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*J. Virol.* 2012, 86(18):9666. DOI: 10.1128/JVI.00958-12.  
Published Ahead of Print 27 June 2012.

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# Key Molecular Factors in Hemagglutinin and PB2 Contribute to Efficient Transmission of the 2009 H1N1 Pandemic Influenza Virus

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**Animal influenza viruses pose a clear threat to public health. Transmissibility among humans is a prerequisite for a novel influenza virus to cause a human pandemic. A novel reassortant swine influenza virus acquired sustained human-to-human transmissibility and caused the 2009 influenza pandemic. However, the molecular aspects of influenza virus transmission remain poorly understood. Here, we show that an amino acid in hemagglutinin (HA) is important for the 2009 H1N1 influenza pandemic virus (2009/H1N1) to bind to human virus receptors and confer respiratory droplet transmissibility in mammals. We found that the change from glutamine (Q) to arginine (R) at position 226 of HA, which causes a switch in receptor-binding preference from human  $\alpha$ -2,6 to avian  $\alpha$ -2,3 sialic acid, resulted in a virus incapable of respiratory droplet transmission in guinea pigs and reduced the virus's ability to replicate in the lungs of ferrets. The change from alanine (A) to threonine (T) at position 271 of PB2 also abolished the virus's respiratory droplet transmission in guinea pigs, and this mutation, together with the HA Q226R mutation, abolished the virus's respiratory droplet transmission in ferrets. Furthermore, we found that amino acid 271A of PB2 plays a key role in virus acquisition of the mutation at position 226 of HA that confers human receptor recognition. Our results highlight the importance of both the PB2 and HA genes on the adaptation and transmission of influenza viruses in humans and provide important insights for monitoring and evaluating the pandemic potential of field influenza viruses.**

Sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes of influenza virus have been isolated from aquatic birds, and it is from these viruses that new subtypes of influenza A virus enter the human population. In the last 2 decades, avian influenza viruses of three subtypes—H5, H7, and H9—have caused human infections (7, 32, 37, 39, 48), posing a substantial pandemic threat. Pandemic preparedness plans have been implemented worldwide to limit the impact of influenza pandemics. However, a major obstacle to improving influenza pandemic preparedness is our lack of knowledge regarding what makes an influenza virus transmissible in humans.

The transmissibility of influenza viruses can be affected by the virus, environmental factors, and host factors. The binding of influenza viruses to their target cells is mediated by viral HA, which recognizes cell surface glycoconjugates containing terminal sialic acid (Sia) residues. The affinity of the HA protein for Sia linked to galactose by  $\alpha$ -2,6 linkages ( $\alpha$ -2,6 glycan) is essential for the transmission of the 1918 H1N1 influenza virus between ferrets (38) and for that of H5N1 virus between guinea pigs (9). The viral polymerase complex is also involved in determining viral host range, replication, and pathogenicity (8, 12, 13, 20, 34) and plays a role in transmission (9, 35, 41). Cold and dry environmental conditions favor the transmission of human influenza virus in guinea pigs (21, 22). Under experimental conditions, avian influenza viruses do not replicate efficiently in humans (2), and human viruses do not replicate efficiently in ducks (27). These findings, together with the fact that the current H5N1 and H9N2 viruses transmit efficiently in poultry but not easily among humans, suggest that host factors are also important for influenza virus transmission.

In the spring of 2009, a novel triple-reassortant H1N1 virus (2009/H1N1) emerged in the human population in the United States and Mexico (4) and then quickly spread worldwide, leading the World Health Organization to declare a pandemic (44). This 2009/H1N1 virus bears the predominant feature of efficient transmissibility among humans and increased virulence, as shown in animal models (16, 23, 26) and in some humans (40) relative to the seasonal H1N1 influenza viruses (11). Several studies have determined the virulence factors for the 2009/H1N1 pandemic influenza virus (46, 49, 51); however, the viral factors contributing to the efficient transmission of this virus in humans remain largely unknown. In the present study, we explore the genetic requirements for the efficient transmission of the 2009/H1N1 virus by generating a series of mutants in the SC/09 virus, A/Sichuan/1/2009 (SC/09), background and evaluating their aerosol transmission in guinea pigs and ferrets. Our results indicated that two amino acids in the HA and PB2 are key determinants for efficient aerosol transmission of the 2009/H1N1 virus, and emphasized the effect of coevolution of the PB2 and HA genes on the adaptation and transmission of influenza viruses in humans.

Received 18 April 2012 Accepted 20 June 2012

Published ahead of print 27 June 2012

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doi:10.1128/JVI.00958-12

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## MATERIALS AND METHODS

**Ethics statements.** The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (approval numbers BRDW-TS-09 for guinea pigs and BRDW-XD-09 for ferrets).

**Facility.** Studies with the 2009 pandemic influenza viruses were conducted in a biosecurity level 2+ laboratory approved by the Chinese Ministry of Agriculture. All animal studies were approved by the Review Board of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

**Viruses.** H1N1 virus A/Sichuan/1/2009 (SC/09) was isolated from the first human case of the 2009 influenza pandemic in China. The mutants HA/226R, PB2/271T, PB2/590G+591Q, and HA/226R+PB2/271T were generated by using reverse genetics (15, 28, 33). A site-directed mutagenesis kit (Invitrogen) was used to create specific mutations in the HA and PB2 genes (primer sequences available upon request). Virus stock was propagated in specific-pathogen-free chicken eggs.

**Receptor-binding analysis using hemagglutination assays.** Hemagglutination assays using resialyated chicken red blood cells (cRBCs) were performed as described previously (30, 31) with minor modifications. cRBCs were enzymatically desialyated with *Vibrio cholerae* neuraminidase (VCNA; Roche), and then resialylated by using either  $\alpha$ 2-6-*N*-sialyltransferase or  $\alpha$ 2-3-*N*-sialyltransferase (Calbiochem) and CMP-sialic acid (Sigma).

