The evolution of H5N1 influenza viruses in ducks in southern China

H. Chen*†‡, G. Deng*†, Z. Li*, G. Tian*, Y. Li*, P. Jiao*, L. Zhang*, Z. Liu*, R. G. Webster§, and K. Yu*¶

*Animal Influenza Laboratory of the Ministry of Agriculture and †National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, People's Republic of China; and §Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38105

Contributed by R. G. Webster, May 21, 2004

The pathogenicity of avian H5N1 influenza viruses to mammals has been evolving since the mid-1980s. Here, we demonstrate that H5N1 influenza viruses, isolated from apparently healthy domestic ducks in mainland China from 1999 through 2002, were becoming progressively more pathogenic for mammals, and we present a hypothesis explaining the mechanism of this evolutionary direction. Twenty-one viruses isolated from apparently healthy ducks in southern China from 1999 through 2002 were confirmed to be H5N1 subtype influenza A viruses. These isolates are antigenically similar to A/Goose/Guangdong/1/96 (H5N1) virus, which was the source of the 1997 Hong Kong ''bird flu'' hemagglutinin gene, and all are highly pathogenic in chickens. The viruses form four pathotypes on the basis of their replication and lethality in mice. There is a clear temporal pattern in the progressively increasing pathogenicity of these isolates in the mammalian model. Five of six H5N1 isolates tested replicated in inoculated ducks and were shed from trachea or cloaca, but none caused disease signs or death. Phylogenetic analysis of the full genome indicated that most of the viruses are reassortants containing the A/Goose/Guangdong/1/96-like hemagglutinin gene and the other genes from unknown Eurasian avian influenza viruses. This study is a characterization of the H5N1 avian influenza viruses recently circulating in ducks in mainland China. Our findings suggest that immediate action is needed to prevent the transmission of highly pathogenic avian influenza viruses from the apparently healthy ducks into chickens or mammalian hosts.

Avian influenza viruses are zoonotic agents recognized as a continuing threat to both veterinary and human public health. During the past 6 years, infection of humans with avian influenza viruses of three subtypes (H5, H7, and H9) has been detected on multiple occasions (1, 2). In 1997, H5N1 avian influenza viruses transmitted from birds to humans in Hong Kong caused the deaths of 6 of 18 infected persons (3, 4). The virus was eradicated by the slaughter of all poultry in Hong Kong, but new genotypes of H5N1 virus continued to emerge in poultry in Hong Kong in 2000 and 2001 (5, 6), and in 2003, antigenically and biologically novel H5N1 influenza virus killed one of two infected humans (7).

Subtype-H9N2 avian influenza virus was isolated from two sick children in Hong Kong in 1999 (8) and from six patients in mainland China (9); all recovered from the infection. The H9N2 variant isolated from humans on the mainland also was isolated from pigs in southern China in 1999 (10, 11), but there was no serologic evidence that a stable virus lineage had become established in pigs or humans. The H9N2 viruses have continued to circulate in poultry throughout Europe and Asia, and are now considered to be enzootic throughout the entire region.

The H7N7 avian influenza virus that caused highly pathogenic avian influenza on 225 poultry farms in Holland in 2003 was associated with conjunctivitis in 347 humans (12). Infection with H7N7 influenza virus was confirmed in 87 of these cases; one person, a veterinarian, died of the infection. There was also human-to-human transmission of the virus and serologic evidence of H7N7 infection of pigs (13). This outbreak was controlled by culling and quarantine of infected poultry; to prevent the emergence and spread of human–avian virus reassortants, the poultry workers were given human influenza vaccine and antineuraminidase drugs.

The transmission of H5N1 "bird flu" to humans in 1997 first established the ability of avian influenza viruses to be transmitted to humans despite their preferential binding to avian sialic acid receptors (i.e., those with a terminal α2,3Gal linkage) (14). Before 1997, there had been isolated reports of human infection with H7N7 influenza virus (usually causing conjunctivitis) (12), but there was no convincing evidence of repeated transmission of avian viruses to humans. How did avian influenza viruses come to acquire a progressively greater capacity to infect mammals? Here, we characterize a series of 21 H5N1 influenza viruses isolated from apparently healthy domestic ducks in coastal provinces and cities of southern China from 1999 through 2002. These isolates were highly lethal to chickens and demonstrated the progressive acquisition of pathogenicity to mice (a mammalian host). They were genomically heterogenous, having acquired multiple gene segments and deletions in their nonstructural (NS) and neuraminidase (NA) genes. We propose a hypothetical mechanism to explain the selection of H5N1 viruses with increasing pathogenicity to mice.

Materials and Methods

Virus Isolation and Identification. After A/Goose/Guangdong/1/96 (H5N1)(GSGD/1/96) virus was identified as an important precursor of the Hong Kong 1997 (HK/97) avian influenza virus, routine influenza virus surveillance of apparently healthy poultry was started in the coastal Chinese towns and provinces of Guangdong, Guangxi, Fujian, Zhejiang, and Shanghai. Cloacal samples were obtained from apparently healthy ducks on farms. Viruses were isolated in embryonated chicken eggs as described in ref. 15. A total of 21 H5N1 viruses were identified by hemagglutination inhibition (HI) and neuraminidase inhibition tests with a panel of antisera provided by Office International Des Épizooties Reference Laboratory (Veterinary Laboratory Agency, Surrey, U.K.). Each virus was biologically cloned by three rounds of limiting dilution in embryonated specific-pathogen-free eggs. All experiments with the H5N1 isolates were performed in a biosafety-level 3 laboratory facility, and animal experiments were performed in high-efficiency particulate air-filtered (HEPA-filtered) isolators.

