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The Polymerase Acidic Protein Gene of Influenza A Virus Contributes to Pathogenicity in a Mouse Model▽

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Adaptation of influenza A viruses to a new host species usually involves the mutation of one or more of the eight viral gene segments, and the molecular basis for host range restriction is still poorly understood. To investigate the molecular changes that occur during adaptation of a low-pathogenic avian influenza virus subtype commonly isolated from migratory birds to a mammalian host, we serially passaged the avirulent wild-bird H5N2 strain A/Aquatic bird/Korea/W81/05 (W81) in the lungs of mice. The resulting mouse-adapted strain (ma81) was highly virulent (50% mouse lethal dose = 2.6 $\log_{10}$ 50% tissue culture infective dose) and highly lethal. Nonconserved mutations were observed in six viral genes (those for PB2, PB1, PA, HA, NA, and M). Reverse genetic experiments substituting viral genes and mutations demonstrated that the PA gene was a determinant of the enhanced virulence in mice and that a Thr-to-Iso substitution at position 97 of PA played a key role. In growth kinetics studies, ma81 showed enhanced replication in mammalian but not avian cell lines; the PA_{97I} mutation in strain W81 increased its replicative fitness in mice but not in chickens. The high virulence associated with the PA_{97I} mutation in mice corresponded to considerably enhanced polymerase activity in mammalian cells. Furthermore, this characteristic mutation is not conserved among avian influenza viruses but is prevalent among mouse-adapted strains, indicating a host-dependent mutation. To our knowledge, this is the first study that the isoleucine residue at position 97 in PA plays a key role in enhanced virulence in mice and is implicated in the adaptation of avian influenza viruses to mammalian hosts.

Migratory waterfowl are the natural reservoir of influenza A viruses (11, 53). The viruses replicate efficiently in their natural hosts but replicate poorly if at all in other species (53). However, these viruses can undergo adaptation or genetic reassortment to infect other hosts (43, 44, 53), including humans. Since 1997, the World Health Organization has documented more than 400 laboratory-confirmed cases of human infection with H5N1 avian influenza virus (54).

The molecular basis of influenza virus host range restriction and adaptation to a new host species is poorly understood. Mutations associated with cross-species adaptation are thought to be associated with increased virulence (30). Therefore, studies in animal models have attempted to identify the viral molecular determinants of virulence in specific hosts. Reverse genetics (Rg) methods have also identified genetic differences that affect virus virulence and host range, including changes in the viral internal proteins. Experimental infection of mouse lungs is an effective approach for understanding influenza virus virulence and adaptation (reviewed by A. C. Ward [51]). To acquire virulence in mice, influenza A viruses usually must adapt to these hosts over several consecutive generations (serial passages) in the lungs or brain (1, 25, 30). Previous studies have found that the acquisition of virulence during adaptation in the mouse model is associated with mutations in the HA, NP, NA, M, and NS genes and one or more polymerase genes (2, 3, 18, 36, 42, 51). The polymerase basic protein 2 (PB2) gene is a particularly well-characterized polymerase subunit (7, 23, 40, 46). The PB1 and polymerase acidic protein (PA) genes have been implicated in mouse lung virulence (5, 18, 36, 39, 49) but have shown no evidence of having acquired mutations during adaptation (52). However, the many studies conducted to date have focused mainly on highly pathogenic avian influenza (HPAI) viruses such as the H1N1, H5N1, and H7N7 subtypes (7, 23, 48, 50).

Various low-pathogenic avian influenza (LPAI) viruses are considered to be potential genetic contributors to the next pandemic strain. Lee et al. (2009) recently reported the presence of avian-like LPAI H5N2 viruses in a number of Korean swine and proposed that the efficient transmissibility of the swine-adapted H5N2 virus could facilitate spread of the virus. They suggested that this adapted virus could potentially serve as a model for pandemic outbreaks of HPAI (e.g., H5N1 and H7N7) virus or could become a pandemic strain itself (21). These findings prompted our interest in the adaptation of an LPAI virus often harbored by wild migratory birds of South Korea. In our ongoing surveillance from 2004 to 2008, approximately 27% of the viruses isolated were of the H5N2 subtype (unpublished data). Studies show that influenza viruses with

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different genetic backgrounds can acquire different mutations during adaptation in mice. Therefore, we sought to determine whether this common H5N2 virus (nonlethal in mice) would undergo changes different from those observed in highly virulent viruses during adaptation in mice. Wild-bird influenza virus strain A/Aquatic bird/Korea/W81/05 (W81) was adapted in mice over 11 passages and became highly virulent. To identify molecular determinants of this adaptation and altered virulence, we used Rg-generated recombinant viruses to compare the parental and mouse-adapted strains. Here we show that the PA subunit of the polymerase complex, independently of PB2, contributed to adaptation and increased virulence in our mammalian model.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were grown in minimum essential medium (MEM) with Eagle salts containing 5% fetal bovine serum (FBS), and 293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS. Chicken embryonic fibroblasts (CEF) were digested from 18-day-old chicken embryos using buffered saline containing 2.5% trypsin and then cultured in RPMI 1640 medium with 10% FBS. DF-1 chicken fibroblast cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS. LA-4 mouse lung adenoma cells were maintained in RPMI 1640 medium containing 10% FBS. Human bronchial epithelial (HBE135-E6E7) cells were grown in keratinocyte serum-free medium that included 5 ng/ml human recombinant epidermal growth factor, 0.05 mg/ml bovine pituitary extract, 0.005 mg/ml insulin, and 500 ng/ml hydrocortisone. Media and additives were purchased from Gibco. All cells were incubated at 37°C in 5% CO_2.

Viruses. Virus strain A/Aquatic bird/Korea/W81/05 (W81, H5N2) was isolated in 2005 from routine surveillance of influenza virus activity among wild birds of Korea. It was serially passaged 11 times in mice until it generated a mouse-adapted, highly virulent strain termed ma81. The ma81K virus is phenotypically and genetically similar to the ma81 virus except for an E627K substitution in its PB2. These two virulent viruses were isolated by plaque purification, described below, and were selected based on their phenotypes and sequence differences relative to the parental wild-type virus.

Plaque purification and selection. To isolate single-phenotype viruses that cause mortality similarly to the mouse-adapted virus, we plaque-purified lung isolates of the virulent mouse-adapted strain in MDCK cells (9). Supernatants of lung tissue homogenates were serially diluted 10-fold in appropriate media. MDCK cells were infected with the dilution samples in six-well plates. After 1 h of incubation, the cells were washed with PBS and overlaid with a 0.7% agarose-medium mixture with L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin. Sixty hours later, eight single-plaque colonies were picked, resuspended in medium, and injected into 11-day-old embryonated chicken eggs. After 48 h of incubation, viruses were harvested and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated by the method of Reed and Muench (33). Virulence of the eight plaque-purified viruses (5.5 $TCID_{50}$) was reevaluated in mice. Based on the highly virulent phenotype and sequence analysis, two out of the eight purified plaques were selected for further studies.

Plasmids and rescue of Rg viruses. To clone all eight genes of the W81 and ma81 viruses and the PB2 gene of the ma81K virus, we amplified each segment by reverse transcription-PCR from isolated viral RNAs and cloned them into pHW2000 vector as described previously (12, 13). All recombinant and point mutation viruses (see Fig. 4 and 5) were rescued in a six-well plate of cocultured 293T and MDCK cell mixtures (3:1 ratio) transfected with the corresponding eight viral plasmids, each containing 1 μg of the respective gene segment, using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Transfection medium was removed after 6 h and replaced with Opti-MEM I (Gibco) containing 0.3% bovine serum albumin and 0.01% FBS. After 30 h, 1 ml of Opti-MEM I containing 0.2 μg/ml of TPCK-trypsin was added to the transfected cells. Supernatant was harvested after 48 h and injected into 11-day-old embryonated chicken eggs for virus propagation. We fully sequenced all rescued viruses to ensure the absence of unwanted mutations.

