Minimal molecular constraints for respiratory droplet transmission of an avian–human H9N2 influenza A virus

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Pandemic influenza requires interspecies transmission of an influenza virus with a novel hemagglutinin (HA) subtytpe that can adapt to its new host through either reassortment or point mutations and transmit by aerosolized respiratory droplets. Two previous pandemics of 1957 and 1968 resulted from the reassortment of low pathogenic avian viruses and human subtypes of that period; however, conditions leading to a pandemic virus are still poorly understood. Given the endemic situation of avian H9N2 influenza with human-like receptor specificity in Eurasia and its occasional transmission to humans and pigs, we wanted to determine whether an avian–human H9N2 reassortant could gain respiratory transmission in a mammalian animal model, the ferret. Here we show that following adaptation in the ferret, a reassortant virus carrying the surface proteins of an avian H9N2 in a human H3N2 backbone can transmit efficiently via respiratory droplets, creating a clinical infection similar to human influenza infections. Minimal changes at the protein level were found in this virus capable of respiratory droplet transmission. A reassortant virus expressing only the HA and neuraminidase (NA) of the ferret-adapted virus was able to account for the transmissibility, suggesting that currently circulating avian H9N2 viruses require little adaptation in mammals following acquisition of all human virus internal genes through reassortment. Hemagglutinin inhibition (HI) analysis showed changes in the antigenic profile of the virus, which carries profound implications for vaccine seed stock preparation against avian H9N2 influenza. This report illustrates that aerosolized respiratory transmission is not exclusive to current human H1, H2, and H3 influenza subtypes.

aerosol | ferrets | contact | pandemic | preparedness

H5, H7, and H9 avian influenza subtypes top the World Health Organization's (WHO) list with the greatest pandemic potential. A transition from avian-like α2,3-linked sialic acid (SAα2,3) receptors to human-like α2,6-linked sialic acid (SAα2,6) receptors appears to be a crucial step for avian influenza viruses to replicate efficiently and transmit in humans (1). An increasing number of contemporary avian H9N2 viruses contain leucine (L) at position 226 in the hemagglutinin (HA) receptor-binding site (RBS), supporting the preferential binding to SAα2,6 receptors and the ability to replicate efficiently in human respiratory epithelial cells and in the ferret model, an in vivo model which closely resembles human airway epithelium and clinical infection (2–5). Since the mid-1990's, H9N2 influenza viruses have become endemic in poultry throughout Eurasia and have occasionally transmitted to humans and pigs (6–8). In addition to possessing human virus-like receptor specificity, avian H9N2 viruses induce typical human flu-like illness, which can easily go unreported, and therefore have the opportunity to circulate, reassort, and improve transmissibility. Seroepidemiological studies in Asia suggest that the incidence of human H9N2 infections could be more prevalent than what has been reported and possible human-to-human transmission cannot be completely excluded (9–11). These direct infections with avian H9N2 confirm that interspecies transmission of H9N2 from avian species to mammalian hosts occurs and it is not uncommon. Reassortment between the current human epidemic strain and an avian virus of a different subtype is postulated to generate the next pandemic strain. Given the receptor specificity of avian H9N2 viruses and their repeated introduction into humans, as recent as December 2008 (Vietnam Partnership on Avian and Human Influenza (PAHI) http://www.avianinfluenza.org.vn/), the opportunity for their reassortment and/or adaptation for human-to-human transmission is ever present. However the question remains what is missing for the H9N2 virus to transmit from human-to-human and possibly lead to the next pandemic.

In our previous study (4), we showed that human virus-like receptor specificity, specifically leucine (L) at position 226 in the HA RBS, is critical for direct transmission of avian H9N2 viruses in ferrets. Creation of an H9N2 avian–human reassortant virus led to increased replication, direct transmission, and expanded tissue tropism in ferrets compared to the parental avian H9N2 virus. The reassortant, 2WF10:6M98, contained the surface genes [HA and neuraminidase (NA)] of A/guinea fowl/Hong Kong/WF10/99 (H9N2) [WF10] and the internal genes (PB2, PB1, PA, NP, M, NS) of A/Memphis/14/98 (H3N2) [M98](4). This reassortant however, lacked the ability to transmit via respiratory droplets despite clinical signs including high titers in nasal washes and sneezing, indicating that additional traits are needed. The transmission modes postulated for natural influenza A infections include large droplets, direct contact, and aerosols with aerosol transmission having obvious implications for pandemic influenza (12). The ferret is an ideal model for this study as aerosolization is the main mode of influenza A transmission in this species (13–16). As a result, we began adapting this avian–human reassortant, 2WF10:6M98 in ferrets and after 10 passages achieved respiratory droplet transmission. Here we show for the first time an avian–human H9N2 reassortant that can transmit efficiently in respiratory droplets. We have identified key changes in the surface proteins that are critical for respiratory droplet transmission and also play important roles in antigenic variation. Our studies provide valuable information for pandemic preparedness against H9N2 strains.

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Data deposition: The nucleotide sequences of the RCP10 virus, A/ferret/Maryland/P10-UMD/08 (H9N2), have been deposited at NCBI's GenBank (accession nos. CY036274–CY036281).

