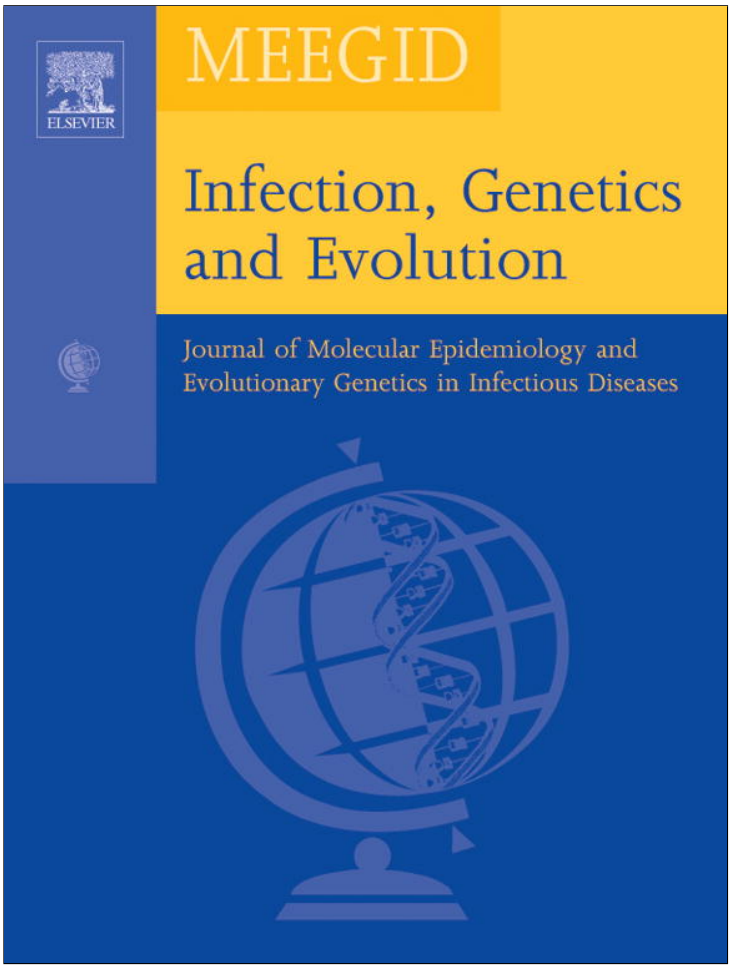

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Evolution of complementary nucleotides in 5′ and 3′ untranslated regions of influenza A virus genomic segments

Yoshiyuki Suzuki [a,*], Yuki Kobayashi [b]

[a] Graduate School of Natural Sciences, Nagoya City University, 1 Yamanohata, Mizuho-cho, Mizuho-ku, Nagoya-shi, Aichi-ken 467-8501, Japan
[b] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom

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ABSTRACT

The genome of influenza A virus comprises 8 segments (segments 1–8) of single-stranded RNA (virion RNA: vRNA) with negative-polarity. All vRNAs share 13 and 12 terminal nucleotides in the 5′ and 3′ untranslated regions (UTRs), respectively, which are partially complementary and constitute panhandle and corkscrew structures. Here, it is shown, from the analysis of genomic sequences for 506 strains of influenza A virus, that the number of contiguous complementary nucleotides in the 5′ and 3′ UTRs varies from 4 to 7 among segments. Complementary nucleotides were segment specific and highly conserved in all segments except for segment 6, where in the phylogenetic analysis co-evolution was observed to have occurred between and within subtypes of neuraminidase (NA). Mutations in the terminal sequences sometimes appeared to have caused convergence between subtypes, involving changes in multiple nucleotide positions. These observations suggest that intra-segmental (homologous) recombinations may have taken place for transferring terminal sequences in segment 6.

