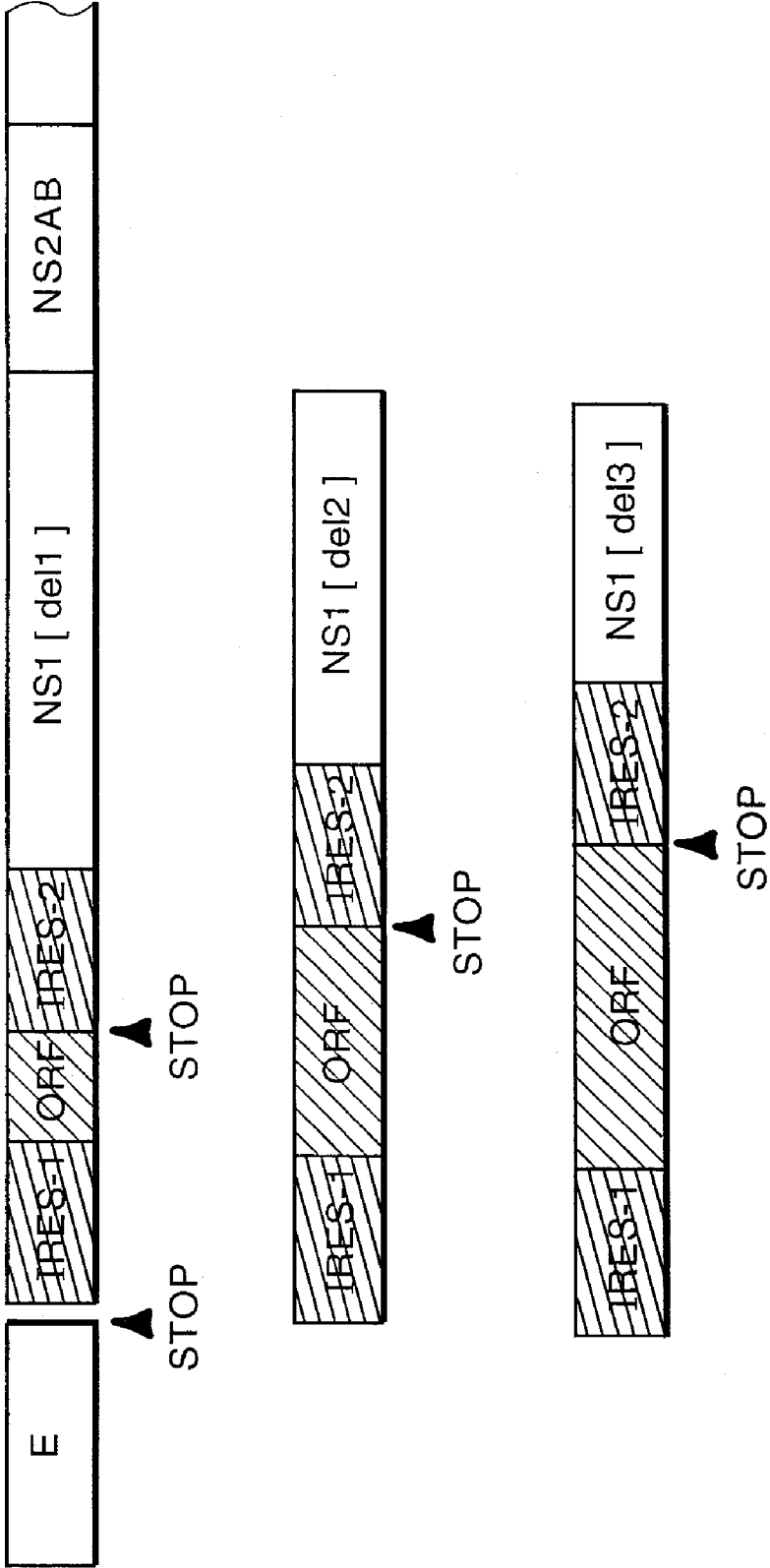


FIG. 22

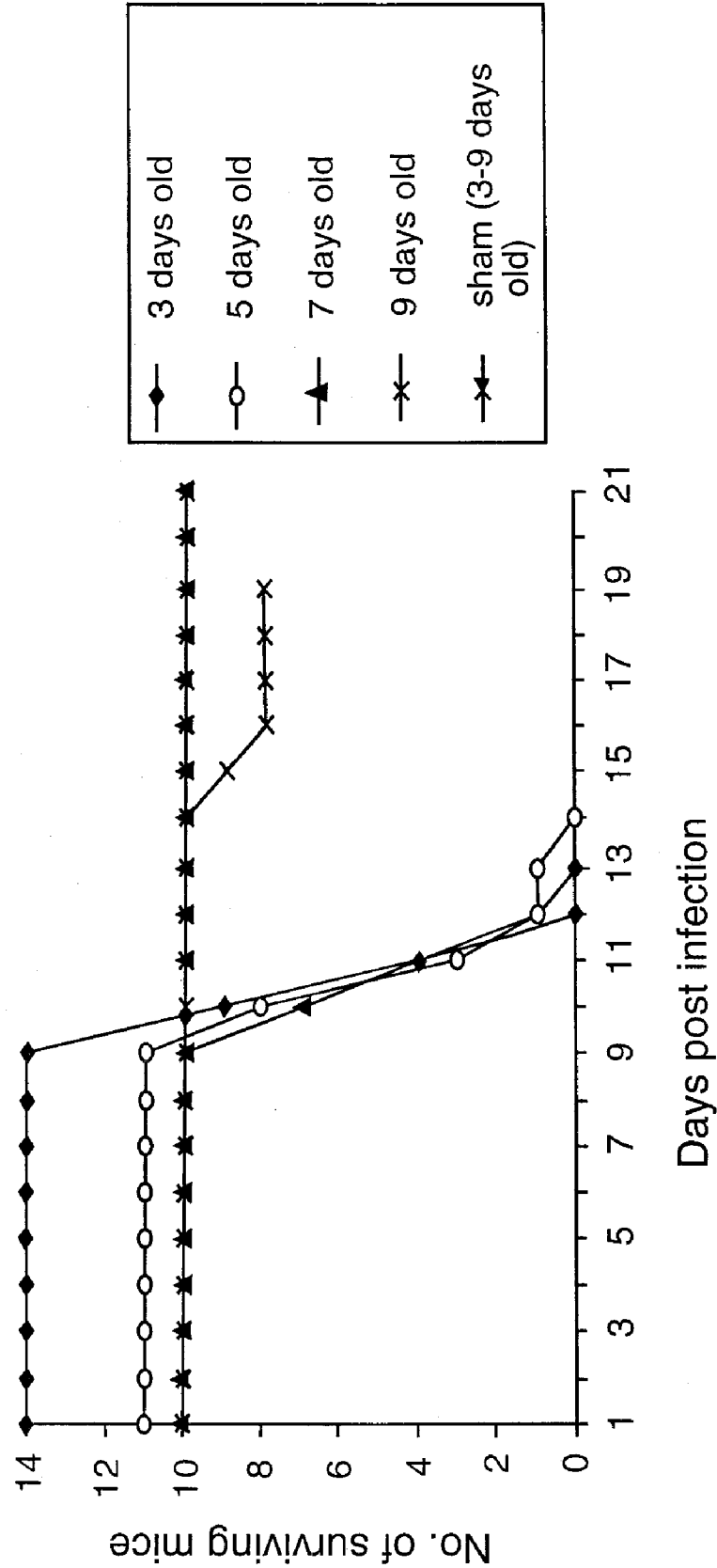


FIG. 23

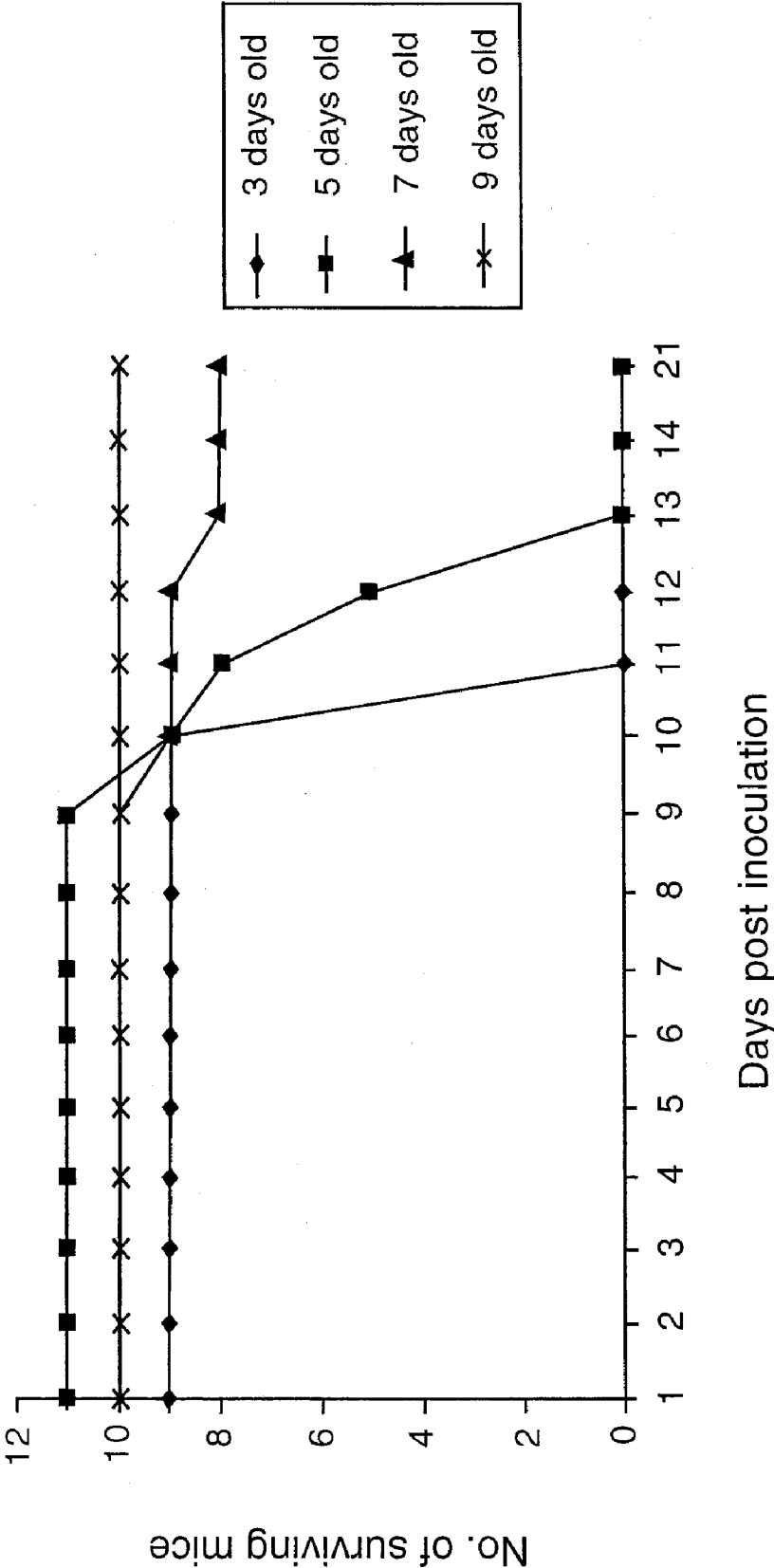


FIG. 24A

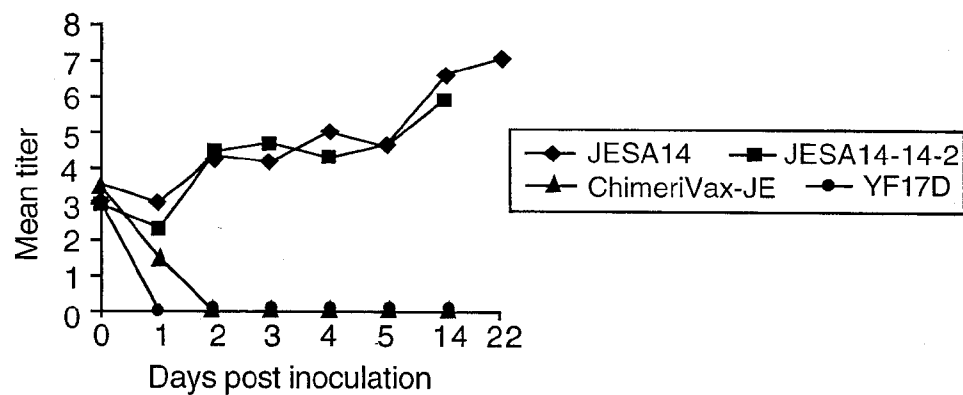


FIG. 24B

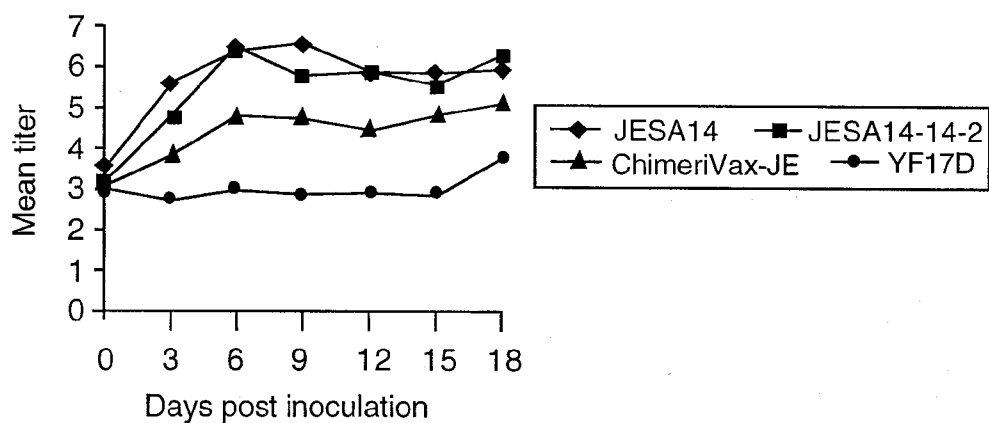


FIG. 24C

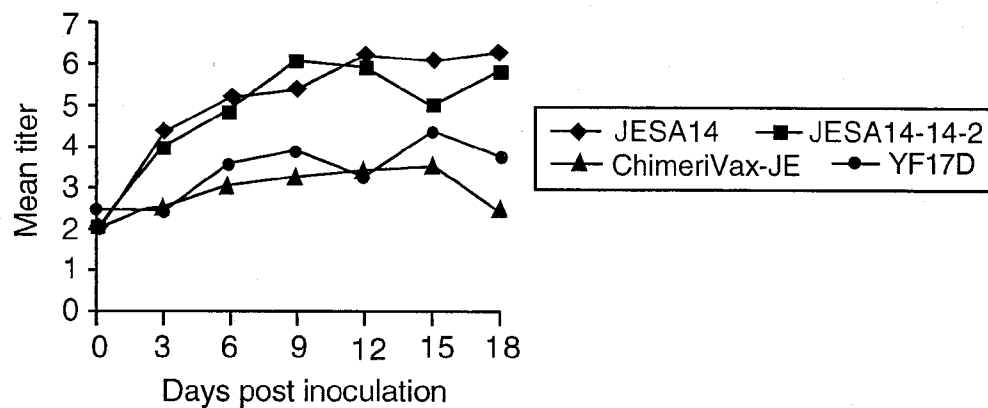


FIG. 25A

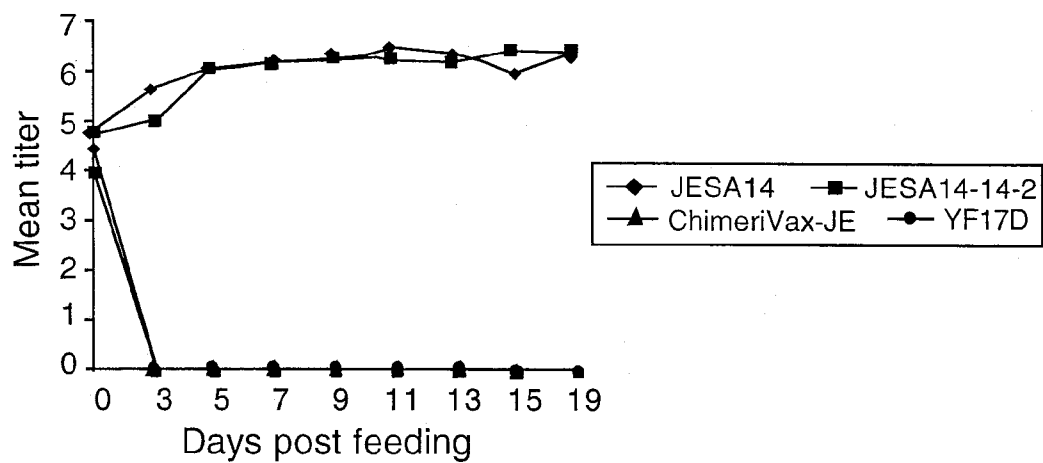


FIG. 25B

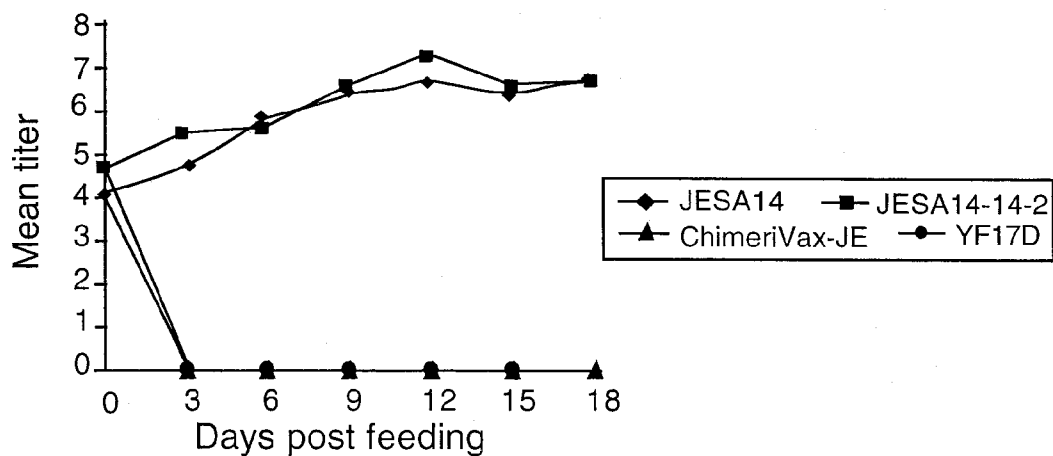


FIG. 25C

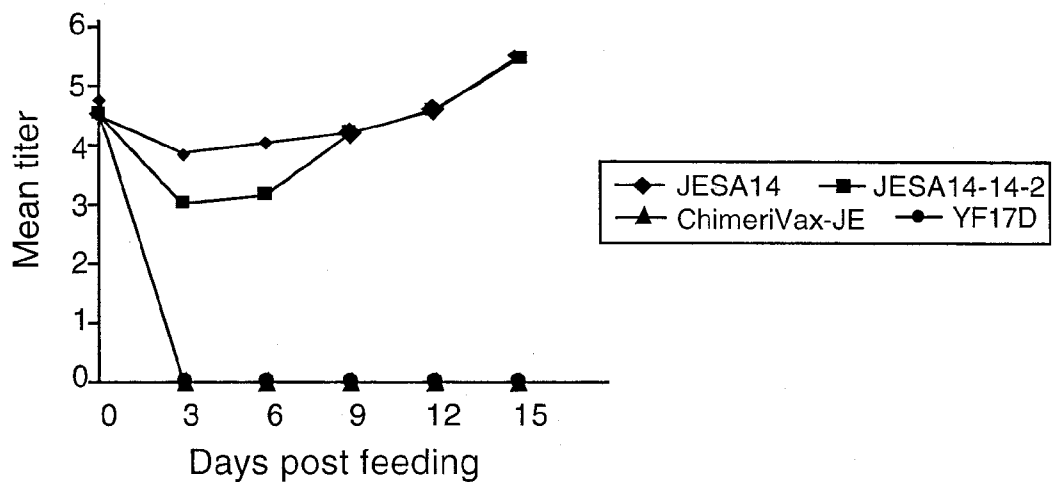


FIG. 26A

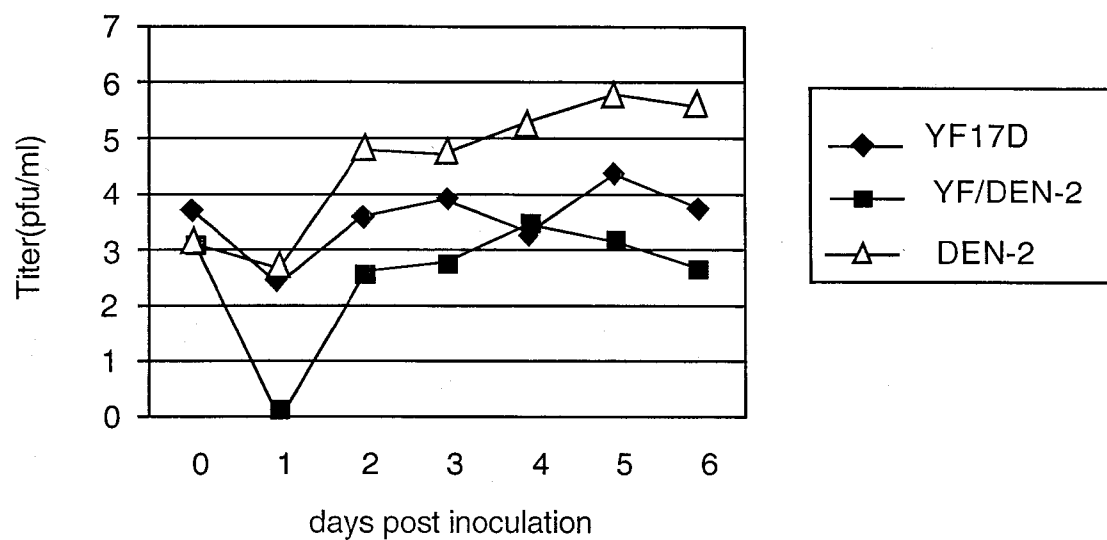


FIG. 26B

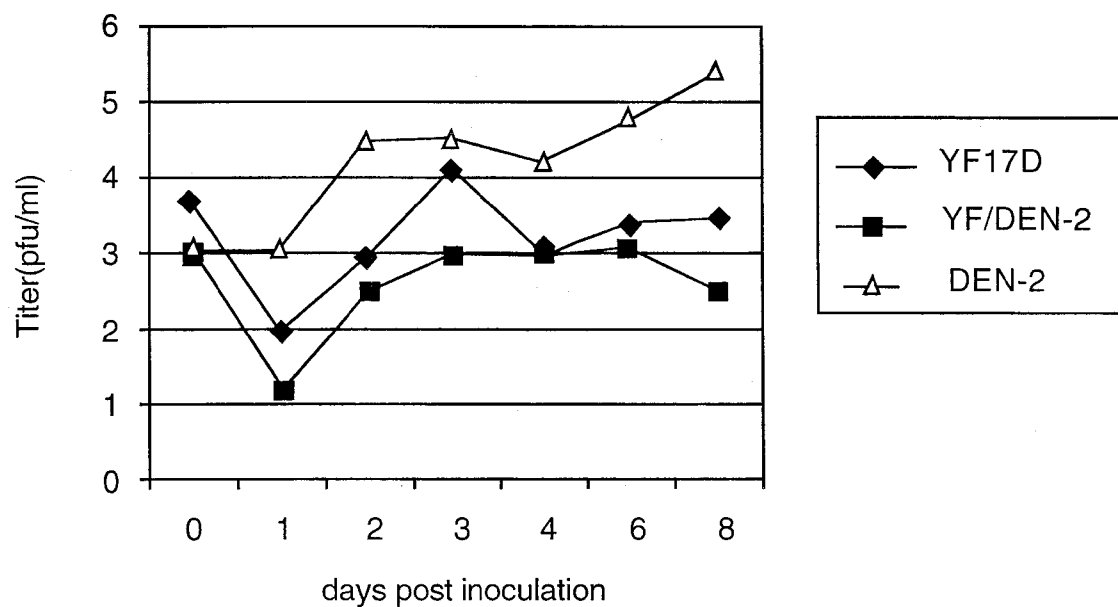


FIG. 27

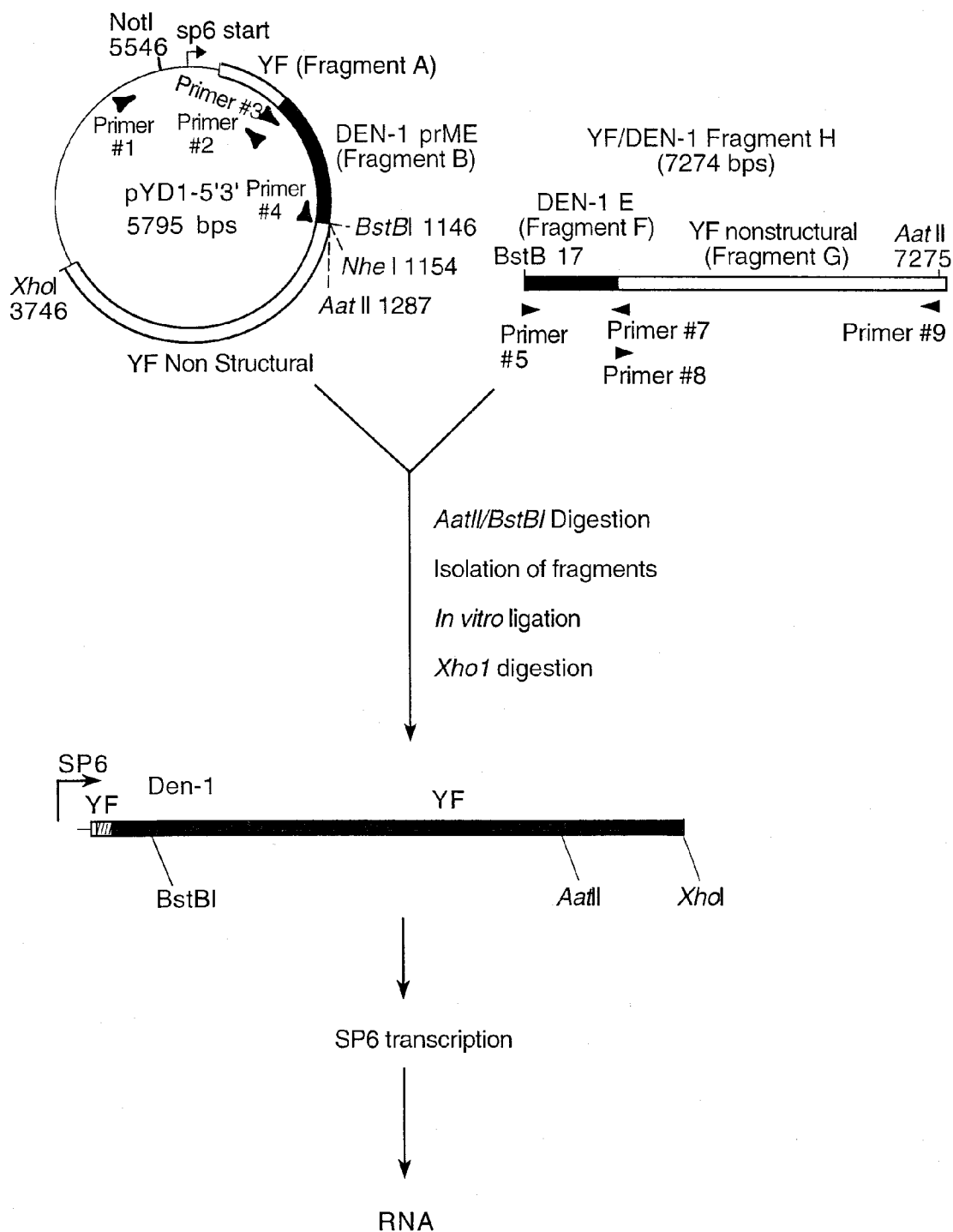


FIG. 28

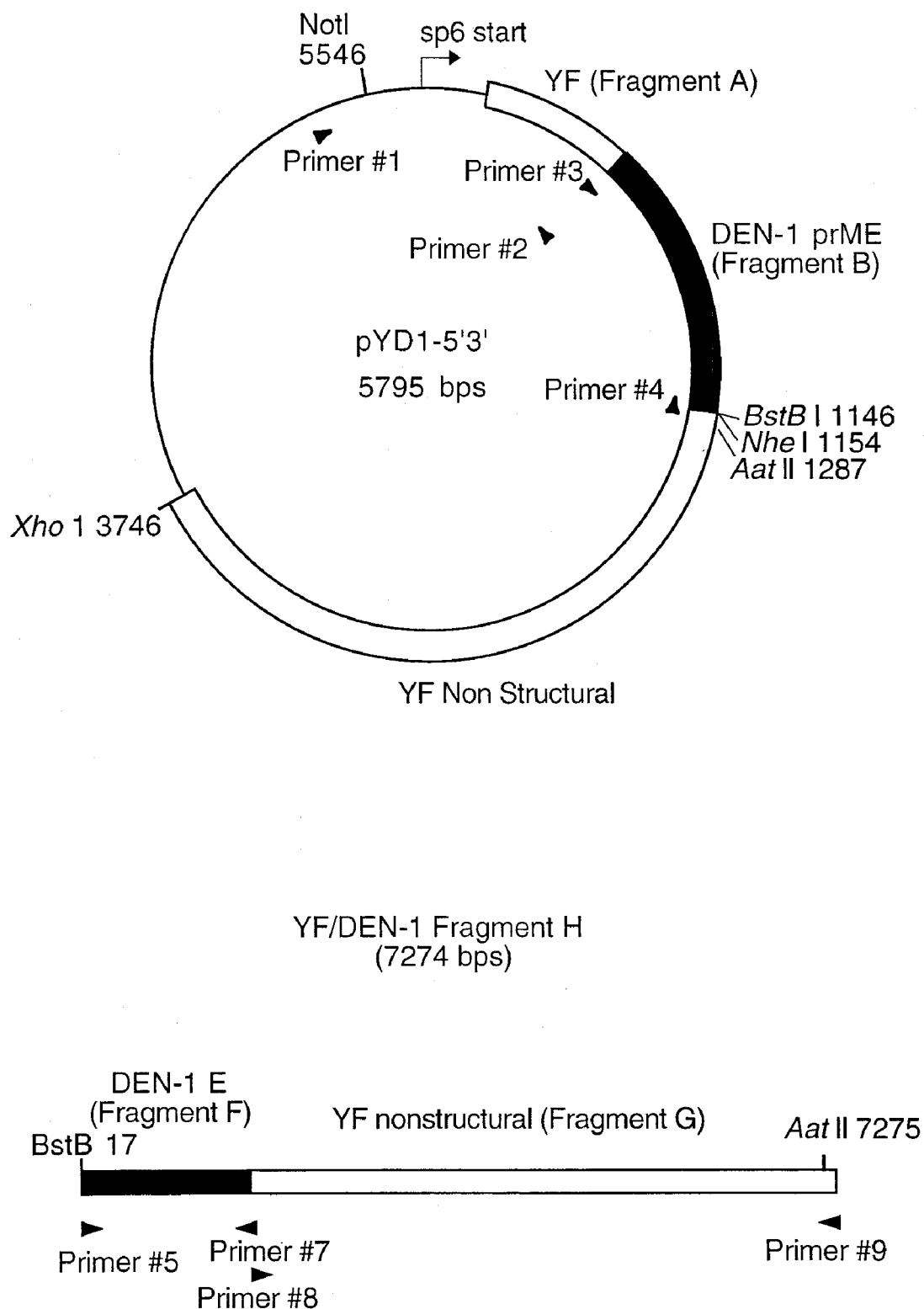


FIG. 29

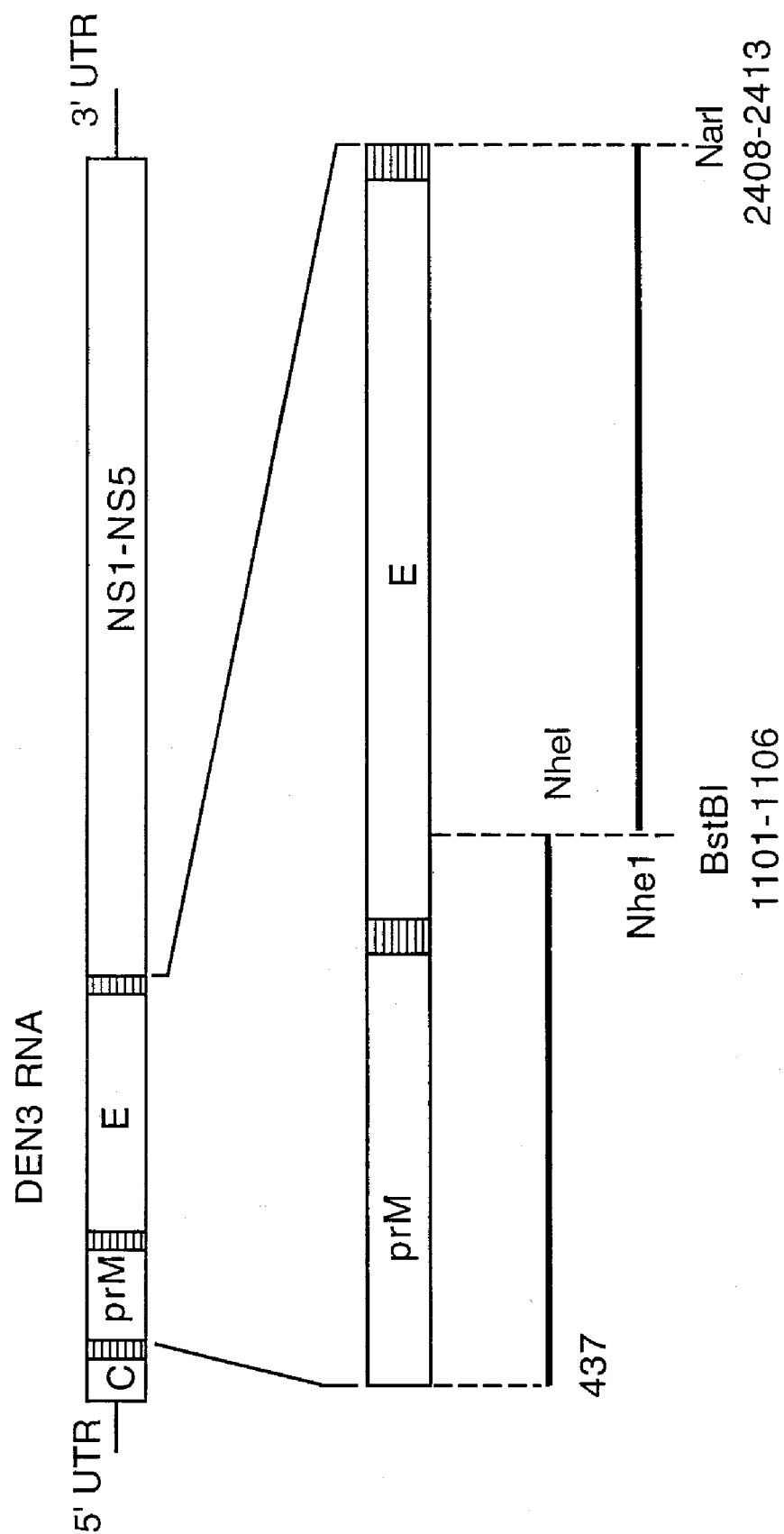


FIG. 30

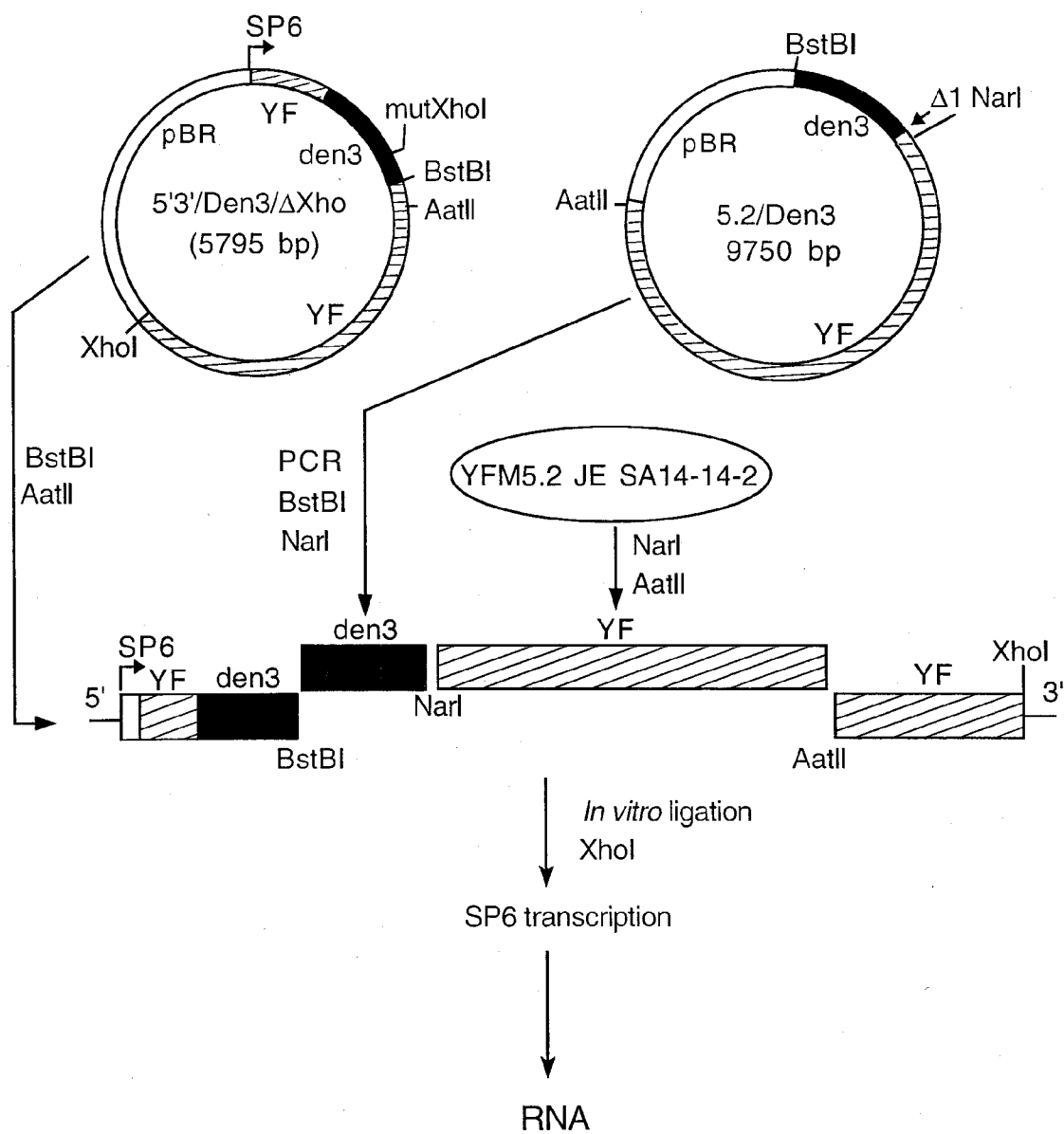


FIG. 31

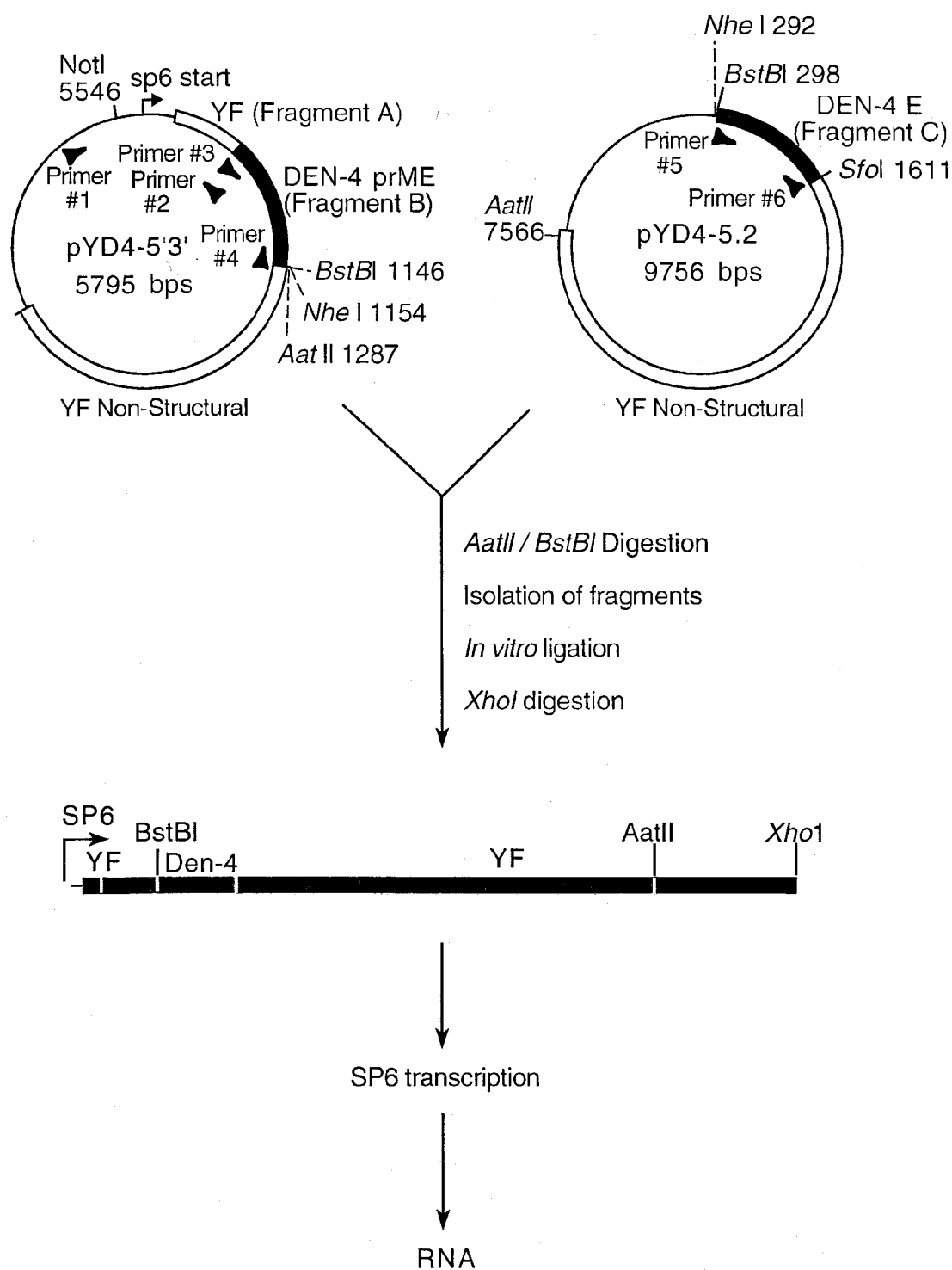
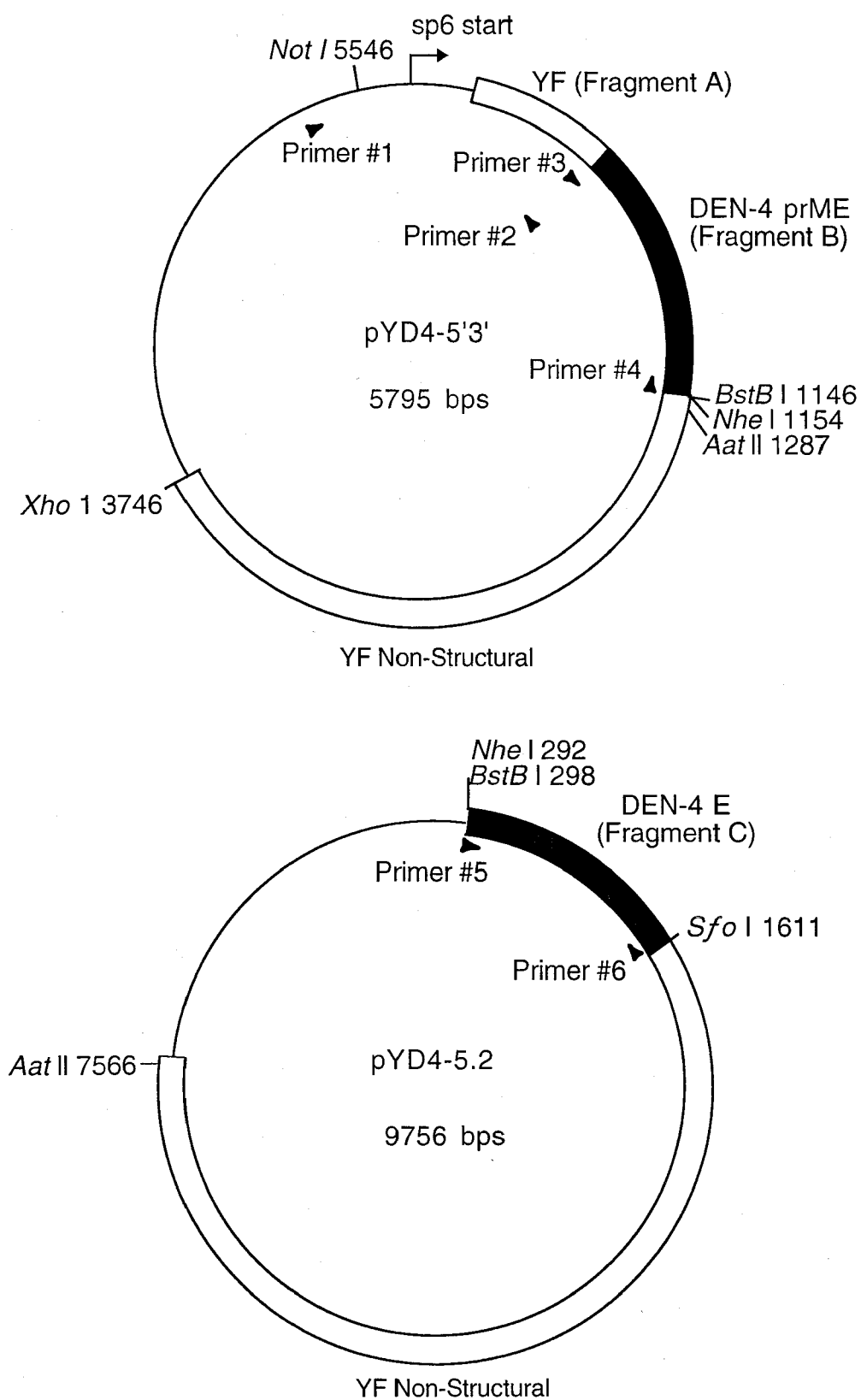


FIG. 32



CHIMERIC FLAVIVIRUS VACCINES

[0001] This is a continuation of, and claims priority from, U.S. Ser. No. 12/423,566, filed Apr. 14, 2009 (abandoned), which is a continuation of U.S. Ser. No. 10/701,122, filed Nov. 4, 2003 (abandoned), which is a continuation of U.S. Ser. No. 09/452,638, filed Dec. 1, 1999 (U.S. Pat. No. 6,696,281), which is a continuation-in-part of U.S. Ser. No. 09/121,587, filed on Jul. 23, 1998 (U.S. Pat. No. 6,962,708), which is a continuation-in-part of PCT/US98/03894, filed on Mar. 2, 1998, which is a continuation-in-part of U.S. Ser. No. 09/007,664, filed on Jan. 15, 1998 (abandoned), which is a continuation-in-part of U.S. Ser. No. 08/807,445, filed on Feb. 28, 1997 (abandoned).

