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MINISTERIO DE COMERCIO INDUSTRIA Y
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DIVISION DE NUEVAS CREACIONES

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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REFERENCIA : SOLICITUD DE REGISTRO DE LA PATENTE USO
DE FLAVIVIRUS PARA LA EXPRESION
EPITOPOS DE PROTEINA Y DESARROLLO DE
NUEVOS VIRUS VIVOS ATENUADOS
VACUNANTES PARA INMUNIZAR CONTRA
FLAVIVIRUS Y OTROS AGENTES
INFECCIOSOS EXPEDIENTE No. 03-000274.

CODIGO : 01-01-08

LAURA MICHELSEN NIÑO, mayor de edad, domiciliada en la ciudad de Bogotá D.C, República de Colombia, abogada titulada e identificada como aparece al pie de mi firma, en mi calidad de apoderada de la sociedad FUNDAÇÃO OSWALDO CRUZ - FIOCRUZ, existente y organizada conforme a las leyes del Brasil, con domicilio en la ciudad de Rio de Janeiro, Brasil, por medio del presente escrito me permito dar respuesta al oficio No. 5927, notificado por fijación en lista de fecha 22 de mayo de 2009, y conforme a la prórroga presentada oportunamente a su Despacho, el día 21 de agosto de 2009, me permito manifestar y anexar lo siguiente:

1. Manifiesto

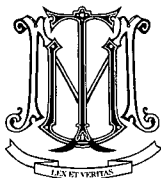
1.1 En materia de claridad de la solicitud, el examinador se refiere a que el capítulo reivindicatorio tiene defectos de forma y no comprende el alcance de lo que pretende protegerse, de manera que no es conciso. En respuesta a lo requerido, nos permitimos dar respuesta a dichas observaciones sobre lo siguiente:

1.1.1 El examinador define que la reivindicación 1 se refiere a un método pero no define claramente los pasos o etapas que se deban seguir para conformar dicho proceso.

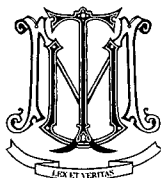
En consecuencia, ha sido modificada la reivindicación 1 con el propósito de definir claramente los pasos necesarios para conformar dicho proceso. Adicionalmente, dicha reivindicación se ha dividido mediante el término "CARACTERIZADO POR QUE", de tal forma que sea posible apreciar el preámbulo y la parte característica de la invención, estableciendo claramente la materia que se considera novedosa y sobre la cual se solicita protección.

1.1.2 A partir de este primer cambio, es posible apreciar que el proceso de la invención comprende el paso de: (a) introducción y expresión de secuencias de genes foráneos dentro de sitios de inserción, caracterizado por que el sitio de inserción comprende la región de hilos-β f y g que incluye el lazo fg que forma parte de la lámina β antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus.

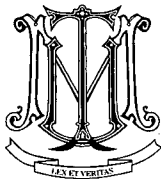
1.1.3 Adicionalmente, se han redefinido las reivindicaciones dependientes de ésta primera, eliminando la cláusula 2 y reenumerando las subsiguientes, ubicando claramente el preámbulo y su parte característica en cada una.



- 1.2 El señor examinador también se refiere a que las reivindicaciones que se refieren a la construcción de ADN no definen claramente lo que desea proteger; que las reivindicaciones 37 a 51 reclaman una cantidad indeterminada de posibles flavivirus y que no están sustentados en la descripción; que las cláusulas 52 a 57 reclaman una composición según virus no definidos; y, por último, se refiere a que la reivindicación 58 reclama un flavivirus sin definición clara y concreta, respecto a lo que cabe señalar lo siguiente:
 - 1.2.1 Es preciso destacar que se considera novedoso es la identificación de los sitios de inserción de la proteína E del virus de la fiebre amarilla, por lo que después de la inserción de varios epítopos heterólogos en la proteína, su estructura no sufre modificación, de manera que lo novedoso del flavivirus, es el sitio de inserción que según la descripción comprende la región de hilos- β f y g que incluye el lazo fg que forma parte de la lámina antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus. Ello es la materia que se considera aparte del arte conocido.
- 1.3 Para aclarar aún más la solicitud de la referencia, es preciso señalar que ésta invención se encuentra dirigida a un método para la producción de Flavivirus como un vector para los antígenos heterólogos. Para obtener un virus de vacuna basada en Flavivirus expresando antígenos foráneos para el desarrollo de nuevas vacunas vivas, utilizando técnicas de recombinación del ADN, de lo que resulta necesario, acumulativamente:
 - 1.3.1 Diseñar estrategias que permiten la introducción de antígenos extranjeros sin comprometer la estructura y la función del virus;
 - 1.3.2 Para asegurar que la construcción cDNA infecciosa y las transcripciones de RNA generadas derivadas de los mismos darán lugar a un virus que es no patógeno y que no tiene el potencial para convertirse a una forma patógena; y que la secuencia de antígeno foráneo es genéticamente estable una vez se ha integrado en el genoma viral;
 - 1.3.3 El Flavivirus generado a partir del cDNA clonado, además de ser atenuadas, deben conservar sus propiedades inmunológicas y presentar el antígeno foráneo expresado que cause la respuesta inmune adecuada.
 - 1.3.4 Por lo tanto, a fin de producir el virus (17D) de vacuna YF para expresar a nivel de su envoltura, los epítopos de proteínas de otros agentes patógenos que suscitan una respuesta inmune específica al patógeno parental, los inventores han desarrollado el método de la presente invención, que consiste en la introducción y la expresión de secuencias de genes extranjeros en un sitio de inserción en el nivel de la proteína envolvente de cualquier Flavivirus, en el que los sitios son estructuralmente aparte de las sabidas zonas que interfieren con los sitios de la proteína E del Flavivirus general y que comprenda que:
 - 1.3.4.1 Se encuentren en la superficie exterior del virus para proporcionar acceso a los anticuerpos;



