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ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

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ERNESTO CAVELIER

Asunto: Radicación: 8-23024- -11
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Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2006/029575				
Fecha de Presentación Internacional	28/07/2006				

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:	Radicado No. 08-023024-00000-0000	4/Marzo/2008
Reivindicaciones:	Radicado No. 08-023024-00000-0000	4/Marzo/2008
	Radicado No. 08-023024-00005-0000	18/Diciembre/2012
Dibujos:	Radicado No. 08-023024-00000-0000	4/Marzo/2008
Prioridad:	US 60/705,808	4/Agosto/2005

2. Análisis de la Prioridad

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

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio (Radicado No. 08-023024-00005-0000) porque no se amplía el objeto de la solicitud.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso, se tiene en cuenta la totalidad del nuevo capítulo reivindicatorio (Radicado No. 08-023024-00005-0000) para el examen de patentabilidad.

Título de la solicitud: “VIRUS DE INFLUENZA MODIFICADO PARA CONTROL Y MEJORAMIENTO DE LA EFICACIA DE LA VACUNA”.

El problema planteado en esta solicitud consiste en la necesidad de disponer de vacunas para los virus de influenza emergentes.

Para solucionar este problema técnico la solicitud en estudio suministra una molécula de hemaglutinina modificada del virus de influenza H5, haciéndola más antigénica respecto a anticuerpos específicos.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/145; C07K14/11; C07H 21/04; C12N 7/00; C12Q 1/70.

5. No es una invención (Art 15 D 486 literal (b))

El objeto de la reivindicación 27 no se considera una invención, de acuerdo con el Art citado, porque si bien encabeza la reivindicación con la palabra “método”, reclama un antisuero reactivo obtenido de un animal vacunado.

6. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

Las reivindicaciones 41 – 43 no son patentables, de acuerdo con el Art 14, porque hacen referencia al uso de la composición para su administración por vía intramuscular, oral o por las mucosas.

7. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 – 22 carecen de concisión porque utilizan las expresiones “comprende uno o más cambios de aminoácidos”, “una molécula de hemaglutinina (HA)”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se sugiere definir la molécula de HA, sobre la que ocurre la modificación, por su secuencia de aminoácidos.

La reivindicación 23 hace referencia a un “conjunto para ensayos de inhibición de hemaglutinina (...)”, cuando en realidad se trata de un kit-de-partes, el cual está conformado por preparaciones combinadas de componentes individuales, donde tales componentes forman una unidad funcional (combinación verdadera) dirigida a un único propósito. Sin embargo, en la reivindicación 23, dicho conjunto para ensayos no se encuentra caracterizado por las preparaciones combinadas.

Las reivindicaciones 24 y 25 carecen de concisión porque utilizan la expresión “un ácido nucleico aislado” lo cual dificulta determinar la extensión del objeto que se

busca proteger. Se recuerda a la solicitante que un ácido nucleico se caracteriza por la secuencia de polinucleótidos.

Las reivindicaciones 26 – 35 carecen de concisión porque utilizan las expresiones “un vector recombinante”, “un sistema de genética inversa”, “introducción de un vector recombinante”, “reaccionar in vitro”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda a la solicitante que un ácido nucleico se caracteriza por la secuencia de polinucleótidos y que un método se caracteriza por una serie de etapas en un orden lógica y las condiciones en las cuales se llevan a cabo. Sin embargo, en los métodos reclamados no se describen las condiciones en que se llevan a cabo las etapas de introducción del vector y de reacción *in vitro*.

Las reivindicaciones 36 – 55 carecen de concisión porque utilizan las expresiones “un vehículo”, “una molécula inmunoestimuladora”, “un portador membranoso inmunopotenciador”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se sugiere definir los componentes por sus características técnicas, así como sus proporciones dentro de la composición.

Se requiere a la solicitante hacer las aclaraciones necesarias.

8. 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: agosto 4 de 2005, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D2	Virology, Vol. 334, Págs. 276-283	“Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain”	10/Abril/2005
D3	PNAS, Vol. 97, Págs. 6108-6113	“A DNA transfection system for generation of influenza A viru from eight plasmids”	23/Mayo/2000

D2¹ divulga las propiedades de unión al receptor de aislados del virus de influenza de aves y mamíferos usando conjugados poliméricos de sialo-oligosacáridos modificados (Resumen).

D3² enseña un sistema de transfección de ocho plásmidos para el rescate de virus de influenza A infecciosos de cDNA clonado (Resumen).

9. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en una molécula de hemaglutinina (HA) del virus de influenza recombinante H5, donde dicha molécula H5HA:

- a. Comprende uno o más cambios de secuencias de aminoácidos comparados a los que le ocurren a la molécula HA que comprende un aminoácido diferente a la asparagina en el lugar correspondiente a la posición 223 en la HA del H5,
- b. Comprende el aminoácido asparagina en el lugar correspondiente a la posición 223 en la HA del H5,

1 <http://www.ncbi.nlm.nih.gov/pubmed/15780877>

2 <http://www.ncbi.nlm.nih.gov/pubmed/10801978>

- c. Posee una reactividad aumentada con los antisueros obtenidos de un animal expuesto a un virus de influenza H5 o una vacuna contra la influenza comparada con la molécula HA de tipo silvestre y
- d. No es una molécula AIHKI213/03 HA

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2
Una molécula de hemaglutinina (HA) del virus de influenza recombinante H5, donde dicha molécula H5HA: a. Comprende uno o más cambios de secuencias de aminoácidos comparados a los que le ocurren a la molécula HA que comprende un aminoácido diferente a la asparagina en el lugar correspondiente a la posición 223 en la HA del H5, b. Comprende el aminoácido asparagina en el lugar correspondiente a la posición 223 en la HA del H5, c. Posee una reactividad aumentada con los antisueros obtenidos de un animal expuesto a un virus de influenza H5 o una vacuna contra la influenza comparada con la molécula HA de tipo silvestre y d. No es una molécula AIHKI213/03 HA	Molécula de hemaglutinina (HA) del virus de influenza recombinante H5 en la que el cambio en la estructura de HA, específicamente alrededor del aminoácido 222 del HA del virus de influenza podría dar lugar a una interacción favorable o a una interferencia estérica con la unión al receptor (Resumen y Pág. 280, Col. derecha, Párr. 2)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque enseña que el cambio en la estructura de HA, específicamente alrededor del aminoácido 222 del HA del virus de influenza podría dar lugar a una interacción favorable o a una interferencia estérica con la unión al receptor, resultando en un efecto sobre la capacidad de unión al receptor entre las cepas de influenza (Pág. 280, Col. derecha, Párr. 2). El documento D2 también enseña que un análisis comparativo de la HA secuenciada de diferentes virus ha mostrado la presencia de diferencias sustanciales en la composición de aminoácidos de la región del loop 210-213.

La diferencia entre la materia reclamada en la reivindicación 1 y el documento D2 consiste en que en la molécula de la solicitud se sustituye el aminoácido 223 por una asparagina, mientras que en el documento D2 se sustituyen aminoácidos alrededor de la posición 222, sin indicar que se incluya asparagina.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la molécula de hemaglutinina reclamada, puesto que en ambos casos se logra que la hemaglutinina tenga una reactividad aumentada.

El problema técnico objetivo resuelto por la invención consiste en obtener una hemaglutinina alternativa con reactividad aumentada.

Sin embargo, la sustitución del aminoácido 223 para colocar asparagina, tiene el mismo efecto que la sustitución del aminoácido 222 reportada en el documento D2, esto es, modificar un sitio de glicosilación con la repercusión en la capacidad de unión del virus a su receptor del ácido siálico. De esta manera, la persona del oficio normalmente versada en la materia incluiría sustituciones en esta región con el fin de obtener una molécula de HA modificada.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y en las reivindicaciones 2 – 25 es obvia y no tiene nivel inventivo.

La reivindicación 26 consiste en un método para hacer un virus que incluye la molécula de HA de la reivindicación 1, método que incluye la introducción de un vector recombinante que expresa la molécula HA del virus de influenza de la reivindicación 1 en un sistema de genética inversa.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Un método para hacer un virus que incluye la molécula de HA de la reivindicación 1, método que incluye la introducción de un vector recombinante que expresa la molécula HA del virus de influenza de la reivindicación 1 en un sistema de genética inversa.	Molécula de hemaglutinina (HA) del virus de influenza recombinante H5 en la que el cambio en la estructura de HA, específicamente alrededor del aminoácido 222 del HA del virus de influenza podría dar lugar a una interacción favorable o a una interferencia estérica con la unión al receptor. (Resumen y Pág. 280, Col. derecha, Párr. 2)	Un método para hacer un virus que incluye la introducción de un vector recombinante en un sistema de genética inversa. (Pág. 6108, Col. derecha, Párr. 3)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 26 porque enseña que el cambio en la estructura de HA, específicamente alrededor del aminoácido 222 del HA del virus de influenza podría dar lugar a una interacción favorable o a una interferencia estérica con la unión al receptor, resultando en un efecto sobre la capacidad de unión al receptor entre las cepas de influenza (Pág. 280, Col. derecha, Párr. 2).

La diferencia entre la materia reclamada en la reivindicación 26 y el documento D2 consiste en que el método comprende la introducción de un vector recombinante en un sistema de genética inversa.

El efecto que logra el incluir un sistema de genética inversa para la producción del virus es que permite la manipulación del genoma viral.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo producir el virus conocido en el documento D2 de manera que se pueda manipular el genoma viral.

Sin embargo, la utilización de un sistema de genética inversa para la producción de un virus de influenza ya está divulgada en el documento D3 que se refiere a un un método para hacer un virus H5N1 del virus de influenza A que incluye la introducción de un vector recombinante en un sistema de genética inversa (Pág. 6108, Col. derecha, Párr. 3), en el que se usa un vector recombinante (Pág. 6109 Col. izquierda) que codifica proteínas estructurales que presentan sustituciones (Pág. 6112). El documento D3 también enseña que el método permite desarrollar vacunas eficientes (Pág. 6113, Col. izquierda, Párr .1).

En consecuencia, el la persona del oficio normalmente versada en la materia, incluiría el método del documento D3 para producir el virus del documento D2 para llegar así al objeto de la reivindicación 26 de la solicitud. Por lo cual se considera

obvia.

De manera que la solución propuesta por esta solicitud en la reivindicación 26 y en las reivindicaciones 28 – 40 y 44 – 55 es obvia y no tiene nivel inventivo.

10. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	Virology, Vol. 334, Págs. 276-283	1 – 26, 28 – 40 y 44 – 55	Nivel inventivo
D3	PNAS, Vol. 97, Págs. 6108-6113	26, 28 – 40 y 44 – 55	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 del día siguiente 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 de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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.



