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Role of Quail in the Interspecies Transmission of H9 Influenza A Viruses: Molecular Changes on HA That Correspond to Adaptation from Ducks to Chickens

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H9 influenza viruses have become endemic in land-based domestic poultry in Asia and have sporadically crossed to pigs and humans. To understand the molecular determinants of their adaptation to land-based birds, we tested the replication and transmission of several 1970s duck H9 viruses in chickens and quail. Quail were more susceptible than chickens to these viruses, and generation of recombinant H9 viruses by reverse genetics showed that changes in the HA gene are sufficient to initiate efficient replication and transmission in quail. Seven amino acid positions on the HA molecule corresponded to adaptation to land-based birds. In quail H9 viruses, the pattern of amino acids at these seven positions is intermediate between those of duck and chicken viruses; this fact may explain the susceptibility of quail to duck H9 viruses. Our findings suggest that quail provide an environment in which the adaptation of influenza viruses from ducks generates novel variants that can cross the species barrier.

H9N2 influenza A viruses circulate worldwide (1). In North America, H9 viruses are found mainly in shore birds but also in wild ducks (19, 34). Since 1966 (16, 27), H9 viruses have caused disease outbreaks in turkeys in the United States (reviewed in reference 2). There is no evidence that H9 viruses have established stable lineages in turkeys, although as many as 16 outbreaks of H9-associated disease in turkeys were documented between 1981 and 1996 (2, 12).

No H9-associated disease has been reported in chickens in North America. In Asia, however, H9 viruses (mostly of the N2 NA subtype) have caused disease outbreaks and have established stable lineages in chickens and other land-based poultry, such as quail, pheasant, chukar, and other minor domestic poultry (3, 9, 28). Phylogenetic analysis of Asian H9N2 viruses suggests that they have been transmitted from aquatic to land-based birds multiple times (8). Interestingly, the natural avian reservoir of H9 viruses in Asia has not been identified. Surveillance studies in the 1970s identified H9 viruses in domestic ducks (24, 35, 36). The available evidence suggests that H9 viruses did not appear in chickens until the early 1990s. Since the mid-1990s, H9 viruses have become adapted to land-based birds and have crossed sporadically to pigs and humans, causing mild respiratory disease (7, 8, 22, 31, 38). Importantly, some of the currently circulating H9N2 viruses bind to sialic acid receptors linked to galactose in the α-2,6 conformation, which is the preferential binding pattern of human influenza viruses (26). Thus, these H9N2 viruses possess one of the key elements needed to establish stable lineages in humans.

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The 1997 outbreak of H5N1 avian influenza in humans in Hong Kong demonstrated that avian influenza viruses can be directly transmitted to humans. The H5N1 viruses were found to be cocirculating with H9N2 and H6N1 viruses in the Hong Kong poultry markets. Sequence analysis suggested that the 1997 H5N1 virus was a reassortant containing genes from an H6N1 and/or an H9N2 virus. Both viruses are endemic in quail in Hong Kong (4, 8, 14). Thus, since 1997, there has been increased interest in characterizing the incidence and subtypes of avian influenza A virus infection in quail.

Few major influenza outbreaks have been observed in quail (37). The first reported outbreak was in Italy. Nardelli et al. (29) found quail to be infected with influenza A virus that caused respiratory disease and was lethal to young quail (<3 months old). More recently, Tashiro et al. (39) showed that quail infected with an H5N3 virus that was highly pathogenic to turkeys were resistant to disease but could transmit the lethal virus to chickens. Interestingly, we have recently shown that quail are highly susceptible to infection with highly pathogenic H5N1 viruses isolated from geese. These viruses cause disease in quail; however, infected quail have a longer disease period than do chickens and thus are more likely to transmit the virus (40).

The isolation of three H9N2 viruses from quail in 1988 (this report; W. Lim, unpublished data) was the first evidence of H9 virus in land-based poultry in Asia. More recently, quail in Hong Kong have shown a high incidence of infection with influenza A viruses, particularly H9N2 viruses; 16% of quail in the Hong Kong markets were found to be positive for H9N2 viruses (8). In this study, we compared the susceptibility of quail and chickens to H9 viruses and investigated the molecular determinants of replication and transmission of H9 viruses in quail and chickens. We identified molecular features in the

