

# Mutations to PB2 and NP Proteins of an Avian Influenza Virus Combine To Confer Efficient Growth in Primary Human Respiratory Cells

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## ABSTRACT

Influenza pandemics occur when influenza A viruses (IAV) adapted to other host species enter humans and spread through the population. Pandemics are relatively rare due to host restriction of IAV: strains adapted to nonhuman species do not readily infect, replicate in, or transmit among humans. IAV can overcome host restriction through reassortment or adaptive evolution, and these are mechanisms by which pandemic strains arise in nature. To identify mutations that facilitate growth of avian IAV in humans, we have adapted influenza A/duck/Alberta/35/1976 (H1N1) (dk/AB/76) virus to a high-growth phenotype in differentiated human tracheo-bronchial epithelial (HTBE) cells. Following 10 serial passages of three independent lineages, the bulk populations showed similar growth in HTBE cells to that of a human seasonal virus. The coding changes present in six clonal isolates were determined. The majority of changes were located in the polymerase complex and nucleoprotein (NP), and all isolates carried mutations in the PB2 627 domain and regions of NP thought to interact with PB2. Using reverse genetics, the impact on growth and polymerase activity of individual and paired mutations in PB2 and NP was evaluated. The results indicate that coupling of the mammalian-adaptive mutation PB2 E627K or Q591K to selected mutations in NP further augments the growth of the corresponding viruses. In addition, minimal combinations of three (PB2 Q236H, E627K, and NP N309K) or two (PB2 Q591K and NP S50G) mutations were sufficient to recapitulate the efficient growth in HTBE cells of dk/AB/76 viruses isolated after 10 passages in this substrate.

## IMPORTANCE

Influenza A viruses adapted to birds do not typically grow well in humans. However, as has been seen recently with H5N1 and H7N9 subtype viruses, productive and virulent infection of humans with avian influenza viruses can occur. The ability of avian influenza viruses to adapt to new host species is a consequence of their high mutation rate that supports their zoonotic potential. Understanding of the adaptation of avian viruses to mammals strengthens public health efforts aimed at controlling influenza. In particular, it is critical to know how readily and through mutation to which functional components avian influenza viruses gain the ability to grow efficiently in humans. Our data show that as few as three mutations, in the PB2 and NP proteins, support robust growth of a low-pathogenic, H1N1 duck isolate in primary human respiratory cells.

Influenza A viruses circulate in a wide range of animal hosts (1). The major reservoir for these viruses comprises wild waterfowl of the orders Anseriformes (mainly ducks, geese, and swans) and Charadriiformes (mainly shorebirds, terns, and gulls) (2, 3). Influenza A viruses adapted to avian hosts occasionally infect human, swine, and other mammalian species (4). Such zoonotic transmission events do not typically initiate outbreaks due to the host specificity of influenza A viruses: avian strains are poorly adapted for growth and transmission in mammalian hosts (5, 6). Infection of mammals with avian influenza viruses does, however, create the opportunity for these viruses to adapt to a new species. Such adaptation can occur through the acquisition of point mutations arising through polymerase errors and/or the reassortment of gene segments between viruses derived from different hosts (2, 7–9). When these evolutionary processes lead to escape from host restriction and efficient growth and transmission in the new host species, outbreaks ensue (10). In humans, this process leads to pandemics.

An understanding of how influenza viruses overcome host species barriers is important for pandemic preparedness. The identification before an outbreak or early in its course of influenza viruses with genetic changes known to enhance fitness in humans can inform public health responses by indicating the urgency with which containment measures should be put in place. For this reason, and with the aim of understanding influenza virus ecology at a fundamental level, much research effort has been spent on identifying viral determinants of host range (10, 11).

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Strain differences in hemagglutinin (HA) attachment and fusion have each been found to play a role in defining influenza virus host range. The specificity of HA receptor binding differs between human- and avian-adapted influenza viruses, with avian strains binding preferentially sialic acids with  $\alpha$ 2,3 linkages to galactose and human strains binding predominantly  $\alpha$ 2,6-linked sialic acids. Although both receptor types are found in the target tissues of human and avian hosts, their distributions differ (12–14), and changes to the HA receptor binding pocket that improve recognition of human-type receptors have been shown to increase the growth and/or transmission of avian influenza viruses in mammalian hosts (15–18). Similarly, the precise pH at which HA proteins undergo the conformational changes required for fusion has been implicated in host range restriction, with avian influenza viruses tending to have a higher pH of fusion than human strains (15, 16, 19–21).

To achieve efficient attachment to and release from host cells, influenza virus HA and neuraminidase (NA) proteins must be in balance. Thus, host-adaptive changes in HA binding are often associated with alteration of NA (22). Polymorphisms in the M segment have also been shown to alter NA activity and may be important for maintaining HA/NA balance following host species jumps (23).

The viral polymerase complex, comprised of PB2, PB1, and PA polymerase proteins plus NP, must be well adapted to the host cell to mediate efficient replication and transcription of the viral genome (24). The importance of the replication machinery in host adaptation is underlined by the fact that most host-associated genetic signatures identified among natural isolates (25, 26) and mouse-adapted variants (27–29) lie in the polymerase and NP genes. Most notably, a few mutations that alter amino acid charge in the so-called 627 domain of PB2 have been found to have potent effects on the polymerase activity, replication, virulence, and transmission of avian influenza viruses in mammalian substrates and hosts (18, 30–38). While the precise mechanism(s) by which these mutations act have not been established conclusively, various reports suggest potentially overlapping effects of (i) differential temperature sensitivity of polymerase activity (39, 40), (ii) differences in the strength of interactions between PB2 and NP (30, 41–44), (iii) differences in polymerase binding to the viral promoter (45, 46), and (iv) changes in the strength of interactions between PB2 or viral ribonucleoproteins (vRNPs) and human isoforms of importin- $\alpha$  (47–50). The mammalian adaptive mutation NP N319K was also shown to enhance the interaction of monomeric NP with human importin- $\alpha$ 1 (47). Perhaps due to the multiple interactions between NP and cellular factors (51–57), this protein has been found repeatedly to play a role in host restriction (58–61).

