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Compatibility among Polymerase Subunit Proteins Is a Restricting Factor in Reassortment between Equine H7N7 and Human H3N2 Influenza Viruses▽

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Reassortment is an important driving force for influenza virus evolution, and a better understanding of the factors that affect this process could improve our ability to respond to future influenza pandemics and epidemics. To identify factors that restrict the generation of reassortant viruses, we cotransfected human embryonic kidney cells with plasmids for the synthesis of viral RNAs of both A/equine/Prague/1/56 (Prague; H7N7) and A/Yokohama/2017/03 (Yokohama; H3N2) viruses together with the supporting protein expression plasmids. Of the possible 256 genotypes, we identified 29 genotypes in 120 randomly plaque-picked reassortants examined. Analyses of these reassortants suggested that the formation of functional ribonucleoprotein (RNP) complexes was a restricting factor, a finding that correlated with the activities of RNP complexes composed of different combinations of the proteins from the two viruses, as measured in a minigenome assay. For at least one nonfunctional RNP complex (i.e., Prague PB2, Prague PB1, Yokohama PA, and Prague NP), the lack of activity was due to the inability of the three polymerase subunit proteins to form a heterotrimer. Adaptation of viruses possessing a gene encoding a chimera of the PA proteins of the two viruses and the remaining genes from Prague virus resulted in compensatory mutations in the PB2 and/or PA protein. These results indicate substantial incompatibility among the gene products of the two test viruses, a critical role for the RNP complex in the generation of reassortant viruses, and a functional interaction of PB2 and PA.

Influenza A viruses cause disease in humans, pigs, horses, and a number of avian species (47). The genomes of influenza A viruses are composed of eight single-stranded, negative-sense RNA segments that allow gene reassortment between viruses that coinfect the same cell. Prime examples of how deadly the consequences of reassortment can be include the 1957 H2N2 "Asian influenza" and 1968 H3N2 "Hong Kong influenza" pandemics, in which avian virus PB1 and HA or HA and NA genes were introduced into the circulating human viruses (25, 27). Multiple reassortment events have also occurred among H3N2 viruses that belong to different lineages (42). Furthermore, reassortment is an important mechanism in the evolution of H5N1 avian influenza viruses, which continue to pose a potential pandemic threat (3, 4, 13).

Despite these reassortment events, there is evidence to suggest that gene exchange does not occur freely between two viruses. Maines et al. (28) reported that viruses with certain gene combinations between A/Victoria/3/75 (H3N2) and A/Hong Kong/486/97 (H5N1) viruses are not produced. Similarly, we reported the inability to generate single-gene reassortants containing the PB1, PA, HA, or NA segment from A/Memphis/8/88 (H3N2) when the remaining genes came from A/mallard/New York/6750/78 (H2N2) virus (18). Since reassortment is one of the most important events in the emergence of influenza pandemics and epidemics, it is important that we identify the underlying factors that restrict gene exchange during influenza virus reassortment.

In this study, we generated reassortant viruses by cotransfecting 293T cells with plasmids encoding the viral RNAs (vRNAs) of both A/equine/Prague/1/56 (Prague; H7N7) and A/Yokohama/2017/03 (Yokohama; H3N2) viruses. The diversities in each segment of these two viruses (80.8% to 85.4% homologies for genes other than HA and NA at the nucleotide level) provided us with an opportunity to clearly define the restricting factors for reassortment. Our results suggest a critical role for the ribonucleoprotein (RNP) complex in the generation of reassortant viruses.

MATERIALS AND METHODS

Cells and viruses. 293 and 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) containing 5% newborn calf serum and antibiotics. After infection with influenza virus, the MDCK cells were maintained in MEM containing 0.3% bovine serum albumin (BSA). The Yokohama virus was propagated in MDCK cells. The Prague virus was maintained in our repository and propagated in 10-day-old embryonated chicken eggs.

Construction of plasmids. The cDNAs of the Prague and Yokohama viruses were synthesized by reverse transcription of vRNAs with an oligonucleotide (Uni 12 [5′-AGCAAAAGCAGG-3′]) complementary to the conserved 3′ end of the vRNA, as previously described (24). The cDNAs were amplified by PCR with gene-specific oligonucleotide primers and then sequenced. The generation of plasmid constructs for vRNA production, containing the genes of the Prague and

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Yokohama viruses flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator (referred to as pPolI plasmids), is described in a previous publication (33). The protein expression vector pCAGGS/BsmBI was generated to clone the genes for the expression of the RNP complex proteins. This plasmid was constructed by insertion of the hybridized linkers 5′-AATTCGGGGGAGGAGACGGTACCGTCTCCAATAACCA-3′ and 5′-GATCTGGTTATTGGAGACGGTACCGTCTCCTCCCCCCG-3′ (the BsmBI site is shown by underlining) into the expression vector pCAGGS/MCS (26), which was previously digested with EcoRI and BglII. The full-length cDNAs of PB2, PB1, PA, and NP were then cloned into pCAGGS/BsmBI that had been digested with BsmBI.

To generate the NA vRNA reporter carrying the firefly luciferase gene, the region from the initiation codon of the enhanced green fluorescent protein gene to the StuI site on pPolI WSN-NA(183)GFP(157) (9) was replaced with the restriction enzyme sites of PstI, XbaI, EcoRI, SalI, PvuII, HindIII, NciI, BsrGI, SacI, BamHI, AvaI, and XhoI. The ATATG sequences at nucleotide positions 113 to 117 and 161 to 165 were mutated to GCGCG to modify the ATG codon in this pentanucleotide. The firefly luciferase gene was cloned between the BamHI and XhoI sites of this vector, and the resulting product was named pPolWSNNA F-Luc.