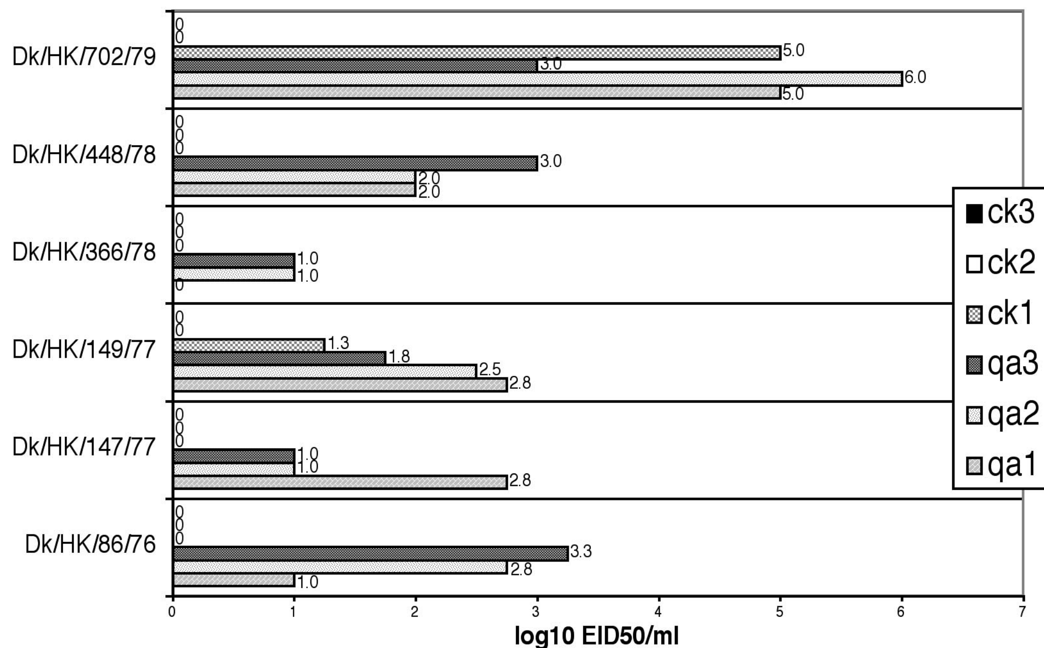

FIG. 1. Replication of duck H9 influenza viruses in quail and chickens. The replication of five H9N2 viruses (Dk/HK/702/79, Dk/HK/448/78, Dk/HK/366/78, Dk/HK/149/77, and Dk/HK/86/76) and one H9N6 virus (Dk/HK/147/77) was monitored by taking tracheal swabs collected during 9 days after inoculation. Tracheal swabs were inoculated in embryonated chicken eggs to determine the presence of H9 viruses and were titrated by EID_{50} (*x* axis). The graph represents the amount of virus shedding for each inoculated bird at 3 days postinoculation, which is the time when maximum viral shedding was observed. The *y* axis shows the abbreviated names of viruses used. Dk, duck; HK, Hong Kong; ck, chicken; qa, quail. Numbers to the right of the horizontal bars show virus titers, with zero indicating a lack of virus shedding as determined in our assay.

HA molecule of H9 viruses associated with their becoming endemic in land-based poultry in Asia. Our findings further emphasize the role played by quail in the evolution of influenza A viruses; quail provide an environment in which influenza viruses from ducks can adapt and generate variants with the capacity to infect other avian species.

MATERIALS AND METHODS

Viruses. The H9N2 influenza A viruses used in this study were obtained from the repositories at The University of Hong Kong and at St. Jude Children's Research Hospital and were propagated in 10-day-old embryonated chicken eggs.

Animals and experimental infections. Four-week-old Japanese quail (*Coturnix coturnix*) (B & D Game Farm, Harrah, Okla.), 4-week-old mallard ducks and white Peking ducks (IDEAL Poultry and Breeding Farms, Inc., Cameron, Tex.), and 4-week-old specific-pathogen-free white Leghorn chickens (Spafas, North Franklin, Conn.) were used. Groups of three birds were inoculated orally, intranasally, and intratracheally with 5×10^6 50% egg infective doses (EID_{50}) of avian influenza viruses/ml. One milliliter and 0.5 ml of virus inocula were used for chickens and quail, respectively; 2 drops (~100 μl) were introduced through the nares, and the rest of the virus dilution was equally distributed between oral and tracheal inoculations. Tracheal and cloacal swabs were obtained daily for 12 days after inoculation. Swab samples were diluted in 1 ml of freezing medium (50% glycerol in phosphate saline buffer) containing antibiotics, as described previously (9). Swab samples were titrated for infectivity in embryonated chicken eggs by the method of Reed and Muench (32). Undiluted positive samples with no hemagglutinin (HA) activities at the 10^{-1} dilution in EID_{50} assays were scored as positive with the notation of "<1.0 EID_{50}/ml." The birds were weighed daily and observed for overt signs of disease. Samples from ducks and chickens were obtained and analyzed essentially as described above. In the transmission experiments, uninfected birds were placed in direct, aerosol, and fecal contact with inoculated birds 1 day after inoculation (where indicated). Trays between the cages were removed to allow efficient aerosol and fecal transmission. Animal work was performed under BL3+ biosafety conditions at St. Jude Children's Research Hospital.

Isolation of RNA, reverse transcription-PCR amplification, and sequencing. Extraction of viral RNA, synthesis of cDNA, and PCR were performed as described by Hoffmann et al. (15), with minor modifications. Sequencing was performed by the Hartwell Center for Biotechnology at St. Jude Children's Research Hospital by using the rhodamine dye-terminator cycle sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems Inc., Foster City, Calif.).

Cloning and generation of viruses by reverse genetics. The eight genes of A/Duck/Hong Kong/448/78 and A/Guinea fowl/Hong Kong/WF10/99 and the HA and neuraminidase (NA) genes of A/Quail/Hong Kong/A28945/88 were cloned as described previously (13). Plasmids were sequenced as described above, and the sequences were compared to the sequences generated from the wild type virus. Only clones that exactly matched the parental virus sequence were used for virus rescue by reverse genetics. Viruses were rescued by using the eight-plasmid system with minor modifications (13). Briefly, eight plasmids (1 μg each) were incubated for 45 min with 18 μl of Trans-LTI (Panvera, Madison, Wis.) in 1 ml of Optimem (Invitrogen). Subsequently, the DNA was used to transfect a 1:1 mixture of 293-T and MDCK cells as described previously (13). Supernatant was collected from transfected cells after 72 h and was used to inoculate 10-day-old embryonated chicken eggs. Allantoic fluid containing virus was collected, titrated to determine the EID_{50}, and stored at −70°C. The titers of the viruses generated by reverse genetics were 10^8 to $10^{8.75}$ EID_{50}/ml. The HA and NA genes of the recombinant viruses were sequenced to confirm the lack of spurious mutations. Reverse genetic viruses were generated and handled under BL3+ biosafety conditions.

Phylogenetic and protein sequence analyses. Phylogenetic analysis of the H9 HA genes was based on a 993-nucleotide region (positions 84 to 1077) of the HA1 portion of the molecule. The Lasergene software package (DNAstar, Madison, Wis.) was used to edit and translate all sequence data. Sequence data were analyzed by using PAUP (phylogenetic analysis using parsimony) software, version 4.0b10. A heuristic search was used to generate the shortest phylogenetic tree. The deduced amino acid sequences were aligned by using ClustalX, version 1.81 (18).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study are available from GenBank under accession numbers AY206671 to AY206680.

