

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

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Abogado
Emilio Ferrero

ASUNTO	Radicación	08-23024
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	008

1 2 4 6 4

Patente de Invención	☐	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☐	Cap. I	☐ Cap. II ☐
No. Sol. Internacional		PCT/US2006/029575	
Pub. Internacional		WO 2007/019094	

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 le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 85 a 149	04/03/2008
<u>Reivindicaciones:</u>	Folios: 150 a 156	04/03/2008
<u>Figuras:</u>	Folios: 157 a 159	04/03/2008
<u>Prioridad:</u>	US 60/705,808	04/08/2005

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 las prioridades reivindicadas.

3. Objeto de la invención

Título de la solicitud: "Virus de influenza modificado para control y mejoramiento de la eficacia de la vacuna".

El objeto de la presente solicitud es suministrar un grupo de subtipos de virus de influenza que presentan incremento de la antigenicidad y/o la inmunogenicidad.

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

Expediente 08- 023024 1er Art.45

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

El objeto de la solicitud definida anteriormente se clasificó en la IPC: A61K 39/145.

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

El objeto de las reivindicaciones 23 a 27 es un método de diagnóstico aplicado a humano y animales, y no es patentable de acuerdo con el Art citado.

Para que dicho método sea aceptado debe referirse explícitamente a un método *in-vitro*, e indicar cada uno de sus etapas con los elementos involucrados y los procesos realizados, de manera que sea evidente que se realiza *in-vitro*.

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

El uso de las reivindicaciones 14, 34 a 37 no es patentable, de acuerdo con el Art 14.

6. De la solicitud de Patente

Reivindicaciones (artículo 30 D 486)

El capítulo reivindicatorio presentado carece de claridad y concisión por cuanto:

- 1) Varias reivindicaciones se refieren al resultado a alcanzar con la invención, pero no se indica como se logra dicho resultado definiendo específicamente el producto modificado o indicando el método para prepararlo (reivindicaciones 1-3, 17-25, 28, 31);
- 2) La molécula de hemaglutinina (HA) del virus de influenza de la reivindicación 1 debe ser definida indicando la secuencia de la misma y la modificación específica que contiene (identificación de la sustitución realizada y la posición de esta);
- 3) Se recomienda definir el origen del virus de influenza, indicando el serotipo y la variante y evitando especificarlo a partir de la variante que no es su origen. Lo anterior, puesto que la definición de la reivindicación 3 además de no ser clara, da lugar a múltiples opciones de variantes las cuales no tienen sustento en la descripción. Se recomienda en su lugar, incluir la información de la reivindicación 6 y 7;
- 4) El sistema de genética inversa de la reivindicación 11 debe ser definido a partir de cada uno de los elementos que lo componen, identificándolos además por sus respectivas secuencias;
- 5) El método para hacer el virus de la reivindicación 16 debe ser definido por cada una de las etapas que permiten obtener el virus modificado, indicando las secuencias de todos los elementos involucrados, el tipo de vector empleado, entre otras. Lo mismo aplica para la reivindicación 19;
- 6) Hay múltiples reivindicaciones independientes que se refieren a un método, pero que no definen dicho método por las etapas del mismo sino por el resultado a alcanzar o el uso que se le va a dar al método. Se recomienda eliminar dichas

reivindicaciones puesto que no aportan características técnicas de la invención (reivindicaciones 17, 19, 20, 23, entre otras);

- 7) El virus para vacuna de influenza de la reivindicación 28 debe ser definido por el número de depósito del mismo o identificándolo de manera precisa, resaltando las modificaciones exactas que contiene;
- 8) El ácido nucleico de la reivindicación 29 y 30 debe ser definido a partir de su secuencia nucleótida (SEQ ID N°);

De esta manera, el capítulo reivindicatorio presentado no cumple con los requisitos establecidos en el art. 30 de la Decisión 486.

7. Determinación del Estado de la Técnica

Para determinar el estado de la técnica se ha tenido en cuenta la fecha de presentación de la solicitud prioritaria reivindicada con la solicitud: 04/08/2005.

Teniendo en cuenta la fecha indicada se realizó búsqueda en el estado de la técnica de documentos relacionados con la solicitud, encontrándose los siguientes documentos relevantes a la misma:

Nº	Documento	Título	Fecha de prioridad
D1	Both, <i>et.al.</i> PNAS, vol 80: 6996-7000	"Hemagglutinin of swine influenza virus: A single amino acid change pleiotropically affects viral antigenicity and replication"	Nov 1983
D2	Gambaryan A., <i>et.al.</i> Virology, vol 334: 276-283	"Receptor specificity of influenza viruses from birds and mammals: New data on involvement of the inner fragments of the carbohydrate chain"	March, 2005
D3	Hoffman <i>et.al.</i> PNAS, vol 97: 6108-6113	"A DNA transfection system for generation of influenza A virus from eight plasmids"	Mayo, 2000

Resumen de los documentos citados:

D1: Se divulga la secuencia completa del gen de la hemaglutinina H1 de un mutante de alta producción (H) de la cepa A/NJ/11/76(H1N1) del virus de la influenza porcina en estudios de reagrupamiento viral teniendo este gen (resumen, folio 169).

D2: Se 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, folio 172);

D3: Se ha desarrollado un sistema de transfección de ocho plásmidos para el rescate de virus de influenza A infecciosos de cDNA clonado (resumen, folio 173).

8. Análisis de patentabilidad (artículo 14 D486)

Nivel inventivo (artículo 18 D486)

La reivindicación 1 de la solicitud reclama una molécula de hemaglutinina (HA) del virus de influenza que contiene una sustitución de un aminoácido en el sitio de fijación en el receptor que hace a la molécula de HA más antigénica respecto a anticuerpos específicos que la molécula carente de sustitución del aminoácido en el sitio de fijación de su receptor.

El documento D1 divulga la secuencia de nucleótidos de hemaglutinina obtenida del virus de influenza porcina de la cepa A/NJ/11/76(H1N1) (resumen). Este documento revela las mutaciones en la secuencia que presentan los virus de influenza tipo H y tipo L, que representan fenotipos de alta y baja productividad, respectivamente (resumen).

