

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

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Abogada
EMILIO FERRERO

ASUNTO	Radicación	07-044491
	Trámite	11
	Evento	378
	Actuación	519
	Oficio	
	Folios	8 . 07799

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐

No. Solicitud Internacional PCT/US2005/035785

Fecha de Presentación Internacional 4/Octubre/2005

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 233 a 329	4/Mayo/2007
Reivindicaciones:	Folios 309 a 327	4/Mayo/2007
Dibujos:	Folios 329 a 410	4/Mayo/2007
Listado de Secuencias:	Folios 411 a 493	4/Mayo/2007
Prioridad:	US 10/958,267	5/Octubre/2004

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Vectores quiméricos".

El problema planteado en esta solicitud consiste en la necesidad de suministrar alternativas para el suministro eficiente de los replicones Semliki Forest (SFV) *in vivo*.

Expediente 07-44491 1er Art 45

Para resolver este problema técnico la solicitud en estudio proporciona vectores de virus de viruela recombinantes y virus que expresan secuencias heterólogas de interés de replicón viral capaz de amplificación como ARN.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 15/863.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 65 – 104 no es patentable de acuerdo con el Art citado.

5. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque ésta hace referencia a un vector quimérico de virus de la viruela aviar recombinado, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio carece de concisión porque utiliza las expresiones “una secuencia de ácido nucleico”, “un promotor de viruela”, “un promotor vírico”, “una secuencia heteróloga de interés”, “una polimerasa específica”, “vacuna antivírica”, “promotor de viruela aviar H6”, “un promotor de viruela aviar sintético”, “se muta el promotor de viruela aviar”, “un locus de inserción C6”, “secuencias C6L y C6R de viruela”, “un locus de inserción F8”, “un alfavirus”, “virus Semliki Forest”, “un virus de ARN monocatenario de sentido positivo”, “poliovirus”, “rinovirus”, “promotor 26S”, “se muta la polimerasa nsP2 alfavírica”, “un mutante condicional”, “un antígeno”, “un fragmento antigénico de una proteína”, “un agente terapéutico”, “una citocina”, “una toxina”, “un inmunomodulador”, “un ARN antisentido”, “un ARN catalítico”, “ARN pequeño de interferencia”, “una proteína”, “un péptido”, “un anticuerpo”, “un fragmento de unión a antígeno de un anticuerpo”, “un adyuvante”, “un plásmido donante”, “una endonucleasa de restricción”, “una célula permisiva”, “un gen reportero”, lo cual dificulta determinar la extensión del objeto que se busca proteger.

Se recuerda al solicitante que una molécula de ácido nucleico y un polipéptido se caracterizan por sus secuencias de polinucleótidos y aminoácidos, respectivamente. Un vector y sus regiones promotoras, locus y secuencias heterólogas se caracterizan por una secuencia de polinucleótidos. En el caso de un anticuerpo, éste se debe caracterizar por la secuencia de aminoácidos de las regiones variables de la cadena ligera y pesada o las secuencias de los 6 CDRs de las cadenas pesada y ligera.

En la reivindicación 41 se utiliza el término “Alinear”. Se sugiere al solicitante sustituir este término por uno más apropiado, como “Linearizar”, debido a que es la razón por la cual se utiliza una endonucleasa de restricción.

Se requiere al Solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Octubre 5 de 2004 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	US20030119182	Alphavirus replicon vector systems	26/Junio/2003
D2	US20040029279	Packaging of positive-strand RNA virus replicon particles	12/Febrero/2004

D1<http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en> EP&FT=D&date=20030626&CC=US&NR=2003119182A1&KC=A1 divulga un vector recombinante de poxvirus como Vaccinia Ankara modificado (párrafos 8-25, 44 y ejemplo 9).

D2<http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en> EP&FT=D&date=20040212&CC=US&NR=2004029279A1&KC=A1 divulga un vector recombinante de poxvirus como Vaccinia Ankara modificado, NYVAC o avipoxvirus (párrafos 18, 25, 19, 25, 103, 122 y Figura 21).

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un vector de virus de la viruela aviar recombinado que comprende una secuencia de ácido nucleico operablemente ligada a un promotor de viruela aviar que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón vírico transcrito capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés, y una polimerasa específica para multiplicación del replicón vírico.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un vector de virus de la viruela aviar recombinado que comprende: a) una secuencia de ácido nucleico operablemente ligada a un promotor de viruela aviar que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón vírico transcrito capaz de amplificación como ARN,	Un vector recombinante de poxvirus como Vaccinia Ankara modificado que comprende una secuencia de ácido nucleico operablemente ligada a un promotor que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón alfaviral (VEE, Sindbis o SFV) capaz de amplificación como ARN, (Párrafos 8-25 y ejemplo 9)	Un vector recombinante de poxvirus como Vaccinia Ankara modificado, NYVAC o avipoxvirus que comprende una secuencia de ácido nucleico operablemente ligada a un promotor poxviral que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón viral (virus RNA de cadena positiva como alfavirus, como VEE o poliovirus) capaz de amplificación como ARN, (Párrafos 18, 19, 25, 122)
b) en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una	donde el replicón vírico transcrito comprende al menos un promotor vírico (promotor 26S) operablemente ligado a	Donde el replicón viral transcrito comprende al menos un promotor viral operablemente ligado a una secuencia

secuencia heteróloga de interés, y una polimerasa específica para multiplicación del replicón vírico.	una secuencia heteróloga de interés, y una secuencia que codifica una polimerasa específica para la replicación del replicón viral. (Párrafo 44)	heteróloga de interés (que codifica una citocina, un antígeno). (Párrafos 25 y 103)
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Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga un vector recombinante de poxvirus como Vaccinia Ankara modificado que comprende una secuencia de ácido nucleico operablemente ligada a un promotor que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón alfaviral (VEE, Sindbis o SFV) capaz de amplificación como ARN, (párrafos 8-25 y ejemplo 9), donde el replicón vírico transcrito comprende al menos un promotor vírico (promotor 26S) operablemente ligado a una secuencia heteróloga de interés, y una secuencia que codifica una polimerasa específica para la replicación del replicón viral (párrafo 44).

