H5N1 Hybrid Viruses Bearing 2009/H1N1 Virus Genes Transmit in Guinea Pigs by Respiratory Droplet

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Science **340**, 1459 (2013);
DOI: 10.1126/science.1229455

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Acknowledgments: The authors thank R. Min and P. Oh for technical assistance; M. Sellars for a critical reading of the manuscript; J. M. Weiss, J. J. Lafaille, and A. Y. Rudensky for their advice; and E. Newman (NYU School of Medicine, New York) for providing surgical specimens. *Foxp3*$^{ires\text{-}mrfp}$ mice were provided by R. A. Flavell (Yale University School of Medicine, Howard Hughes Medical Institute) and subject to a Materials Transfer Agreement. A patent application related to the work in this paper has been filed (S.V.K. and D.R.L. as inventors). The data in this paper are tabulated in the main paper and in the supplementary materials, and are deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GSE45773). S.V.K. was supported by the Irvington Institute fellowship program of the Cancer Research Institute and by NIH National Research Service Award grant 1T32AI100853-01. Y.Y. was supported by the Arthritis National Research Foundation. D.R.L. is a Howard Hughes Medical Institute Investigator. This work was supported in part by National Cancer Institute (NCI), NIH, grant 5P30CA016087-32 (BioRepository Center, Genome Technology Center, and Histopathology Core), NCI-NIH grant 5P30CA016087-33 (Cytometry and Cell Sorting Core), National Center for Research Resources, NIH, grant UL1RR029893 (BioRepository Center), and NCI-NIH P30 CA016087-30 (for data collected on the Affymetrix GeneChip System).

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1237013/DC1
Materials and Methods
Figs. S1 to S13
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25 February 2013; accepted 17 April 2013
Published online 9 May 2013;
10.1126/science.1237013

H5N1 Hybrid Viruses Bearing 2009/H1N1 Virus Genes Transmit in Guinea Pigs by Respiratory Droplet

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In the past, avian influenza viruses have crossed species barriers to trigger human pandemics by reassorting with mammal-infective viruses in intermediate livestock hosts. H5N1 viruses are able to infect pigs, and some of them have affinity for the mammalian type α-2,6-linked sialic acid airway receptor. Using reverse genetics, we systematically created 127 reassortant viruses between a duck isolate of H5N1, specifically retaining its hemagglutinin (*HA*) gene throughout, and a highly transmissible, human-infective H1N1 virus. We tested the virulence of the reassortants in mice as a correlate for virulence in humans and tested transmissibility in guinea pigs, which have both avian and mammalian types of airway receptor. Transmission studies showed that the H1N1 virus genes encoding acidic polymerase and nonstructural protein made the H5N1 virus transmissible by respiratory droplet between guinea pigs without killing them. Further experiments implicated other H1N1 genes in the enhancement of mammal-to-mammal transmission, including those that encode nucleoprotein, neuraminidase, and matrix, as well as mutations in H5 *HA* that improve affinity for humanlike airway receptors. Hence, avian H5N1 subtype viruses do have the potential to acquire mammalian transmissibility by reassortment in current agricultural scenarios.

Avian influenza viruses continue to evolve and spread, perpetuating the fear of an influenza pandemic if they acquire the ability to transmit efficiently among humans. The influenza virus genome comprises eight gene segments: basic polymerase 2 (*PB2*), basic polymerase 1 (*PB1*), acidic polymerase (*PA*), hemagglutinin (*HA*), nucleoprotein (*NP*), neuraminidase (*NA*), matrix (*M*), and nonstructural protein (*NS*). Hemagglutinin and neuraminidase are integral membrane proteins. The *HA* of human-infective influenza subtypes preferentially recognizes α-2,6-linked sialic acids (SAs) (humanlike receptor), whereas the *HA* of avian-infective influenza subtypes preferentially recognizes α-2,3-linked SAs (avian-like receptor) (*1*). Combinations of amino acid changes such as 158D/224K/226L, 196R/226L/228S, or 110Y/160A/226L/228S (H3 numbering used throughout; see fig. S6) in HA protein can allow H5N1 viruses to recognize α-2,6-linked SAs, thereby conferring viral transmission between ferrets (*2*–*4*).

When two different influenza viruses infect the same cell, their genes can reassort to produce new viral strains. Historically, such reassortment has led to the emergence and spread of pandemic viruses in immunologically naïve human populations (*5*–*8*). A previous study with an H5N1 virus and a human H3N2 virus suggested that reassortments between these two subtypes to produce a dangerous virus would be rare (*9*). However, both avian H5N1 and human 2009/H1N1 viruses have been found in pigs (*10*–*14*), so we asked: Could an H5N1 reassortant between avian H5N1 and the highly transmissible 2009/H1N1 virus become transmissible among mammals and potentially cause a human pandemic?