Abbreviations: DK, duck; eID_{50}, egg 50% infective dose; FJ, Fujian; GD, Guangdong; GS, goose; GX, Guangxi; HA, hemagglutinin; HK, Hong Kong; IVPI, i.v. pathogenicity index; MDT, mean time to death; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; SH, Shanghai; ZJ, Zhejiang; i.n., intranasally.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY585357–AY585524).

‡To whom correspondence should be addressed at: Harbin Veterinary Research Institute, 427 Maduan Street, Harbin 150001, People's Republic of China. E-mail: hlchen1@yahoo.com.

¶Present address: National Animal Husbandry and Veterinary Service of the Ministry of Agriculture, 20 Maizidian Street, Beijing 100026, People's Republic of China.

Table 1. Mortality rate and MDT of chickens inoculated with 21 H5N1 avian influenza viruses isolated from ducks and with A/goose/Guangdong/1/96

Virus titer (log_{10} eID_{50}/ml)	i.v. inoculation: No. dead/ inoculated	i.v. inoculation: MDT, days	i.v. inoculation: IVPI	i.n. inoculation: No. dead/ inoculated	i.n. inoculation: MDT, days
GSGD/1/96 (8.5)	9/10	>5.9	2.1	6/10	>8.3
DKGX/07/99 (8.4)	5/10	>7.2	1.2	2/7*	>8.8
DKFJ/19/00 (9.0)	10/10	2.7	2.6	5/10	>8.7
DKGD/12/00 (9.5)	10/10	5.3	2.1	10/10	6.5
DKZJ/11/00 (9.5)	10/10	1.1	3.0	10/10	3.3
DKZJ/52/00 (8.6)	10/10	3.3	2.3	10/10	3.8
DKGD/07/00 (8.3)	10/10	2.7	2.4	6/10	>8.7
DKGD/40/00 (8.2)	10/10	1.3	2.9	10/10	4.0
DKGD/1/01 (8.3)	10/10	1.0	3.0	10/10	2.1
DKSH/8/01 (8.3)	10/10	1.0	3.0	10/10	3.8
DKSH/13/01 (7.5)	10/10	1.0	3.0	10/10	2.2
DKFJ/17/01 (7.2)	10/10	1.4	3.0	10/10	3.1
DKGX/22/01 (7.5)	10/10	1.6	2.9	10/10	4.0
DKGX/35/01 (7.4)	10/10	1.0	3.0	10/10	2.1
DKSH/38/01 (8.0)	10/10	1.0	3.0	10/10	2.0
DKGX/50/01 (8.6)	10/10	1.0	3.0	10/10	2.0
DKFJ/01/02 (8.5)	10/10	1.6	2.9	10/10	2.7
DKFJ/13/02 (8.5)	10/10	1.1	3.0	10/10	1.7
DKGD/22/02 (8.5)	10/10	1.5	2.9	10/10	2.5
DKSH/35/02 (7.5)	10/10	1.0	3.0	10/10	2.7
DKSH/37/02 (8.7)	10/10	1.5	3.0	10/10	1.8
DKGX/53/02 (8.0)	10/10	4.1	2.1	8/10	>6.5

*Three chickens were killed on day 3 postinoculation.

Animal Experiments. ***Chickens.*** To determine the pathogenicity of the virus isolates, the i.v. pathogenicity index (IVPI) was tested according to the recommendation of the Office International Des Épizooties. Groups of 10, specific-pathogen-free 6-week-old White Leghorn chickens housed in isolator cages were inoculated i.v. with 0.2 ml of a 1:10 dilution of bacteria-free allantoic fluid containing virus (virus titers are shown in Table 1). Ten additional chickens were inoculated intranasally (i.n.) with 10^6 egg 50% infective dose (eID_{50}) of each virus in a 0.1-ml volume; on day 3, three birds in each group were killed, and virus was titrated in samples of lung, bursa, kidney, thymus, thyroid gland, brain, heart, pancreas, muscular stomach, spleen, and trachea. Similar tests were performed on birds that had died. These tissues also were studied histopathologically; they were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Unfixed samples were titrated for virus infectivity in embryonated eggs.

Mice. Groups of eight 6- to 8-week-old female BALB/c mice (Beijing Experimental Animal Center, Beijing) were lightly anesthetized with CO_2 and inoculated i.n. with $10^{6.0}$ eID_{50} of H5N1 influenza virus in a volume of 50 μl. Control mice were inoculated with PBS. On day 3, three of the eight mice were killed for virus titration of the lungs, kidneys, spleen, and brain. The remaining five mice were monitored daily for weight loss and mortality. The mouse 50% minimal lethal dose (MLD_{50}) was determined for viruses that caused lethal infection of mice by i.n. inoculation of groups of five mice. The MLD_{50} was calculated by the method of Reed and Muench (16).

Ducks. Groups of five 3-week-old ducks (a local outbred strain of sheldrake) were inoculated i.n. with $10^{7.85}$ to $10^{8.5}$ eID_{50} of influenza virus in 0.1 ml. One day later, three contact ducks were introduced into each group. Ducks were observed daily for 10 days for signs of disease. Oropharyngeal and cloacal swab specimens were collected from all ducks 3 and 5 days after inoculation for titration of virus in eggs.

Genetic and Phylogenic Analysis. Viral RNA was extracted from allantoic fluid by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and was reverse-transcribed. PCR amplification was performed by using fragment-specific primers (primer sequences available on request). The PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced by using the CEQ DTCS-Quick Start Kit on a CEQ 8000 DNA sequencer (Beckman Coulter). Sequence data were compiled with the SEQMAN program (DNASTAR, Madison, WI), and the nucleotide sequences were compared and the phylogenetic trees were generated with the MEGALIGN program (DNASTAR) by using the Clustal alignment algorithm.