La diferencia entre la invención, definida en la reivindicación 1 y el vector de D1 consiste en que D1 no menciona que el vector recombinante de poxvirus que comprende una secuencia de ácido nucleico está operablemente ligada a un promotor de viruela aviar que dirige la transcripción del replicón viral.

D2 divulga un vector recombinante de poxvirus como Vaccinia Ankara modificado, NYVAC o avipoxvirus que comprende una secuencia de ácido nucleico operablemente ligada a un promotor poxviral que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón viral (virus RNA de cadena positiva como alfavirus, como VEE o poliovirus) capaz de amplificación como ARN, (párrafos 18, 19, 25, 122), donde el replicón viral transcrito comprende al menos un promotor viral operablemente ligado a una secuencia heteróloga de interés (que codifica una citocina, un antígeno) (párrafos 25 y 103). D2 también divulga que la secuencia heteróloga de interés se encuentra corriente debajo de la polimerasa (Figura 21).

La diferencia entre la invención, definida en la reivindicación 1 y el vector de D2 consiste en que la polimerasa no es un mutante condicional.

El efecto que logra el incluir un promotor de viruela aviar es que permite la transcripción del replicón viral.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el vector recombinante conocido en D1 para dirigir la transcripción de un replicón RNA alfaviral".

Sin embargo, la utilización de un promotor poxviral para dirigir la transcripción de un replicón RNA alfaviral, ya está divulgada en el documento D2 que se refiere a un vector recombinante de poxvirus que comprende una secuencia de ácido nucleico operablemente ligada a un promotor poxviral que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón viral (virus RNA de cadena positiva como alfavirus, como VEE o poliovirus) capaz de amplificación como ARN, (párrafos 18, 19, 25, 122), donde el replicón viral transcrito comprende al menos un promotor viral operablemente ligado a una secuencia heteróloga de interés (que

codifica una citocina, un antígeno) (párrafos 25 y 103).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el promotor poxviral de D2, en el contexto de un vector poxviral que comprende el replicón de D1 para llegar así al objeto de la reivindicación 1 de la solicitud, más aún teniendo en cuenta que es bien conocido en el estado del arte¹ (Resumen, Pág. 3605 y 3608) que es posible utilizar polimerasas mutantes nsP2 sensibles a la temperatura, para controlar la síntesis de cadenas negativas. Por lo cual se considera obvia.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y sus dependientes 2 – 20 y las reivindicaciones 21 – 40 es obvia y no tiene Nivel Inventivo.

La reivindicación 41 consiste en un método para producir un vector de virus de la viruela aviar recombinado, que comprende las etapas de:

- a. Alinear un plásmido donante con una endonucleasa de restricción, en donde el plásmido donante comprende sitios de clivaje de endonucleasa de restricción o un sitio de clonación múltiple; y
- b. Ligar al menos una secuencia de ácido nucleico que comprende
 - i. Un promotor operablemente ligado a la molécula de ácido nucleico que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón vírico transcrito capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés y una polimerasa específica para multiplicación del replicón vírico
 - ii. Una secuencia de promotor de viruela aviar, y
 - iii. Flanqueamiento de las secuencias de inserción (i) y (ii) que tienen sitios de clivaje de endonucleasa de restricción complementaria al plásmido donante a la secuencia de ácido nucleico,

que produce por lo tanto el vector de virus de la viruela aviar recombinado.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un método para producir un vector de virus de la viruela aviar recombinado, que comprende las etapas de: a) Alinear un plásmido donante con una endonucleasa de restricción, en donde el plásmido donante comprende sitios de clivaje de endonucleasa de restricción o un sitio de clonación múltiple; y	Un método para producir un vector recombinante de poxvirus como Vaccinia Ankara modificado, que comprende linealizar un plásmido donante con una endonucleasa de restricción, en donde el plásmido donante comprende sitios de clivaje de endonucleasa de restricción o un sitio de clonación múltiple; y (ejemplos 1-5)	Un método para producir un vector recombinante de poxvirus como Vaccinia Ankara modificado, NYVAC o avipoxvirus que comprende linealizar un plásmido donante con una endonucleasa de restricción, en donde el plásmido donante comprende sitios de clivaje de endonucleasa de restricción o un sitio de clonación múltiple; y (ejemplo 1)

1. D. Sawicki and S. Sawicki. A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. Journal of Virology, Vol. 67, No. 6, Junio 1993.

