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Adaptive Mutations Resulting in Enhanced Polymerase Activity Contribute to High Virulence of Influenza A Virus in Mice[▽]

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High virulence of influenza virus A/Puerto Rico/8/34 in mice carrying the Mx1 resistance gene was recently shown to be determined by the viral surface proteins and the viral polymerase. Here, we demonstrated high-level polymerase activity in mammalian host cells but not avian host cells and investigated which mutations in the polymerase subunits PB1, PB2, and PA are critical for increased polymerase activity and high virus virulence. Mutational analyses demonstrated that an isoleucine-to-valine change at position 504 in PB2 was the most critical and strongly enhanced the activity of the reconstituted polymerase complex. An isoleucine-to-leucine change at position 550 in PA further contributed to increased polymerase activity and high virulence, whereas all other mutations in PB1, PB2, and PA were irrelevant. To determine whether this pattern of acquired mutations represents a preferred viral strategy to gain virulence, two independent new virus adaptation experiments were performed. Surprisingly, the conservative I504V change in PB2 evolved again and was the only mutation present in an aggressive virus variant selected during the first adaptation experiment. In contrast, the virulent virus selected in the second adaptation experiment had a lysine-to-arginine change at position 208 in PB1 and a glutamate-to-glycine change at position 349 in PA. These results demonstrate that a variety of minor amino acid changes in the viral polymerase can contribute to enhanced virulence of influenza A virus. Interestingly, all virulence-enhancing mutations that we identified in this study resulted in substantially increased viral polymerase activity.

Influenza virus infections continue to represent a major public health threat. Epidemics caused by influenza A viruses (FLUAV) occur regularly, often leading to excess mortality in susceptible populations, and may result in devastating pandemics for humans (37). An avian FLUAV originating from Asia and currently circulating among domestic birds in many countries has the potential to infect and kill people. If further adaptation to humans occurs, this virus strain might become the origin of a future pandemic (57). Although influenza viruses are well characterized, the molecular determinants governing cross-species adaptation and enhanced virulence of emerging virus strains in humans are presently not well understood. The known viral virulence factors are the envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA), the nonstructural proteins NS1 and PB1-F2, and the polymerase complex. HA and NA are of key importance for host specificity and virulence because they determine specific receptor usage and efficient cell entry, as well as formation and release of progeny virus particles. NS1 is a multifunctional protein with interferon-antagonistic activity able to suppress host innate immune responses (11, 15). The small proapoptotic protein PB1-F2 induces more-severe pulmonary immunopathology and increases susceptibility to secondary bacterial pneumonia (3, 30). Recent evidence indicates that the polymerase complex consisting of the three subunits PA, PB1, and PB2 is also a determinant of virulence. Analyses of the 1918 pandemic virus showed that PB1 contributed to the high virulence of this deadly strain (38, 54, 56). Likewise, PB1 also contributed to the unusually high virulence of the pandemic viruses of 1957 and 1968 (23, 47). Interestingly, in recent avian-to-human transmissions of H5N1 and H7N7 viruses, the PB2 subunit was found to play a critical role (32, 40). Molecular studies revealed that an E-to-K exchange at position 627 of PB2 facilitates efficient replication of avian viruses in human cells (24, 33) and determines pathogenicity in mammals (18, 32, 51). Furthermore, recent analyses of highly pathogenic H5N1 viruses demonstrated that PA is involved in high virulence of these avian strains for both avian and mammalian hosts (21, 27).

Moderately pathogenic FLUAV strains can be rendered more pathogenic by repeated passages in experimentally infected animals (2, 13, 16, 49, 55). During such adaptations, the evolving viruses frequently seem to acquire virulence-enhancing mutations in the polymerase genes. We recently characterized a virus pair with strikingly different virulences in mice and showed that the virulence-enhancing mutations of the highly virulent strain mapped to the HA, NA, and polymerase genes (13). The two A/Puerto Rico/8/34 (A/PR/8/34) strains are referred to here as high-virulence A/PR/8/34 (hvPR8) and low-virulence A/PR/8/34 (lvPR8). Interestingly, hvPR8 is also highly virulent in mice that carry functional alleles of the Mx1 resistance gene (17), most likely because it replicates rapidly enough to evade the innate immune response of naïve hosts (13).

Here, we systematically analyzed which mutations in the three viral polymerase genes contribute to enhanced virulence of hvPR8. We found that two conservative mutations, one in

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PB2 (I504V) and one in PA (I550L), account for the high-virulence phenotype and that each single mutation considerably increases the activity of the reconstituted polymerase complex. Interestingly, in a new mouse adaptation experiment, the same I504V mutation in PB2 was acquired again by a highly virulent isolate as the only change in the polymerase complex. In contrast, another virulent, mouse-adapted isolate acquired two different mutations in PA and PB1. In this case, the change in PA had a greater impact on both enhanced polymerase activity and enhanced virulence than the mutation in PB1. These data demonstrate that increased polymerase activity contributes to high virus virulence and that human FLUAV have a range of options to achieve this goal.