Nucleotide Sequence Accession Numbers. The nucleotide sequences obtained in this study are available from GenBank (accession nos. AY585357–AY585524).

Results

Antigenic Analysis. HI and neuraminidase inhibition tests with reference antisera showed each of the viruses to be of the H5N1 subtype. The viruses had a similar pattern of HI and neuraminidase inhibition reactivity that indicated they were most closely related to GSGD/1/96 (results not shown). The 21 H5N1 viruses isolated from 1999 through 2002 showed no evidence of antigenic drift; i.e., their heterologous HI titers were <4-fold lower than the homologous HI titers (data not shown).

Studies in Chickens. We used the IVPI and the criteria recommended by the Office International Des Épizooties to assay the pathogenicity of the virus isolates in chickens. All of the isolates were pathogenic by these criteria (IVPI >1.2) (Table 1). However, one (Table 1) of the earlier isolates (DKGX/07/99) killed only half of the i.v. inoculated chickens, and the mean time to death (MDT) of two of the early isolates was 8 days after i.n. inoculation. The year 2000 H5N1 isolates showed some diversity in MDT (3.3 to >8.7) after i.n. inoculation, whereas 2001 and 2002 isolates had an MDT between 1.7 and 4 after i.n. inoculation (the only exception is that DKGX/53/02 had an MDT >6.5). There was an upward trend in the IVPI over time. In the seven 1999 and 2000 isolates, the IVPI was 1.2–3.0, whereas in 13 of the 14 viruses isolated in 2001 and 2002, the IVPI was 2.9–3.0. The exception was DKGX/53/02, whose IVPI (2.1) and MDT (>6.5 days) values indicated much lower pathogenicity.

All chickens that died on day 2 or 3 after inoculation had lesions indicating systemic replication of virus. These comprised moderate necrosis of brain tissues, severe histiocytic interstitial pneumonia with edema, severe necrosis of the pancreas, mild necrosis of the

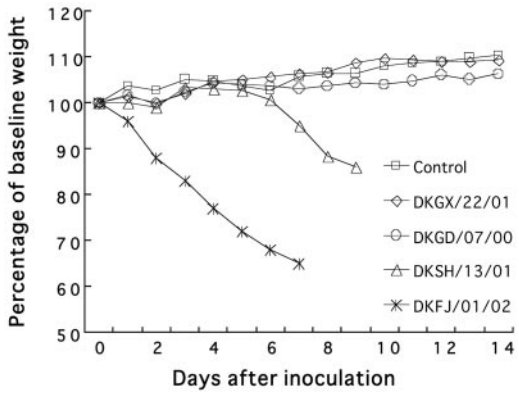

Fig. 1. Weight change of mice inoculated with H5N1 duck influenza viruses representative of the four pathotypes. Groups of five mice were inoculated i.n. with 10^6 eID_{50} (in 50 μl) of DKGX/22/01 (◇) (nonpathogenic), DKGD/07/00 (○) (low pathogenicity), DKSH/13/01 (△) (medium pathogenicity), or DKFJ/01/02 (∗) (high pathogenicity) virus or with PBS as control (□) and weighed daily for 14 days.

MICROBIOLOGY

Table 2. Replication and virulence of the H5N1 viruses in mice

Viruses	Virus replication in organs, $log_{10}eID_{50}/ml$*				Seroconversion†	MLD_{50}, log_{10} eID_{50}	Pathotype‡	Genotype§
	Lung	Spleen	Kidney	Brain				
GSGD/1/96	−	−	−	−	No	>6.5	Non	A
DKGX/07/99	−	−	−	−	No	>6.5	Non	B
DKFJ/19/00	−	−	−	−	No	>6.5	Non	C
DKGD/12/00	−	−	−	−	No	>6.5	Non	A
DKZJ/11/00	−	−	−	−	No	>6.5	Non	A
DKZJ/52/00	−	−	−	−	No	>6.5	Non	D
DKGX/22/01	−	−	−	−	Yes	>6.5	Non	D
DKGD/07/00	3.7 ± 0.1	−	−	−	Yes	>6.5	Low	A
DKGD/1/01	1.8 ± 0.7	−	−	−	Yes	>6.5	Low	D
DKFJ/17/01	1.4 ± 0.4	−	−	−	Yes	>6.5	Low	D
DKGX/53/02	3.2 ± 0.5	−	−	−	Yes	>6.5	Low	I
DKGD/40/00	3.9 ± 0.5	+	−	−	Yes	6.4	Middle	E
DKGX/50/01	4.0 ± 0.7	+	−	−	Yes	6.4	Middle	G
DKSH/13/01	2.7 ± 1.4	−	−	−	Yes	5.0	Middle	D
DKSH/38/01	3.4 ± 1.2	1.8 ± 0.7	−	−	Yes	5.8	Middle	F
DKSH/8/01	5.9 ± 0.4	1.7 ± 1.2	+	+	ND	4.7	Middle	A
DKGD/22/02	3.2 ± 1.0	−	−	−	ND	4.8	Middle	D
DKSH/37/02	4.8 ± 0.6	+	−	−	ND	5.3	Middle	H
DKGX/35/01	5.1 ± 2.2	1.3 ± 0.8	+	+	ND	1.5	High	D
DKSH/35/02	5.6 ± 0.1	+	+	+	ND	2.3	High	F
DKFJ01/02	6.5 ± 0.3	2.0 ± 0.6	+	1.6 ± 0.5	ND	<0.5	High	I
DKFJ/13/02	6.6 ± 0.2	1.2 ± 0.2	1.4 ± 0.1	+	ND	<0.5	High	H

*Six-week-old BALB/c mice were infected i.n. with 10^6 eID_{50} of each virus in a 50-μl volume. Organs were collected on day 3 postinoculation, and clarified homogenates were titrated for virus infectivity in eggs at initial dilutions of 1:10 (lung), 1:2 (other tissues), or undiluted if negative at the lowest dilution. + and −, virus was detected or not detected, respectively, in the undiluted samples.