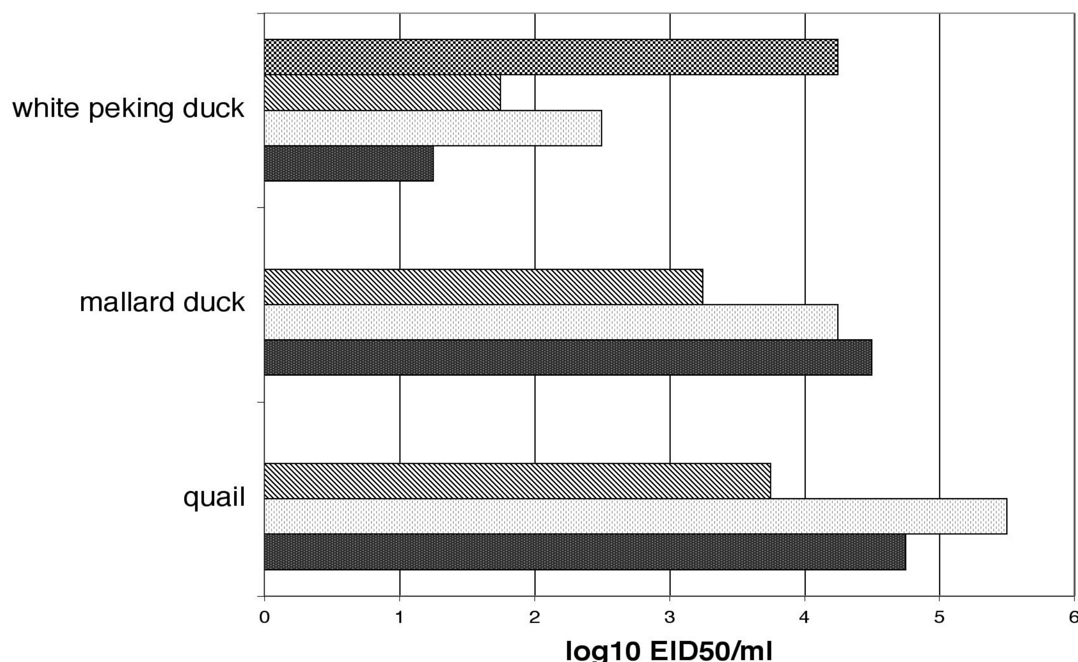

FIG. 2. Replication of Duck/Hong Kong/702/79 (H9N2) virus in quail and ducks. Tracheal swabs collected 2 to 4 days after inoculation were inoculated in embryonated chicken eggs and titrated by EID_{50}. Each bar shows the maximum titer for each inoculated bird (n = 3 quail, 3 mallard ducks, and 4 white Peking ducks). Virus titers were maximal at 3 days postinoculation in quail, 4 days postinoculation in mallard ducks, and 2 days postinoculation in white Peking ducks.

RESULTS

Replication and transmission of duck H9N2 viruses in chickens and quail. To better understand the biological and molecular features that have enabled H9 viruses to become established in land-based poultry, we compared the replication and transmission of five H9N2 viruses and one H9N6 virus in quail and chickens; all of these viruses had been isolated from domestic ducks in Hong Kong between 1976 and 1979. Groups of three 4-week old white Leghorn chickens and quail (*C. coturnix*) were inoculated by oral, tracheal, and nasal inoculations. Virus replication was monitored daily for 9 days, starting 1 day after inoculation. Quail were markedly susceptible to infection with the six duck viruses (Fig. 1). The viruses replicated predominantly in the respiratory tract of the quail but were isolated occasionally from the cloaca (data not shown). Maximum virus shedding was observed between days 3 and 4 postinoculation, with traces of virus shedding by 7 days postinoculation. In contrast, a single chicken inoculated with the Duck/HK/149/77 (H9N2) virus shed virus from the trachea at a low titer (Fig. 1), and a single chicken inoculated with the Duck/HK/702/79 (H9N2) virus shed as much as 10^5 EID_{50} of virus/ml from the trachea (Fig. 1) and the cloaca (data not shown) 3 days postinoculation. Duck/HK/702/79 virus was shed by the positive chicken for an additional 2 days at titers lower than that obtained at day 3 postinoculation. No evidence of Duck/HK/149/77 virus replication was observed in any of the chickens infected with this virus at 4 days postinoculation. No evidence of virus replication was observed in the other chickens inoculated with the other H9 viruses, and no signs of disease or changes in body weight were observed in any of the birds. Interestingly, in two additional separate experiments involving the Duck/HK/702/79 and Duck/HK/149/77 viruses and three chickens per group, no evidence of virus replication was observed in any of the inoculated chickens (data not shown); which suggests that chickens are mostly refractory to these viruses. Nevertheless, our results highlight the complexity of biological systems and show that in nature the species barrier may be overcome by the presence of unusually suscep-

TABLE 1. Transmission of H9N2 viruses in quail and chickens

Virus	No. of positive tracheae/total no. of birds (log_{10} EID_{50}/ml for each bird in the group)					
	Quail				Chickens	
	Inoculated[a]	Direct contact	Aerosol contact	Fecal contact	Aerosol contact	Fecal contact
Dk/HK/448/78	3/3 (2.5, 2.5, 1.0)	0/3	0/3	0/3	0/3	0/3
Dk/HK/702/79	3/3 (3.8, 2.5, 4.0)	2/3 (2.8, 2.3)	1/3 (3.8)	1/3 (3.8)	0/3	0/3
Qa/HK/A28945/88	3/3 (5.5, 5.5, 5.0)	3/3 (5.5, 5.5, 1.5)	3/3 (4.5, 5.0, 4.0)	3/3 (5.5, 5.5, 1.3)	0/3	3/3 (2.3, 2.0, 1.0)

[a] For inoculated quail, titers in parentheses correspond to each positive bird in the group 3 days after inoculation. For all other groups, titers in parentheses correspond to each positive bird in the group 5 to 7 days after inoculation.

