Reassortment and Mutation of the Avian Influenza Virus Polymerase PA Subunit Overcome Species Barriers

Andrew Mehle,[a]* Vivien G. Dugan,[b]* Jeffery K. Taubenberger,[b] and Jennifer A. Doudna[a,c,d]

Department of Molecular and Cell Biology,[a] and Department of Chemistry,[c] University of California, Berkeley, California, USA; Viral Pathogenesis and Evolution Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[b]; and Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California, USA[d]

The emergence of new pandemic influenza A viruses requires overcoming barriers to cross-species transmission as viruses move from animal reservoirs into humans. This complicated process is driven by both individual gene mutations and genome reassortments. The viral polymerase complex, composed of the proteins PB1, PB2, and PA, is a major factor controlling host adaptation, and reassortment events involving polymerase gene segments occurred with past pandemic viruses. Here we investigate the ability of polymerase reassortment to restore the activity of an avian influenza virus polymerase that is normally impaired in human cells. Our data show that the substitution of human-origin PA subunits into an avian influenza virus polymerase alleviates restriction in human cells and increases polymerase activity *in vitro*. Reassortants with 2009 pandemic H1N1 PA proteins were the most active. Mutational analyses demonstrated that the majority of the enhancing activity in human PA results from a threonine-to-serine change at residue 552. Reassortant viruses with avian polymerases and human PA subunits, or simply the T552S mutation, displayed faster replication kinetics in culture and increased pathogenicity in mice compared to those containing a wholly avian polymerase complex. Thus, the acquisition of a human PA subunit, or the signature T552S mutation, is a potential mechanism to overcome the species-specific restriction of avian polymerases and increase virus replication. Our data suggest that the human, avian, swine, and 2009 H1N1-like viruses that are currently cocirculating in pig populations set the stage for PA reassortments with the potential to generate novel viruses that could possess expanded tropism and enhanced pathogenicity.

Seasonal influenza virus infections are punctuated by the emergence of pandemic viral strains. These pandemic viruses are introduced from animal reservoirs into the human population, rapidly become the dominant circulating virus, and are ultimately established as attenuated variants that contribute to the next generation of seasonal viruses (34). The natural reservoir of influenza A viruses is numerous wild bird species, predominantly from the orders *Anseriformes* and *Charadriiformes*, although a large number of diverse mammalian hosts have been identified (47). Viral transmission from one species to the next and the establishment of a pandemic virus are multifactorial processes influenced by viral evolution and the complex interplay between viral and host genes.

Influenza A virus evolution proceeds via reassortment and mutation. The viral genome is composed of eight separate negative-sense single-stranded RNA segments. The genome can undergo reassortment when two distinct viruses coinfect the same cell and exchange one or more of their gene segments to produce progeny possessing a new genome constellation. Pigs have long been thought of as a "mixing vessel" where human and avian influenza virus reassortment could occur; viruses containing genes from both avian and human sources have been isolated from naturally and experimentally infected pigs, and several different reassortant viral lineages are stably circulating in pig populations (24, 54). The 2009 H1N1 pandemic (pH1N1) was a clear example where reassorted viruses containing genes derived from avian, human, classical swine, and Eurasian swine isolates were circulating in pigs prior to transmission to humans (12, 41). Evolution via mutation is a consequence of the selection and fixation of mutations generated via the error-prone viral RNA (vRNA)-dependent RNA polymerase, which mediates genome replication (20). Mutations are important for adaption to new host environments, escape from immune pressure, and the emergence of drug-resistant viruses.

Mutations can also lower the barrier to cross-species transmission. For example, point mutations in avian viral genes circulating in pigs have partially adapted these proteins for replication in humans (10, 19). The unique interaction between viral genes and the host environment rapidly and continually drives the selection of well-adapted combinations of mutations and different gene constellations.

As a major determinant of species tropism and pathogenicity, the influenza virus polymerase is a frequent target of mutation and reassortment (reviewed in reference 31). The polymerase is a heterotrimer composed of the subunits PB1, PB2, and PA. The polymerase binds nucleoprotein (NP) and viral RNA to form the viral ribonucleoprotein (vRNP) complexes required for replication and transcription. The origin of the polymerase genes can alter substantially the activity of the influenza virus polymerase in a host-specific fashion (1). Viruses with avian polymerases generally have reduced transmissibility in animal models, replicate at lower levels with reduced pathogenicity in mice and ferrets, and display low levels of polymerase activity in human cells (23, 27, 35, 38, 44, 49). To overcome this natural restriction, avian viruses

acquire mutations that lead to enhanced activity in mammalian hosts. A well-characterized mutation is the conversion of PB2 amino acid (aa) 627 from the avian-signature glutamic acid to the human-signature lysine (45). PB2 K627 is correlated with enhanced polymerase activity, virus replication, transmissibility, and, in some cases, pathogenicity and mortality in humans (14, 30, 38, 44). Interestingly, the 2009 pH1N1 virus retained the avian-signature PB2 residue E627. It exploited at least one alternative adaptive strategy whereby the SR polymorphism, named for the introduction of serine and arginine at PB2 residues 590 and 591, increases polymerase activity in human cells and infection in animal models despite the presence of E627 (27, 51). We have proposed that adaptive mutations in the domain containing residue 627 permit escape from an inhibitory activity present in human cells that targets avian polymerases, although the molecular mechanism(s) controlling this defect is unclear (23, 28, 29, 35).

The reassortment of the polymerase plays a role in regulating host-specific functions. Polymerases from the 1957, 1968, and 2009 pandemics were reassortants (12, 22, 40). Whereas PA is essential for polymerase function, whether the reassortment of PA subunits with different viral origins affects species tropism and pathogenicity is poorly understood. Here we investigated the potential for gene exchange between avian and human influenza virus polymerases, including those of 2009 pH1N1, to rescue the activity of an avian polymerase in human cells. We show that an avian polymerase containing a PA subunit from human isolates overcomes restriction in human cells. Those with PA subunits from 2009 pH1N1 possessed the highest levels of activity. We mapped this enhancing activity in multiple seasonal influenza viruses to a single amino acid, PA amino acid 552, which was identified previously by bioinformatic analysis as a residue marking host shifts (4, 8, 11, 46, 48). Viruses created with these reassorted polymerases had enhanced replication kinetics in culture and enhanced pathogenicity in animal infections. Thus, the exchange of PA genes between human and avian viruses, both of which currently circulate in pigs (24), could preadapt viruses for growth in humans and lower the barrier to cross-species transmission.