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MATERIALS AND METHODS

Viruses. hvPR8 and lvPR8 strains were described previously (13). Reassortant viruses were generated by reverse genetics as described previously (13). Virus stocks were produced in 9-day-old embryonated chicken eggs. Virus titers were determined by infecting MDCK cells with 10-fold serial dilutions of virus. Fluorescent cell foci detected after staining with a rabbit antiserum specific for NP were counted. Virus titers are expressed as focus-forming units (FFU).

Generation of recombinant viruses. Mutations were introduced into the cDNA by PCR. Resulting cDNA was cloned into the ambisense expression vector pDZ (41), which contains a human RNA polymerase I promoter and a chicken β-actin promoter. For the rescue of recombinant viruses, a mixture of eight pDZ plasmids (0.5 μg each) was transfected into cocultures of 293T and MDCK cells by using Lipofectamine 2000 (Invitrogen). Recombinant viruses in the supernatant of transfected cells were plaque purified on MDCK cells. Virus stocks were prepared in embryonated chicken eggs and stored at −80°C.

Mice. Standard C57BL/6 mice with defective *Mx1* alleles were purchased from Harlan, The Netherlands. Congenic B6.A2G-*Mx1* mice (50) carrying intact *Mx1* alleles on the C57BL/6 background ($Mx1^{+/+}$) were bred locally. Six- to eight-week-old animals were used for the challenge experiments which were performed in accordance with the local Animal Care Committee and the Regierungspraesidium, Freiburg, Germany. For infection, animals were anesthetized by intraperitoneal injection of a mixture of ketamine (100 μg per gram of body weight) and xylazine (5 μg per gram of body weight) and infected intranasally with the indicated doses of virus in 50 μl of phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin. Animals were euthanized if severe symptoms developed or if weight loss approached 30%. Fifty percent lethal doses (LD_{50}) were calculated as described previously (42).

Virus adaptation experiments. Serial virus passages were performed by intranasal infection of B6.A2G-*Mx1* mice with 1,000 FFU of PR, a reassortant virus carrying segments 1, 2, and 3 of lvPR8 and the other five segments from hvPR8. After 24 h, lungs were removed and virus titers were determined. For the following passages, samples of lung homogenates containing 1,000 FFU of virus were used for intranasal infections.

Stocks of mouse-adapted viruses were propagated in embryonated eggs. To determine the nucleotide sequences of the passaged viruses, reverse transcription was performed using random hexamer primers and murine leukemia virus reverse transcriptase. Suitable cDNA fragments were amplified with *Pfx* DNA polymerase (Invitrogen) by using primers corresponding to the 3′ and 5′ noncoding regions of the A/PR/8/34 segments, and the products were sequenced.

Determining virus titers in lungs. Lung homogenates were prepared by grinding the tissue using a mortar and sterile quartz sand. Homogenates were suspended in 1 ml PBS, and tissue debris was removed by low-speed centrifugation. Virus titers in supernatants were determined on MDCK cells by serial tenfold dilutions in PBS containing 0.3% bovine serum albumin and staining with specific antibodies as described above.

Minireplicon assay. Murine L929, human A549, chicken LMH, and quail QM-7 cells were transfected using Nanofectin (PAA Laboratories, Pasching, Austria) with pDZ expression plasmids for segments 1, 2, 3, and 5, encoding PB2, PB1, PA (0.25 μg each), and NP (0.5 μg), respectively, and with pPOLI-FFLuc-RT (0.1 μg) encoding firefly luciferase in the negative-sense orientation under the control of either the noncoding regions of FLUAV segment 8. In this construct, expression of the viral minigenome is under the control of either the murine, human, or chicken RNA polymerase I promoter and terminator (5, 28, 36). In addition, the cells were transfected with an expression plasmid (0.01 μg) encoding renilla luciferase under the control of the simian virus 40 promoter (pRL-SV40). At 24 h after transfection, the cells were harvested and luciferase activities were determined using a dual-luciferase assay system (Promega). Firefly luciferase activity was normalized to the renilla luciferase activity.

Nucleotide sequence accession numbers. GenBank accession numbers for hvPR8 segments are EF190971 to EF190978, and those for lvPR8 segments are EF190979 to EF190986.

