

## RAPID COMMUNICATION

## H9N2 Influenza A Viruses from Poultry in Asia Have Human Virus-like Receptor Specificity

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H9N2 influenza A viruses are currently widespread in chickens, quail, and other poultry in Asia and have caused a few cases of influenza in humans. In this study, we found that H9N2 viruses from Hong Kong live bird markets have receptor specificity similar to that of human H3N2 viruses. In addition, the neuraminidase of poultry H9N2 viruses has mutations in its hemadsorbing site, a characteristic resembling that of human H2N2 and H3N2 viruses but differing from that of other avian viruses. Peculiar features of surface glycoproteins of H9N2 viruses from Hong Kong suggest an enhanced propensity for introduction into humans and emphasize the importance of poultry in the zoonotic transmission of influenza viruses.

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**Introduction.** The 1997 influenza outbreak in Hong Kong caused by "bird flu" H5N1 viruses suggested that influenza viruses circulating in poultry can be potential precursors of human pandemic viruses. This hypothesis has been further strengthened by recent cases of human infection by H9N2 viruses in the Guangdong province of China (8) and in Hong Kong, SAR (19). Genetic characterization of the Hong Kong virus isolates from humans indicated a close relationship between these isolates and avian H9N2 viruses from Hong Kong live bird markets (7, 12). The six genes encoding internal components of human H9N2 virus isolates and of poultry H9N2 viruses from one distinct lineage are similar to those of human H5N1 viruses isolated in 1997; this finding suggests that such viruses could have an enhanced ability to infect humans. In addition to properties of internal proteins, receptor specificity of the virus hemagglutinin (HA) is believed to be the factor that may limit the generation of human pandemic viruses from avian precursors (Ref. (15) and references therein). Analysis of the H5N1 chicken and human viruses indicated that their HA receptor-binding site (RBS) and receptor specificity have not yet acquired changes typical of human viruses (14). Significantly, Lin *et al.* (12) observed different amino acid substitutions in the conserved positions of the RBS of human and avian H9N2 viruses from Hong Kong. In particular, Leu-226 of these viruses is typical of that

found in human H2 and H3 pandemic strains but not in avian viruses (13, 15). This unexpected finding prompted us to study how mutations in the RBS affected the receptor-binding properties of H9N2 viruses and, in particular, to determine whether some of these avian viruses have acquired a human virus-like receptor specificity.

**Results. Structural variation of the RBS of H9N2 viruses.** To specify patterns of mutations in the RBS of H9N2 viruses of distinct evolutionary lineages and to select representative strains for receptor-binding studies, we searched for two types of amino acid substitutions in H9 HA sequences from GenBank. The first type included mutations in the highly conserved positions of the RBS that are believed to be essential for the maintenance of strict receptor specificity of influenza viruses in wild aquatic birds (13). The second type included substitutions of less conserved amino acids in or near the RBS.

The available H9 HA sequences can be separated into the three main lineages (Fig. 1). Lineages I and II contain viruses of wild aquatic birds and viruses isolated from poultry. With two exceptions all of these viruses contained each of the 41 amino acids in the HA globular head, which are conserved among most other HA subtypes of viruses in wild aquatic birds (13). One virus from each lineage had a mutation in the RBS: Gly225Asp; analogous mutations in this position had previously been shown to affect the receptor specificity of H1 influenza viruses (15). (For the location of mutations on the three-dimensional structure of HA, see Fig. 4A.) The third H9 HA lineage was represented exclusively by poultry viruses from Asia. This lineage included two distinct

