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Molecular Basis of Replication of Duck H5N1 Influenza Viruses in a Mammalian Mouse Model

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We recently analyzed a series of H5N1 viruses isolated from healthy ducks in southern China since 1999 and found that these viruses had progressively acquired the ability to replicate and cause disease in mice. In the present study, we explored the genetic basis of this change in host range by comparing two of the viruses that are genetically similar but differ in their ability to infect mice and have different pathogenicity in mice. A/duck/Guangxi/22/2001 (DKGX/22) is nonpathogenic in mice, whereas A/duck/Guangxi/35/2001 (DKGX/35) is highly pathogenic. We used reverse genetics to create a series of single-gene recombinants that contained one gene from DKGX/22 and the remaining seven gene segments from DKGX/35. We find that the PA, NA, and NS genes of DKGX/22 could attenuate DKGX/35 virus to some extent, but PB2 of DKGX/22 virus attenuated the DKGX/35 virus dramatically, and an Asn-to-Asp substitution at position 701 of PB2 plays a key role in this function. Conversely, of the recombinant viruses in the DKGX/22 background, only the one that contains the PB2 gene of DKGX/35 was able to replicate in mice. A single amino acid substitution (Asp to Asn) at position 701 of PB2 enabled DKGX/22 to infect and become lethal for mice. These results demonstrate that amino acid Asn 701 of PB2 is one of the important determinants for this avian influenza virus to cross the host species barrier and infect mice, though the replication and lethality of H5N1 influenza viruses involve multiple genes and may result from a constellation of genes. Our findings may help to explain the expansion of the host range and lethality of the H5N1 influenza viruses to humans.

Avian influenza viruses of the H5N1 subtype are likely to cause the next human influenza pandemic (28). In 1996, a highly pathogenic H5N1 influenza virus, A/goose/Guangdong/1/96 (GSGD/96), was isolated from a sick goose in southern China (3, 29). In 1997, highly pathogenic H5N1 avian influenza virus that derived its hemagglutinin (HA) gene from GSGD/96-like viruses and its other seven genes from the H6N1 subtype A/teal/Hong Kong/W312/97-like virus caused disease outbreaks in chickens in Hong Kong (6) and were transmitted to humans and caused the deaths of 6 of 18 people infected (7, 24). In 2003, H5N1 virus infection was confirmed in two members of a Hong Kong family, one of whom died (18). Later in 2003 and 2004, H5N1 influenza viruses began to spread and caused disease outbreaks in China, Japan, South Korea, Thailand, Vietnam, Indonesia, Cambodia, Malaysia, and Laos, resulting in the destruction of hundreds of millions of poultry, including chickens, ducks, and geese. In Vietnam (27), Thailand, and Cambodia, H5N1 viruses were transmitted to humans and caused death in 50 of the 80 confirmed cases. The 33% to 73% human mortality rate caused by H5N1 viruses has sparked great interest in pandemic preparedness and in understanding the genetic determinants that allow them to cross the avian-mammalian host species barrier and the molecular basis of their pathogenicity in mammals.

In the 1997 Hong Kong H5N1 incident, 16 viruses were isolated from the 18 infected humans. These viruses could replicate in mice without adaptation, but their virulence for mice varied (9, 16). Hatta et al. selected a pair of model viruses, the highly pathogenic A/Hong Kong/483/97 and the nonlethal A/Hong Kong/486/97, to explore the molecular basis of the virulence of these viruses for mice. Using reverse genetics, they discovered that a mutation at position 627 in the PB2 protein influenced the outcome of infection in mice and that the high cleavability of the hemagglutinin glycoprotein was an essential requirement for lethal infection (11). Other studies suggested that glutamic acid at position 92 of the NS1 protein enables the H5N1 viruses to resist the antiviral effects of interferons and tumor necrosis factor alpha and may be crucial to the pathogenicity of the viruses in pigs (22).

We recently characterized a series of 21 H5N1 influenza viruses isolated from healthy ducks in southern China from 1999 through 2002 (4). These viruses (along with GSGD/96) were divided into nine genotypes (A to I) on the basis of genomic diversity. Studies in mice clearly showed a progressive increase in the pathogenicity of the viruses in each genotype over time, especially genotypes A and D. Most of the viruses isolated before 2001 were unable to replicate in mice, whereas several of those isolated in 2001 to 2002 replicated systemically

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and were highly lethal in mice. We have now compared two of these viruses that have similar genomes but display disparate pathogenicity to identify the genetic determinants that allow H5N1 viruses to cross the species barrier and infect mammals. The A/duck/Guangxi/22 (DKGX/22) and A/duck/Guangxi/35 (DKGX/35) viruses were isolated in Guangxi Province, People's Republic of China. Both belong to genotype D, but DKGX/22 fails to replicate in mice, whereas DKGX/35 causes lethal systemic infection in mice following intranasal administration.