The plasmid pPolI $Pr_{(Yok\ 1–100)}$PA contains the 5′- and 3′-noncoding regions of the Prague PA vRNA segment to allow the synthesis of negative-sense vRNAs of Prague PA in which the region encoding the first 100 amino acids is replaced with the corresponding region of Yokohama PA. To generate this plasmid, we used overlap extension PCR (20). Briefly, using plasmid pPolI YokPA as the template, primer pair Ba-PrPA-1/Yok 100 (5′-CACACAGGTCTCCGGGAGCGAAAGCAGGTACTAATTCAAAATGGAAGAT-3′ and 5′-AATCTAGGTTTCTCAGCTCCAGTAGTGTTG-3′) amplified the fragment including the 3′-noncoding region of Prague vRNA and the region encoding Yokohama amino acids 1 to 100. Similarly, using pPolI PrPA as the template, the primer pair Pr 101/Ba-PrPA-2233R (5′-TACTGGAGCTGAGAAACCTAGATTCCTTC-3′ and 5′-CACACAGGTCTCCTATTAGTAGAAACAAGGTACTTTTTTGGAC-3′) amplified the region from amino acid 101 to the 5′ end of the Prague PA vRNA. The primers Yok 100 and Pr 101 overlapped by 24 nucleotides, so the two PCR fragments shared a small common region. The primers Ba-PrPA-1 and Ba-PrPA-2233R, both of which contained a BsaI site, were used in a second PCR, using the two PCR fragments obtained above as templates. The final chimeric PA PCR products, containing the Prague 3′- and 5′-noncoding regions, the region encoding Yokohama amino acids 1 to 100, and the region encoding Prague amino acids 101 to 716, were digested with BsaI and cloned into the BsmBI site of the pPolI vector to generate the chimeric PA vRNA or pCAGGS/BsmBI vector for protein expression. Using the same strategy, we generated a series of pPolI and pCAGGS constructs that contained portions of the Yokohama PA coding regions in addition to the noncoding regions and the remaining portions of the Prague PA coding regions (see Fig. 3). These chimeric PAs were named according to the coding regions of the Prague and Yokohama PA proteins that they contained; $Pr_{(Yok\ 101–716)}$PA, for example, contains the noncoding regions and the coding regions for amino acids 1 to 100 of Prague PA, as well as the coding region for amino acids 101 to 716 of Yokohama PA.

The mutations shown in Table 4 were introduced into PrPB2 or chimeric PA in the pPolI vector by using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and then were subcloned into the pCAGGS/BsmBI vector. A complete list of oligonucleotides used to generate the point mutations is available upon request.

To generate plasmids expressing Prague PA (PrPA), Yokohama PA (YokPA), and 12 chimeric PAs with a FLAG tag at the C terminus, pCAGGS PrPA, pCAGGS YokPA, and the respective protein expression plasmids, e.g., pCAGGS $Pr_{(Yok\ 1–100)}$PA, were used as templates. PCR amplification was performed using the following primer pairs: Bm-PA-1 (5′-CACACACGTCTCCGGGAGCGAAAGCAGGTAC-3′) and Bm-PrPA-c-FLAG (5′-CACACACGTCTCCTATTCTACTTATCGTCGTCATCCTTGTAATCAGCGGCAGCCTCTAGTGCATGTTTAAG-3′) or Bm-PA-1 and Bm-YokPA-c-FLAG (5′-CACACACGTCTCCTATTCTACTTATCGTCGTCATCCTTGTAATCAGCGGCAGCTTTTAATGCATGTGTCAG-3′). The PCR products were cloned into the pCAGGS/BsmBI vector.

All of the above constructs were fully sequenced to ensure the absence of unwanted mutations.

Genotyping of plaque-purified viruses. The eight pPolI plasmids for both Prague and Yokohama vRNA synthesis (16 plasmids in total), together with the four WSN protein expression plasmids (pCAGGS-WSN PB2, pCAGGS-WSN PB1, pCAGGS-WSN PA, and pCAGGS-WSN NP), were mixed with Trans IT LT-1 (Panvera) (2 μl/μg) and transfected into 1×10^6 293T cells. Six hours later, the DNA and transfection reagent were replaced with Opti-MEM. After a 24-h incubation, the transfection supernatant was harvested and subjected to plaque assay on MDCK cells cultured in 60-mm dishes by standard procedures. Plaques in dishes with fewer than 10 plaques were picked and suspended in 500 μl of 1× MEM-BSA. Each of the plaque-purified viruses was then inoculated into one well of MDCK cells in 12-well plates and incubated for 48 to 72 h. The cells were then observed for cytopathic effect, and hemagglutination (HA) assay (using 0.5% chicken red blood cells) was performed on the supernatant to detect virus.

vRNA was extracted from the culture supernatant by using an RNeasy Mini kit (Qiagen) and was reverse transcribed by using Uni12 with Superscript II (Invitrogen) for 1 h at 37°C. To distinguish the gene origins of the plaque-purified viruses, 16 pairs of identifying primers were synthesized based on the sequence diversities of all eight segments shared between the Prague and Yokohama viruses (primer sequences are available upon request). PCR amplification was performed by using the 16 pairs of strain-specific primers, and the gene origin of each segment was determined by the presence or absence of the corresponding band. The gene origins of some plaque-purified viruses were also confirmed by sequencing.

Minigenome assay. To compare the activities of viral RNP complexes, a dual-luciferase reporter assay system (Promega) was used according to the manufacturer's instructions. Briefly, the construct pPolWSNNA F-Luc (0.05 μg) was transfected into 2×10^5 293 cells cultured in 12-well plates together with the four protein expression plasmids (pCAGGS PB2, pCAGGS PB1, pCAGGS PA, and pCAGGS NP [0.5 μg of each]) for each of the 16 RNP combinations of Prague and Yokohama virus proteins. At 48 hours posttransfection, the luciferase activities were measured on a GloMax 96 microplate luminometer (Promega) according to the manufacturer's instructions. As an internal control for the dual-luciferase assay, pGL4.74[hRluc/TK] (Promega) was used (37).

Generation of influenza viruses with chimeric PAs and sequence analysis. Prague viruses containing one of a series of chimeric PAs were rescued by the use of eggs. Briefly, 0.1 μg of each of the seven Prague pPolI plasmids for the generation of vRNA segments other than the PA segment and one of the pPolI PA plasmids encoding chimeras between the Prague and Yokohama PA proteins [e.g., pPolI $Pr_{(Yok\ 1–100)}$PA], together with 1 μg each of the protein expression plasmids for WSN PB2, PB1, PA, and NP, was mixed with Trans IT LT-1 and transfected into 293T cells. At 6 hours posttransfection, the DNA transfection mixture was replaced with Opti-MEM. Forty-eight hours later, aliquots of the supernatant were used to inoculate 10-day-old embryonated chicken eggs. The eggs were incubated at 33°C for 72 h, and the presence of virus was confirmed by HA assay with 0.5% chicken red blood cells.

A second passage in eggs was performed for those viruses that could not be detected by HA assay after the first passage in embryonated eggs. For these viruses, the PB2, PB1, PA, and NP genes were sequenced.

