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The Avian-Origin PB1 Gene Segment Facilitated Replication and Transmissibility of the H3N2/1968 Pandemic Influenza Virus

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ABSTRACT

The H2N2/1957 and H3N2/1968 pandemic influenza viruses emerged via the exchange of genomic RNA segments between human and avian viruses. The avian hemagglutinin (HA) allowed the hybrid viruses to escape preexisting immunity in the human population. Both pandemic viruses further received the PB1 gene segment from the avian parent (Y. Kawaoka, S. Krauss, and R. G. Webster, J Virol 63:4603–4608, 1989), but the biological significance of this observation was not understood. To assess whether the avian-origin PB1 segment provided pandemic viruses with some selective advantage, either on its own or via cooperation with the homologous HA segment, we modeled by reverse genetics the reassortment event that led to the emergence of the H3N2/1968 pandemic virus. Using seasonal H2N2 virus A/California/1/66 (Cal) as a surrogate precursor human virus and pandemic virus A/Hong Kong/1/68 (H3N2) (HK) as a source of avian-derived PB1 and HA gene segments, we generated four reassortant recombinant viruses and compared pairs of viruses which differed solely by the origin of PB1. Replacement of the PB1 segment of Cal by PB1 of HK facilitated viral polymerase activity, replication efficiency in human cells, and contact transmission in guinea pigs. A combination of PB1 and HA segments of HK did not enhance replicative fitness of the reassortant virus compared with the single-gene PB1 reassortant. Our data suggest that the avian PB1 segment of the 1968 pandemic virus served to enhance viral growth and transmissibility, likely by enhancing activity of the viral polymerase complex.

IMPORTANCE

Despite the high impact of influenza pandemics on human health, some mechanisms underlying the emergence of pandemic influenza viruses still are poorly understood. Thus, it was unclear why both H2N2/1957 and H3N2/1968 reassortant pandemic viruses contained, in addition to the avian HA, the PB1 gene segment of the avian parent. Here, we addressed this long-standing question by modeling the emergence of the H3N2/1968 virus from its putative human and avian precursors. We show that the avian PB1 segment increased activity of the viral polymerase and facilitated viral replication. Our results suggest that in addition to the acquisition of antigenically novel HA (i.e., antigenic shift), enhanced viral polymerase activity is required for the emergence of pandemic influenza viruses from their seasonal human precursors.

Influenza A viruses are single-stranded negative-sense RNA viruses. Their genome consists of 8 RNA segments, each of which encodes 1 to 3 proteins (1). Pandemic influenza has been occurring at irregular intervals for at least 500 years, causing significant, occasionally catastrophic mortality in the human population (2, 3). Genetic data available for the last four pandemic viruses (H1N1/1918, H2N2/1957, H3N2/1968, and H1N1/2009) indicate that they all contained an antigenically novel hemagglutinin (HA) which was derived from animal influenza A viruses (reviewed in references 2–5). The origin of the other seven gene segments of the pandemic viruses varied (3, 4, 6). The source of the non-HA gene segments of the H1N1/1918 virus is not clear due to the lack of sequence data for coexistent avian and mammalian influenza A viruses. The precursor of the H1N1/2009 virus emerged and circulated in pigs; this virus was transmitted and adapted to humans as a whole. Two other pandemic viruses emerged via gene mixing (reassortment) between animal viruses and contemporary human viruses and contained either 5 gene segments (H2N2/1957) or 6 gene segments (H3N2/1968) of the human virus parent.

Avian influenza viruses do not replicate efficiently in humans. It is believed that exchange via reassortment of nonadapted gene segments from an avian virus by segments from a human-adapted parent might allow the emerging pandemic virus to overcome host range restriction (2, 7, 8). Surprisingly, both 1957 and 1968 pandemic viruses contained the avian PB1 segment (gene segment 2) (9). This segment encodes three proteins, polymerase basic protein 1 (PB1), PB1-F2, and PB1-N40, which are translated from the start codons 1, 4, and 5, respectively (1, 10). PB1 is the core subunit of the trimeric viral RNA-dependent RNA polymerase complex (PB1, PB2, and PA), which is responsible for transcription and replication of the viral genome (1, 11). PB1-F2 and PB1-N40 are not essential for virus replication, but they can affect replication efficiency and contribute to pathogenicity (10, 12).

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The presence of the avian PB1 segment in 1957 and 1968 pandemic viruses may indicate that there was no significant host range restriction on this segment in humans, so it was included simply by chance (8). Alternatively, the avian PB1 segment could have provided some selective advantage to the reassortant pandemic virus, either by itself or via cooperation with the avian HA or NA segments (9). Several pieces of evidence argue in favor of the selective advantage hypothesis. First, studies on the compatibility of polymerase proteins from human and avian influenza viruses showed that replacement of the human PB1 by its avian counterpart can enhance polymerase activity in minigenome reporter assays (13–15). Second, analyses of single-gene reassortants demonstrated an important role of the PB1 segment for the high virulence of the pandemic H1N1/1918 virus in mice and ferrets (16, 17). Third, vaccine strains of influenza viruses prepared by gene reassortment between wild-type (wt) viruses and the high-growth donor strain A/PR/8/1934 often contain, in addition to mandatory wild-type HA and neuraminidase (NA) segments, the wt PB1 segment (18). Some of such "3 + 5" reassortant viruses replicated more efficiently than their "2 + 6" counterparts lacking wild-type PB1 (19, 20). Also, Chen and colleagues studied reassortment between contemporary avian H5N1 and human H3N2 viruses and found that a 3 + 5 reassortant virus which included the avian PB1, HA, and NA segments was highly virulent in mice, whereas its 2 + 6 counterpart was significantly less pathogenic (21). These results (18–21) support the hypothesis that there is cooperation between homologous PB1 and HA/NA. Indeed, it was shown recently that the selection of the high-growth 3 + 5 reassortants of A/PR/8/1934 could be explained by favorable interactions between the homologous vRNA segments encoding PB1 and NA during the packaging of vRNAs into virus particles (22).

The outcome of reassortment events between two influenza viruses strongly depends on the nature of the parental virus strains (for a review, see reference 23). Therefore, the data obtained in the aforementioned studies cannot unambiguously explain the biological significance of the presence of an avian virus-derived PB1 in the 1957 and 1968 pandemic viruses. To address this question, we replayed in this study the emergence of the 1968 pandemic virus from its putative human and avian precursors and tested whether the avian-origin PB1 gene segment has provided the reassortant virus with selective replicative advantage. We also tested the hypothesis that homologous PB1 and HA segments cooperated during reassortment.

MATERIALS AND METHODS

Cells. 293T and MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (pen-strep), and 2 mM glutamine. Calu-3 cells (human bronchial adenocarcinoma; ATCC HTB-55) were cultured in DMEM-F12 Ham (1:1) (Gibco) with 10% FCS, pen-strep, and 2 mM glutamine. Infections were performed using DMEM with 0.1% bovine serum albumin (PAA Laboratories), pen-strep, and 2 mM glutamine. Cell cultures were maintained and all viral replication and reverse genetics experiments were performed at 37°C with 5% CO_2 if not indicated otherwise.

Viruses and plasmids. Viruses A/California/1/1966 (H2N2) (Cal) and A/Hong Kong/1/1968 (H3N2) (HK) were kindly provided by Alexander Klimov, CDC, Atlanta, GA, USA, and Earl Brown, University of Ottawa, Canada, respectively. RNA was isolated from Cal using the QIAamp viral RNA minikit (Qiagen); each viral gene segment was amplified by reverse transcription-PCR (RT-PCR) using a set of universal primers (24). PCR products were ligated into pHW2000 polymerase I (Pol I)/Pol II reverse genetics plasmid (kindly provided by Robert Webster, St. Jude Children's Research Hospital, Memphis, TN, USA). The pHW2000 plasmids expressing gene segments of HK were prepared previously (25). To generate plasmids U4-$PB1_{Cal}$ and $PB1_{Av}$, mutations were introduced into $PB1_{Cal}$ and $PB1_{HK}$ plasmids, respectively, using a site-directed mutagenesis kit (QuikChange; Stratagene).

