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Authors
Mehle, Andrew
Doudna, Jennifer A

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Adaptive strategies of the influenza virus polymerase for replication in humans

Andrew Mehle[a] and Jennifer A. Doudna[a,b,c,1]

[a]Departments of Molecular and Cell Biology and [b]Chemistry, Howard Hughes Medical Institute, University of California, Berkeley, CA 94705; and [c]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Contributed by Jennifer A. Doudna, October 19, 2009 (sent for review September 28, 2009)

Transmission of influenza viruses into the human population requires surmounting barriers to cross-species infection. Changes in the influenza polymerase overcome one such barrier. Viruses isolated from birds generally contain polymerases with the avian-signature glutamic acid at amino acid 627 in the PB2 subunit. These polymerases display restricted activity in human cells. An adaptive change in this residue from glutamic acid to the human-signature lysine confers high levels of polymerase activity in human cells. This mutation permits escape from a species-specific restriction factor that targets polymerases from avian viruses. A 2009 swine-origin H1N1 influenza A virus recently established a pandemic infection in humans, even though the virus encodes a PB2 with the restrictive glutamic acid at amino acid 627. We show here that the 2009 H1N1 virus has acquired second-site suppressor mutations in its PB2 polymerase subunit that convey enhanced polymerase activity in human cells. Introduction of this polymorphism into the PB2 subunit of a primary avian isolate also increased polymerase activity and viral replication in human and porcine cells. An alternate adaptive strategy has also been identified, whereby introduction of a human PA subunit into an avian polymerase overcomes restriction in human cells. These data reveal a strategy used by the 2009 H1N1 influenza A virus and identify other pathways by which avian and swine-origin viruses may evolve to enhance replication, and potentially pathogenesis, in humans.

2009 A(H1N1) | PB2 | species barriers

Influenza viruses circulating in animal reservoirs represent a significant public health threat as a potential source of pandemic viruses. Highlighting these concerns are the occasional infection of humans with highly pathogenic H5N1 viruses circulating in avian populations, and recently, the emergence of a 2009 swine-origin H1N1 influenza A virus [2009 A(H1N1)] in humans (1, 2). The 2009 A(H1N1) virus was first detected in humans in early 2009 and has since been declared a pandemic by the World Health Organization. The processes regulating emergence of viruses into the human population involve a complex interplay between virus and host (3). Understanding the mechanisms by which influenza viruses acquire the ability to infect multiple species is thus imperative to controlling future outbreaks.

A major determinant of viral tropism is the influenza virus polymerase (4). The polymerase, composed of viral proteins PB1, PB2, and PA, assembles with viral RNA and nucleoprotein (NP) to mediate transcription and replication of the viral genome. A single residue in the PB2 subunit of the influenza polymerase, amino acid 627, regulates polymerase activity in a species-specific fashion (5). PB2 derived from human viral isolates almost exclusively possesses a lysine at position 627 (K627), whereas glutamic acid (E627) at this position predominates in avian viruses (6). PB2 K627 correlates with enhanced polymerase activity, virus replication, transmission, and in certain cases, pathogenicity and mortality in mammals (5, 7–16). PB2 K627 also correlates with moderately enhanced influenza virus replication in pigs, consistent with pigs serving as an intermediary viral reservoir between birds and humans (17). Conversely, the presence of a glutamic acid at this position severely attenuates replication efficiency and pathogenicity in mammalian systems (7, 9). Our recent results suggest that the glutamic acid-to-lysine mutation facilitates escape from an inhibitory factor that restricts the function of avian-derived polymerases in human cells (11). The identity of the putative inhibitor and the molecular basis for the activity associated with changes at amino acid 627 have not yet been established.

Strikingly, approximately two-thirds of the H5N1 viruses recovered from human infections retain the inhibitory avian-like E627 (Table S1). The polymerase from this virus has been identified as a virulence determinant, and PB2 E627 correlates with reduced pathogenicity and transmissibility in animal models, and possibly humans (7, 8, 10, 15). Yet, the current mortality rate for confirmed H5N1 infections is 59.3%*. Moreover, all of the 2009 A(H1N1) isolates to date possess the avian-signature E627, but this virus replicates and is efficiently transmitted in humans and animal models (1, 2, 18–20). We therefore sought to determine alternative adaptive strategies used by the influenza virus polymerase to escape restriction in human cells. Here, we identify and characterize two mechanisms that mediate enhanced polymerase activity in human cells for viruses containing the restricted PB2 E627: evolution of a second-site suppressor polymorphism in the PB2 subunit of 2009 A(H1N1) influenza polymerases and reassortment of a human PA subunit into an avian polymerase.

