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A 27-Amino-Acid Deletion in the Neuraminidase Stalk Supports Replication of an Avian H2N2 Influenza A Virus in the Respiratory Tract of Chickens▽†

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The events and mechanisms that lead to interspecies transmission of, and host adaptation to, influenza A virus are unknown; however, both surface and internal proteins have been implicated. Our previous report highlighted the role that Japanese quail play as an intermediate host, expanding the host range of a mallard H2N2 virus, A/mallard/Potsdam/178-4/83 (H2N2), through viral adaptation. This quail-adapted virus supported transmission in quail and increased its host range to replicate and be transmitted efficiently in chickens. Here we report that of the six amino acid changes in the quail-adapted virus, a single change in the hemagglutinin (HA) was crucial for transmission in quail, while the changes in the polymerase genes favored replication at lower temperatures than those for the wild-type mallard virus. Reverse genetic analysis indicated that all adaptive mutations were necessary for transmission in chickens, further implicating quail in extending this virus to terrestrial poultry. Adaptation of the quail-adapted virus in chickens resulted in the alteration of viral tropism from intestinal shedding to shedding and transmission via the respiratory tract. Sequence analysis indicated that this chicken-adapted virus maintained all quail-adaptive mutations, as well as an additional change in the HA and, most notably, a 27-amino-acid deletion in the stalk region of neuraminidase (NA), a genotypic marker of influenza virus adaptation to chickens. This stalk deletion was shown to be responsible for the change in virus tropism from the intestine to the respiratory tract.

Of the 16 known hemagglutinin (HA) subtypes, only 3 (H1, H2, and H3) have established stable lineages in humans. The H2N2 virus caused a pandemic in 1957 and circulated in the human population until reassortment of the H2N2 virus with an avian H3 virus resulted in the H3N2 pandemic of 1968 (36). Since then, H2N2 viruses have been absent from the human population; however, the H2 subtype has been repeatedly isolated in wild-bird surveillance, and its HA has been found to be antigenically similar to the H2 pandemic virus HA (23, 25, 36). An H2 influenza virus containing human-like receptor specificity was recently isolated as an H2N3 avian-swine reassortant. This virus caused disease and was transmitted in swine and ferrets (24), indicating that this subtype continues to circulate and mutate and can cross the species barrier to mammals. The repeat introduction of a novel H1N1 pandemic this past year (12, 37) highlights the need to understand the mechanisms of introduction, adaptation, and transmission of avian H2N2 influenza viruses in terrestrial birds and potentially mammalian species.

Our previous study built on reports that Japanese quail (*Coturnix coturnix*) play an important role as an intermediate host in the adaptation of avian influenza viruses to land-based birds (38). Japanese quail are typically more susceptible to aquatic influenza viruses than other terrestrial poultry. These viruses establish infection in the respiratory tract, and shedding occurs via aerosol (2, 19, 26, 34, 38, 43). Quail have been implicated in the transmission of avian influenza viruses, such as H5N1 and H9N2 viruses, which have crossed the species barrier to infect humans (9, 14, 15, 22, 28). The susceptibility of quail to multiple subtypes and their role in interspecies transmission led to their removal from live-bird markets in Hong Kong in 2000; however, they continue to be an integral part of live-bird markets throughout the world. Their role as potential intermediate hosts requires further study to identify important molecular markers in the adaptation via quail of avian viruses to other terrestrial poultry, and possibly to humans.

The molecular determinants of the host range and pathogenesis of influenza A viruses have been linked to multiple regions of the 11 genes, most notably those encoding the viral surface glycoproteins (HA and neuraminidase [NA]) and the polymerase proteins (PB2, PB1, PA, and NP). However, a comprehensive map of the various determinants remains incomplete, and the molecular mechanisms involved are unclear. In our previous report, we demonstrated that through the use of quail as an intermediate host, a mallard H2N2 influenza virus, A/mallard/Potsdam/178-4/83 (mall/178), which in its wild-type (wt) form was unable to be transmitted in quail or to establish an efficient infection in chickens, was able, in its adapted form (qa-mall/178), not only to be transmitted to

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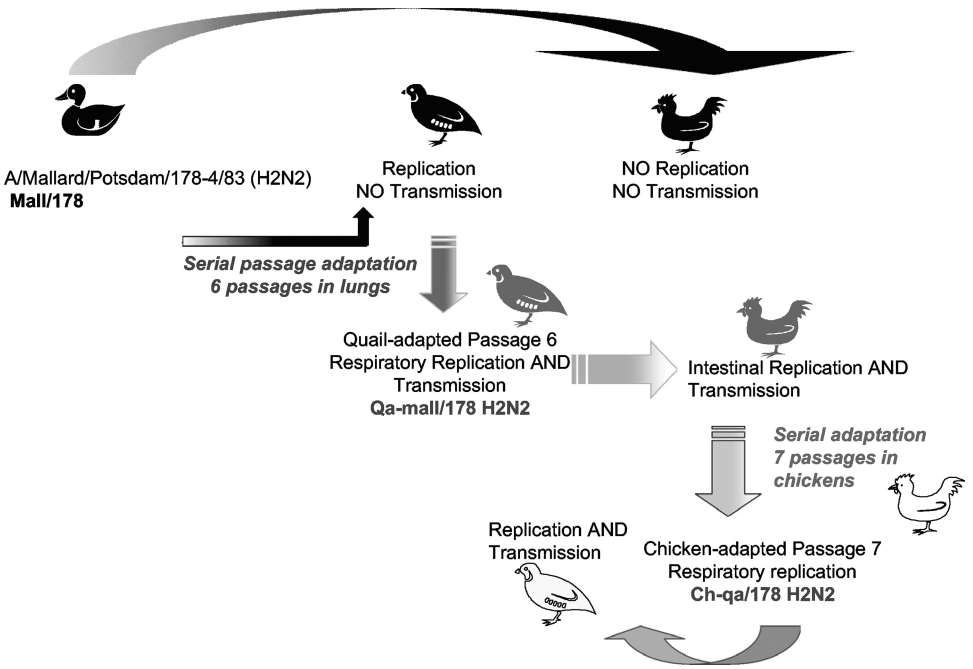