**Dose-dependent direct binding to different glycans of H1N1 influenza viruses.** Receptor specificity was analyzed by using a direct solid-phase assay. Briefly, a streptavidin-coated, high-binding capacity 96-well plate (Pierce) was rinsed with phosphate-buffered saline (PBS). Then, 50  $\mu$ l of a 2.4 mM solution of biotinylated glycans in PBS was added to each well, and the plate was incubated overnight at 4°C. Two  $\alpha$ -2,6 glycans (6'SLN, Neu5Aca2-6Galb1-4GlcNAcb-SpNH-LC-LC-biotin; 6'S-Di-LN, Neu5Aca2-6[Galb1-4GlcNAcb1-3]<sub>2</sub>b-SpNH-LC-LC-biotin) and two  $\alpha$ -2,3 glycans (3'SLN, Neu5Aca2-3Galb1-4GlcNAcb-SpNH-LC-LC-biotin; 3'S-Di-LN, Neu5Aca2-3[Galb1-4GlcNAcb1-3]<sub>2</sub>b-SpNH-LC-LC-biotin), kindly provided by the Consortium for Functional Glycomics (Scripps Research Institute, Department of Molecular Biology, La Jolla, CA), were tested. The plate was subsequently washed with cold PBS to remove any excess glycans. Virus was inactivated by adding 0.1% (vol/vol)  $\beta$ -propiolactone and incubating for 3 days at 4°C. Virus binding to the glycan-coated wells was assessed by adding serially diluted virus in PBS containing 1% bovine serum albumin to each well, followed by an overnight incubation at 4°C. After being rinsed with PBS containing 0.05% Tween 20 to remove excess virus, the wells were incubated with chicken antisera against SC/09 virus for 5 h at 4°C. The wells were then extensively washed and subsequently incubated with horseradish peroxidase-linked goat anti-chicken antibody (Sigma-Aldrich) for 2 h at 4°C. The wells were washed again with PBS containing 0.05% Tween 20 and incubated with *O*-phenylenediamine in substrate solution containing 0.01% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature. The reaction was stopped by adding 50  $\mu$ l of 1 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance was determined at 492 nm.

**Animal studies.** Hartley strain female guinea pigs weighing 300 to 350 g (Vital River Laboratories, Beijing, China) and 4-month-old female ferrets (Wuxi Cay Ferret Farm, Jiangsu, China) serologically negative for influenza viruses were used in these studies. Ketamine (20 mg/kg) and xylazine (1 mg/kg) were used to anesthetize animals by intramuscular injection.

To investigate the replication of influenza viruses, groups of three guinea pigs or two ferrets were anesthetized and inoculated i.n. with 10<sup>6</sup> 50% egg infectious doses (EID<sub>50</sub>) of test virus in a 300- $\mu$ l volume (150  $\mu$ l per nostril) for guinea pigs or in a 500- $\mu$ l volume (250  $\mu$ l per nostril) for ferrets. The animals were euthanized on day 3 postinoculation (p.i.), and

nasal washes, tonsils (ferrets), and lungs were collected for virus titration in eggs.

For the respiratory droplet transmission studies, groups of five guinea pigs or three ferrets were inoculated intranasally (i.n.) with 10<sup>6</sup> EID<sub>50</sub> of test virus and housed in a cage placed inside an isolator. After 24 h, five naive guinea pigs or three naive ferrets were placed in an adjacent cage (4 cm away), separated by a double-layered net divider. Nasal washes were collected at 2-day intervals, beginning on day 2 p.i. (1 day postexposure [p.e.]) and titrated in eggs; sera were collected from all animals on day 21 p.i. for hemagglutinin inhibition (HI) antibody detection. The ambient conditions for these studies were set at 20 to 22°C and 30 to 40% relative humidity. The airflow in the isolator was horizontal with a speed of 0.1 m/s; the airflow direction was from the inoculated animals to the exposed animals.

**Histological study.** Lungs were collected from ferrets on day 3 after virus or PBS inoculation and were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4- $\mu$ m sections. The sections were stained with hematoxylin-eosin (H&E) or used in immunohistochemical (IHC) assays with mouse antiserum against the SC/09 virus and a goat anti-mouse IgG as the secondary antibody (Millipore, Temecula, CA).

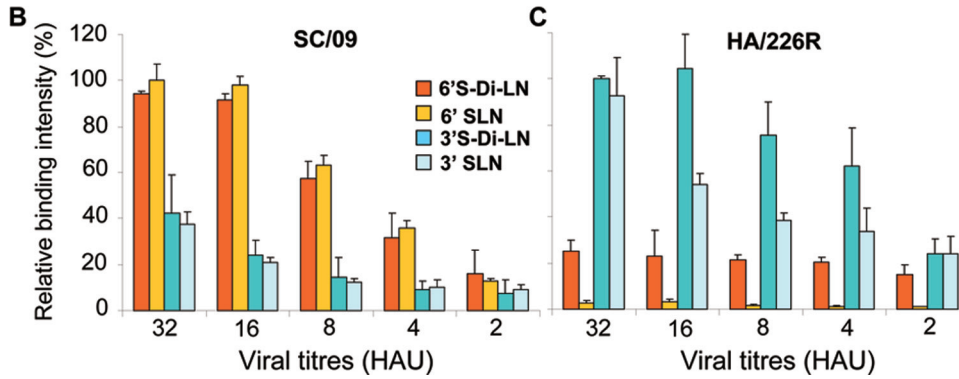
**Antibody detection.** Sera were treated with *Vibrio cholerae* (Denka-Seiken) receptor-destroying enzyme before being tested for the presence of HI antibody with 0.5% (vol/vol) chicken erythrocytes. The antigen used was the wild-type SC/09 virus.