†Sera were collected on day 14 postinoculation, and seroconversion was tested by agar gel precipitation and HI tests. ND, all mice in the indicated group died before day 14.

‡Pathotypes were determined on the basis of replication and lethality in mice, as described in *Materials and Methods.* Non, nonpathogenic; Low, low pathogenicity; Middle, medium pathogenicity; High, high pathogenicity.

§Genotypes were determined on the basis of the diversity of the PBI, PA, NP, M, and NS nucleotide sequences, as described in Fig. 3.

spleen, mild to moderate nephrosis, and mild to severe necrosis of the bursa. Only DKGX/07/99 indicated nonsystemic replication (only the lungs were involved).

Studies in Mice. Most highly pathogenic avian influenza viruses of the Eurasian clade isolated since 1997 are able to replicate in mammals (4, 5, 15). Before testing their pathogenicity in mice, we biologically cloned each isolate by three limiting dilution cycles in specific-pathogen-free embryonated chicken embryos. No blind passage in mice was attempted. Our data were obtained from a single i.n. passage in mice.

On the basis of their ability to replicate and cause weight loss and death (Table 2 and Fig. 1), the H5N1 isolates were divided into four groups. When the virus could not be detected from any organs of the mice 3 days after inoculation with 10^6 eID_{50} and no weight loss and death of the mice were observed, the virus will be regarded as nonpathogenic in mice. The viruses that could replicate in mice were further divided into low ($MLD_{50} > 6.5$ $log_{10}eID_{50}$), medium (3 $log_{10}eID_{50} < MLD_{50} \leq 6.5$ $log_{10}eID_{50}$), or high ($MLD_{50} \leq 3$ $log_{10}eID_{50}$) pathogenicity groups on the basis of their lethality in mice. The nonpathogenic group comprised seven viruses (including GSGD/1/96) that did not replicate in the mice and caused no weight loss; only one virus (DKGX/22/01) induced seroconversion. The low-pathogenicity group contained four viruses that replicated to modest titers only in the lungs; all of these mice seroconverted by day 14 after inoculation. A high dose of these viruses ($>10^{6.5}$ eID_{50}) was required to kill the mice. All seven viruses in the medium-pathogenicity group replicated in the lungs of mice; low levels of two viruses were detected in the spleen, and one isolate (DKSH/8/01) was detected at very low levels in the brain. All of these viruses caused lethal infection in mice; but high doses of virus (10^4 to 10^6 eID_{50}) were required, and the MLD_{50} ranged from 4.7 to 6.4 $log_{10}eID_{50}$. The four highly pathogenic viruses replicated to high titers in the lungs of mice, and virus was detected in spleen, kidney, and brain, indicating systemic infection. The MLD_{50} of viruses in this group was <2.3 $log_{10}eID_{50}$.

Table 3. Replication and transmission of the H5N1 viruses in domestic ducks

Virus	Virus shedding on day 3 postinoculation				Virus shedding on day 5 postinoculation			
	Oropharyngeal		Cloacal		Oropharyngeal		Cloacal	
	No. birds shedding/total	Virus titer, $log_{10}eID_{50}/ml$	No. birds shedding/total	Virus titer, $log_{10}eID_{50}/ml$	No. birds shedding/total	Virus titer, $log_{10}eID_{50}/ml$	No. birds shedding/total	Virus titer, $log_{10}eID_{50}/ml$
DKGD/12/00	0/5	ND	0/5	ND	0/5	ND	0/5	ND
DKZJ/11/00	3/5	≤2.0–4.3	2/5	≤2.0–2.7	0/5	ND	0/5	ND
DKFJ/17/01	1/5	≤2.0–2.7	0/5	ND	2/5	≤2.0–3.0	1/5	≤2.0–2.7
DKGX/35/01	2/5	≤2.0–4.0	3/5	≤2.0–4.0	1/5	≤2.0–3.0	1/5	≤2.0–2.7
DKSH35/02	4/5	≤2.0–3.5	2/5	≤2.0–4.3	0/5	ND	0/5	ND
DKFJ/01/02	0/5	ND	1/5	≤2.0–3.5	0/5	ND	0/5	ND

Three-week-old sheldrake ducks were studied; no deaths or disease signs were observed in the infected ducks. ND, no detectable virus.