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

Características esenciales	D1
Molécula de hemaglutinina (HA) del virus de influenza	Molécula de hemaglutinina (HA) del virus de influenza (resumen)
contiene una sustitución de un aminoácido en el sitio de fijación en el receptor, que hace a la molécula de HA más antigénica respecto a anticuerpos específicos que la molécula carente de sustitución del aminoácido en el sitio de fijación de su receptor	Revela varias sustituciones de aminoácido, una de ellas en la posición 155 (Gly por Glu) adyacente al sitio de unión del receptor por lo que afecta la replicación del virus (resumen). Sugiere que sustitución cambia la antigenicidad (Pág 7000 col izq último párr.)

Este documento identifica el cambio de un aminoácido ubicado en el residuo 155 (aminoácido 158 en la estructura HA de Wiley), cambio de Gly a Glu como el responsable del cambio de fenotipo del virus de tipo H a tipo L y adyacente al sitio de unión del receptor, por lo que dicha sustitución puede repercutir sobre la replicación del virus (resumen). Además, se divulgan otras sustituciones conocidas para este virus que involucran aminoácidos en otras posiciones (Tabla 2, Fig 4).

D1 también menciona que el virus de influenza B tiene variabilidad antigénica, con diferente tropismo sobre la célula huésped (pág. 7000 col der). Este documento también realiza las pruebas de inhibición de hemaglutinina con los virus que presentan la sustitución en HA, mostrando que al usar estos virus se incrementa la sensibilidad del ensayo (pág. 7000 col izq, tabla 2).

La diferencia entre la materia reclamada y D1, es que la modificación de un aminoácido en la solicitud provoca que la HA sea más antigénica, mientras que en D1 este cambio se reporta como una Ha de un virus de baja productividad.

El efecto que produce esta diferencia no resulta aparente puesto que en ambos casos lo que se obtiene es una molécula Ha que no se une a su receptor y un virus de menor capacidad infectiva.

El problema técnico objetivo a resolver es obtener un virus de influenza que sea más antigénico a partir de la variación de la HA.

Sin embargo, a partir de la información divulgada en D1 resultaba evidente que el virus obtenido con la sustitución en el residuo 155 (aminoácido 158 en la estructura HA de Wiley), cambio de Gly a Glu era de fenotipo del virus de tipo L. Además que dicho virus tenía una capacidad disminuida para replicarse y D1 indicaba que esto podría estar asociado a un cambio antigénico (Pág. 7000 col izq, último párr). De esta manera, a partir de la información divulgada en D1 resultaba obvio desarrollar el virus de influenza que tenía una mayor antigenicidad y una menor capacidad infectiva.

El documento D1 contiene la información necesaria para que la persona del oficio normalmente versada en la materia pudiera desarrollar la molécula reclamada en la reivindicación 1, 20 a 22 de la solicitud, por lo que carece de nivel inventivo e incumple el artículo 18 de la D486.

Las reivindicaciones dependientes 2 a 6 reclaman que la sustitución de aminoácido ocurre en la posición 223, en la HA de un virus H5 que no sea aislado de humano y en donde dicha sustitución altera un sitio de glicosilación.

El documento D2 divulga 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 y cambio en la composición del loop 210-230, resultando en un efecto sobre la capacidad de unión al receptor entre las cepas de influenza (Pág. 280 col der).

D2 también divulga que la molécula de HA no se origina en el aislado de virus del humano (Pág. 280 col der) y es un virus del subtipo H5 (Pág. 227 col. der). Adicionalmente, D2 enseña que el cambio en la región de glicosilación de la molécula HA en el mismo loop 210-230 podía cambiar la capacidad de unión del virus al receptor (Pág. 280 col der).

De esta manera, a partir de la información divulgada en D1 y D2, la persona del oficio podía desarrollar la invención tal como es reclamada en las reivindicaciones 1-9 de la solicitud, por lo que la misma carece de nivel inventivo.

La reivindicación 10 de la solicitud reclama un virus recombinante de influenza que contiene la molécula HA del virus de influenza de la reivindicación 1.

La reivindicación 11 de la solicitud reclama un sistema de genética inversa para hacer un virus que contiene la molécula HA del virus de influenza de la reivindicación 1.

El documento D3 enseña un virus de influenza recombinante y un sistema de genética inversa para hacer el virus (Pág. 1608 col der, último párr). Este sistema usa un vector recombinante (Pág. 6109 col. izq) el cual codifica proteínas estructurales con sustituciones (Pág. 6112).

D3 enseña el uso del virus H5N1 de los virus de influenza A (Pág. 6111 col izq). De esta manera, a partir de la información divulgada en D1 sobre las sustituciones en HA del virus de influenza y su efecto sobre la unión al receptor y la información de D3 sobre la obtención de un virus recombinante y el sistema de genética inversa, la persona del oficio normalmente versada en la materia podía llevar a cabo la invención tal como es reclamada.

Puesto que las reivindicaciones dependientes no mencionan características inventivas, dichas reivindicaciones no se considera que superen la objeción de nivel inventivo planteada con las anterioridades mencionadas.

9. Conclusión

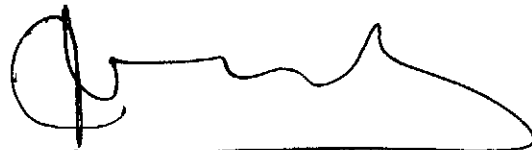
Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Both, <i>et.al.</i> PNAS, vol 80: 6996-7000	1-37	Nivel inventivo
D2	Gambaryan A., <i>et.al.</i> Virology, vol 334: 276-283	1-9	Nivel inventivo
D3	Hoffman <i>et.al.</i> PNAS, vol 97: 6108-6113	10-19	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 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 N° 10 de 2001.



JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

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

18 JUL 2012

Elaboró: N. Lamprea
Revisó: Consuelo Leguizamón
Aprobó: Jesús F. García



Hemagglutinin of swine influenza virus: A single amino acid change pleiotropically affects viral antigenicity and replication

(antigenic variation/point mutation/nucleotide sequence/viral genetics/virulence)

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Contributed by Edwin D. Kilbourne, August 8, 1983

ABSTRACT The complete nucleotide sequence has been obtained of the H1 hemagglutinin (HA) gene of a high-yielding (H) mutant of the A/NJ/11/76(H1N1) strain of swine influenza virus in studies of a viral reassortant (X-53a) bearing this gene. This determination has permitted comparison with human influenza H1N1 prototype viruses A/WSN/33 and A/PR/8/34, with which 80% and 94% amino acid homology was found between HA1 and HA2, respectively. Partial sequences have been determined for other viral reassortants containing either H or L (low-yielding phenotype) genes derived from A/NJ/11/76. Sequence of the HA1 region of an L mutant prototype was virtually completed and differed from that of the H mutant by only four amino acid changes. Sequence analysis of four other viruses was restricted to regions of the HA with which monoclonal antibodies capable of distinguishing L and H mutants are presumed to react. Therefore, changes in these sequences are relevant to changes in viral phenotype. Change at residue 155 from Gly to Glu is associated with change from L to H HA phenotype. This site, structurally equivalent to amino acid 158 on the Wiley *et al.* HA model [Wiley, D. C., Wilson, I. A. & Skehel, J. J. (1981) *Nature (London)* 289, 373-378] is near the tip of the HA monomer adjacent to the proposed receptor binding site and therefore credibly could influence both viral antigenicity and replication. Because both L and H variants exist in nature and because revertants may be selected in the laboratory as replication variants in the absence of immunoselection, these studies provide evidence for fortuitous antigenic change in association with change in biological function, which is determined by a single base change.

Hemagglutinin (HA) L and H mutants of contemporary strains of swine influenza virus differ pleiotropically in their capacity to replicate in chicken embryos, Madin-Darby canine kidney cells (MDCK cells), and the respiratory tract of swine (1-3). Although antigenically identical in tests with conventional polyclonal antibodies (1), these mutants can be distinguished with certain cross-absorbed heterotypic antisera or with monoclonal antibodies (4). The L and H mutants have been intensively studied because (i) they are commonly found in field isolates from swine as dimorphic variants, in which one may be detected in primary passage in the absence of the other (2), or in early passage isolates from humans (1, 2) or turkeys (unpublished data); (ii) genetic evidence suggests that the variants reflect point mutations in the HA gene (1); and (iii) it has seemed important to identify a mutational site that concomitantly influences both the antigenicity of virus and replication in its natural host.

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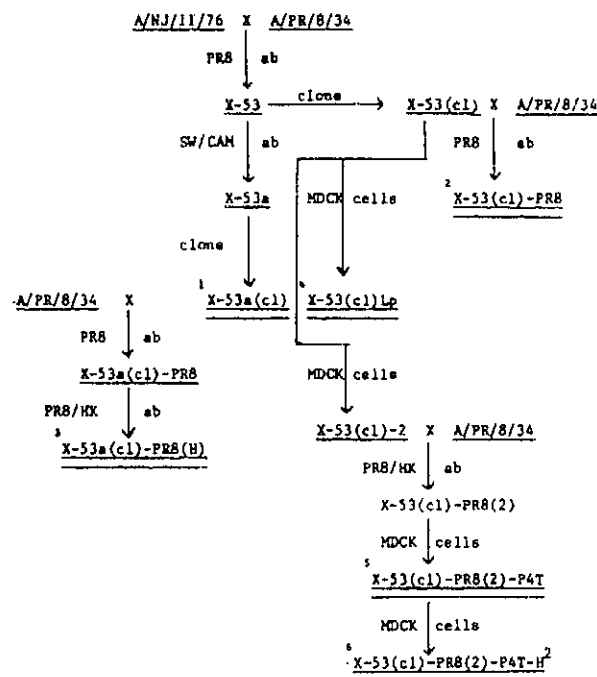


FIG. 1. Genealogy of reassortant viruses on which nucleotide sequence analysis of the HA gene has been performed (numbered, double-underlined viruses). The details of passage history are shown in Table 1. All reassortants are definitively derived from uncloned A/NJ/11/76(H1N1) swine influenza virus and A/PR/8/34(H1N1). PR8 ab, antibody to PR8 virus; SW/CAM ab, antibody to A/SW/CAM/39(H1N1) virus; PR8/HK ab, antibody to reassortant of PR8 and Hong Kong (H0N2) viruses containing PR8 HA and Hong Kong neuraminidase. X-53 viruses (L phenotype) are on the right and X-53a (H phenotype) are on the left. Repeated reassortment with PR8 virus has yielded viruses (designated by PR8 suffix) containing all PR8 genes, except for that coding for the (swine) HA. When not specified (i.e., MDCK cells) passage has been in chicken embryos.

MATERIALS AND METHODS

Viruses. The derivation and properties of the basic reassortant viruses X-53 and X-53a used in the present experiments have been presented in detail (1). These viruses were derived from genetic reassortment of the A/PR/8/34 (H1N1) (hereafter referred to as PR8) and A/NJ/11/76 (H1N1) (swine) influenza viruses. X-53 (L phenotype) and X-53a (H phenotype) viruses

Abbreviations: HA, hemagglutinin; HI, hemagglutination-inhibition.

Table 1. Detailed passage history of viruses on which nucleotide sequence analysis of portions of the HA gene was performed

Virus	Immediate antecedent*	Passage history†‡§
1	X-53a	E E E X-53a(cl) E E E E E → RNA (1)
2	X-53	E E E E E X-53(Cl) × PR8 E E E → X-53(cl)-PR8 E E → RNA (P) (P) (P) (2) (P) (P)
3	X-53a(cl) × PR8	E E E E → X-53a(cl)-PR8 E E E E E E E X-53a(cl)-PR8(H) E → RNA (P) (P) (P) (P) (3)
4	X-53(cl)	E E E M M M E X-53(cl)Lp → RNA (4)
5	X-53(cl)	M M E M E E X-53(cl)(2) × PR8 E E → X-53(cl)(2)PR8(2) M M M M E X-53(cl)-PR8(2) (P) (P) p4t ↓ RNA
6	X-53(cl)-PR8(2)-p4t	M M E E M M E X-53(cl)-PR8(2)-p4t-H ² E → RNA (5) (6)

Note: × PR8 indicates reassortment with A/PR/8/34 virus during dual infection of chicken embryo allantoic sac.

* See Fig. 1.

† The letter E designates a single passage in the allantoic sac of the chicken embryo.

‡ P designates passage with antibody to the PR8 (A/PR/8/34) virus HA.