FIG. 1. Adaptation scheme for mall/178 H2N2. Quail and White Leghorn chickens were infected with mall/178 H2N2 and were then tested for replication and transmission. Mall/178 replicated but was not transmitted in quail; in chickens, it neither replicated nor was transmitted. Steps were taken to adapt this mall/178 virus by passage in quail, using lung homogenates from the previous passage as the viral inoculum. At the sixth passage of lung homogenates, transmission in quail was tested. Respiratory transmission was observed, and this quail-adapted virus was designated qa-mall/178. Chickens were then infected with qa-mall/178, and both replication and transmission were tested. Qa-mall/178 not only replicated but also was transmitted in chickens. This virus was transmitted via the fecal-oral route, and therefore, steps were taken to adapt this virus in chickens so as to produce respiratory shedding. After seven passages, a chicken-adapted qa-mall/178 virus (ch-qa/178) was isolated; it was found to replicate in the lungs and to be transmitted via the respiratory route in chickens while maintaining its ability to replicate and be transmitted in quail.

sentinel quail but also to replicate to efficient levels in the chicken intestinal tract and to be transmitted to sentinel cagemates via the fecal-oral route. This adaptation was the result of six serial passages of lung homogenates in quail that led to six amino acid changes in four genes (38). Here we present data confirming the role that Japanese quail play in the transmission of this mall/178 H2N2 virus in land-based birds. Reverse genetics studies confirmed that the amino acid changes produced during the adaptation in quail were necessary for the infection of chickens with this virus and for its transmission in chickens. Further adaptation of the qa-mall/178 H2N2 virus in chickens, aimed at establishing replication in the respiratory tract, resulted in a deletion in the stalk region of the NA, which supported replication in the chicken trachea and lung. This 27-amino-acid deletion in the stalk region of the N2 NA is characteristic of the adaptation of aquatic influenza viruses to domestic poultry, particularly chickens (3, 5, 29). Our work indicates that through the use of quail as an intermediate host, this mallard H2N2 virus is able to further adapt within an additional terrestrial poultry species, potentially improving its chances of expanding its host range further.

MATERIALS AND METHODS

Viruses and cells. A/Mallard/Potsdam/178-4/83 (H2N2) (mall/178) was kindly provided by Robert Webster from the repository of St. Jude's Children's Research Hospital. The virus was propagated as described previously (38). Mall/178 was then adapted in Japanese quail by using serial lung homogenates, as reported previously (38). Briefly, quail were infected with mall/178, and 3 days postinfection (dpi), virus was isolated from the lungs and was used for dosing quail for the next passage. Serial passage was continued to passage 6, when transmission of the quail-adapted virus (qa-mall/178) was tested and confirmed (Fig. 1). Further adaptation of qa-mall/178 in White Leghorn chickens led to the isolation of the H2N2 virus ch-qa/178 (Fig. 1). Briefly, qa-mall/178 was adapted in chickens by using cloacal swabs collected 3 dpi (qa-mall/178 was shed solely via the fecal-oral route), from which the virus was grown once in embryonated chicken eggs. At each passage, lungs were collected and tested for the presence of the virus. Virus was isolated at passage 4, and lung homogenates were prepared and used to adapt the virus in chickens through passage 7, when the chicken-adapted virus was labeled ch-qa/178 (H2N2). Both qa-mall/178 and ch-qa/178 were grown and titrated in embryonated chicken eggs as described previously (38).

Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) containing 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). 293T human embryonic kidney cells were cultured in Opti-MEM I (GIBCO, Grand Island, NY) containing 5% FBS. Chicken embryo kidney (CEK) cells were isolated from 18-day-old chicken embryos and were maintained in medium M199 (GIBCO, Grand Island, NY) containing 5% FBS and 2.5% chicken serum.

Viral sequencing. Total RNA was extracted from allantoic fluid containing virus by using the RNeasy kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Swab samples and lung homogenates were directly used for RNA extraction to confirm the presence or absence of a virus mutation(s) in the samples. Reverse transcription was carried out with the uni12 primer (5′-AGCAAAAGCAAGG-3′) and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) (18). PCR amplification was performed using the universal primers described by Hoffmann et al. (18) as well as specific primers (sequences available upon request). The PCR products were sequenced using the BigDye Terminator protocol, version 3.1 (Applied Biosystems, Foster City, CA). Full-length sequences were obtained for all genes of the viruses used in these studies.

Cloning and generation of viruses by reverse genetics. The genes of mall/178, qa-mall/178, and ch-qa/178 (H2N2) were cloned using a previously described set

of universal primers (17, 18). Viruses generated using reverse genetics are referred to as RG mall/178, RG qa-mall/178, and RG ch-qa/178. Cloned genes were sequenced, and their sequences were compared with the corresponding viral sequences in order to determine that the clones did not carry spurious mutations. Sequences were generated using the BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems, Foster City, CA) and a model 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

Viruses were generated as described previously (17, 33). Briefly, the day prior to transfection, confluent 293T and MDCK cells were trypsinized, and cells were then cocultured 1:1 in Opti-MEM I. The next day, 1 μg of each plasmid (~8 μg) was mixed with TransIT LT-1 (Mirus, Madison, WI). After a 45-min incubation at room temperature, the mixture was added to the cells. Six hours later, the DNA-transfection mixture was replaced by Opti-MEM I. Thirty hours after transfection, 1 ml of Opti-MEM I containing 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin was added to the cells. Viruses were propagated in 10-day-old embryonated chicken eggs and were titrated by 50% egg infective doses (EID_{50}). The recovery of recombinant viruses was verified by sequencing using specific primers.

Site-directed mutagenesis. Mutations in selected genes were introduced by site-directed mutagenesis using a commercially available kit, the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol. Mutations were introduced one at a time into the wild-type mallard virus PB1 to create single, double, and triple mutant viruses containing the quail-adaptive mutations. The mutant viruses were generated as described above, and the genes were sequenced to verify the presence of the desired mutations.

Animals and experimental infections. Four- to 6-week-old Japanese quail (*Coturnix coturnix*; McMurray Hatchery, Webster City, IA) and 3- to 4-week-old White Leghorn chickens (Charles River Laboratories, Wilmington, MA) were used. Groups of three birds were inoculated intraocularly, intranasally, and intratracheally with 5×10^6 EID_{50} of virus/ml (a 1-ml dose for chickens and a 0.6-ml dose for quail), and tracheal and cloacal swabs were collected as described in our previous report (38). Birds were observed and scored daily for clinical signs of infection and general well-being. They were evaluated for appetite, activity, fecal output, and signs of distress, including cyanosis of the comb, tongue, or legs, ruffled feathers, and respiratory distress. For transmission experiments, three contact birds were introduced 1 dpi to the cage where the infected birds were kept. Water and food bowls, as well as cage liners, were changed in order to prevent transmission of the virus via contaminated water or food. Experiments were carried out under biosafety level 2 (BSL2) conditions, with investigators wearing appropriate personal protective equipment, in compliance with Institutional Animal Care and Use Committee (IACUC)-approved protocols, and under Animal Welfare Act (AWA) regulations.