Immunoprecipitation assay. 293T cells growing in 60-mm dishes were transfected with 2 μg each of the indicated combinations (see Fig. 2) of protein expression plasmids. Cells were harvested at 48 h posttransfection. After three washes with cold phosphate-buffered saline, cell pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, one Complete Mini protease inhibitor cocktail tablet [Roche]/50 ml) on ice. The supernatants were then mixed and rocked at 4°C for 3 h with goat polyclonal anti-PB2 antibodies (Santa Cruz). Protein G-Sepharose beads were added, and the mix was rocked at 4°C overnight. The IgG-Sepharose beads were washed three times with 1 ml of lysis buffer, and immunoprecipitated proteins were separated by 4% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes by use of an iBlot dry blotting system (Invitrogen), followed by Western blotting with mouse anti-PB2 or anti-PB1 monoclonal antibodies (15, 16). PA proteins fused to the FLAG tag were detected with a mouse monoclonal anti-FLAG antibody (Sigma).

RESULTS

Genotyping of plaque-purified viruses. To understand the molecular basis for the restriction of genetic reassortment among influenza A viruses, we designed experiments to identify incompatibilities among the genes of two viruses. The Prague and Yokohama viruses were originally isolated from a horse and a human, respectively, and are genetically different in each segment (80.8% to 85.4% homologies for genes other than HA and NA at the nucleotide level). These viruses were chosen because they are phylogenetically distantly related, thereby maximizing the possibility of finding more restrictions

TABLE 1. Genotypes of reassortant viruses between Prague (H7N7) and Yokohama (H3N2) viruses[a]

Genotype	Gene origin[b]								Frequency (no. of viruses)
	PB2	PB1	PA	HA	NP	NA	M	NS	
A1	Y	Y	P	Y	P	Y	P	Y	6
A2	Y	Y	P	Y	P	Y	Y	Y	5
A3	Y	Y	P	Y	P	P	P	P	4
A4	Y	Y	Y	Y	P	Y	Y	Y	11
A5	Y	Y	Y	P	P	Y	P	Y	1
A6	Y	Y	Y	Y	P	Y	P	Y	23
A7	Y	Y	Y	Y	P	P	P	P	6
A8	Y	Y	Y	Y	P	P	Y	Y	2
A9	Y	Y	P	Y	P	Y	P	P	4
A10	Y	Y	Y	Y	P	Y	P	P	14
A11	Y	Y	P	Y	P	Y	Y	P	1
A12	Y	Y	Y	Y	Y	P	Y	Y	3
A13	Y	Y	Y	Y	P	P	P	Y	14
A14	Y	Y	P	Y	Y	Y	P	P	1
A15	Y	P	P	Y	P	P	Y	Y	2
A16	Y	P	P	Y	P	Y	P	Y	1
A17	Y	Y	Y	Y	Y	P	Y	P	1
A18	Y	Y	Y	P	P	P	P	Y	1
A19	Y	Y	Y	Y	Y	P	P	Y	1
A20	Y	Y	Y	Y	Y	Y	P	P	2
A21	Y	P	P	Y	P	Y	P	P	1
A22	Y	Y	Y	Y	P	Y	Y	P	1
A23	Y	Y	P	Y	P	P	P	Y	4
A24	Y	Y	Y	Y	Y	Y	Y	Y	4
A25	Y	Y	Y	Y	Y	P	P	P	1
A26	Y	Y	Y	Y	Y	Y	P	Y	1
A27	Y	P	P	Y	P	P	P	Y	3
A28	Y	Y	P	Y	P	P	Y	Y	1
A29	Y	P	P	Y	Y	P	P	P	1
No. of viruses possessing the respective Yokohama segment/ total no. of viruses	120/120	112/120	86/120	118/120	15/120	76/120	31/120	83/120	

[a] 293T cells were transfected with plasmids for the generation of vRNAs for both the Prague and Yokohama viruses together with the four protein expression plasmids of WSN. The cotransfection supernatants were plaque purified on MDCK cells. The genotypes of the plaque-purified viruses were determined by using strain-specific PCRs with identifying primers.

[b] P, Prague; Y, Yokohama.

of reassortment than if we had chosen viruses that were highly related. The eight vRNA expression plasmids derived from both the Prague and Yokohama viruses, together with the four protein expression plasmids, were cotransfected into 293T cells. The supernatants of plasmid-transfected cells were then used for plaque assays using MDCK cells. Both the Prague and Yokohama viruses can form visible plaques on these cells. Plaque-purified viruses were genotyped by using two sets of primers capable of identifying the gene origins. The 120 plaque-purified viruses we examined were classified as having 29 different genotypes, designated A1 to A29 (Table 1). Wild-type Prague virus was not detected among the 120 plaque-purified viruses, and there were only four plaques (genotype A24) that represented wild-type Yokohama virus. Some genotypes were present with high frequencies, such as genotypes A4, A6, A10, and A13, while others were rare. Theoretically, there are a total of 256 (2^8) possible genotypes between two influenza A viruses. However, our experiments yielded only 29 genotypes, indicating that these genotypes are competitive in the reassortment process, whereas other genotypes are noncompetitive or nonviable, possibly due to incompatibilities at the protein and/or genomic level.

Interestingly, all plaque-purified viruses possessed the Yokohama PB2 segment. Similarly, viruses possessing the Yokohama PB1 or HA gene were present at high frequencies (93.3% and 98.3%, respectively). Only eight viruses possessed the Prague PB1 gene. It is interesting that whenever the Prague PB1 gene was found in a virus, the PA gene also originated from the Prague virus. On the other hand, most of the NP and M genes (87.5% and 74.2%, respectively) of the reassortant viruses were of Prague virus origin. We therefore found a differential segregation phenomenon in which the progeny viruses obtained certain segments from one parent and other segments from the other parent. With respect to the PA, NA, and NS genes, no predominance of one parent over the other was found.

Although 16 combinations of proteins forming the RNP complex (PB2, PB1, PA, and NP) are possible between two influenza A viruses, only 6 combinations were detected among the 120 plaque-purified viruses (Table 2); the RNP combination $Y_{PB2}Y_{PB1}Y_{PA}P_{NP}$ (Yokohama PB2, PB1, and PA and Prague NP; genotypes A4, A5, A6, A7, A8, A10, A13, A18, and A22) was especially dominant.

