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Abogado
LUIS FELIPE CASTILLO GIBSON

ASUNTO	Radicación	08-067535
	Trámite	11
	Evento	378
	Actuación	519
	Oficio	
	Folios	7

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Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Via Nacional	☐		
Via PCT (Fase Nacional)	☒	Capítulo I	☒
		Capítulo II	☐
No. Solicitud Internacional	PCT/US2006/045859		
Fecha de Presentación Internacional	01/Diciembre/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:	Folios 4 a 111	2/Julio/2008
Reivindicaciones:	Folios 112 a 117	2/Julio/2008
Dibujos:	Folios 118 a 127	2/Julio/2008
Listado de Secuencias:	Folios 128 a 143	2/Julio/2008
Prioridad:	US 60/741,833	2/Diciembre/2005
	US 60/802,864	22/Mayo/2006

2. Análisis de la Prioridad

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

3. Objeto de la invención

Título de la solicitud: "Virus quiméricos que presentan proteínas de superficie no nativas y uso de los mismos".

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El problema planteado en esta solicitud consiste en la necesidad de suministrar un virus para la inmunización en contra de dos agentes infecciosos.

Para resolver este problema técnico la solicitud en estudio proporciona virus quiméricos ARN de hebra negativa.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/00, 39/15.

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

El objeto de las reivindicaciones 32 y 33 no es patentable de acuerdo con el Art citado.

5. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque hace referencia al uso, el cual no se considera materia patentable, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual hace difícil definir cuál es el objeto que se busca proteger. Se sugiere al solicitante combinar la reivindicación 13 con la reivindicación 1, la reivindicación 14 con la reivindicación 2, la reivindicación 15 con la reivindicación 3, la reivindicación 16 con la reivindicación 4, la reivindicación 17 con la reivindicación 6 y la reivindicación 18 con la reivindicación 7, redefiniendo las respectivas dependencias. Por ejemplo, la diferencia entre las reivindicaciones 1 y 13 consiste en que la reivindicación 1 reclama "salvo influenza", mientras que la reivindicación 13 menciona la expresión "diferente de la influenza".

El capítulo reivindicatorio no es conciso porque utiliza las expresiones "un segmento NA empaquetado de virus de influenza", "que codifica una proteína de fusión neuroaminidasa", "nucleótidos que codifican un ectodominio de un antígeno neuroaminidasa de un agente infeccioso", "un segmento HA empaquetado de virus de influenza", "que codifica una proteína de fusión hemaglutinina", "nucleótidos que codifican un ectodominio de un antígeno enlazante/fusogénico del receptor de un agente infeccioso", "un primer marco de lectura abierto que codifica una proteína hemaglutinina de influenza aviar", "un segundo marco de lectura abierto que codifica una proteína de fusión de hemaglutinina", "un primer marco de lectura abierto que codifica una proteína neuraminidasa de influenza aviar", "un segundo marco de lectura abierto que codifica una proteína de fusión neuraminidasa", "nucleótidos que codifican un ectodominio de un antígeno HN de NDV", "un segmento empaquetado del gen NS1", "un segmento HA que tiene un marco de lectura abierto modificado (...)", "una molécula de ADN recombinante que codifica el segmento (...)", "una

secuencia nucleótida que codifica una proteína de fusión F que tiene los dominios (...)", "un epítipo de un ectodominio de un agente protector de un agente infeccioso diferente de NDV o de un antígeno asociado con una enfermedad", "una secuencia que codifica una proteína F", "una secuencia nucleótida que codifica una proteína de fusión HN", "una secuencia nucleótida que codifica una proteína HN", lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que un polipéptido y un ácido nucleico se caracterizan por sus secuencias de aminoácidos y nucleótidos, respectivamente.

Se requiere al Solicitante hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 2 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

- 1° Un virus quimérico de influenza aviar. Reivindicaciones 1 – 23.
- 2° Un NDV quimérico. Reivindicaciones 24 – 30.

No se guarda un concepto común inventivo porque los Grupos 1 y 2 están dirigidos a virus cuyos genomas tienen características estructurales diferentes. Mientras el genoma del virus de la influenza es segmentado, el genoma del virus de la enfermedad de Newcastle (NDV) no lo es.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. 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: Diciembre 2 de 2005, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	<u>Journal of Virology, Vol. 79, Pág. 6674-6679</u>	Live Bivalent Vaccine for Parainfluenza and Influenza Virus Infections	Junio/2005
D2	<u>Vaccine, Vol. 19, Pág. 1616-1627</u>	Generation of a recombinant chimeric Newcastle disease virus vaccine that allows serological differentiation between vaccinated and infected animals	8/Febrero/2001
D3	<u>Journal of Virology, Vol. 79, Pág. 10467-10477</u>	A Chimeric Respiratory Syncytial Virus Fusion Protein Functionally Replaces the F and HN Glycoproteins in Recombinant Sendai Virus	Agosto/2005

D1 <http://jvi.asm.org/content/79/11/6674> divulga un virus quimérico de influenza aviar (Resumen).

D2 <http://www.ncbi.nlm.nih.gov/pubmed?term=11166884> divulga un NDV quimérico (Resumen).

D3 <http://jvi.asm.org/content/79/16/10467> divulga un RSV quimérico en el que el ectodominio de la proteína F reemplaza el ectodominio de la proteína F del virus Sendai (SeV) (Resumen).

8. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

Grupo Inventivo 1

La **reivindicación 1** consiste en un virus quimérico de influenza aviar, que comprende un segmento NA empaquetado de virus de influenza que codifica una proteína de fusión neuroaminidasa, en el cual el marco de lectura abierto NA es modificado de modo que los nucleótidos que codifican el ectodominio NA son reemplazados por nucleótidos que codifican un ectodominio de un antígeno neuroaminidasa de un agente infeccioso, salvo influenza, que está anclado por el extremo-N, de modo que la proteína de fusión neuraminidasa es expresada e incorporada en el virus quimérico de influenza aviar.

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

Características esenciales	D1
Un virus quimérico de influenza aviar, que comprende un segmento NA empaquetado de virus de influenza que codifica una proteína de fusión neuroaminidasa,	Un virus quimérico de influenza aviar, que comprende un segmento NA que codifica una proteína de fusión neuroaminidasa, (Resumen)
en el cual el marco de lectura abierto NA es modificado de modo que los nucleótidos que codifican el ectodominio NA son reemplazados por nucleótidos que codifican un ectodominio de un antígeno neuroaminidasa de un agente infeccioso, salvo influenza, que está anclado por el extremo-N, de modo que la proteína de fusión neuraminidasa es expresada e incorporada en el virus quimérico de influenza aviar.	en el cual el marco de lectura abierto NA es modificado de modo que los nucleótidos que codifican el ectodominio NA son reemplazados por nucleótidos que codifican un ectodominio de HN de parainfluenza. (Resumen)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque D1 divulga un virus quimérico de influenza aviar, que comprende un segmento NA que codifica una proteína de fusión neuroaminidasa en el cual el marco de lectura abierto NA es modificado de modo que los nucleótidos que codifican el ectodominio NA son reemplazados por nucleótidos que codifican un ectodominio de HN de parainfluenza (Resumen).

La diferencia entre la invención, definida en la reivindicación 1 y el virus quimérico de D1 consiste en que el marco de lectura abierto NA es modificado de modo que codifican un ectodominio de un antígeno neuroaminidasa.

El efecto que logra el incluir un ectodominio de un antígeno neuroaminidasa es que permite obtener una vacuna ambivalente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar el virus quimérico de influenza conocido en D1 para obtener una vacuna ambivalente”.

Sin embargo, el mismo documento D1 enseña que el marco de lectura abierto NA es modificado de modo que los nucleótidos que codifican el ectodominio NA son reemplazados por nucleótidos que codifican un ectodominio de HN de parainfluenza, el cual tiene funcionalidad hemaglutinina (HN) y neuroaminidasa (NA) (Resumen), para obtener una vacuna bivalente.

En consecuencia, la persona del oficio normalmente versada en la materia, buscaría expresar proteínas de no-influenza en lugar de proteínas nativas, como NA heteróloga para proveer un virus quimérico capaz de inducir una respuesta inmune. La persona del oficio normalmente versada en la materia se vería motivada a modificar el virus quimérico de D1 porque el virus quimérico es capaz de expresar de forma estable el ectodominio HN del virus de parainfluenza, el cual es un Paramixovirus, para obtener una vacuna bivalente, la cual puede ser atenuada al introducir mutaciones en proteínas conocidas en el estado del arte como NS1¹.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y en las reivindicaciones 2 – 23, es obvia y no tiene Nivel Inventivo.

Grupo Inventivo 2

La **reivindicación 24** consiste en un NDV quimérico que comprende un genoma empaquetado, que comprende una secuencia nucleótida que codifica una proteína de fusión F que tiene los dominios de transmembrana y citoplásmico de una proteína F y al menos un epítipo de un ectodominio de un agente protector de un agente infeccioso diferente de NDV o de un antígeno asociado con una enfermedad, que está anclado por el extremo C, de modo que la proteína de fusión de la proteína F es expresada e incorporada en el NDV quimérico.

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

Características esenciales	D2	D3
Un NDV quimérico que comprende un genoma empaquetado, que comprende una secuencia nucleótida que codifica una proteína de fusión F que tiene los dominios de	Un NDV quimérico que comprende una secuencia nucleótida que codifica una proteína de fusión HN que tiene los dominios de transmembrana y citoplásmico de una proteína	Un RSV quimérico en el que el ectodominio de la proteína F reemplaza el ectodominio de la proteína F del virus Sendai (SeV)

1 J. Schickli, et al. Plasmid-only rescue of influenza A virus vaccine candidates. Philosophical transactions of the Royal Society of London, Vol. 356, Pág. 1965-1973. 29/Diciembre/2001.
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transmembrana y citoplásmico de una proteína F y al menos un epítipo de un ectodominio de un agente protector de un agente infeccioso diferente de NDV o de un antígeno asociado con una enfermedad, que está anclado por el extremo C, de modo que la proteína de fusión de la proteína F es expresada e incorporada en el NDV quimérico.	HN de NDV y el dominio globular inmunogénico HN de un paramixovirus aviar tipo 4 (APMV 4). (Resumen)	(Resumen)
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Los documentos D2 y D3 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 24 porque:

D2 divulga un NDV quimérico que comprende una secuencia nucleótida que codifica una proteína de fusión HN que tiene los dominios de transmembrana y citoplásmico de una proteína HN de NDV y el dominio globular inmunogénico HN de un paramixovirus aviar tipo 4 (APMV 4) (Resumen).

La diferencia entre la invención, definida en la reivindicación 24 y el NDV quimérico de D2 consiste en la sustitución del ectodominio de la proteína F de NDV por al menos un epítipo de un ectodominio de un agente protector de un agente infeccioso diferente de NDV.

El efecto que logra el incluir la sustitución del ectodominio de la proteína F de NDV es que permite obtener una vacuna ambivalente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el NDV quimérico conocido en D1 para obtener una vacuna ambivalente".

Sin embargo, la utilización del ectodominio de la proteína F de forma heteróloga en otro virus para generar una vacuna ambivalente ya está divulgada en el documento D3 que se refiere a un RSV quimérico en el que el ectodominio de la proteína F reemplaza el ectodominio de la proteína F del virus Sendai (SeV) (Resumen), donde los virus RSV y SeV pertenecen, al igual que el virus NDV, a la familia Paramixovirus y donde la sustitución no inhibe la habilidad de SeV de infectar las células, debido al papel de la proteína F de RSV en unión a receptor y subsecuente infección.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría la sustitución del ectodominio de la proteína F del Paramixovirus de D3 en el NDV quimérico de D2 para llegar así al objeto de la reivindicación 24 de la solicitud, por lo cual se considera obvia. La persona del oficio normalmente versada en la materia se vería motivada a modificar el NDV quimérico de D2 porque D2 enseña que es necesario diferenciar animales vacunados de aquellos infectados mediante la utilización de vacunas marcadoras, que comprenden la inclusión de secuencias heterólogas, sin afectar la habilidad del virus de afectar las células, y porque D3 enseña que la sustitución del ectodominio de la proteína F en otros Paramixovirus

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permite la obtención de un virus quimérico, sin afectar la habilidad del virus de infectar las células.

De manera que la solución propuesta por esta solicitud en la reivindicación 24 y en las reivindicaciones 25 – 30, es obvia y no tiene Nivel Inventivo.

9. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	<u>Journal of Virology,</u> <u>Vol. 79, Pág. 6674-</u> <u>6679</u>	1 – 23	Nivel Inventivo
D2	<u>Vaccine, Vol. 19,</u> <u>Pág. 1616-1627</u>	24 – 30	Nivel Inventivo
D3	<u>Journal of Virology,</u> <u>Vol. 79, Pág.</u> <u>10467-10477</u>	24 – 30	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

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

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


JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 25 JUL. 2012

Elaborado por: Juan David Moncaleano P.
Revisado por: Consuelo Leguizamón. 

Live Bivalent Vaccine for Parainfluenza and Influenza Virus Infections

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Influenza and human parainfluenza virus infections are of both medical and economical importance. Currently, inactivated vaccines provide suboptimal protection against influenza, and vaccines for human parainfluenza virus infection are not available, underscoring the need for new vaccines against these respiratory diseases. Furthermore, to reduce the burden of vaccination, the development of multivalent vaccines is highly desirable. Thus, to devise a single vaccine that would elicit immune responses against both influenza and parainfluenza viruses, we used reverse genetics to generate an influenza A virus that possesses the coding region for the hemagglutinin/neuraminidase ectodomain of parainfluenza virus instead of the influenza virus neuraminidase. The recombinant virus grew efficiently in eggs but was attenuated in mice. When intranasally immunized with the recombinant vaccine, all mice developed antibodies against both influenza and parainfluenza viruses and survived an otherwise lethal challenge with either of these viruses. This live bivalent vaccine has obvious advantages over combination vaccines, and its method of generation could, in principle, be applied in the development of a “cocktail” vaccine with efficacy against several different infectious diseases.

Influenza and human parainfluenza virus infections rank high among human respiratory diseases worldwide. Influenza accounts for 3 to 5 million cases of severe illness each year, 250,000 to 500,000 of which are fatal, while an estimated 16,300 to 96,500 people are hospitalized because of human parainfluenza virus infections (see the World Health Organization website at <http://www.who.int/>). Considerable effort has been expended to develop useful vaccines against influenza and human parainfluenza viruses. Inactivated influenza virus vaccines are safe and effective in preventing influenza virus-related hospitalization and death, but the immune response triggered by these vaccines is suboptimal and short lived, requiring annual vaccination (12). By contrast, live attenuated influenza virus vaccines elicit robust humoral and cellular responses that are predicted to be long lived (6). For human parainfluenza viruses, neither inactivated nor live vaccines are available. Although no data are available from studies with humans, formalin-inactivated vaccines for parainfluenza virus in animals aggravate the symptoms of infection upon challenge with homologous virus (23), as previously shown in humans and animal models given inactivated vaccines for respiratory syncytial virus (RSV) (a close relative of parainfluenza virus) (5, 9). This enhancement of pathogenicity was not observed for a live attenuated candidate vaccine, which proved to be effective in animal studies (22). Thus, for the prevention of both influenza

and human parainfluenza virus infections, live attenuated vaccines are likely to be more effective than inactivated vaccines.

Multivalent vaccines containing protective antigens against several infectious agents have been developed as a means to reduce the burden of repeated childhood vaccinations (1). With the exception of the MMR (measles, mumps, and rubella) preparation, they are based on inactivated vaccines because propagation of one vaccine virus strain might interfere with the growth of the other vaccine component(s) (24). A possible solution to this problem would be to produce a single vaccine virus possessing several antigens that elicit protection against two or more infectious agents.

Influenza A virus (family *Orthomyxoviridae*, characterized by a segmented, negative-strand RNA genome) is considered an attractive vaccine vector candidate. It elicits strong humoral and cellular responses (6), its genome is amenable to genetic modification (7, 21), and the availability of 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes (HA and NA are the major viral antigens) would allow repeated immunization. However, early attempts to express foreign proteins from recombinant influenza viruses failed due to the genetic instability of the foreign genes (15). This limitation was recently overcome when we identified regions in influenza viral RNA (vRNA) segments that govern the efficient incorporation of vRNA into virions (8, 33). These incorporation signals, which reside in the coding regions, have been exploited for the stable expression of foreign proteins from influenza viruses (27, 33).

Thus, using our knowledge of influenza vRNA incorporation signals, we aimed to create a bivalent live vaccine that would provide protection against both influenza and parainfluenza virus infections. This objective was pursued by generating an influenza A virus in which the ectodomain of the NA was

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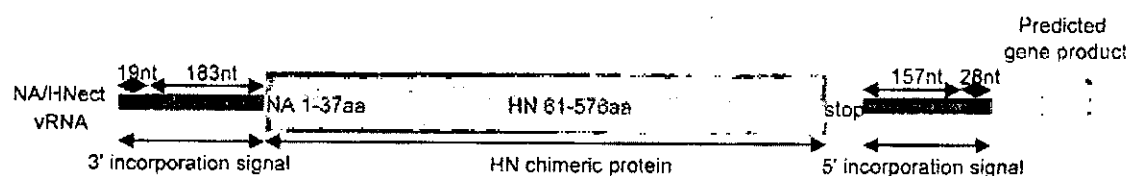


FIG. 1. Schematic diagram of NA/HNect vRNA. NA/HNect vRNA encodes the 3' noncoding region of NA vRNA (19 nucleotides), 183 nucleotides of the NA coding frame (the start codon located in this region has been replaced by GCG), amino acids 1 to 37 of WSN NA (encoding the cytoplasmic tail, the transmembrane region, and a portion of the NA stalk), amino acids 61 to 576 of SeV HN (encoding the SeV HN ectodomain), two sequential stop codons (TAA TAG), 157 nucleotides of the NA reading frame, and the 5' noncoding region of NA vRNA.

replaced by that of the HN, a parainfluenza virus protective antigen, and then evaluating the protective potential of the vaccine in a mouse model.

MATERIALS AND METHODS

Cells. 293T human embryonic kidney cells (a derivative of the 293 line into which the gene for simian virus 40 T antigen was inserted) and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and in minimum essential medium containing 5% newborn calf serum, respectively. All cells were maintained at 37°C in 5% CO₂.

Viruses. Influenza A/WSN/33 (H1N1) virus (WSN) was generated by plasmid-based reverse genetics as previously described (21) and propagated in 10-day-old embryonated chicken eggs. A murine parainfluenza virus type 1, Sendai virus (SeV; strain Enders) was propagated in 10-day-old embryonated chicken eggs.

