

Señora

Alix Carmenza Céspedes de Vergel

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 03-097535- -00009-0000

Fecha: 2007-12-19 16:04:35 Dep. 2020 NUECREACIONE
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI
Act 444 RESPUESTAREQUE Folios: 15

Referencia: Expediente No. 03.097.535

Solicitud de Patente de Invención titulada: "Vacunas de ácido nucléico para
prevención de la infección de flavivirus"Solicitante: The Government of the United States of America as represented by the
Secretary of the Department of Health and Human Services, Centers for
disease control and prevention

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 348
DEL 21 DE SEPTIEMBRE DE 2007, OFICIO NO. 11661 (EXAMEN DE PATENTABILIDAD)

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término legal:

1. Para dar respuesta la primera observación del Examinador relacionada con el sustento experimental para demostrar un efecto técnico para todas las unidades transcripción que resultan de combinar todas las secuencias señal de un 1er flavivirus con todos los posibles antígenos derivados de flavivirus, indico que en las páginas 126 a 173 de la descripción se revelan amplios ensayos y los resultados de los mismos para las vacunas de la presente invención.

2. En respuesta a las objeciones relacionadas con las reivindicaciones, el solicitante pone a consideración del Examinador un nuevo capítulo reivindicatorio el cual se encuentra

totalmente sustentado en la descripción originalmente presentada. Por ejemplo, para los antígenos del flavivirus quiméricos, antígenos quiméricos que comprenden una secuencia de amino ácidos de más de un flavivirus, y antígenos quiméricos que comprenden una secuencia de aminoácidos desde un primer flavivirus y un segundo flavivirus (reivindicación 1 corregida y nuevas reivindicaciones 45 a 47) se encuentra sustento en las páginas 23 (líneas 16-26), 24 (líneas 1-9), 31 (líneas 8-15), 32 (líneas 7-18), 55 (líneas 25-25), 56 (líneas 11-14), 57 (líneas 24-26), 58 (líneas 8-15) y el ejemplo 20 de la especificación desde la página .

Mención
no sustento !!

3. De igual manera, para las proteínas quiméricas E (nueva reivindicación 48) y proteínas quiméricas que comprenden una porción de un carboxi-terminal de un virus japonés de encefalitis (nuevas reivindicaciones 50-54), se encuentra sustento en la página 32 (líneas 20-26), 33 (líneas 1-4), 57 (líneas 15-16) y 58 (líneas 9-10). Para las unidades transcripcionales que comprenden una secuencia de señal de un virus de encefalitis japonés y un antígeno quimérico que comprende una secuencia de aminoácidos de un virus de encefalitis japonés y un segundo flavivirus (reivindicación 49) se encuentra sustento en la página 32 (líneas 7-14) y 59 (líneas 1-3). Las secuencias de señal modificadas (nueva reivindicación 44) tienen sustento en la página 59 (líneas 3-16).

Mención no sustento

4. En respuesta a la objeción relacionada con la amplitud de las reivindicaciones, el solicitante considera que cada una de las secuencias de aminoácidos se encuentran definidas en el apartado de listado de secuencias, y en esa medida no es necesario incluir apartes de las secuencias en las reivindicaciones. Así mismo, el solicitante desea modificar las reivindicaciones 3 a 19 para incluir el virus de encefalitis St. Louis.

no

5. El solicitante no comparte la apreciación del Examinador en cuanto a la falta de unidad de invención de las reivindicaciones, por cuanto la invención gira alrededor del único concepto inventivo que es el ácido nucleico aislado de la reivindicación 1.

6. En cuanto a la reivindicación 38, insisto en esta reivindicación por cuanto corresponde al antígeno que se produce por el ácido nucleico de la reivindicación 1 el cual contienen manipulación humana para su protección. Este antígeno es la sustancia que finalmente producirá el anticuerpo para combatir los virus mencionados en la invención. Sustento de la reivindicación 38 se encuentra en las páginas 34 y 35 de la especificación.

7. Finalmente, en cuanto a las reivindicaciones 18 a 27, 31, 33, 35 que reclaman métodos de tratamiento que pueden ser aplicados a seres humanos y las reivindicaciones 39, 41 a 43 reclaman un método de diagnóstico de la presencia de flavivirus en una muestra, que puede llevar a determinar la condición de infección en un paciente; el solicitante dejarlas de la manera en que se encuentran redactadas actualmente, y si es del caso, solicito que ese Despacho otorgue el privilegio de patente a las reivindicaciones que según su criterio sean patentables bajo la legislación colombiana.

En virtud de lo anterior, solicito que se acepten las razones expuestas en el presente documento y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,

ÁLVARO CORREA ORDÓÑEZ

C.C.No.79.143.366 de Usaquén

T.P.20.281

Anexos:

Capítulo reivindicatorio modificado
Formulario de modificación
Recibo

BOGATM/BOGDMS-98650.DOC

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Industria y Comercio
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES

1		SOLICITUD DE	
☐ Corrección de Error Material		☒ Modificación de una solicitud	
2 SOLICITANTE	Nombre:	The Government of the United States of America as represented by the Secretary of the Department of Health and Human Services, Centers for disease control and prevention	
	Dirección:	Technology Transfer Office, 1600 Clifton Road, N.E., MS E-67, Atlanta, Georgia 30333, Estados Unidos de América	
	Nacionalidad o Domicilio:	Estadounidense	
	Lugar de Constitución:	Georgia, Estados Unidos de América	
	Teléfono:	Fax:	
	E-Mail		
	IDENTIFICACION		
	C.C.	☐	NIT ☐
	C.E.	☐	Otro ☐
	Numero		Cual
3 REPRESENTANTE O APODERADO	Nombre:	ALVARO CORREA ORDOÑEZ	
	Dirección:	Avenida 82 No.10-62 Piso 60.	
	Teléfono:	634 1500	Fax: 376 2211
	E-Mail:	patents.bogota@bakernet.com	
	IDENTIFICACION		
	C.C.	☒	NIT ☐
	C.E.	☐	Otro ☐
	Numero 79.143.366		TP 20.281
4 Numero de expediente			
03.097.535			
5 Descripción de la corrección o modificación solicitada			
Se presenta modificación voluntaria al capítulo reivindicatorio según el memorial adjunto.			
6 Comprobante de Pago No. Fecha			
7 Anexos			
☒ Comprobante de pago de la tasa.			
☐ Poderes, si fuere el caso.			
☐ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.			
8			
NOMBRE ALVARO CORREA ORDOÑEZ		FIRMA	
		C.C. 79.143.366	T.P. 20.281

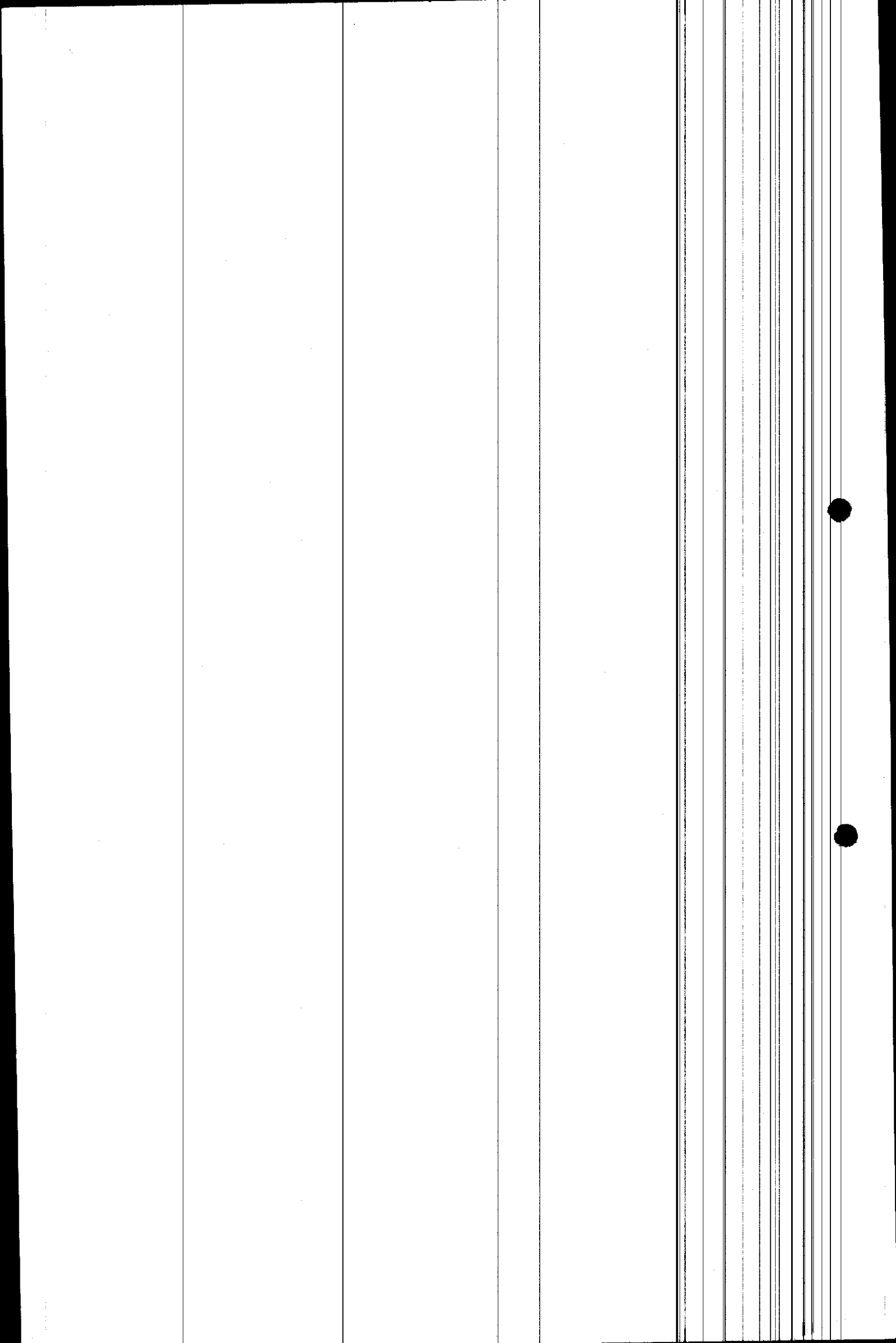
Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

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VACUNAS DE ÁCIDO NUCLÉICO PARA PREVENCIÓN DE LA
INFECCIÓN DE FLAVIVIRUS

Lo que se reivindica es:

1. Un ácido nucléico aislado que comprende una unidad transcripcional que codifica una secuencia de señal de una proteína estructural de un primer flavivirus y un antígeno de flavivirus inmunogénico, en donde el antígeno es de un segundo flavivirus, o el antígeno es un antígeno quimérico que comprende una secuencia de aminoácidos de más de un flavavirus, en donde la unidad transcripcional dirige las síntesis del antígeno.
2. El ácido nucléico de la reivindicación 1, en donde la secuencia de señal es una secuencia de señal de virus de encefalitis japonesa.
3. El ácido nucléico de la reivindicación 1, en donde el antígeno del flavivirus inmunogénico es de un flavivirus seleccionado entre el grupo que consiste de virus de fiebre amarilla, virus dengue serotipo 1, virus dengue serotipo 2, virus dengue serotipo 3, virus dengue serotipo 4, virus de encefalitis japonesa, virus Powassan, virus de encefalitis St. Louis y virus West Nile.
4. El ácido nucléico de la reivindicación 1, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus West



Nile.

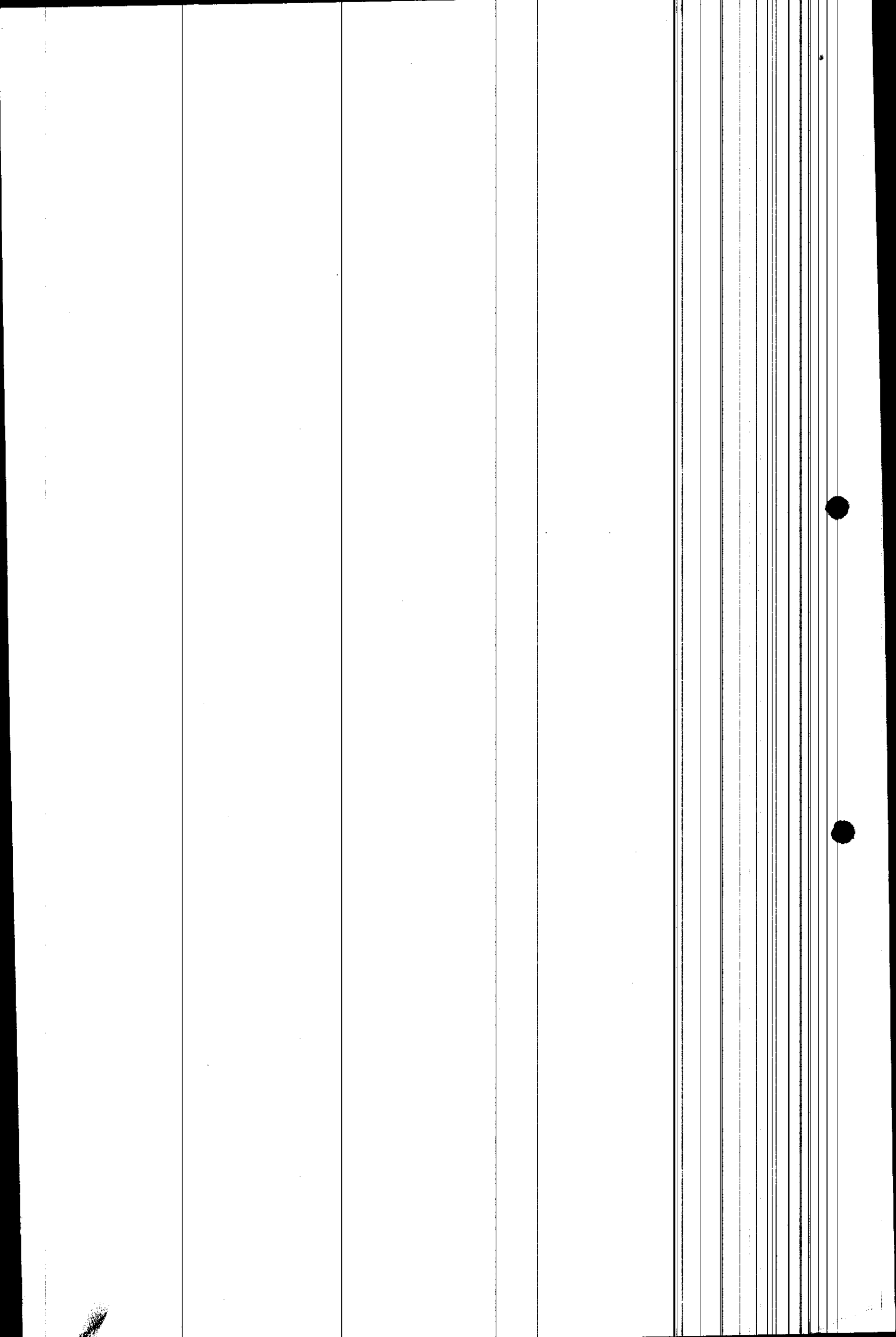
5. El ácido nucléico de la reivindicación 1, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus de fiebre amarilla.

6. El ácido nucléico de la reivindicación 1, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus de encefalitis St. Louis.

7. El ácido nucléico de la reivindicación 1, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus Powassan.

8. El ácido nucléico de la reivindicación 1, en donde el antígeno se selecciona entre el grupo que consiste de una proteína M, una proteína E, tanto una proteína M como una Proteína E, una parte de una proteína M, una parte de una proteína E y tanto una porción de una proteína M como una porción de una proteína E o una combinación de las mismas.

9. El ácido nucléico de la reivindicación 8, en donde el antígeno es tanto la proteína M como la proteína E.



10. El ácido nucléico de la reivindicación 1, en donde el ácido nucléico es DNA.
11. El ácido nucléico de la reivindicación 10, que comprende una secuencia nucleótida seleccionada entre el grupo que consiste de SEQ ID NO:15, SEQ ID NO:19, SEQ ID NO:21 y SEQ ID NO:23.
12. El ácido nucléico de la reivindicación 1, en donde la unidad transcripcional comprende una secuencia de control dispuesta apropiadamente de forma tal que controla en forma operable la síntesis del antígeno.
13. El ácido nucléico de la reivindicación 12, en donde la secuencia de control es el promotor precoz inmediato del citomegalovirus.
14. El ácido nucléico de la reivindicación 1, que comprende una secuencia de consenso Kozak localizada en el sitio de inicio translacional para un polipéptido que comprende el antígeno codificado por la unidad transcripcional.
15. El ácido nucléico de la reivindicación 1, en donde la unidad transcripcional comprende un terminador poli-A.
16. Una célula que comprende el ácido nucléico de la reivindicación 1.

17. Una composición que comprende el ácido nucleico de la reivindicación 1 y un portador aceptable desde el punto de vista farmacéutico.

18. Un método de inmunizar a un sujeto contra infección por flavivirus, que comprende administrarle una cantidad eficaz de la composición de la reivindicación 17.

19. El método de la reivindicación 18, en donde el antígeno del flavivirus es de un flavivirus o flaviviruses seleccionado entre el grupo que consiste del virus de fiebre amarilla, virus dengue serotipo 1, virus dengue serotipo 2, virus dengue serotipo 3, virus dengue serotipo 4, virus de encefalitis japonesa, virus Powassan, virus de encefalitis St. Louis y virus West Nile, o una combinación de los mismos.

