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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. By using reverse genetics, we systemically 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 study showed that both polymerase PA gene and non-structural protein NS gene of H1N1 virus made the H5N1 virus transmissible by respiratory droplet between guinea pigs, without death. Further experiments implicated other H1N1 genes in the enhancement of mammal-to-mammal transmission, including nucleoprotein (NP), neuraminidase (NA) and matrix (M), as well as mutations in H5 HA that improve affinity for human-like 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 genes, basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural protein (NS). The HA gene and the NA gene encode hemagglutinin and neuraminidase, respectively, which are integral membrane proteins. The HA of human-infective influenza subtypes preferentially recognizes α-2,6-linked sialic acids (SAs) (human-like 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, 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 (see 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 701 (701N) in PB2 and the alanine residue at 160 (160A) in HA (*19*). The mutation of 160A, resulting in the absence of glycosylation at 158–160 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 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 (see 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 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 were either not generated or did not grow efficiently because of genomic incompatibility, for example, virus bearing 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 their 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}) 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 10^3EID_{50} or higher doses of DK/35(H5N1), but was only detected in the brain of one of three mice that were inoculated with 10^6EID_{50} of SC/09(H1N1) (fig. S2C). We did not measure the MLD_{50} of all 127 viruses; instead, we infected mice with 10^3EID_{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 euthanized 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 based on 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 euthanized on day 5 p.i., 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 euthanized on day 5 p.i., all the remaining five mice died [mean time to death (MDT) = <10 days] and all lost 10%–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°C 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°C 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, NP with SC/09(H1N1) PA. At 37°C, the RNP complex activity of wild-type DK/35(H5N1) was about 35% of 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 PA gene of SC/09(H1N1) and the PB1 gene of DK/35(H5N1) combination 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 the same HA, NA, M, and NS genes of DK/35(H5N1). Two guinea pigs were intranasally (i.n.) inoculated with 10^6EID_{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 10^6EID_{50} of the test virus and housed in separate cages within an isolator. Twenty-four hours later, three naïve guinea pigs were placed in adjacent 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 (HI) 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, including 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). So, we 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 (Fig. 1, compare panels 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 non-polymerase 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 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). Other reassortants lethal to mice may also be transmissible by respiratory droplet between guinea pigs, which is supported by the fact that r3678 transmitted with high efficiency in guinea pigs (Fig. 3F). Four viruses (r35, r678, r8, and r3678) were detectable in the naïve animals by day 1 p.e, indicating that these viruses 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 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(HA226L+228S), containing two amino acid changes at 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(HA226L+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 see if the gene combinations of the highly transmissible virus in guinea pigs would also make the DK/35(HA226L+228S) mutant transmissible in ferrets, because a moratorium on such studies was applied between 20th January 2012 and 20th 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. Since 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*).

