Tyr82 Amino Acid Mutation in PB1 Polymerase Induces an Influenza Virus Mutator Phenotype

Tadasuke Naito,^a Kazumasa Shirai,^b Kotaro Mori,^a Hidetaka Muratsu,^a Hiroshi Ushirogawa,^a Ryosuke L. Ohniwa,^{c,d} Kousuke Hanada,^b Mineki Saito^a

^aDepartment of Microbiology, Kawasaki Medical School, Okayama, Japan

^bDepartment of Bioscience and Bioinformatics, Kyusyu Institute of Technology, Fukuoka, Japan

^cDivision of Biomedical Science, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

^dCenter for Biotechnology, National Taiwan University, Taipei, Taiwan

ABSTRACT In various positive-sense single-stranded RNA viruses, a low-fidelity viral RNA-dependent RNA polymerase (RdRp) confers attenuated phenotypes by increasing the mutation frequency. We report a negative-sense single-stranded RNA virus RdRp mutant strain with a mutator phenotype. Based on structural data of RdRp, rational targeting of key residues, and screening of fidelity variants, we isolated a novel low-fidelity mutator strain of influenza virus that harbors a Tyr82-to-Cys (Y82C) single-amino-acid substitution in the PB1 polymerase subunit. The purified PB1-Y82C polymerase indeed showed an increased frequency of misincorporation compared with the wild-type PB1 in an *in vitro* biochemical assay. To further investigate the effects of position 82 on PB1 polymerase fidelity, we substituted various amino acids at this position. As a result, we isolated various novel mutators other than PB1-Y82C with higher mutation frequencies. The structural model of influenza virus polymerase complex suggested that the Tyr82 residue, which is located at the nucleoside triphosphate entrance tunnel, may influence a fidelity checkpoint. Interestingly, although the PB1-Y82C variant replicated with wild-type PB1-like kinetics in tissue culture, the 50% lethal dose of the PB1-Y82C mutant was 10 times lower than that of wild-type PB1 in embryonated chicken eggs. In conclusion, our data indicate that the Tyr82 residue of PB1 has a crucial role in regulating polymerase fidelity of influenza virus and is closely related to attenuated pathogenic phenotypes *in vivo*.

IMPORTANCE Influenza A virus rapidly acquires antigenic changes and antiviral drug resistance, which limit the effectiveness of vaccines and drug treatments, primarily owing to its high rate of evolution. Virus populations formed by quasispecies can contain resistance mutations even before a selective pressure is applied. To study the effects of the viral mutation spectrum and quasispecies, high- and low-fidelity variants have been isolated for several RNA viruses. Here, we report the discovery of a low-fidelity RdRp variant of influenza A virus that contains a substitution at Tyr82 in PB1. Viruses containing the PB1-Y82C substitution showed growth kinetics and viral RNA synthesis levels similar to those of the wild-type virus in cell culture; however, they had significantly attenuated phenotypes in a chicken egg infection experiment. These data demonstrated that decreased RdRp fidelity attenuates influenza A virus *in vivo*, which is a desirable feature for the development of safer live attenuated vaccine candidates.

KEYWORDS fidelity, mutator, RNA polymerases, influenza

RNA viruses exhibit higher mutation frequencies than do DNA viruses (1–3). The rate of accumulation of viable mutations in the genome is markedly higher for RNA than for DNA viruses because the polymerases of the vast majority of RNA viruses lack the

Citation Naito T, Shirai K, Mori K, Muratsu H, Ushirogawa H, Ohniwa RL, Hanada K, Saito M. 2019. Tyr82 amino acid mutation in PB1 polymerase induces an influenza virus mutator phenotype. *J Virol* 93:e00834-19. <https://doi.org/10.1128/JVI.00834-19>.

Editor Adolfo García-Sastre, Icahn School of Medicine at Mount Sinai

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Tadasuke Naito, tadanaito@med.kawasaki-m.ac.jp, or Mineki Saito, mineki@med.kawasaki-m.ac.jp.

Received 16 May 2019

Accepted 19 August 2019

Accepted manuscript posted online 28 August 2019

Published 29 October 2019

mechanisms of proofreading and mismatch repair that are available for high-fidelity DNA replication. The mutation rate during viral RNA genome replication is generally in the range of 10^{-4} to 10^{-5} substitutions per nucleotide copies, implying that 0.1 to 1 misincorporation occurs per round of genome replication (4, 5). The high error rate of viral RNA-dependent RNA polymerases (RdRps) leads to a naturally maintained level of genetic variation within a population, referred to as a “mutant spectrum” or “quasi-species” (6). The high genetic diversity of RNA viruses provides them with the capacities of immune escape, acquisition of drug resistance, and infection of new host species (7–11). The mutation rate is a critical parameter for viral evolution, and the error threshold inherent to each virus might determine optimal proliferation. A study by Holland et al. revealed that the mutation rate of a lytic RNA virus could be increased by only about 2.5-fold by chemical mutagens (12). To understand the biological significance of viral diversity fluctuations in pathogenesis, numerous studies have used fidelity variants, in which an amino acid substitution is introduced in the viral RNA polymerase to alter fidelity (13, 14). These variants have either a low (high-fidelity viruses) or a high (mutator viruses) mutation rate compared to that of the wild-type virus.