20. El método de la reivindicación 18, en donde el antígeno se selecciona entre el grupo que consiste de una proteína M, una proteína E, tanto una proteína N como una proteína E, una porción de una proteína M, una porción de una proteína E y tanto una porción de una proteína M como una porción de una proteína E, o cualquier combinación de las mismas.

21. El método de la reivindicación 20, en donde el antígeno es tanto la proteína M como la proteína E de un flavivirus, y donde una célula dentro del cuerpo del sujeto, después de incorporar el ácido nucleico dentro de ella, secreta partículas subvirales que comprenden la

proteína M y la proteína E.

22. El método de la reivindicación 18, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus West Nile.

23. El método de la reivindicación 18, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus de fiebre amarilla.

24. El método de la reivindicación 18, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína N y una proteína E del virus de encefalitis St. Louis,

25. El método de la reivindicación 18, en donde la unidad transcripcional codifica una secuencia de señal del virus de encefalitis japonesa y una proteína M y una proteína E del virus Powassan.

26. El método de la reivindicación 18, que comprende administrar la composición al sujeto en una sola dosis. *ref. #6*

27. El método de la reivindicación 18, en donde la composición se

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administra vía parenteral. *met. to.*

28. El ácido nucléico de la reivindicación 1, en donde el antígeno comprende un antígeno de virus de encefalitis St. Louis.

29. El ácido nucléico de la reivindicación 18, en donde el antígeno comprende un antígeno del virus de encefalitis St. Louis.

30. El ácido nucléico de la reivindicación 1, en donde el antígeno comprende un antígeno de virus de encefalitis japonesa.

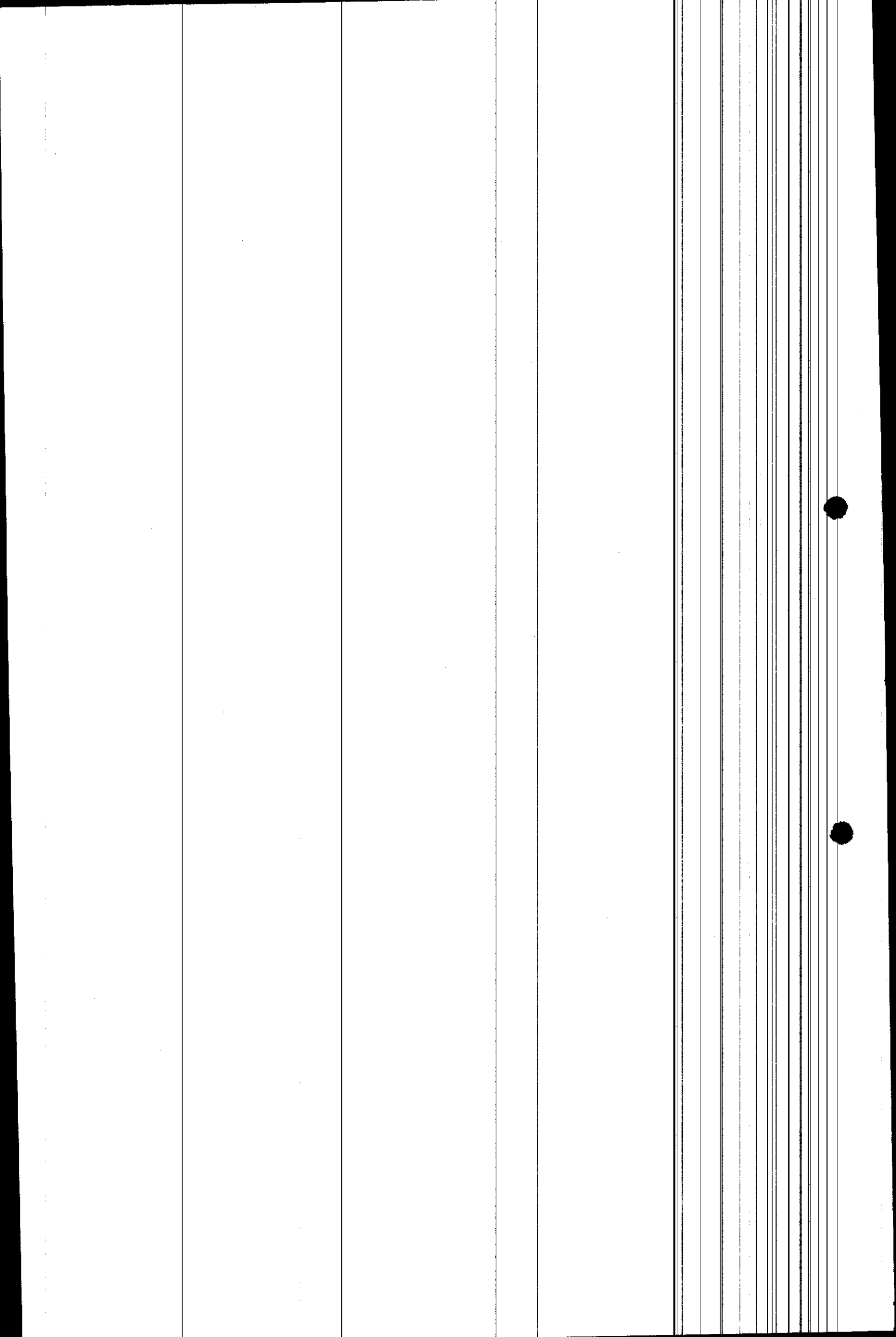
31. El método de la reivindicación 18, en donde el antígeno comprende un antígeno del virus de encefalitis japonesa.

32. El ácido nucléico de la reivindicación 1, en donde el antígeno comprende un antígeno de virus de fiebre amarilla.

33. El método de la reivindicación 18, en donde el antígeno comprende un antígeno de virus de fiebre amarilla.

34. El ácido nucléico de la reivindicación 1, en donde el antígeno comprende un antígeno de virus dengue.

35. El método de la reivindicación 18, en donde el antígeno comprende un antígeno del virus dengue.



36. El ácido nucléico de la reivindicación 1, en donde el antígeno comprende un antígeno de virus West Nile.

37. El método de la reivindicación 18, en donde el antígeno comprende un antígeno de virus West Nile.

38. El antígeno producido a partir del ácido nucléico de la reivindicación 1.

39. Un método de detectar un anticuerpo de flavivirus en una muestra, que comprende:

(a) poner en contacto la muestra con el antígeno de la reivindicación 38 bajo condiciones por medio de las cuales se pueda formar un antígeno/anticuerpo complejo; y

(b) detectar la formación del antígeno/anticuerpo complejo, detectando así un anticuerpo de flavivirus en la muestra.

40. Un anticuerpo producido en respuesta a la inmunización con el antígeno de la reivindicación 38.

41. Un método de detectar un antígeno de flavivirus en una muestra, que comprende:

(a) poner en contacto la muestra con el anticuerpo de la reivindicación 40 bajo condiciones por medio de las cuales se pueda formar un antígeno/anticuerpo complejo; y

(b) detectar la formación del antígeno/anticuerpo complejo,

detectando por ello un antígeno de flavivirus en una muestra.

42. Un método de diagnosticar una infección de flavivirus en un sujeto, que comprende:

(a) poner en contacto una muestra del sujeto con el antígeno de la reivindicación 38 bajo condiciones por medio de las cuales se pueda formar un antígeno/anticuerpo; y

(b) detectar la formación del antígeno/anticuerpo complejo, diagnosticando así una infección de flavivirus en un sujeto.

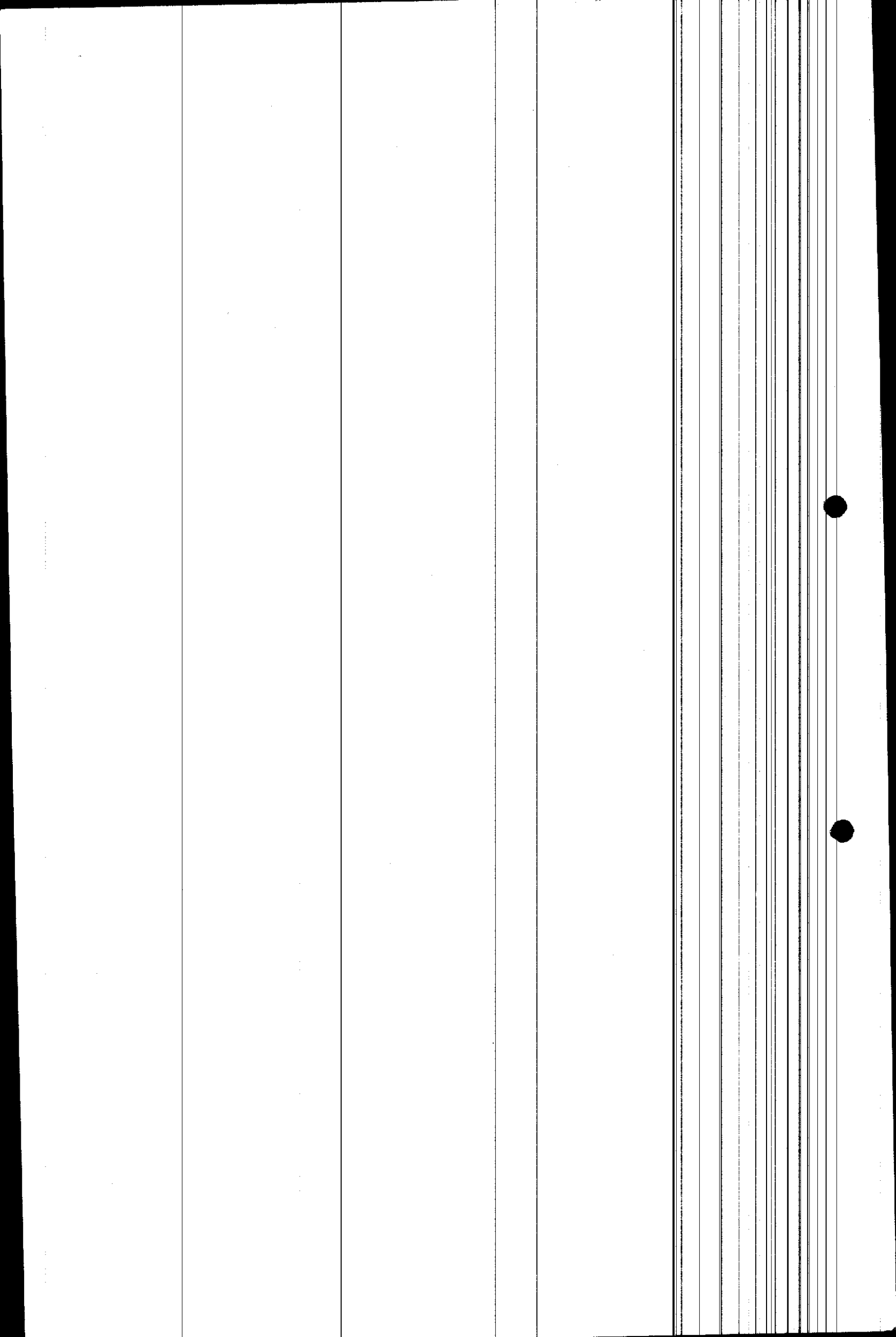
43. Un método de diagnosticar una infección de flavivirus en un sujeto, que comprende:

(a) poner en contacto una muestra del sujeto con el anticuerpo de la reivindicación 40 bajo condiciones por medio de las cuales se pueda formar un antígeno/anticuerpo complejo; y

(b) detectar la formación del antígeno/anticuerpo complejo, diagnosticando así una infección de flavivirus en un sujeto.

44. El ácido nucleico de la reivindicación 2, en donde la secuencia de señal es una secuencia de señal modificada de un virus de encefalitis japonés.

45. El ácido nucleico de la reivindicación 1, en donde el antígeno de



flavivirus inmunogénico es un antígeno quimérico que comprende una secuencia de amino ácidos a partir de mas de un flavvivirus, en donde los flaviviruses se seleccionan entre virus de fiebre amarilla, virus dengue serotipo 1, virus dengue serotipo 2, virus dengue serotipo 3, virus dengue serotipo 4, virus de encefalitis japonesa, virus de encefalitis St. Louis, virus Powassan y virus West Nile.

46. El ácido nucleico de la reivindicación 1, en donde el antígeno de flavivirus inmunogénico es un antígeno quimérico que comprende una secuencia de amino ácidos a partir de el primer flavivirus y un segundo flavivirus, en donde los flaviviruses se seleccionan entre virus de fiebre amarilla, virus dengue serotipo 1, virus dengue serotipo 2, virus dengue serotipo 3, virus dengue serotipo 4, virus de encefalitis japonesa, virus de encefalitis St. Louis, virus Powassan y virus West Nile.

47. El ácido nucleico de la reivindicación 46, en donde el primer flavivirus es virus de encefalitis japonesa.

48. El ácido nucleico de la reivindicación 46, en donde el antígeno de flavivirus inmunogénico comprende una proteína E quimérica.

49. El ácido nucleico de la reivindicación 46, en donde el antígeno de flavivirus inmunogénico comprende una proteína M a partir del segundo flavivirus y una proteína E quimérica que comprende una

secuencia de amino ácido a partir del virus de encefalitis japonesa y el segundo flavivirus.

50. El ácido nucleico de la reivindicación 49, en donde la proteína E quimérica comprende una porción carboxi-terminal de virus de encefalitis japonesa, en donde la porción carboxi-terminal es alrededor de 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50% o 75% de la proteína E quimérica.

51. El ácido nucleico de la reivindicación 50, en donde la porción carboxi-terminal es alrededor de 10% de la proteína E quimérica.

52. El ácido nucleico de la reivindicación 51, en donde la porción carboxi-terminal es alrededor de 20% de la proteína E quimérica.

53. El ácido nucleico de la reivindicación 49, en donde el segundo flavivirus es un virus dengue.

54. El ácido nucleico de la reivindicación 49, en donde el segundo flavivirus es un virus de encefalitis St. Louis.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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RECIBO OFICIAL DE CAJA : 07 - 82,080
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Fecha 2007-10-05 10:14:00 Det. 2020 NUECREACIONE
DE 11 PATENTILYII LEX 379 FASENACIONALII
ART 444 RESPUESTAQUE Folios: 15

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
RAISBECK LARA RODRIGUEZ	CONSIGNACION	BANCO POPULAR	050-00110-6	69421920	05/10/2007	51,962,080.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
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		TOTAL :	\$ 99,000.00

SON: NOVENTA Y NUEVE MIL PESOS

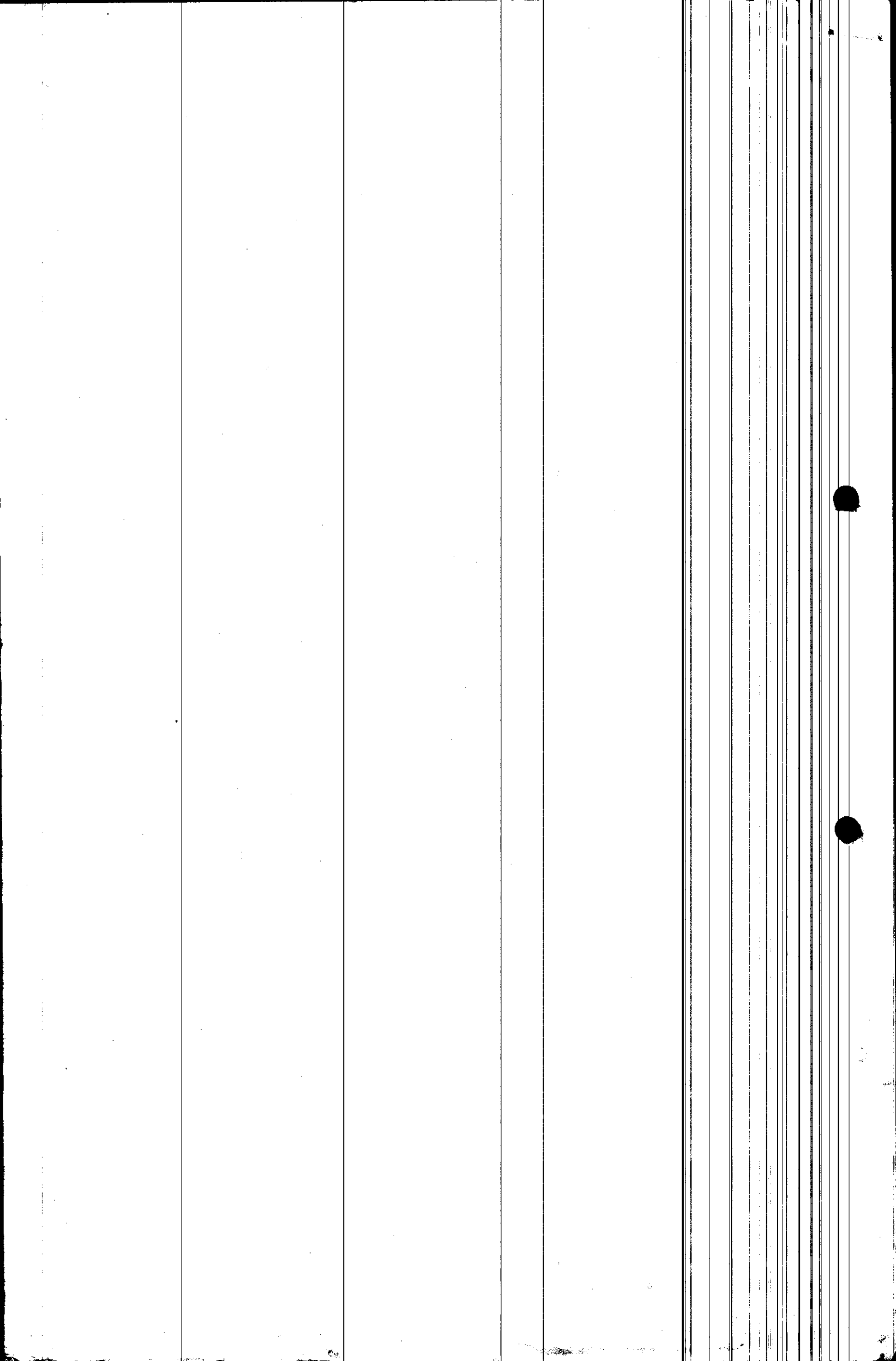
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RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

