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Combination of PB2 271A and SR Polymorphism at Positions 590/591 Is Critical for Viral Replication and Virulence of Swine Influenza Virus in Cultured Cells and *In Vivo*

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Triple reassortant swine influenza viruses (SIVs) and 2009 pandemic H1N1 (pH1N1) virus contain an avian-origin PB2 with 271A, 590S, 591R, and 627E. To evaluate the role of PB2 271A, 590S, and 591R in the replication and virulence of SIV, single (1930-TX98-PB2-271T)-, double (1930-TX98-PB2-590A591A)-, and triple (1930-TX98-PB2-271T590A591A)-mutated viruses were generated in the background of the H1N1 A/swine/Iowa/15/30 (1930) virus with an avian-origin PB2 from the triple-reassortant A/swine/Texas/4199-2/98 (TX98) virus, called the parental 1930-TX98-PB2. Compared to parental virus and single- and double-mutated viruses, the triple-mutated virus replicated less efficiently in cell cultures and was attenuated in mice. These results suggest that a combination of 271A with the 590/591 SR polymorphism is critical for pH1N1 and triple-reassortant SIVs for efficient replication and adaptation in mammals.

Both triple-reassortant swine influenza viruses (SIVs) circulating in North American swine herds and the 2009 pandemic H1N1 (pH1N1) virus contain an avian-origin PB2 with 271A, 590S, 591R, and 627E (14, 18, 20). Avian-origin PB2 with 627E was introduced into North American SIVs more than 10 years ago, and this avian-like signature (i.e., 627E) remains stable rather than changing to the mammalian-like signature (i.e., 627K) (8). The pH1N1 viruses have circulated in humans for more than 2 years and have been transmitted to other animal species (1, 3, 11, 15), yet almost all of the isolates still contain the avian-like signature 627E instead of the mammalian-like signature 627K in their PB2 proteins. The PB2 271A of pH1N1 contributes to enhance viral polymerase activity and growth in mammalian cells (2). Furthermore, a recent study in cultured cells showed that the 590/591 (SR) polymorphism in PB2 helps pH1N1 overcome host restriction by enhancing its polymerase activity (9).

So far, no *in vivo* studies have been conducted to confirm the roles of the 590/591 (SR) polymorphism or of PB2 271A of the pH1N1 and triple-reassortant SIVs, so their effects on viral replication and pathogenicity are poorly understood. In this study, 1930-TX98-PB2, used as the parental virus, was generated in the backbone of the classical H1N1 A/swine/Iowa/15/30 (1930) virus by replacing its swine-origin PB2 (containing 271T, 590G, 591Q, and 627K) with an avian-origin PB2 (containing 271A, 590S, 591R, and 627E) from a triple-reassortant A/swine/Texas/4199-2/98 (TX/98) H3N2 virus; 1930-TX98-PB2 has been shown to be able to induce severe disease and lung lesions in mice at low doses (8). To explore the potential roles of these three amino acids in the replication and virulence of SIVs, we generated recombinant viruses with single (1930-TX98-PB2-271T), double (1930-TX98-PB2-590A591A), or triple (1930-TX98-PB2-271T590A591A) amino acid substitutions and evaluated their replication and virulence compared to those of the parental virus in cultured cells and *in vivo*. Our study demonstrates that a combination of 271A with the 590/591 SR polymorphism (not single 271A or single 590/591SR) is critical for the replication and virulence of SIV and compensates for the absence of 627K.

The single (1930-TX98-PB2-271T)-, double (1930-TX98-PB2-590A591A)-, and triple (1930-TX98-PB2-271T590A591A)-mutated viruses were generated in the background of the parental 1930-TX98-PB2 virus by replacing its TX98-PB2 with mutated PB2 containing single, double, or triple amino acid substitutions (see the materials and methods in the supplemental material). Plaque assays showed that the double-mutated 1930-TX98-PB2-590A591A and triple-mutated 1930-TX98-PB2-271T590A591A formed smaller plaques than did either the parental 1930-TX98-PB2 or the single-mutated viruses (Fig. 1A). The triple-mutated 1930-TX98-PB2-271T590A591A grew to significantly lower titers in MDCK cells than did the parental and single- and double-mutated viruses; no significant difference was observed between the parental and single- or double-mutated viruses (Fig. 1B). Similarly, the triple-mutated virus grew to significantly lower virus titers in human A549 cells than did the parental and double-mutated viruses (Fig. 1C). The single-mutated virus had lower virus titers at 24 and 36 h postinfection (hpi), but at 48 hpi the titers were similar to that of the parental and double-mutated viruses. In swine PK15 cells, the parental virus grew to significantly higher titers than the triple-mutated virus did (Fig. 1D). Significant differences also were observed between the single- and triple-mutated viruses at 12 and 36 hpi and between double- and