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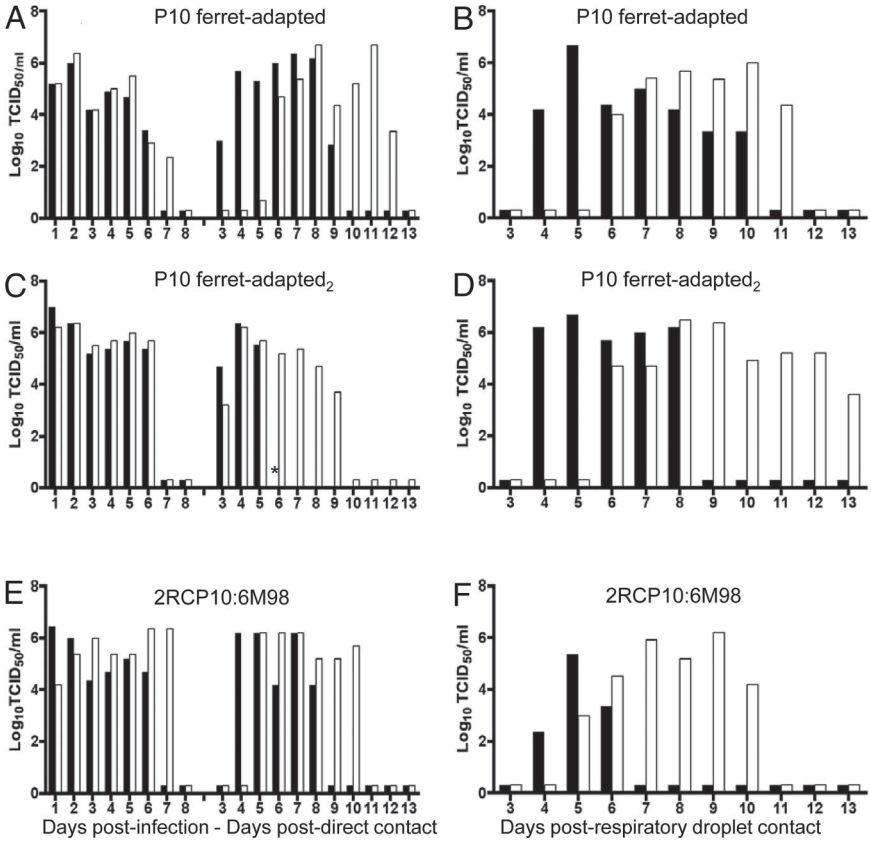

Fig. 1. Respiratory droplet transmission of H9N2 avian–human reassortant viruses. Ferrets were inoculated with 10^6 $TCID_{50}$ of P10 ferret-adapted H9N2 virus (*A* and *C*) or 2RCP10:6M98 reassortant virus (*E*). Direct contact ferrets (*A*, *C*, and *E*) and respiratory droplet contact ferrets for P10 (*B* and *D*) and 2RCP10:6M98 (*F*) were introduced at 24 h p.i. and nasal washes were collected daily. Black and white bars represent individual ferrets. In *C*, day 6 p.c., the direct contact from the group represented by the black bars died, as noted by an asterisk in the bar graph. Titers are expressed as $\log_{10}$ values of $TCID_{50}$/mL with the limit of detection at 0.699 $\log_{10}TCID_{50}$/mL.

Results

Respiratory Droplet Transmission of a H9N2 Avian–Human Reassortant. The generation of a H9N2 avian–human reassortant, 2WF10:6M98, containing the surface genes of A/guinea fowl/Hong Kong/WF10/99 (H9N2) [WF10] and the internal genes of A/Memphis/14/98 (H3N2) [M98] led to increased replication, direct transmission, and tissue tropism when compared to the parental WF10 virus. Clinical signs displayed were similar to those observed during infection with the full human M98 H3N2 virus and included high viral titers in nasal washes and sneezing, yet no transmission to respiratory droplet contacts occurred (4). To determine the key components necessary for efficient respiratory droplet transmission we began adapting the 2WF10:6M98 H9N2 virus in ferrets. Ferrets were infected intranasally (i.n.) with 10^6 tissue culture infectious dose 50 ($TCID_{50}$) of 2WF10:6M98 (passage 1); nasal washes were collected 3 days postinfection (p.i.), pooled, and used as the dose for the following passage of ferrets. Respiratory droplet contact ferrets were introduced at passages 1 and 2; however, no transmission was observed. After 9 passages of nasal wash, we arbitrarily tested the transmissibility of this virus during the 10th passage, herein referred to as P10. Within 3 days postcontact (p.c.), direct contact ferrets were shedding virus and were able to transmit to respiratory droplet contact ferrets by days 4 and 6 p.c. (Fig. 1 *A* and *B*). All ferrets, including respiratory droplet contacts, shed virus up to 6–7 days and displayed clinical signs, including sneezing and fever, similar to that of a human virus infection (4) and showed high antibody titers to the homologous virus (Tables 1 and 2). The transmission phenotype of the P10 virus was confirmed in additional groups of ferrets (Fig. 1 *C* and *D*), consistently resulting in efficient respiratory droplet transmission and clinical signs. It is necessary to note that in the second round of experiments, 1 of the 2 direct contact ferrets died on day 6 p.c. (Fig. 1*C*); however, postmortem examination was inconclusive. Our adaptation study shows that our H9N2 avian–human virus is able to sustain efficient, reproducible respiratory droplet transmission in ferrets causing an infection similar in duration and clinical signs to typical human H3N2 strains. These results suggest that current H9N2 viruses circulating in poultry require little adaptation in mammals, following reassortment and acquisition of human internal genes, to cause respiratory droplet transmission.