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A Single Intramuscular Injection of Recombinant Plasmid DNA Induces Protective Immunity and Prevents Japanese Encephalitis in Mice

GWONG-JEN J. CHANG,* ANN R. HUNT, AND BRENT DAVIS

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service,
U.S. Department of Health and Human Services, Fort Collins, Colorado 80522

Received 10 November 1999/Accepted 1 February 2000

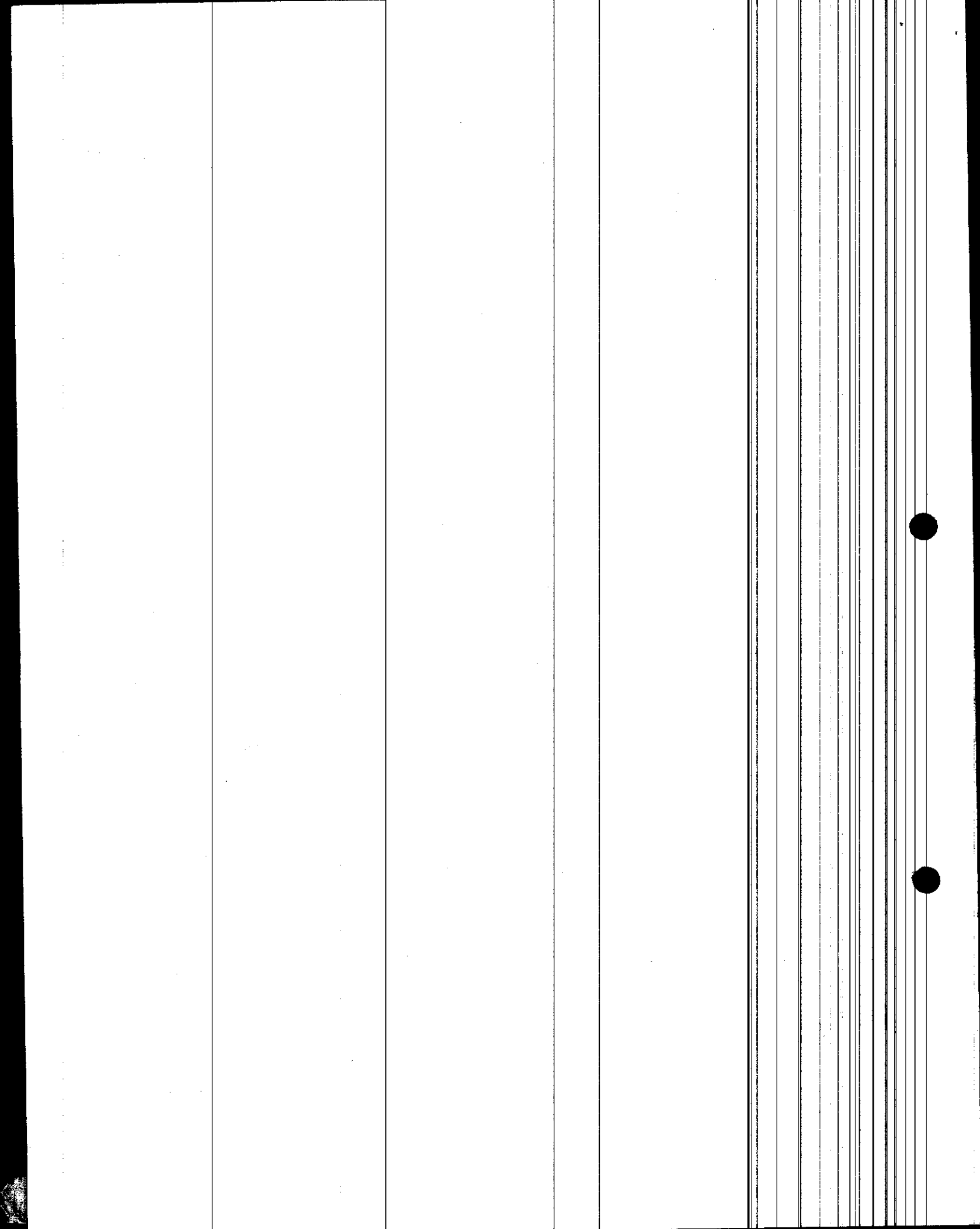
Plasmid vectors containing Japanese encephalitis virus (JEV) premembrane (prM) and envelope (E) genes were constructed that expressed prM and E proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50,000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs *in vitro* is an effective vaccine.

Japanese encephalitis (JE) is a mosquito-borne viral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually (52). *Japanese encephalitis virus* (JEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. More than 70 species in the *Flavivirus* genus have been genetically and serologically classified (29). Other important human pathogenic flaviviruses include yellow fever, dengue type 1 to 4 (DEN1 to DEN4), tick-borne encephalitis (TBE), and St. Louis encephalitis (SLE) viruses. Vaccination has been an effective mechanism for prevention of flavivirus infection in humans and domestic animals. Three JEV vaccines are in widespread production and use (52). These are inactivated virus from infected mouse brain, inactivated virus from primary hamster kidney cells, and a live attenuated SA14-14-2 vaccine. Only inactivated JEV vaccine, JEVAX, produced in mouse brain is distributed commercially and available internationally (52). Inactivated, mouse brain-derived whole virus vaccine is costly to prepare and carries the risk of allergic reaction to murine encephalitogenic basic proteins or gelatin stabilizer (45; M. M. Andersen, and T. Ronne, Letter, Lancet 337:1044, 1991). Since 1989, an unusual number of systemic reactions characterized by generalized urticaria and/or angioedema following JEVAX immunization have been reported from Australia, Canada, and Denmark (36). A major problem associated with use of the inactivated mouse brain vaccine is the failure to stimulate long-term immunity (39). Multiple immunization is recommended to provide adequate protection (28, 39). The attenuated JEV vaccine, SA14-14-2, is undergoing clinical trials (31). However, because of regulatory issues this vaccine has not found wide acceptance outside the People's Republic of China (11).

Several experimental recombinant virus, attenuated virus, and subunit JEV vaccines have been reported. Recombinant baculovirus vector that contained the JEV envelope (E) protein gene has been used to infect insect cells and produce E protein that has been studied as a biosynthetic immunogen (33). Recombinant vaccinia viruses expressing the JEV genes extending from premembrane (prM) to NS2B proteins have been the most promising candidate vaccines. These candidate vaccines produced extracellular virus-like particles (EPs) in infected cell culture that induced high titers of neutralizing and hemagglutination-inhibiting antibodies and protective immunity in mice (19-21, 47, 54). Recombinant vaccinia viruses expressing the same JEV genes based on the attenuated vaccinia virus strain, NYVAC-JEV, or canarypox, ALVAC-JEV, were tested in phase I human trials (18). In this trial, only 1 in 10 ALVAC-JEV recipients developed detectable viral neutralizing antibody, and vaccinia virus-preimmune recipients had a significantly lower humoral immune response.

Inoculation of animals with purified plasmid vectors (DNA) by the intramuscular (i.m.) or intradermal route leads to expression of the recombinant vector-encoded protein in transfected cells, resulting in stimulation of a protein-specific immune response. Plasmid DNA vaccines provide an alternative to attenuated, inactivated, or virus-vectored subunit vaccines. Flavivirus DNA vaccines for Murray Valley encephalitis, DEN2, JE, SLE, and TBE (Central European encephalitis and Russian spring summer encephalitis) viruses have been developed and tested in the mouse model (4, 17, 24, 30, 38, 49). All of these plasmid DNA constructs contained similar transcriptional regulatory elements and a flavivirus gene cassette. Vaccination of mice with these plasmid DNA vaccines induced a virus-specific antibody response, as detected by enzyme-linked immunosorbent assay (ELISA). However, production of neutralizing antibody leading to 100% protection of vaccinated animals from virus challenge was observed only after multiple immunizations or delivery of DNA to the epidermis by particle

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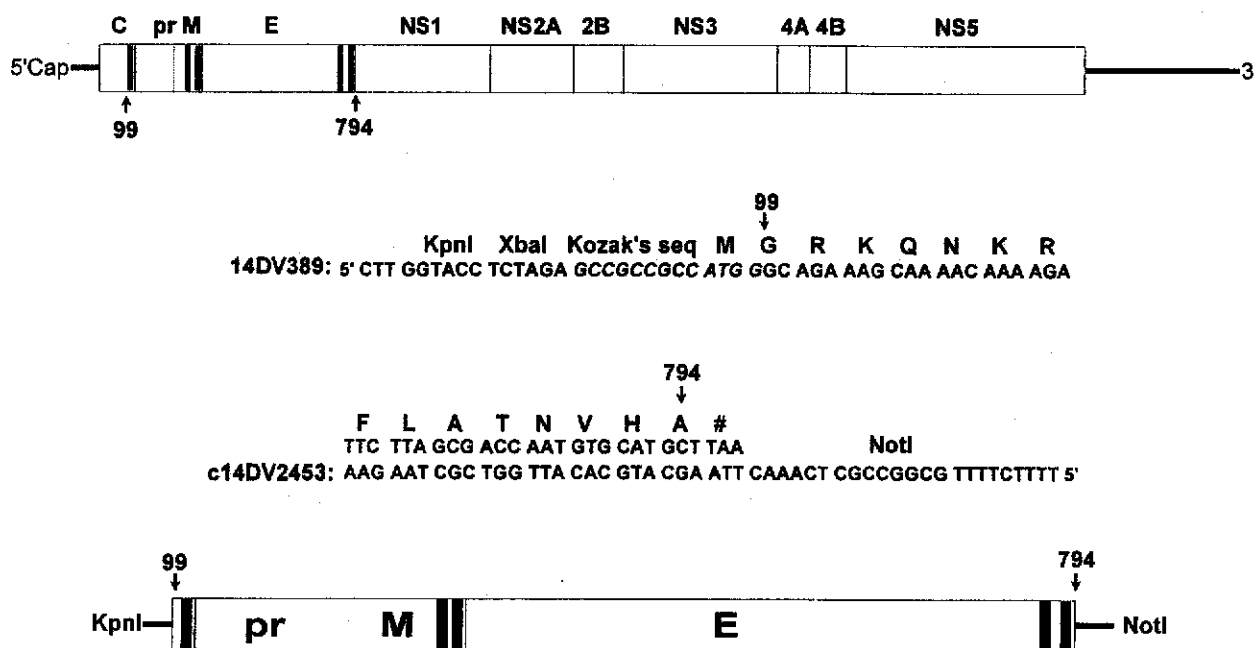


FIG. 1. Map of the JEV genomic structure (top) and the DNA sequence of oligonucleotides used in RT-PCR to construct the transcription unit for the expression of prM-E protein coding regions (bottom). Potential transmembrane helices of viral polyprotein are indicated by blackened areas.

bombardment (4, 24, 49). In this study, we constructed a JEV prM and E gene cassette that incorporates an extended signal peptide sequence at the NH₂ terminus of the prM gene and Kozak's sequence, an optimal translation enhancing element surrounding the AUG site. JEV protein expression was characterized using six different recombinant vectors containing the same insert. The humoral immune response and protection from virulent JEV challenge following immunization with the recombinant plasmid DNAs were compared to findings for the human vaccine, JEVAX, licensed by the U.S. Food and Drug Administration, in outbred ICR mice.

MATERIALS AND METHODS

Cell culture and virus strain. COS-1, COS-7, and SV-T2 cells (1650-CRL, 1651-CRL, and 163.1-CCL; American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 7.5% NaHCO₃ (30 ml/liter), penicillin (100 U/ml), streptomycin (100 µg/ml). COS-1 and COS-7 cells were derived from simian virus 40 (SV40) transformed CV1 cells which have an African green monkey kidney cell origin. SV-T2 cells were derived from SV40-transformed mouse fibroblasts. Vero cells were grown under the same conditions except that 5% fetal calf serum without nonessential amino acid was used. C6/36 cells (13) were grown at 28°C in the same medium used for the COS-1 cells. The SA14 strain of JEV, propagated by intracranial inoculation into suckling mouse brain, was used for animal challenges and plaque reduction neutralization tests (PRNT). The SA14 virus used in ELISA and Western blot experiments was propagated in C6/36 cells and purified by ultracentrifugation on 30% glycerol-45% potassium tartrate gradients (37).

Construction of plasmids expressing JEV prM and E gene proteins. Genomic RNA was extracted from 150 µl of SA14 mouse brain JEV by using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). RNA was adsorbed on a silica membrane, eluted in 80 µl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated water, and used as a template for amplification of JEV prM and E genes. Primer sequences were obtained from the published data (35). A single cDNA fragment containing genomic nucleotides (nt) 389 to 2478 was amplified by reverse transcriptase-mediated PCR (RT-PCR). Restriction enzyme sites for *KpnI* and *XbaI* and Kozak's sequence for an optimal translation initiation (25, 26) were engineered at the 5' terminus of the cDNA by primer 14DV389. An in-frame translation termination codon, followed by a *NotI* restriction site, was introduced at the 3' terminus of the cDNA by primer

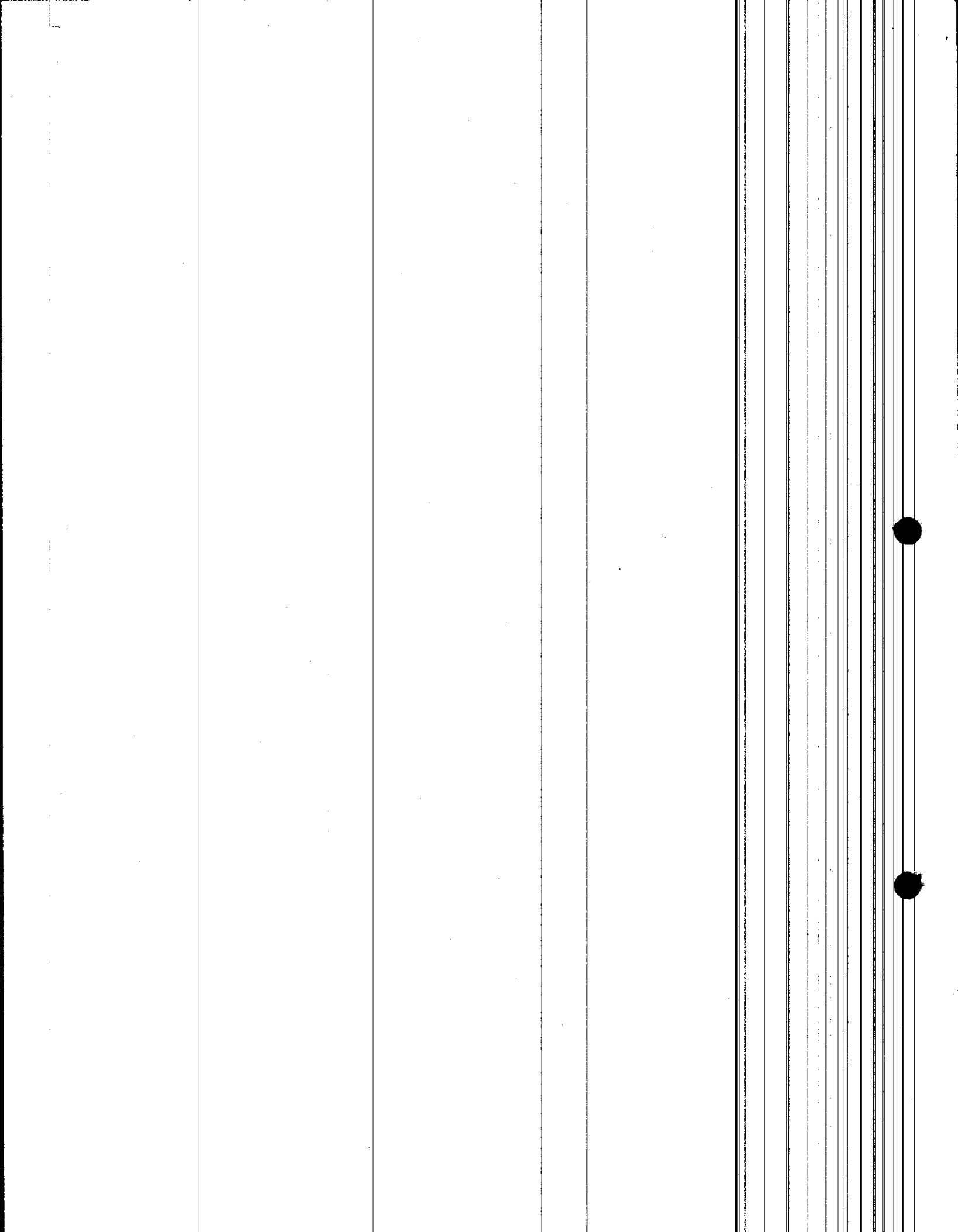
c14DV2453 (Fig. 1). A single-tube RT-PCR was performed using a Titan RT-PCR Kit (Roche Molecular Biochemical, Indianapolis, Ind.). The RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen), and the DNA was eluted with 50 µl of 1 mM Tris-HCl (pH 7.5).