Características esenciales	D1	D2
b) Ligar al menos una secuencia de ácido nucleico que comprende 1. Un promotor operablemente ligado a la molécula de ácido nucleico que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón vírico transcrito capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés y una polimerasa específica para multiplicación del replicón vírico 2. Una secuencia de promotor de viruela aviar, y 3. Flanqueamiento de las secuencias de inserción (i) y (ii) que tienen sitios de clivaje de endonucleasa de restricción complementaria al plásmido donante a la secuencia de ácido nucleico, que produce por lo tanto el vector de virus de la viruela aviar recombinado.	Ligar al menos una secuencia de ácido nucleico que comprende: i) un promotor operablemente ligado a la molécula de ácido nucleico que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón alfaviral (VEE, Sindbis o SFV) capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés y una polimerasa específica para multiplicación del replicón vírico ii) Una secuencia de promotor, y iii) Flanqueamiento de las secuencias de inserción (i) y (ii) que tienen sitios de clivaje de endonucleasa de restricción complementaria al plásmido donante a la secuencia de ácido nucleico, que produce por lo tanto el vector de virus de la viruela aviar recombinado. (ejemplos 1-5)	Ligar al menos una secuencia de ácido nucleico que comprende: i. un promotor operablemente ligado a la molécula de ácido nucleico que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón viral (virus RNA de cadena positiva como alfavirus, como VEE o poliovirus) capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés (que codifica una citocina, un antígeno) y una polimerasa específica para multiplicación del replicón vírico ii. Una secuencia de promotor poxviral, y iii. Flanqueamiento de las secuencias de inserción (i) y (ii) que tienen sitios de clivaje de endonucleasa de restricción complementaria al plásmido donante a la secuencia de ácido nucleico, que produce por lo tanto el vector de virus de la viruela aviar recombinado. (ejemplo 1)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga un método para producir un vector recombinante de poxvirus como Vaccinia Ankara modificado que comprende linealizar un plásmido donante con una endonucleasa de restricción, en donde el plásmido donante comprende sitios de clivaje de endonucleasa de restricción o un sitio de clonación múltiple (ejemplos 1-5); y ligar al menos una secuencia de ácido nucleico que comprende: i) un promotor operablemente ligado a la molécula de ácido nucleico que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón alfaviral (VEE, Sindbis o SFV) capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés y una polimerasa específica para multiplicación del replicón vírico; ii) una secuencia de promotor, y iii) flanqueamiento de las secuencias de inserción (i) y (ii) que tienen sitios de clivaje de endonucleasa de restricción complementaria al plásmido donante a la secuencia de ácido nucleico, que produce por lo tanto el vector de virus de la viruela aviar recombinado (ejemplos 1-5).

La diferencia entre la invención, definida en la reivindicación 41 y el método de D1 consiste en que D1 no menciona que el vector recombinante de poxvirus que comprende una secuencia de ácido nucleico y que está operablemente ligada a un promotor de viruela aviar que dirige la transcripción del replicón viral.

D2 divulga un método para producir un vector recombinante de poxvirus como Vaccinia Ankara modificado, NYVAC o avipoxvirus que comprende linealizar un plásmido donante con una endonucleasa de restricción, en donde el plásmido donante comprende sitios de clivaje de endonucleasa de restricción o un sitio de clonación múltiple (ejemplo 1); y ligar al menos una secuencia de ácido nucleico que comprende: i) un promotor operablemente ligado a la molécula de ácido nucleico que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón viral (virus RNA de cadena positiva como alfavirus, como VEE o poliovirus) capaz de amplificación como ARN, en donde el replicón vírico transcrito comprende al menos un promotor vírico operablemente ligado a una secuencia heteróloga de interés (que codifica una citocina, un antígeno) y una polimerasa específica para multiplicación del replicón vírico; ii) una secuencia de promotor poxviral; y iii) flanqueamiento de las secuencias de inserción (i) y (ii) que tienen sitios de clivaje de endonucleasa de restricción complementaria al plásmido donante a la secuencia de ácido nucleico, que produce por lo tanto el vector de virus de la viruela aviar recombinado (ejemplo 1).

La diferencia entre la invención, definida en la reivindicación 41 y el método de D2 consiste en que la polimerasa no es un mutante condicional.

El efecto que logra el incluir un promotor de viruela aviar es que permite la transcripción del replicón viral.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el método conocido en D1 para obtener un vector de virus de viruela aviar recombinado que comprenda un promotor para dirigir la transcripción de un replicón RNA alfaviral".

Sin embargo, la utilización de un promotor poxviral para dirigir la transcripción de un replicón RNA alfaviral, ya está divulgada en el documento D2 que se refiere a un vector recombinante de poxvirus que comprende una secuencia de ácido nucleico operablemente ligada a un promotor poxviral que dirige la transcripción de la secuencia de ácido nucleico para generar un replicón viral (virus RNA de cadena positiva como alfavirus, como VEE o poliovirus) capaz de amplificación como ARN, (párrafos 18, 19, 25, 122), donde el replicón viral transcrito comprende al menos un promotor viral operablemente ligado a una secuencia heteróloga de interés (que codifica una citocina, un antígeno) (párrafos 25 y 103).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el promotor poxviral de D2, en el contexto de un método para preparar el vector poxviral que comprende el replicón de D1 para llegar así al objeto de la reivindicación 41 de la solicitud.

De manera que la solución propuesta por esta solicitud en la reivindicación 41 y sus dependientes 42 – 64 es obvia y no tiene Nivel Inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US20030119182	1 - 64	Nivel Inventivo
D2	US20040029279	1 - 64	Nivel Inventivo

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 (60) 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.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
13 MAYO 2012

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Revisado por: Consuelo Leguizamón.