RNP complexes composed of different combinations of polymerase and NP proteins differ in activity in a minigenome assay. Since only a limited number of protein combinations for the RNP complex were detected in our reassortant viruses, we

TABLE 2. Gene combinations of RNP complexes in reassortant viruses between Prague (H7N7) and Yokohama (H3N2) viruses[a]

Combination no.	RNP gene origin				Frequency (no. of reassortant viruses)
	PB2	PB1	PA	NP	
1	Y	P	P	P	7
2	P	Y	P	P	–
3	P	P	Y	P	–
4	P	P	P	Y	–
5	Y	Y	P	P	25
6	Y	P	Y	P	–
7	Y	P	P	Y	1
8	P	Y	Y	P	–
9	P	Y	P	Y	–
10	P	P	Y	Y	–
11	Y	Y	Y	P	73
12	Y	Y	P	Y	1
13	Y	P	Y	Y	–
14	P	Y	Y	Y	–
15	Y	Y	Y	Y	13
16	P	P	P	P	–

[a] Frequencies are based on the characterization of 120 plaque-purified viruses (see Table 1). P, Prague; Y, Yokohama. –, the RNP gene combination was not present among the plaque-purified viruses.

tested the role of the RNP complex in restricting the generation of reassortant viruses by using a minigenome assay. Plasmid pPolWSNNA F-Luc, expressing a virus-like RNA encoding firefly luciferase, was cotransfected with the protein expression plasmids for PB2, PB1, PA, and NP derived from either the Prague or Yokohama virus. We found that the activities of the heterogeneous RNP complexes varied substantially (Fig. 1). The Yokohama virus RNP complex ($Y_{PB2}Y_{PB1}Y_{PA}Y_{NP}$) showed the highest activity, and six hybrid RNP combinations ($Y_{PB2}P_{PB1}P_{PA}P_{NP}$, $P_{PB2}Y_{PB1}P_{PA}P_{NP}$, $Y_{PB2}Y_{PB1}P_{PA}P_{NP}$, $Y_{PB2}P_{PB1}P_{PA}Y_{NP}$, $Y_{PB2}Y_{PB1}Y_{PA}P_{NP}$, and $Y_{PB2}Y_{PB1}P_{PA}Y_{NP}$) possessed higher activities than the Prague virus RNP complex ($P_{PB2}P_{PB1}P_{PA}P_{NP}$). Among the four possible combinations of Prague PB2 and Yokohama PA, three ($P_{PB2}P_{PB1}Y_{PA}P_{NP}$, $P_{PB2}P_{PB1}Y_{PA}Y_{NP}$, and $P_{PB2}Y_{PB1}Y_{PA}Y_{NP}$) entirely lacked replicative activity. Another combination, $P_{PB2}Y_{PB1}Y_{PA}P_{NP}$, showed substantially reduced activity compared to that of the $P_{PB2}P_{PB1}P_{PA}P_{NP}$ combination. Hence, Prague PB2 may not cooperate efficiently with Yokohama PA. As expected, all six RNP combinations that were identified in the 120 plaque-purified viruses showed considerable activity in this minigenome assay, suggesting that the activity of the RNP complex is one of the underlying restricting factors for reassortment. However, the RNP combination found most frequently ($Y_{PB2}Y_{PB1}Y_{PA}P_{NP}$) did not show the highest replicative ability, suggesting that other factors (such as compatibilities of the RNP components with other viral proteins) contribute to the outcome of reassortment events as well.

Cooperation of PB2 and PA proteins. The RNP combinations $P_{PB2}P_{PB1}Y_{PA}P_{NP}$ and $P_{PB2}P_{PB1}Y_{PA}Y_{NP}$ showed no activity in the minigenome assay. Given that functional viral polymerase complexes are formed by the interaction of PB1 with both PB2 and PA (5), we asked whether the defect in replicative activity of the $P_{PB2}P_{PB1}Y_{PA}P_{NP}$ and $P_{PB2}P_{PB1}Y_{PA}Y_{NP}$ complexes was due to a lack of heterotrimer formation. To address this question, we performed immunoprecipitation assays with lysates prepared from 293T cells transfected with expression plasmids for the three polymerase subunits and antibodies against PB2, PB1, and PA. PB1 and PA coimmunoprecipitated with PB2 for the polymerase combinations $P_{PB2}P_{PB1}P_{PA}$ and $P_{PB2}Y_{PB1}Y_{PA}$, although the amount of Yokohama PA immunoprecipitated with Prague PB2 was lower than the amount of Prague PA (Fig. 2A, lanes 1 and 3), consistent with the difference in RNP activity in the minigenome assay (compare $P_{PB2}P_{PB1}P_{PA}P_{NP}$ and $P_{PB2}Y_{PB1}Y_{PA}P_{NP}$ in Fig. 1). In contrast, Yokohama PA was not immunoprecipitated with Prague PB2 in cells expressing the polymerase combination $P_{PB2}P_{PB1}Y_{PA}$ (Fig. 2A, lane 2), indicating that these three proteins are unable to form a heterotrimeric complex. Since formation of the polymerase complex is essential for vRNA replication and transcription (21), the inability of the $P_{PB2}P_{PB1}Y_{PA}$ proteins to form a heterotrimeric complex is likely responsible for the loss of activity for the $P_{PB2}P_{PB1}Y_{PA}P_{NP}$ and $P_{PB2}P_{PB1}Y_{PA}Y_{NP}$ combinations and the failure to generate a single-gene reassortant virus possessing the Yokohama PA gene and the remaining genes from the Prague virus.

Region important for Prague PA to form a functional RNP complex. Yokohama PA was not immunoprecipitated with Prague PB2 and PB1 in cells expressing the polymerase combination $P_{PB2}P_{PB1}Y_{PA}$. To determine the region in PA responsible for this deficiency, we constructed a series of chimeric PAs in which the noncoding region originated from Prague PA and the coding region of Prague PA was replaced with various portions of Yokohama PA. In total, 12 PA chimeras were generated (Fig. 3). In RNP combination $P_{PB2}P_{PB1}P_{(Y1\text{-}100)PA}P_{NP}$, as an example, amino acids 1 to 100 of Prague PA were replaced with the corresponding region of Yokohama PA. To verify the expression levels of these chimeric PA proteins, we constructed protein expression plasmids that expressed chimeric PAs with a FLAG tag at the C terminus and performed Western blotting with a mouse monoclonal anti-FLAG antibody. All PA chimeras were expressed at levels similar to that of wild-type Prague PA (data not shown). We then performed