MATERIALS AND METHODS

Cells and viruses. Human embryonic kidney cells (293T) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and were incubated at 37°C in 5% CO_2. In this study, we used two wild-type H5N1 avian influenza viruses, DKGX/22 and DKGX/35, which were isolated from healthy ducks in Guangxi Province in southern China and which we characterized previously (4). A total of 50 amino acid differences are observed between these two viruses (Table 1). Virus stocks were propagated in 10-day-old specific-pathogen–free embryonated chicken eggs and stored at −70°C until they were used.

Construction of plasmids. We used an eight-plasmid reverse genetics system for virus rescue (9, 11, 14, 19). To generate an mRNA-viral RNA bidirectional transcription vector (pBD), we inserted the polymerase I-SapI-ribozyme cassette of the plasmid pPolI-SapI-ribozyme (19) into the XbaI site of plasmid pCI (Promega, Madison, WI) in the sequence polymerase II (cytomegalovirus)–ribozyme–SapI–polymerase I–simian virus 40 polyadenylation signal. The SapI sites were used to clone the cDNA of influenza virus genes. We then inserted the cDNA derived from DKGX/22 or DKGX/35 viral genes between the ribozyme and promoter sequence of polymerase I.

Because the PB2, PB1, and PA genes have SapI cleavage sites, we could not use the strategy of adding the SapI sequence to the PCR primer for subsequent cloning. Therefore, a binucleotide cloning strategy was used for all pBD cDNA construction. Briefly, we used a set of primers with two extra nucleotides (CC and TT, respectively) at the 5′ ends of the forward and reverse primers to amplify the full-length cDNAs of the viruses. The primer sequences are listed in Table 2.

We treated the PCR products with T4 polymerase (New England Biolabs, Beverly, Massachusetts) in the presence of 100 mM dTTP and dCTP for 10 min at 12°C to generate a CC and a TT overhang in the two ends, respectively. We cut plasmid pBD with SapI (New England Biolabs) and then partially filled it by treatment with Klenow fragment in the presence of 30 mM dATP and dGTP at 25°C for 30 min to create the two ends 5′GG and 5′AA to match the ends of the PCR products. The chimeric PB2 genes were generated by cutting and switching the NsiI and HindIII fragments of the plasmids pBD-DKGX/22PB2 and pBD-DKGX/35PB2. Mutations were introduced into the PB2 gene by using Invitrogen site-directed mutagenesis kits with the following set of primers: DKGX/35 PB2 K699N: 5′TAATTCTAGGCAATGAGAACAAAAGATATG/CATATCTTTTGTTCTCATTGCCTAGAATTA; DKGX/35 PB2 N701D: 5′TAATTCTAGGCAAGGAGGACAAAAGATATG/CATATCTTTTGTCCTCCTTGCCTAGAATTA; DKGX/22 PB2 N699K: 5′TAATTCTAGGCAAGGAGGACAAAAGATATG/CATATCTTTTGTCCTCCTTGCCTAGAATTA; DKGX/22 PB2 D701K: 5′TAATTCTAGGCAATGAGAACAAAAGATATG/CATATCTTTTGTTCTCATTGCCTAGAATTA. The plasmids used for transfection were prepared by using the Perfectprep Plasmid mini kit (Eppendorf, Hamburg, Germany). All of the constructs were completely sequenced to ensure the absence of unwanted mutations.

Virus rescue. When monolayers were approximately 80% to 90% confluent, 293T cells in six-well plates were transfected with 5 μg of the eight plasmids (about 0.6 μg of each plasmid) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (25). Briefly, DNA and transfection reagent were mixed (2 μl Lipofectamine 2000 per μg of DNA), incubated at room temperature for 30 min, and added to the cells. Sixteen hours later, the mixture was replaced with Opti-MEM (GIBCO/BRL) containing 0.3% bovine serum albumin and 0.01% fetal bovine serum. After 48 h, the supernatant was harvested and injected into embryonated eggs for virus propagation. Because helper virus was not required for this procedure, we analyzed the recovered transfectant viruses without further purification. Virus was detected by hemagglutination assay, and viruses were fully sequenced to ensure the absence of unwanted mutations.

