The Effect of the PB2 Mutation 627K on Highly Pathogenic H5N1 Avian Influenza Virus Is Dependent on the Virus Lineage

Jason S. Long,^{a,b} Wendy A. Howard,^b Alejandro Núñez,^b Olivier Moncorgé,^a Samantha Lycett,^c Jill Banks,^b Wendy S. Barclay^a

Section of Virology, Division of Infectious Disease, Imperial College London, London, United Kingdom^a; Animal Health and Veterinary Laboratories Agency, Weybridge, Surrey, United Kingdom^b; Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, United Kingdom^c

Clade 2.2 Eurasian-lineage H5N1 highly pathogenic avian influenza viruses (HPAIVs) were first detected in Qinghai Lake, China, in 2005 and subsequently spread through Asia, Europe, and Africa. Importantly, these viruses carried a lysine at amino acid position 627 of the PB2 protein (PB2 627K), a known mammalian adaptation motif. Previous avian influenza virus isolates have carried glutamic acid in this position (PB2 627E), commonly described to restrict virus polymerase function in the mammalian host. We sought to examine the effect of PB2 627K on viral maintenance in the avian reservoir. Viruses constructed by reverse genetics were engineered to contain converse PB2 627K/E mutations in a Eurasian H5N1 virus (A/turkey/Turkey/5/2005 [Ty/05]) and, for comparison, a historical pre-Asian H5N1 HPAIV that naturally bears PB2 627E (A/turkey/England/50-92/1991 [50-92]). The 50-92 PB2 627K was genetically unstable during virus propagation, resulting in reversion to PB2 627E or the accumulation of the additional mutation PB2 628R and/or a synonymous mutation from an A to a G nucleotide at nucleotide position 1869 (PB2 A1869G). Intriguingly, PB2 628R and/or A1869G appeared to improve the genetic stability of 50-92 PB2 627K. However, the replication of 50-92 PB2 627K in conjunction with these stabilizing mutations was significantly restricted in experimentally infected chickens, where reversion to PB2 627E occurred. In contrast, no significant effects on viral fitness were observed for Ty/05 PB2 627E or 627K in *in vitro* or *in vivo* experiments. Our observations suggest that PB2 627K is supported in Eurasian-lineage viruses; in contrast, PB2 627K carries a significant fitness cost in the historical pre-Asian 50-92 virus.

Historically, highly pathogenic (HP) H5N1 avian influenza viruses (AIVs) have caused sporadic outbreaks of severe disease restricted to gallinaceous poultry (1). The pathogenic nature of these viruses in poultry is attributed to the presence of the multi-basic cleavage site in the hemagglutinin (HA) protein (2–4). Since the emergence of the H5N1 HPAIV in Asia, the epidemiology of HP avian influenza has altered substantially, such that it has now reached panzootic proportions (5). The foundation of this significant epidemiological change can be traced back to the 1997 H5N1 poultry outbreaks in Hong Kong, which coincided with the first human cases (6, 7). These resultant Asian-lineage H5N1 HPAIVs have caused ongoing outbreaks of severe disease in poultry and humans and have undergone continual evolution and reassortment, leading to the emergence of the genotype Z H5N1 viruses (8). Subsequently, an unprecedented outbreak of H5N1 HPAIV in wild aquatic birds occurred in 2005 at Qinghai Lake, China (9–11).

Descendants of these Qinghai Lake-like viruses spread into Europe and Africa, thus establishing the Eurasian-lineage Z viruses (clade 2.2) (5, 12–15). Strikingly, the Qinghai Lake isolates and descendants possessed Lys at amino acid position 627 (627K) on the polymerase basic 2 (PB2) protein, a motif previously associated with adaptation to mammals (10, 11, 16). In contrast, H5N1 viruses isolated from avian hosts prior to 2005 typically possessed a Glu at position 627 (627E), emblematic of the majority of other avian influenza virus subtypes.

The PB2 protein, together with PB1 and PA, form the influenza virus polymerase complex. The polymerase complex interacts with the influenza virus nucleoprotein (NP) and host cellular factors to transcribe and replicate the virus genome (17). The PB2 627K mutation was present in the 1918 Spanish influenza virus and was then maintained during reassortment events in all human-adapted influenza A viruses that circulated in the 20th cen-

tury (18). Interestingly, viruses from the 2009 H1N1 pandemic had PB2 627E but carried other PB2 mutations, 590-91SR and 271A, that appeared to compensate for PB2 627E and adapt the viruses for efficient replication in mammals (19–21). Avian species-derived influenza virus polymerases bearing PB2 627E are restricted in mammalian cells, including cells of human, murine, primate, and porcine origin (22–26). Despite recent developments, the exact mechanism for this host-specific restriction by PB2 627 remains unclear. It has been suggested that position 627-dependent restriction is due to an incompatibility with a host intracellular protein(s) that results in synthesis of a dysfunctional cRNA template and thus blocks viral RNA replication (27).

With regard to H5N1 HPAIV infection, the acquisition of PB2 627K by avian H5N1 viruses has been shown to be important for infection of the human and mouse host (28–31). However, this observation is not absolute, as H5N1 viruses bearing PB2 627E may also successfully infect the mouse (32, 33) and ferret (34, 35), likely when they carry other compensatory mutations that overcome the block to polymerase activity, such as D701N in PB2 (36). PB2 627K was one of the few mutations necessary to support H5N1 respiratory droplet transmission in the ferret host (37).

In the avian host, the polymerase genes of H5N1 have previ-

Received 24 May 2013 Accepted 30 June 2013

Published ahead of print 10 July 2013

Address correspondence to Wendy S. Barclay, w.barclay@imperial.ac.uk, or Jason S. Long, jason.long08@imperial.ac.uk.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.01399-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JVI.01399-13

ously been ascribed to be markers of pathogenesis. By comparison of closely related low-pathogenicity (LP) AIVs (LPAIVs) and H5N1 HPAIVs, Leung et al. (2010) suggested that differences between PA and PB2 of these two phenotypically distinct viruses were responsible for increases in pathogenicity by means of increased polymerase activity (38). In contrast, the comparison by Hulse-Post et al. (2007) of different plaque isolates concluded that changes in PA and PB1 that did not affect polymerase activity were responsible for altered pathogenesis; all the viruses compared in that study carried the PB2 627K mutation, so any role of that mutation in the avian host could not be examined (39). More experimentation is needed to understand the significance and mechanisms whereby genetic variation in polymerase genes affects the outcome of infection in the avian host.

Indeed, the implication of the PB2 627K mutation for the avian host is little understood. Previously, it was widely considered irrelevant following *in vitro* analysis. Polymerase function and growth of PB2 627E/K viruses in avian species-derived cells have demonstrated no host specificity (29, 40, 41). However, recent work by Schat et al. (2012) suggested a small fitness cost associated with H5N1 PB2 627K in the avian host (42). The biological significance of this observation remains unclear, as this single piece of work used a human H5N1 isolate that might have contained other genetic adaptations, and the effect was apparent only in a genetically modified virus background from which the multibasic cleavage site in HA was removed. In a separate study, Bogs et al. (2011) studied the reversion of PB2 627E to the K genotype using a Eurasian-lineage clade 2.2 virus in mammalian cells (41). They found that in MDCK cells this occurred frequently but only in the presence of the cognate NP. In contrast, reversion from E627 to K was not observed for any genotype constellation in avian cells, suggesting no fitness gain by the PB2 627K protein for the avian host (41).

To further examine the effect of the PB2 627K mutation in the avian host, we used reverse genetics to generate viruses bearing either PB2 627K or 627E. As an example of a historical pre-Asian-lineage H5N1 HPAIV bearing the typical avian virus signature PB2 627E, we used A/turkey/England/50-92/1991 (50-92) virus, reportedly restricted to *Galliformes* (43, 44). We compared this to a modern Eurasian-lineage virus (HA clade 2.2, A/turkey/Turkey/05/2005 [Ty/05]) that naturally bears PB2 627K and that is known to infect a variety of avian hosts, including *Anseriformes* (45). The converse mutations were engineered, resulting in an isogenic pair of viruses for each lineage differing only at position 627 in the PB2 protein. Our observations suggest that modern H5N1 HPAIVs tolerate either genotype at PB2 position 627 with no obvious fitness cost or advantage, in contrast to 50-92, which was intolerant of the PB2 627K mutation.

(This study was performed as part of a joint Imperial College London-Animal Health and Veterinary Laboratories Agency [AHVLA] Ph.D. higher education degree by Jason S. Long.)