RESULTS

Polymerase subunits PB2 and PA determine the phenotype of hvPR8. Previous studies suggested an enhanced polymerase activity of hvPR8 that contributes to enhanced virulence (13). As hvPR8 was generated by multiple passages in the lungs of laboratory mice, we first analyzed whether this elevated activity is an exclusive feature of the viral polymerase expressed in murine cells. For this, we employed a minireplicon system which allows quantification of the activity of reconstituted viral polymerase complexes of hvPR8 compared to that of a low-virulence variant, lvPR8. In this system, mouse L929 cells, human A549 cells, and two avian cell lines, LMH and QM-7, were transfected with expression constructs that code for the three polymerase subunits and NP and with an influenza virus-like minigenome carrying the firefly luciferase gene. Twenty-four hours later, reporter gene expression in cell lysates was determined. Reconstituted polymerase complexes containing all three subunits of hvPR8 were about 8- to 11-fold more active in L929 than complexes made of subunits derived from lvPR8 (Fig. 1A). The activity of the complexes was not influenced substantially by the origin of NP (data not shown). Enhanced polymerase activity of hvPR8 was also found in transfected A549 cells (Fig. 1A). However, in avian cells, the activities of the polymerase complexes of hvPR8 and lvPR8 did not differ significantly (Fig. 1A), indicating that the enhanced activity of hvPR8 is a mammalian cell type-specific effect.

To determine the contributions of the individual polymerase subunits to the high-level activity, a systematic analysis of all possible combinations of the polymerase subunits was performed with L929 cells. The minireplicon system revealed that the reconstituted polymerase complexes were highly active if they contained the PB2 subunit of hvPR8 (Fig. 1B). In this assay, the PA subunit of hvPR8 also enhanced the activity of the viral polymerase complex, although its contribution was less strong than that of PB2 (Fig. 1B). These data indicate that PB2 contributed most to the enhanced polymerase activity of hvPR8.

To define the importance of the various polymerase subunits for the high virulence in $Mx1^{+/+}$ mice, we generated a series of different reassortant viruses in which segments 4 and 6 (coding for HA and NA, respectively) were always derived from hvPR8 while segments 1, 2, and 3 (coding for the polymerase subunits) were derived from either hvPR8 or lvPR8 (Table 1). This particular experimental strategy was chosen because we knew from previous work that the virulence-enhancing effects of polymerase mutations can be observed only in the presence of the HA and NA genes of hvPR8, which mediate efficient delivery of the viral genome into the cytoplasm of cells (13). All