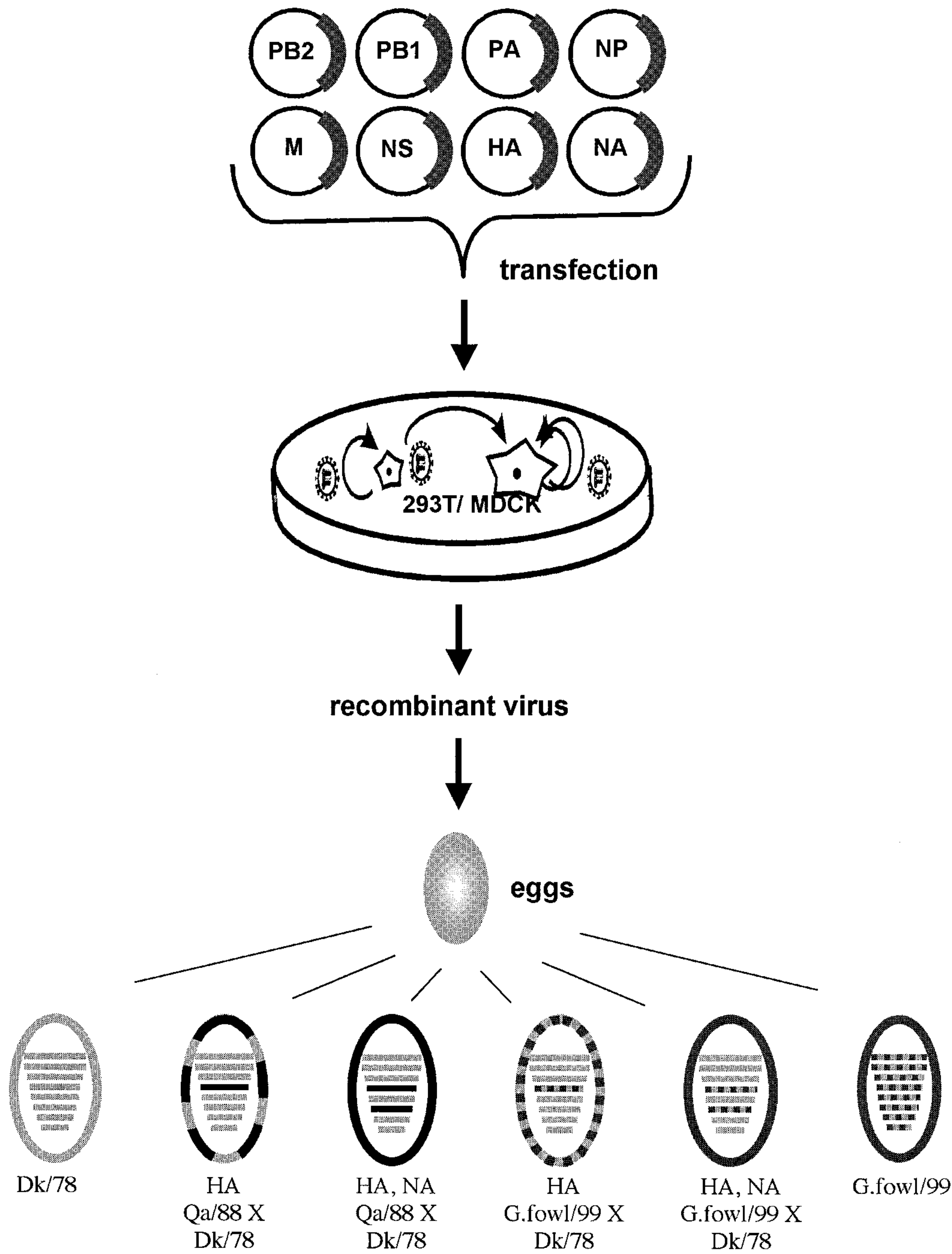

FIG. 3. Generation of H9N2 influenza viruses by reverse genetics. A/Duck/Hong Kong/448/78 (H9N2) (Dk/78) and A/Guinea fowl/Hong Kong/wf10/99 (H9N2) (Gfowl/99) influenza viruses were generated by reverse genetics using the 8-plasmid system. Plasmids were transfected into 293T cells cocultured in the presence of MDCK cells, as described by Hoffmann et al. (13). Rescued viruses were grown once in embryonated chicken eggs and titrated to determine the EID_{50}. Recombinant H9N2 viruses generated by using the 8-plasmid strategy contained the internal genes of Dk/78 and the HA or the HA and NA genes of A/Quail/Hong Kong/A28945/88 (H9N2) (Qa/88) or Gfowl/99.

tible animals. Virus replicated as efficiently in the respiratory tracts of quail as in the respiratory tracts of white Peking ducks or mallard ducks, although both duck species shed virus mainly from the cloaca (data not shown). Figure 2 shows representative results obtained with the Duck/HK/702/79 (H9N2) virus tested in three quail, three mallard ducks, and four white Peking ducks. Similar results were obtained when we compared the replication of the Duck/HK/448/78 (H9N2) virus in quail, mallard ducks, and white Peking ducks, although the virus replicated less efficiently in the tracheae of the three bird species tested (data not shown). Taken together, our results suggest that quail are more susceptible than chickens to infection with the duck H9N2 viruses.