A Second Nonstructural Protein Functions in the Regulation of Alphavirus Negative-Strand RNA Synthesis

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Previous studies (D. L. Sawicki, D. B. Barkhimer, S. G. Sawicki, C. M. Rice, and S. Schlesinger, *Virology* 174:43-52, 1990) identified a temperature-sensitive (*ts*) defect in Sindbis virus nonstructural protein 4 (nsP4) that reactivated negative-strand synthesis after its normal cessation at the end of the early phase of replication. We now report identification of two different *ts* alterations in nsP2 of Ala-517 to Thr in *ts*17 or Asn-700 to Lys in *ts*133 that also reactivated negative-strand synthesis. These same mutations caused severely reduced protease processing by nsP2 and recognition of the internal promoter for subgenomic mRNA synthesis and were responsible for the conditional lethality and RNA negativity of these mutants. Reactivation of negative-strand synthesis by mutations in nsP2 resembled that in nsP4: it was a reversible property of stable replication complexes and did not require continuation of viral protein synthesis. Recombinant viruses expressing both mutant nsP2 and nsP4 reactivated negative-strand synthesis more efficiently than did either mutant protein alone, consistent with the hypothesis that both nsP2 and nsP4 participate in template recognition. We propose that these alterations cause nsP2 and nsP4 to switch from their normal preference to recognize negative strands as templates to recognize positive strands and thereby mimic the initial formation of a replication complex.

Four members of the Sindbis superfamily (1, 7, 8), Sindbis virus (SIN) and Semliki Forest virus of the *Alphavirus* genus of the *Togaviridae* family, tobacco mosaic virus of the *Tobamoviridae* family, and brome mosaic virus of the *Bromoviridae* family, selectively cease negative-strand RNA synthesis at the end of the early phase of the replication cycle (15, 19, 27, 30). For alphaviruses, inhibition of translation during the early phase inhibits negative-strand synthesis and limits positive-strand transcription to the rate existing at the time translation was inhibited (27, 30). During the late phase, when negative-strand synthesis is no longer detectable, inhibition of translation has no effect on transcription (27, 30). Thus, both the positive- and negative-strand polymerases are composed of viral nonstructural proteins (nsPs) synthesized early in infection, but only the negative-strand polymerase is unstable and needs to be continuously produced. SIN nsPs, numbered according to their gene order, are translated initially as polyproteins nsP123 and nsP1234, the latter arising from read-through of an opal codon at the end of the nsP3 open reading frame, before cleavage by the nsP2 protease to yield nsP1, nsP2, nsP3, and nsP4 or the fusion protein nsP34 (3, 4, 12, 13, 22, 34, 35, 38).

The mechanism responsible for cessation of negative-strand synthesis is unknown. The existence of SIN temperature-sensitive (*ts*) mutant *ts*24 and its revertants (e.g., 24R1), which were capable of turning back on negative-strand synthesis at 40°C late in infection either in the absence of new protein synthesis (28, 31, 32) or in the presence of wild-type nsPs (25), suggested that cessation did not involve an irreversible loss of negative-strand polymerases shortly after their synthesis or a *trans*-active viral protein that turned off negative-strand synthesis. Although the mutation responsible for *ts*24 conditional lethality mapped to nsP2 (10), the mutation conferring *ts* reactivation of negative-strand synthesis was in nsP4, which contains a conserved

XGDD replicase sequence (14, 16, 21, 37) and functions in template recognition and elongation (25). Two other A complementation group mutants, *ts*17 and *ts*133, exhibited a similar *ts* reactivation of negative-strand synthesis (28) that, however, was not retained by their respective revertants.

Analysis of nonstructural polyprotein cleavage patterns led de Groot and colleagues (3, 11) and Strauss and Strauss (38) to suggest that the accumulation of nsP2 proteases would lead to cessation of negative-strand synthesis because polyprotein processing would be altered late in infection and prevent production of nsP4 but, in turn, favor production of nsP34. Our results (28) with *ts*17 were interpreted to support this model because loss of 26S mRNA synthesis and reactivation of negative-strand synthesis after a shift to 40°C would occur from nsP34 cleavage and release of nsP4. We set out to test directly their model with use of *ts*17 and *ts*133 by mapping the responsible mutations and determining whether an unstable nsP, such as nsP34, played a role in reactivation.

The mutations in *ts*17 and *ts*133 map to nsP2. Reversion frequencies suggested that a single-point mutation in *ts*17 and one or possibly two mutations in *ts*133 (31) were responsible for *ts* reactivation of negative-strand synthesis. To map the mutation in *ts*17, we used hybrid cDNA clones originally constructed by Hahn et al. (10) (Table 1). They reported that only Toto:*ts*17B- and Toto:*ts*17B1-derived viruses were *ts* for plaque formation, RNA negativity, and nonstructural polyprotein cleavage. We confirmed these results (Table 1; data not shown) and, furthermore, found that only *ts*17, Toto:*ts*17B, and Toto:*ts*17B1 viruses had *ts* 26S mRNA synthesis and reactivated negative-strand synthesis (Table 1). Sequencing of Toto:*ts*17B1 cDNA independently identified a single mutation at nucleotide (nt) 3228 and its true reversion in a *ts*17 revertant genome (data not shown; 10). Our findings thus extend previous analyses by showing that the predicted change of Ala-517 to Thr in nsP2 was responsible for all phenotypes of *ts*17.

By creating similar recombinant clones, the *ts*133 mutation was mapped to the B1 region (Table 1), which contained

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TABLE 1. Analysis of ts17 and ts133 constructs

Parental virus or recombinant clone	Replaced fragment (nt)	Phenotype ^a	% of labeled RF RNA in negative strands ^b
SIN HR		wt	5.8
ts17		ts	16.1
Toto:ts17A ^c	504-2713	wt	4.6
Toto:ts17B	2713-5262	ts	17.5
Toto:ts17B1	2713-4280	ts	17.7
Toto:ts17C	5262-7334	wt	5.7
ts133		ts	21.2
Toto:ts133B1	2288-4280	ts	19.6
Toto:ts133CX ^d	2713-3594	wt	5.9
Toto:ts133XA ^d	3594-4280	ts	14.8