The first high-fidelity variant of an RNA virus was isolated by serially passaging poliovirus in the presence of ribavirin to select for resistant variants (15). A poliovirus variant encoding mutated high-fidelity RdRp (3D^{pol}) had reduced genetic diversity and a restricted mutant spectrum and was impaired in its ability to invade the central nervous system in susceptible mice (11). Since this study, numerous high-fidelity RdRp variants have been isolated in various virus families. Influenza virus H5N1 and H3N2 high-fidelity strains showed strong attenuation in mice (16), and chikungunya virus (17, 18), West Nile virus (19), St. Louis encephalitis virus (20), enterovirus 71 (21–23), coxsackievirus B3 (24, 25), foot-and-mouth disease virus (FMDV) (26–28), porcine reproductive and respiratory syndrome virus (29), and norovirus (30) have been converted to attenuated phenotypes by increasing their replication fidelity.

The first low-fidelity RdRp variant was established in FMDV, in a screen for resistance to ribavirin (31). The mutant harbored an M296I substitution in RdRp, and the mutation frequency of this strain was 1.5-fold higher than that of wild-type FMDV. Biochemical experiments revealed that purified mutant viral polymerase with M296I tended to show a lower capacity to use ribavirin triphosphate as a substrate than the wild-type polymerase. Further, low-fidelity variants of poliovirus (32), coxsackievirus B3 (33), chikungunya virus (34), Sindbis virus (35), West Nile virus (19), and Venezuelan equine encephalitis virus (36) have been isolated by mutagen screening or introducing an amino acid change in RdRp.

To understand the structural difference between low- and high-fidelity RdRps, Castro et al. generated site-directed mutagenesis of targeted residues near the catalytic site of RdRp in polioviruses (37). They identified six motifs (designated polymerase motifs A to F) that are crucial to the enzymatic activity in RdRps (38, 39). The amino acid residues in the identified motifs are associated with the binding of two metal ions, nucleoside triphosphates (NTPs), and viral RNA, all of which are critical for the nucleotidyltransferase reaction (40). All nucleic acid polymerases use a general acid for nucleotidyl transfer, which in the case of poliovirus 3D^{pol} is the Lys residue at position 359 (K359). Mutation of this Lys to Arg (K359R) resulted in a 5-fold increase in replication fidelity compared to that of the wild-type enzyme (37, 41). Likewise, it has been suggested that the K359H substitution might confer a high-fidelity poliovirus polymerase (37). This residue, located in motif D, functions as a proton donor during nucleotidyl transfer and is conserved in all RdRps. Evaluating the effect of a Lys-to-Arg or Lys-to-His mutation of this conserved residue in other viruses might therefore reveal a general mechanism of viral replication fidelity control.

The influenza virus polymerase is a heterotrimeric complex consisting of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). The PB1 subunit plays a central role in the catalysis of the polymerization of the RNA chain and contains polymerase motifs that are common to RdRps (42, 43). Structural and amino acid sequence alignment data of the influenza A virus RdRp

FIG 1 Importance of the PB1-Lys481 residue in viral RNA synthesis and proliferation. (A) Cartoon representations of poliovirus polymerase (left) and influenza virus PB1 (right) structures. Polymerase motifs A, B, C, D, E, and F are represented in red, green, yellow, blue, magenta, and orange, respectively. Poliovirus polymerase Lys359 residue (cyan), PB1-Lys479 (white), PB1-Lys480 (dark gray), and PB1-Lys481 (cyan) are indicated in a space-filling Corey-Pauling-Koltun model. (B) The Lys residue in polymerase motif D that functions as a general acid is conserved in poliovirus and influenza virus RNA polymerases. Sequence alignments of motif D from the indicated picornaviral and orthomyxoviral RNA polymerases are shown. Conserved Lys residues (poliovirus, Lys359; influenza virus, Lys481) are underlined. (C) The effect of a Lys residue substitution on viral RNA polymerase activity using a minireplicon reporter assay system for the influenza virus genome replication. The 293T cells were transfected with pRL-SV40, pHH-vNS-Luc, pCAGGS-PB2, pCAGGS-PA, pCAGGS-NP, and either pCAGGS-PB1 wild type (PB1-wt) or pCAGGS-PB1 mutants. Cells were incubated at 37°C for 20 h, harvested, and subsequently assayed for luciferase activity. The luciferase activity was normalized as that relative to *Renilla* luciferase activity. The quantitative result is presented as the average and standard deviation of results from at least four independent experiments. Significance was determined using Student's *t* test (*, $P = 4.6 \times 10^{-5}$; **, $P = 4.6 \times 10^{-5}$). (D) Growth kinetics of PR8-PB1 mutant viruses in egg passage. HA titers of viruses that were isolated and passaged in eggs ($n = 3$ or 4) were determined from the first to third passages (E1 to E3, respectively).