TABLE 1. Amino acid differences between avian influenza viruses DKGX/22 and DKGX/35

Gene	Amino acid position	Amino acid in virus:	
		DKGX/22	DKGX/35
PB2	105	T	A
	108	A	T
	153	A	D
	699	N	K
	701	D	N
PB1	103	F	L
	292	S	N
	372	K	M
	439	D	G
	533	N	S
PA	20	T	A
	277	P	S
	315	L	F
	355	P	S
HA	2	K	E
	11	V	I
	99	T	A
	154	Q	L
	157	P	S
	204	T	I
	529	I	T
NP	9	P	S
	117	G	R
	291	L	F
	353	A	V
	365	I	V
	372	E	D
	377	S	N
	450	N	S
	496	Y	F
	497	D	E
NA	7	V	I
	16	V	I
	41	G	E
	42	D	N
	50	S	N
	80	V	A
	94	V	I
	99	I	V
	220	M	R
	235	S	N
	289	I	T
	338	M	V
M1	196	S	N
NS1	43	I	L
	66	G	E
	90	I	L
	98	F	L
	192	T	I
NS2	48	L	F

Mouse study. Groups of 11 6-week-old female BALB/c mice (Beijing Experimental Animal Center) were lightly anesthetized with CO_2 and inoculated intranasally with $10^{6.0}$ 50% egg infectious doses (EID_{50}) of H5N1 influenza virus in a volume of 50 μl. Three mice in each group were euthanized on days 4 and 6 for virus titration of the lungs, kidneys, spleen, and brain. Organs were collected and homogenized in 1 ml of cold phosphate-buffered saline. Solid debris

TABLE 2. Primers used for pBD cDNA construction with a binucleotide cloning strategy to amplify full-length cDNAs of the viruses

Gene segment	Forward primer	Reverse primer
PB2	5′CCAGCAAAAGCAGGTCAATTAT	5′TTAGTAGAAACAAGGTCGTTTTTAAACAAT
PB1	5′CCAGCAAAAGCAGGCAAACCA	5′TTAGTAGAAACAAGGCATTTTTTCAT
PA	5′CCAGCAAAAGCAGGTACTGATCCA	5′TTAGTAGAAACAAGGTACTTTTTTGGA
HA	5′CCAGCGAAAGCAGGGGTATAATC	5′TTAGTAGAAACAAGGGTGTTTTTAACT
NP (DKGX/35)	5′CCAGCAAAAGCAG GGTTGATAAT	5′TTAGTAGAAACAAGGGTAT
NP (DKGX/22)	5′CCAGCGAAA GCAGGGTAGATAAT	5′TTAGTAGAAACAAGGGTAT
NA	5′CCAGCAAAAGCAGGAGTTTAAAATGAAT	5′TTAGTAGAAACAAGGAGTTTTTTGAACAA
M (DKGX/35)	5′CCAGCAAAAGCAGGTAGATGTTGAAAGATG	5′TTAGTA GAAACAAGGT AGTTTTTTACTC
M (DKGX/22)	5′CCAGCGAAAGCAGGTAGATGTTGAAAGATG	5′TTAGTAGAAACAAGGTAGTTTTTTACTC
NS (DKGX/35)	5′CCAGCGAAAGCAGGGTGACAA	5′TTAGTAGAAACAAGGGTGTTTTT TATCAT
NS (DKGX/22)	5′CCAGCAAAAGCAGGGTGACAA	5′TTAGTAGA AACAAGGGTGTTTTTTATCAT

was pelleted by centrifugation, and undiluted and 10-fold serially diluted supernatants were titrated for virus infectivity in eggs. The lower limit of virus detection was 0.5 $\log_{10}$ EID_{50} per ml tissue homogenate. The remaining five mice were monitored daily for 14 days for weight loss and mortality.

To determine the 50% mouse lethal dose (MLD_{50}) of viruses that caused lethal infection of mice, six groups of five mice were inoculated intranasally with 10-fold serial dilutions contains 10^1 to 10^6 EID_{50} virus in a 50-μl volume (4). The MLD_{50} was calculated by the method of Reed and Muench (20). The rescued viruses R-DKGX/22 and R-DKGX/35 were included as controls for all experiments.

