Co-incorporation of the PB2 and PA polymerase subunits from human H3N2 influenza virus is a critical determinant of the replication of reassortant ribonucleoprotein complexes

Koyu Hara, Yoko Nakazono, Takahito Kashiwagi, Nobuyuki Hamada and Hiroshi Watanabe

Division of Infectious Diseases, Department of Infectious Medicine, Kurume University School of Medicine, Fukuoka, Japan

Received 2 April 2013
Accepted 9 August 2013

The influenza virus RNA polymerase, composed of the PB1, PB2 and PA subunits, has a potential role in influencing genetic reassortment. Recent studies on the reassortment of human H3N2 strains suggest that the co-incorporation of PB2 and PA from the same H3N2 strain appears to be important for efficient virus replication; however, the underlying mechanism remains unclear. Here, we reconstituted reassortant ribonucleoprotein (RNP) complexes and demonstrated that the RNP activity was severely impaired when the PA subunit of H3N2 strain A/NT/60/1968 (NT PA) was introduced into H1N1 or H5N1 polymerase. The NT PA did not affect the correct assembly of the polymerase trimeric complex, but it significantly reduced replication-initiation activity when provided with a vRNA promoter and severely impaired the accumulation of RNP, which led to the loss of RNP activity. Mutational analysis demonstrated that PA residues 184N and 383N were the major determinants of the inhibitory effect of NT PA and 184N/383N sequences were unique to human H3N2 strains. Significantly, NT PB2 specifically relieved the inhibitory effect of NT PA, and the PB2 residue 627K played a key role. Our results suggest that PB2 from the same H3N2 strain might be required for overcoming the inhibitory effect of H3N2 PA in the genetic reassortment of influenza virus.

INTRODUCTION

The genome of influenza A virus is composed of eight segments of negative-sense, ssRNA (Palese & Shaw, 2007). This segmented genome structure facilitates genetic reassortment with other influenza strains that co-infect the same cells, and has played a pivotal role in the emergence of influenza pandemics. The pandemic strains of the past century have incorporated the avian genes expressing the superficial haemagglutinin and neuraminidase (NA) glycoproteins, giving rise to new subtypes with novel surface antigens. Besides the introduction of genes for superficial glycoproteins, at least one internal gene of RNA polymerase from the avian strains has been concurrently incorporated into human strains [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009; Itoh et al., 2009; Kawaoka et al., 1989]. Recent studies have suggested that the RNA polymerase has a potential role in the generation of reassortant viruses. The introduction of avian PB2 into human strains has been observed and is known to produce reassortant viruses (Chen et al., 2008; Li et al., 2010), suggesting that the avian PB2 may interfere with human polymerase during genetic reassortment (Nakazono et al., 2012). Co-incorporation of PB2 and PA from the same virus of origin appears to be important for efficient virus replication (de Wit et al., 2010; Labadie et al., 2007; Treanor et al., 1994), particularly in the reassortment between human H3N2 strains and other influenza strains (Chen et al., 2008; Li et al., 2010). Interestingly, the 1968 H3N2 (Hong Kong flu) strain simultaneously incorporated PB2 and PA genes from the 1957 H2N2 (Asian flu) strain, which was originally derived from the 1918 H1N1 strain (Spanish flu) (Fig. 1a) (Wright et al., 2007). Similarly, natural-reassortant H3N2 viruses isolated from swine possess PB2 and PA genes from the same virus of origin (Fan et al., 2012; Olsen, 2002). Simultaneous incorporation of PB2 and PA was also found in the 2009 H1N1 strain [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009; Itoh et al., 2009; Peiris et al., 2009]. These observations imply the importance of functional cooperation between PB2 and PA in the generation of viable reassortant viruses. However, the mechanism of this phenomenon remains poorly understood. In this study, we investigated the molecular basis of the relationship between the PB2 and PA subunits from a human H3N2 strain during genetic reassortment. We
reassorted the H3N2 polymerase with subunits from other influenza strains and reconstituted the influenza ribonucleoprotein (RNP) in 293T cells. Our data showed that H3N2 PA had a significant inhibitory effect on RNP activity when introduced into the polymerases of other influenza strains. The inhibitory effect was specifically relieved by H3N2 PB2, suggesting that co-incorporation of PB2 and PA subunits from the same H3N2 strain might be a prerequisite for generation of viable reassortant viruses.