Plasmid construction. Plasmid pPolINA(61)NA/HNect (Fig. 1), which was used for the production of a negative-sense vRNA containing the coding sequence for an NA-HN chimeric protein between the 3' and 5' NA vRNA incorporation signals (8), was generated as follows. The region corresponding to the WSN NA cytoplasmic tail, transmembrane domain, and a portion of the stalk (i.e., amino acids 1 to 37 of WSN NA) was amplified by PCR. Likewise, we amplified the region corresponding to the SeV HN ectodomain (i.e., amino acids 61 to 576 of SeV HN), followed by two consecutive stop codons. The resultant PCR products were inserted between the 3' and 5' NA vRNA incorporation signals (i.e., the 3' noncoding region plus the first 183 nucleotides [nt] of the coding region with replacement of the start codon, ATG by GCG, and the 5' noncoding region plus the last 157 nt of the coding region). The recombinant influenza viral cDNA thus produced was inserted between RNA polymerase I promoter and terminator sequences.

Generation of influenza virus expressing SeV HN antigen. To generate influenza virus expressing SeV HN protein, designated FluH/SeVHN, we performed reverse genetics as previously described (21). Briefly, eight RNA polymerase I-driven plasmids for transcription of all eight influenza vRNA segments, including the NA/HNect segment, were transfected into 293T cells as previously described (21). We then cotransfected cells with five eukaryotic protein expression plasmids encoding NA, as well as the PA, PB1, PB2, and NP proteins, which are required for replication and transcription of vRNAs. Two days posttransfection, virus was collected from the supernatant of transfected cells, grown once in 10-day-old embryonated chicken eggs, and stored at -80°C until used in experiments.

Experimental infection. The pathogenicity of FluH/SeVHN was tested by intranasal infection of 4-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) that were anesthetized with isoflurane. Survival times and body weights were monitored daily for 14 days after infection. Three days postinfection, three mice per group were euthanized to determine virus titers in nasal turbinates and lungs. The dose lethal to 50% of mice (MLD₅₀) infected with wild-type WSN or SeV was determined by intranasally inoculation of 8-week-old female BALB/c mice with 10-fold serial dilutions of virus under anesthetization; infected mice were observed daily for 14 days.

To evaluate the protective efficacy of FluH/SeVHN against challenge with lethal doses of wild-type viruses, we infected each mouse intranasally with FluH/SeVHN (10^{5.5}, 10^{6.5}, or 10^{7.5} 50% egg infectious doses [EID₅₀]/mouse). As controls, we infected mice intranasally with formalin-inactivated virus (equivalent to 10^{6.5} EID₅₀/mouse) or phosphate-buffered saline (PBS). Twenty-seven days later, serum samples as well as trachea-lung and nasal washes were collected from a subset of mice and examined for virus-specific immunoglobulin A (IgA) or IgG antibodies by an enzyme-linked immunosorbent assay (ELISA) (13).

Briefly, wells of Immulon 2HB (Thermo Labsystems, Franklin, MA) plates were coated with purified and detergent-treated WSN or SeV and blocked with PBS containing 10 mg/ml bovine serum albumin. After incubation of virus-coated wells with test samples, bound antibodies were detected with goat anti-mouse IgA or IgG conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Statistical analyses were performed with Student's *t* test. Twenty-eight days after infection, the remaining mice were challenged intranasally with 10 MLD₅₀ of wild-type WSN or SeV and monitored daily for survival and body weight for 28 days. Virus titers were determined in organs from five mice per group at 3 days postchallenge.

RESULTS

Generation and characterization of an influenza A virus expressing SeV HN antigen. To generate an influenza virus-based live, bivalent vaccine, we chose the influenza A/WSN/33 (H1N1) virus and the Enders strain of SeV, a murine type 1 parainfluenza virus, since both cause lethal infection in mice, providing a useful animal model with which to evaluate vaccine efficacy. Moreover, we recently mapped the sequences that mediate efficient WSN NA vRNA virion incorporation to 183 nt, corresponding to the amino-terminal coding region of NA, and to 157 nt, corresponding to the carboxy-terminal coding region of this protein, in addition to the 3' and 5' noncoding regions (8). With this information, we were able to generate a recombinant WSN virus expressing the ectodomain of SeV HN (designated FluH/SeVHN), as described in Materials and Methods. To determine if FluH/SeVHN expressed both the SeV HN and WSN HA glycoproteins, we infected MDCK cells with this virus or wild-type WSN (as a control) and stained cells 48 h later with antibodies to SeV HN, WSN HA, or influenza virus NP. Cells infected with influenza virus expressed HA and NP proteins (Fig. 2) but not SeV HN, whereas cells infected with FluH/SeVHN expressed both influenza virus HA and NP proteins and the SeV HN protein.

To determine whether the NA-HN chimeric protein was expressed on the cell surface, we performed a flow cytometry analysis of HN antigen on 293T cells transfected with pPolINA(61)NA/HNect plasmid, encoding the chimeric HN protein and those expressing the three polymerase and NP proteins. HN antigens were detected on the surface of these cells but not on control cells, which did not receive the polymerase I plasmid. Next, to determine whether FluH/SeVHN virus particles possess the HN antigen, we carried out ELISA. Briefly, wells of Immulon 2HB plates were coated with purified and detergent-treated FluH/SeVHN, WSN, or SeV; SeV HN and influenza virus NP were detected by monoclonal antibodies. The wells coated with FluH/SeVHN or SeV reacted with antibodies against SeV HN protein, while those coated with WSN did not (data not shown). By contrast, the wells coated

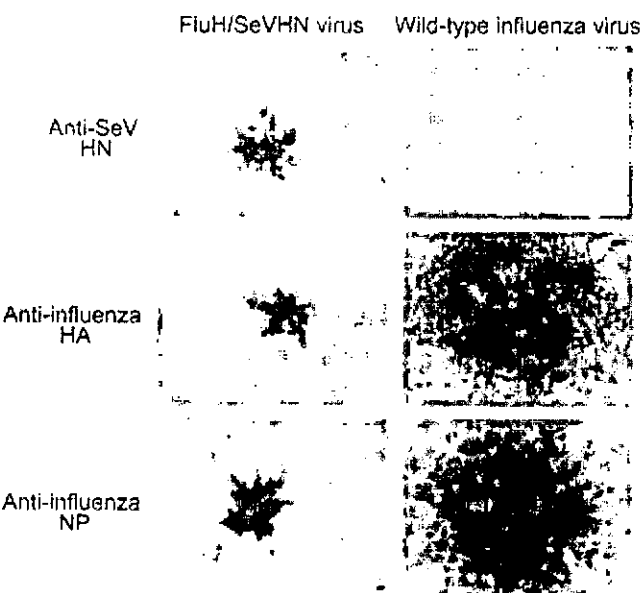


FIG. 2. Expression of SeV HN and WSN HA by FluH/SeVHN. MDCK cells were infected with FluH/SeVHN or wild-type WSN and overlaid with 1.0% agarose. Infected cells were incubated for 2 days at 37°C, fixed with formalin, and treated with 0.1% Triton X-100 in PBS. Viral antigens were detected by immunostaining with anti-SeV HN monoclonal antibodies, anti-WSN HA monoclonal antibodies, or an anti-influenza virus NP monoclonal antibody.

with FluH/SeVHN or WSN, but not SeV, reacted with an antibody against influenza virus NP. These results demonstrate that SeV HN antigen is present in FluH/SeVHN virions.

For vaccine production, the virus must grow to high titers. We therefore evaluated the titers of the recombinant virus grown in MDCK cells or embryonated chicken eggs. In MDCK cells, FluH/SeVHN replication was attenuated compared with that of wild-type influenza virus (1.0×10^6 PFU/ml versus 1.6×10^8 PFU/ml), resulting in pinpoint plaques only (Fig. 2). In eggs, however, FluH/SeVHN replicated to titers comparable to those of wild-type influenza virus ($10^{9.3}$ EID₅₀/ml for the former versus $10^{8.3}$ EID₅₀/ml for the latter).

The stability of FluH/SeVHN was assessed by passaging the virus 10 times in embryonated chicken eggs. During 10 serial passages, FluH/SeVHN virus grew well, with titers ranging from $10^{8.5}$ EID₅₀/ml to $10^{9.8}$ EID₅₀/ml. Even after 10 serial passages in eggs, 88% of the plaques expressed SeV HN when tested in MDCK cells, and only one mutation (Asn to Lys at amino acid position 229) was found in the NA/HNect vRNA. Since not all virions of even wild-type influenza virus contain all eight RNA segments (17), the finding that not all of the FluH/SeVHN plaques expressed the NA/HNect was anticipated. These results demonstrate that influenza vRNA packaging signals can be exploited to stably express foreign genes.

Pathogenicity of FluH/SeVHN virus in mice. Attenuation in animals is a critical requirement for live vaccines. We therefore determined the pathogenicity of FluH/SeVHN virus in mice by intranasally infecting them with $10^{5.3}$, $10^{6.3}$, or $10^{7.3}$ EID₅₀/mouse of recombinant virus that had been passed once in eggs. Mice inoculated with $10^{7.3}$ EID₅₀ or less showed no overt symptoms of illness. Thus, the MLD₅₀ of FluH/SeVHN virus was calculated to be $>10^{7.3}$ EID₅₀/mouse, while that of wild-

TABLE 1. Replication of FluH/SeVHN in mice^a

Amt of FluH/SeVHN virus/mouse (EID ₅₀)	Virus titer (mean log ₁₀ EID ₅₀ ± SD/g) in:	
	Nasal turbinates	Lungs
$10^{5.3}$	4.0 ± 1.2	4.4 ± 0.58
$10^{6.3}$	4.0 ± 0.66	4.3 ± 0.66
$10^{7.3}$	3.9 ± 0.29	4.6 ± 0.88

^a BALB/c mice were intranasally inoculated with FluH/SeVHN virus. Three mice per group were euthanized on day 3 postinoculation for virus titration.

type WSN virus in mice of the same age was $10^{3.0}$ EID₅₀/mouse (16). Virus titers in the lungs of mice (three mice/group) inoculated with $10^{5.3}$, $10^{6.3}$, or $10^{7.3}$ EID₅₀ of FluH/SeVHN virus ranged from $10^{4.3}$ to $10^{4.6}$ EID₅₀/g at 3 days postinfection (Table 1), while those in nasal turbinates ranged from $10^{3.9}$ to $10^{4.0}$ EID₅₀/g, lower than titers measured after inoculation with $10^{5.9}$ EID₅₀ of wild-type influenza virus ($10^{7.2}$ EID₅₀/g for lung and $10^{5.3}$ EID₅₀/g for nasal turbinates) (16). Considered together, the results indicate that FluH/SeVHN virus is highly attenuated in mice.

Antibody responses of mice immunized with FluH/SeVHN virus. To determine if infection of mice with FluH/SeVHN induces antibodies against both SeV and WSN, we intranasally inoculated the animals with $10^{5.3}$, $10^{6.3}$, or $10^{7.3}$ EID₅₀ of virus. Mice intranasally inoculated with PBS or a dose equivalent to $10^{6.3}$ EID₅₀ of formalin-inactivated vaccine virus served as controls. Twenty-seven days after inoculation, mice were euthanized, and their sera, as well as trachea-lung and nasal washes, were tested for IgA and IgG antibody levels against both viruses by ELISA (Fig. 3). Compared to mice infected with PBS or formalin-inactivated virus, those infected with $10^{6.3}$ or $10^{7.3}$ EID₅₀ of FluH/SeVHN virus had elevated antibody levels to SeV in trachea-lung washes (both IgA and IgG) and in sera (IgG), but not in nasal washes. For mice inoculated with $10^{5.3}$ EID₅₀ of vaccine virus, antibody titers to SeV did not increase significantly. For the antibody response to WSN, both IgA and IgG antibody titers were significantly elevated ($P < 0.01$) in all samples derived from mice infected with FluH/SeVHN. Mice inoculated with PBS or formalin-inactivated vaccine virus did not show any significant IgA or IgG antibody response to either WSN virus or SeV. These results indicate that $\geq 10^{6.3}$ EID₅₀/mouse of FluH/SeVHN virus induces significant antibody responses against both WSN and SeV in mice.

Protective efficacy of FluH/SeVHN. To evaluate the protective efficacy of FluH/SeVHN, we immunized mice with live virus ($10^{5.3}$, $10^{6.3}$, or $10^{7.3}$ EID₅₀), formalin-inactivated vaccine virus (equivalent to $10^{6.3}$ EID₅₀), or PBS and challenged them with 10 MLD₅₀ of wild-type SeV ($10^{6.5}$ PFU/mouse) or WSN ($10^{5.5}$ PFU/mouse) at 28 days postimmunization. Mice immunized with $10^{6.3}$ or $10^{7.3}$ EID₅₀ of live FluH/SeVHN virus were completely protected from challenge with a lethal dose of SeV, while immunization with $10^{5.3}$ EID₅₀ provided only partial protection (Table 2). By contrast, control animals immunized with formalin-inactivated virus or PBS died on days 6 to 10 postchallenge. For mice immunized with $10^{7.3}$ EID₅₀ of live vaccine virus, SeV titers in lungs and nasal turbinates were lower than in mice immunized with PBS on day 3 postchallenge (Student's *t* test; $P < 0.05$). However, virus titers in the

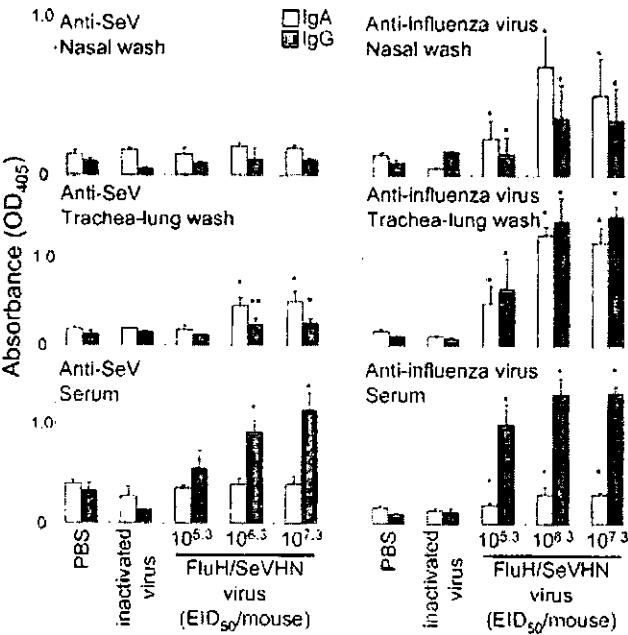


FIG. 3. Detection of virus-specific antibodies in samples of immunized mice. Mice were intranasally inoculated with live FluH/SeVHN (10^{5.3}, 10^{6.3}, or 10^{7.3} EID₅₀), inactivated FluH/SeVHN (equivalent to 10^{6.3} EID₅₀), or PBS. Samples (nasal wash, trachea-lung wash, or serum) from five mice per group were obtained 27 days postimmunization. IgA (white bars) and IgG (gray bars) levels in the samples of individual mice were detected by ELISA, as described in Materials and Methods. Results are expressed as the mean absorbances ($\pm$ standard deviations) of undiluted samples (nasal and trachea-lung washes), 1:2 diluted samples (detection of anti-SeV antibodies in sera), or 1:100 diluted samples (detection of anti-WSN antibodies in sera). Asterisks (*) or (**) indicate significance difference from samples derived from mice immunized with PBS (*, $P < 0.01$; **, $P = 0.012$).

organs of mice immunized with lower doses were not significantly lower than those in the control group, in spite of the protection observed in the immunized groups (Table 2). Conceivably, viral clearance at later time points postchallenge might have been more efficient in the immunized animals. All mice that survived SeV challenge lost body weight with a maximum loss of approximately 26% occurring by day 6 or 7 postchallenge (Fig. 4). After challenge with lethal doses of WSN, none of the mice immunized with live vaccine virus died or showed weight loss; by contrast, mice immunized with for-

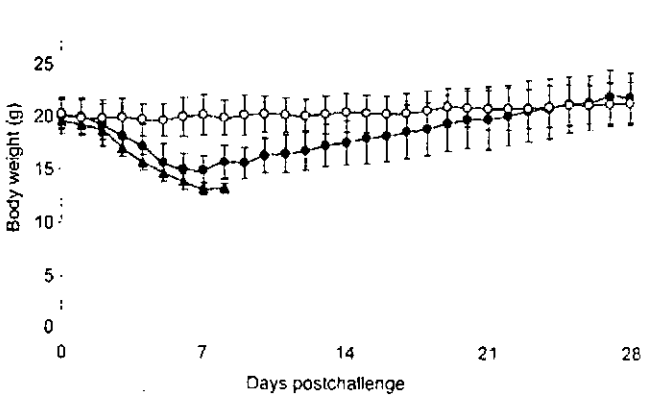


FIG. 4. Body weights of immunized mice after challenge with lethal doses of wild-type SeV. PBS-treated control mice ($n = 6$) and mice immunized with 10^{7.3} EID₅₀ of FluH/SeVHN ($n = 7$) were challenged with 10 MLD₅₀ of SeV at 28 days postimmunization (day 0 on graph). Closed circles, mice immunized with 10^{7.3} PFU of FluH/SeVHN virus; closed triangles, PBS-treated mice (note that all animals in this group had died by day 8 postchallenge, compared with none in the vaccine group); open circles, nonchallenged control mice.

malin-inactivated virus or PBS succumbed to infection (Table 3). WSN titers in lungs and nasal turbinates of mice immunized with vaccine virus were below the detection limits (<100 PFU/g) on day 3 postchallenge. Taken together, these data indicate that FluH/SeVHN provides efficient protection of mice against challenge with lethal doses of SeV or WSN.