Site-directed mutagenesis. The characteristic mutations found in influenza virus strain ma81 were introduced into the plasmid constructs of W81 or vice versa by site-directed mutagenesis using the GeneTailor site-directed mutagenesis system (Invitrogen) according to the manufacturer's instructions. To ensure that no unwanted mutations were introduced into the cDNA, we had the entire sequences of cDNAs of the rescued viruses resequenced by Cosmo GeneTech (Seoul, Korea).

Virulence and virus replication in mice. Groups of 10 5-week-old female BALB/c mice were anesthetized with ether, inoculated intranasally (i.n.) with 5.5 $\log_{10}$ $TCID_{50}$ of infectious virus, and monitored daily for weight loss and mortality for 12 days. Three mice in each group were euthanized on days 3 and 5 (most mice inoculated with strain ma81 died before day 5), and virus was titrated in the lungs, kidneys, spleens, and brains. Organs were homogenized (1 g/ml) in cold 1× phosphate-buffered saline (PBS) containing antibiotics (0.1% penicillin-streptomycin; Gibco). The supernatants were serially diluted 10-fold and inoculated into 11-day-old embryonated chicken eggs for virus titration ($\log_{10}$ 50% egg infective dose [EID_{50}] per gram of tissue).

To determine the 50% mouse lethal dose (MLD_{50}) of viruses that were lethal, we inoculated groups of six mice i.n. with 10-fold serial dilutions containing 10^1 to 10^6 $TCID_{50}$ of virus in a 30-μl volume (4, 23). The MLD_{50} was expressed as $\log_{10}$ $TCID_{50}$. All $TCID_{50}$, EID_{50}, and MLD_{50} calculations were done by the method of Reed and Muench (33).

Viral growth in animals. We compared the growth of influenza virus strains W81, ma81, W81 WPA_{97I}, and ma81-$maPA_{97T}$ in vivo in groups of mice (Samtaco, Seoul, South Korea) and chickens (CAvac, Daejeon, South Korea). Four groups of 15 mice were inoculated i.n. with 5.5 $\log_{10}$ $TCID_{50}$ of infectious virus in 30 μl of PBS. Three mice were euthanized on days 1, 3, 5, 7, and 9 postinoculation (p.i.) for lung virus titration. Lung tissues were homogenized (1 g/ml) in cold 1× PBS with antibiotics. The supernatants were serially diluted 10-fold, and virus was titrated ($\log_{10}$ EID_{50} per gram of tissue) in 11-day-old embryonated chicken eggs.

Four groups of five 4-week-old White Leghorn chickens were infected i.n. and intratracheally with 500 μl of PBS containing 5.5 $\log_{10}$ $TCID_{50}$ of parental and recombinant viruses. Tracheal swabs were collected on days 1, 3, 5, 7, and 9 p.i. and resuspended in 1 ml of 1× PBS with antibiotics. Samples were serially diluted, and virus was titrated ($\log_{10}$ EID_{50}/ml) in embryonated chicken eggs. Hemagglutination (HA) assays were performed with 0.5% chicken red blood cells. All EID_{50}s in these experiments were calculated by the method of Reed and Muench (33).

Histology and immunohistochemistry. The lungs of mice inoculated with strains W81 and ma81 were harvested on day 5 p.i., fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned (4 μm), and examined in the pathology laboratory of Chungbuk National University Hospital. Histological assessment used standard hematoxylin and eosin staining and light microscopy (magnification, ×100). For immunohistochemistry, the slides were incubated for 3 h at 4°C with influenza A virus NP goat polyclonal antibody (Abcam, Cambridge, MA) at a dilution of 1:500 after antigen unmasking and blocking steps. The slides were rinsed with PBS three times and incubated with anti-mouse horseradish peroxidase secondary antibody (Jackson Laboratories) for 2 h at room temperature. After washing, the sections were stained with 3,3′-diaminobenzidine (DAB) (Vector) and observed at ×400 magnification.

Replication in avian and mammalian cells. Confluent MDCK, DF-1, Vero, HBE135-E6E7, and LA-4 cells were inoculated with strains W81, ma81, and ma81K at a multiplicity of infection of 10^{-4} and incubated at 37°C in appropriate medium containing 0.2% bovine serum albumin and TPCK-treated trypsin or 10% normal allantoic fluid. After 24 h, additional TPCK-trypsin was added. Supernatants were collected at 12, 24, 48, 72, and 96 h p.i., and virus was titrated ($\log_{10}$ $TCID_{50}$) in MDCK cells by the method of Reed and Muench (33).

Viral polymerase activity. Luciferase activity was measured as described by Salomon et al. (37). A luciferase reporter plasmid was constructed by replacing the open reading frame of enhanced green fluorescent protein (EGFP) in the pHW72-EGFP plasmid with the luciferase gene. 293T cells were transfected with 0.3 μg of pHW72-Luc, pHW2000-PB2, pHW2000-PB1, pHW2000-PA, pHW2000-NP, and pCMV-β-gal plasmids using TransIT-LT1 transfection reagent as directed. After 6 h, transfection medium was replaced with fresh complete medium. After 36 h, cells were washed with PBS and lysed for 30 min with 100 μl of lysis buffer (Promega). Cells were then harvested, and luciferase activity was assayed in triplicate by using the Promega luciferase assay system. Results were normalized to β-galactosidase activity. Student's *t* test was used for statistical comparisons.

RESULTS

Mouse-adapted H5N2 virus is highly virulent and pathogenic in mice. Strain A/Aquatic bird/Korea/W81/05 (H5N2) is an LPAI virus that does not bear a polybasic amino acid motif at the HA proteolytic cleavage site. The virus was isolated in