Plaque assays and immunostaining. The temperature phenotype of the recombinant viruses was examined by plaque assay in primary CEK cells at 37°C and 41°C as described previously (42). Briefly, confluent cell monolayers in 6-well plates were infected with 10-fold dilutions of virus in a total volume of 0.4 ml of M199 medium (Invitrogen Corp., Grand Island, NY) for 1 h at 37°C. Cells were washed twice and were then covered with an overlay of M199 containing 1.8% agar, 0.02% bovine serum albumin (BSA), 1% glutamine, and 1 μg/ml TPCK-trypsin. The plates were then incubated at 37°C or 41°C under 5% CO_2. After 3 days of incubation, the overlays were removed, and the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The potential endogenous peroxidase activity was destroyed by incubation with 1% H_2O_2-methanol. After being blocked with 1% BSA in phosphate-buffered saline (PBS), the cells were incubated with a mouse anti-WF10 polyclonal antibody prepared in our laboratory, followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). The viral antigen was visualized by incubating the cells in a solution of aminoethylcarbazole (Dakocytomation, Carpinteria, CA). The size and number of plaques obtained at each temperature were compared among viruses. The restrictive temperature was defined as the lowest temperature at which the titer was reduced 100-fold or more from that at 37°C.

Minigenome assay for viral polymerase activity. A model viral RNA (vRNA), consisting of the *Gaussia* luciferase (GLuc) open reading frame flanked by the noncoding regions of the influenza virus nonstructural (NS) segment, was used to assess polymerase activity in a minigenome reconstitution assay. Briefly, 293T cells were seeded in 6-well plates, transfected with 1 μg of the reporter plasmid along with 1 μg of each of the polymerase plasmids (PB2, PB1, PA, and NP), and mixed with TransIT-LT1 (Mirus, Madison, WI). After a 45-min incubation at room temperature, the mixture was added to the cells. Six hours later, the DNA-transfection mixture was replaced by Opti-MEM I, and cells were either kept at 37°C or moved to 41°C. In addition, the pCMV/SEAP plasmid, encoding a secreted alkaline phosphatase (SEAP) gene, was cotransfected into the cells in order to normalize the transfection efficiency. At 5, 24, 36, and 48 h posttransfection, the supernatant from transfected cells was harvested and assayed for both luciferase and secreted alkaline phosphatase activities by using the BioLux *Gaussia* luciferase assay kit (New England Biolabs, Ipswich, MA) according to the manufacturer's recommendations. Relative polymerase activity was calculated as the ratio of luciferase luminescence to SEAP luminescence. Experiments were performed in triplicate.

Statistical analysis. The statistical significance of differences was evaluated by Student's *t* test using GraphPad Prism software, version 5.00 (GraphPad Software Inc., San Diego, CA). Differences were considered significant at a *P* value of <0.05.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study are available from GenBank under Sequin numbers DQ017486 to DQ017501 (38) and CY067271 to CY067274.

TABLE 1. Comparison of viruses with adaptive amino acid changes in quail and chickens with the wild-type mall/178 H2N2 virus

Gene	Amino acid change(s) (compared to mall/178)		
	Qa-mall/178	Ch-qa/178[a]	Qa-mall/178_2[b]
PB2	A588V	A588V	R318K, S688F
PB1	Q268R, D398E, S654I	Q268R, D398E, S654I	
HA[c]	*N155D*[d]	*N155D*,[d] ***K303Q***	HA1: *N155D*,[d] T188K[d] HA2: F63L, M84V
NP	A234T	A234T	V242A
NA		***Deletion of aa 54–81***	T171A, V231I, G235R

[a] All changes for quail adaptation were maintained in chicken adaptation. Additional changes are italicized and shown in boldface.

[b] The 2nd adaptation was a completely independent adaptation of mall/178 (H2N2) in quail. The change common to the two quail adaptations is italicized and underlined.

[c] Based on the PDB 2FHU structure corresponding to A/Japan/305/57 (H2N2), by O. Spiga et al. (RCSB-PDB) (http://www.pdb.org/pdb/files/2fhu.pdb).

[d] Known position for escape mutants.

RESULTS

A single amino acid change on the HA of mall/178 is crucial for transmission in quail. Our previous work demonstrated that adaptation of A/Mallard/Potsdam/178-4/83 (H2N2) (referred to below as mall/178) in quail resulted in the creation of a virus with increased transmissibility and an extended host range (38). The adaptation of mall/178 required six serial lung passages and created a virus, quail-adapted mall/178 (qa-mall/178), that was transmitted via respiratory contact to sentinel quail and was capable of replication and fecal-oral transmission in chickens, whereas the wild type mall/178 virus was not (Fig. 1). Sequence analysis indicated that only six amino acid changes were present in four gene segments (Table 1).

To fully understand the role that each amino acid played in the adaptation of this qa-mall/178 H2N2 virus, we used reverse genetics to create viruses containing single-gene reassortants in a 7:1 ratio (Table 2). These viruses were used to determine whether the transmissibility of qa-mall/178 in quail was multigenic. The PB2, PB1, HA, and NP genes from the qa-mall/178 viruses were inserted one at a time into the mall/178 backbone in order to determine whether a single gene from the qa-mall/178 virus was responsible for the transmission phenotype (the PA, NA, M, and NS genes did not undergo changes resulting in amino acid mutations during the adaptation of mall/178 [H2N2] in quail). The reverse was also done in order to deter-

TABLE 2. List of reassortant viruses made for transmission studies in quail and chickens[a]