- 1.3.4.2 No interrumpir o desestabilizar significativamente la estructura tridimensional de la proteína E y no interferir con la formación de la red de proteína E dentro de la envoltura viral.
- 1.3.5 La principal preocupación acerca de la inserción de epítopes en la proteína E del Flavivirus, en particular en la proteína YF 17D E, apunta al hecho que esta proteína es el objetivo principal de la respuesta humoral neutralizante, siendo esta la proteína donde se hemaglutinan y neutralizan los epítopes, y es la proteína que sirve de receptor de reconocimiento de mediador celular y la debida penetración de la célula, por lo tanto, el objetivo del virus contra células específicas. Mediante la inserción de un nuevo inmunoprotector en algún lugar en la proteína E de un determinado Flavivirus, una o varias de estas propiedades podrían cambiarse a menos que el análisis de la estructura 3D permita la identificación de posibles sitios de inserción estructuralmente aparte de zonas sabidas que median de uno o varios de estos procesos.
- 1.4 Por lo señalado en los numerales que anteceden, fue modificada la cláusula 1 de la presente solicitud, y ahora está limitada a los pasos necesarios para la producción de un Flavivirus como un vector para los antígenos heterólogos. Además, el sitio de inserción de las secuencias de genes foráneos se definió en dicha reivindicación 1.
- 1.5 En consecuencia, solicito muy respetuosamente al Examinador, tener en cuenta que la reivindicación 1 define un problema técnico para resolverse, de manera que se soluciona el problema técnico y el resultado (mediante un método) que ahora está definido en la cláusula 1.
- 1.6 Respecto, a la observación de que la construcción de ADN no está definida por una secuencia, nos permitimos señalar que:
- 1.6.1 Las reivindicaciones 19 al 28 se dirigen a una construcción de ADN obtenida por el método tal como se define en reclamaciones anteriores.
- 1.6.2 La construcción del ADN está formado por bloques individuales de elementos genéticos, cada elemento fundamentalmente constituido por tres componentes:
- (i) Promotor
 - (ii) La resistencia del virus conferida por un elemento genético
 - (iii) Terminador.
- 1.6.3 Por lo tanto, las reivindicaciones respecto a la construcción de ADN, están estrechamente vinculadas a las reivindicaciones del método, por lo que consideramos que están bien definidos.
- 1.7 Para tal efecto, y para precisar la invención y lo que se considera novedoso, se propone un pliego de nuevas cláusulas que define el Flavivirus y lo aparta del arte anterior, donde el Flavivirus como un vector para antígenos heterólogos, está caracterizado por que el sitio de inserción comprende la región de hilos- β f y g que incluye el lazo fg que forma parte de la lámina antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus.



- 1.8 Así las cosas, presento ante ése Despacho un nuevo capítulo reivindicatorio, restringiendo aún más la invención, donde se ha definido el preámbulo y se ha limitado la parte característica. Es preciso aclarar que dicho nuevo capítulo reivindicatorio no implica ampliación alguna de la invención sino por el contrario, su intención es precisar la invención con base en las apreciaciones del señor examinador.
- 1.9 Por todo lo anterior, me permito en conclusión solicitar sea de nuevo revisada la presente invención y verificar las diferencias aquí expuestas, y en consecuencia se practique el estudio de fondo en los términos y el tenor de las nuevas cláusulas del capítulo reivindicatorio.
2. Anexo
- 2.1 En consecuencia de lo anterior, me permito anexar el **nuevo capítulo reivindicatorio**, por medio del cual se evidencian los ajustes realizados, conforme al requerimiento que acá se contesta.
3. Petición

Conforme a lo señalado anteriormente, y a los documentos que acompañan el presente escrito, cumpliendo lo requerido en el oficio que acá se responde, reitero mi respetuosa petición de que la presente invención sea nuevamente evaluada, y en consecuencia, se compruebe que no existe ningún impedimento para su correspondiente concesión.

De la Señora Jefe,

LAURA MICHELSEN NIÑO
C.C. No. 39.783.287 de Usaquén
T.P.A. 67.897 de Min. Justicia

REIVINDICACIONES

1. Método para la producción de Flavivirus como un vector para antígenos heterólogos, donde el método comprende el paso de:
 - (a) introducción y expresión de secuencias de genes foráneos dentro de sitios de inserción en el nivel de la proteína de envoltura de cualquier Flavivirus, en donde los sitios están a parte estructuralmente de las áreas conocidas para interferir con la estructura de la proteína E del Flavivirus general; **CARACTERIZADO POR QUE** el sitio de inserción comprende la región de hilos- β f y g que incluye el lazo fg que forma parte de la lámina β antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus.
2. Método de acuerdo con la reivindicación 1 **CARACTERIZADO POR QUE** el sitio es el área de lazo entre los hilos- β f y g que forma parte de la lámina β antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus.
3. Método de acuerdo con la reivindicación 1 **CARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 196 a 215 con referencia a la secuencia de virus de encefalitis tick-borne descrita en la figura 2.
4. Método de acuerdo con la reivindicación 2 **CARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 205 a 210 con referencia a la secuencia de virus de encefalitis tick-borne descrita en la figura 2.
5. Método para la producción de Flavivirus como un vector para antígenos heterólogos que comprende los pasos de (a) introducción y expresión de secuencias de genes foráneos dentro de sitios de inserción en el nivel de la proteína de envoltura de cualquier Flavivirus, en donde los sitios están a parte estructuralmente de las áreas conocidas para interferir con la estructura de la proteína E del Flavivirus general **CARACTERIZADO POR QUE** el sitio comprende la región de los hilos E_0 y F_0 que incluye el lazo E_0F_0 que forma parte del barril- β de 8 ramificaciones del dominio I.

6. Método de acuerdo con la reivindicación 5 **CHARACTERIZADO POR QUE** el sitio es el área de lazo entre los hilos E_0 y F_0 que forma parte del barril- β de 8 ramificaciones del dominio I.
7. Método de acuerdo con la reivindicación 5 **CHARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 138 a 166 con referencia a la secuencia de virus de encefalitis tick-borne descrita en la figura 2.
8. Método de acuerdo con la reivindicación 6 **CHARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 146 a 160 con referencia a la secuencia de virus de encefalitis tick-borne descrito en la figura 2.
9. Método de acuerdo con cualquiera de las reivindicaciones 1 a 8 **CHARACTERIZADO POR QUE** el Flavivirus se selecciona a partir del grupo que consiste de cualquier Flavivirus que incluye el virus de fiebre amarilla, el virus de encefalitis tick-borne, el virus del dengue y el virus de encefalitis japonesa.
10. Método de acuerdo con la reivindicación 9 **CHARACTERIZADO POR QUE** el virus es un virus recombinante o atenuado del tipo salvaje o natural.
11. Método de acuerdo con las reivindicaciones 9 y 10 **CHARACTERIZADO POR QUE** el Flavivirus es un virus de fiebre amarilla.
12. Método de acuerdo con la reivindicación 11 **CHARACTERIZADO POR QUE** el virus es un virus de fiebre amarilla recombinante.
13. Método de acuerdo con la reivindicación 12 **CHARACTERIZADO POR QUE** el virus de fiebre amarilla es la cepa de virus YF17D y las sub-cepas del mismo.
14. Método de acuerdo con las reivindicaciones 1 a 13 **CHARACTERIZADO POR QUE** el epítipo foráneo es una secuencia del gen malarial.
15. Método de acuerdo con la reivindicación 14 **CHARACTERIZADO POR QUE** la secuencia de gen malarial es el (NANP)₃ epítipo humoral.