To determine whether replication of the duck H9 viruses in quail leads to their efficient transmission to other quail and/or chickens, we designed a transmission experiment using two of

TABLE 2. Transmission of recombinant H9N2 viruses in quail and chickens

Virus	No. of positive tracheae/total no. of birds ($\log_{10}$ EID_{50}/ml for each bird)			
	Quail		Chickens	
	Inoculated[a]	Direct contact[b]	Inoculated[a]	Direct contact[b]
RG HA Qa/88 × Dk/78	3/3 (3.8, 1.8, 2.5)	3/3 (3.5, 5.3, 5.0)	3/3 (1.3, <1.0, <1.0)	1/3 (<1.0)
RG HA, NA Qa/88 × Dk/78	3/3 (4.8, 4.3, 2.8)	3/3 (5.5, 5.3, 5.5)	3/3 (2.8, <1.0, 3.5)	0/3
RG HA Gfowl/99 × Dk/78	3/3 (1.8, 1.5, 4.8)	3/3 (2.3, 2.5, 1.0)	3/3 (<1.0, <1.0, <1.0)	0/3
RG HA, NA Gfowl/99 × Dk/78	3/3 (3.8, 3.8, 4.8)	3/3 (5.3, 5.5, 3.8)	3/3 (1.5, <1.0, 2.8)	0/3
RG Gfowl/99	6/6 (4.0 ± 1.0)[c]	6/6 (3.5 ± 0.5)[c]	6/6 (4.0 ± 1.0)[c]	4/6 (2.5 ± 0.5)[c]
RG Dk/78	3/3 (2.5, 2.0, 3.0)	0/3	0/3	0/3

[a] Titers in parentheses correspond to each bird in the group 3 days after inoculation.
[b] Titers in parentheses correspond to each bird in the group 5 to 7 days after inoculation.
[c] Results of two independent experiments. Mean titers and standard deviations are shown.

the duck H9N2 viruses. Groups of three quail were inoculated by the oral, intratracheal, and intranasal routes. One day after inoculation, each group was placed in direct contact with three uninfected quail, in fecal contact with three uninfected quail and three uninfected chickens, and in aerosol contact with three uninfected quail and three uninfected chickens. Only the Duck/HK/702/79 (H9N2) virus was transmitted to other quail, albeit inefficiently, and neither virus was transmitted from quail to chickens (Table 1). In addition, we tested the transmission of these viruses from chickens to chickens. As expected, the poor replication of these viruses in chickens did not allow their efficient transmission to other chickens (data not shown). Therefore, although quail are susceptible to infection with the duck H9N2 viruses, efficient transmission to other quail or to chickens would require adaptation.

Characterization of an early H9N2 virus isolated from quail in Hong Kong. In 1988, we isolated three H9N2 viruses from dead quail on one Hong Kong farm (40,000 birds) where there was an outbreak lasting 2 to 3 months. Five thousand young birds 5 to 7 days old suffered from respiratory diseases and died (W. Lim, unpublished data). These isolates were the first evidence of H9 viruses in land-based poultry in Asia. Previous studies have shown that viruses currently circulating in quail may acquire the capacity to cross to other species, including chickens and humans (3, 7, 8, 9, 22, 28). Therefore, we sought to determine whether the H9N2 viruses isolated from quail in 1988 had acquired the capacity to replicate and be transmitted in chickens and quail. We tested one of these isolates, the A/Quail/Hong Kong/A28945/88 (H9N2) virus. The virus caused no signs of disease in quail and chickens. It replicated in the tracheae of chickens and quail and was transmitted efficiently from quail to quail and from quail to chickens (Table 1). The three quail in each contact group (direct, aerosol, and fecal) became infected with the A/Quail/Hong Kong/A28945/88 (H9N2) virus and shed virus at titers similar to those of inoculated quail (mean titer, 10^5 EID_{50}/ml). This level of replication in the contact quail was similar to that observed with currently circulating quail H9 viruses, suggesting that the A/Quail/Hong Kong/A28945/88 (H9N2) virus is fully adapted to quail and may have been circulating in quail before 1988. Surprisingly, the virus was transmitted to chickens only via fecal contact, despite its preferential replication in the respiratory tract. Titers of virus shed in the tracheae were lower for chickens ($\sim10^{2.5}$ EID_{50}/ml) than for quail or for chickens infected with other H9N2 viruses adapted to chickens (10). Our results clearly showed that a virus adapted to quail is able to cross the species barrier a second time.

Gene alterations required for transmission of H9N2 viruses in quail and chickens. The host range of influenza A viruses is thought to be polygenic, determined by both surface and internal viral gene products (reviewed in reference 17). This hypothesis has been confirmed by numerous studies, particularly those investigating the replication of avian-mammalian reassortant viruses in birds and mammals (5, 33). In the case of interspecies transmission of influenza viruses among nonnatural avian hosts (such as land-based poultry), the factors that determine host range are not clear. To better understand these factors in the host range of H9N2 viruses, we used reverse genetics to generate the virus Duck/Hong Kong/448/78 (Dk/78), which is not transmitted in quail and does not replicate in chickens. We then generated recombinants that had the Dk/78 genetic background and the HA gene or the HA and NA genes of the A/Quail/Hong Kong/A28945/88 virus (Qa/88), which transmits well in quail and chickens (Fig. 3), and we tested their replication and transmission in quail and chickens (Table 2). Replacing the HA gene of Dk/78 with the HA gene of Qa/88 was sufficient to allow efficient replication and transmission of the recombinant in quail. The addition of the N2 NA gene of Qa/88 to this recombinant did not appreciably affect the results. In contrast, both recombinants replicated poorly in chickens and were not transmitted in chickens. Similar results were obtained with two recombinant viruses carrying the HA or the HA and NA genes of A/Guinea fowl/Hong Kong/WF10/99 (H9N2) (Gfowl/99) in the background of the Dk/78 virus (Table 2 and Fig. 3). The wild-type Gfowl/99 virus is related phylogenetically to H9N2 influenza viruses isolated from chickens (e.g., A/Chicken/Pakistan/2/99), quail (e.g., A/Quail/Hong Kong/G1/97), and humans (e.g., A/Hong Kong/1074/99). The HA and the HA and NA Gfowl/99 × Dk/78 virus (H9N2) recombinants replicated and transmitted efficiently in quail but not in chickens. In contrast, the wild-type Gfowl/99 (H9N2) virus generated by reverse genetics replicated and was transmitted in chickens (Table 2). Taken together, these results suggest that molecular changes in the internal genes are required to allow H9N2 viruses to replicate efficiently in chickens. In contrast, alteration of only the HA gene can render these viruses capable of replication and transmission in quail.