References and Notes

1. G. N. Rogers, J. C. Paulson, Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**, 361 (1983). doi:10.1016/0042-6822(83)90150-2 Medline
2. M. Imai *et al*., Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **486**, 420 (2012). Medline
3. S. Herfst *et al*., Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **336**, 1534 (2012). doi:10.1126/science.1213362 Medline
4. L. M. Chen *et al*., In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity. *Virology* **422**, 105 (2012). doi:10.1016/j.virol.2011.10.006 Medline
5. C. Scholtissek, W. Rohde, V. Von Hoyningen, R. Rott, On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* **87**, 13 (1978). doi:10.1016/0042-6822(78)90153-8 Medline
6. Y. Kawaoka, S. Krauss, R. G. Webster, Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* **63**, 4603 (1989). Medline
7. S. E. Lindstrom, N. J. Cox, A. Klimov, Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957-1972: Evidence for genetic divergence and multiple reassortment events. *Virology* **328**, 101 (2004). doi:10.1016/j.virol.2004.06.009 Medline
8. R. J. Garten *et al*., Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **325**, 197 (2009). doi:10.1126/science.1176225 Medline
9. T. R. Maines *et al*., Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 12121 (2006). doi:10.1073/pnas.0605134103 Medline
10. Q. Zhu *et al*., A naturally occurring deletion in its NS gene contributes to the attenuation of an H5N1 swine influenza virus in chickens. *J. Virol.* **82**, 220 (2008). doi:10.1128/JVI.00978-07 Medline
11. C. A. Nidom *et al*., Influenza A (H5N1) viruses from pigs, Indonesia. *Emerg. Infect. Dis.* **16**, 1515 (2010). doi:10.3201/eid1610.100508 Medline
12. T. Pasma, T. Joseph, Pandemic (H1N1) 2009 infection in swine herds, Manitoba, Canada. *Emerg. Infect. Dis.* **16**, 706 (2010). doi:10.3201/eid1604.091636 Medline
13. D. Sreta *et al*., Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. *Emerg. Infect. Dis.* **16**, 1587 (2010). doi:10.3201/eid1610.100665 Medline
14. J. H. Yan *et al*., Pandemic (H1N1) 2009 virus circulating in pigs, Guangxi, China. *Emerg. Infect. Dis.* **18**, 357 (2012). doi:10.3201/eid1802.111346 Medline
15. See supplementary materials on *Science* Online.
16. R. A. Fouchier *et al*., Pause on avian flu transmission research. *Science* **335**, 400 (2012). doi:10.1126/science.1219412 Medline
17. R. A. Fouchier, A. García-Sastre, Y. Kawaoka, Pause on avian flu transmission studies. *Nature* **481**, 443 (2012). doi:10.1038/481443a Medline
18. Z. Li *et al*., Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. *J. Virol.* **79**, 12058 (2005). doi:10.1128/JVI.79.18.12058-12064.2005 Medline
19. Y. Gao *et al*., Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. *PLoS Pathog.* **5**, e1000709 (2009). doi:10.1371/journal.ppat.1000709 Medline
20. J. Stevens *et al*., Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity. *J. Mol. Biol.* **381**, 1382 (2008). doi:10.1016/j.jmb.2008.04.016 Medline
21. Y. Zhang *et al*., Key molecular factors in hemagglutinin and PB2 contribute to efficient transmission of the 2009 H1N1 pandemic influenza virus. *J. Virol.* **86**, 9666 (2012). doi:10.1128/JVI.00958-12 Medline
22. E. Hoffmann, G. Neumann, Y. Kawaoka, G. Hobom, R. G. Webster, A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6108 (2000). doi:10.1073/pnas.100133697 Medline
23. G. Neumann *et al*., Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9345 (1999). doi:10.1073/pnas.96.16.9345 Medline
24. S. Pleschka *et al*., A plasmid-based reverse genetics system for influenza A virus. *J. Virol.* **70**, 4188 (1996). Medline
25. L. M. Chen, C. T. Davis, H. Zhou, N. J. Cox, R. O. Donis, Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. *PLoS Pathog.* **4**, e1000072 (2008). doi:10.1371/journal.ppat.1000072 Medline
26. C. Li *et al*., Reassortment between avian H5N1 and human H3N2 influenza viruses creates hybrid viruses with substantial virulence. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4687 (2010). doi:10.1073/pnas.0912807107 Medline
27. Y. Li *et al*., Continued evolution of H5N1 influenza viruses in wild birds, domestic poultry, and humans in China from 2004 to 2009. *J. Virol.* **84**, 8389 (2010). doi:10.1128/JVI.00413-10 Medline
28. X. Lu *et al*., A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J. Virol.* **73**, 5903 (1999). Medline