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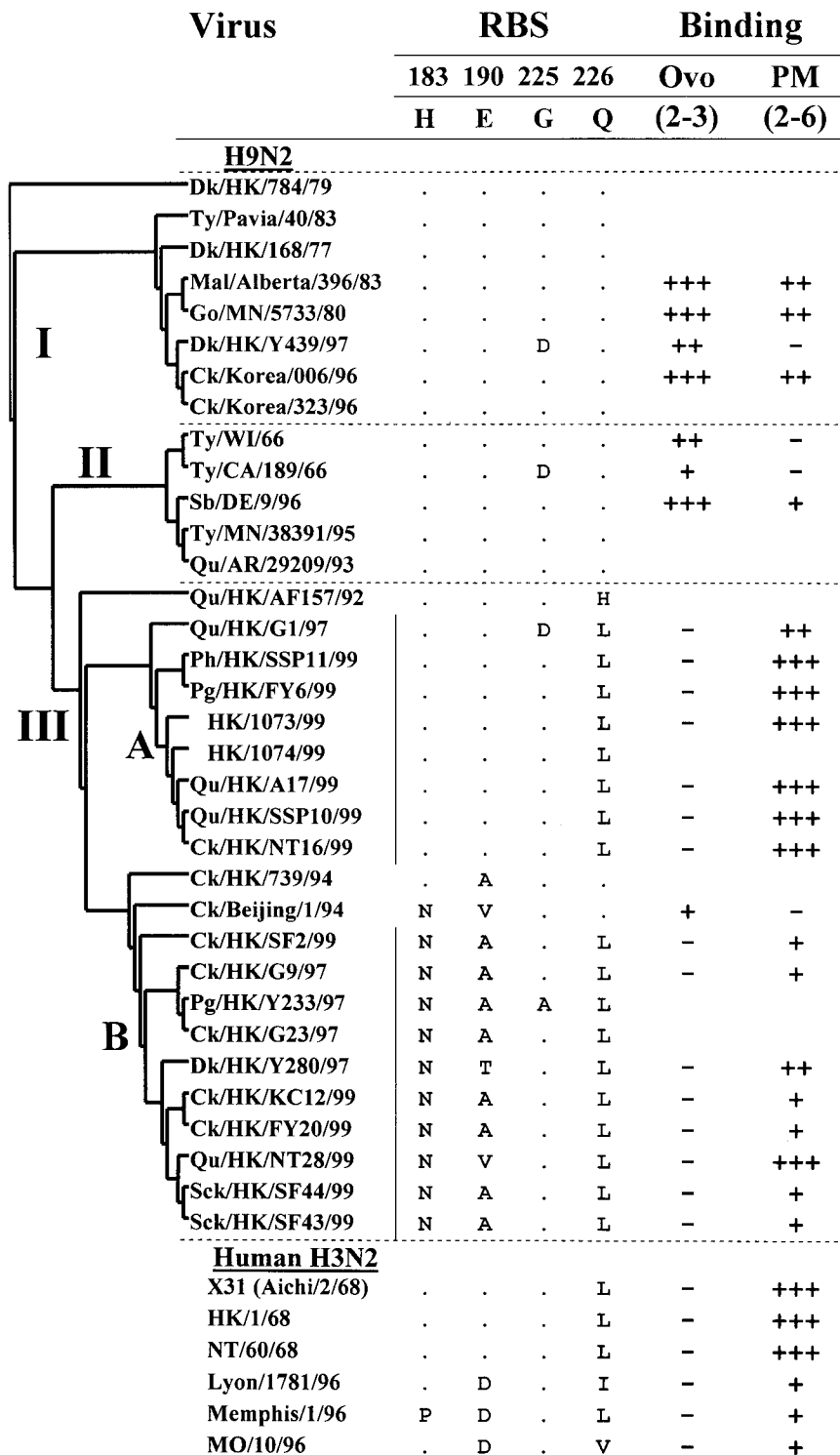


FIG. 1. Amino acid substitutions in the RBS of HA and receptor specificity of H9N2 and H3N2 viruses. Virus, virus names and phylogenetic relationships among H9 HAs based on the nucleotide sequences of HA1. Abbreviations: Ck, chicken; Dk, duck; Go, goose; Mal, mallard; Qu, quail; Pg, pigeon; Ph, pheasant; Sb, shorebird; Sck, silky chicken; Ty, turkey. The vertical lines to the right of the virus names indicate those viruses that are part of group A or B. RBS, amino acids at positions 183, 190, 225, and 226 in the receptor-binding site; the letters in bold (top line) indicate the amino acids that are conserved at these positions in the avian virus consensus sequence. Dots indicate no change. Binding, relative binding affinity for ovomucoid (Ovo) and pig macroglobulin (PM). The symbols -, +, ++ and +++ indicate that the relative binding affinity was, respectively, 0-5, 5-20, 20-50, and 50-150% of that of the reference strain. Blank cells indicate that receptor-binding properties were not tested.