Although significant progress has been made toward understanding how influenza A viruses adapt to new host species, the picture remains incomplete. Surveillance of influenza viruses circulating in wild birds and domestic animals, as well as full-genome sequencing of isolates derived from that surveillance, has increased dramatically in recent years. Nevertheless, it remains difficult based on sequencing data alone to estimate the zoonotic potential of these viruses. Here, we sought to broaden our knowledge of mutations that facilitate the growth of avian influenza viruses in humans. To this end, we adapted an avian influenza virus, A/duck/AB/35/1976 (H1N1) (dk/AB/76), to primary human respiratory cell cultures and sequenced the genomes of intact

viral isolates. Reintroduction of specific sequence changes acquired during adaptation using reverse genetics allowed us to investigate their phenotypic impact. Our results support the concept that the PB2 E627K mutation acts in concert with NP to improve virus multiplication in mammalian cells and suggest a similar relationship between NP and PB2 proteins carrying polymorphisms at position 591. Data from polymerase reconstitution assays confirm that the PB2 E627K and Q591K mutations greatly enhance polymerase activity in mammalian cells but suggest that the contributions of adaptive mutations in NP to increased viral fitness occur through a different mechanism. Finally, our data reveal novel host-adaptive mutations acquired in a relevant human substrate.

## MATERIALS AND METHODS

**Cells.** Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (Gibco) supplemented with 10% fetal bovine serum and penicillin-streptomycin and were used for plaque assays. 293T cells were maintained in Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% fetal bovine serum.

Human tracheo-bronchial epithelial (HTBE) cells were purchased from Lonza and passed twice to generate a stock of cells stored in liquid nitrogen. Fully differentiated HTBE cells were used for viral infections. For differentiation, cells were amplified once in standard cell culture flasks and then seeded onto 12-mm-diameter transwell filters (Corning) coated with collagen from human placenta (Sigma-Aldrich). Once confluent monolayers were formed on the filters, medium was removed from the apical chamber, and cells were fed fresh medium only through the basolateral surface. At this time, retinoic acid (Sigma) at a final concentration of 50 nM was added to the medium. Cells were maintained in this way, with medium refreshed on Monday, Wednesday, and Friday, for at least 4 weeks to allow differentiation. For the initial amplification of HTBE cells, bronchial epithelial growth medium (BEGM), trypsin-EDTA, and HEPES-buffered saline solution from Lonza were used, according to the manufacturer's instructions. Upon seeding onto transwell filters, cells were maintained in Gray's medium, a 1:1 mixture of bronchial epithelial basal medium (BEBM) and DMEM (Gibco), supplemented with growth factors and antimicrobials included in a SingleQuots kit (Lonza).

**Viruses.** RNA derived from the A/duck/Alberta/35/1976 (H1N1) (dk/AB/76) biological isolate was a kind gift of Terrence Tumpey (Centers for Disease Control and Prevention). To generate a reverse genetics system for this virus, the dk/AB/76 genome was cloned into the pDZ ambisense vector (62) using standard methods. Multiple plasmid clones were sequenced to obtain a consensus for each segment, and plasmids encoding the consensus sequence were used for rescue. The PB2 and NP segments were subsequently subcloned into the pPOL1 reverse genetics plasmid to facilitate site-directed mutagenesis. Where pPOL1 PB2 or NP plasmids were used for rescue, pCAGGS influenza A/WSN/1933 (WSN) PB2 and/or pCAGGS WSN NP plasmids were also included as appropriate. Viruses were obtained by standard reverse genetics techniques (63, 64). Briefly, 293T cells in DMEM were cotransfected with 8 to 10 plasmids. One day after transfection, 293T cells and associated medium were collected and used to inoculate 10-day-old embryonated hens' eggs. Viruses were recovered by harvesting allantoic fluid 2 days after infection and were titrated by plaque assay on MDCK cells.

The A/Panama/2007/1999 (Pan/99) (H3N2) virus used was generated by reverse genetics as previously described (9, 31). The A/Netherlands/602/2009 (NL/09) (H1N1) virus used was also generated by reverse genetics, and this rescue system was generously provided by Ron Fouchier (Erasmus Medical Center) (65).

**Serial passage and isolation of viral clones.** The reverse genetics-derived dk/AB/76 virus was used to initiate three independent lineages of HTBE cell passage. The lineages were labeled A, B, and C. At each passage, infection was carried out with 0.1 PFU/cell in one well per lineage. Re-

leased virus was collected from the apical surface of HTBE cells by incubation in 200  $\mu$ l of phosphate-buffered saline (PBS) for 30 min at either 48 h or 72 h after infection. For the initial passages, virus was collected at 72 h; after passage 5, virus was collected at 48 h. Samples were aliquoted, stored at  $-80^{\circ}\text{C}$ , and titrated by plaque assay on MDCK cells prior to the next passage.

Viral clones were derived from the passage 10 (P10) populations by performing plaque assays on MDCK cells and collecting agar plugs from well-isolated plaques. These isolates were then amplified once on differentiated HTBE cells to generate virus stocks for growth and sequencing analyses. Clones A3 and A4 were derived from lineage A, clone B4 was from lineage B, and clones C3, C4, and C7 were from lineage C. Clones were selected for further analysis based on (i) a high titer upon amplification in HTBE cells and (ii) a diverse set of plaque phenotypes since variable plaque morphologies were seen in MDCK cells.

**Sequencing.** Full-genome sequences were determined for six clonal isolates derived from the dk/AB/76 passage 10 populations. For this purpose, viral RNA (vRNA) was extracted from 140  $\mu$ l of each HTBE cell-amplified virus stock using a QIAamp Viral RNA Extraction kit (Qiagen). Reverse transcription was performed using segment-specific universal primers complementary to the 3' end of the vRNA (sequences are given in Hoffmann et al. [66]) and Transcriptor reverse transcriptase (Roche). PCR amplification of each segment was then achieved using paired segment-specific universal primers [66]. PCR products were separated by agarose gel electrophoresis, extracted from the gel using a QIAquick Gel Extraction kit (Qiagen), and sequenced directly by Genewiz. Lasergene software was used for sequence analysis, and mutations were identified by alignment of amino acid sequences obtained for the passage 10 isolates with that of the reverse genetics-derived dk/AB/76 virus.

**Analysis of growth in HTBE cells.** HTBE cells on 12-mm-diameter filters were washed three times with PBS to remove mucus and then infected via the apical surface in a 100- $\mu$ l volume of PBS containing virus diluted to either 0.1 or 0.01 PFU/cell. Following a 45-min incubation at  $37^{\circ}\text{C}$ , the inoculum was removed, and the apical surface was washed three times with PBS. Released virus was collected immediately after the removal of inoculum (0 h) and at 8, 12, 24, 48, 72, and, in some cases, 96 h postinfection by the addition of 200  $\mu$ l of PBS to the apical surface and incubation at  $37^{\circ}\text{C}$  for 30 min. Samples were stored at  $-80^{\circ}\text{C}$  and titrated by plaque assay on MDCK cells. Unless otherwise indicated, growth analyses were performed in triplicate. Statistical significance was evaluated using Student's *t* test.