BACKGROUND OF THE INVENTION

[0002] This invention relates to infectious, attenuated viruses useful as vaccines against diseases caused by flaviviruses.

[0003] Several members of the flavivirus family pose current or potential threats to global public health. For example, Japanese encephalitis is a significant public health problem involving millions of at risk individuals in the Far East. Dengue virus, with an estimated annual incidence of 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever worldwide, has emerged as the single most important arthropod-transmitted human disease.

[0004] Other flaviviruses continue to cause endemic diseases of variable nature and have the potential to emerge into new areas as a result of changes in climate, vector populations, and environmental disturbances caused by human activity. These flaviviruses include, for example, St. Louis encephalitis virus, which causes sporadic, but serious, acute disease in the midwest, southeast, and western United States; West Nile virus, which causes febrile illness, occasionally complicated by acute encephalitis, and is widely distributed throughout Africa, the Middle East, the former Soviet Union, and parts of Europe; Murray Valley encephalitis virus, which causes endemic nervous system disease in Australia; and Tick-borne encephalitis virus, which is distributed throughout the former Soviet Union and eastern Europe, where its Ixodes tick vector is prevalent and responsible for a serious form of encephalitis in those regions.

[0005] Hepatitis C virus (HCV) is another member of the flavivirus family, with a genome organization and replication strategy that are similar, but not identical, to those of the flaviviruses mentioned above. HCV is transmitted mostly by parenteral exposure and congenital infection, is associated with chronic hepatitis that can progress to cirrhosis and hepatocellular carcinoma, and is a leading cause of liver disease requiring orthotopic transplantation in the United States.

[0006] The Flaviviridae family is distinct from the alphaviruses (e.g., WEE, VEE, EEE, SFV, etc.) and currently contains three genera, the flaviviruses, the pestiviruses, and the hepatitis C viruses. Fully processed mature virions of flaviviruses contain three structural proteins, envelope (E), capsid (C), and membrane (M), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Immature flaviviruses found in infected cells contain pre-membrane (prM) protein, which is the precursor to the M protein.

[0007] After binding of virions to host cell receptors, the E protein undergoes an irreversible conformational change upon exposure to the acidic pH of endosomes, causing fusion

between the envelope bilayers of the virions and endocytic vesicles, thus releasing the viral genome into the host cytosol. PrM-containing tick-borne encephalitis (TBE) viruses are fusion-incompetent, indicating that proteolytic processing of prM is necessary for the generation of fusion-competent and fully infectious virions (Guirakhoo et al., J. Gen. Virol. 72(Pt. 2):333-338, 1991). Using ammonium chloride late in the virus replication cycle, prM-containing Murray Valley encephalitis (MVE) viruses were produced and shown to be fusion incompetent. By using sequence-specific peptides and monoclonal antibodies, it was demonstrated that prM interacts with amino acids 200-327 of the E protein. This interaction is necessary to protect the E protein from the irreversible conformational changes caused by maturation in the acidic vesicles of the exocytic pathway (Guirakhoo et al., Virology 191:921-931, 1992).

[0008] The cleavage of prM to M protein occurs shortly before release of virions by a furin-like cellular protease (Stadler et al., J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and incorporated into the virus lipid envelope together with the E protein.

[0009] Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera (Pletnev et al., J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo et al., 1991, supra; Guirakhoo et al., 1992, supra; Heinz et al., Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith et al., ASTMH meeting, Dec. 7-11, 1997). Deletion mutants replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in lethal mouse and hamster models and shown to be attenuated; in non-human primates it caused 100% seroconversion and protected all immunized monkeys from a lethal challenge.

SUMMARY OF THE INVENTION

[0010] The invention features chimeric, live, infectious, attenuated viruses that are each composed of:

[0011] (a) a first yellow fever virus (e.g., strain 17D), representing a live, attenuated vaccine virus, in which the nucleotide sequence encoding the prM-E protein is either deleted, truncated, or mutated so that the functional prM-E protein of the first flavivirus is not expressed, and

[0012] (b) integrated into the genome of the first flavivirus, a nucleotide sequence encoding the viral envelope (prM-E) protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed from the altered genome of the first flavivirus.

[0013] The chimeric virus is thus composed of the genes and gene products responsible for intracellular replication belonging to the first flavivirus and the genes and gene prod-

ucts of the envelope of the second flavivirus. Since the viral envelope contains antigenic determinants responsible for inducing neutralizing antibodies, the result of infection with the chimeric virus is that such antibodies are generated against the second flavivirus.

[0014] A preferred live virus for use as the first yellow fever virus in the chimeric viruses of the invention is YF 17D, which has been used for human immunization for over 50 years. YF 17D vaccine is described in a number of publications, including publications by Smithburn et al. ("Yellow Fever Vaccination," World Health Org., p. 238, 1956), and Freestone (in Plotkin et al., (Eds.), *Vaccines*, 2nd edition, W.B. Saunders, Philadelphia, 1995). In addition, the yellow fever virus has been studied at the genetic level (Rice et al., *Science* 229:726-733, 1985) and information correlating genotype and phenotype has been established (Marchevsky et al., *Am. J. Trop. Med. Hyg.* 52:75-80, 1995). Specific examples of yellow fever substrains that can be used in the invention include, for example, YF 17DD (GenBank Accession No. U17066), YF 17D-213 (GenBank Accession No. U17067), YF 17D-204 France (X15067, X15062), and YF-17D-204, 234 US (Rice et al., *Science* 229:726-733, 1985; Rice et al., *New Biologist* 1:285-296, 1989; C 03700, K 02749). Yellow Fever virus strains are also described by Galler et al., *Vaccine* 16 (9/10):1024-1028, 1998.

[0015] Preferred flaviviruses for use as the second flavivirus in the chimeric viruses of the invention, and thus sources of immunizing antigen, include Japanese Encephalitis (JE, e.g., JE SA14-14-2), Dengue (DEN, e.g., any of Dengue types 1-4; for example, Dengue-2 strain PUO-218) (Gruenberg et al., *J. Gen. Virol.* 67:1391-1398, 1988) (sequence appendix 1; SEQ ID NO:50; nucleotide sequence of Dengue-2 insert; Pr-M: nucleotides 1-273; M: nucleotides 274-498; E: nucleotides 499-1983) (sequence appendix 1; SEQ ID NO:51; amino acid sequence of Dengue-2 insert; Pr-M: amino acids 1-91; M: amino acids 92-166; E: amino acids 167-661), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE) (i.e., Central European Encephalitis (CEE) and Russian Spring-Summer Encephalitis (RSSE) viruses), and Hepatitis C (HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. As is discussed further below, the second flavivirus sequences can be provided from two different second flaviviruses, such as two Dengue strains.

[0016] It is preferable to use attenuated inserts, for example, in the case of inserts from neurotropic viruses, such as JE, MVE, SLE, CEE, and RSSE. In the case of non-neurotropic viruses, such as dengue viruses, it may be preferable to use unmodified inserts, from unattenuated strains. Maintenance of native sequences in such inserts can lead to enhanced immunogenicity of the proteins encoded by the inserts, leading to a more effective vaccine.

[0017] In a preferred chimeric virus of the invention, the prM-E protein coding sequence of the second flavivirus is substituted for the prM-E protein coding sequence of the live yellow fever virus. Also, as is described further below, the prM portion of the protein can contain a mutation or mutations that prevent cleavage to generate mature membrane protein. Finally, as is discussed in detail below, the chimeric viruses of the invention include the prM signal of yellow fever virus.

[0018] Also included in the invention are methods of preventing or treating flavivirus infection in a mammal, such as a human, by administering a chimeric flavivirus of the invention to the mammal; use of the chimeric flaviviruses of the invention in the preparation of medicaments for preventing or treating flavivirus infection; nucleic acid molecules encoding the chimeric flaviviruses of the invention; and methods of manufacturing the chimeric flaviviruses of the invention.

[0019] The invention provides several advantages. For example, because they are live and replicating, the chimeric viruses of the invention can be used to produce long-lasting protective immunity. Also, because the viruses have the replication genes of an attenuated virus (e.g., Yellow Fever 17D), the resulting chimeric virus is attenuated to a degree that renders it safe for use in humans.

[0020] Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1A is a schematic representation of processing events at the C/prM junction of parental viruses that can be used in the invention.

[0022] FIG. 1B is a schematic representation of the sequences in the capsid, prM signal, and prM regions of flaviviruses that can be used in the invention. Sequence identification numbers are assigned as follows: YF (SEQ ID NOs: 54 and 55); JE (SEQ ID NO: 56); TBE (SEQ ID NOs: 57 and 58); MVE (SEQ ID NOs: 59 and 60); SLE (SEQ ID NOs: 61 and 62); DEN1 (SEQ ID NOs: 63 and 64); DEN2 (SEQ ID NOs: 65 and 66); DEN3 (SEQ ID NOs: 67 and 68); and DEN4 (SEQ ID NOs: 69 and 70).

[0023] FIG. 2 is a schematic representation of the approach to making chimeric flaviviruses at the prM signal region (DEN4, SEQ ID NO: 71 and TBE, SEQ ID NO: 72) used by C. J. Lai (WO 93/06214).

[0024] FIG. 3 is a schematic representation of an attempt to use the method of C. J. Lai (WO 93/06214) with a yellow fever backbone (YF, SEQ ID NO: 73 and DEN2, SEQ ID NO: 74).

[0025] FIG. 4 is a schematic representation illustrating that the viability of flavivirus chimeras depends on the choice of signal.

[0026] FIG. 5 is a schematic representation of the cloning method used in the present invention, at the prM signal region (YF, SEQ ID NO: 75 and DEN2, SEQ ID NOs: 76 and 77).

[0027] FIG. 6 is a schematic representation of the C, prM, E, and NS1 regions and junction sequences of a YF/JE chimera of the invention. The amino acid sequences flanking the Signalase junction between the C and prM regions for the indicated viruses are assigned as follows: JE (SEQ ID NO: 78); YF (SEQ ID NO: 79); and YF/JE (SEQ ID NO: 80). The amino acid sequences flanking the NS2B-3 protease junction between the C and prM regions are assigned as follows: JE (SEQ ID NO: 81); YF (SEQ ID NO: 82); and YF/JE (SEQ ID NO: 82). The amino acid sequences flanking the Signalase junction between the E and NS1 regions are assigned as follows: JE (SEQ ID NO: 83); YF (SEQ ID NO: 84); and YF/JE (SEQ ID NO: 85).

[0028] FIG. 7 is a schematic representation of genetic manipulation steps that were carried out to construct a Yellow-Fever/Japanese Encephalitis (YF/JE) chimeric virus of the invention.

[0029] FIG. 8 is a set of growth curves for chimeric YF/JE viruses of the invention in cell cultures of vertebrate and mosquito origin.

[0030] FIG. 9 is a growth curve of RMS (Research Master Seed, YF/JE SA14-14-2) in Vero and LLC-MK2 cells.

[0031] FIG. 10 is a graph showing a growth comparison between RMS (YF/JE SA14-14-2) and YF-Vax® (Yellow Fever 17D Vaccine) in MRC-5 cells.

[0032] FIG. 11A is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA14-14-2 (0.01 MOI) in FRhL cells.

[0033] FIG. 11B is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA14-14-2 (0.1 MOI) in FRhL cells.

[0034] FIG. 12 is a graph and a table showing the results of a mouse neurovirulence analysis carried out with a YF/JE chimeric virus of the invention.

[0035] FIG. 13 is a graph showing the neutralizing antibody response of mice immunized with a YF/JE SA14-14-2 chimeric vaccine of the invention. Three week old mice were immunized, and samples for testing were taken at 6 weeks.

[0036] FIG. 14A is a graph showing the results of neurovirulence testing of YF-Vax® (Yellow Fever 17D Vaccine) in 4 week old ICR mice by the i.c. route.

[0037] FIG. 14B is a graph showing the results of neurovirulence testing of YF/JE SA14-14-2 in 4 week old ICR mice by the i.c. route.

[0038] FIG. 15 is a set of graphs showing the results of PRNT analysis of neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. The results in the top graph are 3 weeks post immunization, and the results in the bottom graph are 8 weeks post immunization.

[0039] FIG. 16 is a series of graphs showing the serological responses of mice immunized with a single dose of the live viruses indicated in the figure.

[0040] FIG. 17 is a set of graphs showing viremia and GMT of viremia in 3 rhesus monkeys inoculated with CHIMERIVAX™ (chimeric flavivirus vaccine) or YF-Vax® (Yellow Fever 17D Vaccine) by the i.c. route.

[0041] FIG. 18 is a graph showing the PRNT neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculation with a single dose of YF-Vax® (Yellow Fever 17D Vaccine) or CHIMERIVAX™ (chimeric flavivirus vaccine) vaccines by the i.c. route.

[0042] FIG. 19 is a graph showing the results of neurovirulence testing of YF/JE SA14-14-2 (E-138 K→mutant).

[0043] FIG. 20 is a schematic representation of a two plasmid system for generating chimeric YF/DEN-2 virus. The strategy is essentially as described for the YF/JE chimeric virus.

[0044] FIG. 21 is a schematic representation of the structure of modified YF clones designed to delete portions of the NS1 protein and/or express foreign proteins under control of an internal ribosome entry site (IRES). The figure shows only the E/NS1 region of the viral genome. A translational stop codon is introduced at the carboxyl terminus of the envelope (E) protein. Downstream translation is initiated within an intergenic open reading frame (ORF) by IRES-1, driving expression of foreign proteins (e.g., HCV proteins E1 and/or E2). The second IRES (IRES-2) controls translational initiation of the YF nonstructural region, in which nested, truncated NS1 proteins (e.g., NS1del-1, NS1del-2, or NS1del-3) are expressed. The size of the NS1 deletion is inversely proportional to that of the ORF linked to IRES-1.

[0045] FIG. 22 is a graph showing the neurovirulence phenotype of CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

[0046] FIG. 23 is a graph showing the neurovirulence phenotype of 17D vaccine (YF-Vax® (Yellow Fever 17D Vaccine)) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.

[0047] FIGS. 24A-C are graphs showing the growth of JE SA14, JE SA14-14-2, CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and YF 17D intrathoracically inoculated mosquitoes: (A) *Cx. tritaeniorhynchus* mosquitoes, (B) *Ae. albopictus* mosquitoes, and (C) *Ae. aegypti*. Mean titer=geometric mean of the titers of three individual mosquitoes; log₁₀ pfu/mosquito.

[0048] FIGS. 25A-C are graphs showing the growth of JE SA14, JE SA14-14-2, CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and YF 17D IT orally exposed mosquitoes: (A) *Cx. tritaeniorhynchus* mosquitoes, (B) *Ae. albopictus* mosquitoes, and (C) *Ae. aegypti* mosquitoes. Mean titer=geometric mean of the titers of three individual mosquitoes; log₁₀ pfu/mosquito.

[0049] FIGS. 26A and B are graphs showing the growth of virus in IT inoculated *Ae. aegypti* (A) and *Ae. albopictus* (B) mosquitoes.

[0050] FIG. 27 is a schematic representation of an overview of construction of a YF/DEN1 chimera of the invention.

[0051] FIG. 28 is a schematic representation of a plasmid and fragment map relating to construction of a YF/DEN1 chimera of the invention.

[0052] FIG. 29 is a schematic representation of RT-PCR amplification of the prM-E region of the PaH881/88 DEN3 virus genome. The virus genome is shown on the top diagram. Regions encoding hydrophobic signals for corresponding downstream proteins are shadowed. The prM-E region was amplified in two fragments (black solid lines). Restriction sites introduced for subsequent in-frame in vitro ligation into YF backbone (BstBI and NarI) and cloning (NheI) are indicated.

[0053] FIG. 30 is a schematic representation of construction of a YF/DEN3 chimera of the invention. YF- and DEN3-specific sequences are shown as shadowed and black boxes, respectively. The chimeric YF/DEN3 genome was reconstituted by in vitro ligation of three fragments: the large BstBI-AatII portion of 5'3'/Den3/DXho plasmid, a PCR fragment containing the DEN3-specific part of 5.2/Den3 without the one nucleotide deletion (D1) digested with BstBI and EheI (an isoschizomer of NarI), and the large EheI-AatII fragment of YFM5.2 JE SA14-14-2. Ligation products were linearized with XhoI and then transcribed in vitro with SP6 RNA polymerase. Vero PM cells were transfected with in vitro RNA transcripts to recover the chimeric virus.

[0054] FIG. 31 is a schematic representation of an overview of construction of a YF/DEN4 chimera of the invention.

[0055] FIG. 32 is a schematic representation of a plasmid and fragment map relating to construction of a YF/DEN4 chimera of the invention.

DETAILED DESCRIPTION

[0056] The invention provides chimeric flaviviruses that can be used in vaccination methods against flavivirus infec-

tion. Construction and analysis of chimeric flaviviruses of the invention, such as chimeras of yellow fever virus and Japanese Encephalitis (JE), Dengue types 1-4 (DEN 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses are described as follows.

[0057] Yellow fever (YF) virus is a member of the Flaviviridae family of small, enveloped positive-strand RNA viruses. Flavivirus proteins are produced by translation of a single long open reading frame to generate a polyprotein, and a complex series of post-translational proteolytic cleavages of the polyprotein by a combination of host and viral proteases, to generate mature viral proteins (Amberg et al., *J. Virol.* 73:8083-8094, 1999; Fields, "Flaviviridae," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, p. 937). The virus structural proteins are arranged in the order C-prM-E, where "C" is capsid, "prM" is a precursor of the viral envelope-bound M protein, and "E" is the envelope protein. These proteins are present in the N-terminal region of the polyprotein, while the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are located in the C-terminal region of the polyprotein. The amino termini of prM, E, NS1, and NS4B are generated by host signalase cleavage within the lumen of the endoplasmic reticulum (ER), while most cleavages within the non-structural region are mediated by a viral protease complex known as NS2B-NS3 (Fields, "Flaviviridae," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, p. 937). In addition, the NS2B-NS3 protease complex is responsible for mediating cleavages at the C terminus of both the C protein and the NS4A protein (Amberg et al., *J. Virol.* 73:8083-8094, 1999).

[0058] Several research efforts suggest a regulatory role for NS2B-NS3-mediated cleavage at the C terminus of the capsid protein. This site is the only site in the structural region of polyprotein that is cleaved by the NS2B-NS3 protease and, in addition, it includes a highly conserved dibasic-site motif of flaviviruses, which indicates a functional role (Amberg et al., *J. Virol.* 68:3794-3802, 1994; Yamshchikov et al., *J. Virol.* 68:5765-5771, 1994). In vitro data from various flavivirus models suggest that efficient generation of the prM protein, an early step in proper release of structural proteins, is dependent on the function of the viral protease at the capsid protein site (see summary in Amberg et al., *J. Virol.* 73:8083-8094, 1999).

[0059] Maintenance of this mechanism of coordinate cleavages by NS2B-NS3 at the C terminus of the capsid protein and signalase at the N terminus of prM in the chimeras described below is central to the present invention. In particular, in the chimeras of the present invention, the length of the so-called "prM signal," which separates the two cleavage sites by 20 amino acids in YF (FIGS. 1A and 1B), is substantially maintained, to ensure polyprotein proteolytic processing and subsequent growth of chimeric viruses that are created in a YF backbone. A hydrophobic domain within this signal serves to direct the translocation of prM into the ER lumen, where efficient signalase cleavage occurs only after cleavage at the NS2B-NS3 site in the capsid protein (Amberg et al., *J. Virol.* 73:8083-8094, 1999; FIGS. 1A and 1B).

[0060] In the chimeras of the present invention, only the regions encoding the membrane and envelope proteins (i.e., the prME region) of a non-yellow fever flavivirus are used to replace the corresponding genes in a yellow fever virus clone. The prM signal of the yellow fever virus backbone is maintained. Another method, described in a patent application by

C. J. Lai, WO 93/06214, suggests a universal approach to constructing chimeric flaviviruses, involving cloning the prME region of a donor virus into the backbone of an acceptor virus, such that the prM signal sequence is contributed by the incoming prM protein gene. This approach was illustrated using dengue 4 virus as the backbone (acceptor) and tick-borne encephalitis as the donor prME gene. As is illustrated in FIG. 2, the approach described in WO 93/06214 suggests that variability in this cloning strategy, with other chimeric models using flaviviruses as backbone, will have no effect on proper processing of the resulting polyprotein. That is, that flavivirus prM signals are exchangeable when producing viable chimeric viruses. However, attempts to use this approach with YF as a backbone for the insertion of prME genes of dengue 2 virus to create a chimera in which dengue 2 sequences were inserted at the 5' NS2B/NS3 cleavage site (e.g., by R. Galler, see FIG. 3) failed to produce a viable virus, and the use of the 14 amino acid signal of the dengue 2 virus prM in this construct explains why. When the cloning strategy was altered to maintain the YF prM signal of 20 amino acids (i.e., only the Den 2 prME region was replaced), it was successful. The failure to make a viable chimera in YF using the shorter signal of dengue prM demonstrates that the approach described in WO 93/06214 does not work for all chimeras, such as those including a YF backbone.

[0061] The explanation of the success of the approach described in WO 93/06214, using a dengue virus backbone, is that both the viral protease and the prM signal of dengue viruses were maintained. The dengue prM signal is 6-8 amino acids shorter than that of other flaviviruses, such as YF, TBE, MVE, and JE. Dengue, and chimeric flaviviruses with a dengue backbone, rely on dengue NS2B-NS3 protease complex for eventual signalase cleavage at the prM signal. Possibly dengue strain evolution favors a short signal sequence for optimum cascade-event processing of structural viral proteins, proper assembly, and virus production. If a longer signal is cloned in a dengue chimera, the amino acid additions favors translocation of prME, and perhaps cleavage, but not necessarily optimal viral growth. On the other hand, YF, TBE, MVE, and JE have evolved using a long prM signal, and cloning of a shorter signal in any of these backbones obliterates C-prM-E processing and viral growth (FIG. 4).

[0062] Thus, central to the present invention is the length of the prM signal (FIG. 5). The cloning of the prME region of any flavivirus into a YF backbone according to the invention always takes place after the prM signal sequence (i.e., LMTGG/VTL for yellow fever); in this way, all prME chimeras encode the yellow fever prM signal, thus ensuring proper processing of the polyprotein. In addition, it is preferable to maintain the length and sequence of the YF prM signal in the chimeras of the invention. That is, preferably, the length of the prM signal is 20 amino acids. Less preferably, the length of the prM signal is 15, 16, 17, 18, 19, or more than 20 amino acids in length. Also, it is preferable that the amino acid sequence of the YF prM signal is maintained in the chimeras of the invention, although this sequence can be modified using, for example, conservative amino acid substitutions. Preferably, the sequence of the prM signal is 100%, less preferably, 90%, 80%, 70%, 60%, 50%, or 40% identical to the YF prM signal.

[0063] As an example of construction of a chimera of the invention, FIG. 6 illustrates a YF/JE chimera in which the YF NS2B-NS3 protease recognition site is maintained. Thus, the recognition site for cleavage of the cytosolic from membrane-

associated portions of capsid is homologous for the YF NS2B-NS3 enzyme. At the C/pr-M junction, the portion of the signalase recognition site upstream of the cleavage site is that of the backbone, YF, and the portion downstream of the cleavage site is that of the insert, JE. At the E/NS1 junction, the portion of the signalase recognition site upstream of the cleavage site is similar to that of the insert, JE (four of five of the amino acids are identical to those of the JE sequence), and the portion downstream of the cleavage site is that of the backbone, YF. It is preferable to maintain this or a higher level of amino acid sequence identity to the viruses that form the chimera. Alternatively, at least 25, 50, or 75% sequence identity can be maintained in the three to five amino acid positions flanking the signalase and NS2B-NS3 protease recognition sites.

[0064] Also possible, though less preferable, is the use of any of numerous known signal sequences to link the C and pre-M or E and NS1 proteins of the chimeras (see, e.g., von Heijne, *Eur. J. Biochem.* 133:17-21, 1983; von Heijne, *J. Mol. Biol.* 184:99-105, 1985) or, for example, using the known sequences for guidance, one skilled in the art can design additional signal sequences that can be used in the chimeras of the invention. Typically, for example, the signal sequence will include as its last residue an amino acid with a small, uncharged side chain, such as alanine, glycine, serine, cysteine, threonine, or glutamine. Other requirements of signal sequences are known in the art (see, e.g., von Heijne, 1983, *supra*; von Heijne, 1985, *supra*).

[0065] Following the approach described above, we have succeeded in making viable YF chimeric constructs for JE (section 1) and dengue serotypes 1 through 4 (sections 2-5, respectively). Construction and characterization of these, as well as other, constructs are described further below.

1.0 Construction of cDNA Templates for Generation of YF/JE Chimeric Virus

[0066] The derivation of full-length cDNA templates for YF/JE chimeras of the invention described below employed a strategy similar to that earlier workers used to regenerate YF 17D from cDNA for molecular genetic analysis of YF replication. The strategy is described, e.g., by Nestorowicz et al. (*Virology* 199:114-123, 1994).

[0067] Briefly, derivation of a YF/JE chimera of the invention involves the following. YF genomic sequences are propagated in two plasmids (YF5'3'IV and YFM5.2), which encode the YF sequences from nucleotides 1-2,276 and 8,279-10,861 (YF5'3'IV) and from 1,373-8,704 (YFM5.2) (Rice et al., *The New Biologist* 1:285-296, 1989). Full-length cDNA templates are generated by ligation of appropriate restriction fragments derived from these plasmids. This method has been the most reliable for ensuring stable expression of YF sequences and generation of RNA transcripts of high specific infectivity.

[0068] Our strategy for construction of chimeras involves replacement of YF sequences within the YF5'3'IV and YFM5.2 plasmids by the corresponding JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In addition to cloning of JE cDNA, several steps were required to introduce or eliminate restriction sites in both the YF and JE sequences to permit *in vitro* ligation. The structure of the template for regenerating chimeric YF (C)/JE (prM-E) virus is shown in FIG. 7. A second chimera, encoding the entire JE structural region (C-prM-E) was engineered

using a similar strategy. The second chimera was not able to generate RNA of high infectivity.

1.1 Molecular Cloning of the JE Virus Structural Region

[0069] Clones of authentic JE structural protein genes were generated from the JE SA 14-14-2 strain (JE live, attenuated vaccine strain), because the biological properties and molecular characterization of this strain are well-documented (see, e.g., Eckels et al., *Vaccine* 6:513-518, 1988; JE SA14-14-2 virus is available from the Centers for Disease Control, Fort Collins, Colo. and the Yale Arbovirus Research Unit, Yale University, New Haven, Conn., which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA14-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK₂ cells to obtain sufficient amounts of virus for cDNA cloning. The strategy used involved cloning the structural region in two pieces that overlap at an NheI site (JE nucleotide 1,125), which can then be used for *in vitro* ligation.

[0070] RNA was extracted from monolayers of infected LLC-MK₂ cells and first strand synthesis of negative sense cDNA was carried out using reverse transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71) containing nested XbaI and NarI restriction sites for cloning initially into pBluescript II KS(+), and subsequently into YFM5.2(NarI), respectively. First strand cDNA synthesis was followed by PCR amplification of the JE sequence from nucleotides 1,108-2,471 using the same negative sense primer and a positive sense primer (JE nucleotides sequence 1,108-1,130) containing nested XbaI and NsiI restriction sites for cloning into pBluescript and YFM5.2(NarI), respectively. JE sequences were verified by restriction enzyme digestion and nucleotide sequencing. The JE nucleotide sequence from nucleotides 1 to 1,130 was derived by PCR amplification of negative strand JE cDNA using a negative sense primer corresponding to JE nucleotides 1,116 to 1,130 and a positive sense primer corresponding to JE nucleotides 1 to 18, both containing an EcoRI restriction site. PCR fragments were cloned into pBluescript and JE sequences were verified by nucleotide sequencing. Together, this represents cloning of the JE sequence from nucleotides 1-2,471 (amino acids 1-792).

1.2 Construction of YF5'3'IV/JE and YFM5.2/JE Derivatives

[0071] To insert the C terminus of the JE envelope protein at the YF E/NS1 cleavage site, a unique NarI restriction site was introduced into the YFM5.2 plasmid by oligonucleotide-directed mutagenesis of the signalase sequence at the E/NS1 cleavage site (YF nucleotides 2,447-2,452, amino acids 816-817) to create YFM5.2(NarI). Transcripts derived from templates incorporating this change were checked for infectivity and yielded a specific infectivity similar to the parental templates (approximately 100 plaque-forming units/250 nanograms of transcript). The JE sequence from nucleotides 1,108 to 2,471 was subcloned from several independent PCR-derived clones of pBluescript/JE into YFM5.2(NarI) using the unique NsiI and NarI restriction sites. YF5'3'IV/JE clones containing the YF 5' untranslated region (nucleotides 1-118) adjacent to the JE prM-E region were derived by PCR amplification.

[0072] To derive sequences containing the junction of the YF capsid and JE prM, a negative sense chimeric primer spanning this region was used with a positive sense primer

corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript vector sequence upstream of the EcoRI site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector) from nucleotide 477 (N-terminus of the prM protein) through the NheI site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the NotI and EcoRI restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the NheI site (JE nucleotide 1,125) required for in vitro ligation.

1.3 Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for In Vitro Ligation

[0073] To use the NheI site within the JE envelope sequence as a 5' in vitro ligation site, a redundant NheI site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction fragments and introduced into YFM5.2(Nare)/JE by exchange of an NsiI/NarI fragment encoding the chimeric YF/JE sequence.

[0074] To create a unique 3' restriction site for in vitro ligation, a BspEI site was engineered downstream of the AatII site normally used to generate full-length templates from YF5'3'IV and YFM5.2. (Multiple AatII sites are present in the JE structural sequence, precluding use of this site for in vitro ligation.) The BspEI site was created by silent mutation of YF nucleotide 8,581 (A C; serine, amino acid 2,860), and was introduced into YFM5.2 by exchange of appropriate restriction fragments. The unique site was incorporated into YFM5.2/JE by exchange of the XbaI/SphI fragment, and into the YF5'3'IV/JE(prM-E) plasmids by three-piece ligation of appropriate restriction fragments from these parent plasmids and from a derivative of YFM5.2 (BspEI) deleting the YF sequence between the EcoRI sites at nucleotides 1 and 6,912.

1.4 Exchange of JE Nakayama cDNA into YF/JE Chimeric Plasmids

[0075] Because of uncertainty about the capacity of the PCR-derived JE SA14-14-2 structural region to function properly in the context of the chimeric virus, we used cDNA from a clone of the JE Nakayama strain that has been extensively characterized in expression experiments and for its capacity to induce protective immunity (see, e.g., McIida et al., *Virology* 158:348-360, 1987; the JE Nakayama strain is available from the Centers for Disease Control, Fort Collins, Colo., and the Yale Arbovirus Research Unit, Yale University, New Haven, Conn.). The Nakayama cDNA was inserted into the YF/JE chimeric plasmids using available restriction sites (HindIII to PvuII and BpmI to MunI) to replace the entire prM-E region in the two plasmid system except for a single amino acid, serine, at position 49, which was left intact in order to utilize the NheI site for in vitro ligation. The entire JE region in the Nakayama clone was sequenced to verify that the replaced cDNA was authentic (Table 1).

1.5 Generation of Full-Length cDNA Templates, RNA Transfection, and Recovery of Infectious Virus

[0076] Procedures for generating full-length cDNA templates are essentially as described in Rice et al. (*The New Biologist* 1:285-96, 1989; also see FIG. 7). In the case of chimeric templates, the plasmids YF5'3'IV/JE (prM-E) and

YFM5.2/JE are digested with NheI/BspEI and in vitro ligation is performed using 300 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with XhoI to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of ³H-UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice et al. (supra) for YF 17D. In initial experiments, LLC-MK₂ cells were used for transfection and quantitation of virus, since we have determined the permissiveness for replication and plaque formation of the parental strains of YF and JE. Table 2 illustrates typical results of transfection experiments using Lipofectin (GIBCO/BRL) as a transfection vehicle. Vero cell lines have also been used routinely for preparation of infectious virus stocks, characterization of labeled proteins, and neutralization tests.

[0077] Amplification products from Vero cells were sent to the FDA (CBER) for preparation of the RMS in diploid, Fetal Rhesus lung cells. Fetal rhesus lung cells were received from the ATCC as cultured cells and were infected with YF/JE SA14-14-2 (clone A-1) at an MOI of 1.0. After 1 hour of incubation at 37° C., the inoculum was aspirated and replaced with 50 ml of EMEM, containing 2% FBS. Virus was harvested 78 hours later, aliquoted into 1 ml vials (a total of 200 vials) and frozen at -70° C. Virus titers were determined in Vero, LLC MK2, and CV-1 cells using a standard plaque assay. Titers (pfu/ml) were 1.6×10⁶ in Vero cells, 1.25×10⁶ in LLC MK2 cells, and 1.35×10⁵ in CV-1 cells.

1.6 Nucleotide Sequencing of Chimeric cDNA Templates

[0078] Plasmids containing the chimeric YF/JE cDNA were subjected to sequence analysis of the JE portion of the clones to identify the correct sequences of the SA14-14-2 and Nakayama envelope protein. The nucleotide sequence differences between these constructs in comparison to the reported sequences (McAda et al., supra) are shown in Table 1.

[0079] Five amino acid differences at positions 107, 138, 176, 264, and 279 separate the virulent from the attenuated strains of JE virus. Amino acid differences map to three subregions of Domains I and II of the flavivirus E protein model (Rey et al., *Nature* 375:291-298, 1995). These include the putative fusion peptide (position 107), the hinge cluster (positions 138, 279), the exposed surface of Domain I (positions 176 and 177), and the alpha-helix located in the dimerization Domain II (position 264). Changes at position 107, 138, 176, and 279 were selected early in the passage history, resulting in attenuation of JE SA14-14-2, and remained stable genetic differences from the SA₁₄-14-2 parent (Ni et al., *J. Gen. Virol.* 75:1505-1510, 1994), showing that one or more of these mutations are critical for the attenuation phenotype. The changes at positions 177 and 264 occurred during subsequent passage, and appear to be genetically unstable between two SA14-14-2 virus passages in PHK and PDK cells, showing that this mutation is less critical for attenuation.

[0080] The nucleotide sequence of the E protein coding region of the RMS was determined to assess potential sequence variability resulting from viral passage. Total RNA was isolated from RMS-infected Vero cells, reversed transcribed, and PCR amplified to obtain sequencing templates. Several primers specific for SA14-14-2 virus were used in

individual sequencing reactions and standard protocols for cycle sequencing were performed.

[0081] Sequence data revealed two single nucleotide mutations in the RMS E protein, when compared to the published SA14-14-2 JE strain sequence data. The first mutation is silent, and maps to amino acid position 4 (CTT to CTG); the second is at amino acid position 243 (AAA to GAA) and introduces a change from lysine to glutamic acid. Both mutations identified are present in the sequence of the JE wild type strains Nakayama, SA14 (parent of SA14-14-2), and JaOArS982 (Sumiyoshi et al., *J. Infect. Dis.* 171:1144-1151, 1995); thus, they are unlikely to contribute to virulence phenotype. We conclude that in vitro passage in FRhL cells to obtain the RMS did not introduce unwanted mutations in the E protein.

1.7 Structural and Biological Characterization of Chimeric YF/JE Viruses

[0082] The genomic structure of chimeric YF/JE viruses recovered from transfection experiments was verified by RT/PCR-based analysis of viral RNA harvested from infected cell monolayers. These experiments were performed to eliminate the possibility that virus stocks were contaminated during transfection procedures. For these experiments, first-pass virus was used to initiate a cycle of infection, to eliminate any possible artifacts generated by the presence of residual transfected viral RNA. Total RNA extracts of cells infected with either the YF/JE (prM-E)-SA14-14-2 or YF/JE (prM-E)-Nakayama chimera were subjected to RT/PCR using YF and JE-specific primers that allowed recovery of the entire structural region as two PCR products of approximately 1 kilobase in size. These products were then analyzed by restriction enzyme digestion using the predicted sites within the JE SA14-14-2 and Nakayama sequences that allow differentiation of these viruses. Using this approach, the viral RNA was demonstrated to be chimeric and the recovered viruses were verified to have the appropriate restriction sites. The actual C-prM boundary was then verified to be intact at the sequence level by cycle sequence analysis across the chimeric YF/JE C-prM junction.

[0083] The presence of the JE envelope protein in the two chimeras was verified by both immunoprecipitation with JE-specific antisera and by plaque reduction neutralization testing using YF and JE-specific antisera. Immunoprecipitation of ³⁵S-labeled extracts of LLC-MK₂ cells infected with the chimeras using a monoclonal antibody to the JE envelope protein showed that the JE envelope protein could be recovered as a 55 kDa protein, while the same antisera failed to immunoprecipitate a protein from YF-infected cells. Both JE and YF hyperimmune sera demonstrated cross-reactivity for the two envelope proteins, but the size difference between the proteins (YF=53 kDa, unglycosylated; JE=55 kDa, glycosylated) could reproducibly be observed. Use of YF monoclonal antibodies was not satisfactory under the immunoprecipitation conditions, thus, the specificity was dependent on the JE monoclonal antibodies in this analysis.

[0084] Plaque reduction neutralization testing (PRNT) was performed on the chimeric viruses and the YF and JE SA14-14-2 viruses using YF and JE-specific hyperimmune ascitic fluid (ATCC) and YF-specific purified IgG (monoclonal antibody 2E 10). Significant differences in the 50% plaque reduction titer of these antisera were observed for the chimeras when compared to the control viruses in these experiments (Table 3). The YF/JE SA14-14-2 chimeric vaccine candidate,

as well as the Nakayama chimera and SA14-14-2 viruses, were neutralized only by JE ascitic fluid, whereas YF 17D was neutralized in a specific fashion by YF ascites and the monoclonal antibody (Table 3). Thus, epitopes required for neutralization are expressed in the infectious chimeric YF/JE viruses, and are specific for the JE virus.

1.8 Growth Properties in Cell Culture

[0085] The growth capacity of the chimeras has been examined quantitatively in cell lines of both primate and mosquito origin. FIG. 8 illustrates the cumulative growth curves of the chimeras on LLC-MK₂ cells after low multiplicity infection (0.5 plaque-forming units/cell). In this experiment, YF.5.2iv (cloned derivative) and JE SA14-14-2 (uncloned) viruses were used for comparison. Both chimeric viruses reached a maximal virus yield of approximately one log higher than either parental virus. In the case of the YF/JE SA₁₄-14-2 chimera, the peak of virus production occurred 12 hours later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE Nakayama chimera exhibited considerably more cytopathic effects than the YF/JE SA14-14-2 chimera on this cell line.

[0086] A similar experiment was carried out in C6/36 cells after low multiplicity infection (0.5 plaque-forming units/cell). FIG. 8 also illustrates the growth kinetics of the viruses in this invertebrate cell line. Similar virus yields were obtained at all points used for virus harvest in this experiment, further substantiating the notion that chimeric viruses are not impaired in replication efficiency.

[0087] Additional experiments showing the growth properties of RMS are shown in FIG. 9. Briefly, Vero cells were grown in EMEM, 1% L-Glutamine, 1% non-essential amino acid, and 10% FBS buffered with sodium bicarbonate. LLC-MK2 cells were purchased from the ATCC (CLL-7.1, passage 12) and were grown in the same medium as Vero cells. Cells were inoculated with the RMS virus at an MOI of 0.1. Supernatant fluid was sampled at 24 hour intervals for 7 days and frozen at -70° C. for subsequent plaque assay. Plaque assays were performed in 6-well plates. The RMS reached more than 8 log₁₀ pfu/ml in 5 days. In LLC-MK2 cells, the RMS grew slower and peaked (6 log₁₀ pfu/ml) at about 6 days.

1.9 Comparison of Growth Kinetics of the RMS (YF/JE SA14-14-2) with YF 17D Vaccine in MRC-5 Cells

[0088] An experiment was performed to assess the ability of the vaccine candidate to propagate in a cell line acceptable for human vaccines. Commercial Yellow Fever 17D vaccine (YF-VAX® (Yellow Fever 17D Vaccine) was obtained from Connaught Laboratories, Swiftwater, Pa. MRC-5 (diploid human embryonal lung cells) were purchased from ATCC (171-CCL, Batch#: F-14308, passage 18) and grown in EMEM, 2 mM L-Gln, Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 10% FBS.

[0089] To compare growth kinetics of RMS (sequence appendices 2 and 3; Research Master Seed, YF/JE SA14-14-2; nucleotide sequence of ORF; C: nucleotides 119-421; Pr-M: nucleotides 422-982; E: nucleotides 983-2482; and Non-structural proteins: 2483-10381); (amino acid sequence of ORF; C: amino acids 1-101; Pr-M: amino acids 102-288; E: amino acids 289-788; and Non-structural proteins: amino acids 789-3421); (nucleotide sequence of RMS; the coding sequence is from nucleotide 119 to nucleotide 10381)) with YF-VAX® (Yellow Fever 17D Vaccine), cells were grown to 90% confluency and infected with RMS or YF-VAX® (Yel-

low Fever 17D Vaccine) at an MOI of 0.1 pfu. Since MRC-5 cells generally grow slowly, these cells were kept for 10 days post inoculation. Samples were frozen daily for 7-10 days and infectivity determined by plaque assay in Vero cells. YF-VAX® (Yellow Fever 17D Vaccine) and the YF/JE chimera grew to modest titers in MRC-5 cells (FIG. 10). The peak titer was $\sim 4.7 \log_{10}$ pfu for YF-VAX® (Yellow Fever 17D Vaccine) achieved on the second day and was slightly lower, $4.5 \log_{10}$ pfu, for the RMS after 6 days.

1.10 Growth Curve of YF/JE SAJ4-14-2 in FRhL Cells with and without IFN-Inhibitors

[0090] Fetal rhesus lung cells were obtained from the ATCC and propagated as described for MRC-5 cells. Growth kinetics of the RMS were determined with and without interferon inhibitors.

[0091] Double-stranded RNA appears to be the molecular species most likely to induce interferon (IFN) in many virus infected cells. Induction of interferon apparently plays a significant role in the cellular defense against viral infection. To escape cellular destruction, many viruses have developed strategies to down-regulate induction of interferon-dependent activities. Sindbis virus and vesicular stomatitis virus have been shown to be potent IFN inducers. Using chick embryo cells, mouse L cells, and different viral inducers of IFN, it was shown that 2-aminopurine (2AP) and indomethacin (IM) efficiently and reversibly inhibit IFN action (Sekellick et al., J. IFN Res. 5:651, 1985; Marcus et al., J. Gen. Virol. 69:1637, 1988).

[0092] To test whether inhibition of IFN (if present) in FRhL cells will increase the virus yield, we added 2AP at a concentration of 10 mM or IM at a concentration of 10 mg/ml to the FRhL cells at the time of infection with 0.1 or 0.01 MOI of RMS. Samples were taken daily and frozen for determination of virus infectivity by plaque assay. As shown in FIG. 11A, virus titers peaked on day 4 in the presence or absence of inhibitors. When cells were infected at 0.01 MOI (FIG. 11A), virus titer reached 2.65×10^7 pfu/ml on day 4 in the absence of inhibitors. In cells infected in the presence of IM, virus titer was increased about 2-fold, to 5.95×10^7 pfu/ml on day 4. This increase was more dramatic (4-fold) when 2AP was used (9.7×10^7 pfu/ml). Addition of IM did not increase virus yield when cells were infected at a higher MOI (0.1). A titer of 5.42×10^7 was reached without inhibitor and 3.45×10^7 was achieved in the presence of IM. Addition of 2AP increased virus yields to 1.1×10^8 pfu/ml by day 4 and only $1 \log_{10}$ pfu was lost in the following 3 days (9.5×10^6 pfu/ml on day 7) (FIG. 11B). We conclude from this experiment that the YF/JE SA14-14-2 vaccine candidate replicates to titers of $\sim 7.5 \log_{10}$ /ml in an acceptable cell substrate. The addition of interferon inhibitors can result in a modest increase in yields, but is not a requirement for vaccine production.

1.11 Neurovirulence Testing in Normal Adult Mice

[0093] The virulence properties of the YF/JE SA14-14-2 chimera was analyzed in young adult mice by intracerebral inoculation. Groups of 10 mice (4 week old male and female ICR mice, 5 each per group) were inoculated with 10,000 plaque-forming units of the YF/JE SA14-14-2 chimera, YF 17D 5.2iv, or the Chinese vaccine strain JE SA14-14-2 and observed daily for 3 weeks. The results of these experiments are illustrated in FIG. 12. Mice receiving the YF5.2iv parent succumbed by approximately one week post-inoculation. No mortality or illness was observed among mice receiving either the JE SA14-14-2 parent or the chimera. The inocula

used for the experiments were titrated at the time of injection and a subgroup of the surviving mice were tested for the presence of neutralizing antibodies to confirm that infection had taken place. Among those tested, titers against the JE SA14-14-2 virus were similar for animals receiving either this strain or the chimera.

[0094] The results of additional experiments investigating the neurovirulence of the YF/JE SA14-14-2 chimera in mice are illustrated in Table 4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-8 days. In contrast, none of the mice inoculated with YF/JE SA14-14-2 died during two weeks of post-inoculation observation.

[0095] The results of experiments investigating the neuro-invasiveness and pathogenesis of YF/JE chimeras are illustrated in Table 5. In these experiments, the chimeric viruses were inoculated into 3 week old mice at doses varying between 10,000 and 1 million plaque-forming units via the intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or YF/JE SA14-14-2 died during three weeks of post-inoculation observation, indicating that the virus was incapable of causing illness after peripheral inoculation. Mice inoculated with YF/JE SA14-14-2 developed neutralizing antibodies against JE virus (FIG. 13).

[0096] In additional experiments testing the neurovirulence phenotype and immunogenicity of the RMS, 4-week old ICR mice (n=5) were inoculated by the i.e. route with 0.03 ml of graded doses of the RMS or YF-VAX® (Yellow Fever 17D Vaccine) (Table 6). Control mice received only diluent medium by this route. Mice were observed daily and mortality rates were calculated.