All vector constructions and analyses were carried out using standard techniques (46). RT-PCR-amplified cDNA was digested with enzymes *KpnI* and *NotI* and inserted into the *KpnI-NotI* site of eukaryotic expression plasmid vector pCDNA3 (Invitrogen, Carlsbad, Calif.). Electroporation-competent *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, Calif.) were transformed by electroporation (Gene Pulser; Bio-Rad Laboratories, Hercules, Calif.) and plated on Luria broth (LB) agar plates that contained carbenicillin (100 µg/ml; Sigma). Clones were picked and inoculated into 3 ml of LB containing carbenicillin (100 µg/ml). Plasmid DNA was extracted from a 14-h LB culture by using a QIAprep Spin Miniprep kit (Qiagen). Automated DNA sequencing was performed as recommended on an ABI Prism 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Both strands of the cDNA were sequenced and compared to the published SA14 virus sequence (35).

The pCDNA3 fragment from nt 1289 to nt 3455, which contained the fl-encoded eukaryotic origin of replication (ori), SV40 ori, neomycin coding region, and SV40 poly(A) elements, was deleted by *PvuII* digestion and then self-ligated to generate plasmid pCBamp. The pCBamp vector, which contained a chimeric intron insertion at the *NcoI-KpnI* site of the pCB vector, was constructed by excising the intron sequence from pCI (Promega, Madison, Wis.) by digestion with *NcoI* and *KpnI*. The resulting 566-bp fragment was cloned into *NcoI-KpnI*-digested pCBamp to replace its 289-bp fragment. Figure 2 shows a schematic drawing of plasmids pCDNA3, pCBamp, and pCBamp.

The DNA fragment containing the JEV coding region in the recombinant plasmid pCDJE2-7, derived from the pCDNA3 vector, was excised by *NotI* and *KpnI* or *XbaI* digestion and cloned into the *KpnI-NotI* sites of pCB, pCIB, pCEP4 (Invitrogen), and pREP4 (Invitrogen) and into the *SpeI-NotI* site of the pRE/RSV (Invitrogen) expression vector to create pCBIE1-14, pCIBIE14, pCEJE, pREJE, and pRCJE, respectively. Both strands of the cDNA from each plasmid vector were sequenced, and recombinant clones with a correct nucleotide sequence were identified. Plasmid DNA for in vitro transformation or mouse immunization was purified by anion-exchange chromatography using an Endo-Free Plasmid Maxi kit (Qiagen).

IFA. Expression of JEV-specific gene products by the various recombinant expression plasmids was evaluated by indirect immunofluorescence antibody assay (IFA) in the transient expression system using COS-1, COS-7, and SV-T2 cells. For transformation, cells were grown to 75% confluence in 150-cm² culture flasks, trypsinized, and resuspended in 4°C phosphate-buffered saline (PBS) to a final density of 1×10^7 to 2×10^7 cells/ml. Five hundred microliters of cell suspension was then electroporated with 10 µg of plasmid DNA, using a Bio-Rad Gene Pulser II set at 250 V and 960 µF. Cells were diluted with 25 ml of fresh medium after electroporation and seeded into one 75-cm² flask. Forty-eight hours after transformation, the medium was removed, and the cells were



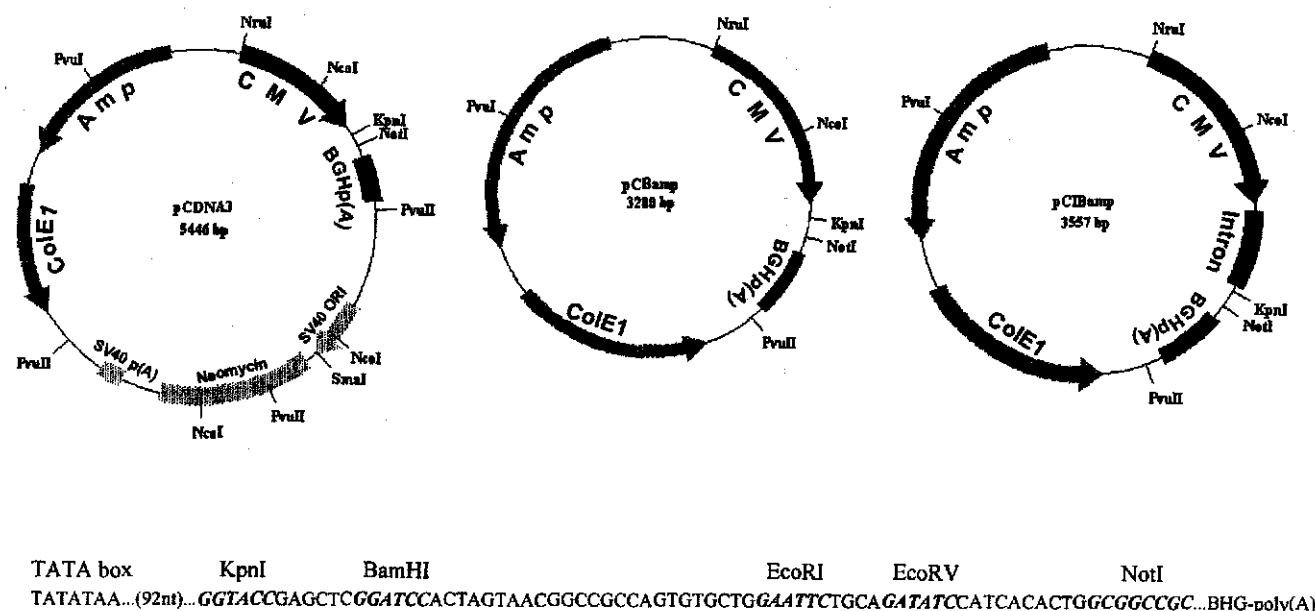


FIG. 2. Schematic representations of plasmid vectors pCDNA3, pCBamp, and pCIBamp. These plasmids include the CMV promoter/enhancer element, BGH poly(A) signal and transcription termination sequence [BGHp(A)], ampicillin resistance gene (Amp), and ColE1 ori for selection and maintenance in *E. coli*. The fl ori for single-stranded rescue in *E. coli* cells, SV40 ori, neomycin coding region, and SV40 poly(A) [SV40 p(A)] sequences were deleted from pCDNA3 to generate pCBamp. An intron sequence was inserted in the *NcoI*-*KpnI* site of pCBamp to generate pCIBamp. The multiple cloning site for the insertion of JEV genes, located between the TATA box of the CMV promoter/enhancer and BGH poly(A) site, is shown.

trypsinized and resuspended in 5 ml of PBS with 3% normal goat serum. Ten-microliter aliquots of the cell suspension were then spotted onto slides, air dried, and fixed with acetone at 4°C for 10 min. Immunofluorescent mapping of the E protein-specific epitopes was performed using a panel of murine monoclonal antibodies (MAbs) (15, 42, 55) and JEV-specific hyperimmune mouse ascitic fluid (HIAF). All antibodies were tested at 1:400 dilution in PBS.

Selection of an *in vitro*-transformed stable cell line constitutively expressing JEV-specific gene products. COS-1 cells transformed with 10 µg of pCDJE2-7 DNA by electroporation were incubated in nonselective culture medium for 24 h and then treated with neomycin (G418; 0.5 mg/ml; Sigma). G418-resistant colonies, which became visible after 2 to 3 weeks, were cloned by limited dilution in G418-containing medium. Expression of the JEV proteins was determined by IFA using JEV HIAF. One IFA-positive (JE-4B) and one IFA-negative (JE-5A) clone were selected for further analysis and maintained in medium containing 200 µg of G418 per ml. These stably transformed cells secreted antigen in the form of EPs (A. Hunt and G. J. Chang, unpublished data).

Antigen capture ELISA for detection of E protein secreted into culture fluid. The antigen capture ELISA, a modification of the procedure described by Guirakhoo et al. (8), was used to detect E protein from transiently transformed cells or JE-4B culture fluid. Flavivirus group-reactive MAb 4G2 was used to capture the JEV antigens (7). The 4G2-captured antigen was detected using horseradish peroxidase-conjugated MAb 6B6C-1 by incubation for 1 h at 37°C. Enzyme activity on the solid phase was detected with 3,3',5,5'-tetramethylbenzidine ELISA substrate (Life Technologies, Grand Island, N.Y.); the reaction was stopped with the addition of 2 M H₂SO₄, and the optical density was measured at 450 nm.

Mouse experiments. Three-day-old mixed-sex or 3-week-old female ICR outbred mice were vaccinated i.m. with 50 or 100 µg of plasmid DNA at a concentration of 1 µg/µl in PBS or subcutaneously (s.c.) with 1/10 or 1/5 of the adult human dose of JEVAX (manufactured by the Research Foundation for Microbial Disease of Osaka University and distributed by Connaught Laboratories, Swiftwater, Pa.). The chloramphenicol acetyltransferase (CAT) protein expression plasmid pCDNA3/CAT (Invitrogen) was used as the vaccination control. Selected groups of mice were boosted 3 weeks later with an additional dose of plasmid vaccine or JEVAX. Mice were bled from the retro-orbital sinus; serum samples were evaluated for JEV antibody by ELISA and Western blotting using purified JEV and by PRNT.

Mice vaccinated at 3 days of age were challenged intraperitoneally (i.p.) 7 weeks postvaccination with JEV strain SA14 (50,000 PFU/100 µl) and observed for 3 weeks. To evaluate passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following their vaccination with plasmid DNA at 3 weeks of age. Pups were challenged by the i.p. route 3 to 15 days after birth with SA14 virus (5,000 PFU/100 µl) and observed daily for 3 weeks. Postchallenge serum was collected from survivors and tested for reactivity with JEV antigens by ELISA and Western blotting.

Serological tests. Postvaccination and postchallenge serum samples were tested for the ability to bind to purified JEV by ELISA, neutralize JEV infectivity by PRNT, or recognize JEV proteins by Western blotting (12, 41, 48). The PRNT assay was performed by incubating ~200 PFU of SA14 virus in 100 µl of Dulbecco's modified Eagle medium containing 5% bovine serum albumin and 20 mM HEPES buffer (pH 8.0) with serial twofold dilutions of serum specimens, started at 1:10, in 100 µl of the same buffer in 96-well trays at 4°C overnight. Serum specimens were heat inactivated at 56°C for 30 min before use. Duplicate 100-µl aliquots were assayed for infective virus by plaque formation on Vero cell monolayers. The percent plaque reduction was calculated relative to virus controls without serum. Titers were expressed as the reciprocal of serum dilutions yielding a 90% reduction in plaque number (PRNT₉₀).

RESULTS

Effect of the promoter and poly(A) signal on the efficiency of JEV prM and E protein expression. Four eukaryotic cell expression plasmids that contained the JEV coding region extending from genomic nt 390 to nt 2478 were constructed. This region of the genome encoded the prM and E genes. The Kozak sequence for the eukaryotic translation initiation site (underlined) of -9 to +4, GCCGCCGCCATGG, at the 5' terminus (2, 25, 26, 27) and the in-frame translation termination sequence at the 3' terminus of cDNA were incorporated directly into cDNA by RT-PCR using viral RNA as a template. Transcription of the JEV genes in plasmid pCDJE2-7 was controlled by the human cytomegalovirus (CMV) early 1A gene promoter/enhancer. The resulting mRNA is terminated and stabilized by a bovine growth hormone (BGH) transcript ion terminator and a poly(A) signal, respectively. The transcriptional control elements in pREJE were replaced by the Rous sarcoma virus (RSV) long terminal repeat promoter and SV40 poly(A). The pCEJE and pRCJE plasmids contain CMV plus SV40 poly(A) and RSV plus BGH poly(A), respectively (Table 1).

To determine the influence of the promoter and poly(A) elements on JEV prM and E protein expression, recombinant plasmids pCDJE2-7, pCEJE, pRCJE, and pREJE were ini-

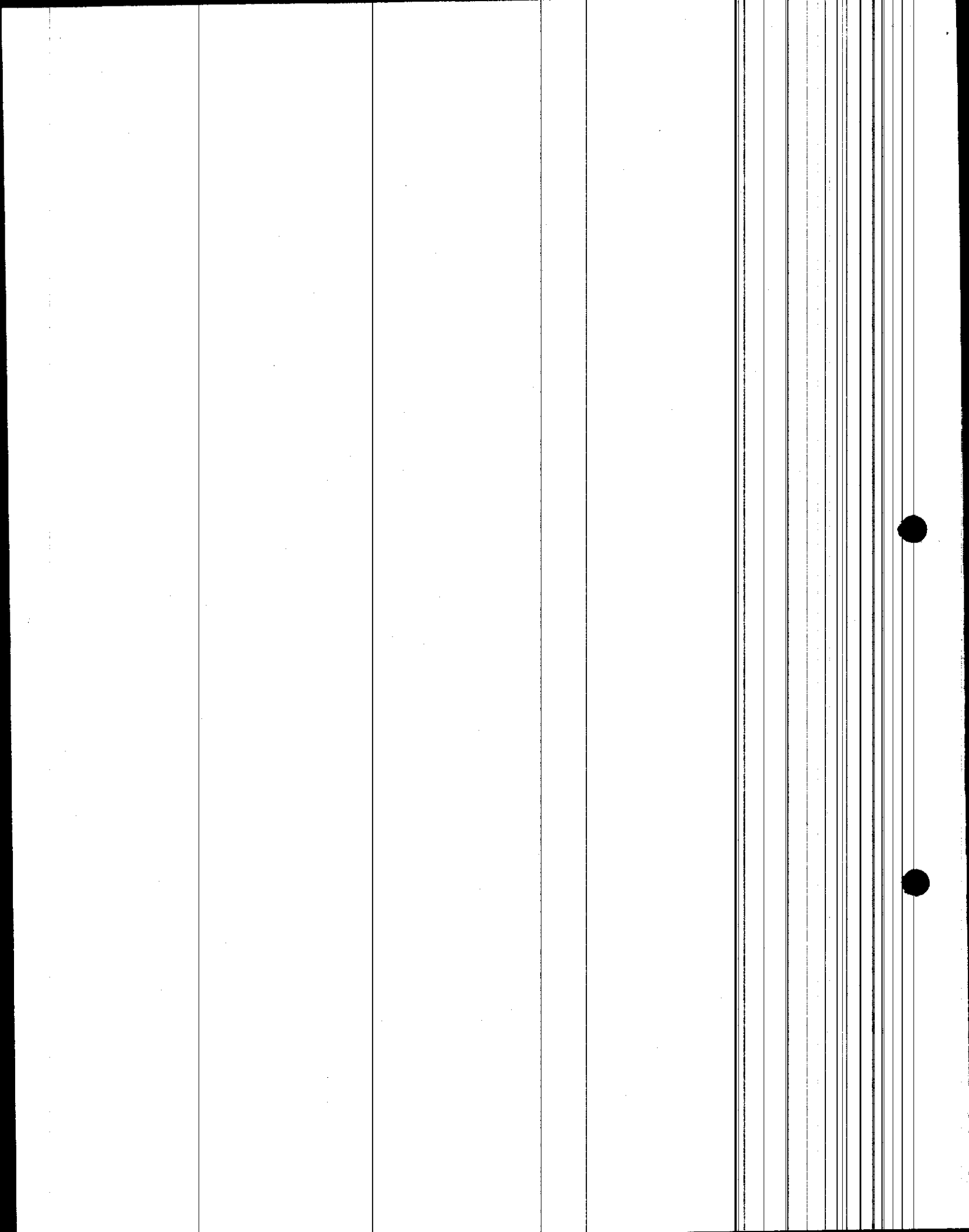


TABLE 1. Transient expression of JEV prM and E proteins by various recombinant plasmids in two transformed cell lines

Name	Promoter	Intron	Poly(A)	Ori	Recombinant plasmid	IFA intensity/% positive ^a	
						COS-1	COS-7
pCDNA3	CMV	No	BGH	SV40	pCDJE2-7	3+/40	3+/35
pCBamp	CMV	No	BGH	No	pCBE1-14	3+/45	ND
pCIBamp	CMV	Yes	BGH	No	pCIBES14	3+/39	ND
pCEP4	CMV	No	SV40	OriP	pCEJE	2+/4	2+/3
pREP4	RSV	No	SV40	OriP	pREJE	1+/3	1+/2
pRc/RSV	RSV	No	BGH	SV40	pRCJE	1+/3	1+/3
pCDNA3	CMV	No	BGH	SV40	pCDNA3/CAT	—	—

^a Various cell lines were transformed with pCDNA3/CAT (negative control), pCDJE2-7, pCBE1-14, pCIBES14, pCEJE, pREJE, or pRCJE. Cells were trypsinized 48 h later and tested by IFA with JEV HIAF. Data are presented as the intensity (scale of 1+ to 4+) and percentage of IFA-positive cells. pCDNA3/CAT-transformed cells were used as the negative control. ND, not determined. —, negative.

tially tested for the ability to express JEV prM and E proteins following transformation of various mammalian cells. COS-1, COS-7, and SV-T2 cells were transiently transformed with equal amounts of pCDJE2-7, pCEJE, pRCJE, or pREJE plasmid DNA. The SV-T2 cell line was excluded from further testing after preliminary results showed that less than 1% of pCDJE2-7-transformed SV-T2 cells were expressing JEV antigen.