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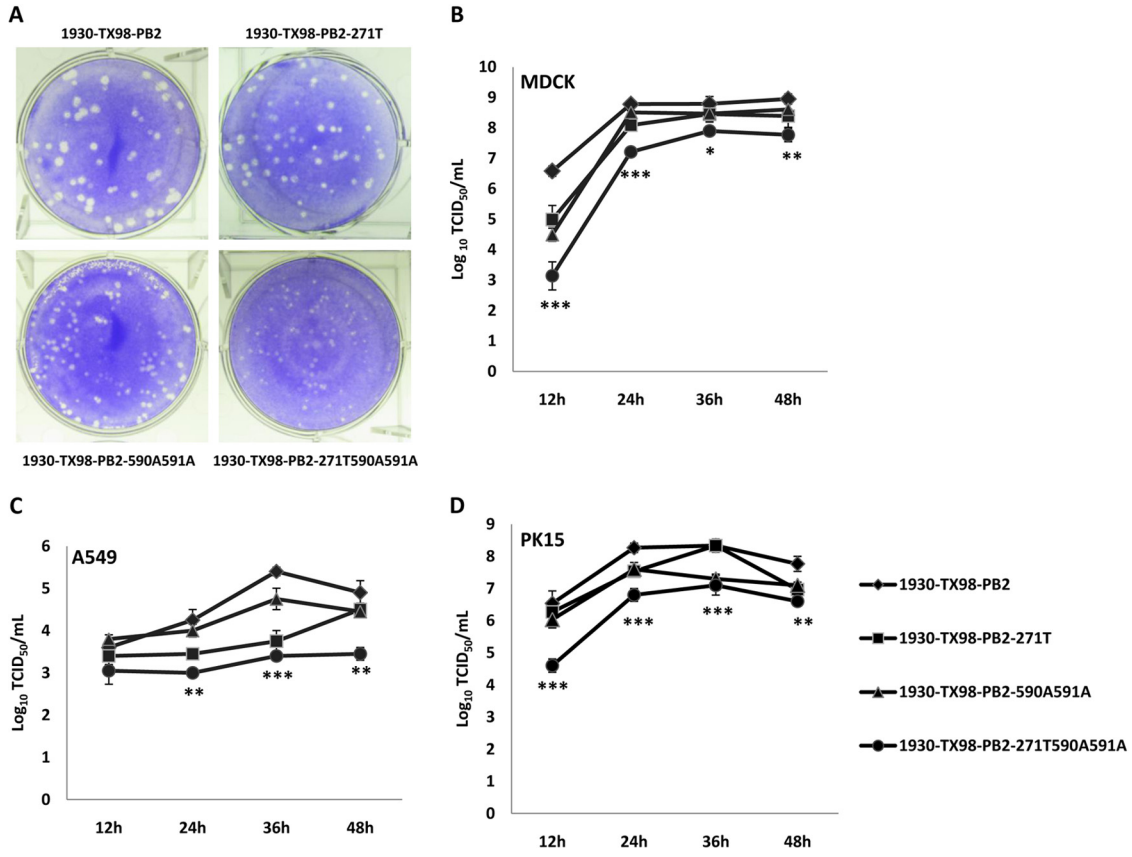

FIG 1 Growth properties of the parental and mutated viruses. (A) Plaque size in MDCK cells 2 days after infection. Growth curve in MDCK cells that were infected with each virus at an MOI of 0.001 (B) and in A549 cells (C) and in PK15 cells (D) that were infected with each virus at an MOI of 0.1. Each data point on the curve indicates the means from three independent experiments, and error bars indicate the standard errors of the means (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

triple-mutated viruses at 12 hpi. In conclusion, the triple-mutated virus replicates less efficiently in three different cell lines of human, canine, and swine origin than the parental, single- and double-mutated viruses.