**RESULTS**

**H3N2 PA impairs RNP activity when reconstituted with H1N1 or H5N1 polymerase**

Previously, we have shown that the PA subunit of H3N2 strain A/NT/60/1968 (NT PA) severely impaired RNP activity when introduced into the human-isolated H5N1 strain A/HongKong/156/1997 (HK) (Fig. 2, lane 20) or the closely related A/Vietnam/1194/2004 (VN) (Nakazono et al., 2012). To generalize this finding to other influenza strains, we introduced NT PA into two human H1N1 strains, A/WSN/1933 (WSN) and A/Kurume/K0910/2009 (SW). The RNP-containing hybrid polymerase was reconstituted in human 293T cells in a background of WSN-derived nucleoprotein (NP) and NA vRNA, and the steady-state levels of NA reporter mRNA, vRNA and cRNA were measured by primer extension. The levels of all three RNA species were reduced significantly, or decreased to essentially inactive levels when NT PA alone was introduced into the WSN or SW polymerase, respectively [20 ± 2.9 % of mRNA in WSN (mean ± SD, n = 3), 7 ± 2.8 % of mRNA in SW; Fig. 2, lanes 4 and 12]. This result provides further support for the inhibitory effect of NT PA on the reassortment of the polymerase. It is also noteworthy that this impaired RNP activity appears to be restored slightly (24 ± 14.4 % of mRNA in WSN; Fig. 2, compare lane 6 with 4), or significantly (69 ± 23.2 % of mRNA in SW, 118 ± 11.6 % of mRNA in HK; Fig. 2, compare lane 14 with 12, and lane 22 with 20, respectively) when the PB2 was replaced with the NT PB2. This suggests that the co-incorporation of NT PB2 and NT PA is important for the reassortent RNP activity. In addition, the NT PB2 alone appears to enhance the RNP activity when reconstituted with SW (180 ± 26.7 % of mRNA; Fig. 2, compare lane 11 with 9) or HK (148 ± 53.4 % of mRNA; Fig. 2, compare lane 19 with 17) polymerase.

**Impaired RNP activity is rescued by mutations at positions 55, 86, 184 and 383 in the NT PA**

To identify the amino acid residues of NT PA responsible for the impaired RNP activity, we aligned the PA sequences of four influenza strains and found 15 amino acids that differ only in NT PA (Fig. 3a). We speculated that substitutions of these amino acids in NT PA to the sequences conserved in the other three strains might rescue the impaired RNP activity. In particular, we focused on the N-terminal region of PA (55–404), because several functional domains have been mapped (Dias et al., 2009; Hara et al., 2009). We included positions 86, 91, 114, 277 and 388, because these positions are highly conserved in two or three strains. We excluded positions 85, 87, 115, 127, 148 and 389, because these positions are highly conserved in two or three strains. We excluded positions 385 and 268, due to the similar basic (R and K) amino acid or complete loss of RNP activity by mutation, respectively. The single mutations D66G, N142K, F277S and Y321N did not rescue the impaired RNP activity. In particular, we focused on the N-terminal region of PA (35–404), because several functional domains have been mapped (Dias et al., 2009; Hara et al., 2009). We included positions 86, 91, 114, 277 and 388, because these positions are highly conserved in two or three strains. We excluded positions 85, 87, 115, 127, 148 and 389, because these positions are highly conserved in two or three strains. We excluded positions 385 and 268, due to the similar basic (R and K) amino acid or complete loss of RNP activity by mutation, respectively. The single mutations D66G, N142K, F277S and Y321N did not rescue the impaired RNP activity (Fig. 3b, lanes 4–7). However, the single mutation N383D showed a slight increase in the RNP activity (40 ± 5.7 % of mRNA, 13 ± 2.3 % of cRNA and 23 ± 5.6 % of vRNA) when compared with NT PA WT (20 ± 2.9 % of mRNA, 6.7 ± 3.8 % of cRNA and 12 ± 2.3 % of vRNA; Fig. 3b, compare lane 8 with 3). Although the RNP activity was decreased again by the additional mutation E114K (Fig. 3b, compare lane 14 with 12), the RNP activity was gradually increased by the additional mutations N55D (38 ± 7.0 % of mRNA, 7.6 ± 2.0 % of cRNA and 17 ± 8.0 % of vRNA) and M86I (59 ± 4.5 % of mRNA, 24 ± 3.0 % of cRNA and...
We further asked whether the substitution at four positions, 55, 86, 184 and 383, in the NT PA are responsible for the impaired RNP activity. This finding was further supported by the observations that the NT PA carrying mutations only at positions 55, 86, 184 and 383 showed a significant RNP activity (87 ± 1.7 % of mRNA, 56 ± 8.2 % of cRNA and 66 ± 3.4 % of vRNA; Fig. 3b, lane 23), comparable to that of sevenfold mutant (N55D/M86I/V91I/E114K/N184S/N383D) (Fig. 3b, compare lane 3 with 22). In addition, mutation at only two positions, 184 and 383, significantly rescued the impaired RNP activity (50 ± 11.0 % of mRNA, 15 ± 7.3 % of cRNA and 25 ± 5.7 % of vRNA; Fig. 3b, lane 25).