Recombinant viruses. Viruses were generated using the eight-plasmid reverse genetics system (26). In brief, 293T cells in T25 flasks were transfected with a set of eight different plasmids (1 μg each) using Lipofectamine 2000 (Invitrogen). Six hours later, the transfection medium was replaced with DMEM containing 0.1% bovine serum albumin (BSA). After 2 days of incubation, supernatants were collected from 293T cells and treated with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 μg ml^{-1}) for 1 h. Rescued viruses were amplified in MDCK cells, clarified by low-speed centrifugation, aliquoted, and stored at −80°C. Three different stocks of pHW2000-PB1 plasmids and two different stocks of recombinant viruses were prepared and used in replicate experiments. The identity of all plasmids was confirmed by sequencing. Sequence identities were confirmed for all viral gene segments of the first set of stocks and for HA, NA, and PB1 segments of the second set of stocks.

Biocontainment. All experiments with A/California/1/66 (H2N2) and its recombinant variants were conducted in a biosafety level 3 (BSL3) laboratory in accordance with German law (Gesetz zur Regelung der Gentechnik and Verordnung über die Sicherheitsstufen und Sicherheitsmaßnahmen bei Gentechnischen Arbeiten in Gentechnischen Anlagen).

Virus titration in MDCK cells. Titers of virus stocks were determined as PFU per ml using plaque assay in 6-well plates under the low-viscosity overlay medium Avicel RC/CL (27). In all other experiments, virus suspensions were titrated using a single-cycle focus assay (25), and the titers were expressed as focus-forming units (FFU) per ml.

Analyzing the composition of virus mixtures by sequencing. Virus-containing fluids were clarified by low-speed centrifugation. RNA was isolated using the QIAamp viral RNA minikit (Qiagen), reverse transcribed, and PCR amplified with PB1-specific primers PB1-590-for (5′-GAAGAGTAAGAGACAACATGACC-3′) and PB1-1123-rev (5′-GTGTTCGGAGCTTCATGCTC-3′) using a OneStep RT-PCR kit (Qiagen). The primers were designed to amplify an arbitrarily chosen part of the PB1 segment between nucleotides (nt) 590 and 1123 (message sense, 5′ to 3′); each primer bound to a conserved sequence shared by the PB1 genes of Cal and HK. Generated cDNAs were sequenced using the PB1-590-for primer, and the sequence chromatograms were analyzed in the region between nucleotides 785 and 845 in the middle of the cDNA amplicon where the PB1 sequences of Cal and HK differed by 10 nucleotide substitutions, as shown in Fig. 2a. The heights (h) of $PB1_{HK}$-specific and $PB1_{Cal}$-specific peaks (h_{HK} and h_{Cal}, respectively) were measured on printed chromatograms using a ruler. Ratios of $h_{HK}/(h_{HK} + h_{Cal})$ were calculated for each of 10 polymorphic positions and averaged.

Viral competitive replication fitness in cell culture. Eight replicate cultures of Calu-3 cells in 12-well plates were inoculated with 1:1 mixtures of two viruses differing by the origin of the PB1 segment (either $PB1_{Cal}$ or $PB1_{HK}$). Mixtures were prepared based on viral infectious titers and inoculated at a total multiplicity of infection (MOI) of 0.002 PFU per cell. Aliquots used for preparation of the mixtures were plaque titrated in MDCK cells on the same day to confirm that equal amounts of viruses were inoculated. Culture supernatants were collected from infected Calu-3 cells after 3 days of incubation, titrated, and passaged two more times in Calu-3 cells at an MOI of 0.002 FFU per cell. Compositions of the original inoculated mixtures and passage 3 supernatants were analyzed by sequencing.

Competitive reverse genetics experiments. Recombinant viruses were generated by reverse genetics as described above using a 1:1 mixture of $PB1_{Cal}$ and $PB1_{HK}$ plasmids instead of individual PB1 plasmids and

Calu-3 cells instead of MDCK cells for virus amplification (see Fig. 4a). In brief, 293T cell cultures in 12-well plates were transfected with 2 μg of the mixture of pHW2000-PB1 plasmids $PB1_{Cal}$ and $PB1_{HK}$, 1 μg of either pHW2000-HA_{Cal} or pHW2000-HA_{HK} plasmid, and six pHW2000 plasmids (1 μg of each), which encode the remaining six gene segments of Cal. At 48 h posttransfection, supernatants were collected and used for infection of Calu-3 cells. Virus-containing supernatants were collected from Calu-3 cells at 3 days postinfection, clarified by low-speed centrifugation, and aliquoted. Four to eight replicate competitive rescues were performed on the same day, and the experiments were repeated on different days using different stocks of plasmids.

The composition of the generated virus mixtures was determined by sequencing as described above, with modifications which served to destroy remaining plasmid DNA. To this end, isolated viral RNA was treated with 1 U μl^{-1} DNase I (Thermo Scientific) at 37°C for 3 h, purified with an RNeasy minikit, and reverse transcribed using RevertAid H minus Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas) and a primer complementary to the conserved 12 nt at the 3′ end of the vRNA (Uni12 primer) (24). Generated cDNAs were amplified by PCR using *Pfu* polymerase (Thermo Scientific) and PB1-specific primers PB1-590-for and PB1-1123-rev, followed by sequencing. To confirm complete removal of plasmid DNA, RNA samples were PCR amplified without prior reverse transcription, and the absence of PCR products was controlled by gel electrophoresis.

Contact transmission experiments in guinea pigs. Experiments were performed as previously described (28, 29). In brief, 5- to 6-week-old female Hartley strain guinea pigs weighing 300 to 350 g were obtained from Charles River Laboratories. For each experiment, four guinea pigs were inoculated intranasally with 10^4 PFU of a 1:1 mixture of Cal-HA_{HK} and Cal-$(HA+PB1)_{HK}$ virus in 300 μl of Opti-MEM. Prior to intranasal inoculation and nasal washings, guinea pigs were anesthetized intramuscularly with a mixture of ketamine (30 mg kg^{-1} of body weight) and xylazine (2 mg kg^{-1}). Four noninfected animals were cohoused with inoculated animals starting from 24 h postinoculation. At days 2, 4, 5, 6, and 8 postinoculation, nasal washings were collected and stored at −80°C until analysis. Virus titers in nasal washings were determined using a focus assay. Compositions of virus mixtures in the inoculum and nasal washings were determined by sequencing.

Animal experiments were performed according to the guidelines of the German animal protection law (TierSchG). The animals were housed and handled in accordance with good animal practice as defined by FELASA (www.felasa.eu/) and the national animal welfare body GV-SOLAS (www.gv-solas.de/). All animal experiments were approved by the animal welfare committees of the local authorities (permit G-11/80). All animal procedures were conducted under BSL3 conditions in accordance with the local guidelines.

Luciferase minigenome reporter assay. Human 293T cells in 24-well plates were transfected with four pHW2000 plasmids containing the gene segments PB1, PB2, PA, and NP (1 μg each) together with 1 μg of the reporter plasmid pPolI-NP-Luc, expressing firefly luciferase in negative-sense orientation flanked by the noncoding regions of NP of A/WS/33 virus (kindly provided by Thorsten Wolff, Robert Koch Institute, Berlin, Germany). To normalize for transfection efficiency, 1 μg of the *Renilla* luciferase plasmid pGL4.73[hRluc/SV24] (Promega) was cotransfected. As a negative control, we omitted the pHW2000-PB1 plasmid and used the double amount of pHW2000-PB2 plasmid. After 24 h of incubation at 37°C, the cells were lysed and luciferase activity was measured with the dual-luciferase reporter assay system (Promega) using a Berthold Centro LB 960 luminometer.

Phylogenetic analyses. To identify the putative human virus precursor of the 1968 pandemic virus, H3N2 viruses isolated in the first year of the 1968 pandemic and H2N2 viruses which circulated in humans between 1965 and 1968 were studied. Fifty-one corresponding viral genome sets for which full-length sequences were available for all 6 gene segments shared by H3N2 and H2N2 viruses were downloaded from the NCBI Influenza Virus Resource (30). Nucleotide sequences were trimmed to include coding regions and concatenated in the order PB2-PA-NP-NA-M-NS. A phylogenetic tree was generated using MEGA software, version 6, with the minimum evolution method (31).