MATERIALS AND METHODS

Plasmids, antibodies, and cells. Expression vectors for influenza virus polymerase proteins and NPs from A/California/07/2009 (CA07), A/Utah/01/2009 (UT1), A/Utah/02/2009 (UT2), A/green-winged teal/OH/175/1983 (S009), A/Brevig Mission/1918 (1918), and A/New York/312/2001 (NY312) were created as previously described (27). PA mutations were engineered by site-directed mutagenesis and confirmed by sequencing. Vectors expressing proteins from A/WSN/1933 were a kind gift of E. Fodor. The vRNA-luciferase reporter plasmid pHH21-vNA-luc contains the luciferase-encoding sequence in the antisense orientation in the context of the vNA gene segment from the A/WSN/33 isolate (36). PA proteins were cloned into the bidirectional pBD vector for virus rescue. Antibodies recognizing PB1, PB2, and PA were created in rabbits (28, 36). 293T, MDCK, A549, and DF1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS).

Polymerase activity assays. 293T cells were transfected in triplicate with TransIt-LT1 (Mirus) with vectors expressing the indicated PB1, PB2, PA, and NP proteins along with the vNA-luc reporter construct. Luciferase activity was measured 20 to 24 h after transfection by using BrightGlo reagent (Promega). Viral proteins were detected by Western blotting.

Multicycle replication assay. Recombinant virus was produced in 293T cells transfected by using TransIT-LT1 (Mirus) with plasmids pTMΔRNP and pBD encoding the PB1, PB2, and NP genes derived from S009 and the indicated PA proteins (27). The virus titer was determined

on MDCK cells by a plaque assay, and the virus was used to infect A549 cells at 33°C at a multiplicity of infection (MOI) of 0.1. Three independent infections were performed for each virus. Aliquots were recovered, and titers were determined on MDCK cells.

Mouse infections. Animal experiments were performed in an enhanced animal biosafety level 3 (BSL3) laboratory at the National Institutes of Health under an NIH Animal Care and Use Committee-approved animal study protocol. Groups of 8- to 10-week-old female BALB/c mice ($n = 5$) were inoculated intranasally with 2×10^5 PFU of virus under light isoflurane anesthesia. Body weight was monitored daily, and mice losing >20% of their original body weight were humanely euthanized.

RESULTS

Avian influenza virus polymerases generally function poorly in human cells, especially those that retain the restrictive glutamic acid at residue 627 in PB2 (23, 27, 38, 45). The avian polymerase cloned from the A/green-winged teal/Ohio/175/1986 (H2N1) viral isolate (9) (here termed S009) possesses the avian-signature PB2 residue E627 and exhibits low levels of activity in human cells compared to polymerases from human influenza viruses (27). We have previously shown that reassorted S009 polymerases containing a PA subunit from seasonal human influenza virus isolates rescued the activity of this otherwise severely restricted polymerase in human cells (27). We therefore sought to test the generality of our initial observations and assess the ability of polymerase subunits from 2009 pH1N1 isolates to create polymerase reassortants that function in human cells.

Polymerase activity assays were performed with human 293T cells expressing a luciferase-based viral reporter construct, the viral nucleoprotein (NP), and the polymerase subunits PB1, PB2, and PA (28, 36). Reassortants were created by the replacement of one subunit of the polymerase or NP with that from a human isolate. The replacement of S009 PB1 with that from a human viral isolate significantly reduced polymerase activity, regardless of whether PB1 was derived from the laboratory-adapted isolate A/WSN/33 (H1N1) (WSN), the primary viral isolates A/Brevig Mission/1918 (H1N1) (1918) and A/New York/312/2001 (H1N1) (NY312), or the 2009 pandemic isolate A/Utah/01/2009 (H1N1) (UT1), A/Utah/02/2009 (H1N1) (UT2), or A/California/07/2009 (H1N1) (CA07) (Fig. 1A). In agreement with data from previous reports, these data suggest that PB1 reassortants are less likely to be functional, possibly due to multiple constraints placed on PB1, including three discrete protein-protein interactions (PB1 binding to NP, PB2, and PA), two RNA binding events (PB1 binding to the 5′ and 3′ untranslated regions [UTRs] of genomic RNA), and catalysis (5, 25, 27). This stands in contrast to the 1957 and 1968 pandemic viruses, where the PB1 subunit was exchanged by reassortment, although in this case, it was the introduction of an avian PB1 into a human influenza virus polymerase (22). NP reassortants displayed variable levels of activity (Fig. 1B). NPs from WSN and 1918 slightly increased the activity by ~2- to 4-fold, whereas NP from NY312 modestly reduced activity to 63% of that of S009. Replication complexes with the swine-origin 2009 pH1N1 NP proteins from UT1, UT2, and CA07 showed no significant difference from those composed wholly of S009 subunits.