Sequence analysis. The plasmids used for virus rescue and the rescued viruses were fully sequenced to confirm the absence of unexpected mutations. Viral RNA was extracted from allantoic fluid by using the RNeasy mini kit (QIAGEN, Valencia, CA) and was reverse-transcribed with an oligonucleotide (Uni 12) complementary to the conserved 3′ end of viral RNA, as described (13). PCR amplification used fragment-specific primers (shown in Table 2). The PCR products were purified with the QIAquick PCR purification kit (QIAGEN). Plasmids and PCR products were sequenced by using the CEQ DTCS-Quick Start kit on a CEQ 8000 DNA sequencer (Beckman Coulter) with a set of gene-specific primers (sequences available upon request).

RESULTS

Rescued H5N1 viruses retained the biological properties of the wild-type viruses. We inserted cDNAs of each full-length RNA segment of the DKGX/22 and DKGX/35 viruses into the viral RNA-mRNA bidirectional expression plasmid pBD as described in Materials and Methods. Using these plasmids, we rescued the DKGX/22 and DKGX/35 viruses, designated R-DKGX/22 and R-DKGX/35, respectively. After confirmation by sequence analysis, we grew the virus stocks in 10-day-old, specific-pathogen–free embryonated chicken eggs and tested their replication and lethality in mice. The rescued R-DKGX/22 virus, like its wild-type counterpart, did not kill mice or replicate in any of the organs tested (Fig. 1 and Table 3). R-DKGX/35 replicated systemically to titers similar to those of the wild-type virus (Table 3) and remained highly pathogenic, although the MLD_{50} was higher than that of wild-type DKGX/35 (2.3 versus 1.8 $\log_{10}$ EID_{50}). These results indicated that the rescued viruses maintained the biological properties of the wild-type viruses.

PB2 gene of DKGX/35 virus alters the host range of the DKGX/22 virus. To identify the genes that contributed to replication in mice, we generated eight single-gene recombinants, each containing one gene derived from DKGX/35 and seven genes derived from DKGX/22, as described by Hatta et al. (11), and tested their replication and pathogenicity in mice. Only the recombinant virus that contained the PB2 gene of DKGX/35 (DKGX/22-35PB2) was able to replicate in mice. This recombinant was lethal to the infected mice (MLD_{50}, 4.3). High titers of virus were detected in the lungs on days 4 and 6; virus titers in spleen and brain were 1.3 and 3.3 $\log_{10}$ EID_{50}, respectively, on day 6, but virus was not detected in the kidneys (Fig. 1 and Table 3). These results indicated that the PB2 gene of DKGX/35 alters the ability of the DKGX/22 virus to replicate in mice.