Results

SR Polymorphism of 2009 A(H1N1) Polymerases Helps Evade Restriction in Human Cells. The PB2 subunit from the 2009 A(H1N1) viruses contains a glutamic acid at position 627, which is normally correlated with impaired polymerase activity in human cells. Nonetheless, 2009 A(H1N1) viruses replicate and cause disease in humans and animal models (1, 2, 18–21). We therefore tested the activity of polymerases derived from 2009 A(H1N1) isolates. Polymerase activity assays were performed in human 293T cells expressing a luciferase-based reporter construct, NP, and the polymerase subunits PB1, PA, and WT or mutant PB2. Viral genes were cloned from the primary human isolates A/Brevig Mission/1918 (1918), A/New York/312/2001 (NY312), A/Utah/01/2009 (UT1), A/Utah/02/2009 (UT2), A/California/07/2009 (CA07), and the avian isolate A/Green-winged Teal/Ohio/175/1986. Western blot analysis confirmed that WT and mutant polymerases for each isolate were expressed at similar levels (Fig. 1*A*). Polymerases derived from 1918 and NY312 viruses displayed high levels of activity when compared to their cognate PB2 K627E mutants. Conversely, the avian S009 poly-

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: doudna@berkeley.edu.

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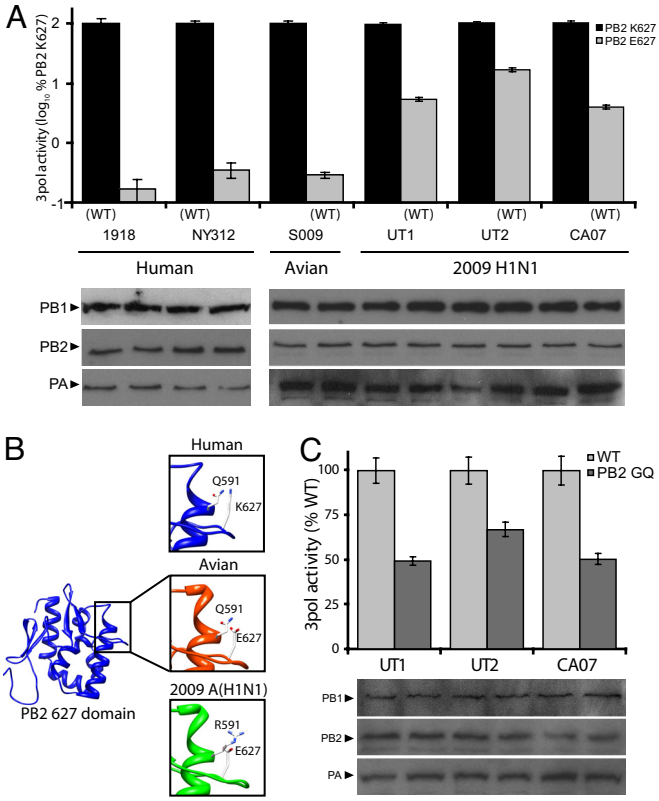

Fig. 1. Identification of an adaptive strategy utilized by the polymerase of 2009 A(H1N1) in human cells. (*A*) Cell-based activity assays for influenza polymerases from human, avian, and 2009 A(H1N1) primary isolates containing WT or mutant PB2. Activity assays were performed in 293T cells and normalized to PB2 K627 for comparison. The identity of PB2 amino acid 627 for the naturally occurring (WT) and mutant variants for each isolate are indicated. Western blot analyses were performed on lysates used in the polymerase activity assays with the indicated antibodies. (*B*) Structural model of the SR polymorphism in the PB2 627 domain. Structures of the human and avian PB2 627 domains (27) are shown along with a model of the 2009 A(H1N1) domain. (*C*) The SR polymorphism contributes to polymerase activity for 2009 A(H1N1) isolates in human cells. Assays were performed as in *A* using WT PB2 or the GQ mutant (S590G/R591Q). Polymerase proteins were detected by Western blot analysis. For all activity assays, $n = 3 \pm$ standard deviation.

merase displayed low levels of activity compared to S009 containing a PB2 E627K mutation, which was approximately 800-fold more active. In a similar fashion, introducing the PB2 E627K mutation into 2009 A(H1N1) polymerases significantly increased activity compared to the native polymerase. Thus, despite efficient replication in humans and animal models (1, 2, 18–20), the 2009 A(H1N1) polymerases remain partially restricted by the presence of a glutamic acid at position 627.