MATERIALS AND METHODS

Ethics statement. The animal experiments were evaluated and approved by the AHVLA Ethics Committee for Animal Studies and was carried out in accordance with the United Kingdom 1986 Animal Scientific Procedure Act and the AHVLA Code of Practice for Performance of Scientific Studies Using Animals (available upon request). In all *in vivo* experiments, animals deemed moribund following H5N1 infection were killed humanely.

Safety/biosecurity. All infectious experiments were performed in bio-safety level 3+ (BSL-3+) facility (United Kingdom Department of Envi-

ronmental, Food and Rural Affairs [Defra] specific animal pathogen order [SAPO] 4; Advisory Committee on Dangerous Pathogens [ACDP] 3) facilities at AHVLA, Weybridge, United Kingdom.

Plasmid constructs. PolI and pCAGGS plasmids for A/Puerto Rico/8/1934 50-92, Ty/05 (supplied by R. Fouchier, Erasmus University, Rotterdam, Netherlands), and A/England/195/2009 (pH1N1) viruses have been described previously (26, 46). The 50-92, Ty/05, and pH1N1 PB1, PB2, PA, and NP genes were subcloned into the pCAGGS expression plasmid as described previously (25, 26). Mutagenesis of PB2 genes was performed by overlapping PCR as described previously (25, 26). Polymerase assays utilized the minigenome firefly reporter plasmid pChicken-PolI-Firefly or pHuman-PolI-Firefly as described previously (25). All plasmid constructs were verified by DNA sequencing.

Cell culture. Primary chicken embryo fibroblast (CEF) cells were generated from 9-day-old chicken embryos using 0.25% trypsin (Invitrogen). CEF, human embryonic kidney (293T), chicken fibroblast (DF-1), and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; Biosera) and 1% penicillin-streptomycin (PS; Invitrogen). The cell lines were maintained at 37°C in a 5% CO₂ atmosphere.

Generation of recombinant viruses. Viruses were generated by reverse genetics using a 12-plasmid system as described previously (26). Briefly, all 12 plasmids were transfected into 293T cells in 12-well plates using the Fugene HD transfection reagent (Roche Diagnostics). After 24 h, the transfected cells were removed from the wells, mixed with MDCK cells, and cocultured in 25-ml flasks using DMEM containing 10% serum. Six to 8 h later, the cells were briefly washed in serum-free DMEM and the medium was replaced with serum-free DMEM. For generation of viruses with a single basic HA cleavage site, serum-free DMEM was supplemented with 2.5 µg/ml trypsin (Worthington). Reverse transcription-PCR (RT-PCR) was carried out on the cell supernatant of the recovered viruses, the PB2 627K region was amplified, and cDNAs were sequenced to confirm their genotype. Virus stocks were propagated in 9-day-old embryonated chicken eggs incubated at 37°C. Infectious titers were determined by plaque assay on MDCK cells or by determination of the 50% egg infectious dose (EID₅₀).

Chicken experiments. A standard intravenous pathogenicity index (IVPI) test on Ty/05 with a K at PB2 residue 627 (Ty/05/K), Ty/05 with an E at PB2 residue 627 (Ty/05/E), 50-92 bearing K at residue 627 and nt 1869G (50-92/gKR), and 50-92 bearing E at residue 627 and nt 1869A (WT) (50-92/aEQ) was performed, according to OIE guidelines (47).

Oropharyngeal swab, cloacal swab, and blood samples were taken from all chickens prior to infection to test for previous influenza virus infection. Samples revealed no previous exposure to H5 infection by matrix (M) gene quantitative RT-PCR (qRT-PCR) or by hemagglutination inhibition tests using A/turkey/England/50-92/1991 antigen (AHVLA, Weybridge, United Kingdom). Seven 10-week-old White Leghorn specific-pathogen-free chickens (Cherry Valley Farms Ltd., United Kingdom) were infected oculonasally with 10⁶ EID₅₀ units of either 50-92/aEQ or 50-92/gKR. Virus shedding in oropharyngeal and cloacal swab samples and feather samples from the brow was measured. Clinical signs were recorded for each bird at multiple times daily.

Duck experiments. High-health-status Pekin ducks (*Anas platyrhynchos*) were acquired from Cherry Valley Farms Ltd., United Kingdom. Oropharyngeal swab, cloacal swab, and blood samples were taken from all ducks to test for previous influenza virus infection. Samples revealed no previous exposure to H5 infection by H5 gene qRT-PCR or by hemagglutination inhibition tests using A/turkey/Turkey/1/2005 antigen (AHVLA, Weybridge, United Kingdom). Twelve 12-week-old Pekin ducks were each oculonasally inoculated with 10⁶ EID₅₀ units of either Ty/05/K or Ty/05/E. Five 4-week-old naive contact Pekin ducks were cohoused at 1 day postinfection (d.p.i.). Five 12-week-old ducks were randomly selected and humanely culled at 2 d.p.i. for postmortem examination. Oropharyngeal and cloacal swab specimens were taken daily from all birds.

IHC. At postmortem, tissue samples (including nasal turbinates, air sac, bursa, spleen, and gonad) were harvested and fixed in 10% buffered formalin. Four-micrometer-thick sections were stained with hematoxylin-eosin or used for immunohistochemical detection of influenza A virus nucleoprotein as previously described (48). Briefly, sections for immunohistochemistry (IHC) were dewaxed in xylene and passed through graded alcohols to Tris-buffered saline (TBS) solution (0.005 M Tris, pH 7.6, 0.85% [wt/vol] NaCl). Endogenous peroxidase activity was quenched with a methanol-hydrogen peroxide block (BDH) and treated with protease XXIV. Samples were incubated at room temperature with an anti-influenza A virus nucleoprotein primary antibody (Statens Serum Institute, Denmark) at a 1/4,000 dilution in TBS for 1 h and Dako Envision polymer for 30 min. Sections were washed three times with TBS between incubations. The immunohistochemical signal was visualized using 3,3'-diaminobenzidine (Sigma-Aldrich), and sections were counterstained in Mayer's hematoxylin (Surgipath, United Kingdom).

Viral RNA isolation. Viral RNA was isolated from oropharyngeal swab, cloacal swab, and feather samples, egg allantoic fluid, and cell supernatant using an automated QIAquick RNA extraction method (Qia-gen) as previously described (45). Samples were stored at -70°C .

Quantification of viral RNA. 50-92 and Ty/05 viral RNA was quantified by influenza virus matrix and H5 gene qRT-PCR, respectively, as previously described (45). The numbers of relative EID₅₀ units (REU) were calculated by quantitative standards of five 10-fold dilutions of RNA extracted from 10^6 EID₅₀s of Ty/05 or 50-92.

Growth kinetics *in vitro*. CEF cells in 6-well plate format were inoculated at a multiplicity of infection (MOI) of 10^{-3} (50-92) or 10^{-4} (Ty/05), washed once in phosphate-buffered saline (PBS), and incubated in 2 ml DMEM supplemented with 2% FCS and 1% PS at 37 or 41°C. At the times indicated, cell supernatant was collected and the viral titer was determined by qRT-PCR.

Minigenome assay. PB1, PB2, PA, and NP expression plasmids were transfected together with the minigenome firefly reporter plasmid pChicken-PolI-Firefly into avian DF-1 cells or together with pHuman-PolI-Firefly into human 293T cells as previously described (26). Cells were incubated at 37, 39, or 41°C. At 20 h after transfection, cells were lysed with 300 μl of passive lysis buffer (Promega), and firefly luciferase activity was measured using a FLUOstar Omega plate reader (BMG Labtech).

Sequence analysis. H5N1 PB2 sequences ($n = 1,325$) and HA sequences ($n = 2,392$) from avian, human, and other hosts were downloaded from the NCBI Influenza Virus Resource, April 2013 (49). Fifty-nine additional human H5N1 genomes and 1 avian genome were also obtained through the Global Initiative on Sharing All Influenza Data (GISAID; see Table S1 in the supplemental material). The nucleotide sequences were aligned by codon using the Muscle tool in the MEGA v5.05 program and manually adjusted. HP isolates were identified by the presence of a multibasic cleavage site on the HA protein sequences. A neighbor-joining tree was used to identify the virus clades in HA according to the WHO H5 nomenclature (http://www.who.int/influenza/gisrs_laboratory/h5n1_nomenclature/en/b). The tree was generated in MEGA from the downloaded HPAIV HA nucleotide sequences, together with the sequences used in the WHO tree (http://www.who.int/influenza/gisrs_laboratory/201101_h5smalltreealignment.txt), using the Tamura-Nei model, which allows site and branch heterogeneity. HP isolates from the Hong Kong 97-like clade, clade 2.2, and the time periods from 1996 to 2004 and 2005 to the present (excluding isolates in the Hong Kong 97-like clade and clade 2.2) were labeled manually using the FigTree program, and duplicate isolates were removed. The available PB2 sequences corresponding to the labeled isolates (final number after matching and deduplication, 963) were analyzed for PB2 E627K mutations by host type and clade using custom R scripts.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism v6 software.