[0097] Mice inoculated with YF-VAX® (Yellow Fever 17D Vaccine) started to die on day 7 (FIG. 14A). The $icLD_{50}$ of unpassaged YF-VAX® (Yellow Fever 17D Vaccine), calculated by the method of Reed and Muench, was $1.62 \log_{10}$ and the average survival time (AST) at the highest dose ($4.2 \log_{10}$ pfu) was 8.8 days. In contrast, all mice receiving the RMS survived challenge at all doses (FIG. 14B), indicating that the virus is not neurovirulent for mice. None of the mice inoculated with YF-VAX® (Yellow Fever 17D Vaccine) or the RMS by the peripheral (subcutaneous) route (as shown in Table 6) showed signs of illness or death. Thus, as expected, yellow fever 17D virus was not neuroinvasive.

1.12 Comparison of Immunogenicity of YF/JE RMS with YF 17D Vaccine

[0098] The immunogenicity of the of the RMS was compared with that of the YF 17D vaccine in outbred ICR mice. Groups of five 4 week-old mice received graded doses of the vaccines shown in Table 6. Mice were inoculated with 100 μ l of each virus dilution by the s.c. route. For comparison, two groups of mice received two weekly doses of commercial inactivated JE vaccine prepared in mouse brain tissue JE-VAX® (inactivated Japanese encephalitis virus vaccine) at 1:30 and 1:300 dilution, representing $10 \times$ and $1 \times$ the human equivalent dose based on body weight, respectively. Animals were bled 3 and 8 weeks later and neutralizing antibody titers were measured in heat-inactivated sera against homologous viruses by PRNT. End-point titers were the highest dilution of sera that reduced the number of viral plaques by 50% compared to a normal mouse serum control.

[0099] The highest N antibody titers were observed 8 weeks after immunization in mice receiving $5 \log_{10}$ pfu of the RMS (FIG. 15 and Table 7). The geometric mean N antibody titer in these mice was 5,614. N antibody responses induced by YF/JE SA14-14-2 vaccine against JE were higher than N

antibody responses against YF induced by YF 17D vaccine. Interestingly, the highest concentration of the YF 17D vaccine did not induce significant titers of neutralizing antibodies 3 or 8 weeks post immunization, but antibodies were elicited at lower doses.

[0100] Very low doses (1.4-2.4 log₁₀ PFU) of YF 17D vaccine elicited an immune response in mice 8 weeks after inoculation (Table 7). This result may indicate delayed replication of the vaccine in mice receiving low virus inocula. In contrast, the YF/JE SA14-14-2 chimeric vaccine in this dose range was not immunogenic. It is likely that the chimeric vaccine is somewhat less infectious for mice than YF 17D. However, when inoculated at an infective dose, the chimera appears to elicits a higher immune response. This may be due to higher replication in, or altered tropism for, host tissues. Animals that received two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) did not mount a significant antibody response. Only one animal in the 1:30 dose group developed a neutralizing titer of 1:10 eight weeks after immunization. This might be due to the route (s.c.) and dilution (1:30) of the vaccine.

1.13 Protection of YF/JE SA 14-14-2 RMS Immunized Mice against Challenge with Virulent JE

[0101] The YF/JE SA14-14-2 RMS and other viruses were evaluated for immunogenicity and protection in C57/BL6 mice in collaboration with Dr. Alan Barrett, Department of Pathology, University of Texas Medical Branch, Galveston. Experimental groups are shown in Table 8. Ten-fold dilutions (10²-10⁵) of each virus were inoculated by the s.c. route into groups of 8 mice. Mice were observed for 21 days, at which time surviving animals were bled from the retro-orbital sinus and serum frozen for neutralization tests. The 50% immunizing dose (ID₅₀) for each virus and GMT was determined (see below).

[0102] Surviving mice that received viruses by the s.c. route were challenged on day 28 by i.p. inoculation of 158 LD₅₀ (2,000 PFU) of JE virus (JaOArS982, IC37). Animals were observed for 21 days following challenge. Protection is expressed as the proportion of mice surviving challenge (Table 9).

[0103] As expected, YF 17D virus afforded minimal cross-protection against JE challenge. The YF/JE SA14-14-2 RMS chimera was protective at doses $\geq 10^3$ PFU. The 50% protective dose of the chimeric vaccine was 2.32 log₁₀ PFU. Animals that received 3 doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) were solidly protected against challenge. Mice given a single dose of the SA14-14-2 vaccine were poorly protected. Wild-type Nakayama virus was lethal for a proportion of animals, in a dose-dependent fashion; survivors were poorly protected against challenge indicating that the lethal dose was close to the infecting dose for this virus.

[0104] The YF/JE_{Nakayama} chimeric virus was somewhat more virulent than the Nakayama strain, in that all mice given 10⁵ of the chimera died after inoculation. This is in contrast to earlier studies in outbred mice, in which this virus was not neuroinvasive, confirming the increased susceptibility of C57/BL6 mice to peripheral challenge with JE viruses. Survivors were fully protected against challenge, showing that the infection established by the chimeric virus was more active (immunogenic) than infection by Nakayama virus without the YF replication background. These results show that the combination of viral envelope determinants of a neurovirulent strain (Nakayama) with a replication-efficient

virus (YF 17D) can enhance virulence of the recombinant, emphasizing the need for genetic stability of the mutations conferring attenuation in the YF/JE_{Nakayama} chimera.

1.14 Serological Response

[0105] Sera from mice in groups shown in Table 8 were tested 21 days after immunization for neutralizing antibodies. N tests were performed as follows. Six-well plates were seeded with Vero cells at a density of 10⁶ cells/well in MEM alpha containing 10% FBS, 1% nonessential amino acids, buffered with sodium bicarbonate. One hundred μ l of each test serum (inactivated at 60° C. for 30 minutes) diluted two-fold was mixed with an equal volume of virus containing 200-300 PFU. The virus-serum mixtures were incubated at 4° C. overnight and 100 μ l added to each well after removal of growth medium. The plates were overlaid after 1 hour incubation at 37° C. with 0.6% agarose containing 3% fetal calf serum, 1% L-glutamine, 1% HEPES, and 1% pen-strep-amphotericin mixed 1:1 with 2xM199. After 4 days of incubation at 37° C., 5% CO₂, a second overlay containing 3% Neutral red was added. After appearance of plaques, the monolayer was fixed with 1% formaldehyde and stained with crystal violet. The plaque reduction titer is determined as the highest dilution of serum inhibiting 50% of plaques compared with the diluent-virus control.

[0106] Results are shown in Table 10 and FIG. 16. NT antibody responses in mice immunized with the YF/JE SA14-14-2 chimera showed a dose response and good correlation with protection. At doses of 4-5 logs, the chimeric vaccine elicited higher N antibody responses against JE than either SA14-14-2 virus or wild-type Nakayama virus. Responses were superior to those elicited by YF-VAX® (Yellow Fever 17D Vaccine) against YF 17D virus. No prozone effect was observed in animals receiving the chimera or infectious-clone derived YF 5.2iv; responses at the highest vaccine dose (5 logs) were higher than at the next lower dose (4 logs). In contrast, mice that received SA14-14-2, Nakayama, and YF-VAX® (Yellow Fever 17D Vaccine) at the highest dose responded less well than animals inoculated with diluted virus.

1.15 Safety and Immunogenicity of CHIMERIVAX™ (Chimeric Flavivirus Vaccine) in Monkeys

[0107] The safety of RMS was tested in monkeys, essentially as described in WHO Biological Standards for YF 17D vaccine with minor modifications (see below). Two groups (N=3) of rhesus monkeys were bled and shown to be free from HI antibodies to YF, JE, and SLE. Group 1 received undiluted CHIMERIVAX™ (chimeric flavivirus vaccine) (Vero-passage 2) by the I.C. route (frontal lobe). Group 2 (N=3) received 0.25 ml of 1:10 diluted commercial YF 17D vaccine (YF-VAX® (Yellow Fever 17D Vaccine) by the same route. The virus inocula were frozen, back titrated, and shown to contain 7.0 and 5.0 log₁₀ pfu/0.25 ml of YF/JE SA14-14-2 and YF-VAX® (Yellow Fever 17D Vaccine), respectively.

[0108] Monkeys were observed daily for clinical signs and scored as in WHO standards. Sera were collected daily for 7 days after inoculations and tested for viremia by plaque assay in Vero cells. Blood collected 2 and 4 weeks post inoculation and tested for NT antibodies to the homologous viruses. None of the monkeys showed sign of illness. Monkeys were euthanized on Day 30, and brains and spinal cords were examined for neuropathology as described in the WHO standards. A

sample of the brain and spinal cord from each animal was collected and stored frozen for virus isolation attempts and immunocytochemistry experiments.

[0109] As shown in FIG. 17, a low level viremia was detected in all animals in both groups, and lasted for 2-3 days for the RMS and 1-2 days for YF-VAX® (Yellow Fever 17D Vaccine). All viruses were cleared from the blood by Day 4. According to the WHO standards, monkeys receiving 5,000-50,000 ($3.7-4.7 \log_{10}$) pfu should not have viremia greater than 165,000 pfu/ml (approximately 16,500 mL_{D50}). None of the monkeys in the experiments had viremia of more than 15,000 pfu/ml, despite receiving $6 \log_{10}$ pfu of the RMS.

[0110] Neutralizing antibody titers were measured at 2 and 4 weeks post inoculation (FIG. 18). All monkeys seroconverted and had high titers of neutralizing antibodies against the inoculated viruses. The level of neutralizing antibodies in 2 of 3 monkeys in both groups exceeded a titer of 1:6,400 (the last dilution of sera tested) at 4 weeks post inoculation. The geometric mean antibody titers for CHIMERIVAX™ (chimeric flavivirus vaccine) were 75 and 3,200 after 2 and 4 weeks respectively and were 66 and 4971 for the YF-VAX® (Yellow Fever 17D Vaccine) for the same time points (Table 11).

[0111] Histopathological examination of coded specimens of brain and spinal cord were performed by an expert neuropathologist (Dr. I. Levenbook, previously CBER/FDA), according to the WHO biological standards for yellow fever vaccine. There were no unusual target areas for histopathological lesions in brains of monkeys inoculated with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins. Mean lesion scores in discriminator areas were similar in monkeys inoculated with YF-VAX® (Yellow Fever 17D Vaccine) (0.08) and monkeys inoculated with a 100-fold higher dose of (CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (0.07). Mean lesion scores in discriminator+target areas were higher in monkeys inoculated with YF-VAX® (Yellow Fever 17D Vaccine) (0.39) than in monkeys inoculated with a 100-fold higher dose of (CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (0.11). These preliminary results show an acceptable neurovirulence profile and immunogenicity for (CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) vaccine. A summary of the histopathology results is provided in Table 22.

1.16 Efficacy of YF/JE Chimera in Protecting Monkeys Against Intracerebral Challenge

[0112] The YF/JE chimera were given to adult rhesus monkeys without pre-existing flavivirus immunity by the subcutaneous route. Three monkeys received 4.3 log pfu and three monkeys received 5.3 log pfu of YF/JE SA14-14-2 virus. All 6 monkeys developed very low level (1-2 log/ml) viremias. All animals developed neutralizing antibodies by day 15 (earliest time tested) and titers rose by day 30. Five of six animals survived a very severe intracerebral challenge with a highly virulent JE virus (100,000 mouse LD₅₀ were injected IC 60 days after immunization). None of 4 sham immunized monkeys survived; all died between days 8-10 after challenge. The single death in the immunized group was a pregnant female; pregnancy could have suppressed the cellular immune response to the vaccine. The results show the immu-

nogenicity and protective efficacy of the vaccine, while validating safety with respect to low vaccine viremia. The results of these experiments are illustrated in Tables 12-15.

1.17 Genetic Stability of the RMS

[0113] The E protein of the attenuated SA14-14-2 virus used to construct the YF/JE chimera differs from its virulent parent (SA14 or Nakayama) at 6 positions: 107, 138, 176, 177, 264, and 279. Because the presence of a single residue controlling virulence would be a disadvantage for any vaccine candidate because of the potential for reversion, studies are being undertaken to determine which residue(s) are responsible for attenuation and in particular whether a single residue is responsible for the difference.

1.18 Position 138 on the E Protein

[0114] A single mutation of an acidic residue glutamic acid (E) to a basic residue, lysine (K) at position 138 on the E protein of JE virus results in attenuation (Sumiyoshi et al., J. Infect. Dis. 171:1144, 1995). Experiments were carried out to determine whether the amino acid at position 138 of the JE envelope protein (K in the vaccine chimera and E in the virulent Nakayama chimera) is a critical determinant for neurovirulence in mice. Chimeric YF/JE SA14-14-2 (K 138→E) virus containing the single reversion of K→E at position 138 was generated from an engineered cDNA template. The presence of the substitution and the integrity of the entire E protein of the resulting virus was verified by RT/PCR sequencing of the recovered virus. A standard fixed-dose neurovirulence test of the virus was conducted in 4-week-old outbred mice by i.c. inoculation with 10^4 pfu of virus. The YF/JE SA14-14-2 and YF/JE Nakayama chimeric viruses were used as controls. The virulence phenotype of YF/JE SA14-14-2 (K→E) was indistinguishable from that of its attenuated parent YF/JE SA14-14-2 in this assay, with no morbidity or mortality observed in the mice during the observation period (FIG. 19).

[0115] We conclude that the single mutation at position 138 to the residue found in the JE-Nakayama virus does not exert a dominant effect on the neurovirulence of the YF/JE SA14-14-2 chimera, and that one or more additional mutations are required to establish the virulent phenotype.

1.19 Other Putative Attenuation Loci

[0116] Additional experiments to address the contributions of the other 6 residues (mentioned above) using the format described here were conducted. The mutant viruses constructed by site directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The E proteins of these viruses were sequenced and confirmed to contain the desired mutations. Upon inoculation into weanling mice by the I.C. route it is possible to determine those residues involved in attenuation of the vaccine.

[0117] Additional experiments to address the contributions of other residues are underway. The mutant viruses constructed to date by site-directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The methodology is as described above. Results to date confirm that at least two and possibly more than 2 mutations are responsible for the attenuation phenotype of YF/JE SA14-14-2 virus (Table 23).

1.20 Stability of the RMS in Tissue Cultures: Characterization of Genetic Changes, Neurovirulence and Immunogenicity, Serial Passages In Vitro

[0118] The RMS was used to inoculate a T75 flask of FRhL2 cells at an m.o.i. of 0.1. Subsequent passages were

carried out in T75 flasks and harvested 3 days post-inoculation. At each passage, the culture supernatant was assumed to hold 10^7 pfu/ml and an aliquot corresponding to an moi of approximately 0.1 was added to a fresh flask of cells. The remainder of the culture supernatant was stored at -80°C . for later characterization.

1.21 Quasispecies and DNA Sequencing

[0119] The chimeric JE vaccine is an RNA virus. Selective pressure can cause rapid changes in the nucleic acid sequences of RNA viruses. A mutant virus that invades FRhL cells more rapidly, for example, may gain a selective advantage by competing more effectively with the original vaccine virus and take over the culture. Therefore, mutant strains of the vaccine that grow better than the original vaccine may be selected by subculturing in vitro. One concern that addressed experimentally is whether such selective pressures might lead to mutant vaccine viruses with increased virulence.

[0120] In theory, molecular evolution should occur more rapidly for RNA viruses than DNA viruses because viral RNA polymerases have higher error rates than viral DNA polymerases. According to some measurements, RNA virus mutation rates approach one mutation per replication event. This is why an RNA virus can be thought of as a family of very closely related sequences (or “quasispecies”), instead of a single unchanging sequence (a “classical species”).

[0121] Two different approaches can be taken to determine the sequence of an RNA virus:

[0122] 1) purify viral genomic RNA from the culture supernatant, reverse-transcribe the RNA into cDNA and sequence this cDNA. This is the approach we have taken. It yields an averaged, or consensus sequence, such that only mutations which represent a large proportion (roughly, $>20\%$) of the viruses in the culture can be detected.

[0123] 2) Alternatively, cDNA can be cloned and individual clones sequenced. This approach would reveal the quasispecies nature of the vaccine by identifying individual mutations (deviations from the consensus sequence) in some proportion of the clones.

1.22 Biological Characterization of Serially Passaged RMS

[0124] As stated above, we demonstrated experimentally that the selective pressures exerted by serial passaging of the RMS does not lead to mutant vaccine viruses with increased virulence. Here, three biological properties of Passages 10 and 18 (P10 and P18) were examined. First, neurovirulence was tested by inoculating mice i.c. with graded doses of P1 as well as P10 and P18. Second, immunogenicity was compared by inoculating mice s.c. with graded doses of the RMS, P10 and P18. Blood was drawn from these mice 30 days post inoculation and serum neutralizing titers were determined and compared. Finally, the growth kinetics of the RMS and of P10 and P18 were compared by inoculating FRhL cells at moi's of 0.1 and 0.01 and collecting samples of culture supernatant daily. The titers in each flask were plotted as a function of time and compared.

1.23 Stability of prM and E Genes

[0125] The M and E genes of P10 and P18 were sequenced completely from base 642 to base 2454. Both sequences were identical and carried only one mutation (A $\rightarrow$ G) resulting an amino acid substitution from H to R at position 394 on the E protein. This means that selective pressures did not lead to the

loss of any of the attenuating mutations of the E gene. Codon H394 (CAC) encodes a Histidine in the RMS but we have found that the second base of this codon is mutated to a G in a significant proportion of the viruses, leading to the expression of Arginine. It is important to emphasize that a mixture of A and G are observed at this position in the sequence data. The ratio of A to G (A/G) was also determined for P1, P4, and P8. Interestingly, the ratio decreases steadily from P1 to P10, but at P18 it is back to the value seen at P8. One possible explanation for this observation is that a mutant bearing the H394R mutation gradually became as abundant as the original virus but was then out-competed by a new mutant bearing other mutations not present in the M or E genes and therefore, only detected as a rebound in the A/G ratio. We are reproducing these results by doing a second passaging experiment under identical conditions. It must also be noted that duplicate samples of viral genomic RNA were isolated, reverse-transcribed, amplified, and sequenced in parallel for each passage examined. Reported results were seen in both duplicate samples, arguing against any RT-PCR artifacts obscuring the data.

[0126] These observations show that minor genetic changes (one nucleotide substitution in the entire envelope E and M genes) have occurred in the JE sequences of the chimeric vaccine upon passaging, but that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene.

1.24 Neurovirulence Phenotype of Passages 10 and 18

[0127] Groups of five female ICR mice, 3 to 4 weeks-old, received 30 μl i.c. of undiluted, P1, P10, or P18, as well as 30 μl of 10-fold dilutions. None of the mice injected with P1, P10, or P18 (doses $\geq 7 \log_{10}$ pfu) showed any sign of illness over a five-week period. As determined by back-titration, the doses administered (pfu) were measured as shown in Table 17.

1.25 Immunogenicity of Passages 10 and 18

[0128] Groups of five female ICR mice were injected subcutaneously (s.c.) with 100 μl of undiluted virus stock of either the RMS or P10 or P18, as well as with doses of 10^5 and 10^4 pfu (see Table 18, results of back-titration).

1.26 Growth Kinetics of Passages 10 and 18

[0129] Monolayers (90% confluent) of FRhL cells were infected with an moi of 0.1 or 0.01 of RMS, P10, or P18. Time points were then taken daily for seven days and the titer of each time point was determined by plaque assay. Visual observation of cytopathic effects (CPE) on FRhL cells used in this growth curve experiment show that later passages of the RMS have different growth properties than the RMS itself. CPE is clearly greater for P18 and P10 than for the RMS at 4 days postinfection showing that these viruses might replicate much faster than the RMS.

[0130] Other observations also show that the growth properties of P10 and P18 differ from those of the RMS. The titers of P1, P10, and P18 are $\sim 2 \times 10^7$, 2×10^8 , and 3×10^8 , respectively. The relative yields of RT-PCR products suggest higher titers of P10 and P18 compared to P1. Although the PCR data are not necessarily quantitative, they are consistent with the observed titers.

[0131] These results raise the possibility that we have discovered a variant of the vaccine that is immunogenic, attenu-

ated for mouse neurovirulence, and that grows to titers ten-fold higher than the original vaccine (RMS) in tissue culture. Such a mutant may have value for manufacturing.

[0132] Finally, the sequences of the entire genomes of the RMS and p18 were determined and found to be identical, except for the E-H394 mutation (Table 25). There are 6 nucleotide (NT) differences (NT positions are shaded) between the published YF 17D sequences and RMS shown in bold letters. Changes in positions 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitution, whereas changes in positions 4025 (ns2a) and 7319 (ns4b) result in amino acid substitutions from V to M and from E to K, respectively. Amino acid Methionine (M) at position 4025 is unique for RMS and is not found in any other YF strains, including parent Asibi virus and other yellow fever 17D strains (e.g., 204, 213, and 17DD), whereas Lysine (K) at position 7319 is found in 17D204F, 17D213, and 17DD, but not in 17D204US or Asibi strain. Since the RMS is more attenuated than YF 17D with respect to neurovirulence, and thus has better biological attributes as a human vaccine, it is possible that the amino acid differences at positions 4025 and 7319 in the nonstructural genes of the yellow fever portion of the chimeric virus contribute to attenuation. Other workers have shown that the nonstructural genes of yellow fever virus play an important role in the attenuation of neurovirulence (Monath, "Yellow Fever," in Plotkin et al., (Eds.), *Vaccines*, 2nd edition, W.B. Saunders, Philadelphia, 1998).

1.27 Experiment to Identify Possible Interference Between YE 17D and YF/JE SA14-14-2

[0133] It is well-established that yellow fever virus encodes antigenic determinants on the NS1 protein that induce non-neutralizing, complement-fixing antibodies. Passive immunization of mice with monoclonal anti-NS1 antibodies confers protection against challenge. Active immunization with purified or recombinant NS1 protects mice and monkeys against lethal challenge. The mechanism of protection is presumed to involve antibody-mediated complement-dependent cytotoxicity.

[0134] In addition to protective determinants on NS1, CTL epitopes on other nonstructural proteins, including NS3, NS2a, and possibly NS5 may be involved in protection. Thus, infection with the YF/JE chimeric virus may stimulate humoral or cellular anti-yellow fever immunity. It is possible, therefore, that use of the chimeric vaccine may interfere with subsequent immunization against YF 17D, or that prior immunization with YF 17D may interfere with seroconversion to YF/JE SA14-14-2. Against this hypothesis is a substantial body of data showing that reimmunization with YF 17D results in a boost in yellow fever N antibodies. Those data show that it should be possible to successfully immunize against JE in an individual with prior YF immunity and vice versa.

[0135] To investigate possible interference effects, the experiment shown in Table 19 was initiated. Mice are immunized with one vaccine and subsequently boosted with the heterologous vaccine. Mice are bled every 30 days and sera tested for neutralizing antibodies against heterologous and homologous viruses.

1.28 Seroconversion Rate and Antibody Titers After Primary Immunization

[0136] Three groups (n=8) of 3-4 weeks old female outbred ICR mice were immunized with a single dose (5.3 log₁₀ pfu)

of CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins (YF/JE SA14-14-2), three groups (n=8) were immunized with two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (0.5 ml of a 1:5 dilution of reconstituted vaccine) and three groups (n=8) were immunized with a single dose of YF-VAX® (inactivated Japanese Encephalitis virus vaccine) (0.1 ml of a 1:2 dilution of reconstituted vaccine, containing 4.4 log₁₀ pfu, previously determined to induce the highest immune response to YF virus). Six groups (n=4) of mice (similar age, 3-4 weeks old) were kept as controls for booster doses at 3, 6, and 12 months post primary immunization.

[0137] All mice were bled 4 and 8 weeks after primary immunization and their neutralizing antibody titers were measured against homologous viruses in a plaque assay. 21/24 (87.5%) of the animals immunized with a single dose of CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) developed anti-JE neutralizing antibodies 1 month after immunization; at 2 months, 18/24 (75%) were seropositive. Geometric mean increased somewhat between 1 and 2 months post inoculation. In contrast, only 25%-33% of the mice immunized with YF-VAX® (Yellow Fever 17D vaccine) seroconverted and antibody responses were low. These results show that YF 17D virus and chimeric viruses derived from YF 17D are restricted in their ability to replicate in the murine host; however, when the envelope of JE virus is incorporated in the chimeric virus, the ability to replicate in and immunize mice is apparently enhanced. Mice receiving two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) developed high neutralizing titers against parent Nakayama virus, and titers increased between 1 and 2 months post immunization.

1.29 Secondary Immunization of CHIMERIVAX-JE™ (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis Virus prM and E Proteins) and JE-VAX® (Inactivated Japanese Encephalitis Virus Vaccine) Immunized Mice With YF-VAX® (Yellow Fever 17D Vaccine)

[0138] Three months and six months after primary immunization with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), mice were inoculated with YF-VAX® (Yellow Fever 17D Vaccine) (1:2 dilution of a human dose containing 4.4 log₁₀ pfu). Control mice not previously immunized and of identical age received CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) only or YF-VAX® (Yellow Fever 17D Vaccine) (Groups 10-13). One month later, mice were tested for presence of YF-specific neutralizing antibodies.

[0139] At the 3 month time point, none of the control mice or mice previously immunized with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) or JE-VAX® (inactivated Japanese Encephalitis virus vaccine) seroconverted to YF-VAX® (inactivated Japanese Encephalitis virus vaccine), again confirming the poor immunogenicity of YF-VAX® (inactivated Japanese Encephalitis virus vaccine) at the dose used. However, all mice immunized with YF-VAX® (inactivated Japanese Encephalitis virus vaccine) 6 months after primary immunization with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and 7/8 mice previously immunized with JE-VAX® (inactivated Japanese Encephalitis virus vaccine), seroconverted after immunization with YF-VAX® (Yellow

Fever 17D Vaccine) (Table 24). There was no difference in seroconversion rate or GMT in mice with and without prior immunization with either JE vaccine.

1.30 Secondary Immunization of YE-VAX® (Yellow Fever 17D Vaccine) Immunized Mice with CHIMERIVAX-JE™ (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis virus prM and E Proteins)

[0140] All mice previously immunized with YF-VAX® (Yellow Fever 17D Vaccine) and reimmunized with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) 3 months later developed neutralizing antibodies to JE (group 7, Table 10). None of the controls seroconverted. Five of 6 mice (83%) previously immunized to YF-VAX® (Yellow Fever 17D Vaccine) and reimmunized with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) 6 months later seroconverted to JE (group 8, Table 10, as did all controls (group 13)), and the GMTs were similar across these groups.

[0141] There was no evidence for cross-protection between YF and JE viruses or limitation of antibody response to sequential vaccination with these viruses. Yellow fever 17D vaccine elicits a poor antibody response in the mouse; while this limited interpretation of the data somewhat, it provided a sensitive test of any restriction in replication and immunogenicity of YF 17D virus in mice previously immunized with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). The fact that all mice immunized with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) responded 6 months later to immunization with YF-VAX® (Yellow Fever 17D Vaccine) and that the GMT and range of neutralizing antibody titers were similar to controls suggests that the chimeric vaccine imposed no significant barrier to yellow fever immunization.

2.0 Construction of cDNA Templates for Generation of Yellow Fever/Dengue (YF/DEN) Chimeric Viruses

[0142] Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is described as follows, which, in principle, is carried out the same as construction of the YF/JE chimeras described above. Other flavivirus chimeras can be engineered with a similar strategy, using natural or engineered restriction sites and, for example, oligonucleotide primers as shown in Table 20.

2.1 Construction of YF/DEN Chimeric Virus

[0143] Although several molecular clones for dengue viruses have been developed, problems have commonly been encountered with stability of viral cDNA in plasmid systems, and with the efficiency of replication of the recovered virus. We chose to use a clone of DEN-2 developed by Dr. Peter Wright, Dept. of Microbiology, Monash University, Clayton, Australia, because this system is relatively efficient for regenerating virus and employs a two-plasmid system similar to our own methodology. (See Table 21 for a comparison of the sequences of Dengue-2 and YF/Den-2₂₁₈ viruses; YF/Den-2₂₁₈ contains the nucleotide and amino acid sequences of PUO-218. The NGC and PR-159 strains, which are also listed in Table 21, are other wild strains of dengue that differ from PUO-218 and can be used in the chimeras of the invention.) The complete sequence of this DEN-2 clone is available and facilitated the construction of chimeric YF/DEN templates because only a few modifications of the YF clone were required. The relevant steps are outlined as follows.

[0144] Similar to the two plasmid system for YF5.2iv and YF/JE viruses, the YF/DEN system uses a unique restriction site within the DEN-2 envelope protein (E) as a breakpoint for propagating the structural region (prM-E) within the two plasmids, hereinafter referred to as YF5'3'IV/DEN (prM-E') and YFM5.2/DEN (E'-E) (see FIG. 20). The two restriction sites for in vitro ligation of the chimeric template are AatII and SphI. The recipient plasmid for the 3' portion of the DEN E protein sequence is YFM5.2(NarI[+]SphI[-]). This plasmid contains the Nan site at the E/NSI junction, which was used for insertion of the carboxyl terminus of the JE E protein. It was further modified by elimination of an extra SphI site in the NS5 protein region by silent site-directed mutagenesis. This allowed insertion of DEN-2 sequence from the unique SphI site to the NarI site by simple directional cloning. The appropriate fragment of DEN-2 cDNA was derived by PCR from the DEN-2 clone MON310 furnished by Dr. Wright. PCR primers included a 5' primer flanking the SphI site and a 3' primer homologous to the DEN-2 nucleotides immediately upstream of the signalase site at the E/NSI junction and replacing the signalase site by substitutions that create a novel site, but also introduce a NarI site. The resulting 1,170 base-pair PCR fragment was then introduced into YFM5.2(NarI[+]SphI[-]).

[0145] The 5' portion of the YF/den-2 clone, including the C/prM junction was engineered by PCR. The C/prM junction was created by incorporating a TfiI restriction site at the junction using synthetic oligonucleotides. A 5' PCR fragment encompassing the flanking YF sequence 5' untranslated and capsid sequence and a 3' TfiI site, together with a 3' PCR fragment beginning with a TfiI site at the amino terminus of the dengue-2 prM protein and the flanking dengue-2 prM protein sequence, were ligated into the YF5'3'IV plasmid after intermediate construction in pBluescript. Screening with TfiI was used to confirm correct assembly of the chimeric junction in the final plasmid YF5'3'IV/DEN(prM-E).

2.2 Construction of Chimeric YF/DEN Viruses Containing Portions of Two DEN Envelope Proteins

[0146] Since neutralization epitopes against DEN viruses are present on all three domains of the E protein, it is possible to construct novel chimeric virus vaccines that include sequences from two or more different DEN serotypes. In this embodiment of the invention, the C/prM junction and gene encoding the carboxyl terminal domain (Domain III) of one DEN serotype (e.g., DEN-2) and the N-terminal sequences encoding Domains I and II of another DEN serotype (e.g., DEN-1) are inserted in the YF 17D cDNA backbone. The junctions at C/prM and ENS1 proteins are retained, as previously specified, to ensure the infectivity of the double-chimeras. The resulting infectious virus progeny contains antigenic regions of two DEN serotypes and elicits neutralizing antibodies against both.

2.3 Transfection and Production of Progeny Virus

[0147] Plasmid YF5'3'IV/DEN(prME) and YFM5.2/DEN (E'-E) were cut with SphI and AatII restriction enzymes, appropriate YF and dengue fragments were isolated and ligated in vitro using T4 DNA ligase. After digestion with XhoI to allow run-off transcription, RNA was transcribed (using 50 ng of purified template) from the SP6 promoter and its integrity was verified by non-denaturing agarose gel electrophoresis. Vero cells were transfected with YF/Den-2 RNA

using Lipofectin (Gibco/BRL), virus was recovered from the supernatants, amplified twice in Vero cells, and titrated in a standard plaque assay on Vero cells. The virus titer was 2×10^6 PFU/ml.

2.4 Nucleotide Sequencing of YF/Den-2 Chimera

[0148] Vero cells were infected with YF/DEN-2 (clone 5.75) at an MOI of 0.1. After 96 hours, cells were harvested with Trizol (Life Technologies, Inc.). Total RNA was primed with a YF-5' end NS1 minus oligo, and reverse transcribed with Superscript II RT following a long-RT protocol (Life Technologies, Inc.). Amplification of cDNA was achieved with XL-PCR kit (Perkin Elmer). Several primers specific for dengue type 2 strain PUO-218 were used in individual sequencing reactions and standard protocols for cycle sequencing were performed. Sequence homology comparisons were against the PUO-218 strain prME sequence (GenBank accession number D00345).

[0149] Sequencing showed that the YF/DEN-2 chimera prME sequence is identical to that of PUO-218 (Gruenberg et al., J. Gen. Virol 69:1391-1398, 1988). In addition, a NarI site was introduced at the 3' end of E, resulting in amino acid change Q494G (this residue is located in the transmembrane domain and not compared in Table 21). In Table 21, amino acid differences in the prME region of YF/Den2 is compared with prototype New Guinea C (NGC) virus and the attenuated dengue-2 vaccine strain PR-159 S1 (Hahn et al., Virology 162:167-180, 1988).

2.5 Growth Kinetics in Cell Culture

[0150] The growth kinetics of the YF/Den-2 chimera were compared in Vero and FeRhL cells (FIG. 16). Cells were grown to confluency in tissue culture flask (T-75). FeRhL cells were grown in MEM containing Earle's salt, L-Glu, non-essential amino acids, 10% FBS and buffered with sodium bicarbonate, and Vero cells were grown in MEM-Alpha, L-Glu, 10% FBS (both media purchased from Gibco/BRL). Cells were inoculated with YF/Den2 at 0.1 MOI. After 1 hour of incubation at 37° C., medium containing 3% FBS was added, and flasks were returned to a CO₂ incubator. Every 24 hours, aliquots of 0.5 ml were removed, FBS was added to a final concentration of 20%, and frozen for determination of titers in a plaque assay. Forty-eight hours post infection CPE was observed in FeRhL cells and reached 100% by day 3. In Vero cells, CPE was less dramatic and did not reach 100% by the completion of the experiment (day 5). As shown, the YF/Den2 reached its maximum titer ($7.4 \log_{10}$ pfu/ml) by day 3 and lost about one log ($6.4 \log_{10}$ pfu/ml) upon further incubation at 37° C., apparently due to death of host cells and virus degradation at this temperature. The maximum virus titer in Vero cells was achieved by day 2 ($7.2 \log_{10}$ pfu/ml) and only half log virus ($6.8 \log_{10}$ pfu/ml) was lost on the following 3 days. This higher rate of viable viruses in Vero cells may be explained by incomplete CPE observed in these cells. In sum, the chimera grows well in approved cell substrate for human use.

2.6 Neurovirulence Phenotype in Suckling Mice

[0151] Although wild-type unpassaged dengue viruses replicate in brains of suckling mice and hamsters inoculated by the intracerebral route (Brandt et al., J. Virol 6:500-506, 1970), they usually induce subclinical infection and death occur only in rare cases. However, neurovirulence for mice

can be achieved by extensive passage in mouse brain. Such neuroadapted viruses can be attenuated for humans. For example, the New Guinea C (NGC), the prototype dengue 2 virus isolated in 1944 and introduced into the Americas in 1981, is not neurovirulent for suckling mice; however after sequential passage in mouse brain it became neurovirulent for mice, but was attenuated for humans (Sabin, Am. J. Trop. Med. Hyg., 1:30-50, 1952; Sabin et al., Science 101:640-642, 1945; Wisseman et al., Am. J. Trop. Med. Hyg. 12:620-623, 1963). The PUO-218 strain is a wild type dengue 2 virus isolated in 1980 epidemic in Bangkok. It is closely related to the NGC strain by nucleotide sequencing (Gruenberg et al., J. Gen. Virol 69:1391-1398, 1988). When the prME genes of the PUO-218 strain were inserted into the neuroadapted NGC backbone, the chimeric virus was attenuated for 3-days old mice inoculated by the I.C. route (Peter Wright, Xth International Congress of Virology, Jerusalem, Israel, 1996). The PUO218 virus differs from NGC in one amino acid in prM (residue 55 is F in NGC and is L in PUO218) and 6 amino acids in the E protein (71 D→E, 126K→E, 141I→V, 164 I→V, 402I→F, and 484 V→I) (see Table 21). All amino acid differences (except residue E-126) are also present in PR S1 strain (attenuated vaccine strain), indicating that they may not be involved in attenuation. Only residue 126 on the E protein is different between these viruses. This residue was shown to be responsible for the neurovirulent phenotype of the mouse adapted NGC (Bray et al., J. Virology 72:1647-1651, 1998). Although mouse neurovirulence does not predict virulence/attenuation of dengue viruses for humans, it is important to determine the neurovirulence of a YF/Den-2 chimeric virus. YF 17D retains a degree of neurotropism for mice, and causes (generally subclinical) encephalitis in monkeys after IC inoculation. For vaccine development of a den/YF chimera it will be necessary to show that the construct does not exceed YF 17D in neuroinvasiveness and neurovirulence. Ultimately safety studies in monkeys will be required. In initial studies, we determined if insertion of the prME of the PUO218 into YF 17D vaccine strain will affect its neurovirulence for suckling mice (Table 24). Groups of 3, 5, 7, and 9 days old suckling mice were inoculated by the I.C. route with 10,000 pfu of YF/Den-2 or YF/JE SA14-14-2 chimera and observed for paralysis or death for 21 days. For controls similar age groups were inoculated either sham with medium (I.C. or I.P.) or with 1,000 pfu of unpassaged commercial YF vaccine YF-VAX® (Yellow Fever 17D Vaccine) by the I.P. route (it is not necessary to inoculate suckling mice with YF-VAX® (Yellow Fever 17D Vaccine) by the I.C. route because we have previously shown that this vaccine is virulent for 4-weeks old mice by this route).

[0152] As shown in FIG. 22, all suckling mice (3 to 7 days old) inoculated by the I.C. route with the YF/Den2 chimera died between 11 and 14 days post inoculation, whereas 8 out of 10 suckling mice (9 days old) survived. Similarly, all suckling mice (3-5 days old) inoculated with YF-VAX® (Yellow Fever 17D Vaccine) by the I.P. route, with a dose which was 10-fold lower than the YF/Den2 chimera, died between 11 to 13 days post inoculation (FIG. 23). All nine day old, as well as 8 out of 9 seven day old, mice inoculated with the YF-VAX® (Yellow Fever 17D Vaccine) survived. Similar results to the YF/Den2 chimera obtained with suckling mice inoculated with the YF/JE SA14-14-2 chimera.

[0153] As is mentioned above, when prME genes of the PUO218 strain were inserted into the NGC backbone the chimeric virus was not neurovirulent for 3 days old suckling

mice inoculated by the I.C. route. In contrast, when these genes were inserted into the 17D backbone, the resulting YF/Den2 chimera demonstrated a neurovirulence phenotype (for suckling mice) similar to the YF/ JE SA14-14-2. This experiment also demonstrated that the replacement of the prME genes of the YF 17 D with prME genes of the Dengue 2 PUO218 resulted in a chimeric virus that was less neurovirulent than the 17D parent strain.

[0154] Unlike most flaviviruses, there is no correlation between neurovirulence of dengue viruses in mice and humans. Currently the most suitable animal models for dengue infection are Old World monkeys, New World monkeys, and apes that develop subclinical infection and viremia. There is, however, no animal model for the most severe illness (DHF) in humans, which occurs when individuals become infected with a heterologous serotype due to antibody dependent enhancement of infection. Today it is generally accepted that a tetravalent vaccine is required to induce protective immunity in human beings against all four serotypes to avoid sensitizing vaccinee to more severe illness DHF. For the last fifty years, many approaches have been undertaken to produce effective dengue vaccines and although dengue viruses have been satisfactory attenuated (e.g., PR-159/S-1 for Dengue 2) in many cases in vitro or in vivo correlation of attenuation were not reproducible in humans. A current strategy is to test selected live virus vaccine candidates stepwise in small numbers of human volunteers. Many laboratories around the world are exploring various strategies to produce suitable vaccine candidates. These range from subunit vaccines including prME (protein vaccine or DNA vaccine) of dengue viruses to live attenuated whole viruses (produced by tissue culture passage or recombinant DNA technology). Although some of these candidates have shown promise in preclinical and human volunteers, development of a successful dengue vaccine remained to be implemented.

[0155] Evaluating the immunogenicity and protective efficacy of the YF/Den2 chimera in monkeys should shed light on selection of appropriate prME genes (from wild type or attenuated strain) for construction of all 4 serotypes of chimeric dengue viruses.

2.7 Stability of prME Genes of CHIMERIVAX-DEN2™ (Chimeric Flavivirus Vaccine Comprising Dengue 2 prM and E Proteins) Virus In Vitro

[0156] The CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) virus at passage 2 post transfection was used to inoculate a 25 cm² flask of Vero cells. Total RNA was isolated and the complete nucleotide sequence of the CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) was determined (P3) and compared to the published sequence of the YF 17D virus (Rice et al., Science 229:726-733, 1985). There was one nucleotide difference: at position 6898 there was an A in the chimera (P3), which was a C in the 17D nucleotide sequence. No difference in the prME region was found when the sequence of CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) was compared to its parent dengue 2 virus (PUO218 strain). Also, no mutations were found in the prME genes of the chimera upon 18 passages in VeroPM cells. Within the YF genes, however, there was one silent mutation in position 6910 (C to A), and at position 3524 the P18 virus appeared to be heterozygous (both parent nucleotides, G and mutant A, were present). This would translate into a mixture of E and K amino acids at position 354 of the NS1 protein.

[0157] Similar to the passage 3 virus, the passage 18 virus was not neurovirulent for 4 week old outbred mice inoculated by the IC route (5 log₁₀ pfu was the highest dose tested). Passage 3, passage 5, passage 10, and passage 18 of CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) were inoculated into mice by SC and IC routes, and antibody responses were compared. There were no significant differences in production of anti-dengue 2 neutralizing antibodies across 18 passages (Table 26).

2.8 Viremia, Immunogenicity, and Protective Efficacy of CHIMERIVAX-DEN2™ (Chimeric Flavivirus Vaccine Comprising Dengue 2 prM and E Proteins) in YF Immune Monkeys

[0158] Because CHIMERIVAX™ (chimeric flavivirus vaccines)-viruses contain core and NS genes of the YF 17D virus, it is important to determine if preimmunity to the 17D vaccine interferes with vaccination with CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) virus. As is discussed above, in the case of CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), there was no significant interference between YF 17D immunity and CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), virus, measured by production of neutralizing antibodies in mice.

[0159] Since YF17D vaccine and both CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) were not highly immunogenic in mice inoculated by the SC route (see also table 26), non-human primates were used, which are more susceptible/relevant for evaluation of flavivirus vaccines for humans. Sixteen rhesus monkeys (some of which were previously immunized with YF 17D vaccine) received CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins), YF 17D vaccine, or a wild type dengue 2 virus (strain S16803). As is shown in Table 27, all YF immune monkeys seroconverted to the YF/dengue 2 or wild type dengue 2 virus, demonstrating a lack of vector immunity. These monkeys were also protected from viremia after challenge with wild type dengue 2 virus. In contrast, YF 17D immunized monkeys, as well as non-immunized animals, became viremic after challenge with wild type dengue 2 virus. Wild type dengue 2 viruses produce a high level of viremia (3-5 logs) in rhesus monkeys, which lasts between 3-6 days. Attenuation of dengue 2 viruses can therefore be estimated by comparing the level and duration of viremia with reference wild-type strains. These experiments clearly showed that core and non-structural proteins of YF 17D virus present in CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) do not interfere with CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) immunization.

2.9 Dose Response Effectiveness of CHIMERIVAX-DEN2™ (Chimeric Flavivirus Vaccine Comprising Dengue 2 prM and E Proteins) in Monkeys

[0160] The goals of this experiment were to (i) determine the viremia profile of the vaccine candidate, using YF 17D

and wild type dengue 2 virus controls, (ii) compare neutralizing antibody responses to the vaccine candidate and wild-type virus, and (iii) determine minimum dose required for protection against challenge with wild type dengue-2 virus. It was anticipated that these experiments would define the viremia profile of the CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) virus in non-YF immune monkeys, and would determine whether immunization with a single dose results in protection of animals against challenge with a wild type dengue 2 virus. Protection in these experiments is defined as reduction of viremia in test monkeys compared to control viruses.

[0161] As is shown in table 28, all monkeys became viremic, and the duration of viremia was dose-dependent. The peak level of viremia for CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) was between 1.3 to 1.6 log₁₀ pfu, which was significantly lower than that of the wild type dengue virus (3.6 log₁₀ pfu).

[0162] All monkeys developed anti-dengue 2 neutralizing antibodies by day 15. Lower dose of the vaccine resulted in lower GMTs, however, by day 30 post-immunization, all monkeys developed high titers of neutralizing antibodies, independent of the dose they received. Upon challenge, no viremia was detected in any immunized monkeys, demonstrating that even at its lowest dose (2 log₁₀ pfu), CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) had protected these animals from dengue infection (Table 29).

2.10 Growth Characteristics of CHIMERIVAX-JE™ (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis Virus prM and E Proteins) Virus in *Culex Tritaeniorhynchus*, *Aedes Albopictus*, and *Aedes Aegypti* Mosquitoes

[0163] As is described above, CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), which consists of a YF 17D virus backbone containing the prM and E genes from the JE vaccine strain SA14-14-2, exhibited restricted replication in non-human primates, producing only a low level viremia following peripheral inoculation. Although this reduces the likelihood that hematophagous insects could become infected by feeding on a vaccinated host, it is prudent to investigate the replication kinetics of the vaccine virus in mosquito species that are known to vector the viruses from which the chimera is derived. In this study, CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), virus was compared to its parent viruses (YF17D and JE SA14-14-2), as well as to wild type JE SA14 virus, for its ability to replicate in *Culex tritaeniorhynchus*, *Aedes albopictus*, and *Aedes aegypti* mosquitoes. Individual mosquitoes were exposed to the viruses by intrathoracic (IT) virus inoculation or by oral ingestion of a virus-laden blood meal.

2.11 Intrathoracic Inoculation

[0164] Mosquitoes were inoculated 7-10 days post-emergence with 0.34 ml of approximately 6.0 log₁₀ pfu/ml virus suspension (~5.5 log₁₀ pfu/mosquito). This route of inoculation was chosen to avoid variables, such as threshold titer, that might limit midgut infection and subsequent dissemination of the viruses. Three mosquitoes per virus were collected either at 24 hour intervals for 5-10 days or at 72 hour intervals for 18 days. Individual mosquitoes were triturated in 1 ml of M199

media (Gibco BRL, Grand Island, N.Y.) supplemented with 5% fetal calf serum, clarified by brief centrifugation, and then titrated in Vero cells to monitor virus replication.