We then tested the effect of individual genes derived from

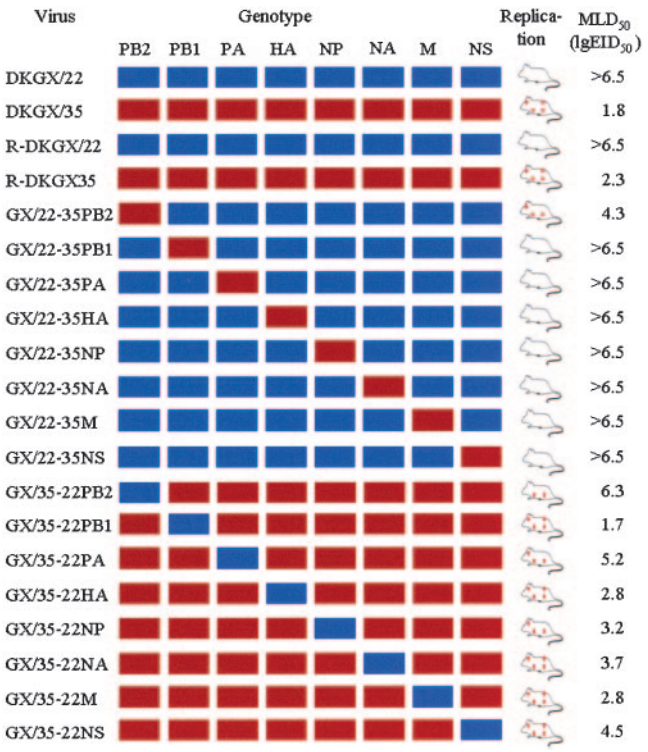

FIG. 1. Replication and virulence of rescued viruses. Colored bars indicate the origin of the viral gene: blue, A/duck/Guangxi/22/01 (DKGX/22); red, A/duck/Guangxi/35/01 (DKGX/35). The red dots in the mouse figures indicate tissue tropism (upper left, brain; lower left, lung; upper right, kidney; lower right, spleen). The MLD_{50} was determined by inoculating groups of five mice with 10-fold serial dilutions containing 10^1 to 10^6 EID_{50} of the virus in a 50-μl volume (4) and calculated by the method of Reed and Muench (20). The MLD_{50} values of DKGX/35 and R-DKGX/35 are the means of three experiments, and the values of other viruses were obtained from a single experiment.

TABLE 3. Replication of rescued viruses in BALB/c mice inoculated intranasally with 10^6 EID_{50} (50 μl)[a]

Virus[b]	Mean virus titer (log_{10} EID_{50}/ml) ± SD							
	Lung		Spleen		Kidney		Brain	
	Day 4 p.i	Day 6 p.i	Day 4 p.i	Day 6 p.i	Day 4 p.i	Day 6 p.i	Day 4 p.i	Day 6 p.i
W-DKGX/22								
W-DKGX/35	6.5 ± 0.1	6.4 ± 0.4	1.9 ± 0.6	1.4 ± 0.2	1.4 ± 0.3	1.9 ± 0.4	2.4 ± 1.0	5.7 ± 0.2
R-DKGX/22								
R-DKGX/35	6.5 ± 0.1	5.9 ± 1.0	3.0 ± 0.4	2.2 ± 0.7	1.8 ± 1.0	1.6 ± 0.4	2.2 ± 0.4	5.0 ± 1.1
GX/22-35PB2	4.4 ± 0.4	4.7 ± 0.8		1.3 ± 0				3.3 ± 1.9
GX/35-22PB2	5.0 ± 1.6	3.8 ± 2.0	1.3 ± 0.1[d]	2.4 ± 0.4				
GX/35-22PB1	4.5 ± 2.7	6.0 ± 0.4	2.0 ± 0.7	1.9 ± 0.1		1.5 ± 0.4	1.3 ± 0.1	3.5 ± 0.5
GX/35-22PA	5.4 ± 0.4[d]	5.5 ± 0.1		1.9 ± 0.9				3.6 ± 1.3
GX/35-22HA	6.5 ± 0.3	6.5 ± 0.3	3.0 ± 0.4	2.8 ± 0.1	1.6 ± 0.5	1.3 ± 0	3.0 ± 1.7	4.8 ± 0.8
GX/35-22NP	6.4 ± 0.1	6.3 ± 0.5	2.8 ± 0.4	2.6 ± 0.1			2.0 ± 0.7	5.1 ± 0.5
GX/35-22NA	5.1 ± 0.5[d]	4.5 ± 0.3	1.5 ± 0.4[c]	1.3 ± 0.1		1.2 ± 0.1	2.0 ± 1.3	3.2 ± 1.8
GX/35-22M	5.0 ± 1.5	6.1 ± 0.6	2.2 ± 0.9	2.3 ± 0.7	1.2 ± 0.1	2.0 ± 1.3	1.4 ± 0.3	2.8 ± 0.8[c]
GX/35-22NS	5.5 ± 0.7	5.9 ± 0.3	1.7 ± 0.9	3.6 ± 0.6		1.4 ± 0.1		4.5 ± 0.7
Chimera 1								
Chimera 2	4.5 ± 0.5	4.9 ± 0.5	1.2 ± 0.1					2.5 ± 1.1
Chimera 3	2.8 ± 1.8[c]	2.0 ± 1.3[c]						
Chimera 4	6.2 ± 0.7	5.3 ± 1.4	1.6 ± 0.5[c]	1.3 ± 0.1	1.3 ± 0.1		2.6 ± 1.8	2.6 ± 1.4
22/PB2-699K								
22/PB2-701N	3.6 ± 0.6	2.4 ± 2.0	1.6 ± 0.7					
35/PB2-699N	5.2 ± 0.1[d]	4.2 ± 2.6	1.5 ± 0.3[d]	2.3 ± 0.9	1.2 ± 0.1		1.3 ± 0.1[c]	1.7 ± 0.9[c]
35/PB2-701D	2.5 ± 1.3[d]	6.1 ± 1.2		1.5 ± 0.2				

[a] Three mice from each group were killed on days 4 and 6 postinoculation (p.i.), and virus in the organs was titrated in eggs. Blank cells indicate no virus detected.
[b] Reassortant viruses bearing the PB1, PA, HA, NP, NA, M, and NS genes in the DKGX/22 background were not recovered from any organ of the inoculated mice on day 4 or day 6, and therefore, the data are not shown.
[c] $P < 0.05$ compared with the titers in the corresponding organs of the R-DKGX/35-inoculated mice.
[d] $P < 0.01$ compared with the titers in the corresponding organs of the R-DKGX/35-inoculated mice.