groups of viruses (A and B, Fig. 1), which were isolated in Hong Kong live bird markets in 1997–1999; two H9N2 isolates from humans (group III-A); and three single isolates from 1992 and 1994. A unique feature of all viruses from lineage III is that they contain amino acid substitutions that have never been seen in avian viruses. Thus, group A and B viruses had the substitution Gln226Leu, whereas group B viruses contained the additional mutation His183Asn and a substitution at position 190 (Ala, Thr, or Val). In addition, group A and B viruses differ from each other and from the rest of H9 viruses at several other positions on the rim of the RBS (amino acids 137, 141, 156–158) and on the tip of the HA (amino acids 159–160, 196–198) (data not shown). Because of their location (Fig. 4A), substitutions at these positions may affect virus interactions with cell-surface receptors. Finally, one more characteristic of group A strains that is not found in other H9 viruses was the presence of two potential glycosylation sites at positions 95 and 198 of the HA globular head; this feature is typical of poultry viruses but not of aquatic bird viruses (13, 14).

Thus, despite occasional virus transmission between wild aquatic birds and poultry, HAs of group I and II viruses retained structural features typical of aquatic bird viruses. In contrast, HAs of poultry viruses from Hong Kong, all of which belong to the different phylogenetic lineage, underwent significant evolutionary changes in the region of the RBS.

**Receptor Specificity of H9N2 Viruses.** Representative strains of the three H9 HA phylogenetic lineages were compared with human H3N2 viruses for their binding to ovomucoid (Ovo) and pig macroglobulin (PM) (Fig. 1). These sialylglycoproteins contain sialic acid moieties linked to the penultimate sugar residue by the 2–3 linkage and the 2–6 linkage, respectively, and were used previously for the characterization of the receptor-binding specificity of avian and human influenza viruses (14).

All H9 avian viruses that carried the avian virus-like Gln-226 (i.e., each strain from lineages I and II and Ck/Beijing/1/94 from lineage III) bound to Ovo better than they bound to PM, thus, displaying a typical avian virus-like receptor specificity. Among viruses with Gln-226, strains with substitutions in other conserved positions of the RBS (Gly225Asp; His183Asn and Glu190Val) bound to Ovo and to PM more weakly than other viruses. Notably, two of these low-affinity viruses were isolated from turkeys and chickens. The third low-affinity virus, Dk/HK/Y439/97, was isolated in the live poultry market (6) where it could have been transmitted to duck from other bird species. This notion correlates with the previous conclusion that the receptor-binding affinity of poultry H5 viruses is weaker than that of H5 viruses of wild aquatic birds (14).

Human H3N2 viruses bound to PM but not to Ovo, in accord with their known preference for 2–6-linked sialic

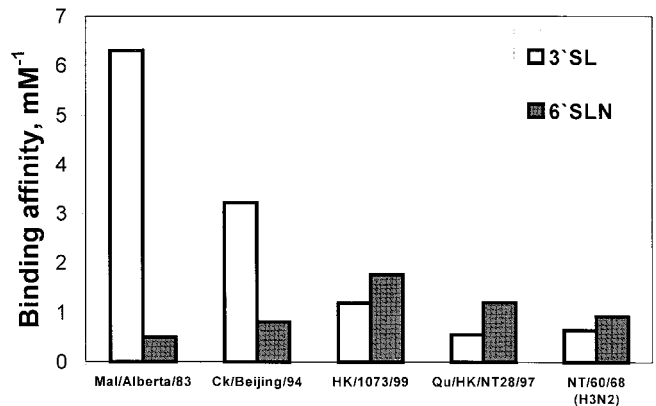


FIG. 2. Binding affinity constants of virus complexes with 3'sialyllactose (Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc, open bars) and with 6'sialyl(*N*-acetyllactosamine) (Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc, dark bars).

acid species and their poor binding to 2–3-linked receptor moieties. Three human virus strains isolated in 1996 displayed a lower binding affinity than viruses isolated at the beginning of the 1968 pandemic. This feature of recently isolated human H3 viruses is probably related to the substantial alterations of their RBS after 1992, in particular, the substitutions at amino acids 190, 194, and 226 (3).