JEV antigens were expressed in COS-1 and COS-7 cells transformed by all four recombinant plasmids, thus confirming that the CMV or RSV promoter and BGH or SV40 poly(A) elements were functionally active. However, the percentage of transformed cells and the level of JEV antigens expressed, as determined by the number of IFA-positive cells and IFA intensity, respectively, differed significantly (Table 1). A significantly higher percentage of pCDJE2-7-transformed COS-1 cells expressed JEV proteins with greater IFA intensity at a level equal to that observed with JEV-infected cells. Cells transformed with the pCEJE, pREJE, or pRCJE vector, on the other hand, showed a lower percentage of antigen-expressing cells as well as a lower IFA intensity. Vectors containing the CMV promoter and BGH poly(A) were selected for further analysis (Fig. 2).

To determine whether the enhanced expression of JEV proteins by the pCDJE2-7 vector was influenced by the SV40 ori, we constructed the pCBE1-14 vector in which a 2,166-bp fragment containing the fl ori, SV40 ori, neomycin coding region, and SV40 poly(A) elements was deleted. A chimeric intron was then inserted into pCBE1-14 to generate pCIBES14. Plasmid pCIBES14 was used to determine whether the expression of JEV proteins could be enhanced by an intron sequence. Following transformation, both pCBE1-14 and pCIBES14 vectors resulted in cells expressing levels of JEV proteins similar to that observed with the pCDJE2-7 vector (Table 1). These results indicated that expression of the JEV proteins was influenced only by the transcriptional regulatory elements encoded in the recombinant plasmid. Neither the SV40 ori nor the intron sequence enhanced JEV protein expression in the cells used.

Epitope mapping of E protein expressed by a stably transformed cell line constitutively expressing JEV-specific gene products. Authenticity of the JEV E protein expressed by the JE-4B clone was demonstrated by epitope mapping by IFA using a panel of JEV E-specific murine MAbs. JEV HIAF and one irrelevant mouse ascitic fluid were used as positive and negative antibody controls, respectively. Four JEV-specific, six flavivirus subgroup-specific, and two flavivirus group-reactive MAbs reacted similarly with the 4B clone and with JEV-infected COS-1 cells (Table 2).

Detection of JEV E protein secreted by the JE-4B COS-1 cell line. An antigen capture ELISA, employing flavivirus group-reactive, anti-E MAbs 4G2 and 6B6C-1, was used to detect JEV E proteins that were secreted into the culture fluid by the COS-1 cell clone JE-4B. Antigen could be detected in the culture fluid the first day following seeding of the cells with maximum ELISA titers that ranged from 1:16 to 1:32.

Comparison of immune responses in mice vaccinated with pCDJE2-7 genetic vaccine and JEVAX. Plasmid pCDJE2-7 was used as a nucleic acid vaccine to induce an antibody response in mice by immunizing groups of five 3-week-old female ICR outbred mice. Mice were bled at 3, 6, 9, 23, 40, and 60 weeks after immunization, and antibody titers were determined by ELISA or by PRNT. As expected, sera from animals in the pCDNA3/CAT control group did not contain JEV antibody. All animals immunized with pCDJE2-7 and JEVAX seroconverted by 3 weeks after the first vaccination (Table 3). The antibody titers were similar irrespective of the number of doses

TABLE 2. Epitope mapping of E protein expressed by JE-4B, a pCDJE2-7 stably transformed clone of COS-1 cells, with JEV-reactive antibodies^a

MAb or antiserum	Biological activity of MAb		IFA intensity of cells	
	Specificity	Biological function	JEV infected	4B
MAbs				
MC3	JEV specific	HI, N	2+	2+
2F2	JEV specific		4+	4+
112	JEV specific		4+	4+
503	JEV specific	N	4+	3+
109	Subgroup	HI	2+	1+
N.04	Subgroup	HI, N	3+	4+
201	Subgroup	HI	1+	1+
203	Subgroup		4+	3+
204	Subgroup		2+	2+
301	Subgroup		2+	2+
504	Flavivirus		4+	4+
6B6C-1	Flavivirus		2+	2+
3B4C-4	VEE		—	—
HIAF				
Anti-JEV			4+	3+
Anti-WEE			—	—
PBS				
			—	—

^a VEE, Venezuelan equine encephalomyelitis virus; WEE, Western equine encephalomyelitis virus. —, negative.

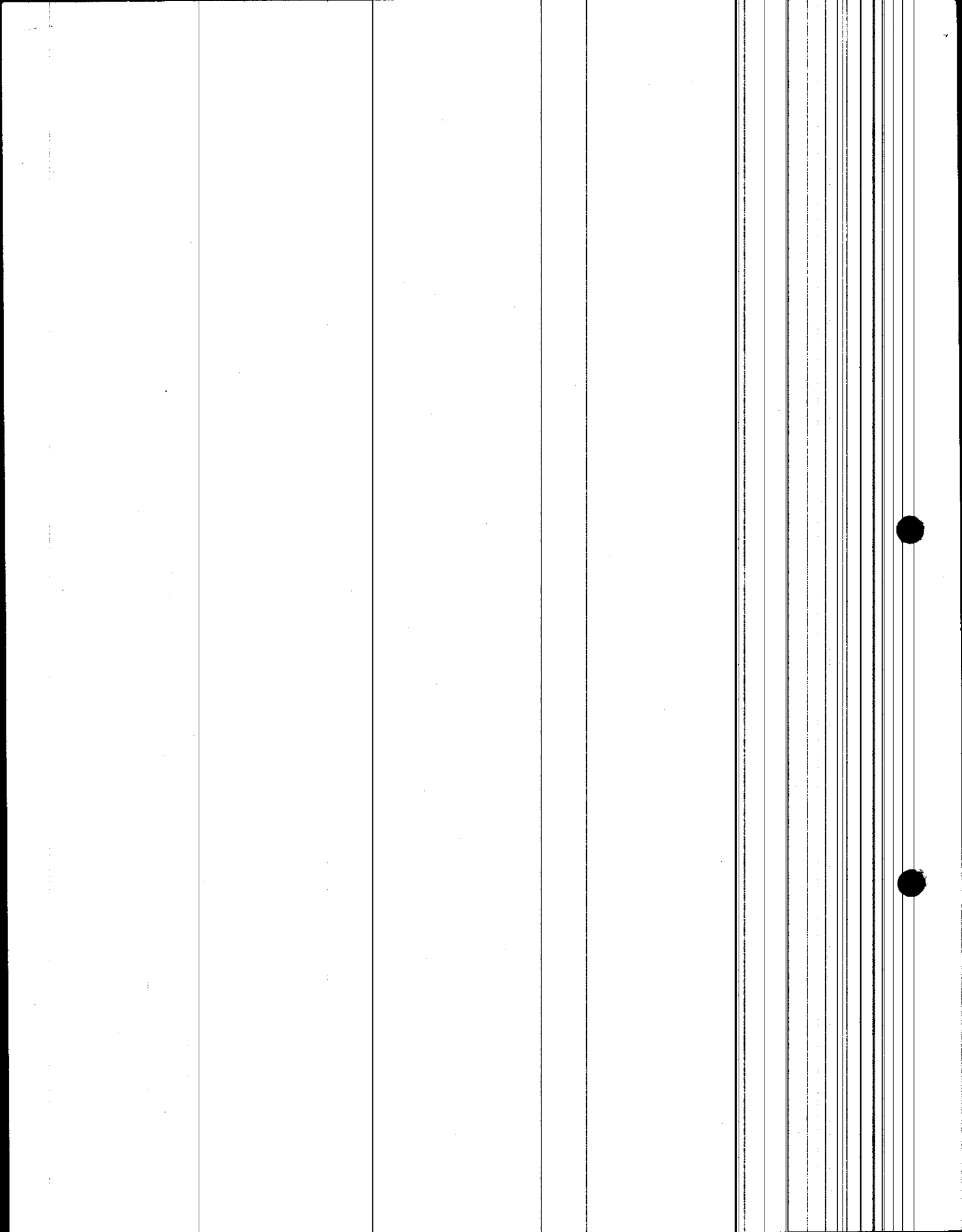


TABLE 3. Persistence of the immune response in mice (five per group) immunized with pCDJE2-7 or JEVAX

Inoculation ^a	ELISA titer (log ₁₀)						PRNT ₅₀ titer		
	3 ^b	6	9	23	40	60 ^c	3	6	9
pCDJE2-7									
1 dose	2.6–3.2	3.8–5.0	3.8–4.4	>3.2	>3.2	2.4, 2.4, 3.8, 4.4	<20	20	40–160
2 doses	2.6–3.8	4.4	3.8–4.4	>3.2	>3.2	2.6, 3.8, 3.8	<20	20–40	40–160
JEVAX, 2 doses	2.6–3.8	4.4–5.0	3.8–5.6	>3.2	>3.2	<2, <2, <2, 4.4	<20	20–40	20–160
pCDNA3/CAT, 2 doses	<100	<100	<100	ND ^d	ND	ND	<20	<20	<20

^a Three-week-old mice were inoculated i.m. with one or two 100-μg doses of plasmid DNA or twice s.c. with one-fifth of the human dose of JEVAX.

^b Weeks postimmunization.

^c Individual serum titers.

^d ND, not determined.

of pCDJE2-7 or JEVAX given. Mouse serum samples collected 9 weeks after immunization were also tested by Western blotting using purified JEV. Serum specimens from DNA-vaccinated mice, which had reactivity similar to that of JEV HIAF, detected E and prM proteins (Fig. 3). However, mouse serum from JEVAX-immunized mice reacted only with E protein. Comparable ELISA antibody titers were maintained in DNA-vaccinated groups for up to 60 weeks, at which time the experiment was terminated. Only one of four mice in the JEVAX group remained JEV antibody positive at 60 weeks postinoculation. These results demonstrated that one dose of JEV-specific nucleic acid vaccine was more effective in maintaining JEV antibody levels in mice than the commercially available vaccine JEVAX.

Comparison of various nucleic acid vaccine constructs and JEVAX for ability to induce JEV-reactive antibody in different age groups of mice. Similar amounts of JEV protein were expressed by COS-1 cells transformed by either pCDJE2-7, pCBJE1-14, or pCIBJES14. JEV antibody induction by these nucleic acid constructs was compared to results for JEVAX in two different age groups of mice. Three-day-old mixed-sex or 3-week-old female ICR outbred mice, 10 per group, were vac-

inated i.m. with 50 or 100 μg of plasmid DNA or s.c. with 1/10 or 1/5 of the adult human dose of JEVAX, respectively. Serum specimens were collected at 7 weeks after immunization and tested at 1:400 or 1:1,600 by ELISA. Ninety to 100% of all 3-week-old mice that received pCBJE1-14, pCDJE2-7, pCIBJES14, or JEVAX had antibody titers of ≥1:1,600. However, a significant difference in antibody response was observed in 3-day-old groups that received various vaccines. None of the 3-day-old JEVAX-vaccinated mice had antibody titers higher than 1:400. All 3-day-old mice vaccinated with pCBJE1-14 had antibody titers higher than 1:1,600. Seroconversion of 100% was observed at 1:400 in 3-day-old mice that received pCDJE2-7 or pCIBJES14, but only 60% of both mouse groups were positive at 1:1,600. pCBJE1-14 was the most effective of three DNA constructs tested. The minimum dose of this DNA construct capable of providing 100% seroconversion (1:400 by ELISA) by i.m. immunization in 3-week-old mice was determined to be 25 μg (data not shown).

Protective immunity conferred by the nucleic acid vaccine. Mice immunized at 3 days of age were challenged by the i.p. route at 7 weeks postvaccination with the SA14 strain of JEV (50,000 PFU/100 μl) and observed for 3 weeks. One hundred percent of the animals that received various nucleic acid vaccine constructs were protected. In contrast, only 40 and 30% of mice that received JEVAX and pCDNA3/CAT, respectively, survived virus challenge (Fig. 4). These results suggested that the DNA vaccine could be effective as a neonatal vaccine. In contrast, JEVAX was not as effective in neonatal animals.

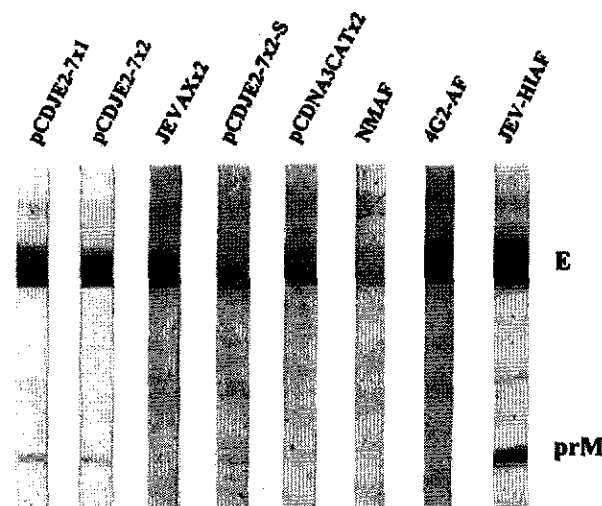


FIG. 3. JEV-specific reactivity of prechallenge and postchallenge serum samples obtained from mice immunized with DNA vaccine or JEVAX. Serum specimens collected from the mice used in the experiments represented in Tables 3 and 4 were randomly selected and tested at 1:1,000 dilution by Western blot analysis using purified JEV as the antigen. pCDJE2-7x2-S was the serum from one of the mice challenged at 4 days of age (Table 4). NMAF, 4G2-AF, and JEV-HIAF were the mouse ascitic fluids included as normal mouse, E-specific, and JEV hyperimmune controls, respectively.

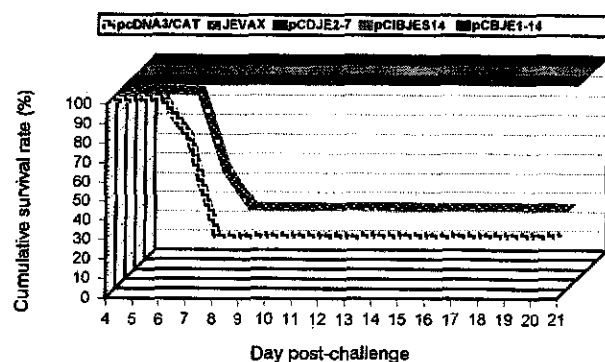


FIG. 4. Postchallenge survival rates of mice (10 per group) that were immunized with pCDJE2-7, pCBJE1-14, pCIBJES14, pCDNA3/CAT, or JEVAX at 3 days of age and challenged i.p. with 50,000 PFU of JEV (SA14) 7 weeks postimmunization. A *P* value of 0.003 was obtained by Fisher's exact test when the survival rate of the JEV DNA-immunized groups was compared with that of the pCDNA3/CAT or JEVAX group.

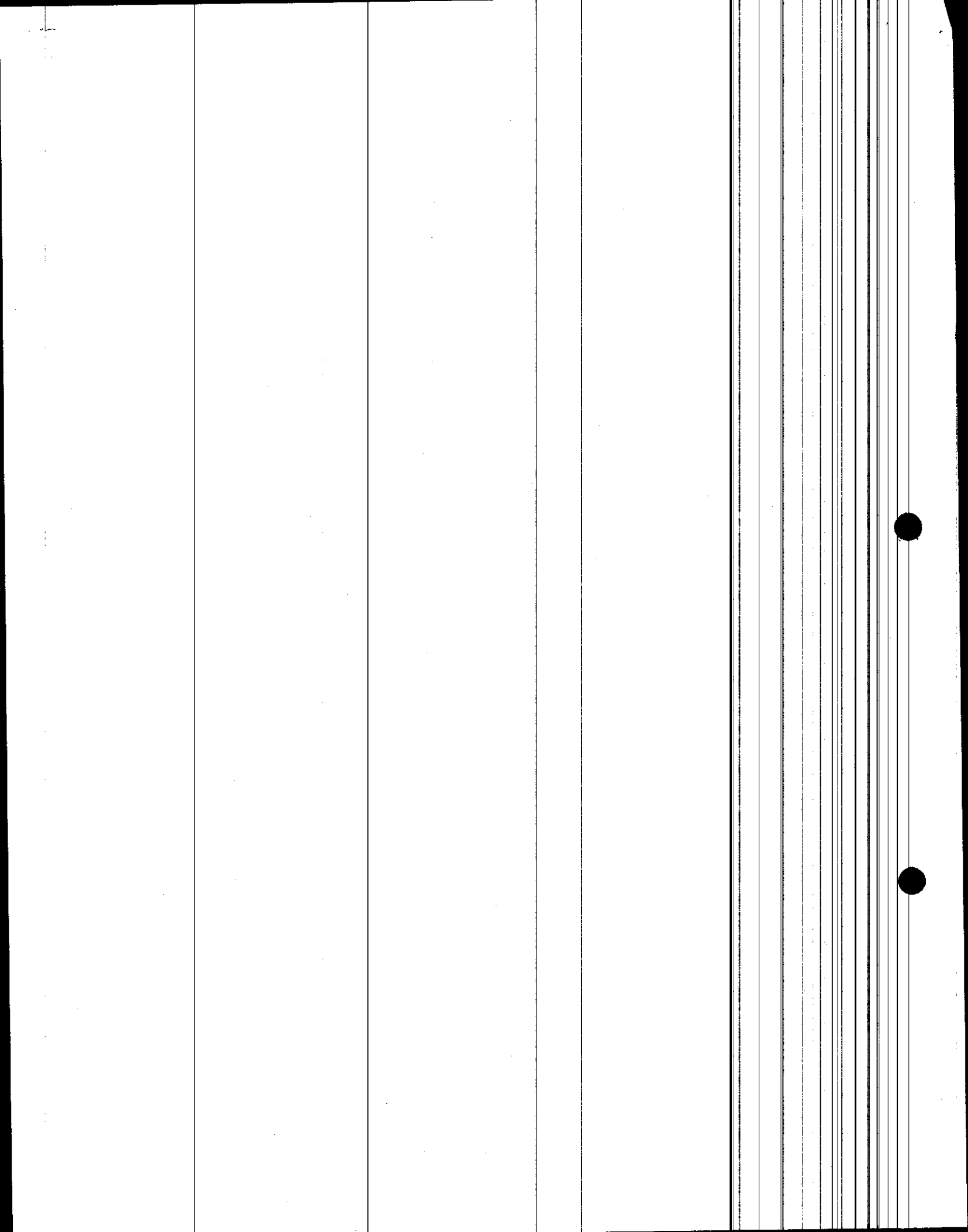


TABLE 4. Ability of maternal antibody from JEV nucleic acid-vaccinated female mice to protect their pups from fatal JE

Vaccinated mother ^a		JEV-challenged pups			
Vaccine	PRNT ₉₀	Age (days)	No. of survivors/total in litter	Avg survival time (days)	ELISA ^b
1 × pCDJE2-7	40	4	0/11	5.27	12/12
2 × pCDJE2-7	80	4	12/12	NA ^c	
2 × JEVAX	20	3	0/16	4.75	
2 × pCDNA3/CAT	<10	5	0/14	4.00	
1 × pCDJE2-7	20	15	5/11	10.0	5/5
2 × pCDJE2-7	40	14	8/12	13.75	7/8
2 × JEVAX	80	13	5/5	NA	5/5
2 × pCDNA3/CAT	<10	14	0/14	6.14	

^a Mice were inoculated i.m. with one or two 100-μg doses of pCDJE2-7 DNA or twice s.c. with one-fifth of the adult human dose of JEVAX. Serum samples were collected 9 weeks postvaccination for PRNT testing prior to mating with nonimmune male.