**Polymerase reconstitution assays.** To evaluate viral polymerase activity, 293T cells in six-well plates were cotransfected with pCAGGS dk/AB/76 PB2, pGAGGS dk/AB/76 PB1, pCAGGS dk/AB/76 PA, pCAGGS dk/AB/76 NP, pPOL1 NP luc (encoding, in the negative sense, firefly luciferase flanked by the NP untranslated regions of influenza A/WSN/1933 virus), and pRL-TK, encoding *Renilla* luciferase under the control of a thymidine kinase promoter. Transfections lacking pCAGGS dk/AB/76 PB2 were included as a negative control. To test the impact of adaptive mutations in NP and PB2 proteins, plasmids encoding the wild-type open reading frames (ORFs) were replaced with those carrying the relevant mutation(s). Per well, 0.5  $\mu$ g of each plasmid was used. X-tremeGENE (Roche) transfection reagent was used according to the manufacturer's instructions. Cells were incubated at  $37^{\circ}\text{C}$  for 24 h after transfection and then washed, collected in fresh DMEM, and reseeded into 96-well plates. Cells from each well of a six-well plate were divided among eight wells of the 96-well plate. Cells were then incubated for a further 24 h at  $37^{\circ}\text{C}$  and then lysed for quantification of luminescence using a Dual-Glo luciferase assay system (Promega), according to the manufacturer's instructions, and a Bio-Tek Synergy plate reader. Each assay was run at least twice, with each transfection set up in duplicate (yielding  $2 \times 8$ , or 16, data points for each set of plasmids in each assay). Data were analyzed by calculating the average of all 16 data points, dividing the average firefly activity by the average *Renilla* activity (to address variation in transfection efficiency),

and then normalizing all values to those obtained for the dk/AB/76 wild-type polymerase.

## RESULTS

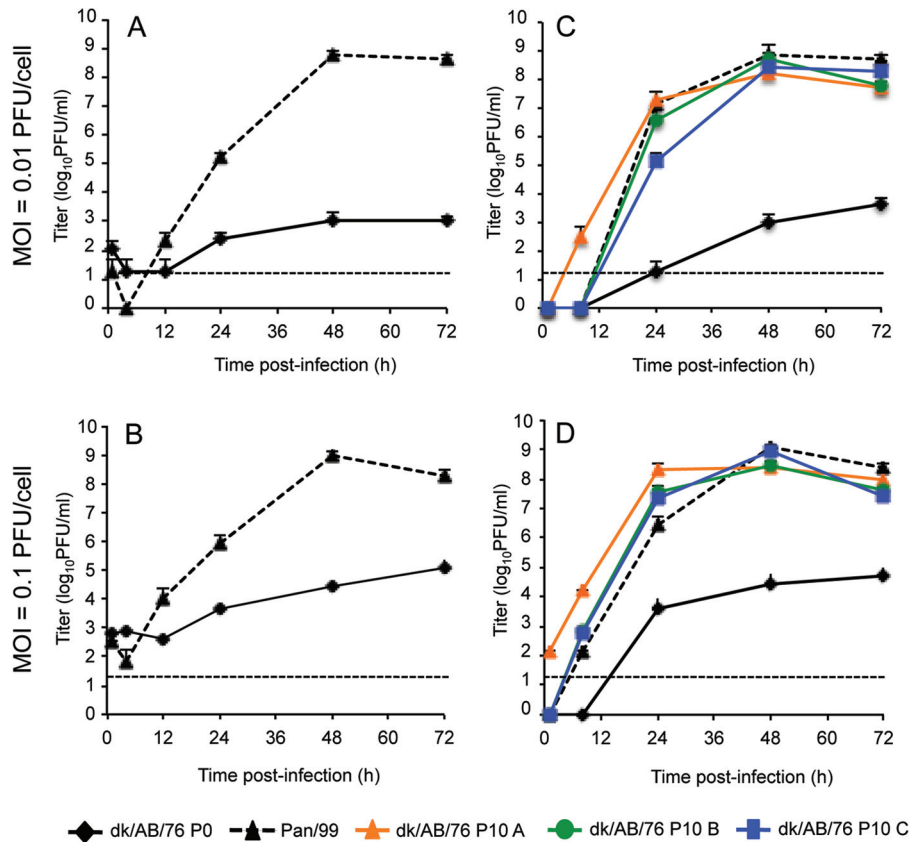
**Adaptation of dk/AB/76 virus to HTBE cells.** To establish parameters for serial passage, we first evaluated the growth of the wild-type dk/AB/76 virus in HTBE cells. Growth was compared to the human seasonal strain, Pan/99, from multiplicities of infection (MOIs) of 0.01 and 0.1 PFU/cell. As shown in panels A and B of Fig. 1, dk/AB/76 virus grew very poorly, reaching titers of approximately  $1 \times 10^3$  and  $1 \times 10^5$  PFU/ml at the MOIs of 0.01 and 0.1 PFU/cell, respectively. Pan/99 virus, by contrast, yielded maximal titers of  $6 \times 10^8$  and  $9 \times 10^8$  PFU/ml, respectively, under the same conditions. Based on these results, an MOI of 0.1 PFU/cell was selected for serial infections with dk/AB/76 virus, and, for the first five passages, infections were allowed to proceed for 72 h prior to the collection of virus. Passaging was performed in triplicate such that three independent lineages (A, B, and C) were generated. Following 10 passages, the growth properties of the three passage 10 populations were compared to those of wild-type dk/AB/76 and Pan/99 viruses, again from MOIs of 0.01 and 0.1 PFU/cell (Fig. 1C and D). The results indicated very robust adaptation in all three dk/AB/76 virus lineages. Yields of populations A, B, and C were approximately  $10^4$ - and  $10^5$ -fold greater than those of dk/AB/76 virus at MOIs of 0.1 and 0.01 PFU/cell, respectively, and were similar to those of Pan/99 virus.

**Sequencing of HTBE cell-adapted dk/AB/76 viruses.** For sequencing, plaque isolates were derived from each of the passage 10 populations. We chose to sequence clonal virus stocks rather than to determine a consensus sequence for the mixed populations in order to learn which mutations arose together in an intact viral genome. Six clones were selected for sequencing: two from lineage A (A3 and A4), one from lineage B (B4), and three from lineage C (C3, C4, and C7). The growth of these isolates was evaluated from an MOI of 0.01 PFU/cell to confirm that they exhibited high-growth phenotypes in HTBE cells (Fig. 2).