16. Método de acuerdo con la reivindicación 14 **CHARACTERIZADO POR QUE** la secuencia de gen malarial es el DYENDIEKKI del epítoto de linfocitos T citotóxicos (CTL).
17. Método de acuerdo con la reivindicación 14 **CHARACTERIZADO POR QUE** la secuencia de gen malarial es el SYVPSAEQI del epítoto de linfocitos T citotóxicos (CTL).
18. Método de acuerdo con la reivindicación 1 **CHARACTERIZADO POR QUE** uno o más residuos de glicina son insertados en la región inmediatamente aguas arriba y aguas abajo del epítoto foráneo.
19. Una construcción de DNA que consiste esencialmente de un vector seleccionado del grupo de pACNR1180 y pBeloBACII; un genoma de Flavivirus generalmente estable seleccionado de cualquier Flavivirus incluyendo el virus de fiebre amarilla, el virus de encefalitis tick-borne, el virus del dengue y el virus de encefalitis japonesa; y, una secuencia de genes foráneos introducida en un sitio de inserción, **CHARACTERIZADO POR QUE** la construcción de DNA es obtenida de acuerdo con cualquiera de las reivindicaciones 1 a 18.
20. La construcción de DNA de acuerdo con la reivindicación 19 **CHARACTERIZADO POR QUE** dicho vector es seleccionado a partir del grupo que comprende plásmidos de números de alta copia.
21. La construcción de DNA de acuerdo con la reivindicación 19 **CHARACTERIZADO POR QUE** el genoma de Flavivirus estable genéticamente se deriva de cualquier sepa YF 17D.
22. La construcción de DNA de acuerdo con la reivindicación 21 **CHARACTERIZADO POR QUE** el genoma de Flavivirus estable genéticamente es el genoma YF que lleva la secuencia completa establecida en la SEQ ID NO:1 o secuencias equivalentes funcionalmente de la misma.
23. La construcción de DNA de acuerdo con la reivindicación 19 **CHARACTERIZADO POR QUE** la secuencia de gen foráneo derivada de la

malaria, fiebre amarilla, dengue, encefalitis japonesa, encefalitis tick-borne e infecciones fungales.

24. La construcción de DNA de acuerdo con la reivindicación 23 **CARACTERIZADO POR QUE** la secuencia de gen foráneo es una secuencia de gen malarial.
25. La construcción de DNA de acuerdo con las reivindicaciones 22 a 24 **CARACTERIZADO POR QUE** la secuencia del gen malarial es el epítoto humoral (NANP)₃.
26. La construcción de DNA de acuerdo con la reivindicación 24 **CARACTERIZADO POR QUE** la secuencia de gen malarial es el DYENDIEKKI del epítoto de linfocitos T citotóxicos.
27. La construcción de DNA de acuerdo con la reivindicación 24 **CARACTERIZADO POR QUE** la secuencia de gen malarial es el SYVPAEQI del epítoto de linfocitos T citotóxicos.
28. La construcción de DNA de acuerdo con la reivindicación 25 **CARACTERIZADO POR QUE** el plásmido pYF17D/8.
29. Construcción de DNA **CARACTERIZADO POR QUE** tiene la estructura del plásmido pYFE200.
30. Construcción de DNA **CARACTERIZADO POR QUE** tiene la estructura del plásmido pYFE200/1.
31. Construcción de DNA **CARACTERIZADO POR QUE** tiene la estructura del plásmido pYFE200/13.
32. Construcción de DNA **CARACTERIZADO POR QUE** tiene la estructura del plásmido pYFE200/8.
33. Un Flavivirus como un vector para antígenos heterólogos que comprende secuencias de gen foráneo insertadas en los sitios en el nivel de su proteína de envoltura, en donde los sitios están estructuralmente a parte de las áreas

conocidas para interferir con la estructura de la proteína E del Flavivirus general; **CARACTERIZADO POR QUE** el sitio de inserción comprende la región de hilos- β f y g que incluye el lazo fg que forma parte de la lámina antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus.

34. Un Flavivirus de acuerdo con la reivindicación 33 **CARACTERIZADO POR QUE** el sitio es el área de lazo entre los hilos-5 de f y g que forma parte de la lámina antiparalela de 5 ramificaciones de dominio II de la proteína de envoltura del Flavivirus.
35. Un Flavivirus de acuerdo con la reivindicación 33 **CARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 196 a 215 con referencia a la secuencia de virus de encefalitis tick-borne descrita en la figura 2.
36. Un Flavivirus de acuerdo con la reivindicación 34 **CARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 205 a 210 con referencias al virus de encefalitis tick-borne descrita en la figura 2.
37. Un Flavivirus como un vector para antígenos heterólogos que comprende secuencias de gen foráneo insertadas en los sitios en el nivel de su proteína de envoltura, en donde los sitios están estructuralmente a parte de las áreas conocidas para interferir con la estructura de la proteína E del Flavivirus general; **CARACTERIZADO POR QUE** el sitio comprende la región de los hilos E_0 y F_0 que incluye el lazo E_0F_0 que forma parte del barril- β de 8 ramificaciones del dominio I.
38. Un Flavivirus de acuerdo con la reivindicación 37 **CARACTERIZADO POR QUE** el sitio es el área de lazo entre los hilos E_0 y F_0 que forma parte del barril- β de 8 ramificaciones del dominio I.
39. Un Flavivirus de acuerdo con la reivindicación 37 **CARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 138 a 166 con referencia a la secuencia de virus de encefalitis tick-borne descrita en la figura 2.

40. Un Flavivirus de acuerdo con la reivindicación 38 **CARACTERIZADO POR QUE** la secuencia foránea ha sido insertada en la región de los aminoácidos 146 a 160 con referencia a la secuencia de virus de encefalitis tick-borne descrito en la figura 2.
41. Un Flavivirus de acuerdo con la reivindicación 37 **CARACTERIZADO POR QUE** el Flavivirus se selecciona a partir del grupo que consiste de cualquier Flavivirus que incluye el virus de fiebre amarilla, el virus de encefalitis tick-borne, el virus del dengue y el virus de encefalitis japonesa.
42. Un Flavivirus de acuerdo con la reivindicación 41 **CARACTERIZADO POR QUE** el virus es un virus recombinante o atenuado del tipo salvaje o natural.
43. Un Flavivirus de acuerdo con la reivindicación 41 ó 42 **CARACTERIZADO POR QUE** el Flavivirus es un virus de fiebre amarilla.
44. Un Flavivirus de acuerdo con la reivindicación 43 **CARACTERIZADO POR QUE** el virus es un virus de fiebre de amarilla recombinante.
45. Un Flavivirus de acuerdo con la reivindicación 43 **CARACTERIZADO POR QUE** el virus de fiebre amarilla es la sepa de YF 17D y las sub-sepas del mismo.
46. Un Flavivirus de acuerdo con cualquiera de las reivindicaciones 33 a 45 **CARACTERIZADO POR QUE** el epítipo foráneo es una secuencia del gen malarial.
47. Una composición de vacuna para inmunización contra el Flavivirus y otros agentes infecciosos **CARACTERIZADO POR QUE** dicha composición consiste de una cantidad suficiente de un virus como el definido en las reivindicaciones 33 a 46, donde el Flavivirus es un virus de fiebre amarilla y los otros agentes infecciosos que son agentes causativos de malaria; y, un vehículo farmacéuticamente aceptable.
48. Una composición de vacuna de acuerdo con la reivindicación 47 **CARACTERIZADO POR QUE** dicha secuencia de gen malarial es el epítipo humoral (NANP)₃.