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El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-03-04

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Receptor specificity of influenza viruses from birds and mammals: New data on involvement of the inner fragments of the carbohydrate chain

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Abstract

We studied receptor-binding properties of influenza virus isolates from birds and mammals using polymeric conjugates of sialooligosaccharides terminated with common Neu5Ac α 2-3Gal β fragment but differing by the structure of the inner part of carbohydrate chain. Viruses isolated from distinct avian species differed by their recognition of the inner part of oligosaccharide receptor. Duck viruses displayed high affinity for receptors having β 1–3 rather than β 1–4 linkage between Neu5Ac α 2-3Gal-disaccharide and penultimate N-acetylhexosamine residue. Fucose and sulfate substituents at this residue had negative and low effect, respectively, on saccharide binding to duck viruses. By contrast, gull viruses preferentially bound to receptors bearing fucose at N-acetylglucosamine residue, whereas chicken and mammalian viruses demonstrated increased affinity for oligosaccharides that harbored sulfo group at position 6 of (β 1–4)-linked GlcNAc. These data suggest that although all avian influenza viruses preferentially bind to Neu5Ac α 2-3Gal-terminated receptors, the fine receptor specificity of the viruses varies depending on the avian species. Further studies are required to determine whether observed host-dependent differences in the receptor specificity of avian viruses can affect their ability to infect humans.

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Keywords: Influenza virus; Hemagglutinin; Receptor specificity; Sialyloligosaccharides; Glycopolymers

Introduction

Until recently, the receptor specificity of influenza viruses has been mainly defined in terms of their recognition of sialic acid species (Neu5Ac, Neu5Gc, Neu4,5Ac₂, etc.) and the type of glycosidic linkage between sialic acid and penultimate galactose (α 2–3 or α 2–6). In contrast to type A and B influenza viruses, which bind to Neu5Ac/Gc, influenza C virus binds to 9-

O-acetylated sialic acid (Herrler et al., 1985). Human influenza B viruses and H1 subtype influenza A viruses do not bind 4-O-acetylated sialic acid (Neu4,5Ac₂), whereas 4-O-acetylation does not affect binding of H2 and H3 subtype viruses (Matrosovich et al., 1992). Equine H3N8 viruses, in contrast to closely related duck viruses, lost the ability to bind to Neu4,5Ac₂ (Matrosovich et al., 1998). The ability to recognize N-glycolyl form of sialic acid (Neu5Gc) was reported for a number of H3 subtype viruses from humans, ducks, pigs and horses raising a hypothesis that this ability is adaptive (Ito et al., 2000; Masuda et al., 1999; Matrosovich et al., 1997; Suzuki et al., 1997).

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It was shown that inner monosaccharide moieties of carbohydrate chain can modulate the binding affinity of human viruses. Indeed, modern non-egg-adapted human H1N1 and H3N2 influenza A and influenza B viruses bound Neu5Ac α 2-6Gal β 1-4GlcNAc (6'SLN) significantly stronger than Neu5Ac α 2-6Gal β 1-4Glc (6'SL) (Gambaryan et al., 1997, 1999; Mochalova et al., 2003).

Avian influenza viruses were generally considered as a uniform group in terms of their Neu5Ac α 2-3Gal receptor specificity (Paulson, 1985). It was shown that receptor-binding site (RBS) of avian virus HA is evolutionally very stable, and that it undergoes changes after virus transmission and adaptation to humans. These changes can be structurally different for hemagglutinins of different subtypes, but they always lead to the same functional result enabling HA binding to receptors with terminal trisaccharide 6'SLN (Matrosovich et al., 1997, 2000).

The alteration of receptor-binding phenotype of avian viruses in humans correlates with the notion that α 2–6 sialylgalactose motif is predominantly expressed on epithelial cells of human respiratory epithelium, whereas α 2–3-linked sialic acid is mainly expressed on human mucins (Couceiro et al., 1993). The differences in receptor specificity of avian and human influenza viruses raised a hypothesis that avian viruses cannot infect humans (Ito et al., 1998). Indeed, very high doses of virus were found to be required for the replication of avian influenza viruses in volunteers (Beare and Webster, 1991). It was suggested that avian viruses can transmit to humans only via an intermediate animal host, most likely, pig (Scholtissek, 1994). This hypothesis is supported by the finding of both 3-linked and 6-linked sialic acids in pig tracheal epithelium (Ito et al., 1998). Furthermore, influenza viruses that circulate in pigs display increased affinity for Neu5Ac α 2-6Gal β 1-4GlcNAc receptors, resembling by this property human viruses (Ito et al., 1998; Matrosovich et al., 2000; Rogers and D'Souza, 1989).

The human infections caused by H5N1 chicken viruses in Hong Kong in 1997 (Claas et al., 1998; Subbarao et al., 1998) demonstrated for the first time that avian viruses can infect humans directly without intermediate evolution in pigs. The ability of H5 viruses to replicate in humans despite their avian virus-like receptor specificity (Matrosovich et al., 1999) may be explained by the finding that human airway epithelium harbors (2–3)-linked sialic acids on ciliated cells (Matrosovich et al., 2004).

More recently, avian H9N2 viruses were isolated from humans (Guo et al., 1999; Peiris et al., 1999), numerous cases of conjunctivitis caused by chicken virus H7N7 were documented in the Netherlands (Fouchier et al., 2004), and new human infections with H5N1 avian viruses took place in South-Eastern Asia (Peiris et al., 2004). It is important that all human infections were caused by viruses of terrestrial poultry rather than by feral bird's viruses.

The principal natural reservoir of influenza viruses are wild aquatic birds. All viruses in other species originate

from this reservoir, with all mammalian and chicken evolution branches usually being dead ends (Webster et al., 1992). For example, duck viruses transmitted to poultry evolve as rapidly as in mammals indicative of their adaptive evolution in terrestrial birds (Suarez, 2000). Moreover, it seems that even wild aquatic birds are not uniform as influenza virus hosts. Thus, subtype H13 is a typical dead end branch adapted to gulls (Chambers et al., 1989). Acceleration of virus evolution was noted also during transmission and adaptation of Asian duck H2 viruses to gulls and shorebirds in North America (Makarova et al., 1999). As a rule, virus introduction into a new host is accompanied by a change of receptor-binding phenotype. For example, surface glycoproteins of chicken and turkey viruses differ from glycoproteins of related duck viruses by the presence of additional glycosylation sites near the RBS of the HA, deletions in the stem region and accumulation of mutations in the neuraminidase (Banks et al., 2001; Matrosovich et al., 1999, 2001). These alterations in both the HA and the NA of chicken viruses likely indicate that sialic acid-containing receptors utilized by the virus in chickens differ from its receptors in ducks. Indeed, we have shown recently that H5N1 chicken and human viruses isolated in 1997 in Hong Kong differed from H5 duck viruses by extraordinary high affinity to sulfated trisaccharide Neu5Ac α 2-3Gal β 1-4(6-HSO₃)GlcNAc β (Gambaryan et al., 2003, 2004). Moreover, one of the H9N2 virus lineages in terrestrial poultry acquired receptor specificity, which is similar to receptor specificity of human influenza viruses (Matrosovich et al., 2001). Comparison of influenza viruses from ducks and gulls also revealed differences in their receptor specificity. For gull viruses, the role of galactose moiety in binding the receptor is less significant, the affinity for the different sialooligosaccharides is more uniform and the fucose-substitution at the position 3 of glucosamine does not interfere with the binding (Yamnikova et al., 2003).

In this study, we wished to characterize receptor specificity of influenza viruses from different hosts in more detail. To this end, we tested virus binding to a wide range of oligosaccharides containing terminal Neu5Ac α 2-3Gal moiety but differing by the composition of more distant (inner) part of the carbohydrate chain.

Results and discussion

To characterize fine receptor-binding specificity of avian and mammalian influenza viruses, we tested their binding to 17 distinct oligosaccharides conjugated with polyacrylamide (OS-PAA, see Table 1 for structural formula and abbreviations). Eleven oligosaccharides shared the same terminal Neu5Ac α 2-3Gal moiety but differed either by the bond type between galactose and the penultimate sugar residue (β 1–3 or β 1–4), or the nature of this residue (Glc β , GlcNAc β , GalNAc α), or by substituent at this residue (fucose, sulfo group or sialic acid). All studied oligosaccharides with the

Table 1
Structures of oligosaccharides attached to polyacrylamide

Saccharide	Abbreviation
Neu5Ac α 2-3Gal β	3-SiaGal
Neu5Ac α 2-3GalNAc α	3-SiaGalNAc α
Neu5Gc α 2-3Gal β 1-4GlcNAc β	3'-(Neu5Gc)LN
Neu5Ac α 2-3Gal β 1-4Glc β	3'SL
Neu5Ac α 2-3Gal β 1-4GlcNAc β	3'SLN
Neu5Ac α 2-3Gal β 1-4-(6-O-Su)GlcNAc β	6-O-Su-3'SLN
Neu5Ac α 2-3Gal β 1-3GlcNAc β	SiaLe ^c
Neu5Ac α 2-3Gal β 1-3-(6-O-Su)GlcNAc β	6-O-Su-SiaLe ^c
Neu5Ac α 2-3Gal β 1-3GalNAc α	SiaTF
Neu5Ac α 2-3Gal β 1-3-(6-O-Su)GalNAc α	6-O-Su-SiaTF
Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-6)GalNAc α	3',6-Sia ₂ TF
Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β	SiaLe ^x
Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)(6-O-Su)GlcNAc β	6-O-Su-SiaLe ^x
Neu5Gc α	Neu5Gc
(Neu5Ac α 2-) ₂ -3,6-GalNAc α	3,6-Sia ₂ GalNAc α
Neu5Ac α 2-8Neu5Ac α 2-8Neu5Ac α	Sia ₃
3'-O-Su-Gal β 1-3GlcNAc β	3'-O-Su-Le ^c

exception of 3-SiaGalNAc α and 3,6-Sia₂GalNAc α have been found in glycoproteins or glycolipids of mammals (Varki et al., 1999). The virus binding to OS-PAA was determined in a competitive solid-phase assay, and expressed in terms of binding affinity constants (Table 2). To explain the differences in binding affinity in molecular terms, we modeled putative disposition of oligosaccharides in the receptor-binding site of the avian H5 HA (Ha et al., 2001) (Fig. 1).

Recognition of oligosaccharides with modified Neu5Ac α 2-3Gal motif

Four OS-PAA, that lacked terminal Neu5Ac α 2-3Gal (3'-O-Su-Le^c, Neu5Gc, Sia₃ and 3,6-Sia₂GalNAc α) did not bind to any of the viruses tested (data not shown). This result is consistent with the current concept that disaccharide Neu5Ac α 2-3Gal represents the minimal receptor determinant recognized by avian influenza viruses (Ha et al., 2001; Matrosovich et al., 1997).

3-SiaGalNAc α differs from 3-SiaGal β by the presence of acetamido group at C-2 of Gal and by the α instead of β configuration of the spacer group. All tested H1, H2, H4, H7, H11, H12 and H13 viruses bound to 3-SiaGalNAc α much weaker than they bound to 3-SiaGal β (Table 2). It should be noted that some of the H5 duck viruses displayed only marginal differences in their affinity for these two OS-PAA.

3'SLN and 3'-(Neu5Gc)LN represent oligosaccharide structures harboring two major natural sialic acid species, Neu5Ac and Neu5Gc, respectively. All avian and mammalian viruses with H3 and H7 HAs bound to these OS-PAA with similar affinity. This finding agrees with the previous reports on Neu5Gc potentially playing an important role in the receptor recognition by H3 subtype duck and equine viruses (Ito et al., 2000; Suzuki et al., 2000). The viruses of other subtypes (H2, H4, H5, H6, H9, H11, H12 and H14)

bound to 3'-(Neu5Gc)LN weakly if at all. These data suggest that binding to Neu5Gc is not essential for the replication of the majority of avian virus subtypes.