^a Viruses having a phenotype indicated as wild type (wt) gave similar numbers of plaques at 30°C and 40°C, parental virus levels of overall RNA synthesis when infected cells were maintained at 40°C, and continued synthesis of 26S mRNA after a shift to 40°C. The latter was determined from the molar ratio of incorporation into 49S and 26S RNAs obtained after electrophoresis of cell lysates on agarose gels and cutting out and counting the respective two areas of the gels.
^b Negative-strand synthesis was determined as described previously (30).
^c The ts17 recombinant clones are those described by Hahn et al. (10).
^d Toto:ts133CX is the recombinant clone containing the *Cla*I (nt 2713)-to-*Xmn*I (nt 3594) fragment from ts133 cDNA, and Toto:ts133XA is that containing the *Xmn*I-to-*Avr*II (nt 4280) fragment from ts133. The clones were constructed as described previously (23, 25) and sequenced (24).

base changes at nt 2605 (A → T), 3579 (A → G), 3779 (C → G), and 3897 (C → G) from the Totol101 sequence. A revertant of ts133 (28, 31) retained the changes at nt 2605 and 3897 but reverted nt 3579 to the parental base and restored Asn-700 by changing the G at nt 3779 to a U instead of the parental C. Only recombinant virus containing the sequence from nt 3594 to 4280 reproduced the phenotypes of ts133 (Table 1). We conclude that in ts133, a single mutation at nt 3779, predicting a change of Asn-700 to Lys in nsP2, was necessary and sufficient for its ts phenotypes, and unlike the case for ts24, conditionally lethal mutations led to ts reactivation of negative-strand synthesis by ts17 and ts133. Because the ts133 (28) and ts24 (10, 28) mutations had reversion frequencies (10⁻⁷ to 10⁻⁸) lower than expected for a single mutation in an RNA genome, sequences surrounding these nucleotides may influence reversion to the wild-type sequence. As shown in Fig. 1, the region of nsP2 containing the ts133 mutation is relatively highly conserved among alphaviruses and near the mutation in ts24, which also makes the protease ts. It contains a pattern of leucine residues that,

TABLE 2. Reversibility of 26S mRNA synthesis

Incubation conditions	Cycloheximide	49S/26S RNA molar ratio	
		ts17	ts133
Maintained 30°C	—	0.10	
	+	0.06	0.11
Shifted to 40°C at 6 h p.i.	+	2.73	1.31
Shifted to 40°C at 6 h p.i., returned to 30°C at:			
7 h p.i.	+	0.06	0.14
8 h p.i.	+	0.06	0.16
9 h p.i.	+	0.07	0.15

while not heptadic, is present in the otherwise nonconserved rubella virus sequence (5) and may have a structural role in hydrophobic interactions. The ts17 change in nsP2 is not in a highly conserved region (10), and thus critical residues or overall structure may be important.
Reversal of ts RNA phenotypes is independent of new protein synthesis. If expression of the two phenotypes were coupled to nsP34 cleavage at 40°C, restoration of the 30°C patterns of high 26S mRNA synthesis and shutdown of negative-strand synthesis would require translation of new nsP34 proteins at 30°C. Therefore, we determined whether ts17- and ts133-infected cells shifted to 40°C late in infection continued negative-strand synthesis and reduced mRNA synthesis after a return to 30°C in the absence of new protein synthesis. This approach permitted us to assess the activities of viral proteins synthesized prior to the shift, since previous studies indicated that these proteins reactivated negative-strand synthesis (28, 31). The assay was performed as follows. Cycloheximide (100 µg/ml) was added beginning at 6 h postinfection (p.i.) when appropriate. Labeling was from 9.5 to 10.5 h p.i. by refedding the cultures with Dulbecco modified Eagle medium (DMEM) containing 200 µCi of [³H]uridine per ml and 20 µg of actinomycin D per ml in the presence or absence of cycloheximide and at the respective temperatures. Shifting to 40°C or back to 30°C was accomplished by two washes with DMEM at the respective temperature before refedding with complete DMEM. For both ts17 and ts133, a shift to 40°C led to decreased 26S mRNA synthesis and increased molar ratios of 49S to 26S RNAs (Table 2). Upon a return to 30°C in the absence of protein synthesis, restoration of 26S mRNA synthesis and of normal 30°C molar ratios was observed. Reversibility was not de-

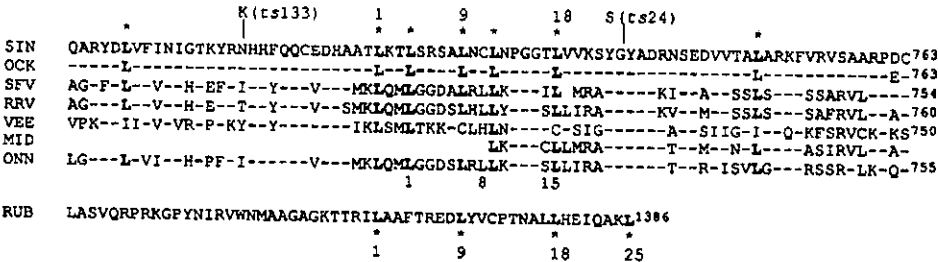


FIG. 1. Comparison of alphavirus and rubella virus amino acid sequences in a region of nsP2 encompassing the ts133 and ts24 mutations. Certain leucine residues are in bold, and patterns of leucine residues are indicated by numbers that refer to their relative spacing; a three-heptad repeat was noted in the O'Nyong-nyong sequence. Sequence data are from Strauss et al. (37) for SIN, Shirako et al. (33) for Ockelbo virus (OCK), Takkinen (39) for Semliki Forest virus (SFV), Faragher et al. (6) for Ross River virus (RRV), Kinney et al. (18) for Venezuelan equine encephalitis virus (VEE), Hahn et al. (10) for Middleburg virus (MID), Strauss et al. (36) for O'Nyong-nyong virus (ONN), and Dominguez et al. (5) for rubella virus (RUB).