H5N1 influenza viruses were handled in the enhanced animal biosafety laboratory level 3 (ABSL3+) facility at the Harbin Veterinary Research Institute, China (*15*). All experimental studies with live H5N1 viruses were performed before the moratorium on such studies was in place (*16*, *17*). Details of the biosafety and biosecurity measures taken and the dates on which the experiments were performed are provided in the supplementary materials.

We used two influenza viruses isolated in China: the H5N1 virus A/duck/Guangxi/35/2001 [DK/35(H5N1)] and the H1N1 virus A/Sichuan/1/2009 [SC/09(H1N1)]. DK/35(H5N1) is highly pathogenic for both chickens and mice (*18*). It transmits by direct contact among guinea pigs when they are housed together (*19*) but does not transmit between guinea pigs by respiratory droplet (Fig. 1A). We previously identified two molecular changes that are critical for the contact transmission of DK/35(H5N1) among guinea pigs: the asparagine residue at position 701 (701N) in *PB2* and the alanine residue at position 160 (160A) in *HA* (*19*). The mutation of 160A, resulting in the absence of glycosylation at positions 158 to 160 in *HA*, permits virus binding to α-2,6-linked SAs (*19*, *20*). Receptor specificity testing, using a solid-phase binding assay with four different glycans, indicated that DK/35(H5N1) binds to both α-2,3-linked SAs and α-2,6-linked SAs, and that its affinity to α-2,3-linked SAs is higher than to α-2,6-linked SAs (fig. S1A). SC/09(H1N1) was the first virus isolated in China during the 2009 influenza pandemic and transmits efficiently among guinea pigs by respiratory droplet (Fig. 1B) (*21*).

Using plasmid-based reverse genetics (*22*–*24*), we generated all possible reassortants possessing the H5 *HA* gene [i.e., 127 hybrid viruses between DK/35(H5N1) and SC/09(H1N1), 2^7 minus one

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parent virus]. We numbered the gene segments derived from SC/09(H1N1) as follows: 1, *PB2*; 2, *PB1*; 3, *PA*; 5, *NP*; 6, *NA*; 7, *M*; and 8, *NS*. For example, "r123" denotes the reassortant containing SC/09(H1N1) *PB2*, *PB1*, and *PA*, with the other five segments from the DK/35(H5N1) virus. In previous studies (*25*, *26*), certain H5 reassortants with H3N2 either were not generated or did not grow efficiently because of genomic incompatibility; for example, virus bearing the *NP* gene of human H3N2 virus and *M* and *NS* gene of H5N1 avian virus cannot replicate efficiently (*25*). By contrast, in this study, reassortants with the H5N1 *HA* were easily generated and all of them grew efficiently in chicken eggs, with viral titers ranging from 10^7 to 10^{10} 50% egg infectious doses (EID_{50}) (tables S1 to S3).

H5N1 virus virulence in mice correlates with its virulence in humans (*27*, *28*), but this correlation has not been extended to ferrets (*29*). We used BALB/c mice to evaluate the virulence of our H5N1 reassortants by inoculating groups of eight mice with different doses of DK/35(H5N1) or SC/09(H1N1): DK/35(H5N1) had a mouse lethal dose (MLD_{50}; a dose lethal to 50% of mice) of 2.6 $\log_{10}$ EID_{50} (fig. S2A), whereas the MLD_{50} of SC/09(H1N1) was 6.2 $\log_{10}$ EID_{50} (fig. S2B), indicating a milder infection. Virus replication was detected in the brains of mice that received 10^3 EID_{50} or higher doses of DK/35(H5N1) but was detected in the brain of only one of three mice that were inoculated with 10^6 EID_{50} of SC/09(H1N1) (fig. S2C). We did not measure the MLD_{50} of all 127 viruses; instead, we infected mice with 10^3 EID_{50} of each reassortant and scored them for replication in the brain and their effect on mouse mortality. On day 5 post-inoculation (p.i.), three mice in each group of eight were killed and their brain tissues were collected for virus titration; the other five mice were observed for a total of 14 days for body weight changes and death.