WT 2009 A(H1N1) polymerases demonstrated 4–16.5% the activity of a K627-containing mutant. This was significantly higher than the <1% activity observed for other polymerases containing PB2 E627 (i.e., WT S009 or the PB2 K627E mutants for NY312 and 1918) (Fig. 1*A*). This observation raises the possibility that other sequence polymorphisms within 2009 A(H1N1) polymerases might increase polymerase activity. Sequence analysis of PB2 genes identified a pair of amino acid variants conserved in 2009 A(H1N1) viruses that are exceedingly rare in previous human influenza isolates: serine at position 590 and arginine at position 591, termed the SR polymorphism (Fig. S1). This paired polymorphism is present in only three of the 2,849 PB2 sequences derived from human isolates before 2009 (Table S1). Two of these isolates were from individuals that had documented exposure to swine and were infected by "triple reassortant" viruses containing human PB1, avian PB2 and PA, and classic swine NP, similar to the 2009 A(H1N1 viruses) (22, 23). The SR polymorphism is more prevalent in pigs, present in >20% of the sequenced isolates, but only appears after the establishment of the triple reassortant swine viruses that emerged in 1998–1999 (24, 25). For all isolates, the SR polymorphism occurs only when there is also the E627 variant. Modeling these variants onto the PB2 627 domain structure predicts the juxtaposition of R591 and E627, suggesting that these two residues might interact (Fig. 1*B*).

We investigated the role of the SR polymorphism in polymerase function using a polymerase activity assay. The SR polymorphism in PB2 from 2009 A(H1N1) isolates was mutated to the consensus sequence of G590Q591 (PB2 SR→GQ). Removal of the SR polymorphism reduced polymerase activity by approximately 50% for all three primary isolates (Fig. 1*C*), suggesting a potential role in host-specific adaptation. Mutation of the SR polymorphism did not affect polymerase protein steady-state levels (Fig. 1*C*). Similar results obtained in polymerase activity assays performed at 33 °C (Fig. S2*A*) indicate that the effect of the SR polymorphism on polymerase function also occurs at the temperature of the upper respiratory tract in humans. Comparing UT1 PB2 mutants in a single experiment confirms that WT polymerase is twice as active as the PB2 SR→GQ mutant and shows that the PB2 E627K mutation increases activity an additional 12.4-fold over WT (Fig. S2*B*). The fully humanized UT1 double mutant, which lacks the SR polymorphisms and has a lysine at position 627 (termed GQ+K), was the most active. The SR polymorphism enhanced the activity of polymerases containing PB2 E627, but did not further increase the activity of polymerases containing PB2 K627, suggesting that the SR polymorphism functions primarily to alleviate the restriction associated with PB2 E627 (Fig. S2*B*). Experiments in animal models are required to confirm the role of the SR polymorphism in vivo. These results identify the SR polymorphism as a regulator of polymerase activity in human cells and begin to provide an explanation for the unexpected activity associated with 2009 A(H1N1) polymerases.

SR Polymorphism Rescues Activity of an Avian Influenza Polymerase. On rare occasions, humans are directly infected with avian influenza virus, including H5N1 viruses (26). Most of the H5N1 viruses isolated from humans contain a glutamic acid at position 627, but none possesses the SR polymorphism (Table S1). We therefore tested whether the effects of the SR polymorphism are unique to 2009 A(H1N1) viruses or whether they represent a general adaptive strategy that can function in a heterologous avian virus isolate. PB2 from the avian isolate S009 contains the consensus GQ sequence at amino acids 590 and591 and a glutamic acid at amino acid 627. As such, activity of this polymerase is severely restricted in human cells (Fig. 1*A*).