To determine the effect of single, double, and triple substitutions in TX98-PB2 on replication and transcription rates of viral RNAs (vRNA), the presence of vRNA, cRNA, and mRNA was investigated by using primer extension assays to detect viral negative-strand and positive-strand RNAs derived from the PB1 gene segment as described previously (12) after virus infection of MDCK cells at a multiplicity of infection (MOI) of 3 (see the materials and methods in the supplemental material). The expression of the three viral RNA species of all four viruses appeared to increase during the course (4, 8, and 12 h) of infection (Fig. 2A). In agreement with the growth curve, the viral RNA levels in cells infected with the triple-mutated 1930-TX98-PB2-271T590A591A virus were the lowest of the four viruses throughout the course of infection (Fig. 2A). Differences were observed in vRNA (1.2- to 2.4-fold), mRNA (1.4- to 2.7-fold), and cRNA (1.3- to 2.1-fold) levels between the triple-mutated virus and the parental and single- and double-mutated viruses at the indicated hpi. Single- and double-mutated viruses produced amounts of viral RNAs comparable to that of the parental virus (Fig. 2A). These data suggest that SR polymorphism at positions 590/591 and position 271A in the avian-origin PB2 are critical for the replication and transcription of viral RNAs of SIVs.

To further understand how triple amino acid substitutions in PB2 affect virus replication, viral PB2 and PB1 proteins were examined by Western blot analysis after the virus infection of MDCK cells at an MOI of 3 (see the materials and methods in the supplemental material). The amounts of both PB2 and PB1 proteins in cells infected with the triple-mutated 1930-TX98-PB2-271T590A591A were the lowest of the four viruses at all tested hours postinfection (Fig. 2B), which is consistent with viral RNA levels shown in primer extension assays. The triple-mutated virus expressed 1.5- to 3.1-fold less PB2 protein at the indicated hpi in infected cells than parental and single- and double-mutated viruses did (Fig. 2B). Similarly, 1.1- to 3.1-fold differences were observed in PB1 protein production by the triple-mutated virus compared to the parental and single- and double-mutated viruses. Notably, single- and double-mutated viruses expressed amounts of both PB1 and PB2 proteins comparable to that of the parental virus. Furthermore, both PB2 and PB1 proteins in these viruses were expressed at almost equal levels (Fig. 2B). Taken together, these results indicate that triple amino acid substitutions directly affect the expression of PB2 and PB1, resulting in decreased viral replication and transcription.

Mice infected with 10^6 50% tissue culture infective doses ($TCID_{50}$) of the parental or mutated viruses (see the materials and methods in the supplemental material) experienced body weight loss starting 2 days postinfection (dpi) and had clinical symptoms, including ruffled fur, lethargy, anorexia, and dyspnea. The triple-