The 127 viruses were categorized into three groups according to their replication in the brain and lethality in mice: (i) 54 viruses showed similar pathogenicity to DK/35(H5N1); some viruses were lethal, but virus was not detected in the brains of all three mice. Mice infected with these viruses showed diversity in body weight changes at the end of the observation period (Fig. 2A, viruses shown in black, and table S1); (ii) 38 viruses were less pathogenic than DK/35(H5N1), they were not detected in the brain of any mouse killed on day 5 p.i., and the mice survived the infection and gained body weight (except those infected with the r23678 virus) (Fig. 2A, viruses shown in blue, and table S2); and (iii) 35 viruses were more pathogenic than DK/35(H5N1), they were detected in the brains of all three mice killed on day 5 p.i., all the remaining five mice died [mean time to death <10 days], and all lost 10 to 30% of their body weight (Fig. 2A, viruses shown in red, and table S3).

The activity of the influenza virus ribonucleoprotein (RNP) complex (i.e., the products of the *PB2*, *PB1*, *PA*, and *NP* genes) is important for virus replication and virulence (*30*). The levels of RNP activity at 33° and 37°C correlate with viral replication efficiency in the upper and lower respiratory tracts, respectively (*31*, *32*). One study suggests that replication efficiency of influenza virus in the upper respiratory tract favors viral transmission (*31*). Therefore, we tested virus RNA replication in human cells at both 33° and 37°C to determine the activities of the 16 combinations of RNP complex arising from DK/35(H5N1) and SC/09(H1N1). In general, these RNP complexes showed less activity at 33°C than at 37°C (Fig. 2B), except for two combinations that showed equivalent activity at both temperatures: (i) DK/35(H5N1) *PB2* and *NP* with SC/09(H1N1) *PB1* and *PA*, and (ii) DK/35(H5N1) *PB2*, *PB1*, and *NP* with SC/09(H1N1) *PA*. At 37°C, the RNP complex activity of wild-type DK/35(H5N1) was about 35% that of wild-type SC/09(H1N1) (Fig. 2B). All seven hybrid RNP combinations containing the SC/09(H1N1) *PA* were more active than the RNP of the parent SC/09(H1N1) at 37°C. All hybrid RNP combinations containing the SC/09(H1N1) *PB1* and the DK/35(H5N1) *PA* were less active than the RNP of wild-type DK/35(H5N1) at 37°C. Other combinations had intermediate activity.

A comparison of tables S1 to S3 with Fig. 2 reveals that 29 of the 32 viruses with an RNP containing the SC/09(H1N1) *PB1* and the DK/35(H5N1) *PA* were less pathogenic than DK/35(H5N1) (Fig. 2A, viruses shown in blue). All viruses with greater pathogenicity than DK/35(H5N1) contained an RNP that included the SC/09(H1N1) *PA* (Fig. 2A, viruses shown in red). The components of the RNP complex therefore contribute to the virulence of the hybrid viruses in mice, and the combination of the *PA* gene of SC/09(H1N1) and the *PB1* gene of DK/35(H5N1) elevates the virulence of the reassortants.

We next determined whether any of these reassortants were transmissible in mammals. Guinea pigs and ferrets have been widely used as animal models for influenza virus transmission studies (*2*, *3*, *19*, *21*, *33*, *34*), and human influenza viruses transmit similarly in these two models (*21*, *35*, *36*). Respiratory droplet transmission is restricted in ferrets if the virus does not exclusively or preferentially bind to α-2,6-linked SAs (*37*). Guinea pigs have both avian and mammalian types of airway receptor, but they did not support the respiratory droplet transmission of influenza virus that only binds to α-2,3-linked SAs (*21*). We used guinea pigs to test for respiratory droplet transmission of our H5N1 reassortants. We compared SC/09(H1N1) with DK/35(H5N1) plus reassortants r1, r12, r123, r125, r13, r135, r15, r2, r23, r235, r25, r3, r35, r5, and r1235 (the viruses listed in the first row of Fig. 2A). These H5N1 viruses contain different RNPs but have the same *HA*, *NA*, *M*, and *NS* genes of DK/35(H5N1). Two guinea pigs were intranasally (i.n.) inoculated with 10^6 EID_{50} of each virus, and nasal washes and lung tissues from each guinea pig were collected on day 3 p.i. for viral titration. All viruses were detected in the nasal washes and lungs of these guinea pigs (Table 1).