§ The letter M designates a single passage in MDCK cells.

have only HA and neuraminidase genes from the swine influenza virus parent (1, 5). In other reassortants (X-53-PR8 and X-53a-PR8) the swine influenza virus HA RNA has been segregated into viruses deriving all other RNAs from A/PR/8/34 virus. The genealogy or derivation from the above viruses of viruses with different biologic characteristics or passage history is shown in Fig. 1. The details of passage of the six viruses of which the HA RNAs were subjected to nucleotide sequence analysis are presented in Table 1. These viruses will be identified below by the numerical designations shown in Tables 1 and 2 and in Fig. 1.

Antibody Preparations and Antigenic Analysis. The nature of antibody preparations employed and the methods for viral antigenic analyses have been described (4).

Plaques and Plaque Inhibition. Plaques and plaque inhibition of viruses was carried out in MDCK cells with trypsin-containing minimal essential medium by methods previously described (6, 7).

Definition of Viral Phenotype. Criteria for the definition of the antigenic and biologic phenotypes of the mutant viruses

have been presented in earlier papers (1, 4). In brief, reassortant viruses of L phenotype are relatively low yielding in chicken embryos (HA titers < 1:2,048) and produce small plaques (1–3 mm in diameter) in MDCK cells. The H phenotype is the reciprocal; though the plaque sizes overlap, the mean plaque size is greater with H reassortants. Very few L and H mutant viruses have been studied in swine, but L viruses are more infectious for swine than H mutants by a factor of at least 1,000-fold (2). Antigenic phenotype is defined with monoclonal antibodies, as indicated in Table 2 and specifically described in ref. 4.

Cloning and Nucleotide Sequence Analysis of Swine Influenza HA Genes. A DNA copy of the HA gene for virus 1 was synthesized and cloned as described (8). This was subjected to sequence analysis either directly (9) or by the "dideoxy" method (10) after subcloning fragments into the bacteriophage M13 mp7.1 (11). The cloned HA gene was also digested with suitable restriction endonucleases to generate DNA fragments that were used as primers for cDNA synthesis by reverse transcriptase by using genomic RNA from viruses 1–6 as template (12). By the

Table 2. Summary of representative reassortant viruses on which nucleotide sequence analysis of HA RNA has been carried out

Number	Virus Name	log ₂ HI titers with antibody				Antigenic phenotype	Biologic phenotype	HA residue in H1 antigenic regions				
		SW/CAM*	9C8†	PR8/HK‡	Sa-13‡			153§	155¶	159	228	84
1	X-53a(cl)	<1	1	2	7	H	H	Lys	Glu	Pro	Ser	
2	X-53(cl)-PR8	2	6	<1	4	L	L	Glu	Gly	Pro	Asn	(Lys → Asn)
3	X-53a(cl)-PR8(H)	<1	1	<1	<1	Non-L	H	Lys	Glu	Thr	Asn	
4	X-53(cl)-Lp	6	5	<1	1	L	L**	Lys	Gly	Pro	Asn	
5	X-53(cl)-PR8(2)-p4t (recloned)	5	7	<1	<1	L	L	Lys	Gly	Pro	Asn	
6	X-53(cl)-PR8(2)-p4t-H ² (L to H revertant)	<1	1	—	7	H	H	Lys	Glu	Pro	Asn	

HI, hemagglutination-inhibition.

* Polyclonal antibody (rabbit serum).

† X-53-PR8 monoclonal antibody (mouse ascites fluid).

‡ Monoclonal antibody (mouse ascites fluid).

§ H1 antigenic region Sb.

¶ H1 antigenic region Sa.

‡ Structurally equivalent to residue 158 in H3 model.

** Large plaque L mutant not yet tested in swine for definitive biophenotype.

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1  --UAGAAACA AGGGUGUUUU UUCUCAUGUC UCUGAAAUCC UAGUUUUAAA UACAUAUUCU
61 GCACUGUAAA GACCCAUUAG AGCACAUCCA AAAACUGAUU GCCCCCAGGG AGCCACACAG
121 UACCAUUGAA CUGGCGACAG UUGAAUAGAU CGCCAAAACU UGGUAAAUUC UUGUUGACUC
181 CACUUUUAUC CCAUCUAUCU CCUCUCUGUU UAGCUUUGAU UCUUCUGAGU ACUUUGGGUA
241 AUCAUAAGUC CCAUUUUUGA CGCUCUCCAU GCAUGUAUCA UCACAUUUUG GAUAGAAUUC
301 AAGGAGCCCA UUUCCAAUUU CUUUGGCAUU GUUCCUUGAC UGGCUUCUUA CUUUCUCAUA
361 CAGGUUCUUU ACUUUCGAGU CGUGGAAAUU CAGAGUUCUU UCAUUUUCCA AUAGAACCAC
421 CAGUUCUGCA UUGUUGUCC AAUAUUCAG AAAACCAUCA UCAACCUUUU UGUUUAAAUU
481 CUCUAUUUUU UUUUCCAGU GGUUGAAUUC UUUACCCACU GCCGUGAAUU GCGUGUUCAU
541 CUUUUCAAUA ACAGAAUUUA CUUUGUUAGU GAUCCCGUCA AUGGCAUUUU GUGUGUCCU
601 UUGGUCCGCU GCAUUAUCCU AUCCUGUUC AUUCUGAUGG UGAUAACCAU ACCAUCCAUC
661 UAUAUUCUU GUCCAUCUCC CCUCAUUAU ACCAGCAUUG GCUCCAAACA GACCUUAGA
721 UUGAAUAGAC GGGCAUUCU UAAGUCCGU AGCCAAUUCU AAUUUUUGUG UUUUGACUA
781 UUUUGGACAU UUCUCAAUUG UGACUGGAGU UAUAUUCUGA AAUGGGAGGC UGGUGUUUAU
841 GGCACCCUUG GGUGUUUGAC ACUUCGUGUU ACAUACAUGA ACUGGUGCAU CCCAAUUUAU
901 AAUACCAAGU CCAGAACUUC UAUAUUAUUG GAAGCAUAU CUUGGUACCA CUAGAUVUCC
961 AGUUGCUUUC AAUUAUUAU UGUUCCAGG UUCUAUUAGU GUCCAGUAU AGCUCAUCCU
1021 CCGUUGUUGA CCUCUACCU UGGGUUUUGC UGCUAUUUUCU GGUUGAUAU UUCUGUUAU
1081 UUUUGAUGAC CCUACAAAA CAUAGGCAUC UGCAUUUUUG UAGAGACUUU GUUGAUCAGU
1141 ACUGGUAGGU GGAUGRUGAA UGCCCCAUG CACRAGGACU UCCUUCUCCU UAUGUUUAAC
1201 AUAAGGAUUU CUGAGCUUUG GGUUAGAAU UUCUUUUUUC ACCAGCCUAU UUAUUUUUCU
1261 UUGGAAGCUG UUUUCUCCAG CAUAAGGGCA UGCUGCCGUC ACACCUUGU UGUUUUUAU
1321 AUUGGGCCAC GAAUUGUCU UGGGGAUAU CUCGAUUCU UCRAAUGAUG ACACUGAGCU
1381 CACUGGUCU CUCAGCUCU CAUAUUUGU GAARUCUCCU GGGUACAUU UCCCAUUGU
1441 UGAUUUCGUA GUUUCACAA UGUAGACCA UGAGCUUACU GUGAGUAGU AUUCACAUU
1501 UGGGUUUUCC AAAAGCCUAC CGGCAUUAU ACAUUUACCC AAGUGCAUG GGGCAUCCC
1561 CCCCAGUUUA CAUAGUUUCC CGUUAUGUCU GUCUUCAGA AGAUUAACAG AGUGUGUUAU
1621 UGUUACAUCU UUUUCUAGU CUGUAUCAC AGUGUCAGU GAUUUUUUU CAUGAUACC
1681 UAUAUAUAGU GUGUCUGCAU UUGUGGUGC AAUAUAUCA AACAGACUA AUAGUAUUGC
1741 CUUCAUUUCG UUGGCUUUUA UUUUCCUUG CUUUUGCU