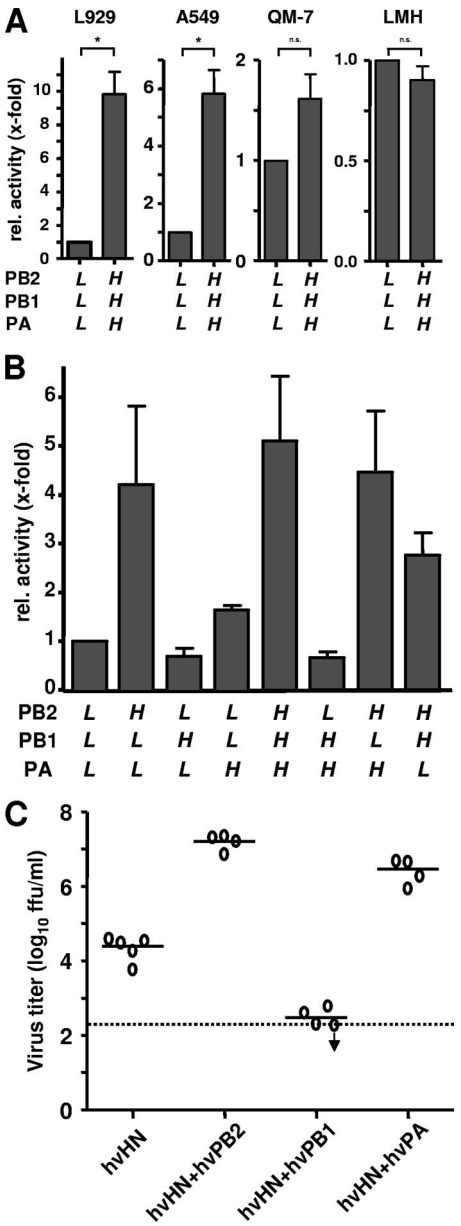

FIG. 1. Contribution of polymerase subunits to high virulence of hvPR8. rel., relative. (A) Murine L929, human A549, quail QM-7, or chicken LMH cells were transfected with a firefly luciferase-encoding viral minigenome construct and expression constructs encoding PB2, PB1, and PA of either lvPR8 (*L*) or hvPR8 (*H*) as indicated. Transfection mixtures further contained an expression plasmid encoding NP of lvPR8 and plasmid pSV40-RL encoding renilla luciferase. Cells were lysed at 24 h posttransfection, and firefly and renilla luciferase activities were determined. For the calculation of the relative activities of the reconstituted viral polymerase complexes, firefly luciferase activities were normalized to renilla luciferase activities. The value obtained with the lvPR8 plasmid set was set to 1. Mean values from three independent experiments are shown. *, $P < 0.0001$; n.s., not significant. (B) PB2 contributes the most to enhanced polymerase activity. L929 cells were transfected with all combinations of the reassortant polymerase complex constructs and analyzed as described above. The activities of polymerase complexes containing PB2 or PA of hvPR8 were significantly increased ($P < 0.02$) compared to that of the complex of lvPR8. (C) C57BL/6 mice were infected intranasally with 1,000 FFU of the indicated reassortant viruses, and viral titers in lung homogenates were determined at 20 h postinfection. hvHN contains segment exchanges were tolerated well, and the various recombinant viruses grew to similar titers in 9-day-old embryonated chicken eggs (data not shown). As expected, the viruses differed markedly in virulence when used to infect *Mx1*$^{+/+}$ mice (Table 1). The LD_{50} of hvHN, a virus that contains segments 4 and 6 of hvPR8 and the other six segments of lvPR8, was 2×10^4 FFU. The LD_{50} of hvHN+hvPPP, a virus that contains the three polymerase subunits as well as segments 4 and 6 of hvPR8, was below 100 FFU (Table 1). If only the PB2 polymerase subunit of hvPR8 (gene segment 1) was introduced into hvHN (hvHN+hvPB2), the LD_{50} similarly decreased below 100 FFU. If only the PA subunit (gene segment 3) was exchanged (hvHN+hvPA), the LD_{50} also decreased, although less strongly. By contrast, no virulence-enhancing effect was observed if the PB1 subunit (gene segment 2) of lvPR8 was exchanged with that of hvPR8 (hvHN+hvPB1). In fact, the LD_{50} of hvHN+hvPB1 was even higher than that of hvHN (Table 1). The LD_{50}s in *Mx1*$^{+/+}$ mice inversely correlated with the replication capacity of the various viruses in the lungs of C57BL/6 mice. Within 20 h of infection, viruses carrying gene segments encoding PB2 and PA of hvPR8 grew to titers that were significantly higher than that of the parental hvHN virus (Fig. 1C). As expected from the LD_{50} study, we found that hvHN+hvPB1 grew to only low titers in mouse lungs. Taken together, the data for the recombinant viruses, which correlate with the results of the minireplicon analyses, demonstrate that PB2 and to a lesser extent PA contribute to enhanced virulence of hvPR8.

Residues in PB2 and PA that confer virulence to hvPR8. The PB2 polymerase subunit of hvPR8 differs from its counterpart in lvPR8 at only three amino acid positions. The changes in PB2 of hvPR8 are I105M, N456D, and I504V (13). To determine their relative levels of importance, we mutated the critical amino acids individually and used appropriate expression constructs to reconstitute viral polymerase complexes in transfected L929 cells. Using the viral minireplicon system, we observed that viral polymerase complexes with the I504V exchange in PB2 almost reached the activity level of polymerase complexes in which the complete PB2 subunit was derived from hvPR8 (Fig. 2A). No increased activity of reconstituted polymerase complexes was observed if the I105M or N456D change was introduced into PB2 of lvPR8 (Fig. 2A).

To determine which of the two mutations in PA might contribute most strongly to the virulence of hvPR8, we introduced the Q193H and the I550L mutations (13) individually into an expression construct coding for PA of lvPR8. Reconstituted polymerase complexes containing the PA subunit with the I550L mutation were about twofold more active than complexes without this change (Fig. 2A). By contrast, the Q193H

segments 4 and 6 of hvPR8 and all other segments of lvPR8. hvHN+hvPB2, hvHN+hvPB1, and hvHN+hvPA carry segment 1, 2, and 3, respectively, of hvPR8 in addition to segments 4 and 6. Each open circle represents the data derived from one animal. Mean lung virus titers of each group are indicated by a horizontal bar in the figure. The titers differed significantly from that of the hvHN control virus ($P < 0.05$). The dotted line indicates the detection limit of 200 FFU per ml of lung extract, and the arrow indicates a titer below the detection limit.

