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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, 2000*a*). 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*. 2000*a*,*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	C177T	silent	T60A	C10S
G342A	M105I	C182G	A53G	T228A	silent	G140A	silent
A779G	K251R	T421C	silent	A366G	silent	C469A	T146N
C852T	silent	G435A	silent	C501A	silent	Δ471–473**	Δ147K
A923G	K299R	A549C	K175N	G546A	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	A1442T	H463L	C1629T	silent	T1045C	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		
A2238G	silent			G2052T	silent		
				A2106G	silent		

NP		NA		M		NS	
nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid	nucleotide	amino acid
T54C	silent	T43C	I8T	T58A	silent	T173C	silent
T282G	silent	G403A	R128K	C136T	silent	T176C	silent
G306A	silent	T860C	silent	C535T	silent	C299T	silent
T528C	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.* 1998*a*; Talon *et al.* 2000*a*). In addition, viruses with mutated NS1 proteins have an intermediate level of attenuation (Talon *et al.* 2000*b*). 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)

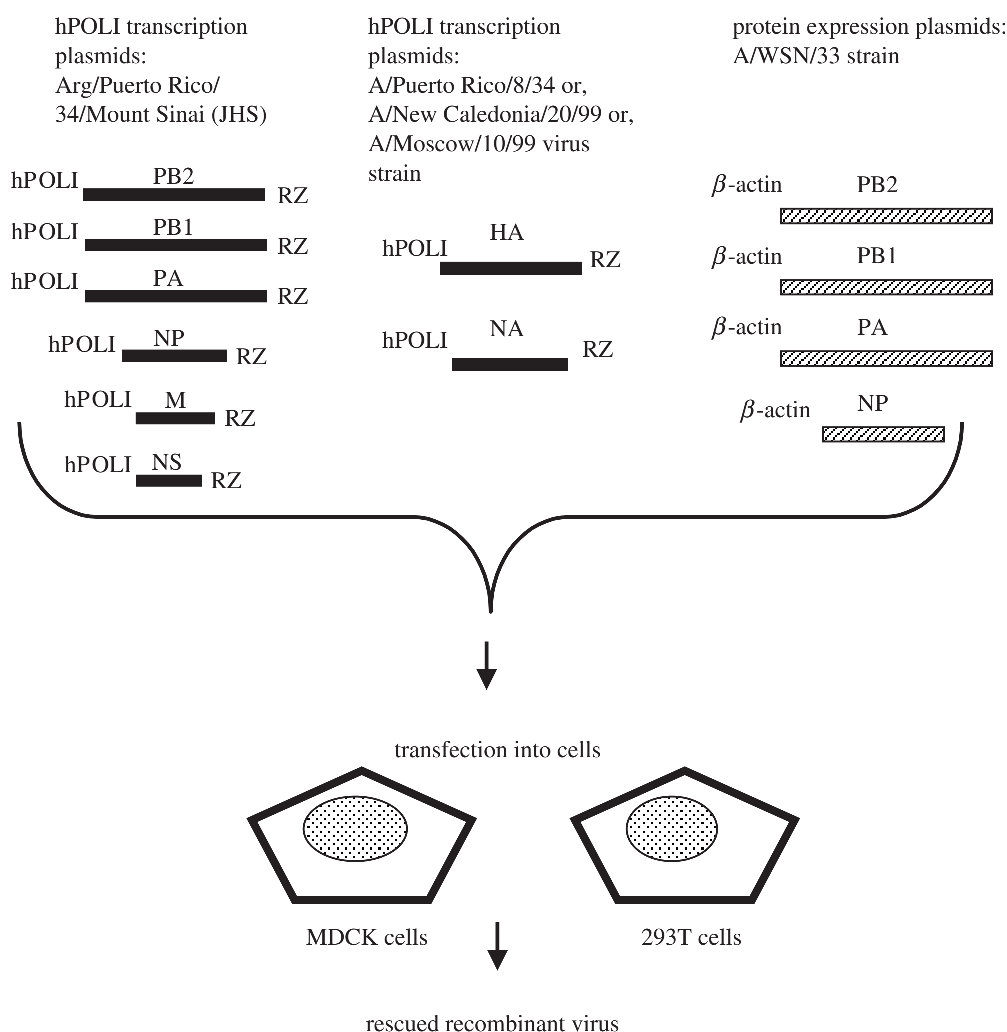

Figure 1. Strategy to rescue recombinant influenza virus containing six gene segments from the high-yield A/Puerto Rico/8/34 virus with HA and NA gene segments from either the A/Puerto Rico/8/34, the A/New Caledonia/20/99 or the A/Moscow/10/99 strain. MDCK and 293T cells were transfected with Lipofectamine 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/8/34/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 10^6 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