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FIG. 2. Nucleotide sequence of the HA gene of swine influenza virus strain A/NJ/11/76 (H mutant) from reassortant virus X-53a.

use of dideoxynucleoside triphosphates (10), sequence information from the same region of the HA gene was obtained and compared for the various mutants.

RESULTS

Sequence of the H-Phenotype Swine Influenza Virus HA Gene. The complete nucleotide sequence was determined for the HA gene of H-phenotype virus 1 (Fig. 2) by the strategy shown in Fig. 3. Swine influenza HA has been grouped with human isolates such as A/PR/8/34 and A/WSN/33 in the H1 subtype (13). There is approximately 80% and 94% amino acid homology in HA1 and HA2, respectively, between virus 1 and these strains. The differences among them are summarized in Fig. 4. The nucleotide and inferred amino acid sequences of the H-phenotype virus 1 provide the basis for comparison with other viruses with different antigenic and biological phenotypes.

Sequence Analysis of HA RNAs from Other Related Viral Reassortants. Considering the available information on the structure, function, and antigenic regions of the influenza HA

and the conserved nature and primarily structural role of HA2 (16), we concentrated our sequence analysis efforts on the HA1 region of the L phenotype HA RNAs. To compare HAs of the L and H phenotypes, partial nucleotide sequences for the HA genes of viruses 2–6 were determined indirectly by using primer DNA fragments from the cloned virus 1 (12). The sequence of the HA1 region of the gene of L-prototype virus 2 was virtually completed, except for nucleotides 1–65, 235–305, 800–890, and 1,048–1,061, which encode amino acids –1 to –11 (in the signal peptide), 51–74, 239–266, and 322–326, respectively. Comparison of the nucleotide sequences encoding HA1 for viruses 1 and 2 revealed point mutations at bases 335 (A → C), 540 (A → G), 547 (A → G), and 766 (C → A), resulting in changes at amino acid residues 84, 153, 155, and 228, respectively (Table 2). Determination of the nucleotide sequences encoding these residues in other closely related L-phenotype viruses 4 and 5 showed no differences among them with respect to residues 153 (Lys) or 228 (Asn). Therefore, interest focused on amino acid 155 as the probable determinant of L or H viral phenotype. All viruses of L phenotype had Gly at this site and the

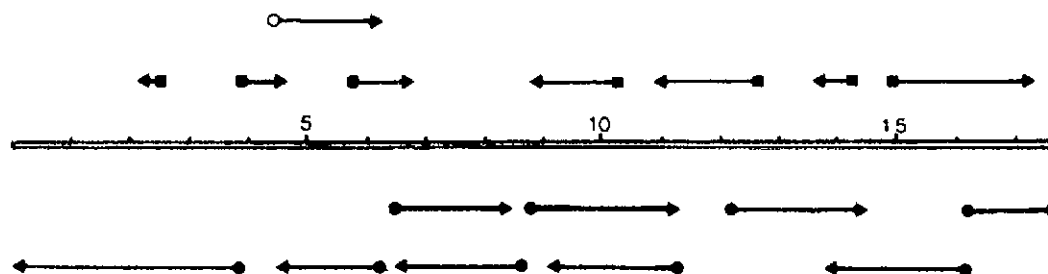


FIG. 3. Strategy for determining the nucleotide sequence of the cloned DNA copy of the swine influenza HA gene. The numbers refer to the nucleotides $\times 10^{-3}$. The sequence was determined either by copying cDNA from swine influenza genome RNA (○) (12), by the method of Maxam and Gilbert (■) (9), or by the dideoxy method (10) after subcloning fragments into bacteriophage M13 mp7.1 (●) (11).

WSN/33 PR/8/34 NJ/11/76	M K A K L L V L L Y A F V A T D A D T I C I G T H A N N S T D T V D T I F E K N	23
	I C T A N L Y	
WSN/33 PR/8/34 NJ/11/76	V A V T H S V N L L E D R H N G K L C K L K G I A P L Q L G K C R C T G W L L G	63
	T S R G H A	
WSN/33 PR/8/34 NJ/11/76	N P E C D S L L P A R S W S Y I V E T P N S E N G A C Y P G D F I D Y E E L R E	103
	P V S S K D T N	
WSN/33 PR/8/34 NJ/11/76	Q L S S V S S L E R F E I F P K E S S W P N H T F N G V T V S C S H R G K S S	143
	F T E N R A A A P Y A A N	
WSN/33 PR/8/34 NJ/11/76	P Y R N L L W L T K K C D S Y P K L T N S Y V N N K C K E V L V L W G V H H P S	183
	I V E H S K K O I P	
WSN/33 PR/8/34 NJ/11/76	S S D E Q Q S L Y S N C N A Y V S V A S S N Y N R R F T P E I A A R P K V K D Q	223
	N K D N I Q E V T R R G K K R G	
WSN/33 PR/8/34 NJ/11/76	H G R M N Y Y W T L L E P G D T I I F E A T G N L I A P W Y A F A L S R C F E S	263
	A S I O T V V R M N S C	
WSN/33 PR/8/34 NJ/11/76	C I I T S N A S M H E C N T K C Q T P Q G S I N S N L P F Q N I H P V T I G E C	303
	I W D P V D L A S T S	
WSN/33 PR/8/34 NJ/11/76	P K Y V R S T K L R M V T C L R N I P S I Q Y	
	K A A V S	