DKGX/22 virus on the replication and virulence of DKGX/35 virus. We generated eight single-gene recombinant viruses, each containing one gene from DKGX/22 and the remainder from DKGX/35. The viruses that carried the PB1, HA, or M gene of DKGX/22 were as virulent as the DKGX/35 virus and replicated in all four organs studied (Fig. 1, Table 3). The recombinants that contained the PA, NP, NA, or NS gene of DKGX/22 were less lethal than the wild-type DKGX/35 virus (MLD_{50}, 3.2 to 5.2) but replicated in multiple organs (Fig. 1). Although the recombinant virus bearing the PB2 gene of DKGX/22 in a background of genes from the DKGX/35 virus replicated in the lungs and spleen, the virus was dramatically attenuated in virulence (MLD_{50}, 6.3). Therefore, the PB2, PA, and NS genes are important for attenuation, although all single-gene recombinant viruses were detectable in organs and were able to replicate in mice (Table 3).

Amino acid at position 701 of PB2 plays an important role in the host range of the DKGX/22 virus. The predicted PB2 protein sequences of the DKGX/22 and DKGX/35 viruses differ by five amino acids (Table 1, Fig. 2). To pinpoint the amino acid(s) that enabled DKGX/22 to replicate in mice or that attenuated the DKGX/35 virus, we generated four viruses that had chimeric PB2 genes (Fig. 2) and tested them in mice. Chimera 1 (DKGX/22 virus possessing the amino-terminal portion of the DKGX/35 PB2 gene) did not kill mice and was not detected in any organ tested. Chimera 2 (DKGX/22 virus possessing the carboxy-terminal portion of the DKGX/35 PB2 gene) was recovered from the lungs, spleens, and brains of infected mice and killed mice at high doses (MLD_{50}, 4.9). When the carboxy-terminal portion of the DKGX/35 PB2 gene was replaced with the corresponding DKGX/22 PB2 gene seg-

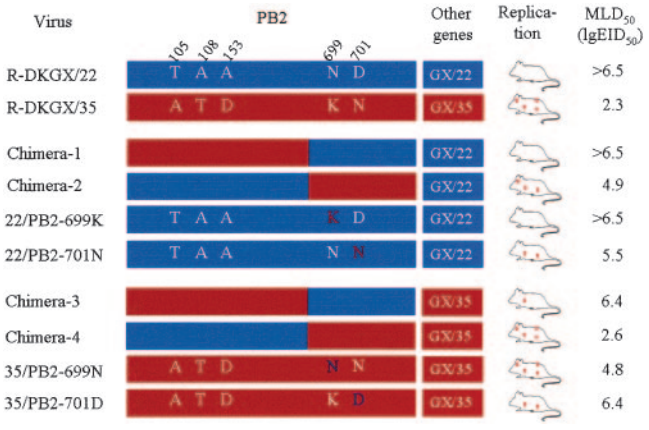

FIG. 2. PB2 mutant viruses and their virulence in mice. Colors indicate the origin of the gene, gene region, or encoded amino acid: blue, DKGX/22; red, DKGX/35. The amino acid differences between DKGX/22 and DKGX/35 PB2 are shown as single letters with the positions numbered at the top. The red dots in the mouse figures indicate tissue tropism (upper left, brain; lower left, lung; upper right, kidney; lower right, spleen). The MLD_{50} was determined by inoculating groups of five mice with 10-fold serial dilutions contain 10^1 to 10^6 EID_{50} of the virus in a 50-μl volume (4) and calculated by the method of Reed and Muench (20). The MLD_{50} value of R-DKGX/35 is the mean of three experiments, and the values of other viruses were obtained from single experiment. Amino acid abbreviations: T, Thr; A, Ala; N, Asn; D, Asp; K, Lys.

ment (chimera 3), within the context of remaining genes from DKGX/35, the pathogenicity of DKGX/35 in mice was greatly attenuated; the chimeric virus replicated only in the lungs, and the titers were 3.7 log less than those of the wild-type DKGX/35 virus (Table 3). However, chimera 4 (DKGX/35 with the amino-terminal portion of the DKGX/22 PB2 gene) was as lethal as the wild-type DKGX/35 virus and replicated systemically in mice (Fig. 2, Table 3). These results indicated that the carboxy terminus of the PB2 gene determines the host range of the DKGX/22 virus and affects the virulence of the DKGX/35 virus.