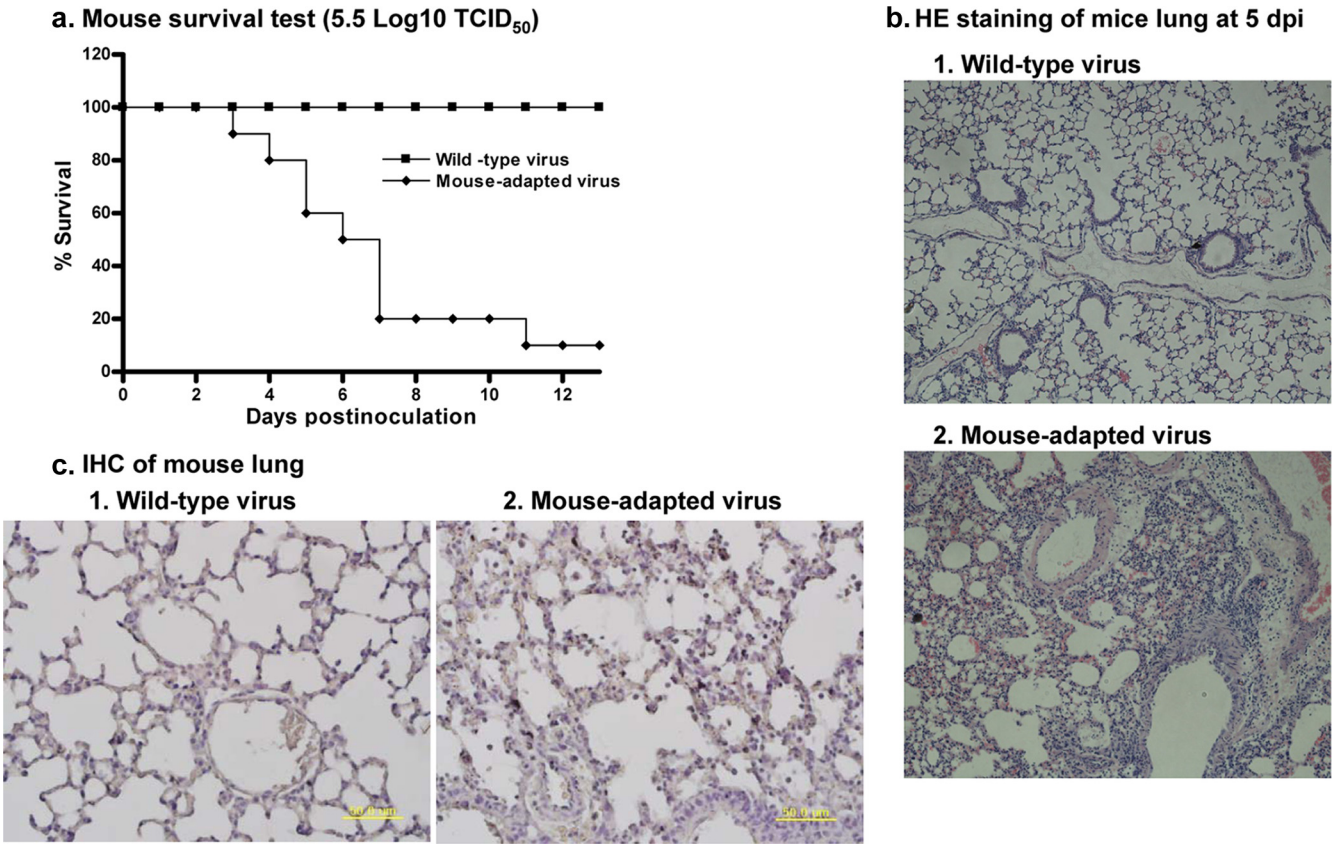

FIG. 1. Virulence of wild-type and mouse-adapted viruses in mice. Survival (a) and histopathological findings (b and c) of mice infected with 5.5 $\log_{10}$ $TCID_{50}$ of W81 or ma81 virus. (a) Groups of 10 mice were inoculated i.n. with serially diluted virus, and survival was monitored daily for 12 days. (b) Lung histopathology at 5 days p.i. (hematoxylin and eosin stain; magnification, ×100). (c) Viral infection was confirmed by immunohistochemistry with influenza A virus NP goat polyclonal antibody. Magnification, ×400.

2005 during routine surveillance in migratory wild birds. It is able to infect but is otherwise avirulent in mice (data not shown). Interestingly, however, we observed that a mouse-adapted variant obtained after serial lung-to-lung passages in mice replicated well and was lethal. To directly compare the virulence of the wild-type and mouse-adapted strains, we experimentally infected mice (Fig. 1). After inoculation i.n. with 5.5 $\log_{10}$ $TCID_{50}$ of the parental virus, no disease signs or deaths were observed during 12 days of observation. In contrast, an equivalent inoculation with the mouse-adapted virus caused death as early as day 3 p.i. (Fig. 1a). Infected mice showed prominent signs of infection, such as weight loss, ruffled hair, hunched back, and heavy/labored breathing. Lung virus titers of the virulent strain were at least twofold higher than those of the parental strain on day 5 p.i. (data not shown). However, no virus was detected in the spleens, livers, kidneys, or brains of mice in either group. These results suggest that the mouse-adapted virus acquired its high virulence during serial passages in mice, although virus replication was limited to the lungs.

To correlate the virulence of the viruses with pathogenicity in this host, we observed the severity of lesions in the lungs of infected mice. After inoculation with strain W81, histological sections of lung tissue on day 5 p.i. revealed moderate signs of virus infection but intact tissue structure (Fig. 1b1); in contrast, the mouse-adapted, highly virulent virus caused severe lung tissue damage with disrupted epithelial cells and extensive macrophage and neutrophil infiltration of the alveoli and perivascular spaces (Fig. 1b2). Therefore, the virulence observed appears to be related to pathogenicity in mice. These results were confirmed by immunohistochemical analysis showing more of the virulent virus than the wild-type parental virus in lung tissue sections (Fig. 1c).

Selection, plaque purification, and sequencing of highly virulent, mouse-adapted viruses. To isolate the highly virulent, mouse-adapted strain, we plaque-purified viruses recovered from mouse lungs in MDCK cells. The plaques formed by the two viruses were morphologically similar, but those formed by the mouse-adapted strain were considerably larger (data not shown). Eight plaques were purified. Two of the eight samples were highly lethal (>90%) and were chosen for further study. Full-length sequences of all eight segments of these two purified viruses were compared with those of the wild-type parental virus (W81). Several base differences were observed between the parental and mouse-adapted viruses, affecting six of the eight viral gene segments. Specifically, we noted substitutions in the RNA-dependent RNA polymerases (three in PB2, one in PB1, and four in PA), in the surface glycoproteins (three each in HA and NA), and in the matrix 1 protein (two) (Table 1). One virus strain bearing these mutations was designated ma81. The other plaque-purified virus contained an additional

TABLE 1. Amino acid differences between the parental wild-type avirulent strain W81 (H5N2) and the highly virulent mouse-adapted strain ma81[a]

Gene	Amino acid position	Amino acid encoded in indicated strain	
		W81	ma81
PB2	63	I	V
	288	Q	R
	578	E	D
	627	E	K[b]
PB1	10	L	S
PA	22	K	R
	97	T	I
	155	M	T
	216	D	N
HA	172	A	T
	224	R	K
	393	K	R
NA	106	I	V
	316	H	Y
	436	V	A
M1	129	G	D
	249	P	Q

[a] No amino acid differences were observed between the NP and NS genes of the two viruses.
[b] Found only in the ma81K virus.

E627K substitution in PB2, which has a well-documented role in host specificity (46) and replication efficiency (28, 40). This virus was designated ma81K. No mutations were observed in the nonstructural (NS) and nucleoprotein (NP) segments.

The virulent phenotype of the two purified viruses was confirmed in mice. Mice inoculated with these viruses did not survive the 12-day observation period (Fig. 2). All viruses were detected only in the lungs, but the titers of the mouse-adapted viruses were >1 $\log_{10}$ higher than those of the parental strain. The ma81K strain, bearing lysine (K) at position 627 in PB2, had a slightly higher mean virus titer (an approximately 0.3 $\log_{10}$ $TCID_{50}$ difference) than ma81 (Table 2). Interestingly, the ma81 variant lacking this mutation had comparable lethality and replication kinetics in mice, suggesting that other genetic factors contributed to its virulence.

Growth characteristics in avian and mammalian cells. To determine whether the genetic alterations we observed were host restriction factors, we compared the replication kinetics of the W81, ma81, and ma81K viruses in various avian and mammalian cell lines. Infection of the wild-type virus (W81) and both mouse-adapted viruses (ma81 and ma81K) in avian fibroblast cell lines CEF and DF-1 indicated similar growth kinetics without notable differences at peak titers between 3 and 3.7 $TCID_{50}$ at 48 h p.i. (Fig. 3a and b). In MDCK cells, all three viruses produced comparable titers at the indicated time points (Fig. 3c). MDCK cells are known to support productive growth of a wide variety of influenza A viruses regardless of their passage history (47). However, in Vero monkey kidney cells (Fig. 3d), human bronchial epithelial cells (HBE135-E6E7) (Fig. 3e), and mouse lung adenoma cells (LA-4) (Fig. 3f), the mouse-adapted viruses (ma81 and ma81K) grew faster and yielded titers approximately 100 to 1,000 times the titers of W81. These differences demonstrate that the serially passaged viruses had a considerable growth advantage in mammalian cells but not in avian cells.