Three guinea pigs were inoculated i.n. with 10^6 EID_{50} of the test virus and housed in separate cages within an isolator. Twenty-four hours later, three naïve guinea pigs were placed in ad-

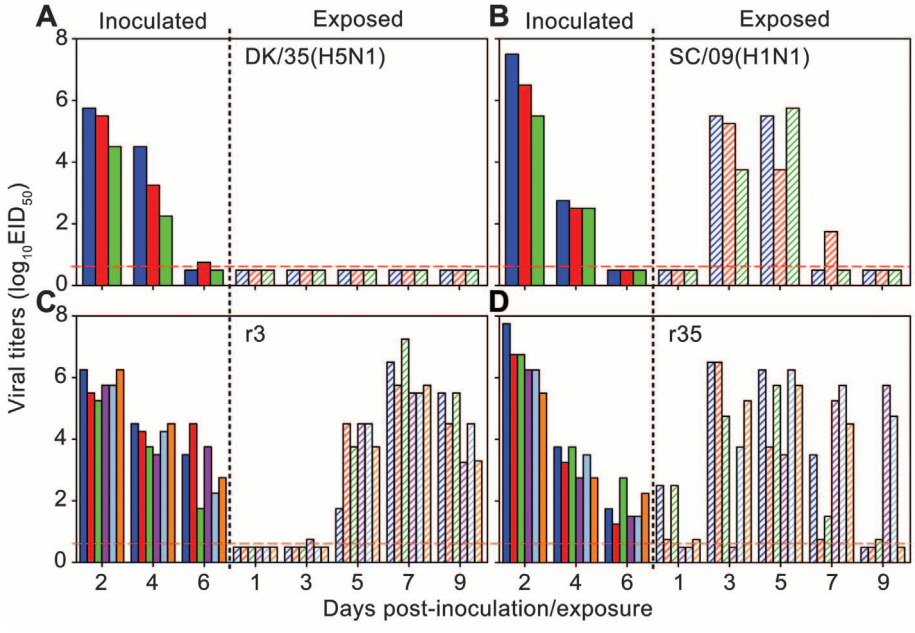

Fig. 1. Respiratory droplet transmission in guinea pigs of the DK/35 (H5N1) virus and its reassortants bearing different RNP complexes. (**A**) DK/35(H5N1); (**B**) SC/09(H1N1); (**C**) r3; (**D**) r35. Data for the 13 viruses that did not transmit are presented in fig. S3, A to M. Each color bar represents the virus titer from an individual animal. The dashed red lines indicate the lower limit of detection.

jacent cages. The six animals were separated by a double-layered net divider (4 cm apart) (fig. S3). Nasal washes were collected on days 2, 4, and 6 p.i. from the inoculated animals or on days 1, 3, 5, 7, and 9 post exposure (p.e.) from the exposed animals and titrated in eggs to test for respiratory droplet transmission. Sera were collected from all animals on day 21 p.i. for hemagglutinin inhibition antibody detection. Respiratory droplet transmission was confirmed when virus was detected in the nasal washes and by seroconversion of the naïve exposed animals at the end of the 3-week observation period.

Virus was detected in all directly infected animals (Fig. 1 and fig. S4) and in all three animals exposed to the guinea pigs that had been inoculated with SC/09(H1N1), r3 [containing the *PA* of SC/09(H1N1)], and r35 [containing the *PA* and *NP* of SC/09(H1N1)] (Fig. 1, B to D). Virus was detected later in the r3-exposed animals than in the r35-exposed animals, but no virus was detected in any animal exposed to those inoculated with DK/35(H5N1) (Fig. 1A) or the 13 hybrid viruses containing SC/09 *PB2* or *PB1* in their RNP complex (Table 1 and fig. S4). Six of these reassortants (r123, r1235, r13, r135, r23, and r235) had RNP complex activities comparable to or higher than those of SC/09(H1N1) at 33°C (Fig. 2B). We therefore repeated the transmission experiments with these six viruses and the r3 and r35 reassortants and obtained the same results (the combined data from both experiments are shown in Fig. 1, C and D, and fig. S4, E to J). Of note, r35 was detected earlier than wild-type SC/09(H1N1) in the exposed animals (compare Fig. 1, B and D). Seroconversion occurred in the virus-inoculated animals and in all exposed animals that were virus-positive (Table 1). None of the guinea pigs died. These results indicate that the *PA* gene of SC/09(H1N1) alone can make DK/35(H5N1) transmissible by respiratory droplet between guinea pigs. Furthermore, the addition of the *NP* of SC/09(H1N1) appears to promote the transmission of r3 by respiratory droplet, although the *NP* of SC/09(H1N1) alone did not support virus transmission. Viruses bearing the *PB2* or *PB1* of SC/09(H1N1) in their RNP complexes were not transmissible, even though some also possessed the *PA* of SC/09(H1N1).

We also investigated the contributions of the nonpolymerase genes of SC/09(H1N1) to transmission by testing r678 and r1235678. Both viruses replicated well in the nasal cavities and lungs of guinea pigs (Table 1), and r1235678 and r678 were transmitted by respiratory droplet to two and three naïve animals, respectively (Fig. 3, A and B).