## RESULTS

**The amino acid at position 226 of HA protein is important for the receptor binding preference of SC/09 virus.** The binding of influenza viruses to their target cells is mediated by viral HA, which recognizes cell surface glycoconjugates containing terminal sialic acid residues. Previous studies have shown that two amino acids at positions 190 and 225 (H3 numbering) in the 1918 pandemic influenza virus HA determine this virus's receptor binding specificity and are important for its transmissibility (10, 38, 41). These two amino acids are highly conserved in the H1N1 swine and human influenza viruses. The amino acid at position 226 (H3 numbering) of HA plays a key role in receptor binding in H2-, H3-, H4-, H5-, and H9-subtype influenza viruses (1, 9, 24, 36, 43). Two different amino acids, arginine (R) and glutamine (Q), have been detected at this position of the HA protein of H1N1 swine and human influenza viruses, and most 2009/H1N1 influenza viruses have 226Q in their HA proteins. To investigate the contribution of the amino acid at position 226 of HA to the receptor specificity and transmission of the 2009/H1N1 virus, we generated a mutant, HA/226R, by introducing the Q-to-R mutation at position 226 (Q226R) in the HA protein of the A/Sichuan/1/2009 (H1N1) (SC/09) virus by using reverse genetics. We examined the receptor-binding specificity of SC/09 and HA/226R by using hemagglutination assays with resialyated cRBCs. SC/09 agglutinated only cRBCs resialyated with  $\alpha$ -2,6-glycans, whereas HA/226R agglutinated only cRBCs resialyated with  $\alpha$ -2,3-glycans (Fig. 1A). We also tested the binding of the HAs of the SC/09 and HA/226R viruses to different glycans by using a dose-dependent direct binding assay. The SC/09 virus preferentially bound to both short and long  $\alpha$ -2,6-linked glycans (6'SLN and 6'S-Di-LN), and bound to  $\alpha$ -2,3-linked glycans (3'SLN and 3'S-Di-LN) to a substantially lesser degree (Fig. 1B). In contrast, HA/226R exhibited preferential binding to both short and long  $\alpha$ -2,3-linked glycans, and some binding to long  $\alpha$ -2,6-linked glycans (Fig. 1C); its binding to short  $\alpha$ -2,6-linked glycans was very limited (Fig. 1C). These results indicate that 226Q in HA plays an important role in 2009/H1N1 virus binding to  $\alpha$ -2,6 receptors.

**A** Haemagglutination assays of H1N1 influenza viruses using chicken red blood cells (cRBCs) with different treatments.

Virus	Amino acid at position 226 of HA	Haemagglutination titre		
		$\alpha$ 2,3 cRBCs	$\alpha$ 2,6 cRBCs	cRBCs
SC/09	Q	<2	64	128
HA/226R	R	32	<2	64



**FIG 1** Receptor binding specificity of SC/09 and HA/226R. (A) Hemagglutination assay of two viruses with 0.5% cRBCs treated as follows: cRBCs, untreated;  $\alpha$ -2,3 cRBC, *Vibrio cholerae* neuraminidase (VCNA) treated and resialylated with  $\alpha$ -2,3 glycans;  $\alpha$ -2,6 cRBC, VCNA treated and resialylated with  $\alpha$ -2,6 glycans. (B) Glycan binding specificity of SC/09. (C) Glycan binding specificity of HA/226R.

**The amino acid at position 226 of HA affects the replication of the SC/09 virus in ferrets.** We then evaluated the replication of SC/09 and HA/226R in guinea pigs and ferrets. Groups of three guinea pigs or two ferrets were i.n. inoculated with  $10^6$  EID<sub>50</sub> of each virus, and nasal washes and lung tissue from the guinea pigs, or nasal wash, lung lobes, and tonsils from the ferrets were collected from the euthanized animals on day 3 p.i. for viral titration. The SC/09 and HA/226R viruses replicated equally well in the respiratory tract of guinea pigs (Table 1); however, their replication in ferrets was different. High titers of virus were detected in all of the organs tested in the SC/09-infected ferrets (Table 2). However, HA/226R replication was only detected in one lung lobe of one ferret, although virus was detected in the nasal washes and tonsils of both ferrets infected with this virus, and the titers in the nasal washes were >10-fold higher than those of the SC/09 virus-inoculated ferrets (Table 2). The HA/226R-inoculated ferrets had less extensive bronchopneumonia than that experienced by the SC/09-inoculated ferrets (Fig. 2). Previous studies have shown that the  $\alpha$ -2,6 receptor predominated in the lung of ferrets (42,

45). The reduced ability of the HA/226R virus to replicate in the lungs of ferrets thus correlated with the receptor distribution in the respiratory tract of ferrets and is in agreement with similar observations with other H1N1 influenza viruses (45).

**The Q226R mutation in HA abolishes the respiratory droplet transmission of the SC/09 virus in guinea pigs.** We then tested the respiratory droplet transmission of SC/09 and HA/226R in guinea pigs. As shown in Fig. 3A, in the SC/09-inoculated group, virus was detected in the nasal washes of all five inoculated guinea pigs on days 2 and 4 and was also detected in the nasal washes of all five exposed animals between days 5 and 7 postexposure (p.e.). In the HA/226R-inoculated group, virus was detected in the nasal washes of all five inoculated guinea pigs on days 2 and 4 p.i., with peak titers >10-fold lower than those in the SC/09 virus-inoculated animals but not in any of the exposed guinea pigs (Fig. 3B). Seroconversion occurred in all inoculated groups and in the SC/09-exposed group but not in the HA/226R-exposed group (Table 1).