The low levels of S009 polymerase activity in human cells were previously mapped to PB2 (27). As expected, the replacement of the PB2 subunit with that from human viral isolates relieved restriction and significantly increased the polymerase activity (Fig. 1C). PB2 subunits from the WSN, 1918, and NY312 viruses, which contain the human-signature residue K627, increased activity ≥3
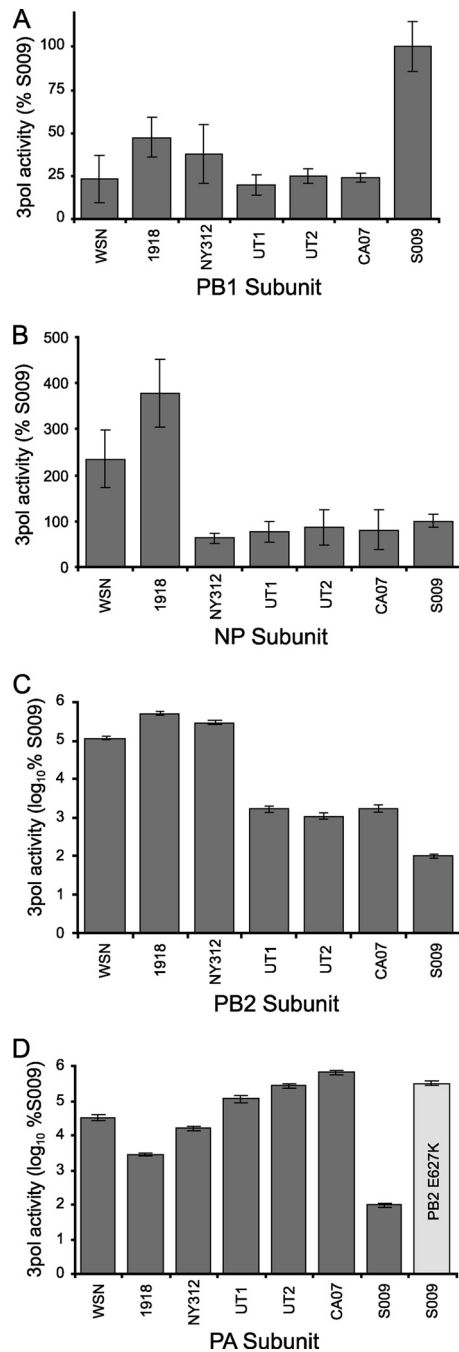
FIG 1 Human PA subunits enhance the activity of an avian influenza virus polymerase. Polymerase activity assays were performed with human 293T cells by transfecting vectors expressing S009 polymerase subunits, NP, and a vNA-based luciferase reporter. Reassortants were created by substituting the indicated human PB1 (A), NP (B), or PA (C) subunits. Data were normalized to the activity observed for the polymerase (3pol) composed completely of S009 subunits and are presented on a log scale in the case of panel C ($n = 3 \pm$ standard deviation [SD]).

logs. The substitution of PB2 from 2009 pH1N1 isolates UT1, UT2, and CA07, which contain the SR polymorphism, conveyed a smaller increase in activity, consistent with the less-potent effect of the SR polymorphism than that of the E627K mutation (27, 51).

S009 polymerase reassortants containing PAs from WSN,

1918, and NY312 displayed dramatic increases in activity (Fig. 1D), consistent with our previous results (27). We next assessed the abilities of 2009 pH1N1 PA clones to reconstitute polymerase activity (Fig. 1D). These PA proteins are of avian origin and thought to have been introduced into swine ca. 1992 (40), yet surprisingly, PAs from UT1, UT2, and CA07 demonstrated the most robust enhancement, with levels of activity 1,120 to 6,460 times higher than that of S009. These PA subunits created polymerases with activity comparable to that of the S009 PB2 E627K mutant or the reassortants with PB2 from WSN, 1918, or NY312, suggesting that the polymorphisms in PA might completely overcome the restriction associated with an avian PB2 (Fig. 1D). Furthermore, these results suggest that 2009 pH1N1 PA underwent adaptive changes while circulating in pigs prior to transmission to humans, providing functional data to support similar conclusions from recent evolutionary modeling studies (46). Changes at PA residues 85, 186, and 336 are almost exclusive to 2009 pH1N1 viruses and contribute to its adaption to humans (3). Thus, even though the PA subunits in 2009 pH1N1 viruses are of recent avian origin, they function efficiently in human cells to rescue the activity of the S009 polymerase in human cells.

Polymorphisms in PA influencing pathogenicity and host adaptation have been identified throughout the protein (reviewed in reference 31). Adaptive changes in 2009 pH1N1 PA have been mapped to the N- and C-terminal domains (3). To localize the contribution of discrete changes in PA from 1918, NY312, and WSN, we created chimeric proteins. Biochemical and structural studies showed that PA forms a distinct N-terminal endonuclease domain and a C-terminal domain that binds PB1 (Fig. 2A) (7, 13, 16, 32, 52). Chimeric PA proteins were created by fusing coding sequences from isolates S009, NY312, and 1918 at amino acid 257, the proteolytic site separating the N- and C-terminal domains (13) (Fig. 2A). Polymerases were reconstituted in 293T cells with native PA proteins or their cognate chimeras, and activity was measured. As before, the polymerase containing PA from WSN, 1918, and NY312 possessed increased activity (Fig. 2B). Chimeras with N-terminal human and C-terminal S009 domains (PA NY312$_N$-S009$_C$ and PA 1918$_N$-S009$_C$) displayed reduced polymerase activity compared to that of full-length S009 PA, 53% and 22%, respectively, and over 30-fold lower levels of activity than those of polymerases containing full-length NY312 or 1918 PA. This finding suggests that the N terminus of S009 might contain polymorphisms required for full polymerase activity. By contrast, chimeras with N-terminal S009 and human C-terminal domains (PA S009$_N$-NY312$_C$ and S009$_N$-1918$_C$) increased the polymerase activity to 78- and 67-fold higher than that of S009. The levels of activity of these PA S009$_N$:human$_C$ chimeras are higher than those of their full-length human counterparts, providing further support for enhancing mutations in the N terminus of S009 and suggesting potential additive effects of the polymorphisms present in the different domains. The rescue afforded by chimeric PAs with a human C-terminal domain is better than that of the S009 polymerase containing the PB2 SR polymorphism, which is partly responsible for the adaptation of the 2009 pH1N1 PB2 segment (27, 51), but remains substantially less than that of S009 PB2 E627K (Fig. 2). These data suggest that the majority of the polymorphisms contributing to enhanced polymerase activity reside in the C-terminal domain of human PA proteins.

