Rewiring the RNAs of influenza virus to prevent reassortment

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Influenza viruses contain segmented, negative-strand RNA genomes. Genome segmentation facilitates reassortment between different influenza virus strains infecting the same cell. This phenomenon results in the rapid exchange of RNA segments. In this study, we have developed a method to prevent the free reassortment of influenza A virus RNAs by rewiring their packaging signals. Specific packaging signals for individual influenza virus RNA segments are located in the 5′ and 3′ noncoding regions as well as in the terminal regions of the ORF of an RNA segment. By putting the nonstructural protein (NS)-specific packaging sequences onto the ORF of the hemagglutinin (HA) gene and mutating the packaging regions in the ORF of the HA, we created a chimeric HA segment with the packaging identity of an NS gene. By the same strategy, we made an NS gene with the packaging identity of an HA segment. This rewired virus had the packaging signals for all eight influenza virus RNAs, but it lost the ability to independently reassort its HA or NS gene. A similar approach can be applied to the other influenza A virus segments to diminish their ability to form reassortant viruses.

chimeric RNA segment | live attenuated virus | packaging signals | reverse genetics

Influenza viruses are classified as members of the family *Orthomyxoviridae* with three known types: A, B, and C. The A- and B-type viruses each possess eight RNA segments, whereas type C viruses have seven RNAs. A and B viruses have the potential to cause epidemic/pandemic human disease, and C viruses result in only mild upper respiratory tract illness (1). Understanding the influenza virus replication cycle within infected cells, in particular how the different genomic RNA segments interact, sort, and package into one viral particle, is of great significance. A previous study showed that soon after budding, the influenza A virus ribonucleoprotein (RNP) complexes inside the virions form a specific architecture: a central segment surrounded by the seven other vRNPs (2), which suggests that influenza viral RNA (vRNA) packaging is not a random process. Moreover, segment-specific RNA packaging sequences have been identified on each segment of the influenza A/WSN/33 virus (3–11). A common feature of these packaging signals is that both the 3′ and 5′ noncoding regions (NCRs), as well as coding sequences at the two ends of each open reading frame (ORF) are important [(3–11) and Fig. 47.23 in reference (1)]. Surprisingly, except for the 12 conserved nucleotides at the 3′ end and the 13 nucleotides at the 5′ end of the vRNA, which are required for both polymerase recognition and binding (1), no other conserved motif has been identified in the remainder of the packaging sequences. In addition, the minimum lengths required for efficient packaging, either at the 3′ or 5′ ends of the vRNA, differ from one segment to another (3–11). Although much is known about the role of these packaging sequences in influenza RNA packaging, their mechanism has yet to be fully elucidated. However, what is currently known about these packaging sequences can be used to incorporate foreign genes into the virus genome. This adds an approach for the development of influenza virus as a bivalent vaccine or gene delivery vector (8, 12–15).

In this study, we attempted to rewire the influenza vRNAs by changing the packaging sequences to prevent reassortment of a specific segment. The packaging sequences of the influenza A/PR/8/34 hemagglutinin (HA) and nonstructural protein (NS) segments, which include both NCRs and coding regions at the 3′ and 5′ ends (3, 8), were used to flank the ORFs of the NS and HA proteins, respectively. The recombinant virus carrying both chimeras was successfully rescued and exhibited efficient growth, and each chimeric segment was still able to reassort with wild-type virus. However, when the original packaging sequences from the ORFs of the HA and NS segments were eliminated by synonymous mutations—so that each segment carried only one set of segment-specific packaging sequences—the chimeric segments lost their ability to reassort. We hypothesize that a similar approach can be applied to other segments and an influenza virus lacking the capability to reassort can be made. This strategy is useful for engineering improved live attenuated influenza vaccines.

Results

A Chimeric Influenza Virus RNA, Which Contains a Wild-Type ORF and a Set of Flanking Packaging Sequences Derived from Another Segment, Maintains Its Ability to Form Reassortant Virus. In this study, we attempted to change the packaging signals of the HA and NS segments of influenza A/PR/8/34 virus to prevent free reassortment of these RNAs. It was shown earlier that foreign genes can be packaged into influenza viruses by adding flanking packaging sequences (8, 12–15). For example, a green fluorescent protein (GFP) segment was incorporated into influenza virus particles using the packaging signals of the neuraminidase segment (15). We hypothesized that we could create a reassortment-deficient influenza virus by incorporating a chimeric segment containing the ORF of the HA gene and the packaging signals from the NS gene, and a reverse segment containing the ORF of the NS gene and the packaging sequences of the HA gene. Such a virus having the HA gene with an NS packaging identity and the NS gene with an HA packaging identity would not freely reassort its HA or NS gene with a wild-type virus. The resulting virus acquiring a wild-type HA gene would have no NS gene-specific packaging sequences because of its chimeric NS gene (ORF from the NS gene and packaging signals from the HA gene); likewise, the resulting virus acquiring a wild-type NS gene would have no HA gene-specific packaging sequences because of its chimeric HA gene (ORF from the HA gene and packaging signals from the NS gene).

To do this, the wild-type HA ORF was amplified by polymerase chain reaction (PCR), and ligated to the flanking NS packaging sequences that include: the 3′ and 5′ NCRs, the 3′ 77 nucleotides (nt), and the 5′ 102 nt of the NS ORF. This generated the chimeric NS-HAwt-NS construct of 1,941 nt in length (Fig.