1. Introduction

The genome of influenza A virus comprises 8 segments (segments 1–8) of single-stranded RNA (virion RNA: vRNA) with negative-polarity. All vRNAs share 13 and 12 terminal nucleotides in the 5′ and 3′ untranslated regions (UTRs), respectively (Fig. 1). These nucleotides are partially complementary and constitute a panhandle structure, which may be recognized by the polymerase. Binding of the polymerase may induce a conformational change of these nucleotides to form a corkscrew structure, which exhibits a promoter activity (Fodor et al., 1993; Tiley et al., 1994; Cheong et al., 1996; Noble et al., 2011). Both the 5′ terminal 10 nucleotides (positions 1–10) and the 3′ terminal 9 nucleotides (positions 1–9) constitute stem-loops in the corkscrew structure, which may be necessary for synthesis of polyadenylated mRNA (Pritlove et al., 1999) and endonuclease activity (Leahy et al., 2001), respectively. The nucleotides at positions 11–13 in the 5′ UTR and 10–12 in the 3′ UTR are $^{5'}$AGG$^{3'}$ and $^{3'}$UCC$^{5'}$, respectively, which are complementary and form a duplex in both the panhandle and corkscrew structures. The duplex formation is necessary for cap primer utilization (Hagen et al., 1994), transcription initiation and termination (Fodor et al., 1994), polyadenylation (Poon et al., 1998), and packaging (Hsu et al., 1987).

Another shared sequence among all vRNAs is a uracil stretch located at positions 17–21 in the 5′ UTR, which acts as a polyadenylation signal (Fig. 1) (Li and Palese, 1994). Binding of the polymerase to the 5′ terminus of template vRNA throughout transcription may cause steric hindrance and stuttering of the polymerase while it transcribes the uracil stretch, leading to reiterative transcription of uracil and synthesis of polyadenylated tail of mRNA.

Although the functions of positions 1–13 and 17–21 in the 5′ UTR of vRNA have been characterized as described above, those of positions 14–16 have not been well understood (Fig. 1) (Furuse and Oshitani, 2011). It has only been reported that some of these positions are segment specific and complementary to positions 13–15 in the 3′ UTR, constituting a contiguous complementary region together with positions 11–13 in the 5′ UTR (Fodor et al., 1994). Since the length and sequence of the complementary region, as well as the length and location of the uracil stretch, may affect transcription and replication of vRNA (Luo et al., 1991; Lee and Seong, 1998), it is interesting to characterize these regions in more detail for understanding functions. The purpose of the present study was to examine the overall architecture and the evolutionary mechanism of these regions for each segment of influenza A virus.

2. Materials and methods

2.1. Sequence data

Genomic sequences for 10,854 strains (86,832 sequences) of influenza A virus, excluding laboratory strains and vaccine strains, were retrieved from the Influenza Virus Resource at the National Center for Biotechnology Information on May 31, 2012 (Bao et al.,

* Corresponding author. Tel./fax: +81 52 872 5821.
E-mail address: yossuzuk@nsc.nagoya-cu.ac.jp (Y. Suzuki).

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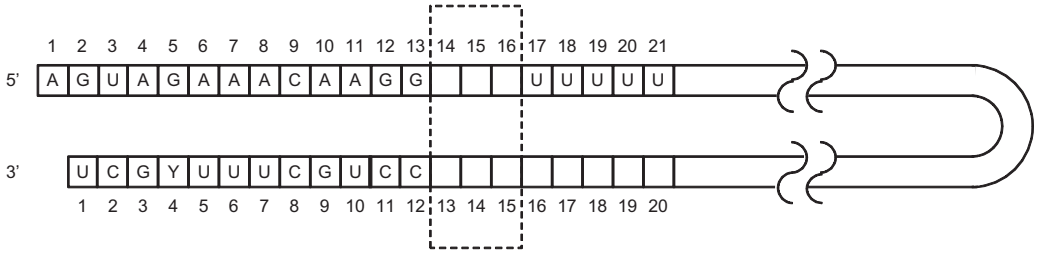

Fig. 1. Schematic diagram for the general structure of influenza A virus genomic segments (vRNAs). The vRNA is a single-stranded RNA with negative-polarity. All vRNAs share 13 and 12 terminal nucleotides in the 5′ and 3′ UTRs, respectively, which are partially complementary and constitute panhandle and corkscrew structures. The nucleotides at positions 11–13 in the 5′ UTR and 10–12 in the 3′ UTR are $^{5'}$AGG$^{3'}$ and $^{3'}$UCC$^{5'}$, respectively, which are complementary and form a duplex in both the panhandle and corkscrew structures. Another shared sequence among all vRNAs is a uracil stretch located at positions 17–21 in the 5′ UTR, which acts as a polyadenylation signal. Positions 14–16 in the 5′ UTR and 13–15 in the 3′ UTR are surrounded by a broken rectangle.