PA gene plays an important role in the virulence of ma81 virus. To investigate the genetic determinants of virulence, we first inserted the full-length cDNAs of the ma81 and W81 RNA segments into the bidirectional expression plasmid pHW2000 (12, 13) and generated the viruses by Rg. To generate the ma81K recombinant virus, we cloned the PB2 gene segment bearing the E627K mutation and combined it with the other ma81 gene segments. This variant was included for comparison in most of the succeeding experiments. The Rg W81, ma81, and ma81K viruses were rescued and designated R-W81, R-ma81, and R-ma81K, respectively. After the sequences of the rescued viruses were confirmed, virus stocks were prepared in 10-day-old embryonated chicken eggs, and their replication and lethality were tested in mice. The three viruses yielded lung virus titers and lethality similar to those of the original viruses (Table 2 and Fig. 2), indicating that they retained the biological properties of the original strains.

By using appropriate plasmid combinations, we then created a series of single-gene reassortant W81 and ma81 virus strains, each containing one gene from the other virus, with the exception of the NS and NP genes. We named the Rg W81 virus possessing the PB2 gene of ma81 "W81-maPB2" and named the other Rg viruses similarly (Table 2). Figure 4 shows the MLD_{50} values of the viruses. The parental virus MLD_{50}s were >5.5 (R-W81) and 2.7 $\log_{10}$ (R-ma81) $TCID_{50}$, respectively, indicating a clear disparity in virulence. Recombinant Rg ma81 viruses carrying the PB2, PB1, HA, or M gene of W81 retained the virulent phenotype (MLD_{50} = 2.4 to 3.0 $\log_{10}$ $TCID_{50}$), and the Rg virus carrying the NA gene of W81 showed moderately attenuated virulence (3.5 $\log_{10}$ $TCID_{50}$). Interestingly, the ma81-WPA virus showed the most dramatic de-

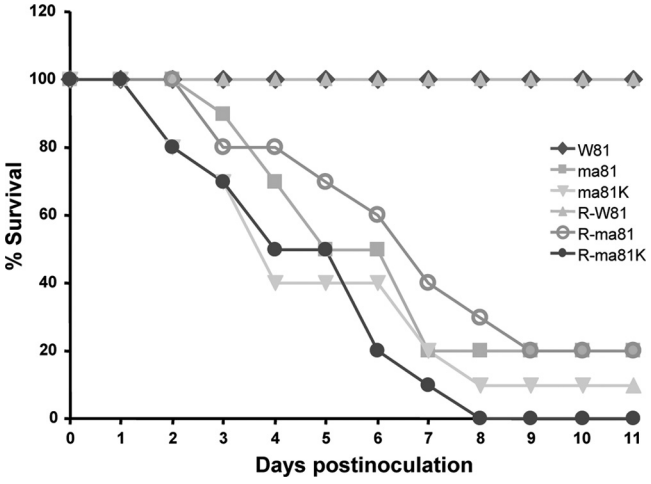

FIG. 2. Survival of mice inoculated with parental and Rg-generated viruses. The virulence of the Rg viruses confirmed that the viruses retained the biological properties of the parental viruses. Groups of 10 5-week-old BALB/c mice were inoculated intranasally with 5.5 $\log_{10}$ $TCID_{50}$ of virus, and weight and survival were monitored daily for 12 days.

TABLE 2. Replication of rescued viruses in BALB/c mice inoculated intranasally with 5.5 $\log_{10}$ $TCID_{50}$ of virus[a]

Virus strain	Mean virus titer ($\log_{10}$ EID_{50}/g) ±SD	
	Day 3 p.i.	Day 5 p.i.
ma81	4.6 ± 0.5	5.7 ± 0.5
R-ma81	4.6 ± 0.3	6.0 ± 0.0
ma81-WPA	3.6 ± 0.3	3.6 ± 0.3
ma81-WNA	4.1 ± 0.9	5.5 ± 0.6
ma81-maPA$_{97T}$	3.4 ± 0.3	4.0 ± 0.3
W81-WPA$_{97I}$	3.5 ± 0.4	4.5 ± 0.5
W81K-WPA$_{97I}$	3.9 ± 0.3	4.7 ± 0.2
W81	3.3 ± 0.3	3.6 ± 0.3
R-W81	2.9 ± 0.3	3.3 ± 0.5
W81-maPA	3.8 ± 0.3[b]	4.2 ± 0.3
W81-maNA	3.1 ± 0.5	3.5 ± 0.4
ma81K[c]	4.9 ± 0.5	6.0 ± 0.4
W81K	4.0 ± 0.4	4.3 ± 0.3
W81-WNA$_{316Y}$-WPA $_{97I}$	3.4 ± 0.5	4.0 ± 0.9

[a] Three mice in each group were euthanized on days 3 and 5 p.i., and virus was titrated in lung tissue. All animal experiments undertaken in this study were performed in accordance with relevant policies and guidelines of the Animal Use and Care by the Laboratory Animal Research Center (LARC) in Chungbuk National University, a member of the International Animal Care and Usage Committee (IACUC). All viruses and animal studies were handled in an enhanced biosafety level 3 (BSL-3+) containment facility approved by the Korea Centers for Disease Control and Prevention. ma, mouse-adapted; W, wild type; R, reverse genetics generated.

[b] $P < 0.05$; compared virus titers of W81-maPA relative to W81-inoculated mice at 3 days p.i.

[c] ma81K also reflects similar virus titers obtained from R-ma81K-infected mice.

crease in virulence (the MLD_{50} increased from 2.7 to >5.5 $\log_{10}$ $TCID_{50}$). Conversely, the virulence of recombinant W81-maPA virus was enhanced but was considerably lower than that of ma81 (4.7 versus 2.7 MLD_{50}). The remainder of the W81 Rg viruses bearing a single ma81 gene had MLD_{50}s comparable to that of the parental R-W81 ($MLD_{50} > 5.5$ $\log_{10}$ $TCID_{50}$), with the exception of W81-maNA ($MLD_{50} = 5.4$ $\log_{10}$ $TCID_{50}$). These results suggest that the PA gene was a determinant of the high virulence of ma81 in mice. Furthermore, Rg W81 virus carrying both the maPA and maNA genes showed greater virulence ($MLD_{50} = 4.1$ $\log_{10}$ $TCID_{50}$) than the single-gene recombinants (Fig. 4); therefore, the NA gene appeared to have an additive effect on virulence. When W81 PA was substituted in the ma81 background, mean lung virus titers in mice were onefold to twofold lower than those of parental virus; when ma81 PA was substituted in the W81 background, mean titers were about 0.5-fold higher than titers of parental virus. In contrast, the exchange of NA segments did not substantially alter the replication kinetics of the viruses (Table 2).