Molecular characterization of H9N2 viruses. We analyzed the HA genes of the H9N2 viruses used in this study to determine their phylogenetic relationships. To identify molecular

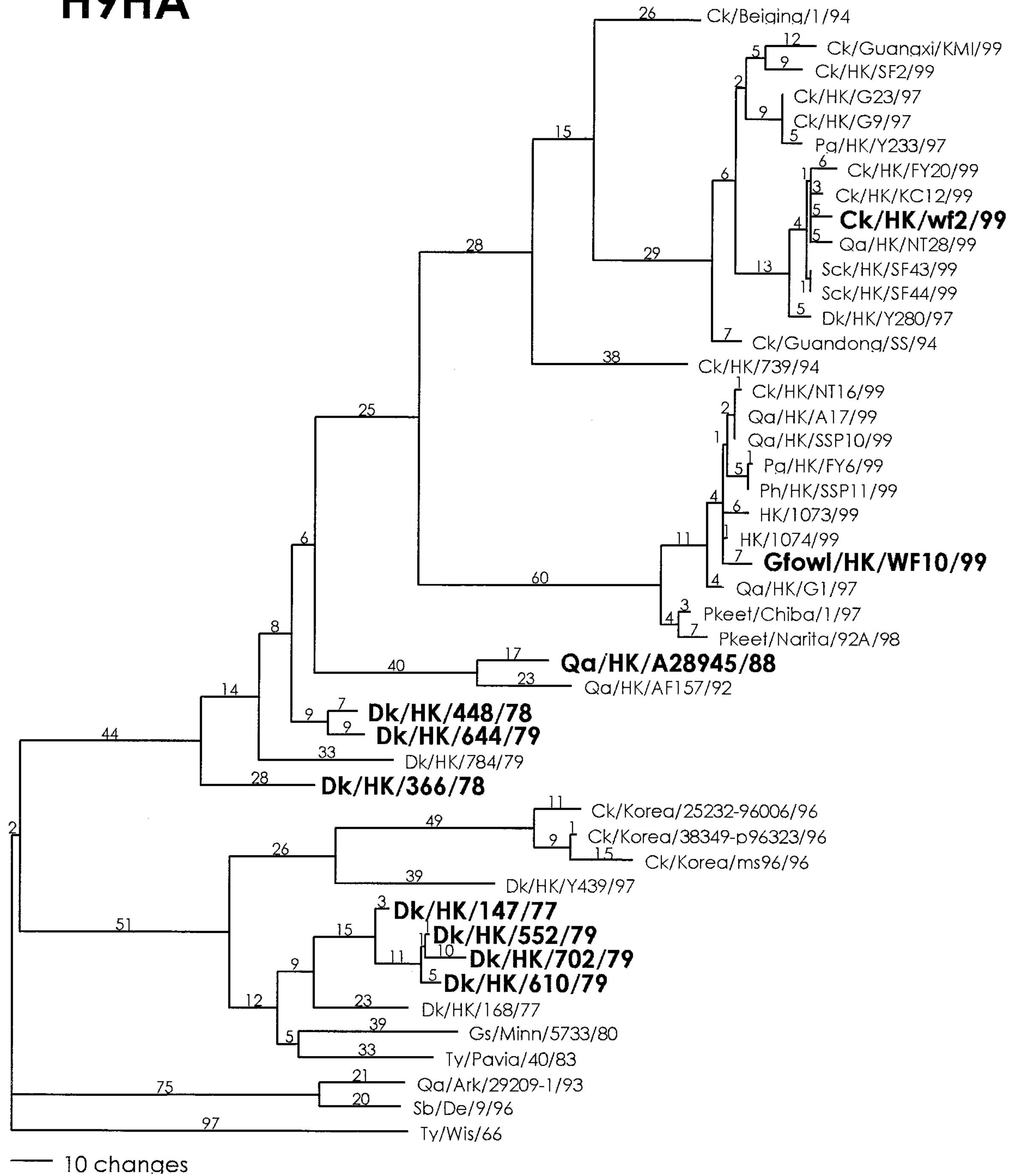

FIG. 4. Phylogenetic tree for H9 viruses. The 993-nucleotide sequences corresponding to the HA1 regions of H9 viruses were analyzed by the PAUP program, using a maximum-parsimony algorithm. The tree is rooted to A/Turkey/Wisconsin1/66. Numbers on horizontal lines indicate the number of nucleotide differences required to join the nodes. Boldface indicates viruses analyzed in this study; other virus sequences were obtained from the Influenza Sequence Database at Los Alamos National Laboratories (23) (accession numbers available on request). Ck, chicken; Dk, duck; Gs, goose; Pg, pigeon; Ph, pheasant; Pkeet, parakeet; Qa, quail; Sb, shorebird; Sck, silkie chicken; Ty, turkey; Ark, Arkansas; De, Delaware; Minn, Minnesota; HK, Hong Kong; Wis, Wisconsin.

factors in the viruses' adaptation and transmissibility in land-based birds, we also aligned the viruses' HA protein sequences with the predicted open reading frames. The HA genes of the 1970s duck H9N2 viruses formed two sublineages (Fig. 4). One is close to the root of the ancestor of the H9N2 viruses currently circulating in land-based birds in Hong Kong. The other contains viruses that are closer to H9N2 viruses currently circulating in ducks in Hong Kong.