TABLE 1 Prevalence of the PB2 627K mutation in HP H5N1 virus isolates^a

Asian H5N1 HPAIV isolate	% (no.) of sequences with the following amino acid at PB2 position 627:	
	E	K
Avian		
Hong Kong 97-like	100 (13)	0
1996–2004	98.9 (173)	1.1 (2)
2005–present (excluding clade 2.2)	97.8 (396)	2.2 (9)
2005–present (clade 2.2)	11.3 (24)	88.7 (188)
Human		
Hong Kong 97-like	60 (3)	40 (2)
1996–2004	68.8 (11)	31.2 (5)
2005–present (excluding clade 2.2)	80.2 (93)	19.8 (23)
2005–present (clade 2.2)	0	100 (21)

^a The analysis was performed using full-length PB2 sequences of avian and human H5N1 HPAIVs from the NCBI Influenza Virus Resource database and human H5N1 HPAIVs from GISAID, April 2013.

RESULTS

Prevalence of the PB2 627K mutation in HPAI H5N1 isolates. Of the two PB2 sequences of HPAI H5N1 available prior to 1996 (A/chicken/Scotland/1959 and A/turkey/England/50-92/1991), neither harbored the PB2 627K mutation. To determine the prevalence of the PB2 627K mutation in H5N1 HPAIVs isolated between 1996 and 2013 from avian and human hosts in Asia, Europe, and Africa, viruses were first assigned to the WHO H5 nomenclature by building an H5N1 HA neighbor-joining tree and then identifying the corresponding PB2 mutations. This analysis demonstrated a high prevalence of the PB2 627K mutation in clade 2.2 viruses compared to its prevalence in other H5N1 isolates (Table 1). Human isolates displayed a higher proportion of PB2 627K, indicative of the potential for positive selection of this residue in the human host (Table 1).

Rescue of recombinant viruses altered in PB2 at residue 627 and spontaneous selection of additional mutations upon passage of pre-Asian H5N1 50-92 PB2 627K. To examine the effect of the 627K genotype in an H5N1 avian influenza virus that circulated before 1996, the mutation was engineered into the PB2 gene of a pre-Asian-lineage H5N1 virus, 50-92, isolated in 1991. The corresponding virus pair bearing either E or K at this position was rescued by reverse genetics and termed 50-92/aEQ (wild type [WT]) and 50-92/aKQ. Similarly, an isogenic pair of recombinant Eurasian-lineage H5N1 viruses that differed only by E or K at PB2 residue 627 was rescued and termed Ty/05/K (WT) and Ty/05/E. Viruses generated by reverse genetics were passaged in 9- or 10-day-old embryonated chicken's eggs and sequenced through the PB2 627 region at the time of rescue and at each egg passage. No mutations were observed in Ty/05/K, Ty/05/E, or 50-92/aEQ virus (Fig. 1A to C). In contrast, 50-92/aKQ readily mutated, either acquiring a synonymous mutation from an A to a G nucleotide at nucleotide position 1869 (A1869G) or reverting to 627E. Moreover, viral RNA could not be amplified and sequenced from all 50-92/aKQ-infected eggs due to insufficient virus titer. A recombinant virus carrying the synonymous A1869G mutation was subsequently engineered and was termed 50-92/gKQ (Fig. 1E). Carried out in duplicate, one 50-92/gKQ virus displayed genetic

FIG 1 Rescue of recombinant viruses altered in PB2 at residue 627 and spontaneous selection of additional mutations upon passage of pre-Asian H5N1 50-92 PB2 627K virus. Viruses generated by reverse genetics (RG) were generated by one passage in MDCK cells. Viral RNA of virus generated by reverse genetics was extracted, amplified by RT-PCR, and sequenced in the PB2 627 region. (A) DNA sequencing chromatograms of the sequence from nucleotides 1867 to 1884 of the PB2 gene of viruses generated by reverse genetics (Ty/05/E, Ty/05/K, 50-92/aEQ, 50-92/aKQ) and/or passage mutants generated by reverse genetics (50-92/gKQ, 50-92/gKR). Nucleotide changes are shaded in gray. Mutations introduced by site-directed mutagenesis are underlined by a solid line. Mutations detected upon virus passage are underlined by a dashed line. (B to F) Viruses generated by reverse genetics were blind passaged in embryonated chicken eggs up to egg passage 3 (EP3), and the PB2 627 region was identified by Sanger sequencing ($n = 7$). Eggs were inoculated with <4 hemagglutination units determined by hemagglutination assay using turkey red blood cells and incubated at 37°C . (E and F) 50-92/aKQ and 50-92/gKQ were twice retested using separate viruses generated by reverse genetics displayed on the left and right. (F) 50-92/gKQ input has an unknown mix of 50-92/gKQ and 50-92/gKR viruses after generation through one MDCK passage.

instability at the point of generation, resulting in a change from Gln to Arg at residue 628, while it retained the engineered 627K mutation as well as the synonymous 1869G mutation during MDCK passage. The resultant 50-92/gKR virus was maintained during egg passage (Fig. 1F). The second recombinant 50-92/gKQ virus also selected the 628R mutation, albeit less readily in 1/7 eggs

by the second egg passage (EP2) (Fig. 1F). Indeed, mutation of Q628R was observed in the context of E627K on multiple separate occasions during egg or MDCK cell passage of 50-92 recombinant viruses (data not shown). It was not possible to generate a pure 50-92/aKQ virus of sufficient titer for further experimentation; therefore, subsequent *in vivo* experiments were carried out with

FIG 2 H5N1 50-92/gKR virus is attenuated *in vivo*. Seven 10-week-old chickens were oculonasally inoculated with 10^6 EID₅₀ units of 50-92/aEQ or 50-92/gKR. Viral RNA was extracted from oropharyngeal swab, cloacal swab, or feather samples, and the virus titer was measured by qRT-PCR of the matrix gene, displayed as the mean log₁₀ number of REU. (A) Oropharyngeal virus shedding; (B) cloacal virus shedding; (C) area-under-the-curve (AUC) analysis of shed virus up to 36 h.p.i.; (D) virus titer of the basal calamus of feather samples taken from the brow of chickens at 36 h.p.i.; (E) percent survival of chickens; (F) average number of clinical signs observed per bird at chosen time points. Log values (plus an arbitrary 0.01 to allow log transformation of 0 values) were used for suitable statistical analysis by two-way analysis of variance corrected by the Bonferroni posttest (A, B, C, F) or by two-tailed paired *t* test (D). Error bars display the SEMs. *, *P* < 0.05; **, *P* < 0.01.

50-92/gKR virus, as it appeared to be the most stable during egg passage.

H5N1 50-92/gKR virus is attenuated *in vivo*. To assess the effect of the PB2 627K mutation on pathogenicity in the chicken host, the IVPI assay was used to assign a pathogenicity index (47). In accordance with OIE guidelines, a highly pathogenic virus has an IVPI greater than 1.2. For each virus, ten 6-week-old chickens were intravenously inoculated with virus, and clinical signs were measured daily for 10 days. Both Ty/05 viruses scored a maximum IVPI of 3.00, regardless of the sequence at PB2 residue 627. On the

other hand, 50-92/aEQ, with the typical avian residue, scored a higher pathogenicity index than 50-92/gKR, 2.88 and 1.59, respectively. In addition, sequencing of samples taken from surviving birds at the experiment endpoint revealed that the 50-92/gKR virus had undergone PB2 K627E reversion but retained the 628R mutation during the experiment (data not shown). To further examine the lower pathogenicity in chickens associated with the 627K mutation in 50-92, seven 10-week-old White Leghorn chickens were inoculated oculonasally with 10^6 EID₅₀ units of either 50-92/aEQ or 50-92/gKR and virus shedding was quantified

in both groups. Oropharyngeal virus shedding was significantly higher at 36 h postinfection (h.p.i.) for 50-92/aEQ (2.75 log₁₀ REU) than 50-92/gKR (0.49 log₁₀ REU) (Fig. 2A). Cloacal virus shedding was significantly higher at 36 h.p.i. for 50-92/aEQ (4.03 log₁₀ REU) than 50-92/gKR (1.55 log₁₀ REU) (Fig. 2B). Area-under-the-curve analysis was performed on oropharyngeal and cloacal virus shedding in the first 36 h of infection. Values were higher for 50-92/aEQ than 50-92/gKR, and this difference was statistically significant for cloacal virus shedding (Fig. 2C). Viral titers in the basal calamus of feathers plucked from birds at 36 h.p.i. were significantly higher for 50-92/aEQ (3.94 log₁₀ REU) than 50-92/gKR (1.62 log₁₀ REU), suggesting that 50-92/gKR virus disseminated through the chicken host but replicated to lower titers (Fig. 2D). In addition to higher shedding, 50-92/aEQ caused greater mortality in the first 72 h.p.i. The mean survival time across the experiment was less for chickens infected with 50-92/aEQ (3 days) than those infected with 50-92/gKR (5 days) (Fig. 2E), although this difference was not statistically significant. The number of clinical signs was observed and recorded for both groups. Chickens inoculated with 50-92/aEQ virus demonstrated earlier onset of clinical signs, coinciding with mortality. Taken together, the data show that 50-92/gKR virus replicated less efficiently in chickens than wild-type 50-92/aEQ virus.