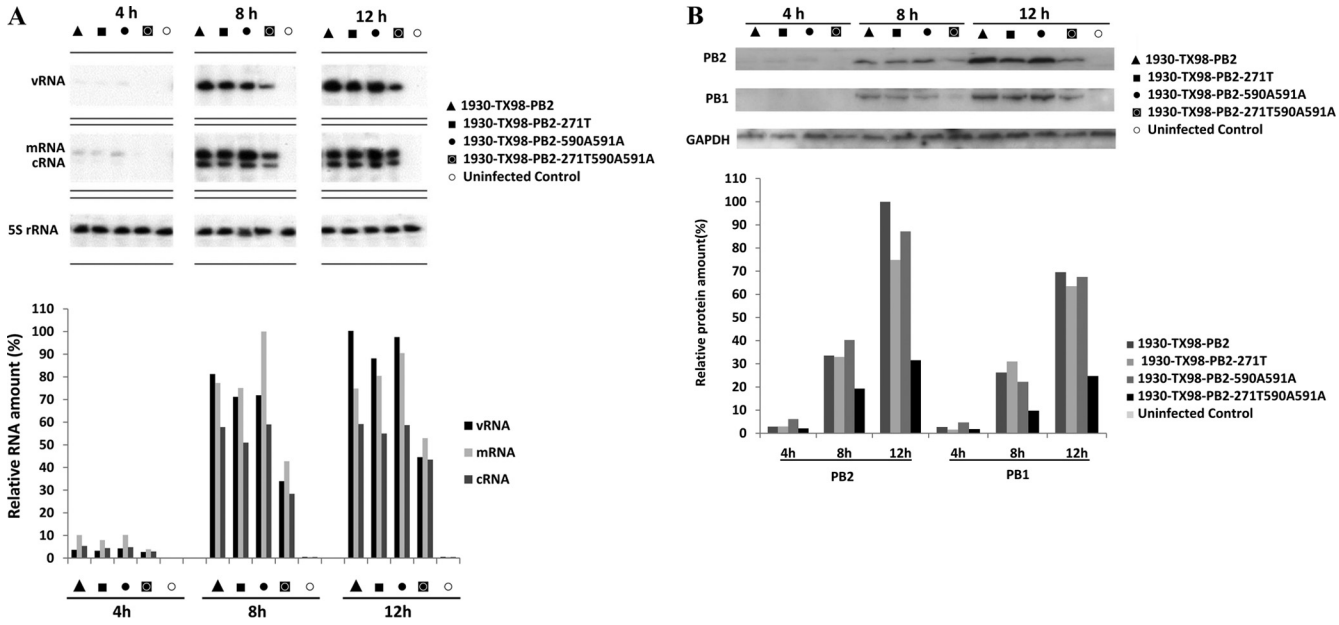

FIG 2 Viral RNA accumulation and Western blots. MDCK cells were infected with the indicated viruses at an MOI of 3. (A) Total RNA was isolated at the indicated time points and analyzed by performing primer extension assays. The 5S rRNA was used as a loading control. The quantification of the relative RNA amount was calculated and normalized using ratios to 5S rRNA. (B) Infected cells were harvested and lysed at the indicated time points and analyzed by conducting Western blotting using polyclonal antibodies against influenza PB2 and PB1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative PB2 and PB1 amounts were calculated and normalized using ratios to GAPDH.

mutated 1930-TX98-PB2-271T590A591A virus caused less weight loss than did the other three viruses (Fig. 3A); there was no significant difference in weight loss caused by the parental and the single- or double-mutated viruses. The single-mutated 1930-TX98-PB2-271T and double-mutated 1930-TX98-PB2-590A591A viruses showed virulence similar to that of the parental virus (100% mortality rate). The triple-mutated 1930-TX98-PB2-271T590A591A was attenuated and caused slower disease onset and less mortality (62.5%) than did the parental, single-mutated, and double-mutated viruses (Fig. 3B). These data indicate that the triple-mutated 1930-TX98-PB2-271T590A591A virus was significantly attenuated, which is consistent with the findings in cultured cells.

All viruses replicated very efficiently (virus titers range from $10^{5.8}$ to $10^{6.6}$ $TCID_{50}$/100 μg) in mouse lungs without prior adaptation (see Fig. S1A in the supplemental material). The parental and mutated viruses induced pneumonia in infected mice (see Fig. S1B and S2 in the supplemental material). The single-mutated 1930-TX98-PB2-271T and triple-mutated 1930-TX98-PB2-271T590A591A viruses caused significantly fewer microscopic lung lesions in infected mice at 3 dpi than did the parental virus. At 5 dpi, the triple-mutated 1930-TX98-PB2-271T590A591A virus induced significantly fewer lung lesions in mice than the other viruses; no significant difference was observed among the groups inoculated with the parental, single-, or double-mutated virus (see Fig. S1B and S2).