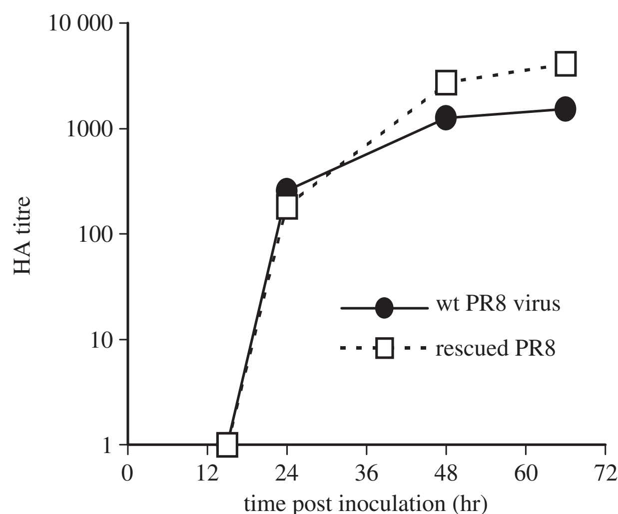

Figure 2. Growth curves of rescued influenza A/PR/8/34 virus and wt A/PR/8/34 virus in eggs. Eggs were inoculated with 5 PFU of either rescued A/PR/8/34 or wt A/PR/8/34 virus, in triplicate. At 15, 24, 48 and 66 h post-inoculation, 100 μl allantoic fluid were removed and the titre was measured by haemagglutination 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 10^8 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 500 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 10^8 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 3*a*. 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 3*a*. 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 PB1 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 PB1 gene segments amplified by the primers PB1,1245 and PB1,1836 followed by digestion with the restriction enzyme Sal I confirmed that the PB1 gene of the recombinant was derived from the A/PR/8/34 virus (figure 3*b*).