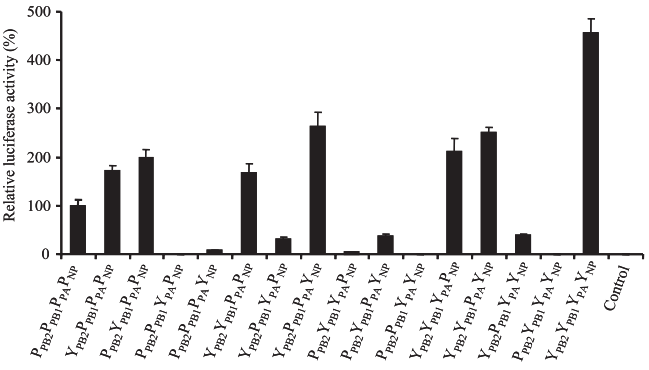

FIG. 1. Activities of 16 RNP combinations measured in a minigenome assay. Four protein expression plasmids (PB2, PB1, PA, and NP [0.5 μg of each]) for the 16 RNP combinations were transfected into 293 cells together with pPolWSNNA F-Luc, which encodes a vRNA possessing a reporter firefly luciferase gene. At 48 h posttransfection, a dual-luciferase assay was performed in which the relative firefly luciferase activity was normalized to the internal control, *Renilla* luciferase activity (see Materials and Methods). The values shown are means ± standard deviations for three independent experiments and are standardized to the activity of $P_{PB2}P_{PB1}P_{PA}P_{NP}$ (100%). P, Prague. Y, Yokohama.

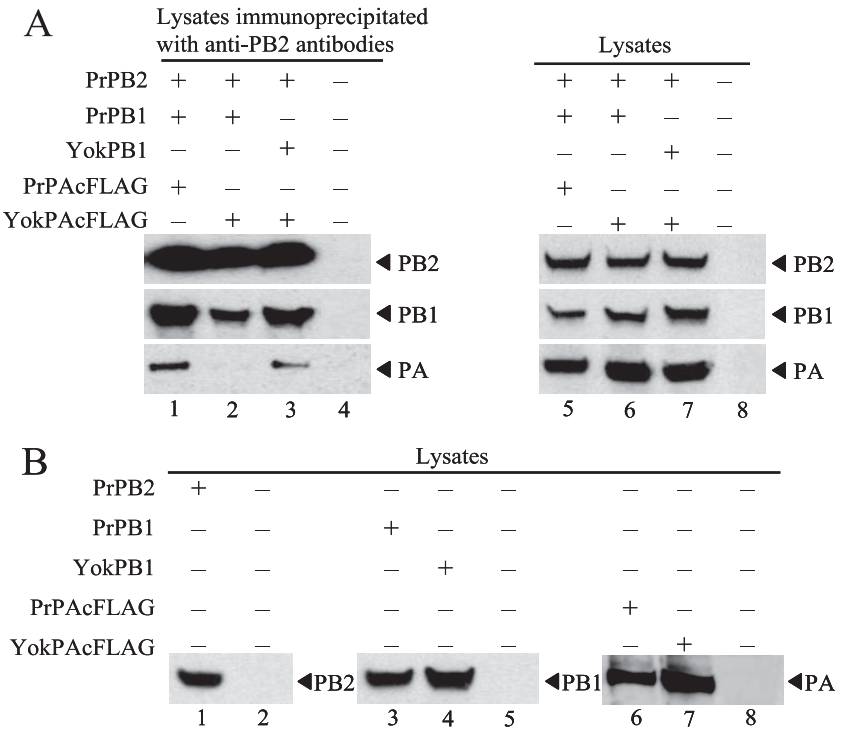

FIG. 2. Interactions among RNA polymerase subunits. (A) 293T cells were cotransfected with 2 μg each of protein expression plasmids, as indicated (+). At 48 h posttransfection, cell lysates were incubated with a goat polyclonal anti-PB2 antibody (Santa Cruz) (lanes 1 to 4). The agarose bead-bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted with mouse monoclonal anti-PB2, anti-PB1, or anti-FLAG antibodies. The cell lysates were also directly Western blotted with mouse monoclonal anti-PB2, anti-PB1, and anti-FLAG antibodies, as shown in lanes 5 to 8. (B) 293T cells were transfected with 2 μg of protein expression plasmids encoding Prague PB2, Prague PB1, Yokohama PB1, Prague PA, and Yokohama PA. At 48 h posttransfection, cell lysates were prepared and Western blotted with mouse monoclonal antibodies (anti-PB2 [lanes 1 and 2], anti-PB1 [lanes 3 to 5], and anti-FLAG [lanes 6 to 8]).

minigenome assays with the chimeric PA proteins. We observed that the RNP activities decreased dramatically when N-terminal portions of Prague PA were replaced with the respective Yokohama PA sequences. Overall, Prague and Yokohama PA proteins share 91.2% homology at the amino acid level (62 amino acid differences). There are 13 amino acid differences in the N-terminal 100 amino acids of the Prague and Yokohama PA proteins. The RNP activity of $P_{PB2}P_{PB1}P_{(Y1\text{-}100)PA}P_{NP}$ decreased approximately 370 times compared to that of the parental combination $P_{PB2}P_{PB1}P_{PA}P_{NP}$, suggesting that the N-terminal 100 amino acids of Yokohama PA are important for its failure to function in combination with the Prague PB1 and PB2 proteins. Almost no activity was detected when more than 400 N-terminal amino acids of Prague PA were replaced with Yokohama PA sequences. Conversely, the RNP activity decreased only slightly (<25% reduction compared with the parental Prague PA protein) when the C-terminal 216 amino acids of Prague PA were replaced with Yokohama PA [$P_{PB2}P_{PB1}P_{(Y501\text{-}716)PA}P_{NP}$ and $P_{PB2}P_{PB1}P_{(Y601\text{-}716)PA}P_{NP}$]. The RNP activity decreased substantially, however, when the replacements extended further toward the N terminus of Prague PA [compare the activities of $P_{PB2}P_{PB1}P_{(Y401\text{-}716)PA}P_{NP}$, $P_{PB2}P_{PB1}P_{(Y301\text{-}716)PA}P_{NP}$, $P_{PB2}P_{PB1}P_{(Y201\text{-}716)PA}P_{NP}$, and $P_{PB2}P_{PB1}P_{(Y101\text{-}716)PA}P_{NP}$]. Collectively, these results show that the N-terminal half of Prague PA, especially the region of amino acids 1 to 400, is important for the formation of a functional RNP complex.