Recognition of the Neu5Ac α 2-3Gal motif in a context of the penultimate saccharide residue

The effect of fragments penultimate to the terminal Neu5Ac α 2-3Gal on the binding was assessed by comparing virus affinity for 3-SiaGal, 3'SL, 3'SLN, SiaLe^c and SiaTF.

Most of the viruses bound with similar affinity to 3'SL, 3'SLN, and to 3-SiaGal that lacks third sugar moiety. Thus, Glc or GlcNAc residues linked to preceding 3-SiaGal moiety via β 1-4-bond, either do not substantially contribute to the binding, or slightly interfere with it. In SiaLe^c and SiaTF, the terminal Neu5Ac α 2-3Gal moiety is connected to penultimate GlcNAc or GalNAc, respectively, via β 1-3-bond. All viruses have similar affinity for corresponding OS-PAA, suggesting that configuration of C-4 of the third saccharide residue is not critical for the recognition and that the spacer orientation at the third saccharide unit (β in SiaLe^c, α in SiaTF) does not affect the binding affinity.

Duck viruses as a rule displayed a higher affinity for SiaTF than for 3'SLN, indicating that these viruses are adapted to preferential recognition of sialosaccharides with 1-3 bond between galactose and the penultimate carbohydrate residue. On the contrary, chicken viruses bind 3'SLN better than SiaTF, suggesting that they are adapted to receptors with 1-4 bond between galactose and penultimate sugar. The relative affinity of gull viruses for these glycoconjugates varied with the virus subtype. H13 subtype gull viruses were likely transmitted from duck to gulls a long time ago and underwent significant adaptation to this species (Chambers et al., 1989). These viruses, in contrast to duck viruses, bound to 3'SLN stronger than to SiaTF. H4 and H14 gull viruses are more closely related to duck viruses of corresponding subtypes. Accordingly, the viruses of these recently emerged gull lineages, similar to duck viruses, displayed a higher affinity for SiaTF than for 3'SLN.

Role of substituents at the penultimate carbohydrate residue

Additional substituents at the third carbohydrate unit of sialosaccharide produced variable effects on binding (from negative to positive) depending on the position of the substituent and on the virus strain. Most viruses displayed decreased affinity for 3',6-Sia₂TF, the tetrasaccharide with Neu5Ac substituent at position 6 of N-acetylgalactosamine. This effect seems to be due to inability of 6-linked sialic acid to bind to the avian RBS and by its concomitant steric interference with the binding of Neu5Ac α 2-3Gal fragment.

Sulfo-substituent

A strong host-specific differences were observed between viruses with respect to their binding to four oligosaccharides with sulfo group at position 6 of N-

Table 2
Binding affinity (K_{ass} , mM^{-1}) of virus with sialoglycoconjugates OS-PAA

Influenza viruses from different hosts		Saccharides ^a											
		3-SiaGal	3-SiaGalNAc α	3'(Neu5Gc)LN	3'SLN ^b	6-O-Su-3'SLN	SiaLe ^c	6-O-Su-SiaLe ^c	SiaTF	6-O-Su-SiaTF	SiaLe ^x	6-O-Su-SiaLe ^x	3',6-Sia ₂ TF
<i>Duck viruses</i>													
Dk/France/46/82	H1N1	200	<5	<5	100	100	200	200	200	200	20	20	100
Pintail/Primorie/695/76	H2N3	50	5	<10	100	100	100	100	500	500	100	200	10
Dk/Buryatia/652/88	H3N8	200	15	100	100	100	200	200	400	400	20	20	100
Dk/Buryatiya/664/88	H3N8	300	10	100	100	–	300	–	500	500	20	–	500
Dk/Buryatiya331/78	H4N6	100	<5	<5	50	–	50	–	100	–	5	–	–
Dk/Kyrgyzstan/1047/87	H4N6	70	<5	<5	50	50	50	50	100	100	7	10	20
Dk/Buryatiya/1905/00	H4N6	100	<5	<5	50	50	100	100	150	150	10	10	20
Dk/Primorie/2483/01	H4N6	100	<5	<5	100	–	200	200	500	–	10	–	–
Dk/Astrakhan/3091/02	H4N8	100	<5	<5	100	–	200	200	400	–	10	–	–
Dk/Pennsylvania/10218/84	H5N2	100	100	<5	100	100	100	100	200	200	20	40	100
Dk/HoChiMinh/014/78	H5N3	200	200	<5	100	100	100	100	200	200	20	50	10
Dk/MN/1525/81	H5N1	200	50	<5	100	100	100	100	100	100	10	20	200
Dk/Primorie/2634/01	H5	200	10	<5	100	100	–	–	200	100	25	–	20
Dk/Primorie/2632/01	H5	100	100	<5	100	200	100	–	200	200	30	–	100
Dk/Primorie/2635/01	H5	200	100	<5	100	100	100	–	200	100	25	–	20
Dk/Primorie/3/82	H9N2	150	50	<10	100	150	150	200	200	300	10	40	200
Dk/Primorie/3628/02	H9N2	–	20	<10	50	100	50	50	50	–	<10	–	–
Dk/England/56	H11N6	50	<5	<10	50	50	–	–	–	–	–	–	–
Dk/Alberta/60/76	H12N5	200	<5	<10	50	–	–	–	–	–	–	–	–
Mallard/Gurjev/244/82	H14N6	400	<50	<10	200	200	200	200	300	300	200	–	100
<i>Gull viruses</i>													
Tern/Buryatiya/1901/00	H4N6	200	<5	<5	200	500	200	400	400	800	200	–	300
Gull/Buryatiya/2407/01	H4N6	200	<5	<5	100	300	100	200	300	400	100	–	200
Gull/Astrakhan/3528/02	H4N8	200	<5	<5	150	–	50	50	200	–	150	–	–
Tern/South Africa/61	H5N1	25	25	<5	25	40	25	–	25	–	25	–	10
Gull/Astrakhan/165/86	H6N5	50	–	<10	50	–	50	–	30	–	100	–	–
Gull/Astrakhan/1421/74	H13N6	15	<5	<5	25	20	20	20	10	10	100	100	10
Gull/Astrakhan/227/82	H13N6	50	<5	<5	100	100	50	50	30	30	100	100	10
Gull/Astrakhan/1808/98	H13N6	30	<5	<5	50	50	30	–	20	–	50	–	10
Gull/Astrakhan//267/82	H14N5	200	40	–	200	–	200	–	400	–	200	–	–
<i>Chicken viruses</i>													
Chicken/HK/220/97	H5N1	400	<5	<5	400	4000	100	500	300	1000	15	50	300
Chicken/HK/728/97	H5N1	500	30	<5	300	3000	100	500	200	500	10	50	100
Chicken/HK/786/97	H5N1	1000	10	<5	1000	10,000	300	1000	500	1000	10	500	–
FPV/Rostok/34	H7N1	500	<5	100	300	600	100	100	100	200	500	3000	20
<i>H5N1 human isolate</i>													
HK/481/97	H5N1	500	–	–	500	5000	100	500	200	500	10	50	–
<i>Mammalian viruses</i>													
Swine/Iowa/31	H1N1	–	–	<5	50	20	30	50	–	–	50	200	–
Swine/Finistere/82	H1N1	–	–	<5	100	100	100	100	–	–	50	500	–
Swine/Kazakhstan/48/82	H3N6	–	–	100	100	300	100	100	–	–	20	300	–
Equine/Miami/1/63	H3N8	–	<10	100	300	2000	100	500	100	500	50	500	–
Seal/Massachusetts/1/80	H7N7	–	<10	200	200	200	50	50	50	–	200	800	–

–, Not tested.

The data were averaged from 3 sets of experiments. Standard errors did not exceed 50% of the mean values.

^a Data for 3'-O-Su-Le^c, Neu5Gc α , 3,6-Sia₂GalNAc α , Sia₃ are not shown, see Results and discussion.

^b None of the viruses displayed statistically significant difference in its affinity for 3'SLN and 3'SL, therefore, data for 3'SL omitted.

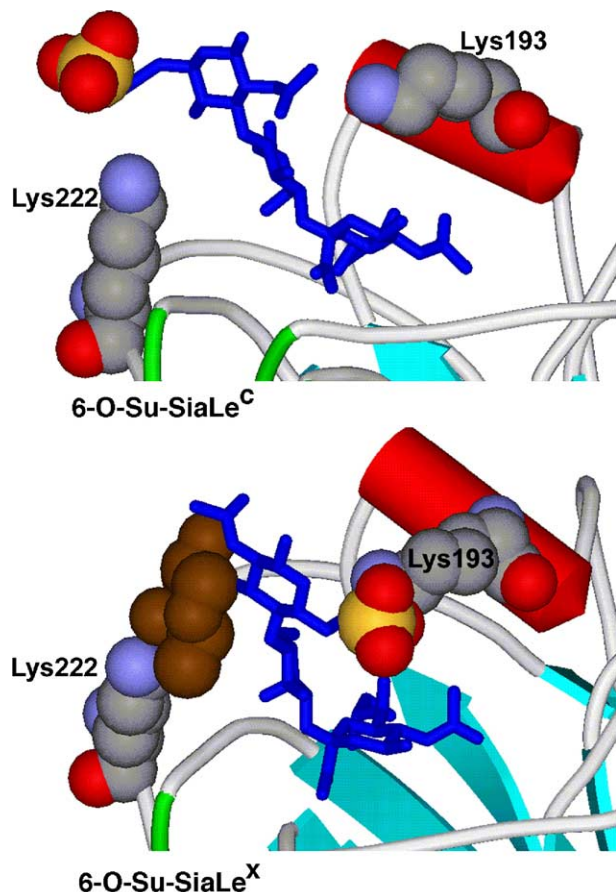


Fig. 1. Putative disposition of fucose and 6-O-sulfo substituents of sialosaccharide in the receptor-binding site of H5 influenza virus. The receptor was fitted into the RBS of H5 HA complexed with LSTa (1JSN, Brookhaven Protein Databank) by superimposing sialic acid residue of the different analogs over the sialic acid residue of LSTa. The molecular model of 6-O-Su-SiaLe^c (top panel) was build based on LSTa (1JSN). The molecular model of 6-O-Su-SiaLe^x (bottom panel) was build based on SiaLe^x (2KMB structure, Brookhaven Protein Databank). The major part of the oligosaccharide is shown as blue stick model with the fucose and sulfo-group depicted in spacefill mode; fucose is colored brown. Lys193 and Lys222 of the HA are represented in spacefill mode. The modeling was performed and figures were generated using Discovery Studio ViewerPro5.0 (Accelrys Inc.) software.

acetylhexosamine, 6-O-Su-3'SLN, 6-O-Su-SiaTF, 6-O-Su-SiaLe^c and 6-O-Su-SiaLe^x. Presence of sulfate typically did not affect the binding of gull and duck viruses. However, in the case of chicken, swine, equine and seal viruses sulfate at this position increased the binding affinity. In particular, the affinity of chicken H5 viruses and equine H3 virus for 6-O-Su-3'SLN was drastically increased comparing to 3'SLN. H7 viruses from chicken and seal, as well as all swine viruses displayed an enhanced affinity for 6-O-Su-SiaLe^x, which is the analog of 6-O-Su-3'SLN with additional fucose. In the model of putative disposition of sulfo analogs in the RBS of H5 virus (Fig. 1), the sulfo group of 6-O-Su-SiaLe^x is located closely to positively charged Lys193, whereas in case of 6-O-Su-SiaLe^c, it is directed towards the solution. Our data suggest that RBS of chicken viruses

supports the energetically favorable interaction between 193 amino acid and negatively charged sulfo group of 6-O-Su-SiaLe^x and 6-O-Su-3'SLN.