We further asked whether the substitution at four positions, 55, 86, 184 and 383, in WSN PA or HK PA with the NT PA sequence would impair RNP activity. In the case of WSN PA, there was only a slight effect on the RNP activity by a single mutation at positions 86, 184 and 383 (Fig. 3c, compare lanes 3, 4, 5 with 6). However, the RNP activity was severely reduced by a double mutation at positions 184 and 383 (66 ± 7.9 % of mRNA, 32 ± 7.1 % of cRNA and 41 ± 3.0 % of vRNA; Fig. 3c, compare lane 6 with 2), and further decreased by the additional mutation at positions 55 (33 ± 3.2 % of mRNA, 11 ± 1.9 % of cRNA and 13 ± 2.6 % of vRNA; Fig. 3c, compare lane 7 with 6) and 86 (22 ± 5.4 % of mRNA, 5.3 ± 2.1 % of cRNA and 7.8 ± 1.3 % of vRNA; Fig. 3c, compare lane 8 with 7), comparable to that of NT PA (Fig. 3b, lane 3). In the case of HK PA, we did not mutate position 86, because this residue is identical to that of NT PA (Fig. 3a).

The RNP activity was markedly reduced to almost inactive levels by double mutation at positions 184 and 383 or by triple mutation at positions 55, 184 and 383 (Fig. 3c, lanes 12 or 13, respectively). Taken together, we suggest that four positions, 55, 86, 184 and 383, in NT PA are involved in the optimal activity of the reassortant polymerase. In particular, two positions, 184 and 383, are the major determinants.

### NT PA impairs the replication-initiation activity and accumulation of the RNP

To address the reason for the functional loss of RNP activity by NT PA, we examined: (i) the assembly of the trimeric complex of PB1, PB2 and PA; (ii) the in vitro polymerase activities; and (iii) the RNP assembly. We partially purified the reassortant polymerase or RNP using C-terminally tandem-affinity purification (TAP)-tagged PB2. In the NT PA reassortant polymerase, the amount of PB2-TAP and PB1-PA co-purifying with PB2-TAP were comparable to those of the WSN polymerase (Fig. 4a, left panel, compare lane 2 with 1). Similar levels of the three subunits were observed in the NT PA mutant (Fig. 4a, left panel, lane 3) that possessed significant RNP activity (Fig. 3b, lane 18). This indicates that the NT PA does not appear to impair the correct assembly of the trimeric complex. Interestingly, the NT PA WT migrated faster than WSN PA (Fig. 4a, left panel, compare lane 2 with 1), as observed before (Kashiwagi et al., 2008) and thus these bands in our conditions (Maier et al., 2009). However, the NT PA mutations caused a decrease in its migration rate (Fig. 4a, left panel, lane 3).