TABLE 3. Reversibility of negative-strand synthesis

Expt	Incubation conditions	% of labeled RF RNA in negative-strand RNA ^a			
		ts17	ts133	SIN HR	24R1
1	Maintained at 30°C	1.9	1.8		
	Shifted to 40°C at 6 h p.i.	13.8	13.6		
	Shifted to 40°C at 6 h p.i., returned to 30°C at:				
	7 h p.i.	0.9	3.7		
	8 h p.i.	1.1			
2	9 h p.i.	0.9	4.3		
	Maintained at 30°C	2.0		1.4	4.8
	Shifted to 40°C at 6 h p.i.	11.2		1.8	16.7
	Shifted to 40°C at 6 h p.i., returned to 30°C at 9 h p.i.	2.4		<1.0	3.6

^a The values shown are averages of multiple hybridizations of RFs from two to five experiments.

pendent on the length of time spent at 40°C, since similar levels were reached after 1, 2, or 3 h of incubation.

To test the reversibility of negative-strand synthesis, cells were infected with each virus at a multiplicity of infection of 100 and were treated and labeled as described above except that cultures in experiment 1 were labeled from 9.5 to 10.5 h p.i. and cultures in experiment 2 were labeled from 9.5 to 11.5 h p.i. The cells were harvested by lysis at 0°C in buffer containing 1% Triton X-100 and scraped from the dish, the nuclei were removed by centrifugation at 1,000 rpm, and the supernatant was adjusted to 1% lithium dodecyl sulfate–100 µg of proteinase K per ml before extraction with phenol and chloroform and ethanol precipitation of the nucleic acids. The viral replicative forms (RFs) were purified, and the amount of labeled minus strands in the RF RNA was determined as described previously (25). Reactivation of ts17 and ts133 negative-strand synthesis was also temperature dependent and fully reversible in cultures treated with cycloheximide from the time of the shift to 40°C (Table 3). Reversal did not depend on the length of time spent at 40°C before shift-down. Table 3 also shows that this pattern resembled that for the nsP4 mutant 24R1.

Polypeptide processing. Although nsP1234 and nsP123 polypeptides synthesized at 40°C by ts133 (Fig. 2) and ts17 (data not shown; 11, 28) were not cleaved or poorly cleaved at 40°C compared with parental proteins, processing occurred when the cultures were shifted down and chased at 30°C. Because proteolytic processing of the nsPs was reversible, these mutations most likely affect the activity of nsP2 protease rather than the cleavage sites in the polypeptide. To mimic conditions used to demonstrate reactivation of negative-strand synthesis, we determined whether nonstructural polypeptides synthesized at 30°C were cleaved at 40°C to yield mature nsPs. As shown in Fig. 3A, the nsP2 defects in ts17 and ts133 allowed efficient processing of polypeptides at 30°C but reduced processing at 40°C. Moreover, immunoprecipitation (Fig. 3B) and quantitation of labeled ts17 nsPs found that compared with the 30°C chase period, only 18 to 50% as much nsP34, nsP3, and nsP4 were present after the 40°C chase, while nsP2 levels were unchanged from levels present at the end of the 30°C pulse period. In summary, our analysis did not support the concept (3) that reactivation of negative-strand synthesis resulted from specific cleavage at 40°C of ts17 polypeptides.

Recombinants possessing both mutant nsP2 and nsP4. Our data suggested that nsP2 and nsP4 interact functionally,

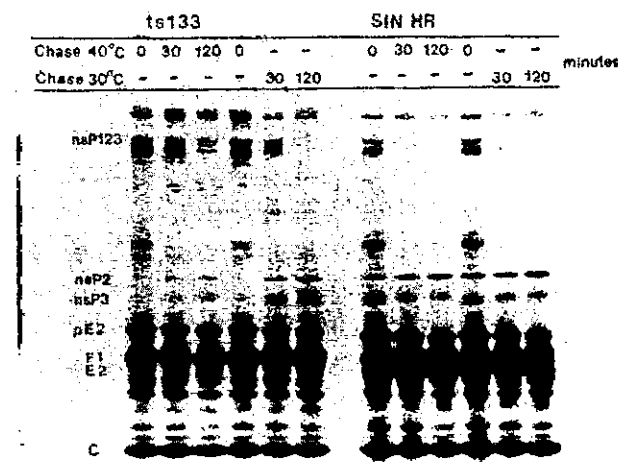


FIG. 2. Assay determining whether polypeptides produced at 40°C by ts133-infected cells were cleaved at 30°C but not at 40°C. Chicken embryo fibroblast cells infected with ts133 or SIN HR were maintained at 30°C until 6 h p.i., when they were shifted to 40°C, treated with hypertonic salt medium (0.335 M NaCl in DMEM lacking methionine and containing 5% dialyzed fetal bovine serum and 22 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.4]) for 40 min, and given a 30-min pulse of [³⁵S]methionine (50 µCi/ml). Duplicate cultures were chased at either 40 or 30°C for the times indicated. Samples were electrophoresed on 5 to 10% polyacrylamide gels in Laemmli buffer (20) and fluorographed. An autoradiograph of the gel is shown.