Consistent Isolation of Ferret-Adapted P10 H9N2 Virus in Lung Tissue. The consistency of obtaining respiratory droplet transmission in multiple rounds of transmission led us to compare tissue tropism of the P10 virus to the parental 2WF10:6M98 and WF10 viruses (4). Ferrets were also mock infected with PBS as a negative control. Tissues were collected on day 5 p.i., homogenized, and virus titrations performed as previously described (4). While 2WF10:6M98 was able to replicate and expand tissue tropism compared to WF10, the P10 virus shows over 1.5 $\log_{10}$ higher viral

Table 1. Clinical signs, virus replication, and seroconversion associated with H9N2 reassortant viruses in infected ferrets

	Infected ferrets		
Virus	Weight Loss (%)*	Sneezing (day of onset)	Serum (HI titer)†
2WF10:6M98	5.1 ± 0.85	2/2 (2, 2)	2560, 2560
P10‡	4.01 ± 1.2	4/4 (3, 5, 7)	2560, 2560, 2560, 2560
2RCP10:6M98‡	4.67 ± 1.7	3/4 (5, 6)	2560, 2560, 2560, 2560
RCP10 (A189, G192)‡	5.0 ± 2.48	4/4 (5, 6)	2560, 2560, 2560, 1280
RCP10 (T189, R192) ‡	3.69 ± 1.43	4/4 (5, 6)	2560, 2560, 1280, 1280
2WF10:6RCP10‡	1.9 ± 1.0	4/4 (5, 6)	1280, 1280, 2560, 1280

*Average body weight loss is shown as average ± standard deviation.
†At 2 weeks p.i. convalescent sera was collected and used with the homologous virus in HI assays to detect anti-H9 antibodies.
‡Two independent experiments with 2 infected, 2 direct, and 2 respiratory droplet ferrets each.

Table 2. Clinical signs, virus replication, and seroconversion associated with H9N2 reassortant viruses in direct contact and respiratory-droplet contact ferrets

	Direct contacts			Respiratory-droplet contacts		
Virus	Weight loss (%)*	Sneezing (day of onset)	Serum (HI titer)†	Weight loss (%)*	Sneezing (day of onset)	Serum (HI titer)†
2WF10:6M98	1.65 ± 0.50	2/2 (4, 5)	1280, 2560	ND	0/2	<10, <10
P10‡	5.36 ± 0.1	4/4 (5, 7)	2560, 2560, 2560, 2560	7.91 ± 1.98	4/4 (7, 8, 9)	2560, 2560 1280, 2560
2RCP10:6M98‡	2.79 ± 1.43	4/4 (7, 9)	1280, 1280, 1280, 1280	2.07 ± 0.59	4/4 (6, 7, 8)	1280, 1280, 640, 640
RCP10 (A189, G192)‡	1.67 ± 0.82	4/4 (5, 7)	1280, 1280, 2560, 2560	ND	0/4	<10, <10, <10, <10
RCP10 (T189, R192)‡	8.65 ± 5.16	4/4 (6, 7)	1280, 2560, 2560, 2560	ND	0/4	<10, <10, <10, <10
2WF10: 6RCP10‡	2.3 ± 1.4	4/4 (6, 7)	1280, 2560, 1280, 1280	1.2 ± 0.4	0/4	<10, 40, 640, 40

*Average body weight loss is shown as average ± standard deviation.
†Homologous virus was used in HI assays to detect anti-H9 antibodies (sera collected at 2 weeks p.c.).
‡Two independent experiments with 2 infected, 2 direct, and 2 respiratory droplet ferrets each. ND, not determined, because no viral replication occurred in ferrets.

titers than 2WF10:6M98 (Fig. 2). We also isolated virus from the brain of the P10 ferrets, suggesting that in addition to improving its transmissibility phenotype, this virus has the potential to become more virulent. However, we must note that this study focuses largely on the molecular features that alter the transmission phenotype of an H9N2 virus in ferrets. The molecular markers that modulate virulence of this virus in ferrets are beyond the scope of the present report and are currently being evaluated.