DISCUSSION

Here, we describe the development of a live bivalent vaccine against two major respiratory infections: influenza virus and parainfluenza virus. Using reverse genetics, we produced a recombinant influenza virus, FluH/SeVHN, that expresses the ectodomain of SeV HN (an antigen that elicits protection against SeV infection) instead of the influenza virus NA, which is not as important as the HA for induction of a protective immune response (19). FluH/SeVHN protected mice against lethal challenge with influenza and murine parainfluenza viruses. It also replicated efficiently in eggs, stably expressed the HN antigen, was highly attenuated in mice, and induced protective antibodies against both parainfluenza and influenza

TABLE 2. Protection of mice immunized with FluH/SeVHN virus against wild-type SeV challenge^a

Vaccine	Amt of vaccine virus/ mouse (EID ₅₀)	No. of survivors/no. tested	Virus titer (mean $\pm$ SD log ₁₀ PFU/g)	
			Nasal turbinates	Lungs
Live FluH/SeVHN virus	10 ^{5.3}	3/7	7.1 $\pm$ 0.1	6.8 $\pm$ 0.4
	10 ^{6.3}	7/7	6.7 $\pm$ 0.2	6.9 $\pm$ 0.2
	10 ^{7.3}	7/7	6.5 $\pm$ 0.1	6.7 $\pm$ 0.3
Formalin-inactivated FluH/ SeVHN virus	Equivalent to 10 ^{6.3}	0/7	7.3 $\pm$ 0.2	7.1 $\pm$ 0.2
PBS		0/6	7.1 $\pm$ 0.3	7.1 $\pm$ 0.0

^a BALB/c mice were intranasally infected under anesthesia with 10 MLD₅₀ of wild-type SeV on day 28 postimmunization and monitored for 28 days after challenge. Five mice from each virus-immunized group were euthanized on day 3 postchallenge for virus titration.

TABLE 3. Protection of mice immunized with FluH/SeVHN against wild-type WSN challenge^a

Vaccine	Amt of vaccine virus/ mouse (EID ₅₀)	No. of survivors/no. tested	Virus titer (mean + SD log ₁₀ PFU/g) ^b	
			Nasal turbinates	Lungs
Live FluH/SeVHN virus	10 ^{5.3}	7/7	ND	ND
	10 ^{6.3}	7/7	ND	ND
	10 ^{7.3}	7/7	ND	ND
Formalin-inactivated FluH/SeVHN virus	Equivalent to 10 ^{6.3}	0/7	5.7 ± 0.2	8.0 ± 0.2
PBS		0/6	5.7 ± 0.2	8.3 ± 0.2

^a BALB/c mice were intranasally infected under anesthesia with 10 MLD₅₀ of wild-type WSN at 28 days postimmunization and monitored for 28 days after challenge. Five mice from each virus-immunized group were euthanized on day 3 postchallenge for virus titration.
^b ND, not detectable (detection limit, <100 PFU/g).

viruses in these animals. Hence, it meets the basic requirements for a live attenuated vaccine.

The development of viral vector systems for vaccine delivery is being vigorously pursued. DNA viruses and positive-sense RNA viruses have attracted the most attention as possible vaccine vectors, although with the advent of reverse genetics, the potential of negative-sense RNA viruses can be explored as well. In contrast to DNA viruses, the genetic material of RNA viruses (with the exception of members of the *Retroviridae* family) cannot be integrated into the host cell genome, adding to their biological safety. Among the group of nonsegmented negative-sense RNA viruses, Newcastle disease virus (20), vesicular stomatitis virus (14, 26), rinderpest virus (32), and Sendai virus (18, 30) have been tested as vaccine vectors. The use of segmented negative-sense RNA viruses as vaccine vectors has been impeded by the difficulty of artificially generating viruses that contain multiple gene segments (eight for influenza A virus). Moreover, early attempts to express foreign genes from a ninth segment were not encouraging, due to genetic instability (15). These limitations have been overcome with the establishment of a plasmid-based reverse genetics system for influenza virus (21) and our recent identification of segment-specific virion incorporation signals in the influenza vRNA segments (8, 33). Here, we extended our earlier findings by generating an influenza A virus that contains a parainfluenza virus HN ectodomain and by demonstrating the protective efficacy of this new vaccine candidate in a mouse model.

The high mutation rate and the potential reassortment of influenza viruses have raised some concern over their use as live vaccines. The recently approved live attenuated influenza virus vaccine relies on a limited number of amino acid replacements to confer the requisite cold-adapted, temperature-sensitive, and attenuated phenotypes; hence, point mutations could result in the loss of such phenotypes. By contrast, the vaccine virus described in this report is attenuated through replacement of the NA ectodomain with that of SeV HN, so that point mutations are highly unlikely to result in a loss of attenuation.

Combined vaccination, in which recipients are immunized with vaccines for multiple pathogens at once, is highly desirable in the clinic. However, simultaneous immunization with two or more live vaccine viruses can result in immunization failure if one virus affects the replication and/or viral pathogenicity of the other viruses (24). With vector systems, immune responses against vector components can present major obsta-

cles, prohibiting repeated immunization with the same vector backbone, as noted for adenoviral vectors (25). In our approach, the immunogenicity of the influenza virus vector is considered beneficial, since it would elicit immune responses against a societally and economically important pathogen. Indeed, we found that an influenza virus expressing the SeV HN ectodomain induced significant antibody levels against both influenza and parainfluenza viruses and fully protected immunized mice from challenge with lethal doses of the parental viruses. Swayne et al. (29), using a strategy similar to ours, generated a recombinant Newcastle disease virus that expressed the HA protein of an avian influenza virus; however, the vaccine failed to fully protect chickens from challenge with lethal doses of Newcastle disease or influenza virus. This discrepancy may reflect differences in the immunogenic potential of the two viruses, the doses used for protection and/or challenge, or the type of animal model used.

The known protective effect of the SeV HN ectodomain during viral infection led us to choose it as an SeV antigen. In addition, we anticipated that its sialidase activity would compensate for the lack of this enzyme in the neuraminidase-deficient influenza A virus vector. Efficient replication of WSN NA/HNect virus in eggs and poor replication in eggs of a recombinant virus expressing green fluorescent protein instead of NA (hence lacking sialidase activity; data not shown) substantiated this concept. The effect of balanced HA and NA activities on influenza virus replication depends on culture system (i.e., mammalian cell culture and embryonated chicken eggs) (3, 31). For example, WSN viruses with deletions in the stalk region of the NA protein replicated as well as wild-type WSN virus in cultured cells; however, some of these mutant viruses replicated poorly in embryonated chicken eggs (2). For FluH/SeVHN virus, we replaced the NA ectodomain with that of SeV HN, likely affecting the balance between the receptor-binding and -destroying activities of the viral glycoproteins and resulting in attenuated phenotypes in cell culture. By contrast, the imbalanced HA and NA activities were overcome in eggs, allowing efficient FluH/SeVHN replication.

The FluH/SeVHN virus we generated appears to have the essential properties of an ideal live vaccine. It is safe, genetically stable, and strongly immunogenic, and its growth profile in vitro is compatible with the requirements for efficient vaccine production. Hence, our results could open a new avenue for the production of multivalent vaccines. For example, the live influenza virus vaccine currently in use is a mixture of two

type A viruses (subtypes H1N1 and H3N2) and a type B virus. Thus, by replacing the NA of these influenza viruses with three different genes encoding the HNs of human parainfluenza virus types 1 and 3 (the two most common human parainfluenza viruses) and the G or F of RSV, one could generate a protective cocktail vaccine against six types of respiratory tract infections. Human parainfluenza virus and RSV infection primarily occur in the first year of life, and influenza can also occur in patients under 6 months of age (10). Although live influenza virus vaccine is not currently recommended for children under 5 years of age, the safety and efficacy of live attenuated influenza virus vaccines in infants under 1 year of age have been shown (4, 11, 28). Moreover, the safety and efficacy of the current live attenuated influenza virus vaccine can be further improved by using reverse genetics. Thus, we believe that influenza virus-based vaccines for younger children will be used in the future. Ultimately, by exploiting the known packaging signals of influenza vRNAs, it may be possible to add genes encoding the protective antigens of any infectious agent.

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Plasmid-only rescue of influenza A virus vaccine candidates

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The potential threat of another influenza virus pandemic stimulates discussion on how to prepare for such an event. The most reasonable prophylactic approach appears to be the use of effective vaccines. Since influenza and other negative-stranded RNA viruses are amenable to genetic manipulation using transfection by plasmids, it is possible to outline new reverse genetics-based approaches for vaccination against influenza viruses. We suggest three approaches. First, we use a plasmid-only rescue system that allows the rapid generation of high-yield recombinant vaccine strains. Second, we propose developing second-generation live influenza virus vaccines by constructing an attenuated master strain with deletions in the NS1 protein, which acts as an interferon antagonist. Third, we suggest the use of Newcastle disease virus recombinants expressing influenza virus haemagglutinin proteins of pandemic (epizootic) strains as novel vaccine vectors for use in animals and possibly humans.

Keywords: influenza A virus; interferon; Newcastle disease virus; vaccine; reverse genetics; virus vectors

1. INTRODUCTION

Influenza A virus causes widespread respiratory disease in humans and animals, placing great demands on the health-care system during outbreaks. Influenza virus is an enveloped virus with eight segments of negative-sense RNA encoding genes for at least 10 proteins. The haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins contain the major antigenic determinants responsible for the induction of neutralizing immune responses. Subtypes of influenza A viruses have been defined based on the antigenicity of the HA and NA surface glycoproteins. Small antigenic changes in HA and NA can cause antigenic drift, and reassortment between two strains may lead to antigenic shift with possibly a change in subtype. Both mechanisms, antigenic drift and antigenic shift, allow the virus to escape the protective immune system of the human host. For these reasons, influenza virus strains are globally monitored and vaccines are re-evaluated on an annual basis. Since 1977, both H3N2 and H1N1 subtypes have been circulating in the human population (Hayden & Palese 1997; Baseler *et al.* 2001).

Current influenza virus vaccines use formalin-inactivated preparations of viruses. If the circulating strain grows poorly or is particularly virulent, then manufacturing procedures might involve making antigenically similar reassortant viruses (which requires selection of one particular reassortant from a mixture) (Kilbourne *et al.* 1971) or growing multiple passages to select for a virus suitable for vaccine production. These procedures are time-consuming and unpredictable.

Live attenuated vaccines have shown promise of inducing a longer-lasting protective immune response. Nasal administration of an attenuated cold-adapted influenza virus has been reported to induce IgA antibodies in the respiratory tract as well as a humoral antibody response and provides effective protection from homotypic influenza virus infection in field trials (Belshe *et al.* 1998, 2000a). For use in vaccines, live attenuated viruses also need to be subjected to reassortant procedures to incorporate the HA and NA of circulating influenza virus strains (Maassab & Bryant 1999).

To overcome the difficulties of selecting reassortants and time-consuming multiple passaging of viruses, we suggest the use of a plasmid-only system to rapidly generate vaccine strains. Recently, plasmid-based reverse genetics systems were developed to generate infectious influenza viruses (Fodor *et al.* 1999; Neumann *et al.* 1999; Hoffmann *et al.* 2000a,b). The plasmids include four protein-expression plasmids for the three polymerase proteins (PB1, PB2 and PA) and nucleoprotein (NP) proteins plus eight hPOLI transcription plasmids that encode the eight viral gene segments (Neumann *et al.* 1994; Fodor *et al.* 1999). Here, we demonstrate use of the plasmid-based system to generate influenza A viruses with the HA and NA subtypes of currently circulating strains: one is an H1N1 and the other is an H3N2 subtype virus. The remaining six gene segments are provided by the influenza A/Puerto Rico/8/34 (PR8) virus that grows to high yield in embryonated chicken eggs and has been used to generate high-yield reassortant vaccine strains (Kilbourne *et al.* 1971). We obtained the recombinant vaccine strain as early as 48–72 h following transfection of cells with the 12 plasmids.

As an alternative to the development of cold-adapted influenza viruses for live attenuated vaccines, we suggest

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Table 1. Comparison of nucleotide and amino-acid sequences of influenza A/Puerto Rico/8/34/Mount Sinai and published PR8 viruses.

The eight vRNA segments of Arg/Puerto Rico/8/34/Mount Sinai (JHS)* were cloned into hPOLI plasmids and then sequenced at the Mount Sinai School of Medicine DNA Sequencing Core Facility. The nucleotide or amino acid as it appears in the GenBank database for A/Puerto Rico/8/34 viruses (left side of number) is compared with the corresponding nucleotide or amino acid as sequenced in the hPOLI plasmids used for virus rescue (right side of number). Accession numbers for the A/Puerto Rico/8/34/Mount Sinai virus genes are PB2, AF3891; PB1, AF389116; PA, AF389117; HA, AF389118; NP, AF389119; NA, AF389120; M, AF389121; and NS, AF389122. Accession numbers for the published A/Puerto Rico/8/34 virus genes are PB2, GI: 60484; PB1, GI: 324897; PA, GI: 60808; HA, GI: 62290; NP, GI: 324709; NA, GI: 324507; M, GI: 60788; and NS, GI: 324833.

PB2		PB1		PA		HA	
nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid
C279A	silent	C99T	silent	G177T	silent	T60A	C10S
G342A	M105I	C182G	A53G	T228A	silent	G140A	silent
A779G	K231R	T421C	silent	A366G	silent	C469A	T146N
C852T	silent	G435A	silent	C501A	silent	Δ471-473**	Δ147K
A923G	K299R	A549C	K175N	G346A	silent	C499A	A156E
G953A	G309D	G647A	R208K	A1107G	silent	G587A	silent
T1269C	silent	A651G	silent	T1233A	silent	T630C	S200P
C1309T	silent	G924C	L300F	C1257T	silent	T644A	D204E
C1422A	silent	C1002A	silent	T1299C	silent	G838T	R269M
G1527T	silent	T1152C	silent	A1431G	silent	T958A	F309Y
C1530T	silent	A1142T	H163L	C1629T	silent	T1015C	I338T
G1537A	V504I	T1551C	silent	C1672A	L550I	C1130T	silent
G1815A	silent	T1563G	silent	G1722A	silent	T1224A	S398T
C2001T	silent	G1574T	S507I	T1776C	silent		
C2055T	silent			T1896C	silent		
G2132A	R702K			T1962C	silent		
T2149C	silent			T2049C	silent		
A2238C	silent			G2052T	silent		
				A2106G	silent		

NP		NA		M		NS	
nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid
T34C	silent	T43C	I8T	T58A	silent	T173C	silent
T282G	silent	G403A	R128K	G136T	silent	T176C	silent
G306A	silent	T860C	silent	C535T	silent	C299T	silent
T328C	silent	A960G	K314E	G655A	silent	A329C	E101D
A630G	silent	G1142T	silent	T829C	silent	A404G	silent
G924A	silent	G1187A	silent			A575G	silent
G1350A	silent	A1229G	I403M			G763A	V89I in NEP***
		C1372G	T451S			A849G	silent

* rg indicates that the virus was obtained by reverse genetics.

** Δ471-473 indicates a deletion in nt471, 472, 473 compared with HA, GI: 62290.

*** NEP refers to the nuclear export protein encoded by the NS gene.

using a virus with deletions in the non-structural (NS)-1 protein as a donor strain. We have found that influenza virus mutants that do not express the NS1 protein are severely attenuated in interferon (IFN)-competent systems, but can grow in STAT 1^{-/-} mice and Vero cells (García-Sastre *et al.* 1998a; Talon *et al.* 2000a). In addition, viruses with mutated NS1 proteins have an intermediate level of attenuation (Talon *et al.* 2000b). A probable advantage of a deletion mutant for use as an attenuated vaccine is the reduced possibility of reversion.

A third approach to improve currently available influenza virus vaccines may be to incorporate the antigenically important HA and NA genes into a heterologous vaccine vector. To explore this approach, we have put an influenza virus HA into Newcastle disease virus (NDV),

that may be used as an avian, or possibly human, vaccine. In this manner, an anti-HA immune response can be induced while avoiding exposure to highly virulent influenza virus strains.

These three approaches can be used to rapidly, reproducibly and safely generate vaccines expressing the HA and/or NA of circulating influenza A virus strains for use in formalin-inactivated, live attenuated or heterologous virus vaccine preparations.

2. PLASMID-ONLY RESCUE OF INFLUENZA A/PR/8/34 VIRUS

Reverse transcription-polymerase chain reaction (RT-PCR) products encoding the eight viral RNA (vRNA)

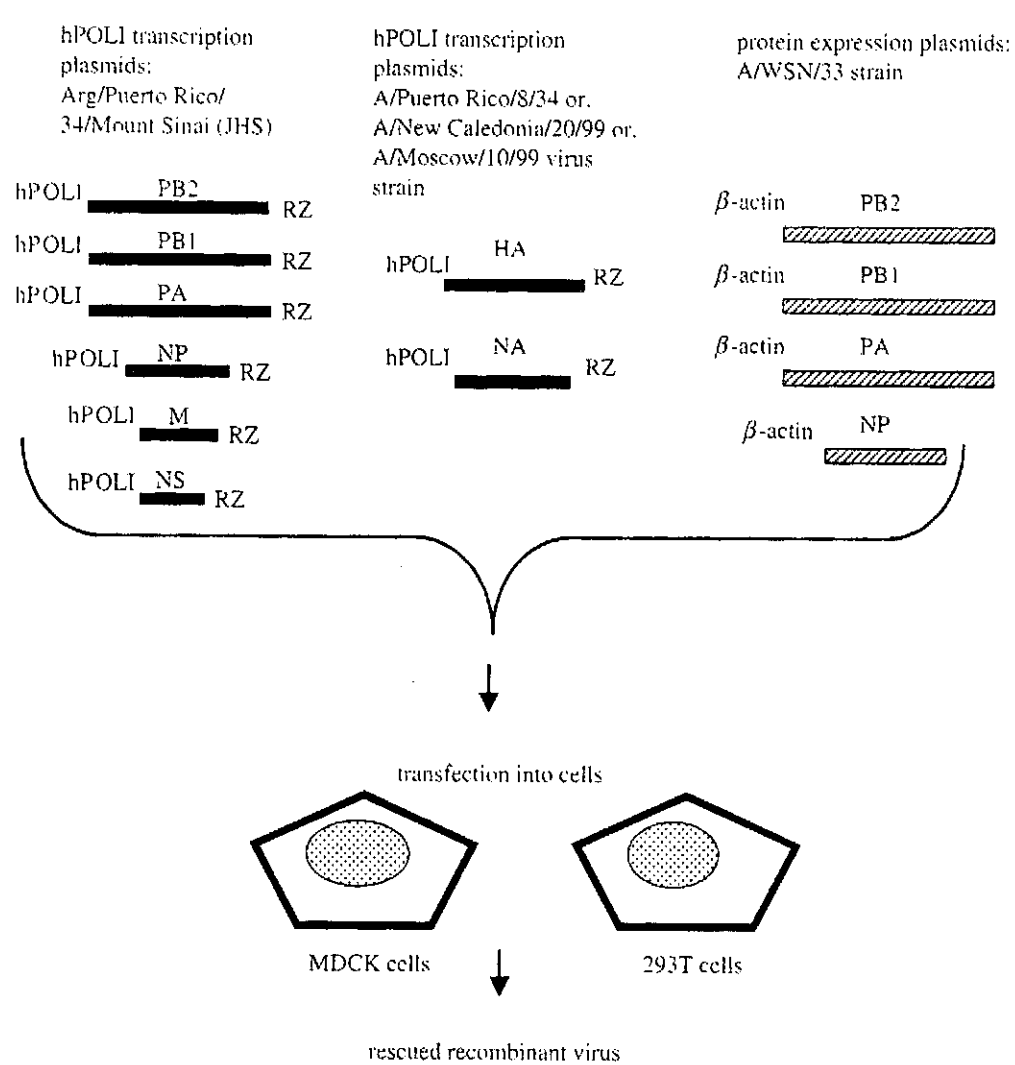


Figure 1. Strategy to rescue recombinant influenza virus containing six gene segments from the high-yield A/Puerto Rico/8/34 virus with HA and NA gene segments from either the A/Puerto Rico/8/34, the A/New Caledonia/20/99 or the A/Moscow 10/99 strain. MDCK and 293T cells were transfected with Lipofectamine 2000 with 12 plasmids. The 12 plasmids included six hPOLI transcription plasmids encoding the indicated gene segments of the influenza A/Puerto Rico/8/34 strain, two hPOLI transcription plasmids encoding the HA and NA gene segments of either the A/Puerto Rico/8/34, the A/New Caledonia/20/99 or the A/Moscow/10/99 strain and four protein-expression plasmids encoding PB2, PB1, PA, and NP genes of the influenza A/WSN 33 strain. Forty-eight to 72 h following transfection, rescued virus in the supernatant was detected by haemagglutination titre.