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Conflict of interest statement: Mount Sinai School of Medicine has submitted a provisional patent application on altering the packaging signals of influenza viruses to prevent reassortment.

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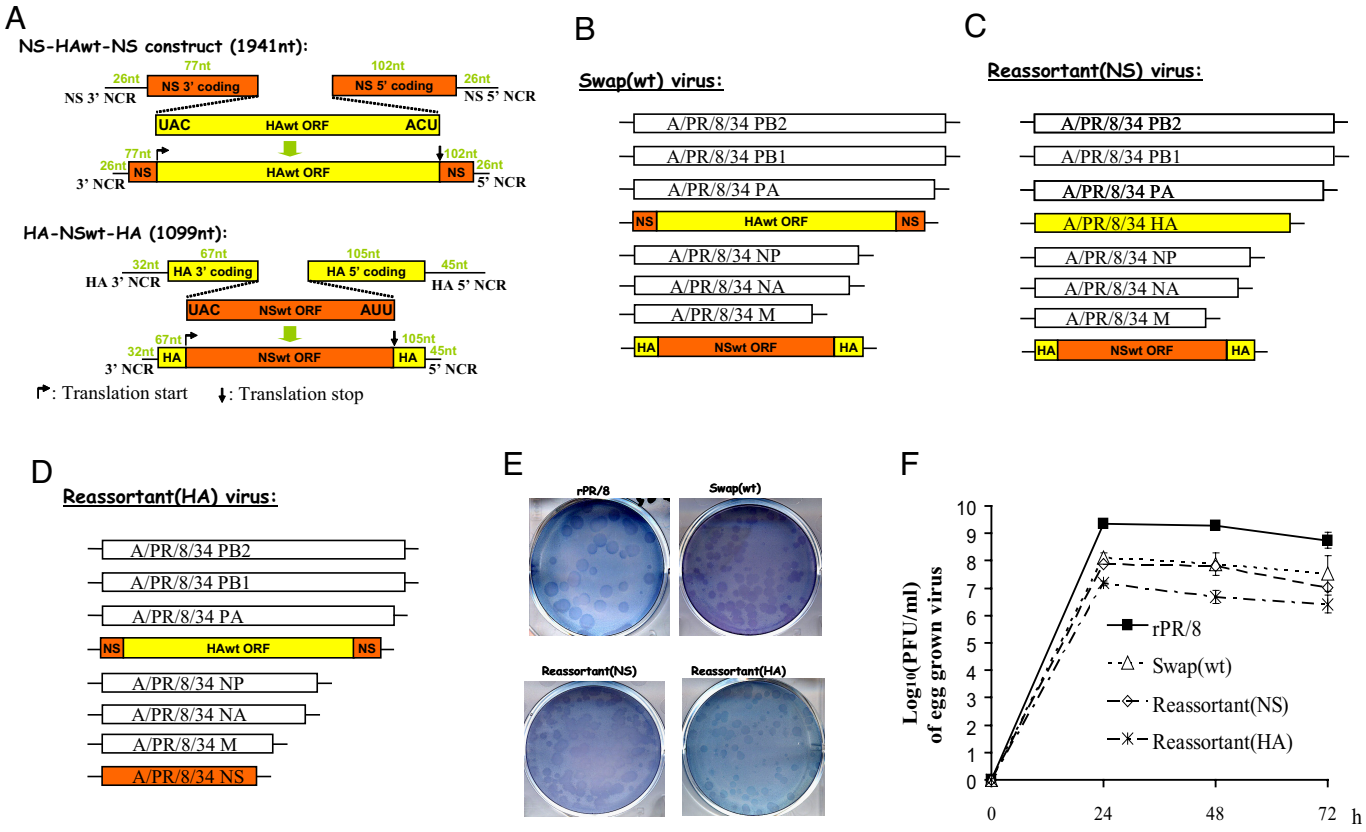

Fig. 1. Generation of the recombinant Swap(wt) virus carrying HA and NS chimeric segments which can independently form reassortant viruses. (*A*) NS-HAwt-NS and HA-NSwt-HA constructs. The A/PR/8/34 HA wild type (HAwt) ORF (in yellow) was flanked by the NS 3′ and 5′ NCRs and the 77 nt and 102 nt of NS ORF packaging signals (in red), generating the 1,941 nt long NS-HAwt-NS construct; likewise, the NS wild type (NSwt) ORF (in red) was flanked by the HA 3′ and 5′ NCRs and the 67 nt and 105 nt of HA ORF packaging signals (in yellow), generating the 1,099 nt long HA-NSwt-HA construct. The ATGs (in positive sense) upstream of the HA and NS translation start codons were all mutated to TTGs (in positive sense). The 5′ splice site on the 77-nt part of NS packaging signals in the NS-HAwt-NS construct was also mutated (see *Materials and Methods*). (*B*) Genome structure of the Swap(wt) virus. Six A/PR/8/34 ambisense plasmids (15, 19, 22), and the NS-HAwt-NS and HA-NSwt-HA constructs were used to generate the Swap(wt) virus. (Sequencing of the NS-HAwt-NS RNA segment revealed one G81U mutation in the 3′ end. No nucleotide changes were identified for the HA-NSwt-HA RNA segment). (*C*) Genome structure of the Reassortant(NS) virus which contains seven A/PR/8/34 RNAs and the HA-NSwt-HA RNA. (*D*) Genome structure of the Reassortant(HA) virus which contains seven A/PR/8/34 RNAs and the NS-HAwt-NS segment. (*E*) Immunostaining of the plaques formed in MDCK cells by the recombinant viruses. (*F*) Growth rates of the recombinant viruses in eggs at 37 °C.