TABLE 3. Molecular markers for species specificity of H9 HA genes

Amino acid position	Function/location	Amino acid in:		
		Chickens	Quail	Ducks[a]
8	Signal peptide	T	T (A)	A
129 (109 HA1)	Bottom of globular head/pH of fusion?	S (T)	S, N, R (K)	N, R (K)
166 (146 HA1)	Adjacent receptor binding site	Q	Q, H	H
337 (317 HA1)	−4 position, HA cleavage site	R	K, R	T, A, V
339 (319 HA1)	−2 position, HA cleavage site	S	S (N)	D, N, G
475 (135 HA2)	Viral membrane-interacting residue?	K	K	N
500 (160 HA2)	Viral membrane-interacting residue?	R	R	Q

[a] Includes viruses isolated from domestic ducks, shorebirds, and turkeys.

An increased number of glycosylation sites on the HA protein have been linked to the adaptation of H5 and H7 viruses to land-based poultry (25). Although H9 viruses vary in the number of HA glycosylation sites (from six in duck H9 viruses to as many as eight in quail and chicken H9 viruses), no single specific glycosylation site corresponds to adaptation to land-based poultry. The alignment of 53 H9 HA protein sequences revealed seven amino acid positions that correspond to the adaptation of H9 viruses in land-based birds (Table 3 and Fig. 5). Interestingly, in quail H9 viruses, the amino acids at these seven positions corresponded to those found in chicken and/or duck H9 viruses; this finding may explain the susceptibility of quail to the duck H9N2 viruses used in this study. One of those sites (residue 146) is adjacent to residues involved in receptor binding (11, 20, 30). Chicken H9 viruses contain glutamine at position 146, while duck H9 viruses contain histidine at this location. Quail H9 viruses contain either glutamine or histidine at position 146. The recent discovery that chickens possess both α-2′3′ and α-2′6′ sialic acid receptors explains the emergence of chicken H9 viruses with human-like receptor specificity (6). Our results are consistent with this observation and suggest that changes on the periphery of the receptor-binding site contribute to species specificity.

Residue 109 is located at the bottom of the globular head of the HA1 portion of H9 HA. In chicken H9 viruses, this position is occupied almost exclusively by serine, whereas in duck H9 viruses, it is usually occupied by asparagine, arginine, or, less often, lysine. Interestingly, quail H9 viruses either carry serine at position 109 (as do chicken viruses) or carry asparagine, arginine, or lysine at this position (as do duck viruses).

Two other sites related to species adaptation are at the HA1–HA2 cleavage site. Residues 317 and 319 are 4 residues and 1 residue, respectively, upstream of the HA1–HA2 cleavage site and possess distinctive biophysical properties that depend on the virus's origin. An arginine (basic amino acid) is present at position 317 in H9 viruses from chickens and quail, but alanine or valine (hydrophobic amino acids) occupy this position in most duck H9 viruses. Likewise, position 319 is occupied by serine in chicken H9 viruses and most quail H9 viruses; however, aspartic acid, asparagine, or glycine occupies this position in duck H9 viruses and in some quail H9 viruses. In the H9 viruses, as in the H5 and H7 viruses adapted to chickens, the presence of basic amino acids at the HA1–HA2 cleavage site appears to be related to the cleavability of the HA protein by host proteases during virus maturation. However, H9 viruses do not appear to accumulate extra basic amino acids at this site, as do H5 and H7 viruses.

Amino acid position 8, within the signal peptide region of the H9 HA, is also related to the ability of H9 viruses to adapt to chickens and quail. Position 8 is occupied by threonine in all chicken and quail H9 viruses but by alanine in duck H9 viruses. Analysis of the signal peptide sequences of all avian HA subtypes reveals that threonine at position 8 is unique to H9 viruses from land-based birds (data not shown). Amino acid residues 135 and 160 in the H9 HA2 molecule are also related to species specificity. These two residues are spatially positioned close to the viral membrane (Table 3), but further studies are needed to ascertain their biological significance in the adaptation of H9 viruses to land-based birds.

We found four exceptions to this pattern: three H9N2 viruses isolated from chickens in Korea in 1996 (21) and one duck isolate, Duck/Hong Kong/Y280/97. However, the three Korean chicken isolates still differ from all duck isolates at two HA positions: they carry tyrosine at the −2 position in the cleavage site, and they carry lysine at the 135 (HA2) position, as do the other chicken isolates. Duck/Hong Kong/Y280/97 was isolated from the live poultry markets in Hong Kong at a time when all poultry species were mixed, and it may well be the result of reverse transmission of a chicken virus to a duck.

DISCUSSION

The mechanism responsible for the establishment of H9 viruses in land-based poultry is poorly defined. In this study, we found that H9N2 viruses isolated from ducks in Hong Kong in the 1970s replicate more readily in quail than in chickens and that infection is established mostly in the respiratory tract. The latter observation is consistent with the premise that infection of quail can promote a change in the tissue tropism of avian influenza A viruses, allowing the emergence of variants that transmit by aerosol. Unlike quail, chickens were generally refractory to infection with these viruses. The greater susceptibility of quail is consistent with the fact that quail were the first land-based birds in Asia from which H9N2 viruses were isolated. Interestingly, by 1988, H9N2 viruses isolated from quail had already acquired some of the molecular markers associated with established virus lineages in land-based birds (Table 3). The transmissibility of the 1988 quail virus from quail to chickens (Table 1) further supports the hypothesis that quail can act as an intermediate host in the interspecies spread of influenza viruses. We also demonstrated that changes on the surface of the HA protein are sufficient to allow efficient replication and transmission in quail (Table 2). In contrast, viruses may have to undergo molecular changes in their internal genes