49. Una composición de vacuna de acuerdo con la reivindicación 47 **CARACTERIZADO POR QUE** dicha secuencia de gen malarial es el DYENDIEKKI del epítotope de linfocitos T citotóxico.
50. Una composición de vacuna de acuerdo con la reivindicación 47 **CARACTERIZADO POR QUE** dicha secuencia de gen malarial es el SYVPSAEQI del epítotope de linfocitos T citotóxicos.



US006171854B1

(12) **United States Patent**
Galler et al.

(10) **Patent No.: US 6,171,854 B1**
 (45) **Date of Patent: Jan. 9, 2001**

(54) **YELLOW FEVER INFECTIOUS CDNA AND PLASMIDS**

(75) Inventors: **Ricardo Galler; Marcos Da Silva Freire**, both of Niteroi (BR)

(73) Assignee: **Fundaco Oswaldo Cruz-Fiocruz**, Rio de Janeiro (BR)

(*) Notice: Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **09/058,411**

(22) Filed: **Apr. 10, 1998**

(30) **Foreign Application Priority Data**

Apr. 11, 1997 (BR) 9701774

(51) Int. Cl.⁷ **C12N 15/40**

(52) U.S. Cl. **435/320.1; 536/23.72**

(58) Field of Search **536/23.72; 435/320.1**

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ABSTRACT

The present invention is related to a vaccine composition for humans against YF infections-consisting-essentially-of-a recombinant-YF-virus-YFiv5.2/DD, which is regenerated from YF-infectious-cDNA. There are provided new plasmids, pYF 5'3' IV/G1/2 and pYFM 5.2/T3/27, which together, have the complete sequence of said YF infectious cDNA. The method for producing recombinant YF virus and the Original, Primary and Secondary Seed Lots are other embodiments of the present invention.

8 Claims, 8 Drawing Sheets

3

and 17D-204 genomic sequences (see Hahn, C. S. et al, 1987). The comparison between the nucleotide sequences of 17DD and 17D-213 substrains (see Duarte dos Santos et al, 1995) and the nucleotide sequence of 17D-204 substrain (see Rice et al, 1985) showed that not all changes are common and thus not confirmed as being 17D-specific. Therefore, the 17D-substrain specific changes observed are very likely not related to attenuation but may reflect differences in behavior of these strains in monkey neurovirulence tests. In consequence, the number of changes likely to be associated with viral attenuation were reduced by 26%, i.e., to 48 nucleotide changes. From these 48 nucleotide sequence changes which are scattered along the genome, 26 are silent mutations and 22 led to amino acid substitutions. More important are the alterations noted in the E protein because it is the main target for humoral neutralizing response, i.e., it is the protein where hemagglutination and neutralization epitopes are located, and it mediates cell receptor recognition and cell penetration, therefore targeting the virus to specific cells. Importantly, E protein accumulate the highest ratio of nonconservative to conservative amino acid changes. Altogether, eleven nucleotide substitutions were observed in the E protein gene leading to 8 amino acid changes at positions 52, 170, 173, 200, 299, 305, 331 and 380 (respectively nucleotides 1127, 1482, 1491, 1572, 1870, 1887, 1965 and 2112 from the RNA 5' end).

~~Alterations at amino acids 52 and 200 are located in domain A of E protein (domain II in 3-D structure proposed for Flaviviruses E protein—R  y, F. A.; Heinz F. X.; Mandl, C.; Kunz, C. and Harrison, S. C. (1995). "The envelope glycoprotein from tick-borne encephalitis virus at 2   resolution". Nature. 375: 291–298) which is conserved among Flaviviruses and contains cross-reactive epitopes as shown by Mandl, C. W. et al (Mandl, M. W.; Guirakhoo, F.; Holzmann, H.; Heinz, F. X. and Kunz, C. (1989). "Antigenic structure of the flavivirus envelope E protein at the molecular level using tick-borne encephalitis virus as a model". J. Virol. 63: 564–571). This domain II is highly crosslinked by disulphide bonds and undergoes low pH transition which is related to exposing a strictly conserved and hydrophobic stretch of amino acids which are supposed to be involved in the fusion of the viral envelope to the endosome membrane.~~

Alterations at amino acids 299, 305, 331 and 380 are located in the B domain (domain III in the 3-D structure—see Rey, F. A. et al). This domain was suggested to be involved in viral attachment to a cellular receptor and consequently being a major determinant both of host range and cell tropism and of virulence/attenuation. The 4 amino acid changes reported for YF are located on the distal face of domain III. This area has a loop which is a tight turn in tick-borne encephalitis virus but contains 4 additional residues in all mosquito-borne strains. Because viruses replicate in their vectors, this loop is likely to be a host range determinant. This enlarged loop contains an Arginine-Glycine-Aspartic Acid (Arg-Gly-Asp) sequence in all 3 YF 17D vaccine strains. This sequence motif is known to mediate a number of cell interactions including receptor binding and is absent not only in the parental virulent Asibi strain but also in other 22 strains of YF wild type virus (Lepiniec, L.; Dalgarno, L.; Huong, V. T. Q.; Monath, T. P.; Digoutte, J. P. and Deubel, V. (1994). "Geographic distribution and evolution of yellow fever viruses based on direct sequencing of genomic DNA fragments". J. Gen. Virol. 75: 417–4123). Such a fact suggests that the mutation from Threonine (Thr) to Arginine (Arg), creating a Arg-Gly-Asp motif, is likely to be relevant for the attenuated phenotype of the YF 17D strain. Consistently, Lobigs et al (Lobigs, M.;

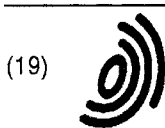
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Usha, R.; Nesterowicz, A.; Marschall, I. D.; Weir, R. C. and Dalgarno, L. (1990). "Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse neurovirulence". Virology. 176: 587–595) identified a Arg-Gly-Asp sequence motif (at amino acid 390) which led to the loss of virulence of Murray Valley encephalitis virus for mice.