[0165] Both JE SA14 and JE SA14-14-2 viruses replicated in *Cx. tritaeniorhynchus* following IT inoculation, reaching titers at day 14 of 6.7 and 6.0 log₁₀ pfu/mosquito, respectively (FIG. 24A). Additionally, IFA conducted on head squashes from JE SA14 and JE SA14-14-2-inoculated *Cx. tritaeniorhynchus* mosquitoes was positive for detection of JE virus antigen. In contrast, YF 17D and CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) did not replicate in *Cx. tritaeniorhynchus* mosquitoes. Virus titers declined rapidly following inoculation, and no virus was detectable by plaque titration assay in YF 17D or CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)-inoculated mosquitoes by days 1 and 2, respectively (FIG. 24A). IFA analysis of head squashes from *Cx. tritaeniorhynchus* mosquitoes inoculated with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) or YF 17D was negative for JE or YF virus antigens, supporting our observation that neither the chimera nor YF 17D replicate in this mosquito species.

[0166] CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) did replicate in IT-inoculated *Ae. albopictus* mosquitoes, reaching a titer of 5.2 log₁₀ pfu/mosquito at day 18 (FIG. 24B) and IFA results were weakly positive for both JE virus and YF virus antigens. The JE SA14 and JE SA14-14-2 viruses also replicated in *Ae. albopictus* mosquitoes, reaching maximum titers of 6.3 and 6.0 log₁₀ pfu/mosquito, respectively. YF 17D virus did not replicate to high titers in *Ae. albopictus* mosquitoes, however, a low level of detectable virus was maintained (3.8 log₁₀ pfu/mosquito at day 18) (FIG. 24B) and IFA-stained head squashes were weakly positive for YF virus antigen. CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and YF 17D inoculated IT into *Ae. aegypti* mosquitoes replicated at low levels over the 18 day incubation period (FIG. 24C). Peak titers of 3.6 and 4.4 log₁₀ pfu/mosquito, respectively, were reached on day 15. IFA staining of head tissues from CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and YF 17D IT-inoculated mosquitoes was weakly positive. Both JE SA14 and JE SA14-14-2 viruses proliferated in *Ae. aegypti* mosquitoes, reaching peak titers of 6.3 and 6.1 log₁₀ pfu/mosquito on days 9 and 18, respectively.

2.12 Oral Infection

[0167] Seven to ten day old *Ae. albopictus* and *Ae. aegypti* mosquitoes were orally exposed to an artificial virus-containing blood meal that was prepared from equal parts of washed calf red blood cells (Colorado Serum Company, Denver, Colo.) and freshly harvested virus. The blood/virus mixture was heated to 37° C. immediately prior to feeding. Mosquitoes were starved for 48-72 hours prior to feeding on virus/blood soaked cotton pledgets. *Cx. tritaeniorhynchus* mosquitoes are reluctant to feed from blood-soaked pledgets, and were therefore fed using a membrane feeder. Mosquitoes were allowed to feed for 15-30 minutes, after which fully engorged mosquitoes were collected. Three mosquitoes per virus were harvested at 48-72 hour intervals over a 15-18 day

period, or, in a second experiment, all mosquitoes were harvested at 22 days after feeding.

[0168] FIG. 25A illustrates growth of the viruses in orally exposed *Cx. tritaeniorhynchus* mosquitoes. Individuals that were fed a blood meal containing $6.9 \log_{10}$ pfu/ml CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) virus did not become infected. Similar results were observed in mosquitoes that had ingested a blood meal containing YF 17D virus. In contrast, high virus titers were detected in *Cx. tritaeniorhynchus* mosquitoes that had ingested JE SA14 or JE SA14-14-2 viruses.

[0169] FIGS. 25B and 25C illustrate growth of the viruses in orally exposed *Ae. albopictus* and *Ae. aegypti* mosquitoes, respectively. Only JE SA14 and JE SA14-14-2 viruses successfully infected and replicated in these species. For example, in *Ae. aegypti* mosquitoes on day 15, the titers of JE SA14 and JE SA14-14-2 viruses were 5.4 and 5.5 $\log_{10}$ pfu, respectively. In contrast, mosquitoes that had ingested 4.7 $\log_{10}$ pfu/mosquito of YF17D virus or 4.5 $\log_{10}$ pfu/mosquito of CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) virus failed to become infected.

[0170] In a separate experiment, *Ae. aegypti* and *Ae. albopictus* mosquitoes were orally exposed to JE SA14-14-2, YF 17D, and CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) viruses and processed after 22 days extrinsic incubation to permit growth to maximum virus titers. The results of this experiment are summarized in Table 30. Only JE SA14-14-2 virus was detectable in mosquitoes. Because CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) did not grow in any of the mosquito species tested, transmission studies were not performed.

[0171] Viruses recovered from *Ae. Albopictus* after IT or oral inoculation, or from *Ae. Aegypti* after IT inoculation, were identical to their parent CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) virus (Vero2FrhL1) in the prME region. 2.13 Amplification and Sequencing of the "Late Replicating" CHIMERIVAX-JE™ (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis Virus prM and E Proteins) Viruses Isolated from Mosquitoes

[0172] *Ae. albopictus* mosquitoes inoculated with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by IT or oral routes and *Ae. aegypti* inoculated with CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by IT route, were harvested on day 15 post-inoculation. After triturating in 1 ml of M199 (supplemented with 5% fetal calf serum), samples were clarified by centrifugation, filtered through a 0.2 micron filter, and used to inoculate a T-25 cm² flask of VeroPM cells, passage 144 (0.5 ml/flask). After 1 hour, virus adsorption at 37° C., 5 ml MEM-containing 5% FBS was added and flasks returned to the 37° C. CO₂ incubator. Viruses were harvested from supernatants 4 days later (at 2+CPE) and kept frozen at -70° C. Total RNA was extracted from infected monolayers by the use of the Trizol® reagent (Gibco/BRL), reverse-transcribed, amplified by XL PCR (Perkin-Elmer), and the prME region was sequenced. Viruses recovered from *Ae. Albopictus* after IT or oral inoculation, or from *Ae. Aegypti* after IT inoculation, were identical to their parent CHIMERIVAX-JE™ (chi-

meric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) virus (Vero2FrhL1) in the prME region.

2.14 Growth Characteristics of CHIMERIVAX-DEN2™ (Chimeric Flavivirus Vaccine Comprising Dengue 2 prM and E Proteins) Virus in *Aedes Albopictus* and *Aedes Aegypti* Mosquitoes

[0173] Similar experiments were carried out in *Ae. albopictus* and *Ae. aegypti* mosquitoes with CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) virus. For controls, the YF17D vaccine and a dengue 2 wild type virus were used. Dengue 2 wild type virus grew to more than 5 $\log_{10}$ pfu/ml in both mosquito species inoculated by IT or oral routes. The growth of YF17D vaccine was lower than the wild type dengue 2 virus, and did not exceed 4 $\log_{10}$ pfu/ml. Interestingly, the CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) virus revealed the most restricted growth in both mosquito species inoculated by either route (its titer did not exceed 3 $\log_{10}$ pfu/ml) (FIG. 26).

2.15 Summary of JE and Dent Experiments in Mosquitoes

[0174] In summary, CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) virus did not replicate following ingestion by any of the three mosquito species. Additionally, replication was not detected after IT inoculation of CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) in the primary JE virus vector, *Cx. tritaeniorhynchus*. CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) exhibited moderate growth following IT inoculation into *Ae. aegypti* and *Ae. albopictus* mosquitoes, reaching titers of 3.6-5.0 $\log_{10}$ pfu/mosquito. There was no change in the virus genotype associated with replication in mosquitoes. Similar results were observed in mosquitoes of all three species that were IT inoculated or had orally ingested the YF 17D vaccine virus. In contrast, all mosquitoes either IT inoculated with, or orally fed, wild type and vaccine JE viruses became infected, reaching maximum titers of 5.4-7.3 $\log_{10}$ pfu/mosquito. The growth of CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) in both *Ae. albopictus* and *Ae. aegypti* mosquitoes inoculated by IT or oral routes was also significantly lower than its parent wild type dengue 2 and YF 17D vaccine viruses.

[0175] These results showed that CHIMERIVAX-JE™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and CHIMERIVAX-DEN2™ (chimeric flavivirus vaccine comprising Dengue 2 prM and E proteins) viruses are restricted in their abilities to infect and replicate in these mosquito vectors. The low viremia caused by the viruses in primates and poor infectivity for mosquitoes are safeguards against secondary spread of the vaccine virus.

[0176] 3.0 Construction of CHIMERIVAX-DEN1™ (Chimeric Flavivirus Comprising Dengue 1 prM and E Proteins)

[0177] A yellow fever/dengue 1 (YF/DEN-1) chimeric virus was constructed using a novel technology, which differs from the approaches used to construct Yellow fever/Japanese encephalitis (YF/JE) chimeric viruses as described by Chambers et al. (J. Virol. 73:3095-4101, 1999; see above), and the construction of YF/DEN-4 chimera (see below). We used the

same two plasmid system used to create YF/DEN-4. These plasmids first encoded the yellow fever (YF) genome as created by Rice et al. (New Biol. 1:285-296, 1989). Later, the structural membrane precursor and envelope protein genes, i.e., the prME region, of the YF genome plasmids was replaced with those of the JE SA14-14-2 sequence, and the resulting plasmids were used to produce RNA in vitro, which was then transfected into cells to produce live YF/JE chimeric virus. Although the two-plasmid system was suitable for the construction of JE, DEN-2, and DEN-4 chimeras in a YF backbone, the marked instability of one of the plasmids created with DEN-1 sequences was such that we opted for a PCR alternative to replace it.

[0178] Here we describe in detail the procedures for construction of the YF/DEN-1 chimera (FIGS. 27 and 28). Dengue 1 (strain PU0359 isolated in 1980 in Thailand) was passed once in C6/36 and total RNA was isolated. The dengue 1 prME region was first amplified and sequenced using primers derived from consensus sequences (Genbank). The sequence data created was applied to primer design and was used, with the cDNA produced earlier, as a PCR starting point for assembly of chimeric YF/DEN-1 virus. A dengue 1 PCR product encoding prM and the 5' end of E was then used as a template, along with template encoding the capsid (C) of yellow fever derived from plasmid pYF5'3'IV/JE SA 14-14-2, in an overlap extension PCR to result in a single fusion product, which was then cloned into a vector fragment of pYFM5'3'IV in which JE sequences were deleted. In contrast to the construction of the YF/DEN-4 (see below) and YF/JE SA 14-14-2 (see above) plasmid systems, the 3' end of the DEN-1 envelope was fused to the YF non-structural genes normally present in the pYFM5.2/JE SA 14-14-2 plasmid using an overlap extension PCR similar to that used to construct the fusion of YF capsid to the DEN-1 prM and envelope gene 5' end. Next, the pYD1-5'3' plasmid was transformed into *E. coli* strain MC 1061 (RecA-) for amplification, followed by midi-scale plasmid purification, while the DEN-1Env/YF5.2 (Fragment H) was gel purified. In vitro ligation of the plasmid to the PCR product resulted in full-length virus cDNA template for RNA transcription. All steps involving cDNA fragments, plasmids, and PCR products were carried out in a BL-2 lab designated for recombinant DNA work. Steps involving manipulations of infectious RNA and virus were carried out in a limited access BL-2+ virus lab.

3.1 Amplification of Dengue 1 Sequence

[0179] Dengue 1 cDNA was synthesized from RNA using the Superscript II™ method. All primers for this experiment were synthesized by Life Technologies and are listed in Table 31. Upon arrival as lyophilized material, they were dissolved to 250 μM stock solutions using RODI-water. From this, 25 μM working solutions were made. The fragment encoding the SP6 promoter and the yellow fever capsid (Fragment A) was amplified using XL-PCR Reaction Kit™ (Perkin-Elmer Part#N808-0192), with 0.5 μl (250 ng) of pYF5'3'IV plus 3.5 μl RODI-water as template and primers 1 and 2 (see Table 31). The fragment encoding dengue 1 prM and 5' end of E (Fragment B) was amplified using the XL-PCR Reaction Kit™ (Perkin-Elmer Part#N808-0192) and primers 3 and 4. The fragment encoding the 3' end of the Dengue 1 envelope gene (Fragment F) was amplified using the same protocol, but with primers 5 and 7. The fragment encompassing the YF portion of pYFM5.2 (Fragment G) was amplified using the same protocol, but with primers 8 and 9 and 1 μl of pYFM5.2/2 with

39 μl water. The PCR for fragments F and G required an annealing temperature of 50 C and an extension time of 6.5 minutes. The PCR reaction was performed using the following master mixes for each reaction.

Upper Mix (UM)	
3.3 × buffer	18 μl
RTth	1.25 μl
H ₂ O	1 μl
Volume	20.25 μl
Lower Mix (LM)	
3.3 × buffer	12 μl
10 mM dNTPs	8 μl
5' primer	1 μl
3' primer	1 μl
Mg(OAc) ₂	4 μl
H ₂ O	14 μl
Volume	40 μl
cDNA Mix	
H ₂ O	36 μl
Dengue 1 cDNA	4 μl
Volume	40 μl

[0180] The LM was added to a Perkin-Elmer thin-walled 0.2 ml tube. Next, Ampliwax 100 (Perkin-Elmer) was added to the tube, which was then placed in a Perkin-Elmer 2400 Thermal Cycler and heated to 80° C. for 5 minutes, and then cooled to 4° C. The cDNA and UM were then added to the top of the wax layer. The tube was then cycled in a Perkin-Elmer 2400 as follows: 94° C., 1 minute; repeat 30× (94° C., 15 seconds; 53° C., 15 seconds; 68° C., 3 minutes), 72° C., 4 minutes; 4° C., hold. The expected sizes for the fragments are as follows.

Fragment	Approximate Size (kb)
A	0.94
B	0.65
F	1.3
G	6.0

[0181] Forty μl of each fragment was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentrations of the purified fragments were determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment A	0.0116	0.0260	0.4453	2.2457	52 ng/μl
Fragment B	0.0076	0.0202	0.3782	2.6440	40.4 ng/μl
Fragment F	0.0160	0.0335	0.4785	2.0898	67 ng/μl
Fragment G	0.0199	0.0380	0.5242	1.9076	76 ng/μl

3.2 Recombinant PCR

[0182] To create a fusion between the yellow fever capsid and DEN-1 prM, a recombinant PCR technique known as

overlap-extension PCR was used to create Fragment E. The same basic UM and LM were used, and primers 1 and 4 replaced earlier primers. The same approach was used to create a fusion between fragment F and G, resulting in fragment H. For this, primers 5 and 9 were used. The cDNA mixes were as follows:

	Fragment E	Fragment B control	Fragment A control
H ₂ O	37.82 μ l	38.97 μ l	38.85 μ l
Fragment A	1.15 μ l	0 μ l	1.15 μ l
Fragment B	1.03 μ l	1.03 μ l	0 μ l
Volume	40 μ l	40 μ l	40 μ l

	Fragment H	Fragment F control	Fragment G control
H ₂ O	38.9 μ l	39.7 μ l	39.2 μ l
Fragment F	0.3 μ l	0.3 μ l	0 μ l
Fragment G	0.8 μ l	0 μ l	0.8 μ l
Volume	40 μ l	40 μ l	40 μ l

[0183] The same protocol that was used for creation of fragments A, B, F, and G was used, except that only $\frac{1}{2}$ the cDNA, UM, and LM were used for the control reactions. The tubes were cycled in a Perkin-Elmer 2400 as follows: 94° C., 1 minute; repeat 30 $\times$ (94° C., 15 seconds; 55° C., 15 seconds; 68° C., 2 minutes; 72° C., 7 minutes; 4° C., hold, for Fragment C and its controls. For Fragment H and its controls, the annealing temperature was 50° C. and the extension time was 6.5 minutes. The expected sizes were as follows:

Fragment	Approximate Size (kb)
E	1.59
H	7.3

[0184] Forty μ l of Fragment E and 50 μ l of Fragment H was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentration of the purified fragment was determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment E	0.0334	0.0516	0.6473	1.5449	103.2 ng/ μ l
Fragment H	0.0035	0.0068	0.5088	1.9655	13.6 ng/ μ l

3.3 Cloning of Fragment E into the Yellow Fever Vector

[0185] The capsid-prME fusion was cloned into the yellow fever plasmid needed, and after digestion of the purified Fragment E, as well as pYF5'3'IV, with the appropriate enzymes. The digested plasmid resulted in two bands. Lower bands seen contain a fragment of Japanese encephalitis virus equivalent to Fragment E. All restriction enzymes, buffers, and 100 $\times$ BSA were from New England Biolabs. All the digestions were incubated in a Perkin-Elmer 480 cyclor set to hold at 37° C. overnight.

Fragment E Digest	
Fragment E (600 ng)	5.8 μ l
NEB Buffer 4	4 μ l
10 $\times$ BSA	4 μ l
H ₂ O	24.2 μ l
Not I	1 μ l
Nhe I	1 μ l
Volume	40 μ l

pYFMIV5'3' Digest	
pYFMIV5'3' (1.02 μ g)	2 μ l
NEB Buffer 4	2 μ l
10 $\times$ BSA	2 μ l
H ₂ O	12 μ l
Not I	1 μ l
Nhe I	1 μ l
Volume	20 μ l

3.4 Vector Dephosphorylation

[0186] Calf Intestinal Phosphatase (CIP) from New England Biolabs (cat#290S) was diluted 1:10 in 1 $\times$ CIP Buffer. One μ l of this dilution was then added to the pYFMIV5'3' digest, and incubated for 1 hour at 37° C. Then, 0.8 μ l 125 mM EDTA was added to the tube, which was placed at 75° C. for 10 minutes to deactivate CIP.

3.5 Gel Excision

[0187] The digested fragment E and the digested plasmid were separated on a 1.0% Agarose/TAE gel, and were purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704).

3.6 Ligations

[0188] The digested Fragment E and pYF5'3'IV were ligated using T4 DNA Ligase (New England Biolabs cat#202S) to create pYD1-5'3'. All ligation reactions were incubated in a Perkin-Elmer 480 cyclor set to hold at 16° C. overnight.

PYD1-5'3' Ligation	
Fragment E (97.5 ng)	12.8 μ l
pYFM5'3' (50 ng)	3.0 μ l
H ₂ O	1.2 μ l
10 $\times$ T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l

pYFM-5'3' Control Ligation	
Fragment E (97.5 ng)	0 μ l
pYFM5'3' (50 ng)	3.0 μ l
H ₂ O	14 μ l
10 $\times$ T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l

3.7 Transformations

[0189] Ligation reactions were individually transformed into *E. coli* strain MC 1061 (recA-). Briefly, an aliquot of MC

1061 was removed from storage at -80°C . and allowed to thaw on ice for one to two minutes. 0.9 ml of cold 0.1 M CaCl_2 was added to the cells. One hundred μl of cells was aliquoted into three 12 ml culture tubes on ice. Ten μl of each ligation reaction was added to each culture tube, leaving the third tube as a no DNA control. Culture tubes were left on ice for 30 minutes. The tubes were heat shocked in a water bath at 42°C . for 45 seconds, and then were put back on ice for 2 minutes. 0.9 ml SOC medium was added to each culture tube and incubated at 225 rpm in a shaking incubator at 37°C . for 1 hour. Each transformation mix was aliquoted into 1.5 ml microcentrifuge tubes. One hundred μl of each mix was spread onto LB/Agar-Amp (100 $\mu\text{g}/\text{ml}$) plates and labeled as "neat." Each tube was spun at 14,000 rpm in a microcentrifuge for 2-3 seconds to pellet the cells. The supernatant was poured into the waste container and the pellet resuspended in the residual broth by pipetting up and down. This material was plated (approximately 100 μl) onto LB/Agar-Amp (100 $\mu\text{g}/\text{ml}$) plates and labeled as 10x. All plates were inverted in a 37°C . incubator overnight.

3.8 Transformant Screening

[0190] Resulting bacterial colonies were patch-plated onto fresh LB/Agar-Amp (100 $\mu\text{g}/\text{ml}$) and placed inverted in a 37°C . incubator overnight. The following day, 50 μl of RODI-water was aliquoted into 0.5 ml tubes. Using a sterile plastic pick, a small amount of each patch was scraped into one of the 0.5 ml tubes containing water. These were then placed at 95°C . for 10 minutes, and spun at 14,000 rpm for 10 minutes in a microcentrifuge. To identify the proper insert in pYD1-5'3', colonies were screened by PCR using Taq Polymerase (Promega) and primers 4 and 10. The PCR reaction was performed using the following master mix.

Component	4 rxns	10 rxns	15 rxns	20 rxns	25 rxns	30 rxns
RODI-water	161.5 μl	355.5 μl	516.8 μl	678.3 μl	839.8 μl	1001.3 μl
H_2O	25 μl	55 μl	80 μl	105 μl	130 μl	155 μl
MgCl_2	10 μl	22 μl	32 μl	42 μl	52 μl	62 μl
P1 (100 μM)	1 μl	2.2 μl	3.2 μl	4.2 μl	5.2 μl	6.2 μl
P2 (100 μM)	1 μl	2.2 μl	3.2 μl	4.2 μl	5.2 μl	6.2 μl
dNTPs (10 mM)	40 μl	88 μl	128 μl	168 μl	208 μl	248 μl
Taq polymerase	1.5 μl	3.3 μl	4.8 μl	6.3 μl	7.8 μl	9.3 μl

[0191] Forty eight μl of each master mix was added to a 0.5 ml tube along with 5 μl of the template prepared from the colony patches. Additionally, a tube using RODI-water as a template was also made as a negative control. These tubes were then placed in the Ericomp Delta Cyclor and cycled as follows: 96°C ., 4 minutes; $30\times$ (94°C ., 30 seconds; 50°C ., 1 minute; 72°C ., 1 minute 25 seconds), 72°C ., 4 minutes; 4°C ., Hold. The PCR products were then run on 1.5% Agarose/TAE gels to check for positive colonies.

3.9 Glycerol Stocks

[0192] One hundred twenty ml of LB-Amp (100 $\mu\text{g}/\text{ml}$) was then inoculated from a patch pYD1-5'3'/2 and shaken at 225 rpm overnight at 37°C . Two $\times$ 1 ml of this culture was then at 14 Krpm for 2-3 seconds to pellet the cells. These were resuspended in LB-Glycerol (30%) and frozen at -80°C .

3.10 MIDI Plasmid Preparation

[0193] Qiagen Midi-Prep was performed on the remaining culture using the following modified protocol.

- [0194]** 1. Spin 150 ml of each culture at 7 Krpm in GSA rotor for 10 minutes to pellet.
- [0195]** 2. Decant Supernatant
- [0196]** 3. Resuspend pellet in 4 ml P1 buffer; Transfer to 50 ml Falcon tube.
- [0197]** 4. Rinse centrifuge bottle with 1 ml P1 buffer and transfer to the Falcon tube.
- [0198]** 5. Add 5 ml P2 buffer; invert gently; incubate 5 minutes at room temperature or until lysed (no more than 12 minutes).
- [0199]** 6. Add 5 ml P3 buffer; mix as above; incubate 10 minutes on ice.
- [0200]** 7. Transfer supernatant to Qiagen Syringe Filter; Let sit for 10 minutes.
- [0201]** 8. Equilibrate Q-100 tip with 4 ml QBT.
- [0202]** 9. Gently push plunger to filter supernatant onto Q-100 tip.
- [0203]** 10. Allow to drain by gravity.
- [0204]** 11. Wash with 10 ml $2\times$ QC.
- [0205]** 12. Elute into 50 ml polypropylene centrifuge tube with 5 ml QF.
- [0206]** 13. Add 14 ml 100% EtOH (or 8 ml isopropanol); Place in dry ice/ethanol bath for 10 minutes (or at -20°C . for 2 hours or at 4°C . overnight).
- [0207]** 14. Spin at 15 K $\times$ G for 30 minutes at 4°C .
- [0208]** 15. Wash with 5 ml 70% ethanol; Spin 15 K $\times$ G for 20 minutes.
- [0209]** 16. Air Dry.
- [0210]** 17. Resuspend in 150 μl EB.

DNA concentration was measured as before.

Sample	A280	A260	280/260	260/280	Concentration
pYD1-5'3'/2	0.1708	0.3010	0.5675	1.7621	602 $\mu\text{g}/\text{ml}$

3.11 In Vitro Ligation to Create Full-Length cDNA Chimeric Template and RNA Production Digestion with AatII and BstBI

[0211] The plasmid and fragment H were then digested with Aat II and BstB I (NEB) in a sequential digest as follows.

pYD1-5'3'/2 (AatII digest)	
pYD1-5'3'/2 (10 µg)	16.6 µl
Buffer 4 (NEB)	5 µl
Aat II (NEB)	4 µl
H ₂ O	24.4 µl
Volume	50 µl
Fragment H (AatII digest)	
Fragment H (0.7 µg)	51.0 µl
Buffer 6 (NEB)	5 µl
Aat II (NEB)	3 µl
H ₂ O	0 µl
Volume	60 µl

[0212] Both reactions were incubated in a 37° C. block overnight. Five µl of each digestion was run out on a 1.5% Agarose/TAE gel to check for complete digestion. The digestion was then incubated at 65° C. for 20 minutes to inactivate the enzyme. 2.5 µl Bst BI (NEB) was added to each reaction and placed at 65° C. overnight. The expected results of the digest are as follows:

pYD1-5'3'/2	Fragment H
5.6 kb	7.2 kb
0.14 kb	0.1 kb

[0213] The largest band from each reaction was gel excised and the UV concentration was determined (as previously described).

Sample	A280	A260	280/260	260/280	Concentration
pYD1-5.2 fragment	0.0089	0.0154	0.5777	1.7309	30.8 ng/µl
Fragment H	0.0020	0.0018	1.1333	0.8824	3.6 ng/µl

[0214] There was not enough fragment H for the ligation. Another 50 µl of fragment H was cleaned over a Qiagen Qiaquick column and digested with Aat II and Bst BI as described previously. The digested fragment was then gel excised as before and the UV concentration determined.

Sample	A280	A260	280/260	260/280	Concentration
Fragment H	0.0000	0.0021	0.0000	NA	4.2 ng/µl

3.12 Precipitation of Fragments

[0215] The following was prepared in a 1.5 ml tube.

pYD1-5'3'/2 fragment (298 ng)	9.7 µl
Fragment H (3.6 ng/µl)	38 µl

-continued

Fragment H (4.2 ng/µl)	32 µl
Water	20.3 µl
Volume	100 µl

[0216] The fragments were then ethanol precipitated and resuspended in 43.5 µl of water to facilitate the ligation reaction.

3.13 Ligation

[0217] The following ligation reaction was setup using high concentration T4 DNA ligase (NEB). The ligations were incubated at 16° C. overnight.

Precipitated fragments	43.5 µl
100x BSA (NEB)	0.5 µl
T4 DNA ligase buffer (NEB)	5 µl
*T4 DNA ligase	1 µl
Volume	50 µl

*heat reaction at 37° C. for 5 minutes, then briefly chill on ice before adding ligase

3.14 Linearization of Template

[0218] The ligation was heat inactivated at 65° C. for 10 minutes. The ligated material was then linearized at the 3' end of the Yellow Fever sequence to allow proper RNA transcription. 5.5 µl of Buffer 2 (NEB) was added to the ligation, followed by 1.5 µl XhoI (NEB), and then the reaction was put at 37° C. for 2 hours.

3.15 Linear cDNA Extraction (RNAse Free Phase)

[0219] 1. Add H₂O to 100 µl total volume.

[0220] 2. Add 1/10th volume 3 M Sodium Acetate

[0221] 3. Add 100 µl Phenol/Chloroform/Isoamyl Alcohol and spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNAse-free 1.5 ml tube. Repeat once.

[0222] 4. Add 100 µl RNAse-free Chloroform. Spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNAse-free 1.5 ml tube. Repeat once.

[0223] 5. Add 200 µl 100% RNAse-free ethanol.

[0224] 6. Place on dry-ice/ethanol bath for 10 minutes.

[0225] 7. Spin at 14 Krpm for 20 minutes in a microcentrifuge.

[0226] 8. Wash with 200 µl 70% ethanol (RNAse-free).

[0227] 9. Repeat 70% ethanol wash two more times.

[0228] 10. Dry in Speed-Vac for 8 minutes (or until no more ethanol is present).

[0229] 11. Resuspend in 22 µl nuclease free water from the SP6 kit listed below.

3.16 SP6 Transcription

[0230] The following reaction was setup using the SP6 transcriptase kit (Epicentre) and Rnasin (Promega) in an RNAse-free 1.5 ml tube using RNAse-free tips in a BL-2 hood. The reaction was then placed in a 40° C. water bath for 1 hour.

Capping NTP solution	6 μ l
10x buffer	2 μ l
20 mM Cap Analog	3 μ l
100 mM DTT	2 μ l
Linearized DNA	5 μ l
Rnasin	0.5 μ l
SP6 transcriptase	2 μ l
Volume	20.5 μ l

3.17 Transfection with RNA

[0231] Three six well tissue culture plates were seeded with Vero-PM cells (p#162 from Cell Culture Facility) (2 plates at 1×10^6 cells/well and 1 plate at 2×10^6 cells/well) in growth media (Minimum Essential Media, Sodium Pyruvate, Non-Essential Amino Acids, Penicillin/Streptomycin, and 5% Fetal Bovine Serum) and placed in a 37° C. CO₂ incubator until confluent.

[0232] The following transfection reactions were made using Lipofectin (Life Technologies) and RNase-free PBS (Sigma).

YF/DEN-1	
PBS	250 μ l
Lipofectin	20 μ l
YF/DEN-1 RNA	10 μ l
Volume	280 μ l
Total RNA control	
PBS	250 μ l
Lipofectin	20 μ l
YF/JE total RNA	10 μ l
Volume	280 μ l
Lipofectin control	
PBS	260 μ l
Lipofectin	20 μ l
Volume	280 μ l

[0233] 1. Allow reactions to sit at room temperature for 10 minutes, and then remove Media from the six well plates.

[0234] 2. Wash 3 times with PBS.

[0235] 3. Remove last of PBS.

[0236] 4. Overlay with each lipofectin reaction (add the YF/DEN-1 RNA to the 2×10^6 cells/well plate). Add 280 μ l media to the remaining wells.

[0237] 5. Rock for 10 minutes at room temperature.

[0238] 6. Wash 2 times with media.

[0239] 7. Add 2 ml of media to each well and place in the 37° C. CO₂ incubator for 4 days or more.

3.18 Harvest of the First Vero-PM Passage (P1)

[0240] The supernatant from YF/DEN-1 was harvested on day 6 by splitting the 2 ml of supernatant between two cryovials (each containing 1 ml FBS) that were labeled YF/DEN-1 (PCR) (P1). The cell monolayer was harvested with 1 ml Trizol into a 1.5 ml tube. All vials and tubes were then placed at -80° C.

3.19 Amplification of YF/DEN-1

[0241] Vero-PM Passage #2

[0242] 1. Three T-25 flasks containing Vero-PM cells (p#166) were obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-1 (PCR) (P1) was removed from the -80° C. freezer, thawed, and then placed on ice. The same was done for an aliquot of YF/JE (frozen stock from the P1 control transfection) to be used as a control.

[0243] 2. Media was removed from each T-25 flask.

[0244] 3. One ml of YF/DEN-1 (PCR) (P1) was added to the first flask, 1 ml of media only (the same as was used in the transfection) was added to the second flask, and 1 ml of YF/JE (P1) was added to the third flask.

[0245] 4. Each flask was then put in the 37° C., 5% CO₂ incubator for 1 hour with rocking every 10 minutes.

[0246] 5. Four ml of media was then added to each flask.

[0247] 6. The flasks were then placed in the 37° C., 5% CO₂ incubator for 4 days.

[0248] Harvest P2 and Passage #3

[0249] 1. The supernatant from YF/DEN-1 flask was harvested on day 7.

[0250] 2. Five hundred ml of YF/DEN-1 (P2) was harvested and subsequently overlaid onto a T-25 flask containing Vero-PM cells (p#168 from the Cell Culture Facility).

[0251] 3. Five hundred ml of media (same as used for transfection) was added to the monolayer.

[0252] 4. One ml of media only was added to a control flask.

[0253] 5. The flasks were placed in a 37° C. CO₂ incubator and rocked every 15 minutes for 1 hour.

[0254] 6. Meanwhile, the remaining YF/DEN-1 (P2) was harvested into 4 cryovials containing 1 ml FBS and 1 cryovial containing 0.5 ml FBS and labeled as YF/DEN-1 (P2). The cell monolayer was harvested with 3 ml Trizol into 1.5 ml tubes. All vials were placed at -80° C. in a box labeled YF/DEN-1.

[0255] 7. After infection (Step 5), 4 ml of media was added to each flask and were transferred to the incubator for 4 or more days.

[0256] Harvest of P3

[0257] The supernatant from YF/DEN-1 (P3) was harvested on day 5 by splitting the 5 ml of supernatant between five cryovials (each containing 1 ml FBS), which were labeled YF/DEN-1 (P3). The cell monolayer was harvested with 3 ml Trizol into 1.5 ml tubes. All vials and tubes were then placed at -80° C.

3.20 Virus Identification

[0258] The RNA from P3 was extracted using Trizol methods according to the manufacturer's protocol, RT-PCR was performed followed by sequencing of the YF/DEN-1 prME region 5', 3' junctions, inclusive. The expected sequence of the prME region was confirmed.

4.0 Construction of CHIMERIVAX-DEN3™ (Chimeric Flavivirus Comprising Dengue 3 Virus and prM and E Proteins)

[0259] A viable yellow fever/dengue type 3 chimera (YF/DEN3) was constructed that contains the pre-membrane (prM) and envelope (E) genes of dengue type 3 virus (DEN3) replacing the corresponding prM-E region of the genome of the attenuated 17D yellow fever virus (YF). Virion RNA of wild-type DEN3 (strain PaH881/88) was used as a template to synthesize by RT-PCR two cDNA fragments that cover the DEN3 prM-E region. These fragments were cloned and sequenced. A modified protocol was used to prepare infec-

tious YF/DEN3 in vitro RNA transcripts in which three appropriate DNA fragments were ligated in vitro followed by linearization with XhoI and in vitro transcription with SP6 RNA polymerase (standard ChimeriVax protocol employs two-fragment ligation). Following transfection of Vero PM cells with the RNA transcripts, virus-specific CPE was detectable as early as on day 5 post-transfection (and on day 3 post-infection in subsequent passages). The presence of the chimeric virus in the post-transfection (postinfection) media and the DEN3-specificity of its prM-E region were confirmed by RT-PCR and sequencing.

[0260] These results demonstrate that a chimeric YF virus containing DEN3-specific envelope is readily recoverable. The obtained YF/DEN3 chimera is a candidate part of our proposed tetravalent vaccine directed against all four dengue virus serotypes (dengue types 1-4).

[0261] The purpose of these experiments was to determine whether it is possible to create a viable YF/DEN3 chimera containing DEN3-specific envelope. At the same time, the proposed chimera was designed to contain the prM-E region from a pathogenic wild type strain of DEN3 (a prerequisite for high immunogenicity) in a backbone of the 17D vaccine strain of YF that includes the 5' and 3' UTRs, the C gene, and the nonstructural protein genes, NS1-5, (a prerequisite for safety).

[0262] To engineer a YF/DEN3 chimera containing the prM-E cassette from DEN3 in place of the prM-E cassette of YF we first wanted to use the two-plasmid approach that was successful in previous studies where 17D YF virus (Rice et al., New Biol. 1:285-296, 1989) and the YF/JE chimera (Chambers et al., J. Virol. 73:3095-3101, 1999) were recovered following in vitro transcription and transfection. The DEN3 (strain PaH881/88) prM-E region was RT-PCR amplified in two adjacent fragments (FIG. 29). To determine consensus sequence of this region of the parental virus, the RT-PCR fragments were directly sequenced in both directions. Since oligonucleotide primers used to synthesize these fragments were designed based on the published sequence of the H87 reference strain of DEN3 (Osatomi et al., Virology 176: 643-647, 1990), actual viral sequences in the primer areas (at the beginning of prM, nucleotides 437-459; at the junction between the two fragments, nucleotides 1079-1131; and at the end of E, nucleotides 2385-2413) could not be determined. A total of 83 nucleotide changes were found compared to H87 strain. The rate of nucleotide differences, 4.44%, was similar to that (4.5%) reported previously by Delenda et al. (J. Gen. Virol. 75:1569-1578, 1994) who sequenced roughly 80% of PaH881/88 E gene. Although the majority of nucleotide differences in the 80% E area coincided with those found by Delenda et al. (V. Deubel, personal communication) (53 changes coincided), there were 4 additional changes that were not found by Delenda et al. In addition, we did not observe 3 of the changes reported by these authors. The PaH881/88 virus (a starting material in our experiments) was isolated from a patient by single amplification in mosquito AP61 cells. We propagated this virus in C6/36, another mosquito cell line. Sequences of both our P1 and P3 viruses were found to be identical indicating that there was no selective pressure on the virus in C6/36 cells. Therefore, it is more likely that the few discrepancies were due to sequencing mistakes in (Delenda et al., J. Gen. Virol. 75:1569-1578, 1994). They resulted in three discrepancies in the amino acid sequence of the corresponding region of E protein (C-terminally truncated E; compare Table 32 below

with Table 1 in Delenda et al., J. Gen. Virol. 75:1569-1578, 1994). A complete list of amino acid differences we found in the prM-E region between the 1187 and PaH881/88 strains is given in Table 33 (a total of 12 changes).

[0263] The RT-PCR fragments were used to replace corresponding JE-specific sequences in YFM5'3'IV JE SA14-14-2 and YFM5.2 JE SA 14-14-2 plasmids, which resulted in 5'3'/Den3 and 5.2/Den3 plasmids (FIG. 30). Inserts of both plasmids were sequenced. Since an extra XhoI site was found in the DEN3-specific region of 5'3'/Den3, the site was ablated by silent mutagenesis that resulted in 5'3'/Den3/DXho plasmid. The insert of this plasmid was also sequenced to ensure the absence of mutations introduced by PCR.

[0264] Difficulties were encountered in obtaining high quality 5.2/Den3 plasmid without mutations within its DEN3-specific region. Different growth conditions of plasmid-containing cultures and modifications in the extraction protocol (e.g., reduction of alkali concentration in the lysis buffer), as well as growth of the plasmid in different *E. coli* strains were examined to overcome these difficulties. Finally, a clone of the plasmid (#26) was selected (propagated in ABLE C cells) that was of high quality and contained no nucleotide changes except for a single one nucleotide deletion at the 3' end of the DEN3 insert which could be easily eliminated by PCR (other sequenced clones contained more mutations). Therefore, the standard ChimeriVax procedure for preparation of infectious in vitro RNA transcripts that employs two fragment ligation prior to in vitro transcription was modified. According to the standard protocol, the large BstBI-AatII fragment from 5.2/Den3 would be ligated with the large BstBI-AatII fragment of 5'3'/Den3/DXho (see in FIG. 30). Instead, to correct the deletion, three-fragment ligation was done (FIG. 30). The DEN-3 part of 5.2/Den3 was PCR-amplified on the #26 clone template with high-fidelity LA Taq polymerase and digested with BstBI and EheI (isoschizomer of NarI). The opposite PCR primer was expected to correct the deletion. Second fragment, corresponding to the NarI-AatII part of 5.2/Den3, was derived by digestion of YFM5.2 JE SA14-14-2 with EheI and AatII. The two fragments were ligated with the large BstBI-AatII fragment of 5'3'/Den3/Xho. Ligation products were digested with XhoI and transcribed in vitro with SP6 RNA polymerase.

[0265] Vero PM cells (at passage 149) grown in 6 well plates were transfected with the in vitro RNA transcripts. A first indication that the expected YF/DEN3 chimera was present was the appearance of CPE characteristic of other chimeras created to date based on the YF backbone. It was first noticeable on day 5 post-transfection and became apparent (~10% of detached and rounded cells) on day 7 when virus-containing medium was harvested (P1). Subsequent P2 and P3 viruses were obtained by infecting fresh monolayers of Vero PM cells (at passages 150 and 151, respectively) with the P1 and P2 viruses (1 and 0.5 ml of the viruses were used for each infection, respectively) and harvesting the virus when apparent CPE (~10%) was observable (on days 3 and 4 for P2, and day 3 for P3).

[0266] The presence and DEN3-specificity of the YF/DEN3 chimera was confirmed by RT-PCR with YF- and/or DEN3-specific primers using P1 and P2 virion and intracellular RNAs as templates. All these reactions yielded specific RT-PCR products of expected sizes. As was expected, when DEN3-specific primers were used for RT-PCR on a control YF/JE RNA template, no product was recovered. The entire prM-E region of the P2 virus was sequenced. This also

confirmed that the chimera contained the DEN3 envelope glycoprotein genes. In addition to the silent mutation introduced to ablate the XhoI site in the E gene, only one more silent nucleotide change was detected in the virus (A G at nucleotide 2341 in the chimeric genome that corresponds to nucleotide 2296 in the parental DEN3). Since one of the DNA fragments used in the three-fragment ligation was synthesized by PCR (albeit on a plasmid template with known sequence), it is possible that the P1-3 viruses described here contain minor subpopulations with mutations introduced by PCR. To ensure homogeneity, these viruses can be plaque-purified and then sequenced. In addition, we are currently developing alternative cloning techniques that, if necessary, will allow recreation of the YF/DEN3 genome without using the intermediate PCR step. For instance, the DEN3-specific BstBI-NarI fragment of 5.2/Den3 plasmid was recently cloned without any mutations in low-copy number vectors (pCL and pACYC series). This fragment can be excised from the new plasmids and used instead of the PCR fragment in the three-fragment ligation to regenerate the chimera.

[0267] In conclusion, the prM-E region of the PaH881/88 DEN3 was sequenced and cloned. We demonstrate that a recombinant flavivirus genome (e.g., YF/DEN3 in this study) can be reconstituted in vitro by using three-fragment ligation (instead of two-fragment ligation used previously to create other YF chimeras). This approach can be helpful in overcoming technical difficulties that are often encountered during cloning of genetic material from many flaviviruses in *E. coli* (especially dengue viruses). A viable 17D YF/DEN3 chimeric virus was recovered which is yet another successful example of the usefulness of the approach developed by Chambers et al. (J. Virol. 73:3095-3101, 1999; see above), in which the prM-E cassette of a heterologous flavivirus is inserted into the YF backbone such that the hydrophobic signal for prM remains YF-specific.

[0268] The materials and methods used to make and characterize the YF/DEN3 chimera are described as follows.

4.1 Virus and Cells

[0269] DEN3 strain PaH881/88 was isolated from a patient by single amplification in AP61 (mosquito) cells. C6/36 cells were maintained in MEM (Gibco, Cat. #11095-072) supplemented with 10% FBS (HyClone, Cat. #SH30070103) and 1× non-essential amino acids (Sigma, Cat. #M7145) (OraVax ML-8 medium, Lot #108H2308) at 28° C. under 5% CO₂. DEN3 was passaged two times by infecting monolayers of C6/36 at an unknown MOI and harvesting virus-containing growth media on day 7 post-infection (P1 and P2) and one time by infecting C6/36 cells with the P2 virus at an MOI of 0.01 pfu/cell and harvesting the medium on day 6 (P3; pronounced virus-specific CPE was observed in P3). Virus-containing media were mixed with an equal volume of FBS, aliquoted and stored at 70° C. Following transfection/infection, Vero PM cells were maintained in MEM (Gibco, Cat. #11095-080, Lot #1017611) supplemented with 5% heat-deactivated FBS (OraVax Lot #AGE6578) and penicillin/streptomycin (100 U/0.1 mg per ml; Sigma, Cat. #P-0781, Lot #78H2386) at 37° C. under 5% CO₂.

4.2 RNA Extraction

[0270] DEN3 virion RNA was extracted from 0.5 ml of clarified P3 virus-containing medium using TRI Reagent-LS (Molecular Research Center, Inc., Cat. #TS-120) according to

the manufacturer's procedure and redissolved in 10 µl H₂O. Alternatively, (e.g., to confirm the presence of YF/DEN3 chimera), intracellular RNA from infected cells was extracted using TRI Reagent (Molecular Research Center, Cat. #TR-118).

4.4 Reverse Transcription, PCR, and Sequencing

[0271] First strand cDNA syntheses were done in a total volume of 20 µl using 2.5 µl of DEN3 virion RNA as a template, indicated oligonucleotide primers (see below) and SuperScript II reverse transcriptase (Gibco, Cat. #18064-014) according to the manufacturer's procedure. Prior to PCR, RT products were treated with RNase H (Promega). High-fidelity PCR on RT products or indicated plasmid DNAs as templates was done using the GeneAmp XL PCR kit (Perkin Elmer, Cat. #N808-0192), or the TaKaRa LA Taq polymerase kit (PanVera, Cat. #TAK RR002M) according to the protocols provided by the kit manufacturers with a GeneAmp 2400 thermocycler (Perkin Elmer). Sequencing of indicated PCR products or plasmid DNAs was done using the ABI Prism dRhodamine Terminator Cycle Sequencing kit (Perkin Elmer, Cat. #403042). Sequencing reaction products were resolved with ABI Prism 310 automated sequencer (Perkin Elmer). Data were analyzed using Sequencer 3.0 software and stored on the Internet (ORAVAX/VOLTEMP/GROUPS/LABTECH/KOSTIA/ folder "KP sequencing data"/Exp.##). With each area of interest, both DNA strands were sequenced and analyzed. Oligonucleotide primers are listed in Table 32.

[0272] Primers were ordered from Custom Primers (Life Technologies/GibcoBRL). In the names of primers, numbers indicate approximate localization of oligos on the DEN3 genome and "+/-" indicates orientation of each primer, with the following exceptions: oligo 5 is colinear with a region of YFM5'3' series of plasmids upstream from the NotI cloning site; oligos 6 (opposite) and 7 (direct) are YF-specific; the former corresponds to the end of YF C gene; oligos 15 (direct) and 16 (opposite) were designed for amplification and sequencing of inserts in the YFM5.2 series of plasmids and correspond to regions of the plasmids located within ~60 nucleotides upstream and downstream from the corresponding inserts, respectively; oligo 8 (direct) was used to mutate the XhoI site at nucleotide 1052 of the recombinant YF/DEN3 genome (within 5'3'/Den3 plasmid); and oligo 17 is colinear with the SP6 promoter and a few of the 5' terminal nucleotides from YF.

4.3 DNA Manipulations

[0273] Standard molecular biology techniques were in accordance with Maniatis et al., Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1992. All restriction enzymes, except for Ehel (Fermentas) and T4 DNA ligase, were from New England Biolabs.