Minor Sequence Changes Observed During Ferret Respiratory Droplet Adaptation. Viruses collected from the nasal washes of respiratory droplet contacts, A/ferret/Maryland/P10-UMD/08 (H9N2) [RCP10], were directly sequenced to determine the molecular changes supporting respiratory droplet transmission. Sequence analysis of nasal washes collected on days 5 and 8 p.c., from 4 independent respiratory droplet contacts, revealed the same 5 amino acid changes from 2WF10:6M98 to the RCP10 virus, indicating their selection during respiratory droplet transmission. Three amino acid changes were found on the surface proteins while 2 were found on the internal proteins. Two changes occurred on the HA, one on the HA1 portion of the molecule at position 189 (H3 numbering) within antigenic site B and in close proximity to the RBS (Fig. 3). This amino acid change from threonine (T) to alanine (A) has been documented before (17) and is also found in naturally occurring isolates (18). However, the combination of key amino acid residues at the RBS found in the RCP10 ferret-adapted virus; i.e., histidine (H) 183, A189, glutamic (E) 190, and L226 has yet to be identified in nature (Fig. 3*D*). The available human H9N2 sequences from the NCBI database that have yet to show sustained human-to-human transmission, contain H183 or asparagine (N) 183, T189, E190, and L226. The only major difference in these viruses and the RCP10 viruses is at position 189. The second change is located on the HA2 at position 192 (H3 numbering), a change from glycine (G) to arginine (R), 3 amino acids away from the transmembrane region of the HA2. Unfortunately, this amino acid change lies within a region that has not been resolved by crystallography and therefore cannot be mapped structurally. The change in the NA at position 28, isoleucine (I) to valine (V), is located in the transmembrane domain. This domain has been reported to participate in virus assembly and/or shedding (19). The 2 remaining changes, L to I and H to tyrosine (Y), at positions 374 and 110 of PB2 and M1, respectively, map to regions of unassigned functions within these 2 proteins.

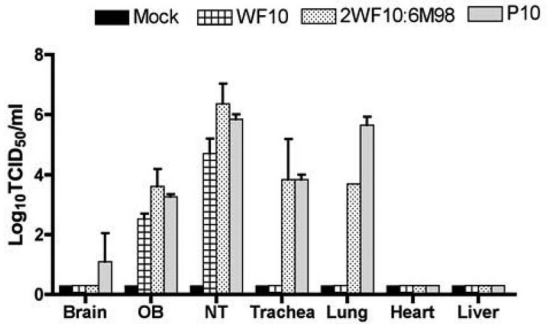

Fig. 2. Consistent isolation of P10 H9N2 virus in lung tissue. Two ferrets were infected with the ferret-adapted P10 virus or mock infected with PBS. Data are compared to the 2WF10:6M98 and WF10 viruses published in ref. 4. Tissues were collected at 5 dpi. *Note only 1 of 2 ferret lungs were positive for virus in the 2WF10:6M98 group. Titers are expressed as $\log_{10}$ values of $TCID_{50}$/mL with the limit of detection at 0.699 $\log_{10}TCID_{50}$/mL. OB, olfactory bulb; NT, nasal turbinate.

To establish whether the amino acid changes observed in RCP10 occurred before passage 10, we sequenced the P10 inoculum virus [P10] (nasal wash from passage 9 ferrets used to infect passage 10), P9, and P8 inoculum viruses (nasal wash from passages 8 and 7 ferrets used to infect passages 9 and 8, respectively) and nasal wash from respiratory droplet contacts from the second transmission study of P10 [$RCP10_2$] (Fig. 1 *C* and *D*). Sequence analysis of passages 8–10 revealed that changes observed in the PB2 and NA occurred either before or at passage 8 with the M1 having a mixed population at P8 selecting for Y at P9. Interestingly the 2 changes observed on the HA were not present until RCP10 and nasal washes collected from $RCP10_2$ respiratory droplet contacts revealed the same 2 changes in the HA gene, implying these changes were selected during adaptation and are perhaps necessary for respiratory droplet transmission (Table 3). The sequence analysis shows minor changes are necessary to support respiratory droplet transmission, one of which alters the RBS of the HA and most likely results in the observed transmission phenotype.

Adaptive Mutations on RCP10 Surface Proteins Support Respiratory Droplet Transmission. A majority of the adaptive amino acid changes occurred before passage 10, with the exception of changes found on the HA. Therefore we wanted to determine whether the amino acid changes on the surface proteins alone are sufficient for respiratory droplet transmission in the background of the M98 backbone. Using reverse genetics, we created a reassortant virus, 2RCP10:6M98, which contains the HA and NA genes from the ferret-adapted RCP10 virus and the internal genes from the human M98 virus. We found that the changes in the surface proteins alone are indeed sufficient for respiratory droplet transmission (Fig. 1 *E* and *F*) with direct contacts shedding virus days 4 and 5 p.c. and transmission to respiratory droplet contacts on the same day with similar titers to P10. Clinical signs were also similar to those observed during the P10 infection, highlighting the role of the surface protein changes on transmissibility (Tables 1 and 2). Respiratory droplet transmission of 2RCP10:6M98 was confirmed in a second, independent study in which 1 out of 2 respiratory droplet contacts became positive for virus shedding (Table 4). Although the M98 back-

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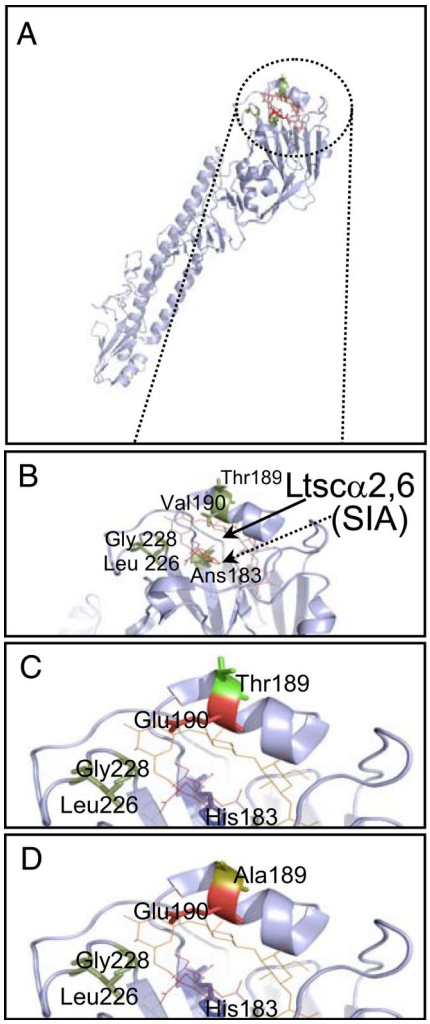