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

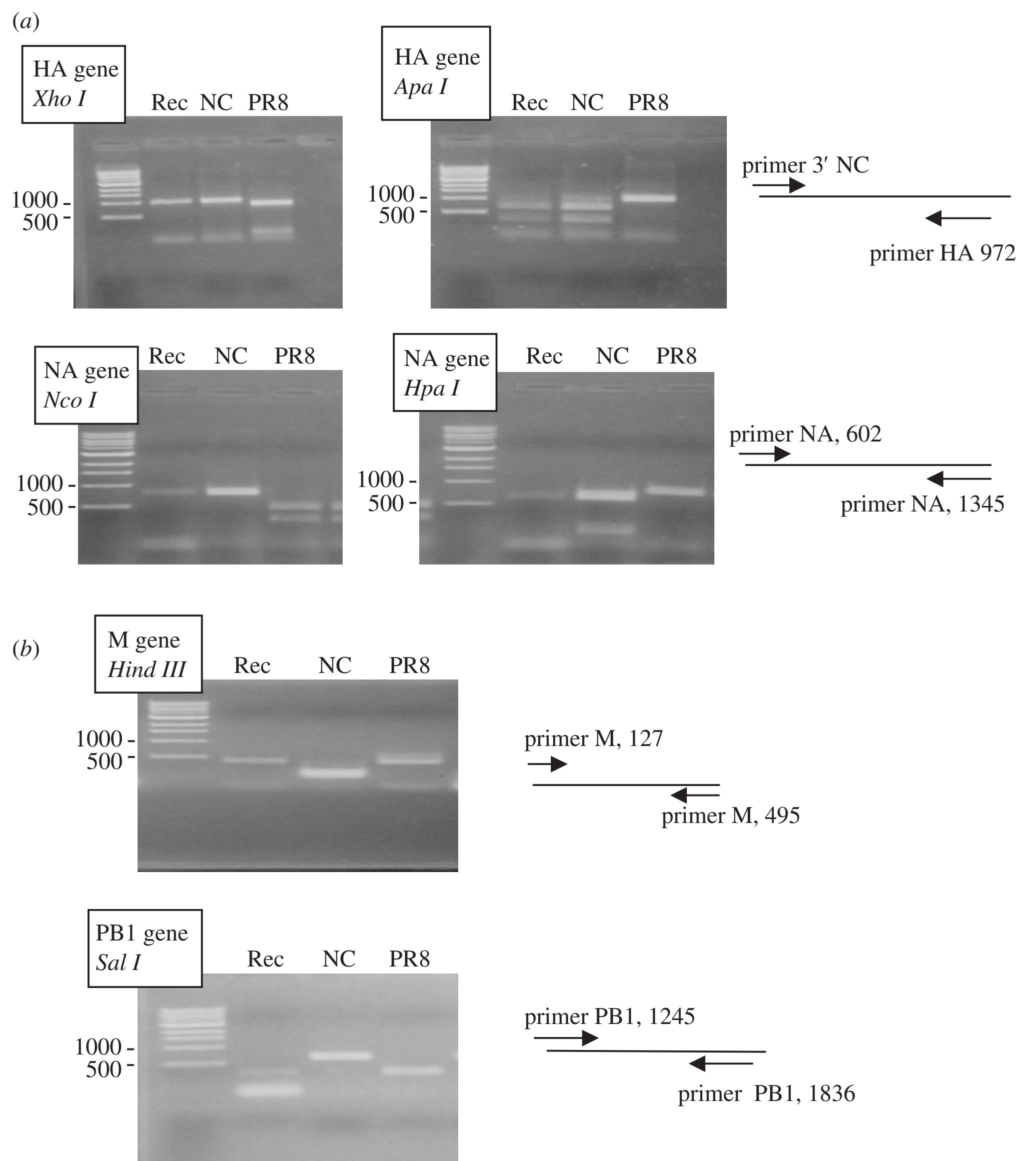

Figure 3. Characterization of the rescued PR8/New Caledonia/20/99 (6 + 2) recombinant virus. RT-PCR was performed on rescued PR8/New Caledonia/20/99 (6 + 2) recombinant (Rec) virus, the wt A/New Caledonia/20/99 (NC) virus and rescued A/Puerto Rico/8/34 (PR8) virus as described in the text (§ 3). The 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 4*a*). In addition, RT-PCR products with primers HA,229 and 5′ 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