Alterations at amino acids 170 and 173 in domain C (domain I of the E protein in the 3-D structure) map very close to the position that a neutralization epitope was identified for tick-borne encephalitis (TBE) virus (see Mandl, C. W. et al). A mutation at position 171 of TBE virus E protein was shown to affect the threshold of fusion-activating conformational change of this protein and the 2 changes observed for YF 17D virus may be related to same phenomenon. It is conceivable that a slower rate of fusion may delay the extent of virus production and thereby lead to a milder infection of the host. It is noteworthy that the recent development of infectious cDNA for Japanese encephalitis (JE) virus made by Sumiyoshi, H. et al (Sumiyoshi, H.; Hoke, C. H. and Trert, D. W. (1992). "Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates". J. Virol. 66: 5425–5431) allowed the identification of a mutation (Lys for Glu) at amino acid 136 of the E protein which resulted in the loss of neurovirulence for mice (see Sumiyoshi, H.; Tignor, G. H. and Shope, R. E. (1996). "Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA". J. Infect. Dis. 171: 1144–1151). ~~This means that domain I is an important area which contains a critical determinant of JE virus virulence in contrast to most of the data obtained from the analyses of virulence for several other flaviviruses for which it is suggested that domain III would be the primary site for virulence/attenuation determinants. Nevertheless, such analyses of the E protein provides a framework for understanding several aspects of flavivirus biology and suggests that it should be possible to engineer viruses for the development of new live flavivirus vaccine.~~

The issue of virulence/attenuation is of special interest for vaccine development but conceivably viral attenuation can result from genetic modification in one or more viral functions. YF virus is the ideal system to study flavivirus virulence and attenuation because: (i) there is a virulent strain (Asibi) from which an extremely well characterized vaccine strain was derived (17D) and has been successfully used for human vaccination for over 50 years; (ii) there is an animal system which reflects human infection; (iii) the complete nucleotide sequences from both virulent and attenuated strains have been determined and (iv) cDNA clones from which infectious RNA can be synthesized are available.

Holland, J. et al (Holland, J.; Spindler, K.; Horodyski, H.; Grabau, E.; Nichol, S. and VandePol, S. (1982). "Rapid evolution of RNA genomes". Science. 215: 1577–1585) described the fact that viral RNA genomes evolve rapidly. Therefore, a given viral population including YF vaccine viruses is likely to consist of a major type sequence population in which genetic variants can be detected. For YF 17D virus, this is easily seen when the virus is plaqued on cultured cells under an semi-solid overlay in which plaques of different sizes are observed. Previous genomic variability analysis using oligonucleotide fingerprinting suggested a high degree of genetic similarity between vaccines produced worldwide with an estimated sequence homology of 98–100%. However, genetic changes were detected and may have occurred within 1–2 passages possibly due to the



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Description**Field of the Invention**

[0001] The present invention relates, in general, to recombinant viable chimeric flaviviruses and to vaccines for dengue virus and other flaviviruses, including tick-borne encephalitis virus (TBEV). The invention further relates to cDNA sequences encoding the RNA transcripts to direct the production of recombinant dengue type 4 virus, recombinant mutant dengue type 4 virus chimeric dengue viruses, chimeric dengue viruses incorporating mutations to recombinant DNA fragments generated therefrom, and the cells transformed therewith.

Background Information

[0002] The family *Flaviviridae* includes approximately 60 enveloped, positive strand RNA viruses, most of which are transmitted by an insect vector. Many members of this family cause significant public health problems in different regions of the world (Monath, T.P. (1986) In: **The Togaviridae and Flaviviridae**. S. Schlesinger et al., eds. pp. 375-440. Plenum Press, New York). The genome of all flaviviruses sequenced thus far has the same gene order: 5'-C-preM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' in which the first three genes code for the structural proteins the capsid (C), the premembrane protein (pre M) and the envelope protein (E).

[0003] Dengue is a mosquito-borne viral disease which occurs in tropical and sub-tropical regions throughout the world. The dengue virus subgroup causes more human disease than any other member of the flavivirus family. Dengue is characterized by fever, rash, severe headache and joint pain. Its mortality rate is low. However, over the past few decades, a more severe form of dengue, characterized by hemorrhage and shock (dengue hemorrhagic fever/dengue shock syndrome; DHF/DSS) has been observed with increasing frequency in children and young adults. DHF/DSS occurs most often during dengue virus infection in individuals previously infected with another dengue virus serotype. This has led to the suggestion that immune enhancement of viral replication plays a role in the pathogenesis of the more severe form of disease (Halstead, S.B. (1988) *Science* 239, 476-481).

[0004] Dengue epidemics are a major public health problem in many tropical and subtropical areas where the vector mosquito species are abundant. Despite 40 years of intensive research, safe and effective vaccines for dengue virus disease are not available. The WHO has assigned dengue virus as a high priority target for accelerated research and vaccine development.

[0005] Soon after their isolation in 1944, dengue viruses were passaged repeatedly in mouse brain, resulting in the selection of mouse neurovirulent mutants (Sabin, A.B. (1952) *Amer. J. Trop. Med. Hyg.* 1:30-50). Interestingly, studies performed in volunteers showed that mouse brain-adapted neurovirulent mutants of three strains of type 1 or type 2 dengue virus were attenuated, but still immunogenic for humans (Sabin, A.B. (1952) *Amer. J. Trop. Med. Hyg.* 1:30-50; Sabin, A.B. (1955) *Amer. J. Trop. Med. Hyg.* 4:198-207; Sabin, A.B. (1955) *Amer. J. Trop. Med. Hyg.* 4:198-207; Schlesinger, R.W. et al. (1956) *J. Immunol.* 77:352-364; Wiseman, C.L. et al. (1963) *Amer. J. Trop. Med.* 12:620-623). However, the mutants were not developed further as candidate vaccine strains because of concern for mouse brain antigens in the vaccine preparations. Since that time, virus mutants that: (i) exhibited the small plaque size phenotype, and/or (ii) were temperature sensitive, and/or (iii) were adapted to cell cultures derived from an unnatural host (i.e., host range mutants), have been selected and evaluated as candidates for inclusion in a live attenuated virus vaccine (Harrison, V.R. et al. (1977) *Infect. Immun.* 18:151-156; Hoke, C.H. et al. (1990) *Am. J. Trop. Med. Hyg.* 43:219-226; Bhamarapravati, N. et al. (1987) *Bull. WHO.* 65:189-195). However, despite 25 years of such efforts, safe, effective dengue vaccines are still not available for general use. Inactivated whole dengue virus vaccines have been shown to be insufficiently immunogenic. Live virus vaccines attenuated by serial passage in cell culture have suffered from genetic instability under attenuation or poor immunogenicity. The present invention represents a technical breakthrough by providing chimeric dengue and flavivirus vaccines.

[0006] These four serotypes of dengue viruses (type 1 to type 4) are distinguishable by plaque reduction neutralization using serotype-specific monoclonal antibodies and by less specific tests using polyclonal sera (Bankcroft, W. M. et al. (1979) *Pan Am. Hlth. Org. Sci. Publ.* 375:175-178; Henchal, E.A. et al. (1982) *Am. J. Trop. Med. Hyg.* 31: 548-555). The existence of serotypes was first discovered during early studies in human volunteers, which showed that infection with one dengue serotype induced durable homotypic immunity, whereas heterotypic immunity lasted only 3 to 5 months (Sabin, A.B. (1952) *Amer. J. Trop. Med. Hyg.* 1:30-50). An effective dengue vaccine that contains all four serotypes in order to induce broad immunity to dengue viruses in general would help to preclude the occurrence of DHF/DSS.