segments of the strain influenza A/Puerto Rico/8/34 (H1N1) (PR8) were cloned into hPOLI transcription plasmids that have a truncated human polymerase I promoter at the 5' vRNA end and a hepatitis delta virus ribozyme positioned downstream to cleave precisely at the 3' vRNA end of the transcribed RNA. This type of plasmid has previously been used to successfully express model influenza virus genes (Neumann *et al.* 1994, 1999; Pleschka *et al.* 1996; Fodor *et al.* 1999). DNA sequencing revealed several nucleotide differences between our eight vRNA segments cloned into the hPOLI transcription plasmids and the A/PR/8/34 virus sequences available in GenBank (table 1). Comparing all eight vRNA segments, we found 94 nucleotide differences, resulting in 30 predicted amino-acid differences. It should be noted that the sequences used for the comparison were obtained in the years 1980–1982 when sequencing may not have been as reliable as today. Also, genetic drift during passage of the PR8 virus (in eggs) could account for the differences. Finally, the PR8 viruses used in different laboratories

may indeed possess different genotypes. The cloned sequences have been submitted to the GenBank database under the name Arg/PuertoRico/8/31/Mount Sinai (JHS). Using standard cloning procedures, the open reading frames of the genes of PB1, PB2, PA and NP of influenza A/WSN 33 virus were cloned into protein-expression plasmids under a chicken β-actin promoter (Niwa *et al.* 1991). These four proteins are the minimal set required for encapsidation, transcription and replication of the eight vRNAs (Huang *et al.* 1990; Pleschka *et al.* 1996).

For virus rescue, all 12 plasmids were transfected into a 1:1 mixture of 293T and MDCK cells, as depicted in figure 1. For transfection, 0.5 µg of each plasmid (6 µg total) were transfected into 10⁶ cells using 6 µl Lipofectamine 2000 (GIBCO, Grand Island, NY, USA). We consistently achieved transfection efficiencies of 80–90% in 293T cells (as estimated by co-transfection of a GFP plasmid under a CMV promoter). Because influenza A viruses grow to high titres in MDCK cells, the mixture

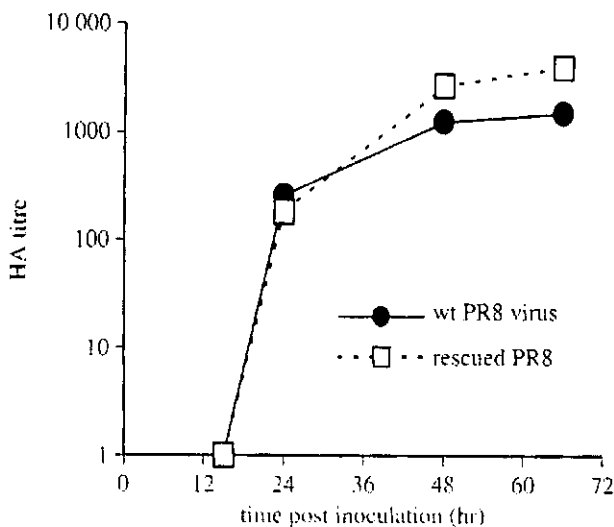


Figure 2. Growth curves of rescued influenza A/PR/8/34 virus and wt A/PR/8/34 virus in eggs. Eggs were inoculated with 5 PFU of either rescued A/PR/8/34 or wt A/PR/8/34 virus, in triplicate. At 15, 24, 48 and 66 h post-inoculation, 100 µl allantoic fluid were removed and the titre was measured by haemagglutination of chicken red blood cells. Each point is the average haemagglutination titre of three eggs.

of 293T and MDCK cells provides a good growth environment for the rescued virus. Sixteen hours post-transfection the cells were fed with Dulbecco's modified Eagle's medium (GIBCO) containing 10 mM Hepes (GIBCO), 0.3% bovine albumin (ICN Biomedicals, Aurora, OH, USA), 200 U ml⁻¹ penicillin G sodium with 200 µg ml⁻¹ streptomycin sulphate (GIBCO), and 1 µg ml⁻¹ TPCK-trypsin (Sigma, St. Louis, MO, USA). Forty-eight to 72 h post-transfection the supernatant typically showed a haemagglutination titre of approximately 100 as measured by haemagglutination of 0.25% chicken red blood cells. The supernatant was used to inoculate 10-day-old embryonated chicken eggs. Forty-eight hours after inoculation, virus titre in the allantoic fluid was measured by haemagglutination and/or plaque formation in MDCK cells. The virus titre of rescued A/PR/8/34 virus was typically 3000 and the supernatant had approximately 10³ plaque forming units (PFU) ml⁻¹.

In order to confirm that the rescued A/PR/8/34 virus grew like wild-type (wt) A/PR/8/34 virus, 10-day-old embryonated chicken eggs were inoculated with 5 or 300 PFU per egg, in triplicate. Allantoic fluid was extracted at roughly 12 h intervals for 66 h and haemagglutination titres were measured to quantify virus in allantoic fluids. As seen in figure 2, the rescued and the wt A/PR/8/34 viruses had comparable growth kinetics, both reaching a haemagglutination titre of approximately 3000 by 48 h after inoculation with 5 PFU per egg.

3. PLASMID-ONLY RESCUE OF RECOMBINANT H1N1 VIRUS

For the 2000–2001 and the 2001–2002 influenza virus seasons, the recommendation for influenza virus vaccines includes an influenza A/New Caledonia/20/99-like H1N1 strain. Lyophilized influenza A/New Caledonia/20/99

(H1N1) virus (CDC no. 99044140, from passage E4/E1, 8/30/99) was kindly provided by Alexander Klimov at the Center for Disease Control (CDC) in Atlanta, GA, USA). The virus was reconstituted in sterile water and passaged once in 10-day-old embryonated chicken eggs. Virus from the allantoic fluid was directly used in RT-PCR to amplify the HA and NA genes using primers corresponding to the non-coding regions of the vRNA. (Sequences of all primers used are available upon request.) Standard cloning techniques were employed to insert the cDNA segments into hPOLI transcription plasmids. As with the rescue of A/PR/8/34 virus described above (§ 2), rescue of high-titre recombinant PR8/New Caledonia (6+2) virus was achieved by transfecting 12 plasmids into a mixture of 293T and MDCK cells. The rescued virus grew to high titres (typically haemagglutination titres of 3000) in eggs as well as in MDCK cells (typically 10³ PFU ml⁻¹).

To confirm that the HA segment in the rescued PR8/New Caledonia (6+2) virus was derived from the New Caledonia strain, RNA was extracted from the recombinant virus, A/PR/8/34 virus and New Caledonia/20/99 virus grown in eggs. RT-PCR was done with the primers 3'NC and HA,972 as depicted in figure 3a. These primers amplified the HA gene segments of both A/PR/8/34 and A/New Caledonia/20/99 strains. A set of controls without the RT enzyme had no products, which confirmed that the PCR products were derived from rescued virus, as opposed to residual plasmid cDNA from the transfection procedure (data not shown). (Similar controls without RT enzyme were done for all subsequent RT-PCR reactions described.) Unique Xho I and Apa I restriction enzyme sites confirmed that the HA gene of the recombinant virus was derived from the A/New Caledonia/20/99 virus. Similarly, we confirmed that the NA segment in the PR8/New Caledonia (6+2) virus was derived from the A/New Caledonia/20/99 strain. RT-PCR was done with the primers NA,602 and NA,1345 that were chosen because they recognize regions conserved between the NA genes of both the A/PR/8/34 and the A/New Caledonia/20/99 strains as depicted in figure 3a. The restriction fragment length polymorphism (RFLP) patterns using the restriction enzymes Nco I and Hpa I confirmed that the NA gene of the recombinant virus was derived from the A/New Caledonia/20/99 virus.

To demonstrate that the rescued virus was a PR8/New Caledonia (6+2) recombinant, RT-PCR was done on the matrix protein (M) and PBI genes as well. RT-PCR products from primers M,127 and M,495 were digested by Hind III, which cut only the product derived from the M gene of A/New Caledonia/20/99 virus. Similarly, the PBI gene segments amplified by the primers PBI,1245 and PBI,1836 followed by digestion with the restriction enzyme Sal I confirmed that the PBI gene of the recombinant was derived from the A/PR/8/34 virus (figure 3b).

4. PLASMID-ONLY RESCUE OF RECOMBINANT H3N2 VIRUS

Since 1968, influenza A viruses of the H3N2 subtype have been circulating in humans. The current H3N2 strain A/Moscow/10/99 (CDC no. 99006887, passage E2 E3, 8.26.99) was kindly provided by Alexander

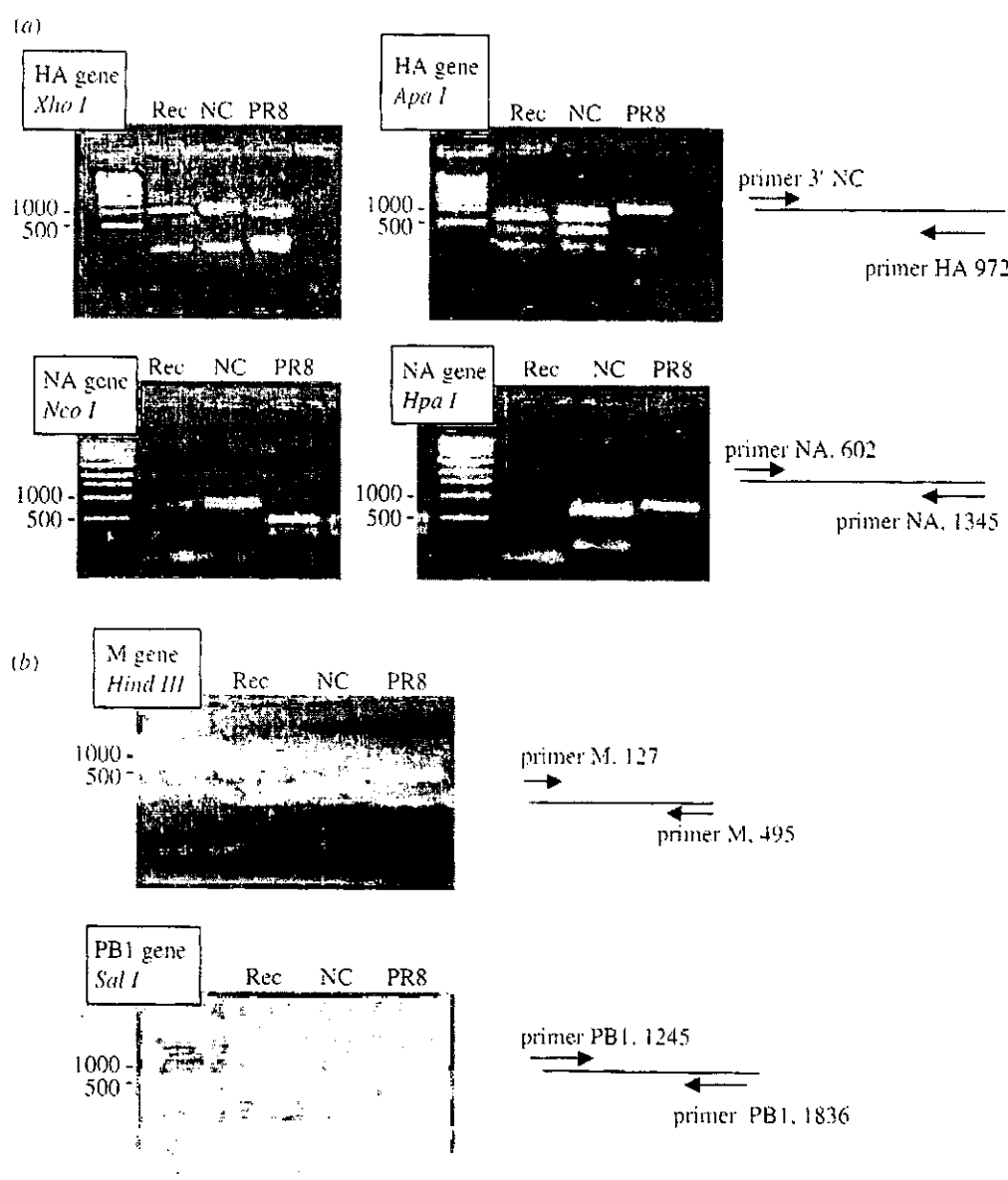


Figure 3. Characterization of the rescued PR8/New Caledonia/20/99 (6 + 2) recombinant virus. RT-PCR was performed on rescued PR8/New Caledonia/20/99 (6 + 2) recombinant (Rec) virus, the wt A/New Caledonia/20/99 (NC) virus and rescued A/Puerto Rico/8/34 (PR8) virus as described in the text (§ 3). The PCR products were digested by the indicated restriction enzymes. (a) Analysis of the HA and NA gene segments of the Rec virus, NC virus and PR8 virus. (b) Analysis of the M and PB1 gene segments of the Rec virus, NC virus and PR8 virus.

Klimov at the CDC. The lyophilized virus was reconstituted in sterile water and passaged once in 10-day-old embryonated chicken eggs. To clone the HA gene, we designed primers using nucleotides in the cleavage site that is conserved among HA proteins of the H3 subtype and in the non-coding region of the 3' and 5' ends of the vRNA common to all of the influenza virus segments. With these primers we introduced an *Mlu* I site in the cleavage site without changing the amino-acid sequence. In order to clone the NA gene, we first determined the sequences of the non-coding regions. Using that sequence information, primers specific for the A/Moscow/10/99 NA gene were designed. Standard cloning procedures were subsequently used to clone the PCR products into the hPOLI vector as described above (§ 2).

As described for the other two viruses, recombinant PR8/Moscow (6 + 2) virus was readily rescued 48–72 h

following transfection. To confirm that the NA and HA segments in the recombinant PR8/Moscow (6 + 2) virus were derived from the influenza A/Moscow/10/99 strain, RT-PCR was again done on the two parental viruses as well as the recombinant virus. This time, however, RT-PCR was performed with two sets of primers: one set unique for A/Moscow/10/99 and one set unique for A/Puerto Rico/8/34 virus. RFLP patterns confirmed that the NA and HA gene segments of the recombinant virus were derived from A/Moscow/10/99 virus (figure 4a). In addition, RT-PCR products with primers HA₂₂₉ and 3' NC were cleaved by the restriction enzyme *Mlu* I, indicating that the rescued virus was derived from the transfected plasmids (data not shown).

The other six gene segments were derived from A/PR/8/34 virus. This was confirmed by amplification using PR8-specific primers for each of the NP, M, NS (figure

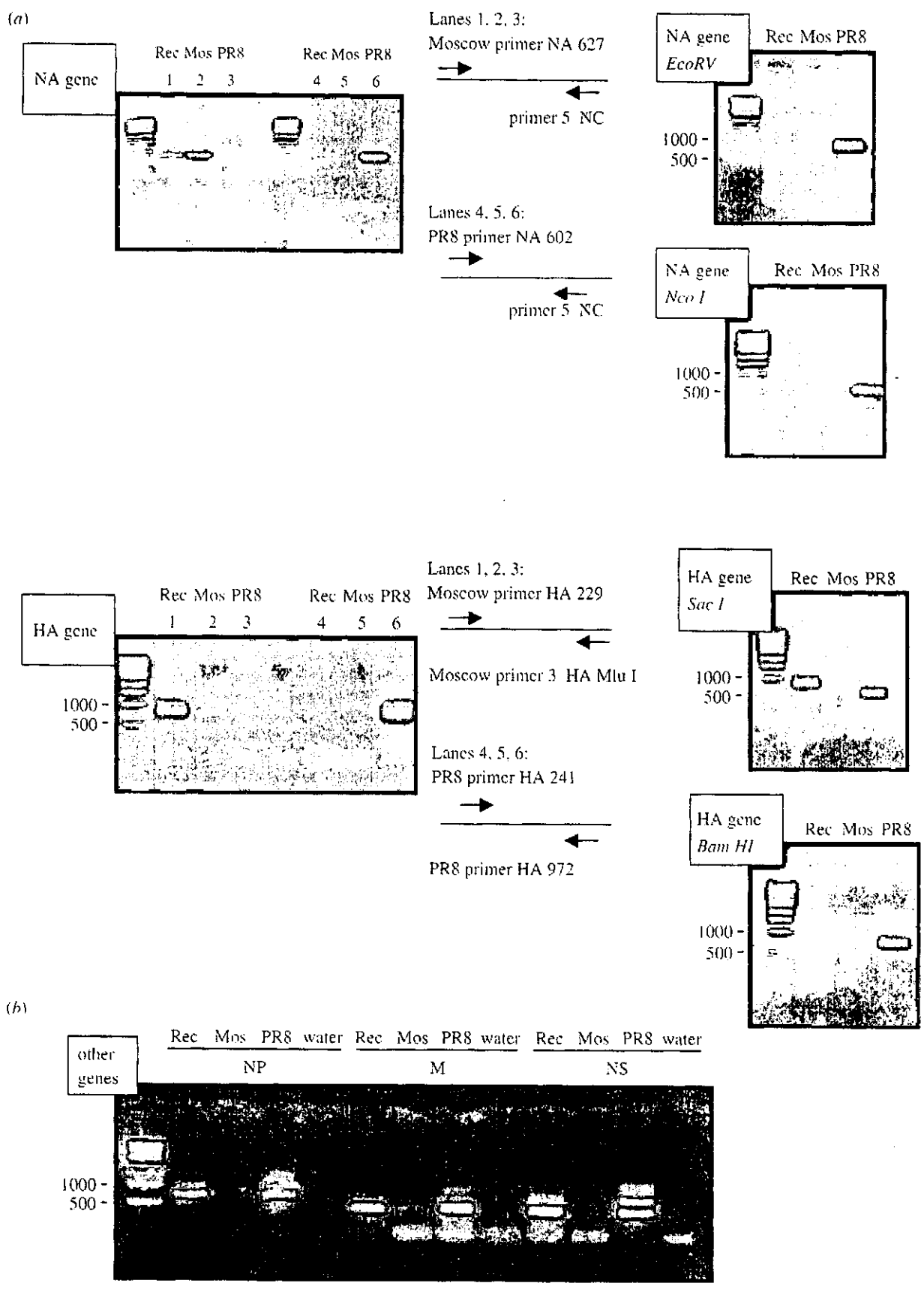


Figure 4. Characterization of the rescued PR8/Moscow/10/99 (6-2) recombinant virus. RT-PCR was performed on rescued PR8/Moscow/10/99 (6-2) recombinant (Rec) virus, the wt A/Moscow/10/99 (Mos) virus and rescued A/Puerto Rico/8/34 (PR8) virus as described in the text [§4]. The PCR products were digested by the indicated restriction enzymes. (a) Analysis of the NA and HA gene segments of the Rec virus, Mos virus and PR8 virus. (b) Analysis of the NP, M and NS segments of the Rec virus, Mos virus and PR8 virus.