**The Q226R mutation in HA impairs the respiratory droplet transmission of the SC/09 virus in ferrets.** We also tested the

**TABLE 1** Replication and seroconversion of guinea pigs inoculated with or exposed to different H1N1 influenza viruses

Virus	Mean virus titer (log <sub>10</sub> EID <sub>50</sub> ) $\pm$ SD <sup>a</sup>		Seroconversion (positive/total) <sup>b</sup>		Respiratory droplet transmission
	Nasal wash	Lung	Inoculated (HI titer)	Exposed (HI titer)	
SC/09	5.2 $\pm$ 0.4	3.6 $\pm$ 0.6	5/5 (40–2,560)	5/5 (40–640)	Efficient
HA/226R	5.2 $\pm$ 0.4	3.2 $\pm$ 1.7	5/5 (320)	0/5 (<10)	None
PB2/271T	5.3 $\pm$ 0.4	3.3 $\pm$ 1.1	5/5 (80–160)	0/5 (<10)	None
PB2/590G+591Q	4.9 $\pm$ 0.3	3.8 $\pm$ 0.3	5/5 (20–160)	5/5 (40–320)	Efficient

<sup>a</sup> Groups of three guinea pigs were i.n. inoculated with  $10^6$  EID<sub>50</sub> of each virus and euthanized on day 3 p.i.; nasal washes and the indicated organs were collected for virus titration in eggs.

<sup>b</sup> Sera were collected from the animals 3 weeks after the virus inoculation or exposure; these animals are the same ones that were used for the transmission studies shown in Fig. 3 and 4.

TABLE 2 Replication and seroconversion of ferrets inoculated with or exposed to different H1N1 influenza viruses

Virus	Viral titer ( $\log_{10}$ EID <sub>50</sub> ) <sup>a</sup>								Seroconversion (positive/total) <sup>b</sup>		Respiratory droplet transmission
	Left lung				Right lung				Inoculated (HI titer)	Exposed (HI titer)	
	Nasal wash	Lower	Middle	Upper	Lower	Middle	Upper	Tonsil			
SC/09	5.3, 5.8	6.0, 8.3	6.0, 6.3	6.3, 3.8	6.3, 6.8	6.5, 4.8	4.5, 5.5	3.3, 2.8	3/3 (1,280)	3/3 (1,280–2,560)	Efficient
HA/226R	7.0, 6.8	<, <	<, <	<, <	2.5, <	<, <	<, <	2.5, 1.3	3/3 (1,280–2,560)	3/3 (2,560)	Less efficient
PB2/271T	4.3, 6.3	4.5, <	5.5, <	5.3, <	5.3, 5.5	4.8, <	5.5, 3.5	3.5, 3.8	3/3 (1,280)	3/3 (1,280–5,120)	Efficient
HA/226R+PB2/271T	5.3, 7.0	<, <	<, <	<, <	2.8, <	<, <	<, <	2.3, 0.8	3/3 (1,280)	0/3 (<10)	None

<sup>a</sup> Groups of two ferrets were i.n. inoculated with  $10^6$  EID<sub>50</sub> of each virus and euthanized on day 3 p.i.; nasal washes and the indicated organs were collected for virus titration in eggs. The individual titers of each ferret are presented. <, not detected in 0.1 ml of the undiluted nasal washes.

<sup>b</sup> Sera were collected from the animals 3 weeks after the virus inoculation or exposure; these animals are the same ones that were used for the transmission studies shown in Fig. 3 and 4.

respiratory droplet transmission of SC/09 and HA/226R in ferrets. In both the SC/09- and the HA/226R-inoculated groups, virus was detected in the nasal washes of the inoculated ferrets between days 2 and 6 p.i. In the exposed animals, SC/09 was detected as early as day 1 p.e. in one of the ferrets, and until day 9 in another animal, with peak titers on day 3 for all three animals ranging from 7.2 to 7.3  $\log_{10}$  EID<sub>50</sub>. However, HA/226R was first detected on day 5 p.e., with peak titers in the three animals ranging from 3.8 to 4.3  $\log_{10}$  EID<sub>50</sub>, which were >1,000-fold lower than the peak titer in the SC/09-exposed animals (Fig. 4A and B). Seroconversion occurred in all inoculated and exposed animals (Table 2). These results suggest that 226R in HA eliminates the respiratory droplet

transmission of the SC/09 virus in guinea pigs and partially impairs the transmission of the SC/09 virus in ferrets.

**The A271T mutation in PB2 eliminates the respiratory droplet transmission of the SC/09 virus in guinea pigs, but not in ferrets.** Two amino acids in PB2, the position 627 lysine (627K) and the position 701 asparagine (701N), make significant contributions to the adaptation and transmission of influenza viruses in guinea pigs (8, 9, 12, 20, 35). However, the 2009/H1N1 influenza viruses do not bear either of these amino acids in their PB2 gene. Recently, it was reported that the amino acid alanine (A) at position 271 of PB2 enhances the polymerase activity of influenza A viruses in mammalian host cells (3) and that serine (S) at position