To identify the putative avian precursor of the PB1 segment of the H3N2/68 pandemic virus, we used all full-length nonredundant PB1 sequences of avian influenza viruses isolated between 1925 and 1985 available from NCBI Influenza Virus Resource, accessed on 4 January 2014 (312 sequences), and 16 full-length PB1 sequences of pandemic virus strains isolated in 1968. The nucleotide sequences were combined and edited using BioEdit 7.1.11 (Tom Hall), and a phylogenetic tree was generated using MEGA6 with the minimum evolution method. About 40 avian sequences clustering with the human sequences were selected for further analysis (see Fig. 7). PB1 proteins encoded by the avian sequences were compared with PB1 proteins of the 1968 pandemic viruses to identify amino acid substitutions separating human PB1s from the avian consensus sequence.

Statistics. Analyses were done using R 3.1.1 (32). Mean values and mean differences of peak ratios $[h_{HK}/(h_{HK} + h_{Cal})]$ were bootstrapped using 10,000 replications. *P* values for the two-sided alternative hypothesis also were determined by bootstrapping (33). Bootstrapping was employed because the distribution of peak ratios was not symmetric and could be bi- or trimodal (separating individual replicates where there was enrichment, depletion, or balanced replication of the HK virus, with complicated transitions). Numbers given in the text and figure legends are mean values (or mean differences) with their 95% confidence intervals (separated by a comma) given in brackets.

Figures 3 to 6 and 8 show the data together with the bootstrap means and their 95% confidence intervals. *P* values shown on top of a group were obtained from testing the null hypothesis that the expected peak ratio is 0.5. *P* values shown between groups are obtained from testing the null hypothesis that the expected mean difference between the groups is zero. The mean difference between PB1 and HA+PB1 shown in Fig. 3 is adjusted for the difference in the peak ratio measured in the original 1:1 mixtures. Practically, they are estimated by bootstrapping the interaction of the two-factorial model of replication (before/after passaging) and virus pair (PB1 and HA+PB1).

The polymerase activity data (see Fig. 9) were adjusted for interassay variability by using a two-factorial model accounting for the assay replication. Because the ratios were approximately normally distributed, the results and estimates were taken directly from the linear model assuming normal errors (no bootstrapping).

RESULTS

Modeling of the gene reassortment event that led to the 1968 pandemic virus. The virus which caused the 1968 pandemic contained HA and PB1 gene segments from an unknown avian H3 virus and 6 other segments from a human H2N2 virus (Fig. 1a). Phylogenetic analyses suggest that the gene reassortment between the parental human and avian viruses occurred between 1966 and 1968 either in some intermediate host, such as swine, or in humans (4, 34). We decided to reconstruct this reassortment event using reverse genetics and to test whether substitution of the PB1 segment of a human parent by that of an avian parent enhances viral replication. Because exact identities of parental viruses and the original human-avian H3N2 reassortant virus are not known, we focused on the closest available viruses. Our analysis of published full-genome sequences of human H2N2 viruses showed that A/California/1/66 (Cal) displayed the highest similarity of the six non-HA non-PB1 segments with the corresponding segments of the 1968 pandemic viruses (Fig. 1b; Table 1). Therefore, we used Cal as a model of the human virus parent. As a source of avian-origin HA and PB1 gene segments, we used one of the ear-

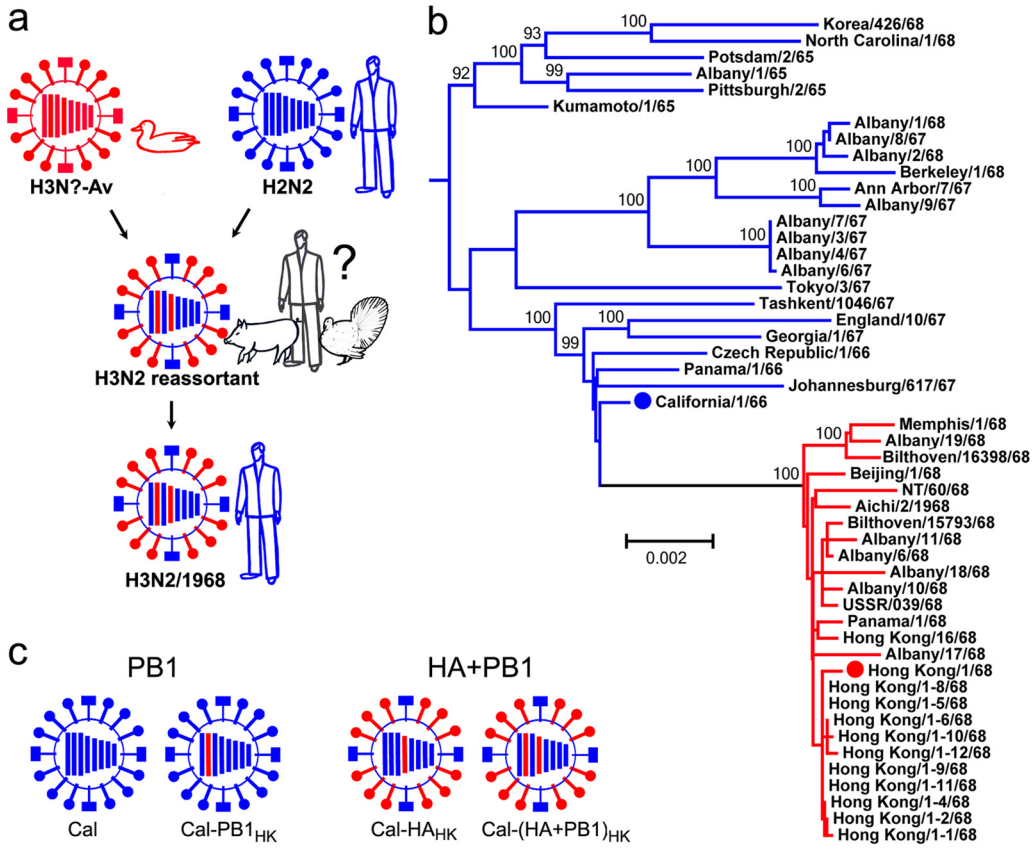

FIG 1 Modeling of the gene reassortment event which led to the emergence of the 1968 pandemic influenza virus. (a) Genesis of the pandemic virus. An avian-origin H3 subtype virus (H3N?-Av) reassorted with a seasonal human H2N2 virus in undefined host species, producing the H3N2 reassortant virus with HA and PB1 gene segments from the avian parent (red) and the remaining 6 gene segments from the human parent (blue). This virus could have circulated in mammals for 1 to 2 years (4) before it caused the 1968 pandemic (H3N2/1968). (b) Phylogenetic tree of concatenated nucleotide sequences of 6 gene segments (PB2, PA, NP, NA, M, and NS) of human influenza viruses which circulated in 1965 to 1968. Blue branches represent seasonal human H2N2 viruses; the red branch represents pandemic H3N2 viruses isolated in 1968. Blue and red dots depict virus strains A/California/1/66 (H2N2) and A/Hong Kong/1/68 (H3N2), which were used in this study as donors of human and avian gene segments, respectively. (c) Two pairs of recombinant viruses prepared to study effects of the PB1 segment on viral replication. Viruses of each pair differed by the origin of PB1 and shared either 7 gene segments of Cal (PB1 pair) or 6 gene segments of Cal and the HA gene segment of HK (HA+PB1 pair).

liest H3N2 pandemic virus strains, A/Hong Kong/1/68 (HK), which inherited these segments from the avian virus parent.

We cloned the gene segments of Cal and HK into reverse genetics plasmid pHW2000 (26) and generated two pairs of recombinant viruses (Fig. 1c). The virus counterparts of each pair differed solely by the origin of the PB1 segment. The first pair of viruses, Cal and Cal-PB1$_{HK}$, shared 7 segments of Cal. This virus pair was used to infer whether avian-origin PB1 alone could affect replication of the reassortant human virus. The viruses of the second pair, Cal-HA$_{HK}$ and Cal-(HA+PB1)$_{HK}$, shared 6 segments of Cal and the HA segment of HK. This pair of viruses was used to test the hypothesis that cooperation exists between homologous HA and PB1 segments.