TABLE 1. Virulence of reassortant PR8 viruses in *Mx1*$^{+/+}$ mice

Virus[a]	Gene type or mutation at indicated segment[b]								LD_{50}[c] (FFU)
	1	2	3	4	5	6	7	8	
hvHN	L	L	L	H	L	H	L	L	2×10^4
hvHN+hvPPP	H	H	H	H	L	H	L	L	$<10^2$
hvHN +hvPB2	H	L	L	H	L	H	L	L	$<10^2$
hvHN+hvPB1	L	H	L	H	L	H	L	L	2.5×10^5
hvHN+hvPA	L	L	H	H	L	H	L	L	5×10^2
hvHN+lvPB2(I504V)	I504V	L	L	H	L	H	L	L	10^2
hvHN+lvPA(I550L)	L	L	I550L	H	L	H	L	L	6×10^2

[a] The reference virus hvHN contains gene segments 4 and 6 of hvPR8 and the six remaining segments of lvPR8.
[b] H, hvPR8 type; L, lvPR8 type.
[c] LD_{50}s were determined by infecting groups of *Mx1*$^{+/+}$ mice with various doses of the indicated viruses. Animals were killed if they were severely ill or if weight loss approached 30%.

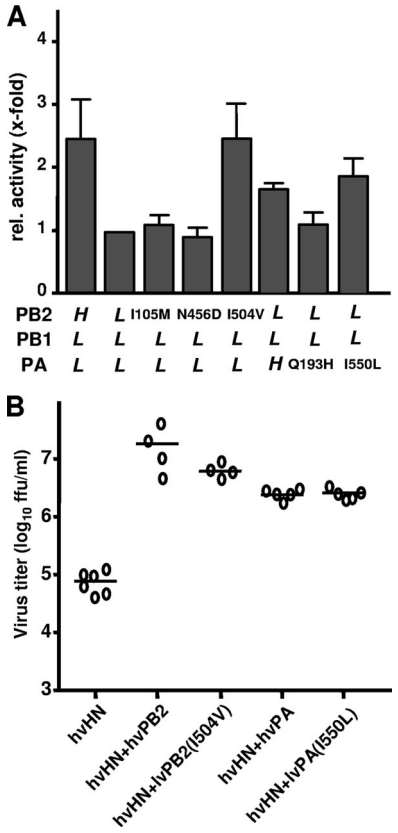

FIG. 2. Identification of residues in PB2 and PA which determine virulence of hvPR8. (A) The activities of reconstituted polymerase complexes consisting of unmodified subunits from either lvPR8 (*L*) or hvPR8 (*H*) were compared to the activities of complexes consisting of lvPR8-derived subunits with the indicated single amino acid substitutions. Relative (rel.) polymerase activities were determined using the minireplicon assay used for the experiment whose data are represented in Fig. 1. Mean values of three independent experiments are shown. Statistical analysis of the activity of PB2(I504V) and PA(I550L) showed *P* values of 0.0597 and 0.0392, respectively, in comparisons to the activity of the lvPR8 polymerase. (B) Virus titers in lung homogenates of C57BL/6 mice 20 h after intranasal infection with 1,000 FFU of reassortant viruses with the indicated single amino acid substitutions in either PB2 or PA of lvRP8. Each open circle represents the data derived from one animal. Mean lung virus titers of each group are indicated by a horizontal bar in the figure. The titers differed significantly from that of the hvHN control virus ($P < 0.02$).

mutation in PA did not boost viral polymerase activity to a significant extent (Fig. 2A).

Recombinant viruses with individual changes in either PB2 or PA were generated to assess the contributions of these mutations in the context of infections. Infection studies with C57BL/6 mice showed that viral lung titers at 20 h postinfection were increased nearly 100-fold if the I504V mutation was introduced into the PB2 subunit of lvPR8 (Fig. 2B). A slightly less dramatic increase in viral lung titers was observed if the I550L mutation was introduced into the PA subunit of lvPR8 (Fig. 2B). Interestingly, the viruses with these single mutations grew almost as well as the reassortants that carried the unmodified PB2 or PA segment of hvPR8. Our virus growth studies with lungs of C57BL/6 mice agreed well with the results from survival studies with *Mx1*$^{+/+}$ mice (Table 1). We found that the I504V mutation in PB2 and the I550L mutation in PA rendered the viruses as virulent in *Mx1*$^{+/+}$ mice as those which carried the unmodified PB2 of hvPR8 and those which carried the unmodified PA of hvPR8, respectively. Thus, enhanced polymerase activity which contributes to the high-virulence phenotype of hvPR8 mainly results from the I504V mutation in PB2 and the I550L mutation in PA.

