The nucleoprotein and matrix protein segments of H5N1 influenza viruses are responsible for dominance in embryonated eggs

Kenta Shimizu,1,2 Chengjun Li,3 Yukiko Muramoto,1 Shinya Yamada,1 Jiro Arikawa,2 Hualan Chen4 and Yoshihiro Kawaoka1,3,5,6,7

Since their emergence in 1996 in southern China, highly pathogenic H5N1 avian influenza viruses have spread widely and continue to circulate in some countries. Genetic reassortment has created multiple H5N1 virus lineages, some of which are dominant in nature. However, the mechanism by which certain H5N1 influenza virus lineages (or genotypes) become dominant in avian species remains unknown. Here, we used competitive inoculation and genetic analysis of the resultant viruses to show that the nucleoprotein (NP) and matrix protein (M) segments of Fujian-like viruses (clade 2.3.4), which became predominant in southern China in mid-2006, are responsible for viral dominance in embryonated eggs. We further found that specific residues in the NP and M proteins play key roles in conferring this viral dominance; specifically, a glutamic acid at position 66 in M2 was conserved among the Fujian-like viruses. These results suggest roles for these viral proteins in influenza virus dominance.
multiple sublineages, some of which spread rapidly and become dominant in certain areas, whereas others have disappeared (WHO, 2008). For example, in mid-2005, a Fujian-like sublineage (clade 2.3.4), whose prototype was A/duck/Fujian/1734/2005 (H5N1), emerged in southern China. The Fujian-like viruses rapidly replaced the existing multiple sublineages and became the dominant viruses by mid-2006 (Smith et al., 2006). These viruses continue to circulate in avian species and cause human infections (Shu et al., 2006; Wang et al., 2008; Xu et al., 2009; Li et al., 2010). The mechanism by which the Fujian-like sublineage became dominant remains unclear. Smith et al. (2006) speculated that vaccination may have facilitated the selection of Fujian-like viruses because the vaccine used in China at that time was less effective against these viruses. However, most of the market poultry was not seroconverted (Smith et al., 2006). Furthermore, many unvaccinated wild birds in nature have acquired these viruses, suggesting that other mechanisms are also involved. To address this issue, we attempted to identify the molecular determinants that confer dominance to Fujian-like viruses.

A/wild bird/Anhui/82/2005 (H5N1) (AH), which belongs to the Fujian-like sublineage (clade 2.3.4), and A/chicken/Vietnam/TY31/2005 (H5N1) (TY), a member of a pre-existing sublineage (clade 2.3.2), were used in this study. Recombinant AH and TY strains, as well as the mutant viruses described below, were generated by using reverse genetics, as described previously by Neumann et al., (1999). Stocks of each virus were prepared in 10-day-old embryonated eggs. The infectivity of the viruses was determined in Madin–Darby canine kidney (MDCK) cells according to standard procedures and expressed as p.f.u.; viral titres, determined in embryonated eggs and MDCK cells, were similar.

To evaluate the proliferative abilities of the AH and TY strains, viral growth in embryonated eggs was compared (Fig. 1a). Each virus (10³ p.f.u.) was inoculated into 10 day-old embryonated eggs. Virus-containing allantoic fluid was collected at 6, 12, 18 and 24 h post-inoculation and titrated in MDCK cells. The titres of both strains reached a similar level at 24 h post-inoculation. However, the titres of the AH strain at 12 and 18 h post-inoculation were lower than those of the TY strain belonging to the minor sublineage. In nature, Fujian-like viruses co-existed with viruses of other sublineages and then became dominant. We therefore tested whether the AH strain or its genes became dominant when the AH and TY strains were co-inoculated into embryonated eggs. To this end, we conducted competition experiments (Fig. 1b). The AH and TY strains were mixed at 1 : 1, 1 : 10 or 1 : 100 ratios based on viral infectivity, so that the relative amount of the AH strain in the mixture decreased. Each viral mixture (10³ p.f.u. in 0.1 ml) was inoculated into 10-day-old embryonated eggs and virus-containing allantoic fluid was collected 24 h post-inoculation. Because the infectivity of the collected viruses reached about 10⁸ p.f.u. ml⁻¹, the viruses were diluted 10⁴-fold and reinoculated into a new set of eggs. After five passages, viral RNA was extracted with a Viral RNA Mini kit (Qiagen) and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen) and an oligonucleotide complementary to the conserved 3' end of the viral RNA. The cDNAs were amplified by using PCR with Pfu ultra (Stratagene) and primers specific for each segment. Primer sequences are