Avian PB1 facilitates replication and transmission of the reassortant human viruses. The recombinant viruses of each pair showed similar replication kinetics in Calu-3 cells, a continuous

TABLE 1 Nucleotide and amino acid differences between pandemic virus strain A/Hong Kong/1/68 and the closest H2N2 human viruses

H2N2 virus	Differences between HK and H2N2 viruses (nucleotide/amino acid) in segment[a]:						
	PB2/PB2	PA/PA	NP/NP	NA/NA	M/M1/M2	NS/NS1/NS2	PB1/PB1/N40/PB1-F2
A/California/1/66	*17*/3	*17*/2	*7*/4	*7*/2	*2*/0/0	*3*/2/0	*152*/13/13/11
A/Panama/1/66	*19*/4	*20*/3	*7*/4	*8*/3	*6*/2/2	*5*/2/0	*152*/13/13/11
A/Czech Republic/66	*18*/4	*22*/3	*12*/4	*10*/4	*3*/0/1	*5*/4/0	*157*/13/13/11
A/England/10/67	*25*/6	*29*/5	*15*/4	*14*/3	*10*/2/1	*7*/4/0	*153*/13/13/12
A/Tashkent/1046/67	*27*/4	*31*/6	*16*/8	*11*/5	*4*/0/0	*6*/1/1	*154*/12/12/12
A/Johannesburg/617/67	*22*/4	*27*/5	*13*/5	*17*/7	*4*/0/0	*5*/4/1	*156*/12/12/12
A/Georgia/1/67	*28*/6	*23*/4	*8*/5	*15*/6	*3*/0/1	*6*/3/0	*154*/13/13/14

[a] Nucleotide differences between coding regions of gene segments are shown in italics. Amino acid differences are depicted using regular font.

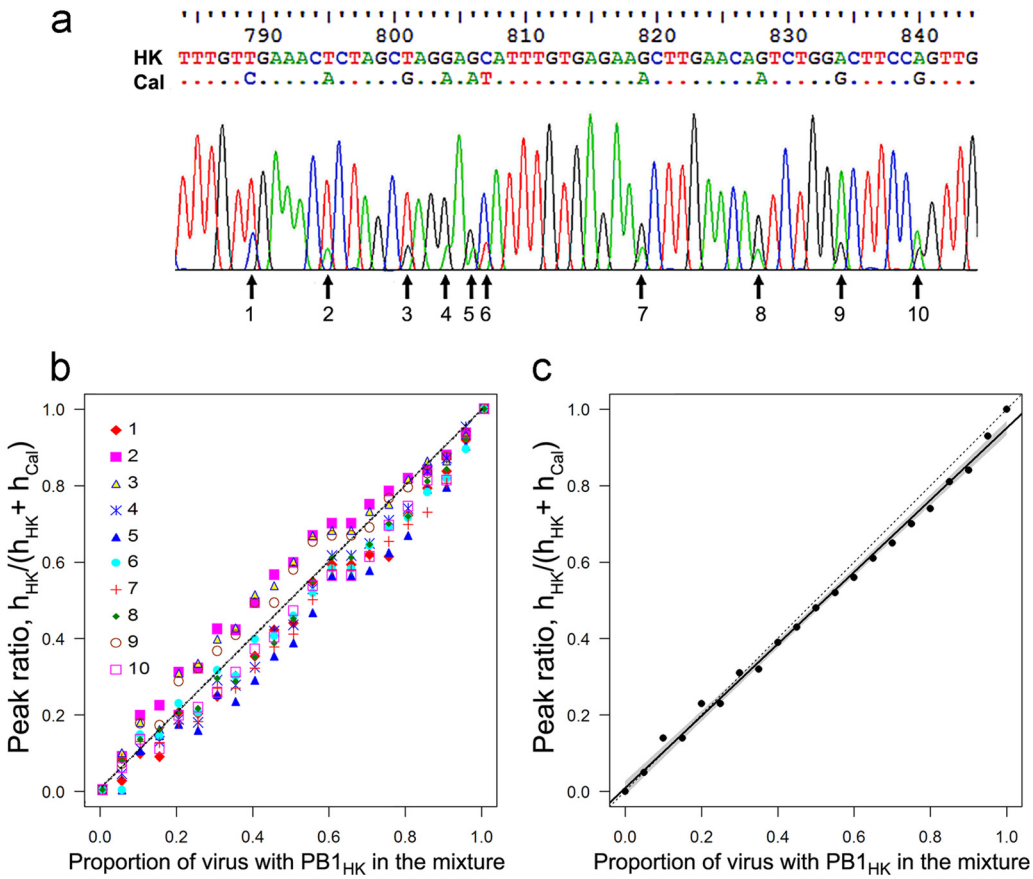

FIG 2 Analysis of the composition of mixtures of viruses differing by PB1 gene segment. (a) Example of sequencing chromatogram of the region of the PB1 containing 10 nucleotide differences between HK and Cal. Ratios of $PB1_{HK}$-specific and $PB1_{Cal}$-specific peaks were calculated for each of 10 polymorphic positions (arrows) by the formula $h_{HK}/(h_{HK} + h_{Cal})$ and averaged. (b and c) Dose-response patterns generated using standard mixtures of viruses Cal-HA_{HK} and Cal-$(HA+PB1)_{HK}$. Panel b illustrates the variation of peak ratios for individual polymorphic positions. The dotted line indicates the identity line. Panel c shows the dose-response curve obtained by averaging peak ratios for all positions. The thick line depicts the linear regression line with a 95% confidence band (gray).

cell line derived from human respiratory epithelium (data not shown). Therefore, we employed a more sensitive replication assay which compares fitness of the viruses under competitive conditions (35, 36). Viruses of each pair were mixed at a 1:1 ratio based on their infectious titers, and the mixtures were passaged 3 times in Calu-3 cells. This number of passages was chosen arbitrarily with the assumption that a single passage may not be sufficient for significant enrichment of the mixture with one virus. Compositions of the mixtures before and after passaging were determined by Sanger sequencing of a nonconservative part of the PB1 segment and calculating the mean ratio of the heights of $PB1_{HK}$-specific peaks and $PB1_{Cal}$-specific peaks at 10 polymorphic positions on the sequencing chromatogram (Fig. 2a). There was some variation of the peak ratios for $PB1_{HK}$ $[h_{HK}/(h_{HK} + h_{Cal})]$ at individual positions (Fig. 2b); however, the mean peak ratio closely correlated with the proportion of the virus with $PB1_{HK}$ in the mixture with a detection limit of as low as 5% of the minor viral population (Fig. 2c).

Passaging of initially equal virus mixtures resulted in enrichment by the virus with the PB1 segment of HK in the majority of replicates (Fig. 3). Effects of similar magnitude were observed for the PB1 and HA+PB1 virus pairs. These results indicated that avian-origin PB1 of HK provided the virus with a replicative advantage in human cells, and that this effect did not significantly depend on the presence of the homologous HA.

To corroborate these findings, we studied competition of the PB1 segments during the process of virus preparation from reverse genetics plasmids (37, 38). Viruses were generated using nine plasmids, namely, seven non-PB1 plasmids required for the preparation of either the PB1 pair or the HA+PB1 pair plus the 1:1 mixture of $PB1_{Cal}$ and $PB1_{HK}$ plasmids (Fig. 4a). All rescued viruses were enriched by variants containing PB1 segment of HK with no detectable effect of the origin of the HA segment (Fig. 4b). These experiments confirmed that the avian-origin $PB1_{HK}$ alone increases competitive fitness of the human virus Cal.