[0007] The complete nucleotide sequences have been determined for dengue virus types 3 and 4 and several strains of type 2 virus including the mouse-neurovirulent New Guinea C, however, only the 5' portion of the type 1 virus genome has been sequenced (Mackow, E. et al. (1987) *Virology* 159:217-228; Zhao, B. et al. (1986) *Virology* 155:77-88; Osatomi, K. & Sumiyoshi, H. (1990) *Virology* 176:643-647; Irie, A. et al. (1989) *Gene* 75:197-211; Mason, P.W. et al.

(1987) *Virology* 161:262-267; Hahn, Y.S. et al. (1988) *Virology* 162:167-180). The results of these studies indicate that the four dengue virus serotypes share a common genome organization. The genome of the dengue type 4 Caribbean strain 814669 was found to contain 10646 nucleotides (Mackow, E. et al. (1987) *Virology* 159:217-228; Zhao, B. et al. (1986) *Virology* 155:77-88). The first 101 nucleotides at the 5' end and the last 384 at the 3' end are non-coding. The remaining sequence codes for a 3386 amino-acid polyprotein which includes the three structural proteins, namely, capsid (C), premembrane (pre-M), and envelope (E), at its N-terminus, followed by seven non-structural proteins in the order, provided above, that is consistent with all Flavivirus genomes identified thus far. The polyprotein is processed to generate 11 or more viral proteins by cell signal peptidase(s) and by viral proteases (Markoff, L. (1989) *J. Virol.* 63: 3345-3352; Falgout, B. et al. (1989) *J. Virol.* 63:1852-1860; Falgout, B. et al. (1991) *J. Virol.* 65:2467-2476; Hori, H. & Lai, C.J. (1990) *J. Virol.* 64:4573-4577).

[0008] Previously we constructed a full-length dengue virus cDNA that could serve as the template for transcription of infectious RNA. We have obtained stably cloned full-length dengue virus cDNA and in vitro RNA transcripts derived from the DNA template were shown to be infectious for cells in culture. However, this infectious construct and infectious RNA transcripts generated therefrom are pathogenic. Moreover, the attenuated dengue viruses generated thus far are genetically unstable and have the potential to revert back to a pathogenic form overtime. Yet, attenuated viruses are desirable since they are generally known to provided long-lasting immunity. Therefore, modifications to this construct or to chimeric constructs that then direct the production of a less pathogenic virus would be a considerable advance to attenuated flavivirus vaccine technology. Accordingly, we have constructed a series of deletions in the 3' non-coding region of cDNA, as disclosed herein, and have recovered viable dengue virus mutants for analysis of growth characteristics.

[0009] Other members of the Flavivirus family are also pathogenic. Examples include tick-borne encephalitis virus and Japanese Encephalitis Virus. Like attenuated dengue virus vaccines, attenuated tick-borne encephalitis virus (TBEV) virus has tended to be genetically unstable and poorly immunogenic. Therefore, other attenuated flavivirus vaccines would also be a considerable advance in the art. Thus, this invention additionally employs modified full-length recombinant cDNA constructs of dengue virus or another flavivirus as a framework for gene manipulation and chimeric virus development for the production of vaccines to other Flaviviruses. Tick-borne encephalitis virus (TBEV) is transmitted exclusively by ticks and can be divided into two serologically distinguishable subtypes: the Eastern subtype (prototype strain Sofjin), prevalent in Siberian and Far Eastern regions of Russia, and the Western subtype (prototype strain Neudorfl), common in eastern and central Europe. TBEV causes a serious encephalitic illness with a mortality rate ranging from 1 to 30%. For a review of TBEV see Calisher, et al. (*J. Gen. Virol.* 70:37-43). Currently, an experimental TBE vaccine produced by formalin inactivation of TBEV is available, but this vaccine has several limitations. For example, the vaccine is not sufficiently immunogenic, therefore repeated vaccinations are required to generate a protective immune response. Even when antibody responses to the vaccine are present, the vaccine fails to provide protective responses to the virus in 20% of the population. Therefore, there remains a need for an improved TBEV vaccine.

Summary of the Invention

[0010] In one aspect, the present invention provides a recombinant DNA construct containing nucleic acid derived from at least two flaviviruses. The construct includes a region of nucleic acid operably encoding no more than two structural proteins from tick-borne encephalitis virus (TBEV). This region is operably linked to a region of nucleic acid encoding structural proteins from a flavivirus other than TBEV. Preferably, the construct includes a region of nucleic acid operably encoding flavivirus capsid protein operably linked to the region of nucleic acid operably encoding no more than two structural proteins from tick-borne encephalitis virus. The regions of nucleic acid encoding capsid protein and non-structural proteins are preferably from dengue virus, such as dengue type 4 virus. In a preferred form, the construct contains at least one mutation in the nucleic acid derived from at least two flaviviruses. In one embodiment of this preferred form, the mutation ablates NS1(1) protein glycosylation. In another embodiment of this form, the mutation prevents production of the mature flavivirus membrane protein. The mutation preferably affects viral growth rate. The invention also includes RNA transcripts corresponding to these recombinant DNA constructs are also included within the present invention.

[0011] In another aspect of the present invention, there is provided a chimeric virus having a genome derived from a flavivirus. This chimeric virus include a region of nucleic acid encoding no more than two structural proteins from tick-borne encephalitis virus, and a region of nucleic acid encoding nonstructural proteins from another flavivirus. Preferably, the virus includes a region of nucleic acid encoding capsid protein from the other flavivirus. The nucleic acid from tick-borne encephalitis virus can encode any of a number of proteins, such as pre-membrane protein and/or the envelope protein. The nucleic acid encoding structural proteins from another flavivirus is preferably from dengue virus, such as type 4 dengue virus. In one embodiment, the chimeric virus of this aspect of the invention contains at least one mutation in the nucleic acid. This mutation can take any of a number of forms, such as a mutation that ablates NS1(1) protein glycosylation, or one that prevents production of the mature flavivirus membrane protein. The mutation

preferably affects viral growth rate.

[0012] Another aspect of the present invention provides a chimeric virus that includes region of nucleic acid encoding the premembrane and envelope proteins of tick-borne encephalitis virus, and a region of nucleic acid encoding the capsid protein from another flavivirus, and a region of nucleic acid encoding non-structural proteins from the same flavivirus. The flavivirus can be, for example, a dengue virus, such as dengue virus type 4. In this aspect of the invention,

a preferred form of the chimeric virus includes at least one mutation in the region of nucleic acid encoding premembrane and envelope protein of tick-borne encephalitis virus. The mutation can be one that prevents production of the mature flavivirus membrane protein. In another preferred form, the chimeric virus includes at least one mutation in the regions of nucleic acid from the flavivirus. This mutation can be one that ablates NS1(1) protein glycosylation.