4.4 Construction of 5'3'/Den3/DXho Plasmid

[0274] The 5' terminal part of the DEN3 prM-E region was synthesized on purified virion RNA of the P3 virus by RT-high-fidelity PCR (XL PCR) using oligonucleotide primers 1 and 2. It starts precisely at the beginning of the coding region for prM protein (at nucleotide 437; DEN3 nucleotide numbering is according to the sequence of H87 reference strain (Osatomi et al., Virology 176:643-647, 1990)) that is gener-

ated by signalase and ends at nucleotide 1106 and thus contains the entire prM and approximately one-seventh of the E gene. In addition to DEN3 sequences, the resulting RT-PCR product contains the last 23 nucleotides of the YF C gene for subsequent overlapping PCR (at its 5' end). The last six nucleotides of DEN3 sequence (nucleotides 1101-1106) are changed to a BstBI site by introduction of three silent nucleotide changes for subsequent in vitro ligation, which is followed by a NheI site for cloning.

[0275] To combine the RT-PCR product with the upstream YF-specific sequences, a fragment of YFM5'3'IV JE SA14-14-2 plasmid (an analog of 5'3'/Den3 plasmid used to generate a similar YF/JE chimera (Chambers et al., J. Virol. 73:3095-3101, 1999; see above)) containing SP6 RNA polymerase promoter followed by the 17DYF 5' UTR and C gene (first 481 nucleotides of YF genome) was amplified by XL PCR with oligos 5 and 6. For overlapping PCR, the resulting DNA fragment was mixed with the RT-PCR product and XL PCR amplified with oligos 2 and 5. Consensus sequence of the dengue type 3 region was determined by sequencing the RT-PCR and the overlapping PCR products in both directions using oligos 1, 2, 5, 7, 9, and 10.

[0276] The overlapping PCR product was used to replace the short NotI-NheI fragment in YFM5'3'IV JE SA14-14-2. The replaced region of the resulting 5'3'/Den3, which is a pBR322-based plasmid maintained in *E. coli* MC 1061 RecA-cells, was sequenced using oligos 1, 2, 9, 10, and 17, and a correct clone (#3) was selected, which does not have any mutations compared to the consensus sequence.

[0277] Sequencing revealed that the DEN3-specific portion of 5'3'/Den3 contains an additional XhoI site located in the beginning of E gene (nucleotides 1007-1012 in DEN3 genome). Another XhoI site used for linearization prior to in vitro transcription (see below) is located at the end of YF sequence in 5'3'/Den3. The additional site was destroyed by silent oligonucleotide-directed mutagenesis (LA PCR; DEN3-specific C at nucleotide 1009 was changed to G) using oligo 8, resulting in a plasmid 5'3'/Den3/DXho. The entire region of the plasmid replaced during mutagenesis was sequenced with oligos 1, 2, 9, 10, and 17 and a clone (#10) was selected that does not have any mutations, except for the desired C to G nucleotide change.

4.5 Construction of 5.2/Den3 Plasmid

[0278] The 3' terminal part of DEN3 prM-E region was RT-PCR amplified (XL PCR) on the P3 virion RNA template using primers 3 and 4. It starts with BstBI site introduced at nucleotides 1101-1106 for in-frame ligation with 5'3'/Den3/DXho plasmid and ends with a NarI site introduced precisely at the 3' end of E gene (nucleotides 2408-2413) for in-frame ligation with YF NS1. The NarI site that leads to Q to G change of the penultimate amino acid residue in the DEN3 E was used previously to generate YF/JE chimera (Chambers et al., J. Virol. 73:3095-3101, 1999; see above). An NheI cloning site was placed upstream from the BstBI site. The consensus sequence of this DEN3 region was determined by sequencing of the RT-PCR product in both orientations using oligos 3, 4, 11, 12, 13, and 14.

[0279] The PCR product was cloned in place of the short NheI-NarI fragment in YFM5.2 JE SA14-14-2 plasmid (Chambers et al., J. Virol. 73:3095-3101, 1999; see above), resulting in the 5.2/Den3 plasmid. Originally it was propagated in *E. coli* MC1061RecA-cells, and the DNA extracted from these cells was of poor quality (partially denatured),

which hindered restriction digestions. Subsequently, several of the extracted plasmid clones were propagated in *E. coli* ABLE C cells (Stratagene), and good quality DNAs were recovered. Six of these clones were thoroughly analyzed by restriction analysis and then sequenced. For sequencing, the DEN3-specific insert with adjacent regions of the vector was PCR amplified using oligos 15 and 16, and the product was sequenced in both orientations with oligos 11, 12, 13, 14, 15, and 16. Clone #26 was chosen for subsequent manipulations. It contains no mutations, compared to the consensus sequence, except for a single nucleotide deletion (nucleotide 2407, in the region of the opposite PCR primer 4).

4.6 In vitro Transcription and Transfection

[0280] These techniques were essentially as described by Rice et al. (New Biol. 1:285-296, 1989). Approximately 1 µg total of equimolar amounts of appropriate gel-purified DNA fragments were ligated overnight in 20 µl volume at 40° C. T4 DNA ligase was heat-inactivated (10 minutes, 65° C.). Ligation products were digested with XhoI to provide run-off transcription, phenol-chloroform extracted, and precipitated with ethanol. The resulting DNA was transcribed in vitro with SP6 RNA polymerase in the presence of m7G(5')ppp(5')G cap analog and Rnasin in a 20 µl reaction (AmpliScribe SP6 Kit With Cap Analog; Epicentre Technologies; Cat. #AS2606C2). RNA transcripts were analyzed by electrophoresis of 2 µl aliquots in 1% agarose gel. Monolayers of Vero PM cells grown in 6 well tissue culture plates were transfected with RNA transcripts using Lipofectin reagent (Gibco, Cat. #18292-011). Following transfections, cells were incubated as is described above, and virus-containing media were harvested on indicated days post-transfection, mixed with equal volume of FBS, aliquoted and stored at -70° C.

5.0 Construction of CHIMERIVAX-DEN4™ (Chimeric Flavivirus Comprising Dengue 4 Virus prM and E Proteins)

[0281] The purpose was to generate yellow fever/dengue 4 (YF/DEN-4) chimeric virus as a dengue vaccine candidate (see FIGS. 31 and 32). To attain this, we used a technology derived from the construction of Yellow fever/Japanese encephalitis (YF/JE SA 14-14-2) chimeric virus (Chambers et al., J. Virol. 73:3095-3101, 1999). It consists of a plasmid system which originally encoded the yellow fever (YF) genome. These YF plasmids were created by Charlie Rice (Rice et al., New Biol. 1:285-296, 1989). The structural membrane precursor and envelope protein genes, i.e., the prME portion, of the YF genome plasmids with that of JE SA14-14-2 sequence and used the resulting plasmids to produce RNA in vitro, which was then transfected into cells to produce live YF/JE chimeric virus. The system seemed suitable to construct other flavivirus chimeras using YF as backbone and here we describe the use of dengue 4 as a start point. The dengue 4 strain, #1228 isolated in 1978 in Indonesia and passaged twice in mosquitoes, was passed once in C6/36 and total RNA was isolated to synthesize cDNA for PCR of the prME region as needed for cloning. Here we describe in detail the procedures for construction of the YF/DEN-4 chimera. The dengue 4 prME region was first amplified and sequenced using primers derived from consensus sequences (Genbank). The sequence data created was applied to primer design which were used, with the cDNA produced earlier, as PCR starting point for assembly of the two-plasmid system for dengue 4 (i.e., by replacing the corresponding prME JE sequences in each plasmid). A PCR product encoding dengue

prM and 5' end of E was used as template, along with template encoding the capsid (C) gene of yellow fever derived from the plasmid pYF5'3'IV/JE SA 14-14-2, in an overlap extension PCR to result in a single fusion product which was then cloned into a fragment of pYFM5'3'IV where JE sequences were deleted. The 3' end of dengue 4 envelope protein gene was also amplified and then cloned into a vector fragment of pYFM5.2/JE SA 14-14-2, resulting in replacement of JE sequence with that of dengue 4. Both plasmids were then transformed into *E. coli* strain MC 1061 (RecA-) and mid-scale plasmid cultures were grown. In vitro ligation of the two plasmids resulted in full-length virus DNA template of YF/DEN-4 for RNA transcription. All steps involving cDNA fragments and plasmids were carried out in a BL-2 lab designated for recombinant DNA work. Steps involving manipulations of infectious RNA and virus were carried out in a limited access BL-2+ virus lab.

5.1 Amplification of Dengue 4 Sequence

[0282] Dengue 4 cDNA was synthesized from RNA using the Superscript II™ method. All primers for this experiment were synthesized by Life Technologies and are listed in Table 34. Upon arrival as lyophilized material, primers were dissolved to 250 mM stock solutions in RODI-water. From this, 25 mM working solutions were made. The fragment encoding the SP6 promoter and yellow fever capsid (Fragment A) was amplified using the XL-PCR Reaction Kit™ (Perkin-Elmer Part #N808-0192), 0.5 ml (250 ng) of template pYF5'3'IV plus 3.5 ml RODI-water, and primers 1 and 2. The fragment encoding dengue 4 prM and the 5' end of E (Fragment B) was amplified using the XL-PCR Reaction Kit™ (Perkin-Elmer Part #N808-0192) and primers 3 and 4. The fragment encoding the 3' end of dengue 4 envelope (Fragment C) was amplified using the same protocol but using primers 5 and 6. Each PCR reaction was performed as indicated in master mixes (see section 3.1, above).

[0283] For each reaction, the lower mix (LM) was added to a Perkin-Elmer thin-walled 0.2 ml tube. Next, Ampliwax 100 (Perkin-Elmer) was added to the tube, which was then placed in a Perkin-Elmer 2400 Thermal Cycler and heated to 80° C. for 5 minutes, and then cooled to 4° C. The cDNA and UM were then added to the top of the wax layer. The tube was then cycled in a Perkin-Elmer 2400 as follows: 94° C., 1 minute; repeat 30× (94° C., 15 seconds; 53° C., 15 seconds; 68° C., 3 minutes), 72° C., 4 minutes; 4° C., hold. The expected sizes of the PCR fragments for cloning were as follows:

Fragment	Approximate Size (kb)
A	0.940
B	0.650
C	1.300

[0284] Forty µl of each fragment was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat. #28704). Next, the concentration of the purified fragments was determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment A	0.0247	0.0340	0.7271	1.3754	68 ng/µl
Fragment B	0.0086	0.0152	0.5643	1.7721	30 ng/µl
Fragment C	0.0099	0.0179	0.5536	1.8065	36 ng/µl

5.2 Recombinant PCR

[0285] To create a fusion between the yellow fever capsid gene and the 5' end of the dengue 4 prM, a recombinant PCR technique, Overlap-extension PCR was used to create Fragment E. The same basic UM and LM were used and primers 1 and 4 replaced earlier primers; the cDNA templates used were mixes shown below:

	Fragment E	Fragment A control	Fragment B control
H ₂ O	37.8 µl	39.1 µl	38.7 µl
Fragment A	0.9 µl	0.9 µl	0 µl
Fragment B	1.3 µl	0 µl	1.3 µl
Volume	40 µl	40 µl	40 µl

[0286] The same protocol that was used for creation of Fragments A, B, and C was used except that only ½ of the cDNA, UM, and LM were used for the control reactions. The tube was then cycled in a Perkin-Elmer 2400 cycler as follows: 94° C., 1 minute; repeat 30× (94° C., 15 seconds; 55° C., 15 seconds; 68° C., 2 minutes; 72° C., 7 minutes; 4° C., hold. The expected sizes were as follows.

Fragment	Approximate Size (kb)
E	1.59
A control	no product
B control	no product

[0287] Forty µl of Fragment E was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat. #28704). Next, the concentration of the purified fragment was determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment E	0.0049	0.0110	0.4489	2.2276	22 ng/µl

5.3 Cloning of Fragments C and E into Yellow Fever Vectors

[0288] The fragments were then cloned into the yellow fever two-plasmid system by digestion of the purified Fragments C and E as well as the plasmids pYF5'3'IV and pYFM5.2/2 with appropriate restriction enzymes as shown below. The digested plasmids resulted in two bands. The smaller bands contain a fragment of Japanese encephalitis corresponding to either Fragment C or Fragment E for the new dengue 4 constructs. All restriction enzymes, buffers, and 100× BSA were from New England Biolabs. All the digestions were carried in a Perkin-Elmer 480 cycler set to hold at 37° C. overnight.

Fragment E digest	
Fragment E (528 ng)	27 μ l
NEB buffer 4	4 μ l
10x BSA	4 μ l
H ₂ O	3 μ l
Not I	1 μ l
Nhe I	1 μ l
Volume	40 μ l
pYF5.2 digest	
pYF5.2 (1 μ g)	9.5 μ l
NEB buffer 2	2 μ l
10x BSA	2 μ l
H ₂ O	4 μ l
Sfo I	1.5 μ l
Nhe I	1 μ l
Volume	20 μ l
pYFMIV5'3' digest	
pYFMIV5'3' (1.02 μ g)	2 μ l
NEB buffer 4	2 μ l
10x BSA	2 μ l
H ₂ O	12 μ l
Not I	1 μ l
Nhe I	1 μ l
Volume	20 μ l
Fragment C digest	
Fragment C (710 ng)	20 μ l
NEB buffer 2	4 μ l
10x BSA	4 μ l
H ₂ O	9.5 μ l
Sfo I	1.5 μ l
Nhe I	1 μ l
Volume	40 μ l

5.4 Vector Dephosphorylation

[0289] Calf Intestinal Phosphatase (CIP) from New England Biolabs (cat. #290S) was diluted 1:10 in 1x CIP Buffer. One μ l of this dilution was then added to the pYFMIV5'3' digest. 0.62 μ l of stock CIP was added directly to the pYF5.2 digest. Both were incubated for 1 hour at 37° C. Then, 0.8 μ l 125 mM EDTA was added to the two tubes and placed at 75° C. for 10 minutes to inactivate CIP.

5.5 Gel Excision

[0290] The digested PCR fragments were separated on a 1.0% Agarose/TAE gel, while the digested plasmids were separated on a 0.8% Agarose/TAE gel. All were purified using the QIAquick Gel Extraction Kit (Qiagen cat. #28704).

5.6 Ligations

[0291] The digested Fragment E and pYF5'3'IV were ligated using T4 DNA Ligase (New England Biolabs cat. #202S) to create pYD4-5'3'. The digested Fragment C and pYFM5.2 were ligated to create pYD4-5.2. All ligation reactions were incubated in a Perkin-Elmer 480 cyclor set to hold at 16° C. overnight.

PYD4-5'3' Ligation	
Fragment E (97.5 ng)	9.5 μ l
pYFM5'3' (50 ng)	3.6 μ l
H ₂ O	3.9 μ l
10x T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l
PYD4-5.2 Ligation	
Fragment C (56 ng)	4.5 μ l
pYFM5.2 (70 ng)	8.8 μ l
H ₂ O	3.7 μ l
10x T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l
pYFM-5'3' Control Ligation	
Fragment E	0 μ l
pYFM5'3' (50 ng)	3.6 μ l
H ₂ O	13.4 μ l
10x T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l
pYF5.2 Control Ligation	
Fragment C	0 μ l
pYFM5.2 (70 ng)	8.8 μ l
H ₂ O	8.2 μ l
10x T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l

5.7 Transformations

[0292] All four ligation reactions were transformed into *E. coli* strain MC1061 (recA-). An aliquot of MC1061 (OraVax Notebook 661-4) was removed from storage at -80° C. and allowed to thaw on ice for one to two minutes. 0.9 ml of cold 0.1 M CaCl₂ was added to the cells. One hundred μ l of cells was aliquoted into five 12 ml culture tubes on ice. Ten μ l of each ligation reaction was added to each culture tube, leaving the fifth tube as a negative (no DNA) control. Culture tubes were left on ice for 30 minutes. The tubes were heat shocked in a water bath at 42° C. for 45 seconds. The tubes were put back on ice for 2 minutes. 0.9 ml SOC medium was added to each culture tube and incubated at 225 pm in a shaking incubator at 37° C. for 1 hour. Each transformation mix was aliquoted into 1.5 ml microcentrifuge tubes. One hundred μ l of each was spread onto LB/Agar-Amp (100 μ g/ml) plates and labeled as "neat." Each tube was spun at 14 Krpm in a microcentrifuge for 2-3 seconds to pellet the cells. The supernatant was poured into the waste container and the pellet resuspended in the residual broth by pipetting up and down. This material was plated (approximately 100 μ l) onto LB/Agar-Amp (100 μ g/ml) plates and labeled as 10x. All plates were inverted in a 37° C. incubator overnight.

5.8 Transformant Screening

[0293] The resulting bacterial colonies were patch-plated onto fresh LB/Agar-Amp (100 μ g/ml) and placed inverted in a 37° C. incubator overnight. The following day, 50 μ l of RODI-water was aliquoted into 0.5 ml tubes. Using a sterile

plastic pick, a small amount of each patch was scraped into one of the 0.5 ml tubes containing water. These were then placed at 95° C. for 10 minutes and spun at 14,000 rpm for 10 minutes in a microcentrifuge. To identify the proper insert in pYD4-5'3', colonies were screened by PCR using Taq Polymerase (Promega) and primers 4 and 7. The pYD4-5.2 was screened using primers 5 and 6. The PCR reaction was performed using the following master mix.

Component	4 rxns	10 rxns	15 rxns	20 rxns	25 rxns	30 rxns
RODI-water	161.5 µl	355.5 µl	516.8 µl	678.3 µl	839.8 µl	1001.3 µl
H ₂ O	25 µl	55 µl	80 µl	105 µl	130 µl	155 µl
MgCl ₂	10 µl	22 µl	32 µl	42 µl	52 µl	62 µl
P1 (100 µM)	1 µl	2.2 µl	3.2 µl	4.2 µl	5.2 µl	6.2 µl
P2 (100 µM)	1 µl	2.2 µl	3.2 µl	4.2 µl	5.2 µl	6.2 µl
dNTPs (10 mM)	40 µl	88 µl	128 µl	168 µl	208 µl	248 µl
Taq Polymerase	1.5 µl	3.3 µl	4.8 µl	6.3 µl	7.8 µl	9.3 µl

[0294] Forty eight µl of each master mix was added to a 0.5 ml tube along with 5 ml of the template prepared from the colony patches. Additionally, a tube using RODI-water as a template was also made as a negative control. These tubes were then placed in the Ericomp Delta Cycler and cycled as follows: 96° C., 4 minutes; 30x (94° C., 30 seconds; 50° C., 1 minute; 72° C., 1 minute 25 seconds), 72° C., 4 minutes; 4° C. hold. The PCR products were then run on 1.5% Agarose/TAE gels to check for positive colonies.

5.9 Glycerol Stocks

[0295] Five ml of LB-Amp (100 µg/ml) was then inoculated from a patch pYD4-5'3'/2 or pYD4-5.2/1 and shaken at 225 rpm overnight at 37° C. One ml of this culture was then spun at 14 Krpm for 2-3 seconds to pellet the cells. This was then resuspended in LB-Glycerol (30%) and frozen at -80° C.

5.10 MIDI Plasmid Preparation

[0296] Fifty µl of each glycerol stock was added to 150 ml LB-Amp (100 µg/ml) in separate 4 L flasks and shaken at 225 rpm overnight at 37° C. Qiagen Midi-Prep (Qiagen) was performed using the following modified protocol.

[0297] 1. Spin 150 ml of each culture at 7 Krpm in GSA rotor for 10 minutes to pellet.

[0298] 2. Decant Supernatant.

[0299] 3. Resuspend pellet in 4 ml P1 Buffer; transfer to 50 ml Falcon tube.

[0300] 4. Rinse centrifuge bottle with 2 ml P1 buffer and transfer to the Falcon tube.

[0301] 5. Add 6 ml P2 buffer; invert gently; 5 minutes at room temperature or until lysed (no more than 12 minutes).

[0302] 6. Add 6 ml P3; mix as above; 10 minutes on ice.

[0303] 7. Transfer supernatant to Qiagen Syringe Filter; let sit for 10 minutes.

[0304] 8. Equilibrate Q-100 tip with 4 ml QBT.

[0305] 9. Gently push plunger to filter supernatant onto Q-100 tip.

[0306] 10. Allow to drain by gravity.

[0307] 11. Wash with 2x10 ml QC.

[0308] 12. Elute into 50 ml polypropylene centrifuge tube with 5 ml QF.

[0309] 13. Add 14 ml 100% ETOH (or 8 ml isopropanol); Place in dry ice/ethanol bath for 10 minutes (or at -20° C. for 2 hours or at 4° C. overnight).

[0310] 14. Spin at 15 KxG for 30 minutes at 4° C.

[0311] 15. Wash with 5 ml 70% ethanol; Spin 15 KxG for 20 minutes.

[0312] 16. Air Dry.

[0313] 17. Resuspend in 150 W EB.

DNA concentrations were measured as before.

Sample	A280	A260	280/260	260/280	Concentration
pYD4-5'3'/2	0.0516	0.0939	0.5498	1.1817	1.87.8 ng/µl
pYD4-5.2/1	0.0900	0.1407	0.6399	1.5629	281.4 ng/µl

5.11 In Vitro Ligation to Create Full-Length cDNA Chimeric Template and RNA Production

[0314] Digestion with AatII and BstBI

[0315] Plasmids pYD4-5'3'/2 and pYD4-5.2/1 were digested with AatII and BstBI (NEB) in a sequential digest as follows.

YD4-5'3'/2 (AatII digest)	
pYD4-5'3'/2 (8 µg)	42.5 µl
Buffer 4 (NEB)	5 µl
AatII (NEB)	2 µl
H ₂ O	0.5 µl
Volume	50 µl
YD4-5.2/1 (AatII digest)	
pYD4-5.2 (10 µg)	35.5 µl
Buffer 4 (NEB)	5 µl
AatII (NEB)	2 µl
H ₂ O	7.5 µl
Volume	50 µl

[0316] Both reactions were incubated in a 37° C. block for 2 hours. Five µl of each digest was run out on a 1.5% Agarose/TAE gel to check for complete digestion. The pYD4-5'3'/2 digest did not cut completely so the reaction was cleaned over a Qiaprep spin column (Qiagen). The digest was repeated using this material and 3 µl of Aat II. In addition, 3 µl of Aat II was added to the existing pYD4-5.2/1 reaction. Both were incubated in a 37° C. block, overnight. After confirmation of digest on another gel (as previously described), 2.5 µl Bst BI (NEB) was added to each reaction and placed at 65° C. for 3 hours. The results of the digest were as follows.

PYD4-5'3'/2	PYD4-5.2/1
5.6 kb	7.2 kb
	2.0 kb
0.14 kb	0.4 kb

[0317] The largest band from each reaction was gel excised as and the UV concentration was determined (as previously described).

5.12 Ligation

[0318] The following ligation reaction was setup using high concentration T4 DNA ligase (NEB). The ligations were incubated at 16° C. overnight.

H ₂ O	13.5 µl
pYD4-5.2 fragment (600 ng)	23 µl
pYD4-5'3' fragment (300 ng)	7 µl
100x BSA (NEB)	0.5 µl
T2 DNA ligase buffer (NEB)	5 µl
*T4 DNA ligase	1 µl
Volume	50 µl

*Heat reaction at 37° C. for 5 minutes, then briefly chill on ice before adding ligase

5.13 Linearization of Template

[0319] The ligation was heat inactivated at 65° C. for 10 minutes. The ligated material was then linearized at 3' end of the yellow fever sequence to allow proper RNA transcription, as follows: 5.5 ml Buffer 2 (NEB) was added to the ligation, followed by 1.5 µl XhoI (NEB), and this reaction mixture was placed at 37° C. for 2 hours.

5.14 Linear cDNA Extraction (RNAse Free Phase)

- [0320] 1. Add H₂O to 100 µl total volume.
 [0321] 2. Add 1/10th volume 3 M Sodium Acetate.
 [0322] 3. Add 100 µl Phenol/Chloroform/Isoamyl Alcohol and spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNAse-free 1.5 ml tube. Repeat once.
 [0323] 4. Add 100 µl RNAse-free Chloroform. Spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNAse-free 1.5 ml tube. Repeat once.
 [0324] 5. Add 200 µl 100% RNASE-free ethanol.
 [0325] 6. Place on dry-ice/ethanol bath for 10 minutes.
 [0326] 7. Spin at 14 Krpm for 20 minutes in a microcentrifuge.
 [0327] 8. Wash with 200 µl 70% ethanol (RNAse-free).
 [0328] 9. Repeat 70% ethanol wash two more times.
 [0329] 10. Dry in Speed-Vac for 8 minutes (or until no more ethanol is present).
 [0330] 11. Resuspend in 22 µl nuclease free water from the SP6 kit listed below.

5.15 SP6 Transcription

[0331] The following reaction was setup using the SP6 transcriptase kit (Epicentre) and Rnasin (Promega) in an RNAse-free 1.5 ml tube using RNAse-free tips in a BL-2 hood. The reaction was then placed in a 40° C. water bath for 1 hour.

Capping NTP solution	6 µl
10x buffer	2 µl
20 mM Cap Analog	3 µl
100 mM DTT	2 µl
Linearized DNA	5 µl
Rnasin	0.5 µl
SP6 transcriptase	2 µl
Volume	20.5 µl

5.16 RNA Transfection

[0332] Two six well tissue culture plates were seeded with Vero-PM (p#153 OraVax notebook#743-7) cells at 7.4×10⁵ cells/well in growth media (Gibco MEM; Sodium Pyruvate; NEAA; Penicillin/Streptomycin; 5% fetal bovine serum), and placed in a 37° C. CO₂ incubator until confluent.

[0333] The following transfection reactions were made using Lipofectin (Life Technologies) and RNAse-free PBS (Sigma), and allowed to sit at room temperature for 10 minutes.

YF/DEN-4	
PBS	250 µl
Lipofectin	20 µl
YF/DEN-4 RNA	10 µl
Volume	280 µl
Total RNA control	
PBS	250 µl
Lipofectin	20 µl
YF/JE total RNA	10 µl
Volume	280 µl
Lipofectin control	
PBS	260 µl
Lipofectin	20 µl
Volume	280 µl

- [0334] 1. Remove Media from the 6 well plates.
 [0335] 2. Wash 3 times with PBS.
 [0336] 3. Remove last of PBS.
 [0337] 4. Overlay with each lipofectin reaction. Add 280 µl PBS for the remaining wells.
 [0338] 5. Rock for 10 minutes at room temperature.
 [0339] 6. Wash 2 times with media (MEM, Sodium Pyruvate, NEAA, P/S, 5% FBS).
 [0340] 7. Add 2 ml of media to each well and place in the 37° C. CO₂ incubator for 4 days or more.

5.17 Chimeric Virus Harvest

[0341] The supernatant from YF/DEN-4 was harvested on day 6 by splitting the 2 ml of supernatant between two cryovials (each containing 1 ml FBS), which were labeled YF/DEN-4 (P1). The cell monolayer was harvested with 1 ml Trizol into a 1.5 ml tube. All vials and tubes were then placed at -80° C.

5.18 Amplification of YF/DEN-4

[0342] Passage #2

[0343] 1. Three T-25 flasks containing Vero-PM cells (p#149) were obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-4 (P1) was removed from the -80°C . freezer, thawed, then placed on ice. The same was done for an aliquot of YF/JE (frozen stock from the P1 control transfection).

[0344] 2. Media was removed from each T-25 flask.

[0345] 3. Five hundred μl of YF/DEN-4(P1) was added to the first flask, 500 μl of media (MEalphaM, NEAA, Sodium Pyruvate, 5% FBS, P/S) was added to the second flask, and 500 μl of YF/JE(P1) was added to the third flask.

[0346] 4. Two hundred μl of the media was added to each flask to ensure complete coverage.

[0347] 5. Each flask was then put in the 37°C . CO_2 incubator for 1 hour with rocking every 15 minutes.

[0348] 6. 4.5 ml of media was then added to each flask.

[0349] 7. The flasks were then placed in the 37°C . CO_2 incubator for 4 days.

[0350] Harvest P2

[0351] The supernatant from YF/DEN-4 was harvested on day 4 by splitting the 5 ml of supernatant between five cryovials (each containing 1 ml FBS), which were labeled YF/DEN-4 (P2). The cell monolayer was harvested in 3 ml Trizol and aliquoted into 3 tubes. All vials and tubes were then placed at -80°C .

Passage#3 (Titration of P2)

[0352] 1. A 12 well tissue culture plate containing Vero-PM cells (p#163) was obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-4 (P2) was removed from the -80°C . freezer, thawed, and then placed on ice.

[0353] 2. Serial dilutions were made in a 24 well tissue culture plate ending at 10^{-4} in a total volume of 300 μl . The plate was then placed on ice.

[0354] 3. The media was removed from each well of the 12 well plate and 100 μl of virus stock, as well as each dilution, was added to the wells in duplicate. One hundred μl of media only was added to the last set of wells as a negative control.

[0355] 4. The plate was then put in the 37°C . CO_2 incubator for 1 hour with rocking every 20 minutes.

[0356] 5. The 1st overlay was made by preheating 25 ml M199(2 $\times$), 1.5 ml FBS, and 0.5 ml PSA at 42°C . in a 50 ml Falcon tube (tube #1). Additionally, 23 ml Agarose (0.6% in water) was heated at 42°C . in a 50 ml Falcon tube (tube #2). At the end of the 1 hour incubation, tube #1 was added to tube #2 and mixed thoroughly.

[0357] 6. One ml of this overlay was then added to the edge of each well.

[0358] 7. The plate was then put in the 37°C . CO_2 incubator for 4 days.

[0359] 8. The 2nd overlay was made by preheating 25 ml M199(2 $\times$), 1.5 ml FBS, 1.5 ml Neutral Red, and 0.5 ml PSA at 42°C . in a 50 ml Falcon tube (tube #1). Additionally, 21.5 ml Agarose (0.6% in water) was heated at 42°C . in a 50 ml Falcon tube (tube #2). Tube #1 was added to tube #2 and mixed thoroughly.

[0360] 9. One ml of this overlay was added to the center of each well.

[0361] 10. It was then placed in the 37°C . CO_2 incubator

[0362] Titration of P2 Results

[0363] Instead of titer determination, plaques were picked for purification to segregate a mixed population of large and small plaques observed. The RNA from P2 was extracted using Trizol methods according to the manufacturer's protocol, RT-PCR was performed followed by sequencing of the YF/DEN-4 prME region 5', 3' junctions, inclusive. The expected sequence of the prME was confirmed.

6.0 Construction of Chimeric Templates for Other Flaviviruses

[0364] Procedures for generating full-length cDNA templates encoding chimeric YF/MVE, YF/SLE, YF/WN, YF/TBE viruses are similar to those described above for the YF/DEN-2 system. Table 20 illustrates the features of the strategy for generating YF 17D-based chimeric viruses. The unique restriction sites used for in vitro ligation, and the chimeric primers for engineering the C/prM and E/NS1 junctions are also shown. Sources of cDNA for these heterologous flaviviruses are readily available (MVE: Dalgarno et al., J. Mol. Biol. 187:309-323, 1986; SLE: Trent et al., Virology 156:293-304, 1987; TBE: Mandl et al., Virology 166:197-205, 1988; Dengue 1: Mason et al., Virology 161:262-267, 1987; Dengue 2: Deubel et al., Virology 155:365-377, 1986; Dengue 3: Hahn et al., Virology 162:167-180, 1988; Dengue 4: Zhao et al., Virology 155:77-88, 1986).

[0365] An alternative approach to engineering additional chimeric viruses is to create the C/prM junction by blunt end ligation of PCR-derived restriction fragments having ends that meet at this junction and 5' and 3' termini that flank appropriate restriction sites for introduction into YF5'3'IV or an intermediate plasmid such as pBS-KS(+). The option to use a chimeric oligonucleotide or blunt-end ligation will vary, depending on the availability of unique restriction sites within the envelope protein coding region of the virus in question.

6.1 Construction of YF Viruses Encoding HCV Antigens

[0366] Because the structural proteins E1 and E2 of HCV are not homologous to the structural proteins of the flaviviruses described above, the strategy for expression of these proteins involves insertion within a nonessential region of the genome, such that all of these proteins are then co-expressed with yellow fever proteins during viral replication in infected cells. The region to be targeted for insertion of the proteins is the N terminal portion of the NS1 protein, since the entire NS1 protein is not required for viral replication. Because of the potential problems with stability of the YF genome in the presence of heterologous sequence exceeding the normal size of the genome (approximately 10,000 nucleotides), the detection strategy described below can be used. In addition, deletion of NS1 may be advantageous in the chimeric YF/Flavivirus systems described above, because partial deletion of this protein may abrogate the immunity to YF associated with antibodies against NS1, and thus avoid problems with vector immunity if more than one chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had been previously given or needed at a future point.

[0367] The strategy involves creating a series of in-frame deletions within the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering a translational termination codon at the end of E, and a series of two IRESs (internal ribosome entry sites). One IRES is immediately downstream of the termination codon and allows for expression of an open reading frame within the region between E and NS1. The

second IRES initiates translation from truncated NS1 proteins, providing expression of the remainder of the YF non-structural polypeptide. These derivatives are tested for recovery of infectious virus and the construct with the largest deletion is used for insertion of foreign sequences (e.g., HCV proteins) in the first IRES. This particular construct can also serve as a basis for determining whether deletion of NS1 will affect vector-specific immunity in the context of YF/Flavivirus chimeric viruses expressing prM-E, as described above.

[0368] The insertion of nucleotides encoding E1, E2, and/or E1 plus E2 HCV proteins is limited by the size of the deletion tolerated in the NS1 protein. Because of this, truncated HCV proteins can be used to enhance stability within the modified YF clone. The HCV proteins are engineered with an N-terminal signal sequence immediately following the IRES and a termination codon at the C terminus. This construction will direct the HCV proteins into the endoplasmic reticulum for secretion from the cell. The strategy for this construction is shown schematically in FIG. 21. Plasmids encoding HCV proteins of genotype I can be used for these

cleavage site in flavivirus infectious clones of interest, such as dengue, TBE, SLE, and others can be mutated by site-directed mutagenesis. Any or all of the amino acids in the cleavage site, as set forth above, can be deleted or substituted. A nucleic acid fragment containing the mutated prM-E genes can then be inserted into a yellow fever virus vector using the methods described above. The prM deletion can be used with or without other attenuating mutations, for example, mutations in the E protein, to be inserted into the yellow fever virus. These mutants have advantages over single substitution mutants as vaccine candidates, because it is almost impossible to revert the deleted sequence and restore virulence.

[0370] The following chimeric flaviviruses of the invention were deposited with the American Type Culture Collection (ATCC) in Rockville, Md., U.S.A. under the terms of the Budapest Treaty and granted a deposit date of Jan. 6, 1998: Chimeric Yellow Fever 17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593) and Chimeric Yellow Fever 17D/Japanese Encephalitis SA14-14-2 Virus (YF/JE A1.3; ATCC accession number ATCC VR-2594).

TABLE 1

Sequence comparison of JE strains and YF/JE chimeras										
Virus	E 107	E 138	E 176	E 177	E 227	E 243	E 244	E 264	E 279	E 315
JE SA14-14-2	F	K	V	T	S	K	G	H	M	V
YF/JE SA14-14-2	F	K	V	A	S	E	G	H	M	V
YF/JE Nakayama	L	E	I	T	P	E	E	Q	K	A
JE Nakayama	L	E	I	T	P	E	E	Q	K	A
JE SA14	L	E	I	T	S	E	G	Q	K	V

constructions, for example, HCV plasmids obtained from Dr. Charles Rice at Washington University (Grakoui et al., J. Virology 67:1385-1395, 1993), who has expressed this region of the virus in processing systems and within a replication-complement full-length HCV clone.

6.2 PrM Cleavage Deletion Mutants as Attenuating Vaccine Candidates for Flaviviruses

[0369] Additional chimeric viruses included in the invention contain mutations that prevent prM cleavage, such as mutations in the prM cleavage site. For example, the prM

TABLE 2

Characterization of YF/JE chimeras					
Clone	Yield (μg)	Infectivity plaques/100 ng LLC-MK2	PBS log titer VERO	RNAse log titer VERO	DNAse log titer VERO
YF5.21v	5.5	15	7.2	0	7
YF/JE-S	7.6	50	6.2	0	6.2
YF/JE-N	7	60	5	0	5.4

TABLE 3

Plaque reduction neutralization titers on YF/JE chimeras					
Virus	non-immune ascitic fluid	YF ascitic fluid	JE ascitic fluid	non-immune IgG	YF IgG
YF5.21v	<1.3	3.7	<1.3	<2.2	>4.3
JE SA14-14-2	<1.3	<1.3	3.4	<2.2	<2.2
YF/JE SA14-14-2	<1.3	<1.3	3.1	<2.2	<1.9
YF/JE Nakayama	<1.3	<1.3	3.4	<2.2	<2.2
JE Nakayama virus	<1.3	<1.3	3.4	not determined	not determined

TABLE 4

Neurovirulence of YF/JE SA14-14-2 Chimera 3 week old male ICR mice			
	log dose I.C.	% Mortality	
YF5.2iv	4	100	(7/7)
YF/JE SA14-14-2	4	0	(0/7)
YF/JE SA14-14-2	5	0	(0/7)
YF/JE SA14-14-2	6	0	(0/8)

TABLE 5

Neuroinvasiveness of YF/JE Chimeras 3 week old male ICR mice			
	log dose (intraperitoneal)	% mortality	
YF/JE Nakayama	4	0	(0/5)
YF/JE Nakayama	5	0	(0/4)
YF/JE Nakayama	6	0	(0/4)
YF/JE SA14-14-2	4	0	(0/5)
YF/JE SA14-14-2	5	0	(0/4)
YF/JE SA14-14-2	6	0	(0/4)

TABLE 6

Doses and routes of virus inoculation into groups of 4-week-old ICR mice					
Group	YF/JE s.c.	YF/JE i.c.	YF-VAX ® (Yellow Fever 17D Vaccine) s.c.	YF-VAX ® (Yellow Fever 17D Vaccine) i.c.	mice
	log ₁₀ pfu	log ₁₀ pfu	log ₁₀ pfu	log ₁₀ pfu	
1	5	4.5	4.7	4.2	20
2	4	4	4.4	3.9	20
3	3	3	3.4	3.4	20
4	2	2	2.4	2.4	20
5	1	1	1.4	1.4	20
6	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) (BIKEN) 1:30, day 0, 7, s.c.				5
7	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) (BIKEN) 1:300, day 0, 7, s.c.				5
8	control s.c. (medium +10% FBS)				5
9	control i.c. (medium +10% FBS)				5

TABLE 7

Geometric mean neutralizing antibody titers 3 and 8 weeks after immunization, outbred mice inoculated with graded doses of vaccines by the s.c. route					
Vaccine	log ₁₀ PFU	Antibody titer (GMT ± SD) vs.			
		JE		YF 17D	
		3 w	8 w	3 w	8 w
YF/JE	5.0	151 ± 93	5,614 ± 3514		
	4.0	38 ± 60	127 ± 247		
	3.0	19 ± 65	43 ± 560		
	2.0	7 ± 12	3 ± 71		
	1.0	2 ± 8	0		
YF 17D	4.7			2 ± 4	18 ± 13
	4.4			35 ± 24	250 ± 109
	3.4			9 ± 20	54 ± 179
	2.4			1 ± 0	53 ± 22
	1.4			0	46 ± 18

TABLE 8

Immunogenicity and protection vs. challenge Mice were immunized on Day 0 with live vaccines and on days 0, 7, and 20 with JE-VAX ® (inactivated Japanese Encephalitis virus vaccine), bled on day 21 and challenged on day 28.				
Virus	No./group	Dose (pfu)	Route	Total no. mice
1. YF/JE (SA14-14-2 RMS)*	8	10 ² -10 ⁵	sc	32
2. YF 17D (iv5.2) (Vero)	8	10 ² -10 ⁵	sc	32
3. YF 17D (PMC)	8	10 ² -10 ⁵	sc	32
4. JE Nakayama	8	10 ² -10 ⁵	sc	32
5. JE SA14-14-2 (BHKP1)**	8	10 ² -10 ⁵	sc	32
6. YF/JE (Nakayama)#	8	10 ² -10 ⁵	sc	32
7. JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) Connaught lot EJN*151B	8	100 ul 1:300 dilution on Day 0, 7 and 100 ul 1:5 dilution on D 20	sc	8

TABLE 8-continued

Immunogenicity and protection vs. challenge Mice were immunized on Day 0 with live vaccines and on days 0, 7, and 20 with JE-VAX ® (inactivated Japanese Encephalitis virus vaccine), bled on day 21 and challenged on day 28.				
Virus	No./group	Dose (pfu)	Route	Total no. mice
8. None (challenged)	8	—	ip	8
9. None (unchallenged)	8	—	—	8

*YF/JE SA14-14-2 vaccine candidate

**Chinese live vaccine, passed once in BHK cells

#Chimeric YF/JE virus, with prM-E insert of wild-type JE Nakayama

TABLE 9

Protection of C57/BL6 mice by a single SC inoculum of graded doses of live virus vaccines against IP challenge with 158 LD50 of wild-type JE virus (IC-37). Mice were challenged 28 days after immunization.							
Number of survivors/number challenged (% survivors) by vaccine dose (log ₁₀ pfu)							
Vaccine	None	1	2	3	4	5	Other
Yellow fever 17D (YF-VAX ® (Yellow Fever 17D vaccine) unpassaged)	NT*		3/8 (37.5%)	1/8 (12.5%)	1/8 (12.5%)	2/8 (25%)	
Yellow fever 17D (YF5.2iv infectious clone)	NT		0/8 (0%)	1/8 (12.5%)	1/8 (12.5%)	1/8 (12.5%)	
Yellow fever/JE SA14-14-2 chimera		1/8 (12.5%)	2/8 (25%)	7/7 (100%)	7/8 (87.5%)	7/7 (100%)	
Chinese JE vaccine		NT	1/8 (12.5%)	1/8 (12.5%)	0/8 (0%)	3/8 (37.5%)	
SA14-14-2 (BHK1)							
Wild-Type JE (Nakayama)#		NT	2/7 (29%)	1/6 (17%)	1/3 (33%)	1/4 (25%)	
YF/JE (Nakayama)			3/3 (100%)	5/5 (100%)	3/3 (100%)		
Mouse brain vaccine (JE-VAX ® (inactivated Japanese Encephalitis virus vaccine))**							7/8 (87.5%)
Control (challenge)	1/8 (12.5%)						
Control (no challenge)	8/8 (100%)						

*Not tested;

#Some mice died as a result of inoculation of the wild-type virus at high doses, thus fewer mice remained for challenge;

**Three doses at 1 week intervals;

^No mice survived initial inoculation at this dose

TABLE 10

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE- VAX TM (inactivated Japanese Encephalitis virus vaccine).			
Vaccine	Dose (log ₁₀ PFU)	Antibody titer (GMT ± SD) vs.	
		JEV	YF 17D
YF/JE SA14-14-2	5	44.8 ± 25.2	
	4	26.5 ± 23.1	
	3	6.2 ± 4.9	

TABLE 10-continued

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE- VAX TM (inactivated Japanese Encephalitis virus vaccine).			
Vaccine	Dose (log ₁₀ PFU)	Antibody titer (GMT ± SD) vs.	
		JEV	YF 17D
	2	1.1 ± 0.35	
	1	1 ± 0	

TABLE 10-continued

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE-VAX TM (inactivated Japanese Encephalitis virus vaccine).				
Vaccine	Dose (log ₁₀ PFU)	Antibody titer (GMT ± SD) vs.		
		JEV	YF 17D	
SA14-14-2(BHK1)	5	2.5 ± 4.3		
	4	3.5 ± 20.5		
	3	4.7 ± 15.5		
	2	1 ± 0		
JE Nakayama	5	1.32 ± 1		
	4	4 ± 4.0		
	3	1.6 ± 1.8		
	2	1 ± 0		
YF/JE-Nakayama	5	10 ± 70*		
	4	102.5 ± 45.7		
	3	76.8 ± 63.9		
	2	19.8 ± 8.1		
JE-VAX [®] (inactivated Japanese Encephalitis vaccine) (mouse brain)	3 doses**	2.8 ± 6.5		
YF-VAX [®] (Yellow Fever 17D vaccine)	5		11 ± 9.6	
	4		13.8 ± 19.1	
	3		4.3 ± 11.7	
	2		1 ± 0	
YF5.2iv (17D infect. clone)	5		29.3 ± 47.1	
	4		11 ± 15.2	
	3		8 ± 19.4	
	2		2.1 ± 3.2	
Controls	0	1 ± 0		

*only 2/8 mice survived immunization with this virus; the low antibody titers in these animals probably reflect low level virus replication consistent with survival.

**3 doses on days 0, 7, 20; animals were bled on day 21, 1 day after their third immunization. The day 20 boost was performed with a higher dose of vaccine, thus antibody titers pre-challenge are expected to be higher than those shown here.