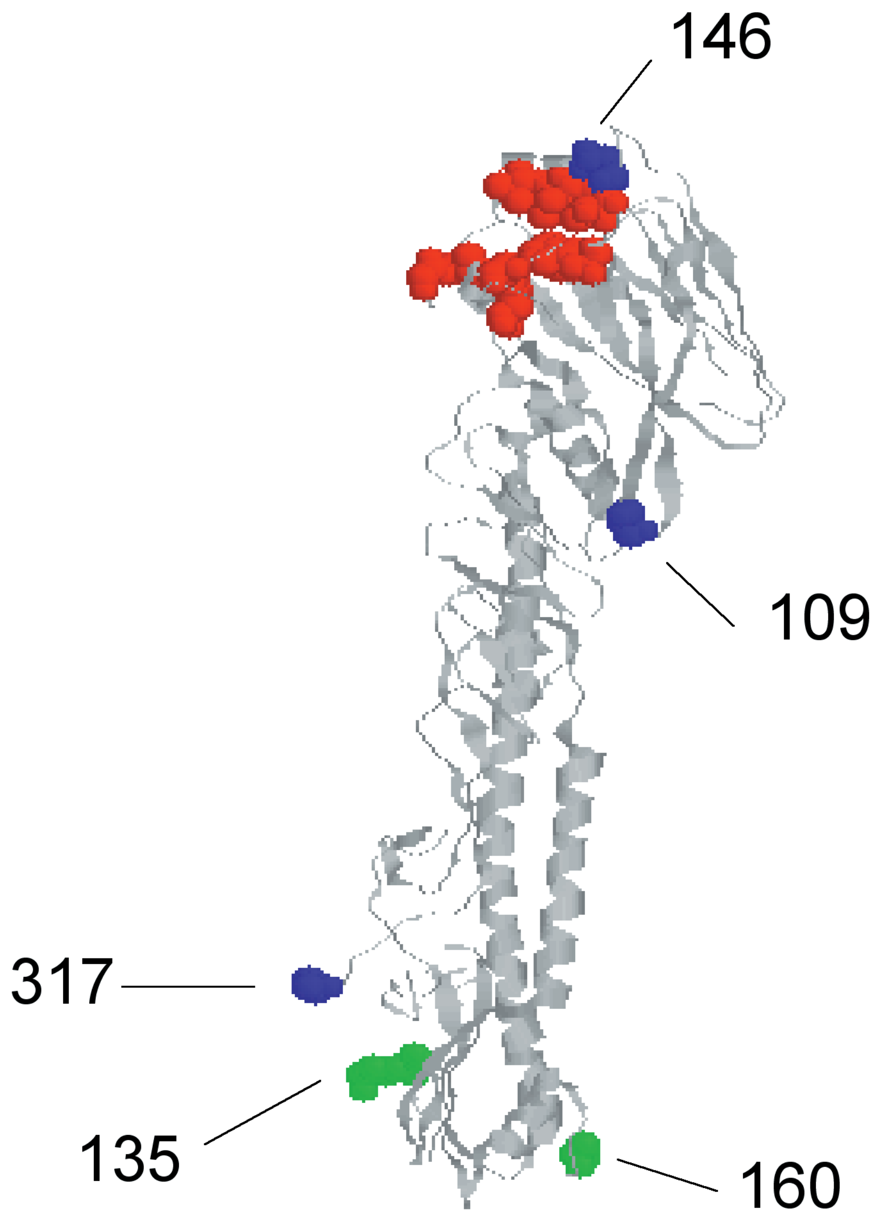

FIG. 5. Amino acid positions in the HA molecule that correspond to adaptation of H9 viruses to land-based birds. Seven amino acid positions correspond to adaptation of H9 viruses to chickens and quail (five are shown). Space-filling amino acids correspond to residues involved in receptor binding (red) (11), species-specific residues on the HA1 portion of the H9 HA molecule (blue), and species-specific residues found on the HA2 portion of the H9 HA molecule (green). Numbers indicate the amino acid positions in the H9 HA1 or HA2 region. Two species-specific residues are removed by posttranslational modification (residue 8 within the signal peptide region and residue 319 of HA1 at the HA1–HA2 cleavage site).

before they are able to replicate and be transmitted in chickens.

We identified seven amino acids on the H9 HA glycoprotein that correspond to the adaptation of H9 viruses to land-based birds. Because these amino acids are found in viruses that have become endemic in chickens after multiple introductions from the aquatic bird reservoir (10), we speculate that they can confer important biological advantages. Interestingly, quail viruses have amino acids at these seven positions that correspond to those of chicken and/or duck viruses (Table 3). One of these amino acids (at position 146) is near the receptor-binding site and is likely to influence the binding of the HA molecule to the sialic acid receptor (Fig. 5). Two other residues are at positions −4 and −2 of the HA1–HA2 cleavage site, where the basic amino acids arginine (or lysine) at −4 and serine at −2 are characteristic of chicken and quail viruses. Our results suggest that H9 viruses that contain an aspartic acid at position −2 of the HA1–HA2 cleavage site replicate poorly in chickens and quail. The remaining four amino acid residues related to species specificity occupy positions that have not been recognized as important host range markers, although three of these positions (109 in HA1 and 135 and 160 in HA2) could influence the pH of fusion of the HA molecule to the host endosomal membrane. Ongoing studies will reveal

the biological importance of these residues in the host range of H9 viruses. Our results support the hypothesis that quail play an important role in the evolution of influenza viruses by acting as intermediate hosts in which avian influenza viruses can be amplified and transmitted to other animal species. It is possible that programs aimed at the prevention of influenza pandemics should include influenza virus surveillance in quail and related species, not only in China but also in other parts of the world.

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