We then tested the effect of the PB1 segment on viral replication and transmission in guinea pigs in two independent experiments (Fig. 5). All guinea pigs which were inoculated intranasally with the 1:1 mixture of viruses Cal-HA_{HK} and Cal-$(HA+PB1)_{HK}$ became infected and shed the virus. Efficient virus transmission to all contact-exposed animals that were cohoused with the inoculated group was observed (Fig. 5a). Sequencing of the viruses in nasal washings collected from inoculated animals at 2 days postinfection revealed a slight but significant enrichment of Cal-$(HA+PB1)_{HK}$ (Fig. 5b). This effect was more pronounced on days

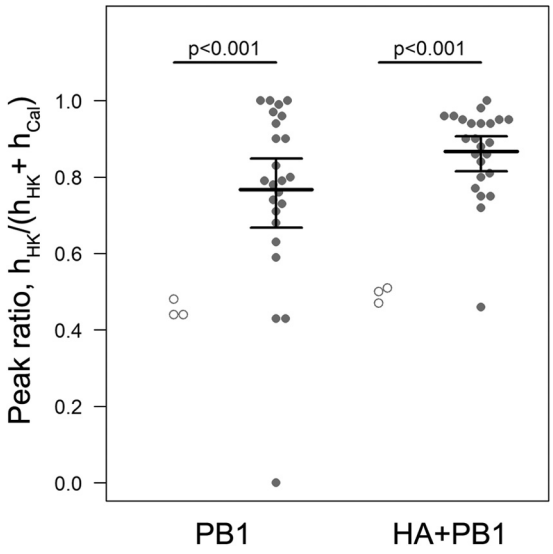

FIG 3 Comparison of viral fitness in competitive replication experiments. One-to-one mixtures of viruses Cal and Cal-$PB1_{HK}$ (PB1 pair) and Cal-HA_{HK} and Cal-$(HA+PB1)_{HK}$ (HA+PB1 pair) were passaged 3 times in Calu-3 cells. The composition of the original mixtures (open circles) and mixtures after passaging (closed circles) was characterized by sequencing and expressed as mean peak ratios for $PB1_{HK}$. Data represent combined results from three experiments performed on different days with a total of 24 replicate mixtures for each pair of viruses. Mean adjusted difference of PB1 pair versus the HA+PB1 pair, 0.06 (−0.04, 0.17); $P = 0.25$.

4 and 6, although all animals still shed detectable amounts of Cal-HA_{HK}. The dominance of Cal-$(HA+PB1)_{HK}$ over Cal-HA_{HK} was particularly pronounced in the nasal washings of contact animals, as no Cal-HA_{HK} could be detected in the nasal washings of 3 and 4 out of 8 contact guinea pigs analyzed on days 4 and 6 postinfection, respectively (Fig. 5b). These results indicated that the virus with avian-origin PB1 of HK replicated and transmitted in guinea pigs more efficiently than the virus containing the Cal-PB1 counterpart.

Effects of substitutions in the 3′NCR and in the PB1 protein. The coding sequences of eight influenza virus RNA segments are flanked by noncoding regions (NCRs). NCRs play several important functions in the virus replication cycle, such as binding of the polymerase complex, transcription initiation, and packaging of segments into the virion. Apart from a U/C polymorphism of the fourth 3′NCR nucleotide, 12 terminal nucleotides at the 3′ end and 13 terminal nucleotides at the 5′ end are conserved and segment independent (1). In accord with the common practice, we generated genomic cDNAs of Cal and HK using a set of previously described cloning primers designed to match the conserved parts of the NCRs (24). However, subsequent sequencing of the NCRs of wild-type Cal and HK revealed a single-nucleotide difference between the wild-type viruses and their recombinant variants: the PB1 segment of wt Cal harbored U4 in the 3′NCR, whereas the generated $PB1_{Cal}$ plasmid coded for C4. The 3′NCR of the PB1 segment of wild-type HK contained C4, and this nucleotide was correctly encoded by the $PB1_{HK}$ plasmid. To test whether the U4/C4 polymorphism in the 3′NCR of $PB1_{Cal}$ affected the results of our experiments, we generated mixtures of viruses with $PB1_{Cal}$ and $PB1_{HK}$ by reverse genetics as described in the legend to Fig. 4a using either C4-$PB1_{Cal}$ plasmid or U4-$PB1_{Cal}$ plasmid. All rescued virus mixtures were enriched to the same extent by viruses containing $PB1_{HK}$ (Fig. 6), indicating that the U/C variation in position 4 of $PB1_{Cal}$ had no significant effect on competitive virus fitness.

The sequences of the PB1 segment of the 1968 pandemic viruses differ from PB1 sequences of the closest known avian viruses. As a result, the PB1 protein of the pandemic viruses harbors 3 amino acid substitutions with respect to the putative avian ancestor sequence, namely, K121R, L212V, and R327K (Fig. 7). PB1 of Cal contained avian-like amino acids in these positions. It is not known whether these substitutions in the PB1 protein of H3N2/1968 viruses occurred before the emergence of the original reassortant virus (H3N2 reassortant in Fig. 1a) or whether they were acquired in the postreassortment period of virus evolution preceding the 1968 pandemic. The first scenario was tested in the

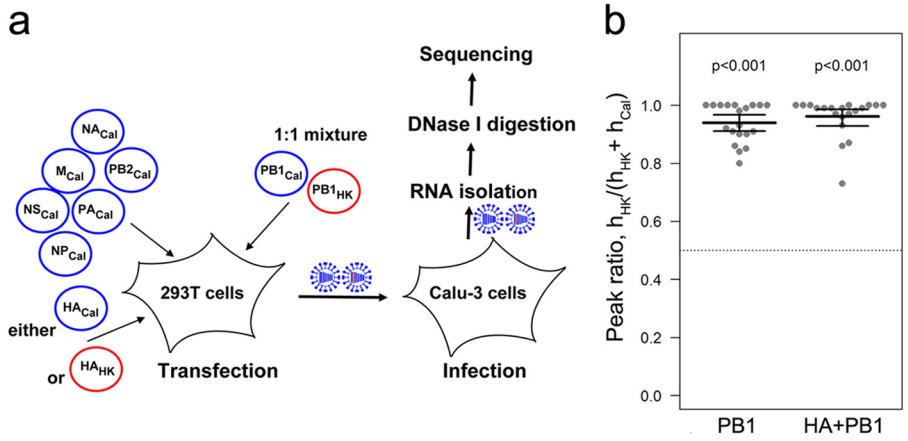

FIG 4 Comparison of viral fitness in competitive reverse genetics experiments. (a) Mixtures of two viruses differing by PB1 segment were generated by transfecting 293T cells with a 1:1 mixture of $PB1_{HK}$ and $PB1_{Cal}$ plasmids, either HA_{Cal} plasmid (PB1 pair) or HA_{HK} plasmid (HA+PB1 pair), and plasmids of 6 other gene segments of Cal. Rescued viruses were amplified in Calu-3 cells and characterized by sequencing. (b) Combined results from three experiments performed on different days with a total of 20 replicate mixtures for each pair of viruses. The dotted line depicts the expected peak ratio in the absence of viral competition (0.5). *P* values refer to the differences between observed and expected peak ratios. Mean difference between PB1 and PB1+HA pairs, 0.02 (−0.02, 0.06); $P = 0.3$.