Remarkably, all H9 viruses recently isolated from Hong Kong bird markets did not bind to Ovo but bound to PM; i.e., these viruses displayed a human virus-like but not an avian virus-like receptor specificity. This effect clearly correlated with the presence of Leu-226 in the RBS of these viruses. Most group III-A viruses and one group III-B virus with Val-190 (Qu/HK/NT28/99) bound to PM with affinities comparable to those of the human H3N2 strains isolated during the 1968 pandemic. Qu/HK/G1/97 (Asp-225) and Dk/HK/Y280/97 (Thr-190) displayed intermediate affinity. Group III-B viruses with Ala-190 showed the lowest affinity for PM, and their Ovo and PM binding patterns and affinities were indistinguishable from those of three human H3N2 viruses isolated in 1996. An additional common trait of the low-affinity H9 viruses with Ala-190 and the recently isolated human viruses with Asp-190 was their poor agglutination of chicken erythrocytes (data not shown).

To further characterize the receptor-binding properties of H9 viruses, we determined the binding affinities of a few representative strains for the low-molecular-weight receptor analogs 3'-sialyllactose (3'SL) and 6'-sialyl(*N*-acetyllactosamine) (6'SLN) (Fig. 2). Consistent with their pattern of binding to Ovo and PM, two H9 viruses with Gln-226 bound more strongly to 3'SL than to 6'SLN, whereas a human H3N2 virus and two H9 viruses with Leu-226 had the opposite binding specificity. These results also support the placement of poultry H9 viruses from Hong Kong into the same receptor-binding group as human viruses and clearly indicate their separation from other avian viruses.

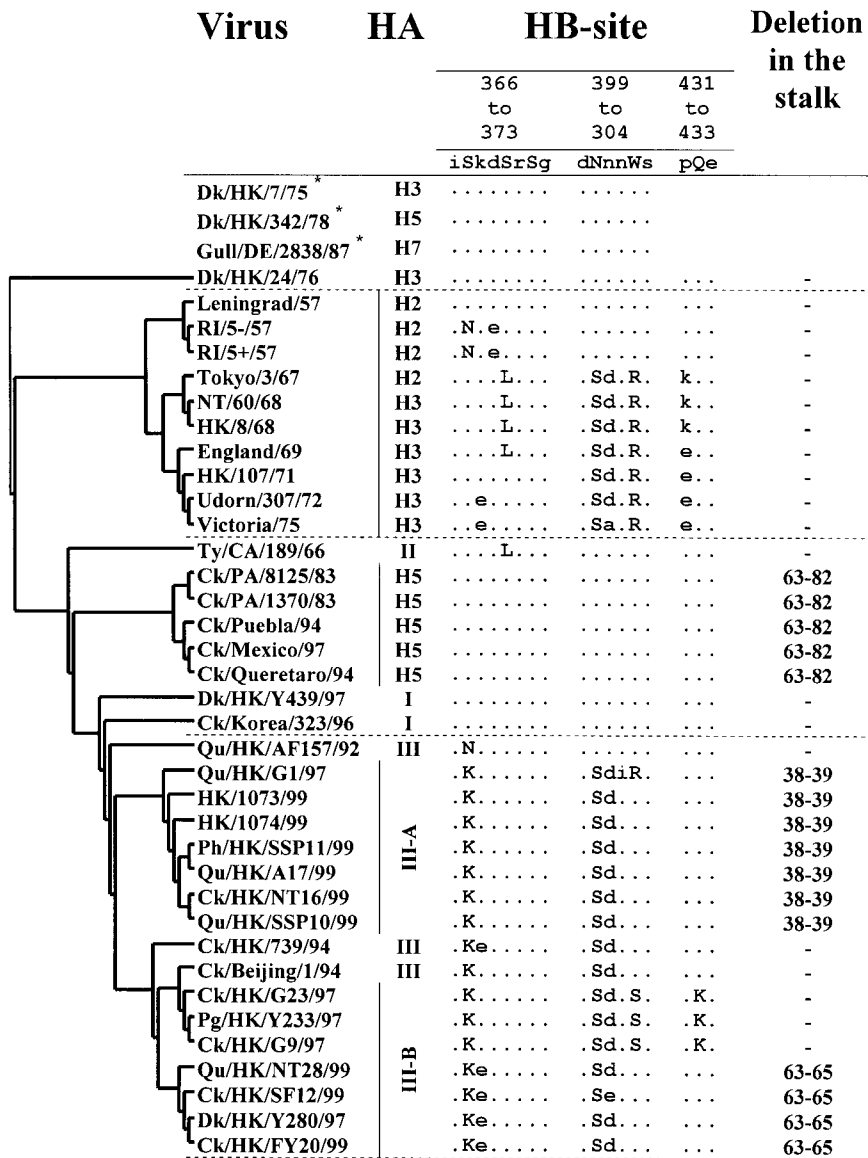
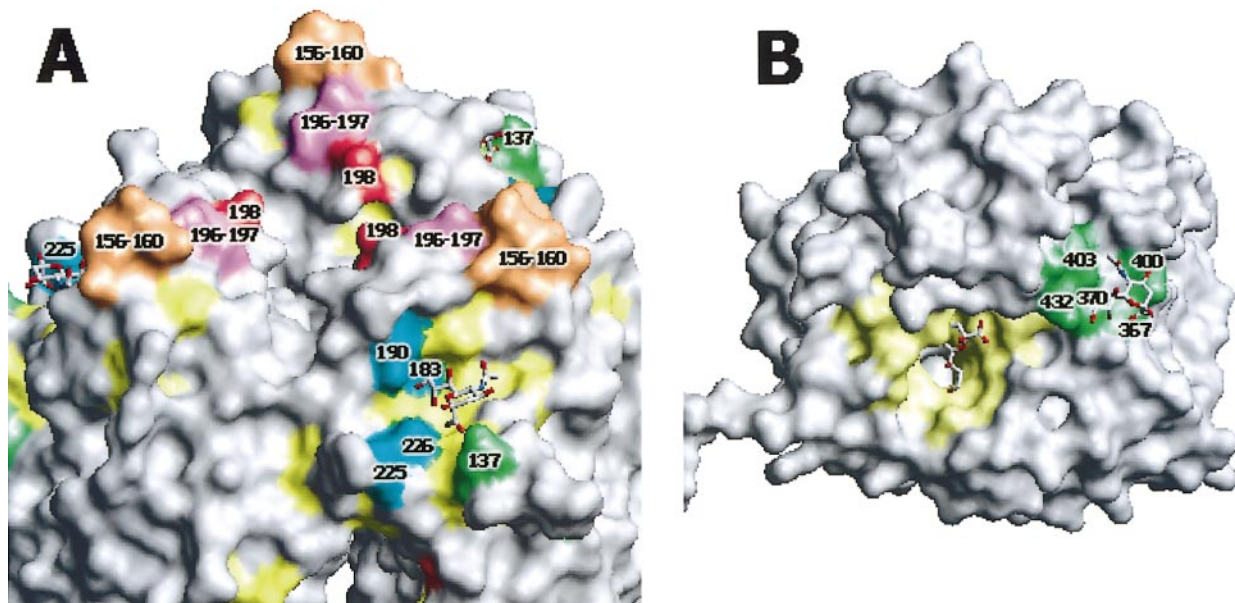