PA amino acid at position 97 alters the virulence of ma81. The deduced amino acid sequences encoded by the PA genes of W81 and ma81 indicated that the genes differ by four residues. To pinpoint which of these characteristic mutations enhanced virulence, we generated recombinant viruses with point mutations in the PA gene via site-directed mutagenesis and tested their virulence in mice (Fig. 5). Mutant ma81 viruses containing the single substitutions observed in W81 at position 22 (R22K), 155 (T155M), or 216 (N216D) of PA retained high virulence (≥2.5 MLD_{50}). Remarkably, however, when threo-

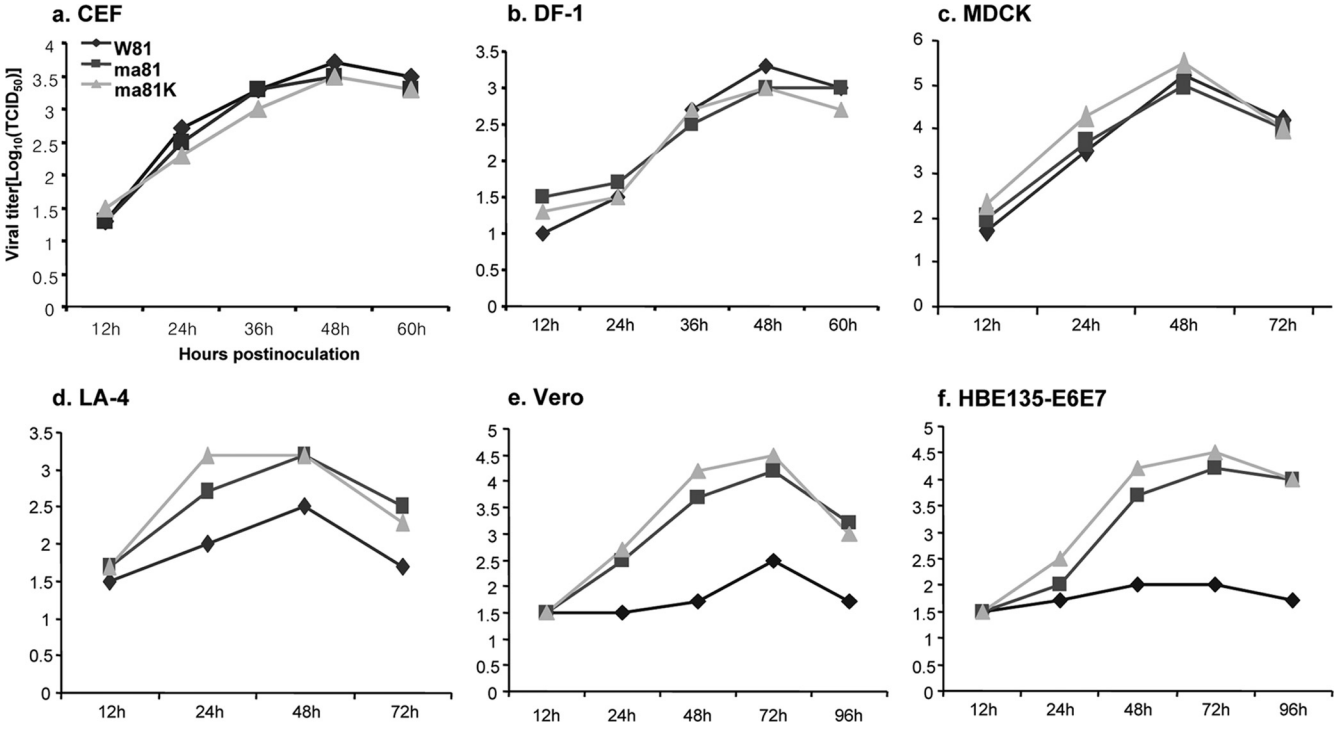

FIG. 3. Viral growth kinetics in avian and mammalian cells. Shown are growth curves of virus strains W81 (diamonds), ma81 (squares), and ma81K (triangles) in CEF cells (a), chicken fibroblast (DF-1) cells (b), MDCK cells (c), mouse lung adenoma (LA-4) cells (d), monkey kidney (Vero) cells (e), and HBE135-E6E7 cells (f). Cells were infected at a multiplicity of infection of 10^{-4}. Virus titers were determined in MDCK cells at the indicated time points.

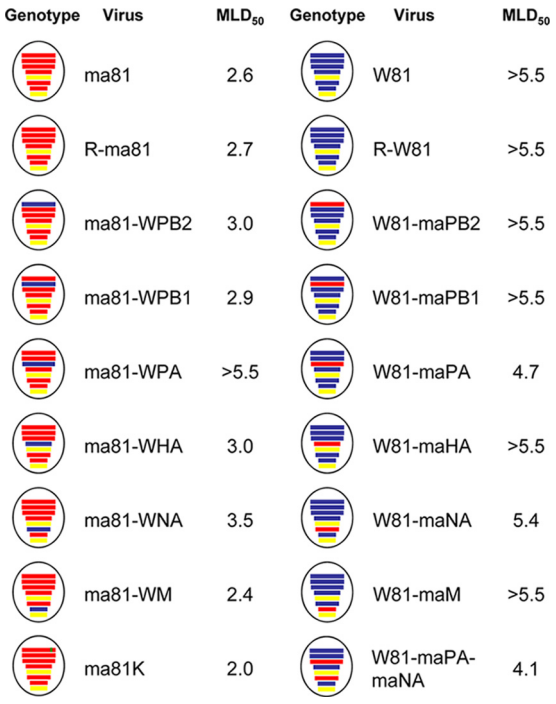

FIG. 4. Virulence of rescued recombinant Rg viruses in mice. The bars representing each segment indicate the origin of the viral gene as follows: blue, nonpathogenic wild-type A/Aquatic bird/Korea/W81/05 (W81); red, virulent mouse-adapted strain (ma81); yellow, NP and NS (similar for both W81 and ma81). The green dot in the top bar of "ma81K" indicates the $PB2_{627}$ lysine residue, the only notable difference from the ma81 strain. The MLD_{50} (expressed as $\log_{10}$ $TCID_{50}$) was determined by inoculating groups of six mice with 10-fold serial dilutions containing 10^1 to 10^6 $TCID_{50}$ of virus.

nine (T) was substituted for isoleucine (I) at position 97 of the ma81 PA gene, the mutant virus (ma81-$maPA_{97T}$) had no detectable 50% lethal dose ($MLD_{50} > 5.5$ $\log_{10}$ $TCID_{50}$), indicating that the PA 97I mutation is important for the highly virulent phenotype of ma81. When the PA 97T mutation was introduced into the other mutant PA viruses, producing double mutants, it also reversed the virulence of these viruses. In addition, the lethality of ma81K (the most lethal of the viruses tested) was significantly attenuated (the MLD_{50} was reduced from 2.0 to 4.1) when it bore the I97T mutation in maPA (Fig. 5). In contrast, when the PA gene of W81 was mutated to reflect the ma81 residue at position 97 (T97I), the virulence was enhanced and was comparable to that of a mutant W81K virus with the Glu-to-Lys mutation at residue 627 of PB2. However, it remained less virulent (approximately twofold less) than the parental mouse-adapted virus. The results of this mutational analysis of PA strongly suggest that the amino acid isoleucine at position 97 is a crucial virulence factor in mice and that the other mutations do not enhance virulence.

We initially observed that the maNA gene appeared to exert an additive effect on the virulence associated with PA. We therefore investigated the simultaneous impact of the ma81 NA gene and the PA T97I modification in the W81 virus (W81-maNA-WPA_{97I}). This combination resulted in greater virulence than that of the WPA mutant (W81-WPA_{97I}), and the addition of the characteristic mutation in PB2 (W81K-WPA_{97I}) did not increase the virulence (Fig. 5).