Virus	Origin of the following protein:							
	PB2	PB1	PA	HA	NP	NA	M	NS
Mall/178	Mallard	Mallard	Mallard	Mallard	Mallard	Mallard	Mallard	Mallard
Qa-mall/178	Quail	Quail	Quail	Quail	Quail	Quail	Quail	Quail
Mall:Qa_{PB2}	Quail	Mallard	Mallard	Mallard	Mallard	Mallard	Mallard	Mallard
Mall:Qa_{PB1}	Mallard	Quail	Mallard	Mallard	Mallard	Mallard	Mallard	Mallard
Mall:Qa_{HA}	Mallard	Mallard	Mallard	Quail	Mallard	Mallard	Mallard	Mallard
Mall:Qa_{NP}	Mallard	Mallard	Mallard	Mallard	Quail	Mallard	Mallard	Mallard
Qa:$Mall_{PB2}$	Mallard	Quail	Quail	Quail	Quail	Quail	Quail	Quail
Qa:$Mall_{PB1}$	Quail	Mallard	Quail	Quail	Quail	Quail	Quail	Quail
Qa:$Mall_{HA}$	Quail	Quail	Quail	Mallard	Quail	Quail	Quail	Quail
Qa:$Mall_{NP}$	Quail	Quail	Quail	Quail	Mallard	Quail	Quail	Quail

[a] PA, NA, M, and NS underwent no amino acid changes during the adaptation.

mine whether the replacement of one gene in the qa-mall/178 backbone with the corresponding mall/178 gene would eliminate irrelevant amino acid mutations and highlight the minimal combination of amino acids required for transmission in quail. The full mall/178 virus and the full qa-mall/178 virus (RG mall/178 and RG qa-mall/178) were also generated; therefore, 10 reassortant viruses were created (Table 2).

Quail were infected intraocularly, intranasally, and intratracheally, as described above, and sentinel contacts were introduced 1 day postinfection (dpi). Tracheal swabs were collected and titrated in embryonic chicken eggs to determine replication and transmission. No cloacal shedding was observed for any of the viruses tested (data not shown). The RG mall/178 virus displayed a replication and transmission phenotype similar to that of the wild-type (wt) stock virus (Fig. 2A). Virus infection was supported in quail for 3 dpi by replacing the mall/178 PB2 with the qa-mall/178 PB2 (Mall:Qa_{PB2}), but this virus showed no increase in length of shedding and no improvement in transmission over mall/178. When the quail-adapted PB1 or NP gene was inserted into the mall/178 backbone (Mall:Qa_{PB1}, Mall:Qa_{NP}), the virus replicated and shed in quail for 3 days, yet no transmission of either virus was observed. However, when the quail-adapted HA was inserted into the mall/178 backbone (Mall:Qa_{HA}), transmission in quail was observed. The contacts began shedding virus by 3 dpi and shed for at least 4 days. Sequence analysis of the virus isolated from contact quail (tracheal swabs and lungs) indicated that no additional changes occurred during the transmission of Mall:

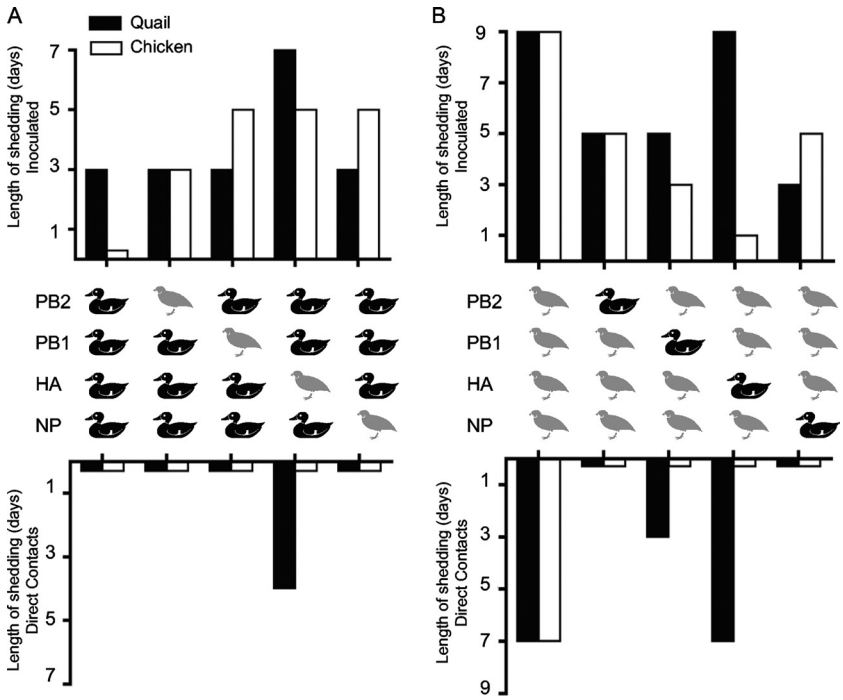

FIG. 2. Replication and transmission of 7:1 reassortants in quail and chickens. Quail and chickens were infected with 5×10^6 EID_{50}/ml of 7:1 reassortants in the mall/178 backbone (A) or the qa-mall/178 backbone (B). Tracheal (quail) or cloacal (chicken) swabs were collected at 1, 3, 5, 7, and 9 dpi. The presence of virus was determined by an HA assay, and the length of shedding was plotted for infected birds and sentinel cagemates (direct contacts) introduced 24 hpi to monitor for transmission of the reassortant viruses tested. The length of shedding is shown for quail (filled bars) and chickens (open bars).

Qa_{HA}. Therefore, the single amino acid change in HA of asparagine to aspartic acid (N155D; numbering based on the HA structure of A/Japan/305/57 [H2N2] predicted by O. Spiga et al., RCSB Protein Data Bank [http://www.pdb.org/pdb/files/2fhu.pdb]) was sufficient to produce a virus that was transmissible in quail. RG qa-mall/178 shed 3 days longer in contact quail than the Mall:Qa_{HA} virus; therefore, it is evident that no 7:1 combination could mimic the RG qa-mall/178 virus. However, it is noteworthy that the single change in HA, N155D, supported replication and efficient transmission.