FIG. 3. Amino acid substitutions in the HB site of N2 NAs. Virus, virus names and phylogenetic relationships based on the NA nucleotide sequences. The asterisks indicate that only partial NA sequences were available for these viruses (11). HA, HA subtype of viruses other than H9; H9 HA lineages are labeled according to their designation in Fig. 1. HB site, substitutions with respect to the consensus sequence (top line). Amino acids that are in contact with the sialic acid moiety in the HB site of the NA-sialic acid complex (Fig. 4B) are shown by capital letters. Deletion in the stalk, positions of deleted amino acids. The symbol - indicates that no deletion occurred in the NA stalk.

*Changes in Neuraminidase.* The main function of influenza virus neuraminidase (NA) is to facilitate virus release and spread by destroying sialic acid receptors on extracellular inhibitors, progeny virions, and infected cells (reviewed in Ref. (1)). Therefore, a balance between the receptor-binding activity of the HA and the receptor-destroying activity of the NA is required for virus fitness (2, 10, 16). To test whether changes in the HAs of poultry H9N2 viruses were accompanied by changes in the functional regions of the NAs, we analyzed N2 NA sequences available from GenBank (Fig. 3).

A most striking feature of the NAs of Hong Kong H9N2 viruses was mutations in the hemadsorbing (HB) site.