Reversion of PB2 K627E and R628Q in mutant non-Asian-lineage H5N1 50-92/gKR upon replication in chickens. The PB2 gene of virus isolates taken at various time points from both groups of infected chickens was sequenced. Viruses from oropharyngeal or cloacal swab samples that were positive for virus by qRT-PCR were sequenced to determine the PB2 amino acid sequence at positions 627 and 628. Five out of seven birds infected with the 50-92/gKR virus showed reversion of the 627K mutation to 627E during the course of the experiment. In 4 of these 5 birds, reversion of the second-site mutation from R back to Q was detected in some samples. The K627E reversion was first detected at 60 h.p.i. in both oropharyngeal and cloacal swab samples (Fig. 3). After this time, the birds inoculated with 50-92/gKR virus showed increased average shedding (Fig. 2A and B) and increased clinical signs (Fig. 2F). Interestingly, a cloacal swab sample from 1 bird carried the aKQ signature at 132 h.p.i.; the biological significance of this is unclear, as the same bird revealed the gER signature in oropharyngeally shed virus at a later time point. In contrast to the genetic instability in birds infected with gKR virus, there was no sequence change in the PB2 gene detected in birds inoculated with 50-92/aEQ virus (data not shown).

PB2 627K does not carry a fitness cost for Eurasian-lineage H5N1 Ty/05. Since poultry were exceptionally susceptible to Ty/05 infection (see the IVPI data above), viral fitness differences between Ty/05/K and Ty/05/E could not be readily measured in this host. Domesticated ducks are a known avian host of modern H5N1 HPAIVs. Ty/05/K WT virus was previously shown to readily infect 12-week-old Pekin ducks and was not lethal in birds of this age, although this virus was 100% lethal to 4-week-old Pekin ducks (45, 48). Therefore, twelve 12-week-old Pekin ducks were infected with 10⁶ EID₅₀ units of either Ty/05/K or Ty/05/E and five 4-week-old naive contact sentinel Pekin ducks were cohoused at 1 d.p.i. to measure contact transmission efficiency. Daily oropharyngeal and cloacal virus shedding was measured in all birds by qRT-PCR. Cloacal virus shedding was absent or extremely limited in 12-week-old ducks (data not shown). There was no significant difference in oropharyngeal virus shedding between the two 12-

FIG 3 Reversion of PB2 K627E and R628Q in mutant non-Asian-lineage H5N1 50-92/gKR upon replication in chickens. At chosen time points, viral RNA was extracted from virus-positive oropharyngeal and cloacal swab samples from chickens infected with 50-92/gKR (Fig. 2), amplified by RT-PCR, and sequenced in the PB2 627 region. Graphs show where 627E was detected; calculated percentages include data for undetectable shedding and dead birds. (A) Oropharyngeal swab samples; (B) cloacal swab samples.

week-old duck groups (Fig. 4A). Similarly, no significant differences in oropharyngeal and cloacal virus shedding were observed in 4-week-old contact ducks, all of whom contracted infection following exposure to directly infected animals (Fig. 4B). Feathers were taken from dead or euthanized 4-week-old ducks, and the virus titer of the basal calamus was measured by qRT-PCR. There was no significant difference in the mean virus titer of the feathers (Fig. 4C). The mean survival time was 7 days for both groups of 4-week-old ducks, with both viruses causing 80% mortality (Fig. 4D). Five randomly selected 12-week-old ducks from each infected group were euthanized at 2 d.p.i., viral loads in tissues were quantified by qRT-PCR, and viral tropism was analyzed by IHC. One to 3 out of 5 birds in each group showed positive qRT-PCR results in some or all tissues, but the loads varied widely and no significant differences were observed (Table 2). No significant gross or histopathological changes were observed in ducks challenged with either viral isolate at 2 d.p.i. The virus distribution was similar in tissues from birds infected with either 627E or 627K virus that were positive by IHC (Fig. 5). Immunolabeling was observed in lymphohistiocytic cells and endothelial cells in the lamina propria in nasal turbinates, epithelial cells in air capillaries and air sacs, medullary cells (lymphocytes, macrophages, and reticular cells) of bursal follicles, splenic red pulp, and stromal cells in ovary (Fig. 5).

All remaining 12-week-old ducks survived the infection, and hemagglutination inhibition of duck sera demonstrated that all surviving ducks seroconverted (data not shown). No mutations

FIG 4 PB2 627K does not carry a fitness cost for Eurasian-lineage H5N1 Ty/05. Twelve 12-week-old Pekin ducks were oculonasally inoculated with 10^6 EID₅₀ units of Ty/05/K or Ty/05/E. Five 4-week-old naive contact Pekin ducks were cohoused at 1 d.p.i. Five 12-week-old ducks were euthanized for postmortem analysis at 2 d.p.i. Virus RNA was extracted from oropharyngeal swab, cloacal swab, or feather samples, and the virus titer was measured by qRT-PCR of the HA gene, displayed as the mean log₁₀ number of REU. (A) Daily oropharyngeal virus shedding of 12-week-old ducks; (B) daily oropharyngeal and cloacal virus shedding of 4-week-old contact ducks; (C) virus titer of the basal calamus of feather samples taken from the breast of dead or euthanized 4-week-old ducks; (D) percent survival of 4-week-old contact ducks. Error bars display the SEMs.

were observed in the 627 region of PB2 in oropharyngeal virus samples from any surviving birds at 9 d.p.i. (data not shown). After detailed analysis, these results did not demonstrate any fitness cost or advantage of PB2 627K for Ty/05 virus in either gallinaceous or anseriform birds.

Growth kinetics in avian cells. To further examine the effect of PB2 627K *in vitro* in the two different virus backbones, multicycle growth curves were performed on primary chicken embryo fibroblast (CEF) cells at both 37 and 41°C, with the latter being more representative of avian body temperature. Viral RNA was extracted from supernatant and quantified by qRT-PCR. At the final time point of the growth curves conducted at 37°C (28 h.p.i.), viral RNA was used to sequence the PB2 gene. The aKQ signature conferred a significant cost to viral replication at both 37 and 41°C in the 50-92 background, and mutation to gKQ was detected at the 28-h.p.i. time point. Surprisingly, viral RNA titers were slightly higher for 50-92/gKQ than 50-92/aEQ at 8 h.p.i. and 37°C, but this pattern was not consistent at later time points (Fig. 6A and B). Ty/05/K and Ty/05/E both replicated to high titers in CEF cells, and there was little difference in the quantity of viral RNA generated by the two viruses (Fig. 6C and D).

Differences in polymerase activity. To understand whether effects on polymerase activity underlie the fitness cost associated with the 50-92 PB2 627K mutation, we performed an *in vitro* polymerase assay using plasmids that expressed WT PB2 (50-92 PB2 627E or Ty/05 PB2 627K) or the corresponding PB2 mutants with their cognate PB1, PA, and NP expression plasmids, together with the minigenome firefly luciferase reporter plasmid pChicken-

Poli-Firefly or pHuman-Poli-Firefly in avian DF-1 or human 293T cells, respectively. For comparison, the polymerase complex and NP of A/England/195/2009 (pH1N1) were also reconstituted in human cells. In this assay, polymerase containing Ty/05/K consistently directed a higher luciferase signal than Ty/05/E polymerase at 37, 39, and 41°C in chicken DF-1 cells, although this was statistically significant only at 37°C (Fig. 7A). Likewise, 50-92 polymerase activity was also increased by the 627K mutation in PB2 at 37°C (Fig. 7A). As expected, polymerases bearing PB2 627E were severely restricted in their ability to support minigenome amplification and expression in human cells, whereas polymerases of both 50-92 and Ty/05 replicated efficiently when carrying the PB2 627K signature (Fig. 7C).