The contributions of the individual *NA*, *M*, and *NS* genes of SC/09(H1N1) to transmissibility were tested using r6, r7, and r8. Respiratory droplet transmission occurred in one of the three naïve animals for the r6 and r7 groups (Fig. 3, C and D). However, r8 transmitted to all three naïve animals by respiratory droplet. The *NA* and *M* genes are important for the highly transmissible phenotype of the 2009/H1N1 virus (*38*–*40*), but the *NS* gene was not previously known to function in influenza virus transmission. We therefore repeated the transmission experiment with r8 and found that the results were reproducible (the combined data from both experiments are shown in Fig. 3E).

Thus, both the *PA* and *NS* genes of SC/09(H1N1) can make DK/35(H5N1) highly transmissible by respiratory droplet between guinea pigs, and the *NA* and *M* of SC/09(H1N1) also promote H5N1 virus transmission through respiratory droplet. Moreover, the *NP* of SC/09(H1N1) accelerates H5N1 virus transmission when combined with the *PA* of SC/09(H1N1). Because r3678 transmitted with high efficiency in guinea pigs (Fig. 3F), other reassortants lethal to mice may also be transmissible by respiratory droplet between guinea pigs. Four viruses (r35, r678, r8, and r3678) were detectable in the naïve animals by day 1 p.e., indicating that these viruses