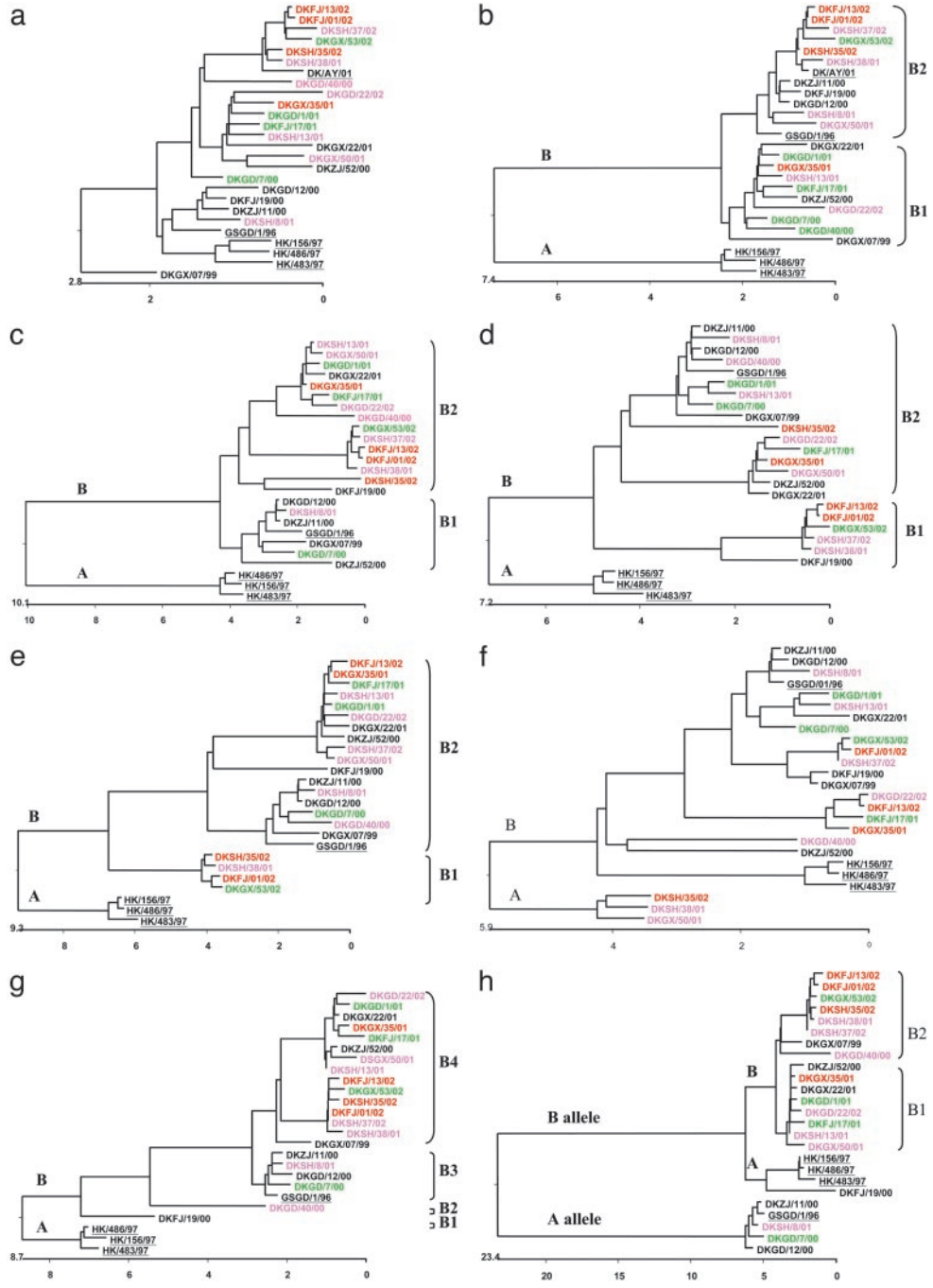

Fig. 2. Phylogenetic trees for the HA (*a*), NA (*b*), PB2 (*c*), PB1 (*d*), PA (*e*), NP (*f*), M (*g*), and NS (*h*) genes of the H5N1 influenza A viruses analyzed. The trees were generated by using MEGALIGN software (DNASTAR) on the basis of the following gene sequences: nucleotides 29–1,732 (1,704 bp) of HA, 21–1,367 or 1,427 (1,347 or 1,407 bp) of NA, 28–2,304 (2,276 bp) of PB2, 25–2,295 (2,271 bp) of PB1, 25–2,172 (2,148 bp) of PA, 45–1,539 (1,495 bp) of NP, 26–781 (756 bp) of M, and 27–708 or 725 (682 or 699 bp) of NS. The length of each pair of branches represents the distance between sequence pairs, and the units at the bottom of the tree indicate the number of substitution events. The colors indicate the pathotypes identified in mice: red, high pathogenicity; pink, medium pathogenicity; green, low pathogenicity; black, nonpathogenic. Table 1 lists the name of the viruses; sequences of the underlined viruses were obtained from GenBank.

We observed an increasing level of pathogenicity to mice with the progression of time: viruses isolated in 1999 and 2000 were less pathogenic to mice than those isolated in 2001 and 2002, although one isolate from 2000 (DKGD/40/00) was in the medium-pathogenicity group and one isolate from 2002 (DKGX/53/02) indicated low pathogenicity.

Studies in Ducks. Wild ducks are the natural hosts of avian influenza viruses. Avian influenza viruses of all subtypes are isolated from wild ducks worldwide, and none of the isolates, including the highly pathogenic H5 and H7 strains, were historically found to cause disease signs or death in ducks (17). Surprisingly, H5N1 viruses isolated from wild waterfowl in Hong Kong in November 2002 caused severe neurological disease and killed ducks in both the field and laboratory (7). We inoculated groups of five ducks with six H5N1 isolates representative of those analyzed in this study, including three viruses from the subgroup highly pathogenic in mice (Table 3). Five of the six H5N1 isolates replicated in the ducks; they

were shed from trachea or cloaca at levels of 2.0–4.3 $\log_{10}$eID$_{50}$/ml. The viruses were not transmitted to contact birds in the isolators, and none of the viruses caused disease signs or death in the ducks.

Molecular and Phylogenetic Analysis. To determine the molecular basis of our observations, the entire genome of each of the 21 isolates was sequenced. The sequences were compared with representative H5N1sequences obtained from GenBank, including those of GSGD/1/96, and human isolates from Hong Kong in 1997 HK/156/97, HK/483/97, and HK/486/97. The hemagglutinin (HA) and NA sequences of A/duck/Anyang/av-1/01 (H5N1)(DK/AY/01), isolated from duck meat exported to South Korea from China, was included in the phylogenetic analysis.