[0013] In another aspect, the invention provides a vaccine for humans against tick-borne encephalitis comprising a chimeric virus of the present invention that is capable of generating a protective immune response in a vertebrate. Thus, the invention also includes a method for preparing a vaccine for humans against a flavivirus comprising. This method includes preparing a DNA construct operably encoding the premembrane and envelope protein from the flavivirus and the capsid and nonstructural protein from dengue virus, generating infectious RNA transcripts from the DNA construct, introducing the RNA transcripts into a cell, expressing the RNA transcripts in the cell, harvesting the virus from the cells, testing the virus in a vertebrate, and inoculating the humans with the virus. The preparing step can include introducing mutations into the DNA construct.

[0014] A preferred aspect of the present invention provides a chimeric virus that includes region of nucleic acid encoding the premembrane and envelope proteins of Japanese encephalitis virus, and a region of nucleic acid encoding the capsid protein from another flavivirus, and a region of nucleic acid encoding non-structural proteins from the same flavivirus, such as dengue virus.

[0015] Another aspect of the present invention provides a chimeric virus having an RNA genome. This genome includes a region of nucleic acid operatively encoding non-structural protein of type 4 dengue virus and a region of nucleic acid operatively encoding structural protein of a type 1 dengue virus, type 2 dengue virus, type 3 dengue virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, or another flavivirus. In one embodiment of the invention, the genome of the chimeric virus is substantially free from nucleic acid operatively encoding type 4 dengue virus structural protein. Preferred embodiments of the chimeric virus can include p2A(D1 WP) or p2A(D2 NGC) RNA. Preferably, the chimeric virus includes a region of nucleic acid operatively encoding non-structural protein of type 1 dengue virus, type 2 dengue virus, or type 3 dengue virus, and also includes a region of nucleic acid operatively encoding structural protein of type 1 dengue virus, type 2 dengue virus, type 3 dengue virus, type 4 dengue virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, or another flavivirus. The nucleic acid operatively encoding non-structural protein is preferably from a different virus than the region of nucleic acid operatively encoding non-structural protein.

[0016] Another aspect of the invention provides a chimeric virus having an RNA genome. This genome includes a region of nucleic acid operatively encoding non-structural protein of yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus or another flavivirus, and a region of nucleic acid operatively encoding structural protein of type 1 dengue virus, type 2 dengue virus, type 3 dengue virus, type 4 dengue virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, or another flavivirus. The region of nucleic acid operatively encoding structural protein is preferably from a different virus than the region of nucleic acid operatively encoding non-structural protein.

[0017] Yet another aspect of the invention provides a vaccine, capable of generating a protective immune response to a virus in a vertebrate. This vaccine includes a safe and immunologically effective amount of any of the viruses of the present invention and a pharmaceutically and immunologically acceptable carrier.

[0018] Still another aspect of the invention provides another vaccine that is capable of generating a protective immune response to a virus in a vertebrate. In this aspect of the invention, the vaccine includes a safe and immunologically effective amount of the following viruses: a) a chimeric virus wherein the genome of the virus includes a region of nucleic acid encoding non-structural protein of type 4 dengue virus and a region of nucleic acid encoding structural protein of type 1 dengue virus, b) a chimeric virus, wherein the genome of the virus includes a region of nucleic acid encoding non-structural protein of type 4 dengue virus and a region of nucleic acid encoding structural protein of type 2 dengue virus, c) a chimeric virus, wherein the genome of the virus includes a region of nucleic acid encoding non-structural protein of type 4 dengue virus and a region of nucleic acid encoding structural protein of type 3 dengue virus, and d) an attenuated type 4 dengue virus.

[0019] A further aspect of the present invention provides a DNA segment that includes a non-structural region of type 4 dengue virus, and a structural region from one of the following viruses: type 1 dengue virus, type 2 dengue virus, type 3 dengue virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, and another flavivirus. In a preferred embodiment of the invention, the DNA segment contains a promoter operably linked to the structural and nonstructural regions. In another preferred embodiment, the DNA segment contains a promoter which is a SP6 or T7 promoter. In yet another preferred embodiment, the DNA segment is p2A(D1 WP) or p2A(D2 NGC).

EP 0 872 553 B1

[0020] Other aspects of the invention provide other chimeric DNA segments. The DNA segments of these aspects of the invention include a non-structural region of type 1 dengue virus, type 2 dengue virus or type 3 dengue virus. These DNA segments further include a structural region from one of the following viruses: type 1 dengue virus, type 2 dengue virus, type 3 dengue virus type, 4 dengue virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, and a flavivirus, with the proviso that the structural region and the non-structural region are not from the same virus.

[0021] A further aspect of the invention provides a DNA segment that includes a non-structural region from one of the following viruses: yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, and another flavivirus, and a structural region from one of the following viruses: type 1 dengue virus, type 2 dengue virus, type 4 dengue virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, and another flavivirus wherein the structural region is from a different virus than the non-structural region.

[0022] Another aspect of the invention provides a DNA segment comprising a non-structural region or a portion thereof of a flavivirus, and a structural region or a portion thereof from another flavivirus.

[0023] Still another aspect of the invention provides a vaccine, capable of generating a protective immune response to a virus in a vertebrate. This vaccine includes a safe and immunologically effective amount of a chimeric virus. The genome of the virus includes nucleic acid operably encoding the structural proteins from a tick-borne encephalitis virus and nucleic acid operably encoding the non-structural protein from a dengue virus.

[0024] Yet another aspect of the invention provides another chimeric virus. In this aspect, the chimeric virus has a genome of the virus operably encodes tick-borne encephalitis structural protein and dengue type 4 virus non-structural protein. In a preferred embodiment, the structural proteins of the virus are derived from tick-borne encephalitis virus.

[0025] Still another aspect of the invention provides a segment of DNA operably encoding tick-borne encephalitis structural protein and dengue type 4 virus non-structural protein.

[0026] A further aspect of the invention provides an isolated DNA fragment that encodes infectious dengue type 4 viral RNA. Still another aspect of the invention provides a recombinant DNA construct comprising the DNA fragment of the present invention, and a vector. In a preferred embodiment, the vector is a plasmid. A further aspect of the invention includes a host cell stably transformed with a recombinant DNA construct according to the present invention in a manner allowing expression of the DNA fragment. In a preferred embodiment, the host cell is a prokaryotic cell.

[0027] Another aspect of the invention provides a method for producing mutants of dengue type 4 virus. This method includes the steps of (i) introducing mutations into the genome of dengue type 4 virus by site-directed mutagenesis, (ii) recovering infectious dengue type 4 viruses harboring the mutations, and (iii) evaluating recovered viruses. In one embodiment of this method, the recovered viruses are evaluated for attenuated or avirulent phenotype.

[0028] Still another aspect of the invention provides a vaccine for humans against dengue type 4 virus. This vaccine includes an avirulent dengue type 4 virus, in an amount sufficient to induce immunization against the disease, and a pharmaceutically acceptable carrier. In a preferred embodiment, the avirulent dengue type 4 virus is obtained by engineering mutations at strategic regions in the viral genome.