Fig. 3. Adaptive mutations in the H9 HA surface protein necessary for respiratory droplet transmission. (*A*) Cartoon representation of the H9 HA monomer as described by Ha et al., (18) binding the Ltsc α2,6 sialic acid analog (orange and red lines) in the RBS. (*B*) Magnification of the globular head of the HA showing stick representations (in green) of key amino acids in the RBS: N183, G228, L226, T189, and V190, binding to α2,6 sialic acid (SIA, red lines). Numbers correspond to amino acid positions based on the H3 HA numbering system. (*C*) H9 HA RBS with amino acids corresponding to the WF10 HA wild-type sequence, which differs from the published crystal structure at 2 positions: H183 (dark blue stick) and E190 (red stick). T at position 189 is represented as a bright green stick. (*D*) H9 HA RBS with amino acids corresponding to the RCP10 HA sequence, which differs from the WF10 HA sequence at A189, represented as an olive green stick. Structures generated using MacPymol (DeLano Scientific).

bone is likely to play a role in transmission, our study indicates that the adaptive changes in PB2 and M1 in the RCP10 virus are not essential for the respiratory droplet transmission phenotype. At most, 3 amino acid changes on the surface proteins of the avian H9N2 support respiratory droplet transmission in the M98 backbone.

Because HA is the major determinant in the transmission of pandemic influenza, and is key for respiratory droplet transmission of RCP10, we wanted to determine whether both changes in the RCP10 HA are required for respiratory transmission. We used site-directed mutagenesis to create RCP10 HA-mutant viruses that carry 1 of the 2 adaptive HA changes. RCP10 (A189, G192) contains the adaptive change of alanine at HA1 189 and the avian glycine at HA2 192 while RCP10 (T189, R192) contains the avian

Table 3. Sequence analysis of avian–human H9N2 viruses obtained through adaptation in ferrets

Gene	Origin	Amino acid position	Parent	P8	P9	P10	RCP10	$RCP10_2$
PB2	Human	374	L	I	I	I	I	I
PB1	Human	No changes*		ND†	ND			
PA	Human	No changes		ND	ND			
HA	Avian	HA1 189	T	T	T	T	A	A
		HA2 192	G	G	***G***/R‡	***G***/R‡	R	R
NP	Human	No changes		ND	ND			
NA	Avian	28	I	V	V	V	V	V
M1	Human	110	H	H/***Y***‡	Y	Y	Y	Y
M2	Human	No changes		ND	ND			
NS1	Human	No changes		ND	ND			
NEP	Human	No changes		ND	ND			

*No amino acid changes detected between the parent and either the RCP10 or the $RCP10_2$ viruses.
†ND, sequencing not done.
‡Bold and italicized letters denotes more prominent residue at particular amino acid position based on electropherograms of sequencing profiles.

threonine at HA1 189 and adaptive arginine at HA2 192. Our transmission studies suggest that both mutations in HA are necessary for respiratory droplet transmission of the avian–human H9N2 reassortant viruses (Fig. 4 *A–D*). Both mutant viruses replicate efficiently and transmit to direct contacts within 5 to 6 days p.c. inducing weight loss and sneezing (Tables 1 and 2); however, transmission to respiratory droplet contacts in neither nasal wash nor serum was detected. Interestingly, we could predict that a change in HA1, in close proximity to the RBS (Fig. 3), would be necessary for respiratory droplet transmission; however, we could not anticipate that a change in the HA2 portion of the molecule would have an impact on transmission. Furthermore, because infection with neither RCP10 (A189, G192) nor RCP10 (T189, R192) resulted in quick selection of strains with respiratory droplet transmission, we must conclude that both the T189A and G192R mutations arose as aleatory mutations during adaptation in the absence of selective immune pressure. Perhaps multiple rounds of infection would be required before a dominant population containing A189 and R192 can emerge from either the RCP10 (A189, G192) or RCP10 (T189, R192) viruses. These studies highlight the complexities associated with transmissibility of influenza viruses and emphasize the need for in vivo studies, like those shown here, to better understand mechanisms of influenza transmission.

To determine the role the adaptive mutations in the internal proteins of RCP10 play in respiratory droplet transmission, we rescued the reassortant H9N2 virus encoding the internal genes of RCP10 and the unadaptive HA and NA of WF10, 2WF10:6RCP10. We found that the virus was able to replicate and transmit efficiently to direct contact ferrets; however, respiratory droplet transmission was observed in only 1 of 4 respiratory droplet contacts, which shed titers roughly 2 logs lower than RCP10 and

Table 4. Summary of reassortant viruses tested for replication and transmission in ferrets

Virus	Replication	Transmission	
		Direct	Respiratory droplet
P10*	4/4	4/4	4/4
2RCP10:6M98	4/4	4/4	3/4
RCP10 (A189, G192)	4/4	4/4	0/4
RCP10 (T189, R192)	4/4	4/4	0/4
2WF10:6RCP10	4/4	3/4†	1/4

*Two separate studies of 2 infected, 2 direct, and 2 respiratory droplet contacts each.
†Minimal shedding for 1 of the 3 positive direct contacts.