Fuco-substituent

Most duck viruses bound SiaLe^x from 5 to 20 times weaker than 3'SLN. In contrast, all tested gull viruses of H4, H5, H6, H13 and H14 subtypes demonstrated affinity for SiaLe^x not lower than that for 3'SLN. On the model of HA-SiaLe^x complex (Fig. 1), fucose moiety located relatively close to the amino acid 222, consequently fine changes in the HA structure in this region could lead to either favorable interactions or steric interference. A comparative analysis of six sequenced HA H13 viruses and other subtype viruses showed presence of substantial differences in the composition of amino acids of the region of 210–230 loop in H13 viruses. In position 222, in all viruses isolated from ducks, there is a bulky amino acid, whereas in HA H13 it is substituted by Gly. The H6N5 virus Gull/Astrakhan/165/86 also has small amino acid Ala at 222 position. H4 duck viruses display maximal affinity for SiaTF and decreased affinity for fucosylated sialosaccharides. At the same time, H4 gull viruses isolated in 2000–2002 have high affinity for SiaLe^x. The H4N6 virus Tern/Buryatiya/1901/00 revealed replacement 205Ser→Pro in comparison to H4N6 duck viruses. Ser205 is located between constant for all subtypes Arg220 and Pro221 of the adjacent monomer of a hemagglutinin, hence replacement Ser→Pro can shift the 222 amino acid. High affinity of all gull viruses and low affinity of all duck viruses for SiaLe^x strongly suggest that duck virus HA adapts to recognize the fucosylated receptor after the virus transmission to and circulation in gulls.

Our data demonstrate clear distinctions in the receptor specificity of influenza viruses from ducks, gulls, and terrestrial poultry (Fig. 2). Although all avian viruses recognize the same terminal Neu5Acα2-3Gal motif, viruses of distinct bird species evolve to additional recognition of more distant parts of the oligosaccharide receptor. Thus, duck viruses preferentially bind to sialosaccharides with 1–3 bond between Neu5Acα2-3Gal and penultimate carbohydrate residue; most of these viruses have maximal affinity for Neu5Acα2-3Galβ1-3GalNAcα. We have demonstrated earlier that duck viruses bind strongly to ganglioside GD1a which carries the same trisaccharide moiety as well as to structurally not yet defined gangliosides from duck intestine (Gambaryan et al., 2002a, 2002b; Matrosovich et al., 1997). One can speculate that gangliosides with terminal Neu5Acα2-3Galβ1-3GalNAc moieties serve as functional receptors of influenza viruses in duck intestinal tissues.

Receptor specificity of chicken viruses differed from that of related duck viruses. Most duck H5 viruses bound to 3-SiaGalNAcα almost as well as to 3-SiaGal. Chicken H5N1 viruses isolated in Hong Kong in 1997 have markedly decreased affinity to 3-SiaGalNAcα, a finding suggesting the different position of galactose in RBS of duck and

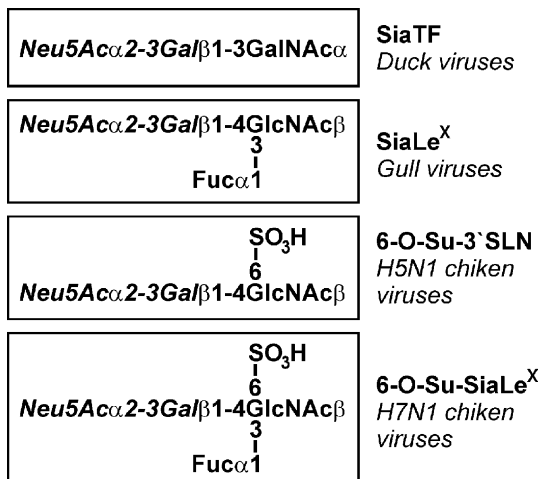


Fig. 2. Optimal receptor determinant for duck, gull, H5N1 and H7N1 chicken viruses.

chicken viruses. Additional 6-sulfo group in composition of sialosaccharide showed marginal or no effect on the binding affinity of duck viruses, while dramatically increased affinity of chicken H5 viruses. Strong binding to 6-O-Su-3'SLN was also observed for H5 viruses isolated from humans (Gambaryan et al., 2004).

Chicken virus A/FPV/Rostok/34 (H7N1) also possesses high affinity for sulfated sialosugar, namely 6-O-Su-SiaLe^x. This oligosaccharide is known to be the ligand of L-selectin.

The mammalian viruses tested here also appear to evolve to recognize sulfated receptors. Each of the three swine viruses tested belongs to an independent HA phylogenetic lineage (H1 classical swine, H1 avian-like and H3 avian-like), and either virus demonstrated increased affinity for 6-O-Su-SiaLe^x. Seal H7 virus also displayed increased affinity for this receptor. The single equine virus isolate tested resembled H5 chicken viruses by its enhanced binding to 6-O-Su-3'SLN.

Thus, mammalian viruses display binding to same oligosaccharides as do chicken viruses. Apparently, sulfated and (or) fucosylated sialoglycans are present in the target tissues of both mammals and chickens. This means that chicken and gull viruses may have a higher affinity than duck viruses have for receptors in epithelial respiratory tissues of mammals. It remains to be determined whether this hypothesis is correct and whether chicken or gull viruses may, therefore, serve as an intermediate host facilitating transmission of duck viruses to mammals.

Materials and methods

Materials

Oligosaccharides conjugated with polyacrylamide (OS-PAA) were synthesized from spacers sialooligosaccharides- ω -aminoglycosides (spacer = -OCH₂CH₂CH₂NH₂, -OCH₂CH₂NH₂, or -NHCOCH₂NH₂) and poly(4-nitrophe-

nylacrylate) having m.w. 30 kDa by the method described in (Bovin et al., 1993; Gordeeva et al., 2000). Spacers oligosaccharides were either synthesized as described earlier (Ovchinnikova et al., 2002; Pazynina et al., 2003; Tuzikov et al., 2000), or obtained from the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>).

Viruses

Influenza A viruses were from the virus repositories of St. Jude Children's Research Hospital (H5N1 viruses) and D.I. Ivanovsky Institute of Virology, Moscow. Viruses were grown in 9-day-old embryonated chicken eggs. The highly pathogenic H5N1 viruses were inactivated by treatment with β -propiolactone. The allantoic and culture fluids were clarified by low-speed centrifugation; the viruses were pelleted by high-speed centrifugation, resuspended in 0.1 M NaCl/0.02 M Tris buffer (pH 7.2) containing 50% glycerol, and stored at -20 °C.

Virus binding to polymeric sialoglycoconjugates

Receptor specificity of influenza viruses was evaluated in a competitive assay based on the inhibition of the binding of solid-phase immobilized virus with bovine fetuin labeled with horseradish peroxidase (Gambaryan and Matrosovich, 1992). The data were expressed in terms of binding constants (K_{ass}) formally equivalent to the association constants of virus-oligosaccharide complexes. For the calculation of the K_{ass} , concentration of the sialic acid residues was used. Each set of experiments presented in the table was repeated three to four times with similar results. Data were averaged from the same set of experiments.

Molecular models

Atomic coordinates of H5 HA complex with LSTa (1JSN, Ha et al., 2001) and of SiaLe^x (2KMB, Ng and Weis, 1997) were obtained from Protein Data Bank. The molecular models were generated with DS ViewerPro 5.0 software (Accelrys Inc.).

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References

- Banks, J., Speidel, E.S., Moore, E., Plowright, L., Piccirillo, A., Capua, I., Cordioli, P., Fioretti, A., Alexander, D.J., 2001. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence

- of highly pathogenic H7N1 avian influenza viruses in Italy. *Arch. Virol.* 146, 963–973.
- Beare, A.S., Webster, R.G., 1991. Replication of avian influenza viruses in humans. *Arch. Virol.* 119, 37–42.
- Bovin, N.V., Korchagina, E.Y., Zemlyanukhina, T.V., Byramova, N.E., Galanina, O.E., Zemlyakov, A.E., Ivanov, A.E., Zubov, V.P., Mochalova, L.V., 1993. Synthesis of polymeric neoglycoconjugates based on N-substituted polyacrylamides. *Glycoconjugate J.* 10, 142–151.
- Chambers, T.M., Yamnikova, S.S., Kawaoka, Y., Lvov, D.K., Webster, R.G., 1989. Antigenic and molecular characterization of subtype H13 hemagglutinin of influenza virus. *Virology* 172, 180–188.
- Claas, E.C., Osterhaus, A.D., Vanbeek, R., Dejong, J.C., Rimmelzwaan, G.F., Senne, D.A., Krauss, S., Shortridge, K.F., Webster, R.G., 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351, 472–477.
- Couceiro, J.N., Paulson, J.C., Baum, L.G., 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res.* 29, 155–165.
- Fouchier, R.A., Schneeberger, P.M., Rozendaal, F.W., Broekman, J.M., Kemink, S.A., Munster, V., Kuiken, T., Rimmelzwaan, G.F., Schutten, M., Van Doornum, G.J., Koch, G., Bosman, A., Koopmans, M., Osterhaus, A.D., 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1356–1361.
- Gambaryan, A.S., Matrosovich, M.N., 1992. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. *J. Virol. Methods* 39, 111–123.
- Gambaryan, A.S., Tuzikov, A.B., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Bovin, N.V., Matrosovich, M.N., 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetylglucosamine). *Virology* 232, 345–350.
- Gambaryan, A.S., Robertson, J.S., Matrosovich, M.N., 1999. Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. *Virology* 258, 232–239.
- Gambaryan, A.S., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Webster, R.G., Matrosovich, M.N., 2002a. Differences in receptor specificity between the influenza A viruses isolated from the duck, chicken, and human. *Mol. Biol. (Moscow)* 36, 542–549.
- Gambaryan, A., Webster, R., Matrosovich, M., 2002b. Differences between influenza virus receptors on target cells of duck and chicken. *Arch. Virol.* 147, 1197–1208.
- Gambaryan, A.S., Tuzikov, A.B., Bovin, N.V., Yamnikova, S.S., Lvov, D.K., Webster, R.G., Matrosovich, M.N., 2003. Differences between influenza virus receptors on target cells of duck and chicken and receptor specificity of the 1997 H5N1 chicken and human influenza viruses from Hong Kong. *Avian Dis.* 47 (Suppl. 3), 1154–1160.
- Gambaryan, A.S., Tuzikov, A.B., Pazynina, G.V., Webster, R.G., Matrosovich, M.N., Bovin, N.V., 2004. H5N1 chicken influenza viruses display a high binding affinity for the Neu5Ac α 2-3Gal β 1-4(6-HSO₃) GlcNAc receptor. *Virology* 326, 310–316.
- Gordeeva, E.A., Tuzikov, A.B., Galanina, O.E., Pochechueva, T.V., Bovin, N.V., 2000. Microscale synthesis of glycoconjugate series and libraries. *Anal. Biochem.* 278, 230–232.
- Guo, Y., Li, J., Cheng, X., Wang, M., Zhou, Y., Li, X.H., Cai, F., Miao, H.L., Zhang, H., Guo, F., 1999. Discovery of humans infected by avian influenza A (H9N2) virus. *Chin. J. Exp. Clin. Virol.* 13, 105–108.
- Ha, Y., Stevens, D.I., Skehel, J.J., Wiley, D.C., 2001. X-ray structures of H5 avian and H9 swine hemagglutinins bound to avian and human receptor analogs. *Proc. Natl. Acad. Sci. U.S.A.* 98, 11181–11186.
- Herrler, G., Rott, R., Klenk, H.D., Muller, H.P., Shukla, A.K., Schauer, R., 1985. The receptor-destroying enzyme of influenza C virus is neuraminidase-O-acetyltransferase. *EMBO J.* 4, 1503–1506.
- Ito, T., Couceiro, J.N., Kelm, S., Baum, L.G., Krauss, S., Castrucci, M.R., Donatelli, I., Kida, H., Paulson, J.C., Webster, R.G., Kawaoka, Y., 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.* 72, 7367–7373.
- Ito, T., Suzuki, Y., Suzuki, T., Takada, A., Horimoto, T., Wells, K., Kida, H., Otsuki, K., Kiso, M., Ishida, H., Kawaoka, Y., 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the α 2,3 linkage is associated with intestinal replication of influenza A virus in ducks. *J. Virol.* 74, 9300–9305.
- Makarova, N.V., Kaverin, N.V., Krauss, S., Senne, D., Webster, R.G., 1999. Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. *J. Gen. Virol.* 80, 3167–3171.
- Masuda, H., Suzuki, T., Sugiyama, Y., Horiike, G., Murakami, K., Miyamoto, D., Jwa Hidari, K.I., Ito, T., Kida, H., Kiso, M., Fukunaga, K., Ohuchi, M., Toyoda, T., Ishihama, A., Kawaoka, Y., Suzuki, Y., 1999. Substitution of amino acid residue in influenza A virus hemagglutinin affects recognition of sialyl-oligosaccharides containing N-glycolylneuraminic acid. *FEBS Lett.* 464, 71–74.
- Matrosovich, M.N., Gambaryan, A.S., Chumakov, M.P., 1992. Influenza viruses differ in recognition of 4-O-acetyl substitution of sialic acid receptor determinant. *Virology* 188, 854–858.
- Matrosovich, M.N., Gambaryan, A.S., Teneberg, S., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Karlsson, K.A., 1997. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology* 233, 224–234.
- Matrosovich, M., Gao, P., Kawaoka, Y., 1998. Molecular mechanisms of serum resistance of human influenza H3N2 virus and their involvement in virus adaptation in a new host. *J. Virol.* 72, 6373–6380.
- Matrosovich, M.N., Zhau, N., Kawaoka, Y., Webster, R., 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* 73, 1146–1155.
- Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M.R., Donatelli, I., Kawaoka, Y., 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J. Virol.* 74, 8502–8512.
- Matrosovich, M., Krauss, S., Webster, R., 2001. H9N2 influenza A viruses from poultry in Asia have human-virus-like receptor specificity. *Virology* 281, 156–162.
- Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D., 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4620–4624.
- Mochalova, L., Gambaryan, A., Romanova, J., Tuzikov, A., Chinarev, A., Katinger, D., Katinger, H., Egorov, A., Bovin, N., 2003. Receptor-binding properties of modern human influenza viruses primarily isolated in Vero and MDCK cells and chicken embryonated eggs. *Virology* 313, 473–480.
- Ng, K.K., Weis, W.I., 1997. Structure of a selectin-like mutant of mannose-binding protein complexed with sialylated and sulfated Lewis(x) oligosaccharides. *Biochemistry* 36, 979.
- Ovchinnikova, T.V., Shipova, E.V., Sablina, M.A., Pazynina, G.A., Popova, I.S., Tuzikov, A.B., Bovin, N.V., 2002. Synthesis of monosulfated saccharides in the spacers form. *Mendelev Commun.* 12, 213–215.
- Paulson, J.C., 1985. Interaction of animal viruses with cell surface receptors. In: Corn, M. (Ed.), *Receptors*, vol. 2. Academic Press, Orlando, pp. 131–219.
- Pazynina, G.V., Sablina, M.A., Tuzikov, A.B., Chinarev, A.A., Bovin, N.V., 2003. Synthesis of complex α 2-3 sialooligosaccharides, including sulfated and fucosylated ones, using Neu5Ac α 2-3Gal as a building block. *Mendelev Commun.* 13, 245–248.
- Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Ip, P.L., Lai, R.W., Orr, W.K., Shortridge, K.F., 1999. Human infection with influenza H9N2. *Lancet* 354, 916–917.

- Peiris, J.S., Yu, W.C., Leung, C.W., Cheung, C.Y., Ng, W.F., Nicholls, J.M., Ng, T.K., Chan, K.H., Lai, S.T., Lim, W.L., Yuen, K.Y., Guan, Y., 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* 363, 582–583.
- Rogers, G.N., D'Souza, B.L., 1989. Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* 173, 317–322.
- Scholtissek, C., 1994. Source for influenza pandemics. *Eur. J. Epidemiol.* 10, 455–458.
- Suarez, D.L., 2000. Evolution of avian influenza viruses. *Vet. Microbiol.* 74, 15–27.
- Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X.Y., Fukuda, K., Cox, N., 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279, 393–396.
- Suzuki, T., Horiike, G., Yamazaki, Y., Kawabe, K., Masuda, H., Miyamoto, D., Matsuda, M., Nishimura, S.I., Yamagata, T., Ito, T., Kida, H., Kawaoka, Y., Suzuki, Y., 1997. Swine influenza virus strains recognize sialylsugar chains containing the molecular species of sialic acid predominantly present in the swine tracheal epithelium. *FEBS Lett.* 404, 192–196.
- Suzuki, Y., Ito, T., Suzuki, T., Holland Jr., R.E., Chambers, T.M., Kiso, M., Ishida, H., Kawaoka, Y., 2000. Sialic acid species as a determinant of the host range of influenza A viruses. *J. Virol.* 74, 11825–11831.
- Tuzikov, A.B., Gambaryan, A.S., Juneja, L.R., Bovin, N.V., 2000. Conversion of complex sialooligosaccharides into polymeric conjugates and their anti-influenza virus inhibitory potency. *J. Carbohydr. Chem.* 19, 1191–1200.
- Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., Marth, J. (Eds.), 1999. *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* 56, 152–179.
- Yamnikova, S.S., Gambaryan, A.S., Tuzikov, A.B., Bovin, N.V., Matrosovich, M.N., Fedyakina, I.T., Grinev, A.A., Blinov, V.M., Lvov, D.K., Suarez, D.L., Swayne, D.E., 2003. Differences between HA receptor-binding sites of avian influenza viruses isolated from Laridae and Anatidae. *Avian Dis.* 47 (Suppl. 3), 1164–1168.

A DNA transfection system for generation of influenza A virus from eight plasmids

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We have developed an eight-plasmid DNA transfection system for the rescue of infectious influenza A virus from cloned cDNA. In this plasmid-based expression system, viral cDNA is inserted between the RNA polymerase I (pol I) promoter and terminator sequences. This entire pol I transcription unit is flanked by an RNA polymerase II (pol II) promoter and a polyadenylation site. The orientation of the two transcription units allows the synthesis of negative-sense viral RNA and positive-sense mRNA from one viral cDNA template. This pol I-pol II system starts with the initiation of transcription of the two cellular RNA polymerase enzymes from their own promoters, presumably in different compartments of the nucleus. The interaction of all molecules derived from the cellular and viral transcription and translation machinery results in the generation of infectious influenza A virus. The utility of this system is proved by the recovery of the two influenza A viruses: A/WSN/33 (H1N1) and A/Teal/HK/W312/97 (H6N1). Seventy-two hours after the transfection of eight expression plasmids into cocultured 293T and MDCK cells, the virus yield in the supernatant of the transfected cells was between 2×10^5 and 2×10^7 infectious viruses per milliliter. We also used this eight-plasmid system for the generation of single and quadruple reassortant viruses between A/Teal/HK/W312/97 (H6N1) and A/WSN/33 (H1N1). Because the pol I-pol II system facilitates the design and recovery of both recombinant and reassortant influenza A viruses, it may also be applicable to the recovery of other RNA viruses entirely from cloned cDNA.

Influenza A virus, a member of the family *Orthomyxoviridae*, is a negative-sense RNA virus with a segmented genome. Its genetic composition allows this virus to evolve by reassortment of gene segments from different strains. Of the 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes circulating in aquatic birds, only the H1N1, H2N2, and H3N2 subtypes are known to have caused pandemics in humans (1). There is evidence that pigs can serve as an intermediate host ("mixing vessel") for the generation of new strains that are pathogenic in humans (2). The H5N1 influenza A outbreak in Hong Kong in 1997 showed that highly pathogenic influenza A viruses can also be transmitted directly from avian species to humans (3–6). The potential of influenza A viruses to generate new pathogenic strains from a vast number of circulating strains in the natural reservoir indicates that disease control requires monitoring these viruses and developing improved antiviral therapies and vaccines.

For influenza A virus, reverse-genetics systems have allowed the manipulation of the viral genome (7, 8). Unlike positive-strand viruses (i.e., poliovirus), the negative-sense viral RNAs (vRNAs) of influenza A viruses are not infectious. Only vRNA molecules encapsidated with the four viral polymerase complex proteins (PB1, PB2, PA, nucleoprotein) are able to initiate a viral replication and transcription cycle. After the ribonucleoproteins (RNPs) penetrate the cell nucleus, the associated proteins begin to transcribe the (–)vRNAs into mRNAs and (+)cRNAs. These cRNAs serve as templates for the synthesis of vRNAs. The first reverse-genetics system to be developed for influenza A virus was the RNP-transfection method (9, 10). After *in vitro* transcription of virus-like vRNA by T3 or T7 RNA polymerase and

reconstitution of viral ribonucleoprotein (vRNP) molecules, genetically altered RNP segments were introduced into eukaryotic cells by transfection. Infection with influenza helper virus resulted in the generation of viruses possessing a gene derived from cloned cDNA. The establishment of the RNA polymerase I (pol I)-driven synthesis of vRNA molecules *in vivo* allowed the intracellular production of RNP complexes (11). In this system, virus-like cDNA was inserted between the pol I promoter and terminator sequences (12). Unlike the mRNA transcripts synthesized by RNA polymerase II (pol II), pol I-generated RNAs lack both a 5' cap and a 3' poly(A) tail. Functional vRNP molecules could be generated either by infection with helper virus or by cotransfection of protein expression plasmids encoding PB1, PB2, PA, or nucleoproteins (11, 13–15). Recent studies demonstrated that the plasmid-driven expression of all eight vRNAs from a pol I promoter and the coexpression of the polymerase complex proteins result in the formation of infectious influenza A virus (16, 17). Because the generation of influenza A virus entirely from plasmids requires no infection with helper virus, no selection system is needed; therefore, all gene segments can be manipulated without technical limitations. Previously, we established a new transcription system that allows the recovery of infectious virus if the PB1 cDNA is inserted between a pol I promoter and a pol II promoter (ref. 18, Fig. 1). Cotransfection of a pol I-pol II promoter PB1 construct with plasmids expressing vRNA and viral proteins resulted in the production of virus.

