PB2 and Hemagglutinin Mutations Are Major Determinants of Host Range and Virulence in Mouse-Adapted Influenza A Virus[▽]

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Serial mouse lung passage of a human influenza A virus, A/Hong Kong/1/68 (H3N2) (HK-wt), produced a mouse-adapted variant, MA, with nine mutations that was >10^{3.8}-fold more virulent. In this study, we demonstrate that MA mutations of the PB2 (D701N) and hemagglutinin (HA) (G218W in HA1 and T156N in HA2) genes were the most adaptive genetic determinants for increased growth and virulence in the mouse model. Recombinant viruses expressing each of the mutated MA genome segments on the HK-wt backbone showed significantly increased disease severity, whereas only the mouse-adapted PB2 gene increased virulence, as determined by the 50% lethal dose ([LD₅₀] >10^{1.4}-fold). The converse comparisons of recombinant MA viruses expressing each of the HK-wt genome segments showed the greatest decrease in virulence due to the HA gene (10²-fold), with lesser decreases due to the M1, NS1, NA, and PB1 genes (10^{0.3}- to 10^{0.8}-fold), and undetectable effects on the LD₅₀ for the PB2 and NP genes. The HK PB2 gene did, however, attenuate MA infection, as measured by weight loss and time to death. Replication of adaptive mutations *in vivo* and *in vitro* showed both viral gene backbone and host range effects. Minigenome transcription assays showed that PB1 and PB2 mutations increased polymerase activity and that the PB2 D701N mutation was comparable in effect to the mammalian adaptive PB2 E627K mutation. Our results demonstrate that host range and virulence are controlled by multiple genes, with major roles for mutations in PB2 and HA.

Although adaptive evolution of influenza A virus (FLUAV) to high virulence in a new host is a common occurrence in nature, the molecular events that control the adaptive process are largely unknown. Evolutionary theory states that adaptive mutations increase replication ability as evident by increased mutant gene frequency. However, adaptive mutations in FLUAV are difficult to identify because of genetic variability among viruses and the involvement of multiple gene and host interactions. Experimental evolution by serial passage in the mouse lung results in the selection of virulent mouse-adapted (MA) variants. Genomic analysis of the A/FM/1/47(H1N1)-MA variant showed the selection of five coding mutations (PB1 D538G, PB2 K482R, HA2 subunit W47G [W47G^{HA2}], neuraminidase [NA] N360I, and M1 T139A) (1). Infection of mice with viruses that differed solely due to the presence of each of these five mutations showed that all mutations contributed both to increased replication in the mouse lung and virulence in the mouse. Thus, experimental evolution by serial mouse lung passage appears to involve strong competitive selection of adapted variants without unselected mutations.

Influenza A viruses are negative-sense, single-stranded, segmented RNA viruses that are classified into 16 hemagglutinin (HA) subtypes and nine neuraminidase (NA) subtypes (9). Wild aquatic birds are known to be the natural reservoirs of these subtypes (22, 42, 54); however, through adaptive evolu-

tion and reassortment, virus variants acquire the ability to transmit among avian and mammalian hosts including humans. In the last 100 years four influenza pandemics have occurred by adaptation of animal and avian viruses or genes, resulting in human viruses, as seen in 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1) (16, 20, 27, 33, 36, 37, 49).

Although the molecular basis of adaptation and virulence of influenza A viruses in new hosts is poorly understood, it is accepted to include changes in multiple genes and to involve host factors. It is generally believed that adaptive mutations involve the restoration of host interactions that were blocked due to molecular differences among hosts. The amino acid at position 627 of the PB2 gene is recognized as a critical mammalian host determinant; the glutamic acid (E) residue is found generally in avian influenza viruses while human viruses have a lysine (K) at this position. The PB2 E627K mutation has been associated with enhanced virus replication, virulence, tissue tropism, and transmission of influenza A viruses in mammals (14, 15, 46, 47). Additionally, the amino acid at position 701 of the PB2 gene has also been known as a determinant of replication, virulence, and transmission. The aspartate (D)-to-asparagine (N) mutation at position 701 of PB2 allowed the avian H5N1 influenza virus to replicate in mice (24), the seal H7N7 influenza virus to adapt in mice (10), and H5N1 influenza virus to transmit in guinea pigs (12). Alternatively, mutations in the HA receptor binding or protease cleavage sites as well as gain or loss of glycosylation sites can also change virulence, replication, tissue tropism, and host range (17, 21, 28, 44, 50, 51). Previous studies have also shown that mutations in other genes, including the PB1-F2, PA, M, and NS genes, can

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enhance replication and virulence in new hosts (4, 7, 19, 25, 29, 45, 53, 55).

In the past decades, genetic mutations in influenza viruses during adaptation in a new host have been studied in mice (1–3, 10, 43), which are considered to offer an ideal model to characterize influenza virus virulence and adaptation in a new host (52). In our study, a human virus, A/Hong Kong/1/68 (H3N2; HK-wt), was serially passaged in the mouse lung, and a highly pathogenic mouse-adapted virus (HK-MA [MA]) was clonally isolated after 20 passages. Genomic sequencing showed that the selection of nine mutations in the HK-MA strain relative to the parental HK strain was responsible for overcoming host resistance. We used reverse genetics to determine the molecular basis for increased virulence in mice and host-dependent replication in cell culture. We found that the D701N mutation in the nuclear localization signal of PB2, the G218W mutation in HA1 (G218W^{HA1}), and a loss of glycosylation site due to T156N in HA2 (T156N^{HA2}) were of primary importance for increased virulence in the mouse-adapted mutant.

MATERIALS AND METHODS

Cells. MDCK, M1, A549, B82, and DF1 cells were maintained in minimum essential medium (MEM), and 293T cells were maintained in Dulbecco's minimal essential medium (DMEM) (Gibco, Invitrogen) supplemented with 10% fetal calf serum (HyClone; Thermo Scientific), penicillin (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. All cells were incubated at 37°C in 5% CO₂.

Viruses. A/Hong Kong/1/68 (H3N2) wild type (HK-wt) and its mouse adaptation have been described previously (3); however, HK-MA was a distinct clonal isolate derived after 20 passages of HK-wt that had not been described previously. Viruses were grown in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs (Canadian Food Inspection Agency, Ottawa, Canada) at 37°C for 48 h and stored at –80°C. Virus titers were determined by plaque assay in MDCK cells.