since mutations in the two proteins show similar phenotypes. Furthermore, the conditional lethality of ts118 requires mutations in both nsP2 and nsP4 (9). To probe such interactions, we created a recombinant genome that contained both the ts17 nsP2 and 24R1 nsP4 lesions and tested them as follows. Infected cultures were shifted to 40°C at 6 h p.i. in the presence of cycloheximide (100 µg/ml) and labeled with [³H]uridine (200 µCi/ml in the presence of 20 µg of actinomycin D per ml) from 6.5 to 8.5 h p.i. Purified RF RNA was denatured and hybridized to an excess of purified SIN virion plus strands as described previously (25). Viruses possessing both mutant nsP2 and nsP4 genes (Toto:ts17:24R1) were viable and expressed all of the ts17 phenotypes (Table 4). The double mutants also resumed negative-strand synthesis when shifted to 40°C but at higher levels typical of 24R1 viruses which possessed only the mutation in nsP4 (31). In cells infected with ts17, 24R1, and Toto:ts17:24R1, negative strands synthesized at 40°C were stable and accumulated as double-stranded RNA at a rate that was linear but higher for Toto:ts17:24R1 and 24R1 than for ts17 (Fig. 4A). For all three viruses, significant fractions of the replicative intermediate and RF populations reactivated negative-strand transcription (Fig. 4B). Consistently, we observed in multiple experiments the accumulation of negative strands by the double mutant Toto:ts17:24R1 that was the sum of the amounts accumulated by 24R1 and ts17, and we concluded there was an additive effect when replication complexes contained both mutant nsP2 and nsP4 and that the nsP2 defect did not interfere with or alter significantly the expression of the nsP4 defect or vice versa. However, replication complexes possessing mutant nsP4 were more efficient at reactivating negative-strand synthesis than were those containing only mutant nsP2. These results suggest that the nsPs

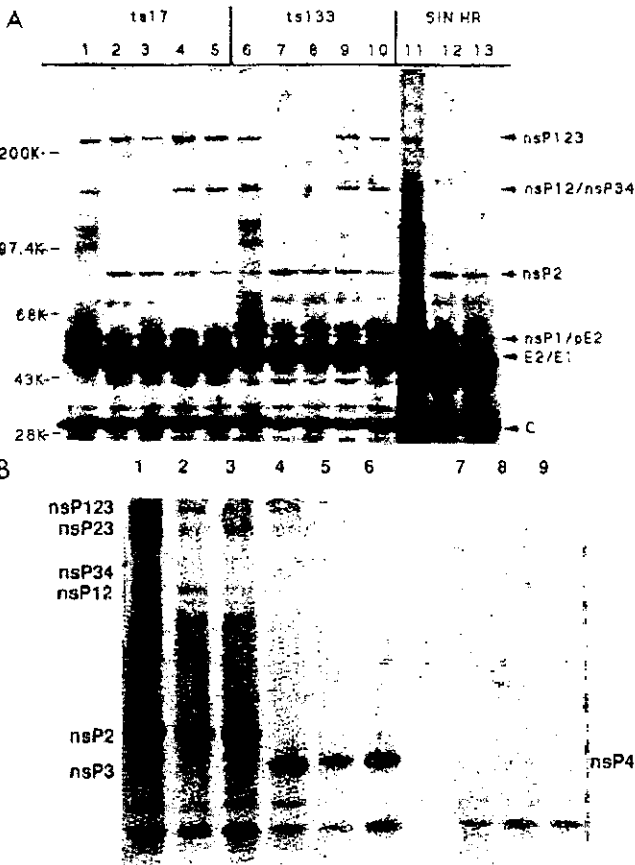


FIG. 3. Assays determining whether polyproteins produced at 30°C by *ts17*- and *ts133*-infected cells were cleaved in the presence or absence of cycloheximide at 30°C but not at 40°C. (A) Chicken embryo fibroblast cells in 35-mm-diameter petri dishes were infected with *ts17*, *ts133*, or SIN HR at a multiplicity of infection of 100 and maintained at 30°C. At 4 h p.i., the cultures were incubated in medium lacking methionine and containing 2 µg of actinomycin D per ml, 5% dialyzed fetal bovine serum, and 22 mM HEPES (pH 7.4) for 2 h and then treated for 90 min with the same medium containing 0.335 M NaCl. Cultures were pulsed with [³⁵S]methionine (50 µCi/ml) for 30 min at 30°C, after which they were either harvested immediately (lanes 1, 6, and 11) or chased for 60 min at 30°C in the absence (lanes 2, 7, and 12) or presence (lanes 3, 8, and 13) of 100 µg of cycloheximide per ml. Duplicate cultures were shifted to 40°C and chased at 40°C for a further 60 min in the absence (lanes 4 and 9) or presence (lanes 5 and 10) of cycloheximide. Samples were electrophoresed together with molecular weight markers on 5 to 10% polyacrylamide gels in Laemmli buffer (20). (B) Immunoprecipitation of *ts17* nsPs. Cultures of *ts17*-infected cells were treated with hypertonic medium from 5 to 6 h p.i. and then subjected to a 1-h pulse of [³⁵S]methionine (200 µCi/ml) at 30°C. Duplicate cultures were harvested immediately after the pulse (lanes 1, 4, and 7) or were chased in the presence of cycloheximide from 7 to 8 h p.i. at 40°C (lanes 2, 5, and 8) or at 30°C (lanes 3, 6, and 9). Samples were denatured and reacted with nsP2 antibodies (lanes 1 to 3), nsP3 antibodies (lanes 4 to 6), or nsP4 antibodies (lanes 7 to 9) as described previously (2). An autoradiograph of the resulting gel is shown.

retain and express individual functions when assembled together in transcriptionally active complexes (2).
To conclude, we report that defective nsP2 proteins in preformed replication complexes were responsible for loss

TABLE 4. Evidence that recombinant viruses with nsP2 and nsP4 mutations expressed conditionally lethal nsP2 phenotypes but levels of negative-strand synthesis typical of mutant nsP4