Sequence analysis of the C-terminal domain (aa 257 to 716) identified several amino acid polymorphisms between S009 and
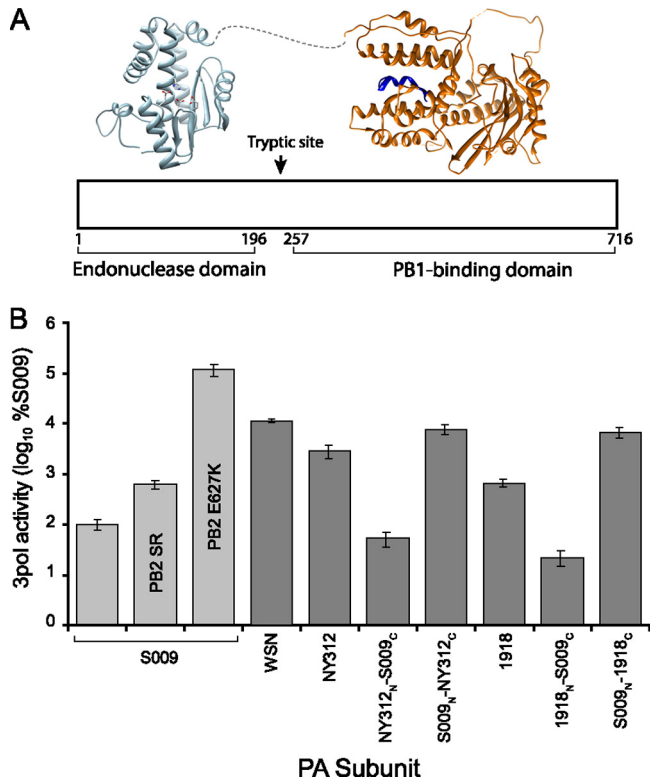
FIG 2 Chimeric PA subunits localize the enhancing activity of PA to the C terminus. (A) Diagram detailing chimeric proteins fused at the native tryptic cleavage site. N- and C-terminal structures were rendered from the structure reported under Protein Data Bank (PDB) accession numbers 3EBJ (52) and 3CM8 (16), respectively. (B) Polymerase activity assays performed with human cells expressing S009 proteins with the indicated point mutations (light gray) or heterologous PA proteins ($n = 3 \pm$ SD).

the human PA proteins used here. We identified 32 polymorphisms, 10 of which were common to the WSN, 1918, and NY312 PA proteins. We compared the frequencies of these polymorphisms in avian versus human isolates and subsequently focused on positions 388, 400, 423, 476, 552, and 630, which showed changes between S009 PA and PA proteins from human influenza viruses circulating prior to 2009 (Table 1). Note that while S009 differs from human isolates at these six positions, the majority of avian viruses differ from human isolates only at residues 400 and 552. Moreover, the polymorphisms present in S009 PA at positions 423, 476, and 630 are rare, even within avian isolates, indicating that this might be an evolutionarily divergent PA subunit. To map the enhancing activity observed for the chimeras, we introduced individual mutations into S009 PA converting avian residues to their human counterparts. The functionality of polymerases with mutant PA subunits was determined by using the polymerase activity assay. In human cells, most of the mutations had modest yet statistically significant ($P < 0.05$ by Student's t test) ~2-fold effects; the PA G388S mutant possessed reduced activity, whereas the P400L, M423I, V476A, and V630E mutants possessed increased activity (Fig. 3). Strikingly, the majority of the enhancing activity was conveyed by the PA T552S mutation, which increased polymerase activity more than 20-fold. Western blotting of human cell lysates confirmed that the PA mutants, PB2, and PB1 were all expressed at comparable levels, eliminating

this variable as a potential explanation for the enhanced activity. (Fig. 3). When the experiments were performed with chicken cells, the activities of all of the mutants were similar to that of the WT (Fig. 3), and the mutants were expressed at comparable levels (data not shown). Whereas a temperature-dependent effect on species-specific polymerase activity and virus replication was reported in certain cases (15, 26), we have not observed similar results with our system (28). As the experiments reported here were performed only at 37°C, we cannot exclude the possibility that temperature might influence the ability of these PA mutants to affect polymerase activity. Thus, the PA T552S mutation selectively enhanced the activity of an impaired avian polymerase in human cells, raising the possibility that mutations in PA are sufficient to overcome this barrier to cross-species transmission.

Polymerase activity is essential for virus replication, and adaptive mutations have been shown to enhance replication, pathogenesis, and transmission (14, 38, 44, 45). However, there is not necessarily an absolute correlation between the polymerase function in the *in vitro* activity assay and virus replication, which is indicative of the complex interplay between the replication machineries throughout the viral life cycle (25). To test the role of PA reassortment during infection, we rescued chimeric WSN viruses containing S009 PB1, PB2, and NP with S009, WSN, 1918, or NY312 PA. Human A549 cells were infected with recombinant virus, and multicycle replication kinetics were determined. Titers were determined at the indicated time points by a plaque assay (Fig. 4A). Cells infected with the virus encoding the S009 polymerase generated moderate viral titers. This finding parallels data from our polymerase activity assays, which showed that the S009 polymerase is impaired in human cells, and supports previous work showing a reduced replication of avian viruses in human cells (23, 35, 38). Conversely, a virus containing a human PA subunit replicated faster and to significantly higher levels than those containing a wholly avian polymerase ($P < 0.05$ for all save NY312 at 72 h by Student's t test) (Fig. 4A). Similar experiments were performed with the PA T552S point mutant. Chimeric virus was rescued with S009 PB1, PB2, NP, and either WT or T552S PA and used to infect A549 cells (Fig. 4B). As before, the virus containing the S009 replication genes replicated to low levels. The introduction of the PA T552S mutation resulted in higher levels of replication, consistent with this mutation relieving the restriction of the S009 polymerase (Fig. 3). These data demonstrate that the acquisition of a human PA subunit, or even just the PA T552S mutation, increases the replication of a virus containing an avian polymerase, possibly as a result of increased polymerase activity.