29. L. A. Zitzow *et al*., Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *J. Virol.* **76**, 4420 (2002). doi:10.1128/JVI.76.9.4420-4429.2002 Medline
30. G. Gabriel *et al*., The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 18590 (2005). doi:10.1073/pnas.0507415102 Medline
31. M. Hatta *et al*., Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog.* **3**, e133 (2007). doi:10.1371/journal.ppat.0030133 Medline
32. P. Massin, S. van der Werf, N. Naffakh, Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. *J. Virol.* **75**, 5398 (2001). doi:10.1128/JVI.75.11.5398-5404.2001 Medline
33. J. Steel, A. C. Lowen, S. Mubareka, P. Palese, Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. *PLoS Pathog.* **5**, e1000252 (2009). doi:10.1371/journal.ppat.1000252 Medline
34. A. C. Lowen, S. Mubareka, T. M. Tumpey, A. García-Sastre, P. Palese, The guinea pig as a transmission model for human influenza viruses. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 9988 (2006). doi:10.1073/pnas.0604157103 Medline
35. S. Chutinimitkul *et al*., Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. *J. Virol.* **84**, 11802 (2010). doi:10.1128/JVI.01136-10 Medline
36. C. W. Seibert *et al*., Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models. *J. Virol.* **84**, 11219 (2010). doi:10.1128/JVI.01424-10 Medline
37. T. M. Tumpey *et al*., A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* **315**, 655 (2007). doi:10.1126/science.1136212 Medline
38. Y. Y. Chou *et al*., The M segment of the 2009 new pandemic H1N1 influenza virus is critical for its high transmission efficiency in the guinea pig model. *J. Virol.* **85**, 11235 (2011). doi:10.1128/JVI.05794-11 Medline
39. S. S. Lakdawala *et al*., Eurasian-origin gene segments contribute to the transmissibility, aerosol release, and morphology of the 2009 pandemic H1N1 influenza virus. *PLoS Pathog.* **7**, e1002443 (2011). doi:10.1128/JVI.05794-11 Medline
40. H. L. Yen *et al*., Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 14264 (2011). doi:10.1073/pnas.1111000108 Medline
41. C. A. Russell *et al*., The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* **336**, 1541 (2012). doi:10.1126/science.1222526 Medline

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Supplementary Materials
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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 the DK/35(H5N1) and SC/09(H1N1) are indicated as D and H, respectively. Viral titers are shown as individual titers for both guinea pigs. Sera were collected from the animals three weeks after virus inoculation or exposure; these animals were used for the transmission studies shown in Figs. 2 and 3.

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.