Two amino acids (627K or 701N) in the PB2 polymerase have been considered to be important molecular markers for the adaptation of avian influenza viruses to mammals (4, 7, 10, 13, 16, 17). To date, most pH1N1 isolates and currently circulating triple-reassortant SIVs possess an avian-origin PB2, which lacks these markers. Notably, both pH1N1 and triple-reassortant SIVs replicate and are transmitted efficiently in human and swine populations. Our previous study showed that a classical SIV containing avian-origin PB2 was able to infect pigs and mice without prior adaptation even if an E occupied position 627 (8). In the current study, a high dose of the parental 1930-TX98-PB2 virus containing an avian PB2 induced 100% mortality in mice, just as the 1930-TX98-PB2-627K virus does. These data suggest that other residues in PB2 compensate for the lack of 627K and/or 701N.

PB2 271A has been shown to enhance the polymerase activity and virus replication of the pH1N1 virus with an avian-like PB2 in cultured cell assays (2). Furthermore, PB2 271A increased the polymerase activity of an avian influenza virus in human cells, but the avian influenza virus with the PB2 271A did not cause significant weight loss and mortality in infected mice (2). Our present study indicates that this single amino acid (i.e., PB2 271A) is insufficient to affect the viral replication and virulence of SIV in cultured cells and *in vivo*, which is consistent with previous findings (2).

A previous study showed that SR polymorphism at positions 590 and 591 in pH1N1 PB2 is important for efficient polymerase activity in cultured cells and enhances reassortant human-avian virus replication in human cells (9). Furthermore, the SR polymorphism increases avian polymerase activity and enhances virus replication in human cells (9). This polymorphism has been considered an adaptive strategy used by influenza viruses to overcome species-specific restriction of replication; however, so far this has not been studied *in vivo*. Structural analysis indicated that the 591R residue in pH1N1 PB2 affects both the shape and charge on the surface of the PB2 protein, which may affect its interaction with other viral or host factors, thus resulting in more efficient virus replication in mammals (19). Consequently, we hypothesized that double substitutions (i.e., S590A and R591A) in the