We further determined if polymerase reassortment increases viral pathogenicity in the mouse model of infection. Six-week-old BALB/c mice were infected in groups of 5 with 2×10^5 PFU of recombinant virus (Fig. 4C). Body weight was monitored for 14 days as a measure of pathogenicity. Mice infected with a virus encoding a human PA displayed rapid weight loss that was significantly faster than the weight loss of those infected with a virus encoding S009 PA ($P < 0.05$ by Student's t test). Specifically, mice infected with reassortants containing the 1918 or NY312 PA gene met endpoint criteria, with a loss of >20% of their body weight, and were euthanized by day 5 postinfection (p.i.) (Fig. 4D). Mice infected with a virus containing WSN PA displayed slightly slower kinetics than did those infected with 1918 and NY312, but they all met endpoint criteria by days 5 to 6 p.i. By contrast, animals infected with viruses containing a complete S009 polymerase dis-

TABLE 1 PA polymorphism frequencies[a]

| | | Polymorphism(s) and frequency (%) | | |
| | | | Human influenza virus | |
aa	S009 residue	Avian influenza virus, pre-2009	Pre-2009	pH1N1 + related
388	G	S = 74.4 G = 19.4 R = 3.9 N = 1.6 C, D, I = ≤0.5	S = 96.8 G = 1.9 C, I, N = ≤0.5	G = 99.9 E = ≤0.5
400	P	P = 38.8 S = 36.4 Q = 18.6 H = 2.3 L = 1.5 A = 0.8 K = 0.6 F, I, M, R, T, Y = ≤0.5	L = 99.2 F, I, M, P, Q, S, V = ≤0.5	P = 99.7 L, S = ≤0.5
423	M	I = 96.2 M = 2.2 V = 1.2 A, L, T = ≤0.5	I = 99.5 L, M, R, V = ≤0.5	I = 100
476	V	A = 99.6 G, P, S, T, V = ≤0.5	A = 100 V = ≤0.5	A = 99.9 V = ≤0.5
552	T	T = 99.9 A, N = ≤0.5	S = 99.0 T = 0.7 G, I, N, R = ≤0.5	T = 99.9 A = ≤0.5
630	V	E = 99.3 A, D, G, K, V = ≤0.5	E = 97.2 D = 2.6 K = ≤0.5	E = 100

[a] Data are for sequence polymorphisms present in PA from pre-2009 avian isolates ($n = 4,108$), pre-2009 human isolates with zoonotic H5N1 infections removed ($n = 3,095$), or 2009 human H1N1 ($n = 2,878$) and highly related isolates. Data were acquired in August 2011 at the Influenza research database (www.fludb.org) from full-length PA proteins accessed by searching for the indicated host and time frame and whether the human viruses were pH1N1 and related viruses.

played a markedly reduced rate of weight loss and decline in survival, with animals in this group reaching the endpoint at days 6, 7, and 9 and with one surviving the challenge. The mouse infections showed enhanced pathogenicity with viruses that contained a human PA subunit. Additional experiments are required to determine the exact degree of pathogenicity of these chimeric viruses (i.e., 50% lethal dose [LD_{50}]) and the effects of the PA T552S mutation in animal models. Nonetheless, results from our infections in culture and in animal models show that the replication and pathogenicity of a virus with an avian polymerase increase upon the substitution of a human PA subunit. Furthermore, these results suggest that reassortment within the polymerase genes might be an effective strategy to broaden the tropism and increase the virulence of an avian virus in mammalian hosts.

DISCUSSION

The stable host switch adaptation of an avian influenza A virus to humans requires the surmounting of barriers to cross-species infectivity and transmissibility that affect steps throughout the viral life cycle. The influenza virus polymerase is a major regulator of viral host tropism and pathogenesis; polymerases derived from avian viruses are restricted in human cells and mammalian models (14, 23, 27, 35, 38, 39, 44). Polymerases likely adapt to new hosts

by both mutation and reassortment. Reassortments of influenza virus gene segments occurred in the last three pandemic outbreaks, and reassortment is a strategy exploited frequently during virus evolution in general (9, 34). Here we assessed the ability of polymerase complexes consisting of different combinations of avian and human influenza virus-derived polymerase genes to rescue the activity of an avian polymerase in human cells. We showed that PA subunits derived from human viruses restored the activity of the impaired avian S009 polymerase, even though the polymerase still included the restrictive PB2 E627 polymorphism. Polymerases reconstituted with the PA proteins derived from 2009 H1N1 isolates were the most active. Data for reassortant viruses confirmed findings from our polymerase activity assays by showing that viruses that contained the avian PB1, PB2, and NP genes but with a human PA subunit replicated faster in culture and had enhanced virulence during mouse infections. Thus, our data suggest that further reassortment between the polymerase genes of avian influenza A viruses and either seasonal human influenza, 2009 pH1N1, or previously swine-adapted viruses, all of which are currently circulating in swine and have already formed new reassortants (24, 50), has the potential to create new viruses with an expanded host range and enhanced pathogenicity.

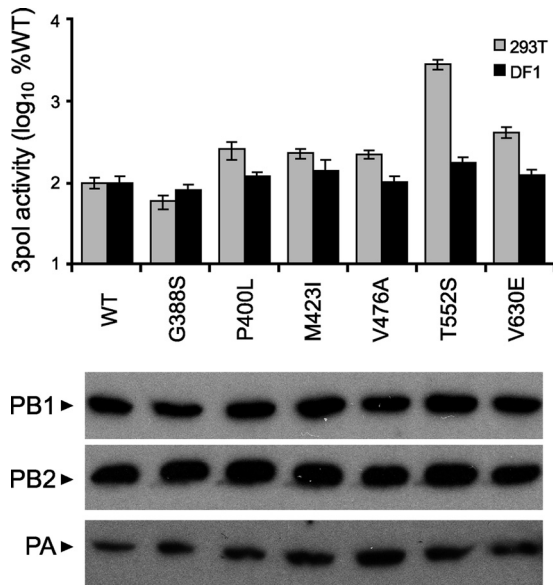The majority of the enhancing activity derived from human-

FIG 3 Introduction of the human-signature residue T552 into avian PA is sufficient to convey enhanced polymerase activity. The activity of the S009 polymerase with the indicated mutations in PA was measured in human (293T) and avian (DF1) cells. Western blots were performed on lysates from human cells using antibodies that detect PB1, PB2, and PA. ($n = 3 \pm$ SD). In 293T cells, all mutants displayed activity that was significantly different from that of the WT ($P < 0.05$ by Student's t test).

adapted influenza virus PA proteins maps to amino acid 552. The conversion of the avian-signature T552 residue to human-signature residue S552 enhanced polymerase function in human but not avian cells. These data demonstrate that the PA T552S mutation was not simply a generalized enhancer of polymerase function but rather was a species-specific regulator that overcame the restriction of the avian polymerase in human cells. PA residue 552 has not been previously shown specifically to play an important role in polymerase function in experimental systems. However, it was identified by several computational strategies as a signature residue indicative of viral origin (4, 8, 11, 46, 48). It has also been shown to undergo characteristic "host shift" changes in amino acid identity as the selective pressures on this residue change from birds to humans (44). Our work shows that PA residue 552 is important as a marker of both viral origin and species-specific polymerase function.