2008). To facilitate identification of the nucleotide positions in the 5′ and 3′ UTRs and comparison of the results from different segments, only the strains whose 8 segments were completely sequenced were selected from those obtained above. After eliminating the strains containing ambiguous nucleotides and those containing premature termination codons in any of the major open reading frames (encoding polymerase basic protein 2 [PB2] for segment 1, PB1 for segment 2, polymerase acidic protein [PA] for segment 3, hemagglutinin [HA] for segment 4, nucleoprotein [NP] for segment 5, neuraminidase [NA] for segment 6, matrix protein 1 [M1] and M2 for segment 7, and non-structural protein 1 [NS1] and NS2 for segment 8), 506 strains (4048 sequences) were retained for the following analysis. The strain names and the accession numbers in the International Nucleotide Sequence Database for the sequences analyzed in the present study are listed in Supplementary Table S1. These strains were originated from avian (420 strains), canine (2 strains), environment (12 strains), equine (1 strain), feline (1 strain), human (24 strains), and swine (46 strains).

2.2. Data analysis

The conservativeness and the complementarity of positions 11–21 in the 5′ UTR and 10–20 in the 3′ UTR were examined for each segment by comparing the vRNA sequences of 506 strains obtained above. For each segment, 506 vRNA sequences were simply aligned from the 5′ and 3′ termini for the study of the 5′ and 3′ UTRs, respectively. The conservativeness was examined by computing the proportions of U, C, A, and G at each position. Since the strains were inter-correlated to various degrees due to shared ancestry, the proportions obtained above may not exactly reflect the conservativeness especially when the sampling of strains is biased. However, this approach may still be useful for identifying highly conserved positions. The complementarity was examined by computing the proportions of U and A, U and G, C and G, A and U, G and U, and G and C at positions 11 in the 5′ UTR and 10 in the 3′ UTR, positions 12 in the 5′ UTR and 11 in the 3′ UTR, and so on, respectively. This approach may also be useful for identifying pairs of highly complementary positions. In the present study, nucleotide positions were operationally considered to be highly conserved or complementary when a particular nucleotide or a complementarity was observed for >90% of strains, respectively.

The phylogenetic analysis of segment 6 for 506 strains was conducted by using the amino acid sequence of NA because the sequences from different subtypes were relatively distantly related to each other (Nei and Kumar, 2000). The multiple alignment of amino acid sequences was made using the computer program MAFFT (version 6.853b) (Katoh et al., 2002), and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with the p distance and the Poisson correction distance in MEGA (version 5.05) (Tamura et al., 2011).

3. Results and discussion

3.1. Conservativeness and complementarity in each segment

The conservativeness and complementarity of positions 11–21 in the 5′ UTR and 10–20 in the 3′ UTR were examined for each segment of influenza A virus by using 506 strains for which complete genomic sequences were available. Influenza A virus has been classified into 17 (H1–H17) and 10 (N1–N10) subtypes according to the antigenic properties of HA and NA, respectively (Tong et al., 2012). The composition of subtypes among 506 strains is shown in Supplementary Table S2. All subtypes of HA and NA, except for H14, H16, H17, and N10, were represented in 506 strains. It should be noted that subtype H17N10 was assigned for the strains distantly related to all other subtypes of influenza A virus. Among 144 possible combinations of H1–H16 and N1–N9, 67 were covered by 506 strains.

In the 5′ UTR, the nucleotides at positions 11–13 were $^{5'}$AGG$^{3'}$ and 17–21 were $^{5'}$UUUUU$^{3'}$ for almost all strains of all segments (Fig. 2A). The nucleotides at positions 14–16 were segment specific and highly conserved in all segments except for segment 6, where these positions were polymorphic. In the 3′ UTR, the nucleotides at positions 10–12 were $^{3'}$UCC$^{5'}$ for almost all strains of all segments (Fig. 2B). The nucleotides at positions 13–20 were segment specific and highly conserved in all segments except for segments 4 and 6, where positions 15–20 and 13–20 were polymorphic, respectively.