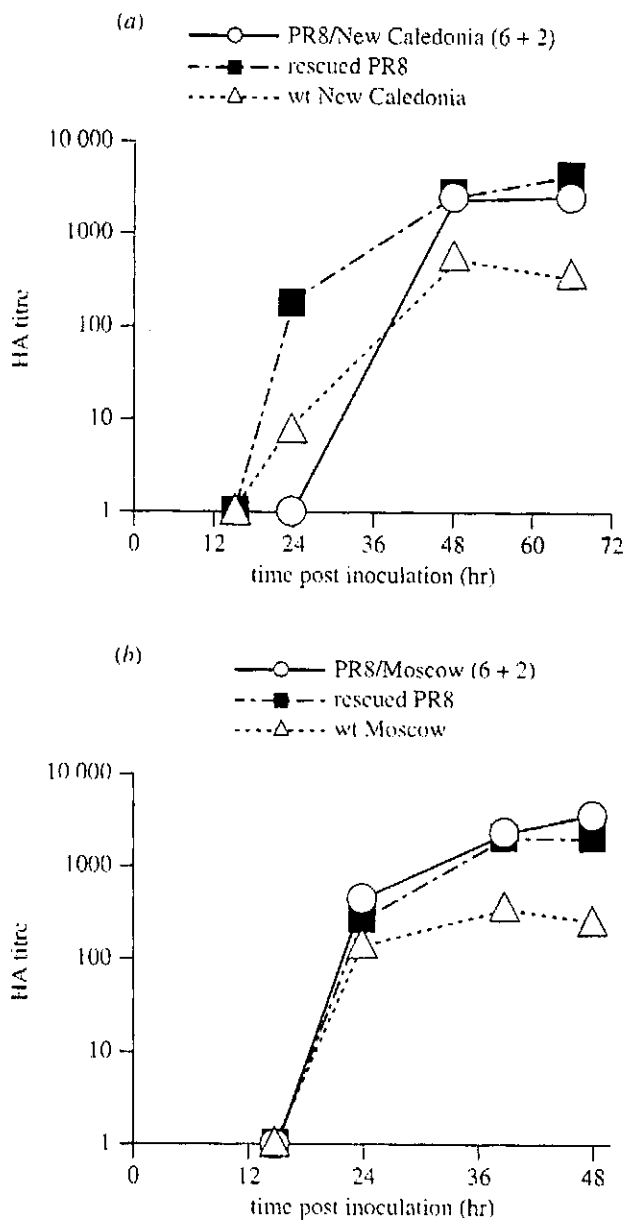


Figure 5. Growth curves of rescued high-yield recombinant viruses. Eggs were inoculated with 20 PFU per egg, in triplicate. At the indicated time-points, 100 μ l allantoic fluid were removed to measure the titre by haemagglutination of chicken red blood cells. (a) The growth curves of A/PR/8/34 virus, A/New Caledonia/20/99 virus and the recombinant PR8/New Caledonia/20/99 (6+2) virus are compared. (b) The growth curves of A/PR/8/34 virus, A/Moscow/10/99 virus and the recombinant PR8/Moscow/10/99 (6+2) virus are compared.

4b) and polymerase (PB1, PB2 and PA) genes (data not shown).

5. GROWTH CURVES OF (6+2) RECOMBINANT VIRUSES

We found that the recombinant viruses grew to high titres in eggs (figure 5) as well as MDCK cells (data not shown). Ten-day-old embryonated chicken eggs were inoculated with 20 PFU (figure 5) or 500 PFU (data not shown) per egg, in triplicate. The rescued recombinant viruses grew slightly better than the wt A/New Caledonia/

20/99 or wt A/Moscow/10/99 viruses. The recombinant viruses grew to haemagglutination titres nearly as high as the influenza A/PR/8/34 virus, suggesting that the 6+2 recombinants made with this plasmid-only rescue system may be candidates for high-yield vaccine strains expressing the HA and NA genes of current H1N1 and H3N2 viruses.

6. LIVE INFLUENZA VIRUS VACCINE STRAINS WITH DELETIONS IN THE NS1 GENE

In an effort to design novel live attenuated vaccines we have explored the characteristics of influenza viruses with mutations in the NS1 gene. DelNS1 influenza A virus, that lacks the NS1 protein, can grow in Vero cells, 6-day-old embryonated chicken eggs and STAT 1^{-/-} mice to titres comparable with that of wt A/Puerto Rico 8/34 virus. However, the delNS1 virus does not grow well in MDCK cells, 10-day-old embryonated chicken eggs or wt (BALB/c or C57BL/6) mice (García-Sastre *et al.* 1998a). Vero cells, young embryonated eggs (less than 8 days old) and STAT 1 knockout mice are all deficient in IFN signalling, that is a potent host defence against virus infection (García-Sastre *et al.* 1998b; Durbin *et al.* 2000). In addition, IFN- α and IFN- β mRNA transcription is induced in mouse embryonic fibroblasts or human 293T cells infected with delNS1 virus, but not A/PR 8/34 virus (Wang *et al.* 2000). The NS1 protein is a viral IFN antagonist and could be a target for engineering live attenuated vaccine strains. The delNS1 virus may be too attenuated in humans to be suitable for vaccines. However, we have found that a truncated NS1 protein, expressing the first 99 or 126 amino acids of the NS1 protein, is an intermediate IFN antagonist and influenza virus NS1-99 expressing the truncated protein stimulates a better antibody response in mice than delNS1 virus (Talon *et al.* 2000b). Thus, an influenza virus with a truncated NS1 protein may be a good master strain for live attenuated viral vaccines engineered to express HA and NA genes of epidemic or pandemic strains.

7. RESCUE OF RECOMBINANT NDV EXPRESSING INFLUENZA VIRUS HA

NDV is a member of the Paramyxoviridae family that can cause disease in birds. Like influenza virus, NDV has a negative-sense RNA genome and reverse genetics procedures have been used to generate infectious virus from cells transfected with cDNA (for reviews, see Palese *et al.* 1996; García-Sastre 1998; Roberts & Rose 1998). NDV has a non-segmented, single-stranded RNA genome of 15186 nucleotides that encodes six proteins: NP, P, M, fusion protein, HN and an RNA-dependent RNA polymerase. The HA gene of influenza A/WSN 33 virus was cloned between the P and M genes of the cDNA of the NDV vaccine strain Hitchner BI and infectious virus was rescued (Nakaya *et al.* 2001). This recombinant rNDV/BI-HA virus was found to express the HA protein (by immunostaining) and grew in embryonated chicken eggs, although to titres roughly 1 log lower than the wt NDV virus. Mice immunized with the rNDV/BI-HA virus developed an anti-HA antibody response and were protected against challenge by influenza A/WSN 33

virus, indicating that NDV may have potential as an effective vaccine vector and that the influenza virus HA protein is immunogenic in the context of a heterologous virus (Nakaya *et al.* 2001).

8. CONCLUSIONS

Currently licensed manufacturing procedures for influenza virus vaccines involve growing virus strains or reassortants expressing the HA and NA of the circulating or antigenically similar strains followed by formalin inactivation. Since 1977, influenza A virus H3N2 and H1N1 subtypes have been circulating in humans. We have shown that the HA and NA genes of the two influenza A virus strains currently recommended for vaccine production can be successfully combined with the six other segments of the high-yield influenza A/PR/8/34 virus to generate a recombinant virus that grows well in eggs, suggesting that this system is suitable for generating candidate vaccine strains. The plasmids encoding the six RNA segments of the donor strain A/PR/8/34 virus provide an immutable genetic background. These plasmids can be easily stored and distributed for use as new strains and subtypes emerge in the human population.

Formalin-inactivated vaccines induce a humoral immune response that does not provide complete protection from influenza in all recipients. Live attenuated vaccines may provide longer-lasting immunity and broader protection against variant strains. An attenuated cold-adapted virus administered intranasally has been shown to be protective in field trials (Maassab & Bryant 1999; Treanor *et al.* 1999; Belshe *et al.* 2000a,b) and it is currently being developed for use in humans. We suggest a second generation live influenza virus vaccine that is based on attenuation afforded by deletion mutations in its NSI gene. Such NSI-modified viruses may have advantages over cold-adapted influenza viruses for use as vaccines.

A live attenuated vaccine virus should replicate well enough in the host to stimulate a protective immune response without causing disease. Viruses with mutations in NSI may be more immunostimulatory compared with viruses expressing an intact NSI protein. Infection with influenza virus induces production of both IFN type I (IFN- α and - β) and type II (IFN- γ), that in turn stimulate a wide range of responses in the host (Henner *et al.* 1992; Muller *et al.* 1994; Van den Broek *et al.* 1995; Welsh *et al.* 1996; Price *et al.* 2000; Durbin *et al.* 2000). IFN- γ stimulates activation of T cells in a manner that enhances a Th1 response, leading to a protective cytolytic T-lymphocyte response. It is likely that NSI-modified influenza viruses induce a higher IFN- α and - β and IFN- γ response than does wt (or cold-adapted) virus.

Consequently, it is possible that influenza virus vaccine strains with mutations in the NSI may induce an enhanced antiviral immunity as compared with live attenuated influenza virus vaccines expressing a wt NSI protein. In addition, the required dose needed per patient of an influenza virus vaccine attenuated by deletions in the NSI gene may be lower than the dose used for cold-adapted vaccines. A lower dose would allow for distribution of lyophilized vaccine formulations as opposed to a cold chain of frozen materials as required for higher-dose

vaccines. Ultimately, these reduced manufacturing costs would enable more people to receive influenza virus vaccines.

The third approach for influenza virus vaccine candidates is incorporating the antigenic HA of influenza A viruses into a vaccine strain of a heterologous virus such as NDV, that is avirulent in humans (Schirmacher *et al.* 1998). A heterologous virus may represent a useful vector for the presentation of antigens of influenza viruses that are particularly virulent. We have introduced the HA gene of influenza A/WSN/33 virus into the genome of the Hitchner BI vaccine strain of NDV. The recombinant rNDV/BI-HA virus induced a protective anti-HA antibody response in mice. Such a recombinant NDV vaccine may be used to inhibit the spread of influenza virus in an avian population and may serve as a vaccine vector in humans.

With all three approaches that we have suggested, only the HA and NA genes need to be cloned as new pandemic (epidemic) strains of influenza A virus arise. Thus, generation of vaccine strains of recombinant influenza viruses or rNDV/BI-HA for use in formalin-inactivated or live attenuated vaccine preparations can be reproducible, safe and rapid.

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A Chimeric Respiratory Syncytial Virus Fusion Protein Functionally Replaces the F and HN Glycoproteins in Recombinant Sendai Virus

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Entry of most paramyxoviruses is accomplished by separate attachment and fusion proteins that function in a cooperative manner. Because of this close interdependence, it was not possible with most paramyxoviruses to replace either of the two protagonists by envelope glycoproteins from related paramyxoviruses. By using reverse genetics of Sendai virus (SeV), we demonstrate that chimeric respiratory syncytial virus (RSV) fusion proteins containing either the cytoplasmic domain of the SeV fusion protein or in addition the transmembrane domain were efficiently incorporated into SeV particles provided the homotypic SeV-F was deleted. In the presence of SeV-F, the chimeric glycoproteins were incorporated with significantly lower efficiency, indicating that determinants in the SeV-F ectodomain exist that contribute to glycoprotein uptake. Recombinant SeV in which the homotypic fusion protein was replaced with chimeric RSV fusion protein replicated in a trypsin-independent manner and was neutralized by antibodies directed to RSV-F. However, replication of this virus also relied on the hemagglutinin-neuraminidase (HN) as pretreatment of cells with neuraminidase significantly reduced the infection rate. Finally, recombinant SeV was generated with chimeric RSV-F as the only envelope glycoprotein. This virus was not neutralized by antibodies to SeV and did not use sialic acids for attachment. It replicated more slowly than hybrid virus containing HN and produced lower virus titers. Thus, on the one hand RSV-F can mediate infection in an autonomous way while on the other hand it accepts support by a heterologous attachment protein.

The membrane glycoproteins of enveloped viruses play a crucial role in the infection process. They mediate virus binding to cellular receptors and induce fusion between the viral and cellular membranes. For several viruses, including members of the families *Orthomyxoviridae* and *Retroviridae*, binding and fusion are functions of the same envelope glycoprotein. A different strategy is accomplished by members of the family *Paramyxoviridae* which have separate attachment and fusion proteins.

The fusion (F) proteins of paramyxoviruses are typical type I fusion proteins. They are synthesized as inactive precursor proteins that have to be proteolytically cleaved into disulfide-linked subunits to be biologically active (19). For most paramyxoviruses, this cleavage occurs at a multibasic cleavage motif that is recognized by the ubiquitous endoprotease furin in the trans-Golgi network. An example for this type of activation is the F protein of respiratory syncytial virus (RSV) (49). In contrast, Sendai virus (SeV) and some apathogenic Newcastle disease virus strains contain a single arginine residue at the cleavage sites and are activated by trypsin-like proteases that are secreted by a restricted subset of cells or by coinfecting bacteria (40, 41). Viruses of this category usually require addition of trypsin for multistep replication in cell culture. What-

ever protease is involved in the processing of the fusion protein, in either case a stretch of hydrophobic amino acids, the so-called fusion peptide, is located at the N terminus of the membrane-anchored subunit. The fusion peptide is probably buried in the native protein unless a conformational change leads to its exposure at the surface of the molecule. Only in this position it interacts with the target membrane and initiates the fusion process (23). For the influenza virus hemagglutinin and other viral fusion proteins, the conformational change required for this process has been shown to occur in response to acidic pH (7, 9, 44). In contrast, fusion by paramyxoviruses takes place at neutral pH and therefore a different trigger for the conformational change must exist. A large body of evidence indicates that the majority of paramyxovirus fusion proteins lack any detectable fusion activity in the absence of the viral attachment protein (1, 6, 11, 14, 26–28, 39). In addition, attempts to replace the homologous attachment protein with one from a related virus were mostly not successful (6, 14, 39, 46). These findings led to the hypothesis that the two proteins act in a cooperative manner. It is believed that binding of the attachment protein to the cellular receptor induces conformational changes in this protein, which in turn trigger conformational changes in the fusion protein (23). Two members of the *Paramyxoviridae* family apparently do not follow this rule. The fusion proteins of simian virus 5 and RSV show fusion activity in the absence of the respective attachment protein (1, 10, 31, 50). In addition, RSV attachment protein G was demonstrated to be dispensable for virus replication in cell culture, indicating

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TABLE 1. Oligonucleotides used for construction of chimeric genes

Primer	5'-sequence-3'
hF-S1	TTTGAATTCACGCGTACAATGGAGTTGC CAATCCTCAAAGC
RSV/SeV-F-AS	GATCGTAATCACAGTATTTGTGGTTGATT TACCAGC
RSV/SeV-F-S	AAATCAACCACAAATACTGTGATTACGAT CATAGTAG
SeV-F-AS	AAAGAATTCCGCGGCTCATCTTTTCTCAG CCATCGCATCAAACC
Tail-AS	CATTGACCTTTTGAGTCTACAGTATAGGA GCAGTCCAACAGCAATTA
Tail-S	GGACTGCTCCTATAGTCTAGACTCAAAGG GTCAATGCTAATGGGTAAT
hF-S2	TTTGTCCGACATGGAGTTGCCAATCCTCA AAGCAAATGC
SeV-F-AS2	TTTTCCTCGAGTTCTCATCTTTTCTCAGCCA TCGCATCAAACCC

* Cleavage sites of restriction endonucleases are underlined. Nucleotides that are part of either the RSV-F or the SeV-F open reading frame are shown in bold.

that RSV-F also has a receptor-binding function (18, 42). Further evidence for this assumption comes from the observation that RSV-F can bind to glycosaminoglycans such as heparin (12). Nevertheless, the G glycoprotein was found to support virus attachment to the cell surface (17, 31, 43).

In this study, we used reverse genetics to analyze RSV-F protein function in the context of infectious SeV. We show that chimeric RSV-F is incorporated into SeV virions and can functionally substitute for the SeV fusion protein and the hemagglutinin-neuraminidase (HN). However, in the presence of HN the intrinsic receptor-binding activity of RSV-F is dominated by the SeV attachment protein.

MATERIALS AND METHODS

Cells. Vero and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Baby hamster kidney cells (BHK-21) were maintained in Earle's minimal essential medium (EMEM) containing nonessential amino acids and 5% FBS.