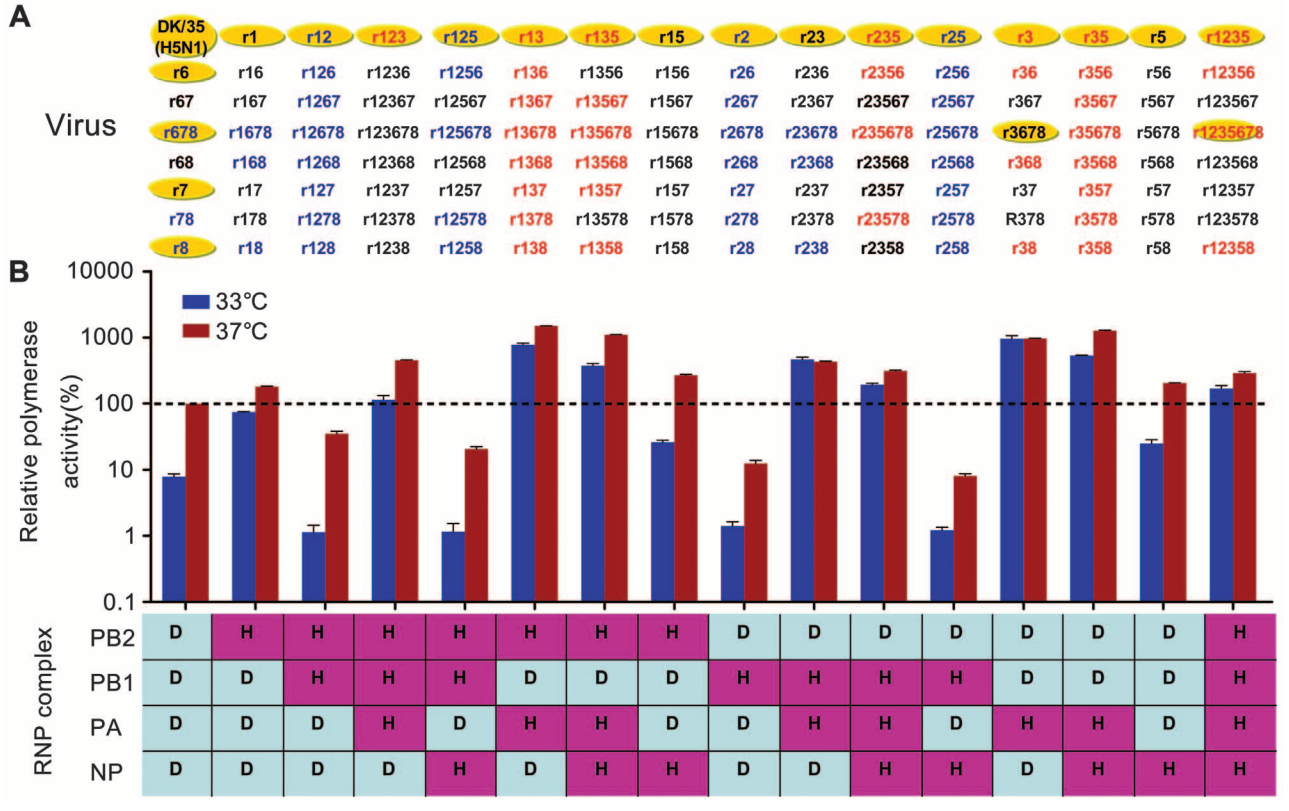

Fig. 2. Correlation of the virulence and the polymerase activity of H5N1 reassortants. (**A**) Pathogenicity of DK/35(H5N1) and SC/09(H1N1) reassortants. The colors of the viruses reflect their virulence in mice relative to DK/35(H5N1) as reported in tables S1 to S3 [red, more pathogenic than DK/35(H5N1); blue, less pathogenic than DK/35(H5N1); black, similar to DK/35(H5N1)]. Viruses highlighted in yellow were tested for respiratory droplet transmission in guinea pigs. (**B**) Polymerase activity of the 16 RNP combinations of DK/35(H5N1) and SC/09(H1N1) viruses. The assay was performed at 37°C (red bars) or 33°C (blue bars). Values shown are means ± SD of three independent experiments and are standardized to those of DK/35(H5N1) measured at 37°C (100%, indicated by the dashed line). The chart below shows the parent subtype origin of the RNP complex. Segments derived from DK/35(H5N1) and those derived from SC/09(H1N1) are denoted D and H, respectively.

transmitted faster than the parent SC/09(H1N1), which was detected by day 3 p.e. (Fig. 1B). These transmission studies indicate that many of the H5N1 hybrid viruses bearing one or more of the *PA*, *NA*, *M*, or *NS* genes of 2009/H1N1 were transmissible in guinea pigs.

Mutations in *HA* that confer exclusive binding to α-2,3-linked SAs eliminate the respiratory droplet transmission of 1918/H1N1 virus among

Table 1. Replication and seroconversion of guinea pigs inoculated with or exposed to the H5N1 virus DK/35 (H5N1) and its reassortants. In the virus name, "r" denotes reassortant. The numbers in the virus name indicate segments derived from the SC/09(H1N1) virus as follows: 1, *PB2*; 2, *PB1*; 3, *PA*; 5, *NP*; 6, *NA*; 7, *M*; and 8, *NS*. The virus segments derived from the DK/35(H5N1) virus were not assigned numbers. Gene segments derived from DK/35(H5N1) and those derived from SC/09(H1N1) are denoted D and H, respectively. Viral titers are shown as individual titers for both guinea pigs. Sera were collected from the animals 3 weeks after virus inoculation or exposure; these animals were used for the transmission studies shown in Figs. 2 and 3. HI, hemagglutinin inhibition.

Virus name	Genotype								Viral titers (log_{10} EID_{50})		Seroconversion (HI titers), positive/total		Respiratory droplet transmission
	PB2	*PB1*	*PA*	*HA*	*NP*	*NA*	*M*	*NS*	Nasal	Lung	Inoculated	Exposed	
DK/35 (H5N1)	D	D	D	D	D	D	D	D	4.8, 3.5	2.8, 2.8	3/3 (40–80)	0/3	None
SC/09 (H1N1)	H	H	H	H	H	H	H	H	4.9, 5.2	3.3, 3.3	3/3 (320–1280)	3/3 (640)	Highly efficient
r1	H	D	D	D	D	D	D	D	3.5, 3.3	2.3, 3.3	3/3 (20–80)	0/3	None
r12	H	H	D	D	D	D	D	D	4.5, 2.5	1.8, 3.8	3/3 (20–40)	0/3	None
r123*	H	H	H	D	D	D	D	D	4.8, 5.3	4.8, 3.5	6/6 (40–80)	0/6	None
r125	H	H	D	D	H	D	D	D	4.5, 3.3	2.3, 3.3	3/3 (40–80)	0/3	None
r13*	H	D	H	D	D	D	D	D	4.8, 5.5	3.3, 1.8	6/6 (40–80)	0/6	None
r135*	H	D	H	D	H	D	D	D	4.3, 4.3	3.3, 2.3	6/6 (20–80)	0/6	None
r15	H	D	D	D	H	D	D	D	2.3, 3.5	4.8, 2.5	3/3 (20–40)	0/3	None
r2	D	H	D	D	D	D	D	D	4.8, 4.5	2.8, 2.5	3/3 (40–80)	0/3	None
r23*	D	H	H	D	D	D	D	D	3.3, 4.5	1.8, 2.8	6/6 (20–80)	0/6	None
r235*	D	H	H	D	H	D	D	D	4.3, 4.5	2.5, 2.8	6/6 (40–80)	0/6	None
r25	D	H	D	D	H	D	D	D	5.5, 5.3	4.3, 4.8	3/3 (20–40)	0/3	None
r3*	D	D	H	D	D	D	D	D	5.5, 4.8	2.8, 3.5	6/6 (80)	6/6 (20–40)	Highly efficient
r35*	D	D	H	D	H	D	D	D	4.3, 5.8	2.8, 2.8	6/6 (40–80)	6/6 (20–40)	Highly efficient
r5	D	D	D	D	H	D	D	D	5.5, 4.8	2.3, 1.3	3/3 (40–80)	0/3	None
r1235*	H	H	H	D	H	D	D	D	4.5, 4.3	2.5, 3.3	6/6 (20–80)	0/6	None
r1235678	H	H	H	D	H	H	H	H	4.8, 4.8	3.3, 2.8	3/3 (40–80)	2/3 (10–20)	Efficient
r678	D	D	D	D	D	H	H	H	4.3, 4.8	4.8, 3.5	3/3 (40–80)	3/3 (10–80)	Highly efficient
r6	D	D	D	D	D	H	D	D	4.3, 4.5	2.3, 3.8	3/3 (80)	1/3 (20)	Less efficient
r7	D	D	D	D	D	D	H	D	4.5, 5.3	4.5, 5.5	3/3 (40–80)	1/3 (40)	Less efficient
r8*	D	D	D	D	D	D	D	H	4.3, 4.8	4.5, 2.3	6/6 (20–160)	6/6 (20–80)	Highly efficient
r3678	D	D	H	D	D	H	H	H	5.5, 4.5	4.3, 3.8	3/3 (40–80)	3/3 (40–80)	Highly efficient