Compensatory mutations in the PB2 and PA proteins. We next tested the compatibility of the PA chimeras with the remaining segments of the Prague virus. Prague virus can grow to high titers in embryonated eggs but grows poorly in MDCK cells. We therefore transfected 293T cells with plasmids for the synthesis of Prague vRNAs (with the plasmid for the PA vRNA segment encoding a hybrid PA protein), together with the four supporting protein expression plasmids. The transfection supernatants were inoculated into embryonated eggs, and the presence of viruses was tested by HA assay after a 72-hour incubation. Five chimeric PA viruses were below the detection threshold of the HA assay (Table 3). Viruses that could not be detected by HA assay after one passage in eggs were passaged once more (Table 3). All five viruses possessing chimeric PA proteins could then be detected by HA assay. In contrast, even after two passages in eggs, we were unable to generate a virus possessing the Yokohama PA gene in the genetic background of Prague virus.

The 12 viruses with chimeric PAs were detected after either one or two passages of the transfection supernatant in eggs. Since the RNP complexes composed of one of these PA chimeras and the Prague PB1 and PB2 proteins showed reduced activities compared to that of the wild-type Prague RNP complex, growth of viruses with chimeric PAs after an additional passage in eggs suggested the presence of adaptive mutations in the viruses. Therefore, we sequenced the genes encoding the proteins in the RNP complexes (i.e., PB2, PB1, PA, and NP) of

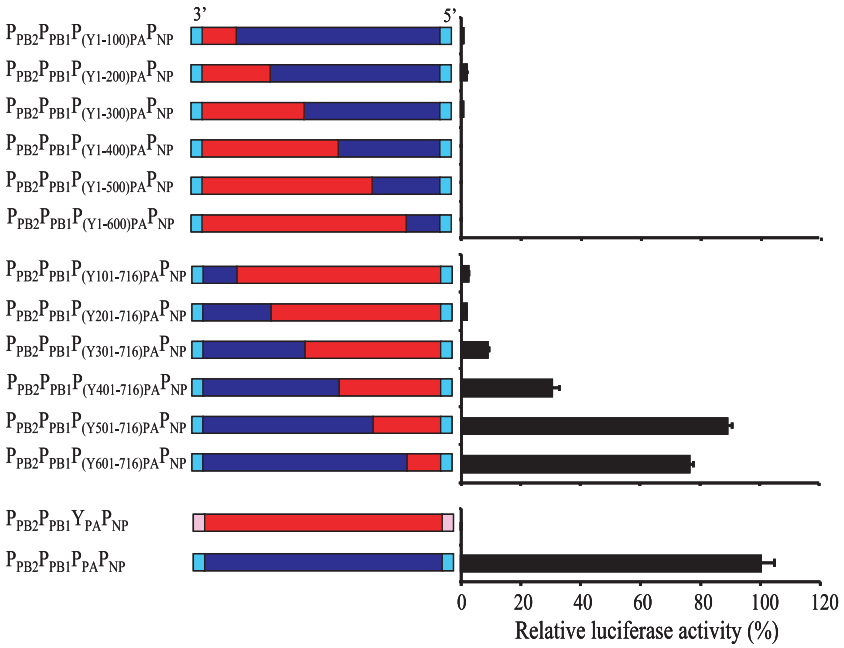

FIG. 3. Schematic diagram of Prague/Yokohama PA chimeras and their effects on RNP activities in a minigenome assay. The 12 PA chimeras were constructed as described in Materials and Methods. The noncoding regions of Prague and Yokohama PA proteins are represented by light blue and pink bars, respectively. The coding regions of Prague and Yokohama PA proteins are shown by blue and red bars, respectively. All constructs are shown in the negative-sense orientation. The RNP combination $P_{PB2}P_{PB1}P_{(Y)PA}P_{NP}$ [Prague PB2 and PB1, chimeric PA, and Prague NP; $P_{(Y)PA}$, chimera of Prague and Yokohama PA] was tested in the minigenome assay described in Materials and Methods. The relative firefly luciferase activity was normalized to the *Renilla* luciferase activity, which served as the internal control. The activity of the RNP combination $P_{PB2}P_{PB1}P_{PA}P_{NP}$ was standardized to 100%. The error bars indicate the standard deviations for three independent experiments. P, Prague. Y, Yokohama. $P_{PB2}P_{PB1}P_{(Y1\text{-}100)PA}P_{NP}$, RNP combination with Prague PB2, Prague PB1, $Pr_{(Yok\ 1–100)}PA$, and Prague NP. Other RNP combinations were similarly named.

the rescued viruses and found mutations in the PB2 and/or PA gene but not in the PB1 and NP genes (Table 4). The majority of mutations occurred in viruses that were rescued only after a second passage in eggs (compare Tables 3 and 4), suggesting that the acquired mutations are critical for viral growth. The mutations were distributed evenly throughout the Prague PB2 protein, whereas the two PA mutations were localized to the introduced Yokohama portion in the PA chimeras. The D253N mutation appeared independently in the PB2 proteins of two chimeric PA viruses, Prague/$Pr_{(Yok\ 1–400)}$PA and Prague/$Pr_{(Yok\ 1–600)}$PA. We then evaluated the contributions of these mutations to the RNP activities in the minigenome assay (Fig. 4). The mutations were tested in the respective backgrounds in which they appeared; for example, the H432R mutation was found in the $Pr_{(Yok\ 201–716)}$PA protein, prompting us to compare the replicative ability of the $Pr_{(Yok\ 201–716)}$PA protein with that of the respective H432R mutant. The results showed that most of the single mutations, i.e., D253N, D256G, N540S, H432R, and T371I in PB2 and A448V in the chimeric PA protein, appreciably increased RNP activities. For four viruses [Prague/$Pr_{(Yok\ 1–500)}$PA, Prague/$Pr_{(Yok\ 1–600)}$PA, Prague/$Pr_{(Yok\ 101–716)}$PA, and Prague/$Pr_{(Yok\ 301–716)}$PA], two mutations were found in PB2 and one each was found in PB2 and PA, affording these viruses the ability to grow to detectable titers. However, we did not observe any apparent cumulative effect of mutations on RNP activities (Fig. 4). These data suggest that the mutations in the chimeric PA viruses played a compensatory role in virus viability by increasing the activity of the RNP complex and that PA and PB2 functionally interact.