Generation and analysis of new viruses with enhanced virulence. We next wished to determine whether the same or different mutations in the viral polymerase genes would appear if we repeated the adaptation experiment with lvPR8 in *Mx1*$^{+/+}$ mice. To ensure that adaptive mutations occurred mainly in the polymerase genes, we used a reassortant virus (designated PR) that carried segments 1 to 3 of lvPR8 and segments 4 to 8 of hvPR8. PR is only moderately virulent in *Mx1*$^{+/+}$ mice, with an LD_{50} of approximately 1×10^4 FFU (Table 2). The rationale for choosing this experimental strategy was that we assumed that minor changes in the polymerase genes acquired during the mouse passages might convert this moderately virulent virus into a highly pathogenic virus. We performed two independent series of rapid passages of PR in lungs of *Mx1*$^{+/+}$ mice, which yielded two new mouse-adapted virus variants designated PR-ma1 and PR-ma2, which replicated to at least 10-fold enhanced titers in mouse lungs (Fig. 3B and data not shown). LD_{50} studies with *Mx1*$^{+/+}$ mice demonstrated that PR-ma1 and PR-ma2 also exhibited substantially enhanced virulence (Table 2).

Sequencing of the complete coding regions of PR-ma1 revealed a single nucleotide change in segment 1, corresponding

TABLE 2. Virulence of virus variants PR-ma1 and PR-ma2 in *Mx1*$^{+/+}$ mice

Virus[a]	Gene type or mutation at indicated segment[b]								LD_{50}[c] (FFU)
	1	2	3	4	5	6	7	8	
PR	L	L	L	H	H	H	H	H	1×10^4
PR-ma1[d]	L	L	L	H	H	H	H	H	$<10^2$
PR-ma2[e]	L	L	L	H	H	H	H	H	3×10^2
Virus with indicated mutation(s)									
PB1(K208R)	L	K208R	L	H	H	H	H	H	3×10^3
PA(E349G)	L	L	E349G	H	H	H	H	H	5×10^2
PB1(K208R)/PA(E349G)	L	K208R	E349G	H	H	H	H	H	3×10^2

[a] The reference virus PR contains gene segments 1, 2, and 3 of lvPR8 and the five remaining segments of hvPR8.
[b] H, hvPR8 type; L, lvPR8 type.
[c] LD_{50}s were determined by infecting groups of *Mx1*$^{+/+}$ mice with various doses of the indicated viruses. Animals were killed if they were severely ill or if weight loss approached 30%.
[d] Bulk sequencing of all coding regions revealed that a single amino acid change, namely I504V in PB2, was acquired during mouse adaptation.
[e] Bulk sequencing of all coding regions revealed that two amino acid changes, namely K208R in PB1 and E349G in PA, were acquired during mouse adaptation.

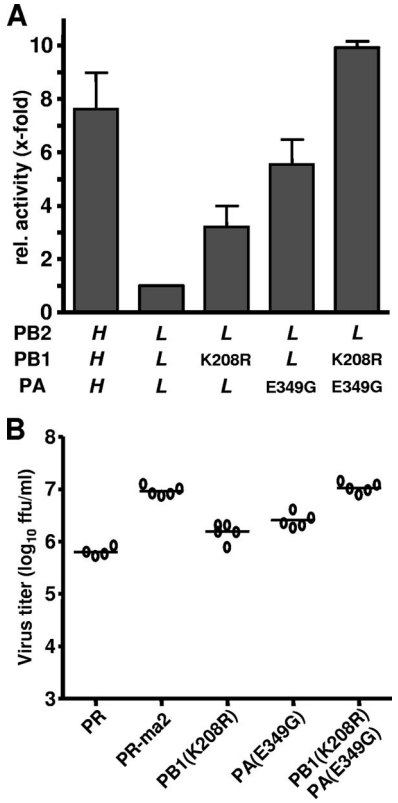

FIG. 3. PB1 and PA contribute to enhanced virulence of mutant PR-ma2. (A) The activities of reconstituted polymerase complexes consisting of unmodified subunits from either lvPR8 (*L*) or hvPR8 (*H*) were compared to the activities of complexes consisting of lvPR8-derived subunits carrying the indicated single amino acid substitutions. Relative polymerase activities were determined using the minireplicon assay used in the experiment whose data are represented in Fig. 1. Differences in the polymerase activities relative to the lvPR8 control were significant ($P < 0.05$). (B) Virus titers in lung homogenates of C57BL/6 mice 20 h after intranasal infection with 1,000 FFU of the indicated viruses. Parental virus PR, highly virulent mutant virus PR-ma2, and PR variants carrying the indicated amino acid substitutions in either PB1 or PA were compared. PR contains all segments of hvPR8 except for the three polymerase segments which originate from lvPR8. Each open circle represents the data derived from one animal. Mean lung virus titers of each group are indicated by a horizontal bar in the figure. The titers differed significantly from that of the PR control virus ($P < 0.01$).

to an amino acid change of isoleucine 504 of PB2 to valine. PR-ma1 thus acquired the very same mutation that also greatly contributes to virulence of hvPR8 (see above).