Relative to the dk/AB/76 wild-type virus, each passage 10 isolate carried between five and nine differences in amino acid sequence throughout its genome. All six isolates had mutations in the NP, HA, and PB2 genes, and none showed changes in M2, PA, PA-X, and PB1-F2 open reading frames. Specific mutations are listed in Table 1 and shown schematically in Fig. 3. We noted that all viruses carried mutations in the PB2 627 domain that have been found previously to confer improved growth in mammalian cells [32, 38]. In addition, all six of the passage 10 clones carried mutations in the regions of NP that have been found to contribute to NP-PB2 binding [24, 67]. The passage 10 HA proteins each carried one mutation in the HA receptor binding site and one within HA2, sites that may impact HA stability and the pH of fusion. For the present manuscript, however, we focused our efforts on the PB2 and NP proteins.

**Growth in HTBE cells of reverse genetics-derived viruses carrying targeted mutations in NP and/or PB2 proteins.** We used reverse genetics to introduce targeted mutations into the dk/AB/76 virus genome with the aim of evaluating their contribution to HTBE cell adaptation. The following amino acid changes were introduced alone or in the combinations indicated in Fig. 4: NP Q12R, NP S50G, NP N309K, NP I425V, NP E454K, PB2 Q236H, PB2 Q591K, and PB2 E627K. Multicycle growth analyses were then performed, again in fully differentiated HTBE cells. Due





**FIG 1** After 10 passages in HTBE cells, dk/AB/76 viruses exhibited growth phenotypes similar to the growth phenotype of the seasonal human strain A/Panama/2007/1999 (H3N2). Viral growth in differentiated HTBE cells is shown. In panels A and C, cells were infected at an MOI of 0.01 PFU/cell. In panels B and D, cells were infected at an MOI of 0.1 PFU/cell. Data shown in panels A and B were generated at the same time and indicate the growth of dk/AB/76 virus prior to passage in HTBE cells compared to that of Pan/99 virus. Data shown in panels C and D were generated at the same time and show the growth of the three independent virus populations (P10 A, B, and C) collected after 10 passages of dk/Ab/76 virus in HTBE cells. The dk/AB/76 passage 0 (P0) and Pan/99 viruses are again included for comparison. The averages of three replicates are plotted, and error bars represent standard deviations. The limit of detection is indicated by a dashed horizontal line.

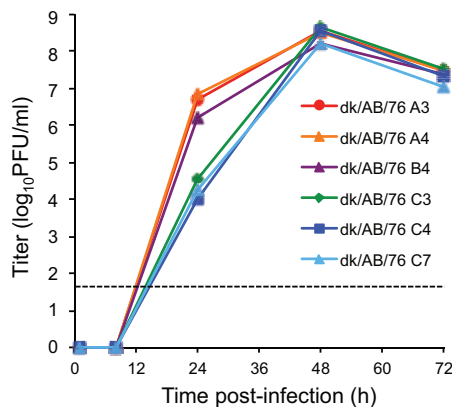
to extraneous circumstances, characterization of the reverse genetics-derived viruses was carried out in HTBE cells derived from a different human donor than those that were used to passage dk/AB/76 virus and to test the growth of the P10 populations and

biological isolates. For this reason, we evaluated the growth of the P10 biological isolates again in the new HTBE cells (Fig. 4A; note that clone C7 is missing due to exhaustion of the virus stock). All five clones showed similar growth phenotypes and >1,000-fold higher growth than the wild-type dk/AB/76 virus. However, in the new HTBE cells, the P10 isolates grew to 10- to 100-fold lower titers than Pan/99 virus.

Since the PB2 627 domain is known to have a potent effect on growth of avian influenza viruses in mammalian cells, we first tested whether the PB2 E627K or PB2 Q591K mutation accounted for the full level of adaptation seen with the biological clones. The dk/AB/76 PB2 E627K and dk/AB/76 PB2 Q591R viruses exhibited similar growth rates, and they showed improved growth relative to wild-type dk/AB/76 virus but markedly slower kinetics of growth than the P10 isolates and the human virus controls, Pan/99 and NL/09 (Fig. 4B).

We also tested the potential for five different NP mutations to improve the growth of dk/AB/76 virus in HTBE cells when the mutations were introduced individually (Fig. 4C). The mutations NP Q12R, NP S50G, and NP E454K each increased peak titers marginally, by approximately 10-fold, relative to dk/AB/76 virus. Mutant viruses carrying NP N309K and NP I425V exhibited very similar growth to the dk/AB/76 virus.

Since five of the seven NP mutations identified fell within the



**FIG 2** Clonal isolates derived from dk/AB/76 passage 10 populations show high-growth phenotypes in HTBE cells. Fully differentiated HTBE cells were infected at an MOI of 0.01 PFU/cell with the six clonal isolates indicated. Titers were determined by plaque assay on MDCK cells. This experiment was performed with a single replicate per virus. The limit of detection is indicated by a dashed horizontal line.