Using partially purified polymerase, we evaluated the promoter-binding activity by a UV cross-linking assay and the replication-initiation activity by the dinucleotide ApG-synthesis assay. In a UV cross-linking analysis, we cannot separate the UV cross-linked PA and PB2-TAP bands in our conditions (Maier et al., 2008) and thus these were assigned as PB2/PA (Fig. 4a, middle panel), even...
though the PA subunit migrated slightly faster or slower on the silver-stained gel. Regardless of the defect in RNP activity, the NT PA reassortant polymerase showed enhanced binding to the vRNA promoter (146% of WSN polymerase), and significant binding to the cRNA promoter comparable to that of WSN polymerase (Fig. 4a, middle panel, lane 2). The binding to the cRNA promoter was significantly reduced to 55% of WSN polymerase in the NT PA mutant (Fig. 4a, middle panel, lane 3), while significant RNP activity was retained (Fig. 3b, lane 18).

Fig. 3. Amino acids responsible for the inhibitory effect of NT PA. (a) Amino acids in NT PA that completely or partially differ from those of WSN, SW or HK were systematically substituted for the WSN PA sequences. n.t., Not tested. (b) The RNP activity which was reconstituted from WSN (W) hybrid polymerase by replacing PA subunit with NT PA WT (N) (lanes 3, 12 and 21) or NT PA mutants (lanes 4–10, 13–19, 22–25). W mut, WSN PB1 with mutation D446Y at the SDD motif of the catalytic site (lane 27). (c) Effects of mutations in WSN PA (lanes 2–8) or HK (H) PA (lanes 10–13) on the RNP activity. The RNP was reconstituted in a background of WSN NP and NA vRNA. The amino acid sequences of WSN PA WT, NT PA WT and HK PA WT are indicated on the left. The numbers indicate the mutated positions in PA.
The NT PA did not largely affect the replication-initiation activity on the cRNA template (92% of WSN PA; Fig. 4a, right panel, lane 2). Notably, however, a remarkable reduction of activity was observed on the vRNA template (47% of WSN polymerase; Fig. 4a, right panel, lane 2), suggesting that the NT PA specifically reduces vRNA synthesis. Nonetheless, the NT PA reassortant polymerase possessed a strong activity to bind the vRNA promoter (146% of WSN PA). Excessive binding to the promoter might impair the promoter clearance, which leads to the abortive initiation of RNA synthesis as previously suggested (Kashiwagi et al., 2009). The reduced replication-initiation activity on the vRNA template was restored to 81% of WSN polymerase in the NT PA mutant (Fig. 4a, right panel, lane 3).

Because we have previously shown that the defect in RNP accumulation leads to the loss of RNP activity (Nakazono et al., 2012), we purified reconstituted RNP using TAP-tagged PB2 and quantified the amount of RNP by Western blotting with NP-specific antibodies. Remarkably, the NT PA reduced the NP signal in the TAP-purified material to 43% of that achieved with WSN RNP (Fig. 4b, lane 2), indicating a significant reduction of RNP accumulation.
Nonetheless, the polymerase trimeric complex was properly formed (Fig. 4b, middle top panel, lane 2) and the overall expression of NP was comparable to that of WSN (Fig. 4b, middle bottom panel, lane 2). The reduced accumulation of RNP was rescued to 69 % and 90 % in the NT PA sixfold mutant and fourfold mutant, respectively (Fig. 4b, middle panel, lanes 3 and 4, respectively). Taken together, these results suggest that the loss of RNP activity was due to a significant reduction of polymerase activity in replication and also to a defect in the accumulation of RNP.

The NP signal was significantly decreased in the NT PA reassortant RNP, but still obtained to some extent (43 %). This suggests the presence of partially formed RNP. It was also possible that the NP signal might represent NP co-purified with polymerase, because NP binds to PB1 and PB2 (Biswas et al., 1998; Poole et al., 2004). To test this, WSN polymerase and NP was expressed in the absence of vRNA (Fig. 4b, lane 5). In addition, we constructed WSN PB1 carrying the D446Y mutation (Biswas & Nayak, 1994; Cauldwell et al., 2013) in the SDD motif that was catalytically inactive (Fig. 3b, lane 27). When the RNP is reconstituted with the PB1 D446Y mutant, the polymerase and NP could be expressed normally, and vRNA could be transcribed by cellular RNA polymerase I from the input pPOLI plasmid and then assembled into RNP, which could not be amplified. TAP-purified material both in the absence of vRNA and in the PB1 D446Y mutant showed the level of NP signal not significantly different from NT PA (55.2 ± 1.8 %, 49.4 ± 9.8 % and 40.9 ± 7.7 %, respectively; Fig. 4b, right top panel, compare lanes 5 and 6 with 2). The overall expression level of NP was not affected in the absence of vRNA or the PB1 D446Y mutant (Fig. 4b, right bottom panel, compare lanes 5 and 6 with 2). These results suggest that the NP signal observed in the NT PA reassortant RNP might primarily represent the NP co-purified with polymerase.