^b Number of JEV ELISA antibody-positive animals (titer ≥ 1:400)/number of survivors. Serum specimens were collected for testing 12 weeks after challenge.

^c NA, not applicable.

Passive protection of neonatal mice correlated with the maternal antibody titer. Female 3-week-old ICR mice were vaccinated with one or two doses of pCDJE2-7 plasmid DNA (100 μg/100 μl) or twice with one-fifth of the adult human dose of JEVAX. For evaluation of passive protection by maternal antibody, pups were obtained from matings of experimental females with nonimmunized male mice. Pups were challenged by the i.p. route at 3 to 5 or 13 to 15 days after birth with SA14 virus (5,000 PFU/100 μl). Survival rates and average survival time correlated with the maternal neutralizing antibody titers (Table 4). One hundred percent of pups nursed by mothers with a PRNT of 1:80 survived viral infection regardless of the type of vaccine received by the mothers. None of the pups from mothers which received pCDNA3/CAT plasmid DNA survived (Table 4). Partial protection (45% [5 of 11 pups] to 67% [8 of 12 pups]) was observed in older pups that were nursed by the mothers which had serum PRNT titers of 1:20 and 1:40, respectively. However, none of the 3-day-old pups survived virus challenge when the mothers had a serum PRNT titer of 1:20 or 1:40. Maternally transferred antibody can only be detected in the circulation of the young mouse up to 40 days after birth. An appreciable level of maternally derived antibody is maintained in the circulation of the young mouse 24 days or more postpartum (1). JEV ELISA antibody detected in the serum of 97% (29 of 30) of the postchallenge pups at 12 weeks after virus challenge was unlikely to be residual maternally transferred antibody. The presence of JEV antibody in the surviving pups challenged at 3 to 4 or 13 to 15 days of age strongly suggested that maternal antibody did not provide sterilizing immunity to the pups. It also indicated that 3- to 4- or 13- to 15-day-old mice could mount an immune reaction to a live-virus challenge. Partial protection in older pups could be explained by the opportunity to accumulate a large quantity of passive antibody due to the length of nursing time before challenge. One randomly selected postchallenge serum sample also reacted with prM and E proteins by Western blotting (Fig. 3).

DISCUSSION

The flavivirus virion contains a capsid protein (C), a membrane protein (M), and an E protein. The prM MABs, exhibiting weak or undetectable neutralizing activity in vitro, can

provide passive protection following DEN2 virus challenge (16). However, the E protein plays a dominant role in generating neutralizing antibodies and providing protective immunity in the host. Passive transfer of JEV E-specific neutralizing MABs has been shown to protect recipients from JEV-induced fatal encephalitis (3, 16, 32, 55). Antigenic and structural analysis using various panels of MABs has shown that most of the E protein epitopes that elicit virus-neutralizing antibodies are conformationally dependent (9, 40). Coexpression of both proteins as type I transmembrane proteins is essential to maintain proper E conformation and prevent the E protein from undergoing irreversible, low-pH-catalyzed conformational changes (8–10, 19, 50). A 2-kb genomic region, from the internal signal peptide at the carboxyl terminus of C to the transmembrane domain at the carboxyl terminus of the E gene, is essential for expressing authentic proteins. These authentic prM and E proteins are able to self-assemble into virus-like particles in cells infected by either recombinant vaccinia virus or alphavirus vector or in cells transformed by recombinant plasmid DNA (4, 19, 22, 48; Hunt and Chang, unpublished data).

A gene cassette including the elements listed above was amplified from SA14 virus by RT-PCR in the present study. Optimal sequence composition surrounding the translation initiation site (–9 to +4) was incorporated into the 14DV398 amplifying primer (2, 26, 27) (Fig. 1). Recombinant plasmids containing the CMV early gene promoter/enhancer and the BHG poly(A) terminator as transcription regulatory elements expressed JEV proteins with the highest efficiency in three different cell lines. Protein expression and the serological response of mice immunized with DNA vaccine were not influenced by the presence or absence of the SV40 ori or an intron sequence in recombinant plasmids. Virus-specific proteins, secreted into culture medium, could be detected by antigen capture ELISA as early as 48 h after plasmid transformation (data not shown). The authenticity of the E protein produced by the pCDJE2-7 stably transformed cell line, JE-4B, was demonstrated by MAB epitope mapping.

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in Tables 5 and 6. All constructs listed had the same transcriptional control elements and similar viral gene cassettes. DEN2 plasmid, which contains prM and 91% of E, is the only exception (Table 6). The JEV DNA vaccine reported in this study is the only construct that stimulated complete protective immunity in mice by a single dose of vaccine given by the i.m. route (Table 5). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs that may contribute to increasing the vaccine potential of our construct (Table 6). Conserved features of the sequences which flank vertebrate translation initiation sites include a strong preference for purine at the –3 position; a higher frequency of G at positions –9, –6, –3, and +4; and a preference for A or C at positions –5, –4, –2, and –1 (2). Instead of the sequence used in previous publications, the sequence used in our construct was –9 · GCCGCCGCC ATGG, which fits the general criteria listed above. Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential (2, 26).

Signal peptides determine translocation and orientation of inserted protein, hence the topology of prM and E. Signal peptide differences in our plasmid construct may account for the efficient translocation and correct topology, thus increasing prM and E secretion. A machine-learning program using neu-

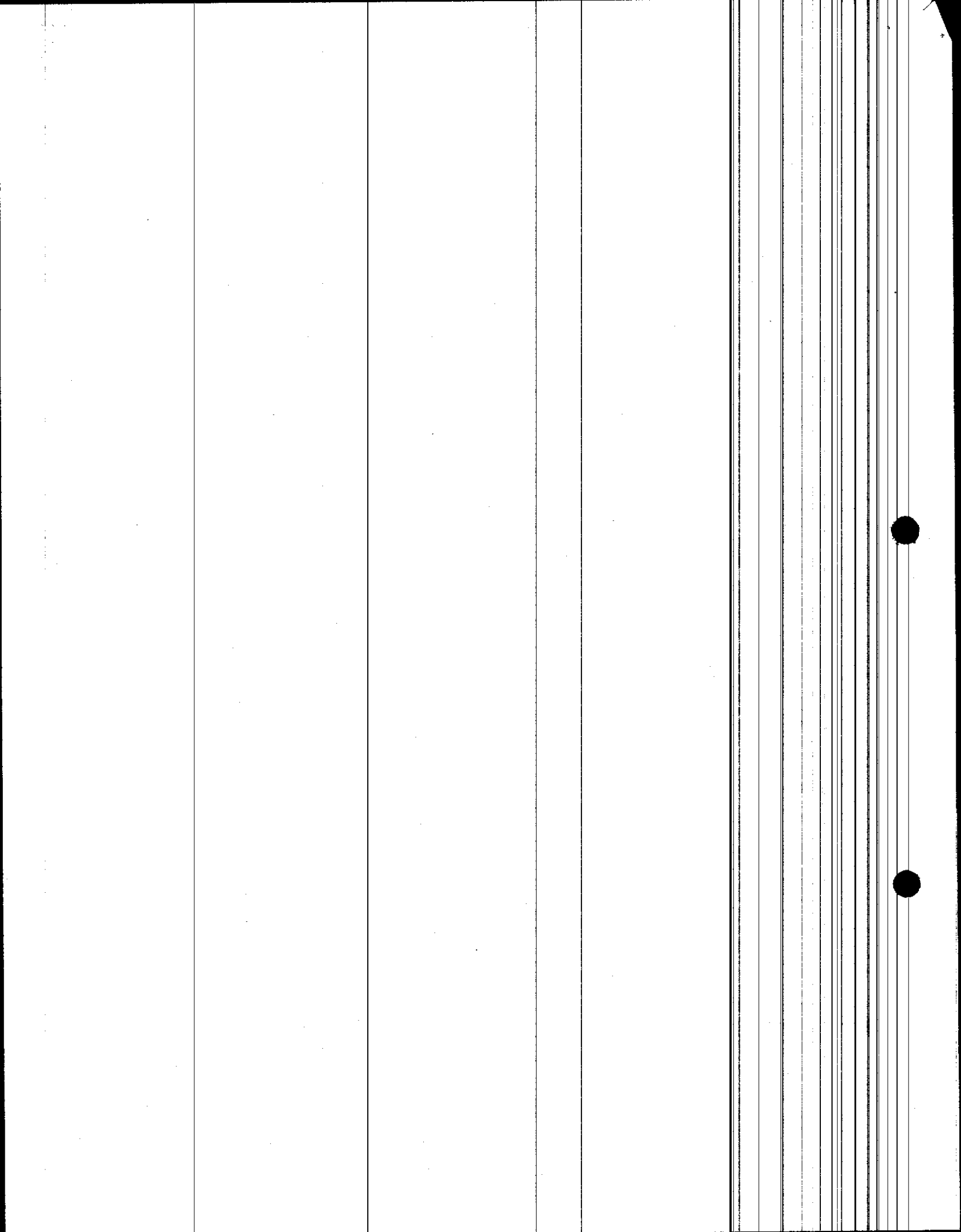


TABLE 5. Vaccine potential of various eukaryotic plasmids that express flavivirus prM and E proteins^a

Virus	In vitro secretion of EPs	Immunization			Protection from virus challenge	Reference
		Dosage	Route/method	Neutralizing antibody ^b		
JE	Yes	25–100 µg × 1	i.m./needle	Yes (1:20–1:160 _{90%})	100%	This report
	ND	100 µg × 2	i.m./needle	No	Partial	30
	ND	10–100 µg × 2	i.m. or i.d./needle	Yes (1:10–1:20 _{90%})	100%	24
MVE	Yes	100 µg × 4	i.m./needle	ND	Partial	4
	Yes	1–2 µg × 2–4	i.d./gene gun	Yes (80–320 _{50%})	100%	4
SLE	ND	100 µg × 2	i.m./needle	No	Partial	38
CEE	ND	1 µg × 1–2	i.d./gene gun	Yes (1:100–1:1,600 _{80%})	100%	49
RSSE	ND	1 µg × 1–2	i.d./gene gun	ND	100%	49
DEN2	ND	200 µg × 3	i.d./needle	Yes (1:10–1:320 _{50%})	None	17

^a MVE, Murray Valley encephalitis; CEE, Central European encephalitis; RSSE, Russian spring-summer encephalitis; i.d., intradermal; ND, not done.

^b Plaque reduction neutralization titer followed by percentage reduction endpoint used in the test.

ral networks trained on eukaryotes (SignalP-NN at <http://www.cbs.dtu.dk/services/>) was applied to test the efficiency of the prM signal peptide sequence in the different plasmid constructs (34) (Table 6). The most probable location and orientation of transmembrane helices in the prM-E protein were then determined by a hidden Markov model-trained computer program (6 [TMHMM at <http://www.cbs.dtu.dk/services/>]). SignalP-NN searches correctly predicted the signal peptidase cleavage site of all constructs. However, a considerable difference in cleavage potential (C score, between 0.578 and 1.000) was observed (Table 6). Cleavage potential differences may be influenced by the amino acid composition and length of the h region in various constructs (44).

The TMHMM program correctly predicted five transmembrane helices encoded in the prM-E protein. Significant difference in the probable orientation of the first transmembrane helix was observed in three JEV constructs (Fig. 5). In our pCDJE2-7 construct, the first 12 amino acids of the n region form a short loop in the cytoplasmic side that causes the following h region (transmembrane helix) to be inserted in a tail orientation. Secretion of JEV protein could be detected by antigen capture ELISA in pCDJE2-7 transient expression studies in which less than 5% of the cells were positive by IFA (data not shown). Thus, there is a high probability that prM and E proteins expressed by pCDJE2-7 would be expressed in the correct orientation, as type I transmembrane proteins (Fig. 5A). There is also a high probability that the prM protein of pDNA3JEME could be expressed as a type II membrane protein with its transmembrane h region inserted in a head orientation because of the absence of positively charged amino acids in its n region (Fig. 5B). Efficient protein synthesis in

conjunction with correct topology of expressed prM and E (Fig. 5A) would most likely enhance EP formation and secretion in transformed cells.

Another characteristic that could explain the excellent vaccine potential of our JEV construct is its ability to produce EPs which have a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells, such as macrophages, dendritic cells, and Langerhans cells (5). When DNA is given by the i.m. route, the majority of antigen is expressed by non-antigen-presenting muscle cells. The efficacy of a DNA vaccine is therefore dependent on transfection of antigen-presenting cells or to reprocessing of antigen derived from other cells. Muscle cells transfected by our construct could conceivably synthesize and secrete EPs, which are highly immunogenic and have been shown to elicit good cellular and humoral responses (22, 23).

Genetic JEV vaccine that induced a completely protective immunity in neonatal mice and a maternally transferable protective immunity in young adult mice by a single i.m. immunization was demonstrated in this study. Additional studies are planned to address the effectiveness of a DNA vaccine in overcoming the potential influence of maternally transferred flavivirus antibodies on the induction of JEV antibody in neonatal mice.

Immunization of pigs is a theoretical means of interrupting transmission and amplification of JEV and thereby preventing human infections (43). The JEV DNA vaccine could also be used as a veterinary vaccine in pregnant sows to prevent JEV-induced stillbirth and abortion (51, 53). Maternally transferred antibody could also interrupt piglets as the JEV-amplifying host and thus reduce human infection.