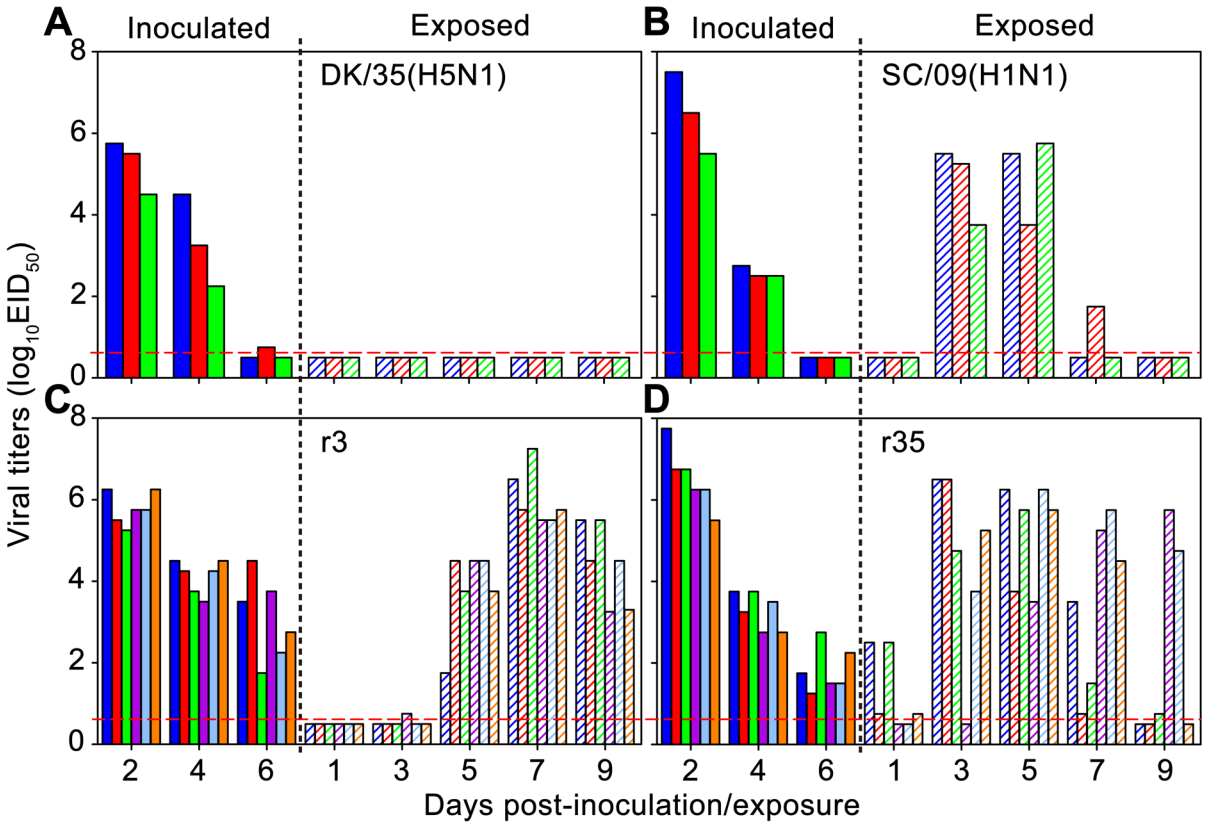

Fig. 1. Respiratory droplet transmission in guinea pigs of the DK/35 (H5N1) virus and its reassortants bearing different RNP complexes. (**A**) DK/35(H5N1); (**B**) SC/09 (H1N1); (**C**) r3; (**D**) r35. Data for the 13 viruses that did not transmit are presented in fig. S3, A to M. Each color bar represents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