[0029] Yet another aspect of the present invention provides a DNA fragment that encodes chimeric flaviviral RNA. The chimeric RNA includes, for example, a member selected from the group consisting of dengue type 1, dengue type 2 and dengue type 3.

[0030] Still another aspect of the invention provides a method for construction of chimeric dengue viruses. This method includes replacing DNA fragments of the dengue type 4 virus DNA according to the method of the present invention with corresponding genes en bloc, or a fraction thereof, from a flavivirus. In a preferred embodiment, the flavivirus is one of the following viruses: dengue type 1, dengue type 2 and dengue type 3. In another preferred embodiment, the dengue type 4 virus contains at least one mutation.

[0031] The present invention includes another aspect which provides a vaccine for humans against dengue virus. In this aspect, the invention includes an infectious chimeric virus, in an amount sufficient to induce immunization against the disease, and a pharmaceutically acceptable carrier. The chimeric virus is, for example, dengue type 1, dengue type 2, dengue type 3 or dengue type 4 virus.

[0032] Yet another aspect of the invention provides a recombinant DNA construct comprising the DNA fragment of the present invention, and a vector. In one preferred embodiment, the vector is a plasmid. Still another aspect of the present invention provides a host cell stably transformed with the recombinant DNA construct according to the method of the present invention, in a manner allowing expression of the DNA. In a preferred embodiment, the host cell is a prokaryotic cell.

[0033] Still another aspect of the invention provides a DNA fragment that encodes a dengue type 4 viral RNA, wherein the DNA fragment contains a substitution mutation in the sequence encoding one or more of eight amino acids at the C terminus of NS1 of the cleavage site of the non-structural protein NS1-NS2A.

[0034] A further aspect of the invention provides a DNA fragment that encodes a dengue type 4 viral RNA, wherein the DNA fragment contains a substitution at the site encoding glycine, which site is at position + 1 following the cleavage site of the non-structural protein NS1-NS2A.

[0035] A still further aspect of the invention provides a DNA fragment that encodes a dengue type 4 viral RNA, wherein the DNA fragment contains a deletion in the 3'-noncoding region. In a preferred embodiment, the deletion is between 30 and 202 nucleotides in length.

[0036] Another aspect of the invention provides a recombinant DNA construct including any of the DNA fragments of the present invention and a vector. Still another aspect of the invention provides an infectious RNA transcript of a DNA fragment of the present invention. A further aspect of the invention provides a host cell transfected with the DNA constructs of the present invention in a manner allowing expression of the DNA fragment. Such a host cell can be a mammalian or an insect cell.

[0037] Still another aspect of the invention provides a vaccine for humans against dengue type 4 virus. In this aspect of the invention, the vaccine includes a mutant dengue type 4 virus exhibiting reduced virulence, in an amount sufficient to induce immunization against the disease. In a preferred embodiment, the mutant dengue type 4 virus is obtained by engineering deletion mutation in the 3'-noncoding region in the viral genome. In another preferred embodiment, the mutant dengue type 4 virus is obtained by engineering substitution mutation in the viral DNA sequence encoding one or more of eight amino acids at the C terminus of NS1 of the cleavage site of the non-structural protein NS1-NS2A. In yet another preferred embodiment, the mutant dengue type 4 virus is obtained by engineering substitution at the viral DNA site encoding glycine, which site is at position + 1 following the cleavage site of the non-structural protein NS1-NS2A.

[0038] Still another aspect of the invention provides a method for construction of chimeric dengue viruses comprising replacing DNA fragments of the dengue type 4 virus DNA with corresponding genes en bloc, or a fraction thereof, from a flavivirus. In a preferred embodiment, the flavivirus is one of dengue type 1, dengue type 2, dengue type 3, Japanese encephalitis and tick-borne encephalitis virus. In another preferred embodiment, the method for construction of chimeric dengue viruses includes replacing DNA fragments of the dengue type 4 virus DNA according to the present invention, with corresponding genes en bloc, or a fraction thereof, from a flavivirus. Preferably, the flavivirus is selected from the group consisting of dengue type 1, dengue type 2, dengue type 3 and Japanese encephalitis. Also preferably, the method for construction of chimeric dengue viruses includes replacing DNA fragments of the dengue type 4 virus DNA according to the present invention, with corresponding genes en bloc, or a fraction thereof, from a flavivirus. In the preferred method, the flavivirus is selected from the group consisting of dengue type 1, dengue type 2, dengue type 3, Japanese encephalitis and tick-borne encephalitis virus.

[0039] One additional aspect of the invention provides a vaccine against dengue virus that is administered in an amount sufficient to induce immunization against the disease. The vaccine includes a chimeric virus exhibiting reduced virulence, wherein the chimeric virus contains a DNA fragment that encodes a dengue type 4 viral RNA containing a deletion in the 3'-non-encoding region. In a preferred embodiment, the chimeric virus is selected from the group consisting of dengue type 2, dengue type 3 and dengue type 4 virus.

[0040] Further aspects of the present invention will become apparent to those of ordinary skill in the art upon reference to the ensuing description of the invention.

Description of the Figures

[0041] Figure 1. Structure of full-length dengue virus cDNAs used for preparation of chimeric dengue viruses.

[0042] Figure 2. Percentage of virus infected cells and titer of virus following transfection with RNA transcripts.

[0043] Figure 3. Serotype analysis of chimeric dengue viruses.

[0044] Figure 4. Polyacrylamide gel analysis of dengue type 1, type 2, or type 4 viral proteins produced by parental or chimeric dengue viruses.

[0045] Figure 5. Morphology of viral plaques on LLC-MK₂ cells.

[0046] Figure 6. Mouse neurovirulence of parental dengue type 2 NGC or dengue type 4 virus or type 2/type 4 chimeric virus.

[0047] Figure 7 provides the terminal sequences of cloned full-length dengue virus cDNA used for transcription of infectious RNA

[0048] The full-length dengue virus cDNA totaling 10646 nucleotides in length was cloned adjacent to the promoter sequence for SP6 polymerase and the unique Asp 718 cleavage site as diagrammed. Note that there is an additional C residue at the position between 10448 and 10449 and an additional G residue between 10470 and 10471, which were missing in the original dengue type 4 virus sequence. The sequences near the predicted 5'-terminus and 3'-terminus of the RNA transcript are also shown. Transcription initiating G was placed preceding the 3'-dengue sequence as shown in bold letters. The Asp 718 cleavage sequence GGTACC was positioned immediately following the 3'-dengue sequence as shown in bold letters.

[0049] Figure 8 diagrams the full-length dengue cDNA clones and the restriction endonuclease digestion pattern.

[0050] The diagram depicts the full-length dengue cDNA clone containing the SP6 promoter sequence at the 5'-end and an Asp 718 cleavage site at the 3'-end. The Bst B1 cleavage site at nucleotide 5069 separates the dengue genome