The reverse study was performed, and single genes from qa-mall/178 were replaced with genes from the mall/178 virus (ratio of qa-mall/178 genes to mall/178 genes, 7:1). The RG qa-mall/178 virus displayed a replication and transmission phenotype similar to that of the qa-mall/178 stock virus (Fig. 2B). Quail infected with either Qa:$Mall_{PB2}$ or Qa:$Mall_{NP}$ shed virus for 5 and 3 days, respectively, without transmission. We observed minimal transmission when quail were infected with Qa:$Mall_{PB1}$, indicating that the three changes found in qa-mall/178 PB1 are not absolutely necessary to support transmission. However, the adaptive mutations in qa-mall/178 PB1 do improve transmission and extend the days of shedding. Analysis of sequences from contact quail shedding Qa:$Mall_{PB1}$ confirmed that no additional mutations arose during the infection and/or transmission. Site-directed mutagenesis studies, performed in the qa-mall/178 backbone, indicate that two of the three changes are crucial for maintaining full qa-mall/178-like transmission (7 days of shedding in contacts), while the Q268R change does not appear to be necessary (data not shown). In addition, and most surprisingly, we found that Qa:$Mall_{HA}$ replicated and was transmitted as efficiently as the RG qa-mall/178 virus. This was unexpected, since the reverse study indicated that the adaptive mutation in HA (N155D) was necessary to support transmission in quail and could do so without any of the additional adaptive mutations. Analysis of sequences from the swabs and lungs of contact quail indicated that an adaptive mutation, K151E (lysine to glutamic acid), occurred four amino acids upstream of the original qa-mall/178 adaptive mutation N155D, and in close proximity based on the predicted protein structure. This change was present in all contact quail. It is interesting that both the N155D and K151E adaptive mutations in this region of the HA are located at the globular head and within the I-A antigenic site (site B in H3) (41, 46). Therefore, given the other five adaptive changes, the quail were able to support a mutation near the original mutation site, indicating its importance in supporting transmission in quail.

Transmission of qa-mall/178 in chickens is dependent on adaptive changes created in quail. Our previous study demonstrated that qa-mall/178 gained the ability to replicate and to be transmitted in White Leghorn chickens via the fecal-oral route (38). Therefore, in this study, chickens were also infected with the 7:1 reassortant viruses discussed above, and transmission was tested. No transmission was observed in any 7:1 viruses created in the mall/178 backbone; however, cloacal replication of Mall:Qa_{PB2} for 3 dpi and of Mall:Qa_{PB1}, Mall:Qa_{HA}, and Mall:Qa_{NP} for 5 dpi (Fig. 2A) was noted. Fecal-oral transmission comparable to that of the qa-mall/178 stock virus was observed for the RG qa-mall/178 virus. The 7:1 combinations Qa:$Mall_{PB2}$, Qa:$Mall_{PB1}$, and Qa:$Mall_{NP}$ were able to establish infection but not transmission in chickens (3 to 5 days of shedding). However, Qa:$Mall_{HA}$ barely replicated (shedding detected for 1 dpi) and failed to be transmitted in chickens, indicating that the N155D change on HA is crucial for establishing an infection in the chicken. It is noteworthy that given the remaining 5 adaptive mutations (Qa:$Mall_{HA}$), quail were able to support an adaptive change, K151E, similar to N155D, allowing transmission to contact quail, a change that chickens were unable to support given the same virus.

Interestingly, during an independent repeat adaptation of mall/178 in quail (serial lung passage and testing of transmission at the sixth passage), the only amino acid change that was consistent for the two quail-adapted viruses was the N155D change in HA (Table 1). An additional mutation was found on the HA at position 188 (T188K), which is also in close proximity to amino acid 155 on the globular head of the molecule and is under immunological pressure (41, 46). The second quail-adapted virus (qa-mall/178_2) was able to replicate and be transmitted in quail as efficiently, and to the same extent, as the original qa-mall/178 virus, yet it could not be transmitted in chickens. Thus, the N155D change on HA is important for the replication and transmission of the mall/178 virus in chickens and quail; however, it works in concert with additional changes to allow for the transmission of qa-mall/178 in chickens.

Lack of correlation between virus shedding and transmissibility. To determine if viral shedding is an indicator of transmissibility, we titrated the tracheal and cloacal swabs collected from the 7:1 reassortant virus-infected quail and chickens, respectively. Each individual animal was swabbed on days 1, 3, and 5 postinfection (p.i.) in order to determine the day(s) of peak shedding. For those viruses that shed between 3 and 7 days, day 3 p.i. was found to be the peak day of shedding. For those viruses that shed for 1 day, titers collected on day 1 p.i. were used as the peak titer (Fig. 3A and B). It is noteworthy that although the RG qa-mall/178 virus was shed to the highest titer in both quail and chickens, the titers of the reassortant 7:1 viruses did not appear to correspond with the length of shedding in infected animals and/or with transmission to contact cagemates. Viruses from the Mall:Qa 7:1 reassortants shed between 3 $\log_{10}$ and 3.7 $\log_{10}$ EID_{50}/ml in quail, compared to a more varied range of 1.5 $\log_{10}$ to 3.5 $\log_{10}$ EID_{50}/ml in chickens. The RG mall/178 virus was shed by quail at the highest titer, 4.5 $\log_{10}$ EID_{50}/ml, even though this virus could not be transmitted in quail (Fig. 4A). Similar observations were made for the Qa:Mall 7:1 reassortant groups. Although the RG qa-mall/178 virus was shed to the highest titers in both quail and chicken swabs (5 $\log_{10}$ to 5.3 $\log_{10}$ EID_{50}/ml), viruses such as Qa:$Mall_{PB2}$ shed to relatively high levels in quail (4 $\log_{10}$ EID_{50}/ml) with decreased length of shedding and no transmission. The level of tracheal shedding for Qa:$Mall_{PB1}$, which was transmitted in quail, was lower than those for all other Mall:Qa and Qa:Mall 7:1 viruses titrated, indicating that the amount of virus shed is not an absolute correlate of transmission (Fig. 4B).

Changes in qa-mall/178 polymerase genes affect replication at 41°C. The ribonucleoprotein (RNP) complex, comprising PB2, PB1, PA, and NP, has been identified as playing a role in the host range of influenza A viruses. These genes contain host-associated markers and have evolved into divergent host-specific lineages (8, 32). Of the 52 host-associated genetic

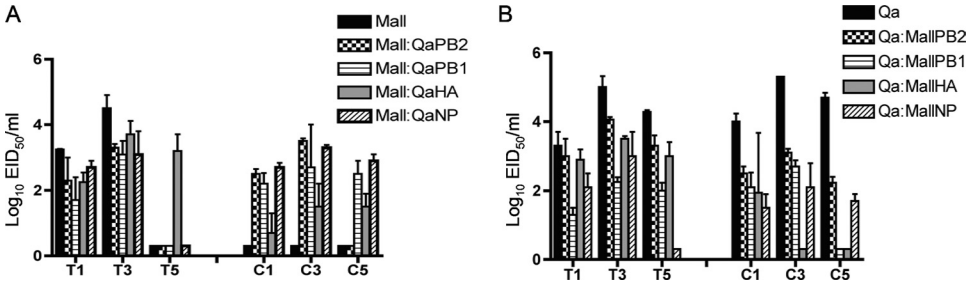

FIG. 3. Peak titers shed by 7:1 reassortants in quail and chickens. Swabs collected on days 1, 3, and 5 p.i. from infected quail and chickens during replication and transmission studies were titrated in embryonated chicken eggs in order to determine the day of peak viral shedding. Results for 7:1 reassortants in the mall/178 (A) and qa-mall/178 (B) backbones are shown. Titrations are averages for three quail/chickens from whom viruses were collected on the specified day ± standard errors of the means. T, tracheal swabs from quail; C, cloacal swabs from chickens.

signatures identified by Chen et al. (8), 35 are located in the RNP. Mutations in polymerase gene segments have been linked to changes in host range, virulence, and optimal replication temperatures (16, 20, 27, 30, 31, 40).