*Each transmission experiment was conducted twice, and the combined data from both experiments are presented in the table.

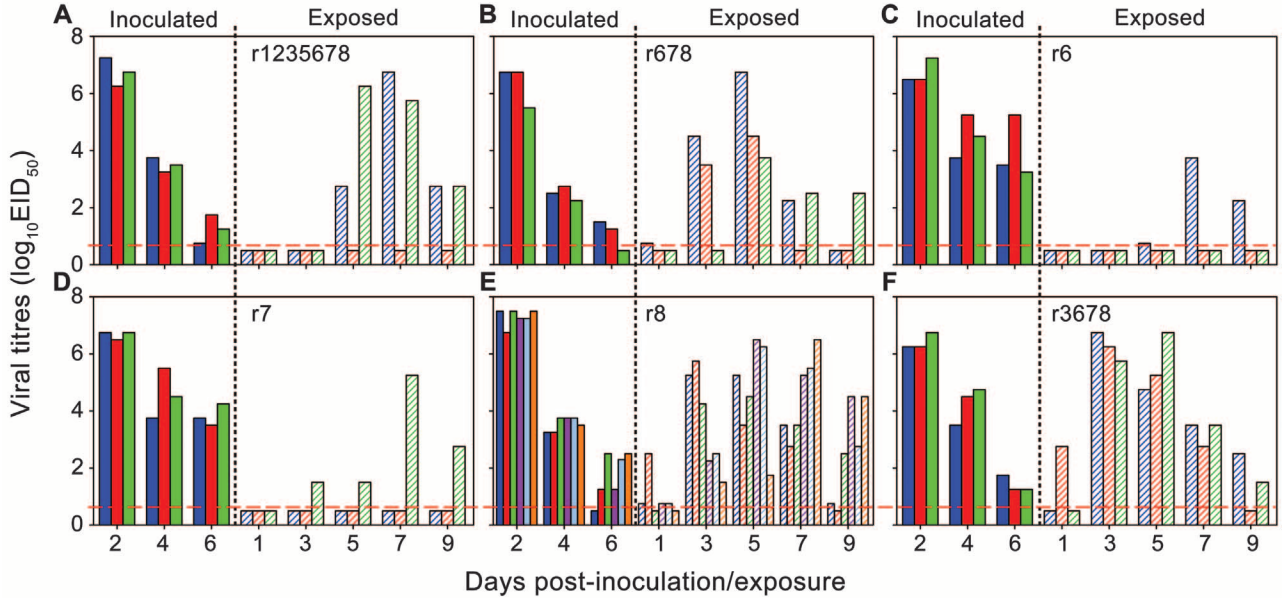

Fig. 3. Contributions of the *NA*, *M*, and *NS* genes of SC/09 (H1N1) to respiratory droplet transmission of H5N1 reassortants in guinea pigs. (**A**) r1235678; (**B**) r678; (**C**) r6; (**D**) r7; (**E**) r8 (data from two experiments); (**F**) r3678. Each color bar represents the virus titer from an individual animal. The dashed red lines indicate the lower limit of detection.