To better understand the adaptation of PA to new hosts, we investigated the emergence of PA residue S552 as viruses adapt from birds to pigs and human populations (Table 1). Serine is completely absent at PA residue 552 in all avian influenza A virus isolates in the database, regardless of their serotype, origin, or time of isolation. The PA of the 1918 pandemic virus had S552, and this was maintained monophyletically in almost all subsequently circulating human influenza H1N1 viruses (excluding 2009 pH1N1-derived sequences [see below]) and H2N2 and H3N2 subtypes (48). Interestingly, even though classical swine viruses are thought to be derived from a 1918 ancestor (47), over 90% of the PA genes cocirculating in swine prior to 2009 (whether classical swine virus, Eurasian avian-like swine virus, or triple-reassortant lineages) contained T552, suggesting that S552 might be a specific adaptation associated with influenza virus in humans rather than a generalized mammalian adaptation. Similarly, equine-adapted influenza
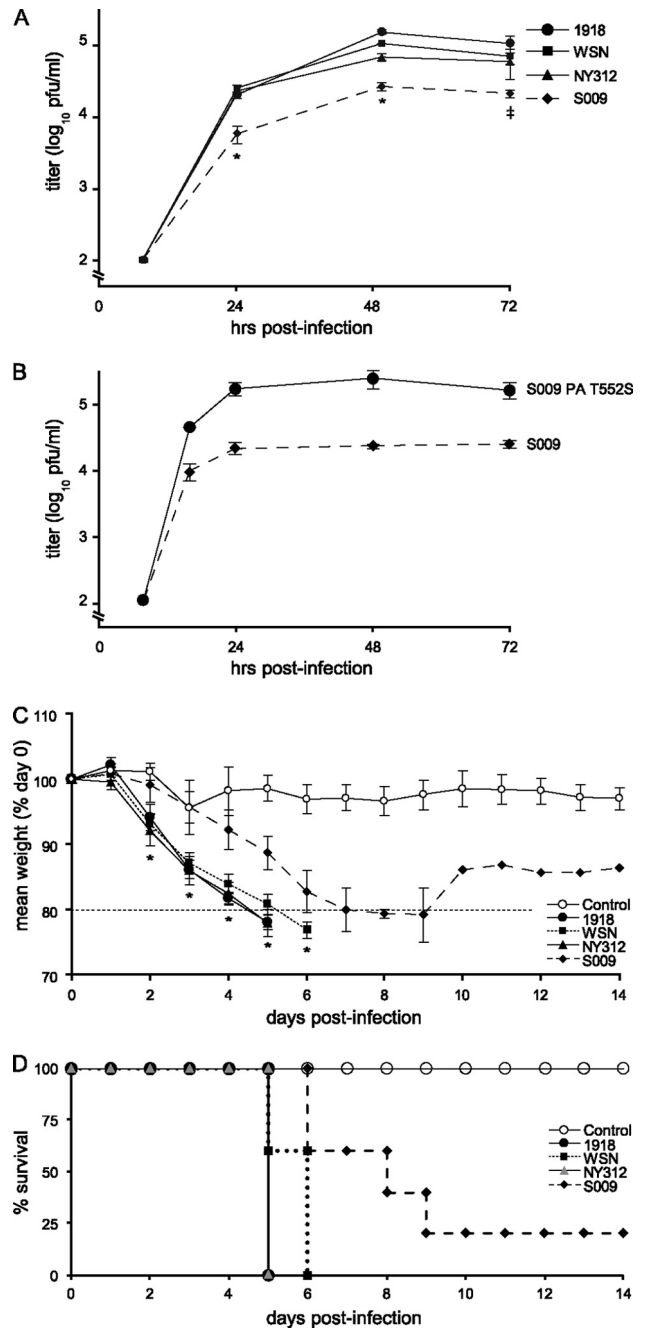

FIG 4 Viruses encoding avian polymerases with a human PA subunit have enhanced replication and pathogenicity in mammalian systems. Recombinant virus containing the S009 polymerase and NP genes in a WSN background was rescued. The PA mutant or reassortants were made as indicated. (A) Multistep growth kinetics were measured in human A549 cells infected at an MOI of 0.1. The titer was determined by a plaque assay on MDCK cells ($n = 3 \pm$ SD). Data collected at 8 h were at or below the level of detection (100 PFU/ml). Differences between S009 and reassorted polymerases were determined to be statistically significant by Student's t test (∗, $P < 0.05$ for all samples; ‡, $P < 0.05$ for 1918 and NY312). (B) Infections were performed, as described above (A), with the PA T552S mutant. Differences between the WT and the mutant were statistically significant at all time points ($P < 0.05$). (C) BALB/c mice were infected intranasally with 2×10^5 PFU of virus, and body weight was measured for 14 days ($n = 5 \pm$ SD). A pairwise statistical comparison was made between mice infected with a virus encoding the S009 polymerase and those infected with a virus encoding a reassorted polymerase (∗, $P < 0.05$ for each comparison, determined by Student's t test). (D) Survival rate of mice infected with virus containing S009 or reassorted polymerases.

viruses also retain T552. However, a discrete subset of recently sequenced isolates encompassing triple-reassortant viruses circulating in Asian swine populations with a PA segment derived by reassortment from human H3N2 viruses maintains the human-associated S552 (24).