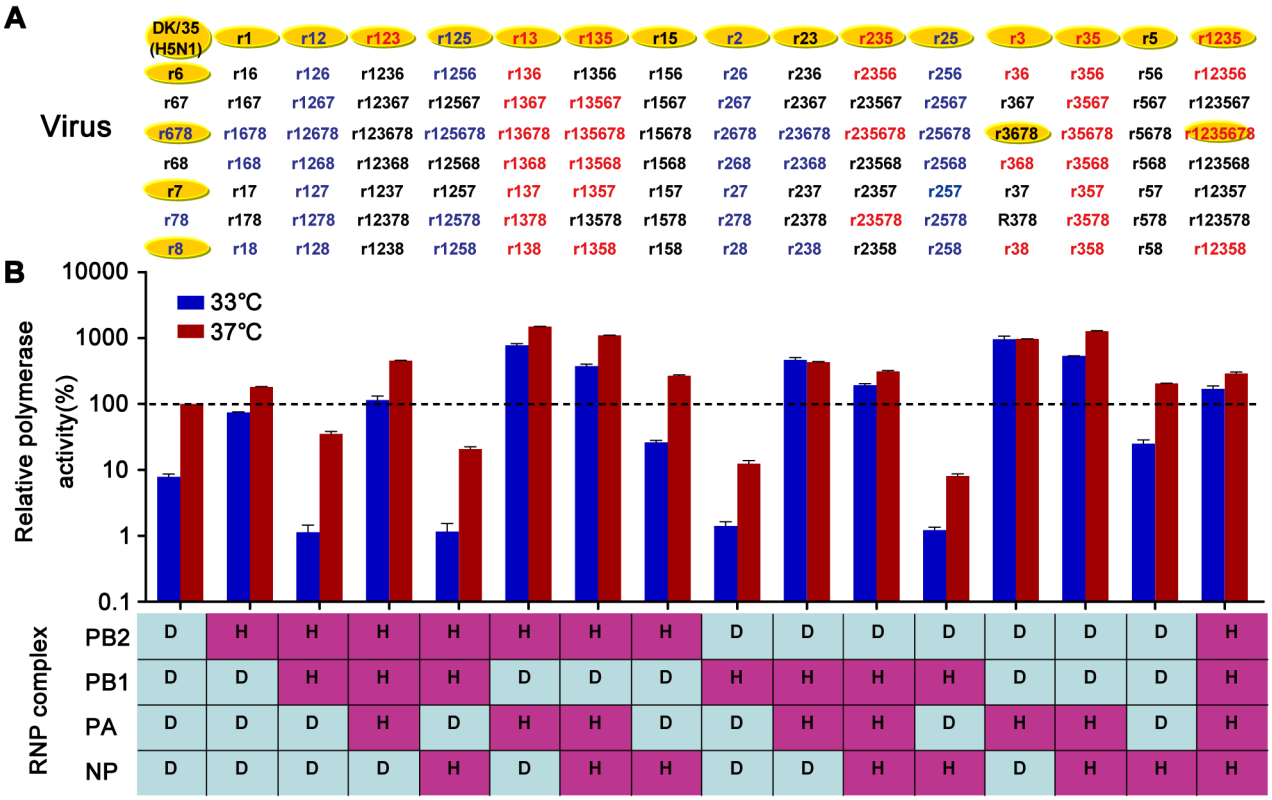

Fig. 2. (**A**) **Pathogenicity of DK/35(H5N1) and SC/09(H1N1) reassortants.** The RNP combinations are shown in the corresponding positions of Fig. 2B. The colors of the viruses reflect their virulence in mice compared with DK/35(H5N1) as reported in tables S1 to S3. Red: more pathogenic than DK/35(H5N1); Blue: less pathogenic than DK/35(H5N1); Black: similar to DK/35(H5N1). Viruses highlighted in yellow were tested for RD 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 the mean ± 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 shows the parent subtype origin of the RNP complex. Segments derived from DK/35(H5N1) are in Cambridge blue with the letter D, and those derived from SC/09 virus are in purple with the letter H.

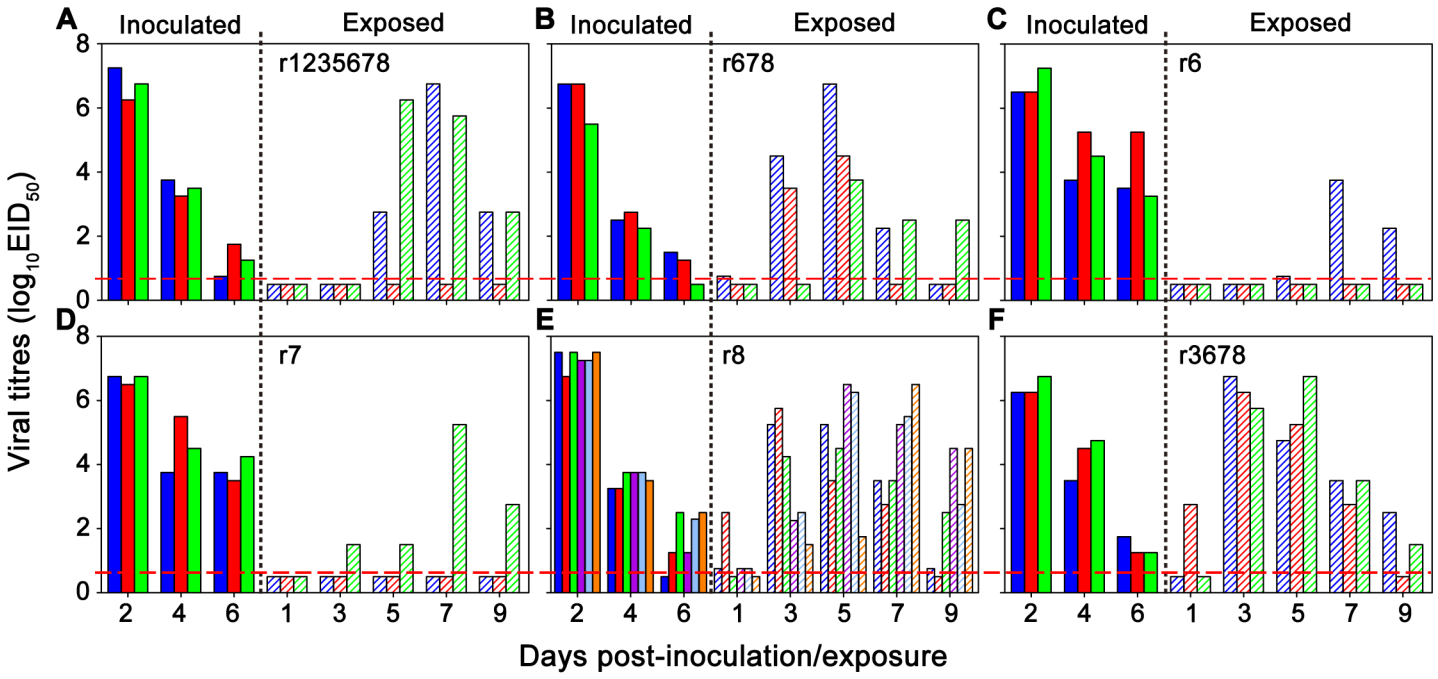

Fig. 3. Contributions of the NA, M, and NS genes of SC/09 (H1N1) to respiratory droplet transmission of H5N1 reassortants in guinea pigs. (**A**) r1235678; (**B**) r678; (**C**) r6; (**D**) r7; (**E**) r8 (data from two experiments); (**F**) r3678. Each color bar represents the virus titer from an individual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

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

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