The two viruses differed at only two amino acid positions (699 and 701) in this region of PB2 (Table 1, Fig. 2). To determine the respective importance of these residues to replication and pathogenicity in mice, we generated and tested four mutant viruses (Fig. 2). A mutant DKGX/22 virus encoding the Asn-to-Lys mutation at position 699 of PB2 (22/PB2-699K) was not lethal and failed to replicate in mice. However, the Asp-to-Asn mutation at position 701 of PB2 (22/PB2-701N) enabled DKGX/22 to replicate in the lungs and spleens of mice and to kill mice at high doses (MLD_{50}, 5.5). When the Lys-to-Asn mutation was introduced at position 699 of the DKGX/35 virus PB2, the mutant virus (35/PB2-699N) was recovered from all organs tested, although its pathogenicity was attenuated (MLD_{50}, 4.8). An Asn-to-Asp substitution at position 701 of PB2 (35/PB2-701D) markedly attenuated DKGX/35 pathogenicity (MLD_{50}, 6.4). This mutant replicated slowly in mice; the lung titer was only 2.5 $\log_{10}$ EID_{50} on day 4 postinfection but increased to 6.1 $\log_{10}$ EID_{50} by day 6, when it was detected in the spleen as well (Fig. 2, Table 3).

The results of this mutational analysis suggest that the amino acid Asn at position 701 of PB2 is one of the important determinants of efficient replication of avian influenza virus in the organs of mice. This finding is further supported by the available sequence data: more than 500 influenza A viruses have Asp at position 701 of PB2, whereas Asn is found at position 701 in only 28 influenza A viruses, most of which were isolated from humans, pigs, and horses (9, 10, 12, 21) The viruses, including DKGX/35-22PB2, chimera 3 virus, and the mutant 35/PB2-701D, that are lacking of Asn at 701 in PB2 are also able to replicate in mice, suggesting that other unidentified amino acids also contribute to the replication of H5N1 viruses in mice.

The 3′ terminus of the PB2 gene (nucleotides 1600 to 2341) of 34 viruses recovered from three chimeric viruses, three mutant viruses, two PB2 recombinant viruses (DKGX/35-22PB2 and DKGX/22-35PB2), and R-DKGX/35 virus-inoculated mouse lung samples were resequenced. Though the viruses were not further purified, the sequencing results clearly indicated that amino acid residues at positions 699 and 701 are stable and the same as in the inoculated viruses. However, it was a surprise to find that mutation of E to K at position 627 occurred. In about 30% of the viruses isolated on day 4 and in over 70% of viruses isolated on day 6 postinfection, K dominated at position 627 of PB2. This mutation may provide an explanation for the increased lung virus titers on day 6 of 35/PB2-701D-inoculated mice. It is interesting that the E-to-K mutation was not observed in any of the viruses recovered from the R-DKGX/35 virus-inoculated mice (data not shown).

DISCUSSION

H5N1 avian influenza viruses have caused the deaths of 57 of 100 infected humans since 1997 and clearly represent a threat to public health. However, the genetic determinants of these viruses' ability to cross the species barrier from birds to mammals remain unknown. We use two duck viruses as models to explore the molecular determinants for H5N1 viruses to cross the avian-mammalian species barrier to transmit and replicate in mice. We found that the host range of H5N1 influenza viruses is largely attributable to PB2. Mutation of a single amino acid residue at position 701 is sufficient to enable replication and virulence in mice.