It is noteworthy that PA from the 2009 pH1N1 lineage does not encode the T552S mutation. After 2 years of widespread human circulation, pandemic H1N1 viruses have maintained T552, suggesting that this change is not necessary for human adaptation in this genetic context, in a manner analogous to this viral lineage continuing to encode PB2 E627. Nonetheless, even without the human-signature residue S552, 2009 pH1N1 PA is a potent activator of avian influenza virus polymerases in human cells and has been shown to be a determinant of adaptation and pathogenesis in mice (Fig. 1D) (3), strongly supporting the independent and polygenic nature of influenza virus host switch events (47).

The 2009 pH1N1 polymerase genes have illuminated a new assemblage of adaptive mutations. The SR polymorphism in PB2 contributes to increased polymerase activity and replication in human cells (27, 51). The activity of the 2009 pH1N1 PA has been attributed to specific residues in the N-terminal domain, I85 and, to a lesser extent, S186, and the C-terminal domain, M336 (3). These polymorphisms are rarely present in PA proteins from human viruses prior to the outbreak and are absent from PA of S009. Reassortment experiments with 2009 H1N1 isolates have also shown that PA is important for the polymerase activity and virulence of A/California/04/2009 in mice, providing further evidence for a crucial role of the PA subunit (33, 43). Finally, mutations in PA that arose during the creation of a highly pathogenic 2009 pH1N1 variant created by serial passage in mice contributed to enhanced polymerase function and, potentially, the highly pathogenic phenotype (18). Whether PA T552 in 2009 pH1N1 viruses will mutate to S552, or if this change increases polymerase activity in 2009 pH1N1, remains to be determined, although this mutation has not arisen during multiple adaptation experiments (18, 53).

Adaptation experiments have identified other polymorphisms in PA that contribute to increased polymerase activity and pathogenicity. The PA I97 mutation arose following the serial passage of a low-pathogenicity avian H5N2 virus in mice and contributed to the high pathogenicity of the newly derived virus (42). The PA I97 mutation increased polymerase activity and replication in mice but not chickens, suggesting that it might influence pathogenicity in a species-specific fashion. Similarly, the adaptation of the laboratory-strain PR8 to a high-virulence form involved PA mutations E349G and I550L (37). The ability of PA mutations to increase virulence is not restricted to experimental settings. An H7N7 virus isolated from a fatal human infection contained the PA L666 mutation, whereas a virus isolated from a nonfatal infection encoded the rare PA F666 mutation. *In vitro* analysis showed that the PA L666 mutation increased polymerase activity preferentially in human versus quail cells, especially when paired with the PB2 K627 mutation (6). 2009 pH1N1 lacks the identified enhancing mutations at residues 97 and 349, suggesting that additional changes might continue to facilitate adaptation to replication in humans. Finally, our results using chimeric PA proteins showed that the S009 N-terminal domain contains residues that increase polymerase activity (Fig. 2B). S009 lacks the enhancing I85, I97, and S186 mutations (3, 42), raising the possibility that other novel polymorphisms in PA might also contribute to the enhanced polymerase activity. Together, these data suggest that there are multiple strategies by which mutations in PA can contribute to enhanced polymerase activity, virus replication, and, possibly, viral host tropism.

The mechanisms by which the PA T552S mutation enhances the activity of polymerases containing the avian-signature PB2 E627 residue and mediates escape from restriction in human cells are poorly defined. The N terminus of PA contains the endonuclease domain responsible for the cleavage of host mRNA during cap snatching and transcription and associates weakly with PB2, while the C terminus binds PB1 (7, 16, 17, 32, 52). PA amino acid 552 maps to a loop that projects away from the core of the C-terminal domain. This loop is not resolved in one of the structures, suggesting a degree of conformational flexibility, and its solvent exposure raises the possibility that it may be involved in protein-protein interactions. The loop is distal from the PB2 and PB1 binding sites, suggesting that polymerase assembly is unaffected by the identity of PA residue 552. This is consistent with our results showing that polymerases with either the PA S552 or T552 residue are equivalently functional in chicken cells. Alternatively, residue 552 might be important for interactions with host proteins, as PA was recently identified as the hub of a large protein binding network between the virus and the host (2). The C terminus of PA, including residue 552, interacts with host MCM proteins to enhance genome replication (21). The T552S mutation may regulate the species-specific association between PA and these or other host factors that could either enhance or inhibit polymerase function.

The 2009 pH1N1 virus emerged as a complex reassortant with a number of novel mutations, highlighting the complicated interplay among viral proteins and between viral proteins and the host. The multisubunit viral polymerase exploits both reassortment and mutation to overcome barriers to cross-species transmission and to adapt to replication in new hosts. Thus, it is not solely the appearance of "host shift" mutations that enhances the host range but the particular combination(s) of polymerase and NP genes in which they appear that is equally important. 2009 pH1N1-like viruses have been reintroduced into pigs and continue to evolve and cocirculate with avian and triple-reassortant swine influenza viruses, where they have already undergone reassortment (50). Therefore, it is important to monitor not only the evolution of enhancing polymorphisms but also the assembly of specific combinations of polymerase genes that could expand the host range and pathogenesis.

REFERENCES

1. **Almond JW.** 1977. A single gene determines the host range of influenza virus. Nature **270**:617–618.
2. **Bradel-Tretheway BG, et al.** 29 June 2011. Comprehensive proteomic analysis of influenza virus polymerase complex reveals a novel association with mitochondrial proteins and RNA polymerase accessory factors. J. Virol. doi:10.1128/JVI.00496-11.

3. Bussey KA, et al. 2011. PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. J. Virol. 85:7020–7028.

4. Chen GW, et al. 2006. Genomic signatures of human versus avian influenza A viruses. Emerg. Infect. Dis. 12:1353–1360.

5. Chen LM, Davis CT, Zhou H, Cox NJ, Donis RO. 2008. Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. PLoS Pathog. 4:e1000072.

6. de Wit E, et al. 2010. Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. J. Virol. 84:1597–1606.

7. Dias A, et al. 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458:914–918.

8. dos Reis M, Tamuri AU, Hay AJ, Goldstein RA. 2011. Charting the host adaptation of influenza viruses. Mol. Biol. Evol. 28:1755–1767.