Primary antibodies. Purified and fluorescein isothiocyanate (FITC)-conjugated goat sera directed to human parainfluenza virus type 1 (anti-PIV1) were purchased from Acris (Hiddenhausen, Germany). Monoclonal antibodies directed to RSV F protein (anti-RSV-F) were purchased from Serotec (Düsseldorf, Germany; clone RSV3216) and Acris (Mab9C5). Monoclonal antibodies Mab19 and Mab13 were kindly provided by Geraldine Taylor (Institute for Animal Health, Compton, United Kingdom). A polyclonal antiserum against SeV (anti-SeV) was produced in rabbits by immunization with sucrose gradient-purified virus. For generation of a monospecific antiserum directed against the SeV-F cytoplasmic domain (anti-SeFTail), the peptide CRHMYTNGGFDAMAEKR was covalently linked by an N-(α -maleimide)-succinimide ester to key-hole limpet hemocyanin (Sigma) as carrier protein and used for immunization of rabbits with ABM-ZK (Lunaris GmbH, Bettingen, Germany) as adjuvant.

Construction of chimeric RSV-F genes. Plasmids pTM1-hF, containing the F gene of human RSV (HRSV; Long strain) (50), and pCDNA3.1-SeV, containing the F gene of SeV (Pushimi strain), were used as templates for construction of chimeric RSV-F gene cassettes by an overlapping-PCR technique. Oligonucleotides used for the construction of the chimeric genes are listed in Table 1. The RSV-F protein ectodomain (amino acids 1 to 524) was amplified from pTM1-hF using *Pfu* DNA polymerase (MBI Fermentas) and the primer pair hF-S1 and RSV/SeV-F-AS. The part of the SeV-F gene encoding the transmembrane and cytoplasmic domains (amino acids 500 to 565) was amplified from pCDNA3.1-SeV-F using the oligonucleotides RSV/SeV-F-S and SeV-F-AS. The two PCR products were purified (QIAGEN PCR purification kit), mixed at a molar ratio of 1:1, and heated for 2 min at 95°C for denaturation. The mixture was then incubated at 60°C for 30 s to allow the two fragments to anneal to each other. Hybridization was mediated by overlapping complementary sequences at

the 5' ends of the oligonucleotides RSV/SeV-F-S and RSV/SeV-F-AS, respectively. A complete double-stranded DNA hybrid was obtained after incubating the mixture with *Pfu* polymerase at 72°C for 4 min. The chimeric F gene (F_{ch}MC) was subsequently amplified with the primers hF-S and SeV-F-AS and cloned into the EcoRI site of pCDNA3.1 (Invitrogen). The same technique was used to generate the F_{ch}C chimera where the cytoplasmic domain of the RSV-F protein was replaced with the corresponding domain of the SeV-F protein. The RSV-F protein ectodomain and the transmembrane domain (amino acids 1 to 550) were amplified from pTM1-hF using primers hF-S and Tail-AS. The region of the SeV-F gene encoding the cytoplasmic domain of the protein (amino acids 524 to 565) was amplified from pCDNA3.1-SeV-F with the oligonucleotides Tail-S and SeV-F-AS. All other steps for generation and cloning of the hybrid gene were performed as described above.

Generation of recombinant SeV. For generation of SeV(RSV-F), SeV(F_{ch}C), and SeV(F_{ch}MC), authentic or chimeric RSV-F genes were inserted into an additional transcription unit located between the P and M genes of a modified SeV genome using MluI and BssHII restriction sites (5). SeV- Δ F(F_{ch}C), SeV- Δ F(F_{ch}MC), SeV- Δ F Δ HN(F_{ch}C/GFP), and SeV- Δ F Δ HN(F_{ch}MC/GFP) genomes were constructed using a genomic cDNA plasmid where the original F and HN open reading frames had been replaced with unique restriction sites (Sall instead of F and XhoI instead of HN), each flanked by the original transcription start and stop signals (4). The F_{ch}C and F_{ch}MC chimeras (see previous section) were amplified by PCR using oligonucleotides hF-S2 and SeV-F-AS2 and inserted into the Sall and XhoI sites of the plasmid pTM1. The authenticity of the chimeric genes was verified by DNA sequencing, and the chimeric genes were then inserted as Sall-XhoI fragments into the compatible Sall site (F position) of the modified SeV genome. Subsequently, the XhoI site (HN position) in the modified genome was filled with the open reading frame of either the enhanced green fluorescent protein (GFP) gene or the HN gene. All resulting recombinant SeV genomes were designed to follow the "rule of six" (8). Recombinant viruses were recovered from transfected HeLa cells according to the previously described procedure (24). To facilitate initial virus rescue, expression plasmids encoding the autologous SeV-F and HN proteins were included in the plasmid mixture used for transfection. Recovered virus was propagated on Vero cells. Virus stocks were prepared from the fifth and sixth passages and used for all following experiments.

Replication kinetics and virus titration. Confluent Vero cell monolayers in six-well plates were inoculated with virus at a multiplicity of infection (MOI) of 0.05. Three wells were infected in parallel with each virus. After adsorption (3 h at 37°C), the inoculum was removed and the cells were washed three times before 2.5 ml of medium (without FBS) was added. In some experiments, acetylated trypsin (Sigma) was added to the medium (1.0 μ g/ml). At 0, 24, 48, and 72 h postinfection, 500- μ l aliquots were taken and replaced with the same volume of fresh medium. The aliquots were flash-frozen in liquid nitrogen and stored at -80°C. Viruses were titrated in duplicate on Vero cells grown in 96-well microtiter plates. The cells were inoculated with 10-fold dilutions of virus (40 μ l/well) for 2 h at 37°C and overlaid with medium containing 2% FBS and 0.9% methylcellulose (Sigma). At day 2 postinfection, the medium was removed and the cells washed twice with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde for 20 min at room temperature. Excess paraformaldehyde was quenched with 0.1 M glycine in PBS for 5 min. The cells were incubated for 90 min at room temperature with anti-PIV1 (1:500 in PBS), washed three times with PBS, and incubated for 1 h at room temperature with a horseradish peroxidase-linked rabbit antiserum directed to goat immunoglobulin (1:500 in PBS; Dako). Following three washing steps, the cells were incubated for 15 min with AEC peroxidase substrate (1.7 mM 3-amino-9-ethylcarbazole, 0.1% H₂O₂ in 50 mM sodium acetate buffer, pH 5.0). Immunostained cells were counted under an inverse light microscope. Titers of infectious recombinant SeV expressing GFP were determined on Vero cells by end point dilution and counting of GFP-expressing cells under a fluorescence microscope.

Plaque reduction assay. Serial dilutions of polyclonal anti-SeV serum or anti-RSV-F monoclonal antibody mixture (Mab13, Mab19, RSV3216) were prepared in DMEM, and 10 μ l of each dilution was incubated with 100 μ l of virus (2 $\times$ 10⁴ PFU/ml) for 30 min at 4°C and then added to confluent Vero cells in 96-well microtiter plates (10 μ l/well, three parallels). Following incubation for 90 min at 37°C, 200 μ l of DMEM containing 0.9% methylcellulose was added to each well. The cells were stained 48 h postinfection as described above. The antibody concentration that produced 50% focus reduction was calculated.

Neuraminidase treatment of cells. BHK-21 cells were seeded into 24-well plates (150,000 cells/well). The following day, the cells were incubated for 60 min at 37°C with 250 μ l of EMEM containing 40 mU of affinity-purified neuraminidase from *Clostridium perfringens* (N-2133; Sigma) (NA⁺ cells). Control cells were left untreated (NA⁻ cells). The cells were washed twice and infected with

recombinant SeV (MOI of 10) for 60 min at room temperature, washed three times, and incubated for 20 h at 37°C with EMEM containing 2% FBS and 0.9% methylcellulose. The cells were stained with FITC-conjugated anti-PIV1 (1:200) for 60 min at 4°C, suspended in PBS, and analyzed by flow cytometry. Infection by SeV- Δ F Δ HN(F_{ch}C/GFP) and SeV- Δ F Δ HN(F_{ch}MC/GFP) was directly monitored taking advantage of the GFP reporter.

Western blot analysis of SeV particles. Confluent monolayers of Vero cells grown in 75-cm² flasks were inoculated with recombinant SeV (MOI of 0.05) for 3 h at 37°C. Thereafter, the cells were washed and maintained for 72 h at 37°C. For SeV(DsRed), SeV(RSV-F), SeV(F_{ch}C), and SeV(F_{ch}MC), medium lacking FBS and supplemented with 1 μ g of acetylated trypsin/ml was used. For all other recombinant SeVs, the cells were incubated with medium containing 2% FBS. The cell culture supernatants were harvested and subjected to low-speed centrifugation (2,000 $\times$ g, 15 min, 4°C) to remove cell debris. An aliquot of each supernatant was taken and titrated by plaque assay as described above. Viruses in the remaining supernatant (about 20 ml) were pelleted through a 25% sucrose cushion by ultracentrifugation (105,000 $\times$ g, 60 min, 4°C) and dissolved in 400 μ l of SDS sample buffer. The volume was adjusted corresponding to the calculated virus titer. Aliquots (5 μ l, corresponding to 5 $\times$ 10⁶ PFU) of the samples were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels under nonreducing conditions and transferred to nitrocellulose by the semidry blotting technique. The nitrocellulose membranes were incubated for 60 min at 4°C with either anti-SeV (1:10,000), anti-hPIV1 (1:1,000), anti-SeV-Ftail (1:500), or anti-RSV-F (1:1,000 each). The blots were washed and subsequently incubated with biotinylated secondary antibodies that were specific for the immunoglobulin G (IgG) fraction of the respective species (1:1,000; Sigma). Finally, the blots were incubated for 60 min with a streptavidin-peroxidase complex (1:1,000; Amersham Biosciences) and the antigens were visualized by enhanced chemiluminescence (Roche Diagnostics).

Immunofluorescence analysis. Vero cells grown on 12-mm-diameter coverslips were infected with recombinant SeVs at an MOI of 10 PFU/cell for 60 min at 37°C, washed once, and maintained in medium with 5% FBS. Twenty-four hours postinfection, the cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature and subsequently incubated for 5 min at room temperature with PBS containing 0.1 M glycine. The cells were stained by incubation with a 1:2,000 dilution of anti-SeV serum followed by incubation in the dark with a 1:500 dilution of FITC-conjugated goat anti-rabbit IgG serum (Sigma). Both incubations were performed at room temperature for 60 min. For detection of RSV-F antigen, the cells were subsequently incubated with anti-RSV-F monoclonal antibody (1:200; Serotec) and sheep Cy3-conjugated anti-mouse IgG serum (1:500; Sigma). Conventional epifluorescence was performed with a Zeiss Axioplan 2 microscope.

Electron microscopy. Immunoelectron microscopic analysis of virus particles was performed as described previously (20, 21), with small modifications. Briefly, a drop of virus suspension in DMEM containing 3% paraformaldehyde was deposited on a Formvar carbon-coated nickel grid for 1 min. Excess fluid was absorbed with filter paper, and the grids were floated for 10 min on a drop of PBS containing 2% bovine serum albumin and 0.2% Tween 20. Indirect immunostaining was performed by incubation of the samples for 60 min either with a mouse monoclonal anti-RSV-F antibody (Mab9C5; 1:50) or with a goat polyclonal anti-PIV1 antibody (1:200) in PBS containing 2% bovine serum albumin and 0.2% Tween 20. The bound antibodies were detected with a donkey anti-mouse IgG antibody conjugated with 12-nm-diameter gold particles (Dianova) and with a rabbit anti-goat IgG antibody conjugated with 10-nm-diameter gold particles (BBInternational), respectively. The samples were washed with PBS, negatively stained with 2% phosphotungstic acid, and examined with a Zeiss 109 electron microscope. Micrographs were scanned using the Leafscan 4S software, and the surface area of the virus particles was determined using the Raytest TINA software. The number of gold particles bound to the virions was calculated from 10 to 20 micrographs at a primary magnification of $\times$ 50,000. The labeling density (mean $\pm$ standard deviation) was expressed as the number of gold particles per 10,000 nm² of virus particle area. Differences in labeling between different virus specimens were statistically evaluated by Student's *t* test.

RESULTS

Generation of recombinant SeV encoding native or chimeric RSV-F. The modular organization of negative-strand RNA virus genomes allows both deletion and insertion of genes. To study the functions of the HRSV fusion protein (RSV-F) in the context of SeV infection, we generated a series of modified

SeV genomes based on the Fushimi strain (Fig. 1). First, an additional transcription unit was assembled into the intergenic region between the P and M genes. Into this transcription unit, four different transgenes were inserted taking into account the "rule of six" (8). We obtained the following virus genomes: (i) SeV(RSV-F) with an unmodified version of the RSV-F open reading frame, (ii) SeV(F_{ch}C) with a chimeric gene encoding the ectodomain and transmembrane domain of RSV-F and the cytoplasmic domain (C) of the SeV fusion protein (SeV-F), (iii) SeV(F_{ch}MC) with a chimeric F gene encoding the RSV-F ectodomain and the SeV-F transmembrane (M) and cytoplasmic domains, and (iv) SeV(DsRed) containing the cDNA of the red fluorescent protein DsRed originating from the marine organism *Discosoma* sp. As DsRed is unrelated to RSV-F and shows no fusion activity, SeV(DsRed) was regarded as a control construct.

The insertion of native and chimeric RSV-F genes into the SeV genome was expected to result in virus particles with two fusion proteins provided the heterologous glycoproteins would be incorporated into the viral envelope (see below). In order to determine whether RSV-F can functionally substitute for SeV-F, we constructed additional recombinant SeV genomes by replacing the SeV-F gene with either of the two chimeric F proteins, resulting in SeV- Δ F(F_{ch}C) and SeV- Δ F(F_{ch}MC) (Fig. 1B). It should be noted that these genomes do not contain an additional transcription unit and are therefore shorter than the modified SeV genomes mentioned above. However, they correspond approximately to the length of an unmodified SeV genome. Since RSV-F has been reported to have receptor-binding activity (12), we wanted to know whether the chimeric F proteins would also substitute for the HN glycoprotein of SeV. To address this question, the HN gene in SeV- Δ F(F_{ch}C) and SeV- Δ F(F_{ch}MC) was replaced with the enhanced GFP, resulting in the genomes SeV- Δ F Δ HN(F_{ch}C/GFP) and SeV- Δ F Δ HN(F_{ch}MC/GFP), respectively. Using a previously published procedure (24), all recombinant SeVs were successfully recovered from the cell culture supernatant of transfected HeLa cells. To monitor RSV-F expression in infected BHK-21 cells, an immunofluorescence analysis using anti-SeV and anti-RSV-F antibodies was performed (Fig. 2). RSV-F was detected at the cell surface of SeV(RSV-F), SeV(F_{ch}C), SeV(F_{ch}MC), and SeV- Δ F(F_{ch}MC) but not in cells that had been infected with SeV(DsRed). Similar results were obtained with SeV- Δ F(F_{ch}C), SeV- Δ F Δ HN(F_{ch}C/GFP), and SeV- Δ F Δ HN(F_{ch}MC/GFP) (data not shown).

Deletion of SeV-F allows chimeric RSV-F protein to be efficiently incorporated into SeV virions. An important aspect of recombinant enveloped viruses that encode heterologous viral glycoproteins is the possibility that these glycoproteins might be incorporated into the virus envelope. To address this question, we purified the recombinant SeV particles from the supernatant of infected Vero cells by ultracentrifugation through a 25% sucrose cushion and analyzed them by immunogold electron microscopy using two different antibodies. The polyclonal anti-PIV1 serum was characterized by Western blot analysis and was found to predominantly react with SeV-F and SeV-HN (for comparison, see Fig. 5c). Figure 3 shows that this antibody strongly reacted with SeV(DsRed), SeV(RSV-F), SeV(F_{ch}MC), and SeV- Δ F(F_{ch}MC) particles, all containing at least one SeV glycoprotein. In contrast, SeV- Δ F Δ HN(F_{ch}MC/

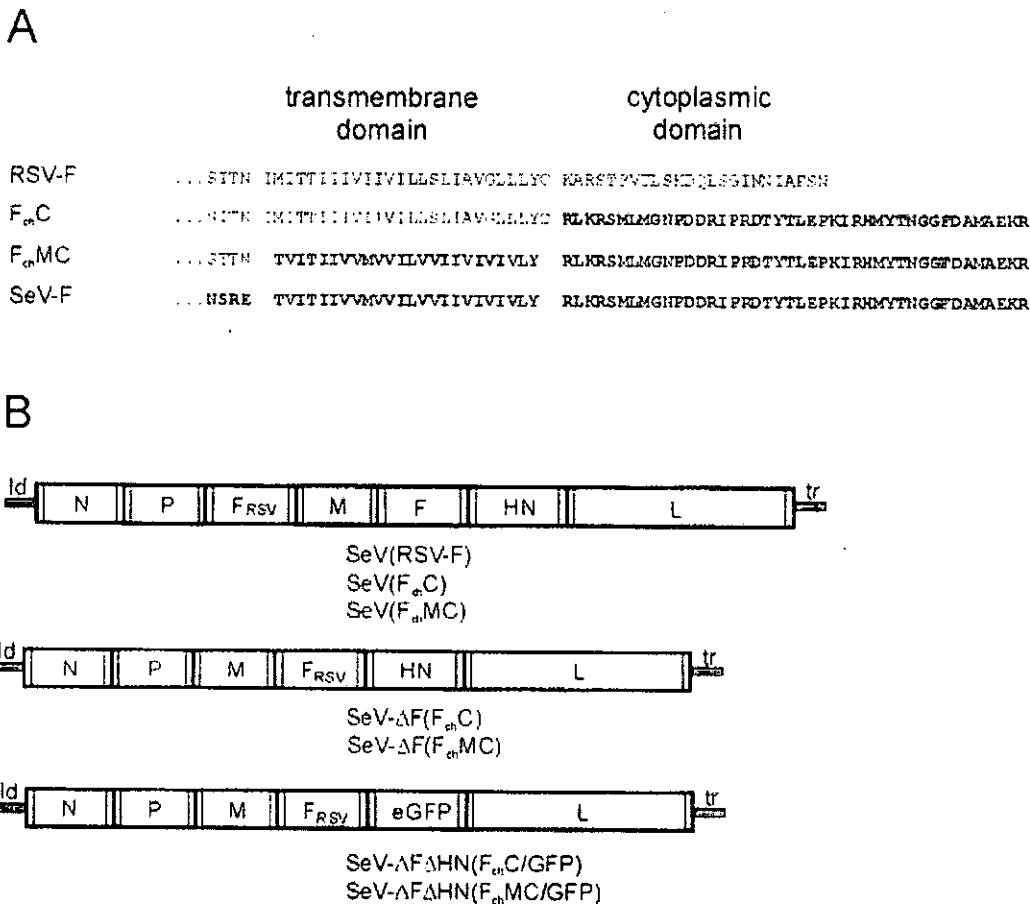


FIG. 1. Generation of recombinant SeV using authentic and chimeric RSV-F genes. (A) The predicted transmembrane and cytoplasmic domains of the fusion proteins from HRSV (Long strain) and SeV (Fushimi strain) and the chimeric fusion proteins F_{ch}C and F_{ch}MC are shown. Sequences derived from SeV-F are in bold type. (B) Schematic representation of the recombinant SeV genomes. The designations of the recombinant viruses that are based on the modified genomes are shown below the schemes.