FIG. 4. Summary of amino acid differences in HA1 between A/WSN/33 (14), A/PR/8/34 (Cambridge) (15), and A/NJ/11/76 (X53a) by using the single-letter amino acid code (12). Boxed residues indicate potential sites for carbohydrate attachment. The NH₂-terminal residue of mature HA is circled. Antigenic regions defined for A/PR/8/34 (Mt. Sinai) (17) are indicated: ▲, Sa; ■, Sb; □, Cb; ●, Ca₂; and ○, CA₁. A/NJ/11/76 (X53a) also differs from A/WSN/33 in HA2 at residues 39 (WSN X53a; K → R), 46 (N → D), 72 (N → H), 77 (M → D), 113 (L → S), 123 (K → R), 127 (N → R), 146 (N → D), 147 (E → T), 153 (R → K), 172 (K → E), 181 (M → T), 182 (G → R), and 183 (V → I).

two H-phenotype viruses had Glu.

Our attention was further directed to this site by parallel studies with monoclonal antibodies to the Sa antigenic site of A/PR/8/34 virus, in which it was found that L and H phenotypes could be antigenically distinguished (4). [Due to the conservation of key residues among influenza virus subtypes (16), amino acid 155 of swine influenza virus HA is structurally equivalent to residue 158 in H3 subtype viruses and lies within the H1 Sa antigenic site (17).] The conclusion that amino acid 155 is indeed the relevant residue is reinforced by phenotypic and biological comparison of viruses 5 and 6. Virus 5 was cloned by five successive plaque-to-plaque passages in MDCK cells (Table 1). After additional MDCK and chicken embryo passages, virus 6 emerged as a large plaque-forming (biologic) mutant of H antigenic phenotype. Corresponding to this change in antigenic and biologic phenotype, amino acid 155 changed from Gly to Glu (Table 2), as a result of a point mutation (C → A) at nucleotide 547.

Virus 3 (non-L, non-H antigenically) was selected with PR8/HK antiserum from H reassortant X-53a(c1)-PR8 (Fig. 1). It is nonreactive with antibodies specific for either L or H mutants. Its biologic phenotype must be provisionally defined as H on the basis of its yield in chicken embryos and large plaque size, but it has not yet been administered to swine. Like viruses of H phenotype, it has Glu at position 155, but, unlike H viruses 1 and 6 (and indeed all of the other viruses studied), as a result of a C → A mutation at nucleotide 558, it has Thr rather than Pro at nearby position 159. The change here at this "anchor residue" (16) might well influence the binding of antibody to the region containing residue 155.

DISCUSSION

The Swine Influenza Virus (X53a) HA Sequence. The complete nucleotide sequence for the H-phenotype swine influenza virus (X-53a) has been determined (Fig. 2). The inferred amino acid sequence revealed, as expected, a high degree of homology with the HA proteins of other H1 subtype viruses. The swine HA gene encodes a signal sequence of 17 amino acids and HA1 and HA2 regions of 326 and 222 amino acids, respectively; a single Arg residue links HA1 and HA2. In comparing the HA amino acid sequences for A/WSN/33, A/PR/8/34, and X-53a, all classified as members of the H1 subtype, it is evident that in HA1 amino acid changes are widely scattered, making the significance of many differences difficult to assess (Fig. 4). However, two features are worth comment: (i) there are notable clusters of amino acid differences, particularly between X-53a and the other viruses, in and adjacent to the regions corresponding to the known antigenic sites of the H1 HA molecule (17)—in particular, the Ca₂, Cb, and Sb sites; (ii) compared with A/WSN/33, the swine HA has lost three carbohydrate attachment sites (at Asn residues 56, 127, and 269) and gained one (at Asn residue 287). Moreover, the Hsw HA retains no attachment sites where the presence of carbohydrate could potentially mask proposed antigenic regions (16, 17). This may explain the drift in the amino acid sequence of the Hsw HA, compared with those of A/WSN/33 and A/PR/8/34 in the region of residues 127–142 and 267–272 (Fig. 4). The latter region, which retains a carbohydrate site in A/PR/8/34 and did not change in laboratory-selected variants (17), is structurally equivalent to H3 residues 269–274—i.e., the C antigenic site (16).

Comparison of L and H Phenotype HAs. Our comparison of nucleotide sequences for the HA1 regions of L and H phenotype reassortants 1 and 2 has demonstrated few differences. It is notable that two of the four differences found are within an antigenic region defined for the closely related PR8 virus and that monoclonal antibodies to this (Sa) region can distinguish between the L and H mutants. When virus 6, the H revertant of L phenotype virus 5, was studied by sequence analysis of the viral RNA through the Sa region, only a single deduced amino acid change was found at position 155, although the revertant virus 6 had changed both in biologic and antigenic phenotype. It is significant that this revertant emerged as a high-yielding (biologic phenotype) variant in MDCK cells and was not selected with antibody.

These data, together with those obtained from study of the other reassortants, indicate that Gly at position 155 predicts L phenotype and Glu predicts H phenotype. An exception is virus 3, which has lost reactivity with H-reactive antibody during anti-H immunoselection, but which retains Glu at position 155. However, the Pro to Thr change at neighboring residue 159 might alter antibody binding to the Sa site without affecting whatever function of Glu determines biologic phenotype.

The large plaque L variant, virus 4, was selected for sequence analysis with the expectation that its changed biologic phenotype might reflect mutation of other viral genes as has been observed with other large plaque mutants of other viruses. In this case, the retention of Gly at position 155 can be correlated with its retained L antigenic phenotype.