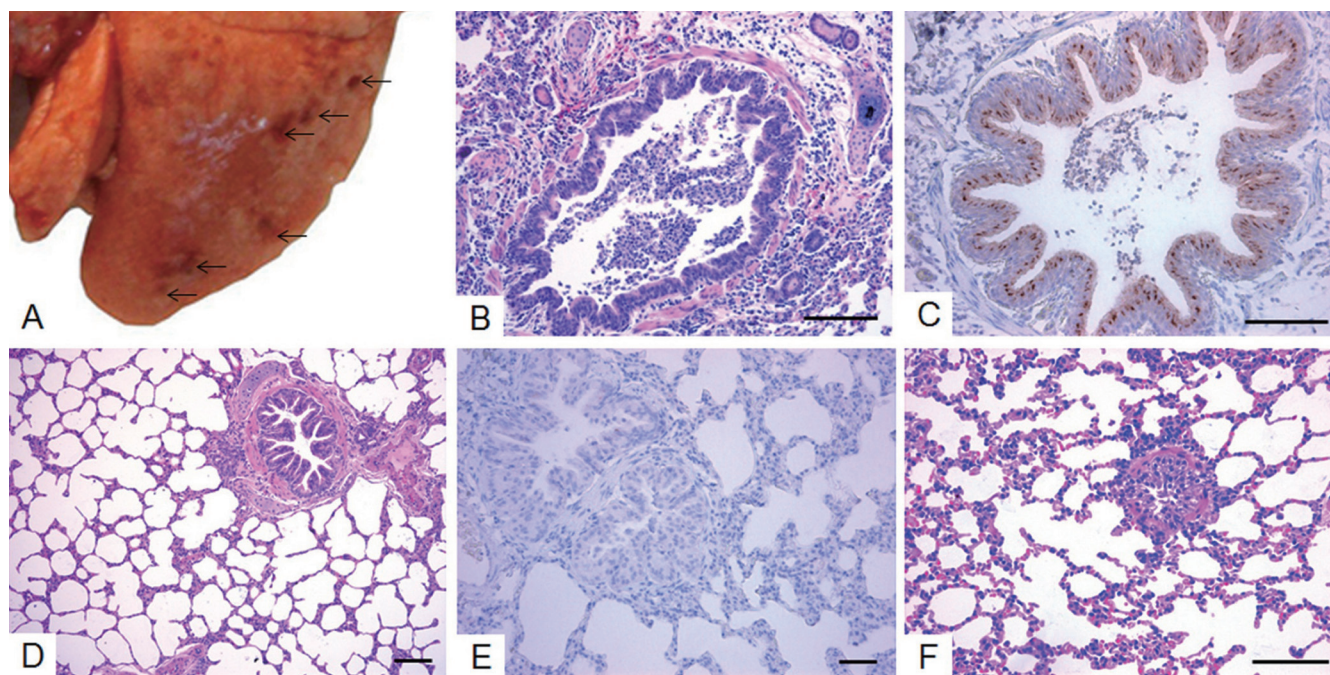
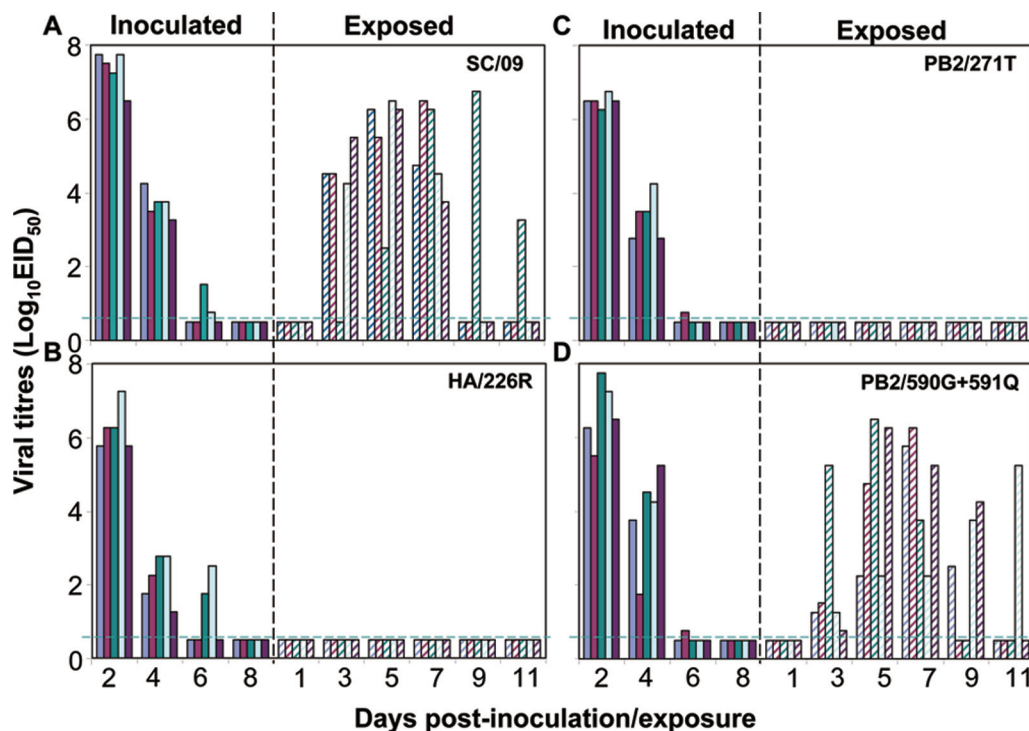


FIG 2 Lung lesions of ferrets caused by the SC/09 and HA/226R viruses. (A) In the SC/09 virus-inoculated ferrets, macroscopic lesions in the lungs and focal discolorations (arrowed) were observed in all of the lobes. (B) Histologically, the consolidated area in the SC/09 virus-infected ferrets was consistent with the prominent features of bronchioalveolar pneumonia with massive recruitment of lymphocytes into the lumen and surrounding alveoli, sloughing of respiratory epithelium in the peribronchiolar areas, and submucosal edema of the bronchiolar wall (H&E staining; scale bar, 100  $\mu$ m). (C) Viral antigen was detected in the bronchiolar epithelial cells (IHC; scale bar, 100  $\mu$ m). (D and E) However, in HA/226R virus-inoculated animals, no obvious histopathological changes were seen in the lung tissues (D) (H&E staining; scale bar, 100  $\mu$ m) compared to the PBS-inoculated animals (E) (H&E staining; scale bar, 100  $\mu$ m). (F) Viral antigens were not detected (IHC; scale bar, 100  $\mu$ m).