GFP) lacking both SeV-F and SeV-HN was rarely recognized by anti-PIV1. The gold labeling of some virions is supposed to be due to a weak reaction of the antibody with the SeV nucleoprotein. HRSV was not stained with anti-PIV1 at all. Monoclonal antibody Mab9C5 recognizes an epitope of the RSV-F ectodomain (data not shown). It showed a strong re-

action with HRSV virions, while staining of SeV(DsRed) particles was not observed. SeV(RSV-F) was rarely labeled by Mab9C5, and only single gold particles were found bound to some virions. Increased labeling was observed with SeV (F_{ch}MC), which expresses a chimeric RSV-F protein, indicating that the SeV-F derived C-terminal sequences of F_{ch}MC

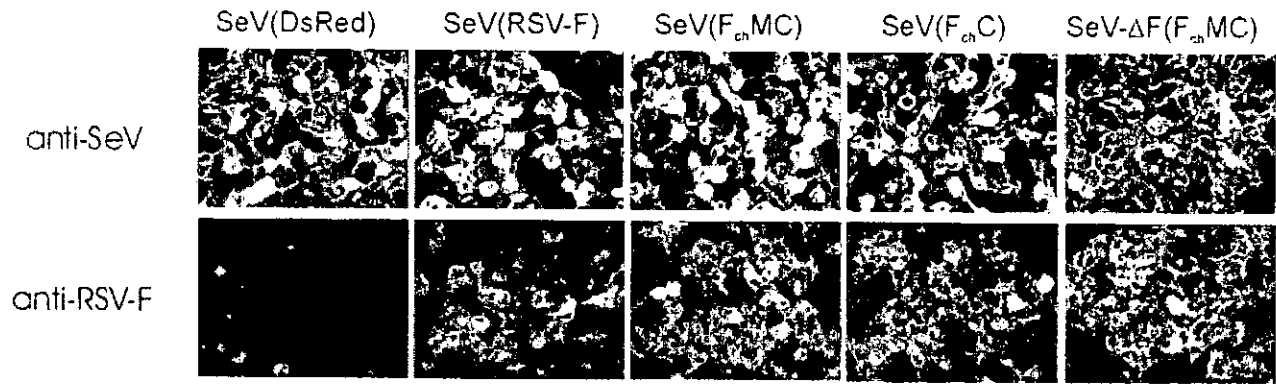


FIG. 2. Indirect immunofluorescence analysis of infected BHK-21 cells. Cells were infected with the indicated recombinant SeV (MOI = 10) and fixed with paraformaldehyde 20 h postinfection. The cells were stained either with a rabbit anti-SeV serum or with a mouse anti-RSV-F antibody as indicated on the left. FITC-conjugated antibodies directed to rabbit IgG or mouse IgG were used as secondary antibodies.

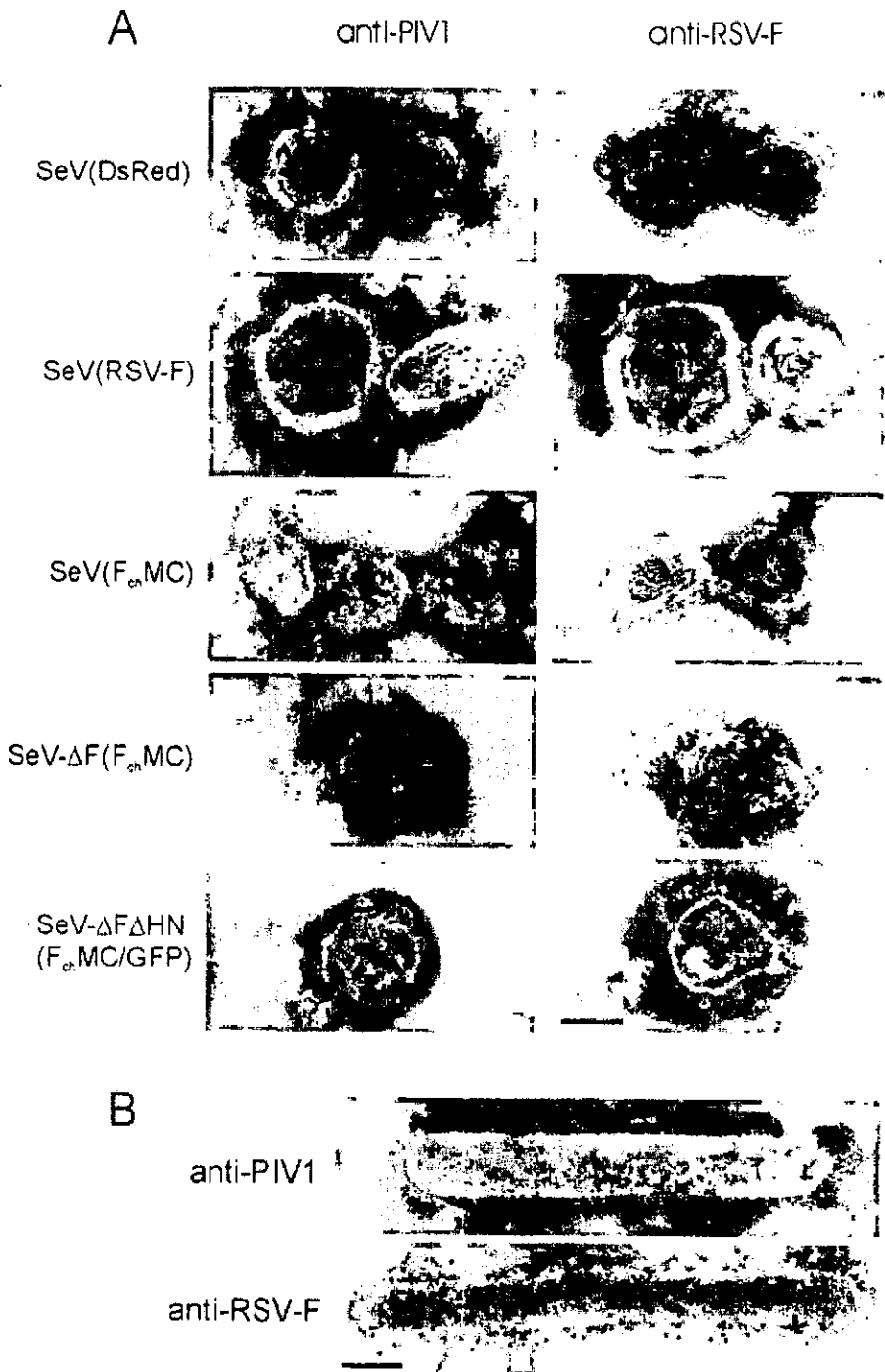


FIG. 3. Immunogold labeling of viruses. The indicated viruses were concentrated from the culture supernatant of infected Vero cells and fixed with 3% paraformaldehyde. The viruses were labeled with the indicated primary antibodies and gold-labeled secondary antibodies as described in Materials and Methods. (A) Labeling of recombinant SeV. (B) Staining of HRSV (Long strain). Bars, 100 nm.

contributed to glycoprotein incorporation. In the absence of SeV-F, the chimeric glycoprotein was most efficiently incorporated, as indicated by the very strong labeling of SeV-ΔF(F_{ch}MC) and SeV-ΔFΔHN(F_{ch}MC/GFP). Figure 4 shows a quantification of the immunogold labeling analysis. A significant difference between SeV-ΔF(F_{ch}MC) and SeV-ΔFΔHN(F_{ch}MC/GFP) was not observed, indicating that the deletion of

SeV-HN did not further improve the incorporation of the chimeric fusion proteins.

Western blot analysis was performed to confirm the results obtained by the immunogold labeling approach. After the concentrated virions had been dissolved in SDS sample buffer, the viral proteins were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, transferred to ni-

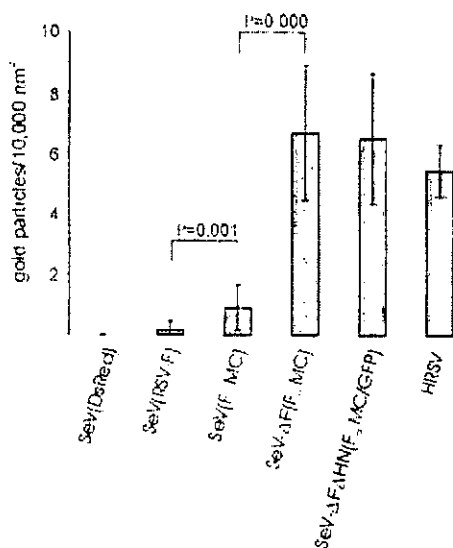


FIG. 4. Quantification of RSV-F antigen in HRSV and recombinant SeV particles. Following staining with a monoclonal anti-RSV-F antibody, the number of gold particles bound to the virions was calculated from 10 to 20 micrographs. The labeling density (mean $\pm$ standard deviation) was expressed as the number of gold particles per 10,000 nm² of virus particle area. Mean values and standard deviations are shown. Significant differences in the labeling were statistically evaluated by Student's *t* test.

trocellulose membranes, and probed with several antibodies (Fig. 5). When a mixture of monoclonal antibodies directed to the RSV-F ectodomain was used (panels a and a'), a small amount of antigen was detected in SeV(F_{ch}C) and SeV(F_{ch}MC) but not in SeV(DsRed) and SeV(RSV-F) particles. The chimeric glycoproteins F_{ch}C and F_{ch}MC were incorporated into the recombinant particles with similar efficiency, suggesting that the SeV-F transmembrane domain in SeV(F_{ch}MC) exerts no promoting effect on glycoprotein uptake (panel a', compare lane 3 with lane 4; panel a, compare lane 5 with lane 6 and lane 7 with lane 8). In agreement with the immunogold labeling presented above, incorporation of either chimeric RSV-F glycoprotein was dramatically improved when the recombinant viruses lacked SeV-F (panel a, lanes 5 to 8). This suggests that in SeV(F_{ch}C) and SeV(F_{ch}MC), SeV-F competes with the chimeric RSV-F proteins for incorporation into the virus envelope. The lower signal obtained with chimeric RSV-F protein in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) (panel a, lanes 5 and 6) compared to the stronger signals detected with SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) (lanes 7 and 8) is probably due to the differential capacity of the antigens to renature on the nitrocellulose membrane following SDS-polyacrylamide gel electrophoresis. In contrast to chimeric RSV-F of SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP), chimeric RSV-F in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) is desialylated by the action of SeV-HN. The lack of sialic acids probably makes renaturation of the glycoproteins more difficult and hampers recognition by the monoclonal antibodies that act in a conformation-dependent manner. In SeV(F_{ch}C) (lane 5) and SeV-ΔFΔHN(F_{ch}C/GFP) (lane 7), an additional band of approximately 140 kDa was detected that probably represents a dimeric form of chimeric RSV-F.

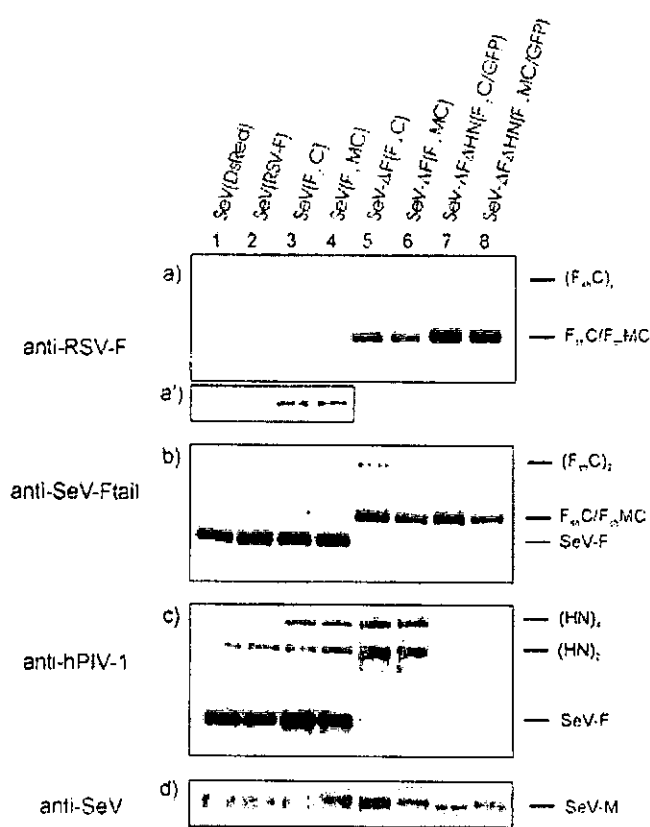


FIG. 5. Incorporation of chimeric RSV-F into SeV particles. Vero cells were infected with the indicated viruses (0.05 PFU/cell) and incubated at 37°C with serum-deficient medium. Virions released into the cell culture supernatant were pelleted through a sucrose cushion by ultracentrifugation, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blot assay using the indicated antibodies. The amount of sample applied to each gel was normalized to infectious virus titers. (Panel a') To allow detection of minor bands, the amount of the samples applied to the gel was scaled up (threefold) and the chemiluminescence signal was collected over a longer time interval. The virus antigens detected are indicated on the right.

The band was not observed with SeV(F_{ch}MC) (lane 6) and SeV-ΔFΔHN(F_{ch}MC/GFP) (lane 8).

As the use of different antibodies for detection of SeV-F on the one hand and the chimeric F proteins on the other hand did not allow us to directly compare the uptake of these glycoproteins, we employed a monospecific antibody that was directed to a linear epitope of the SeV-F cytoplasmic domain (Fig. 5b). In SeV(DsRed), SeV(RSV-F), SeV(F_{ch}C), and SeV(F_{ch}MC) particles (lanes 1 to 4), this antibody recognized a band of 65 kDa representing SeV-F, which was absent in SeV-ΔF(F_{ch}C), SeV-ΔF(F_{ch}MC), SeV-ΔFΔHN(F_{ch}C/GFP), and SeV-ΔFΔHN(F_{ch}MC/GFP) (lanes 5 to 8). In addition to SeV-F, a band of 75 kDa representing chimeric F protein was detected in SeV(F_{ch}C) and SeV(F_{ch}MC) particles (lanes 3 and 4). Compared to SeV-F, these glycoproteins were less efficiently incorporated into the SeV envelope, suggesting that also determinants in the SeV-F ectodomain must exist that are involved in glycoprotein uptake. Like the antibody directed to the RSV-F ectodomain (panel a), the anti-SeV-Ftail antibody strongly reacted with the chimeric F proteins incorporated into SeV-ΔF(F_{ch}C), SeV-ΔF(F_{ch}MC), SeV-ΔFΔHN(F_{ch}C/GFP),

and SeV- $\Delta F\Delta HN(F_{ch}MC/GFP)$ (panel b, lanes 5 to 8), while only a faint band was detectable in SeV($F_{ch}C$) and SeV($F_{ch}MC$) (lanes 3 and 4). This indicates that incorporation of the chimeric glycoproteins is significantly enhanced in the absence of SeV-F. As already observed with anti-RSV-F (panel a), a dimeric form of the chimeric $F_{ch}C$ glycoprotein was also detected with anti-SeV-Ftail (panel b, lanes 5 and 7).

A polyclonal goat antibody directed to human parainfluenza virus type I (hPIV1) was found to primarily react in Western blot assays with the two SeV glycoproteins, SeV-F in its monomeric form with an apparent molecular mass of about 65 kDa and SeV-HN in its dimeric and tetrameric forms with relative molecular masses of about 140 and 280 kDa, respectively. HN was found to be absent in SeV- $\Delta F\Delta HN(F_{ch}C/GFP)$ and SeV- $\Delta F\Delta HN(F_{ch}MC/GFP)$ particles (Fig. 5c, lanes 7 and 8). These two viruses, as well as SeV- $\Delta F(F_{ch}C)$ and SeV- $\Delta F(F_{ch}MC)$, were also proven in this way to contain no SeV-F antigen (panel c, lanes 5 to 8). A very faint band comigrating with SeV-F represents the SeV nucleoprotein, which is also recognized to some extent by the anti-hPIV1 antibody (panel c, lanes 5 to 8). The samples loaded on the gels were normalized to equal infectious titers (approximately 5×10^6 PFU/lane). As a control for the amount of virus particles applied, the matrix protein of SeV was detected using a polyclonal rabbit antibody directed to purified SeV particles (panel d).