**TABLE 1** Amino acid mutations identified in dk/AB/76 clonal virus isolates after 10 serial passages in HTBE cells

Protein	Amino acid change <sup>a</sup>	Clone(s)	Functional domain(s) (reference[s])	Polymorphism(s) in database (frequency [%]) <sup>c</sup>	
				Avian	Human
NS1	M106T	A3	eIF4G/CPSF-30 binding (77, 78)	M (91.7), I (8.1), T (0.10), R (0.031), V (0.042)	M (99.6), I (0.41), L (0.020), V (0.010)
NEP	I32T	C3, C7	None identified (79)	I (99.5), V (0.43), L (0.012), M (0.012), T (0.012)	I (54.8), V (45.2), T (0.033), L (0.022)
M1	T139A	C7	M1-M1 dimer interaction (80)	T (98.6), N (0.88), A (0.47), P (0.05), I (0.01), S (0.01)	T (99.9), A (0.081), S (0.01)
NA	I258V	A4	Near to enzyme active site (81)	M (55.1), I (42.7), V (1.7), L (0.20), N (0.12), T (0.04)	I (99.8), F (0.051), W (0.039), V (0.039), M (0.026)
	R430M	C4, C7	Forms part of second neuraminic acid binding site (HB site) (81)	R (93.6), Q (4.0), K (2.1), L (0.12), W (0.12), G (0.04), P (0.04)	R (99.8), L (0.11), Q (0.041)
NP	G5S	A3, A4	NLS1, PB2 binding (53, 82)	G (99.9), S (0.067)	G (99.99), C (0.011)
	<b>Q12R</b>	C7	NLS1, PB2 binding (53, 82)	Q (99.8), K (0.17), H (0.013), L (0.013), M (0.013)	Q (99.98), E (0.011), P (0.011)
	<b>S50G</b>	C4	PB2 binding (53, 82)	S (97.2), N (21.9), G (0.48), D (0.12), T (0.054), I (0.013)	S (85.4), N (14.6), T (0.033), D (0.011), G (0.011), I (0.011)
	R55K	C3	PB2 binding (53, 82)	R (99.97), G (0.013)	R (100)
	<b>N309K</b>	B4	PB2 binding (53, 82)	N (99.8), K (0.054), S (0.027), T (0.027), D (0.013)	N (85.3), T (14.6), A (0.056), I (0.022)
	<b>I425V</b> <b>E454K</b>	A4 C3, C4	Tail loop, NP oligomerization (83) Insertion groove, NP oligomerization (83)	I (98.8), V (1.1), M (0.027) E (99.02), D (0.9), G (0.09), K (0.01)	V (62.4), I (37.6) E (99.92), D (0.044), G (0.022), V (0.011)
HA1 <sup>b</sup>	T152I	C4, C7	150 loop of receptor binding site (84)	T (49.8), I (45.3), V (4.2), A (0.25)	G (46.9), K (37.2), E (8.2), V (4.9), R (1.1), Q (0.71), M (0.35), A (0.027), H (0.027)
	E219K	A3, A4, B4, C3	220 loop of receptor binding site (84)	K (67.7), Q (15.4), N (8.7), T (4.2), E (2.5), R (1.5)	V (98.4), I (1.3), M (0.16), L (0.08)
HA2	T61I	C4, C7	HA2 loop domain (84)	T (100)	Q (99.92), N (0.027), H (0.027), K (0.027)
	G67S	C3	HA2 loop domain (84)	G (98.0), S (1.2), N (0.25), D (0.25), Q (0.25)	K (99.8), N (0.14), T (0.054), H (0.027)
	D90N	A3, A4, B4	HA2 long alpha helix (84)	D (98.8), N (1.2)	I (69.7), V (30.1), L (0.16)
PB1	E11K	C7	PA binding (85)	K (99.8), R (0.13), Q (0.10), N (0.025)	K (99.9), E (0.035), R (0.023), N (0.011), V (0.011)
	P369S	C7	Near conserved Pol domain (24)	P (98.8), S (0.73), Q (0.11)	P (99.7), S (0.093)
	Y431C	C4	Near conserved Pol domain (24)	T (99.2), H (0.31), C (0.038), S (0.013)	Y (99.7), H (0.10), N (0.012)
PB2	E188K	A4	NP binding (85)	E (99.5), K (0.15), G (0.11), D (0.10), V (0.014)	E (99.8), D (0.046), K (0.035), G (0.023), V (0.011)
	<b>Q236H</b>	A3, A4, B4	NP binding (85)	Q (99.7), H (0.028)	Q (100)
	<b>Q591K</b>	C3, C4, C7	627 domain (30)	Q (97.5), L (0.62), H (0.34), R (0.23), K (0.11), P (0.014)	Q (55.7), R (44.2), H (0.023), L (0.023), K (0.012)
	<b>E627K</b>	A3, A4, B4	627 domain (32)	E (95.2), K (3.9), V (0.69), G (0.028), A (0.014)	K (54.1), E (45.8), R (0.12)

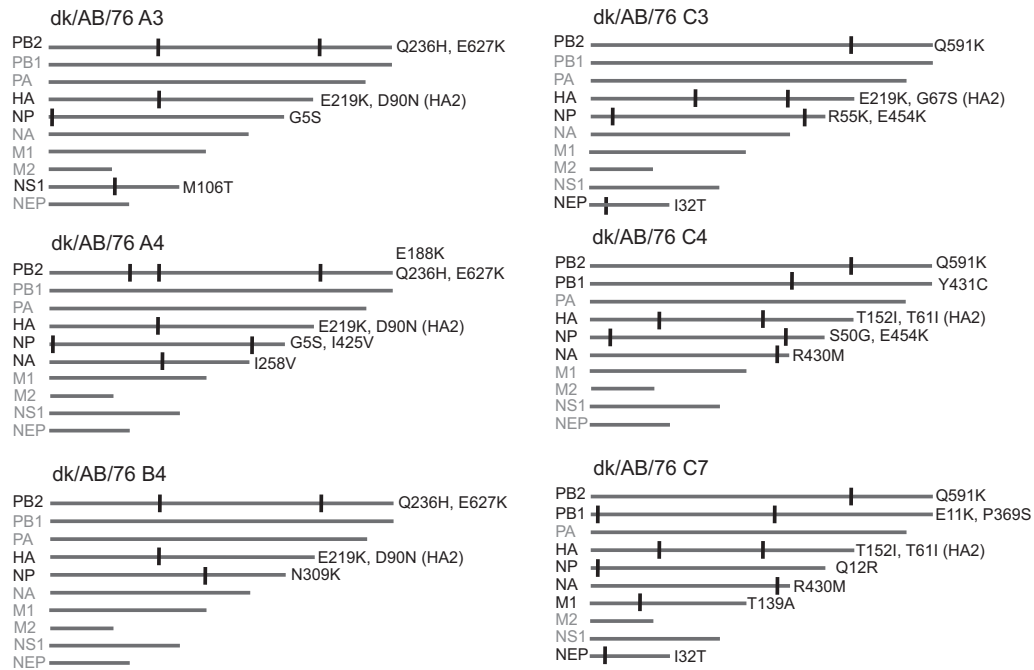
<sup>a</sup> Mutations reintroduced using reverse genetics are shown in bold.

<sup>b</sup> H1 numbering was applied to processed HA1 and HA2 subunits.