**FIG 3** Transmission of the 2009 pandemic H1N1 virus SC/09 and its mutants in guinea pigs. Groups of five guinea pigs were inoculated i.n. with  $10^6$  EID<sub>50</sub> of test virus and, 24 h after the inoculation, five naive guinea pigs were placed in an adjacent cage. Nasal washes were collected every 2 days from all animals beginning 2 days p.i. (1 day p.e.) for the detection of virus shedding. Each color bar represents the virus titer from an individual animal. (A) SC/09 virus; (B) HA/226R virus; (C) PB2/271T virus; (D) PB2/590G+591Q virus. The dashed blue lines in these panels indicate the lower limit of detection.

590 and arginine (R) at position 591 together (25) or 591R alone (46) are important for 2009/H1N1 efficient replication in human cells and mammalian hosts and in mammalian adaptation. We therefore tested whether changes to these amino acids in PB2 affect the transmission of the 2009/H1N1 virus.

We generated two mutants, PB2/271T and PB2/590G+591Q, by introducing the single mutation A271T and the double mutation S590G R591Q, respectively, into the PB2 of the SC/09 virus. As shown in Fig. 3C, the replication of these two mutant viruses in guinea pigs was comparable to that of the wild-type SC/09 virus. In the PB2/271T-inoculated group, virus was detected in the nasal washes of all five inoculated guinea pigs between days 2 and 4 p.i. and in one animal on day 6 p.i. but was not detected in the nasal washes of the exposed guinea pigs (Fig. 3C). In the PB2/590G+591Q-inoculated group, virus was detected in the nasal washes of all five inoculated guinea pigs between days 2 and 4 p.i. and in one animal on day 6 p.i.; virus was also detected in the nasal washes of the exposed animals between days 3 and 11 p.e. (Fig. 3D). These results indicate that 271A in PB2 is necessary for the transmissibility of the SC/09 virus in guinea pigs and that the amino acids 590S and 591R do not contribute to the transmission phenotype of the 2009/H1N1 virus in this animal model.

We next tested the replication and transmission of the PB2/271T virus in ferrets. The replication of PB2/271T in ferrets was partially attenuated compared to that of SC/09, and although high titers of virus were detected in all of the organs of one of the ferrets, virus was only detected in part of the lung samples of another ferret (Table 2). In the transmission study, virus was detected in the nasal washes of all three inoculated ferrets between days 2 and

4 p.i. and was also detected in the nasal washes of the exposed ferrets between days 3 and 9 p.e. (Fig. 4C). Seroconversion occurred in all inoculated and exposed animals (Table 2).

**The HA Q226R mutation and the PB2 A271T mutation together abolish the respiratory droplet transmission of the SC/09 virus in ferrets.** The studies described above indicate that two amino acids, 226Q in the HA gene and 271A in the PB2 gene, are important for the transmission of the 2009/H1N1 virus among guinea pigs but that their contribution to the transmissibility of the 2009/H1N1 virus in ferrets appears to be limited. We next generated a mutant, HA/226R+PB2/271T, by introducing both the Q226R change in HA and the A271T change in PB2 into the SC/09 virus and tested this mutant's replication and transmission in ferrets. As shown in Table 2, virus was detected in the nasal washes and tonsils of both ferrets inoculated, but viral replication in the lungs of ferrets was largely restricted, being detected in only one lung lobe of one ferret (Table 2).

We then tested the transmissibility of the HA/226R+PB2/271T virus in ferrets. The virus was detected in the nasal washes of all three inoculated ferrets between days 2 and 4 p.i. but was not detected in any of the exposed ferrets (Fig. 4D). Moreover, seroconversion was not detected in any of the exposed animals (Table 2). These results indicate that the double mutation of HA Q226R and PB2 A271T completely eliminate the transmission of SC/09 in ferrets, suggesting that the amino acids at these positions in HA and PB2 both make significant contributions to the efficient transmission phenotype of the 2009/H1N1 virus.

We then sought to determine whether the introduced mutations were stably maintained during viral replication in animals;