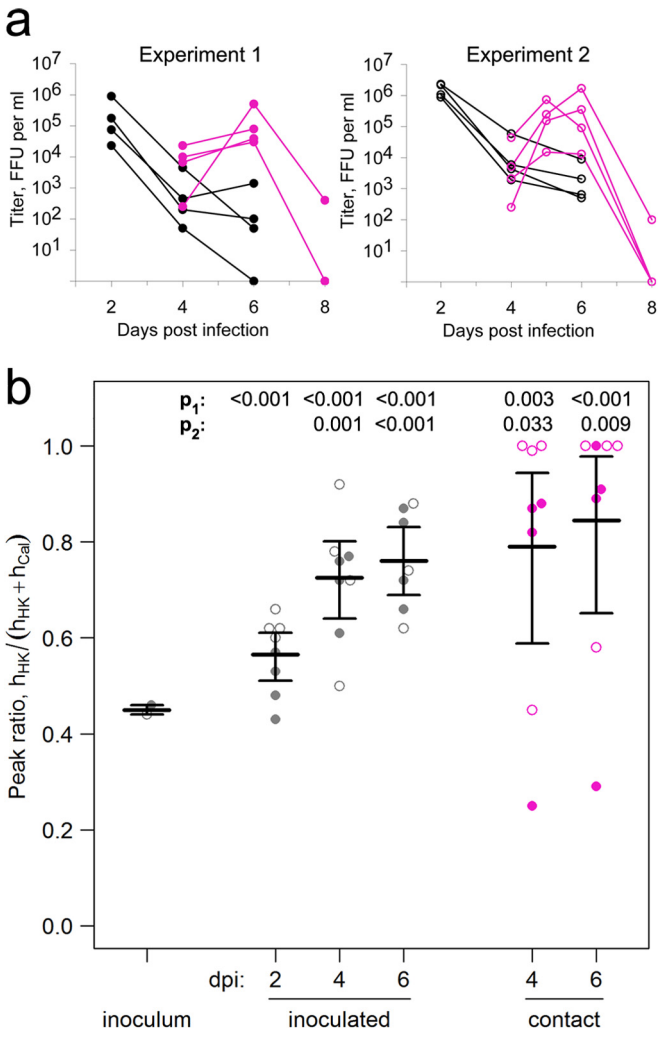

FIG 5 Comparison of viral fitness in competitive transmission experiments in guinea pigs. (a) In each of two experiments, four guinea pigs were inoculated intranasally with a 1:1 mixture of viruses Cal-HA_{HK} and Cal-$(HA+PB1)_{HK}$. Four naive contact guinea pigs were cohoused with inoculated animals starting from day 1 postinfection. Viral titers were determined in nasal washings of directly inoculated animals (black circles) and contact animals (magenta circles). (b) Compositions of the inoculum (black circles), viruses shed by inoculated animals at day 2, 4, and 6 (black circles), and viruses shed by contact animals at days 4 and 6 (magenta circles). Closed and open circles depict data from the first and second experiments, respectively. *P* values refer to the differences with respect to the inoculum (p_1) and with respect to viruses shed by inoculated animals on day 2 (p_2).

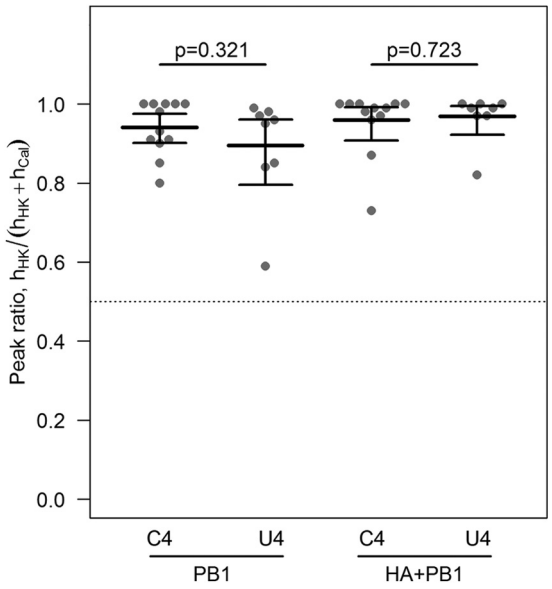

FIG 6 Effects of C4/U4 variation in the 3′NCR of $PB1_{Cal}$ on viral replicative fitness. Mixtures of two viruses differing by PB1 segment ($PB1_{HK}$ and $PB1_{Cal}$) were generated by reverse genetics. Two variants of the $PB1_{Cal}$ plasmid which contained either C4 or U4 in the 3′NCR were used. Peak ratios for all groups were larger than 0.5 ($P < 0.001$). Mean difference of C4 versus U4 in the PB1 group, −0.05 (−0.15, 0.04); mean difference in the HA+PB1 group, 0.01 (−0.05, 0.07).

experiments described above. To account for the second scenario, we generated the $PB1_{Av}$ plasmid by making three mutations in the $PB1_{HK}$ plasmid, R121K, V212L, and K327R, which restored the avian consensus sequence. $PB1_{Av}$ and $PB1_{HK}$ segments then were compared for their ability to compete with the $PB1_{Cal}$ segment in the competitive reverse genetics experiments. The rescues were performed at 33°C to model the temperature of viral infection in the human upper respiratory tract. Viruses containing $PB1_{HK}$ dominated over the viruses with $PB1_{Cal}$ in about half of the replicates [Fig. 8a, PB1(HK) pair and HA+PB1(HK) pair]. The dominance of the viruses with $PB1_{Av}$ was less pronounced [Fig. 8a, PB1(Av) pair and HA+PB1(Av) pair]. As the effect for the PB1(Av) pair did not reach statistical significance after the rescue, we made three additional sequential passages in Calu-3 cells of the replicate rescued PB1(Av) mixtures as well as of the matching PB1(HK) mixtures. The passaging resulted in a further enrichment of the mixtures with the viruses containing $PB1_{Av}$, although to a lower level than that in the case of $PB1_{HK}$ (Fig. 8b). We determined complete sequences of the PB1 segments in the five replicate PB1(Av) mixtures, which contained 100% of the virus with $PB1_{Av}$. No changes in $PB1_{Av}$ sequence were detected, indicating that no adaptive mutations were required for the dominance of $PB1_{Av}$ over $PB1_{Cal}$. Collectively, these experiments showed that in both reassortment scenarios, the avian-origin PB1 segment provided the seasonal human virus with a replicative advantage. Furthermore, the experiments confirmed that neither the U/C variation in position 4 of the 3′NCR of $PB1_{Cal}$ nor the presence of the HA gene segment of HK had significant effects on the outcome of viral competition.

Avian-origin PB1 enhances activity of viral polymerase. The PB1 protein is the key component of the viral polymerase complex, which includes PA, PB1, and PB2 proteins and the viral nucleoprotein (NP). Therefore, we determined effects of the avian PB1 segment on polymerase activity of the reconstituted polymerase complexes using a minigenome luciferase reporter assay. Substitution of the homologous $PB1_{Cal}$ in the Cal polymerase complex by either $PB1_{Av}$ or $PB1_{HK}$ increased polymerase activity (Fig. 9), with the effect of $PB1_{HK}$ being more pronounced than that of $PB1_{Av}$.

DISCUSSION

The factors underlying the formation of pandemic influenza viruses are poorly understood (3, 8). Thus, reasons for the incorporation of an avian-origin PB1 segment into at least two out of four

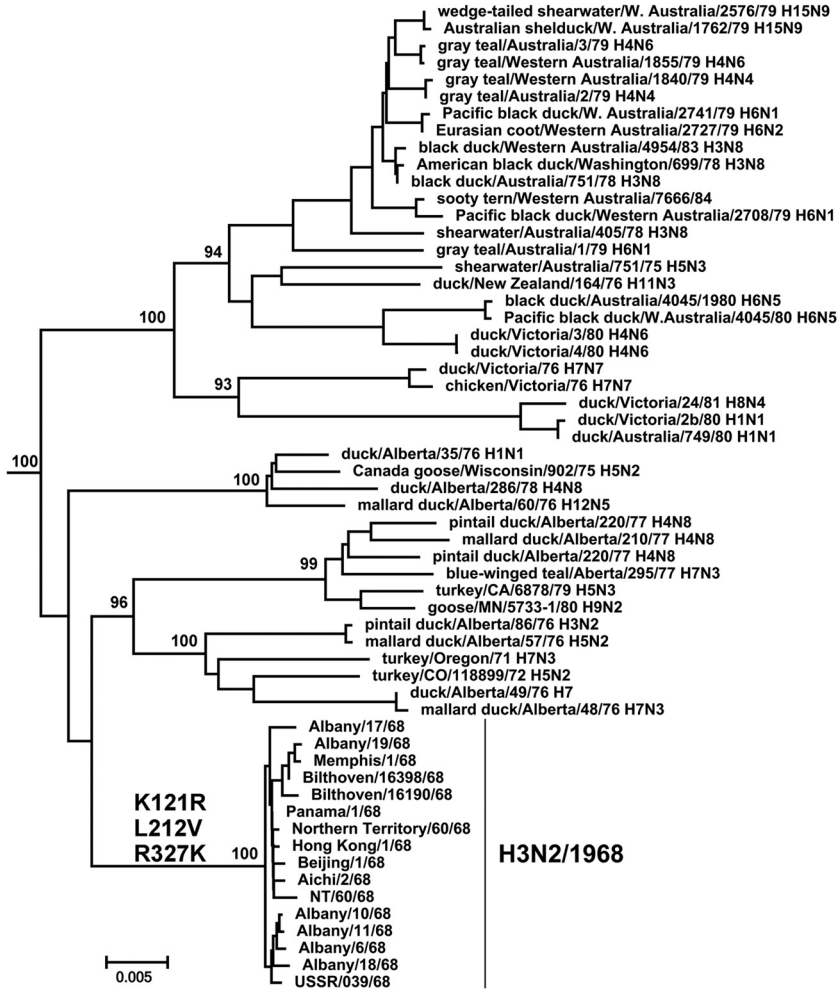

FIG 7 Amino acid differences between the PB1 protein of the 1968 pandemic viruses and its putative avian precursor. Phylogenetic analysis of full-length nucleotide sequences of human and avian PB1 segments was performed as described in Materials and Methods. Shown is a portion of the tree with pandemic virus strains isolated in 1968 and the closest avian viruses. Comparison of amino acid sequences revealed three listed substitutions in the human PB1s with respect to the avian consensus sequence.

characterized pandemic viruses remained enigmatic. Our reconstruction of the 1968 pandemic virus emergence shows that this segment served to facilitate viral replication efficiency. Although the enhancing effect was marginal and could be revealed only in competitive fitness experiments, it can readily explain the selection of the avian PB1-containing virus after natural reassortment between human and avian virus precursors. Deeper insight into the mechanisms responsible for the PB1-mediated enhancement of viral growth will require further studies. Our work indicates, however, that increased activity of the viral polymerase complex containing avian PB1 (Fig. 9) is the most plausible explanation.