Five of the six adaptive changes in qa-mall/178 were found in the polymerase complex. Viral shedding in quail was exclusively respiratory, with virus isolated in the upper and lower respiratory tracts. To determine if changes in the RNP complex, observed in qa-mall/178 and maintained in ch-qa/178, permit viral replication at a lower temperature than that for the mall/178 virus, we performed minigenome and plaque assays at 37°C and 41°C to mimic the respiratory and intestinal temperatures, respectively. Using the minigenome assay, we found a statistically significant difference (P, <0.05 by Student's t test) in polymerase activity between mall/178 and qa-mall/178 at 41°C at each time point tested after 24 h (Fig. 5A). There were however, no statistically significant differences between the polymerase complexes of these viruses at 37°C at the time points tested (Fig. 5B). Thus, mutations in the polymerase complex after adaptation in quail affected polymerase function at 41°C and, to a lesser extent, at 37°C. However, the minigenome assay does not provide sufficient evidence for a role of the polymerase complex in improved replication in either quail or chickens after adaptation in quail.

Plaque assays were performed in CEK cells, which were stained with an anti-NP antibody. A large-plaque phenotype was observed for mall/178 at 41°C, while smaller plaques were observed at the same dilution for the qa-mall/178 virus (Fig. 5C), correlating with the levels of polymerase activity at this temperature. The reverse was true for plaques grown at 37°C. The size and number of plaques for qa-mall/178 were higher than those at 41°C at the same dilution (10^{-6}). However, we were unable even to obtain plaques at a 10^{-6} dilution for mall/178 and had to decrease the dilution by 100-fold in order to obtain small plaques (Fig. 5D), indicating that the adaptive changes in the RNP support replication at a lower temperature.

A 27-amino-acid NA stalk deletion is responsible for viral replication in chicken lungs. Qa-mall/178 replicates and is transmitted efficiently in chickens. However, transmission in chickens occurs through the fecal-oral route, as opposed to respiratory contact transmission in quail. To determine whether this qa-mall/178 virus could adapt within chickens and replicate in their upper respiratory tracts, we further adapted qa-mall/178 in chickens by using serial passages of cloacal swabs collected at peak shedding, as previously described (38). After only 4 passages, we were able to isolate a virus, ch-qa/178, from the lungs. Sequence analysis identified a change in

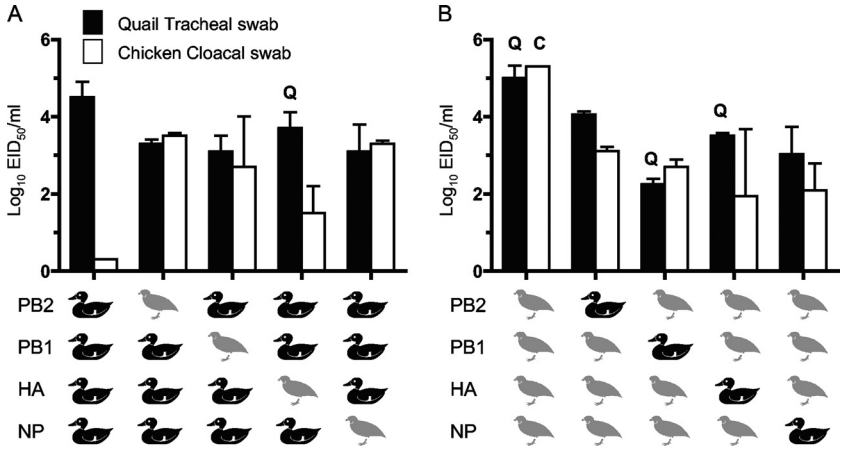

FIG. 4. Titers shed by 7:1 reassortants in quail and chickens. Swabs collected during replication and transmission studies were titrated in embryonated chicken eggs to determine whether the amount of viral shedding was an indicator of transmissibility. Results for 7:1 reassortants in the mall/178 (A) and qa-mall/178 (B) backbones are shown. Titrations are averages for three quail/chickens from whom viruses were collected on the specified day ± standard errors of the means. Q, viruses transmitted in quail; C, viruses transmitted in chickens.