The NT PA inhibitory effect is specifically relieved by NT PB2

We initially observed that the inhibitory effect of the NT PA was relieved by NT PB2 (Fig. 2), suggesting that the co-incorporation of PB2 and PA from the same NT strain was important for polymerase activity. To confirm this, we tested the effect of PB2s from other strains on RNP activity. The impaired RNP activity was not restored by PB2 from WSN or SW (Fig. 5a, lanes 3 and 4), and only marginally restored by VN PB2 in a background of HK polymerase (Fig. 5a, lane 5). In contrast, the RNP activity was significantly restored by NT PB2 (Fig. 5a, lane 6), supporting the requirement for the specific combination of NT PB2 and NT PA for significant RNP activity.

We expected that a significant RNP activity achieved by NT PB2-NT PA would be impaired by mutation in an NT PB2 amino acid which differs from those in other strains. An alignment of PB2 sequences shows that the NT PB2 amino acid sequence differs at positions 41, 34, 35 and 40 from those of HK, VN, WSN and SW, respectively. Among them, we focused on 20 positions (Fig. 5b). First, ten amino acids (44, 67, 82, 338, 344, 358, 382, 394, 463 and 676) that differ only in NT strains were mutated to the sequence conserved in the other four strains. Secondly, five residues (613, 627, 674, 684 and 717) that differ from those in the HK PB2 C-terminal region were mutated to the HK PB2 sequence, since the PB2 C-terminal domain contains important determinants for host adaptation, including residue 627. Thirdly, another five residues (271, 292, 478, 655 and 667) were mutated to the sequence conserved in VN and WSN. The RNP activity was not affected by the serial mutations at positions 44, 67, 82, 338, 344, 358, 382, 394, 463, 676 and 684 (Fig. 5b, lanes 5–9, 11–15 and 17–18). However, the additional mutation K627E markedly reduced the RNP activity to undetectable levels (Fig. 5b, lane 19). This reduced activity was not further affected by additional mutations at positions 674, 717 and 613 (Fig. 5b, lanes 20–22). Importantly, the single mutation K627E dramatically decreased RNP activity (Fig. 6, lane 2), suggesting that the NT PB2 627E is sufficient to impair the RNP activity. The loss of RNP activity was also observed by the K627E mutation in VN PB2 (Fig. 5c, compare lane 7 with 6). Moreover, we tested the effect of mutations except for mutation at position 627. However, in the presence of residue 627K, the serial mutations at positions 44, 67, 82, 338, 344, 358, 382, 394, 463, 676, 684, 674, 717, 613, 271, 292, 478, 655 and 667 did not reduce the RNP activity at all (Fig. 5b, lanes 25–31 and 33–34). Overall, we suggest that PB2 627K plays a pivotal role in overcoming the inhibitory effect of NT PA. This was further supported by the observation that the impaired RNP activity was significantly rescued by E627K mutation in HK PB2 and SW PB2 (Fig. 5c, compare lane 3 with 2 and lane 5 with 4).

The combination of NT PB2 627K and NT PA 184N/383N determines RNP activity

We demonstrated that two positions, 184N and 383N, were the major determinants of the inhibitory effect of NT PA, and the NT PB2 627K was involved in overcoming the inhibitory effect. Amino acid sequences PA 184N and 383N are unique to human H3N2 strains. These findings raise the possibility that the combination of three residues, PA 184N and 383N, and PB2 627K, might be critical for the RNP activity. To test this, we constructed each single mutant NT PB2 K627E, NT PA N184S and N383D, and double mutant NT PA N184S/N383D. The RNP activity was severely impaired by a single 627E mutation in the NT PB2 (Fig. 6, lane 2). However, the impaired RNP activity was partially rescued by a single 184S or 383D mutation in the NT PA (Fig. 6, lanes 3 or 4), and almost fully recovered by a double 184S/383D mutation in the NT PA (95 ± 11.3 % of mRNA, 98 ± 23.4 % of cRNA and 91 ± 17.2 % of vRNA; Fig. 6, lane 5). This suggests that PA 184S/383D are unique to human H3N2 strains. These findings raise the possibility that the combination of three residues, PA 184N/383N determines RNP activity