Chimeric RSV-F protein is functional and mediates trypsin-independent replication of SeV. Each of the recombinant viruses SeV(RSV-F), SeV($F_{ch}MC$), and SeV($F_{ch}C$) contains two genes that encode different fusion proteins. SeV-F is proteolytically activated by secreted trypsin-like enzymes. As many established cell lines do not produce such enzymes, SeV normally requires the addition of a protease to the medium to be infectious. In contrast, RSV-F is cleaved and activated intracellularly by the ubiquitous Golgi protease furin and hence does not require the addition of trypsin to be fusion active. As the chimeric F proteins contain the RSV-F ectodomain, they are also expected to act independently of trypsin. Thus, on the basis of trypsin requirement it should be possible to discriminate between virus infections mediated by one or the other fusion protein. To analyze the function of the heterologous fusion protein in the context of SeV infection, the kinetics of virus replication in the presence or absence of trypsin were studied. Vero cells were infected with the recombinant SeV using an MOI of 0.05 PFU/cell, and aliquots of cell culture supernatant were collected over a 3-day period at 24-h intervals. The virus titers were quantified by a plaque assay facilitated by immunological staining of SeV antigens. Figure 6A shows that SeV-DsRed and SeV-RSV-F replicated well if the medium was supplemented with acetylated trypsin (1 μ g/ml) whereas no significant replication was detected in the absence of trypsin, indicating that expression of native RSV-F does not lead to trypsin-independent replication of recombinant SeV. This finding agrees well with our observation that unmodified RSV-F is not incorporated into SeV particles (see above). Though chimeric fusion proteins were incorporated into SeV particles in addition to SeV-F (see above), analysis of SeV($F_{ch}MC$) and SeV($F_{ch}C$) revealed only very slow trypsin-independent replication (Fig. 6B), suggesting that the fraction of chimeric F protein in the viral envelope is probably too small to support replication. In the presence of trypsin, SeV-F was

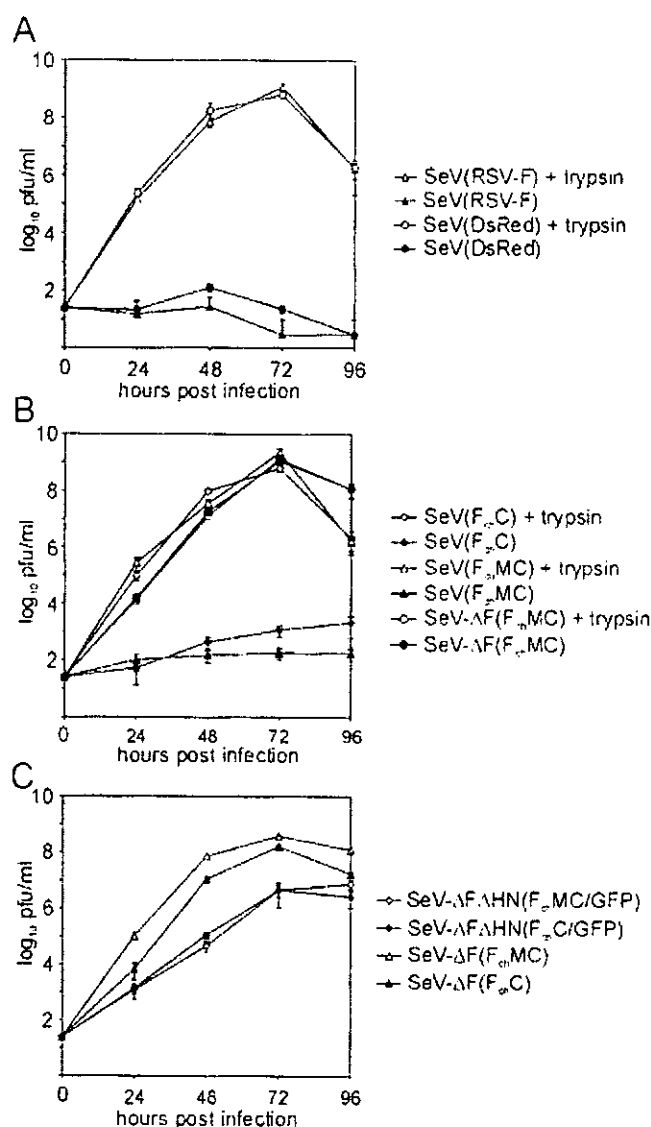


FIG. 6. Multistep replication of recombinant SeV in Vero cells. Triplicate cell monolayers in six-well dishes were infected with the indicated viruses at an MOI of 0.05 and incubated at 37°C with medium in either the absence or the presence of trypsin as indicated on the right-hand side of the figure. Aliquots were taken at the indicated times and titrated in duplicate by plaque assay on Vero cells. Each point shown represents the mean titer from three wells of infected cells. Standard deviations are indicated. For reasons of presentation, the figure has been divided into three parts. (A) Comparison of SeV(DsRed) with SeV(RSV-F); (B) comparison of SeV($F_{ch}C$) and SeV($F_{ch}MC$) with SeV- $\Delta F(F_{ch}MC)$; (C) comparison of SeV- $\Delta F(F_{ch}MC)$ with SeV- $\Delta F(F_{ch}C)$ and SeV- $\Delta F\Delta HN(F_{ch}MC/GFP)$ and SeV- $\Delta F\Delta HN(F_{ch}C/GFP)$.

activated and both viruses showed replication kinetics similar to SeV(DsRed) (compare Fig. 6B with A). In contrast to these viruses, addition of trypsin to the cell culture medium did not affect SeV- $\Delta F(F_{ch}MC)$ replication as this virus lacked the SeV-F gene. Nevertheless, this virus replicated as efficiently as trypsin-activated SeV($F_{ch}MC$) and SeV($F_{ch}C$). When replication of SeV- $\Delta F(F_{ch}MC)$ was compared with SeV- $\Delta F(F_{ch}C)$, it was found that SeV- $\Delta F(F_{ch}MC)$ replicated more efficiently, producing titers that were approximately 1 order of magnitude

TABLE 2. Neutralization of recombinant SeV

Virus	Neutralizing antibody titer ^a	
	Anti-SeV	Anti-RSVF
SeV(DsRed)	2,400	<200
SeV(RSV-F)	2,400	<200
SeV(F _{ch} C)	2,400	<200
SeV(F _{ch} MC)	2,400	<200
SeV-ΔF(F _{ch} C)	2,400	25,600
SeV-ΔF(F _{ch} MC)	2,400	12,800
SeV-ΔF-ΔHN (F _{ch} C/GFP)	<200	51,200
SeV-ΔF-ΔHN (F _{ch} MC/GFP)	<200	51,200
HRSV	<200	51,200

^a A plaque reduction assay was performed to determine the neutralizing titer of polyclonal anti-SeV serum or anti-RSV-F monoclonal antibody mixture. Following preincubation of virus with serial antibody dilutions, the mixture was inoculated with Vero cells and then overlaid with medium containing 0.9% methylcellulose. The cells were stained 48 h postinfection as described in Materials and Methods. The neutralization titers were determined as the reciprocal of the antibody dilution at which 50% focus reduction was observed.

higher than the titers produced by SeV-ΔF(F_{ch}C) (Fig. 6C). In general, the infectious virus titers reached a maximum at 72 h postinfection. From that time on, the titers decreased, probably due to the increasing cytopathic effect.

SeV with chimeric RSV-F protein as the only envelope glycoprotein is infectious. The results presented above demonstrated that a modified RSV-F protein is capable to functionally replace the fusion protein of SeV. Because recent reports have shown that RSV mutants or recombinant RSV lacking attachment protein G are infectious and are able to replicate in cell culture (18, 42), we asked whether chimeric RSV-F will also substitute for SeV attachment protein HN. SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) were used to test this hypothesis. Trypsin-independent replication of these viruses in the presence of FBS showed that the amount of chimeric fusion protein incorporated into the virions is sufficient to support both functions, receptor binding and fusion (Fig. 6C). In this viral background, we did not detect any significant differences between the replication of SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP). However, replication of these viruses resulted in significantly lower virus titers compared to SeV-ΔF(F_{ch}MC), indicating that the HN glycoprotein in the latter viruses supports virus replication.

Neutralizing antibodies differentially affect recombinant SeV. Use of neutralizing antibodies directed to RSV-F provides an additional approach to estimate the contribution of the heterologous fusion protein to SeV replication. Vice versa, recombinant SeV infection dependence on or independence from SeV glycoproteins can be determined using neutralizing antibodies that bind to the SeV envelope glycoproteins. We used a plaque reduction assay to study the effect of serial antibody dilutions on the infection of Vero cells by the recombinant SeVs. The results are summarized in Table 2. Infection with SeV(DsRed) was inhibited by a polyclonal anti-SeV serum, while an antibody mixture containing three different monoclonal antibodies directed against RSV-F had no effect on this virus. The neutralizing potential of anti-RSV-F was demonstrated with HRSV, which was completely inhibited even at very high dilutions of this antibody mixture. As expected, anti-SeV serum showed no inhibitory activity toward

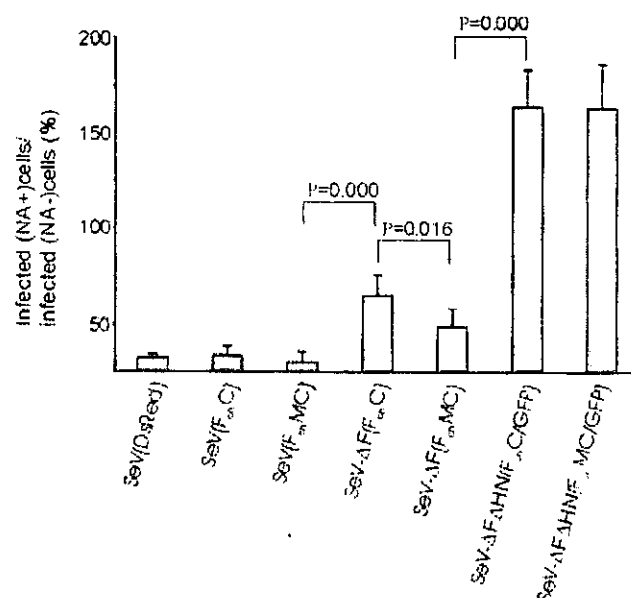


FIG. 7. Importance of sialic acids for infection with recombinant SeV. BHK-21 cells were treated with *C. perfringens* neuraminidase (NA⁺ cells) or left untreated (NA⁻ cells) prior to inoculation with the indicated viruses. Eighteen hours postinfection, the cells were stained with FITC-conjugated anti-PIV1, suspended, and analyzed by flow cytometry (5,000 cells of each sample). Infection with SeV-ΔFΔHN(F_{ch}MC/GFP) and SeV-ΔFΔHN(F_{ch}C/GFP) was monitored by taking advantage of the GFP reporter. The infection rate of (NA⁻) cells was 10% to 20%. The ratio of infected (NA⁺) cells to infected (NA⁻) cells was calculated. Mean values and standard deviations of three to four experiments are shown.

HRSV. Recombinant viruses SeV(RSV-F), SeV(F_{ch}MC), and SeV(F_{ch}C) showed behavior similar to that of SeV(DsRed); i.e., anti-RSV-F had no apparent inhibitory effect. With recombinant SeV lacking both SeV-F and SeV-HN, a phenotype similar to that of HRSV was observed: SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) were neutralized by anti-RSV-F but not by anti-SeV. An interesting phenotype was found with SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) since these recombinants were sensitive to both anti-RSV-F and anti-SeV. A possible explanation for this phenotype is that the polyclonal antibodies bound to HN in the envelope and thereby also blocked RSV-F function by steric hindrance.

SeV-HN dominates over the receptor-binding properties of chimeric RSV-F. Our data provided evidence that RSV-F acts in an autonomous way, mediating attachment and fusion of a heterologous virus in the absence of any other viral glycoprotein. On the other hand, our results also indicate that if HN is present it contributes to replication as the recombinants SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) proliferated much more efficiently than SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) (Fig. 6C). To further assess the role of HN in these hybrid viruses, we studied whether removal of the receptor determinant for HN from the cell surface affects infection and therefore treated BHK-21 cells with bacterial neuraminidase prior to infection with recombinant SeV. Twenty hours later, the cells were analyzed for SeV antigen or GFP expression by flow cytometry (Fig. 7). It turned out that neuraminidase treatment reduced infection with SeV(DsRed), SeV(F-

$\text{SeV}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV}(\text{F}_{\text{ch}}\text{MC})$ by more than 90%. On the other hand, the recombinants $\text{SeV-}\Delta\text{F}\Delta\text{HN}(\text{F}_{\text{ch}}\text{C}/\text{GFP})$ and $\text{SeV-}\Delta\text{F}\Delta\text{HN}(\text{F}_{\text{ch}}\text{MC}/\text{GFP})$, which lacked HN, did not require the presence of sialic acids for infection. Rather, an enhanced infection rate of about 140% was detected following neuraminidase treatment, indicating that sialic acids might have a masking effect on the recognition of cellular receptors by RSV-F. In striking contrast to the latter viruses, removal of sialic acids from cell surface glycoconjugates reduced infection with $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ by 60% and 76%, respectively. Taking into account that about 10% of the remaining receptor-binding activity was still due to HN binding to sialic acids that were not removed, a significant portion of the sialic acid-independent receptor-binding activity was probably mediated by the chimeric RSV-F protein. However, it should be considered that the binding of RSV-F to cellular receptors would probably have been less pronounced if the cells had not been treated with neuraminidase. Together, these findings indicate that the HN protein of $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ contributed significantly to virus attachment.

DISCUSSION

Virus morphogenesis. Cytoplasmic domains of viral glycoproteins have been shown to play an important role in assembly and infectivity of many enveloped negative-strand RNA viruses (15, 32, 35, 36, 38, 47). It is generally believed that during the budding process these domains interact with cytoplasmic viral components, in particular with the matrix proteins, which provide a link between the viral envelope and the nucleocapsid. Interaction between the glycoprotein tail and the matrix protein has been shown for some viruses to be very specific with the consequence that heterologous glycoproteins are not incorporated into the virus particle (36). In some cases, replacement of the cytoplasmic domain with sequences from a homologous virus glycoprotein was found to result in significantly improved uptake of the foreign glycoprotein (25, 36, 48). On the other hand, there are also viruses that incorporate heterologous membrane proteins in a rather unspecific manner. For example, unmodified RSV-F was found to be incorporated into vesicular stomatitis virus with an efficiency similar to that of a chimeric RSV-F glycoprotein that contained the cytoplasmic domain from the vesicular stomatitis virus G glycoprotein (16). In the present work, we found that the RSV-F protein was not incorporated into SeV virions unless its cytoplasmic domain was replaced with the corresponding domain from SeV-F. In agreement with previous reports (13, 37), this result provides further evidence for a crucial role of the SeV-F cytoplasmic domain in SeV particle formation. We found that the presence of the homotypic transmembrane domain did not further improve the uptake of chimeric RSV-F. However, there must be determinants in the SeV-F ectodomain that are involved in glycoprotein uptake. Analysis of $\text{SeV}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV}(\text{F}_{\text{ch}}\text{MC})$ showed that chimeric RSV-F was incorporated into SeV virions with low efficiency in the presence of SeV-F, whereas its uptake was dramatically enhanced if SeV-F was deleted. Since SeV-F and $\text{F}_{\text{ch}}\text{MC}$ share the same cytoplasmic and transmembrane domains, we conclude that the ectodomains of the glycoproteins account for these differences. From the vesicular stomatitis virus glycoprotein, it is known that a

membrane-proximal region in the ectodomain contributes to assembly of this rhabdovirus (30). We cannot exclude the possibility that there is a similar domain present in SeV-F. Besides, it has to be considered that the homotypic parainfluenza virus glycoproteins F and HN are physically associated with each other (6, 14, 33, 39, 46). Therefore, it is possible that SeV-HN contributes to SeV-F uptake by interacting with its ectodomain whereas it does not bind to the unrelated RSV-F ectodomain. However, in the absence of SeV-F these possible recruitment mechanisms cannot compete with uptake of chimeric RSV-F.

Role of chimeric RSV-F in SeV infection. Chimeric RSV-F was shown in this study to mediate trypsin-independent replication of recombinant SeV. This finding indicates that modifications at the RSV-F C terminus are fully compatible with the functions of this glycoprotein. This is remarkable as many other fusion proteins have been found not to tolerate any modifications of their cytoplasmic domains like elongations, truncations, or heterologous sequences (2, 29, 34, 45). Chimeric RSV-F allowed the generation of infectious SeV lacking both SeV-F and SeV-HN, confirming the notion that RSV-F exhibits fusion and receptor-binding activities (18, 42). It is evident that the amount of incorporated chimeric fusion protein was sufficient to mediate attachment of these viruses to cell surface receptors. However, recombinant SeV combining chimeric RSV-F with HN produced significantly higher titers than SeV containing only chimeric RSV-F though both virus types were found to contain similar amounts of chimeric RSV-F. This observation suggests that HN acted as an additional attachment protein and contributed to virus entry and replication. In accordance with this view, infection by $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ for the most part required the presence of sialic acids on the host cell. Based on these findings, we speculate that in the context of $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ the function of chimeric RSV-F differs from its autonomous function in $\text{SeV-}\Delta\text{F}\Delta\text{HN}(\text{F}_{\text{ch}}\text{C}/\text{GFP})$ and $\text{SeV-}\Delta\text{F}\Delta\text{HN}(\text{F}_{\text{ch}}\text{MC}/\text{GFP})$. The reduced receptor-binding activity of chimeric RSV-F in $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ is supposed to be due to the presence of HN, which might lead to a different spatial distribution of the chimeric fusion protein in the viral envelope compared to the situation in $\text{SeV-}\Delta\text{F}\Delta\text{HN}$. In the absence of HN, the chimeric RSV-F molecules probably can more easily cooperate with each other, favoring a multivalent interaction with cellular receptors. In addition, we cannot exclude the possibility that the SeV-F transmembrane and/or cytoplasmic domain in chimeric RSV-F mediates an interaction with HN, which in turn might mask the RSV-F receptor-binding domain. Interestingly, compared to $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$, infection by $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ appeared to be more dependent on HN (Fig. 7), favoring the idea that the transmembrane anchor contributes to this binding. Such an intimate interaction between the two glycoproteins would also explain why anti-SeV polyclonal antiserum showed neutralizing activity toward $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$. When the antibodies bound to HN, they might have blocked the low receptor-binding activity of RSV-F due to steric hindrance. Altogether, it appears that HN performs an accessory function in $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{C})$ and $\text{SeV-}\Delta\text{F}(\text{F}_{\text{ch}}\text{MC})$ similar to that of the RSV-G protein, which supports RSV attachment but nevertheless is dispensable for virus replication in cell culture (31).

Assistance by HN appeared to be more pronounced with SeV-ΔF(F_{ch}MC) compared to SeV-ΔF(F_{ch}C), which replicated to significantly lower titers. This difference was only apparent in the presence of HN, as replication of SeV-ΔFΔHN(F_{ch}MC) and SeV-ΔFΔHN(F_{ch}C) did not significantly differ from each other. The higher fitness of SeV-ΔF(F_{ch}MC) compared to SeV-ΔF(F_{ch}C) is supposed to be related to the transmembrane region in F_{ch}MC. The presence of the homotypic transmembrane domain may provide enhanced virion stability by, for example, supporting interaction of chimeric RSV-F with the HN glycoprotein. However, this phenomenon requires further analysis to confirm this assumption.

In contrast to many other paramyxoviruses, RSV does not use sialic acid residues for attachment to the host cell surface and does not express a neuraminidase. Rather, RSV was found to bind to glycosaminoglycans (22) by virtue of both the attachment protein RSV-G and the fusion protein RSV-F (43). In this study, we found that neuraminidase treatment of target cells rendered them more susceptible to infection by SeV-ΔFΔHN(F_{ch}MC) and SeV-ΔFΔHN(F_{ch}C) while infection by all other SeV recombinants was negatively affected. A similar effect of neuraminidase treatment on RSV fusion and infection was reported by Barretto et al., though they found that infectivity was increased by treating virions with neuraminidase, but not by treating target cells (3). Though the reason for this discrepancy is not known at the moment, recombinant SeV containing chimeric RSV-F as the only glycoprotein represents a useful tool to further analyze the role of RSV-F in virus entry.

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