<sup>c</sup> The single nucleotide polymorphism analysis tool of the Influenza Research Database (fludb.org) was used, and all available amino acid sequences for avian or human influenza viruses isolated from 1970 to 2010 were analyzed. HA sequences were limited to H1 subtype, and NA sequences were limited to N1 subtype.

broad regions of the protein thought to interact with PB2 (67), we hypothesized that a synergistic effect might be seen if certain PB2 and NP mutations were introduced together into the dk/AB/76 background. Thus, a panel of six double mutants was generated, with NP Q12R, NP S50G, or NP N309K coupled with PB2 Q591K or with PB2 E627K. Multicycle growth analyses of these viruses in HTBE cells indicated that the addition of any of the three NP

mutations increased the rate of viral growth relative to viruses carrying either PB2 mutation alone (Fig. 5). The effects of the NP mutations were greatest in the context of PB2 Q591R, where titers at 48 h postinfection were increased by approximately 20- to 200-fold relative to the titer of the PB2 Q591R single mutant (Fig. 5A). Indeed, the mutant dk/AB/76 PB2 Q591K NP S50G exhibited a growth phenotype very similar to that of clone C4, the biological

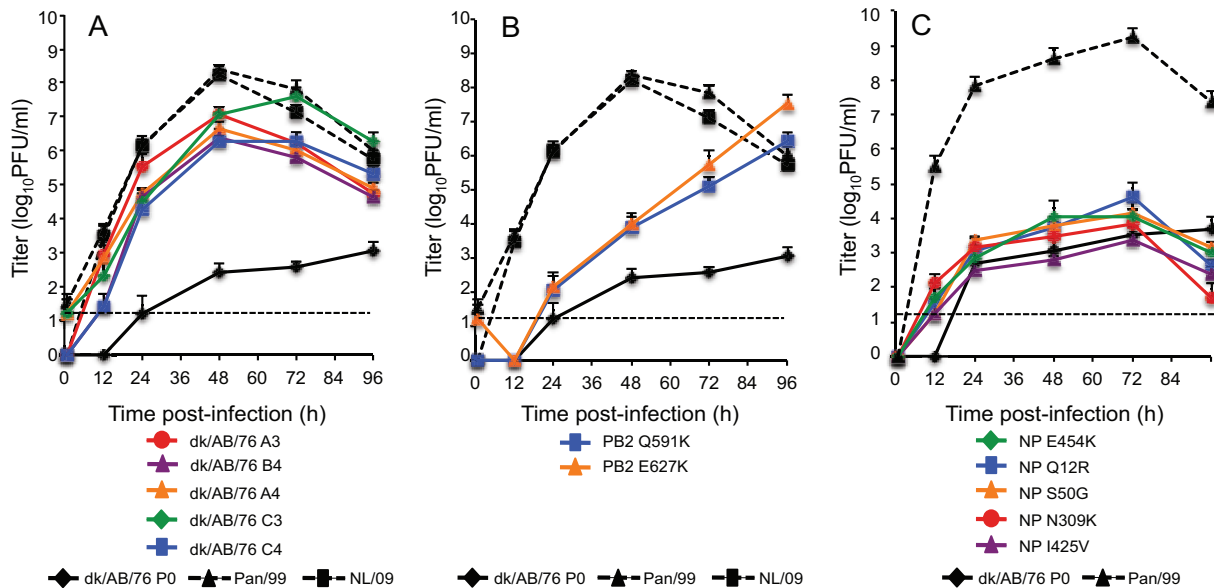


**FIG 3** Genotypes of clonal isolates derived from dk/AB/76 passage 10 populations. Proteins are identified at the left of each schematic diagram, with those containing amino acid changes in black and those that were unchanged in gray. Amino acid changes present in passage 10 clones are indicated at the right of each schematic gene segment.

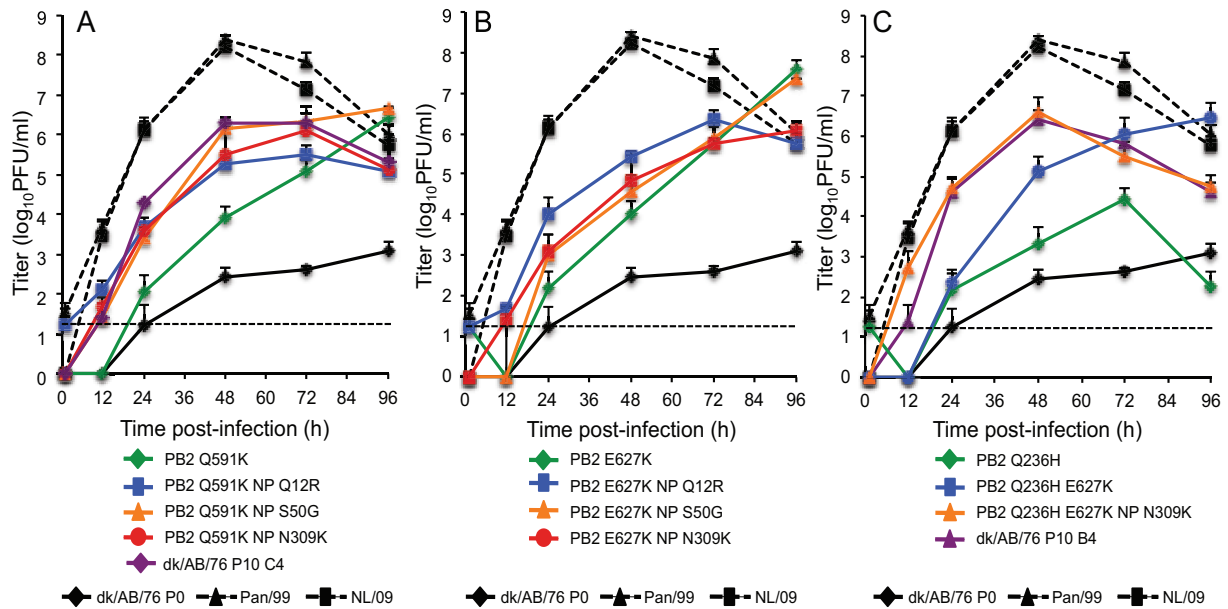
isolate that carried these mutations (Fig. 5A). This result suggests that the remaining five amino acid changes identified in C4 did not contribute significantly to its adaptation to HTBE cells.

In contrast, we noted that the double mutant carrying PB2 E627K and NP N309K did not replicate as efficiently as clone B4. In addition to these two mutations, the replication complex of clone B4 also carried PB2 Q236H. We therefore tested the contri-

bution of PB2 Q236H to mammalian adaptation by constructing mutant viruses carrying (i) PB2 Q236H alone, (ii) PB2 Q236H plus E627K, and (iii) all three mutations found in the replication complex of clone B4, i.e., PB2 Q236H, E627K, and NP N309K. Compared to the dk/AB/76 wild-type virus, PB2 Q236H alone improved growth by approximately 10- to 50-fold (Fig. 5C). Compared to dk/AB/76 PB2 E627K virus, growth of the double



**FIG 4** Growth of PB2 and NP single-mutant viruses in HTBE cells. Differentiated HTBE cells were infected at an MOI of 0.01 PFU/cell with biological clones derived from dk/AB/76 HTBE cell passage 10 populations (A), reverse genetics-derived viruses carrying single amino acid changes in PB2 (B), or reverse genetics-derived viruses carrying single amino acid changes in NP (C). The averages of three replicates are plotted, and error bars represent standard deviations. The limit of detection is indicated by a dashed horizontal line.