TABLE 3. Rescue of Prague viruses with PA chimeras[a]

Virus[b]	Detection of virus in eggs	
	Passage 1	Passage 2
Prague/$Pr_{(Yok\ 1–100)}$PA	+	
Prague/$Pr_{(Yok\ 1–200)}$PA	+	
Prague/$Pr_{(Yok\ 1–300)}$PA	+	
Prague/$Pr_{(Yok\ 1–400)}$PA	+	
Prague/$Pr_{(Yok\ 1–500)}$PA	−	+
Prague/$Pr_{(Yok\ 1–600)}$PA	−	+
Prague/$Pr_{(Yok\ 101–716)}$PA	−	+
Prague/$Pr_{(Yok\ 201–716)}$PA	−	+
Prague/$Pr_{(Yok\ 301–716)}$PA	−	+
Prague/$Pr_{(Yok\ 401–716)}$PA	+	
Prague/$Pr_{(Yok\ 501–716)}$PA	+	
Prague/$Pr_{(Yok\ 601–716)}$PA	+	
Prague/Yok PA[c]	−	−

[a] Prague viruses possessing chimeric PA genes were generated as described in Materials and Methods. The transfection supernatant was inoculated into the allantoic cavities of 10-day-old chicken embryonated eggs. The presence of virus was confirmed by HA assay. A second passage in eggs was performed for those viruses that could not be detected after the first passage in eggs. +, virus was detected by HA assay; −, virus was not detected by HA assay.

[b] The Prague viruses with PA chimeras were named according to the chimeric PAs they contained.

[c] Single-gene reassortant Prague virus containing Yokohama PA.

DISCUSSION

In this study, we attempted to identify restricting factors for reassortment between two genetically diverse influenza A

TABLE 4. Amino acid mutations in the RNP complexes of Prague viruses possessing chimeric PA genes[a]

Virus	Presence of PB2 mutation								Presence of PA mutation	
	D253N	D256G	S279L	T371I	N425T	H432R	N540S	N677T	N444S	A448V
Prague/$Pr_{(Yok\ 1–100)}$PA										
Prague/$Pr_{(Yok\ 1–200)}$PA										
Prague/$Pr_{(Yok\ 1–300)}$PA										
Prague/$Pr_{(Yok\ 1–400)}$PA	+									
Prague/$Pr_{(Yok\ 1–500)}$PA		+						+		
Prague/$Pr_{(Yok\ 1–600)}$PA	+		+							
Prague/$Pr_{(Yok\ 101–716)}$PA					+		+			
Prague/$Pr_{(Yok\ 201–716)}$PA						+				
Prague/$Pr_{(Yok\ 301–716)}$PA				+						+
Prague/$Pr_{(Yok\ 401–716)}$PA									+	
Prague/$Pr_{(Yok\ 501–716)}$PA										
Prague/$Pr_{(Yok\ 601–716)}$PA										

[a] The allantoic fluid of Prague viruses possessing chimeric PA genes was subjected to reverse transcription-PCR, and the PB2, PB1, PA, and NP genes were fully sequenced. The mutations found in the RNP complexes of the Prague chimeric PA viruses are shown. +, mutation present.

viruses, A/equine/Prague/1/56 (H7N7) and A/Yokohama/2017/03 (H3N2). To generate the reassortant viruses, we used cotransfection rather than coinfection of cells because the potential for differences in replicative ability between the two test viruses could have resulted in unequal numbers of gene segments of the two viruses, which would have obscured our results. We found that among 256 possible reassortants, only 29 genotypes (including wild-type Yokohama but not wild-type Prague virus) were identified. Analyses of recovered reassortants suggested that the formation of functional RNP was restricted by and dependent on the combination of proteins from these two viruses. In fact, activity tests of the RNP complexes composed of different proteins from the two viruses in a minigenome assay showed that all RNP combinations found in the recovered reassortants had higher activity than that of the parental Prague RNP, while RNP combinations not found in the reassortants had <40% of the activity of parental Prague RNP, with one exception. These results suggested that the formation of a functional RNP complex is a prerequisite for reassortment between the two influenza A viruses tested in this study under the conditions used.

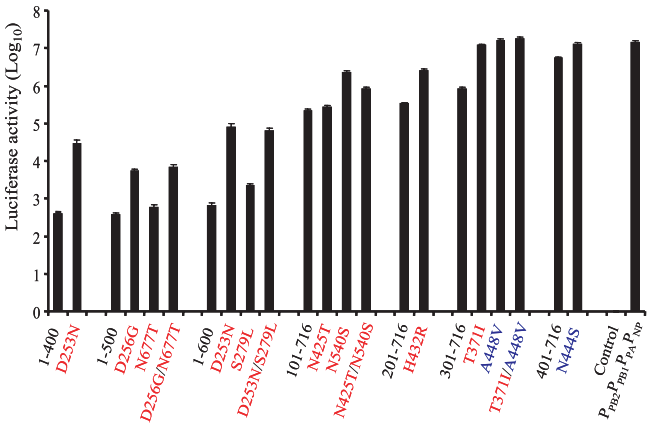

FIG. 4. Activities of $P_{PB2}P_{PB1}P_{(Y)PA}P_{NP}$ RNP combinations possessing mutations in PB2 or the chimeric PA. Four protein expression plasmids (0.5 μg each), including one for Prague PB2 (or mutant PB2), Prague PB1, PA chimera (or mutant PA chimera), and Prague NP, were transfected into 293 cells together with pPolWSNNA F-Luc. At 48 h posttransfection, luciferase assay was performed with a dual-luciferase kit. Firefly luciferase activity is shown as log values. 1-400, RNP combination with Prague PB2, Prague PB1, $Pr_{(Yok\ 1–400)}$PA, and Prague NP; D253N, RNP combination with Prague PB2/D253N, Prague PB1, $Pr_{(Yok\ 1–400)}$PA, and Prague NP. Other RNP combinations were similarly named. Mutations in the PB2 gene are shown in red; those in the PA gene are shown in blue. P, Prague.

We found that the three polymerase subunits Prague PB2, Prague PB1, and Yokohama PA are unable to form a heterotrimeric complex, suggesting that they are structurally incompatible. We constructed 12 PA chimeras between Prague and Yokohama PA proteins and found that amino acid mutations in PB2 and/or chimeric PA proteins increased the activity of the RNP complex, which may have played a compensatory role in the rescue of viruses possessing chimeric PA proteins. These results suggested that RNP complex activity is an important restricting factor for influenza virus reassortment, affecting the viability and/or competitiveness of the reassortant viruses.