Construction of plasmids. To clone all eight gene segments of HK-wt and HK-MA viruses, we amplified each segment by reverse transcription-PCR from isolated viral RNA and inserted each into the pLLB bidirectional vector (26). To generate mutant viruses, PCR-based site-directed mutagenesis with primer pairs containing point mutations was used. All of the constructs were completely sequenced to ensure the absence of unwanted mutations.

Virus rescue. Virus rescue was performed as previously described (24, 39). Briefly, DNA and Lipofectamine 2000 (Invitrogen) were mixed (2 µl of transfection reagent per 1 µg of DNA), incubated at room temperature for 30 min, and added to an 80 to 90% confluent monolayer of 293T cells in six-well plates. Sixteen hours later, the DNA-transfection reagent mixture was replaced by Opti-MEM (Gibco-BRL, Carlsbad, CA) containing tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 µg/ml) (Thermo Fisher Scientific, Waltham, MA). Forty-eight hours after the transfection, the supernatants were harvested and inoculated into 10-day-old SPF embryonated chicken eggs for virus propagation. Viruses were detected by a hemagglutinin assay, and all eight genes were fully sequenced to ensure the absence of unwanted mutations.

Minigenome assay. To compare the activities of viral RNP complexes, a dual luciferase reporter assay system (Promega) was performed in this study (10, 23). Briefly, the reporter plasmids consist of the firefly luciferase open reading frame flanked by noncoding regions of the nucleoprotein (NP) or NA gene of influenza A viruses, under the control of either the human, murine, or chicken RNA polymerase I (Pol I) promoter and terminator. Reporter plasmid (0.06 µg) pHOLI-LUC-NP (human), pmPOLI-LUC-NP (mouse), or pgHH21-vNA-Luc (avian) (31) was transfected into 293T, B82, or DF1 cells in 96-well plates together with 0.06 µg of each of the four pLLB plasmids encoding PB2, PB1, PA, and NP and 0.06 µg of the *Renilla* luciferase expression plasmid pRL-SV40 (where SV40 is simian virus 40) (Promega) as an internal control. At 40 h posttransfection, luminescence was measured using a Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Relative luciferase activities were calculated as the ratio of firefly to *Renilla* luciferase luminescence. Each luciferase activity value is the average of three independent experiments.

Growth curves of recombinant viruses. To analyze viral replication, confluent MDCK, A549, and M1 cells were infected in triplicate with recombinant viruses at a multiplicity of infection (MOI) of 0.01. One hour after incubation at 37°C, the cells were washed once with phosphate-buffered saline (PBS), and fresh MEM with 1 µg/ml TPCK-trypsin was added. Supernatants were collected at 12, 24, 48, and 72 h postinfection (p.i.), and the virus titers in these supernatants were determined by plaque assay.

Mouse infections. To evaluate the viral growth, groups of 12 CD-1 mice (4- to 6-week-old females from Charles River Laboratories, Montreal, Quebec, Canada) were infected intranasally with 5×10^3 PFU of recombinant viruses. Three mice from each group were euthanized at 1, 3, 5, and 7 days postinfection (dpi), and their lungs were suspended in 1 ml of cold sterile PBS and subsequently homogenized. Virus titers were evaluated by plaque assay.

The 50% mouse lethal dose (MLD₅₀) was determined by inoculating groups of five mice with 10-fold serial dilutions containing 10^3 to 10^6 PFU of the virus in a 50-µl volume. Mice were monitored daily for weight loss up to 14 dpi. All mice showing more than 25% body weight loss and respiratory distress were considered to have reached the experimental endpoint and were humanely euthanized. The MLD₅₀ was calculated by using the Karber-Spearman method (32).

For pathological examination, CD-1 mice were inoculated with 10^6 PFU of recombinant viruses, and lungs were collected at 6 dpi and fixed in 10% neutral buffered formalin for 24 h. Subsequently, they were embedded in paraffin, sectioned at a thickness of 4 µm, stained with hematoxylin and eosin (H&E), and examined under light microscopy for histopathologic changes. The images were obtained on a Nikon microscope using a 10× objective lens.

Immunofluorescence staining. Immunofluorescence staining was performed as previously described (21). Briefly, CD-1 mice were inoculated with 10^5 PFU of recombinant viruses; lungs were collected at 2 dpi and fixed and embedded in paraffin for sectioning at a thickness of 4 µm. Viral antigen was detected by incubating the sections with anti-HK primary rabbit antibody diluted (1/500) in buffer (10 mM PBS containing 3% bovine serum albumin and 0.3% Triton X-100). After being washed three times with 10 mM PBS, the slides were incubated with Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch laboratories Inc., ME) diluted (1/500) in the antibody buffer. The slides were then washed, and nuclei were stained by incubation with 100 µl of Hoechst (0.2 µg/ml). Images were taken at ×20 magnification with an Olympus BX50 microscope.

Nucleotide sequence accession numbers. The nucleotide sequences of the HK-MA isolate gene segments employed in this study were deposited in the GenBank under accession numbers HM641145 (PB2), HM641156 (PB1), HM641167 (PA), HM641178 (HA), HM641189 (NP), HM641200 (NA), HM641211 (M), and HM641222 (NS).

RESULTS

Mouse-adapted HK virus (HK-MA) is highly virulent in CD-1 mice. Human influenza A/Hong Kong/1/68 (H3N2) (HK-wt) does not cause disease in CD-1 mice and has a 50% lethal dose [LD₅₀] of $>10^{7.7}$ PFU (3). However, 20 serial lung-to-lung passages of A/Hong Kong/1/68 in CD-1 mice, followed by clonal isolation, resulted in a highly virulent virus called HK-MA. To compare the virulence of HK-wt and HK-MA viruses, CD-1 mice were infected intranasally with 10^5 PFU and monitored for survival. HK-MA-infected mice lost $>25\%$ of body weight and began to die or were euthanized at defined humane endpoints at 5 dpi, with 100% mortality by 8 dpi. In contrast, the mice infected with HK-wt did not show any signs of disease during 14 days of observation (Fig. 1A). Infections with lower dosages showed that HK-MA had an LD₅₀ of $10^{4.0}$ PFU. Consistent with greater disease, viral yield of HK-MA in lungs was $>10^2$ -fold higher at 1 dpi than that of the parental strain at days 3 and 5 p.i. (see below). We could not, however, detect any virus in the brain, spleen, or kidney (data not shown).