Here, we describe a plasmid-based transfection system for the rescue of influenza A virus entirely from cloned cDNA. Unlike established plasmid-based systems, this system for the generation of influenza A virus consists of the construction and transfection of only eight plasmids, each containing one copy of the viral cDNA. This reverse-genetics system reduces the number of plasmids required for the recovery of influenza A viruses and allows the generation of reassortant viruses.

Materials and Methods

Cloning of Plasmids. The plasmid pHW2000 (Fig. 3A) is derived from pHW12 (18). This cloning vector contains 225 bp of the human pol I promoter and 33 bp of the murine terminator sequence separated by two *BsmBI* sites. The pol I promoter and terminator elements are flanked by a truncated immediate-early promoter of the human cytomegalovirus (starting approximately 350 bp upstream of the transcription start site as found in pcDNA3, Invitrogen) and by the polyadenylation signal of the

Abbreviations: HA, hemagglutinin; MDCK cells, Madin-Darby canine kidney cells; NA, neuraminidase; NS, nonstructural; M, matrix; pol I, RNA polymerase I; pol II, RNA polymerase II; RNP, ribonucleoprotein; RT-PCR, reverse transcriptase-PCR; vRNA, viral RNA.

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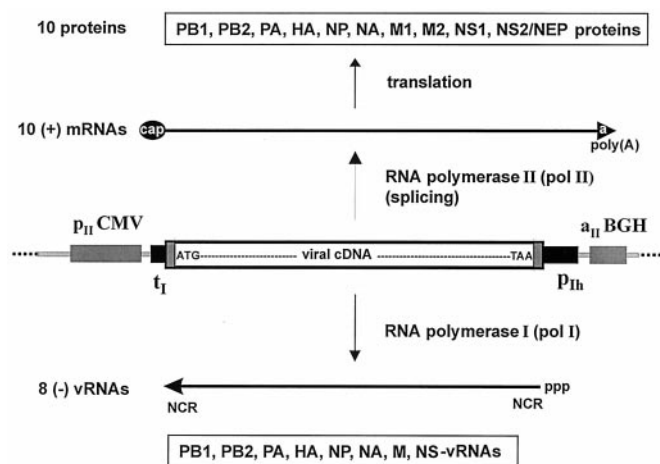


Fig. 1. Schematic representation of the pol I-pol II transcription system for synthesis of vRNA and mRNA. The cDNA of each of the eight influenza virus segments is inserted between the pol I promoter (p_{II}) and the pol I terminator (t_I). This pol I transcription unit is flanked by the pol II promoter (p_{II} CMV) of the human cytomegalovirus and the polyadenylation signal (a_{II} BGH) of the gene encoding bovine growth hormone. After transfection of the eight expression plasmids, two types of molecules are synthesized. From the human pol I promoter, negative-sense vRNA is synthesized by cellular pol I. The synthesized vRNA contains the noncoding regions (NCR) at the 5' and 3' ends. Transcription by pol II yields mRNAs with 5' cap structures and 3' poly(A) tails; these mRNAs are translated into viral proteins. The ATG of the viral cDNA is the first ATG downstream of the pol II transcription start site.

gene encoding bovine growth hormone. The eight plasmids containing the cDNA of the virus A/WSN/33 (H1N1) [pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, and pHW188-NS (NS, were constructed by inserting *Apa*I-*Sal*I fragments (with viral cDNA and pol I promoter and terminator sequences) of the plasmids pPolI-WSN-PB2, pPolI-WSN-PB1, pPolI-WSN-PA, pPolI-WSN-NP, pPolI-WSN-HA, pPolI-WSN-NA (16), pHW127-M, and pHW128-NS (18) into the *Apa*I-*Sal*I vector fragment of pHW2000. The eight plasmids containing the cDNA of A/Teal/HK/W312/97 (H6N1) (pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, and pHW248-NS) were constructed by reverse transcriptase-PCR (RT-PCR) amplification of the viral RNA. The primers used in the PCR reaction contained segment-specific sequences at their 3' ends and *Bsm*BI or *Bsa*I restriction site sequences at their 5' ends. After digestion of the PCR products with *Bsm*BI or *Bsa*I, the fragments were cloned into the vector pHW2000 (Fig. 3A). The sequences of the primers we used are available on request. To ensure that the viral cDNAs derived from RT-PCR amplification in the expression plasmids did not have unwanted mutations, the inserted cDNAs were sequenced.

Viruses and Cell Culture. Influenza viruses A/WSN/33 (H1N1) and A/Teal/HK/W312/97 (H6N1) were propagated in 10-day-old eggs. Madin-Darby canine kidney (MDCK) cells were maintained in MEM containing 10% FBS. 293T human embryonic kidney cells were cultured in Opti-MEM I (Life Technologies, Gaithersburg, MD) containing 5% FBS. For the transfection experiments, six-well tissue culture plates were used. The day before transfection, confluent 293T and MDCK cells in a 75 cm^2 flask were trypsinized, and 10% of each cell line was mixed in 18 ml OptiMEM I; 3 ml of this cell suspension was seeded into one well of a six-well plate. The cocultured MDCK and 293T cells ($0.2 - 1 \times 10^6$ cells per well each) were used for the transfection experiments. TransIT LT-1 (Panvera, Madison, WI)

was used according to the manufacturer's instructions to transfect the cells. Briefly, 2 μ l of TransIT LT-1 per 1 μ g of DNA was mixed, incubated at room temperature for 45 min, and added to the cells. Six hours later, the DNA-transfection mixture was replaced by Opti-MEM I. Thirty hours after transfection, 1 ml of Opti-MEM I containing TPCK-trypsin was added to the cells; this addition resulted in a final concentration of TPCK-trypsin of 0.5 μ g/ml in the cell supernatant. The virus titer of the cell supernatant was determined by titration of the supernatant on MDCK cells.

RNA Isolation and RT-PCR. Viral RNA was isolated from virus particles with the RNeasy-Kit (Qiagen, Valencia, CA), which was used according to the manufacturer's instructions. For characterization of recombinant influenza viruses, the Access RT-PCR kit (Promega) was used according to the protocol provided. The following primers were used in the RT-PCR experiments: Bm-NS#1 (5'-TAT TCG TCT CAG GGA GCA AAA GCA GGG TG-3') and Bm-NS#2 (5'-ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TT-3'). RT-PCR experiments were performed by using the PTC-200 DNA engine (MJ Research, Watertown, MA). The amplification program started with 1 cycle at 48°C for 45 min and 1 cycle at 94°C for 2 min. These cycles were followed by 40 cycles at 94°C for 20 sec, 52°C for 30 sec, and 72°C for 40 sec; the program ended with one cycle at 72°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis and sequenced with the primer Bm-NS#1. The Center for Biotechnology at St. Jude Children's Research Hospital determined the sequence of template DNA by using rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS [Perkin-Elmer/Applied Biosystems (PE/ABI)] and synthetic oligonucleotides. Samples were subjected to electrophoresis, detection, and analysis on PE/ABI model 373, model 373 Stretch, or model 377 DNA sequencers.

Results

Establishment of the pol I-pol II System for the Generation of A/WSN/33 (H1N1). Because the genome of influenza A virus contains eight segments, we reasoned that the insertion of all eight influenza A cDNAs between a pol I promoter and a pol II promoter should result in the transcription of the eight vRNAs, all viral mRNAs, and in the synthesis of all 10 viral proteins (Fig. 1). After assembly of all vRNPs with the structural proteins, infectious influenza A virus should then be formed (Fig. 2).

To test whether we could rescue infectious influenza A virus with this cDNA-bidirectional transcription system, we constructed the eight expression plasmids (pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, and pHW188-NS) containing the eight cDNAs of A/WSN/33 (H1N1). We cotransfected eight plasmids (1 μ g of each plasmid) (Table 1) into transiently cocultured 293T-MDCK cells. Both cell lines were cocultured in one cell-culture well the day before transfection to ensure conditions for high DNA transfection efficiency (293T cells) and for replication efficiency (MDCK cells) of influenza A viruses. After 48 and 72 h, the MDCK cells showed a virus-induced cytopathic effect, but no cytopathic effect was observed after transfection of seven plasmids without the PB1-expression construct (Table 1). The virus titer of the supernatant of transfected cells was determined at different times after transfection by titration in MDCK cells. Twenty-four hours after transfection, the cell supernatant contained 1×10^3 viruses per milliliter; 2×10^7 infectious viruses were generated 72 h after transfection (Table 1). The recovered viruses were passaged two times on MDCK cells. To verify that the generated virus was the designed A/WSN-virus, we pro-