The number of contiguous complementary nucleotides varied from 4 to 7 among segments; 4 for segment 4, 5 for segment 2, 6 for segments 1, 5, 6, 7, and 8, and 7 for segment 3 (Fig. 2C). The sequence of complementary nucleotides also varied among segments, which may influence levels of transcription and replication (Luo et al., 1991; Lee and Seong, 1998). The complementary nucleotides were usually segment specific and highly conserved. However, a variation was observed in segment 6, where positions 14–16 in the 5′ UTR and 13–15 in the 3′ UTRs were polymorphic, as indicated above. In contrast, a variation was not observed in segment 4, where only the 3′ UTR was polymorphic. These results indicate that complementary nucleotides have co-evolved in segment 6. It should be noted that although the segment specificity of complementary nucleotides may also be indicative of co-evolution, it is difficult to assess this possibility because evolutionary relationships among terminal sequences of different segments are unclear. Many of the nucleotides that were not involved in complementarity were highly conserved in a segment specific manner, suggesting that they may be associated with segment specific functions such as packaging (Barclay and Palese, 1995; Odagiri and Tashiro, 1997). The highly conserved nucleotides may be candidates for the target of vaccines and drugs for prophylaxis and treatment against influenza A virus.

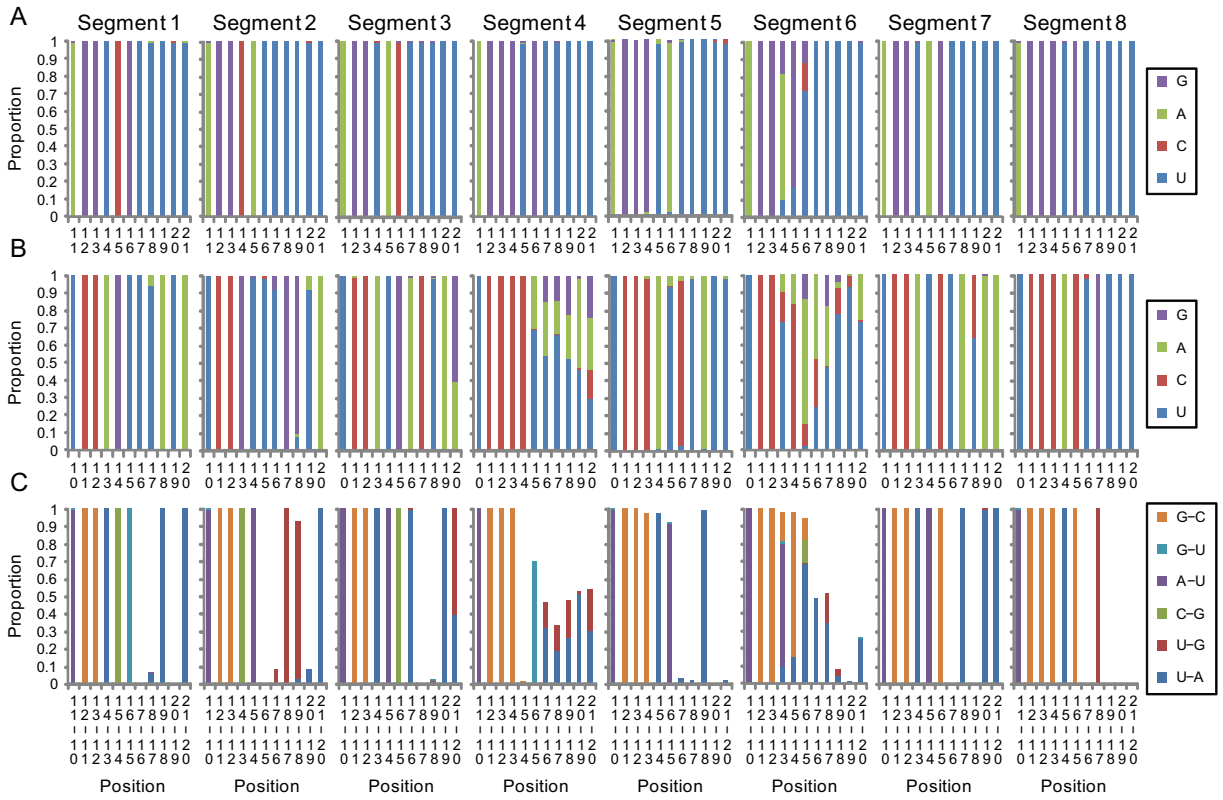

Fig. 2. Conservativeness and complementarity at positions 11–21 in the 5′ UTR and 10–20 in the 3′ UTR for each segment of influenza A virus. (A) For each segment, vRNA sequences for 506 strains were simply aligned from the 5′ terminus and proportions of U, C, A, and G were computed at each of positions 11–21. (B) For each segment, vRNA sequences for 506 strains were simply aligned from the 3′ terminus and proportions of U, C, A, and G were computed at each of positions 10–20. (C) For each segment, proportions of strains possessing U and A (U–A), U and G (U–G), C and G (C–G), A and U (A–U), G and U (G–U), and G and C (G–C) at positions 11 in the 5′ UTR and 10 in the 3′ UTR (11–10), positions 12 in the 5′ UTR and 11 in the 3′ UTR (12–11), and so on, respectively, were computed by using the alignments of 5′ and 3′ termini.