This site is located on the NA surface away from the enzymatic site (Fig. 4B). In the HB site of N9 NA, six amino acid residues on three separate loops interact directly with sialic acid: three serines on the loop comprising amino acids 367 to 372, Asn-400, Trp-403, and Lys-432 (20). The amino acids at these positions were conserved in the N2 NAs of aquatic bird viruses, chicken H5N2 viruses, and three other virus isolates from different hosts (Leningrad/57, Dk/HK/Y439/97, and Ck/Korea/323/96) (Fig. 3). In contrast, all poultry H9N2 viruses from Hong Kong and China, which belong to the group III HA lineage, and most of the human H2N2 and H3N2 viruses contained one to four amino acid substitutions at the



**FIG. 4.** Substitutions in the HA (A) and NA (B) of H9N2 viruses shown on models of an H3 HA trimer and an N9 NA monomer complexed with sialic acid (1HGG and 1MWE structures, respectively, Brookhaven Protein Databank). (A) The regions in yellow indicate amino acids conserved among most subtypes of avian HAs. Different colors depict various substitutions in the H9 HA described in Fig. 1 and in the text. (B) The amino acid residues (yellow) within 5 Å of Neu5Ac in the NA enzymatic site are shown. These residues were conserved among most of the N2 NAs studied. Substitutions (green) in the HB site, listed in Fig. 3, are indicated. The figures were generated by WebLab ViewerPro 3.7 (Molecular Simulations, Inc., San Diego, CA).

positions of the HB site that contact sialic acid. To assess whether mutations in the HB site of H9N2 viruses become fixed under specific selective pressure or whether these mutations simply reflect a higher evolution rate for this lineage, we calculated ratios of nucleotide substitutions per nonsynonymous and synonymous sites ( $dn/ds$ ) for the HB site (codons listed in the Fig. 3) and for the rest of the NA globular head (codons 81 to 461 with the exclusion of the codons that compose the HB site). The  $dn/ds$  ratios for the NA globular head of H5N2 and H9N2 viruses were similar and ranged from 0.09 to 0.12; the similarity in  $dn/ds$  ratios indicates that NAs of both lineages were under similar negative selection pressure. The HB sites of chicken H5N2 viruses and H9N2 viruses Dk/HK/Y439/97 and Ck/Korea/323/96 lacked nonsynonymous substitutions ( $dn/ds < 0.1$ ). In contrast, the  $dn/ds$  ratios for the HB site of poultry H9 viruses ranged from 0.3 to 1.6. These results suggest that the HB site of H9N2 viruses from Asia has been under specific positive selection pressure, i.e., mutations in the HB site improved fitness of these viruses.

We previously reported that NAs of influenza viruses from poultry often contain deletions in the stalk (14). In accord with this notion, two groups of H9N2 viruses from Hong Kong bird markets contained short deletions that occurred independently in these lineages. The third group of viruses that contained a deletion included geographically and historically distinct chicken H5N2 viruses from Pennsylvania and Mexico; these viruses displayed the same deletion of 20 amino acids obviously

present in the NA of their common precursor. In contrast to the variations in the HB site and the occurrence of deletions, no consistent differences in the enzymatic site of the N2 NAs were detected. Each of the 18 amino acids that lay within 5 Å of sialic acid in the crystal structure of N2 NA was conserved among most of the N2 sequences analyzed (Fig. 4B).

*Discussion.* The results of all previous studies of the receptor specificity of human and animal influenza A viruses agreed with the original finding of Paulson's group: avian viruses preferentially bind to 2–3-linked sialic acid moieties, whereas human and pig viruses preferentially bind to 2–6-linked receptor determinants (18). Our study provides the first example of a stable lineage of avian influenza viruses with a human virus-like receptor specificity and an ability to infect different species of poultry (e.g., quail, chicken, pheasant, guinea fowl, pigeon) (6, 7). This finding shows that a strict receptor specificity, which is typical of influenza viruses maintained in wild aquatic birds, may not be an absolute requirement for influenza virus replication in other avian species. Obviously, one reason for this can be host- and tissue-specific differences in the spectrum of the sialic acid receptors on target cells. For example, duck intestinal cells appear to lack Sia2-6Gal-terminated receptors (9), whereas chicken respiratory and intestinal epithelial cells contain both types of Sia-Gal linkages (5; A. S. Gambaryan, personal communication). Thus, the presence of 2–6-terminated receptors in chickens can ex-

plain the susceptibility of chickens to Hong Kong H9N2 viruses. However, it is not clear whether these viruses could initially emerge in chickens, because previous studies have not found lineages of chicken viruses that display a human virus-like receptor specificity, that have mutations in the RBS, or that have both properties (13, 14; M. N. Matrosovich, unpublished data). One possibility is that this H9N2 lineage emerged as a result of initial virus adaptation to a particular avian or mammalian host; subsequently, the virus was introduced into other species of poultry and underwent readaptation. In this case, observed variations in the structure of HA and NA and in receptor specificity of individual phylogenetic groups of H9N2 viruses from Hong Kong may reflect a concerted adaptation of HA and NA to receptors or inhibitors of different avian species.