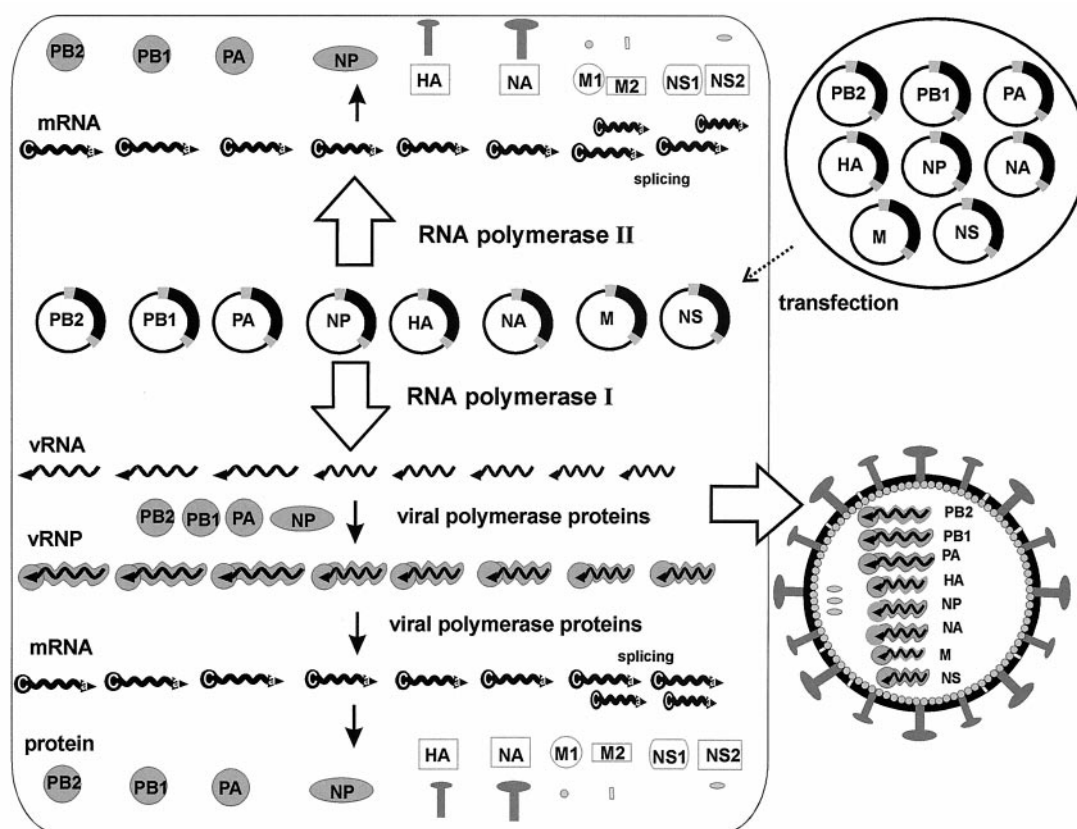


Fig. 2. The eight-plasmid pol I-pol II system for the generation of influenza A virus. Eight expression plasmids containing the eight viral cDNAs inserted between the human pol I promoter and the pol II promoter (see Fig. 1) are transfected into eukaryotic cells. Because each plasmid contains two different promoters, both cellular pol I and pol II will transcribe the plasmid template, presumably in different nuclear compartments, which will result in the synthesis of viral mRNAs and vRNAs. After synthesis of the viral polymerase complex proteins (PB1, PB2, PA, nucleoproteins), the viral replication cycle is initiated. Ultimately, the assembly of all viral molecules directly (pol II transcription) or indirectly (pol I transcription and viral replication) derived from the cellular transcription and translation machinery results in the interaction of all synthesized molecules (vRNPs and the structural proteins HA, NA, M1, M2, NS2/NEP) to generate infectious influenza A virus.

duced the cDNA for the nonstructural (NS) gene by RT-PCR (Fig. 4A, lane 8). The generation of two fragments after digestion with the restriction endonuclease *NcoI* (Fig. 4B, lane 8) and sequence analysis of the amplified fragment confirmed that the recovered virus was indeed the designed A/WSN virus. These findings show that the pol I and pol II-driven

synthesis of vRNA and mRNA from eight templates results in the generation of infectious influenza A virus.

Recovery of A/Teal/HK/W312/97 (H6N1) by Cotransfecting Eight Plasmids. The influenza virus A/WSN/33 (H1N1), originally derived from the human influenza pandemic strain from 1918 (19, 20),

Table 1. Plasmid sets used for the recovery of A/WSN/33 (H1N1) and A/Teal/HK/W312/97 (H6N1) viruses

Segment	A/WSN/33 (H1N1)		A/Teal/HK/W312/97 (H6N1)	
1	pHW181-PB2	pHW181-PB2	pHW241-PB2	pHW241-PB2
2	—	pHW182-PB1	—	pHW242-PB1
3	pHW183-PA	pHW183-PA	pHW243-PA	pHW243-PA
4	pHW184-HA	pHW184-HA	pHW244-HA	pHW244-HA
5	pHW185-NP	pHW185-NP	pHW245-NP	pHW245-NP
6	pHW186-NA	pHW186-NA	pHW246-NA	pHW246-NA
7	pHW187-M	pHW187-M	pHW247-M	pHW247-M
8	pHW188-NS	pHW188-NS	pHW248-NS	pHW248-NS
Virus titer*				
24 h	0	1×10^3	0	0
48 h	0	$2 \times 10^{6\dagger}$	0	2×10^3
72 h	0	$2 \times 10^{7\dagger}$	0	$2 \times 10^{5\dagger}$

*The numbers represent infectious virus particles per milliliter of the supernatant of transfected cells as determined 24, 48, and 72 h after transfection.

†Cytopathic effect in the cocultured MDCK cells was observed.

has been passaged in mouse brain and is well adapted for growth in cell culture. To evaluate the efficiency of the eight-plasmid system for the generation of a virus from cloned cDNA that is not already adapted for growth in cell culture, we sought to generate the virus A/Teal/HK/W312/97 (H6N1) from cloned cDNA alone. This H6N1 virus was isolated from a dead teal during the H5N1 outbreak in Hong Kong in 1997. Genetic analysis of this virus revealed that it has seven segments with more than 97% nucleotide homology to the pathogenic H5N1 virus strains (E.H. and R.G.W., unpublished results). RNA was extracted from infected allantoic fluid, and the RT-PCR-amplified cDNAs were inserted into pHW2000; this insertion resulted in eight expression plasmids (Fig. 3). Seventy-two hours after transfection of pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, and pHW248-NS (1 μ g each) into cocultured 293T-MDCK cells, a virus-induced cytopathic effect was observed in MDCK cells (Table 1). The virus yield was 2×10^5 infectious viruses per milliliter of the supernatant of the transfected cells. As shown in Fig. 4 A and B, lane 2, the identity of the recovered virus was verified by characterization of the NS segment. These results illustrate that this plasmid system requires the cloning of only eight cDNAs into one plasmid vector and that the transfection of the eight expression plasmids allows the recovery of an influenza A virus not already adapted to growth in mammalian cells.

Rescue of Reassortant Influenza A Viruses. We sought to test the utility of the eight-plasmid system for the generation of reassortant viruses. Because this DNA transfection system does not require any selection system, the recovery of reassortant viruses should be achievable by appropriate combinations of expression plasmids in the transfection reactions. Seven expression plasmids containing the cDNA of A/Teal/HK/W312/97 (H6N1) were cotransfected with one expression plasmid containing the cDNA of A/WSN/33 (H1N1) (Table 2). High virus yields were obtained for the reassortant viruses containing seven segments of the teal virus and the M segment or NS segment of the WSN virus. Lower virus yields were obtained for the neuraminidase (NA) and HA-reassortant viruses (Table 2). Because we were able to rescue single reassortant viruses with the eight-plasmid system, we tested whether we could rescue a virus with multiple segments derived from one virus. Therefore, we transfected four expression plasmids containing the cDNA of the RNP-complex genes of the H6N1 virus (pHW241-PB2, pHW242-PB1, pHW243-PA and pHW245-NP) together with the plasmids pHW184-HA, pHW186-NA, pHW187-M, and pHW188-NS containing the cDNA of the WSN virus (Table 2). We recovered 4×10^6 viruses per milliliter of the cell supernatant. As shown in Fig. 4 (lane 10), the amplified NS segment of the quadruple reassortant virus was cleaved by *NcoI*; thus, the NS segment is derived from the WSN virus. These results show that the eight-plasmid transfection system allows the recovery of single and quadruple reassortant viruses.

Discussion

The fact that we were able to rescue influenza A virus after transfection of the eight expression plasmids containing the cDNA of A/Teal/HK/W312/97 (H6N1) or A/WSN/33 (H1N1) proves that the pol I-pol II transcription system provides sufficient amounts of vRNA and viral proteins for the formation of infectious influenza A virus. Two types of mRNAs that differ in their noncoding regions are synthesized (Fig. 1). The mRNA type encoding all viral proteins is directly transcribed by pol II. In addition to the influenza virus sequences of the noncoding regions, these mRNAs contain sequences from the pol I promoter and the murine terminator regions. Importantly, the pol I-pol II expression system we developed contained only 33 bp of

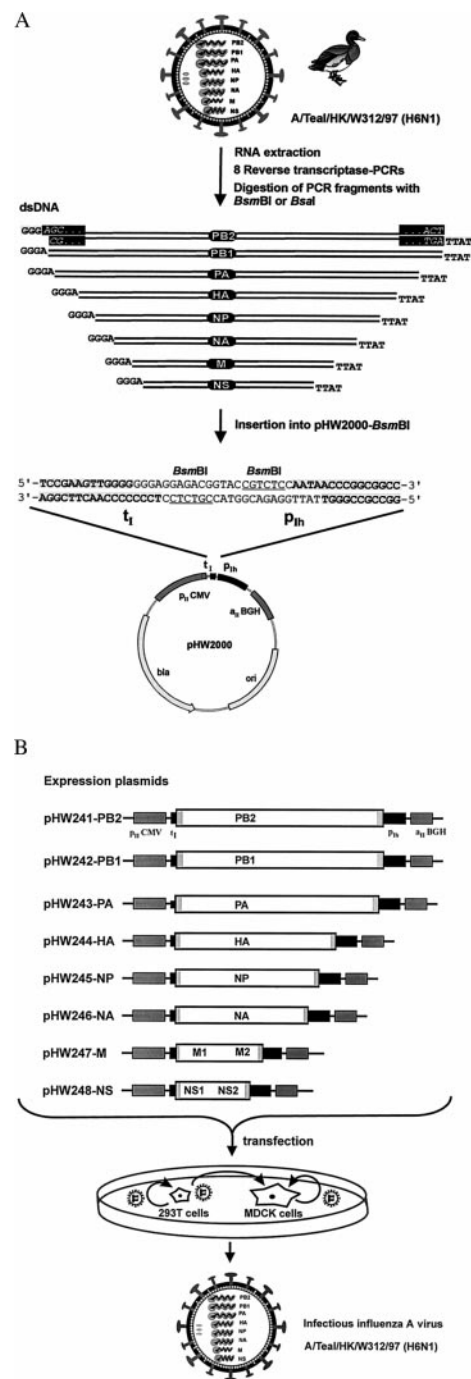


Fig. 3. Schematic representation of the method developed for the construction and transfection of the eight expression plasmids to recover A/Teal/HK/W312/97 (H6N1). (A) Viral RNA was extracted from virus particles. RT-PCR was performed with primers containing segment-specific nucleotides and sequences for the type II restriction endonucleases *BsmBI* or *BsaI*. The eight viral PCR fragments were digested with *BsmBI* or *BsaI* and inserted into pHW2000 (linearized with *BsmBI*). This insertion resulted in eight expression constructs where the viral cDNAs are precisely fused to the pol I promoter and terminator (the viral terminal sequences AGC...ACT are shown for the PB2 segment in the black rectangles). (B) The eight expression plasmids with a pol I promoter and a pol II promoter contain one copy of each of the viral cDNAs of the eight segments. The open reading frames for the 10 viral proteins are flanked by the segment-specific noncoding regions (gray boxes). Because the used human pol I promoter shows high activity only in cell lines derived from humans or related species, we cocultured human 293T cells together with the standard cell line used for influenza A (MDCK cells). Viruses produced in the 293T cells after transfection can then infect MDCK cells and replicate.