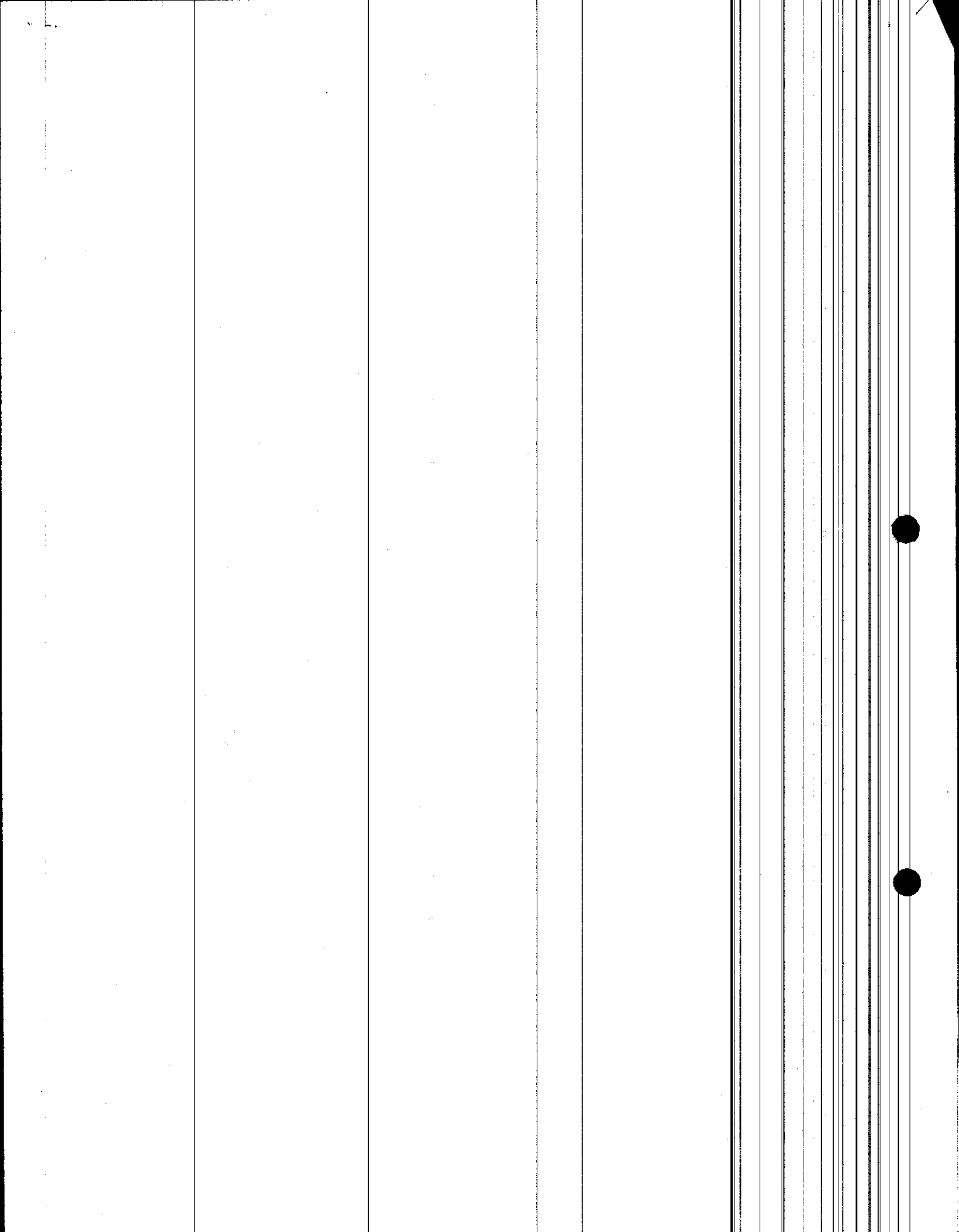
TABLE 6. Characteristics of various eukaryotic plasmids expressing flavivirus prM and E proteins

Virus ^a	Plasmid	Sequence surrounding translation initiation site	Amino acids preceding prM protein ^b	SP potential (C score) ^c	Reference
JE	pCDJE2-7	–9•GCCGCCGCCATGG•+4	MGRKQNKRGNGESIMWLASLAVVIACAGA /MKL	Yes (0.921)	This report
	pJME	–9•GGCTCAATCATGG•+4	MWLASLAVVIACAGA /MKL	Yes (0.578)	30
	pCDNA3JEME	–9•GAATTCACCATGG•+4	MNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	24
MVE	pCDNA3.prM-E	–9•TGATTCAAATGT•+4	MSKKRGGSETSVLMVIFMLIGFAAA /LKL	Yes (0.819)	4
SLE	pSLE1	?	?LDTINRRPSKKRGGTRSLGLAALIGLASS /LQL	Yes (0.709)	38
DEN2	p1012D2ME	?	?AGMIIMLIPTVMA /FHL	Yes (0.646)	17
TBE	SV-PE _{wt}	–9•GCGGCCGCCATGG•+4	MVGLQKRGKRSATDWSWLLVITLLGMTLA /ATV	Yes (1.000)	48
RSSE	pWRG7077	–9•GTAGACAGGATGG•+4	MGWLLVVVLLGVTLA /ATV	Yes (0.762)	50
CEE	pWRG7077	–9•ACGACAGGATGG•+4	MSWLLVITLLGMTIA /ATV	Yes (0.609)	50

^a Abbreviations are as given in Table 5, footnote a.

^b Single amino acid code. Positively charged amino acid is indicated by bold letter. Signal peptidase cleavage site is indicated by /.

^c Cleavage potential of signal peptide (SP) predicted by SignalP-NN at <http://www.cbs.dtu.dk/services/> (34).



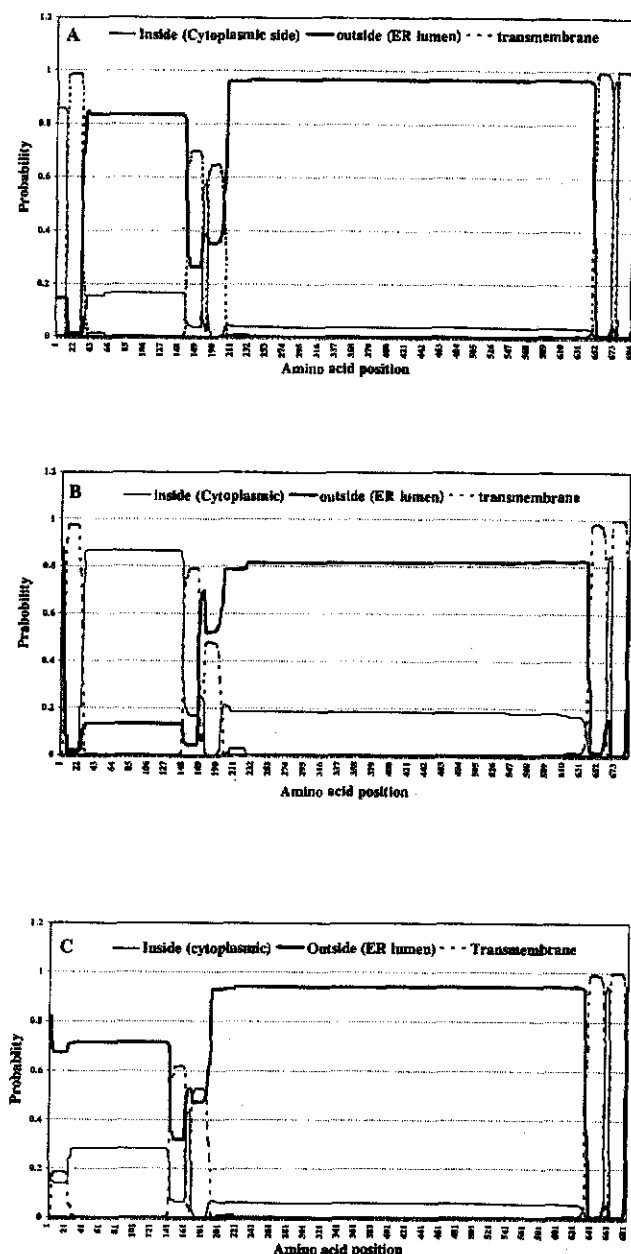


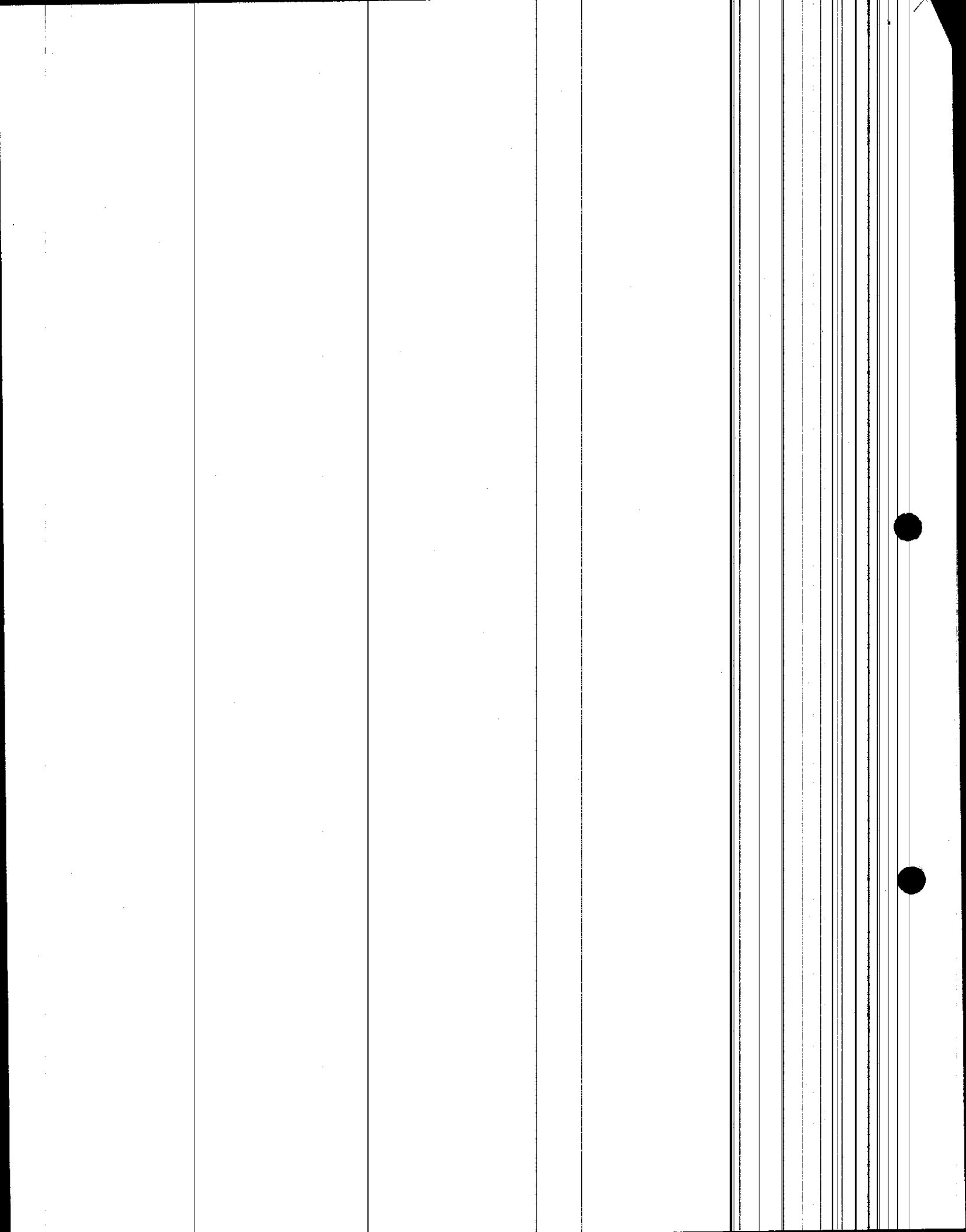
FIG. 5. Graphical representation, generated by the TMHMM program, indicating probable orientations of five transmembrane helices in the prM-E protein expressed by pCDJE2-7 (A), pcDNA3JEME (B), and pJME (C). ER, endoplasmic reticulum.

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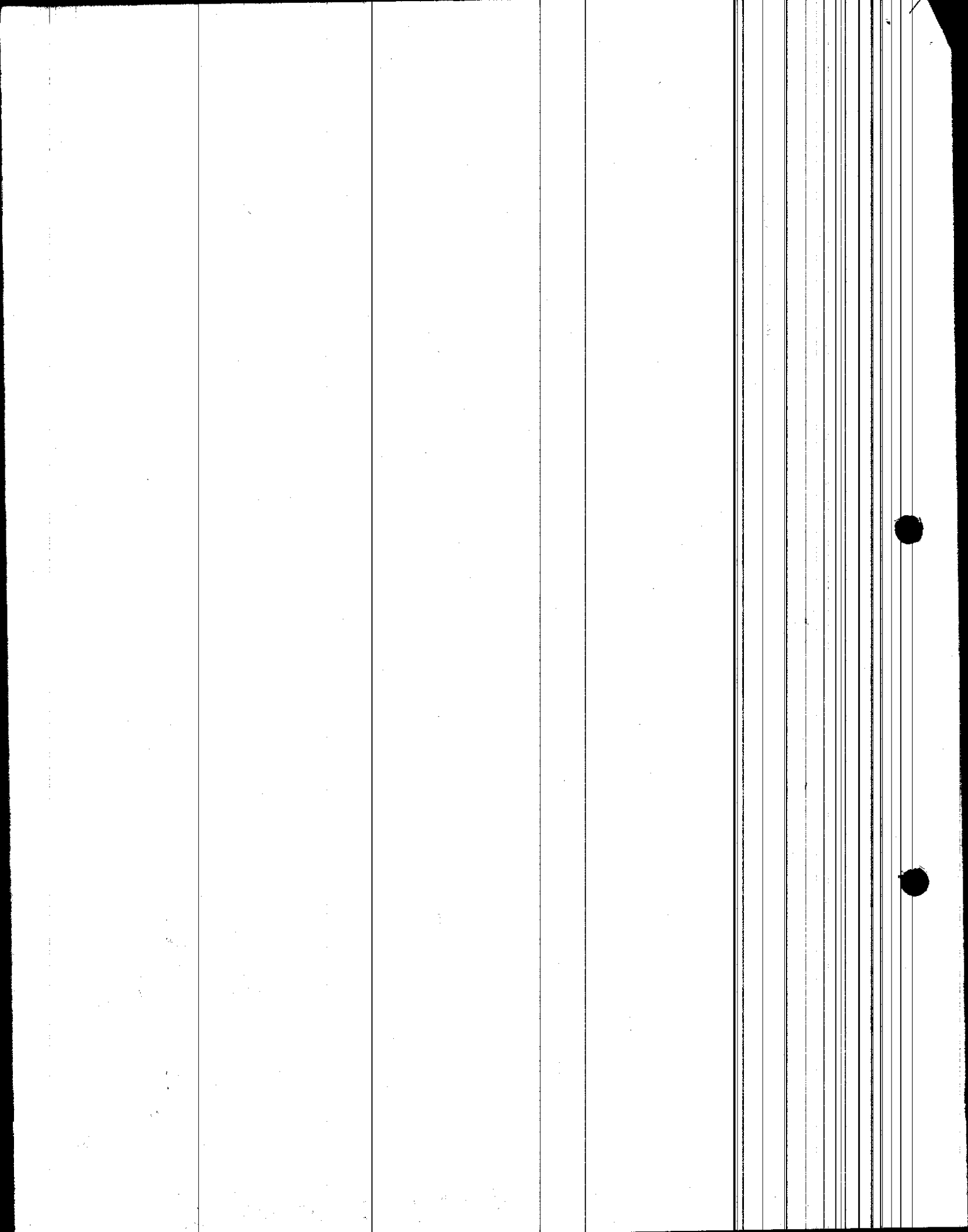
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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES**

2020
Bogotá, D.C., (30, agosto, 2007)

Abogado
ALVARO CORREA ORDOÑEZ

Apoderado
The government of the united States of America represented by the Secretary of the
Department of Health and Human Services Center for Disease Control and
Prevention

ASUNTO	Radicación	03-97535
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	
	Folios	16798

1. Documentos estudiados

- 1.1. Descripción: Folios: 40 a 66 como se radicó inicialmente
- 1.2. Reivindicaciones: 1 a 54 folios 518 A 527 como se radicó inicialmente
- 1.3. Prioridad: US 09/286.115 de abril 4 de 2007.

2. Objeto de la invención

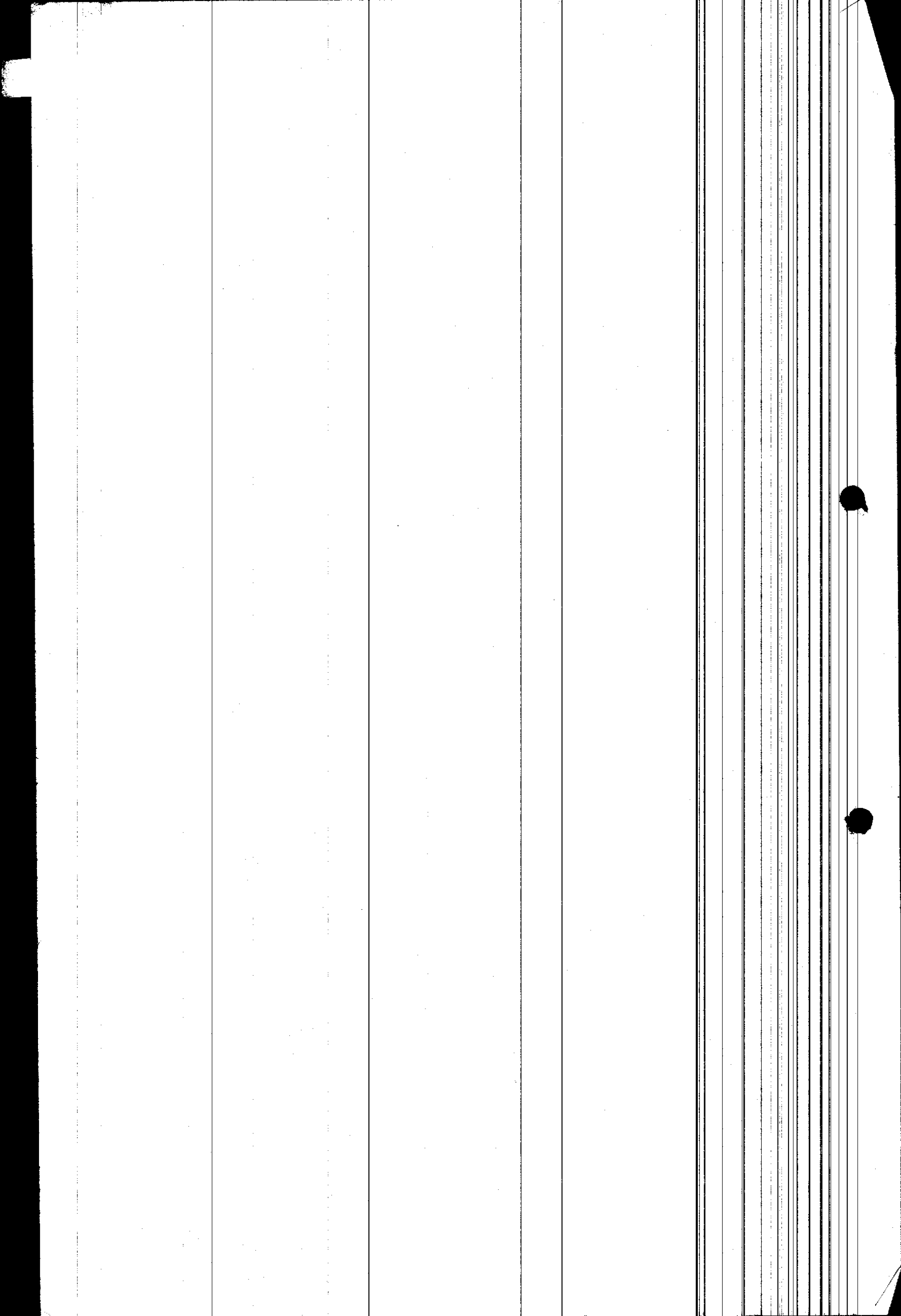
- Título de la solicitud: Vacunas de acido nucleico para la infección de flavivirus.