The combination of NT PB2 627K and NT PA 184N/383N determines RNP activity

We demonstrated that two positions, 184N and 383N, were the major determinants of the inhibitory effect of NT PA, and the NT PB2 627K was involved in overcoming the inhibitory effect. Amino acid sequences PA 184N and 383N are unique to human H3N2 strains. These findings raise the possibility that the combination of three residues, PA 184N and 383N, and PB2 627K, might be critical for the RNP activity. To test this, we constructed each single mutant NT PB2 K627E, NT PA N184S and N383D, and double mutant NT PA N184S/N383D. The RNP activity was severely impaired by a single 627E mutation in the NT PB2 (Fig. 6, lane 2). However, the impaired RNP activity was partially rescued by a single 184S or 383D mutation in the NT PA (Fig. 6, lanes 3 or 4), and almost fully recovered by a double 184S/383D mutation in the NT PA (95 ± 11.3 % of mRNA, 98 ± 23.4 % of cRNA and 91 ± 17.2 % of vRNA; Fig. 6, lane 5). This suggests that PA 184S/383D are involved in overcoming the impaired RNP activity caused by PB2 627E. On the other hand, PB2 627K showed a significant RNP activity in all NT PA mutants (Fig. 6, lanes 6–8), and the highest activity was observed in the presence of PA 184S/
Fig. 5. The inhibitory effect of NT PA is relieved by NT PB2. (a) Effects of PB2 from WSN, SW, HK or VN on the impaired RNP activity. (b) Alignment of amino acid residues selected for mutations of NT PB2 and the effects of mutations in NT PB2 (lanes 5–9, 11–15, 17–22, 24–31, 33–34) on the impaired RNP activity. *, Number of amino acid differences to NT PB2. The PB2 amino acid sequences of NT, HK, WSN and VN are indicated on the left. (c) Effects of mutation at position 627 in HK PB2, SW PB2 and VN PB2 on the RNP activity. The RNP was reconstituted from hybrid polymerase in a background of WSN NP and NA + mRNA in 293T cells, and the RNP activity was measured by primer extension. The numbers in the NT PB2 mutants indicate mutated positions. W, WSN; N, NT; H, HK; V, VN; S, SW.

Recent studies suggest that the co-incorporation of PB2 and PA from the same host origin might be important for the efficient replication of reassortant H3N2 influenza virus. We initially demonstrated that H3N2 PA (A/NT/60/1968) severely impaired the RNP activity when reassorted with H1N1 or H5N1 polymerase. This might be supported by the observation that the introduction of H3N2 PA (A/Yokohama/2017/2003) into H7N7 polymerase (A/equine/Prague/1956) resulted in the loss of RNP activity (Li et al., 2011). We suggest that H3N2 PA plays a key role in the generation of viable reassortant viruses. However, we
cannot rule out the possible contribution of NP to the reassortment (Bean, 1984; Li et al., 2010; Scholtissek et al., 1985; Snyder et al., 1987).

We found that four residues, 55N, 86M, 184N and 383N, in the NT PA were responsible for the inhibitory effect of NT PA. Among them, 184N and 383N were the major determinants and their sequences were found to be unique to human H3N2 strains. Almost all of the human H3N2 strains possess 184N/383N (100% of 100 strains), whereas most of the avian H1N1, avian H3N2 or human H1N1 strains possess 184S/383D (98% of 410 strains; Fig. 1b). The PA 184S/383D sequence from avian strains was probably incorporated into the 1918 H1N1 strain (Taubenberger et al., 2005) and has been maintained in all human H1N1 strains. The PA 184S/383D sequence was also inherited by the 1957 H2N2 strain. Interestingly, however, the S184N/D383N mutation appeared to occur in the late 1950s in human H2N2 strains and became fixed in the 1960s (97% of 38 strains). The resulting 184N/383N sequence has been inherited by the 1968 H3N2 strain and maintained over 40 years. While the significance of acquiring PA residues 184N/383N for the adaptation of influenza viruses to humans is not clear, we provide evidence suggesting that PA residues 184N/383N play a key role in the generation of viable H3N2 reassortant viruses.