Polymerase mutants were constructed that contained the SR polymorphism and/or a lysine at amino acid at 627. Introducing the SR polymorphism to the restricted S009 PB2 increased polymerase activity 26-fold over WT (Fig. 2*A*), a significantly more potent rescue than the 2-fold effect observed for 2009 A(H1N1) (Fig. 1*C*). Polymerase activity was fully restored by the PB2 E627K mutation, increasing activity approximately 800-fold over WT. Mutant polymerase with both the SR polymorphism and K627 (SR+K) did not display additional enhancement over the PB2 E627K mutant. In contrast to results performed in human cells, all PB2 variants displayed approximately equivalent levels of activity when assays were performed in avian cells (Fig. 2*A*). Equivalent expression of subunits for each polymerase complex was confirmed by Western blot analysis. Thus, the SR polymorphism functions in several viral isolates to selectively enhance the activity of restricted polymerases in mammalian cells.

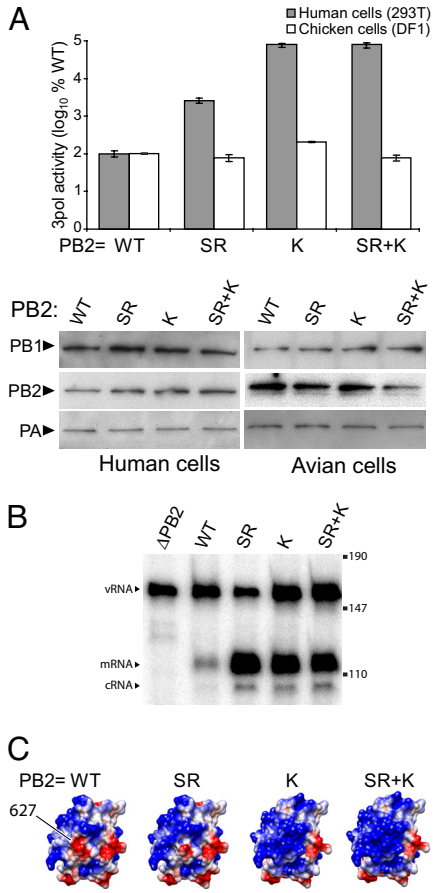

Fig. 2. The SR polymorphism enhances activity of an avian influenza polymerase. (*A*) Polymerase activity assays were performed with the avian S009 polymerase containing WT or mutant PB2. The SR polymorphism was introduced into PB2 SR (G590S/Q591R). The human-signature lysine was introduced into PB2 K (E627K). Activity was measured for polymerases expressed in human (293T) and avian (DF1) cells and normalized to WT PB2. $n = 3 \pm$ standard deviation. PB1, PB2, and PA were detected by Western blot. (*B*) Primer extension analysis of viral RNAs produced by WT or mutant polymerase in 293T cells. Migration of molecular weight standards is indicated in base pairs. (*C*) Electrostatic surface potentials were mapped onto models of S009 PB2 627 domains and colored from −4 kT/e (red) to 4 kT/e (blue).

To directly assess enzyme activity of the variant polymerases, primer extension analysis was used to measure the products of RNA replication [viral RNA (vRNA) and copy RNA (cRNA)] and transcription (mRNA). In agreement with the polymerase activity assays, introducing the SR polymorphism to the S009 polymerase significantly increased viral mRNA production when compared to WT (Fig. 2*B*). The SR polymorphism also rescued RNA replication, as evidenced by the detection of the obligate replication intermediate cRNA. Levels of vRNA were consistently reduced in the presence of the SR mutant, suggesting that the SR mutation may alter the balance of replication products, although further experimentation is required to fully understand this observation. Both PB2 E627K and PB2 SR+K also produced significant amounts of mRNA, cRNA and increased levels of vRNA. These data demonstrate that the SR polymorphism present in the 2009 A(H1N1) polymerase partially rescues activity of an otherwise impaired polymerase by restoring both replication and transcription activities.

Structural analysis of the PB2 627 domain has shown that K627 and E627 variants possess nearly identical structures (27). The primary difference is that a glutamic acid at position 627 disrupts a large positively charged surface on the domain. We modeled the structure of WT and mutant forms of the S009 PB2 627 domain to assess the electrostatic surface potential (Fig. 2*C*). Increased polymerase activity correlates well with the predicted restoration of the positively charged surface of the 627 domain (Fig. 2), raising the possibility that the SR mutation stimulates polymerase function by partially neutralizing E627.