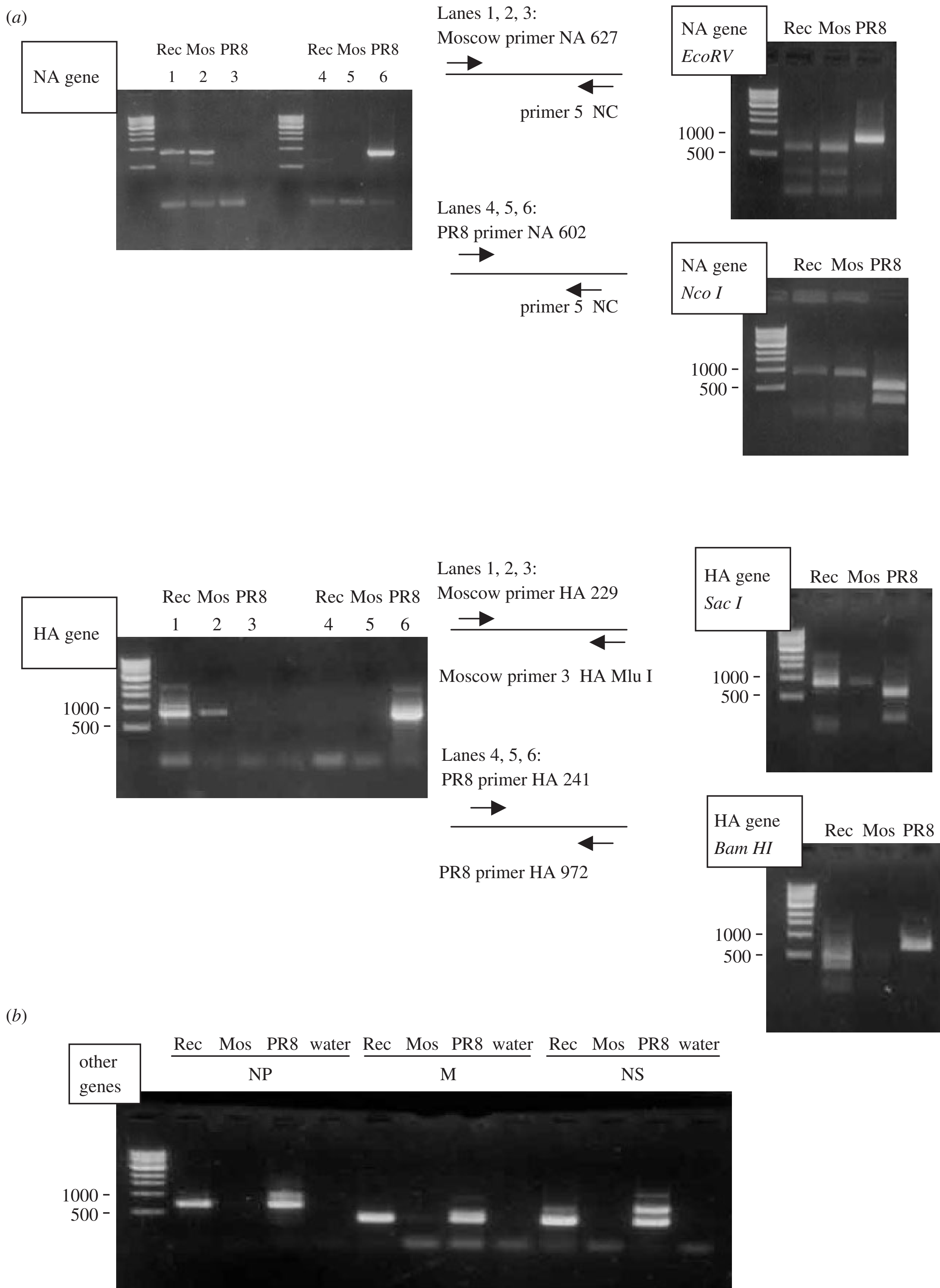

Figure 4. Characterization of the rescued PR8/Moscow/10/99 (6 + 2) recombinant virus. RT-PCR was performed on rescued PR8/Moscow/10/99 (6 + 2) recombinant (Rec) virus, the wt A/Moscow/10/99 (Mos) virus and rescued A/Puerto Rico/8/34 (PR8) virus as described in the text (§ 4). The PCR products were digested 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.

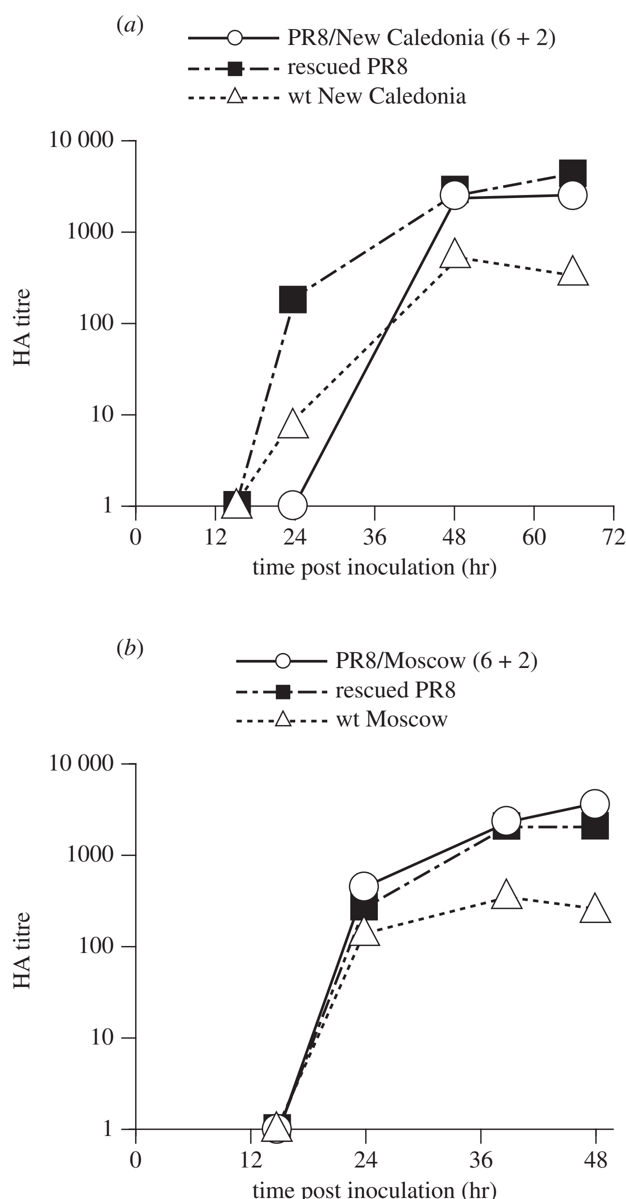

Figure 5. Growth curves of rescued high-yield recombinant viruses. Eggs were inoculated with 20 PFU per egg, in triplicate. At the indicated time-points, 100 µl allantoic fluid were removed to measure the titre by haemagglutination of chicken red blood cells. (*a*) The growth curves of A/PR/8/34 virus, 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.

4*b*) 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.* 1998*a*). 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.* 1998*b*; 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.* 2000*b*). 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 15 186 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 B1 and infectious virus was rescued (Nakaya *et al.* 2001). This recombinant rNDV/B1-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/B1-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*. 2000*a*,*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 NS1 gene. Such NS1-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 NS1 may be more immunostimulatory compared with viruses expressing an intact NS1 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 (Hennet *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 NS1-modified influenza viruses induce a higher IFN-α and -β and IFN-γ response than does wt (or cold-adpated) virus.

Consequently, it is possible that influenza virus vaccine strains with mutations in the NS1 may induce an enhanced antiviral immunity as compared with live attenuated influenza virus vaccines expressing a wt NS1 protein. In addition, the required dose needed per patient of an influenza virus vaccine attenuated by deletions in the NS1 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 (Schirrmacher *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 B1 vaccine strain of NDV. The recombinant rNDV/B1-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 recloned as new pandemic (epidemic) strains of influenza A virus arise. Thus, generation of vaccine strains of recombinant influenza viruses or rNDV/B1-HA for use in formalin-inactivated or live attenuated vaccine preparations can be reproducible, safe and rapid.

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