Asparagine has a structural role in the formation of hydrogen bonds with the peptide backbone near the beginning or end of alpha-helices (Richardson & Richardson, 1989). Acquiring asparagine at positions 184 and 383 in the H3N2 PA may induce structural changes in PA, thereby affecting the assembly of functional RNP. Position 184 is located at the end of helix α6 (Dias et al., 2009; Yuan et al., 2009) and may be linked to the cRNA promoter-binding region: positions 163–178 (Maier et al., 2008). Alternatively, position 184 may be related to position 186, which is involved to some extent in the polymerase activity of A/California/04/2009 (H1N1) at higher temperatures (Bussey et al., 2011). Position 383 is close to positions 349 and 336, which are associated with the polymerase activity and virulence of A/Puerto Rico/8/1934 (H1N1) and A/California/04/2009 (H1N1), respectively (Bussey et al., 2011; Rolling et al., 2009). In addition, positions 55, 86 and 184 are located in the PA-X, which has recently been identified as a second protein, produced from the PA gene by ribosomal frame-shifting (Jagger et al., 2012). PA-X comprises the N-terminal 191 aa of PA fused to 61 aa derived from the +1 ORF. It is unlikely that three residues at positions 55, 86 and 184 affect the function of PA-X, because the putative frame-shift signal of UCC UUU CGU C (PA codons 190–193) is found in all strains used in this study.

We demonstrated that the specific combination of PB2 627K and PA 184N/383N resulted in the loss of RNP activity. However, this defect was overcome by replacement with PB2 627K or PA 184S/383D. This suggests that PB2 627 and PA 184/383 might compensate each other by individually improving the polymerase activity. The compensatory effect of PB2 and PA might be supported by the observations indicating a functional relationship between PB2 and PA (Chen et al., 2008; de Wit et al., 2010; Labadie et al., 2007; Li et al., 2008; Mehle et al., 2012; Treanor et al., 1994). A weak interaction between PB2 and PA has also been proposed recently by using bimolecular fluorescence complementation (BiFC) assay which can detect weak and transient protein–protein interactions in living cells (Hemerka et al., 2009). This compensatory effect might affect the interaction of polymerase with vRNA (Hara et al., 2006; Maier et al., 2008) or host factors (Bradel-Tretheway et al., 2011; Engelhardt et al., 2005; Mayer et al., 2007; Momose et al., 2001; Pérez-González et al., 2006). More recently, PB2 627K was demonstrated to play a role in efficient recruitment of importin-α1 and -α7 to the RNP complex (Hudjetz & Gabriel, 2012). It would...
be interesting to analyse whether PB2 627 and PA 184/383 affect the binding of importin-α to the RNP complex.

Our findings also could explain recent studies pointing out that the human H3N2 strain can efficiently re assort with other strains when the polymerase possesses PB2 and PA subunits from the same H3N2 strain. Li et al. (2010) showed that the combination of H3N2 PA (A/Tokyo/Ut-Sk-1/2007) and H5N1 PB2 (A/chicken/South Kalimantan/UT6028/2006) significantly impaired RNP activity, but this defect was recovered by replacing H5N1 PB2 with H3N2 PB2. We speculate that the loss of RNP activity could be due to the combination of H3N2 PA 184N/383N and H5N1 PB2 627E. Replacing H5N1 PB2 627E with H3N2 PB2 627K could probably lead to overcoming the inhibitory effect of H3N2 PA 184N/383N. However, we cannot fully explain why the RNP activity was impaired when the NT PA alone was introduced into WSN or VN polymerase, in spite of the presence of 627K in WSN PB2 and VN PB2. The amino acid residues of NT PB2 differ at 35 or 34 positions from those of WSN PB2 or VN PB2, respectively (Fig. 5b), and 20 residues of them were tested in this study. We suppose that in addition to 627K, there might be other determinants in NT PB2 that overcame the inhibitory effect of NT PA.