Sequencing of the complete coding regions of PR-ma2 revealed two nucleotide changes that result in an arginine-to-lysine change at position 208 of PB1 and a glutamic acid-to-glycine change at position 349 in PA. To determine the roles of these two amino acid changes in PR-ma2, we first employed the minireplicon system. The mutations were introduced into expression plasmids for lvPR8-derived PB1 and PA, and these constructs were combined with the other polymerase expression constructs. As shown in Fig. 3A, both of these changes increased the activity of the polymerase complex. If the PB1 and PA plasmids with the adaptive mutations were combined, the activity of the reconstituted polymerase complex was as high as that of a complex consisting exclusively of hvPR8-derived components.

To assess the importance of the K208R change in PB1 and of the E349G change in PA for virulence, recombinant viruses were generated on the genetic background of PR with corresponding mutations in segment 2, segment 3, or both. The individual mutations in PB1 and PA moderately increased titers of the virus in the lungs of C57BL/6 mice compared to the parental PR virus. When the two mutations were combined, the virus grew to titers similar to those of the original PR-ma2 isolate (Fig. 3B). Virulence studies with *Mx1*$^{+/+}$ mice yielded a similar picture (Table 2). The K208R change in PB1 enhanced virulence of PR only modestly. The E349G change in PA had a bigger effect. When the two adaptive mutations were combined, the virulence of the resulting virus reached the level of the original PR-ma2 isolate.

DISCUSSION

Here, we identified adaptive mutations which cause enhanced polymerase activity and promote high virulence of FLUAV in mice. Surprisingly, the acquired mutations consisted of minor changes involving conservative amino acid substitutions in one or two of the three polymerase subunits. The experimental system used was based on two new strategies. First, the adaptive gain in virus virulence was tested with *Mx1*$^{+/+}$ mice, which, in contrast to conventional inbred mouse strains, display the full array of innate immunity against influ-

enza virus and other orthomyxoviruses (for a recent review, see reference 17). Second, we used reassortant viruses which were derived from hvPR8 and its low-virulence counterpart (lvPR8) and contained the HA and NA genes of the hvPR8 strain. Previous work has shown that the virulence-enhancing effects of polymerase mutations can be assessed only in the presence of high-virulence virus HA and high-virulence virus NA, which allow efficient delivery of the viral genome into the cytoplasm of cells (13). The results clearly demonstrated that the PB2 and PA subunits were responsible for enhanced virulence, whereas PB1 and PB1-F2 were not.

As hvPR8 gained its increased virulence by multiple passages in mice, we first analyzed whether enhanced polymerase activity resulted from optimal adaptation to murine host factors. Interestingly, the hvPR8 polymerase complex showed high-level activity in mouse cells as well as human cells, but not in cells of avian origin, compared to the activity of the lvPR8 polymerase complex. This result supports the assumption that the hvPR8 polymerase complex is optimized for the use of mammalian cell factors.

The PB2 polymerase subunit of hvPR8 differs at three amino acid positions from the PB2 of lvPR8, while the PAs differ only at two positions (13). Mutational analyses revealed that an isoleucine-to-valine change at position 504 of PB2 and an isoleucine-to-leucine substitution at position 550 of PA were mainly responsible for the high-virulence phenotype. Intriguingly, the very same I504V mutation in PB2 evolved again in a highly virulent isolate (PR-ma1) during an independent mouse adaptation experiment, this time as the only change in the polymerase complex. The strong effect of the conservative amino acid substitution at position 504 of PB2 was unexpected and remains to be explained.

Position 504 is close to the first nuclear localization sequence (NLS) of PB2, which was mapped to positions 449 to 495 (31). The I504V mutation may optimize the interaction of PB2 with the nuclear import machinery and increase accumulation of PB2 in the nucleus, as recently discussed for position 701 in PB2 of a highly virulent mouse-adapted H7N7 virus (10). Furthermore, amino acid residues at position 504 could influence the function of the cap-binding pocket that was mapped to positions 533 to 577 (20, 26) or more recently to positions 318 to 483 by biochemical and structural analyses (7, 14). The recent structural analysis suggests a complex structure of the PB2 subunit composed of independently folded functional domains. Position 504 is located in a putative nonstructured, flexible linker region between the cap-binding region (318 to 483) (14) and the 627 domain (538 to 759) (52) and might therefore be involved in interactions with the other polymerase subunits or cellular partner molecules. Interestingly, a database search revealed that A/PR/8/34 viruses are exceptional in having isoleucine at position 504 (13, 46). Comparisons of PB2 sequences of a large number (n = 2,317) of mammalian and avian FLUAV isolates showed that most strains have a valine at this position (99.7%) and only a few possess an isoleucine (n = 5). Clearly, valine is favored over isoleucine in nature. Our adaptation experiments resulted in a mutation that reversed the rare amino acid of PR8 to the more common residue.