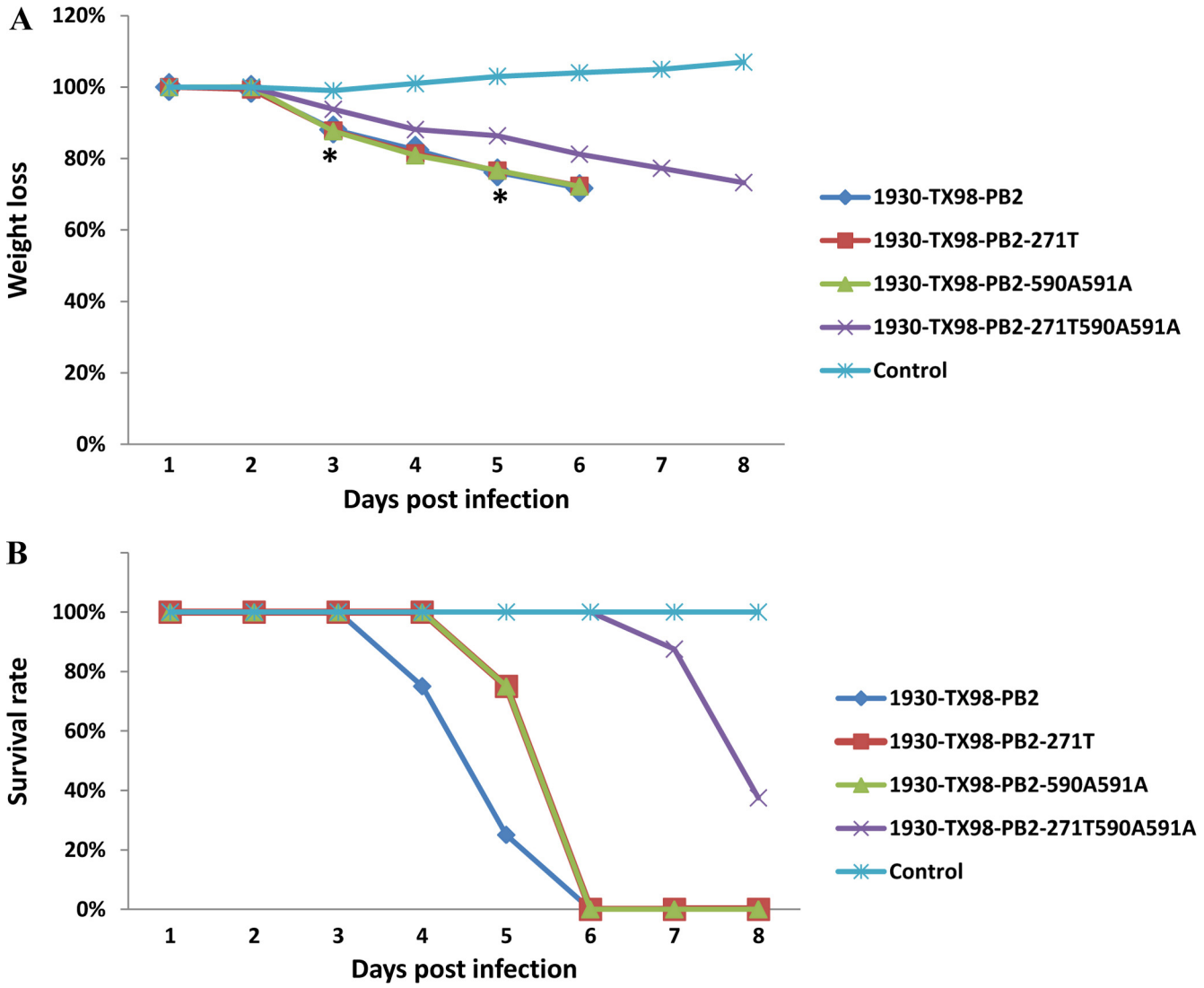

FIG 3 Body weight changes and mortality of mice infected with the parental or mutated viruses. Shown are body weight changes (A) and the survival rate (B) of mice that were infected with either 10^6 $TCID_{50}$ of the indicated viruses or mock infected with 50 μl minimal essential medium (8 mice per group) (*, $P < 0.05$).

avian-origin TX98 PB2 decrease viral replication and attenuate the parental virus. However, the SR polymorphism seems not to be very critical for the replication of the parental 1930-TX98-PB2 virus. In addition, the double-mutated virus had *in vivo* virulence similar to that of the parental and single-mutated viruses, indicating that single (i.e., 271) and double substitutions (i.e., 590/591) are not enough to alter the virulence of the parental virus.

The triple-mutated virus was attenuated in cultured cells and *in vivo*. Our results indicate that a combination of the three residues (i.e., 271, 590, and 591) plays a critical role in virus replication and virulence of SIVs, and this compensates for the lack of 627K or 701N in PB2. These three residues seem to directly contribute to polymerase activity, resulting in decreased virus replication and growth dynamics in three different cell lines and in the mouse study. The three amino acid substitutions in PB2 seem not to affect interactions with host factors because the triple-mutated virus exhibited a similar replication capacity in canine, swine, and human cell lines. Recent studies showed that the PB2 E627K or D701N substitution in 2009 pH1N1 did not lead to enhanced virulence in mice (5, 21) and ferrets (5) or enhanced transmission in ferrets (5); these substitutions actually cause the attenuation of a reassortant virus that contains the 2009 pH1N1 influenza polymerase and NP and the remaining four genes from a recent seasonal H1N1 (A/New York/312/2001) virus in culture cells and mice (6). These findings suggest that the pH1N1 and currently circulating triple-reassortant SIVs use different strategies to achieve efficient replication and adaptation to mammals without the signature PB2 627K or 701N residue. PB2 of the 2009 pH1N1 virus showed 98.8% homology with the TX98 PB2 at the amino acid level, containing the avian-like signatures 627E and 701D. Our study demonstrates that a combination of PB2 271A with SR polymorphism at positions 590 and 591 in the genetic background of a classical H1N1 SIV with an avian-origin PB2 is critical for viral replication and virulence of SIVs in cultured cells and *in vivo*; this suggests that the *in vivo* virulence of pH1N1 resides within the same amino acid positions of PB2. Taken together, the combina-

tion of 271A and 590/591 SR polymorphism in PB2 might be a novel strategy that is used by both pH1N1 and currently circulating triple-reassortant SIVs for efficient replication and adaptation in mammals.

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