**FIG 5** Growth of PB2/NP double- and triple-mutant viruses in HTBE cells. Differentiated HTBE cells were infected at an MOI of 0.01 PFU/cell. Viruses were generated by reverse genetics and carry the indicated mutations, with the exception of dk/AB/76 P10 B4, which is a biological isolate from a dk/AB/76 HTBE cell passage 10 population. The averages of three replicates are plotted, and error bars represent standard deviations. The limit of detection is indicated by a dashed horizontal line.

mutant dk/AB/76 PB2 Q236H E627K was increased only at the 48-h time point (by 13-fold). The triple-mutant virus exhibited growth indistinguishable from that of the biological isolate and showed markedly faster kinetics of growth than the PB2 single- and double-mutant viruses lacking changes to NP (Fig. 5C). Thus, three mutations, in PB2 and NP, were necessary and sufficient to recapitulate the adapted phenotype of clone B4.

**Activity of reconstituted polymerase complexes carrying targeted mutations in NP and/or PB2 proteins.** Toward understanding the mechanism(s) by which the identified adaptive changes augment viral growth in human cells, we evaluated the activity of viral polymerase complexes reconstituted through transient transfection of 293T cells. The dk/AB/76 virus PB2, PB1, PA, and NP proteins were coexpressed with a minigenome RNA comprising a luciferase reporter gene flanked by the untranslated regions of the A/WSN/1933 virus NP segment. When the plasmid encoding the wild-type NP was replaced with that encoding NP Q12R or NP S50G, modest increases in reporter activity, of up to 1.5-fold, were seen. Expression of NP N309K resulted in slightly lower activity than that seen with the wild-type polymerase complex (Fig. 6A). In general, the introduction of adaptive mutations in PB2 yielded much more potent effects than those in NP (Fig. 6B). When introduced alone, PB2 Q236H enhanced reporter activity by approximately 10-fold compared to the wild type. On average, PB2 Q591K increased activity 260-fold, and PB2 E627K showed a 700-fold increase relative to the wild type. Introduction of both the PB2 Q236H and E627K mutations did not increase reporter activity over that seen with PB2 E627K alone. Similarly, the combination of mutations in PB2 with NP S50G or NP N309K did not enhance activity relative to polymerase complexes carrying mutations in PB2 alone (Fig. 6C). These results suggest that, as has been reported previously for E627K and Q591K, the adaptive mutations identified in the HTBE cell-adapted dk/AB/76 virus PB2 protein render the polymerase more efficient in human cells.

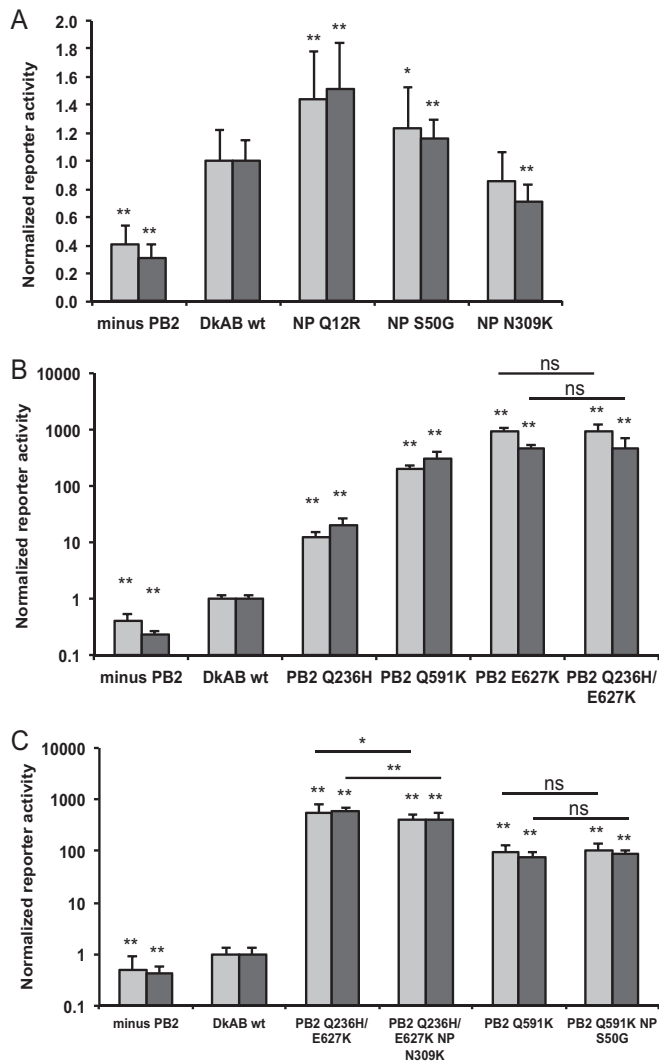
In contrast, the contributions of the adaptive mutations identified in NP to viral fitness appear to occur through a different mechanism.

## DISCUSSION

Serial adaptation of the low-pathogenic dk/AB/76 virus to HTBE cell cultures yielded high-growth variants, all of which carried coding changes in the PB2, HA, and NP proteins. Amino acid changes were also identified in PB1, NA, M1, NS1, and nuclear export protein (NEP) open reading frames, but mutations in each of these proteins arose in only one of the three lineages generated. Mutations were not identified in the PB1-F2, PA, PA-X, or M2 coding regions. To our knowledge, this is the first report of adaptation of an avian influenza virus to growth in primary human respiratory cells. Similar experiments have, however, been performed in mice and revealed trends that are also reflected in our data. Parallel passaging of multiple lineages of influenza A/Hong Kong/1/1968 (H3N2) virus in mice consistently revealed nonsynonymous changes in HA and components of the viral ribonucleoprotein complex (27, 68). Similarly, the mouse-adapted variant, SC35M, of the avian-like SC35 (H7N7) strain was found to carry mutations mainly in the polymerase proteins and NP (28), and mouse adaptation of A/equine/1416/1973 (H7N7) virus yielded changes in PB2, PB1, and PA proteins (29). Reverse genetics-based analyses of mutations identified in each of these mouse-adapted viruses indicated a key role in virulence of substitutions in the PB2 protein, with the D701N or E627K change occurring most frequently.