Mouse lung titers of selected mutant viruses indicated that replication of ma81 with the I97T substitution in PA was lower than that of the parental virus. Likewise, changing the residue at this position (T97I) increased the mean virus yield in mouse lungs. Combining this substitution with $PB2_{627K}$ further increased replication kinetics, yielding titers higher than those observed with the PB2 mutation alone. In contrast, alteration of position 316 of NA did not cause significant elevation of the virus titers above those of the PA_{97I} mutant.

97I residue in PA enhances polymerase activity. The PA protein is a component of the RNA-dependent polymerase complex, although its specific role is poorly defined. We investigated whether the PA97 residue affected polymerase activity by conducting a luciferase reporter gene construct assay in 293T cells with reconstituted ribonucleoprotein (RNP) complexes. The ma81K polymerase gene complex was included as a positive control and comparator. The polymerase activity of ma81 was approximately 1,300% higher than that of W81 (Fig. 6, columns A and E), reflecting substantially higher transcription/replication activity. However, it did not exceed the polymerase activity of the ma81K virus containing the E627K mutation in PB2 (~1,900% higher than that of W81) (Fig. 6, column O), which has been shown to increase polymerase activity (7). To investigate the role of specific polymerase genes in this increased activity, we studied several combinations. The maPA gene elevated the polymerase activity of W81 by 1,169%, whereas maPB2 and maPB1 had no effect (Fig. 6, columns C and D). In contrast, the polymerase activity of ma81 was decreased only by the substitution of WPA, reflecting the importance of PA for enhanced replication (Fig. 6, columns F to H). This finding was also associated with increased virulence of Rg virus strain W81-maPA in mice (Fig. 4). More specifically, the polymerase activity of W81 was greatly increased (by 1,134%) when the T97I mutation was introduced into its PA (Fig. 6, column I); this activity was 100% higher than that of W81K (1,030% activity) (Fig. 6, column P). The stepwise addition of 97I in PA and 627K in PB2, maPB2, and maPB1 to strain W81 increased its polymerase activity but not to the level of ma81K activity (Fig. 6, columns K and M). Conversely, the reverse mutation (I97T) in the PA gene of ma81 decreased the activity of the polymerase complex by almost half (724%) (Fig. 6, column J). To determine whether the PA_{97I} mutation alone is responsible for enhanced polymerase activity, we tested a W81-$maPA_{97T}$ polymerase combination, which did not substantially increase replication (Fig. 6, column L); therefore, the three other mutations at positions 22, 155, and 216 of PA did not increase replication activity. Collectively, these results indicate that the PA gene, particularly the 97I residue of PA, is sufficient to enhance polymerase activity in mammalian cells, consistent with the increased replication we observed in various cell lines (Fig. 3).

Growth in mammalian and avian hosts. To determine whether the properties observed were host specific, we compared the abilities of virus strains W81, ma81, W81-WPA_{97I}, and ma81-$maPA_{97T}$ to replicate in avian (chicken) and mammalian (mouse) hosts. The ma81 and W81-WPA_{97I} viruses grew faster and to higher titers (~100 times) than W81 and ma81-$maPA_{97T}$ viruses in mouse lungs (Fig. 7a). In addition,

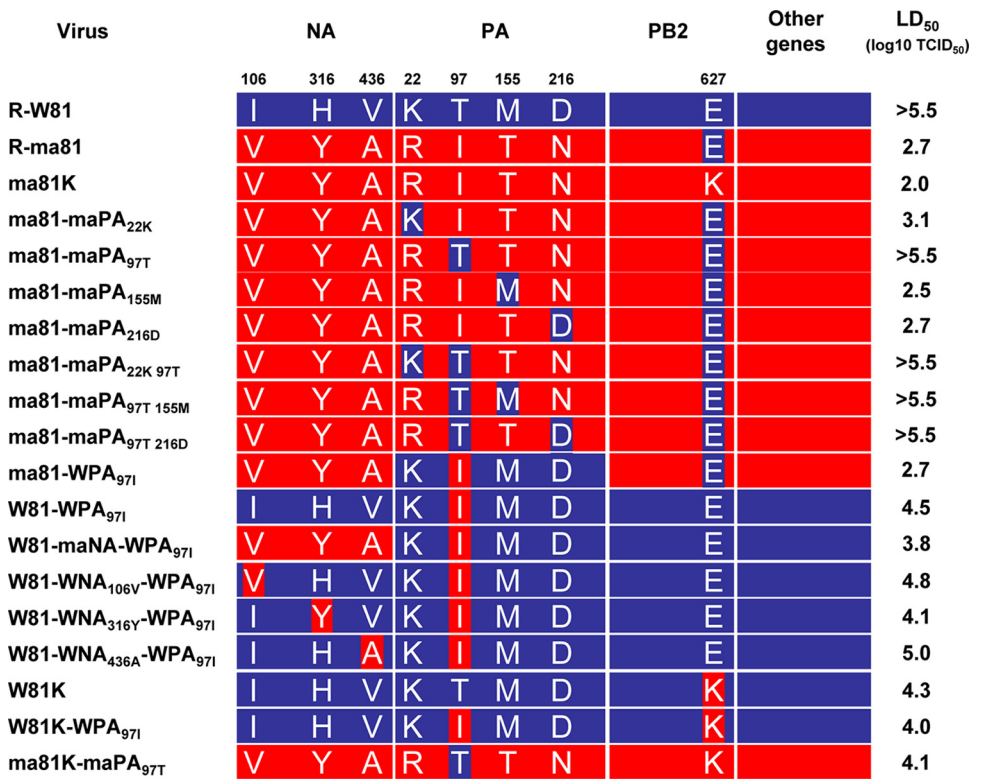

FIG. 5. Virulence, in mice, of viruses with PA, NA, and PB2 mutations. The colors indicate the origin of the gene or the encoded amino acid for W81 (blue) and ma81 (red). The amino acid differences between the NA, PA, and PB2 of virus strains W81 and ma81 are shown as single letters with positions indicated at the top. Strain ma81K is included for comparison. The MLD_{50} (expressed as $\log_{10}$ $TCID_{50}$) was determined by inoculating groups of six mice with 10-fold serial dilutions containing 10^1 to 10^6 $TCID_{50}$ of virus.

some mice began to succumb as early as day 3 p.i., and all but one had died by day 7 p.i., as described above (Fig. 1a). The greater replicative fitness of ma81 than that of W81 in mice was also consistent with its growth kinetics in mammalian cells (Fig. 3). However, none of the viruses tested yielded substantially different lung and tracheal titers in chickens, and the virus titers were lower than those in mice (Fig. 7b). Therefore, the process of mouse adaptation-selected variants with increased replicative fitness in mice and the role of the PA_{97I} mutation in this enhanced fitness appear to be host specific.

DISCUSSION

Serial passage of a virus in an animal model allows competition among all possible mutants and results in the selection of optimal genotypes for replicative fitness in that host. There have been many studies of mouse adaptation, though most are confined to the adaptation of virus strains H1, H2, H3, HPAI H5, or H7 (7, 24, 27, 30, 41). In our surveillance of avian influenza viruses among wild birds in South Korea, the H5N2 subtype is the most abundantly isolated subtype (unpublished data).