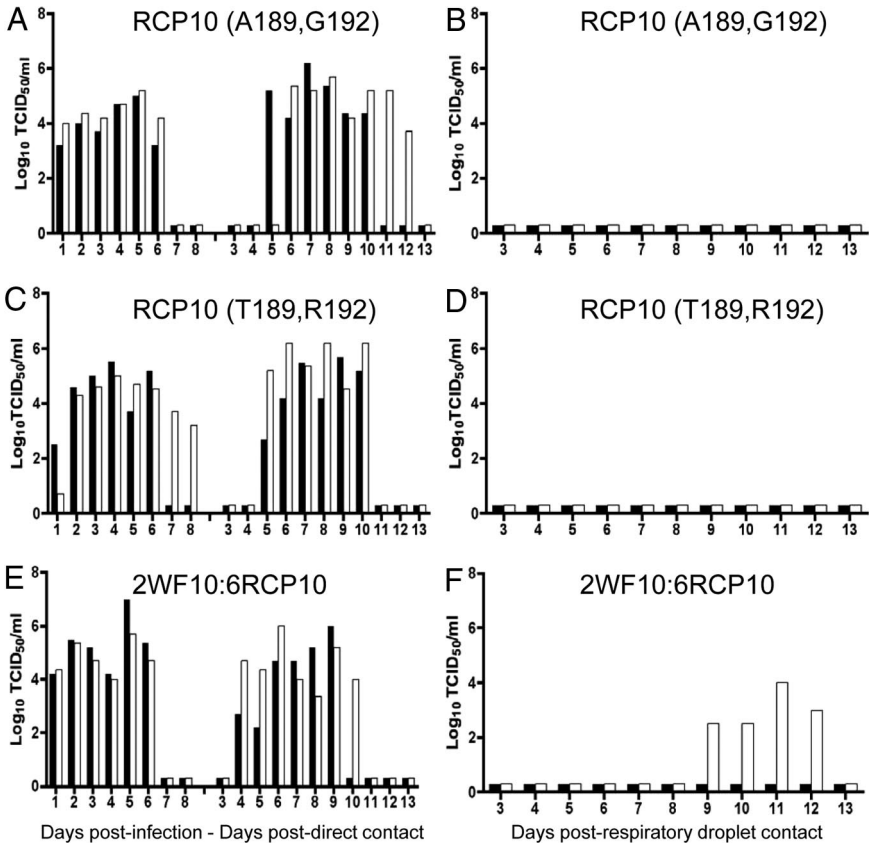

Fig. 4. Transmission phenotype supported through both T189A and G192R changes on the HA. Ferrets were inoculated intranasally (i.n.) with 10^6 $TCID_{50}$ of either RCP10 (A189, G192) (*A*), RCP10 (T189, R192) (*C*), or 2WF10:6RCP10 virus (*E*). Twenty-four hours later, direct contact (*A*, *C*, and *E*) and respiratory droplet contact ferrets (*B*, *D*, and *F*) were introduced and nasal washes collected daily. Black and white bars represent individual ferrets. Titers are expressed as log_{10} values of $TCID_{50}$/mL with the limit of detection at 0.699 $log_{10}TCID_{50}$/mL.

2RCP10:6M98 respiratory droplet contacts (Fig. 4 *E* and *F*, Table 2). Sequence analysis directly from the respiratory droplet contact's nasal wash indicated no adaptive changes on the HA. This result is consistent with the notion that adaptive mutations on the surface proteins are essential for efficient respiratory droplet transmission of the avian–human H9N2 reassortant virus. However we should note that the adaptive changes in PB2 and M1 do play a role in the transmission phenotype noted by the 1 positive respiratory droplet contact in Fig. 4*F* and when comparing the viral titers and length of shedding in respiratory droplet contacts from RCP10 and 2RCP10:6M98. The internal genes have been implicated in transmission not only within avian species (20–22) but also from avian to mammalian species (23, 24). A complete set of viruses tested and their transmission phenotype are listed (Table 4 and supporting information (*SI*) Fig. S1 and Table S1).