**Fig. 1.** The NP and M segments of the AH strain are associated with viral dominance in embryonated eggs. (a) Viral growth of the AH (●) and TY (○) strains in embryonated eggs. Values in the graph are means and SD of the virus titre in the allantoic fluid from three eggs at each time point. (b) Diagram of the competition experiment. Viral mixtures of the AH and TY strains mixed at 1 : 1, 1 : 10 or 1 : 100 ratios were inoculated into embryonated eggs. After five passages, the viral cDNAs of each segment were amplified by using RT-PCR and subjected to direct sequencing to determine the relative proportions of the AH and TY segments. (c) Competition experiment between the AH and TY strains in embryonated eggs.
available upon request. The PCR products were subjected to direct sequencing with a BigDye terminator version 3.1 cycle sequencing kit and an ABI Prism 3100 genetic analyser (Applied Biosystems). To determine the relative proportions of the AH and TY segments, we compared wave levels at positions at which the sequences differ between the two viruses. Preliminary experiments showed that the limit of detection for a minor population was 10–20%, i.e. when the viral RNA of the minor population was approximately 10–20%, we could detect its presence. When the AH and TY strains were mixed at a 1:100 ratio, no AH segments were detected after the passages. At the 1:1 ratio, the proportions of most of the AH segments increased (Fig. 1c). At the 1:10 ratio, the proportions of the NP and M segments of the AH strain dramatically increased despite the lower initial amount of the AH strain. The reproducibility of these results was confirmed (Supplementary Fig. S1, available in JGV Online) and similar results were obtained by genetic analysis of ten clones each of viruses plaque-purified from the fifth passage of the viruses mixed at the 1:1 and 1:10 ratios (Supplementary Table S1, available in JGV Online), indicating the reliability of this method. These results suggest that the NP and M segments of the AH strain confer viral dominance in embryonated eggs under competitive conditions.

Eight amino acid differences in the NP were found between the AH and TY strains (Fig. 2a). To determine which amino acids are involved in dominance, we generated TY-strain-based mutant viruses that contained amino acids of the AH strain in their NP (Fig. 2b). The viral growth in embryonated eggs of each mutant virus was similar (Supplementary Fig. S2a, available in JGV Online). One of the mutants and the TY strain were mixed and passaged in embryonated eggs as described earlier, and the relative proportions of each of the NP segments were evaluated. In the case of TY(AH-NP), which possessed the whole NP segment of the AH strain in the background of the TY genome, the proportion of the NP segment of AH strain was markedly increased (Fig. 2b). TY(AH-NP-N), which had the N-terminal half of the AH NP, also showed a marked increase after passage but TY(AH-NP-C), which had the C-terminal half of the AH NP, did not. There were three amino acid differences between the two viruses in the N-terminal half of NP. We therefore generated TY(AH-NP-34), TY(AH-NP-121) and TY(AH-NP-128), each of which contained an amino acid of the AH strain at positions 34, 121 and 128 in NP, respectively. The competition experiments showed that the proportion of the NP segments of TY(AH-NP-34) and TY(AH-NP-121), but not TY(AH-NP-128), clearly increased. These results demonstrate that the glycine at position 34 and the arginine at position 121 in NP play key roles in conferring dominance.

The M segment encodes proteins M1 and M2; one amino acid in the former and two in the latter differ between the
increased virus fitness for infection of humans. Over the pre-existing sublineages and may lead to a prominent role in the dominance of Fujian-like viruses, we analysed sequences registered in the Influenza Virus Resource at the National Center for Biotechnology Information (Bao et al., 2008). We selected 117 strains of the Fujian-like sublineage and 43 strains of the pre-existing sublineage, which were collected between 2005 and 2006 in Asia and included data on the HA, NP and M segments. The glycine at position 34 in NP was not conserved among the Fujian-like and Qinghai lake sublineages but not among the pre-existing sublineages that circulated before the emergence of the Fujian-like and Qinghai lake sublineages had an alanine residue at this position (data not shown). Therefore, M2-Glu66 may contribute to the viral dominance of both of these major sublineages in nature.

M2 is a transmembrane protein that forms a proton channel (Pinto et al., 1992) that is required for efficient viral growth (Watanabe et al., 2001; Takeda et al., 2002). The amino acid at position 66 is located in the cytoplasmic domain that is indirectly involved in the activity of this channel by stabilizing the structure of the pore (Tobler et al., 1999). In addition, the cytoplasmic domain plays an important role in viral assembly (McCown & Pekosz, 2005, 2006; Iwatsuki-Horimoto et al., 2006). The relationship between the amino acid at position 66 and these functions remains unknown; further studies are necessary to elucidate the mechanistic basis of this relationship.

In conclusion, the NP and M segments are responsible for the viral dominance in embryonated eggs of the AH strain over the TY strain. Glutamic acid at position 66 of the M2 protein may contribute to this viral dominance in avian species in nature as well as to increased virus fitness in humans. A better understanding of the mechanism that confers dominance to newly emerging viruses will be useful for influenza virus control.

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### References


### Table 1. Conservation of amino acids among influenza viruses as identified by using competition experiments

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<th>Virus</th>
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<td>Avian H5N1</td>
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<td>Fujian-like sublineage (clade 2.3.4)</td>
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<td>Pre-existing sublineage (clade 2.3.2)</td>
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