1*A*). The two translation initiation codons and one splice site in the 77 nt of the NS 3′ ORF packaging signal were mutated to allow the HA to translate from its own start codon (Fig. 1*A*). Following the same strategy, a 1,099 nt long HA-NSwt-HA construct was also made (Fig. 1*A*). In this construct, the NS ORF—which encodes both NS1 and NS2 proteins—was flanked by the 3′ and 5′ NCRs of the HA, the 3′ 67 nt, and the 5′ 105 nt of the HA ORF. The three start codons located in the 67 nt of the 3′ ORF packaging region of the HA were also mutated. [Because we used the A/PR/8/34 virus as a backbone and since the currently known HA and NS packaging signals were all identified in the A/WSN/33 virus (3, 8), the flanking packaging sequences used in these experiments were made slightly longer than those identified in A/WSN/33 to assure proper packaging.]

Using previously established methods, the Swap(wt) virus was successfully rescued and was shown to be stable for multiple passages in embryonated chicken eggs (Fig. 1*B*). This virus contains six A/PR/8/34 wild-type segments (PB2, PB1, PA, NP, NA, and M) and two chimeric segments: NS-HAwt-NS and HA-NSwt-HA (Fig. 1*B*). The Swap(wt) virus grew well in eggs, and titers could reach more than 10^8 plaque forming units per mL (PFU/mL) 1 day post-inoculation (Fig. 1*F*). Nevertheless, it was still slightly attenuated in growth compared to the recombinant A/PR/8/34 virus. In Madin-Darby canine kidney (MDCK) cells, the plaques formed by the Swap(wt) virus were slightly smaller than those of A/PR/8/34 virus (Fig. 1*E*), while in eggs, the titers of the Swap(wt) virus were about 10-fold lower than those of A/PR/8/34 virus (Fig. 1*F*).

To determine whether the HA-NSwt-HA and NS-HAwt-NS segments could each freely reassort with wild-type virus genes, we constructed viruses which carried just one of these chimeric genes. To our surprise, we rescued two recombinant viruses: Reassortant(NS) and Reassortant(HA) (Fig. 1 *C* and *D*). The Reassortant(NS) virus contains seven A/PR/8/34 segments (PB2, PB1, PA, HA, NP, NA, and M) and one chimeric HA-NSwt-HA segment (Fig. 1*C*); the Reassortant(HA) virus has seven A/PR/8/34 vRNAs (PB2, PB1, PA, NP, NA, M, and NS) and one chimeric NS-HAwt-NS segment (Fig. 1*D*). Interestingly, the Reassortant(NS) virus exhibited efficient growth (Fig. 1*F*). The plaque sizes in MDCK cells and the titers in eggs were both similar to those of the Swap(wt) virus (Fig. 1 *E* and *F*). The Reassortant(HA) virus was more attenuated, with smaller plaques in MDCK cells and lower titers in eggs (Fig. 1 *E* and *F*). The rescue of both viruses indicated that each of the chimeric segments of the Swap(wt) virus could independently reassort to form a reassortant virus.

Deletion of the Original Packaging Sequences in the ORF Is Critical for the Chimeric Segment To Lose Its Ability to Freely form Reassortant Virus. The ability of the NS-HAwt-NS or HA-NSwt-HA segment to independently form a reassortant virus could be due to the