To assess the pathology caused by HK-MA relative to that of HK-wt, mice were infected with 10^6 PFU, and lungs were collected at 6 dpi for staining and imaging with hematoxylin and eosin. Microscopic images of HK-wt-infected lungs showed

TABLE 1. Amino acids mutations between HK/1/68 and MA viruses

Gene	Mutation site (residue no.)	Amino acid	
		HK/1/68	HK-MA
PB2	701	D	N
PB1	190	R	K
	578	K	T
PA	None		
HA	218 ^{HA1}	G	W
	156 ^{HA2}	T	N
NP	34	D	N
NA	468	P	H
M1	232	D	N
M2	None		
NS1	23	V	A
NS2	None		

minimal signs of infection, whereas the mouse-adapted virus-infected lungs showed extensive interstitial thickening and consolidation, with inflammatory cell infiltrations in the alveolar and bronchiolar regions (Fig. 1B). Therefore, in comparison to the parental strain the MA virus had acquired increased tissue tropism and growth in mouse lungs with associated virulence.

Generation of recombinant HK-wt and HK-MA viruses. Reverse genetics was used to investigate the differences in replication and virulence between HK-wt (HK) and HK-MA (MA). We inserted the eight gene segments of HK and MA into the pLLB vector (26) and, using this eight-plasmid system, rescued the recombinant viruses, r-HK and r-MA. After confirmation by sequence analysis, we tested their replication and lethality in mice. The rescued r-HK and r-MA viruses had LD₅₀ values of >10^{7.3} and 10^{3.8} PFU, respectively (Fig. 1C), indicating that the rescued viruses maintained the same biological properties as the wild-type viruses.

PB2 and HA genes play a key role in determining the virulence of MA virus. Sequence analysis showed that there were nine amino acid differences in MA relative to HK that were distributed among seven proteins, with four proteins without mutations (Table 1). To identify the genetic mutations that contributed to the differences of replication and virulence in

FIG. 1. Genetic and biological characterization of recombinant HK and MA viruses in mice. (A) Mouse adaptation increases virulence in CD-1 mice. Groups of five mice were infected intranasally with 10⁵ PFU of HK-wt (HK) and HK-MA (MA) viruses. Mortality was monitored daily for 14 dpi. (B) Mouse adaptation increased lung pathology in CD-1 mice. Groups of three mice were inoculated intranasally with 10⁶ PFU of HK and MA viruses. Lungs were collected at 6 dpi for staining and imaging with H&E. Magnification, ×100. (C) Virulence of the recombinant viruses (r-HK and r-MA) in mice. HK genome segments were exchanged on the MA backbone and vice versa. Gene segments derived from HK and MA viruses are shown in blue and pink, respectively. The MLD₅₀ was determined by inoculating groups of five CD-1 mice with 10-fold serial dilutions of the stock recombinant viruses in a 50-μl volume. In some instances the MLD₅₀ value was greater than the highest dose tested (indicated by >). The MLD₅₀ was calculated by using the Karber-Spearman method (32). (D) Body weight loss of mice infected with single gene reassortants in the r-HK background. (E) Body weight loss of mice infected with single gene reassortants in the r-MA background. For panels D and E, groups of five mice were intranasally infected with 10⁵ PFU of each of the recombinant viruses. Body weight loss was monitored for 14 dpi.

mice, we generated seven single-gene recombinant viruses, each bearing the PB2, PB1, HA, NP, NA, M, or NS gene from MA and the other seven genes from HK. Groups of five mice were infected with defined dosages of each recombinant virus to determine their LD₅₀s. Only the r-HK virus bearing the PB2 gene of MA (r-HK+MA-PB2) was detected to be more lethal, with an MLD₅₀ of >1.0 log lower than that of r-HK (MLD₅₀, 6.3 versus > 7.3 log PFU/ml) (Fig. 1C). However, all seven recombinant viruses induced increased disease in mice relative to HK-wt, as detected by greater loss of body weight during the first 7 dpi ($P < 0.001$ by paired *t* test), except for r-HK+MA-M that was not statistically different ($P = 0.5$), with the r-HK+MA-PB2 and r-HK+MA-HA recombinants causing the greatest reductions at days 4 and 7 p.i., respectively (Fig. 1D). The effect of individual genes derived from the HK virus on the virulence of MA virus was also examined by generating the alternate combinations of a single HK gene on the MA backbone. The viruses that possessed the PB2, PB1, NP, NA, M, and NS gene of HK caused rapid weight loss; however, the extent of weight loss was significantly less than that of the MA viruses for all single-segment HK-wt recombinants ($P < 0.05$, paired *t* test) (Fig. 1E). Decreased virulence as measured by LD₅₀ was seen on introduction of each HK-wt gene except for PB2 and NP (increases in LD₅₀ values of $10^{0.3}$ for PB1, $10^{0.6}$ for NA, $10^{0.8}$ for M1, and $10^{0.8}$ for NS1), with the greatest effect seen for HK-HA (> $10^{2.0}$ -fold increase in LD₅₀). These combined results for single-segment substitutions of MA mutant or HK-wt genes on the alternate backbone indicated that each of the mutant MA genes played a role in virulence but that the greatest effects were seen for the PB2 and HA genes.

Virulence of MA PB2 plus HA in CD-1 mice. To further determine the effects of MA-PB2 and HA genes on adaptation and virulence, we assessed recombinant viruses carrying both the PB2 and HA genes of MA [MA(PB2+HA)] on the HK backbone in addition to the single MA-PB2 and MA-HA recombinants and vice versa on the MA backbone. To compare the virulence of the recombinant viruses *in vivo*, groups of five mice were infected intranasally with 10^5 PFU of recombinant viruses and monitored daily for lethality and body weight loss. Although the r-HK and single-segment MA-PB2 and MA-HA recombinant viruses did not cause death, the MA(PB2+HA) mutant genes in combination resulted in 40% mortality (Fig. 2A). In the converse combinations, both the r-MA and r-MA+HK-PB2 viruses killed 100% of mice by days 6 and 9, respectively, corresponding to an average time to death of 4.6 and 6.8 days, respectively (Fig. 2A). Thus, although the LD₅₀ was not affected by the HK-PB2 substitution in MA (Fig. 1C), the average survival time was prolonged by 2.2 days ($P = 0.004$), indicating attenuation, which was consistent with the reduction in body weight loss (Fig. 1E). Both the r-MA+HK-HA and r-MA+HK(PB2+HA) viruses resulted in reduced levels of mortality (80% and 40%, respectively) compared to mortality of r-MA (Fig. 2A). These data show that both HK-PB2 and HK-HA reduced virulence when they were introduced into the MA virus.