ferrets (*37*) and that of the 2009/H1N1 virus among both guinea pigs and ferrets (*21*), even if the other genes of these highly transmissible viruses are intact. We performed a solid-phase binding assay to investigate the receptor-binding specificity of the H5N1 viruses by using four different glycans (fig. S1). DK/35(H5N1), r3, r8, r35, r3678, and two H5N1 viruses isolated in nature—A/bar-headed goose/Qinghai/3/2005 and A/duck/Fujian/S4146/2010—bound to both α-2,3-linked SAs and α-2,6-linked SAs (fig. S1, A to G). A/duck/Anhui/1/2006 bound preferentially to α-2,3-linked SAs (fig. S1H). Although the affinity to α-2,6-linked SAs was lower than that to α-2,3-linked SAs, this property has been demonstrated to be required for the transmission of DK/35(H5N1) among guinea pigs by direct contact (*19*). It may therefore also be necessary for respiratory droplet transmission of H5N1 reassortants among guinea pigs.

We previously reported a DK/35(H5N1) mutant, DK/35(*HA*226L+228S), containing two amino acid changes at positions 226 and 228 in *HA* bound exclusively to α-2,6-linked SAs (*19*), and this property was further confirmed by the solid-phase binding assay (fig. S1I). DK/35(*HA*226L+228S), containing the combination of amino acids 160T/226L/228S that presented in the transmissible H5N1 virus reported by Herfst *et al*. (*3*), did not transmit between guinea pigs by respiratory droplet (fig. S4N), although very low virus titer was detected in preliminary experiments in one of three exposed ferrets (fig. S5F). We were unable to determine whether the gene combinations of the highly transmissible virus in guinea pigs would also make the DK/35(*HA*226L+228S) mutant transmissible in ferrets, because a moratorium on such studies was applied between 20 January 2012 and 20 January 2013 (*16*, *17*).

Our studies provide evidence that H5N1 viruses that are capable of respiratory droplet transmission between mammals can be generated by reassortment between mammalian 2009/H1N1 and avian H5N1 viruses. Because the internal genes of these reassortants can already replicate efficiently in mammalian hosts, we predict that similar reassortants could infect humans and subsequently acquire mutations that improve binding efficacy for α-2,6-linked SAs. In fact, several combinations of amino acid changes of 158D, 160A, 224K, 226L, and 228S in *HA* allow the H5N1 virus to bind to α-2,6-linked SAs (*2*, *3*, *19*), and the mutations at 158D, 160A, and 224K have already been detected in H5N1 viruses circulating in nature (*41*).

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Acknowledgments: We thank S. Watson for editing the manuscript, Y. Shu of the China Centers for Disease Control and Prevention for providing the 2009/H1N1 virus A/Sichuan/1/2009, and the Consortium for Functional Glycomics (Department of Molecular Biology, Scripps Research Institute) for providing the glycans. Supported by National Natural Science Foundation of China grant 30825032. Virus sequence data from this study were deposited in GISAID with accession numbers EPI1431518 to EPI1432533 and EPI438129 to EPI438136.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1229455/DC1
Materials and Methods
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29 August 2012; accepted 23 April 2013
Published online 2 May 2013;
10.1126/science.1229455

An Airborne Transmissible Avian Influenza H5 Hemagglutinin Seen at the Atomic Level

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Recent studies have identified several mutations in the hemagglutinin (HA) protein that allow the highly pathogenic avian H5N1 influenza A virus to transmit between mammals by airborne route. Here, we determined the complex structures of wild-type and mutant HAs derived from an Indonesia H5N1 virus bound to either avian or human receptor sialic acid analogs. A cis/trans conformational change in the glycosidic linkage of the receptor analog was observed, which explains how the H5N1 virus alters its receptor-binding preference. Furthermore, the mutant HA possessed low affinities for both avian and human receptors. Our findings provide a structural and biophysical basis for the H5N1 adaptation to acquire human, but maintain avian, receptor-binding properties.

In the past 100 years, only three subtypes of influenza virus have adapted to human populations to cause four pandemics: H1N1 in 1918 and 2009, H2N2 in 1957, and H3N2 in 1968 (*1*–*3*). Other subtypes (e.g., H5N1, H6N1, H7N2, and H9N2) have caused epizootics in domestic poultry in certain regions of the world (*4*), with a recent H7N9 human infection in China (*5*). H5N1 virus, especially, has spread through wild and domestic bird populations across Asia and into Europe, the Middle East, and Africa (*6*, *7*). H5N1 virus has also caused several hundred sporadic cases of human infections with high fatality (*8*–*10*), but it has not acquired the ability to efficiently transmit among humans.

Two recent studies identified several mutations that allow the H5N1 virus to become transmissible by airborne route in a ferret mammalian model system, raising the question of whether these mutations can also confer airborne transmissibility between humans (*11*, *12*). Both reports show that several mutations in hemagglutinin

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