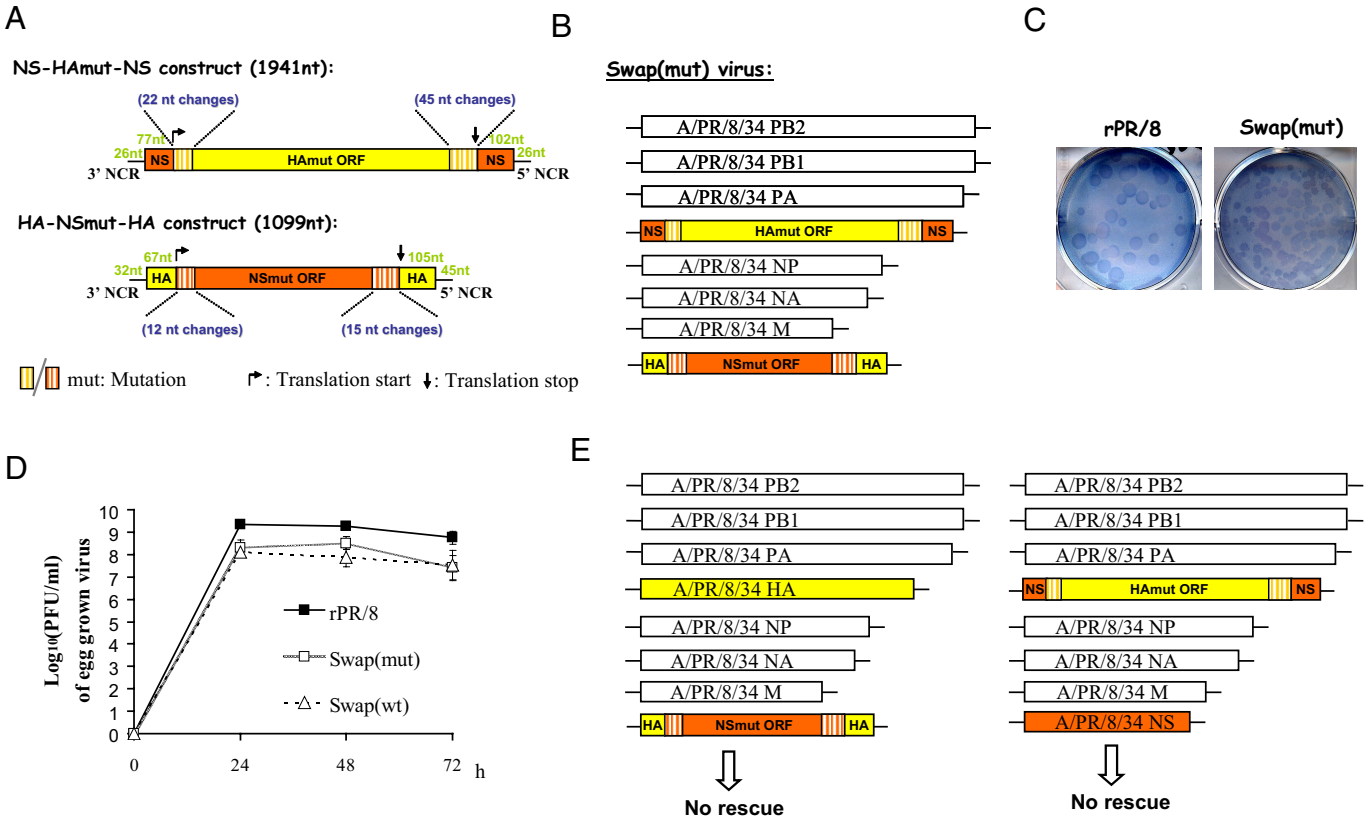

Fig. 2. Generation of the recombinant Swap(mut) virus carrying HA and NS chimeric segments which cannot independently form reassortant viruses. (*A*) NS-HAmut-NS and HA-NSmut-HA constructs. The strategy was the same as that described in Fig. 1*A*, except that the ORF region contained serial synonymous mutations: the NS-HAmut-NS construct carried 22- and 45-nt changes at the 3′ and 5′ ends, respectively; the HA-NSmut-HA construct had 12- and 15-nt changes in the NS ORF. (*B*) Genome structure of the Swap(mut) virus. The genomic composition is similar to that of the Swap(wt) virus (Fig. 1*B*), except that the NS-HAmut-NS and HA-NSmut-HA constructs were substituted for rescue. [Sequencing the NS-HAmut-NS RNA of the Swap(mut) virus revealed eight A to G mutations in the 3′ end. The sequence of the 3′ end 130 nt of the NS-HAmut-NS RNA is: 3′-ucguuuucgucccacuguuucuguauGaccuaggguuugugacacaguucgGGa-gucgaucuaacgGGagaaaccgaacaggcguuugcucaacgucugguucucgGucguacuuucgcuuGGacaaucaa (capitalized Gs designate the changes observed in virus RNA). For the HA-NSmut-HA RNA segment, two conversions on the NS 3′ ORF region were observed: A122G, which results in a Val to Ala amino acid change; and U318C, which is silent.] (*C*) Plaque phenotype of the Swap(mut) virus in MDCK cells. (*D*) Growth rates of the recombinant viruses in 10-day-old embryonated chicken eggs at 37 °C. (*E*) Failure to rescue two hypothetical reassortant viruses. The experiment on the left used seven A/PR/8/34 plasmids (15, 19, 22) and the HA-NSmut-HA construct, and the one on the right used seven A/PR/8/34 plasmids (15, 19, 22) and the NS-HAmut-NS.

fact that two sets of segment-specific packaging signals co-exist on the same vRNA (Fig. 1). The NS-HAwt-NS segment still maintains its original HA-specific packaging sequences in its HA ORF region in addition to the flanking NS packaging signals (Fig. 1*A*). The same is true for the HA-NSwt-HA segment. The original packaging signals may still be functional (Fig. 1*A*). Considering this possibility, we introduced serial synonymous mutations into the 3′ and 5′ ends of the ORFs in these chimeric constructs to force utilization of the flanking packaging signals only (Fig. 2*A*). Previous studies have shown that serial synonymous mutations in the coding region packaging sequences of the HA, NS and other segments indeed diminished the vRNA packaging efficiency (3, 10, 11, 13, 16, 17). In this study, 22- and 45-nt mutations were introduced to the 3′ and 5′ ends of the HA ORF, respectively, forming a construct NS-HAmut-NS (Fig. 2*A*, and *Materials and Methods*); a similar method was applied to the HA-NSwt-HA and 12- and 15-nt mutations were introduced to construct the HA-NSmut-HA (Fig. 2*A*, and *Materials and Methods*).