TABLE 11

Geometric mean neutralizing antibody titers (GMT) in 3 monkeys 2 and 4 weeks post inoculation with a single dose of YF-VAX [®] (Yellow Fever 17D vaccine) or CHIMERIVAX TM (chimeric flavivirus vaccine) by the I.C. route					
Vaccine	Dose (log ₁₀ pfu)	GMT			
		JE		YF	
		2 W	4 W	2 W	4 W
CHIMERIVAX TM (chimeric flavivirus vaccine)	7.0	75	3200		
YF-VAX [®] (Yellow Fever 17D vaccine)	5.0			66	4971

TABLE 12

Immunization and protection: rhesus monkeys Screening III test for flavivirus antibodies: negative					
Group	N	Virus	Dose, route (log ₁₀ PFU/0.5 ml)	JE Challenge Day 60	
1	3	YF/JE SA14-14-2	4.3 SC	5.0 IC	
2	3	YF/JE SA14-14-2	5.3 SC	5.0 IC	
3	4	Saline/sham	—SC	5.0 IC	

Viremia days 1-7 after immunization and challenge

Neutralization test days 0, 15, 30, 45, and 60 after immunization and days 15 and 30 after challenge

Necropsy day 30 post challenge

TABLE 13

Viremia, rhesus monkeys immunized with ChimeriVax TM by the SC route								
Monkey	log ₁₀ PFU	Dose	Day post-inoculation					
			0	1	2	3	4	5
R423	4.3	<1.0*	<1.0	<1.0	<1.0	1.1	1.7	1.0
R073		<1.0	<1.0	<1.0	<1.0	1.0	1.0	<1.0
R364		<1.0	1.0	<1.0	<1.0	1.0	1.0	<1.0
R756	5.3	<1.0	1.0	1.0	1.6	1.0	<1.0	<1.0
R174		<1.0	1.3	1.8	1.6	1.1	<1.0	<1.0
R147		<1.0	2.0	1.6	1.0	1.0	<1.0	<1.0

*log₁₀ PFU/ml

TABLE 14

JE neutralizing antibody responses, rhesus monkeys immunized with ChimeriVax TM by the SC Route 50% PRNT titers, heat-inactivated serum, no added complement				
Monkey	log ₁₀ PFU	Dose	Day post-inoculation	
			Baseline	15
R423	4.3		<10	160
R073			<10	80
R364			<10	160
R756	5.3		<10	20
R174			<10	640
R147			<10	160

TABLE 15

Protection against IC challenge, rhesus monkeys immunized with ChimeriVax TM by the SC route Monkeys challenged IC on Day 60 with 100,000 pfu/mouse LD50		
Vaccine	Dose log ₁₀ PFU	No. survived/No. tested
	4.3	2/3 (67%)*
	5.3	3/3 (100%)
	Sham	0/4 (0%)

*1 monkey that died was a pregnant female

TABLE 16

List of chimeric YF/JE mutants (1 to 9) constructed to identify residues involved in attenuation of the CHIMERIVAX™ (chimeric flavivirus vaccine). Mutated amino acids on the E-proteins are shown in bold letters.													
Positions	Nakayama	CHIMERIVAX™ (chimeric flavivirus vaccine)	Mutant Viruses										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T
227	P	S	S	S	S	S	S	S	S	P	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K	K

TABLE 17

Dose administered i.c. (pfu)			
Group	P1	P10	P18
Neat	$\geq 6 \times 10^4$	1×10^6	2×10^7
10^{-1}	$\geq 6 \times 10^3$	1×10^5	2×10^6

TABLE 18

Dose administered s.c. (pfu)			
Group	RMS	P10	P18
Neat	2×10^5	2×10^7	3×10^7
10^3	1×10^5	5×10^5	5×10^4
10^4	1×10^4	5×10^4	5×10^3

TABLE 19

Design of an experiment to determine cross-protection/interference between YF 17D and YF/JE SA14-14-2				
# of female		2nd vaccine		
ICR mice	1st Vaccine¶	3 months	6 months	12 months
8	YF/JE SA14-14-2	YF-VAX ® (Yellow Fever 17D Vaccine)		
8	YF/JE SA14-14-2		YF-VAX ® (Yellow Fever 17D Vaccine)	
8	YF/JE SA14-14-2			YF-VAX ® (Yellow Fever 17D Vaccine)
8	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine)	YF-VAX ® (Yellow Fever 17D Vaccine)		
8	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine)		YF-VAX ® (Yellow Fever 17D Vaccine)	
8	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine)			YF-VAX ® (Yellow Fever 17D Vaccine)
8	YF-VAX ® (Yellow Fever 17D Vaccine)	YF/JE SA14-14-2		
8	YF-VAX ® (Yellow Fever 17D Vaccine)		YF/JE SA14-14-2	

TABLE 19-continued

Design of an experiment to determine cross-protection/interference between YF 17D and YF/JE SA14-14-2				
# of female		2nd vaccine		
ICR mice	1st Vaccine¶	3 months	6 months	12 months
8	YF-VAX ® (Yellow Fever 17D Vaccine)			YF/JE SA14-14-2
4		YF-VAX ® (Yellow Fever 17D Vaccine)		
4		YF/JE SA14-14-2		
4			YF-VAX ® (Yellow Fever 17D Vaccine)	
4			YF/JE SA14-14-2	
4				YF-VAX ®(Yellow Fever 17D Vaccine)
4				YF/JE SA14-14-2

¶One dose of YF/JE SA14-14-2, 5.3 log₁₀ pfu/mouse, sc.

One dose of YF-VAX ® (Yellow Fever 17D Vaccine), 4.4 log₁₀ pfu/mouse, sc.

Two doses of JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) (PMC), 0.5 ml of 1:5 dilution administered ip at 1 week intervals.

TABLE 20

Engineering of YF/Flavivirus chimeras					
Virus	Chimeric C/prM junction ¹	Chimeric E/NS1 junction ²	5' ligation ³	3' ligation ⁴	Sites ⁵ eliminated or (created)
YF/WN	X-cactgggagagcttgaaggctc (SEQ ID NO: 1)	<u>aaagccagttgcag</u> ccgcggtttaa (SEQ ID NO: 2)	AatII	NsiI	
YF/DEN-1	X-aaggtagactgggtgggtccc (SEQ ID NO: 3)	<u>gatcctcagtagccac</u> ccgcggtttaa (SEQ ID NO: 4)	AatII	SphI	SphI in DEN
YF/DEN-2	X-aaggtagattgggtgtgcattg (SEQ ID NO: 5)	<u>aaccctcagtagccac</u> ccgcggtttaa (SEQ ID NO: 6)	AatII	SphI	
YF/DEN-3	X-aaggtgaattgaagtgtctcta (SEQ ID NO: 7)	<u>acccccagcaccac</u> ccgcggtttaa (SEQ ID NO: 8)	AatII	SphI	XhoI in DEN (SphI in DEN)
YF/DEN-4	X-aaaaggaacagttgttctcta (SEQ ID NO: 9)	<u>accggaagtgtca</u> ccgcggtttaa (SEQ ID NO: 10)	AatII	NsiI	
YF/SLE	X-aacgtgaatagttggatagtc (SEQ ID NO: 11)	<u>accgttgggtgcac</u> ccgcggtttaa (SEQ ID NO: 12)	AatII	SphI	AatII in SLE
YF/MVE	X-aatttcgaaaggtggaaggctc (SEQ ID NO: 13)	<u>gaccggtgttttacag</u> ccgcggtttaa (SEQ ID NO: 14)	AatII	AgeI	(AgeI in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO: 15)	<u>actgggaacctcac</u> ccgcggtttaa (SEQ ID NO: 16)	AatII	AgeI	(AgeI in YF)

^{1,2}The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the NarI site (antisense-cgcggg). This site allows insertion of PCR products into the Yfm5.2 (NarI) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

^{3,4}The unique restriction sites used for creating restriction fragments that can be isolated and ligated in vitro to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

⁵In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient in vitro ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

Summary of histopathology results, monkeys inoculated with YF-VAX ® (Yellow Fever 17D vaccine) or YF/JE SA14-14-2 by the IC route					
YF-VAX ® (Yellow Fever 17D vaccine)			CHIMERIVAX-JE ™ (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)		
Monkey No.	Discriminator area score	Discriminator plus target area score	Monkey No.	Discriminator area score	Discriminator plus target area score
N030	0.21	0.64	N191	0	0.17
N492	0.04	0.36	N290	0.09	0.06
N479	0	0.17	N431	0.13	0.09
Group means	0.08	0.39		0.07	0.11

List of initial chimeric YF/JE mutants constructed to identify residues involved in attenuation of the CHIMERIVAX™ (chimeric flavivirus vaccine). Reverted amino acids on the E-proteins are shown in BOLD													
Protein	Nakayama	CHIMERIVAX™ (chimeric flavivirus vaccine)	MUTANT VIRUSES										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	V	I	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T

List of initial chimeric YF/JE mutants constructed to identify residues involved in attenuation of the CHIMERIVAX™ (chimeric flavivirus vaccine). Reverted amino acids on the E-proteins are shown in BOLD														
Protein	Nakayama	CHIMERIVAX™ (chimeric flavivirus vaccine)	MUTANT VIRUSES											
			1	2	3	4	5	6	7	8	9	10	11	
227	P	S	S	S	S	S	S	S	S	P	P	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K	K	K

TABLE 24

Experiment to determine neurovirulence and neuroinvasiveness phenotypes of vaccine candidates in suckling mice					
Virus	Route	AGE OF MICE (DAYS)			
		3	5	7	9
YF/Den-2	I.C.	10 ⁴ *	10 ⁴	10 ⁴	10 ⁴
YF/JESA14-14-2	I.C.	10 ⁴	10 ⁴	10 ⁴	10 ⁴

TABLE 24-continued

Experiment to determine neurovirulence and neuroinvasiveness phenotypes of vaccine candidates in suckling mice					
Virus	Route	AGE OF MICE (DAYS)			
		3	5	7	9
YF 17D	I.P.	10 ³	10 ³	10 ³	10 ³
MED + 5% FBS	I.C., I.P.	—	—	—	—

*PFU/0.02 ml of inoculum

TABLE 25

Summary of differences between virulent (Asibi) and attenuated
(17D, 17DD, RMS, P18) yellow fever viruses

Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
C	304	G	A	A	A	A	A	A	
	370	T	C	C	C	C	C	C	
non-M	643	A	A	—	—	A	A	G	
M	854	C	T	—	—	T	T	T	L F
	883	A	G	—	—	G	G	A	
E	1127	G	A	—	—	A	A	A	G R
	1140	C	T	—	—	T	T	C	A V
	1431	A	A	—	—	A	C	A	N T
	1436	G	G	—	—	G	G	A	D S
	1437	A	A	—	—	A	A	G	
	1482	C	T	—	—	T	T	T	A V
	1491	C	T	—	—	T	T	T	T I
	1558	C	C	—	—	C	C	A	
	1572	A	C	—	—	C	C	C	K T
	1750	C	T	—	—	T	T	T	
	1819	C	T	—	—	T	T	T	
	1870	G	A	—	—	A	A	A	M I
	1887	C	T	—	—	T	T	T	S F
	1946	C	T	—	—	T	T	C	P S
	1965	A	G	—	—	G	G	G	K R
	2110	G	G	—	—	G	G	A	
	2112	C	G	—	—	G	G	G	T R
	2142	C	A	—	—	A	A	A	P H
	2219	G	A	—	—	A	A	G	A T
	2220	C	C	—	—	C	C	T	T I
NS1	2356	C	T	—	—	T	T	T	
	2687	C	T	T	T	T	T	T	F L
	2704	A	G	G	G	G	G	G	
	3274	G	A	A	A	A	A	A	
	3371	A	G	G	G	G	G	G	V I
	3599	T	T	T	T	T	T	C	
	3613	G	A	A	A	A	A	A	
	3637	C	C	C	C	C	C	T	
ns2a	3817	G.A	G	G	G	G	G	G	
	3860	A	G	G	G	G	G	G	V M
	3915	T.A	T	T	T	T	T	T	
	4007	A	G	G	G	G	G	G	A T
	4013	C	T	T	T	T	T	C	F L
	4022	A	G	G	G	G	G	G	A T
	4025	G	G	A	A	G	G	G	V M
	4054	C	T	T	T	T	T	C	
	5056	C	T	T	T	T	T	T	F S
ns2b	4204	C	C	C	C	C	C	C	
	4289	A	C	C	C	C	C	C	L I
	4387	A	G	G	G	G	G	G	
	4505	A	C	C	C	C	C	C	L I
	4507	T	C	C	C	C	C	C	

TABLE 25-continued

Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, P18) yellow fever viruses								
Gene	NT	Asibi	17D204US	RMS P18	17D204F	17D213	17DD AA	
NS3	4612	T	C	C C	C	C	T	
	4864	G.A	G	G G	G	G	G	
	4873	T	G	G G	G	G	T	
	4942	A	A	A A	A	A	G	
	4957	C	C	C C	C	C	T	
	4972	G	G	G G	G	G	A	
	5115	A	A	A A	A	A	G	O R
	5131	G.T	G	G G	G	G	G	M M.I
	5153	A	G	G G	G	G	A	V I
	5194	T	C	C C	C	C	C	
	5225	A	C	C C	C	C	C	
	5362	C	C	C C	C	T	A	
	5431	C	T	T T	T	T	T	
NS3	5461	T	T	C C	T	T	T	
	5473	C	T	T T	T	T	T	
	5641	G	A	G G	A	G	G	
	6013	C	T	T T	T	T	T	
	6023	G	A	A A	A	A	A	N D
ns4a	6070	C	C	C C	C	C	T	
	6448	G	T	T T	T	T	T	
	6514	T	T	T T	T	T	C	
	6529	T	C	C C	T	T	T	
	6625	A	A	A A	A	C	C	
	6758	A	G	G G	A	A	A	V I
	5829	T	C	C C	C	C	C	
	5876	T	C	C C	C	C	C	A V
ns4b	7171	A	G	G G	G	G	G	M I
	7319	G	G	A A	A	A	A	E K
	7497	T	T	T T	T	C	T	L S
	7571	C	A	A A	A	A	C	
NS5	7580	T	C	C C	C	C	C	H Y
	7642	T	C	C C	C	C	C	
	7701	A	G	G G	G	G	A	R O
	7945	C	T	T T	T	T	T	
	7975	C	C	C C	C	C	T	
	8008	T	C	C C	C	C	C	
	8029	T	T	T T	T	T	C	
	8212	C	C	T T	C	C	C	
	8581	A	A	C C	A	A	A	
	8629	C	T	T T	T	T	T	
	8808	A	A	A A	A	A	G	N S
	9397	A	A	A A	A	A	G	
	9605	A	G	G G	A	A	A	D N
	10075	G.T	G	G G	G	G	G	M M.I
	10142	G	A	A A	A	A	A	K E
	10243	G	A	A A	A	A	A	
	10285	T	C	C C	C	C	C	
	10312	A	G	G G	G	G	G	
	10316	T.C	T	T T	T	T	T	S S.P
3'N	10339	C	G	G G	G	G	G	
	10367	T	C	C C	C	C	C	
	10418	T	C	C C	C	C	C	
	10454	A	G	A A	A	A	A	
	10550	T	C	C C	C	C	T	
	10722	G	G	G G	A	G	G	

TABLE 25-continued

Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, P18) yellow fever viruses								
Gene	NT	Asibi	17D204US	RMS P18	17D204F	17D213	17DD	AA
	10800	G	A	A	A	A	A	A
	10847	A	C	C	C	C	C	C

NT: nucleotide numbers are from 5' terminus of the genome.

Where clonal differences were present, both nucleotides as well as amino acids (if appropriate) are shown. If nucleotide change results in an amino acid substitution, the amino acid (AA) is shown from left to right (e.g. from Asibi to 17D).

—: The genes for prME in RMS (YF17D/JESA14-14-2) and P18 (passage 18th of the RMS) are from JEV strain SA14-14-2, therefore not comparable with YFV sequences.

Sequences for Asibi is taken from Hahn et al. 1987. 17D204US from Rice et al. 1985. 17D204F from Dupuy et al. 1989.

RMS and P18 are unpublished sequences (Ora Vax, Inc.), 17D213 and 17DD from Duarte dos Santos et al. 1994.

Note that there is no sequence difference between RMS and passage 18th.

There are 6 nucleotide differences (nucleotide positions are shaded) between published YF17D sequence and RMS shown in bold letters; Changes in 5461, 5641, 8212 and 8581 are silent and do not result in amino acid substitution.

Changes in positions 4025 and 7319 result in amino acid substitution.

TABLE 26

Immunogenicity of CHIMERIVAX™ (chimeric flavivirus vaccine comprising Dengue-2 virus prM and E proteins) passed in Vero cells for mice.			
Passage level ^a	Dose (Log ₁₀ pfu)	GMT ^b SC	GMT ^b IC
P3	5	1 ± 0 ^c	61 ± 47
	4	1 ± 0	7 ± 15
P5	5	1 ± 0	46 ± 16
	4	1 ± 0	9 ± 20
P10	5	1.8 ± 7.7	46 ± 53
	4	1 ± 0	7 ± 15
P18	5	1 ± 0	53 ± 17
	4	1 ± 0	2 ± 16

^aCHIMERIVAX™ (chimeric flavivirus vaccine comprising Dengue-2 virus prM and E proteins) virus was passaged in Vero_{PM} cells (P141-147) at MOI of 0.1-0.5 and harvested 2-3 days PI.

^bGeometric Mean Titers measured as the last dilution of sera which resulted in 50% reduction in number of virus plaques.

^cTiters less than 1:10

TABLE 27

Immunization and challenge of yellow fever immune monkeys				
1 st Vaccine	2 nd Vaccine	Seroconversion		Viremic after
		Den2	YF	challenge
YF17D	CHIMERIVAX™ (chimeric flavivirus vaccine comprising Dengue-2 virus prM and E proteins)	3/3	3/3	0/3
YF17D	Dengue-2 wt	4/4	4/4	0/4
YF17D	YF 17D	0/3	3/3	3/3
YF17D	None	0/2	2/2	2/2
None	None	0/2	0/2	2/2

TABLE 28

Viremia in rhesus monkeys inoculated SC with graded doses of ChimeriVax™-Den-2 virus.				
Virus	Dose (Log ₁₀ PFU)	No. viremic (%)	Duration (days) mean	Titer mean peak
ChimeriVax™-Den-2	2.0	4/4 (100)	3.2	1.6
	3.0	5/5 (100)	3.8	1.6
	4.0	5/5 (100)	4.0	1.3
	5.0	4/4 (100)	4.3	1.4
Dengue-2 (18603)	4.0	4/4 (100)	5	3.6

TABLE 29

Neutralizing antibody responses in monkeys inoculated with graded doses of ChimeriVax™-Den-2 and protection against wild-type dengue-2 challenge					
Dose	No.	GMT by day			No. Viremic after Challenge
Log ₁₀ PFU	seroconverted	0	15	30	(D60)
2.0	4/4	<10	47	316	0/4
3.0	5/5	<10	112	275	0/5
4.0	5/5	<10	269	316	0/5
5.0	4/4	<10	631	376	0/4
Sham	0/4	<10	<10	<10	4/4

TABLE 30

Mean virus titer in orally exposed mosquitoes 22 days post feeding			
Mosquito Species	Virus	# Infected/# Fed	Mean Titer*
<i>Ae. aegypti</i>	JE SA14-14-2	20/20	6.0
	YF 17D	0/30	<1.0
	ChimeriVax™-JE	0/40	<1.0
<i>Ae. albopictus</i>	JE SA14-14-2	38/38	5.8
	YF 17D	0/44	<1.0
	ChimeriVax™-JE	0/56	<1.0

*log₁₀pfu/ml

TABLE 31

#1) YFM5'3' (4.56)+	(GTGAGCATTGAGAAAGCGCCACGCTTC)	(SEQ ID NO: 17)
#2) YF0.481-	(TCCACCCGTCATCAACAGCATTCCCAAATTAG)	(SEQ ID NO: 18)
#3) 1DE 0.42+	(GAATGCTGTTGATGACGGGTGGATTTCATCTGACCACACGAGGG)	(SEQ ID NO: 19)
#4) 1DE 1.095-	(NheI/BstBI) (GCCGCTAGCTTTTCGAAGGACGGCAGGGTTTGTGACTTC)	(SEQ ID NO: 20)
#5) 1DE 1.102+	(BstBI) (GCCATGCATTTTCGAAAAGTGTGCATCGAAGCTAAATATC)	(SEQ ID NO: 21)
#7) 1DE 2.409FUSE-	(GGCGCATCCTTGATCGGCGCCAACCATGACTCCTAGGTACAG)	(SEQ ID NO: 22)
#8) YF NarI+	(GGCGCCGATCAAGGATGCGCCATC)	(SEQ ID NO: 23)
#9) YF 8.545-	(CCAAGAGGTCATGTACTCAG)	(SEQ ID NO: 24)
#10) SP6YFa+	(ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT)	(SEQ ID NO: 25)

Primers (restriction sites are underlined)

TABLE 32

Oligonucleotide primers.		
No.	SequenceName	
1	3D 0.432+ 5'-GAATGCTGTTGATGACGGGTGGATTCCACTTAACCTCACGAGATGG	(SEQ ID NO: 26)
2	3DE 1.095- 5'-GCCGCTAGCCTTTTCGAAGGGTCGCCAGCTGAGTGGCCTC	(SEQ ID NO: 27)
3	3DE 1.102+ 5'-GCCGCTAGCTTCGAAAGCTATGCATTGAGGGAAAAATTAC	(SEQ ID NO: 28)
4	3DE 2.409- 5'-GCCGCGCGCGCCACACGACCCCGAGATAGAGTG	(SEQ ID NO: 29)
5	YFM5'3' (4.65)+ 5'-GTGAGCATTGAGAAAGCGCCACGCTTC	(SEQ ID NO: 30)
6	YF 0.481- 5'-TCCACCCGTCATCAACAGCATTCCCAAATTAG	(SEQ ID NO: 31)
7	YF 0.2+ 5'-ATGGTACGACGAGGAGTTCGC	(SEQ ID NO: 32)
8	Kpd3/-Xho/+ 5'-GGTTGATGTGGTGCTGGAGCACGGTGGGTGTG	(SEQ ID NO: 33)
9	PsD3/.7+ 5'-TACATCGACATGGGTGAC	(SEQ ID NO: 34)
10	KPsD3/.75- 5'-GACATGGGGAGCTAACGC	(SEQ ID NO: 35)
11	KPsD3/1.5- 5'-CCCGAGGGTTCCATATTCAGG	(SEQ ID NO: 36)
12	KPsD3/1.6+ 5'-GGAACAGGAAAGAGCTTC	(SEQ ID NO: 37)
13	KPsD3/1.9- 5'-GAGTATTGTCCCATGCTG	(SEQ ID NO: 38)
14	KPsD3/2.1+ 5'-GGAATTGGAGACAAAGCC	(SEQ ID NO: 39)
15	KPs5.2 0.23+ 5'-TGGATAGTGGACAGACAGTGG	(SEQ ID NO: 40)
16	KPs5.2 1.66- 5'-CTCTAAATATGAAGATACCATC	(SEQ ID NO: 41)
17	SP6-yfa 5'-ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT	(SEQ ID NO: 42)

TABLE 33

Amino acid differences in the prM-E region of DEN3 H87 and PaH881/88 strains			
(nt/gene) ¹	Position (PaH881/88)	H87	Position H87 (PaH881/88)
	(codon change)	(a.a.) ²	(a.a. change)
599/prM	CAC CTC	168	H L
605/prM	ACC GCC	171	T A
932/prM	ACA GCA	280	T A
1373/E	GAC AAC	427	D N
1394/E	GAA GAC	434	E D
1424/E	TCC CCC	444	S P
1439/E	GCT GTT	449	A V
1607/E	AAA GAG	505	K E
1742/E	ACC AAC	550	T N
1805/E	AAA GAA	571	K E
2105/E	AGG AAG	671	R K
2366/E	ATT GTT	758	I V

¹Positions of nucleotides in the DEN3 genome; numbering is according to (Osatomi et al., Virology 176: 643-647, 1990).

²Positions of amino acid residues in the DEN3 polyprotein; numbering is according to (Osatomi et al., Virology 176: 643-647, 1990).

TABLE 34

#1) YFM5'3' (4.56)+ (GTGAGCATTGAGAAAGCGCCACGCTTC) (SEQ ID NO: 43)
#2) YF0.481- (TCCACCCGTCATCAACAGCATTCCCAAATTAG) (SEQ ID NO: 44)
#3) 4DE 0.432+ (GAATGCTGTTGATGACGGGTGAATTTACCTGTCAACAAGAGACGG) (SEQ ID NO: 45)
#4) 4DEE 1.095- (GCCGCTAGCGGTTTGAATAGGCCACTTCCTTGCGTGT) (SEQ ID NO: 46)
#5) 4DE 1.102+ (GCCGCTAGCTTCGAACCTATTGCATTGAAGCCTCGATATC) (SEQ ID NO: 47)
#6) 4DE 2.409- (GCCGCCGCGCCAACTGTGAAACCTAGAAACACAG) (SEQ ID NO: 48)
#7) sp6YFa+ (ATTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT) (SEQ ID NO: 49)

Other Embodiments

[0371] Other embodiments are within the following claims. For example, the prM-E protein genes of other flaviviruses of medical importance can be inserted into the yellow fever vaccine virus backbone to produce vaccines against other medically important flaviviruses (see, e.g., Monath et al., "Flaviviruses," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, 961-1034). Examples of additional flaviviruses from which genes to be inserted into the chimeric vectors of the invention can be obtained include, e.g., Kunjin, Central European Encephalitis, Russian Spring-Summer Encephalitis, Powassan, Kyasanur Forest Disease, and Omsk Hemorrhagic Fever viruses. In addition, genes from even more distantly related viruses can be inserted into the yellow fever vaccine virus to construct novel vaccines.

Vaccine Production and Use

[0372] The vaccines of the invention are administered in amounts, and by using methods, that can readily be determined by persons of ordinary skill in this art. The vaccines can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, e.g., as a clarified suspension of infected chicken embryo tissue, or a fluid harvested from cell cultures infected with the chimeric yellow fever virus. Thus, the live, attenuated chimeric virus is formulated as a sterile aqueous solution containing between 100 and 1,000,000 infectious units (e.g., plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous, or intradermal routes. In addition, because flaviviruses may be capable of infecting the human host via the mucosal routes, such as the oral route (Gresikova et al., "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Fla., 1988, Volume IV, 177-203), the vaccine virus can be administered by a mucosal route to achieve a protective immune response. The vaccine can be administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

[0373] It may be desirable to use the yellow fever vaccine vector system for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself. Nor will prior immunity to yellow

fever virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against yellow fever. Although YF 17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger et al., J. Immunology 135:2805-2809, 1985), or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is unlikely that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previ-

ously infected with JE virus respond to vaccination with YF 17D similarly to persons without previous JE infection, and (2) individuals who have previously received the YF 17D vaccine respond to revaccination with a rise in neutralizing antibody titers (Sweet et al., Am. J. Trop. Med. Hyg. 11:562-569, 1962). Thus, the chimeric vector can be used in populations that are immune to yellow fever because of prior natural infection or vaccination, and can be used repeatedly, or to immunize simultaneously or sequentially with several different constructs, including yellow fever chimeras with inserts from, for example, Japanese Encephalitis, St. Louis Encephalitis, or West Nile viruses.

[0374] For vaccine applications, adjuvants that are known to those skilled in the art can be used. Adjuvants that can be used to enhance the immunogenicity of the chimeric vaccines include, for example, liposomal formulations, synthetic adjuvants, such as saponins (e.g., QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazine. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a chimeric vaccine delivered via a mucosal route, for example, orally, mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT are useful adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the yellow fever vectors. Thus, genes encoding cytokines, such as α -interferon, GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with heterologous flavivirus genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically towards cellular, humoral, or mucosal responses.

[0375] In addition to vaccine applications, as one skilled in the art can readily understand, the vectors of the invention can be used in gene therapy methods to introduce therapeutic gene products into a patient's cells and in cancer therapy. In these methods, genes encoding therapeutic gene products are inserted into the vectors, for example, in place of the gene encoding the prM-E protein.

[0376] Yellow fever 17D virus targets cells of the lymphoid and reticuloendothelial systems, including precursors in bone marrow, monocytes, macrophages, T cells, and B cells (Monath, "Pathobiology of the Flaviviruses," pp. 375-425, in Schlesinger & Schlesinger (Eds.), "The Togaviridae and Flaviviridae," Plenum Press, New York 1986). The yellow fever 17D virus thus naturally targets cells involved in antigen presentation and immune stimulation. Replication of the virus in these cells, with high-level expression of heterologous genes, makes yellow fever 17D vaccine virus an ideal vector for gene therapy or immunotherapy against cancers of the lymphoreticular system and leukemias, for example. Additional advantages are that (1) the flavivirus genome does not integrate into host cell DNA, (2) yellow fever virus appears to persist in the host for prolonged periods, and (3) that heterologous genes can be inserted at the 3' end of the yellow fever vector, as described above in the strategy for producing a Hepatitis C vaccine. Yellow fever 17D virus can be used as a vector carrying tumor antigens for induction of immune responses for cancer immunotherapy. As a second application, yellow fever 17D can be used to target lymphoreticular tumors and express heterologous genes that have anti-tumor effects, including cytokines, such as TNF- α . As a third application, yellow fever 17D can be used to target heterologous genes to bone marrow to direct expression of bioactive molecules required to treat hematologic diseases, such as, for example, neutropenia; an example of a bioactive molecule that can be used in such an application is GM-CSF, but other appropriate bioactive molecules can be selected by those skilled in the art.

[0377] An additional advantage of the yellow fever vector system is that flaviviruses replicate in the cytoplasm of cells, so that the virus replication strategy does not involve integration of the viral genome into the host cell (Chambers et al., "Flavivirus Genome Organization, Expression, and Replication," In *Annual Review of Microbiology* 44:649-688, 1990), providing an important safety measure.

[0378] All references cited herein are incorporated by reference in their entirety.

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virus

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<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 33

ggttgatgtg gtgctggagc acggtgggtg tg 32

<210> SEQ ID NO 34
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 34

tacatcgaca tgggtgac 18

<210> SEQ ID NO 35
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 35

gacatgggga gctaacgc 18

<210> SEQ ID NO 36
<211> LENGTH: 21
<212> TYPE: DNA

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<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 36

cccgaggggtt ccatattcag g 21

<210> SEQ ID NO 37

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 37

ggaacaggaa agagcttc 18

<210> SEQ ID NO 38

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 38

gagtattgtc ccatgctg 18

<210> SEQ ID NO 39

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 39

ggaattggag acaaagcc 18

<210> SEQ ID NO 40

<211> LENGTH: 21

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 40

tggatagtgg acagacagtg g 21

<210> SEQ ID NO 41

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 41

ctctaaatat gaagatacca tc 22

<210> SEQ ID NO 42

<211> LENGTH: 39

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 42

atttaggtga cactatagag taaatcctgt gtgctaatt 39

<210> SEQ ID NO 43

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 43

gtgagcattg agaaagcgcc acgcttc 27

-continued

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<210> SEQ ID NO 44
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 44

tccacccgtc atcaacagca ttcccaaat tag                               33


<210> SEQ ID NO 45
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 45

gaatgctgtt gatgacgggt gaatttcacc tgtcaacaag agacgg              46


<210> SEQ ID NO 46
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 46

gccgctagcg gttcgaaata gagccacttc cttggctgt                      39


<210> SEQ ID NO 47
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 47

gccgctagct tcgaacctat tgcattgaag cctcgatata                    40


<210> SEQ ID NO 48
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 48

gccgccggcg ccaactgtga aacctagaaa cacag                          35


<210> SEQ ID NO 49
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 49

atttaggtga cactatagag taaatcctgt gtgctaatt                      39


<210> SEQ ID NO 50
<211> LENGTH: 1983
<212> TYPE: DNA
<213> ORGANISM: Dengue-2 virus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(1983)

<400> SEQUENCE: 50

ttc cat cta acc aca cgt aac gga gaa cca cac atg atc gtc agt aga    48
Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Ser Arg
1           5           10           15

caa gag aaa ggg aaa agt ctt ttg ttt aaa aca gag gat ggc gtg aac    96

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Gln	Glu	Lys	Gly	Lys	Ser	Leu	Leu	Phe	Lys	Thr	Glu	Asp	Gly	Val	Asn	
			20					25					30			
atg	tgc	acc	ctc	atg	gcc	atg	gac	ctt	ggg	gaa	ttg	tgt	gaa	gac	aca	144
Met	Cys	Thr	Leu	Met	Ala	Met	Asp	Leu	Gly	Glu	Leu	Cys	Glu	Asp	Thr	
		35					40					45				
atc	acg	tac	aag	tgt	ccc	ctt	ctc	agg	cag	aat	gag	cca	gaa	gac	ata	192
Ile	Thr	Tyr	Lys	Cys	Pro	Leu	Leu	Arg	Gln	Asn	Glu	Pro	Glu	Asp	Ile	
		50				55					60					
gac	tgc	tgg	tgc	aac	tcc	acg	tcc	acg	tgg	gta	acc	tat	ggg	act	tgt	240
Asp	Cys	Trp	Cys	Asn	Ser	Thr	Ser	Thr	Trp	Val	Thr	Tyr	Gly	Thr	Cys	
65					70				75					80		
acc	acc	acg	gga	gaa	cat	aga	aga	gaa	aaa	aga	tca	gtg	gca	ctc	gtt	288
Thr	Thr	Thr	Gly	Glu	His	Arg	Arg	Glu	Lys	Arg	Ser	Val	Ala	Leu	Val	
			85						90					95		
cca	cat	gtg	gga	atg	gga	ctg	gag	acg	cga	act	gaa	aca	tgg	atg	tca	336
Pro	His	Val	Gly	Met	Gly	Leu	Glu	Thr	Arg	Thr	Glu	Thr	Trp	Met	Ser	
			100					105					110			
tca	gaa	ggg	gct	tgg	aaa	cat	gcc	cag	aga	att	gaa	att	tgg	atc	ctg	384
Ser	Glu	Gly	Ala	Trp	Lys	His	Ala	Gln	Arg	Ile	Glu	Ile	Trp	Ile	Leu	
		115					120					125				
aga	cat	cca	ggc	ttc	acc	ata	atg	gca	gca	atc	ctg	gca	tac	acc	ata	432
Arg	His	Pro	Gly	Phe	Thr	Ile	Met	Ala	Ala	Ile	Leu	Ala	Tyr	Thr	Ile	
		130				135					140					
ggg	acg	aca	cat	ttc	cag	aga	gca	ctg	att	ttc	atc	tta	ctg	aca	gct	480
Gly	Thr	Thr	His	Phe	Gln	Arg	Ala	Leu	Ile	Phe	Ile	Leu	Leu	Thr	Ala	
145					150				155					160		
gtc	gct	cct	tca	atg	aca	atg	cgt	tgc	ata	gga	ata	tca	aat	aga	gac	528
Val	Ala	Pro	Ser	Met	Thr	Met	Arg	Cys	Ile	Gly	Ile	Ser	Asn	Arg	Asp	
			165					170						175		
ttt	gta	gaa	ggg	gtt	tca	gga	gga	agc	tgg	gtt	gac	ata	gtc	tta	gaa	576
Phe	Val	Glu	Gly	Val	Ser	Gly	Gly	Ser	Trp	Val	Asp	Ile	Val	Leu	Glu	
		180						185					190			
cat	gga	agc	tgt	gtg	acg	acg	atg	gca	aaa	aac	aaa	cca	aca	ttg	gat	624
His	Gly	Ser	Cys	Val	Thr	Thr	Met	Ala	Lys	Asn	Lys	Pro	Thr	Leu	Asp	
		195					200					205				
ttt	gaa	ctg	ata	aaa	aca	gaa	gcc	aaa	cag	cct	gcc	acc	cta	agg	aag	672
Phe	Glu	Leu	Ile	Lys	Thr	Glu	Ala	Lys	Gln	Pro	Ala	Thr	Leu	Arg	Lys	
		210					215				220					
tac	tgt	ata	gag	gca	aag	cta	acc	aac	aca	aca	aca	gaa	tct	cgt	tgc	720
Tyr	Cys	Ile	Glu	Ala	Lys	Leu	Thr	Asn	Thr	Thr	Thr	Glu	Ser	Arg	Cys	
225					230				235					240		
cca	aca	caa	ggg	gaa	ccc	agc	cta	aat	gaa	gag	cag	gat	aaa	agg	ttc	768
Pro	Thr	Gln	Gly	Glu	Pro	Ser	Leu	Asn	Glu	Glu	Gln	Asp	Lys	Arg	Phe	
			245						250					255		
gtc	tgc	aaa	cac	tcc	atg	gta	gac	aga	gga	tgg	gga	aat	gga	tgt	gga	816
Val	Cys	Lys	His	Ser	Met	Val	Asp	Arg	Gly	Trp	Gly	Asn	Gly	Cys	Gly	
		260					265						270			
tta	ttt	gga	aag	gga	ggc	att	gtg	acc	tgt	gct	atg	ttc	aca	tgc	aaa	864
Leu	Phe	Gly	Lys	Gly	Gly	Ile	Val	Thr	Cys	Ala	Met	Phe	Thr	Cys	Lys	
		275					280					285				
aag	aac	atg	gag	gga	aaa	gtt	gtg	cag	cca	gaa	aac	ttg	gaa	tac	acc	912
Lys	Asn	Met	Glu	Gly	Lys	Val	Val	Gln	Pro	Glu	Asn	Leu	Glu	Tyr	Thr	
		290					295					300				
att	gtg	gta	aca	ccc	cac	tca	ggg	gaa	gag	cat	gcg	gtc	gga	aat	gac	960
Ile	Val	Val	Thr	Pro	His	Ser	Gly	Glu	Glu	His	Ala	Val	Gly	Asn	Asp	
		305				310				315				320		
aca	gga	aaa	cat	ggc	aag	gaa	atc	aaa	gta	aca	cca	cag	agt	tcc	atc	1008

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Thr	Gly	Lys	His	Gly	Lys	Glu	Ile	Lys	Val	Thr	Pro	Gln	Ser	Ser	Ile	
				325					330					335		
aca	gaa	gca	gaa	ttg	aca	ggg	tat	ggc	act	gtc	acg	atg	gag	tgc	tct	1056
Thr	Glu	Ala	Glu	Leu	Thr	Gly	Tyr	Gly	Thr	Val	Thr	Met	Glu	Cys	Ser	
			340					345					350			
ccg	aga	aca	ggc	ctc	gac	ttc	aat	gag	atg	gtg	ttg	ctg	cag	atg	gaa	1104
Pro	Arg	Thr	Gly	Leu	Asp	Phe	Asn	Glu	Met	Val	Leu	Leu	Gln	Met	Glu	
		355					360					365				
aat	aaa	gct	tgg	ctg	gtg	cat	agg	caa	tgg	ttc	cta	gac	ctg	ccg	tta	1152
Asn	Lys	Ala	Trp	Leu	Val	His	Arg	Gln	Trp	Phe	Leu	Asp	Leu	Pro	Leu	
		370				375					380					
cca	tgg	ctg	ccc	gga	gcg	gac	aca	caa	ggg	tca	aat	tgg	ata	caa	aaa	1200
Pro	Trp	Leu	Pro	Gly	Ala	Asp	Thr	Gln	Gly	Ser	Asn	Trp	Ile	Gln	Lys	
				385		390			395						400	
gaa	aca	ttg	gtc	act	ttc	aaa	aat	cct	cat	gcg	aag	aaa	cag	gat	gtt	1248
Glu	Thr	Leu	Val	Thr	Phe	Lys	Asn	Pro	His	Ala	Lys	Lys	Gln	Asp	Val	
			405					410					415			
gtt	gtt	tta	gga	tcc	caa	gaa	ggg	gcc	atg	cac	aca	gca	ctc	aca	ggg	1296
Val	Val	Leu	Gly	Ser	Gln	Glu	Gly	Ala	Met	His	Thr	Ala	Leu	Thr	Gly	
			420				425						430			
gcc	aca	gaa	atc	caa	atg	tca	tca	gga	aac	tta	ctc	ttc	aca	gga	cat	1344
Ala	Thr	Glu	Ile	Gln	Met	Ser	Ser	Gly	Asn	Leu	Leu	Phe	Thr	Gly	His	
		435				440						445				
ctc	aag	tgc	agg	ctg	aga	atg	gac	aag	cta	cag	ctc	aaa	gga	atg	tca	1392
Leu	Lys	Cys	Arg	Leu	Arg	Met	Asp	Lys	Leu	Gln	Leu	Lys	Gly	Met	Ser	
		450				455					460					
tac	tct	atg	tgc	aca	gga	aag	ttt	aaa	gtt	gtg	aag	gaa	ata	gca	gaa	1440
Tyr	Ser	Met	Cys	Thr	Gly	Lys	Phe	Lys	Val	Val	Lys	Glu	Ile	Ala	Glu	
		465			470				475					480		
aca	caa	cat	gga	aca	ata	gtt	atc	agg	gtg	cag	tat	gaa	ggg	gac	ggc	1488
Thr	Gln	His	Gly	Thr	Ile	Val	Ile	Arg	Val	Gln	Tyr	Glu	Gly	Asp	Gly	
			485					490						495		
tct	cca	tgt	aaa	atc	cct	ttt	gag	ata	atg	gat	ttg	gaa	aaa	aga	cat	1536
Ser	Pro	Cys	Lys	Ile	Pro	Phe	Glu	Ile	Met	Asp	Leu	Glu	Lys	Arg	His	
			500				505						510			
gtc	tta	ggg	gcg	ctg	atc	aca	gtc	aac	cca	att	gtg	aca	gaa	aaa	gat	1584
Val	Leu	Gly	Arg	Leu	Ile	Thr	Val	Asn	Pro	Ile	Val	Thr	Glu	Lys	Asp	
		515				520						525				
agc	cca	gtc	aac	ata	gaa	gca	gaa	cct	cca	ttc	gga	gac	agc	tac	atc	1632
Ser	Pro	Val	Asn	Ile	Glu	Ala	Glu	Pro	Pro	Phe	Gly	Asp	Ser	Tyr	Ile	
		530				535					540					
atc	ata	gga	gta	gag	ccg	gga	caa	ctg	aag	ctc	aac	tgg	ttt	aag	aaa	1680
Ile	Ile	Gly	Val	Glu	Pro	Gly	Gln	Leu	Lys	Leu	Asn	Trp	Phe	Lys	Lys	
		545				550				555				560		
gga	agt	tct	atc	ggc	caa	atg	ttt	gag	aca	aca	atg	agg	ggg	gcg	aag	1728
Gly	Ser	Ser	Ile	Gly	Gln	Met	Phe	Glu	Thr	Thr	Met	Arg	Gly	Ala	Lys	
			565					570					575			
aga	atg	gcc	att	ttg	ggg	gac	aca	gcc	tgg	gat	ttt	gga	tcc	ctg	gga	1776
Arg	Met	Ala	Ile	Leu	Gly	Asp	Thr	Ala	Trp	Asp	Phe	Gly	Ser	Leu	Gly	
			580				585						590			
gga	gtg	ttt	aca	tct	ata	gga	aaa	gcc	ctc	cac	caa	gtc	ttt	gga	gca	1824
Gly	Val	Phe	Thr	Ser	Ile	Gly	Lys	Ala	Leu	His	Gln	Val	Phe	Gly	Ala	
		595				600						605				
atc	tat	gga	gct	gcc	ttc	agt	ggg	gtc	tca	tgg	act	atg	aaa	atc	ctc	1872
Ile	Tyr	Gly	Ala	Ala	Phe	Ser	Gly	Val	Ser	Trp	Thr	Met	Lys	Ile	Leu	
		610				615					620					
ata	gga	gtc	att	atc	aca	tgg	ata	gga	atg	aat	tca	cgc	agc	acc	tca	1920

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Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser	
625 630 635 640	
ctg tct gtg tca cta gta ttg gtg gga gtc gtg acg ctg tat ttg gga	1968
Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly	
645 650 655	
ggt atg gtg ggc gcc	1983
Val Met Val Gly Ala	
660	

<210> SEQ ID NO 51
 <211> LENGTH: 661
 <212> TYPE: PRT
 <213> ORGANISM: Dengue-2 virus

<400> SEQUENCE: 51

Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Ser Arg	
1 5 10 15	
Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Glu Asp Gly Val Asn	
20 25 30	
Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu Asp Thr	
35 40 45	
Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu Pro Glu Asp Ile	
50 55 60	
Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys	
65 70 75 80	
Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala Leu Val	
85 90 95	
Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser	
100 105 110	
Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Ile Trp Ile Leu	
115 120 125	
Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr Thr Ile	
130 135 140	
Gly Thr Thr His Phe Gln Arg Ala Leu Ile Phe Ile Leu Leu Thr Ala	
145 150 155 160	
Val Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn Arg Asp	
165 170 175	
Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val Leu Glu	
180 185 190	
His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr Leu Asp	
195 200 205	
Phe Glu Leu Ile Lys Thr Glu Ala Lys Gln Pro Ala Thr Leu Arg Lys	
210 215 220	
Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Thr Glu Ser Arg Cys	
225 230 235 240	
Pro Thr Gln Gly Glu Pro Ser Leu Asn Glu Glu Gln Asp Lys Arg Phe	
245 250 255	
Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly Cys Gly	
260 265 270	
Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr Cys Lys	
275 280 285	
Lys Asn Met Glu Gly Lys Val Val Gln Pro Glu Asn Leu Glu Tyr Thr	
290 295 300	

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Ile Val Val Thr Pro His Ser Gly Glu Glu His Ala Val Gly Asn Asp
305                      310                      315                      320

Thr Gly Lys His Gly Lys Glu Ile Lys Val Thr Pro Gln Ser Ser Ile
                      325                      330                      335

Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu Cys Ser
                      340                      345                      350

Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln Met Glu
                      355                      360                      365

Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu
                      370                      375                      380

Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys
385                      390                      395                      400

Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val
                      405                      410                      415

Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly
                      420                      425                      430

Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His
                      435                      440                      445

Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser
                      450                      455                      460

Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu
465                      470                      475                      480

Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly
                      485                      490                      495

Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His
                      500                      505                      510

Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp
                      515                      520                      525

Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile
                      530                      535                      540

Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys
545                      550                      555                      560

Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys
                      565                      570                      575

Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly
                      580                      585                      590

Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala
                      595                      600                      605

Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu
610                      615                      620

Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser
625                      630                      635                      640

Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly
                      645                      650                      655

Val Met Val Gly Ala
                      660

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<210> SEQ ID NO 52

<211> LENGTH: 10892

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: derived from Yellow Fever virus and Japanese

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Encephalitis virus
 <220> FEATURE:
 <221> NAME/KEY: CDS
 <222> LOCATION: (119) .. (10381)
 <400> SEQUENCE: 52

agtaaatacct gtgtgctaata tgaggtgcat tggctctgcaa atcgagttgc taggcaataa	60
acacatttgg attaatTTTA atcgTtcgtt gagcgattag cagagaactg accagaac	118
atg tct ggt cgt aaa gct cag gga aaa acc ctg ggc gtc aat atg gta Met Ser Gly Arg Lys Ala Gln Gly Lys Thr Leu Gly Val Asn Met Val 1 5 10 15	166
cga cga gga gtt cgc tcc ttg tca aac aaa ata aaa caa aaa aca aaa Arg Arg Gly Val Arg Ser Leu Ser Asn Lys Ile Lys Gln Lys Thr Lys 20 25 30	214
caa att gga aac aga cct gga cct tca aga ggt gtt caa gga ttt atc Gln Ile Gly Asn Arg Pro Gly Pro Ser Arg Gly Val Gln Gly Phe Ile 35 40 45	262
ttt ttc ttt ttg ttc aac att ttg act gga aaa aag atc aca gcc cac Phe Phe Phe Leu Phe Asn Ile Leu Thr Gly Lys Lys Ile Thr Ala His 50 55 60	310
cta aag agg ttg tgg aaa atg ctg gac cca aga caa ggc ttg gct gtt Leu Lys Arg Leu Trp Lys Met Leu Asp Pro Arg Gln Gly Leu Ala Val 65 70 75 80	358
cta agg aaa gtc aag aga gtg gtg gcc agt ttg atg aga gga ttg tcc Leu Arg Lys Val Lys Arg Val Val Ala Ser Leu Met Arg Gly Leu Ser 85 90 95	406
tca agg aaa cgc cgt tcc cat gat gtt ctg act gtg caa ttc cta att Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe Leu Ile 100 105 110	454
ttg gga atg ctg ttg atg acg ggt gga atg aag ttg tcg aat ttc cag Leu Gly Met Leu Leu Met Thr Gly Gly Met Lys Leu Ser Asn Phe Gln 115 120 125	502
ggg aag ctt ttg atg acc atc aac aac acg gac att gca gac gtt atc Gly Lys Leu Leu Met Thr Ile Asn Asn Thr Asp Ile Ala Asp Val Ile 130 135 140	550
gtg att ccc acc tca aaa gga gag aac aga tgt tgg gtt cgg gca atc Val Ile Pro Thr Ser Lys Gly Glu Asn Arg Cys Trp Val Arg Ala Ile 145 150 155 160	598
gac gtc ggc tac atg tgt gag gac act atc acg tac gaa tgt cct aag Asp Val Gly Tyr Met Cys Glu Asp Thr Ile Thr Tyr Glu Cys Pro Lys 165 170 175	646
ctt acc atg ggc aat gat cca gag gat gtg gat tgc tgg tgt gac aac Leu Thr Met Gly Asn Asp Pro Glu Asp Val Asp Cys Trp Cys Asp Asn 180 185 190	694
caa gaa gtc tac gtc caa tat gga cgg tgc acg cgg acc agg cat tcc Gln Glu Val Tyr Val Gln Tyr Gly Arg Cys Thr Arg Thr Arg His Ser 195 200 205	742
aag cga agc agg aga tcc gtg tcg gtc caa aca cat ggg gag agt tca Lys Arg Ser Arg Arg Ser Val Ser Val Gln Thr His Gly Glu Ser Ser 210 215 220	790
cta gtg aat aaa aaa gag gct tgg ctg gat tca acg aaa gcc aca cga Leu Val Asn Lys Lys Glu Ala Trp Leu Asp Ser Thr Lys Ala Thr Arg 225 230 235 240	838
tat ctc atg aaa act gag aac tgg atc ata agg aat cct ggc tat gct Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala 245 250 255	886
ttc ctg gcg gcg gta ctt ggc tgg atg ctt ggc agt aac aac ggt caa	934