Biological Implications of the Mutation. Mutation at residue 155 [corresponding to position 158 on the Wiley *et al.* H3 HA model (16)] is at a credible site to influence viral biologic activity. This residue is near the tip of the HA monomer immediately adjacent to the proposed receptor binding site. Two functions have been unequivocally ascribed to the HA molecule that could be expected to significantly influence viral replication: (i) attachment to cellular receptors and (ii) mediation of infection of the cell after posttranslational modification of the HA by cellular proteases (18). In view of the evidence that the key mutation is at a site at or near the receptor pocket at the top of the HA spike, it seems unlikely that the mutants differ with respect to the second function and probable that the mutation influences cellular attachment in some way. (We have found no difference in the cleavage of HA or protease susceptibility of L and H prototypes.) Accordingly, we have studied the absorption kinetics of L and H reassortants in MDCK cell monolayers (in which replication differences are manifest) but have not found significant differences (data not shown). In studies of attachment of the virus to receptors of erythrocytes of a variety of species we have found differences in hemagglutination pattern only with sheep erythrocytes. L mutants bind with slightly greater avidity to sheep erythrocytes, as reflected by slower elution of virus and disagglutination of cells under conditions in which neuraminidase activity is absent or abolished.

The present experiments provide biochemical confirmation of previously presented genetic evidence (1) that change in viral replicative capacity or virulence (plaque size) may coincidentally be associated with antigenic change related to a single mutation. By inference, minor antigenic variation as defined by highly specific (monoclonal) antibody is not necessarily selected for as a reciprocal of modulation by host antibody but can be

driven by primary selection for changes in the HA that affect its biological function. It should be stressed that H mutants arise in the absence of immunoselection in host systems favoring their replication, in which case the coincident minor antigenic change appears to be essentially fortuitous (19). Minor antigenic variation occurs also in A/NWS/33 (H1N1) HA mutants selected for different trypsin sensitivity (20). Additional evidence for nonimmunologic selection of antigenic variants comes from recent studies of influenza B-virus subpopulations in which antigenicity varies with different host cell tropisms (21). Indeed, the phenomenon may be a general one. Pathogenicity of fixed rabies virus strains for adult mice depends on the presence of an antigenic determinant on the viral glycoprotein (22), whereas HA antigenic variants of reovirus type 3 are changed in neurovirulence (23).

It is not surprising that changes affecting viral replication and virulence might occur at antigenic sites, because such sites must be at the exterior of the molecule in accessible locations for interaction with host cells.

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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 (Herrier 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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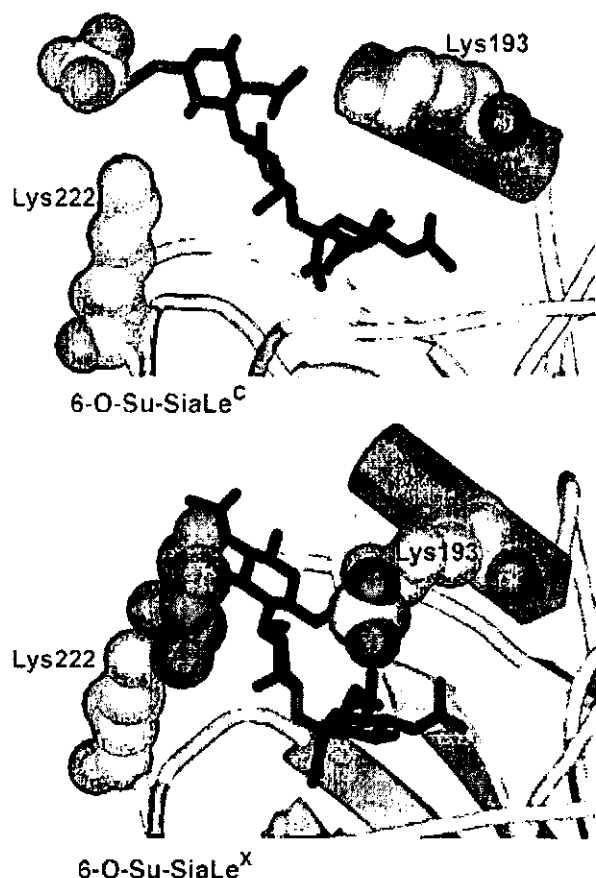


Fig. 1. Putative disposition of fucose and 6-O-sulfo substituents of sialosaccharide in the receptor-binding site of H5 influenza virus. The receptor was fitted into the RBS of H5 HA complexed with LSTa (1J5N; Brookhaven Protein Databank) by superimposing sialic acid residue of the different analogs over the sialic acid residue of LSTa. The molecular model of 6-O-Su-SiaLe^C (top panel) was built based on LSTa (1J5N). The molecular model of 6-O-Su-SiaLe^X (bottom panel) was built based on SiaLe^C (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 model; fucose is colored brown. Lys193 and Lys222 of the HA are represented in spacefill model. 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.

Fucose-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 Tem/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

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 p11W2000 (Fig. 3A) is derived from p11W12 (18). This cloning vector contains 225 bp of the human pol I promoter and 35 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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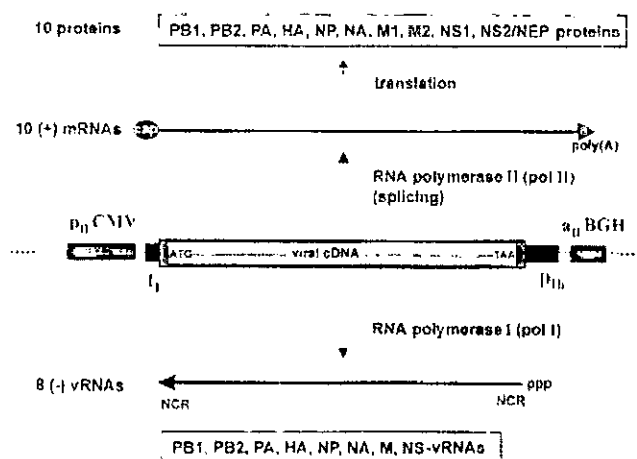