To determine the effect of the 50-92 628R mutation that had emerged during rescue of the recombinant virus, plasmids bearing PB2 628R paired with either 627K or 627E (50-92/KR and 50-92/ER, respectively) were combined with the other 50-92 polymerase constructs and tested for activity in DF-1 cells at 37 and 41°C. Little difference in activity was observed between polymerase constellations with this PB2 change; 50-92/KR caused a small decrease in activity at 37°C compared to 50-92/K (Fig. 7B). These data do not explain the selection of the 628R mutation in the context of the rescued virus.

Altering the genetic constellation of 50-92 prevented reversion of the 627K genotype. Since polymerase activity did not account for the decreased replication *in vitro* and *in vivo* of the 50-92 recombinant virus with the aKQ PB2 genotype, we hypothesized that the selection of the synonymous mutation at PB2 position

TABLE 2 Quantification of viral RNA present in tissues of experimentally infected ducks at 2 d.p.i.^a

Tissue ^a	Ty/05/K			Ty/05/E		
	No. of samples positive/total no. tested ^b	Titer ^c	SD ^d	No. of samples positive/total no. tested	Titer	SD
Nasal turbinate	2/5	4.20	4.12	1/5	1.48	
Lower trachea	2/5	5.93	6.07	1/5	7.00	
Upper trachea	2/5	4.52	4.36	1/5	6.05	
Tracheal swab	2/5	3.73	3.39	1/5	4.06	
Lung	2/5	4.32	4.16	2/5	1.50	0.34
Air sac	3/5	4.98	5.21	1/5	6.98	
Liver	2/5	5.35	5.45	2/5	4.41	4.56
Heart	2/5	2.60	2.04	1/5	4.05	
Kidney	2/5	3.18	2.52	1/5	5.46	
Brain	2/5	6.73	6.88	1/5	5.24	
Spleen	2/5	5.80	5.94	2/5	4.11	4.19
Pancreas	1/5	2.57		1/5	2.41	
Thymus	2/5	4.26	3.99	1/5	5.61	
Bursa	2/5	4.36	4.47	1/5	4.40	
Duodenum	2/5	4.11	4.23	1/5	3.82	
Cecum	2/5	4.17	4.11	1/5	2.58	
Cecal tonsil	2/5	4.16	4.28	1/5	4.98	
Colon	2/5	3.05	3.02	1/5	5.09	
Breast muscle	1/5	2.29		1/5	2.78	
Feather (wing)	0/5			1/5	2.47	

^a Tissues were isolated from 5 randomly selected birds from each group at 2 d.p.i. for postmortem analysis. Viral RNA was extracted and quantified by H5 gene qRT-PCR. Antigen was not detected in the uninfected control bird.
^b The number of samples positive for viral RNA was determined by qRT-PCR.
^c Mean viral RNA titer measured by qRT-PCR (log₁₀ relative EID₅₀ units).
^d Log₁₀ standard deviation.

1869 during passage of this virus indicated an RNA-dependent mechanism. To address this, reassortant viruses were generated by reverse genetics by combining the five internal gene segments of 50-92 virus together with the HA, NA, and M gene segments of PR8 virus (a lab-adapted H1N1 strain) or with the corresponding 3 gene segments from Ty/05 virus. These viruses were termed 50-92/aKQ/PR8_{HA,NA,M} and 50-92/aKQ/Ty/05_{HA,NA,M}, respectively. The PR8 viruses were generated with the aKQ, aEQ, and gKQ PB2 sequences, and the Ty/05 virus carried the aKQ sequence. After three blind passages in embryonated chicken eggs, no changes in the PB2 gene sequence were detected in any of these viruses (data not shown). The introduction of the PR8 or Ty/05 HA, NA, and M genes prevented reversion to gKQ or aEQ, in contrast to the results for 50-92/aKQ virus with its cognate HA, NA, and M gene segments (Fig. 1).

Next, we passaged the recombinant virus panel five times through cells of the MDCK cell line, a mammalian cell line known to select for the 627K mutation (22, 41) (Fig. 8). The sequence of the 627K region of Ty/05/K virus remained the same through each MDCK passage. In contrast, Ty/05/E reverted to PB2 627K by passage 5. Remarkably, the lysine residue was selected by a double nucleotide change (GAA to AAG), despite a single nucleotide being sufficient to switch to the lysine amino acid (GAA to AAA). All the 50-92 recombinant viruses acquired mutations that resulted in the genotype gKQ during passage in MDCK cells, regardless of their starting genotype. Thus, 50-92/aEQ virus mutated to 627K by passage 3 and also acquired the synonymous substitution

A1869G at this time. 50-92/aKQ rescued virus had already gained the synonymous mutation at nucleotide 1869 to become gKQ by the first passage, 50-92/gKR mutated to gKQ at the fourth passage, and 50-92/gKQ remained stable during MDCK cell passage. Taken together, these data provide further evidence of the genetic instability in 50-92 virus caused by the introduction of the 627K mutation. The introduction of the PR8 HA, NA, and M genes into

FIG 5 H5N1 Ty/05/K and Ty/05/E virus antigen distribution in experimentally infected ducks. Immunohistochemical demonstration of influenza A virus nucleoprotein (brown) in tissues of ducks challenged with either Ty/05/K (left) or Ty/05/E (right) at 2 d.p.i. (A) Nasal turbinate. Lymphohistiocytic and endothelial cells in the lamina propria are labeled. (B) Air sac. Note the widespread antigen detection in the epithelium. (C) Spleen. Several positive cells in red pulp are shown. (D) Bursa of Fabricius. Immunolabeling can be seen in lymphocytes, macrophages, and reticular cells in the medulla of bursal follicles. (E) Ovary. Note detection of virus antigen in stromal cells. Bars, 50 μm.

FIG 6 Growth kinetics in avian cells. Primary CEF cells were inoculated with 50-92/aEQ, 50-92/gKR, 50-92/gKQ, or 50-92/aKQ at an MOI of 10^{-3} . Cells were incubated at 37°C (A) or 41°C (B). (A and B) At 28 h postinfection, viral RNA was extracted, amplified by RT-PCR, and sequenced in the PB2 627 region. Reversion from 50-92/aKQ to 50-92/gKQ was detected at 28 h.p.i. (black arrow). In addition, CEF cells were inoculated with Ty/05/K and Ty/05/E at an MOI of 10^{-4} . Cells were incubated at 37°C (C) or 41°C (D). Virus RNA was extracted from the supernatant, and virus titer was measured by qRT-PCR of the matrix (50-92) or HA (Ty/05) gene, displayed as the mean $\log_{10}$ number of REU. Values were determined in triplicate. These trends were reproducible. Error bars display the SEMs. Statistical analysis was by either two-way analysis of variance corrected by the Bonferroni posttest (50-92) or multiple two-tailed *t* test corrected by the Holm-Sidak posttest (Ty/05). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.00001$.

the 50-92 virus background abrogated this genetic instability, and all three of these reassortant viruses, even aEQ virus, remained genetically stable throughout 5 passages in MDCK cells. These observations suggest an RNA incompatibility between the 627K mutation in PB2 and the HA, NA, and/or M gene of 50-92.

DISCUSSION

Since 2005, the unprecedented spread of the Eurasian-lineage H5N1 HPAIVs from Asia to Europe, the Middle East, and Africa has posed a continual threat to domesticated and wild bird populations. In addition, cumulative human cases have been reported in these areas, underscoring the potential for these viruses to give rise to a pandemic. Indeed, although H5N1 has not naturally evolved the ability to spread from person to person, under experimental conditions a clade 2.1 H5N1 virus was shown to acquire mutations that supported transmission between ferrets by the respiratory droplet route, an indicator of pandemic potential (37).

One of the key mutations required for airborne H5N1 transmission in ferrets was the well-known human adaptation motif PB2 627K. Here we confirmed the high prevalence of this mutation in clade 2.2 viruses isolated from both wild and domestic birds compared to that in other H5N1 clades (Table 1), in agreement with the findings of Russell et al. (2012) (50).