Only one amino acid in PB2 has previously been proved to be associated with the host range of influenza viruses. In 1993, Subbarao et al. reported that the amino acid at 627 of PB2 is a determinant of host range and that all avian influenza viruses have glutamic acid at this position, whereas all human influenza viruses (H1N1, H2N2, and H3N2) have lysine (26). Lys at position 627 of PB2 has been observed only in influenza viruses isolated from humans and in those adapted to mammalian cell lines; it has never been reported in an influenza virus isolated from avian species. Our DKGX/22 and DKGX/35 viruses have a Glu at position 627 of PB2. However, it was observed in this study that over 50% of viruses recovered from mouse lungs have an E to K substitution at 627 in PB2. Therefore, the presence of Lys at this position may result from adaptation to mammalian hosts. Our results demonstrated that acquisition of an Asp-to-Asn mutation at position 701 in PB2 enabled an avian influenza virus to cross the host species barrier and replicate in mammals. Most importantly, unlike Lys at position 627 of PB2, which is a host-dependent mutation, the Asp701Asn substitution is a host-independent mutation that occurred naturally in H5N1 viruses in ducks and may facilitate the transmission of these viruses to mammals.

Asparagine is not commonly found at position 701 in the PB2 of avian influenza viruses, and among the 21 H5N1 duck influenza viruses we characterized previously (4), only DKGX/35 virus bears Asn at 701 of PB2, but Asn at 701 of PB2 has been observed in equine influenza viruses (10), swine influenza virus (21), and H5N1 human influenza viruses (12, 15). The Asp-to-Asn mutation at position 701 of PB2 was previously reported in an H7N7 avian influenza virus adapted to a mouse lung cell line (30) and in mouse-adapted human influenza virus (1).

There are conflicting reports on amino acid 701 of PB2 of the A/Hong Kong/156/97 virus. Subbarao et al. reported an Asp at this position (24), while Hiromoto et al. described an Asn at this position in their report (12). Two other viruses, A/Hong Kong/488/97 and A/Hong Kong/97/98 (15), isolated from humans in Hong Kong in 1997 have the amino acid Asn at position 701 of the PB2. Just like the mutant virus 22/PB2-701N, all three viruses are low-pathogenic for mice (15), however, two of them are lethal for humans. Asn was also found at position 701 of PB2 in an H5N1 virus isolated from at least one human in 2004 (A/Viet Nam/3046/2004). Our results suggest that the amino acid Asn at position 701 of PB2 is one of the important factors for the replication and lethality of H5N1 duck virus in mouse model. Human disease is quite different from mouse infection, and therefore, how the amino acid Asn

at position 701 of PB2 affects the virulence of H5N1 viruses in humans needs further exploration.

Numerous factors are reportedly associated with H5N1 pathogenicity. Hatta et al. reported 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 (11). Other studies implicate the NS gene and its ability to modulate the cytokine response (5). Reverse genetics studies have found that residue 92 of the NS1 molecule of the human isolate A/Hong Kong/156/97 (H5N1) is associated with severe pathogenicity in pigs (22). The present study found that PB2 is the most important determinant of the pathogenicity of the H5N1 viruses isolated from ducks in China in mice. The Asn-to-Asp mutation at position 701 attenuated the DKGX/35 virus dramatically, restricting viral replication to the lung and spleen. However, the PA, NA, and NS genes of DKGX/22 also attenuated the DKGX/35 virus, though the amino acids responsible for this effect were not pinpointed, suggesting that the pathogenicity of influenza viruses is a polygenic trait. Importantly, all viruses in this study that contained Asn at position 701 of PB2, with the exception of the 22/PB2-701N mutant, were neurovirulent in mice. Sequence analysis of A/Hong Kong/156/97 clones that demonstrate attenuated virulence in mice show the loss of the Asp701Asn mutation in PB2 (12).

PB2, along with the viral proteins PB1 and PA, makes up the viral RNA polymerase. The amino acid at position 627 has been reported to affect the replicative efficiency of Hong Kong H5N1 influenza A viruses in mice (23). The amino acid at position 701 is located between the cap-binding motif at positions 634 to 650 and the second nuclear localization signal at positions 736 to 739 (2). It remains to be determined whether this mutation affects polymerase function.

Our findings support the contention that residue 701 of the PB2 molecule is associated with interspecies transmissibility of H5N1 influenza viruses from their natural host reservoirs (ducks) to mammals (mice). Interspecies transmission and pathogenicity are interrelated traits that involve multiple genes of both virus and host. Emerging information shows that the viral PB2 gene is important for both pathogenicity (residue 627; Hatta et al. [11]) and host-range (residue 701; this study) of H5N1 influenza viruses. However, more details of the total gene constellations and specific residues of H5N1 viruses must be resolved before the pandemic potential of specific viruses can be predicted.

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