By using the same procedure as that in Fig. 1*B*, we successfully rescued the Swap(mut) virus (Fig. 2*B*), which contains six A/PR/8/34 segments (PB2, PB1, PA, NP, NA, and M) and two chimeric segments NS-HAmut-NS and HA-NSmut-HA (Fig. 2*B*). Right after the rescue, the titer of the Swap(mut) virus was low. After one passage in eggs, the virus grew to higher titers and maintained the same yield over multiple passages. The plaque sizes of the Swap(mut) virus were similar to those of the Swap(wt) virus (Figs. 1*E* and 2*C*). However, in eggs, the Swap(mut) virus grew slightly better than Swap(wt), although it was still slightly attenuated compared to the A/PR/8/34 virus (Fig. 2*D*). It should be noted that eight and two nucleotide conversions were identified on the 3′ ends of the NS-HAmut-NS and HA-NSmut-HA vRNAs of the passaged virus, respectively (see Fig. 2*B* legend).

To determine whether the chimeric genes in Fig. 2*A* are able to independently reassort with wild-type ones, we attempted to rescue two viruses (shown in Fig. 2*E*). The genetic compositions of these two viruses are similar to those of the Reassortant(NS) (Fig. 1*C*) and the Reassortant(HA) (Fig. 1*D*) viruses, except that now the HA-NSmut-HA and NS-HAmut-NS constructs (Fig. 2*A*) have been substituted for their counterparts (see Fig. 2*E*). If each chimeric segment still maintains its ability to reassort freely, then the two viruses in Fig. 2*E* should have been rescued. However, while we were able to easily rescue the Reassortant(NS) (Fig. 1*C*) and Reassortant(HA) viruses (Fig. 1*D*), we were unable to obtain either of the viruses shown in Fig. 2*E*. The failure of the rescue suggests that, unlike HA-NSwt-HA and NS-HAwt-NS, the HA-NSmut-HA and NS-HAmut-NS segments cannot freely reassort with wild-type genes.

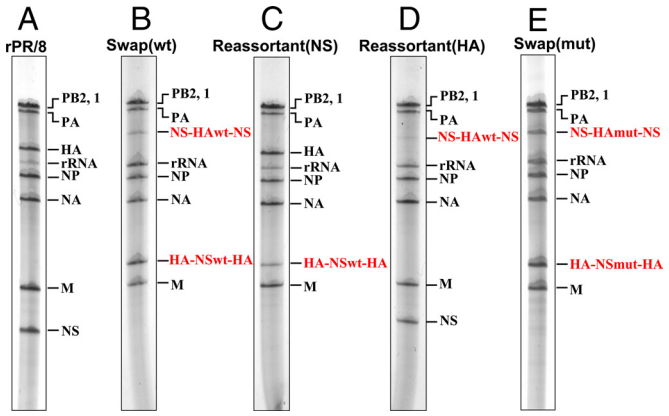

Fig. 3. Analyzing the vRNA genome packaging efficiency of the recombinant viruses. Five recombinant viruses [rA/PR/8/34 (*A*), Swap(wt) (*B*), Reassortant(NS) (*C*), Reassortant(HA) (*D*), and Swap(mut) (*E*)] were grown in eggs at 37 °C and purified viral RNA was separated (0.5 μg/lane) on a 2.8% acrylamide gel and visualized by silver staining. The RNA from the rA/PR/8/34 (*A*) and Swap(mut) (*E*) viruses was separated on one gel, and the RNA from the other three viruses [Swap(wt) (*B*), Reassortant(NS) (*C*), and Reassortant(HA) (*D*)] was separated on another gel.

vRNA Packaging of the Recombinant Viruses. Five recombinant viruses [rA/PR/8/34 (Fig. 3*A*), Swap(wt) (Fig. 3*B*), Reassortant(NS) (Fig. 3*C*), Reassortant(HA) (Fig. 3*D*), and Swap(mut) (Fig. 3*E*)] were grown in eggs and concentrated through a 30% sucrose cushion. RNA was isolated from purified virus and resolved on a 2.8% acrylamide gel to visualize the virus genome composition by silver staining. The NS-HAwt-NS segment of the Swap(wt) virus was inefficiently packaged while the other chimeric segment HA-NSwt-HA has better packaging efficiency (Fig. 3*B*). For the two reasssortant viruses [Reassortant (NS) and Reassortant (HA)], neither chimeric segment [HA-NSwt-HA and NS-HAwt-NS in Fig. 3 *C* and *D*, respectively] was efficiently incorporated. The packaging efficiency of the NS-HAwt-NS segment of the Reassortant(HA) virus was very low (Fig. 3*D*), which might explain the attenuation observed in both MDCK cells and eggs (Fig. 1 *E* and *F*). The two chimeric segments of the Swap(mut) virus were efficiently incorporated compared to the other segments (Fig. 3*E*). The NS-HAmut-NS segment of the Swap(mut) virus (Fig. 3*E*) was incorporated more efficiently than the NS-HAwt-NS segment of the Swap(wt) virus (Fig. 3*B*), suggesting that disruption of the original packaging signals of the HA ORF of the chimeric HA segment is critical to achieve efficient packaging. There was no significant difference in the levels of incorporation between HA-NSwt-HA and HA-NSmut-HA segments and both were packaged efficiently (Fig. 3 *B* and *E*).