Using a reverse genetic approach, we examined the consequence of this mutation for virus replication and transmission in the avian host. We concluded that in the Eurasian-lineage clade

2.2 virus A/turkey/Turkey/05/2005, PB2 627K provided neither a significant fitness cost nor a significant fitness gain and thus did not affect pathogenesis or transmission in ducks or the IVPI score in chickens. The finding that this mutation is neutral in ducks may help refine risk assessments, such as those described by Russell et al. (2012) (50), and indicates the high probability that this mammalian adaptation motif will be maintained in the avian reservoir. Similarly, Schat et al. (2012) (42) concluded that neither disease outcome nor transmission of an HP H5N1 virus in ducks or chickens was altered by PB2 627E or 627K. Their study used a human H5N1 virus isolate of clade 1 (A/Vietnam/1203/2004) which bears 627K. Within the clade 1 virus group, some viruses isolated from human and feline cases contained the 627K mutation, but the majority of natural avian isolates retained 627E (Table 1) (34, 51). Interestingly, Schat and coworkers (42) also performed *in vivo* studies with viruses engineered to be LPAIVs by removal of the multibasic cleavage site in the HA protein. In chickens, the LP virus with PB2 627E induced more profound clinical signs, was detected in more tissues by IHC, and was isolated from more infected bird tissues than the 627K virus. However, the mechanism by which 627E increases replication in chickens in an LP context was not elucidated. Our own data did not conclude any advantage of 627E for polymerase function (Fig. 7) or virus replication (Fig. 6) in chicken cells in either genetic backbone. Schat et al. (2012) (42) did not investigate transmission of the LP viruses between chickens or ducks, so the biological relevance of the ob-

FIG 7 Differences in polymerase activity. Polymerase complex and NP expression plasmids, together with the minigenome firefly luciferase reporter plasmid pChicken-Poli-Firefly or pHuman-Poli-Firefly, were transfected into avian DF-1 or human 293T cells, respectively. Cells were lysed after 20 h, and the level of firefly luciferase activity was measured. (A) Log₁₀ firefly luciferase activity of Ty/05/K, Ty/05/E, 50-92/EQ, and 50-92/KQ with cognate polymerase complex and NP conducted at 37, 39, and 41°C in DF-1 cells; (B) log₁₀ firefly luciferase activity of 50-92/EQ or 50-92/KQ together with the 628R mutation, conducted at 37 and 41°C in DF-1 cells; (C) log₁₀ firefly luciferase activity of Ty/05 and 50-92 PB2 E/K constructs together with pH1N1 human control in 293T cells at 37°C. Mock, transfection of firefly luciferase reporter only. Values were determined in triplicate. These trends were reproducible. Error bars display the SEMs. Statistical analysis was by two-way analysis of variance corrected by the Bonferroni posttest. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

servations in chickens for the maintenance of 627E genotype virus in LP or HP avian influenza viruses in a natural reservoir is not clear.

Clade 2.2 viruses are unusual, as they are shed via the respiratory route in domestic waterfowl, in contrast to the majority of avian influenza viruses, which are shed cloacally (45, 52). The mechanism for this switch in tropism remains unclear. PB2 627K has been shown to confer cold adaptation in mammalian hosts, and it is possible that this mutation provided a small advantage in the cooler temperature of the avian respiratory tract (24). Indeed, we observed an increase in polymerase activity of Ty/05/K compared to that of Ty/05/E at the cooler temperature of 37°C (Fig. 7). However, our *in vivo* experiments showed that Ty/05/E was also shed via the respiratory route, suggesting that other genetic changes may also contribute to the shift in tropism of the clade 2.2 H5N1 viruses.

Indeed, the clade 2.2 Ty/05 HA also naturally carries the mutations T158A, which results in the loss of a glycosylation site, as well as R193 in the receptor binding site; both of these mutations can increase human α 2-6 sialic acid binding, at least when combined with the human receptor-changing mutations Q226/G228 (53, 54). Several poultry species have been shown to contain α 2-6

sialic acid receptors in the respiratory tract (55, 56), which may have selected for the HA mutations and simultaneously supported the 627K genotype in Ty/05 and other clade 2.2 viruses (53). On the other hand, it has also been suggested that such genetic changes have been selected via an intermediate mammalian host or avian species of the Ratitae (e.g., ostrich), before returning to the avian influenza virus reservoir (41, 57). These mutations, combined with the cold-adapting PB2 627K switch, were among those recently shown to confer airborne transmission of HPAI H5N1 virus between ferrets (37, 58). Therefore, our data underscore that in a natural setting the PB2 627K mutation has arisen and is likely to be maintained in a virus whose other genetics support the evolution of a human-transmissible phenotype.

Intriguingly, and in contrast to the results with the modern H5N1 virus, our experiments with a historical H5N1 virus, A/turkey/England/50-92/1991, revealed a significant fitness cost resulting from the genetic instability associated with the nucleotide changes required to generate PB2 627K. Since 50-92 is genetically distinct from the Asian H5N1 viruses, the significance of this finding to currently circulating Asian-lineage H5N1 viruses is difficult to determine, but it may be more pertinent to the observation that most but not all avian isolates naturally maintain a glutamic acid

FIG 8 Altering the genetic constellation of 50-92 prevented reversion of the 627K genotype. Viruses generated by reverse genetics were blind passaged by MDCK cells in T25 flasks ($n = 1$). Cell supernatant was harvested at each passage, viral RNA was extracted, and the PB2 627 region was amplified by RT-PCR and sequenced by Sanger sequencing. Chromatogram trace files for each virus and passage of PB2 from nucleotides 1867 to 1869 and 1879 to 1984 are shown. Solid arrows, nucleotide change.

at PB2 627. Bogs et al. (2011) (41) provided evidence that other viral genes affect the selection of the 627K mutation in PB2. They generated two PB2 gene reassortant viruses with PB2 627E, a Eurasian-lineage (clade 2.2) virus that carried the Hong Kong 1997 (clade 0) H5N1 virus PB2 gene and a Hong Kong 1997 H5N1 virus that carried the Eurasian-lineage PB2 gene. When passaged through MDCK cells, neither of these viruses selected the PB2 627K mutation. In contrast, a Eurasian-lineage virus engineered to bear PB2 627E reverted to 627K by the fifth passage. Further reassortant viruses demonstrated a requirement for the Eurasian-lineage NP to support the 627K reversion (41). Surveillance carried out in Chinese pikas (a small mammal species) near Qinghai Lake revealed two distinct H5N1 virus lineages present in the pika population, a Vietnam-like lineage and a Qinghai Lake-like lineage (59). These virus lineages differed at PB2 627. Only the Qinghai Lake-like viruses carried the PB2 627K mutation and were pathogenic in mice, whereas Vietnam-like viruses retained PB2 627E (41, 59). Since the Vietnam-like-lineage virus did not select

for 627K, this finding may suggest that not all avian influenza viruses favor the 627K genotype, even under natural selective conditions, i.e., in mammals. On the other hand, several reports have described the selection of avian influenza viruses with the PB2 627K mutation, particularly during passage in mice, including H9N2 (60, 61) and H7N7 (62). There are also several reports of viruses that have been stably engineered to possess the 627K mutation (29, 36, 42, 63, 64). Indeed, in the recent outbreak of a novel H7N9 virus in China in 2013, the PB2 627K mutation is often selected for upon human infection (65, 66).

The mechanism driving the observed fitness cost by PB2 627K in the 50-92 genetic background used here remains unclear. Exploration of polymerase activity did not account for *in vitro* and *in vivo* observations, suggesting that the 627K coding change had an effect on the PB2 gene independent of its role in the polymerase complex. We engineered the E627K mutation into 50-92 PB2 by a single nucleotide change, G1879A. In response to this change, we observed the selection of two adenine-to-guanine transitions,

A1869G and A1884G, with the latter encoding a Glu-to-Arg change at amino acid 628. It is unclear why these mutations occurred when a single nucleotide transition, A1879G, was sufficient to revert PB2 to K627E. When the A1869G mutation was engineered into the PB2 gene, the 628R mutation was also selected to various degrees during independent experiments (Fig. 1). The significance of the A1869G mutation was further demonstrated when 50-92/aEQ virus was passaged through MDCK cells and it was selected together with the nonsynonymous mutation that encoded 627K (Fig. 8). Although there are several examples of the 627K mutation being selected for during passage of virus with avian PB2 in cells or mice, the coselection of further mutations synonymous or otherwise in PB2 has not previously been reported (22). The A1869G synonymous change appeared to support the 627K phenotype *in vitro*, as replication of 50-92/gKQ and 50-92/gKR viruses was comparable to that of 50-92/aEQ virus in CEF cells (Fig. 6). However, there was still a significant fitness cost for 50-92/gKR *in vivo*. Interestingly, passage of Ty/05/E virus (Glu codon GAA) through MDCK cells selected for the 627K change as expected but favored a double nucleotide change (Lys codon AAG) rather than a single nucleotide change (Lys codon AAA). This may further demonstrate the importance of the RNA sequence in this region. Virus sequence databases revealed variation in codon use at position 627 among H5N1 viruses, as well as natural examples of the 1869G mutation (data not shown).