9. Dugan VG, et al. 2008. The evolutionary genetics and emergence of avian influenza viruses in wild birds. PLoS Pathog. 4:e1000076.

10. Dunham EJ, et al. 2009. Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses. J. Virol. 83:5485–5494.

11. Finkelstein DB, et al. 2007. Persistent host markers in pandemic and H5N1 influenza viruses. J. Virol. 81:10292–10299.

12. Garten RJ, et al. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201.

13. Hara K, Schmidt FI, Crow M, Brownlee GG. 2006. Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. J. Virol. 80:7789–7798.

14. Hatta M, Gao P, Halfmann P, Kawaoka Y. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293:1840–1842.

15. Hatta M, et al. 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog. 3:1374–1379.

16. He X, et al. 2008. Crystal structure of the polymerase PA(C)-PB1(N) complex from an avian influenza H5N1 virus. Nature 454:1123–1126.

17. Hemerka JN, et al. 2009. Detection and characterization of influenza A virus PA-PB2 interaction through a bimolecular fluorescence complementation assay. J. Virol. 83:3944–3955.

18. Ilyushina NA, et al. 2010. Adaptation of pandemic H1N1 influenza viruses in mice. J. Virol. 84:8607–8616.

19. Ito T, et al. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. 72:7367–7373.

20. Jenkins GM, Rambaut A, Pybus OG, Holmes EC. 2002. Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. J. Mol. Evol. 54:156–165.

21. Kawaguchi A, Nagata K. 2007. De novo replication of the influenza virus RNA genome is regulated by DNA replicative helicase, MCM. EMBO J. 26:4566–4575.

22. Kawaoka Y, Krauss S, Webster RG. 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. 63:4603–4608.

23. Labadie K, Dos Santos Afonso E, Rameix-Welti MA, van der Werf S, Naffakh N. 2007. Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. Virology 362:271–282.

24. Lam TT, et al. 2011. Reassortment events among swine influenza A viruses in China: implications for the origin of the 2009 influenza pandemic. J. Virol. 85:10279–10285.

25. Li C, Hatta M, Watanabe S, Neumann G, Kawaoka Y. 2008. Compatibility among polymerase subunit proteins is a restricting factor in reassortment between equine H7N7 and human H3N2 influenza viruses. J. Virol. 82:11880–11888.

26. Massin P, Rodrigues P, Marasescu M, van der Werf S, Naffakh N. 2005. Cloning of the chicken RNA polymerase I promoter and use for reverse genetics of influenza A viruses in avian cells. J. Virol. 79:13811–13816.

27. Mehle A, Doudna JA. 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. Proc. Natl. Acad. Sci. U. S. A. 106:21312–21316.

28. Mehle A, Doudna JA. 2008. An inhibitory activity in human cells restricts the function of an avian-like influenza virus polymerase. Cell Host Microbe 4:111–122.

29. Moncorge O, Mura M, Barclay WS. 2010. Evidence for avian and human host cell factors that affect the activity of influenza virus polymerase. J. Virol. 84:9978–9986.

30. Munster VJ, et al. 2007. The molecular basis of the pathogenicity of the Dutch highly pathogenic human influenza A H7N7 viruses. J. Infect. Dis. 196:258–265.

31. Naffakh N, Tomoiu A, Rameix-Welti MA, van der Werf S. 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. Annu. Rev. Microbiol. 62:403–424.

32. Obayashi E, et al. 2008. The structural basis for an essential subunit interaction in influenza virus RNA polymerase. Nature 454:1127–1131.

33. Octaviani CP, Ozawa M, Yamada S, Goto H, Kawaoka Y. 2010. High level of genetic compatibility between swine-origin H1N1 and highly pathogenic avian H5N1 influenza viruses. J. Virol. 84:10918–10922.

34. Rambaut A, et al. 2008. The genomic and epidemiological dynamics of human influenza A virus. Nature 453:615–619.

35. Rameix-Welti MA, Tomoiu A, Dos Santos Afonso E, van der Werf S, Naffakh N. 2009. Avian influenza A virus polymerase association with nucleoprotein, but not polymerase assembly, is impaired in human cells during the course of infection. J. Virol. 83:1320–1331.

36. Regan JF, Liang Y, Parslow TG. 2006. Defective assembly of influenza A virus due to a mutation in the polymerase subunit PA. J. Virol. 80:252–261.

37. Rolling T, et al. 2009. Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of influenza A virus in mice. J. Virol. 83:6673–6680.

38. Salomon R, et al. 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J. Exp. Med. 203:689–697.

39. Shinya K, et al. 2004. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320:258–266.

40. Smith GJ, et al. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122–1125.

41. Smith GL, Hay AJ. 1982. Replication of the influenza virus genome. Virology 118:96–108.

42. Song MS, et al. 2009. The polymerase acidic protein gene of influenza A virus contributes to pathogenicity in a mouse model. J. Virol. 83:12325–12335.

43. Song MS, et al. 2011. Virulence and genetic compatibility of polymerase reassortant viruses derived from the pandemic (H1N1) 2009 influenza virus and circulating influenza A viruses. J. Virol. 85:6275–6286.

44. 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:e1000252.

45. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. 67:1761–1764.

46. Tamuri AU, Dos Reis M, Hay AJ, Goldstein RA. 2009. Identifying changes in selective constraints: host shifts in influenza. PLoS Comput. Biol. 5:e1000564.

47. Taubenberger JK, Kash JC. 2010. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe 7:440–451.

48. Taubenberger JK, et al. 2005. Characterization of the 1918 influenza virus polymerase genes. Nature 437:889–893.

49. 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. U. S. A. 106:3366–3371.

50. Vijaykrishna D, et al. 2010. Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science 328:1529.

51. Yamada S, et al. 2010. Biological and structural characterization of a host-adapting amino acid in influenza virus. PLoS Pathog. 6:e1001034.

52. Yuan P, et al. 2009. Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. Nature 458:909–913.

53. Zhou B, et al. 2011. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza A viruses in mice. J. Virol. 85:357–365.

54. Zhou NN, et al. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J. Virol. 73:8851–8856.