Adaptation and Respiratory Droplet Transmission Leads to Changes in Hemagglutinin Inhibition (HI) Profile, Implications for Pandemic Preparedness. Our results suggest that one of the important determinants for respiratory droplet transmission is located in close proximity to the RBS overlapping a major antigenic site of the HA molecule (site B, Fig. 3); this led us to resolve the HI profiles for the reassortant viruses tested. Interestingly we found that the RCP10 virus displays a different antigenic profile from the parental WF10 virus. The HI titers to the WF10 virus are greatly reduced if serum antibodies raised in response to the RCP10 are used instead of those against the parental WF10 virus (Table 5). The RCP10 serum also reacted inefficiently against other H9N2 viruses in HI assays. The opposite is also true: the HI titers to the RCP10 virus are greatly reduced if serum antibodies raised in response to the WF10 virus are used. More importantly, HI titers using anti-RCP10 antiserum were similar for the RCP10 and the RCP10 (A189, G192) viruses, implicating amino acid 189 in antigenicity and in agreement with its position in the tip of the globular head of HA1 (Fig. 3). It has been speculated that given natural conditions, immune pressure can select for variants with altered host specificity and an ability to escape host immunity, which are key factors in the evolution of avian H9N2 viruses. This RCP10 virus, in the absence of immune pressure, created an antigenically variant HA in the ferret. The findings above carry huge implications for vaccine stocks for pandemic preparedness; highlighting the potential discrepancy in antibody protection from the avian field isolate (chosen to prepare the vaccine stock) versus the antigenic makeup of the virus that gains respiratory droplet (or human-to-human) transmissibility. This study also highlights the inherent limitations in the selection of vaccine seed stocks from current avian H9N2 strains, which resembles the WF10 virus in HA sequence. It will be important to determine whether the changes seen in the HA of RCP10, namely amino acid 189 in the HA1, can confer transmissibility in additional H9 HAs and other avian subtypes and whether this should be

Table 5. HI profiles show antigenic differences among H9N2 wild-type and ferret-adapted strains

Ferret sera*/virus	WF10	RCP10	RCP10 (A189, G192)	RCP10 (T189, R192)
WF10	5120, 5120	320, 640	640, 640	1280, 2560
RCP10	1280, 640	5120, 5120	5120, 2560	1280, 640
RCP10 (A189, G192)	1280, 640	5120, 2560	5120, 5120	1280, 640
RCP10 (T189, R192)	2560, 2560	1280, 640	640, 1280	5120, 2560
M98	<10, <10	<10, <10	<10, <10	<10, <10
Dk/Y280†	80, 80	80, 80	ND§	ND
Ch/SF3‡	160, 160	320, 640	ND	ND

*Sera collected at 2 weeks p.i. was used in HI assays against homologous and heterologous viruses.
†Dk/Y280 corresponds to influenza A/duck/Hong Kong/Y280/97 (H9N2).
‡Ch/SF3 corresponds to influenza A/chicken/Hong Kong/SF3/99 (H9N2).
§ND, not done.

considered a critical antigenic site for vaccine candidates. It is interesting to note that residue 189 has been implicated not only in H9 escape mutants but also in escape mutants of the highly pathogenic H5 and pandemic H2 viruses (17, 25–27). However, it must be noted that selection of alanine at position 189 in this study occurred in the absence of preexisting immune pressure in the ferrets.

Discussion

The threat of avian H9N2 strains, and for that matter any avian influenza subtype, becoming a pandemic virus is ever-present. However the key mechanism, human-to-human transmission, is an obstacle yet to be achieved and a process we cannot predict. Insight into the mechanism behind efficient human-to-human transmission can aid in surveillance, countermeasures, vaccine production, and quick reaction/response to outbreaks. Previous studies have compared avian influenza viruses with early pandemic strains, particularly H1 and H3 strains and studied the particular "adaptive mutations" that lead to respiratory droplet transmission using different mammalian and avian animal models (28–31). These and other studies have confirmed the importance of SAα2,6 receptor specificity for sustained transmission in humans (and ferrets) and defined position 226 in the RBS of HA as a key component in influenza host range (32, 33). We have recently shown that L226 in the RBS of the HA of H9 viruses also plays a crucial role in replication in ferrets; however, these viruses have yet to gain the ability to transmit by respiratory droplets regardless of high viral titers and sneezing (4).

Our unique study describes respiratory droplet transmission of an avian–human H9N2 influenza virus in ferrets and pinpoints the minimal changes necessary for respiratory droplet transmission in this model. It is important to note that this particular strain has yet to establish itself in a mammalian host; however, after only 10 passages of nasal washes we were able to establish infection and sustain respiratory droplet transmission that was reproducible in multiple studies. This adaptation resulted in only 5 amino acid changes in the entire genome implying that little is needed for currently circulating avian H9N2 viruses to transmit human-to-human following reassortment with a human strain. Studies to identify the minimal changes necessary indicated the 3 changes in the surface HA and NA as key point mutations essential for respiratory droplet transmission. More importantly, we identified and located a change that dramatically alters the antigenicity of the virus, bringing to light the inherent limitations in the selection of vaccine seed stocks for avian H9N2 viruses and the possible inefficiency regarding the seed stock selection of other avian influenza strains. Whether these changes can affect transmission phenotypes of additional avian H9N2 strains and possibly other influenza subtypes, most notably H5 and H7, is to be determined. However as we have mentioned, changes at position 189 (T189A) have been highlighted in H5 and H2 escape mutants. Our studies show that respiratory droplet transmission in mammals is not an exclusive property of the few virus subtypes that have caused human pandemics (namely H1, H2, and H3 influenza viruses). Other virus subtypes, like H9N2, can also overcome the natural barriers that prevent them from transmitting in a similar manner, provided the ideal host and environmental conditions. It is critical to determine these conditions to develop countermeasures for the impending pandemic whether it is H9, H5, or any other subtype.

Materials and Methods

Viruses and Cells. Viruses were grown and titrated in Madin-Darby Canine kidney (MDCK) cells, as described previously (4). Live viruses were handled under a biosafety level-3^+ containment. The viruses collected from P8, P9, P10, and respiratory droplet contacts, RCP10 and $RCP10_2$, were sequenced directly from the nasal washes without any passages in embryonated chicken eggs or cell culture. Please see online supporting information for a complete description on *Materials and Methods*.