The Chimeric NS Segment of Swap(wt), but Not of Swap(mut), Virus Can Reassort in Infected Cells. Although the rescue of the two viruses in Fig. 1 *C* and *D*, but not of the two hypothetical viruses in Fig. 2*E*, indicates which chimeric segment can freely form reassortant viruses with wild-type segments, these experiments per se do not directly assay for reassortment. To determine whether the chimeric segments can freely reassort in tissue culture, MDCK cells were co-infected with the Swap(wt) [or Swap(mut)] virus and rA/PR/8/34 virus at an moi of 10 for each one (Fig. 4*A*). Single plaques were isolated and subsequently amplified in MDCK cells. RNA was purified from amplified virus and RT-PCR was done to detect the HA and NS segments (Fig. 4*A*). An 824 base pair (bp) product was observed for both the NS-HAwt-NS and NS-HAmut-NS segments, while for the rA/PR/8/34 HA, a 747-bp band was obtained (Fig. 4 *B*, *D*, and *E*). The PCR products for chimeric and wild-type NS segments, on the other hand, were 405- and 326-bp long, respectively (Fig. 4 *C*–*E*). For the Swap(wt) and rA/PR/8/34 co-infection experi-

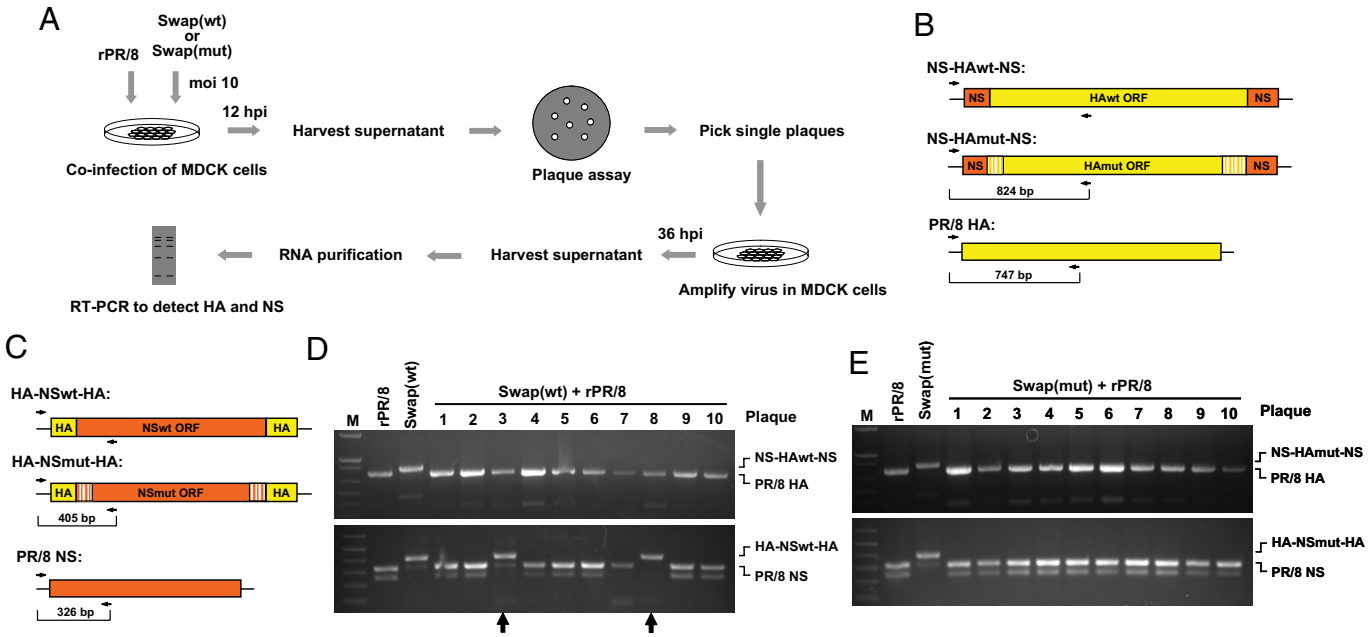

Fig. 4. The chimeric NS segment of the Swap(wt), but not of Swap(mut), virus can reassort in infected cells. (*A*) Diagram of the co-infection experiments. (*B*) RT-PCR primer design to detect the chimeric and wild-type HA segments. The RT-PCR products are 824 bp in length for the NS-HAwt-NS or NS-HAmut-NS segments and 747 bp for the wild-type HA. (*C*) RT-PCR primer design to detect the chimeric and wild-type NS segments. The RT-PCR products for the chimeric and wild-type NS segments are 405 and 326 bp long, respectively. (*D*) The Swap(wt) and rA/PR/8/34 viruses co-infection experiment. Twenty-four single plaques were characterized by RT-PCR (10 shown in the gel) using primers indicated in (*B* and *C*). The rA/PR/8/34 and Swap(wt) viruses were used for RT-PCR control (2nd and 3rd lane). M, marker. (*E*) The Swap(mut) and rA/PR/8/34 co-infection experiment. Forty-eight single plaques were characterized by RT-PCR (10 shown in the gel). The bands below the wild-type or chimeric NS PCR products were artificial by-products of the PCR.

ment, 24 plaques were characterized, and two of them (plaques 3 and 8, indicated by arrows) showed reassortment of the HA-NSwt-HA segment with wild-type virus (Fig. 4*D*). The genetic makeup of these two plaques is the same as the Reassortant(NS) virus (Fig. 1*C*). We did not observe reassortment of the NS-HAwt-NS segment, possibly due to its lower packaging efficiency (Fig. 3*D*). For the Swap(mut) and rA/PR/8/34 coinfection experiment, 48 plaques were picked and they all contained wild-type HA and NS genes, indicating the inability of NS-HAmut-NS or HA-NSmut-HA to reassort freely.