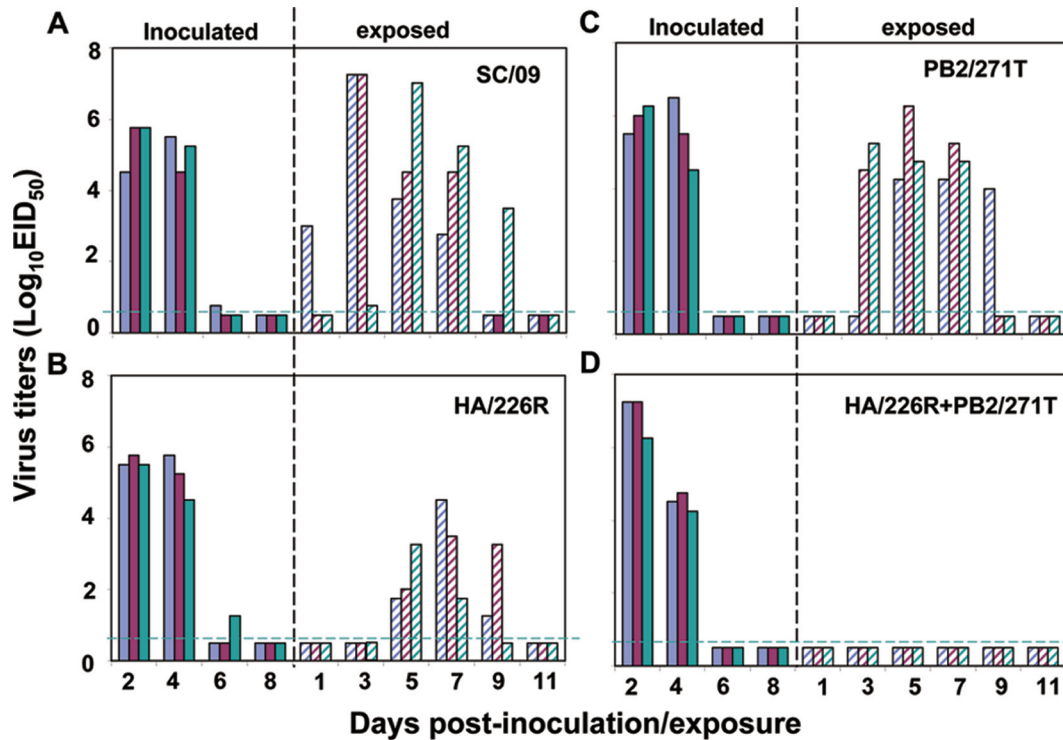


FIG 4 Transmission of the 2009 pandemic H1N1 virus SC/09 and its mutants in ferrets. Groups of three ferrets were inoculated i.n. with  $10^6$  EID<sub>50</sub> of test virus and, 24 h after the inoculation, three naive ferrets were placed in an adjacent cage. Nasal washes were collected every 2 days from all animals beginning 2 days p.i. (1 day p.e.) for the detection of virus shedding. Each bar represents the virus titer from an individual animal. (A) SC/09 virus; (B) HA/226R virus; (C) PB2/271T virus; (D) HA/226R+PB2271T virus. The dashed blue lines in these panels indicate the lower limit of detection.

we extracted viral RNA from the nasal washes collected from virus-inoculated and virus-exposed ferrets and guinea pigs. The PB2 and HA were amplified and cloned into T vectors. Six to ten molecular clones from each sample were randomly selected and sequenced. We found that the PB2 mutations were stably maintained in all of the viruses tested, and no changes were detected at positions 627 or 701 of PB2 (data not shown). However, the 226R

mutation in the HA of HA/226R was not stably maintained during virus replication in animals. In guinea pigs, the R226Q reversion in the HA protein was found in 18 of 30 virus clones recovered on day 4 p.i. and in all of the 12 clones recovered on day 6 p.i. (Table 3). In ferrets, the R226Q reversion in HA was detected in 26 of 30 clones recovered on day 4 p.i. and in all 10 clones recovered on day 6 p.i. This mutation was also detected in all of the virus clones

TABLE 3 Amino acid at position 226 of the HA gene of viruses recovered from the nasal washes of guinea pigs and ferrets<sup>a</sup>

Animal	Sample origin	Virus recovered from animals on different days postinoculation (p.i.) or postexposure (p.e.) <sup>a</sup>			
		Day 2 p.i.	Day 4 p.i.	Day 6 p.i.	Day 5 p.e. <sup>b</sup>
Guinea pig	HA/226R-Guinea pig 1	R (6/6)	Q (6/6)	NA	NA
	HA/226R-Guinea pig 2	R (6/6)	R (3/6) + Q (2/6) + L (1/6)	NA	NA
	HA/226R-Guinea pig 3	R (6/6)	R (3/6) + Q (3/6)	Q (6/6)	NA
	HA/226R-Guinea pig 4	R (6/6)	Q (5/6) + L (1/6)	Q (6/6)	NA
	HA/226R-Guinea pig 5	R (6/6)	R (4/6) + Q (2/6)	NA	NA
Ferret	HA/226R-Ferret 1	R (10/10)	Q (8/10) + L (2/10)	NA	Q (10/10)
	HA/226R-Ferret 2	R (10/10)	Q (9/10) + L (1/10)	NA	Q (10/10)
	HA/226R-Ferret 3	R (10/10)	Q (9/10) + L (1/10)	Q (10/10)	Q (10/10)
	HA226R+PB2/271T-Ferret 1	R (10/10)	R (9/10) + L (1/10)	NA	NA
	HA226R+PB2/271T-Ferret 2	R (10/10)	R (9/10) + L (1/10)	NA	NA
	HA226R+PB2/271T-Ferret 3	R (10/10)	R (10/10)	NA	NA

<sup>a</sup> We extracted viral RNA from the nasal washes collected from virus-inoculated and virus-exposed ferrets and guinea pigs. The PB2 and HA were amplified and cloned into T vectors. Six to ten molecular clones from each sample were randomly selected and sequenced. No changes in PB2 were detected. For values in parentheses, the number on the left of the slash shows the number of clones bearing the indicated amino acid at position 226 of HA, and the number on the right of the slash shows the total number of clones sequenced. NA, not applicable.

<sup>b</sup> All of the samples collected from the exposed animals were sequenced, and the R226Q reversion in HA was detected in all of the clones; only the data from day 5 p.e. are presented in the table.

recovered from the exposed animals (Table 3). We also sequenced 30 virus clones (6 from each organ) recovered from nasal washes, lungs, and tonsils of the two HA/226R virus-inoculated ferrets that were euthanized on day 3 p.i. (Table 2) and detected this R226Q reversion in the HA of 6 of 18 of the clones (1 from the nasal washes, 3 from the lung samples, and 2 from the tonsil sample) that were recovered from one of the ferret. However, we did not detect the R226Q reversion in any of the 12 clones that were recovered from the other ferret (data not shown). It is interesting that the R226Q mutation was not detected in any viruses recovered from the HA/226R+PB2/271T-inoculated ferrets, although two clones (6.7%) had the R-to-L mutation at this position (Table 3). These results indicate that the transmission of the HA/226R virus may have occurred after it acquired the R226Q mutation in its HA protein.