Low-pathogenic viruses, particularly those of subtypes H5 and H7, should not be overlooked as potential pandemic threats. They are known to undergo genetic changes by several mechanisms (8, 15, 19, 31, 45) that can significantly alter their pathogenicity and virulence. Studies of mouse adaptation among these subtypes can shed light on possible virulence factors or potential dangers to mammalian hosts. In this study, we serially passaged a common wild-bird LPAI H5N2 isolate (W81) in mouse lungs to observe the molecular changes that could enhance the virulence of an initially avirulent virus during adaptation. Infection with an isolate of the wild-type virus strain W81 does not cause death or clinical signs of illness. However, adaptation of the virus in mice yielded the virulent ma81 virus, which caused signs of severe disease resulting in >90% mortality and showed enhanced growth in mammalian cells. An amino acid comparison of the virulent, mouse-adapted strain to the wild-type, nonvirulent virus demonstrated approximately 17 base changes spanning six of the eight viral proteins (PB2, PB1, PA, HA, NA, M). However, none of these changes appears to be conserved, and none has been implicated in increased virulence or replication efficiency, with the exception of the E627K mutation in PB2 of the strain ma81K. One particular substitution in the H5 HA1 domain (Ala-156-Thr, H5 numbering) of the mouse-adapted viruses created a potential glycosylation site at Asn-154. The loss of glycosylation sites is known to be a common mechanism for the acquisition of virulence (52); however, the biological importance of the noted mutation on ma81 was not sought in the present study.

By using Rg to produce single-gene reassortant viruses and comparing their 50% lethal dose values in mice, we showed that the PA gene was a strong determinant of the virulence and replication of the lethal ma81 virus. The virulence of ma81 dropped dramatically (>100-fold) when its PA gene was ex-

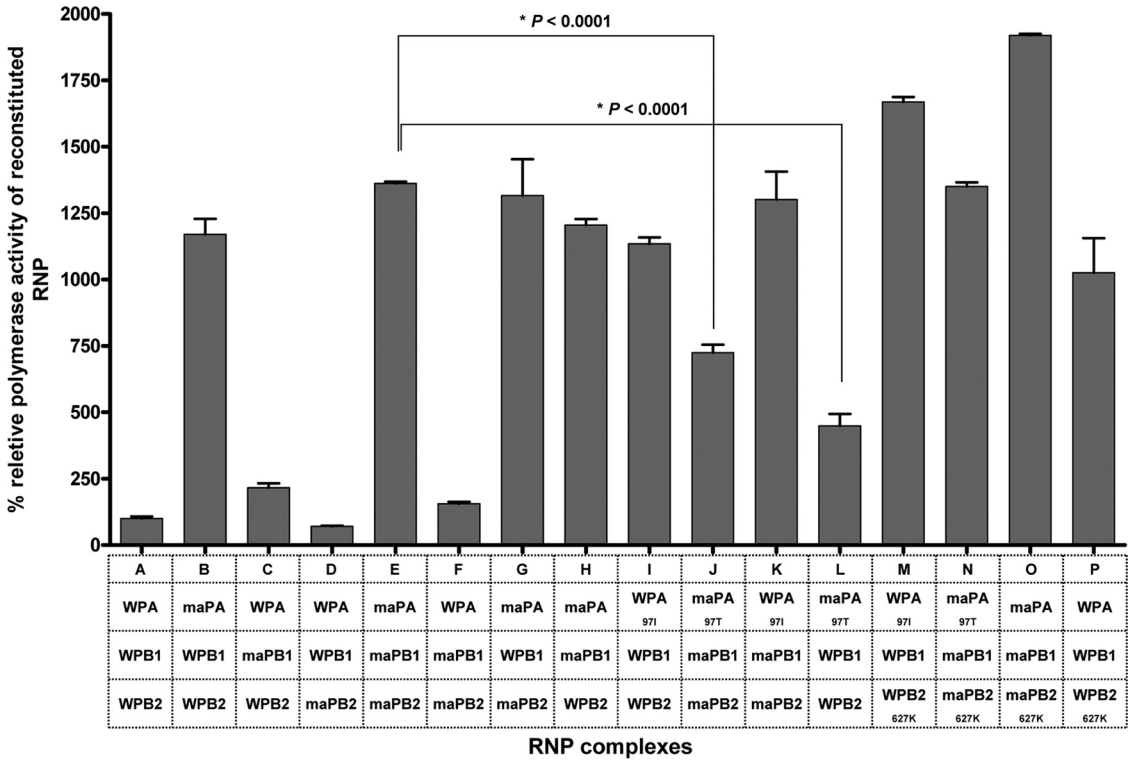

FIG. 6. Enhanced polymerase activity of ma81. Shown are polymerase activities of reconstituted RNP complexes composed of the PB2, PB1, PA, and NP plasmids of virus strains W81 and ma81 and the indicated combinations and single-point mutants. Virus strain ma81K is included for comparison. Note that NP is the same in all complexes, as no mutation was observed in this gene. Luciferase activity values are the means of at least three assays. The asterisk indicates a P value of <0.0001 compared to the polymerase activity of the reconstituted RNP of the homogeneous ma81 complex (Student's t test).

changed for that of W81, and this altered phenotype was largely attributable to the single-residue mutation at PA position 97. A single substitution of the ma81 genes for those of W81 revealed that only maPA substantially increased virulence. However, the considerably lower virulence of the single-gene Rg viruses than that of ma81 indicates that these genes, together with the identified mutations (i.e., a specific gene constellation) are required in combination for a highly virulent phenotype in mice. PA_{97I} alone does not significantly increase virulence when introduced into W81. However, PA_{97I} is essential for ma81 virulence, as substitution with PA_{97T} renders the virus low virulent within the context of the remaining gene mutations. Therefore, PA_{97I} is indispensable for adaptation and acquisition of the virulent phenotype in mice. We did not test the effect of individual mutations in the other segments, and we cannot rule out their possible independent contribution to virulence. Indeed, maNA appears to add to the virulent effect of maPA but not to its replication efficiency. Among the mutations noted in NA, Tyr at position 316 appears to contribute to enhanced virulence in the context of PA_{97I} (Fig. 5 and Table 2).

Of the four mutations observed in PA, only the T97I mutation was sufficient to alter virulence and enhance polymerase activity. Among the polymerase genes, mutations in PB2 have been well characterized for host range restriction, virulence, and polymerase activity (7, 23, 26, 46). Subbarao et al. (46) reported that the E627K amino acid mutation of PB2 is a determinant of host range and enhances virus transcription through efficient polymerase activity (7, 26). Li et al. (23) further contended that residue 701 of PB2 may be associated with interspecies transmissibility of H5N1 influenza viruses from their natural host reservoirs (ducks) to mammals (mice). Our study also discovered a virus bearing the E627K PB2 mutation (designated ma81K) upon plaque purification; this mutation was the only dissimilarity to the ma81 virus. It is not clear whether this mutation preceded or followed the T97I substitution in PA. Nevertheless, the presence of Lys at position 627 of PB2 clearly resulted from adaptation to the mammalian host, as it was not observed in the wild-type avirulent virus. Our results demonstrate that the acquisition of a T97I substitution in PA enabled an avian influenza virus to replicate well, to become virulent, and to cause death in a mammalian model. Like the $PB2_{627K}$ mutation, this mutation appears to be a host-dependent adaptation.

