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

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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.

Influenza A viruses belong to the family Orthomyxoviridae. Their genomes consist of eight single-stranded, negative-sense RNA segments that encode 10 or 11 proteins. Influenza virions are composed of ribonucleoprotein complexes and an envelope. The ribonucleoprotein complex consists of genomic RNA, NP and RNA-polymerase proteins. The envelope that results from budding through the host cell membrane contains the surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), as well as matrix protein 2 (M2). Based on the antigenicity of their HA and NA, influenza A viruses are divided into 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) (Wright et al., 2007).

All subtypes of influenza A viruses are maintained in aquatic birds, their natural reservoir (Webster et al., 1992). The viruses can be transmitted to poultry and mammals, circulate in these hosts following adaptation and form host-specific viral lineages. Because of their segmented genome, novel viral genotypes can emerge by reassortment and multiple viruses of different genotypes can co-circulate. However, what influences the dynamics of viruses in populations is largely unknown.

Highly pathogenic H5N1 avian influenza viruses have continued to circulate in avian species since 1996 (Xu et al., 1999; Li et al., 2010) and still pose a serious threat to human health worldwide. More than 500 human infections with a mortality rate of 60 % have been reported across 15 countries (WHO, 2010). H5N1 viruses have evolved into
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 (10^3 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 (10^3 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 10^8 p.f.u. ml^-1, the viruses were diluted 10^4-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 Pfui 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.](http://www.microbiologyresearch.org/)
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
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The M segment encodes proteins M1 and M2; one amino
acid in the former and two in the latter differ between the

![Fig. 2. Key residues of the NP, M1 and M2
proteins for viral dominance in embryonated
eggs. (a) Amino acid differences between the
NP, M1 and M2 proteins of the AH and TY
strains. (b) Competition experiments between
the TY-strain-based NP mutants and the TY
strain in embryonated eggs. Asterisks indicate
the amino acids of the AH strain. (c) Competition experiments between the TY-
strain-based M1/M2 mutants and the TY strain in embryonated eggs.](http://vir.sgmjournals.org)
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. Sugesttt thhaatt tt h e gluttaa mi ca aci d ta pp o si ti o n6 6i n M2 has a 2009 H1N1 pandemic influenza viruses. These results conserved among human influenza A viruses including clade 2.3.4. Furthermore, the glutamic acid was highly conserved among both 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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<thead>
<tr>
<th>Virus</th>
<th>NP</th>
<th>M2</th>
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<tr>
<td><strong>Avian H5N1</strong></td>
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<td>Fujian-like sublineage (clade 2.3.4)</td>
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<td>AH</td>
<td>G</td>
<td>R</td>
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<td>Consensus</td>
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<td>Pre-existing sublineage (clade 2.3.2)</td>
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<td>TY</td>
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<td>Consensus</td>
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<td><strong>Human</strong></td>
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<td>H1N1 (Seasonal)</td>
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<td>H1N1 (2009 Pandemic)</td>
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<td>H3N2</td>
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