The profound genetic instability of the engineered 50-92 PB2 627K gene was circumvented when the internal gene segments of 50-92 were reassorted with the HA, NA, and M genes of PR8 (H1N1) or of Ty/05. In these mosaic genotype viruses, 50-92/aKQ no longer reverted to PB2 1869G. This suggests an interaction between the PB2 gene segment and other gene segments in these viruses that can be disrupted by mutations around the position 627 coding region.

The only *cis*-acting RNA functions described in influenza virus segments thus far are packaging signals. These have been mapped for individual gene segments in various viruses (reviewed in reference 67), and some work supports the idea that some RNA segments are more dominant than others in directing packaging (68–70). Work by Gog et al. (2007) demonstrates how single nucleotide mutations can substantially reduce incorporation of the gene segment during virus particle assembly (71). However, PB2 codons 623, 627, and 628 were not mentioned in this study, and packaging signals of the noncoding and codon regions for segment PB2 have not been mapped to the position 627 region (72). Disruption of RNA-RNA interactions between segments affects packaging of the viral RNPs (69, 70, 73). In particular, PB2 viral RNA has been shown to interact with NA viral RNA using an H5N2 virus model (70).

We speculate that one or all of the cognate HA, NA, and M viral RNA segments of 50-92 may have interfered with the packaging of the viral RNA carrying the PB2 627K mutation. However, it remains uncertain whether the rescued phenotype observed by the addition of PR8 or Ty/05 HA, NA, and M genes is a result of viral RNA interactions or another unknown mechanism increasing virus fitness.

Viruses of the Qinghai Lake outbreak of 2005 carried an important mutation, PB2 627K, significantly increasing the threat of HP H5N1 viruses to the human population. We have demonstrated no fitness cost associated with this mutation in the clade 2.2. viruses from the Eurasian lineage, offering an explanation as

to why this human-adapting mutation is maintained in the avian reservoir. Such occurrences increase the chance of further mutations that can lead to zoonoses or human transmission. Furthermore, the converse finding in an historic H5N1 virus may suggest an explanation for the paucity of the 627K mutation in other influenza viruses in the avian reservoir.

ACKNOWLEDGMENTS

We thank Amanda Hanna, Christine Russell, Anita Puranik, Susan Collins, Bill Cox, Samantha Watson, Kerry Read, and Christopher Glue (AHVLA, Weybridge, United Kingdom) for their excellent technical assistance. We thank R. Fouchier (Erasmus, Netherlands) for the provision of the Ty/05 reverse genetics system.

This work was funded by the Department for Environment, Food and Rural affairs (Defra).

REFERENCES

- Alexander D, Brown I. 2009. History of highly pathogenic avian influenza. *Rev. Sci. Tech.* 28:19–38.
- Bosch FX, Orlich M, Klenk HD, Rott R. 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. *Virology* 95:197–207.
- Horimoto T, Kawaoka Y. 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *J. Virol.* 68:3120–3128.
- Steinhauer DA. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 258:1–20.
- Alexander DJ. 2007. An overview of the epidemiology of avian influenza. *Vaccine* 25:5637–5644.
- Claas EC, Osterhaus AD, Van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351:472–477.
- Subbarao K. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279:393–396.
- Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepongastie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 430:209–213.
- Chen H, Smith GJ, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JS, Guan Y. 2005. H5N1 virus outbreak in migratory waterfowl. *Nature* 436:191.
- Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang X, Zhao D, Wang G, Feng Y, Ma J, Liu W, Wang J, Gao GF. 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* 309:1206.
- Zhou J-Y, Shen H-G, Chen H-X, Tong G-Z, Liao M, Yang H-C, Liu J-X. 2006. Characterization of a highly pathogenic H5N1 influenza virus derived from bar-headed geese in China. *J. Gen. Virol.* 87:1823–1833.
- Neumann G, Green MA, Macken CA. 2010. Evolution of highly pathogenic avian H5N1 influenza viruses and the emergence of dominant variants. *J. Gen. Virol.* 91:1984–1995.
- Wallace RG, Hodac H, Lathrop RH, Fitch WM. 2007. A statistical phylogeography of influenza A H5N1. *Proc. Natl. Acad. Sci. U. S. A.* 104:4473–4478.
- Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, Guan Y. 2006. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proc. Natl. Acad. Sci. U. S. A.* 103:2845–2850.
- Ducatez MF, Olinger CM, Owoade AA, Tarnagda Z, Tahita MC, Sow A, De Landtsheer S, Ammerlaan W, Ouedraogo JB, Osterhaus AD, Fouchier RA, Muller CP. 2007. Molecular and antigenic evolution and geographical spread of H5N1 highly pathogenic avian influenza viruses in western Africa. *J. Gen. Virol.* 88:2297–2306.
- Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, Qi Q, Tian G, Fan S, Zhao H, Sun Y, Kawaoka Y. 2006. Properties and dissemination of H5N1