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Phe	Leu	Ala	Ala	Val	Leu	Gly	Trp	Met	Leu	Gly	Ser	Asn	Asn	Gly	Gln	
			260					265					270			
cgc	gtg	gta	ttt	acc	atc	ctc	ctg	ctg	ttg	gtc	gct	ccg	gct	tac	agt	982
Arg	Val	Val	Phe	Thr	Ile	Leu	Leu	Leu	Leu	Val	Ala	Pro	Ala	Tyr	Ser	
		275					280					285				
ttt	aat	tgt	ctg	gga	atg	ggc	aat	cgt	gac	ttc	ata	gaa	gga	gcc	agt	1030
Phe	Asn	Cys	Leu	Gly	Met	Gly	Asn	Arg	Asp	Phe	Ile	Glu	Gly	Ala	Ser	
	290					295				300						
ggg	gcc	act	tgg	gtg	gac	ttg	gtg	cta	gaa	gga	gac	agc	tgc	ttg	aca	1078
Gly	Ala	Thr	Trp	Val	Asp	Leu	Val	Leu	Glu	Gly	Asp	Ser	Cys	Leu	Thr	
305					310				315					320		
atc	atg	gca	aac	gac	aaa	cca	aca	ttg	gac	gtc	cgc	atg	att	aac	atc	1126
Ile	Met	Ala	Asn	Asp	Lys	Pro	Thr	Leu	Asp	Val	Arg	Met	Ile	Asn	Ile	
			325						330					335		
gaa	gct	agc	caa	ctt	gct	gag	gtc	aga	agt	tac	tgc	tat	cat	gct	tca	1174
Glu	Ala	Ser	Gln	Leu	Ala	Glu	Val	Arg	Ser	Tyr	Cys	Tyr	His	Ala	Ser	
			340					345					350			
gtc	act	gac	atc	tcg	acg	gtg	gct	cgg	tgc	ccc	acg	act	gga	gaa	gcc	1222
Val	Thr	Asp	Ile	Ser	Thr	Val	Ala	Arg	Cys	Pro	Thr	Thr	Gly	Glu	Ala	
		355					360					365				
cac	aac	gag	aag	cga	gct	gat	agt	agc	tat	gtg	tgc	aaa	caa	ggc	ttc	1270
His	Asn	Glu	Lys	Arg	Ala	Asp	Ser	Ser	Tyr	Val	Cys	Lys	Gln	Gly	Phe	
	370					375					380					
act	gac	cgt	ggg	tgg	ggc	aac	gga	tgt	gga	ttt	ttc	ggg	aag	gga	agc	1318
Thr	Asp	Arg	Gly	Trp	Gly	Asn	Gly	Cys	Gly	Phe	Phe	Gly	Lys	Gly	Ser	
	385				390					395				400		
att	gac	aca	tgt	gca	aaa	ttc	tcc	tgc	acc	agt	aaa	gcg	att	ggg	aga	1366
Ile	Asp	Thr	Cys	Ala	Lys	Phe	Ser	Cys	Thr	Ser	Lys	Ala	Ile	Gly	Arg	
			405						410					415		
aca	atc	cag	cca	gaa	aac	atc	aaa	tac	aaa	gtt	ggc	att	ttt	gtg	cat	1414
Thr	Ile	Gln	Pro	Glu	Asn	Ile	Lys	Tyr	Lys	Val	Gly	Ile	Phe	Val	His	
		420						425					430			
gga	acc	acc	act	tcg	gaa	aac	cat	ggg	aat	tat	tca	gcg	caa	gtt	ggg	1462
Gly	Thr	Thr	Ser	Glu	Asn	His	Gly	Asn	Tyr	Ser	Ala	Gln	Val	Gly		
	435					440					445					
gcg	tcc	cag	gcg	gca	aag	ttt	aca	gta	aca	ccc	aat	gct	cct	tcg	gta	1510
Ala	Ser	Gln	Ala	Ala	Lys	Phe	Thr	Val	Thr	Pro	Asn	Ala	Pro	Ser	Val	
	450					455					460					
gcc	ctc	aaa	ctt	ggc	gac	tac	gga	gaa	gtc	aca	ctg	gac	tgt	gag	cca	1558
Ala	Leu	Lys	Leu	Gly	Asp	Tyr	Gly	Glu	Val	Thr	Leu	Asp	Cys	Glu	Pro	
	465				470				475					480		
agg	agt	gga	ctg	aac	act	gaa	gcg	ttt	tac	gtc	atg	acc	gtg	ggg	tca	1606
Arg	Ser	Gly	Leu	Asn	Thr	Glu	Ala	Phe	Tyr	Val	Met	Thr	Val	Gly	Ser	
			485						490					495		
aag	tca	ttt	ctg	gtc	cat	agg	gag	tgg	ttt	cat	gac	ctc	gct	ctc	ccc	1654
Lys	Ser	Phe	Leu	Val	His	Arg	Glu	Trp	Phe	His	Asp	Leu	Ala	Leu	Pro	
		500						505					510			
tgg	acg	tcc	cct	tcg	agc	aca	gcg	tgg	aga	aac	aga	gaa	ctc	ctc	atg	1702
Trp	Thr	Ser	Pro	Ser	Ser	Thr	Ala	Trp	Arg	Asn	Arg	Glu	Leu	Leu	Met	
		515					520					525				
gaa	ttt	gaa	ggg	gcg	cac	gcc	aca	aaa	cag	tcc	gtt	gtt	gct	ctt	ggg	1750
Glu	Phe	Glu	Gly	Ala	His	Ala	Thr	Lys	Gln	Ser	Val	Val	Ala	Leu	Gly	
	530					535					540					
tca	cag	gaa	gga	ggc	ctc	cat	cat	gcg	ttg	gca	gga	gcc	atc	gtg	gtg	1798
Ser	Gln	Glu	Gly	Gly	Leu	His	His	Ala	Leu	Ala	Gly	Ala	Ile	Val	Val	
	545				550				555					560		
gag	tac	tca	agc	tca	gtg	atg	tta	aca	tca	ggc	cac	ctg	aaa	tgt	agg	1846

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Glu	Tyr	Ser	Ser	Ser	Val	Met	Leu	Thr	Ser	Gly	His	Leu	Lys	Cys	Arg	
				565					570					575		
ctg	aaa	atg	gac	aaa	ctg	gct	ctg	aaa	ggc	aca	acc	tat	ggc	atg	tgt	1894
Leu	Lys	Met	Asp	Lys	Leu	Ala	Leu	Lys	Gly	Thr	Thr	Tyr	Gly	Met	Cys	
			580					585					590			
aca	gaa	aaa	ttc	tcg	ttc	gcg	aaa	aat	ccg	gtg	gac	act	ggc	cac	gga	1942
Thr	Glu	Lys	Phe	Ser	Phe	Ala	Lys	Asn	Pro	Val	Asp	Thr	Gly	His	Gly	
		595					600					605				
aca	gtt	gtc	att	gaa	ctc	tcc	tac	tct	ggg	agt	gat	ggc	ccc	tgc	aaa	1990
Thr	Val	Val	Ile	Glu	Leu	Ser	Tyr	Ser	Gly	Ser	Asp	Gly	Pro	Cys	Lys	
	610					615					620					
att	ccg	att	gtt	tcc	gtt	gcg	agc	ctc	aat	gac	atg	acc	ccc	gtt	ggg	2038
Ile	Pro	Ile	Val	Ser	Val	Ala	Ser	Leu	Asn	Asp	Met	Thr	Pro	Val	Gly	
625					630				635					640		
cgg	ctg	gtg	aca	gtg	aac	ccc	ttc	gtc	gcg	act	tcc	agt	gcc	aac	tca	2086
Arg	Leu	Val	Thr	Val	Asn	Pro	Phe	Val	Ala	Thr	Ser	Ser	Ala	Asn	Ser	
				645				650						655		
aag	gtg	ctg	gtc	gag	atg	gaa	ccc	ccc	ttc	gga	gac	tcc	tac	atc	gta	2134
Lys	Val	Leu	Val	Glu	Met	Glu	Pro	Pro	Phe	Gly	Asp	Ser	Tyr	Ile	Val	
			660					665					670			
gtt	gga	agg	gga	gac	aag	cag	atc	aac	cac	cat	tgg	cac	aaa	gct	gga	2182
Val	Gly	Arg	Gly	Asp	Lys	Gln	Ile	Asn	His	His	Trp	His	Lys	Ala	Gly	
	675					680						685				
agc	acg	ctg	ggc	aag	gcc	ttt	tca	aca	act	ttg	aag	gga	gct	caa	aga	2230
Ser	Thr	Leu	Gly	Lys	Ala	Phe	Ser	Thr	Thr	Leu	Lys	Gly	Ala	Gln	Arg	
	690					695					700					
ctg	gca	gcg	ttg	ggc	gac	aca	gcc	tgg	gac	ttt	ggc	tct	att	gga	ggg	2278
Leu	Ala	Ala	Leu	Gly	Asp	Thr	Ala	Trp	Asp	Phe	Gly	Ser	Ile	Gly	Gly	
	705				710				715					720		
gtc	ttc	aac	tcc	ata	gga	aga	gcc	gtt	cac	caa	gtg	ttt	ggc	ggc	gcc	2326
Val	Phe	Asn	Ser	Ile	Gly	Arg	Ala	Val	His	Gln	Val	Phe	Gly	Gly	Ala	
			725					730					735			
ttc	aga	aca	ctc	ttt	ggg	gga	atg	tct	tgg	atc	aca	caa	ggg	cta	atg	2374
Phe	Arg	Thr	Leu	Phe	Gly	Gly	Met	Ser	Trp	Ile	Thr	Gln	Gly	Leu	Met	
			740					745					750			
ggc	gcc	cta	ctg	ctc	tgg	atg	ggc	gtc	aac	gca	cga	gac	cga	tca	att	2422
Gly	Ala	Leu	Leu	Leu	Trp	Met	Gly	Val	Asn	Ala	Arg	Asp	Arg	Ser	Ile	
	755					760						765				
gct	ttg	gcc	ttc	tta	gcc	aca	gga	ggc	gtg	ctc	gtg	ttc	tta	gcg	acc	2470
Ala	Leu	Ala	Phe	Leu	Ala	Thr	Gly	Gly	Val	Leu	Val	Phe	Leu	Ala	Thr	
	770					775					780					
aat	gtg	ggc	gcc	gat	caa	gga	tgc	gcc	atc	aac	ttt	ggc	aag	aga	gag	2518
Asn	Val	Gly	Ala	Asp	Gln	Gly	Cys	Ala	Ile	Asn	Phe	Gly	Lys	Arg	Glu	
	785				790				795					800		
ctc	aag	tgc	gga	gat	ggc	atc	ttc	ata	ttt	aga	gac	tct	gat	gac	tgg	2566
Leu	Lys	Cys	Gly	Asp	Gly	Ile	Phe	Ile	Phe	Arg	Asp	Ser	Asp	Asp	Trp	
			805					810					815			
ctg	aac	aag	tac	tca	tac	tat	cca	gaa	gat	cct	gtg	aag	ctt	gca	tca	2614
Leu	Asn	Lys	Tyr	Ser	Tyr	Tyr	Pro	Glu	Asp	Pro	Val	Lys	Leu	Ala	Ser	
			820					825					830			
ata	gtg	aaa	gcc	tct	ttt	gaa	gaa	ggg	aag	tgt	ggc	cta	aat	tca	gtt	2662
Ile	Val	Lys	Ala	Ser	Phe	Glu	Glu	Gly	Lys	Cys	Gly	Leu	Asn	Ser	Val	
		835					840					845				
gac	tcc	ctt	gag	cat	gag	atg	tgg	aga	agc	agg	gca	gat	gag	atc	aat	2710
Asp	Ser	Leu	Glu	His	Glu	Met	Trp	Arg	Ser	Arg	Ala	Asp	Glu	Ile	Asn	
	850					855					860					
gcc	att	ttt	gag	gaa	aac	gag	gtg	gac	att	tct	gtt	gtc	gtg	cag	gat	2758

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Ala Ile Phe Glu Glu Asn Glu Val Asp Ile Ser Val Val Val Gln Asp 865 870 875 880	
cca aag aat gtt tac cag aga gga act cat cca ttt tcc aga att cgg Pro Lys Asn Val Tyr Gln Arg Gly Thr His Pro Phe Ser Arg Ile Arg 885 890 895	2806
gat ggt ctg cag tat ggt tgg aag act tgg ggt aag aac ctt gtg ttc Asp Gly Leu Gln Tyr Gly Trp Lys Thr Trp Gly Lys Asn Leu Val Phe 900 905 910	2854
tcc cca ggg agg aag aat gga agc ttc atc ata gat gga aag tcc agg Ser Pro Gly Arg Lys Asn Gly Ser Phe Ile Ile Asp Gly Lys Ser Arg 915 920 925	2902
aaa gaa tgc ccg ttt tca aac cgg gtc tgg aat tct ttc cag ata gag Lys Glu Cys Pro Phe Ser Asn Arg Val Trp Asn Ser Phe Gln Ile Glu 930 935 940	2950
gag ttt ggg acg gga gtg ttc acc aca cgc gtg tac atg gac gca gtc Glu Phe Gly Thr Gly Val Phe Thr Thr Arg Val Tyr Met Asp Ala Val 945 950 955 960	2998
ttt gaa tac acc ata gac tgc gat gga tct atc ttg ggt gca gcg gtg Phe Glu Tyr Thr Ile Asp Cys Asp Gly Ser Ile Leu Gly Ala Ala Val 965 970 975	3046
aac gga aaa aag agt gcc cat ggc tct cca aca ttt tgg atg gga agt Asn Gly Lys Lys Ser Ala His Gly Ser Pro Thr Phe Trp Met Gly Ser 980 985 990	3094
cat gaa gta aat ggg aca tgg atg atc cac acc ttg gag gca tta gat His Glu Val Asn Gly Thr Trp Met Ile His Thr Leu Glu Ala Leu Asp 995 1000 1005	3142
tac aag gag tgt gag tgg cca ctg aca cat acg att gga aca tca Tyr Lys Glu Cys Glu Trp Pro Leu Thr His Thr Ile Gly Thr Ser 1010 1015 1020	3187
gtt gaa gag agt gaa atg ttc atg ccg aga tca atc gga ggc cca Val Glu Glu Ser Glu Met Phe Met Pro Arg Ser Ile Gly Gly Pro 1025 1030 1035	3232
gtt agc tct cac aat cat atc cct gga tac aag gtt cag acg aac Val Ser Ser His Asn His Ile Pro Gly Tyr Lys Val Gln Thr Asn 1040 1045 1050	3277
gga cct tgg atg cag gta cca cta gaa gtg aag aga gaa gct tgc Gly Pro Trp Met Gln Val Pro Leu Glu Val Lys Arg Glu Ala Cys 1055 1060 1065	3322
cca ggg act agc gtg atc att gat ggc aac tgt gat gga cgg gga Pro Gly Thr Ser Val Ile Ile Asp Gly Asn Cys Asp Gly Arg Gly 1070 1075 1080	3367
aaa tca acc aga tcc acc acg gat agc ggg aaa gtt att cct gaa Lys Ser Thr Arg Ser Thr Thr Asp Ser Gly Lys Val Ile Pro Glu 1085 1090 1095	3412
tgg tgt tgc cgc tcc tgc aca atg ccg cct gtg agc ttc cat ggt Trp Cys Cys Arg Ser Cys Thr Met Pro Pro Val Ser Phe His Gly 1100 1105 1110	3457
agt gat ggg tgt tgg tat ccc atg gaa att agg cca agg aaa acg Ser Asp Gly Cys Trp Tyr Pro Met Glu Ile Arg Pro Arg Lys Thr 1115 1120 1125	3502
cat gaa agc cat ctg gtg cgc tcc tgg gtt aca gct gga gaa ata His Glu Ser His Leu Val Arg Ser Trp Val Thr Ala Gly Glu Ile 1130 1135 1140	3547
cat gct gtc cct ttt ggt ttg gtg agc atg atg ata gca atg gaa His Ala Val Pro Phe Gly Leu Val Ser Met Met Ile Ala Met Glu 1145 1150 1155	3592
gtg gtc cta agg aaa aga cag gga cca aag caa atg ttg gtt gga	3637

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Val	Val	Leu	Arg	Lys	Arg	Gln	Gly	Pro	Lys	Gln	Met	Leu	Val	Gly	
1160						1165					1170				
gga	gta	gtg	ctc	ttg	gga	gca	atg	ctg	gtc	ggg	caa	gta	act	ctc	3682
Gly	Val	Val	Leu	Leu	Gly	Ala	Met	Leu	Val	Gly	Gln	Val	Thr	Leu	
1175						1180					1185				
ctt	gat	ttg	ctg	aaa	ctc	aca	gtg	gct	gtg	gga	ttg	cat	ttc	cat	3727
Leu	Asp	Leu	Leu	Lys	Leu	Thr	Val	Ala	Val	Gly	Leu	His	Phe	His	
1190						1195					1200				
gag	atg	aac	aat	gga	gga	gac	gcc	atg	tat	atg	gcg	ttg	att	gct	3772
Glu	Met	Asn	Asn	Gly	Gly	Asp	Ala	Met	Tyr	Met	Ala	Leu	Ile	Ala	
1205						1210					1215				
gcc	ttt	tca	atc	aga	cca	ggg	ctg	ctc	atc	ggc	ttt	ggg	ctc	agg	3817
Ala	Phe	Ser	Ile	Arg	Pro	Gly	Leu	Leu	Ile	Gly	Phe	Gly	Leu	Arg	
1220						1225					1230				
acc	cta	tgg	agc	cct	cgg	gaa	cgc	ctt	gtg	ctg	acc	cta	gga	gca	3862
Thr	Leu	Trp	Ser	Pro	Arg	Glu	Arg	Leu	Val	Leu	Thr	Leu	Gly	Ala	
1235						1240					1245				
gcc	atg	gtg	gag	att	gcc	ttg	ggc	ggc	gtg	atg	ggc	ggc	ctg	tgg	3907
Ala	Met	Val	Glu	Ile	Ala	Leu	Gly	Gly	Val	Met	Gly	Gly	Leu	Trp	
1250						1255					1260				
aag	tat	cta	aat	gca	gtt	tct	ctc	tgc	atc	ctg	aca	ata	aat	gct	3952
Lys	Tyr	Leu	Asn	Ala	Val	Ser	Leu	Cys	Ile	Leu	Thr	Ile	Asn	Ala	
1265						1270					1275				
gtt	gct	tct	agg	aaa	gca	tca	aat	acc	atc	ttg	ccc	ctc	atg	gct	3997
Val	Ala	Ser	Arg	Lys	Ala	Ser	Asn	Thr	Ile	Leu	Pro	Leu	Met	Ala	
1280						1285					1290				
ctg	ttg	aca	cct	gtc	act	atg	gct	gag	gtg	aga	ctt	gcc	gca	atg	4042
Leu	Leu	Thr	Pro	Val	Thr	Met	Ala	Glu	Val	Arg	Leu	Ala	Ala	Met	
1295						1300					1305				
ttc	ttt	tgt	gcc	atg	gtt	atc	ata	ggg	gtc	ctt	cac	cag	aat	ttc	4087
Phe	Phe	Cys	Ala	Met	Val	Ile	Ile	Gly	Val	Leu	His	Gln	Asn	Phe	
1310						1315					1320				
aag	gac	acc	tcc	atg	cag	aag	act	ata	cct	ctg	gtg	gcc	ctc	aca	4132
Lys	Asp	Thr	Ser	Met	Gln	Lys	Thr	Ile	Pro	Leu	Val	Ala	Leu	Thr	
1325						1330					1335				
ctc	aca	tct	tac	ctg	ggc	ttg	aca	caa	cct	ttt	ttg	ggc	ctg	tgt	4177
Leu	Thr	Ser	Tyr	Leu	Gly	Leu	Thr	Gln	Pro	Phe	Leu	Gly	Leu	Cys	
1340						1345					1350				
gca	ttt	ctg	gca	acc	cgc	ata	ttt	ggg	cga	agg	agt	atc	cca	gtg	4222
Ala	Phe	Leu	Ala	Thr	Arg	Ile	Phe	Gly	Arg	Arg	Ser	Ile	Pro	Val	
1355						1360					1365				
aat	gag	gca	ctc	gca	gca	gct	ggc	cta	gtg	gga	gtg	ctg	gca	gga	4267
Asn	Glu	Ala	Leu	Ala	Ala	Ala	Gly	Leu	Val	Gly	Val	Leu	Ala	Gly	
1370						1375					1380				
ctg	gct	ttt	cag	gag	atg	gag	aac	ttc	ctt	ggc	ccg	att	gca	gtt	4312
Leu	Ala	Phe	Gln	Glu	Met	Glu	Asn	Phe	Leu	Gly	Pro	Ile	Ala	Val	
1385						1390					1395				
gga	gga	ctc	ctg	atg	atg	ctg	gtt	agc	gtg	gct	ggg	agg	gtg	gat	4357
Gly	Gly	Leu	Leu	Met	Met	Leu	Val	Ser	Val	Ala	Gly	Arg	Val	Asp	
1400						1405					1410				
ggg	cta	gag	ctc	aag	aag	ctt	ggc	gaa	gtt	tca	tgg	gaa	gag	gag	4402
Gly	Leu	Glu	Leu	Lys	Lys	Leu	Gly	Glu	Val	Ser	Trp	Glu	Glu	Glu	
1415						1420					1425				
gcg	gag	atc	agc	ggg	agt	tcc	gcc	cgc	tat	gat	gtg	gca	ctc	agt	4447
Ala	Glu	Ile	Ser	Gly	Ser	Ser	Ala	Arg	Tyr	Asp	Val	Ala	Leu	Ser	
1430						1435					1440				
gaa	caa	ggg	gag	ttc	aag	ctg	ctt	tct	gaa	gag	aaa	gtg	cca	tgg	4492

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Glu	Gln	Gly	Glu	Phe	Lys	Leu	Leu	Ser	Glu	Glu	Lys	Val	Pro	Trp	
1445						1450					1455				
gac	cag	gtt	gtg	atg	acc	tcg	ctg	gcc	ttg	gtt	ggg	gct	gcc	ctc	4537
Asp	Gln	Val	Val	Met	Thr	Ser	Leu	Ala	Leu	Val	Gly	Ala	Ala	Leu	
1460						1465					1470				
cat	cca	ttt	gct	ctt	ctg	ctg	gtc	ctt	gct	ggg	tgg	ctg	ttt	cat	4582
His	Pro	Phe	Ala	Leu	Leu	Leu	Val	Leu	Ala	Gly	Trp	Leu	Phe	His	
1475						1480					1485				
gtc	agg	gga	gct	agg	aga	agt	ggg	gat	gtc	ttg	tgg	gat	att	ccc	4627
Val	Arg	Gly	Ala	Arg	Arg	Ser	Gly	Asp	Val	Leu	Trp	Asp	Ile	Pro	
1490						1495					1500				
act	cct	aag	atc	atc	gag	gaa	tgt	gaa	cat	ctg	gag	gat	ggg	att	4672
Thr	Pro	Lys	Ile	Ile	Glu	Glu	Cys	Glu	His	Leu	Glu	Asp	Gly	Ile	
1505						1510					1515				
tat	ggc	ata	ttc	cag	tca	acc	ttc	ttg	ggg	gcc	tcc	cag	cga	gga	4717
Tyr	Gly	Ile	Phe	Gln	Ser	Thr	Phe	Leu	Gly	Ala	Ser	Gln	Arg	Gly	
1520						1525					1530				
gtg	gga	gtg	gca	cag	gga	ggg	gtg	ttc	cac	aca	atg	tgg	cat	gtc	4762
Val	Gly	Val	Ala	Gln	Gly	Gly	Val	Phe	His	Thr	Met	Trp	His	Val	
1535						1540					1545				
aca	aga	gga	gct	ttc	ctt	gtc	agg	aat	ggc	aag	aag	ttg	att	cca	4807
Thr	Arg	Gly	Ala	Phe	Leu	Val	Arg	Asn	Gly	Lys	Lys	Leu	Ile	Pro	
1550						1555					1560				
tct	tgg	gct	tca	gta	aag	gaa	gac	ctt	gtc	gcc	tat	ggg	ggc	tca	4852
Ser	Trp	Ala	Ser	Val	Lys	Glu	Asp	Leu	Val	Ala	Tyr	Gly	Gly	Ser	
1565						1570					1575				
tgg	aag	ttg	gaa	ggc	aga	tgg	gat	gga	gag	gaa	gag	gtc	cag	ttg	4897
Trp	Lys	Leu	Glu	Gly	Arg	Trp	Asp	Gly	Glu	Glu	Glu	Val	Gln	Leu	
1580						1585					1590				
atc	gcg	gct	gtt	cca	gga	aag	aac	gtg	gtc	aac	gtc	cag	aca	aaa	4942
Ile	Ala	Ala	Val	Pro	Gly	Lys	Asn	Val	Val	Asn	Val	Gln	Thr	Lys	
1595						1600					1605				
ccg	agc	ttg	ttc	aaa	gtg	agg	aat	ggg	gga	gaa	atc	ggg	gct	gtc	4987
Pro	Ser	Leu	Phe	Lys	Val	Arg	Asn	Gly	Gly	Glu	Ile	Gly	Ala	Val	
1610						1615					1620				
gct	ctt	gac	tat	ccg	agt	ggc	act	tca	gga	tct	cct	att	gtt	aac	5032
Ala	Leu	Asp	Tyr	Pro	Ser	Gly	Thr	Ser	Gly	Ser	Pro	Ile	Val	Asn	
1625						1630					1635				
agg	aac	gga	gag	gtg	att	ggg	ctg	tac	ggc	aat	ggc	atc	ctt	gtc	5077
Arg	Asn	Gly	Glu	Val	Ile	Gly	Leu	Tyr	Gly	Asn	Gly	Ile	Leu	Val	
1640						1645					1650				
ggg	gac	aac	tcc	ttc	gtg	tcc	gcc	ata	tcc	cag	act	gag	gtg	aag	5122
Gly	Asp	Asn	Ser	Phe	Val	Ser	Ala	Ile	Ser	Gln	Thr	Glu	Val	Lys	
1655						1660					1665				
gaa	gaa	gga	aag	gag	gag	ctc	caa	gag	atc	ccg	aca	atg	cta	aag	5167
Glu	Glu	Gly	Lys	Glu	Glu	Leu	Gln	Glu	Ile	Pro	Thr	Met	Leu	Lys	
1670						1675					1680				
aaa	gga	atg	aca	act	gtc	ctt	gat	ttt	cat	cct	gga	gct	ggg	aag	5212
Lys	Gly	Met	Thr	Thr	Val	Leu	Asp	Phe	His	Pro	Gly	Ala	Gly	Lys	
1685						1690					1695				
aca	aga	cgt	ttc	ctc	cca	cag	atc	ttg	gcc	gag	tgc	gca	cgg	aga	5257
Thr	Arg	Arg	Phe	Leu	Pro	Gln	Ile	Leu	Ala	Glu	Cys	Ala	Arg	Arg	
1700						1705					1710				
cgc	ttg	cgc	act	ctt	gtg	ttg	gcc	ccc	acc	agg	gtt	gtt	ctt	tct	5302
Arg	Leu	Arg	Thr	Leu	Val	Leu	Ala	Pro	Thr	Arg	Val	Val	Leu	Ser	
1715						1720					1725				
gaa	atg	aag	gag	gct	ttt	cac	ggc	ctg	gac	gtg	aaa	ttc	cac	aca	5347

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Glu Met 1730	Lys	Glu	Ala	Phe	His 1735	Gly	Leu	Asp	Val	Lys 1740	Phe	His	Thr	
cag gct	ttt	tcc	gct	cac	ggc	agc	ggg	aga	gaa	gtc	att	gat	gcc	5392
Gln Ala 1745	Phe	Ser	Ala	His	Gly 1750	Ser	Gly	Arg	Glu	Val 1755	Ile	Asp	Ala	
atg tgc	cat	gcc	acc	cta	act	tac	agg	atg	ttg	gaa	cca	act	agg	5437
Met Cys 1760	His	Ala	Thr	Leu	Thr 1765	Tyr	Arg	Met	Leu	Glu 1770	Pro	Thr	Arg	
gtt gtt	aac	tgg	gaa	gtg	atc	att	atg	gat	gaa	gcc	cat	ttt	ttg	5482
Val Val 1775	Asn	Trp	Glu	Val	Ile 1780	Ile	Met	Asp	Glu	Ala 1785	His	Phe	Leu	
gat cca	gct	agc	ata	gcc	gct	aga	ggg	tgg	gca	gcg	cac	aga	gct	5527
Asp Pro 1790	Ala	Ser	Ile	Ala	Ala 1795	Arg	Gly	Trp	Ala	Ala 1800	His	Arg	Ala	
agg gca	aat	gaa	agt	gca	aca	atc	ttg	atg	aca	gcc	aca	ccg	cct	5572
Arg Ala 1805	Asn	Glu	Ser	Ala	Thr 1810	Ile	Leu	Met	Thr	Ala 1815	Thr	Pro	Pro	
ggg act	agt	gat	gaa	ttt	cca	cat	tca	aat	ggg	gaa	ata	gaa	gat	5617
Gly Thr 1820	Ser	Asp	Glu	Phe	Pro 1825	His	Ser	Asn	Gly	Glu 1830	Ile	Glu	Asp	
gtt caa	acg	gac	ata	ccc	agt	gag	ccc	tgg	aac	aca	ggg	cat	gac	5662
Val Gln 1835	Thr	Asp	Ile	Pro	Ser 1840	Glu	Pro	Trp	Asn	Thr 1845	Gly	His	Asp	
tgg atc	ctg	gct	gac	aaa	agg	ccc	acg	gca	tgg	ttc	ctt	cca	tcc	5707
Trp Ile 1850	Leu	Ala	Asp	Lys	Arg 1855	Pro	Thr	Ala	Trp	Phe 1860	Leu	Pro	Ser	
atc aga	gct	gca	aat	gtc	atg	gct	gcc	tct	ttg	cgt	aag	gct	gga	5752
Ile Arg 1865	Ala	Ala	Asn	Val	Met 1870	Ala	Ala	Ser	Leu	Arg 1875	Lys	Ala	Gly	
aag agt	gtg	gtg	gtc	ctg	aac	agg	aaa	acc	ttt	gag	aga	gaa	tac	5797
Lys Ser 1880	Val	Val	Val	Leu	Asn 1885	Arg	Lys	Thr	Phe	Glu 1890	Arg	Glu	Tyr	
ccc acg	ata	aag	cag	aag	aaa	cct	gac	ttt	ata	ttg	gcc	act	gac	5842
Pro Thr 1895	Ile	Lys	Gln	Lys	Lys 1900	Pro	Asp	Phe	Ile	Leu 1905	Ala	Thr	Asp	
ata gct	gaa	atg	gga	gcc	aac	ctt	tgc	gtg	gag	cga	gtg	ctg	gat	5887
Ile Ala 1910	Glu	Met	Gly	Ala	Asn 1915	Leu	Cys	Val	Glu	Arg 1920	Val	Leu	Asp	
tgc agg	acg	gct	ttt	aag	cct	gtg	ctt	gtg	gat	gaa	ggg	agg	aag	5932
Cys Arg 1925	Thr	Ala	Phe	Lys	Pro 1930	Val	Leu	Val	Asp	Glu 1935	Gly	Arg	Lys	
gtg gca	ata	aaa	ggg	cca	ctt	cgt	atc	tcc	gca	tcc	tct	gct	gct	5977
Val Ala 1940	Ile	Lys	Gly	Pro	Leu 1945	Arg	Ile	Ser	Ala	Ser 1950	Ser	Ala	Ala	
caa agg	agg	ggg	cgc	att	ggg	aga	aat	ccc	aac	aga	gat	gga	gac	6022
Gln Arg 1955	Arg	Gly	Arg	Ile	Gly 1960	Arg	Asn	Pro	Asn	Arg 1965	Asp	Gly	Asp	
tca tac	tac	tat	tct	gag	cct	aca	agt	gaa	aat	aat	gcc	cac	cac	6067
Ser Tyr 1970	Tyr	Tyr	Ser	Glu	Pro 1975	Thr	Ser	Glu	Asn	Asn 1980	Ala	His	His	
gtc tgc	tgg	ttg	gag	gcc	tca	atg	ctc	ttg	gac	aac	atg	gag	gtg	6112
Val Cys 1985	Trp	Leu	Glu	Ala	Ser 1990	Met	Leu	Leu	Asp	Asn 1995	Met	Glu	Val	
agg ggt	gga	atg	gtc	gcc	cca	ctc	tat	ggc	gtt	gaa	gga	act	aaa	6157
Arg Gly 2000	Gly	Met	Val	Ala	Pro 2005	Leu	Tyr	Gly	Val	Glu 2010	Gly	Thr	Lys	
aca cca	gtt	tcc	cct	ggt	gaa	atg	aga	ctg	agg	gat	gac	cag	agg	6202

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Thr	Pro	Val	Ser	Pro	Gly	Glu	Met	Arg	Leu	Arg	Asp	Asp	Gln	Arg	
2015						2020					2025				
aaa	gtc	ttc	aga	gaa	cta	gtg	agg	aat	tgt	gac	ctg	ccc	gtt	tgg	6247
Lys	Val	Phe	Arg	Glu	Leu	Val	Arg	Asn	Cys	Asp	Leu	Pro	Val	Trp	
2030						2035					2040				
ctt	tcg	tgg	caa	gtg	gcc	aag	gct	ggc	ttg	aag	acg	aat	gat	cgt	6292
Leu	Ser	Trp	Gln	Val	Ala	Lys	Ala	Gly	Leu	Lys	Thr	Asn	Asp	Arg	
2045						2050					2055				
aag	tgg	tgt	ttt	gaa	ggc	cct	gag	gaa	cat	gag	atc	ttg	aat	gac	6337
Lys	Trp	Cys	Phe	Glu	Gly	Pro	Glu	Glu	His	Glu	Ile	Leu	Asn	Asp	
2060						2065					2070				
agc	ggt	gaa	aca	gtg	aag	tgc	agg	gct	cct	gga	gga	gca	aag	aag	6382
Ser	Gly	Glu	Thr	Val	Lys	Cys	Arg	Ala	Pro	Gly	Gly	Ala	Lys	Lys	
2075						2080					2085				
cct	ctg	cgc	cca	agg	tgg	tgt	gat	gaa	agg	gtg	tca	tct	gac	cag	6427
Pro	Leu	Arg	Pro	Arg	Trp	Cys	Asp	Glu	Arg	Val	Ser	Ser	Asp	Gln	
2090						2095					2100				
agt	gcg	ctg	tct	gaa	ttt	att	aag	ttt	gct	gaa	ggc	agg	agg	gga	6472
Ser	Ala	Leu	Ser	Glu	Phe	Ile	Lys	Phe	Ala	Glu	Gly	Arg	Arg	Gly	
2105						2110					2115				
gct	gct	gaa	gtg	cta	gtt	gtg	ctg	agt	gaa	ctc	cct	gat	ttc	ctg	6517
Ala	Ala	Glu	Val	Leu	Val	Val	Leu	Ser	Glu	Leu	Pro	Asp	Phe	Leu	
2120						2125					2130				
gct	aaa	aaa	ggc	gga	gag	gca	atg	gat	acc	atc	agt	gtg	ttc	ctc	6562
Ala	Lys	Lys	Gly	Gly	Glu	Ala	Met	Asp	Thr	Ile	Ser	Val	Phe	Leu	
2135						2140					2145				
cac	tct	gag	gaa	ggc	tct	agg	gct	tac	cgc	aat	gca	cta	tca	atg	6607
His	Ser	Glu	Glu	Gly	Ser	Arg	Ala	Tyr	Arg	Asn	Ala	Leu	Ser	Met	
2150						2155					2160				
atg	cct	gag	gca	atg	aca	ata	gtc	atg	ctg	ttt	ata	ctg	gct	gga	6652
Met	Pro	Glu	Ala	Met	Thr	Ile	Val	Met	Leu	Phe	Ile	Leu	Ala	Gly	
2165						2170					2175				
cta	ctg	aca	tcg	gga	atg	gtc	atc	ttt	ttc	atg	tct	ccc	aaa	ggc	6697
Leu	Leu	Thr	Ser	Gly	Met	Val	Ile	Phe	Phe	Met	Ser	Pro	Lys	Gly	
2180						2185					2190				
atc	agt	aga	atg	tct	atg	gcg	atg	ggc	aca	atg	gcc	ggc	tgt	gga	6742
Ile	Ser	Arg	Met	Ser	Met	Ala	Met	Gly	Thr	Met	Ala	Gly	Cys	Gly	
2195						2200					2205				
tat	ctc	atg	ttc	ctt	gga	ggc	gtc	aaa	ccc	act	cac	atc	tcc	tat	6787
Tyr	Leu	Met	Phe	Leu	Gly	Gly	Val	Lys	Pro	Thr	His	Ile	Ser	Tyr	
2210						2215					2220				
gtc	atg	ctc	ata	ttc	ttt	gtc	ctg	atg	gtg	gtt	gtg	atc	ccc	gag	6832
Val	Met	Leu	Ile	Phe	Phe	Val	Leu	Met	Val	Val	Val	Ile	Pro	Glu	
2225						2230					2235				
cca	ggg	caa	caa	agg	tcc	atc	caa	gac	aac	caa	gtg	gca	tac	ctc	6877
Pro	Gly	Gln	Gln	Arg	Ser	Ile	Gln	Asp	Asn	Gln	Val	Ala	Tyr	Leu	
2240						2245					2250				
att	att	ggc	atc	ctg	acg	ctg	gtt	tca	gcg	gtg	gca	gcc	aac	gag	6922
Ile	Ile	Gly	Ile	Leu	Thr	Leu	Val	Ser	Ala	Val	Ala	Ala	Asn	Glu	
2255						2260					2265				
cta	ggc	atg	ctg	gag	aaa	acc	aaa	gag	gac	ctc	ttt	ggg	aag	aag	6967
Leu	Gly	Met	Leu	Glu	Lys	Thr	Lys	Glu	Asp	Leu	Phe	Gly	Lys	Lys	
2270						2275					2280				
aac	tta	att	cca	tct	agt	gct	tca	ccc	tgg	agt	tgg	ccg	gat	ctt	7012
Asn	Leu	Ile	Pro	Ser	Ser	Ala	Ser	Pro	Trp	Ser	Trp	Pro	Asp	Leu	
2285						2290					2295				
gac	ctg	aag	cca	gga	gct	gcc	tgg	aca	gtg	tac	gtt	ggc	att	gtt	7057

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Asp	Leu	Lys	Pro	Gly	Ala	Ala	Trp	Thr	Val	Tyr	Val	Gly	Ile	Val	
2300						2305					2310				
aca	atg	ctc	tct	cca	atg	ttg	cac	cac	tgg	atc	aaa	gtc	gaa	tat	7102
Thr	Met	Leu	Ser	Pro	Met	Leu	His	His	Trp	Ile	Lys	Val	Glu	Tyr	
2315						2320					2325				
ggc	aac	ctg	tct	ctg	tct	gga	ata	gcc	cag	tca	gcc	tca	gtc	ctt	7147
Gly	Asn	Leu	Ser	Leu	Ser	Gly	Ile	Ala	Gln	Ser	Ala	Ser	Val	Leu	
2330						2335					2340				
tct	ttc	atg	gac	aag	ggg	ata	cca	ttc	atg	aag	atg	aat	atc	tcg	7192
Ser	Phe	Met	Asp	Lys	Gly	Ile	Pro	Phe	Met	Lys	Met	Asn	Ile	Ser	
2345						2350					2355				
gtc	ata	atg	ctg	ctg	gtc	agt	ggc	tgg	aat	tca	ata	aca	gtg	atg	7237
Val	Ile	Met	Leu	Leu	Val	Ser	Gly	Trp	Asn	Ser	Ile	Thr	Val	Met	
2360						2365					2370				
cct	ctg	ctc	tgt	ggc	ata	ggg	tgc	gcc	atg	ctc	cac	tgg	tct	ctc	7282
Pro	Leu	Leu	Cys	Gly	Ile	Gly	Cys	Ala	Met	Leu	His	Trp	Ser	Leu	
2375						2380					2385				
att	tta	cct	gga	atc	aaa	gcg	cag	cag	tca	aag	ctt	gca	cag	aga	7327
Ile	Leu	Pro	Gly	Ile	Lys	Ala	Gln	Gln	Ser	Lys	Leu	Ala	Gln	Arg	
2390						2395					2400				
agg	gtg	ttc	cat	ggc	gtt	gcc	aag	aac	cct	gtg	gtt	gat	ggg	aat	7372
Arg	Val	Phe	His	Gly	Val	Ala	Lys	Asn	Pro	Val	Val	Asp	Gly	Asn	
2405						2410					2415				
cca	aca	gtt	gac	att	gag	gaa	gct	cct	gaa	atg	cct	gcc	ctt	tat	7417
Pro	Thr	Val	Asp	Ile	Glu	Glu	Ala	Pro	Glu	Met	Pro	Ala	Leu	Tyr	
2420						2425					2430				
gag	aag	aaa	ctg	gct	cta	tat	ctc	ctt	ctt	gct	ctc	agc	cta	gct	7462
Glu	Lys	Lys	Leu	Ala	Leu	Tyr	Leu	Leu	Leu	Ala	Leu	Ser	Leu	Ala	
2435						2440					2445				
tct	gtt	gcc	atg	tgc	aga	acg	ccc	ttt	tca	ttg	gct	gaa	ggc	att	7507
Ser	Val	Ala	Met	Cys	Arg	Thr	Pro	Phe	Ser	Leu	Ala	Glu	Gly	Ile	
2450						2455					2460				
gtc	cta	gca	tca	gct	gcc	tta	ggg	ccg	ctc	ata	gag	gga	aac	acc	7552
Val	Leu	Ala	Ser	Ala	Ala	Leu	Gly	Pro	Leu	Ile	Glu	Gly	Asn	Thr	
2465						2470					2475				
agc	ctt	ctt	tgg	aat	gga	ccc	atg	gct	gtc	tcc	atg	aca	gga	gtc	7597
Ser	Leu	Leu	Trp	Asn	Gly	Pro	Met	Ala	Val	Ser	Met	Thr	Gly	Val	
2480						2485					2490				
atg	agg	ggg	aat	cac	tat	gct	ttt	gtg	gga	gtc	atg	tac	aat	cta	7642
Met	Arg	Gly	Asn	His	Tyr	Ala	Phe	Val	Gly	Val	Met	Tyr	Asn	Leu	
2495						2500					2505				
tgg	aag	atg	aaa	act	gga	cgc	cgg	ggg	agc	gcg	aat	gga	aaa	act	7687
Trp	Lys	Met	Lys	Thr	Gly	Arg	Arg	Gly	Ser	Ala	Asn	Gly	Lys	Thr	
2510						2515					2520				
ttg	ggt	gaa	gtc	tgg	aag	agg	gaa	ctg	aat	ctg	ttg	gac	aag	cga	7732
Leu	Gly	Glu	Val	Trp	Lys	Arg	Glu	Leu	Asn	Leu	Leu	Asp	Lys	Arg	
2525						2530					2535				
cag	ttt	gag	ttg	tat	aaa	agg	acc	gac	att	gtg	gag	gtg	gat	cgt	7777
Gln	Phe	Glu	Leu	Tyr	Lys	Arg	Thr	Asp	Ile	Val	Glu	Val	Asp	Arg	
2540						2545					2550				
gat	acg	gca	cgc	agg	cat	ttg	gcc	gaa	ggg	aag	gtg	gac	acc	ggg	7822
Asp	Thr	Ala	Arg	Arg	His	Leu	Ala	Glu	Gly	Lys	Val	Asp	Thr	Gly	
2555						2560					2565				
gtg	gcg	gtc	tcc	agg	ggg	acc	gca	aag	tta	agg	tgg	ttc	cat	gag	7867
Val	Ala	Val	Ser	Arg	Gly	Thr	Ala	Lys	Leu	Arg	Trp	Phe	His	Glu	
2570						2575					2580				
cgt	ggc	tat	gtc	aag	ctg	gaa	ggg	agg	gtg	att	gac	ctg	ggg	tgt	7912