3.2. Conservation and co-evolution for maintaining complementarity

In the above analysis, it was observed that 2 different strategies, conservation and co-evolution, have been used for maintaining complementarity between the 5′ and 3′ UTRs of genomic segments during evolution of influenza A virus. Conservation was adopted by all segments, whereas co-evolution was adopted as an additional mechanism only by segment 6. To examine the evolutionary history of complementary nucleotides in segment 6, the phylogenetic tree was constructed for 506 strains using the amino acid sequences of NA (Fig. 3). In Fig. 3, the phylogenetic tree was constructed by the neighbor-joining method using the p distance. However, the phylogenetic tree with similar topology was also obtained using the Poisson correction distance (data not shown). The phylogenetic relationships among subtypes of NA was consistent with those observed in the previous studies (Russell et al., 2006; Tong et al., 2012), where the root was located on the branch partitioning the clusters consisting of N1, N4, N5, and N8 and N2, N3, N6, N7, and N9. According to the maximum parsimony principle (Fitch, 1971), the ancestral nucleotides at positions 11–16 in the 5′ UTR and 10–15 in the 3′ UTR at the root of the phylogenetic tree were inferred to be $^{5'}$AGGUCA$^{3'}$ and $^{3'}$UCCAGU$^{5'}$, respectively, which were identical to those mainly observed in N1, N2, N4, N5, and N8.

In the phylogenetic tree, co-evolution appeared to have occurred on the ancestral branches of subtypes, such as N3 generating $^{5'}$AGGUGC$^{3'}$ and $^{3'}$UCCACG$^{5'}$, N6, N7, and N9 generating $^{5'}$AGGGUG$^{3'}$ and $^{3'}$UCCCAC$^{5'}$, and N9 generating $^{5'}$AGGGUC$^{3'}$ and $^{3'}$UCCCAG$^{5'}$, in the 5′ and 3′ UTRs, respectively. Co-evolution was also observed within subtypes; complementary sequences mainly observed in N6 and N7 were occasionally found in N3 and N9. Complementarity was sometimes partially disrupted within subtypes, where one of complementary sequences was mutated to the sequence of another subtype or novel sequence. It is interesting to note that, in both cases of co-evolution within subtypes and partial disruption of complementarity, mutations of nucleotide sequences sometimes appeared to have caused convergence of terminal sequences between subtypes, involving changes in multiple nucleotide positions. These observations suggest that intrasegmental (homologous) recombinations may have taken place for transferring terminal sequences in segment 6. It should be noted that these results do not largely depend on the position of the root and the phylogenetic relationships among subtypes.

In the co-evolution of complementary nucleotides, compensatory mutations are required to occur in the 5′ and 3′ UTRs for maintaining complementarity. However, the probability for the occurrence of these mutations at the same time may be small, and it is likely that mutations partially disrupting and restoring complementarity occurred sequentially. The former mutation may be deleterious as long as functional constraints operate on the complementarity, and the degree of deleterious effect should be positively correlated with the strength of functional constraints. Therefore, co-evolution may occur only in the segments with relatively weak functional constraints (Ohta, 1973). It was observed that the complementarity between the 5′ and 3′ UTRs of segment 6 was sometimes partially disrupted among 506 strains (Figs. 2C and 3). In addition, it has been reported that segment 6 is not necessary for packaging of influenza A virus (Gao et al., 2012) and influenza C and thogoto viruses even lack genomic segments encoding NA (Hughes et al., 2000; Gubareva et al., 2002). These observations support the hypothesis that the functional constraint may be relatively weak for segment 6 compared to others.