Fig. 1. Schematic representation of the pol I-pol II transcription system for synthesis of vRNA and mRNA. The cDNA of each of the eight influenza virus segments is inserted between the pol I promoter (p_{H}) and the pol I terminator (t_{H}). This pol I transcription unit is flanked by the pol II promoter (p_{CMV}) of the human cytomegalovirus and the polyadenylation signal (α_{H} 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 *Apal*-*Sall* 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 *Apal*-*Sall* 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 *BsmBI* or *BsaI* restriction site sequences at their 5' ends. After digestion of the PCR products with *BsmBI* or *BsaI*, 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 1 (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 0.8 ml Opti-MEM 1; 3 ml of this cell suspension was seeded into one well of a six-well plate. The cocultured MDCK and 293T cells (0.2×10^6 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 μl of TransIT LT-1 per 1 μg 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 1. Thirty hours after transfection, 1 ml of Opti-MEM 1 containing TPCK-trypsin was added to the cells; this addition resulted in a final concentration of TPCK-trypsin of 0.5 $\mu\text{g}/\text{ml}$ 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 μg 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 1×10^5 viruses per milliliter; 2×10^5 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-

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- Las reivindicaciones 1, 23 y 24 no son claras por que se refieren a un polinucleótido pero lo caracterizan por la proteína codificada. El solicitante debe aportar la secuencia exacta del polinucleótido reclamado. Por otra parte el solicitante debe aclarar cual es la diferencia entre los polinucleótidos de las reivindicaciones 23 y 24, ya que tal como están escritas se refieren a la misma molécula. Por último, se recuerda al solicitante que cualquiera de las macromoléculas mencionadas debe ser caracterizada por su secuencia completa, la cual además debe ser adjuntada en un listado aparte con su respectivo SEQ ID NO, y que no se aceptan definiciones indicando porcentajes de homología o identidad, puesto que esta definición no permite establecer la secuencia aminoácida o nucleótida exacta a la que se refiere, tal como sucede en las reivindicaciones 25 y 26.
- Las reivindicaciones 3 a 6 pretende caracterizar un polinucleótido por su uso, se recuerda al solicitante que los usos son materia no patentable de acuerdo a Art 14 y 21 D 486.
- La reivindicación 8 no es clara por que no especifica cual es la cantidad efectiva de la proteína aislada reclamada.
- La reivindicación 9 no es clara por no especifica la estructura de los promotores y ácidos nucleicos codificantes que conforman el cassette de expresión reclamado.
- El solicitante debe especificar cual es la célula reclamada en las reivindicaciones 10 a 12, suministrando el certificado de depósito para la cepa, y cual es el vector específico reclamado en la reivindicación 13. Se recuerda al solicitante que no es valido reclamar células que hacen parte de un organismo pluricelular como plantas o animales, ya que ello equivale a pedir protección sobre la planta o animal, materia no patentable bajo Art 15 D 486 literal b.
- El método de la reivindicación 19 no es claro por que no especifica cuales son los pasos secuenciales por los cuales las células vegetales transformadas entran en contacto con el insecto. Si dichos pasos están relacionados con el proceso de alimentación del insecto, el método no es patentable ya que este es un proceso que ocurre de forma natural (el insecto se alimenta de una planta) y el solicitante no tiene incidencia sobre el. El hecho de que la planta sea modificada tampoco justifica la patentabilidad del método, ya que necesariamente involucra el proceso natural de alimentación del insecto.
- La reivindicación 21 no es clara por que no identifica la estructura de la sonda que hibridiza con el polinucleótido de interés, ni cual es el método de detección de la hibridación.
- La reivindicación 22 no es clara por que no identifica inequívocamente el anticuerpo utilizado en la detección del polinucleótido ni el método de detección de dicha unión. Es importante mencionar que un anticuerpo debe caracterizarlo a partir de su estructura completa, mencionando las secuencias que lo conforman en la **cadena ligera y cadena pesada**, o las secuencias variables de las cadenas ligera y pesada o las secuencias de los 6 CDR's que conforman **ambas cadenas**.
- El término: "aproximadamente" utilizado a lo largo del capítulo reivindicatorio (reivindicaciones 27, 29 y 30), es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto
- La reivindicación 28 no es clara, ya que se refiere a un composición que comprende un cantidad efectiva de la proteína CryA.105 con SEQ ID NO 2, donde dicha composición se selecciona del grupo que consiste en una célula vegetal, una célula bacteriana, una célula fúngica, un coloide, una emulsión, un recubrimiento para semillas, una carnada y un polvo recubrimiento para

semillas, una carnada y un polvo, sin embargo los miembros del grupo mencionado no corresponden a composiciones. El solicitante debe aclarar cual es el objeto que desea proteger, tanto en esta reivindicación como en las dependientes 29 y 30. Por otra parte, la reivindicación dependiente 29 debe restringir el rango del contenido de Cry1A.105 en la composición, ya que el proporcionado muy amplio (Dos ordenes de magnitud).

- La reivindicación 32 no es clara por que se refiere a una composición pero la caracteriza por la sonda capaz de reconocer el polinucleótido que codifica la proteína de interés, el solicitante debe caracterizar la composición por sus características esenciales: componentes (con su respectiva estructura) y proporciones. De la misma manera la reivindicación 33 no es clara por que pretende caracterizar la composición por un resultado esperado correspondiente a su efecto sobre una plaga.
- Las Reivindicaciones 34 a 36 no son claras porque se refieren a métodos para el control de una infestación de insectos lepidópteros en donde el solicitante no ha realizado ninguna modificación sobre los insectos, sino sobre el cultivo, ya que el método se centra en la el control de la infestación no es claro cual es la mano del inventor sobre el método. Se aclara al solicitante que si su intención es reclamar la planta capaz de expresar la proteína insecticida, esta no es patentable bajo Art 15 D 486 literal b, por otra parte, si la intención del solicitante es reclamar el método de aspersión de dicha proteína insecticida, este tampoco es patentable debido a que se considera un uso (Art 14 y 21 D 486).

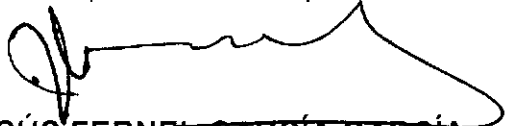
6. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 31/Agosto/2005, y no se encontraron documentos que anticipen el objeto de esta solicitud.

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... 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.



JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha
18 JUL. 2012

Elaborado por: Sandra Carolina Crespo Cárdenas
Revisado Por: Consuelo Leguizamón Leguizamón
Aprobado por: Jesús Fernel García García