The HA genes of our 21 H5N1 isolates shared 97.4–98.9% nucleotide homology with the HA gene of GSGD/1/96 and 96.7–98% homology with the HK/97 isolates. All 21 isolates had a series of basic amino acids at the cleavage site of the HA (-RRKKR-) that is characteristic of influenza viruses that are highly pathogenic in chickens. Phylogenic analysis of the HA genes (Fig. 2*a*) revealed DKGX/07/99 to be on a separate branch from the remainder of the isolates. Four year 2000 duck isolates, together with GSGD/1/96 and the HK/97 isolates, formed one fork in the phylogenetic tree. Most of the 2001 isolates formed a separate branch from the 2002 isolates, and the virus isolated from duck meat in South Korea (DKAY/01) was at the base of the 2002 isolates.

The NA genes of the H5N1 duck isolates were more closely related to those of GSGD/1/96 (96.4–98.3% homology) than to those of the HK/97 isolates (89.5–92% homology). Phylogenetic analysis revealed that the HK/97 NAs form a branch separate from that of the NAs in our duck isolates (Fig. 2*b*). The NAs of the duck isolates were divided into two forks, with DKGX/07/99 at the base of fork B1 and GSGD/1/96 at the base of fork B2. Each of these forks contain viruses isolated from 2000 to 2002. DK/AY/01 is in the fork derived from GSGD/1/96. Analysis of the deduced amino acid sequence revealed that all of the viruses in the fork derived from GSGD/1/96 have a 20-aa deletion in the NA stalk (residues 49–68), whereas GSGD/1/96 itself does not. This NA stalk deletion is distinct from, but overlaps with, the 19-aa deletion found in the HK/97 viruses isolated from humans (residues 54–72). There were no deletions in the NAs of viruses in the fork derived from DKGX/07/99.

The phylogenetic trees of the PB2, PB1, and PA genes of the 22 H5N1 viruses are very similar (Fig. 2 *c–e*). The human HK/97 isolates form a separate branch, whereas the duck isolates and GSGD/1/96 are divided into two main forks in which the earlier isolates are predominantly in one branch. In the phylogenetic tree of PB2, the genes of the two forks in the B branch share very high homology and belong to one lineage. On the other hand, the B1 and B2 forks of the PB1and PA genes share 90–93% homology and can be considered different lineages. The nucleoprotein (NP) phylogenetic tree (Fig. 2*f*) was different; the NP genes of three of the duck isolates (DKSH/35/02, DKSH/38/01, and DKGX/50/01) formed a separate branch, whereas the human HK/97 NP formed a fork and the other H5N1 isolates formed multiple sister forks that did not correspond to time or geographical area. The phylogenetic tree of the M (matrix) gene (Fig. 2*g*) was quite similar to the trees of the polymerase genes, but the M genes of DKFJ/19/00 and DKGD/40/00 were located on different forks in the B branch.

The NS genes of the duck viruses were separated into two alleles (Fig. 2*h*). Four duck viruses and GSGD/1/96 were included in the *A* allele, whereas the remainder, including the human HK/97 viruses, were in the *B* allele, which was further divided into two branches. The deduced NS amino acid sequence of all viruses in the B1 fork of branch B in the *B* allele had a 15-nt deletion resulting in a 5-aa deletion in the NS protein (position 80–84).

On the basis of genomic diversity, the viruses formed nine genotypes, whose evolution and relationships are shown in Fig. 3.

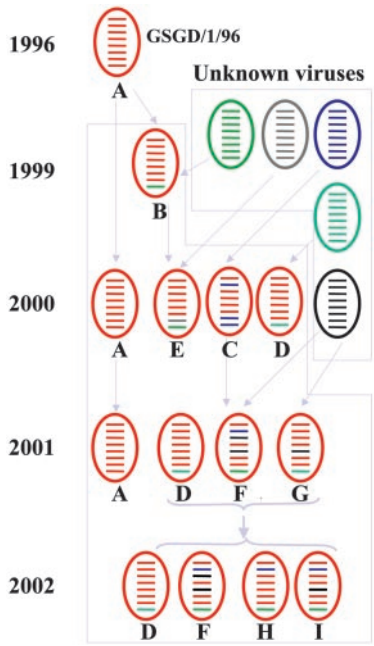

Fig. 3. The genotypic evolution of the H5N1 viruses isolated from ducks in southern China from 1999 through 2002. The eight gene segments in each schematic virus particle are (top to bottom) the PB2, PB1, polymerase (PA), HA, NP, NA, matrix (M), and NS genes. Genes of the same lineage are shown in the same color. The capital letters indicate the genotypes. Table 2 lists the viruses of each genotype.

It is noteworthy that the same PB2, HA, and NA genes are found in different genotypes. There are no strong links between the genotype and the pathotype of these viruses in mice; however, there is a clear temporal pattern in the progressively increasing pathogenicity of the viruses within the same genotypes, especially the genotype A and D viruses. DKSH/38/01 and DKSH/35/02 are in the same genotype (F), but DKSH/35/02 is 10^3-fold more lethal than DKSH/38/01 in mice. The virulence difference of the two viruses within the genotype H and I groups are $>10^5\times$ and $>10^6\times$, respectively. These results suggested that the individual mutations, rather than the genome constellation, may have determined their pathogenic potential.

Discussion

This study describes previously uncharacterized H5N1 influenza viruses isolated from ducks in mainland China since GSGD/1/96 (H5N1) was reported (18, 19). Our findings demonstrate that highly pathogenic H5N1 influenza viruses were isolated from apparently healthy ducks in the coastal provinces of China (between Guangdong and Shanghai) from 1999 through 2002. These H5N1 isolates reacted similarly to antisera and had phylogenetically homogeneous HA genes. All but one of these viruses caused lethal, widespread systemic infection in chickens, and all possessed a series of basic amino acids at the HA cleavage site that is associated with high pathogenicity.