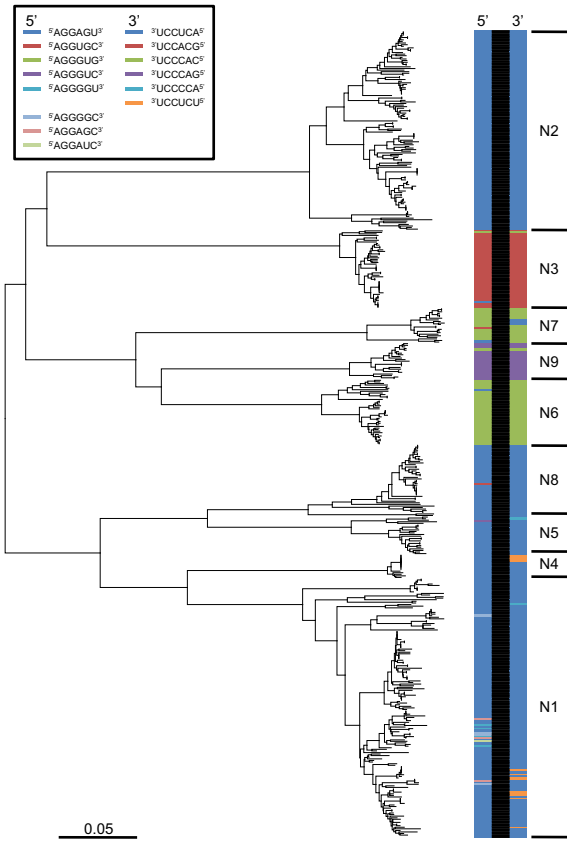

Fig. 3. Phylogenetic tree of segment 6 constructed for 506 strains of influenza A virus by using the amino acid sequence of NA. The phylogenetic tree was constructed by the neighbor-joining method with the p distance. The structure of segment 6 for each strain is described by 2 (5′ and 3′) boxes connected by a thin black line. The 5′ and 3′ boxes are colored according to the nucleotides at positions 11–16 in the 5′ UTR and 10–15 in the 3′ UTR as indicated in the inset, respectively. The same color is used for the 5′ and 3′ boxes with complementary nucleotides. Subtypes of NA (N1–N9) are indicated on the right-hand side. The scale bar denotes the number of amino acid substitutions per site.

3.3. Possibility for occurrence of recombination

In the above analysis, intra-segmental recombination was considered to have taken place for transferring terminal sequences of segment 6. Recombination was apparently confined to terminal ~15 nucleotides in the 5′ and 3′ UTRs (data not shown). Since only ~3 positions are polymorphic among these nucleotides, it may be difficult to detect recombination using statistical approach (Sawyer, 1989). Among 506 strains, the possible recombinants of segment 6 were mostly observed in the strains isolated from aquatic birds (data not shown), which are the natural host of influenza A virus and maintain all subtypes of HA and NA, except for H17 and N10 (Slemons et al., 1974; Webster et al., 1978; Hinshaw et al., 1980).

In general, co-infection of multiple strains has been commonly observed in influenza A virus, providing opportunities for reassortment and recombination (Dugan et al., 2008; Ghedin et al., 2009). Although reassortment is known to occur frequently (Holmes et al., 2005; Nelson et al., 2008; Suzuki, 2010), intra-segmental (Boni et al., 2008, 2010; He et al., 2008, 2009, 2012; Krasnitz et al., 2008) and inter-segmental (non-homologous) (Khatchikian et al., 1989; Bergmann et al., 1992; Orlich et al., 1994; Mitnaul et al., 2000; Suarez et al., 2004; Pasick et al., 2005) recombinations have been reported to occur only with low frequencies. The rate of recombination is believed to be low for single-stranded RNA viruses with negative polarity because of formation of ribonucleoprotein (RNP), which may inhibit the copy-choice of the polymerase (Conzelmann, 1998; Chare et al., 2003).

It should be noted, however, that although the RNP is usually formed by wrapping NP around RNA, that of influenza A virus is formed by wrapping RNA around NP, where NP binds to the phosphate-sugar backbone of RNA and bases are exposed to the surface (Baudin et al., 1994). In addition, since the terminal nucleotides of genomic segments of influenza A virus constitute panhandle and corkscrew structures as well as a duplex, they may be free from NP (Fodor et al., 1993; Tiley et al., 1994; Cheong et al., 1996; Noble et al., 2011). Duplex formation by complementary nucleotides may obstruct the movement of polymerase and may induce recombination during replication of influenza A virus. Therefore, it is possible that recombination occurs at the terminal nucleotides of all genomic segments. However, it may be detectable only in segment 6 because of the existence of variation in complementary nucleotides as a result of co-evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.10.007.

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