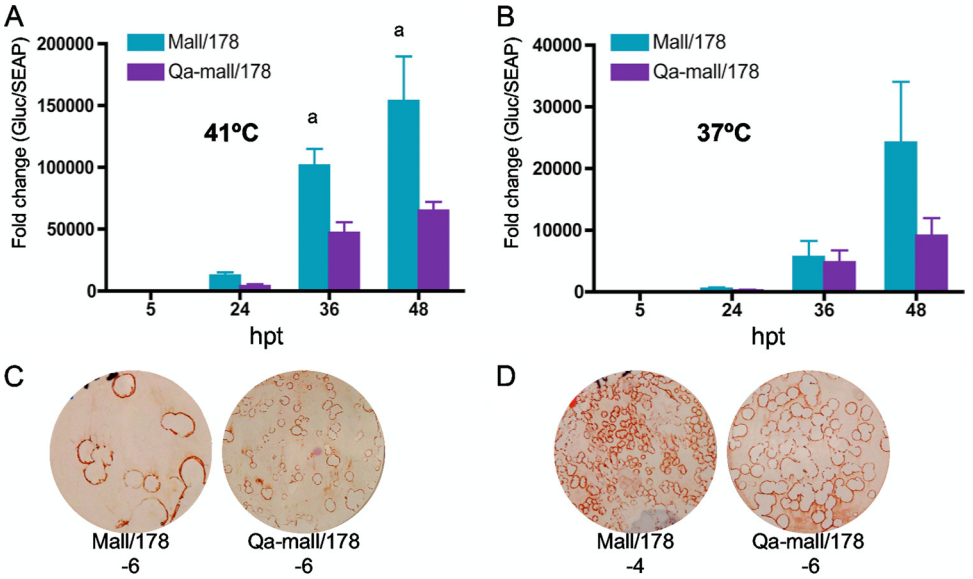

FIG. 5. Minigenome and plaque assays to determine the role of adaptive mutations in the qa-mall/178 polymerase genes. Polymerase activity was measured using a minigenome assay (A and B) and a plaque assay (C and D) at 41°C and 37°C. (A and B) For the minigenome assay, 293T cells were transfected at 41°C (A) or 37°C (B) with PB2, PB1, PA and NP plasmids from either the mall/178 or the qa-mall/178 virus, along with a luciferase reporter plasmid and a pCMV/SEAP plasmid encoding a secreted alkaline phosphatase (to normalize the transfection efficiency). The negative control included an empty vector instead of the plasmid encoding PB1. Luciferase activity was assayed in cell supernatants at the indicated hours posttransfection (hpt). Each result is the average fold change (luciferase/SEAP ratio) ± the standard error of the mean from three independent transfections. A lowercase letter "a" above a bar indicates that the difference between mall/178 and qa-mall/178 is statistically significant ($P, < 0.05$ by Student's t test). (C and D) The RG mall/178 and RG qa-mall/178 viruses were used to infect CEK cells for 1 h at 37°C. Cells were then washed, overlaid with 1.8% agar and MEM (1:1), and incubated at either 41°C (C) or 37°C (D). After 48 h, overlays were removed, and plaques were stained using anti-NP antibodies. Dilutions (-6, 10^{-6}; -4, 10^{-4}) are shown at the bottom.

HA, lysine to glutamine (K303Q), and a 27-amino-acid deletion in the stalk region of the NA (amino acids 54 to 81) (Table 1). Three additional lung passages confirmed the stability of this stalk deletion, since no additional changes/deletions in the genome were observed through passage 7. The ch-qa/178 virus was able to support respiratory contact transmission to sentinel chickens as well as to maintain efficient replication and transmission in quail (Fig. 1).

The ability of this stalk deletion to support viral replication in the lung was investigated through reverse genetics. We generated the ch-qa/178 NA in the mall/178 and qa-mall/178 backbones. We infected chickens with these 7:1 viruses, as well as with the RG mall/178, RG qa-mall/178, and RG ch-qa/178 viruses, and we tested for transmission and measured viral titers in swabs (tracheal and cloacal) and lungs at 3 dpi. RG mall/178 could not be transmitted, as previously reported, and was not isolated in the swabs or lungs. RG qa-mall/178 was isolated from the lungs of only 1 of 3 infected chickens, at a low titer, and was transmitted to contact chickens via the fecal-oral route, as indicated by the swab titers. RG ch-qa/178 was isolated in all lungs collected, at a 6-$\log_{10}$-unit increase over the titer for the single positive qa-mall/178 sample (Fig. 6). This RG ch-qa/178 virus replicated and was transmitted efficiently in chickens, as previously observed (38). This virus not only increased its tropism to replicate in the respiratory tract; it transitioned from a virus transmitted solely by the fecal-oral route to one that is also transmitted via respiratory contact, as evidenced by a 3-fold increase in the viral titers in tracheal swabs and a-4 fold decrease in those in cloacal swabs (Fig. 6).

To determine whether the NA deletion was responsible for the tropism change observed in ch-qa/178, we infected chickens with a Qa:Ch_{NA} or a Mall:Ch_{NA} virus. Ch:$Mall_{NA}$ and Ch:Qa_{NA} were not tested, because they are equivalent to qa-mall/178, except for the single change K303Q in ch-qa/178 HA. Virus was isolated from the lungs of all chickens infected with Mall:Ch_{NA} at titers roughly 3 $\log_{10}$ units higher than those of RG mall/178; however, this single gene was not able to support respiratory contact transmission in chickens. Infection with Qa:Ch_{NA} led to high titers of virus in the lungs, 5 $\log_{10}$ units higher than those of the RG qa-mall/178 virus and only 1 log unit lower than those of RG ch-qa/178, indicating that the stalk deletion in the NA of ch-qa/178 is the main determinant of respiratory tropism for this virus. Importantly, this Qa:Ch_{NA} virus also supported respiratory contact transmission (Fig. 6). Swabs were not titrated for these two RG viruses.

The contrast observed between the replication kinetics of influenza viruses *in vivo* versus *in vitro* is interesting. Published reports indicate that stalk deletions in the NA may prevent viral release or disrupt replication efficiency *in vitro* (4, 7, 13, 28). However, this does not appear to be the case *in vivo*, as evidenced by the fact that the adaptation of duck-origin viruses to terrestrial poultry, particularly chickens, corresponds with NA stalk deletions (5, 6, 29). Therefore, we compared the replication of the five recombinant viruses in chicken lungs to that in chicken fibroblast (DF1) cells. Viruses carrying the NA stalk deletion replicated to lower titers in *in vitro* DF1 cells than their parental counterparts. In chickens, the reverse was observed (Fig. 5), complementing previous reports.

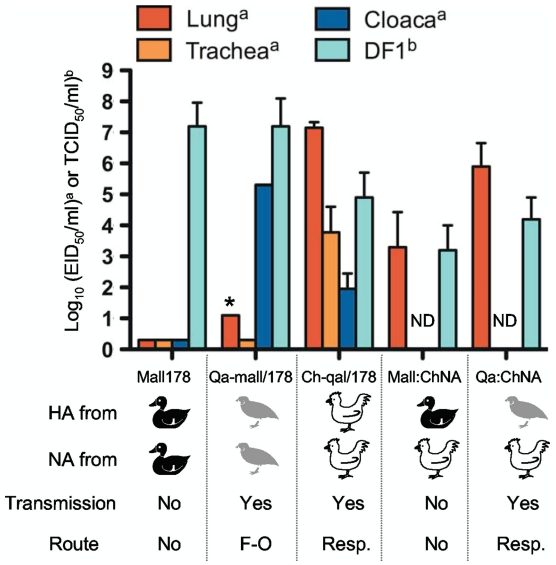

FIG. 6. Replication tropism of NA stalk deletion viruses in chickens. White Leghorn chickens were infected with 5×10^6 EID_{50}/ml of either RG mall/178, RG qa-mall/178, RG ch-qa/178, or the 7:1 reassortant Mall:Ch_{NA} or Qa:Ch_{NA}. Lung homogenates and swabs (tracheal or cloacal) were collected 3 dpi and were titrated in embryonated chicken eggs by EID_{50} (indicated by a superscript "a"). No swabs were collected for chickens infected with either Mall:Ch_{NA} or Qa:Ch_{NA} (ND, not done). The asterisk above the RG qa-mall/178 lung bar indicates that only one lung sample was positive for virus. Replication of recombinant viruses was also tested in DF1 cells. Supernatants were collected at 3 dpi and were titrated using the 50% tissue culture infective dose ($TCID_{50}$) (indicated by a superscript "b"). Titers are expressed in either $\log_{10}$ EID_{50}/ml or $\log_{10}$ $TCID_{50}$/ml, as indicated. The origins of the HA and NA surface genes, the presence or absence of transmission, and the route of transmission are given below the bar graph. F-O, fecal-oral transmission; Resp., respiratory transmission.