Our findings establish that the H5N1 influenza viruses circulating in domestic ducks in China from 1999 through 2002 acquired the ability to replicate in mice and cause systemic infection and death without adaptation. Several investigators have reported that the H5N1 influenza viruses isolated from humans are lethal to mice (20–25). Numerous factors are reportedly associated with H5N1 pathogenicity. Reverse genetics studies have shown that residue 627 of the PB2 protein is crucial to H5N1 pathogenicity in mice, as are a series of basic amino acids at the HA cleavage site (26). Other studies implicate the NS gene and its ability to modulate the

cytokine response (27, 28). Reverse genetics studies have found that residue 92 of the NS1 molecule of the human isolate A/HK/156/97 (H5N1) is associated with the induction of severe pathology in pigs (27). However, all of the H5N1 duck viruses have conserved amino residues of Glu-627 in PB2 and Asp-92 in NS1, and they contain common avian HA1 residues Gln-226 and Gly-228 (H3 numbers) in the receptor-binding site. Therefore, these residues may not contribute to the difference of replication and virulence of the H5N1 duck viruses in mice. Important remaining questions are when and how H5N1 viruses acquired the ability to replicate in mammals.

Our results demonstrate that while circulating in domestic ducks, H5N1 viruses gradually acquired the characteristics that make them lethal in mice. One possible explanation for this finding is the transmission of duck H5N1 viruses to humans, the selective evolution of the viruses in humans, and their subsequent transmission back to ducks. There is limited serological evidence of H5N1 infection of humans and limited evidence of human-to-human transmission of H5N1 viruses (29). The available evidence suggests that since 1997, H5N1 viruses in Asia have caused limited severe or lethal human infection (3, 4) but have not been transmitted from human to human. The transmission of the viruses from humans back to ducks is difficult to envision, despite evidence that H9N2 avian influenza viruses have been transmitted from ducks to other poultry and then back to ducks (30).

The molecular basis of the transmissibility of avian influenza viruses to mammals is not resolved. It certainly involves multiple viral genes, including HA. Some avian gene constellations have been proposed to promote transmission to mammals. The H3N2 influenza viruses that emerged in pigs in the United States in 1997 were triple reassortants that contained gene segments from human, swine, and avian influenza viruses (31). These H3N2 triple-reassortant viruses were highly transmissible among swine and have become widespread in the United States (32). Similarly, the H1N1 swine influenza viruses that are currently widespread in Europe contain avian influenza virus genes that they acquired in 1979. Thus, avian influenza virus genes, when they are a part of certain gene constellations, seem able to promote transmission among mammalian species. The evolution of different genotypes of H5N1 viruses in ducks may have provided the constellation of genes that allow mammalian transmission.

An alternative possibility is involvement of the pig (the postulated intermediate host) in selection. In the region of China where the H5N1 viruses characterized in this study were isolated, pigs and ducks are housed in close proximity, especially in farming villages, where families typically own a small number of pigs and ducks. Our working hypothesis is that H5N1 viruses have gradually acquired the ability to replicate in mammals by means of selection pressure created by possible transmission between pigs and ducks. To date, there are no reports of the isolation of H5N1 viruses from pigs, although pigs have been experimentally infected with H5N1 viruses (33). We have recently obtained preliminary virological and serological evidence of H5N1 virus infection of pigs in Fujian province.

The fact that one of the H5N1 viruses studied here failed to infect inoculated ducks is inconsistent with our hypothesis, as is the absence of transmission to contact ducks. However, laboratory experiments cannot mimic field conditions, which may include stress and coinfection with multiple bacterial and viral agents. Furthermore, the spread of virus to contact ducks is limited in isolator units, where the feces of infected animals fall through screens into pans and the forced-air supply is HEPA-filtered. However, we can conclude from our findings that the H5N1 viruses isolated from ducks in 2002 in this study are not highly lethal to ducks, as were the H5N1 viruses isolated from wild aquatic birds in Hong Kong in November 2002 (7).

The isolation of chicken-lethal H5N1 viruses from apparently healthy ducks explains the presence of H5N1 virus in duck meat exported to Japan and South Korea (34). However, the detailed genotype analysis of our 21 isolates indicates that the H5N1 viruses circulating in ducks in mainland China in 1999–2002 differed from the H5N1 viruses in Hong Kong live bird markets reported by Guan *et al.* (6). The relationship of these duck H5N1 viruses to the H5N1 viruses isolated in China in 2004 remains to be determined.

The HA and PB2 genes seem to play an important role in the replication and pathogenicity of H5N1 viruses in mice (26). Our findings indicate that multiple genotypes of H5N1 influenza viruses have the potential to be pathogenic in mammals. Clearly, H5N1 influenza viruses are continuing to evolve in Asia. Continued surveillance is needed, as is detailed characterization of the gene constellations and specific residues required for transmission to mammals.

K.Y. and H.C. are coprincipal investigators who planned and coordinated these studies; R.G.W. designed part of the experiments and jointly wrote the report with H.C. Z. Li and G.D. contributed equally to the studies in chicken and mice and conducted sequence analysis with the assistance of L.Z., Z. Liu, and P.J.; and G.T. and Y.L. performed the strain surveillance and conducted the duck experiments. We thank Dr. Denis Alexander (Office International Des Épizooties Reference Laboratory) for providing the avian influenza virus reference strains and antisera; Carol Walsh for administrative assistance; and Sharon Naron for editorial assistance. This study was supported by Chinese National Key Basic Research Program (973 Program) Grants G1999011905 and G1999011902; Chinese National S&T "10th Five-Year" Plan Grant 2002BA514A-15; National Institutes of Health Grant AI95357 (to R.G.W.); and the American Lebanese Syrian Associated Charities.