Discussion

Influenza virus RNA packaging seems to be a specific process and each RNA possesses segment-specific packaging sequences that include both the 3′ and 5′ NCRs, as well as coding sequences at the two ends of each ORF [(3–11) and Fig. 47.23 in reference (1)]. Interestingly, for the two chimeric constructs [NS-HAwt-NS and HA-NSwt-HA (Fig. 1*A*)], each contained two sets of segment-specific packaging sequences: the NS-HAwt-NS contained the NS-specific NCRs and ORF packaging regions in addition to the ORF packaging regions of the HA gene; the HA-NSwt-HA contained the HA-specific NCRs and ORF packaging regions in addition to the ORF packaging regions of the NS gene (Fig. 1*A*). The efficient growth of the Swap(wt) virus in both MDCK cells and eggs indicates that two sets of signals can co-exist on one vRNA (Fig. 1 *E* and *F*). It is unclear, however, which set plays the major role during the genome recruitment process.

The levels of the NS-HAwt-NS RNA in the Swap(wt) (Fig. 3*B*) and Reassortant(HA) (Fig. 3*D*) viruses were significantly lower than those of the other segments. This suggests that two sets of signals may interfere with each other during the influenza RNA packaging process if they co-exist on one segment. Previously Liang et al. showed that an artificial GFP vRNA with hybrid ends—the 5′ and 3′ packaging sequences were derived from different segments—was poorly packaged (5). This also suggests the incompatibility of two sets of packaging signals on one segment. The successful rescue of the two reassortant viruses [Reassortant (NS) (Fig. 1*C*) and Reassortant (HA) (Fig. 1*D*)] demonstrates that one virus can incorporate the same packaging signals twice. For example, the Reassortant(NS) virus contains two copies of HA packaging sequences derived from both the wild-type HA segment and the HA-NSwt-HA chimeric segment (Fig. 1*C*); the Reassortant(HA) virus carries two copies of NS packaging signals derived from both the wild type NS segment and the NS-HAwt-NS chimeric segment (Fig. 1*D*). This phenomenon agrees with our previous finding that a nine-segmented influenza virus can incorporate two NS segments (18). However, it is unlikely that under normal circumstances viruses carry an additional (chimeric) segment unless they are required to possess a ninth segment for replication.

In this study, we manipulated the packaging sequences of influenza viral RNAs to prevent reassortment. Our data show that, by simply flanking the ORF with packaging sequences from another segment, inhibition of reassortment cannot be achieved. It was possible to rescue viruses containing a single chimeric gene [the HA-NSwt-HA in the Reassortant(NS) virus (Fig. 1*C*), and the NS-HAwt-NS in the Reassortant(HA) virus (Fig. 1*D*)], and to identify in a reassortment experiment, viruses with a chimeric HA-NSwt-HA segment (Fig. 4*D*). We did not—in the reassortment experiment—isolate viruses with the NS-HAwt-NS segment or the Swap(wt) genotype. This can be explained by the relatively low number of plaques analyzed. Considering the possibility that the ORF terminal packaging signals in the chimeric segments might still be functional, we introduced serial silent mutations into these signals and subsequently, each segment [NS-HAmut-NS or HA-NSmut-HA] lost its ability to freely reassort (Figs. 2 and 4). We hypothesize that the remaining flanking regions of these two chimeric segments then became the main signals for packaging and as a result, the HA is recognized as an NS gene and the NS is recognized as an HA gene. We could not rescue single reassortants with the NS-HAmut-NS or HA-NSmut-HA segment because such viruses would lack an HA or NS packaging signal. Also, in the tissue culture reassortment experiment, no single reassortant was isolated. However, a limitation of our experimental setup holds true for the reassortment between the Swap(mut) and rA/PR/8/34 viruses. Only 48 plaques were isolated and thus we cannot exclude the possibility that a virus with a single rewired segment could be formed. Nevertheless, our data suggest that rewiring of the packaging signals results in a deficiency for reassortment. Only viruses that contain a full complement of all eight packaging signals will grow to high yields. In the case of rewiring one segment by eliminating the original packaging signal, a virus will lose viability which can be regained only by rewiring a second segment to provide the missing packaging sequences. Thus, a virus with an HA gene with the NS packaging identity must also have an NS gene with the HA packaging identity.

A similar approach could be applied to other segments of the virus to create a virus completely lacking the ability to reassort. For example, such a virus would have the PB1 segment with PB2 packaging signal, the PB2 segment with PA packaging signal, and the PA segment with HA packaging signals, etc. Currently, the possibility of live, attenuated influenza vaccines reassorting with circulating wild-type viruses (resulting in the generation of pathogenic strains) remains a concern. Thus, our study offers a method for rewiring the influenza virus RNAs to prevent reassortment. This approach can be used for future live influenza vaccine constructions.