The reciprocal viruses r-HK+MA(PB2+HA) and r-MA+HK(PB2+HA) resulted in very similar rates of lethality of 40 and 60%, respectively. This similarity in virulence was matched by the extent of body weight loss for the first 7 days of infection, with the exceptions of a 1-day delayed onset for the r-HK+MA(PB2+HA) virus, and differed thereafter, with

FIG. 2. Survival and body weight loss in CD-1 mice infected with recombinant viruses possessing combinations of PB2 and HA from HK and MA on the alternate backbone. Groups of five mice were infected intranasally with 10^5 PFU of each of the recombinant viruses as indicated in the figure. Mortality and morbidity were monitored daily for 14 dpi. (A) Survival curve. (B) Body weight loss curve.

the MA(PB2+HA) mutations on the HK backbone resulting in 2 more days of disease before recovery (Fig. 2B). Thus, although all the MA genes contributed to virulence, the two most virulent genes, PB2 and HA, were approximately equivalent in increasing virulence to the combination of the remaining MA genes, PB1, NA, NP, M1, and NS1, with an LD₅₀ of $10^{5.1}$ versus $10^{4.9}$ PFU, respectively (Fig. 1C).

Immunofluorescence staining of infected mouse lungs. To observe the extent of tissue tropism and virus spread in the lung due to the different recombinants, CD-1 mice were infected intranasally with 10^5 PFU. Lungs were collected at 2 dpi, and antigens were detected by immunofluorescence. The r-HK virus showed minimal infection in the lungs, mainly targeting small foci of cells in the bronchioles and surrounding alveolar regions (Fig. 3). However, the r-MA virus spread throughout the lung tissues to infect large regions of alveoli and bronchioles (Fig. 3). The r-HK+MA-PB2 virus enhanced viral infection, involving larger foci of infection in the bronchioles than r-HK virus and increased alveolar infection (Fig. 3). Addition of the MA-HA gene in r-HK+MA(PB2+HA) virus dramatically increased lung infection to encompass more of the alveolar tissues than with infection by r-HK (Fig. 3). Introduction of the HK HA gene into r-MA decreased viral infection of alveoli in r-MA+HK-HA compared to r-MA virus; the double gene substitutions in r-MA+HK(PB2+HA) further decreased viral infection of the alveolar regions compared to r-MA (Fig. 3).

Growth curve of recombinant viruses *in vivo*. To further understand the effects of the MA PB2 and HA mutations on replication, mouse lungs were infected using recombinant vi-

FIG. 3. Immunofluorescent staining of mouse lungs infected with HA and PB2 recombinant viruses. CD-1 mice were infected with 10^5 PFU of the recombinant viruses, and lungs were collected at 2 dpi. Viral antigens were detected by staining lung sections with anti-HK primary antibody followed by Cy3-conjugated secondary antibody (red). Nuclei were stained with Hoechst (blue), and images were taken using a $20\times$ objective.

viruses carrying the wild-type PB2 or HA gene or both the PB2 and HA genes on the virulent MA backbone and vice versa. Infected lungs were collected on days 1, 3, 5, and 7 p.i. and assessed for infectious yield. The mouse-adapted r-MA virus grew to a $>10^2$ -fold-higher titer than the human prototype r-HK at 1 dpi ($P < 0.05$) and was more than 5 logs higher at 7 dpi (Fig. 4A and B).

The substitution of the single polymerase gene PB2 from MA virus into the r-HK backbone enhanced viral growth by 2 logs and 1 log early in infection, at days 1 and 3 p.i., respectively, but growth was slightly reduced at later time points (days 5 and 7 p.i.). The MA-HA gene alone did not enhance replication at day 1 p.i. but did enhance yield at later times (days 3,

FIG. 4. Replication kinetics of HA and PB2 recombinant viruses *in vivo* and *in vitro*. (A) Replication kinetics of the MA reassortants on the r-HK background in mouse lungs. (B) Replication kinetics of the HK reassortants on the r-MA background in mouse lungs. Groups of 12 CD-1 mice were infected intranasally with 5×10^3 PFU. Lungs were collected at 1, 3, 5, and 7 dpi and then homogenized, and virus titers were assessed by plaque assay. (C to H) Replication kinetics of the HA and PB2 reassortants *in vitro*. Monolayers of MDCK, A549, or M1 cells were infected in triplicate with each of recombinant viruses at an MOI of 0.01 in the presence of TPCK-trypsin. Supernatants were collected at 12, 24, 48, and 72 h p.i. and titrated by plaque assay.

5 and 7 p.i.). Addition of MA-HA with MA-PB2 resulted in a virus that had similar growth properties to those of HK+MA-PB2 early in infection but was enhanced at later times (day 7 p.i.). The double substitution of the MA PB2 and HA genes on the HK-wt backbone increased viral growth at all time points compared to r-HK virus (Fig. 4A).

The effects of the reciprocal exchanges of HK-PB2 and HK-HA onto the MA backbone showed that insertion of HK-wt PB2 did not significantly affect yield through 7 days of infection but that there was a trend to decreased yield (0.5 log) at day 5 p.i. However, the HK-HA gene did significantly decrease the growth of the r-MA virus ($P < 0.05$) at all time points, and growth was similar to that of r-MA+HK (PB2+HA) (Fig. 4B).

These results indicate that both the MA PB2 and HA genes function to increase yield in the mouse lung when they are introduced onto the HK backbone but that only the HK-HA gene significantly affected growth in the MA virus backbone. This was consistent with the effects of these genes on disease severity as detected by weight loss versus virulence, as shown in Fig. 1D and E.

Growth of recombinant viruses *in vitro*. To understand the effects of the PB2 and HA genes on host range, we determined their impact on replication *in vitro* in human (A549), mouse (M1), and canine (MDCK) cell lines, using recombinant viruses carrying the PB2 or HA or PB2 and HA wild-type genes on the virulent r-MA backbone and vice versa. Confluent canine, human, and mice epithelial cells were infected with each of the recombinant viruses at an MOI of 0.01. The r-HK virus grew to a higher titer at all time points than r-MA in MDCK and A549 cells; however, r-MA virus grew to a higher titer than r-HK in mouse epithelial cells (Fig. 4C to H). In MDCK cells, r-HK reached a peak titer of 2×10^8 PFU/ml at 24 h p.i.; and although the substitution of the MA-PB2 gene or MA-HA gene alone or in combination did increase yields, this effect was quite small. Surprisingly, the effect of exchanging the HK-HA and HK-PB2 genes on the MA backbone gave a converse effect, whereby the mutations alone or in combination resulted in increased yields in MDCK cells. A549 cells infected with these HK and MA backbone viruses resulted in a similar but more pronounced trend than that in MDCK cell infections. Only the MA-HA gene (but not MA-PB2) on the HK backbone showed enhanced early growth in A549 cells, whereas both HK-PB2 and HK-HA increased growth, at early and later times, respectively, on the MA backbone.