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Arg Gly 2585	Tyr Val Lys Leu 2590	Glu Gly Arg Val Ile 2595	Leu Gly Cys	
ggc cgc Gly Arg 2600	gga ggc tgg tgt tac Gly Gly Trp Cys Tyr 2605	tac gct gct gcg caa Tyr Ala Ala Ala Gln 2610	aag gaa gtg Lys Glu Val	7957
agt ggg Ser Gly 2615	gtc aaa gga ttt act Val Lys Gly Phe Thr 2620	ctt gga aga gac ggc Leu Gly Arg Asp Gly 2625	cat gag aaa His Glu Lys	8002
ccc atg Pro Met 2630	aat gtg caa agt ctg Asn Val Gln Ser Leu 2635	gga tgg aac atc atc Gly Trp Asn Ile Ile 2640	acc ttc aag Thr Phe Lys	8047
gac aaa Asp Lys 2645	act gat atc cac cgc Thr Asp Ile His Arg 2650	cta gaa cca gtg aaa Leu Glu Pro Val Lys 2655	tgt gac acc Cys Asp Thr	8092
ctt ttg Leu Leu 2660	tgt gac att gga gag Cys Asp Ile Gly Glu 2665	tca tca tcg tca tcg Ser Ser Ser Ser Ser 2670	gtc aca gag Val Thr Glu	8137
ggg gaa Gly Glu 2675	agg acc gtg aga gtt Arg Thr Val Arg Val 2680	ctt gat act gta gaa Leu Asp Thr Val Glu 2685	aaa tgg ctg Lys Trp Leu	8182
gct tgt Ala Cys 2690	ggg gtt gac aac ttc Gly Val Asp Asn Phe 2695	tgt gtg aag gtg tta Cys Val Lys Val Leu 2700	gct cca tac Ala Pro Tyr	8227
atg cca Met Pro 2705	gat gtt ctt gag aaa Asp Val Leu Glu Lys 2710	ctg gaa ttg ctc caa Leu Glu Leu Leu Gln 2715	agg agg ttt Arg Arg Phe	8272
ggc gga Gly Gly 2720	aca gtg atc agg aac Thr Val Ile Arg Asn 2725	cct ctc tcc agg aat Pro Leu Ser Arg Asn 2730	tcc act cat Ser Thr His	8317
gaa atg Glu Met 2735	tac tac gtg tct gga Tyr Tyr Val Ser Gly 2740	gcc cgc agc aat gtc Ala Arg Ser Asn Val 2745	aca ttt act Thr Phe Thr	8362
gtg aac Val Asn 2750	caa aca tcc cgc ctc Gln Thr Ser Arg Leu 2755	ctg atg agg aga atg Leu Met Arg Arg Met 2760	agg cgt cca Arg Arg Pro	8407
act gga Thr Gly 2765	aaa gtg acc ctg gag Lys Val Thr Leu Glu 2770	gct gac gtc atc ctc Ala Asp Val Ile Leu 2775	cca att ggg Pro Ile Gly	8452
aca cgc Thr Arg 2780	agt gtt gag aca gac Ser Val Glu Thr Asp 2785	aag gga ccc ctg gac Lys Gly Pro Leu Asp 2790	aaa gag gcc Lys Glu Ala	8497
ata gaa Ile Glu 2795	gaa agg gtt gag agg Glu Arg Val Glu Arg 2800	ata aaa tct gag tac Ile Lys Ser Glu Tyr 2805	atg acc tct Met Thr Ser	8542
tggttt Trp Phe 2810	tat gac aat gac aac Tyr Asp Asn Asp Asn 2815	ccc tac agg acc tgg Pro Tyr Arg Thr Trp 2820	cac tac tgt His Tyr Cys	8587
ggc tcc Gly Ser 2825	tat gtc aca aaa acc Tyr Val Thr Lys Thr 2830	tcc gga agt gcg gcg Ser Gly Ser Ala Ala 2835	agc atg gta Ser Met Val	8632
aat ggt Asn Gly 2840	gtt att aaa att ctg Val Ile Lys Ile Leu 2845	aca tat cca tgg gac Thr Tyr Pro Trp Asp 2850	agg ata gag Arg Ile Glu	8677
gag gtc Glu Val 2855	aca aga atg gca atg Thr Arg Met Ala Met 2860	act gac aca acc cct Thr Asp Thr Thr Pro 2865	ttt gga cag Phe Gly Gln	8722
caa aga	gtg ttt aaa gaa aaa	gtt gac acc aga gca	aag gat cca	8767

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Gln Arg 2870	Val Phe Lys Glu 2875	Lys Val Asp Thr Arg 2880	Ala Lys Asp Pro 2885		
cca gcg Pro Ala 2885	gga act agg aag atc Gly Thr Arg Lys Ile 2890	atg aaa gtt gtc Met Lys Val Val 2895	aac agg tgg ctg Asn Arg Trp Leu 2900	8812	
ttc cgc Phe Arg 2900	cac ctg gcc aga gaa His Leu Ala Arg Glu 2905	aag aac ccc aga Lys Asn Pro Arg 2910	ctg tgc aca aag Leu Cys Thr Lys 2915	8857	
gaa gaa Glu Glu 2915	ttt att gca aaa gtc Phe Ile Ala Lys Val 2920	cga agt cat gca gcc Arg Ser His Ala Ala 2925	att gga gct Ile Gly Ala 2930	8902	
tac ctg Tyr Leu 2930	gaa gaa caa gaa cag Glu Glu Gln Glu Gln 2935	tgg aag act gcc aat Trp Lys Thr Ala Asn 2940	gag gct gtc Glu Ala Val 2945	8947	
caa gac Gln Asp 2945	cca aag ttc tgg gaa Pro Lys Phe Trp Glu 2950	ctg gtg gat gaa gaa Leu Val Asp Glu Glu 2955	agg aag ctg Arg Lys Leu 2960	8992	
cac caa His Gln 2960	caa ggc agg tgt cgg Gln Gly Arg Cys Arg 2965	act tgt gtg tac aac Thr Cys Val Tyr Asn 2970	atg atg ggg Met Met Gly 2975	9037	
aaa aga Lys Arg 2975	gag aag aag ctg tca Glu Lys Lys Leu Ser 2980	gag ttt ggg aaa gca Glu Phe Gly Lys Ala 2985	aag gga agc Lys Gly Ser 2990	9082	
cgt gcc Arg Ala 2990	ata tgg tat atg tgg Ile Trp Tyr Met Trp 2995	ctg gga gcg cgg tat Leu Gly Ala Arg Tyr 3000	ctt gag ttt Leu Glu Phe 3005	9127	
gag gcc Glu Ala 3005	ctg gga ttc ctg aat Leu Gly Phe Leu Asn 3010	gag gac cat tgg gct Glu Asp His Trp Ala 3015	tcc agg gaa Ser Arg Glu 3020	9172	
aac tca Asn Ser 3020	gga gga gga gtg gaa Gly Gly Gly Val Glu 3025	ggc att ggc tta caa Gly Ile Gly Leu Gln 3030	tac cta gga Tyr Leu Gly 3035	9217	
tat gtg Tyr Val 3035	atc aga gac ctg gct Ile Arg Asp Leu Ala 3040	gca atg gat ggt ggt Ala Met Asp Gly Gly 3045	gga ttc tac Gly Phe Tyr 3050	9262	
gcg gat Ala Asp 3050	gac acc gct gga tgg Asp Thr Ala Gly Trp 3055	gac acg cgc atc aca Asp Thr Arg Ile Thr 3060	gag gca gac Glu Ala Asp 3065	9307	
ctt gat Leu Asp 3065	gat gaa cag gag atc Asp Glu Gln Glu Ile 3070	ttg aac tac atg agc Leu Asn Tyr Met Ser 3075	cca cat cac Pro His His 3080	9352	
aaa aaa Lys Lys 3080	ctg gca caa gca gtg Leu Ala Gln Ala Val 3085	atg gaa atg aca tac Met Glu Met Thr Tyr 3090	aag aac aaa Lys Asn Lys 3095	9397	
gtg gtg Val Val 3095	aaa gtg ttg aga cca Lys Val Leu Arg Pro 3100	gcc cca gga ggg aaa Ala Pro Gly Gly Lys 3105	gcc tac atg Ala Tyr Met 3110	9442	
gat gtc Asp Val 3110	ata agt cga cga gac Ile Ser Arg Arg Asp 3115	cag aga gga tcc ggg Gln Arg Gly Ser Gly 3120	cag gta gtg Gln Val Val 3125	9487	
act tat Thr Tyr 3125	gct ctg aac acc atc Ala Leu Asn Thr Ile 3130	acc aac ttg aaa gtc Thr Asn Leu Lys Val 3135	caa ttg atc Gln Leu Ile 3140	9532	
aga atg Arg Met 3140	gca gaa gca gag atg Ala Glu Ala Glu Met 3145	gtg ata cat cac caa Val Ile His His Gln 3150	cat gtt caa His Val Gln 3155	9577	
gat tgt gat gaa tca gtt ctg acc agg ctg gag gca ttg ctc act				9622	

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Asp Cys 3155	Asp Glu Ser Val 3160	Leu Thr Arg Leu Glu Ala 3165	Trp Leu Thr	
gag cac Glu His 3170	gga tgt gac aga Gly Cys Asp Arg 3175	ctg aag agg atg gcg Leu Lys Arg Met Ala 3180	gtg agt gga gac Val Ser Gly Asp	9667
gac tgt Asp Cys 3185	gtg gtc cgg ccc Val Val Arg Pro 3190	atc gat gac agg ttc Ile Asp Asp Arg Phe 3195	ggc ctg gcc ctg Gly Leu Ala Leu	9712
tcc cat Ser His 3200	ctc aac gcc atg Leu Asn Ala Met 3205	tcc aag gtt aga aag Ser Lys Val Arg Lys 3210	gac ata tct gaa Asp Ile Ser Glu	9757
tgg cag Trp Gln 3215	cca tca aaa ggg Pro Ser Lys Gly 3220	tgg aat gat tgg gag Trp Asn Asp Trp Glu 3225	aat gtg ccc ttc Asn Val Pro Phe	9802
tgt tcc Cys Ser 3230	cac cac ttc cat His His Phe His 3235	gaa cta cag ctg aag Glu Leu Gln Leu Lys 3240	gat ggc agg agg Asp Gly Arg Arg	9847
att gtg Ile Val 3245	gtg cct tgc cga Val Pro Cys Arg 3250	gaa cag gac gag ctc Gln Asp Glu Leu 3255	att ggg aga gga Ile Gly Arg Gly	9892
agg gtg Arg Val 3260	tct cca gga aac Ser Pro Gly Asn 3265	ggc tgg atg atc aag Gly Trp Met Ile Lys 3270	gaa aca gct tgc Glu Thr Ala Cys	9937
ctc agc Leu Ser 3275	aaa gcc tat gcc Lys Ala Tyr Ala 3280	aac atg tgg tca ctg Met Trp Ser Leu Met 3285	atg tat ttt cac Tyr Phe His	9982
aaa agg Lys Arg 3290	gac atg agg cta Asp Met Arg Leu 3295	ctg tca ttg gct gtt Leu Ser Leu Ala Val 3300	tcc tca gct gtt Ser Ala Val	10027
ccc acc Pro Thr 3305	tca tgg gtt cca Ser Trp Val Pro 3310	caa gga cgc aca Gln Gly Arg Thr 3315	aca tgg tgc att Trp Ser Ile His	10072
ggg aaa Gly Lys 3320	ggg gag tgg atg Gly Glu Trp Met 3325	acc acg gaa gac Thr Thr Glu Asp Met 3330	ctt gag gtg tgg Leu Glu Val Trp	10117
aac aga Asn Arg 3335	gta tgg ata acc Val Trp Ile Thr 3340	aac aac cca cac Asn Asn Pro His Met 3345	atg gac aag aca Gln Asp Lys Thr	10162
atg gtg Met Val 3350	aaa aaa tgg aga Lys Lys Trp Arg 3355	gat gtc cct tat cta Asp Val Pro Tyr Leu 3360	acc aag aga caa Thr Lys Arg Gln	10207
gac aag Asp Lys 3365	ctg tgc gga tca Leu Cys Gly Ser 3370	ctg att gga atg acc Leu Ile Gly Met Thr 3375	aat agg gcc acc Asn Arg Ala Thr	10252
tgg gcc Trp Ala 3380	tcc cac atc cat Ser His Ile His 3385	tta gtc atc cat cgt Leu Val Ile His Arg 3390	atc cga acg ctg Ile Arg Thr Leu	10297
att gga Ile Gly 3395	cag gag aaa tac Gln Glu Lys Tyr 3400	act gac tac cta aca Thr Asp Tyr Leu Thr 3405	gtc atg gac agg Val Met Asp Arg	10342
tat tct gtg gat gct Tyr Ser Val Asp Ala 3410	gac ctg caa ctg ggt Asp Leu Gln Leu Gly 3415	gag ctt atc tgaacaccca Glu Leu Ile		10391
tctaacagga ataaccggga tacaaccac	gggtggagaa cgggactccc cacaacctga			10451
aaccgggata taaaccacgg ctggagaacc	gggctccgca cttaaaatga aacagaaacc			10511
gggataaaaa ctacggatgg agaaccggac	tcacacatt gagacagaag aagttgtcag			10571

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cccagaaccc cacacgagtt ttgccactgc taagctgtga ggcagtgacg gctgggacag 10631
ccgacctcca ggttgcgaaa aacctgggtt ctgggacctc ccacccaga gtaaaaagaa 10691
cggagcctcc gctaccaccc tcccacgtgg tggtagaaag acggggctca gaggttagag 10751
gagaccctcc agggaacaaa tagtgggacc atattgacgc cagggaaaga ccggagtggt 10811
tctctgcttt tcctccagag gtctgtgagc acagtttgct caagaataag cagaccttg 10871
gatgacaaac acaaaaccac t                                     10892

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<210> SEQ ID NO 53
<211> LENGTH: 3421
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

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<400> SEQUENCE: 53

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Arg Arg Gly Val Arg Ser Leu Ser Asn Lys Ile Lys Gln Lys Thr Lys
20          25          30
Gln Ile Gly Asn Arg Pro Gly Pro Ser Arg Gly Val Gln Gly Phe Ile
35          40          45
Phe Phe Phe Leu Phe Asn Ile Leu Thr Gly Lys Lys Ile Thr Ala His
50          55          60
Leu Lys Arg Leu Trp Lys Met Leu Asp Pro Arg Gln Gly Leu Ala Val
65          70          75          80
Leu Arg Lys Val Lys Arg Val Val Ala Ser Leu Met Arg Gly Leu Ser
85          90          95
Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe Leu Ile
100         105         110
Leu Gly Met Leu Leu Met Thr Gly Gly Met Lys Leu Ser Asn Phe Gln
115         120         125
Gly Lys Leu Leu Met Thr Ile Asn Asn Thr Asp Ile Ala Asp Val Ile
130         135         140
Val Ile Pro Thr Ser Lys Gly Glu Asn Arg Cys Trp Val Arg Ala Ile
145         150         155         160
Asp Val Gly Tyr Met Cys Glu Asp Thr Ile Thr Tyr Glu Cys Pro Lys
165         170         175
Leu Thr Met Gly Asn Asp Pro Glu Asp Val Asp Cys Trp Cys Asp Asn
180         185         190
Gln Glu Val Tyr Val Gln Tyr Gly Arg Cys Thr Arg Thr Arg His Ser
195         200         205
Lys Arg Ser Arg Arg Ser Val Ser Val Gln Thr His Gly Glu Ser Ser
210         215         220
Leu Val Asn Lys Lys Glu Ala Trp Leu Asp Ser Thr Lys Ala Thr Arg
225         230         235         240
Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala
245         250         255
Phe Leu Ala Ala Val Leu Gly Trp Met Leu Gly Ser Asn Asn Gly Gln
260         265         270
Arg Val Val Phe Thr Ile Leu Leu Leu Leu Val Ala Pro Ala Tyr Ser
275         280         285

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Phe	Asn	Cys	Leu	Gly	Met	Gly	Asn	Arg	Asp	Phe	Ile	Glu	Gly	Ala	Ser	290	295	300
Gly	Ala	Thr	Trp	Val	Asp	Leu	Val	Leu	Glu	Gly	Asp	Ser	Cys	Leu	Thr	305	310	315
Ile	Met	Ala	Asn	Asp	Lys	Pro	Thr	Leu	Asp	Val	Arg	Met	Ile	Asn	Ile	325	330	335
Glu	Ala	Ser	Gln	Leu	Ala	Glu	Val	Arg	Ser	Tyr	Cys	Tyr	His	Ala	Ser	340	345	350
Val	Thr	Asp	Ile	Ser	Thr	Val	Ala	Arg	Cys	Pro	Thr	Thr	Gly	Glu	Ala	355	360	365
His	Asn	Glu	Lys	Arg	Ala	Asp	Ser	Ser	Tyr	Val	Cys	Lys	Gln	Gly	Phe	370	375	380
Thr	Asp	Arg	Gly	Trp	Gly	Asn	Gly	Cys	Gly	Phe	Phe	Gly	Lys	Gly	Ser	385	390	395
Ile	Asp	Thr	Cys	Ala	Lys	Phe	Ser	Cys	Thr	Ser	Lys	Ala	Ile	Gly	Arg	405	410	415
Thr	Ile	Gln	Pro	Glu	Asn	Ile	Lys	Tyr	Lys	Val	Gly	Ile	Phe	Val	His	420	425	430
Gly	Thr	Thr	Thr	Ser	Glu	Asn	His	Gly	Asn	Tyr	Ser	Ala	Gln	Val	Gly	435	440	445
Ala	Ser	Gln	Ala	Ala	Lys	Phe	Thr	Val	Thr	Pro	Asn	Ala	Pro	Ser	Val	450	455	460
Ala	Leu	Lys	Leu	Gly	Asp	Tyr	Gly	Glu	Val	Thr	Leu	Asp	Cys	Glu	Pro	465	470	475
Arg	Ser	Gly	Leu	Asn	Thr	Glu	Ala	Phe	Tyr	Val	Met	Thr	Val	Gly	Ser	485	490	495
Lys	Ser	Phe	Leu	Val	His	Arg	Glu	Trp	Phe	His	Asp	Leu	Ala	Leu	Pro	500	505	510
Trp	Thr	Ser	Pro	Ser	Ser	Thr	Ala	Trp	Arg	Asn	Arg	Glu	Leu	Leu	Met	515	520	525
Glu	Phe	Glu	Gly	Ala	His	Ala	Thr	Lys	Gln	Ser	Val	Val	Ala	Leu	Gly	530	535	540
Ser	Gln	Glu	Gly	Gly	Leu	His	His	Ala	Leu	Ala	Gly	Ala	Ile	Val	Val	545	550	555
Glu	Tyr	Ser	Ser	Ser	Val	Met	Leu	Thr	Ser	Gly	His	Leu	Lys	Cys	Arg	565	570	575
Leu	Lys	Met	Asp	Lys	Leu	Ala	Leu	Lys	Gly	Thr	Thr	Tyr	Gly	Met	Cys	580	585	590
Thr	Glu	Lys	Phe	Ser	Phe	Ala	Lys	Asn	Pro	Val	Asp	Thr	Gly	His	Gly	595	600	605
Thr	Val	Val	Ile	Glu	Leu	Ser	Tyr	Ser	Gly	Ser	Asp	Gly	Pro	Cys	Lys	610	615	620
Ile	Pro	Ile	Val	Ser	Val	Ala	Ser	Leu	Asn	Asp	Met	Thr	Pro	Val	Gly	625	630	635
Arg	Leu	Val	Thr	Val	Asn	Pro	Phe	Val	Ala	Thr	Ser	Ser	Ala	Asn	Ser	645	650	655
Lys	Val	Leu	Val	Glu	Met	Glu	Pro	Pro	Phe	Gly	Asp	Ser	Tyr	Ile	Val	660	665	670
Val	Gly	Arg	Gly	Asp	Lys	Gln	Ile	Asn	His	His	Trp	His	Lys	Ala	Gly	675	680	685

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

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1085	1090	1095
Trp Cys Cys Arg Ser Cys Thr Met Pro Pro Val Ser Phe His Gly 1100 1105 1110		
Ser Asp Gly Cys Trp Tyr Pro Met Glu Ile Arg Pro Arg Lys Thr 1115 1120 1125		
His Glu Ser His Leu Val Arg Ser Trp Val Thr Ala Gly Glu Ile 1130 1135 1140		
His Ala Val Pro Phe Gly Leu Val Ser Met Met Ile Ala Met Glu 1145 1150 1155		
Val Val Leu Arg Lys Arg Gln Gly Pro Lys Gln Met Leu Val Gly 1160 1165 1170		
Gly Val Val Leu Leu Gly Ala Met Leu Val Gly Gln Val Thr Leu 1175 1180 1185		
Leu Asp Leu Leu Lys Leu Thr Val Ala Val Gly Leu His Phe His 1190 1195 1200		
Glu Met Asn Asn Gly Gly Asp Ala Met Tyr Met Ala Leu Ile Ala 1205 1210 1215		
Ala Phe Ser Ile Arg Pro Gly Leu Leu Ile Gly Phe Gly Leu Arg 1220 1225 1230		
Thr Leu Trp Ser Pro Arg Glu Arg Leu Val Leu Thr Leu Gly Ala 1235 1240 1245		
Ala Met Val Glu Ile Ala Leu Gly Gly Val Met Gly Gly Leu Trp 1250 1255 1260		
Lys Tyr Leu Asn Ala Val Ser Leu Cys Ile Leu Thr Ile Asn Ala 1265 1270 1275		
Val Ala Ser Arg Lys Ala Ser Asn Thr Ile Leu Pro Leu Met Ala 1280 1285 1290		
Leu Leu Thr Pro Val Thr Met Ala Glu Val Arg Leu Ala Ala Met 1295 1300 1305		
Phe Phe Cys Ala Met Val Ile Ile Gly Val Leu His Gln Asn Phe 1310 1315 1320		
Lys Asp Thr Ser Met Gln Lys Thr Ile Pro Leu Val Ala Leu Thr 1325 1330 1335		
Leu Thr Ser Tyr Leu Gly Leu Thr Gln Pro Phe Leu Gly Leu Cys 1340 1345 1350		
Ala Phe Leu Ala Thr Arg Ile Phe Gly Arg Arg Ser Ile Pro Val 1355 1360 1365		
Asn Glu Ala Leu Ala Ala Ala Gly Leu Val Gly Val Leu Ala Gly 1370 1375 1380		
Leu Ala Phe Gln Glu Met Glu Asn Phe Leu Gly Pro Ile Ala Val 1385 1390 1395		
Gly Gly Leu Leu Met Met Leu Val Ser Val Ala Gly Arg Val Asp 1400 1405 1410		
Gly Leu Glu Leu Lys Lys Leu Gly Glu Val Ser Trp Glu Glu Glu 1415 1420 1425		
Ala Glu Ile Ser Gly Ser Ser Ala Arg Tyr Asp Val Ala Leu Ser 1430 1435 1440		
Glu Gln Gly Glu Phe Lys Leu Leu Ser Glu Glu Lys Val Pro Trp 1445 1450 1455		
Asp Gln Val Val Met Thr Ser Leu Ala Leu Val Gly Ala Ala Leu 1460 1465 1470		

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His	Pro	Phe	Ala	Leu	Leu	Leu	Val	Leu	Ala	Gly	Trp	Leu	Phe	His
1475						1480						1485		
Val	Arg	Gly	Ala	Arg	Arg	Ser	Gly	Asp	Val	Leu	Trp	Asp	Ile	Pro
1490						1495						1500		
Thr	Pro	Lys	Ile	Ile	Glu	Glu	Cys	Glu	His	Leu	Glu	Asp	Gly	Ile
1505						1510						1515		
Tyr	Gly	Ile	Phe	Gln	Ser	Thr	Phe	Leu	Gly	Ala	Ser	Gln	Arg	Gly
1520						1525						1530		
Val	Gly	Val	Ala	Gln	Gly	Gly	Val	Phe	His	Thr	Met	Trp	His	Val
1535						1540						1545		
Thr	Arg	Gly	Ala	Phe	Leu	Val	Arg	Asn	Gly	Lys	Lys	Leu	Ile	Pro
1550						1555						1560		
Ser	Trp	Ala	Ser	Val	Lys	Glu	Asp	Leu	Val	Ala	Tyr	Gly	Gly	Ser
1565						1570						1575		
Trp	Lys	Leu	Glu	Gly	Arg	Trp	Asp	Gly	Glu	Glu	Glu	Val	Gln	Leu
1580						1585						1590		
Ile	Ala	Ala	Val	Pro	Gly	Lys	Asn	Val	Val	Asn	Val	Gln	Thr	Lys
1595						1600						1605		
Pro	Ser	Leu	Phe	Lys	Val	Arg	Asn	Gly	Gly	Glu	Ile	Gly	Ala	Val
1610						1615						1620		
Ala	Leu	Asp	Tyr	Pro	Ser	Gly	Thr	Ser	Gly	Ser	Pro	Ile	Val	Asn
1625						1630						1635		
Arg	Asn	Gly	Glu	Val	Ile	Gly	Leu	Tyr	Gly	Asn	Gly	Ile	Leu	Val
1640						1645						1650		
Gly	Asp	Asn	Ser	Phe	Val	Ser	Ala	Ile	Ser	Gln	Thr	Glu	Val	Lys
1655						1660						1665		
Glu	Glu	Gly	Lys	Glu	Glu	Leu	Gln	Glu	Ile	Pro	Thr	Met	Leu	Lys
1670						1675						1680		
Lys	Gly	Met	Thr	Thr	Val	Leu	Asp	Phe	His	Pro	Gly	Ala	Gly	Lys
1685						1690						1695		
Thr	Arg	Arg	Phe	Leu	Pro	Gln	Ile	Leu	Ala	Glu	Cys	Ala	Arg	Arg
1700						1705						1710		
Arg	Leu	Arg	Thr	Leu	Val	Leu	Ala	Pro	Thr	Arg	Val	Val	Leu	Ser
1715						1720						1725		
Glu	Met	Lys	Glu	Ala	Phe	His	Gly	Leu	Asp	Val	Lys	Phe	His	Thr
1730						1735						1740		
Gln	Ala	Phe	Ser	Ala	His	Gly	Ser	Gly	Arg	Glu	Val	Ile	Asp	Ala
1745						1750						1755		
Met	Cys	His	Ala	Thr	Leu	Thr	Tyr	Arg	Met	Leu	Glu	Pro	Thr	Arg
1760						1765						1770		
Val	Val	Asn	Trp	Glu	Val	Ile	Ile	Met	Asp	Glu	Ala	His	Phe	Leu
1775						1780						1785		
Asp	Pro	Ala	Ser	Ile	Ala	Ala	Arg	Gly	Trp	Ala	Ala	His	Arg	Ala
1790						1795						1800		
Arg	Ala	Asn	Glu	Ser	Ala	Thr	Ile	Leu	Met	Thr	Ala	Thr	Pro	Pro
1805						1810						1815		
Gly	Thr	Ser	Asp	Glu	Phe	Pro	His	Ser	Asn	Gly	Glu	Ile	Glu	Asp
1820						1825						1830		
Val	Gln	Thr	Asp	Ile	Pro	Ser	Glu	Pro	Trp	Asn	Thr	Gly	His	Asp
1835						1840						1845		

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

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2225	2230	2235
Pro Gly Gln Gln Arg Ser Ile Gln Asp Asn Gln Val Ala Tyr Leu 2240 2245 2250		
Ile Ile Gly Ile Leu Thr Leu Val Ser Ala Val Ala Ala Asn Glu 2255 2260 2265		
Leu Gly Met Leu Glu Lys Thr Lys Glu Asp Leu Phe Gly Lys Lys 2270 2275 2280		
Asn Leu Ile Pro Ser Ser Ala Ser Pro Trp Ser Trp Pro Asp Leu 2285 2290 2295		
Asp Leu Lys Pro Gly Ala Ala Trp Thr Val Tyr Val Gly Ile Val 2300 2305 2310		
Thr Met Leu Ser Pro Met Leu His His Trp Ile Lys Val Glu Tyr 2315 2320 2325		
Gly Asn Leu Ser Leu Ser Gly Ile Ala Gln Ser Ala Ser Val Leu 2330 2335 2340		
Ser Phe Met Asp Lys Gly Ile Pro Phe Met Lys Met Asn Ile Ser 2345 2350 2355		
Val Ile Met Leu Leu Val Ser Gly Trp Asn Ser Ile Thr Val Met 2360 2365 2370		
Pro Leu Leu Cys Gly Ile Gly Cys Ala Met Leu His Trp Ser Leu 2375 2380 2385		
Ile Leu Pro Gly Ile Lys Ala Gln Gln Ser Lys Leu Ala Gln Arg 2390 2395 2400		
Arg Val Phe His Gly Val Ala Lys Asn Pro Val Val Asp Gly Asn 2405 2410 2415		
Pro Thr Val Asp Ile Glu Glu Ala Pro Glu Met Pro Ala Leu Tyr 2420 2425 2430		
Glu Lys Lys Leu Ala Leu Tyr Leu Leu Leu Ala Leu Ser Leu Ala 2435 2440 2445		
Ser Val Ala Met Cys Arg Thr Pro Phe Ser Leu Ala Glu Gly Ile 2450 2455 2460		
Val Leu Ala Ser Ala Ala Leu Gly Pro Leu Ile Glu Gly Asn Thr 2465 2470 2475		
Ser Leu Leu Trp Asn Gly Pro Met Ala Val Ser Met Thr Gly Val 2480 2485 2490		
Met Arg Gly Asn His Tyr Ala Phe Val Gly Val Met Tyr Asn Leu 2495 2500 2505		
Trp Lys Met Lys Thr Gly Arg Arg Gly Ser Ala Asn Gly Lys Thr 2510 2515 2520		
Leu Gly Glu Val Trp Lys Arg Glu Leu Asn Leu Leu Asp Lys Arg 2525 2530 2535		
Gln Phe Glu Leu Tyr Lys Arg Thr Asp Ile Val Glu Val Asp Arg 2540 2545 2550		
Asp Thr Ala Arg Arg His Leu Ala Glu Gly Lys Val Asp Thr Gly 2555 2560 2565		
Val Ala Val Ser Arg Gly Thr Ala Lys Leu Arg Trp Phe His Glu 2570 2575 2580		
Arg Gly Tyr Val Lys Leu Glu Gly Arg Val Ile Asp Leu Gly Cys 2585 2590 2595		
Gly Arg Gly Gly Trp Cys Tyr Tyr Ala Ala Ala Gln Lys Glu Val 2600 2605 2610		

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Ser Gly	Val Lys Gly Phe Thr	Leu Gly Arg Asp Gly	His Glu Lys
2615	2620	2625	
Pro Met	Asn Val Gln Ser Leu	Gly Trp Asn Ile Ile	Thr Phe Lys
2630	2635	2640	
Asp Lys	Thr Asp Ile His Arg	Leu Glu Pro Val Lys	Cys Asp Thr
2645	2650	2655	
Leu Leu	Cys Asp Ile Gly Glu	Ser Ser Ser Ser Ser	Val Thr Glu
2660	2665	2670	
Gly Glu	Arg Thr Val Arg Val	Leu Asp Thr Val Glu	Lys Trp Leu
2675	2680	2685	
Ala Cys	Gly Val Asp Asn Phe	Cys Val Lys Val Leu	Ala Pro Tyr
2690	2695	2700	
Met Pro	Asp Val Leu Glu Lys	Leu Glu Leu Leu Gln	Arg Arg Phe
2705	2710	2715	
Gly Gly	Thr Val Ile Arg Asn	Pro Leu Ser Arg Asn	Ser Thr His
2720	2725	2730	
Glu Met	Tyr Tyr Val Ser Gly	Ala Arg Ser Asn Val	Thr Phe Thr
2735	2740	2745	
Val Asn	Gln Thr Ser Arg Leu	Leu Met Arg Arg Met	Arg Arg Pro
2750	2755	2760	
Thr Gly	Lys Val Thr Leu Glu	Ala Asp Val Ile Leu	Pro Ile Gly
2765	2770	2775	
Thr Arg	Ser Val Glu Thr Asp	Lys Gly Pro Leu Asp	Lys Glu Ala
2780	2785	2790	
Ile Glu	Glu Arg Val Glu Arg	Ile Lys Ser Glu Tyr	Met Thr Ser
2795	2800	2805	
Trp Phe	Tyr Asp Asn Asp Asn	Pro Tyr Arg Thr Trp	His Tyr Cys
2810	2815	2820	
Gly Ser	Tyr Val Thr Lys Thr	Ser Gly Ser Ala Ala	Ser Met Val
2825	2830	2835	
Asn Gly	Val Ile Lys Ile Leu	Thr Tyr Pro Trp Asp	Arg Ile Glu
2840	2845	2850	
Glu Val	Thr Arg Met Ala Met	Thr Asp Thr Thr Pro	Phe Gly Gln
2855	2860	2865	
Gln Arg	Val Phe Lys Glu Lys	Val Asp Thr Arg Ala	Lys Asp Pro
2870	2875	2880	
Pro Ala	Gly Thr Arg Lys Ile	Met Lys Val Val Asn	Arg Trp Leu
2885	2890	2895	
Phe Arg	His Leu Ala Arg Glu	Lys Asn Pro Arg Leu	Cys Thr Lys
2900	2905	2910	
Glu Glu	Phe Ile Ala Lys Val	Arg Ser His Ala Ala	Ile Gly Ala
2915	2920	2925	
Tyr Leu	Glu Glu Gln Glu Gln	Trp Lys Thr Ala Asn	Glu Ala Val
2930	2935	2940	
Gln Asp	Pro Lys Phe Trp Glu	Leu Val Asp Glu Glu	Arg Lys Leu
2945	2950	2955	
His Gln	Gln Gly Arg Cys Arg	Thr Cys Val Tyr Asn	Met Met Gly
2960	2965	2970	
Lys Arg	Glu Lys Lys Leu Ser	Glu Phe Gly Lys Ala	Lys Gly Ser
2975	2980	2985	

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Arg 2990	Ala	Ile	Trp	Tyr	Met	Trp 2995	Leu	Gly	Ala	Arg	Tyr 3000	Leu	Glu	Phe
Glu 3005	Ala	Leu	Gly	Phe	Leu	Asn 3010	Glu	Asp	His	Trp	Ala 3015	Ser	Arg	Glu
Asn 3020	Ser	Gly	Gly	Gly	Val	Glu 3025	Gly	Ile	Gly	Leu	Gln 3030	Tyr	Leu	Gly
Tyr 3035	Val	Ile	Arg	Asp	Leu	Ala 3040	Ala	Met	Asp	Gly	Gly 3045	Gly	Phe	Tyr
Ala 3050	Asp	Asp	Thr	Ala	Gly	Trp 3055	Asp	Thr	Arg	Ile	Thr 3060	Glu	Ala	Asp
Leu 3065	Asp	Asp	Glu	Gln	Glu	Ile 3070	Leu	Asn	Tyr	Met	Ser 3075	Pro	His	His
Lys 3080	Lys	Leu	Ala	Gln	Ala	Val 3085	Met	Glu	Met	Thr	Tyr 3090	Lys	Asn	Lys
Val 3095	Val	Lys	Val	Leu	Arg	Pro 3100	Ala	Pro	Gly	Gly	Lys 3105	Ala	Tyr	Met
Asp 3110	Val	Ile	Ser	Arg	Arg	Asp 3115	Gln	Arg	Gly	Ser	Gly 3120	Gln	Val	Val
Thr 3125	Tyr	Ala	Leu	Asn	Thr	Ile 3130	Thr	Asn	Leu	Lys	Val 3135	Gln	Leu	Ile
Arg 3140	Met	Ala	Glu	Ala	Glu	Met 3145	Val	Ile	His	His	Gln 3150	His	Val	Gln
Asp 3155	Cys	Asp	Glu	Ser	Val	Leu 3160	Thr	Arg	Leu	Glu	Ala 3165	Trp	Leu	Thr
Glu 3170	His	Gly	Cys	Asp	Arg	Leu 3175	Lys	Arg	Met	Ala	Val 3180	Ser	Gly	Asp
Asp 3185	Cys	Val	Val	Arg	Pro	Ile 3190	Asp	Asp	Arg	Phe	Gly 3195	Leu	Ala	Leu
Ser 3200	His	Leu	Asn	Ala	Met	Ser 3205	Lys	Val	Arg	Lys	Asp 3210	Ile	Ser	Glu
Trp 3215	Gln	Pro	Ser	Lys	Gly	Trp 3220	Asn	Asp	Trp	Glu	Asn 3225	Val	Pro	Phe
Cys 3230	Ser	His	His	Phe	His	Glu 3235	Leu	Gln	Leu	Lys	Asp 3240	Gly	Arg	Arg
Ile 3245	Val	Val	Pro	Cys	Arg	Glu 3250	Gln	Asp	Glu	Leu	Ile 3255	Gly	Arg	Gly
Arg 3260	Val	Ser	Pro	Gly	Asn	Gly 3265	Trp	Met	Ile	Lys	Glu 3270	Thr	Ala	Cys
Leu 3275	Ser	Lys	Ala	Tyr	Ala	Asn 3280	Met	Trp	Ser	Leu	Met 3285	Tyr	Phe	His
Lys 3290	Arg	Asp	Met	Arg	Leu	Leu 3295	Ser	Leu	Ala	Val	Ser 3300	Ser	Ala	Val
Pro 3305	Thr	Ser	Trp	Val	Pro	Gln 3310	Gly	Arg	Thr	Thr	Trp 3315	Ser	Ile	His
Gly 3320	Lys	Gly	Glu	Trp	Met	Thr 3325	Thr	Glu	Asp	Met	Leu 3330	Glu	Val	Trp
Asn 3335	Arg	Val	Trp	Ile	Thr	Asn 3340	Asn	Pro	His	Met	Gln 3345	Asp	Lys	Thr
Met 3350	Val	Lys	Lys	Trp	Arg	Asp 3355	Val	Pro	Tyr	Leu	Thr 3360	Lys	Arg	Gln
Asp	Lys	Leu	Cys	Gly	Ser	Leu	Ile	Gly	Met	Thr	Asn	Arg	Ala	Thr

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3365	3370	3375
Trp Ala Ser His Ile His Leu Val Ile His Arg Ile Arg Thr Leu		
3380	3385	3390
Ile Gly Gln Glu Lys Tyr Thr Asp Tyr Leu Thr Val Met Asp Arg		
3395	3400	3405
Tyr Ser Val Asp Ala Asp Leu Gln Leu Gly Glu Leu Ile		
3410	3415	3420

<210> SEQ ID NO 54
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 54

Leu Ser Ser Arg Lys Arg Arg Ser His Asp
1 5 10

<210> SEQ ID NO 55
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 55

Val Leu Thr Val Gln Phe Leu Ile Leu Gly Met Leu Leu Met Thr Gly
1 5 10 15
Gly Val Thr Leu
20

<210> SEQ ID NO 56
 <211> LENGTH: 32
 <212> TYPE: PRT
 <213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 56

Gly Arg Lys Gln Asn Lys Arg Gly Gly Asn Glu Gly Ser Ile Met Trp
1 5 10 15
Leu Ala Ser Leu Ala Val Val Ile Ala Tyr Ala Gly Ala Met Lys Leu
20 25 30

<210> SEQ ID NO 57
 <211> LENGTH: 11
 <212> TYPE: PRT
 <213> ORGANISM: Tick-Borne Encephalitis virus

<400> SEQUENCE: 57

Gln Lys Arg Gly Lys Arg Arg Ser Ala Thr Asp
1 5 10

<210> SEQ ID NO 58
 <211> LENGTH: 19
 <212> TYPE: PRT
 <213> ORGANISM: Tick-Borne Encephalitis virus

<400> SEQUENCE: 58

Trp Met Ser Trp Leu Leu Val Ile Thr Leu Leu Gly Met Thr Leu Ala
1 5 10 15
Ala Thr Val

<210> SEQ ID NO 59

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<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Murray Valley Encephalitis virus

<400> SEQUENCE: 59

Gly Lys Lys Gln Lys Lys Arg Gly Gly Ser Glu
1 5 10

<210> SEQ ID NO 60
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Murray Valley Encephalitis virus

<400> SEQUENCE: 60

Thr Ser Val Leu Met Val Ile Phe Met Leu Ile Gly Phe Ala Ala Ala
1 5 10 15

Leu Lys Leu

<210> SEQ ID NO 61
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Saint Louis Encephalitis virus

<400> SEQUENCE: 61

Arg Arg Pro Ser Lys Lys Arg Gly Gly Thr Arg
1 5 10

<210> SEQ ID NO 62
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Saint Louis Encephalitis virus

<400> SEQUENCE: 62

Ser Leu Leu Gly Leu Ala Ala Leu Ile Gly Leu Ala Ser Ser Leu
1 5 10 15

<210> SEQ ID NO 63
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Dengue-1 virus

<400> SEQUENCE: 63

Ile Met Asn Arg Arg Lys Arg Ser Val Thr
1 5 10

<210> SEQ ID NO 64
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Dengue-1 virus

<400> SEQUENCE: 64

Met Leu Leu Met Leu Leu Pro Thr Ala Leu Ala Phe His Leu
1 5 10

<210> SEQ ID NO 65
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Dengue-2 virus

<400> SEQUENCE: 65

Ile Leu Asn Arg Arg Arg Arg Thr Ala Gly
1 5 10

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<210> SEQ ID NO 66
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Dengue-2 virus

<400> SEQUENCE: 66

Met Ile Ile Met Leu Ile Pro Thr Val Met Ala Phe His Leu
1 5 10

<210> SEQ ID NO 67
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 67

Ile Ile Asn Lys Arg Lys Lys Thr Ser Leu
1 5 10

<210> SEQ ID NO 68
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Dengue-3 virus

<400> SEQUENCE: 68

Cys Leu Met Met Met Leu Pro Ala Thr Leu Ala Phe His Leu
1 5 10

<210> SEQ ID NO 69
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Dengue-4 virus

<400> SEQUENCE: 69

Ile Leu Asn Gly Arg Lys Arg Ser Thr Ile
1 5 10

<210> SEQ ID NO 70
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Dengue-4 virus

<400> SEQUENCE: 70

Thr Leu Leu Cys Leu Ile Pro Thr Val Met Ala Phe Ser Leu
1 5 10

<210> SEQ ID NO 71
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Dengue-4 virus

<400> SEQUENCE: 71

Ile Leu Asn Gly Arg Lys Arg Ser Thr Ile Thr Leu Leu Cys Leu Ile
1 5 10 15

Pro Thr Val Met Ala Phe Ser Leu
20

<210> SEQ ID NO 72
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Tick-Borne Encephalitis virus

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<400> SEQUENCE: 72

Gln Lys Arg Gly Lys Arg Arg Ser Ala Val Asp Trp Thr Gly Trp Leu
1 5 10 15
Leu Val Val Val Leu Leu Gly Val Thr Leu Ala Ala Thr Val
20 25 30

<210> SEQ ID NO 73

<211> LENGTH: 30

<212> TYPE: PRT

<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 73

Leu Ser Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe
1 5 10 15
Leu Ile Leu Gly Met Leu Leu Met Thr Gly Gly Val Thr Leu
20 25 30

<210> SEQ ID NO 74

<211> LENGTH: 24

<212> TYPE: PRT

<213> ORGANISM: Dengue-2 virus

<400> SEQUENCE: 74

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

<210> SEQ ID NO 75

<211> LENGTH: 30

<212> TYPE: PRT

<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 75

Leu Ser Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe
1 5 10 15
Leu Ile Leu Gly Met Leu Leu Met Thr Gly Gly Val Thr Leu
20 25 30

<210> SEQ ID NO 76

<211> LENGTH: 7

<212> TYPE: PRT

<213> ORGANISM: Dengue-2 virus

<400> SEQUENCE: 76

Ile Leu Asn Arg Arg Arg Arg
1 5

<210> SEQ ID NO 77

<211> LENGTH: 17

<212> TYPE: PRT

<213> ORGANISM: Dengue-2 virus

<400> SEQUENCE: 77

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

<210> SEQ ID NO 78

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<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 78

Tyr Ala Gly Ala Met Lys Leu
1 5

<210> SEQ ID NO 79
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 79

Met Thr Gly Gly Val Thr Leu
1 5

<210> SEQ ID NO 80
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: derived from Japanese Encephalitis virus and
Yellow Fever virus

<400> SEQUENCE: 80

Met Thr Gly Gly Met Lys Leu
1 5

<210> SEQ ID NO 81
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 81

Asn Lys Arg Gly Gly Asn Glu
1 5

<210> SEQ ID NO 82
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 82

Lys Arg Arg Ser His Asp Val
1 5

<210> SEQ ID NO 83
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 83

Thr Asn Val His Ala Asp Thr Gly Cys Ala
1 5 10

<210> SEQ ID NO 84
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 84

Leu Gly Val Gly Ala Asp Gln Gly Cys Ala

-continued

1 5 10

<210> SEQ ID NO 85
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: derived from Japanese Encephalitis virus and
 Yellow Fever virus

<400> SEQUENCE: 85

Thr Asn Val Gly Ala Asp Gln Gly Cys Ala
 1 5 10

What is claimed is:

1. A chimeric live, infectious, attenuated virus, comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

2. The chimeric virus of claim 1, wherein said second flavivirus is a Japanese Encephalitis (JE) virus, a Dengue virus, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (i.e., a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest Disease virus, or an Omsk Hemorrhagic Fever virus.

3. The chimeric virus of claim 2, wherein said second flavivirus is a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4.

4. The chimeric virus of claim 3, wherein said nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

5. (canceled)

6. The chimeric virus of claim 1, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus;

said nucleotide sequence encoding said prM-E protein of said second, different flavivirus comprises a mutation that prevents prM cleavage to produce M protein;

the prM signal of said chimeric virus is that of yellow fever virus; and/or

the NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

7-9. (canceled)

10. A method of preventing or treating flavivirus infection in a patient, said method comprising administering to said patient a chimeric, live, infectious, attenuated virus comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or

mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

11. The method of claim 10, wherein said second flavivirus is a Japanese Encephalitis (JE) virus, a Dengue virus, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (i.e., a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest Disease virus, or an Omsk Hemorrhagic Fever virus.

12. The method of claim 11 wherein said second flavivirus is a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4.

13. The method of claim 12, wherein said nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

14. (canceled)

15. The method of claim 10, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus;

said nucleotide sequence encoding said prM-E protein of said second, different flavivirus comprises a mutation that prevents prM cleavage to produce M protein;

the prM signal of said chimeric virus is that of yellow fever virus; and/or

the NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

16-18. (canceled)

19. A nucleic acid molecule encoding a chimeric live, infectious, attenuated virus comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

20. The nucleic acid molecule of claim 19, wherein said second flavivirus is a Japanese Encephalitis (JE) virus, a

Dengue virus, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (i.e., a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest Disease virus, or an Omsk Hemorrhagic Fever virus.

21. The nucleic acid molecule of claim **20**, wherein said second flavivirus is a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4.

22. The nucleic acid molecule of claim **21**, wherein said nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

23. (canceled)

24. The nucleic acid molecule of claim **19**, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus;

said nucleotide sequence encoding said prM-E protein of said second, different flavivirus comprises a mutation that prevents prM cleavage to produce M protein;

the prM signal of said chimeric virus is that of yellow fever virus; and/or

the NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

25-27. (canceled)

28. A method of producing a gene product in a cell in a patient, said method comprising introducing into said cell a yellow fever virus vector comprising a gene encoding said gene product.

29. The method of claim **28**, wherein said cell is a cell of the lymphoid system or the reticuloendothelial system, or a precursor thereof.

30. The method of claim **28**, wherein said patient has cancer.

31. The method of claim **30**, wherein said cancer is leukemia.

32. The method of claim **30**, wherein said gene product is a tumor antigen or a cytokine.

* * * * *