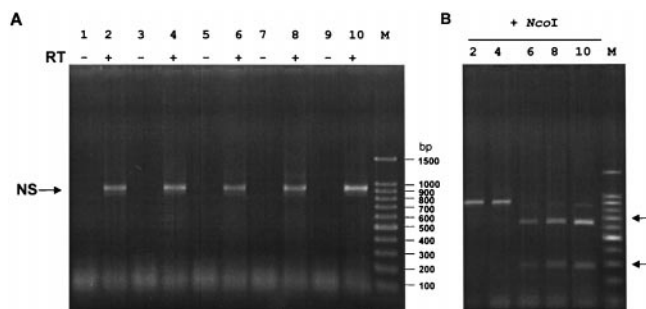


Fig. 4. Characterization of the recovered viruses by RT-PCR. (A) RNA was extracted from virus particles after two passages of the supernatant of transfected cells (see Tables 1 and 2) on MDCK cells. RT-PCR was performed with primers specific for the NS gene segment and with vRNA extracted from virions. The NS primers we used were not strain specific, thus allowing the amplification of any influenza A NS segment. The reaction products were subjected to electrophoresis on a 2% agarose gel. To ensure that the amplified DNA fragments were derived from vRNA and not from plasmid DNA carried over from transfected cells, one reaction was performed without the addition of reverse transcriptase (RT–). Lanes 1 and 2, recombinant A/Teal/HK/W312/97 (Table 1); lanes 3 and 4, M reassortant (Table 2); lanes 5 and 6, NS reassortant (Table 2); lanes 7 and 8, recombinant A/WSN/33 virus (Table 1); lanes 9 and 10, quadruple reassortant (Table 2). (B) *Nco*I digestion of the fragments shown in A (only the WSN-NS-cDNA has an *Nco*I site). The identity of the NS fragments was also verified by sequence analysis of the amplified product (not shown).

the murine terminator sequences. Previous studies using the reporter genes chloramphenicol acetyltransferase and green fluorescent protein showed that sequences in the 174-bp terminator region reduced pol II-mediated expression of protein (21). A second mRNA type is generated after the initiation of the viral replication and transcription process (Fig. 2). This mRNA is synthesized by the viral polymerase proteins and contains a 5' cap structure derived from cellular RNAs by cap snatching preceding the influenza virus noncoding sequences. The structural proteins translated from both mRNAs associate with the RNP complexes to form new virus particles. After the budding of transfectant viruses, the generated virus particles can then replicate in the 293T cells and in the cocultured MDCK cells.

Recently, plasmid-based systems that are designed to express the eight vRNAs and viral mRNAs from separate plasmids have been developed. In the system developed by Neumann *et al.* (16), the eight cDNAs were inserted between a human pol I promoter sequence (407 bp) and a murine terminator sequence (174 bp). The expression of the four RNP-complex proteins was driven by the human cytomegalovirus promoter. Transfection of 12 plasmids into 10^6 293T cells resulted in virus recovery of more than 10^3 plaque-forming units (pfu); this efficiency could be increased

to 5×10^7 pfu after the transfection of 17 plasmids. Fodor *et al.* (17) developed a system in which the eight cDNAs were inserted between a human pol I promoter sequence (250 bp) and a genomic ribozyme sequence of hepatitis δ virus to ensure the precise 3' end of the vRNA. For the expression of the polymerase complex genes, plasmids containing the adenovirus type 2 major late promoter were used. After transfection of the 12 expression plasmids into Vero cells, only one or two infectious viral particles were rescued from 10^6 transfected cells. Unlike these approaches, our method deployed the eight cDNAs in eight plasmids that contained 225 bp of the pol I promoter sequences and 33 bp of the terminator sequences. In the pol I–pol II transcription system, all 10 viral proteins are expressed from a truncated immediate-early promoter of the human cytomegalovirus. The fact that the expression of all structural proteins with the 17-plasmid system (16) and with the 8-plasmid system (this study) resulted in a higher efficiency of virus recovery than did cotransfection of plasmids expressing the RNP complex proteins (16, 17) supports the idea that the generation of infectious influenza A virus is enhanced by providing the HA, NA, M1, M2, and NS2 proteins early after transfection.

The viral replication cycle involves a complex interaction between the viral proteins with each other and with cellular factors (22). Thus, for the generation of infectious virus, the plasmid-driven synthesis of viral molecules should provide optimal concentrations of viral proteins for the initiation of the replication cycle and for the formation of virus-like particles. Although the eight-plasmid system proved to be efficient, it might be possible to further increase the production of virus. It was shown that the ratio of transfected plasmids expressing the RNP complex proteins and the expression of the M1 protein influences the transcriptase activity (14, 23). The efficiency of the formation of virus-like particles also depends on the concentration of structural viral proteins (24–26). The efficiency of the generation of infectious virus with the pol I–pol II system might therefore be increased further by varying the plasmid concentrations used in the transfection reaction or by using expression plasmids with different pol II promoters. Because the splicing efficiency mediated by cellular factors influences the ability of influenza A virus to replicate (27), the use of cell lines other than 293T may increase the virus yield for certain influenza A strains. The high virus yield of the quadruple reassortant (Table 2) is consistent with the finding that the rapid replication of A/WSN/33 (H1N1) in cultured cells is mediated by the HA, NA, and M segments (19, 28, 29).

The fact that we could generate viable reassortants (Table 2) between the avian H6N1 virus and the human H1N1 virus indicates that this H6N1 virus can acquire gene segments from a distantly related virus. Genetic analysis suggested that the pathogenic H5N1 viruses were generated by reassortment (30).

Table 2. Generation of reassortant influenza A viruses between A/Teal/HK/W312 (H6N1) and A/WSN/33 (H1N1) by cotransfecting eight plasmids

Segment*	HA	NA	M	NS	HA-NA-M-NS
1	pHW241-PB2	pHW241-PB2	pHW241-PB2	pHW241-PB2	pHW241-PB2
2	pHW242-PB1	pHW242-PB1	pHW242-PB1	pHW242-PB1	pHW242-PB1
3	pHW243-PA	pHW243-PA	pHW243-PA	pHW243-PA	pHW243-PA
5	pHW245-NP	pHW245-NP	pHW245-NP	pHW245-NP	pHW245-NP
4	pHW184-HA	pHW244-HA	pHW244-HA	pHW244-HA	pHW184-HA
6	pHW246-NA	pHW186-NA	pHW246-NA	pHW246-NA	pHW186-NA
7	pHW247-M	pHW247-M	pHW187-M	pHW247-M	pHW187-M
8	pHW248-NS	pHW248-NS	pHW248-NS	pHW188-NS	pHW188-NS
Virus titer†	2×10^2	2×10^3	2×10^5	2×10^7	4×10^6

*Plasmids containing the cDNA of A/WSN/33 (H1N1) are shown in bold.

†The numbers represent infectious virus particles per milliliter of supernatant of transfected cells as determined 72 h after transfection.

H5N1-like gene segments are found in the H6N1 (E.H. and R.G.W., unpublished results) and H9N2 subtypes (31), a finding indicating that these viruses may have been precursors of the pathogenic H5N1 viruses. Reassortment events that could create new pathogenic influenza viruses are likely to occur in the future. However, the ability to generate and manipulate these viruses by the simplified method developed in this study will help researchers better understand the biological properties of these new viruses and develop efficient vaccines to protect a population against them. The length of the time period between the emergence of a new pathogenic strain and the preparation of a vaccine is a crucial variable in the effectiveness of a vaccination program. The ability to generate viruses by cloning only eight plasmids reduces the time needed for the generation of potential vaccine candidates and improves existing reverse-genetics systems by simplifying virus creation and reducing the overall cost of production of a vaccine.

The concept of introducing viral cDNA between a pol I promoter and a pol II promoter into eukaryotic cells for the

recovery of virus might also be applicable for the generation of other members of the family *Orthomyxoviridae*. For influenza B virus, this strategy would require the construction and cotransfection of eight plasmids; for influenza C, seven; and for Thogotovirus, six. The *in vivo* transcription of 5'-capped mRNA as well as vRNA from the same cDNA template may also simplify plasmid-based systems for other RNA viruses or even facilitate the establishment of pol I-pol II systems for viruses from other families (e.g., *Arenaviridae*, *Bunyaviridae*).

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1. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. (1992) *Microbiol. Rev.* **56**, 152–179.
2. Scholtissek, C., Bürger, H., Kistner, O. & Shortridge, K. F. (1985) *Virology* **147**, 287–294.
3. Claas, E. C., Osterhaus, A. D., van Beek, R., De Jong, J. C., Rimmelzwaan, G. F., Senne, D. A., Krauss, S., Shortridge, K. F. & Webster, R. G. (1998) *Lancet* **351**, 472–477.
4. Suarez, D. L., Perdue, M. L., Cox, N., Rowe, T., Bender, C., Huang, J. & Swayne, D. E. (1998) *J. Virol.* **72**, 6678–6688.
5. Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., *et al.* (1998) *Science* **279**, 393–396.
6. Shortridge, K. F. (1999) *Vaccine* **17** (Suppl 1), S26–S29.
7. Palese, P., Zheng, H., Engelhardt, O. G., Pleschka, S. & Garcia-Sastre, A. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 11354–11358.
8. Neumann, G. & Kawaoka, Y. (1999) *Adv. Virus Res.* **53**, 265–300.
9. Luytjes, W., Krystal, M., Enami, M., Pavin, J. D. & Palese, P. (1989) *Cell* **59**, 1107–1113.
10. Enami, M., Luytjes, W., Krystal, M. & Palese, P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3802–3805.
11. Neumann, G., Zobel, A. & Hobom, G. (1994) *Virology* **202**, 477–479.
12. Zobel, A., Neumann, G. & Hobom, G. (1993) *Nucleic Acids Res.* **21**, 3607–3614.
13. Flick, R., Neumann, G., Hoffmann, E., Neumeier, E. & Hobom, G. (1996) *RNA* **2**, 1046–1057.
14. Pleschka, S., Jaskunas, R., Engelhardt, O. G., Zurcher, T., Palese, P. & Garcia-Sastre, A. (1996) *J. Virol.* **70**, 4188–4192.
15. Zhou, Y., Konig, M., Hobom, G. & Neumeier, E. (1998) *Virology* **246**, 83–94.
16. Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9345–9350.
17. Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. & Garcia-Sastre, A. (1999) *J. Virol.* **73**, 9679–9682.
18. Hoffmann, E., Neumann, G., Hobom, G., Webster, R. G. & Kawaoka, Y. (2000) *Virology* **267**, 310–317.
19. Goto, H. & Kawaoka, Y. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 10224–10228.
20. Reid, A. H., Fanning, T. G., Hultin, J. V. & Taubenberger, J. K. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 1651–1656.
21. Hoffmann, E. (1997) Ph.D. Thesis (Justus Liebig University, Giessen, Germany).
22. Ludwig, S., Pleschka, S. & Wolff, T. (1999) *Viral Immunol.* **12**, 175–196.
23. Perez, D. R. & Donis, R. O. (1998) *Virology* **249**, 52–61.
24. Mena, I., Vivo, A., Perez, E. & Portela, A. (1996) *J. Virol.* **70**, 5016–5024.
25. Gomez-Puertas, P., Mena, I., Castillo, M., Vivo, A., Perez-Pastrana, E. & Portela, A. (1999) *J. Gen. Virol.* **80**, 1635–1645.
26. Neumann, G., Watanabe, T. & Kawaoka, Y. (2000) *J. Virol.* **74**, 547–551.
27. Lau, S. C. & Scholtissek, C. (1995) *Virology* **212**, 225–231.
28. Schulman, J. L. & Palese, P. (1977) *J. Virol.* **24**, 170–176.
29. Yasuda, J., Bucher, D. J. & Ishihama, A. (1994) *J. Virol.* **68**, 8141–8146.
30. Xu, X., Subbarao, K., Cox, N. J. & Guo, Y. (1999) *Virology* **261**, 15–19.
31. Guan, Y., Shortridge, K. F., Krauss, S. & Webster, R. G. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9363–9367.