In mouse M1 cells, the r-HK virus reached maximum titer at 48 hpi (7×10^3 PFU/ml), and introduction of the MA-PB2 gene (alone or with MA-HA) enhanced virus growth by greater than 2 logs at 48 and 72 h p.i. The MA-HA gene resulted in a slightly decreased rate of viral growth but the same yield compared to r-HK. However, all the MA backbone viruses grew to similar titers in mouse epithelial cells at all time points (Fig. 4H), demonstrating a relative insensitivity of the MA backbone to exchange with the HK PB2 or HA gene. This indicates that the adaptive properties of MA PB2 and HA are dispensable from the MA genome in M1 mouse cells (on exchange with the HK PB2 and HA) (Fig. 4H), suggesting that their functions are provided by alternative MA genes such that some adaptive properties are provided by multiple mouse-adapted genes. Although these data showed that the MA-PB2 and MA-HA

genes on the HK backbone enhanced *in vitro* replication in mouse and human cells, respectively, the effects were host and virus backbone dependent: mouse-adapted genes demonstrated the most pronounced gain-of-function effects on growth in mouse cells when introduced onto a human backbone but human genes (HK) showed gain-of-function when introduced onto the MA backbone and grown on human cells. In either instance, human or mouse-adapted genes demonstrated increased function in their corresponding hosts of origin.

Both HA1 and HA2 mutations (G218W^{HA1} and T156N^{HA2}) enhance replication and virulence. The MA-HA gene played a significant role in virulence, as seen by a 10^2 -fold decrease in the LD₅₀ for r-MA+HK-HA virus compared to r-MA that had acquired two point mutations, G218W^{HA1} and T156N^{HA2}. To identify which mutations contributed to increased replication and virulence, we generated recombinant viruses containing each of the individual mutations r-MA-HA(W218G T156N) and r-MA-HA(G218W N156T). We then determined the viral replication *in vivo* in CD-1 mice and *in vitro* using MDCK, A549, and M1 cells. We compared viral growth in CD-1 mice lungs and found that each of the individual MA-HA mutations increased replication at day 1 p.i. such that each of the single mutants grew to levels similar to the level of r-MA virus that possessed both mutations; however, this enhanced growth was not as durable as that of MA virus, and yields were indistinguishable from the yield of MA-HA(218G^{HA1} 156T^{HA2}) at days 3, 5, and 7 p.i. (Fig. 5A). In addition to their partial enhancement of mouse lung replication, they each resulted in a similar loss in body weight. However, these mutations differed in the duration, with the 156N^{HA2} mutation inducing body weight loss associated with disease for 10 days before recovery compared to the 8-day duration of disease for the 218W^{HA1} mutation (Fig. 5B). Thus, we conclude that both the HA 218G^{HA1} 156T^{HA2} mutations resulted in early and transient increases in replication but that they were synergistic in combination, resulting in enhanced replication throughout infection.

Growth yields of the different recombinant viruses were similar in MDCK and M1 cells but with a trend of increased yield due to the 218W^{HA1} mutation (Fig. 5C and E). In A549 cells each of the single 218W^{HA1} 156N^{HA2} mutations in HA did not affect the maximum yield compared to that of HA(218G^{HA1} 156T^{HA2}) virus, but both mutations together were reduced in yield at 48 h p.i. (Fig. 5D). Therefore, we can conclude that both the G218W^{HA1} and T156N^{HA2} mutations determine virulence and replication *in vivo* but that their effects on replication *in vitro* were variable and host dependent.

We also observed that the plaque size was increased for MA virus relative to HK virus (2.7 mm and 1.1 mm, respectively) and assessed the role of the PB2 and HA mutations in controlling plaque size. Using a panel of viruses, we measured the plaque size of recombinants that differed due to individual mutations to show that the MA-HA gene is the primary determinant of plaque size that is controlled by the G218W HA1 mutation but that the MA-PB2 gene also increased plaque size on the HK backbone (Fig. 5F and G).

Host-dependent activity of polymerase mutations in the luciferase minigenome assays. Previous studies suggested that influenza A virus mutations that enhanced polymerase activity contribute to enhanced adaptation and virulence (10, 13, 40).

For this study, minigenome assays were developed for use in human, mouse, and chicken cells by inserting the appropriate Pol I promoters to drive the viral luciferase-expressing minigenomes that were then used to quantify the activity of recombinant viral polymerase complexes in different host cells. In this system, human 293T, mouse B82, and chicken DF1 cells were transfected with three polymerase subunits, with NP, and with an influenza virus-like minigenome carrying the firefly luciferase gene under the control of the corresponding host Pol I promoter plus control plasmid pRL-SV40. Polymerase activities of MA polymerase complex (MA-PB2, MA-PB1, and MA-NP [plus HK-PA]) were about 2.5-fold and 4.5-fold greater than those of HK-wt in 293T and B82 cells, respectively (Fig. 6A and B), but the polymerase activities of the viruses were similar in avian cells (DF1) (Fig. 6C). To investigate the role of the individual polymerase gene in this increased polymerase activity, an analysis of all possible combinations of the polymerase subunits was performed with 293T, B82, and DF1 cell lines. MA-PB2 increased the polymerase activities of HK-wt by 1.5- and 3-fold in 293T and B82 cells, respectively; the PB1 subunit of MA also increased polymerase activity 2- and 2.5-fold in 293T and B82 cells, whereas MA-NP had a small effect that was limited to 293T cells. In contrast, exchange of each of the individual wild-type HK-PB2, HK-PB1, and HK-NP genes decreased the polymerase activities of MA polymerase to some extent, with the exception of HK-PB2 in 293T cells (Fig. 6A). However, there was no significant difference in the polymerase activities of all the different combinations in avian DF1 cells (Fig. 6C). Therefore, MA-PB2 and -PB1 genes acted to enhance the polymerase activity in mammalian cells, but PB2 did not enhance activity in avian cells.