Our experiments have been conducted in human cells, and they were supported by transmission studies in a guinea pig model. However, we cannot exclude that the reassortment between avian and human parents of the pandemic virus occurred in some intermediate host, such as swine or, less likely, species of domestic gallinaceous poultry (Fig. 1a). In this view, it would be interesting to test whether the avian PB1 segment also confers an advantage in these species.

Recent studies revealed that the complete set of eight vRNA segments is incorporated into influenza virus particles as a complex stabilized by a network of interactions between segment-specific packaging signals. Importantly, packaging signals and patterns of intersegment interactions in the network can differ from one virus strain to another, and these differences can affect the outcome of the reassortment between two viruses (for reviews, see references 23, 39, and 40). Thus, strain-specific interactions between gene segments might be responsible for the coselection of two or more homologous segments during the emergence of reassortant pandemic viruses (22, 38). In our experiments, the combination of homologous avian HA and PB1 segments did not significantly enhance fitness of the viruses compared to fitness with single-gene PB1 reassortants. Thus, these results argue against a major role of putative cooperation between the HA and PB1 segments during the incorporation of avian PB1 into the 1968 pandemic virus.

Current knowledge on adaptive changes required for zoonotic transmission and emergence of pandemic influenza viruses mostly are limited to amino acid substitutions in the receptor-binding site of HA which alter viral receptor usage and to substi-

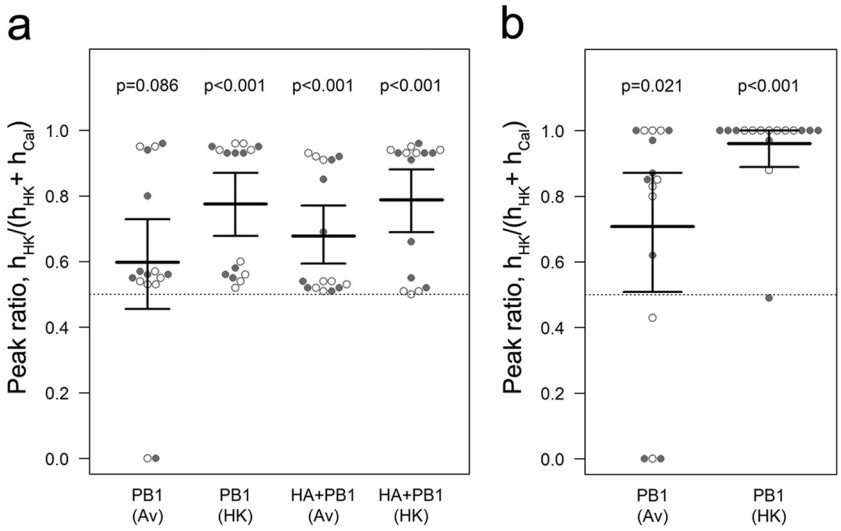

FIG 8 Effects of mutations in PB1 protein on viral replicative fitness. (a) Competitive reverse genetics experiments were performed at 33°C using 1:1 mixtures of $PB1_{Cal}$ plasmid with either $PB1_{HK}$ plasmid [PB1(HK) and HA+PB1(HK)] or $PB1_{Av}$ plasmid [PB1(Av) and HA+PB1(Av)]. Sixteen replicate mixtures were tested for each virus pair. Open and closed circles depict data obtained using $PB1_{Cal}$ plasmids containing C4 and U4 at the 3′NCR, respectively. Mean difference between the PB1(Av) group and HA+PB1(Av) group, 0.08 (−0.08, 0.25); $P = 0.3$. Mean difference between the PB1(HK) group and the HA+PB1(HK) group, 0.01 (−0.12, 0.14); $P = 0.9$. (b) Composition of the rescued virus mixtures PB1(Av) and PB1(HK) after three additional passages in Calu-3 cells at 33°C.

tutions in the PB2 protein which facilitate viral polymerase performance in cells of the upper respiratory tract in humans (for a review, see references 8, 41, and 42). We found here that a combination of 3 amino acid substitutions in the PB1 protein which separate H3N2/1968 virus from its avian precursor increased activity of the polymerase complex in a minireplicon assay and enhanced viral replicative fitness in human cells (Fig. 8 and 9). Thus, these substitutions may represent new markers for avian-to-human adaptation of influenza viruses.

Pandemic influenza viruses differ from seasonal epidemic viruses by carrying an antigenically novel HA envelope, which facilitates viral replication and global spread in a nonimmune population. Our results indicate that enhanced activity of the viral polymerase complex also may be required for the emergence of a pandemic virus from its seasonal virus precursor.

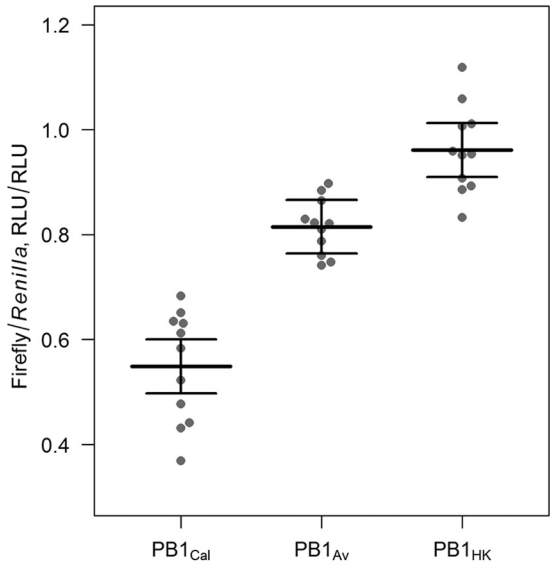

FIG 9 Effects of PB1 on polymerase activity in minigenome reporter assay. 293T cells were cotransfected with firefly luciferase reporter plasmid, *Renilla* luciferase plasmid, and plasmids expressing PB2, PA, and NP of Cal and either $PB1_{Cal}$, $PB1_{av}$, or $PB1_{HK}$. The polymerase activity is expressed as the ratio of the firefly luciferase reading and the *Renilla* luciferase reading. The data represent results from three independent experiments performed on different days using two different stocks of PB1 plasmids. All *P* values are <0.001. RLU, relative light units.

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REFERENCES

1. **Shaw ML, Palese P.** 2013. Orthomyxoviruses, p 1151–1185. *In* Knipe DM, Howley PM, Cohen JI, Griffin DE, Lamb RA, Martin MA, Racaniello VR, Roizman B (ed), Fields virology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. **Kilbourne ED.** 2006. Influenza pandemics of the 20th century. Emerg Infect Dis **12:**9–14. http://dx.doi.org/10.3201/eid1201.051254.
3. **Morens DM, Taubenberger JK.** 2011. Pandemic influenza: certain uncertainties. Rev Med Virol **21:**262–284. http://dx.doi.org/10.1002/rmv.689.
4. **Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, Webster RG, Peiris JS, Guan Y.** 2009. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A **106:**11709–11712. http://dx.doi.org/10.1073/pnas.0904991106.