Analysis of published influenza A virus sequences available through the Influenza Research Database indicated that most of the mutations we identified in dk/AB/76 passage 10 isolates were not highly prevalent among either human or avian natural isolates. The exceptions to this generalization were NP I425V, HA E219K, PB2 Q591K, and PB2 E627K. The increased prevalence of





**FIG 6** Activity of reconstituted polymerase complexes carrying NP and/or PB2 mutations. Viral polymerase complexes, comprising PB2, PB1, PA, and NP proteins plus a firefly luciferase reporter template flanked by the NP untranslated regions of WSN virus, were reconstituted by transient transfection of 293T cells. An expression vector encoding *Renilla* luciferase was included as a control for transfection efficiency, and firefly luciferase activity was normalized to *Renilla* luciferase activity. Omission of PB2 (minus PB2) was used as a negative control. Normalized luciferase activity obtained with the wild-type dk/AB/76 wt run in parallel, unless otherwise indicated. ns, not significant ( $P > 0.05$ ); \*,  $P < 0.05$ ; \*\*,  $P \leq 0.001$ . (A) Activity of polymerase complexes harboring changes to the NP protein only. (B) Activity of polymerase complexes carrying changes in PB2 only. Note that a  $\log_{10}$  scale was used on the y axis. (C) Activity of polymerase complexes carrying changes in PB2 and NP. Note that a  $\log_{10}$  scale was used on the y axis.

NP 425V in human isolates compared to avian strains suggested that this polymorphism might be important in human adaptation. When introduced alone into the dk/AB/76 background, however, the change NP I425V did not improve growth in HTBE cells. The receptor binding site mutation HA E219K, identified in four of our passage 10 isolates, appears to be a reversion to the avian consensus at this site. In addition to our recombinant dk/AB/76

virus, four of six published sequences for the dk/AB/76 HA protein carry 219E (the other two published sequences have 219K). We suspect that HA 219E is an egg adaptation, which reverted upon passage in HTBE cells. The PB2 mutation E627K has been identified repeatedly upon natural or experimental introduction of avian influenza viruses into mammalian host systems (29, 33, 34, 69, 70) and has been found to enhance polymerase activity in mammalian cells and virulence and transmission in mammalian hosts (29, 31, 32, 37, 39, 45, 71). Similarly, PB2 Q591K and the analogous Q591R polymorphism, which is present in isolates of the 2009 H1N1 pandemic, have been shown to improve viral polymerase activity in mammalian cells (30, 38, 71).

Our data show that introduction of either PB2 Q591K or E627K into the dk/AB/76 virus background results in marked improvement in growth in HTBE cells, consistent with previous studies (38, 39, 72). However, these two single-mutant viruses showed slower kinetics of replication than the biological isolates derived from the dk/AB/76 passage 10 population. This growth defect was partially or fully corrected through the introduction of the NP mutation Q12R, S50G, or N309K. In addition, the enhancements in growth conferred by these NP mutations in the context of PB2 Q591K (or PB2 E627K in the case of the NP Q12R mutation) were ~10- to 100-fold greater than those seen when the NP mutations were introduced alone. Thus, the combination of PB2 and NP mutations had a synergistic effect on viral growth.

Evaluation of polymerase activity using a minigenome system indicated that the NP mutations tested do not impact polymerase activity directly and that the enhancement of polymerase activity seen with PB2 E627K and Q591K is not dependent on coincident adaptive changes in NP. The observation that PB2 E627K acts independently of NP is consistent with a recent report in which PB2 627K had higher activity than PB2 627E in a cell-based replication assay that lacks NP (45). Coupled with the observation that adaptations in NP and PB2 act synergistically to increase viral fitness in mammalian cells, our minigenome data suggest that two independent, but complementary, phenomena are at work. Cooperation between PB2 and NP proteins in host adaptation is also evident in a report that describes epitasis between PB2 627 and the NP gene: reversion of PB2 627E to 627K upon passage of A/swan/Germany/R65/2006 (H5N1) virus in mammalian cells required the presence of the cognate NP segment (41). Perhaps for the PB2 E627K change to confer a strong selective advantage, certain permissive mutations must first be present in NP (73).

Multiple studies have reported enhanced PB2-NP binding with the E627K mutation. This effect has been observed in the context of infected cells (43), cells expressing all vRNP components (42, 44), cells expressing only NP and PB2 (44), and *in vitro* with purified NP protein and PB2 627 domain peptides (74). In each case, enhanced NP-PB2 interaction was correlated with enhanced polymerase activity. In contrast, two recent studies suggest that improved polymerase activity of PB2 E627K mutants in mammalian cells is not dependent on NP. In a replication assay that lacks NP, activity of reconstituted viral polymerases was nonetheless reduced in mammalian cells with PB2 627E compared to those with PB2 627K (45). In addition, enhanced coimmunoprecipitation of NP with PB2 627K compared to that with PB2 627E has been shown by some groups to be dependent on the presence of a vRNA template (42, 75). This observation has led to the suggestion that increased PB2-NP interactions with PB2 627K arise not due to an intrinsic improvement in PB2-NP binding but



due to increases in the number active vRNP complexes within the infected cell (75). We have not established herein whether NP-PB2 binding is affected by PB2 E627K or Q591K in the dk/AB/76 virus background. However, it is clear from our data that the adaptive mutations identified in NP do not contribute to improved polymerase activity and therefore do not work in concert with PB2 at the level of replication or transcription.

A candidate mechanism by which the identified NP adaptations may act is enhanced nuclear import of vRNPs. There is some support for this concept in the literature (47, 49), and it is easy to envisage a cooperative effect arising between increased nuclear import (brought about by NP) and higher polymerase activity of nuclear vRNPs (generated by PB2). This model is particularly attractive since the improvement in viral growth seen with our PB2/NP double mutants, relative to the PB2 single mutants, is mainly a kinetic one. In addition, the Q12R mutation identified herein lies within the NP N-terminal nuclear localization signal (76), while the NP N309K mutation is similar to that identified previously by Gabriel et al. to enhance NP binding to human importin- $\alpha$  isoforms (NP N319K) (47). Specifically, NP N319K conferred adaptation to human importin- $\alpha$ 1 and - $\alpha$ 7 isoforms, supporting efficient replication in human lung cell lines especially in combination with PB2 701N or 627K. Although enhanced nuclear import might be expected to increase reporter activity in the minigenome assay, the effect may be apparent only early after transfection, whereas we determined reporter activity relatively late, at 48 h posttransfection.

In summary, our results show that nonsynonymous mutations in PB2, HA, and NP arise consistently during adaptation of an avian influenza A virus to human respiratory cells and that as few as two mutations, in NP and the PB2 627 domain, are necessary and sufficient to confer efficient growth. To understand their potential role in the emergence of pandemic influenza A viruses, it will be important to test the effects of the adaptive changes identified herein on transmission in a mammalian host.

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