The polymerase proteins play important roles in the pathogenicity of influenza A viruses in mammalian species. The human-like amino acid PB2 627K was a principal determinant of high virulence for the 1997 Hong Kong H5N1 influenza viruses (17, 43) and confers on avian H5N1 viruses the advantage of efficient growth in mammals (19). Similarly, the polymerase complex genes for PB2 and PB1 contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04 in mice and ferrets (41). Recently, it was reported that differences in viral transcription and replication levels between mammalian and avian cells are determinants of both host specificity and pathogenicity of an H7N7 virus (10). In our study, we compared the activities of the 16 possible RNP combinations between Prague and Yokohama viruses and found that the activities varied significantly. It is noteworthy that the four RNP combinations containing Prague PB2 and Yokohama PA were either nonfunctional ($P_{PB2}P_{PB1}Y_{PA}P_{NP}$, $P_{PB2}P_{PB1}Y_{PA}Y_{NP}$, and $P_{PB2}Y_{PB1}Y_{PA}Y_{NP}$) or significantly impaired in their activity ($P_{PB2}Y_{PB1}Y_{PA}P_{NP}$). Thus, we have identified an interdependence between PB2 and PA in which Prague PB2 cannot cooperate well with Yokohama PA. The three polymerase subunits, PB2, PB1, and PA, form a compact complex in

order to function in viral replication and transcription (45). Fodor et al. proposed that PB1 and PA are transported into the nucleus as a dimer and that PB2 enters the nucleus as a monomer, where it assembles with the PB1-PA dimer (8). Taking into consideration that there are no direct physical interactions between PB2 and PA (12, 35, 45), our data might suggest that the dimer formation between Yokohama PA and Prague or Yokohama PB1 triggers a conformational change that is not suitable to accommodate Prague PB2.

Hara et al. reported that PA has two distinct domains, one extending from amino acids 1 to 212 and the other extending from amino acid 212 to the C terminus (14). By using the approach of alanine-scanning mutagenesis, they suggested that the N-terminal region of PA is involved in multiple functions of the polymerase, including protein stability, endonuclease activity, cap binding, and promoter binding. Here we constructed 12 chimeras between Prague and Yokohama PA proteins. Our results demonstrated that the N-terminal region of PA, especially the region of amino acids 1 to 400, is critical for the formation of a functional RNP complex. We demonstrated that the hybrid polymerase complex $P_{PB2}P_{PB1}Y_{PA}$ is not functional in replication and/or transcription of the virus-like RNA and that the loss of function is likely due to the inability of these RNA polymerase subunits to form a heterotrimeric complex. The amino acid mutations that occurred in the PB2 and/or PA segment of the chimeric PA viruses apparently play some compensatory role during virus adaptation. Among the mutations present in PB2, the D253N, D256G, T371I, and N540S mutations are located in the proposed cap-binding sites of the PB2 protein (7, 22). The D253N and D256G mutations lie in the N-terminal region of PB2 responsible for PB1 and NP binding (38). The enhancing effect on the luciferase activity of these mutations may be due to their influence on the cap-binding activity or on the formation of functional RNP complexes. The two mutations found in the chimeric PA segment do not localize to known functional domains of the PA molecule. The compensatory mutations found in the PB2 protein are reminiscent of those found in the polymerase proteins of revertants of temperature-sensitive mutants of A/FPV/Rostock/34 virus (29). These findings suggest that suboptimal RNP complexes in reassortants can become fully functional by acquiring mutations in the RNP complex proteins, an event that likely happens during reassortment between human and avian viruses, potentially resulting in the generation of a pandemic strain.

Our data show that the formation of functional RNP complexes is a restricting factor for the generation of reassortants between Prague and Yokohama viruses. However, genes other than those involved in the RNP complex may also restrict this event. Among them, HA is a likely candidate, given that Yokohama HA was dominant in the progeny viruses. The Prague virus grows poorly in MDCK cells but can grow efficiently in eggs. A Yokohama variant possessing Prague HA also grew poorly in MDCK cells (data not shown). Together, these data suggest that Prague HA is another factor restricting efficient virus growth and possibly the reassortment between these two viruses. It is well established that the HA protein plays an important role in the host range restriction of influenza viruses (11, 32, 46). Equine viruses preferentially recognize *N*-glycolyl and *N*-acetyl sialic acid linked to galactose by the α-2,3 linkage (NeuGcα2,3Gal), whereas human viruses bind to NeuAcα2,6Gal (40, 44). Since MDCK cells express these receptors (23), the mechanism by which Prague HA may restrict growth is unclear. It may be that receptor binding is not a restriction factor; rather, later steps, such as fusion or uncoating, may be responsible for the restriction.

It is striking that $Y_{PB2}Y_{PB1}Y_{PA}P_{NP}$ was the most efficient combination of the RNP complex in the reassortant viruses, even though it displayed less RNP activity than $Y_{PB2}Y_{PB1}Y_{PA}Y_{NP}$ in the luciferase assay. The reason for this phenomenon is not clear. The primary function of NP is to encapsidate the virus genome for RNA transcription, replication, and packaging. To fulfill this function, NP interacts with the virus RNA, itself, M1, two subunits of the polymerase (PB2 and PB1), and cellular components, including those of the nuclear import and export apparatus (39, 49). The predominance of Prague NP (105 of 120 reassortants had Prague NP) in the progeny viruses might reflect its advantage not in transcription and replication but in packaging or transport. Correlating with the predominance of Prague NP, the M gene of the reassortant viruses originated mainly from the Prague virus (89 of 120 reassortants). M1 regulates RNP nuclear export (2, 30). It enters the nucleus after synthesis late in the virus replication cycle and interacts with RNPs by binding with NP (34). The viral NEP protein (formerly known as NS2) then binds to M1 (1, 48) and links this complex of proteins with the cellular nuclear export protein CRM1, which mediates RNP export (6, 31, 36). The apparent association of the Prague NP and M segments may originate from their functional interactions as described above.

In conclusion, we identified compatibility among the RNP complex proteins as a crucial restricting factor for reassortment between the two influenza viruses tested. Given that reassortment is an important driving force for influenza virus evolution, a complete understanding of the factors that restrict the reassortment process will provide new insights into the evolution of influenza virus and improve our ability to control influenza pandemics and epidemics in the future.

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