- viruses isolated during an influenza outbreak in migratory waterfowl in western China. *J. Virol.* 80:5976–5983.
17. Naffakh N, Tomoiu A, Rameix-Welti M-A, Van der Werf S. 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. *Annu. Rev. Microbiol.* 62:403–424.
 18. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. 2005. Characterization of the 1918 influenza virus polymerase genes. *Nature* 437:889–893.
 19. Mehle A, Doudna JA. 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc. Natl. Acad. Sci. U. S. A.* 106:21312–21316.
 20. Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T. 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. *J. Virol.* 84:4395–4406.
 21. Liu Q, Qiao C, Marjuki H, Bawa B, Ma J, Guillosoy S, Webby RJ, Richt JA, Ma W. 2012. 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. *J. Virol.* 86:1233–1237.
 22. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.* 67:1761–1764.
 23. Yao Y, Mingay LJ, McCauley JW, Barclay WS. 2001. Sequences in influenza A virus PB2 protein that determine productive infection for an avian influenza virus in mouse and human cell lines. *J. Virol.* 75:5410–5415.
 24. Massin P, Van der Werf S, Naffakh N. 2001. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. *J. Virol.* 75:5398–5404.
 25. Moncorgé O, Mura M, Barclay WS. 2010. Evidence for avian and human host cell factors that affect the activity of influenza virus polymerase. *J. Virol.* 84:9978–9986.
 26. Moncorgé O, Long JS, Cauldwell AV, Zhou H, Lycett SJ, Barclay WS. 2013. Investigation of influenza virus polymerase activity in pig cells. *J. Virol.* 87:384–394.
 27. Mänz B, Brunotte L, Reuther P, Schwemmle M. 2012. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. *Nat. Commun.* 3:802.
 28. Hatta M, Gao P, Halfmann P. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293:1840–1842.
 29. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. 2004. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. *Virology* 320:258–266.
 30. Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pookruk P, Boonnak K, Khanyok R, Thawachsupha P, Kijphati R, Sawanpanyalert P. 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. *J. Gen. Virol.* 86:423–433.
 31. De Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.* 12:1203–1207.
 32. Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y. 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J. Virol.* 73:3184–3189.
 33. Katz JM, Lu X, Tumpey TM, Smith CB, Shaw MW, Subbarao K. 2000. Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *J. Virol.* 74:10807–10810.
 34. Govorkova EA, Reh J, Krauss S, Yen H, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E. 2005. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J. Virol.* 79:2191–2198.
 35. Zitzow LA, Rowe T, Morken T, Shieh W, Zaki S, Katz JM. 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *J. Virol.* 76:4420–4429.
 36. Steel J, Lowen AC, Mubareka S, Palese P. 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. *PLoS Pathog.* 5:e1000252. doi:10.1371/journal.ppat.1000252.
 37. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, De Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336:1534–1541.
 38. Leung BW, Chen H, Brownlee GG. 2010. Correlation between polymerase activity and pathogenicity in two duck H5N1 influenza viruses suggests that the polymerase contributes to pathogenicity. *Virology* 401:96–106.
 39. Hulse-Post DJ, Franks J, Boyd K, Salomon R, Hoffmann E, Yen HL, Webby RJ, Walker D, Nguyen TD, Webster RG. 2007. Molecular changes in the polymerase genes (PA and PB1) associated with high pathogenicity of H5N1 influenza virus in mallard ducks. *J. Virol.* 81:8515–8524.
 40. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog.* 3:1374–1379.
 41. Bogs J, Kalthoff D, Veits J, Pavlova S, Schwemmle M, Mänz B, Mettenleiter TC, Stech J. 2011. Reversion of PB2-627E to -627K during replication of an H5N1 clade 2.2 virus in mammalian hosts depends on the origin of the nucleoprotein. *J. Virol.* 85:10691–10698.
 42. Schat KA, Bingham J, Butler JM, Chen L-M, Lowther S, Crowley TM, Moore RJ, Donis RO, Lowenthal JW. 2012. Role of position 627 of PB2 and the multibasic cleavage site of the hemagglutinin in the virulence of H5N1 avian influenza virus in chickens and ducks. *PLoS One* 7:e30960. doi:10.1371/journal.pone.0030960.
 43. Wood GW, Parsons G, Alexander DJ. 1995. Replication of influenza A viruses of high and low pathogenicity for chickens at different sites in chickens and ducks following intranasal inoculation. *Avian Pathol.* 24:545–551.
 44. Alexander DJ, Lister SA, Johnson MJ, Randall CJ, Thomas PJ. 1993. An outbreak of highly pathogenic avian influenza in turkeys in Great Britain in 1991. *Vet Rec.* 132:535–536.
 45. Löndt BZ, Nunez A, Banks J, Nili H, Johnson LK, Alexander DJ. 2008. Pathogenesis of highly pathogenic avian influenza A/Turkey/Turkey/1/2005 H5N1 in Pekin ducks (*Anas platyrhynchos*) infected experimentally. *Avian Pathol.* 37:619–627.
 46. Howard W, Hayman A, Lackenby A, Whiteley A, Löndt B, Banks J, McCauley J, Barclay W. 2007. Development of a reverse genetics system enabling the rescue of recombinant avian influenza virus A/Turkey/England/50-92/91 (H5N1). *Avian Dis.* 51:393–395.
 47. World Organisation for Animal Health. 2012. Avian influenza. In OIE manual of diagnostic tests and vaccines for terrestrial animals. World Organisation for Animal Health, Paris, France.
 48. Löndt BZ, Núñez A, Banks J, Alexander DJ, Russell C, Richard-Löndt AC, Brown IH. 2010. The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin ducks (*Anas platyrhynchos*) infected experimentally. *Influenza Other Respir. Viruses* 4:17–25.
 49. 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.
 50. Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, Herfst S, Van Boheemen S, Linster M, Schrauwen EJ, Katzelnick L, Mosterin A, Kuiken T, Maher E, Neumann G, Osterhaus AD, Kawaoka Y, Fouchier RA, Smith DJ. 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* 336:1541–1547.
 51. Amonsin A, Payungporn S, Theamboonlers A, Thanawongnuwech R, Suradhat S, Pariyothorn N, Tantilertcharoen R, Damrongwantanapokin S, Buranathai C, Chaisingh A, Songserm T, Poovorawan Y. 2006. Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. *Virology* 344:480–491.
 52. Wasilenko JL, Arafa A, MSelim AA, Hassan MK, Aly MM, Ali A, Nassif S, Elebiary E, Balish A, Klimov A, Suarez DL, Swayne DE, Pantin-Jackwood MJ. 2011. Pathogenicity of two Egyptian H5N1 highly pathogenic avian influenza viruses in domestic ducks. *Arch. Virol.* 156:37–51.
 53. Stevens J, Blixt O, Chen L-M, Donis RO, Paulson JC, Wilson IA. 2008. Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity. *J. Mol. Biol.* 381:1382–1394.
 54. Medeiros R, Naffakh N, Manuguerra J-C, Van der Werf S. 2004. Binding of the hemagglutinin from human or equine influenza H3 viruses to the receptor is altered by substitutions at residue 193. *Arch. Virol.* 149:1663–1671.
 55. Kuchipudi SV, Nelli R, White GA, Bain M, Chang KC, Dunham S. 2009. Differences in influenza virus receptors in chickens and ducks: implications for interspecies transmission. *J. Mol. Genet. Med.* 3:143–151.
 56. Kimble B, Nieto GR, Perez DR. 2010. Characterization of influenza virus sialic acid receptors in minor poultry species. *Virol. J.* 7:365.

57. Shinya K, Makino A, Ozawa M, Kim JH, Sakai-Tagawa Y, Ito M, Le QM, Kawaoka Y. 2009. Ostrich involvement in the selection of H5N1 influenza virus possessing mammalian-type amino acids in the PB2 protein. *J. Virol.* 83:13015–13018.
58. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 486:420–428.
59. Zhou J, Sun W, Wang J, Guo J, Yin W, Wu N, Li L, Yan Y, Liao M, Huang Y, Luo K, Jiang X, Chen H. 2009. Characterization of the H5N1 highly pathogenic avian influenza virus derived from wild pikas in China. *J. Virol.* 83:8957–8964.
60. Li X, Qi W, He J, Ning Z, Hu Y, Tian J, Jiao P, Xu C, Chen J, Richt J, Ma W, Liao M. 2012. Molecular basis of efficient replication and pathogenicity of H9N2 avian influenza viruses in mice. *PLoS One* 7:e40118. doi:10.1371/journal.pone.0040118.
61. Tian J, Qi W, Li X, He J, Jiao P, Zhang C, Liu G-Q, Liao M. 2012. A single E627K mutation in the PB2 protein of H9N2 avian influenza virus increases virulence by inducing higher glucocorticoids (GCs) level. *PLoS One* 7:e38233. doi:10.1371/journal.pone.0038233.
62. Shinya K, Watanabe S, Ito T, Kasai N, Kawaoka Y. 2007. Adaptation of an H7N7 equine influenza A virus in mice. *J. Gen. Virol.* 88:547–553.
63. Ma W, Lager KM, Li X, Janke BH, Mosier DA, Painter LE, Ulery ES, Ma J, Lekcharoensuk P, Webby RJ, Richt JA. 2011. Pathogenicity of swine influenza viruses possessing an avian or swine-origin PB2 polymerase gene evaluated in mouse and pig models. *Virology* 410:1–6.
64. Song M-S, Pascua PNQ, Lee JH, Baek YH, Park KJ, Kwon H, Park S-J, Kim C-J, Kim H, Webby RJ, Webster RG, Choi YK. 2011. Virulence and genetic compatibility of polymerase reassortant viruses derived from the pandemic (H1N1) 2009 influenza virus and circulating influenza A viruses. *J. Virol.* 85:6275–6286.
65. Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, Saito T, Kawaoka Y, Tashiro M. 2013. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. *Euro Surveill.* 18(15):pii=20453. <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20453>.
66. Jonges M, Meijer A, Fouchier R, Koch G, Li J, Pan J, Chen H, Shu Y, Koopmans M. 2013. Guiding outbreak management by the use of influenza A(H7Nx) virus sequence analysis. *Euro Surveill.* 18(16):pii=20460. <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20460>.
67. Hutchinson EC, Von Kirchbach JC, Gog JR, Digard P. 2010. Genome packaging in influenza A virus. *J. Gen. Virol.* 91:313–328.
68. Gao Q, Chou Y-Y, Doğanay S, Vafabakhsh R, Ha T, Palese P. 2012. The influenza A virus PB2, PA, NP, and M segments play a pivotal role during genome packaging. *J. Virol.* 86:7043–7051.
69. Fournier E, Moules V, Essere B, Paillart J-C, Sirbat J-D, Isel C, Cavalier A, Rolland J-P, Thomas D, Lina B, Marquet R. 2012. A supramolecular assembly formed by influenza A virus genomic RNA segments. *Nucleic Acids Res.* 40:2197–2209.
70. Gavazzi C, Isel C, Fournier E, Moules V, Cavalier A, Thomas D, Lina B, Marquet R. 2013. An in vitro network of intermolecular interactions between viral RNA segments of an avian H5N2 influenza A virus: comparison with a human H3N2 virus. *Nucleic Acids Res.* 41:1241–1254.
71. Gog JR, Afonso Edos S, Dalton RM, Leclercq I, Tiley L, Elton D, Von Kirchbach JC, Naffakh N, Escρίου N, Digard P. 2007. Codon conservation in the influenza A virus genome defines RNA packaging signals. *Nucleic Acids Res.* 35:1897–1907.
72. Dos Santos Afonso E, Escρίου N, Leclercq I, Van der Werf S, Naffakh N. 2005. The generation of recombinant influenza A viruses expressing a PB2 fusion protein requires the conservation of a packaging signal overlapping the coding and noncoding regions at the 5' end of the PB2 segment. *Virology* 341:34–46.
73. Fournier E, Moules V, Essere B, Paillart J-C, Sirbat J-D, Cavalier A, Rolland J-P, Thomas D, Lina B, Isel C, Marquet R. 2012. Interaction network linking the human H3N2 influenza A virus genomic RNA segments. *Vaccine* 30:7359–7367.