The properties of the PB2 E627K and D701N mutations. Previous studies have characterized the importance of the PB2 E627K mutation in virulence, mammalian host adaptation, and virus transmission (8, 10, 24, 34, 46, 47). Although the occurrence of the E627K and D701N mutations together is very rare in nature (Table 2), r-MA virus contains both 627K and 701N PB2 mutations. Therefore, to determine the properties of these two mutations with respect to replication in mouse lungs and host-dependent polymerase activity, we rescued four MA viruses that possessed the wild-type and mutant combinations: MA-PB2(627K 701N) (r-MA), MA-PB2(627K 701D) (r-MA+HK-PB2), MA-PB2(627E 701D) and MA-PB2(627E 701N). We then performed growth curves *in vitro* and *in vivo* as well as a virulence assay *in vivo*. In CD-1 mouse lungs, the MA-PB2(627K 701N) and MA-PB2(627K 701D) viruses grew to similar extents at day 1 p.i. while growth of the MA-PB2(627K 701D) virus was slightly decreased in the lungs at later times. The MA-PB2(627E 701D) virus grew very poorly

FIG. 6. Polymerase activity of RNP complex genes of HK and MA in human, mouse, and chicken cell lines. Human 293T, mouse B82, and avian DF1 cells were transfected with the human (H) or mouse-adapted (M) polymerase subunits and NP, with the appropriate influenza virus-like minigenome carrying the firefly luciferase gene. Each luciferase activity value is the average of three independent experiments. (A) 293T cells. (B) B82 cells. (C) DF1 cells (*, $P < 0.05$). The MA-PA has no mutations relative to HK-wt PA (indicated by dashes). The horizontal dashed lines on the graphs represent the baseline polymerase activity of the HK-wt polymerase complex.

FIG. 5. Role of MA HA G218W^{HA1} and T156N^{HA2} mutations in virulence and replication. (A) Viral growth in CD-1 mice. Groups of 12 CD-1 mice were infected intranasally with 5×10^3 PFU of MA backbone viruses that possessed either or both HA mutations. Lungs were collected on days 1, 3, 5, and 7 p.i. and then homogenized, and virus titers were assessed by plaque assay. (B) Body weight loss in CD-1 mice. Groups of five CD-1 mice were intranasally infected with 10^5 PFU of each of the recombinant viruses. Body weight loss was monitored for 14 dpi. (C, D, and E) Replication kinetics of the reassortants *in vitro*. Monolayers of MDCK, A549, or M1 cells were infected with each of recombinant viruses at an MOI of 0.01 in the presence of TPCK-trypsin. Supernatants were collected at 12, 24, 48, and 72 hpi, and virus titers were assessed by plaque assay. (F) Plaque phenotypes of r-HK and r-MA recombinant viruses that differed due to PB2 and individual HA mutations. Plaque assays were produced in MDCK cells under standard conditions and stained with crystal violet. (G) Average plaque diameter for each recombinant virus. The diameter of 10 random plaques was measured for each virus (**, $P < 0.01$; ***, $P < 0.001$). The horizontal dashed line represents the mean plaque diameter of HK-wt.

TABLE 2. PB2 gene substitutions of amino acids 627 and 701 identified in the influenza virus database

Position(s) and amino acid(s)	Frequency of substitution (no. of strains with the substitution/total no. of strains) by virus type							
	Human	Human H1N1 (seasonal flu)	Human H1N1 (pandemic 2009)	Human H3N2	Human H5N1	Avian	Avian H5N1	Swine
627E	1,544/4,503	2/917	2,793/2,796	3/1,962	88/122	2,470/2,693	652/807	177/329
627K	2,954/4,503	915/917	3/2,796	1,959/1,962	31/122	174/2,693	155/807	149/329
701D	4,482/4,503	914/917	2,792/2,796	1,950/1,962	116/122	2,689/2,693	804/807	267/329
701N	16/4,503	3/917	3/2,796	11/1,962	3/122	4/2,693	2/807	61/329
627E 701D	1,540/4,503	1 ^a /917	2,790/2,796	2/1,962	85/122	2,466/2,693	650/807	116/329
627E 701N	4/4,503	1 ^b /917	3/2,796	1/1,962	3/122	4/2,693	2/807	61/329
627K 701D	2,942/4,503	913/917	3/2,796	1,948/1,962	31/122	174/2,693	155/807	149/329
627K 701N	12/4,503	2 ^c /917	0/2,796	10 ^d /1,962	0/122	0/2,693	0/807	0/329

^a A/Iowa/CEID23/2005 (H1N1) (gi: 112456162), a swine virus isolated from a human.

^b A/Thailand/271/2005(H1N1) (gi:118136498), a swine virus isolated from a human in Thailand.

^c A/Cameron/1946 (H1N1) (gi:89903092) and A/PR/8/34 (H1N1) virus passaged in MDCK cells (gi:126599299).

^d All 10 strains are HK/1/68 mouse-adapted viruses.

in mouse lungs at day 1 p.i. (5×10^3 PFU/ml) but began to recover at later time points, reaching a peak titer of 10^6 PFU by day 7 p.i. (Fig. 7A). Growth of MA-PB2 (627E 701N) virus was comparable to that of the 627K mutant early after infection ($>6 \times 10^7$ PFU/ml) and then decreased gradually at later time points but still maintained a higher titer than MA-PB2(627E 701D) (days 3 and 5 p.i.) (Fig. 7A). Disease severity as assayed by body weight loss also showed that the MA-PB2(627K 701D) and MA-PB2(627K 701N) viruses were the most virulent (Fig. 7B); however, the MA-PB2(627E 701D) virus was avirulent in mice as it did not result in significant weight loss or mortality (data not shown), and mice infected with r-MA-PB2(627E 701N) virus lost approximately 20% of their body weight, but they did not die (Fig. 7B). Therefore, we concluded that the PB2 701N mutation was necessary for viral growth and virulence in mouse lungs and played a role in compensating for the decreased growth of a virus containing 627E, observed as a 3-log increase in viral growth at day 1 p.i. (Fig. 7A) and 20% more body weight loss (Fig. 7B).