## DISCUSSION

The 2009/H1N1 virus exhibits the predominant feature of efficient transmissibility among humans and increased virulence, as shown in animal models (16, 23, 26). We found that the amino acid Q at position 226 of HA is important for the 2009/H1N1 virus to bind to  $\alpha$ -2,6 glycans and to replicate efficiently in the lungs of ferrets and that it is critical for the aerosol transmission of the virus in guinea pigs. The Q226R mutation of HA and the A271T mutation of PB2 together abolished the aerosol transmission of the virus in ferrets. The stable maintenance of 226R in the HA gene of the HA/226R+PB2/271T virus provides counterevidence that the amino acid A at position 271 in PB2 plays an important role in the ability of the virus to acquire the R226Q mutation in its HA gene during replication in mammals.

The NA and M genes of the 2009/H1N1 virus promote transmission of other influenza viruses (6, 18, 47), although the precise functional domain(s) or amino acid(s) of their gene products that contribute to the transmission of 2009/H1N1 virus have yet to be defined. Chou et al. (6) reported that reassortant viruses containing the M segment of the 2009/H1N1 virus acquired high transmission efficiency in the guinea pig model and suggested that the M segment plays a critical role in supporting the transmission of the 2009 pandemic virus. Yen et al. (47) reported that the NA gene of the 2009/H1N1 virus improved the aerosol transmission of another swine influenza virus and suggested that the HA-NA balance is the key determinant of the efficient human transmissibility of 2009/H1N1 viruses. Lakdawala et al. (18) reported that the Eurasian-origin NA and M gene segments contribute to the high respiratory droplet transmission of the 2009/H1N1 virus, possibly through increasing its NA activity and altering its pleomorphic phenotype. Our study clearly demonstrates the contribution of both HA and PB2 to the respiratory droplet transmission of the 2009/H1N1 virus. Together, these studies indicate that the efficient transmission of influenza virus results from synergism among multiple viral gene segments.

We used both guinea pig and ferret models in the present study and found that the transmission of influenza viruses differed between the two models. The mutation of A271T in PB2 and Q226R in HA independently abolished the respiratory droplet transmission of the SC/09 virus in guinea pigs, indicating that both 271A in PB2 and 226Q in HA are prerequisites for the efficient respiratory droplet transmission of the 2009/H1N1 virus. This is in agreement with the fact that all of the 2009/H1N1 human isolates to date have 271A in PB2 and >99% of them have 226Q in HA. Although the

TABLE 4 Amino acids at positions 271, 627, and 701 in the PB2 protein of swine H1N1 influenza viruses

Amino acid at position 271 in PB2	Total no. of viruses (lineage)	No. of viruses with amino acid:	
		Lysine (K) at position 627	Asparagine (N) at position 701
Alanine (A)	197 (triple-reassortant and 2009/H1N1)	0	0
	2 (classical)	2	0
	1 (Eurasian avian-like)	0	1
Threonine (T)	254 (classical)	254	0
	120 (Eurasian avian-like)	0	117

PB2/271T mutant transmitted efficiently in ferrets, its transmission in humans is unlikely, since none of the 2009/H1N1 virus isolated from humans contains the 271T in the PB2. If we had only used the ferret model here, the contribution of 271A in PB2 to the transmission of the 2009/H1N1 virus would not have been recognized. Thus, the use of different animal models is important to improve the likelihood of revealing the molecular components that contribute to the transmission of influenza viruses.

Three amino acids—271A, 627K, and 701N—in PB2 were shown to be important for influenza virus transmission in animal models in the present study and in previous studies (9, 35). We compared the amino acids at positions 271, 627, and 701 in the PB2 of 574 H1N1 swine influenza viruses, including 256 classical H1N1 swine influenza viruses, 121 Eurasian avian-like H1N1 swine influenza viruses, and 197 triple-reassortant and 2009/H1N1 swine influenza viruses (Table 4). We found that among 200 H1N1 viruses with 271A in PB2, only two viruses also had 627K and only one had 701N in its PB2 gene; all of the other viruses had 627E and 701D in PB2. However, among the remaining 374 viruses with 271T in their PB2 gene, 254 viruses had 627K, 117 viruses had 701N, and three viruses had 627E and 701D. The lineages of these viruses are listed in Table 4. These findings indicate that the biological functions of these three amino acids in PB2 may be compensatory. We demonstrated here that 271A in PB2 is important to facilitate virus acquisition of  $\alpha$ -2,6 receptor binding property by acquiring the R226Q mutation in HA, which in turn facilitates the ability to transmit in ferrets. Although the underlying mechanism behind how 271A in PB2 facilitates the R226Q mutation in HA during viral replication in animals has yet to be defined, it is clear that mammalian host-adapted mutations in the polymerase help the influenza virus to acquire mutations in other viral proteins to adapt in their mammalian hosts, such as the  $\alpha$ -2,6 receptor binding mutations in HA. Many H5N1 influenza viruses isolated from birds and humans already have 627K or 701N in their PB2 (5, 12, 14, 17, 19, 20). It is of concern that these amino acids may also drive HA mutations that support mammalian host adaptation and transmission when H5N1 viruses replicate in mammalian hosts, such as pigs, which have been infected with H5N1 influenza viruses in several geographical areas (29, 50).

In summary, we identified here key amino acids in the PB2 and HA proteins of the 2009/H1N1 virus that confer efficient respiratory droplet transmission. Our results suggest the importance of both the PB2 and HA genes to the adaptation and transmission of influenza viruses in humans. Our findings provide important insights for monitoring and evaluating the pandemic potential of field influenza viruses.



## ACKNOWLEDGMENTS

We thank Susan Watson for editing the manuscript and the Consortium for Functional Glycomics (Scripps Research Institute, Department of Molecular Biology, La Jolla, CA) for providing the glycans.

This study was supported by the Chinese National Key Basic Research Program (973, 2011CB505000), by the National Natural Science Foundation of China (grant 30825032), by a grant-in-aid for Specially Promoted Research, by the Japan Initiative for Global Research Network on Infectious Diseases from the Ministries of Education, Culture, Sports, Science, and Technology, and by ERATO (Japan Science and Technology Agency).

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