SR Polymorphism Enhances Virus Replication. To determine the role of the SR polymorphism during viral infections, we reconstituted virus containing PB2 variants. For safety reasons, we prepared virus using polymerase and nucleoprotein genes derived from S009. The S009 and 2009 A(H1N1) polymerase proteins are closely related, sharing 94.2–97.6% identity and 98.8–99.2% similarity. The remaining viral genes were derived from human A/WSN/33 (WSN). Multicycle replication kinetics were monitored in human, porcine, and avian cells. Infection of human A549 cells with virus containing a WT S009 polymerase displayed delayed replication kinetics and reduced viral yields (Fig. 3). This recapitulates the restriction of avian viruses in human cells and is in agreement with other studies of viruses containing an avian polymerase (12, 14). Virus with the SR polymorphism replicated 9- to 70-fold higher than WT S009 in A549 cells at the indicated time points. Virus with PB2 SR+K replicated an additional 1.74- to 2.70-fold higher than the single mutant. Infection of porcine PK (15) cells proceeded rapidly with high titers obtained for virus containing WT and SR mutant PB2 (Fig. 3). Still, virus containing the SR polymorphism yielded approximately10-fold more infectious progeny than WT virus. This intermediate level of restriction in porcine cells is consistent with only a moderate level of restriction observed in pigs infected with single-gene reassortant viruses containing either avian or swine PB2 (17). By contrast, similar yields were observed for all viruses from parallel infections performed in chicken DF1 cells (Fig. 3). Thus, the SR polymorphism selectively enhances virus replication in cells that restrict the function of avian influenza polymerases. Together, these data suggest that the SR polymorphism enhances replication in human and porcine cells by partially restoring the activity of a restricted polymerase. This PB2 variant likely contributes to the escape of the 2009 A(H1N1) polymerase from the inhibitory factor present in humans.

PA Reassortment Restores Activity of an Avian Influenza Polymerase. In addition to discrete mutations like the SR polymorphisms, emergent viruses can expand their tropism and adapt to new hosts by reassortment of the eight genomic RNAs. The 2009 A(H1N1) viruses are reassortants with a complex lineage; the virus possesses genes of avian, human, classic swine, and Eurasian swine origin (21, 28). In particular, the polymerase contains PB2 and PA of avian origin, PB1 derived from human viruses, and NP from the classical swine lineage. We tested the ability of gene reassortment to enhance the activity of the severely restricted S009 polymerase. Polymerase reassortants were created by replacing individual subunits of the avian polymerase with cognate subunits from human isolates and subsequently assessed in polymerase activity assays (Fig. 4). Each reassorted polymerase was tested in the context of WT PB2, PB2 with the SR polymorphism, PB2 E627K, and PB2 SR+K. Substitutions with WSN NP had minor effects on polymerase activity for all four PB2 variants. By contrast, introduction of WSN PB1 impaired activity for all polymerases, indicating an incompatibility between this PB1 and the remaining avian subunits. Surprisingly, introduction of WSN PA resulted in a dramatic and selective increase in polymerase activity for the restricted WT- and PB2 SR-containing polymerases. Similar results were demonstrated with PA derived from the primary human isolates A/Brevig Mission/1918 and A/New York/312/2001, suggesting that acquisition of a human influenza