Virus ^a	Growth	RNA negative	26 mRNA synthesis	% labeled RF RNA in negative strands (range) ^b
Totol101	wt ^c	No	wt	4.1 (2–6)
24R1	wt	No	wt	27.6 (19–34)
<i>ts17</i>	<i>ts</i>	Yes	<i>ts</i>	15.9 (12–20)
<i>ts17</i> :24R1.1	<i>ts</i>	Yes	<i>ts</i>	29.2 (17–39)
<i>ts17</i> :24R1.2	<i>ts</i>	Yes	<i>ts</i>	34.1 (32–35)

^a The *ts17*:24R1.1 and *ts17*:24R1.2 viruses were derived from two independently isolated hybrid clones.
^b The range of values is from four individual determinations, whose average values are listed.
^c wt, wild type.

of subgenomic synthesis and reactivation of negative-strand synthesis by *ts17* and *ts133*. The *ts17* and *ts133* mutations did not affect the putative nsP2 helicase activity because transcription was not inhibited after a shift to 40°C (19). Second, reactivation of negative-strand synthesis, once ceased in infected cells, was induced by alterations in either of two different and stable proteins, nsP2 or nsP4 (25), and not by cleavage of a nsP34 precursor as proposed by deGroot et al. (3). Reactivation of negative-strand synthesis caused neither an increase in the rate of positive-strand synthesis nor formation of additional replication complexes (this study; 25, 31). Therefore, we argue that reactivation must be due to the replacement of the preferred template of the replication complex, the negative strand, with a positive-strand template that in turn is copied into a negative strand. Thus, reactivation results from template switching by previously formed replication complexes. In addition, our results suggest that nsP2 and nsP4 function differently in template recognition and initiation of negative-strand synthesis, since the amount of negative-strand synthesis was greater when replication complexes contained both mutant nsP2 and nsP4. Not all nsP2 mutations conferring *ts* 26S mRNA synthesis reactivated negative-strand synthesis (10, 17, 28). Reversibility of reactivation suggests that these particular nsP2 and nsP4 defects induce changes in protein interactions, possibly with limiting host factors (15, 21, 25, 26), or changes in folding or conformation of the nsPs that modify the structure or affinity of the positive-strand polymerase to resemble the negative-strand polymerase and thus allow recognition of the 3' promoter on a positive strand. We interpret such findings as supporting our model (25, 29, 31): a replication complex is formed by the association of newly synthesized nsPs with a positive-strand template and synthesizes a negative strand. The newly created negative strand becomes the preferred template of the replication complex. Both nsP2 and nsP4 would function normally to fix the negative strand as the stable template of alphavirus replication complexes. Because both nsPs are essential for negative-strand synthesis, subsequent modifications that restrict their activity to positive-strand synthesis would shorten the functional half-life of the negative-strand polymerase. This, in turn, would convert the replication complex from negative-strand to positive-strand synthesis and thereby lead to overproduction of positive strands relative to negative strands in infected cells and to cessation of negative-strand synthesis if no new replication complexes form. Given their resemblance to SIN, the mechanism for cessation of negative-strand synthesis may be common to other superfamily members. The

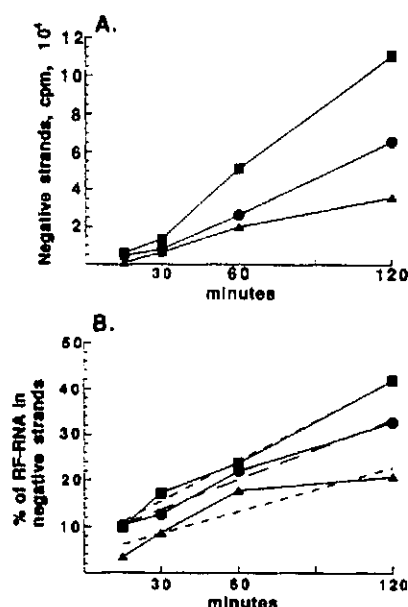


FIG. 4. Accumulation of negative strand RNA at 40°C by *ts17*, *ts24R1*, and the double mutant *Toto:ts17:R1*. Chicken embryo fibroblast cultures in 60-mm-diameter petri dishes were infected at a multiplicity of infection of 100 with *ts17* (▲), *ts24R1* (●), or *Toto:ts17:R1* (■) at 30°C and maintained at 30°C until 6 h p.i., when they were shifted to 40°C and incubated continuously in the presence of 100 µg of cycloheximide per ml. At 6.5 h p.i., the cultures were labeled at 40°C with [³H]uridine (500 µCi/ml, 2 ml per petri dish; ICN, Irvine, Calif.) and were harvested 15, 30, 60, or 120 min later. To measure negative-strand RNA synthesized at 40°C in the presence of cycloheximide, the double-stranded cores (RF RNA) of the viral replicative intermediates were purified by chromatography on CF-11, denatured, and hybridized in the presence of an excess of unlabeled genomic positive strands (25). Typically, 6×10^4 to 8×10^4 cpm in labeled RF RNA per 10^6 infected cells is obtained after a 60-min pulse with 200 µCi of [³H]uridine per ml. The results shown represent the average values from duplicate experiments. (A) Accumulation of incorporation of [³H]uridine in the negative-strand component of RF RNAs; (B) percentage of the total labeled RF RNA that was in labeled negative-strand RNA. Because the replicative intermediates are active also in positive-strand synthesis, a value of 50% of the labeled RF RNA in negative-strand RNA is taken to mean that 100% of RFs contain newly synthesized negative strands.

intriguing aspect of such a mechanism is its stability under normal conditions but its *ts* reversibility in certain SIN nsP2 and nsP4 mutants.

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