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1. Horimoto T, Kawaoka Y (2001) Pandemic threat posed by avian influenza A viruses. *Clin Microbiol Rev* 14(1):129–149.
2. Matrosovich MN, Krauss S, Webster RG (2001) H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* 281(2):156–162.
3. Wan H, Perez DR (2007) Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. *J Virol* 81(10):5181–5191.
4. Wan H, et al. (2008) Replication and transmission of H9N2 influenza viruses in ferrets: Evaluation of pandemic potential. *PLoS ONE* 3(8):e2923.
5. van Riel D, et al. (2006) H5N1 virus attachment to lower respiratory tract. *Science* 312(5772):399.
6. Maines TR, et al. (2008) Pathogenesis of emerging avian influenza viruses in mammals and the host innate immune response. *Immunol Rev* 225:68–84.
7. Peiris JS, et al. (2001) Cocirculation of avian H9N2 and contemporary ''human'' H3N2 influenza A viruses in pigs in southeastern China: Potential for genetic reassortment? *J Virol* 75(20):9679–9686.
8. Peiris M, et al. (1999) Human infection with influenza H9N2. *Lancet* 354(9182):916–917.
9. Jia N, et al. (2008) Increased sensitivity for detecting avian influenza-specific antibodies by a modified hemagglutination inhibition assay using horse erythrocytes. *J Virol Methods* 153(1):43–48.
10. Guo Y, Li J, Cheng X (1999) Discovery of men infected by avian influenza A (H9N2) virus. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 13(2):105–108.
11. Butt KM, et al. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol* 43(11):5760–5767.
12. Tellier R (2006) Review of aerosol transmission of influenza A virus. *Emerg Infect Dis* 12(11):1657–1662.
13. Haff RF, Schriver PW, Stewart RC (1966) Pathogenesis of influenza in ferrets: Nasal manifestations of disease. *Br J Exp Pathol* 47(5):435–444.
14. Herlocher ML, et al. (2001) Ferrets as a transmission model for influenza: Sequence changes in HA1 of type A (H3N2) virus. *J Infect Dis* 184(5):542–546.
15. Renegar KB (1992) Influenza virus infections and immunity: A review of human and animal models. *Lab Anim Sci* 42(3):222–232.
16. Smith H, Sweet C (1988) Lessons for human influenza from pathogenicity studies with ferrets. *Rev Infect Dis* 10(1):56–75.
17. Kaverin NV, et al. (2004) Structural differences among hemagglutinins of influenza A virus subtypes are reflected in their antigenic architecture: Analysis of H9 escape mutants. *J Virol* 78(1):240–249.
18. Ha Y, Stevens DJ, Skehel JJ, Wiley DC (2002) H5 avian and H9 swine influenza virus haemagglutinin structures: Possible origin of influenza subtypes. *EMBO J* 21(5):865–875.
19. Barman S, et al. (2004) Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza a virus neuraminidase in raft association and virus budding. *J Virol* 78(10):5258–5269.
20. Sorrell EM, Perez DR (2007) Adaptation of influenza A/Mallard/Potsdam/178–4/83 H2N2 virus in Japanese quail leads to infection and transmission in chickens. *Avian Dis* 51(Suppl 1):264–268.
21. Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR (2003) Replication and transmission of influenza viruses in Japanese quail. *Virology* 310(1):8–15.
22. Hossain MJ, Hickman D, Perez DR (2008) Evidence of expanded host range and mammalian-associated genetic changes in a duck H9N2 influenza virus following adaptation in quail and chickens. *PLoS ONE* 3(9):e3170.
23. Steel J, Lowen AC, Mubareka S, Palese P (2009) Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. *PLoS Pathog* 5(1):e1000252.
24. Van Hoeven N, et al. (2009) Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. *Proc Natl Acad Sci USA* 106(9):3366–3371.
25. Tsuchiya E, et al. (2001) Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus. *J Gen Virol* 82(Pt 10):2475–2484.
26. Kaverin NV, et al. (2002) Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. *J Gen Virol* 83(Pt 10):2497–2505.
27. Ilyushina N, Rudneva I, Gambaryan A, Bovin N, Kaverin N (2004) Monoclonal antibodies differentially affect the interaction between the hemagglutinin of H9 influenza virus escape mutants and sialic receptors. *Virology* 329(1):33–39.
28. Tumpey TM, et al. (2007) A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* 315(5812):655–659.
29. Taubenberger JK, Morens DM, Fauci AS (2007) The next influenza pandemic: Can it be predicted? *J Am Med Assoc* 297(18):2025–2027.
30. Garcia-Sastre A, Whitley RJ (2006) Lessons learned from reconstructing the 1918 influenza pandemic. *J Infect Dis* 194(Suppl 2):S127–S132.
31. Naeve CW, Webster RG, Hinshaw VS (1983) Phenotypic variation in influenza virus reassortants with identical gene constellations. *Virology* 128(2):331–340.
32. Matrosovich M, et al. (2000) Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol* 74(18):8502–8512.
33. Stevens J, et al. (2006) Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. *J Mol Biol* 355(5):1143–1155.