Minigenome assays were performed to assess the changes of polymerase activity caused by the various combinations of the mutations of residues 627 and 701 of the PB2 gene. Polymerase activity of PB2 627E 701D with human RNP complex in human 293T and murine B82 cells was decreased to very low levels (approximately 2% and 4%, respectively) compared with that of PB2 627K 701D in contrast to chicken cells, where the activity was relatively unaffected. In contrast, with the to PB2 627E 701N mutations, the polymerase activity in 293T and B82 cells was enhanced to 50% and 15% of that of PB2 627K 701D, respectively. In contrast to a decrease, the polymerase activity of PB2 627K 701N increased by 1.4- and 3.2-fold compared to activity of PB2 627K 701D in 293T and B82 cells (Fig. 7D and E). Similar changes were observed as a result of the mutations when they were analyzed in the mouse-adapted RNP complex (Fig. 7D and E). However, the polymerase activity changes caused by mutations at residues 627 and 701 of the PB2 genes did not have significant effects in avian cells (Fig. 7F). Therefore, the minigenome polymerase assay results were consistent with the growth and virulence due to the PB2 mutations at residues 627 and 701.

Because previous studies showed that avian viruses possessing PB2 627E rapidly evolved to 627K in studies of mouse infection, we sequenced the viruses present in the lung samples

collected between days 1 and 7 for the mice infected with MA-PB2(627E 701D) and MA-PB2(627E 701N) to see if PB2 mutations were being selected. The mouse lung growth curves had suggested evolution during the growth assay because the MA-PB2(627E 701D) virus lung titer at 1 day p.i. was less than that of the input virus but increased thereafter to reach levels comparable to those of the other mutants (Fig. 7A). Sequencing of all the PB2 genes of MA-PB2(627E 701D) virus isolated from mouse lung (Table 3), we found that PB2 residue 701 had already mutated from D to N in one of three mouse lungs at day 3 p.i. and that the virus titer was 20-fold higher than that of the other two mice, indicating that this mutation was a primary driver for enhanced polymerase activity of the PB2 627E mutant. Furthermore both 627E and 701D amino acids of PB2 gene were mutated from E to K and D to N, respectively and thus were being selected in all inoculated mouse lungs at days 5 and 7 p.i. This demonstrated that the adaptive properties of the D701N mutation were comparable to those of the E627K mutation. Thus, the growth and polymerase activities due to the E627K and D701N mutations were consistent with the selection of both of these mutations during infection with PB2 627E 701D, with the dominant E627K mutation selected first. We have also shown that the D701N mutation was subsequently selected in mice. This result supports the hypothesis that the D701N mutation can functionally replace E627K during adaptation of avian-like influenza virus to a mammalian host (5).

DISCUSSION

A clonally derived HK prototype H3N2 (clinical isolate) was subjected to serial high-dose passage in mice to select a biological variant that caused fatal viral pneumonia. Genomic sequencing indicated that the MA variant had increased $>10^{3.9}$ -fold in virulence due to the acquisition of nine mutations in seven genes. Although it was clear that this group of nine mutations was responsible for the acquired virulence and change in host range, it was not clear which of these mutations was sufficient or the most relevant to controlling these properties. The impact of each gene substitution between HK-wt and HK-MA on virus replication and virulence was evaluated in a mouse model using single gene reassortants generated by reverse genetics. Whereas all mutant genes were implicated in

FIG. 7. Assessment of the relative roles of PB2 E627K and D701N mutations in MA virus. (A) Viral growth in CD-1 mice. Groups of 12 CD-1 mice were infected intranasally with 5×10^3 PFU of the indicated MA mutants. Lungs were collected at days 1, 3, 5, and 7 p.i. and assessed by plaque assay. (B) Body weight loss in CD-1 mice. Groups of five CD-1 mice were intranasally infected with 10^5 PFU of each of the recombinant viruses. Body weight loss was monitored for 14 dpi. (C) Replication kinetics of the reassortants *in vitro*. Monolayers of MDCK, A549, or M1 cells were infected with each of recombinant viruses at an MOI of 0.01 in the presence of TPCK-trypsin. Supernatants were collected at 12, 24, 48, and 72 hpi, and virus titers were assessed by plaque assay. (D, E, and F). Polymerase activity of reconstituted RNP complexes with mutations of PB2 residues 627 and 701 in 293T, B82, and DF1 cell lines. Human 293T, mouse B82, and avian DF1 cells were transfected with human (H) or MA (M) polymerase subunits (note that the PA sequence is the same for H and M viruses), NP, and the appropriate influenza virus-like minigenome. Each luciferase activity value is the average of three independent experiments (*, $P < 0.05$; ** $P < 0.01$). The horizontal dashed lines represent the baseline polymerase activity of the HK-wt polymerase complex.

increased disease severity on the HK backbone or reduced severity when exchanged with HK-wt genes on the MA backbone (Fig. 1D and E), the greatest effect was seen for the MA-PB2 D701N mutation on the HK backbone (>10 -fold) and the exchange of the HK-HA with MA-HA(G218W^{HA1} T156N^{HA2}) on the MA backbone (10^2 -fold).

Gene interaction and viral backbone effects. We found that the genetic background of the virus was a critical determinant

TABLE 3. PB2 mutations selected in recombinant viruses during mouse lung infection

Virus	No. of days p.i.	Mouse no.	Amino acid at the indicated position of the PB2 gene ^a		Virus titer in mouse lungs (PFU/ml)
			627	701	
MA-PB2(627E 701D)	1	1	E	D	6×10^3
		2	E	D	3×10^2
		3	E	D	5×10^3
	3	1	E	N	1.6×10^6
		2	E	D	1×10^5
		3	E	D	7×10^4
	5	1	E	D/N	7.5×10^4
		2	E/K	D/N	5.5×10^5
		3	K/E	N/D	2.4×10^6
	7	1	K/E	N	1.6×10^6
		2	K/E	D	5.1×10^5
		3	ND	ND	Under detection limit
MA-PB2(627E 701N)	1	1	E	N	1.9×10^7
		2	E	N	1.5×10^7
		3	E	N	4×10^8
	3	1	E	N	3.9×10^6
		2	E	N	4.2×10^6
		3	ND	ND	2.5×10^6
	5	1	E	N	3.4×10^6
		2	E	N	2.8×10^6
	7	1	E	N	1.3×10^5
		2	E	N	4×10^3

^a ND, not done.

of mutant phenotype both *in vivo* (mouse lung) and *in vitro* using cells of human, mouse, and dog origin (Fig. 4). Whereas introduction of PB2 D701N onto the HK backbone resulted in a 2-log increase in replication in the mouse lung, the converse exchange of PB2 N701D into MA did not significantly change replication levels or the LD₅₀ in mice, indicating that this mutation was not critical for virulence on the MA backbone. However, we observed both a decreased loss in body weight (Fig. 1E) and a significantly prolonged time to death due to the HK-PB2 gene (Fig. 2A), indicating reduced pathogenicity. In addition we also saw a trend to reduced replication in mice at late times (0.5-log₁₀ decrease at 5 days postinfection in mouse lungs) (Fig. 4B), and polymerase activity was reduced by >200% relative to MA virus polymerase in mouse cells (Fig. 6B). Therefore, although the HK-PB2 gene had little effect on the lethal dose in MA, this virus was attenuated with respect to disease severity and polymerase activity in mouse cells.