Studies of the earliest virus isolates from human pandemics and swine epidemics demonstrated that these viruses possess a human virus-like receptor specificity and suggested that a switch in receptor specificity is an essential element (in addition to changes in other viral genes) in the initiation of a pandemic (4, 15). Because of these findings and because of the human virus-like receptor specificity of poultry H9N2 viruses, these viruses may be regarded as particularly plausible candidates for the generation of new pandemic strains.

The HB site of influenza virus NA is a second sialic acid-binding pocket on the NA surface with an unknown biological function (20). This site is conserved among aquatic bird viruses and equine viruses, whereas circulation of viruses with avian NA in humans and pigs results in accumulation of nonconservative substitutions in the HB site; these substitutions decrease the viruses' hemadsorption capacity (11, 20). We found that the HB site has been conserved in the NA of chicken H5N2 viruses, but the site appears to be under positive selection pressure to change in H9N2 viruses from poultry in Asia. This pressure probably results from the different spectrum of glycoconjugates in the site of replication in these two distinct virus lineages, from a necessity of a functional match between HA and NA, or from both. Indeed, an association between the HA receptor specificity and the HB site can be observed: the HB site is conserved in viruses with a high affinity for 2–3-linked receptors (i.e., most avian viruses and equine viruses), whereas viruses that preferentially bind to 2–6-linked receptors (i.e., human, swine, and poultry H9N2 viruses) accumulated mutations in key positions in this site. Because of mutations in the HB site, the NAs of poultry H9N2 viruses from Hong Kong and of human pandemic H2N2 and H3N2 viruses have similar amino acid substitutions in this region (Fig. 3).

In summary, human virus-like receptor specificity and human virus-like mutations in the HB site of poultry H9N2 viruses suggest that some species of poultry could serve as an intermediate host in the zoonotic transmission of

influenza viruses from their natural reservoir in aquatic birds to humans.

*Materials and Methods. Viruses.* Isolation and characterization of H9N2 influenza viruses have been described earlier (6, 7, 12). Other viruses were from the repository of St. Jude Children's Research Hospital. Human H3N2 viruses from 1996 were isolated and grown solely in MDCK cells; all other viruses were propagated in 9- to 10-day-old chicken eggs.

*Receptor Specificity.* Binding of peroxidase-labeled chicken egg-white ovomucoid and pig  $\alpha$ 2-macroglobulin was assayed as described by Matrosovich *et al.* (14). Two reference strains, X31 (H3N2) and Mallard/Alberta/396/83 (H9N2), were included in each experiment. The affinity of X31 for PM and the affinity of the mallard virus for Ovo were arbitrarily set at 100%, and the relative affinities of the test viruses with respect to that of each reference strain were calculated. Two to five experiments were performed on different days, and the results were averaged. The binding to oligosaccharides 3'SL and 6'SLN was assessed with the solid-phase fetuin binding inhibition assay and the results were expressed in terms of binding affinity constants (13).

*Analysis of HA and NA Amino Acid Sequences.* All sequences analyzed in this study were obtained from GenBank. Phylogenetic analysis was performed as described (14) by using the PHYLIP software package (J. Felsenstein, 2000. PHYLIP Version 3.6. Distributed by the author. <http://evolution.genetics.washington.edu/phylip/phylip36.html>). The proportions of nonsynonymous substitutions per potential nonsynonymous site to synonymous substitutions per potential synonymous site ( $dn/ds$ ) were calculated by the method of Nei and Gojobori (17) as implemented in the SNAP program (<http://www.hiv-web.lanl.gov>). In this analysis, the NA genes of each H5N2 and H9N2 virus in Fig. 3 were compared with the common ancestral NA sequence of these viruses that was inferred using DnaML program from the PHYLIP 3.6 package.

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