La presente solicitud se refiere a vacunas de acido nucleico de flavivirus que consisten en una unidad transcripcional que codifica para una señal de una proteína estructural de un primer flavivirus y para un antígeno de un segundo flavivirus. Realizaciones preferenciales de la presente invención incluyen aquellas donde la secuencia es una secuencia señal de virus de encefalitis japonesa. (JEV) y el antígeno se selecciona entre el grupo que consiste de virus de fiebre amarilla, virus de serotipos 2,3,4 de encefalitis japonesa, virus Powasan y virus de West Nile.

- Según la Clasificación Internacional de Patentes (IPC) C12Q 1/70, A61K 39/12.

3. De la solicitud de Patente (según el caso)

En el concepto técnico 1516, en cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se requirió al solicitante para que diera respuesta dentro del término de sesenta días a dicha comunicación indicando que en



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caso de no dar respuesta o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegaría la patente.

3.1. Descripción (artículo 28 D. 486)

En el mencionado concepto previo se indicó claramente que en la descripción no se encuentra sustento experimental que pudiese demostrar un efecto técnico para todas las unidades transcripción que resultan de combinar todas las secuencias señal de un 1er flavivirus con todos los posibles antígenos derivados de flavivirus.

A lo anterior el solicitante en su respuesta del 19 de diciembre indica que el sustento experimental se encuentra en las páginas 126 a 173. Sin embargo no cae en la cuenta que la objeción presentada por este despacho se refiere a la comprobación experimental del "efecto técnico" de las unidades transcripcionales reclamadas. Es decir, la sola indicación de los materiales y métodos (bien conocidos en el arte) para la construcción de unidades transcripcionales no es suficiente para llegar a inferir que la totalidad de los flavivirus resultantes van a presentar un efecto sorprendente. Así pues que se mantiene la recomendación de limitar la reclamación a aquellos flavivirus que realmente fueron evaluados y para los cuales se puede evidenciar un efecto sorprendente respecto de los flavivirus quiméricos existentes en el estado de la técnica.

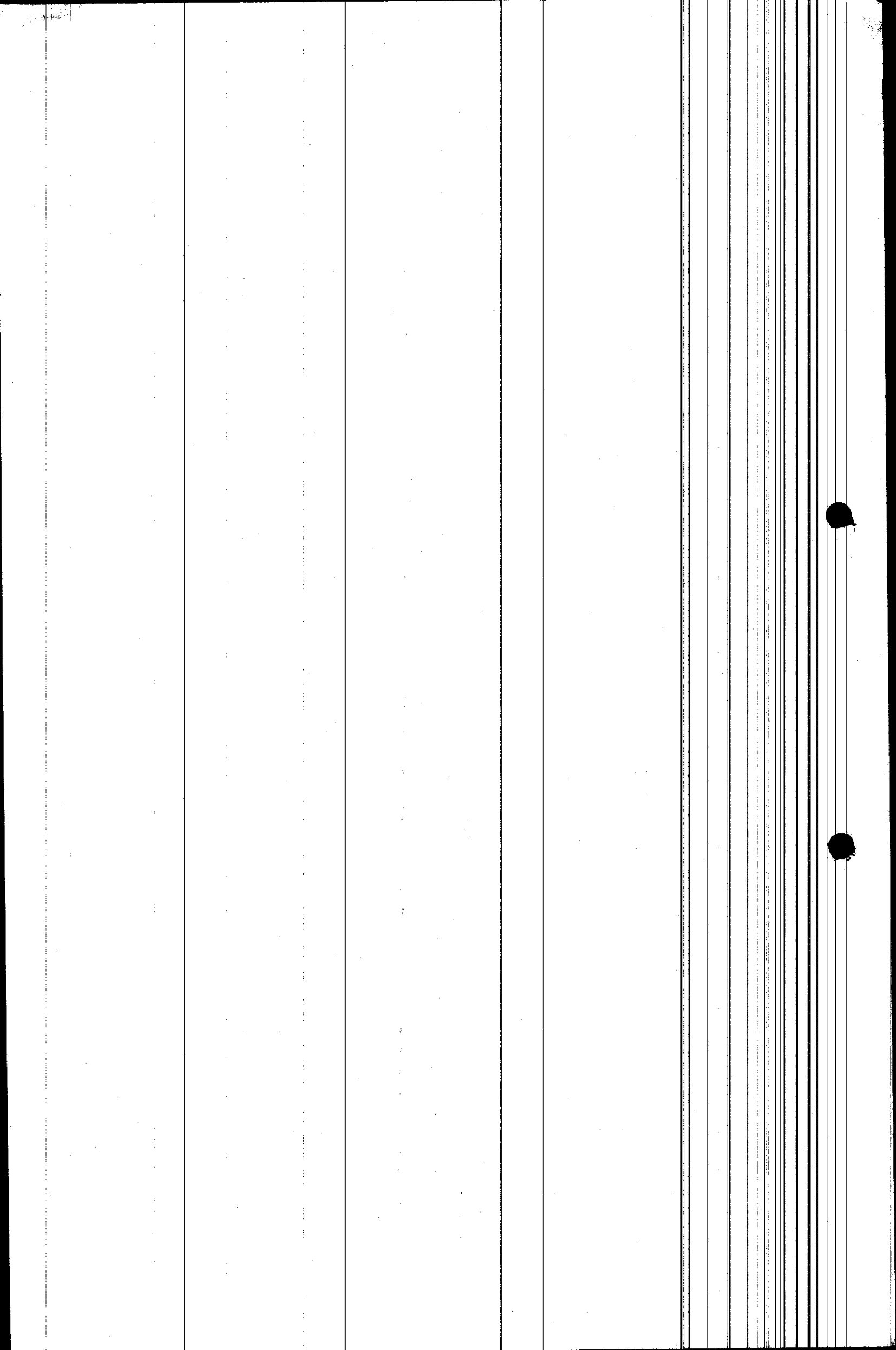
3.2. Reivindicaciones (artículo 30 D 486)

En el concepto anterior se indicó que las reivindicaciones 1 a 10 resultaban demasiado amplias al reclamar un sinnúmero de ácidos nucleicos resultantes de combinar todas las secuencias señal y codificantes de antígenos dentro de todos los tipos de flavivirus. Se mencionó en dicha oportunidad que solo unos pocos presentaban sustento. Sin embargo el solicitante hace caso omiso de lo anterior argumentando que existe soporte para la materia reclamada en algunos apartes de la descripción. Es importante aclarar que una simple mención de lo que se puede llegar desarrollar, no constituye divulgación suficiente para que un tercero pueda repetir la invención. Así pues, que las objeciones en cuanto a claridad y suficiencia efectuadas previamente se mantienen.

La única forma de caracterizar correctamente un constructo de ácido nucleico es mediante su secuencia de nucleótidos, y ésta debe ser definida concretamente en las reivindicaciones que lo reclaman. Para información del señor memorialista las reivindicaciones establecen el alcance de la protección a conferir, deben ser claras por sí mismas y no deben hacer referencia a la descripción u otros apartes de la solicitud. Así pues que no es correcto indicar que las reivindicaciones no son amplias por cuanto las secuencias se encuentran definidas en el aparte de listado de secuencias. En cada cláusula se debe definir las características técnicas de cada objeto reclamado; en este caso cada constructo debe definirse mediante su estructura (SECUENCIA).

Nuevamente se sugiere limitar la reclamación a las unidades transcripcionales que el solicitante verdaderamente desarrolló y evaluó. Por ejemplo PCBJE-1-14 caracterizándola mediante su secuencia de nucleótidos en la reivindicación.

Ahora bien, considerando que en el estado de la técnica ya se han divulgado flavivirus quiméricos que comprenden una secuencia señal de un primer flavivirus junto con una secuencia antígeno de un segundo flavivirus, es sumamente



importante reclamar aquellas unidades que fueron evaluadas y exhiben un efecto sorprendente, pues al mantener las reivindicaciones demasiado generales (actuales reivindicaciones 1 a 10, 12 a 17) se cae en la falta de sustento pero sobretodo se afecta su nivel inventivo por cuanto es imposible demostrar sin datos experimentales que determinado objeto presenta resultados inesperados o sorprendentes.

Las nuevas reivindicaciones 12 a 16 siguen siendo simples selecciones técnicas que cualquier persona medianamente versada en la materia realizaria para el diseño de unidades transcripcionales quiméricas, y que no constituyen los elementos característicos de la invención que contribuyan a su diferenciación de la materia existente en la naturaleza.

Por ultimo se sugiere seguir las recomendaciones efectuadas en el concepto técnico anterior para la redacción de las reivindicaciones en las que se indica como definir cada unidad transcripcional correctamente observando que se cumpla a la vez con el requisito de unidad de invención.

"...se recomienda reclamar en la cláusula independiente las invenciones verdaderamente desarrolladas indicando cuales son las secuencias señales y codificantes de antígenos que la comprenden acompañada de la secuencia de nucleótidos que la define. Por ejemplo: una unidad transcripcional de flavivirus que codifica una secuencia de señal de virus de encefalitis japonesa (SEQ ID _____), una proteína M (SEQ ID _____) y proteína E (SEQ ID No) _____ de virus _____. En las cláusulas dependientes indicar el nombre de los plásmidos que las comprenden y así mismo las composiciones farmacéuticas que lo pueden contener."

Por otra parte cabe resaltar que las cláusulas 28 a 30, 32, 34, 36 siguen reclamando materia indefinida que no permite establecer con exactitud el objeto a proteger y que a pesar de ser objetada no fue modificada, ni retirada del capítulo reivindicatorio. Lo mismo sucede con las cláusulas 44 a 54, en las que no se define una secuencia específica para el ácido nucleico reclamado y de esta forma es imposible efectuar una comparación objetiva con el estado de la técnica. (El origen de los antígenos no define sus características estructurales).

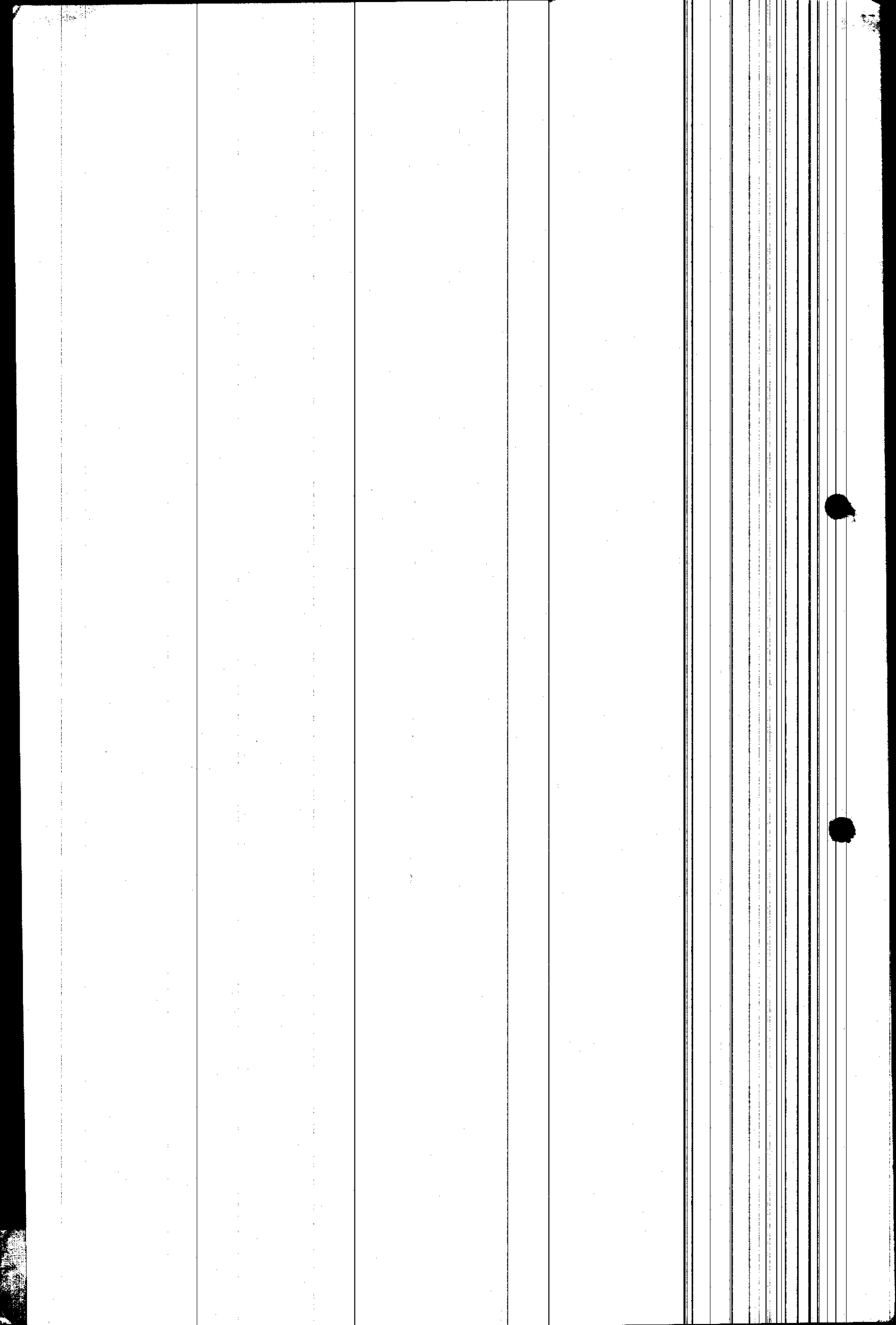
La reivindicación 38 reclama un antígeno que no está definido por sus características estructurales (secuencia), mas aun cuando el acido nucleico que lo codifica tampoco lo está.

La reivindicación 40 reclama un anticuerpo no definido por sus características técnicas propias, si no como resultado de un proceso biológico natural, el cual puede no ser patentable.

4. Unidad de invención (artículo 25 D 486)

Como se ha indicado en varias oportunidades, la gran cantidad de constructos de ácido nucleico que resultan de efectuar las distintas combinaciones propuestas por el solicitante además de no presentar suficiente sustento, carecen de un único concepto común inventivo (estructura y función común), por lo que las cláusulas 1 a 10 y 12 a 16, 28 a 38 y 44 a 54 no guardan unidad de invención entre ellas.

Para la formulación de las nuevas reivindicaciones debe tenerse en cuenta que los distintos constructos (unidades transcripcionales) a reclamar deben presentar un concepto común inventivo, es decir deben cumplir con dos condiciones: i) una secuencia de nucleótidos común y ii) una función común comprobada, para que se puedan tratar como una única invención.



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Debe tenerse en cuenta que el solicitante no es el primero que ha propuesto combinar diferentes antígenos provenientes de distintos flavivirus, para la construcción de unidades quiméricas y por esta razón debe establecerse ¿cuál es la estructura o secuencia nueva e inventiva? que es la responsable de la actividad sorprendente y que constituye el concepto común entre los diferentes objetos reclamados.

5. No es una invención (artículo 15 D 486 literal a)-f)) (según causal)

La reivindicación 40 reclama un anticuerpo producto de un proceso biológico natural, no creado por el solicitante si no por la naturaleza, por lo que se encuentra exceptuado de patentabilidad.

6. Excepción a la patentabilidad (artículo 20 D486 literal a)-d)) (según causal)

Las reivindicaciones 18 a 27, 31, 33, 35 reclaman métodos de tratamiento que pueden ser aplicados a seres humanos. Las reivindicaciones 39, 41 a 43 reclaman un método de diagnóstico de la presencia de flavivirus en una muestra, que puede llevar a determinar la condición de infección en un paciente. La materia comprendida en las anteriores cláusulas se encuentra dentro del alcance del literal d) del artículo 20 de la D.486 y por ende no es patentable.

Hasta el momento el solicitante se ha rehusado a subsanar las objeciones encontradas por este Despacho, por lo que resulta imperativo mencionar que de no efectuarse las correcciones pertinentes, la presente solicitud no podrá ser objeto de patente a la luz de la Decisión 486.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el artículo 8 de la resolución reglamentaria No. 00210 del 15 de enero de 2001, advirtiéndole que contra él no procede recurso alguno en la vía gubernativa.

ALIX CARMENZA CESPEDES DE VERGEL

Jefe de División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha

28 DIC. 2007

CONCEPTO TECNICO No. 2715	EXAMINADOR TECNICO Código No.202063
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