1. Webby, R. & Webster, R. (2003) *Science* **302,** 1519–1522.
2. Subbarao, K. & Katz, J. (2000) *Cell Mol. Life Sci.* **57,** 1770–1784.
3. Claas, E., Osterhaus, A., van Beek, R., De Jong, J., Rimmelzwaan, G., Senne, D., Krauss, S., Shortridge, K. & Webster, R. (1998) *Lancet* **351,** 472–477.
4. Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., *et al.* (1998) *Science* **279,** 393–396.
5. Webster, R., Guan, Y., Peiris, M., Walker, D., Krauss, S., Zhou, N., Govorkova, E., Ellis, T., Dyrting, K., Sit, T., *et al.* (2002) *J. Virol.* **76,** 118–126.
6. Guan, Y., Peiris, J., Lipatov, A., Ellis, T., Dyrting, K., Krauss, S., Zhang, L., Webster, R. & Shortridge, K. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 8950–8955.
7. Sturm-Ramirez, K., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J., Poon, L., Guan, Y., Peiris, M. & Webster, R. (2004) *J. Virol.* **78,** 4892–4901.
8. Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L. S., Lai, R. W. M., Orr, W. K. & Shortridge, K. F. (1999) *Lancet* **354,** 916–917.
9. Guo, Y., Krauss, S., Senne, D., Mo, I., Lo, K., Xiong, X., Norwood, M., Shortridge, K., Webster, R. & Guan, Y. (2000) *Virology* **267,** 279–288.
10. Peiris, J., Guan, Y., Markwell, D., Ghose, P., Webster, R. & Shortridge, K. (2001) *J. Virol.* **75,** 9679–9686.
11. O'Neill, E., Seo, S. H., Woodland, D. L., Shortridge, K. F. & Webster, R. G. (2001) *Int. Congr. Ser.* **1219,** 775–781.
12. Abbott, A. (2003) *Nature* **423,** 5.
13. van Kolfschooten, F. (2003) *Lancet* **361,** 1444.
14. Matrosovich, M., Zhou, N., Kawaoka, Y. & Webster, R. (1999) *J. Virol.* **73,** 1146–1155.
15. Guan, Y., Shortridge, K. F., Krauss, S. & Webster, R. G. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 9363–9367.
16. Reed, L. & Muench, H. (1938) *Am. J. Hyg.* **27,** 493–497.
17. Webster, R., Bean, W., Gorman, O., Chambers, T. & Kawaoka, Y. (1992) *Microbiol. Rev.* **56,** 152–179.
18. Chen, H., Yu, K. & Bu, Z. (1999) *Chin. Agric. Sci.* **32,** 87–92.
19. Xu, X., Subbarao, K., Cox, N. & Guo, Y. (1999) *Virology* **261,** 15–19.
20. Katz, J., Lu, X., Tumpey, T., Smith, C., Shaw, M. & Subbarao, K. (2000) *J. Virol.* **74,** 10807–10810.
21. Dybing, J., Schultz-Cherry, S., Swayne, D., Suarez, D. & Perdue, M. (2000) *J. Virol.* **74,** 1443–1450.
22. Lu, X., Tumpey, T., Morken, T., Zaki, S., Cox, N. & Katz, J. (1999) *J. Virol.* **73,** 5903–5911.
23. Gao, P., Watanabe, S., Ito, T., Goto, H., Wells, K., McGregor, M., Cooley, A. & Kawaoka, Y. (1999) *J. Virol.* **73,** 3184–3189.
24. Shortridge, K., Zhou, N., Gua, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K., *et al.* (1998) *Virology* **252,** 331–342.
25. Gubareva, L., McCullers, J., Bethell, R. & Webster, R. (1998) *J. Infect. Dis.* **178,** 1592–1596.
26. Hatta, M., Gao, P., Halfmann, P. & Kawaoka, Y. (2001) *Science* **293,** 1840–1842.
27. Seo, S., Hoffmann, E. & Webster, R. (2002) *Nat. Med.* **8,** 950–954.
28. Cheung, C., Poon, L., Lau, A., Luk, W., Lau, Y., Shortridge, K., Gordon, S., Guan, Y. & Peiris, J. (2002) *Lancet* **360,** 1831–1837.
29. Buxton, C., Katz, J., Seto, W., Chan, P., Tsang, D., Ho, W., Mak, K., Lim, W., Tam, J., Clarke, M., *et al.* (2000) *J. Infect. Dis.* **181,** 344–348.
30. Li, K., Xu, K., Peiris, J., Poon, L., Yu, K., Yuen, K., Shortridge, K., Webster, R. & Guan, Y. (2003) *J. Virol.* **77,** 6988–6994.
31. Zhou, N., Senne, D., Landgraf, J., Swenson, S., Erickson, G., Rossow, K., Liu, L., Yoon, K., Krauss, S. & Webster, R. (1999) *J. Virol.* **73,** 8851–8856.
32. Webby, R., Swenson, S., Krauss, S., Gerrish, P., Goyal, S. & Webster, R. (2000) *J. Virol.* **74,** 8243–8251.
33. Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K., Kawaoka, Y. & Webster, R. (1994) *J. Gen. Virol.* **75,** 2183–2188.
34. Tumpey, T., Suarez, D., Perkins, L., Senne, D., Lee J., Lee Y., Mo, I., Sung, H. & Swayne, D. (2002) *J. Virol.* **76,** 6344–6355.