The NP D34N mutation may be responsible for compensating for the removal of PB2 D701N because it was the only other mutation that was dispensable for virulence on the MA backbone (LD₅₀ of $10^{3.7}$ PFU versus $10^{3.8}$ PFU for MA) (Fig. 1C). Since PB2 and NP bind each other and since the mutations in each gene are within regions that interact, it is possible that they enhance interaction with each other and thus are each individually expendable on the MA backbone. The PB2 D701N mutation has been shown to result in the removal of a salt bridge that sequesters the nuclear localization signal (NLS) (48), resulting in greater binding to human importin α 1

as well as increased nuclear localization of PB2 and NP in mammalian cells (11). Future studies will address the effects of PB2-NP mutations on their interactions.

Mouse-adaptive polymerase mutations enhance function in multiple hosts. Mutations in the PB2 and PB1 proteins were observed to enhance polymerase activity in the luciferase mini-genome assay in mouse and/or human cells but had little effect in chicken cells. Whereas the PB1 mutation enhanced polymerase activity in all combinations of host (mouse, human, and chicken) and viral genetic backgrounds, the MA-PB2 mutation enhanced RNA Pol activity strongly on both HK and MA backbones in mouse cells (Fig. 6B) but did not affect activity (and was dispensable) for MA polymerase activity in human cells (Fig. 6A). Together, these data showed that the MA RNA polymerase mutations functioned as partially independent of host and virus backbone. We conclude that adaptive mutations have host-independent and host-dependent effects, presumably involving virus-virus as well as virus-host interactions (41).

The role of the E627K and D701N mutations. The polymerase genes, in particular the PB2 gene, have been shown to be important determinants of virulence and host range. Subbarao et al. reported that the amino acid at position 627 of PB2 is a determinant of host range and that all avian influenza viruses have glutamic acid at this position, whereas all human influenza viruses (H1N1, H2N2, and H3N2) have lysine (47). The 2009 pandemic H1N1 strain from swine maintained the PB2 627E due to suppressor mutations at amino acids 590 and 591 (30). Hatta et al. reported that the E627K mutation of PB2 extended the mammalian host range of the 1997 Hong Kong the highly pathogenic avian influenza (HPAI) H5N1 virus, resulting in 10³-fold enhanced virulence in mice (14). Other studies showed that PB2 E627K was the most important mutation for increased virulence and replication in mice for a mouse-adapted A/Equine/London/1416/73 (H7N7) virus (43). However, mouse adaptation of the avian-like A/Seal/Massachusetts/1/1980 (H7N7) resulted in the selection of the PB2 D701N S714R mutations that were an important genetic determinant of polymerase activity and virulence in mice (10). Li et al. also reported that the D701N mutation found in HPAI H5N1 virus controlled its increased pathogenesis in a mouse model (24). Therefore, these data suggest that PB2 mutations E627K and D701N are the first and most effective mutations for a virus to acquire the ability to adapt to increased virulence in a new mammalian host.

Sequence analysis showed that the HK-MA strain possessed both the 627K and 701N mutations in its PB2 gene; however, this combination is very rare in nature because the sequence database showed that these mutations were found only in two laboratory-adapted human strains, A/Cameron/1946 (H1N1) (gi:89903092) and a MDCK cell passage variant of A/PR/8/34 (gi:126599299) (6) (Table 2).

We showed that the MA virus engineered to possess PB2 627E 701D was severely attenuated in mouse lung and within a single infectious cycle selected 627K as well as 701N mutants, indicating that these two mutations were the most adaptive for the mouse lung. Virus with MA-PB2(627E 701N) mutations grew to a much higher titer in mouse lungs than MA-PB2(627E 701D) (i.e., 4 logs higher at day 1 p.i.) due to the PB2 701N mutation alone. This result supports the hypothesis that the D701N mutation can functionally replace E627K dur-

ing avian-like influenza virus adaptation to a mammalian host (5). The polymerase activity assay results correlated with growth in the lung, (Fig. 7D and E). We also showed that the PB2 627E 701D RNA polymerase activity was restored to HK-wt levels when this mutation was combined with the mouse-adapted PB1 and NP genes, indicating that the mouse adapted polymerase possessed other mutations that can compensate for the inhibition due to the PB2 627E mutation in human cells (Fig. 7D).

HA mutations. Each of the HA G218W^{HA1} and T156N^{HA2} mutations operated to enhance the replication and virulence of HK-MA in the mouse lung (Fig. 5). These mutations demonstrated both host-dependent and host-independent effects such that they did not enhance replication in mouse kidney cells but did enhance replication in A549 cells and increased the rate of replication in MDCK cells, resulting in much larger plaques (Fig. 5F). The enhanced adaptive functions due to the MA-HA is consistent with a 0.4-unit reduced pH of fusion as well as increased α 2-3 sialic acid binding (only α 2-3 sialic acid is found in the mouse lung [18]) due to the G218W mutation, as previously shown (21). The HK G218W^{HA1} and T156N^{HA2} mutations on the WSN-HK(HA+NA) backbone also increased virulence in the mouse lung, replication in primary mouse tracheal epithelium cell culture, and lung bronchiolar and alveolar tissues (21). The T156N mutation of HA2, which caused loss of a glycosylation site (3), was similar to G218W of HA1 in increasing virulence in mice (Fig. 5A) and possibly affects molecular rearrangement during fusion but does not alter the pH of fusion (21). Smee et al. showed that elimination of glycosylation sites at position of 63 or 93 of HA1 was selected on mouse adaptation of A/New Caledonia/20/99 (H1N1) virus (44). We along with others (35) have also reported HA G218E^{HA1} and N154T^{HA2} (loss of the same glycosylation site as T156N^{HA2}) mutations in human H3N2 influenza mouse-adapted virus variants that were convergent with the G218E^{HA1} and N154T^{HA2} mutations selected when H3N8 equine influenza adapted to dogs in 2005 (38). This further indicates that common adaptive mutations are selected among different hosts, and thus the mouse model can be used to identify mutations that indicate adaptive selection of influenza viruses in other hosts.

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