The change of isoleucine to leucine, another conservative substitution, at position 550 of PA (I550L) also contributed to enhanced virulence of hvPR8. Again, comparisons of PA sequences of avian and mammalian origin showed that most sequences had a leucine at this position (99.6%). Only 8 out of 2,190 viruses, including A/PR/8/34, have a PA with an isoleucine at position 550. As in the case of PB2, adaptation of lvPR8 resulted in a more wild-type-like PA sequence. The recently published structure of PA (19) locates position 550 at the end of the β6 strand that contributes to a complex structure of seven β-strands and three α-helices forming a putative RNA and nucleotide binding groove. Mutational analysis of this region suggested that this PA subdomain could be important for viral transcriptional activity and might cooperate with the rest of the polymerase complex in endonuclease activity (8). However, position 550 is not positioned to the predicted RNA binding groove and therefore may not be involved in direct contact to the putative ligands. An amino acid exchange in the same region of PA, Q556R, evolved during mouse adaptation of a human H3N2 virus, in addition to two other amino acid exchanges in other subunits of the viral polymerase complex (2).

Finally, rapid mouse lung passages of a reassortant virus expressing the polymerase complex of lvPR8 in an hvPR8 genetic background resulted in a new mouse-adapted virus variant (PR-ma2) with substantially enhanced virulence. Remarkably, this new virus had acquired two novel mutations in the polymerase subunits consisting of a lysine-to-arginine change at position 208 of PB1 (K208R) and a glutamic acid-to-glycine change at position 349 in PA (E349G). The two adaptive mutations complemented each other when introduced together into the polymerase complex of lvPR8 and reconstituted the full polymerase activity and virulence of the original PR-ma2 isolate. Sequence comparisons with other mammalian and avian FLUAV revealed that the two amino acid substitutions identified here are rather uncommon at these positions. Only 17 out of 2,033 PB1 sequences showed an arginine at position 208, whereas most had a lysine residue at this position (99.1%). Among 2,190 viruses analyzed, only two isolates with a glycine at position 349 in the PA subunit were found. In contrast, most sequences have a glutamic acid at this position (99.6%). At the present, position 349 in PA is not located in a region with an assigned function for polymerase activity. Mutations of glutamic acid residues at positions 351 and 352 of PA to alanines had no effect on the activity of the polymerase complex or infectivity of the recombinant viruses (43). Interestingly, however, a four-amino-acid exchange in the PA subunit was correlated with enhanced pathogenicity of an avian H5N1 virus isolated from ducks in China (27). One substitution was at position 355, suggesting a putative functional domain in this region of the molecule. The recently determined crystal structure of the C-terminal 55-kDa domain of PA shows that position 349 is located at the end of helix α2 and is, thus, far from the PB1 interaction site and the putative RNA binding groove (19). The α2-α3 loop, consisting of residues 349 to 363, sticks out of the compact structure of the C terminus of PA in a direction similar to that of the β3-β4 loop. Remodeling the PA subunit into the proposed structure of the polymerase complex determined by electron microscopy (53) localizes the β3-β4 loop to the inside of the trimeric complex (19), suggesting that the exchange of glutamic acid to glycine at position 349 might affect interactions with or functions of the

other polymerase subunits. For instance, PA has been demonstrated to be involved in assembly of the polymerase complex and to enhance the RNA binding activity of the PB1 subunit (22, 25).

The K208R adaptive mutation in PB1 resulted in an only modest enhancement of virulence. The PB1 subunit contains multiple active sites critical for RNA synthesis and interactions with PA and PB2 (1, 12, 39). Position 208 is located within the bipartite NLS motif (positions 186 to 216) of PB1 (35), which is translocated into the nucleus as a heterodimer together with PA (4). The significance of the lysine-to-arginine substitution within the NLS is presently not known. However, it is interesting to note that the PB1 of the hvPR8 strain also contains an arginine at position 208. A comparable amino acid exchange in PB1 has been described for position 207 of two H5N1 virus isolates derived from A/Vietnam/1203/04. In this case, the lysine-to-arginine exchange did not influence viral pathogenicity for ducks and mice (21).

In summary, the present findings suggest that unusually high-level polymerase activity may be a common feature of highly virulent viruses. Increased polymerase activity has also been implicated in the high-virulence phenotype of the 1918 virus (54) and avian viruses adapted to mammalian cells (9, 45, 48). Enhanced activity may be due to optimized collaboration between the three subunits of the viral polymerase complex or to improved usage of cellular factors, such as the nuclear import machinery (4, 10, 44), Hsp90 (34), the cellular RNA polymerase II (6), or others (29).

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