Isoleucine is not commonly found at position 97 in the PA of avian influenza viruses. Sequence comparison with published data, including that in GenBank, showed that the 97T mutation is highly conserved, as it is in strain W81 (Table 3). Most of the strains that bear the T97I characteristic mutation have undergone passages or adaptation in mice, although a few are field isolates (avian, swine, and equine) (22, 25, 35). Therefore, the PA_{97I} mutation is likely to have resulted from adaptation to a mammalian host and could be a determinant of efficient replication and virulence in mice. Lipatov et al. (25) and

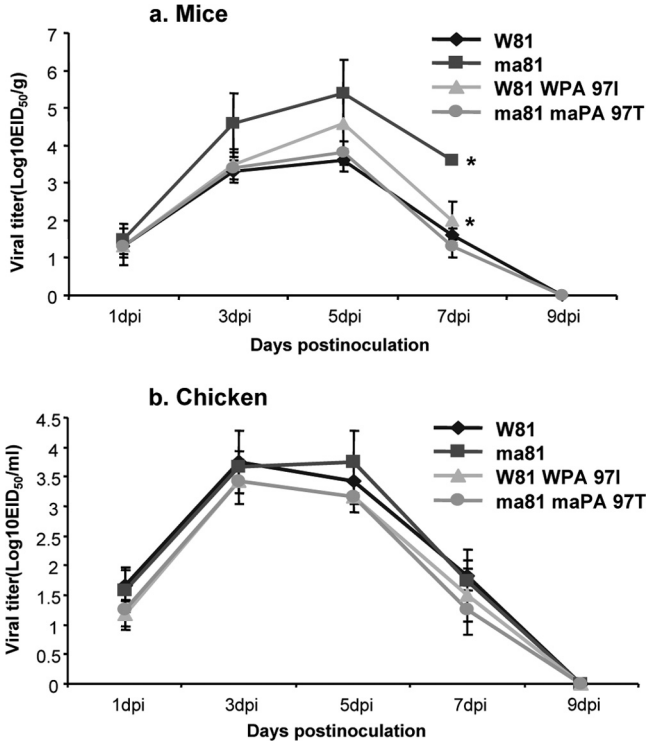

FIG. 7. Virus growth in mammalian and avian species. Growth of W81, ma81, W81 WPA_{97I}, and ma81-$maPA_{97T}$ viruses in mice (a) and chickens (b). Four groups of 15 mice were inoculated i.n. with 5.5 $\log_{10}$ $TCID_{50}$ of virus. Lung virus titers on days 1, 3, 5, 7, and 9 p.i. are expressed as $\log_{10}$ EID_{50} per gram of tissue. Four groups of five 4-week-old chickens were inoculated i.n. and intratracheally with 5.5 $\log_{10}$ $TCID_{50}$ of parental or recombinant virus. Tracheal swabs were collected on days 1, 3, 5, 7, and 9 p.i. for virus titration.

Rigoni et al. (35) recovered viruses that bore the PA_{97I} mutation after experimental infection of mice with HPAI H5N1 and H7N1 viruses, respectively (25, 35). However, the original viruses used in these studies were highly pathogenic viruses isolated during outbreaks among avian species in Hong Kong (25) or in Italy (35), without comparison studies done. Thus, there is insufficient experimental data on the role of this mutation in relation to high levels of virulence in mice. In preliminary studies, we serially passaged another commonly isolated LPAI H7 virus (A/Aquatic bird/Korea/W44/05; H7N3) in mouse lungs. Interestingly, this adaptation also resulted in a T97I substitution in the PA of the virus, and preliminary data indicate that the virulence of the virus is altered (data not shown). Therefore, this adaptive mutation could be a general virulence factor in mice and may not be limited to the ma81 virus or to its combination of genes.

The PA gene is the third subunit of the polymerase complex; its precise function is not well defined. It is proposed to be involved in replication rather than transcription (14, 20, 29), but there is evidence that it plays a role in both (6, 32, 34). Perhaps the best-characterized function of PA is its association with protease activity at the amino-terminal portion of the protein (38). Hara et al. (10) recently reported that the N-terminal region of PA is multifunctional, playing important roles in initiating both transcription and replication through different mechanisms. The PA_{97I} is located in the N-terminal portion of the protein, within the 92 to 117 residues in which amino acids important for polymerase activity are suggested to be clustered (10). The functional role of the PA_{97I} mutation is not fully understood, but its association with the significant increase we observed in polymerase activity, and its likely correspondence to the considerable growth advantage in mammalian cells and replicative fitness in mice, suggest that it plays a role in the adaptation of influenza viruses to a new host. Additional information and studies are needed to determine the characteristic role of the PA_{97I} mutation on polymerase activity of the virus, whether it directly affects replication, transcription, or both. Assays on avian cells using avian promoters will also provide more information on the notable differences in virus replication and polymerase activity between the wild-type and mouse-adapted viruses. Nevertheless, our findings indirectly suggest that this amino acid change may be crucial for the modulation of polymerase activity, which was not affected by the Lys at position 627 of PB2 (Fig. 6). It is not known how the PA_{97I} mutation arises, but it is possible that it results from interaction between the different subunits of the influenza virus polymerase complex and various host cell proteins. The PA subunit is known to interact with hCLE, a cellular transcrip-

TABLE 3. Web search for similar base residue at position 97 of PA in other influenza viruses isolated from various animal species[a]

Virus strain	Accession no.[b]	PA aa at position 97	Origin	Source or reference
A/Hong Kong/1/1968 (H3N2)	**AF348174**	**T**	**WT**	**NIAID Influenza Genome Sequencing Project**
A/Hong Kong/1-8-MA21-2/1968 (H3N2)	CY033142	I	MA	NIAID Influenza Genome Sequencing Project
A/Hong Kong/1-11-MA21-2/1968 (H3N2)	CY033550	I	MA	NIAID Influenza Genome Sequencing Project
A/Hong Kong/1-8-MA21-1/1968 (H3N2)	CY034025	I	MA	NIAID Influenza Genome Sequencing Project
A/Hong Kong/1-8-MA21-3/1968 (H3N2)	CY034033	I	MA	NIAID Influenza Genome Sequencing Project
A/swine/Ohio/23/1935 (H1N1)	CY027296	I	WT	NIAID Influenza Genome Sequencing Project
A/ostrich/Italy/984/00 (H7N1)	DQ991342	I	MA	35
A/mallard/Maryland/789/2002 (H5N2)	CY016616	I	WT	NIAID Influenza Genome Sequencing Project
A/mallard/MD/790/2002 (H5N2)	EU980484	I	WT	NIAID Influenza Genome Sequencing Project
A/chicken/Taiwan/G2/1987 (H6N1)	DQ376799	I	WT	22
A/chicken/Hong Kong/FY150/2001 (H5N1)	**AF509198**	**T**	**WT**	**25**
A/chicken/HongKong/FY150/2001-MB (H5N1)	AY221568	I	MA	25
A/equine/Italy/1062/1991 (H3N8)	CY032378	I	WT	NIAID Influenza Genome Sequencing Project

[a] Boldface type indicates the original isolates from which the mouse-adapted strains were derived. WT, wild type; MA, mouse-adapted; aa, amino acid.
[b] Based on the entry in the NIAID Influenza Genome Sequencing Project.

tional activator-protein homologue that may modulate the synthesis of viral RNAs (16). The adaptive mutation could also have been induced by one or more of the many recently identified influenza virus polymerase-interacting proteins associated with transcription, modification, and translocation (17).

Adaptation is considered to drive evolution by conferring mutations that enhance fitness and natural selection (3). The replacement of the nonvirulent parental virus population by the virulence-adapted variants was defined by increased replication rates (shown in vitro and in vivo) and enhanced polymerase activity that was largely attributable to a distinct mutation in PA. To our knowledge, the residue at position 97 in PA has not previously been characterized or implicated in the evolution of avian influenza viruses. These results add to the growing body of mutational analysis data defining the function of the PA subunit and its role in mammalian host adaptation. They also suggest that changes optimizing the interaction of viral polymerase components with host cellular factors are crucial for adaptation, leading to enhanced replication and transcription and, ultimately, host range determination. Further studies of PA97 are needed to resolve these questions.

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