Materials and Methods

Cells and Viruses. 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS). MDCK cells were grown in Eagle's MEM with 10% FCS. Viruses were grown in 10-day-old specific-pathogen-free chicken embryos (Charles River Laboratories, SPAFAS).

Plasmid Construction. (*i*) Generation of NS-HAwt-NS construct (Fig. 1*A*). The 1.2-kb *Kpn*I fragment from the previously constructed pDZ-NS plasmid (19) was transferred to the *Kpn*I site of pUC18 vector and subjected to site-directed mutagenesis to mutate two ATGs (A27T, A76T), one splice site (G57C), and to generate one *Nhe*I site (A104G, G109C) and one *Xho*I site (G759C, A760G). The 1.2-kb NS *Kpn*I fragment was then transferred back to the pDZ vector (19) (in which the *Nhe*I and *Xho*I sites have been removed), resulting in a plasmid pDZ-NS-ps. The ORF of the A/PR/8/34 HA protein, which is 1,698 bp long, was amplified from an ambisense pDZ-HA plasmid (19) and subjected to mutagenesis to mutate an internal *Xho*I site (C143G). Two restriction sites, *Nhe*I and *Xho*I, were introduced to flank the HA ORF, which was then used to replace the *Nhe*I and *Xho*I fragment of the NS ORF of pDZ-NS-ps plasmid to form the NS-HAwt-NS construct (Fig. 1*A*). (*ii*) Generation of HA-NSwt-HA construct (Fig. 1*A*). Using the same strategy, three ATGs were mutated on the 3′ HA packaging signal (A33T, A79T, and A92T). The ORF of the A/PR/8/34 NS proteins (NS1 and NS2), which is 838 bp long, was amplified and ligated to the HA packaging sequences in a pDZ vector to form the HA-NSwt-HA construct (Fig. 1*A*). (*iii*) Generation of NS-HAmut-NS construct (Fig. 2*A*). The method was the same as described for NS-HAwt-NS (Fig. 1*A*) except that the primers used to amplify the HA ORF carried synonymous mutations. The forward primer is: 5′-ca gctagc atg aaA gcG aaT TtG TtA gtT TtA CtG TCC gcG TtG gcG gcC gcG gaC gca gac aca ata tgt ata ggc tac c-3′; and the two reverse primers are 5′-cca Aaa GGA Aat Cgc Tcc TaA ACT Aac TaG CaA Tac TaA GCT GGA Agc gac agt tga gta gat cgc c-3′ and 5′-gt ctcgag tca Aat Aca Aat CcG Aca Ttg TaG GCT Ccc Gtt GCT Gca cat cca Aaa GGA Aat Cgc Tcc TaA AC-3′. (*iv*) Generation of the HA-NSmut-HA construct (Fig. 2*A*). The method was also the same as described for HA-NSwt-HA (Fig. 1*A*) except that synonymous mutations were introduced into the NS ORF. The forward primer is: 5′-ca gctagc atg gaC ccG aaT acC gtA AGT TCT ttt cag gta gaC tgc ttt ctt tgg cat gtc c-3′; the reverse primer is: 5′-gt ctcgag tta Gat CaA Ttg Gaa GCT Aaa Ggt CcG Gat Ttc Ctg ctc cac ttc aag c-3′. (The capitalized letters in these primer sequences designate mutated nucleotides.)

MICROBIOLOGY

Reverse Genetics for Recombinant Viruses. The method for generating recombinant influenza viruses was slightly modified from previous protocols (15, 19, 20). For the generation of the Swap(wt) and Swap(mut) viruses (Figs. 1*B* and 2*B*), 293T cells were transfected with six A/PR/8/34 plasmids (pDZ-PB2, PB1, PA, NP, NA, and M), and the two chimeric HA and NS constructs [NS-HAwt-NS and HA-NSwt-HA, or NS-HAmut-NS and HA-NSmut-HA] (Figs. 1*A* and 2*A*). For the generation of the Reassortant(NS) virus (Fig. 1*C*), 293T cells were transfected with seven A/PR/8/34 plasmids (pDZ-PB2, PB1, PA, HA, NP, NA, and M), and the HA-NSwt-HA construct. Seven A/PR/8/34 plasmids (pDZ-PB2, PB1, PA, NP, NA, M, and NS), and the NS-HAwt-NS construct were used to rescue the Reassortant(HA) virus (Fig. 1*D*).

Acrylamide Gel Electrophoresis of Purified vRNA. The viruses were grown in 10-day-old eggs at 37 °C and were subsequently processed by using a previously reported method (15). Briefly, virus was purified and RNA was isolated and run on a 2.8% denaturing polyacrylamide gel that was then stained with a silver staining kit (Invitrogen).

Immunostaining of Plaques. Previous methods were followed (15, 21). A rabbit anti-A/PR/8/34 polyclonal antibody (1:2,000 dilution) was used for plaque visualization.

Viral Growth Kinetics. Ten-day-old embryonated chicken eggs were inoculated with influenza viruses (100 PFU/egg) and incubated at 37 °C. At 24, 48, and 72 h post-inoculation, the allantoic fluids were harvested and the titers of the viruses were determined by plaque assay or immunostaining of the plaques in MDCK cells. At each time point, three eggs were analyzed for each virus.

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