The NT PB2 appeared to enhance RNP activity when introduced alone into SW (180% of mRNA) or HK (148% of mRNA) polymerase (Fig. 2, lanes 11 and 19). In the presence of PA 184S/383D, the E→K replacement at PB2 627 slightly increased the RNP activity (135% of mRNA; Fig. 6, compare lane 5 with 8). Indeed, such enhanced activity by H3N2 PB2 has been described in reassortants between A/Tokyo/Ut-Sk-1/2007 (H3N2) and A/chicken/South Kalimantan/UT6028/2006 (H5N1), and A/Wyoming/3/2003 (H3N2) and A/Thailand/16/2004 (H5N1) (Chen et al., 2008; Li et al., 2010).

In summary, we suggest that acquiring both PB2 627K and PA 184N/383N might be a prerequisite step for the generation of viable H3N2 reassortant viruses. Although these findings remain to be tested in the virus rescue experiment, these results could help understand why natural-reassortant H3N2 viruses isolated from human and swine possess PB2 and PA subunits from the same H3N2 strain of origin.

**METHODS**

**Strains and plasmids.** cDNA clones isolated from the following influenza strains were used: A/HongKong/156/1997 (H5N1; abbreviated as HK or H), A/Vietnam/1194/2004 (H5N1; abbreviated as VN or V), A/WSN/1933 (H1N1; abbreviated as WSN or W), A/Kurume/ K0910/2009 (H1N1; abbreviated as SW or S) and A/NTI/60/1968 (H3N2; abbreviated as NT or N) (Kashiwagi et al., 2009). The plasmids expressing the PB1, PB2, PA and NP of different influenza virus strains, and the plasmid expressing vRNA from the WSN NA gene (vNA) have been described previously (Vreede et al., 2004). Mutant PB1, PB2 and PA plasmids were prepared by site-directed mutagenesis and were fully sequenced.

**Analysis of RNP activity.** The RNP activity was analysed by primer extension as described previously (Fodor et al., 2002; Hara et al., 2006). Human embryonic kidney 293T cells were transfected with each of PA, PB1, PB2, NP and vNA expression vectors from each strain (WSN, NT, HK, VN or SW) and total RNA was isolated 30 h post-transfection. Primer extension was performed using three primers labelled with 32P: one for vRNA, one for mRNA and cRNA, and one for host-cell 5S rRNA as an internal control. All assays were carried out at least three times with independently transfected cells.

**Preparation of TAP-tagged polymerase RNP and in vitro assays.** For preparation of the polymerase, 293T cells were transfected with expression vectors containing PB1, TAP-tagged PB2 and the PA subunit of the polymerase. For preparation of the RNP, NP and vNA expression vectors were also transfected simultaneously. The polymerase or RNP was purified by the TAP method as described before (Deng et al., 2005). Briefly, 293T cells transfected with plasmids were harvested 2 days post-transfection, lysed and incubated with IgG-Sepharose (Amersham). After washing, the polymerase or RNP was released from IgG-Sepharose by cleavage with tobacco etch virus protease (Nakalai). The partially purified polymerase or RNP was analysed by 7.5% SDS-PAGE with silver staining (Invitrogen), confirmed by Western blotting with specific antibodies against PB1, PB2, PA and NP. The polymerase was adjusted by quantitative measurements of the levels of PA on silver-stained SDS-PAGE gels, because PA or PB1 levels indicate the level of the trimeric complex. The quantitatively adjusted polymerases were used to test the promoter-binding activity by UV cross-linking assay and replication-initiation activity by dinucleotide initiation of replication assay, as reported previously (Fodor et al., 2002; Hara et al., 2006). Data were quantified by an image analyser (GE Healthcare, Image Quant LAS 4000 mini).

**ACKNOWLEDGEMENTS**

We thank Professor George G. Brownlee and Professor Ervin Fodor (University of Oxford) for helpful discussions and critical reading of the manuscript. This study was supported by a grant from the Promotion of Science and the Ministry of Education, Science, Sports and Culture, Japan, Grant-in-Aid for Scientific Research (C), 24591590, 2012–2015.

**REFERENCES**