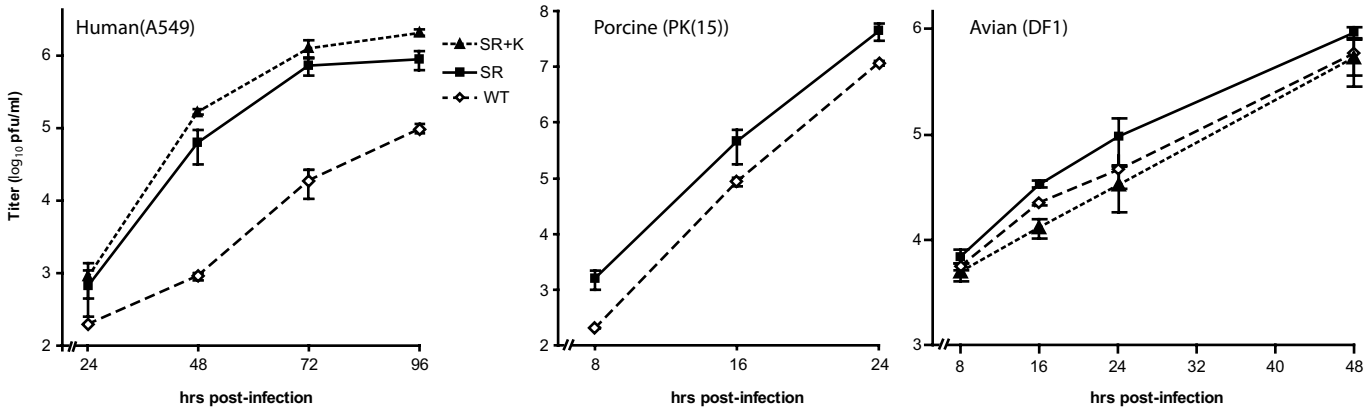

Fig. 3. The SR polymorphism enhances replication in restrictive cells. Multicycle replication kinetics were determined for WT and mutant viruses in human, porcine and avian cells. Recombinant virus encoding WT or mutant forms of the S009 polymerase was used to infect A549 cells (MOI = 0.01) or PK (15) and DF1 cells (MOI = 0.001). Virus yield at the indicated time points was determined by plaque assay. Data represent the mean of three independent infections ± standard deviation.

PA selectively enhances the activity of both an avian polymerase and a swine-like PB2 SR polymerase in restrictive cells.

Discussion

The influenza virus polymerase plays a key role in regulating host-range specificity. Changes within the polymerase complex, either by reassortment or mutation, help to establish infections in a new species. The wholesale acquisition of polymerase genes encoding PB1 has been observed in two prior pandemic outbreaks; viruses responsible for the "Asian" influenza pandemic in 1957 and the "Hong Kong" influenza pandemic in 1968 were reassortants that contained a PB1 subunit of avian origin with the remainder of the polymerase derived from a human virus (29). Experimental analysis has shown that in certain cases, reassorted human influenza polymerases containing an avian PB1 have enhanced activity (30) or support a more pathogenic infection (31), although the potential contribution of PB1-F2 cannot be excluded (32). The current 2009 A(H1N1) pandemic virus is a reassortant as well, containing a replication complex composed of avian-origin PB2 and PA, human-origin PB1, and classic swine-origin NP (21, 28). Discrete mutations within the polymerase have also been associated with an extended host range. A notable example is the species-specific preference for lysine at PB2 position 627 resulting in high levels of viral replication and/or polymerase activity in animal models and human cells (5, 7–16). Other changes within PB2, such as the D701N mutation, are associated with increased host range, polymerase activity, and pathogenicity in mammalian systems and humans (33–35). We have shown here that two additional strategies used by the influenza virus polymerase, the PB2 SR polymorphism present in 2009 A(H1N1) viruses and the acquisition of a human-origin PA subunit by an avian polymerase, overcome species-specific restriction to enhance polymerase activity and replication in human cells.

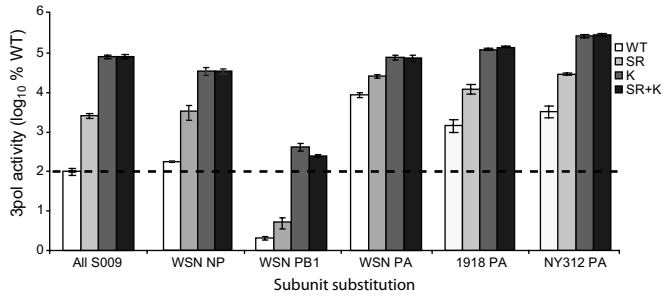

Fig. 4. Acquisition of a human PA rescues activity of reassortant avian polymerases in human cells. Activity assays of reassortant polymerases were performed in 293T cells. Subunits of the S009 polymerase were replaced with those from human virus as indicated. Activity was normalized to WT S009 polymerase. $n = 3$ ± standard deviation.