5. **Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152–179.
6. **Worobey M, Han GZ, Rambaut A.** 2014. Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus. Proc Natl Acad Sci U S A **111:** 8107–8112. http://dx.doi.org/10.1073/pnas.1324197111.
7. **De Jong JC, Rimmelzwaan GF, Fouchier RA, Osterhaus AD.** 2000. Influenza virus: a master of metamorphosis. J Infect **40:**218–228. http://dx.doi.org/10.1053/jinf.2000.0652.
8. **Taubenberger JK, Kash JC.** 2010. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe **7:**440–451. http://dx.doi.org/10.1016/j.chom.2010.05.009.
9. **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.
10. **Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, Digard P.** 2009. A complicated message: identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol **83:**8021–8031. http://dx.doi.org/10.1128/JVI.00826-09.
11. **Fodor E.** 2013. The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virol **57:**113–122. http://dx.doi.org/10.4149/av_2013_02_113.
12. **Chakrabarti AK, Pasricha G.** 2013. An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. Virology **440:**97–104. http://dx.doi.org/10.1016/j.virol.2013.02.025.
13. **Naffakh N, Massin P, Escriou N, Crescenzo-Chaigne B, van der Werf S.** 2000. Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. J Gen Virol **81:**1283–1291.
14. **Li OT, Chan MC, Leung CS, Chan RW, Guan Y, Nicholls JM, Poon LL.** 2009. Full factorial analysis of mammalian and avian influenza polymerase subunits suggests a role of an efficient polymerase for virus adaptation. PLoS One **4:**e5658. http://dx.doi.org/10.1371/journal.pone.0005658.
15. **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. http://dx.doi.org/10.1146/annurev.micro.62.081307.162746.
16. **Pappas C, Aguilar PV, Basler CF, Solorzano A, Zeng H, Perrone LA, Palese P, Garcia-Sastre A, Katz JM, Tumpey TM.** 2008. Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. Proc Natl Acad Sci U S A **105:**3064–3069. http://dx.doi.org/10.1073/pnas.0711815105.
17. **Watanabe T, Watanabe S, Shinya K, Kim JH, Hatta M, Kawaoka Y.** 2009. Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets. Proc Natl Acad Sci U S A **106:**588–592. http://dx.doi.org/10.1073/pnas.0806959106.
18. **Fulvini AA, Ramanunninair M, Le J, Pokorny BA, Arroyo JM, Silverman J, Devis R, Bucher D.** 2011. Gene constellation of influenza A virus reassortants with high growth phenotype prepared as seed candidates for vaccine production. PLoS One **6:**e20823. http://dx.doi.org/10.1371/journal.pone.0020823.
19. **Rudneva IA, Timofeeva TA, Shilov AA, Kochergin-Nikitsky KS, Varich NL, Ilyushina NA, Gambaryan AS, Krylov PS, Kaverin NV.** 2007. Effect of gene constellation and postreassortment amino acid change on the phenotypic features of H5 influenza virus reassortants. Arch Virol **152:** 1139–1145. http://dx.doi.org/10.1007/s00705-006-0931-8.
20. **Wanitchang A, Kramyu J, Jongkaewwattana A.** 2010. Enhancement of reverse genetics-derived swine-origin H1N1 influenza virus seed vaccine growth by inclusion of indigenous polymerase PB1 protein. Virus Res **147:**145–148. http://dx.doi.org/10.1016/j.virusres.2009.10.010.
21. **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. http://dx.doi.org/10.1371/journal.ppat.1000072.
22. **Cobbin JC, Ong C, Verity E, Gilbertson BP, Rockman SP, Brown LE.** 2014. Influenza virus PB1 and neuraminidase gene segments can cosegregate during vaccine reassortment driven by interactions in the PB1 coding region. J Virol **88:**8971–8980. http://dx.doi.org/10.1128/JVI.01022-14.
23. **Steel J, Lowen AC.** 2014. Influenza A virus reassortment. Curr Top Microbiol Immunol **385:**377–401. http://dx.doi.org/10.1007/82_2014_395.
24. **Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol **146:**2275–2289. http://dx.doi.org/10.1007/s007050170002.
25. **Matrosovich M, Matrosovich T, Uhlendorff J, Garten W, Klenk HD.** 2007. Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology **361:**384–390. http://dx.doi.org/10.1016/j.virol.2006.11.030.
26. **Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A **97:**6108–6113. http://dx.doi.org/10.1073/pnas.100133697.
27. **Matrosovich M, Matrosovich T, Garten W, Klenk HD.** 2006. New low-viscosity overlay medium for viral plaque assays. Virol J **3:**63. http://dx.doi.org/10.1186/1743-422X-3-63.
28. **Seibert CW, Kaminski M, Philipp J, Rubbenstroth D, Albrecht RA, Schwalm F, Stertz S, Medina RA, Kochs G, Garcia-Sastre A, Staeheli P, Palese P.** 2010. Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models. J Virol **84:**11219–11226. http://dx.doi.org/10.1128/JVI.01424-10.
29. **Kaminski MM, Ohnemus A, Staeheli P, Rubbenstroth D.** 2013. Pandemic 2009 H1N1 influenza A virus carrying a Q136K mutation in the neuraminidase gene is resistant to zanamivir but exhibits reduced fitness in the guinea pig transmission model. J Virol **87:**1912–1915. http://dx.doi.org/10.1128/JVI.02507-12.
30. **Bao Y, Bolotov P, Dernovoy D, Kiryutin B, Zaslavsky L, Tatusova T, Ostell J, Lipman D.** 2008. The influenza virus resource at the National Center for Biotechnology Information. J Virol **82:**596–601. http://dx.doi.org/10.1128/JVI.02005-07.
31. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:** 2725–2729. http://dx.doi.org/10.1093/molbev/mst197.
32. **R Core Development Team.** 2014. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
33. **Efron B.** 1979. Bootstrap methods: another look at the jackknife. Ann Stat **7:**1–26.
34. **Lindstrom SE, Cox NJ, Klimov A.** 2004. Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957-1972: evidence for genetic divergence and multiple reassortment events. Virology **328:**101–119. http://dx.doi.org/10.1016/j.virol.2004.06.009.
35. **Shimizu K, Li C, Muramoto Y, Yamada S, Arikawa J, Chen H, Kawaoka Y.** 2011. The nucleoprotein and matrix protein segments of H5N1 influenza viruses are responsible for dominance in embryonated eggs. J Gen Virol **92:**1645–1649. http://dx.doi.org/10.1099/vir.0.030247-0.
36. **Brookes DW, Miah S, Lackenby A, Hartgroves L, Barclay WS.** 2011. Pandemic H1N1 2009 influenza virus with the H275Y oseltamivir resistance neuraminidase mutation shows a small compromise in enzyme activity and viral fitness. J Antimicrob Chemother **66:**466–470. http://dx.doi.org/10.1093/jac/dkq486.
37. **Schrauwen EJ, Herfst S, Chutinimitkul S, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Kuiken T, Fouchier RA.** 2011. Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment. Emerg Infect Dis **17:**200–208. http://dx.doi.org/10.3201/eid1702.101268.
38. **Essere B, Yver M, Gavazzi C, Terrier O, Isel C, Fournier E, Giroux F, Textoris J, Julien T, Socratous C, Rosa-Calatrava M, Lina B, Marquet R, Moules V.** 2013. Critical role of segment-specific packaging signals in genetic reassortment of influenza A viruses. Proc Natl Acad Sci U S A **110:**E3840–E3848. http://dx.doi.org/10.1073/pnas.1308649110.
39. **Noda T, Kawaoka Y.** 2010. Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. Rev Med Virol **20:**380–391. http://dx.doi.org/10.1002/rmv.666.
40. **Gerber M, Isel C, Moules V, Marquet R.** 2014. Selective packaging of the influenza A genome and consequences for genetic reassortment. Trends Microbiol **22:**446–455. http://dx.doi.org/10.1016/j.tim.2014.04.001.
41. **Sorrell EM, Schrauwen EJ, Linster M, De Graaf M, Herfst S, Fouchier RA.** 2011. Predicting "airborne" influenza viruses: (trans-)mission impossible? Curr Opin Virol **1:**635–642. http://dx.doi.org/10.1016/j.coviro.2011.07.003.
42. **Klenk HD, Garten W, Matrosovich M.** 2011. Molecular mechanisms of interspecies transmission and pathogenicity of influenza viruses: lessons from the 2009 pandemic. Bioessays **33:**180–188. http://dx.doi.org/10.1002/bies.201000118.