DISCUSSION

The occasional transmission of avian influenza viruses from poultry to humans has been repeatedly documented across multiple avian subtypes; however, adaptation, sustained transmission, and the establishment of a lineage are rare events. This is consistent with findings that multiple host range markers are involved (reviewed in references 8 and 32). The quantity of adaptive mutations necessary for an avian virus of a particular subtype to cross the species barrier is unknown. Our results are consistent with the notion that changes on the surface genes alone are insufficient for the transmission of avian influenza viruses in chickens (19, 26, 34, 35). It should also be noted that the changes that occurred during adaptation in quail were maintained in 7 passages of the virus in chickens and were found to be necessary for transmission. This supports the idea that these changes are biologically relevant for the adaptation of aquatic avian influenza viruses in terrestrial bird species. The single change in HA, N155D, was crucial for transmission in both quail and chickens. This mutation, on its own, was capable of supporting respiratory transmission within quail. The importance of this region in the HA was highlighted when, given the other 5 adaptive mutations, quail supported a mutation to glutamic acid at position K151 (Qa:$Mall_{HA}$), indicating that the change in this region is crucial for efficient transmission. The K151E change is not random and has been found in H2N2 escape mutants (K156E by H3 numbering) (46). Interestingly, the adaptive mutation at amino acid 155, or at any region of the globular head of H2, did not arise in chickens infected with Qa:$Mall_{HA}$, emphasizing the need for quail as the intermediate host. In contrast to the situation in quail, the single change of N155D (Mall:Qa_{HA}) was unable to support transmission in chickens when present alone in the mallard backbone. Therefore, this N155D mutation, found in two independent adaptations of the mallard H2N2 virus in quail, is likely a key marker of increased host range to terrestrial poultry, highlighting the role that quail play as an intermediate host for avian influenza viruses from the wild-bird reservoir.

The adaptation of mall/178 (H2N2) in quail not only permitted transmission within the species; it extended its host range to chickens. Based on our 7:1 reassortant studies, we can conclude that all six amino acid changes derived from the qa-mall/178 virus are necessary to support replication and fecal-oral transmission in chickens. Adaptive changes in the polymerase complex were found to be responsible for supporting replication at a lower temperature, as indicated by plaque assay results at various temperatures. Given these polymerase changes, the virus was able to gain adaptive mutations in the chicken, which allowed a change in tropism from intestinal to respiratory replication. The changes observed in the qa-mall/178 and ch-qa/178 viruses, in PB1 at position 654 and in PB2 at position 588, are located within PB1–PB2 interaction domains, indicating that these changes may have been linked in adapting the mallard virus to quail and chickens. Position 588 in PB2 has previously been defined as a genetic signature separating avian and human strains (8). This change in replication temperature preference to a lower, characteristically respiratory tract temperature is an important step in the adaptation of aquatic influenza viruses to terrestrial poultry and potentially mammalian species.

Dose-dependent virus transmission is a poorly understood concept and differs based on multiple factors, such as host species, virulence, subtype, and strain. Fifty percent infective dose studies, however, have been well documented for a variety of influenza viruses in a variety of hosts. Whether the 50% infectious dose can be correlated to effective viral transmission is not known. The infectivity of a virus includes not only its ability to infect, replicate, and spread in a host but also its ability, when shed from the host, to be transmitted to susceptible species in the environment. This transmissibility can be based on the amount of virus shed and favorable exposure to contact host species. Here we report a lack of correlation between virus shedding and transmissibility. Based on our findings, it appears these particular H2N2 viral titers are unrelated to the potential for virus transmission in quail and chickens. This observation was also made in a study performed with a highly pathogenic H5N1 influenza virus in chickens. The report indicated that the amount of virus shed in infected chickens was unable to predict the transmission of the virus to contact chickens (39). Whether these findings could be relevant to other subtypes and species is not clear; however, it is an important implication for influenza virus transmission in terrestrial poultry.

Many studies have investigated the biological importance of

the neuraminidase stalk length in influenza virus infection and release. The antigenic structure and structure-function relationship of the NA head have been studied (1, 45), yet information regarding the stalk region is limited. Reports that stalk length plays a role in replication and viral release have been published (4, 7); however, there is little information on what role the stalk deletion plays in pathogenesis, transmission, and host range. To date, stalk deletions have been tied to host adaptation of mallard viruses to domestic poultry, including chickens and quail (5, 19). The sizes and sequences of the deletions differ, even within subtypes (10), and this divergence is not correlated to host immune pressure (21). Viruses containing NA stalk deletions without additional glycosylation sites are typically new introductions into poultry and evolve into lineages when mutations are fixed, i.e., when the viruses have acquired glycosylation sites, as previously described (11). Here we show that the 27-amino-acid deletion, arising after only 4 passages of qa-mall/178 in chickens, supports respiratory replication and a shift in viral tropism from intestinal to respiratory replication in the chicken host. This virus also maintained its ability to replicate and be transmitted in quail. Viral tropism, transmission, and the 27-amino-acid stalk deletion were stable for an additional three passages in chickens, suggesting the stability of this stalk deletion and its role in respiratory replication.

Forty years have passed since the H2N2 pandemic, and the naïve population grows larger every year (44). Avian H2 viruses currently circulate in the wild-bird population, and their HA has been shown to be closely related to the 1957 pandemic virus HA (25), while H2 viruses carrying human-like receptor specificity have been isolated in influenza outbreaks among swine as recently as 2007 (24). The recent introduction into the human population of a swine-origin H1N1 virus reminds us that previously established human subtypes can reemerge, using an intermediate host, to evolve into a repeat pandemic or epidemic. The currently circulating avian H2N2 viruses warrant attention and study to further investigate their repeated-pandemic potential.

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