Polymerases derived from 2009 A(H1N1) viruses lack the human-signature PB2 K627 variant, yet these viruses replicate in humans and are efficiently transmitted in humans and animal models (1, 2, 18–20). The SR polymorphism identified in this study acts as a second-site suppressor to partially overcome restriction by enhancing polymerase activity in three different 2009 A(H1N1) isolates. Mutation of the SR polymorphism to the consensus G590Q591 reduces activity in human cells by 50%. The SR polymorphism also functions in the polymerase of an unrelated avian isolate to increase polymerase activity and enhance virus replication in human cells. Thus, the SR polymorphism may represent an additional adaptive strategy utilized by influenza viruses to escape restriction in a new host and reduce the selective pressure for mutations at PB2 amino acid 627. The high prevalence of the SR polymorphism in viruses isolated from swine suggests a large reservoir of virus that is partially adapted for replication in humans (Table S1). Experimental infections in animal models are needed to confirm this hypothesis and demonstrate a role for the SR polymorphism in vivo. Despite the presence of the SR polymorphism, the 2009 A(H1N1) polymerases are still restricted in human cells. Our findings raise the possibility that the 2009 A(H1N1) may further adapt to replication in humans by acquiring a lysine at amino acid 627 in PB2, with a potential concomitant increase in pathogenesis. Continued surveillance for mutations at this position is warranted.

Biochemical and structural models suggest a mode of action for the SR polymorphism. PB2 mutants with positively charged residues at position 627, either lysine or arginine, support equivalent levels of polymerase activity in human cells (11). Conversely, PB2 mutants with the negatively charged glutamic acid or aspartic acid at position 627 are impaired. Structural analysis has shown that K627 is located on a large solvent-exposed face of the protein with a predominant positively charged surface (36). Introduction of the E627 mutation disrupted the positively charged surface without altering the structure. These studies demonstrated a correlation between the presence of a positively charged surface on the 627 domain and polymerase activity in human cells. Structural models of the PB2 627 domain from 2009 A(H1N1) viruses suggest that the SR polymorphism functions by neutralizing E627 and partially re-

storing the positively charged surface of the 627 domain. Sequence analysis of two other swine-origin viruses from humans, A/Indonesia/CDC644/2006 and A/Thailand/271/2005, imply a similar strategy in which a lysine at PB2 position 591 is paired with a glutamic acid at position 627 (37). Thus, other viruses may evade restriction in human cells by introducing mutations into the PB2 627 domain or other regions of the polymerase complex that neutralize a glutamic acid present at PB2 residue 627.

Replacement of the avian PA subunit with a diverse trio of human PA proteins also increased activity for the impaired polymerases. PA substitution did not further increase the activity of polymerases containing the PB2 E627K mutation, suggesting that the reassortment specifically relieved restriction in human cells. These data are in agreement with previous studies showing a genetic linkage between PB2 and PA (38, 39). A variety of differences exist between PA proteins from S009, WSN, 1918, or NY312, including amino acids 55, 100, 382, and 552 which have been identified as key residues that distinguish avian and human polymerases (40). The consequences of these differences are not obvious, as they do not occur in residues important for endonuclease activity or binding to PB1 (41–44). Further investigation is required to determine the residues in PA that contribute to increased polymerase activity. In addition, it will be of great interest to determine if 2009 A(H1N1) viruses also show enhanced adaptation to human cells upon acquisition of a human-lineage PA, or if the circulating 2009 A(H1N1) will be subject to additional reassortment. Alternatively, this strategy may be unique to S009 or other avian virus polymerases which are more restricted in human cells compared to the 2009 A(H1N1). In summary, these findings identify an adaptive mutation in the 2009 A(H1N1) polymerase and reveal additional pathways by which influenza viruses may further evolve to enhance replication, and potentially pathogenesis, in humans.

Methods

Polymerase Proteins. Polymerase and NP genes were cloned from the primary isolates A/California/07/2009, A/Utah/01/2009, and A/Utah/02/2009 (kindly provided by B. Gowan) as described in ref. 45 and sequenced. Plasmids encoding proteins from A/WSN/1933 were shared by E. Fodor and plasmids encoding proteins from A/green-winged teal/OH/175/1983 (S009), A/Brevig Mission/1918 (1918), and A/New York/312/2001 (NY312) were generously provided by J. Taubenberger.

Experiments were performed using standard techniques and are described in detail in the *SI Text*.

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