indicated that the conserved Lys residue controlling polymerase fidelity is localized at position 481 in polymerase motif D of the PB1 subunit (44). To assess fidelity control by Lys481 of PB1 in influenza virus, we generated a recombinant influenza virus by replacing PB1-Lys481 with Arg or His. Recently, Cheung et al. reported that a Val43-to-Ile (V43I) single-amino-acid substitution in PB1 increased selectivity to nucleotides in H3N2 and H5N1 viruses, resulting in increased fidelity of influenza virus polymerase (16). Based on this finding, we previously introduced the PB1-V43I substitution, which increased polymerase fidelity, in an attempt to design a genetically stable high-fidelity H1N1 vaccine strain using A/Puerto Rico/8/1934 (PR8) influenza A virus (45). In this study, we hypothesized that, like PB1-V43I, a PR8-PB1-Lys481 substitution might yield a more faithful influenza virus RNA polymerase, similar to 3D^{pol}-K359R or -K359H mutants.

RESULTS

A highly conserved Lys residue in polymerase motif D determines polymerase activity for viral RNA synthesis. Analysis of the structural model of 3D^{pol} in complex with primer-template RNA and nucleotide showed that Lys359 of the conserved polymerase motif D was located in the vicinity of the α -phosphate of the incoming nucleotide (Fig. 1A, left). Lys359 functions as a general acid, which protonates the

pyrophosphate leaving group of the incoming NTP substrate and contributes to nucleotidyl transfer. This Lys residue is conserved in RdRps, including influenza virus PB1 polymerase (Fig. 1B). Based on sequence alignments, Lys359 in 3D^{pol} is identical to Lys481 in PB1 of influenza virus. The position of Lys481 was plotted on the PB1 polymerase structure of bat/Guatemala/060/2010 (H17N10) (46, 47) (Fig. 1A, right). The Lys481 residue was located near the active site for nucleotide incorporation in the RdRp heterotrimer complex.

We tested whether a Lys-to-Arg or Lys-to-His mutation at position 481 in the PB1 subunit of the PR8 strain would affect its viral polymerase activity and alter replication fidelity. Polymerase activity was evaluated using a minireplicon reporter assay for influenza virus genome replication. In addition, we analyzed the effects of substitution of the Lys479 and Lys480 residues, because these amino acids near Lys481 function as the general acid in the case of PB1 polymerase (46). Consequently, the rates of viral RNA synthesis in PB1-K479R, -K479H, -K480R, and -K480H mutants were reduced by up to 50% of that of wild-type PB1 polymerase, whereas polymerase activity was reduced 10^3 to 10^4 times following K481R or K481H substitution in comparison with that of the wild type (Fig. 1C, K481R, $P = 4.6 \times 10^{-5}$ by Student's *t* test; K481H, $P = 4.6 \times 10^{-5}$ by Student's *t* test). These results suggested that Lys481 is crucial for polymerase activity.

To compare genome replication activities and fidelities of the PB1 variants, we generated reassortant viruses with a K479R, K479H, K480R, K480H, K481R, or K481H substitution in the PB1 segment using a reverse genetics approach. Propagation of each virus in chicken eggs was examined by the hemagglutination (HA) assay (Fig. 1D). HA titers of primary isolates of wild-type PB1 and the PB1-Lys479 and PB1-Lys480 mutant strains were 1,024 to 2,048 HA units, whereas PB1-K481R and PB1-K481H viruses replicated very poorly in eggs, with titers lower than 2 HA units. However, upon continued serial passages of PB1-K481H in eggs, the viral HA titers gradually increased with the number of passages. This result suggested that an additional mutation(s) for functional complementation of a reduced polymerase activity had occurred in the PB1-K481H mutant or that its PB1 sequence was reverted to the wild type by a His-to-Lys mutation at position 481. In contrast, K481R was identified as a lethal mutation.

A Y82C mutation in PB1 restores the replication capacity of the PB1-K481H mutant. To examine whether restorative mutations in the PB1-K481H mutant affect the HA assay, we analyzed the nucleotide sequences of all three polymerase subunit (PB1, PB2, and PA)-specific and nucleoprotein (NP)-specific reverse-transcription PCR (RT-PCR) products generated using RNA collected from egg passage number 2 (E2, 64 HA units) and number 3 (E3, 512 HA units) (Fig. 1D). We found that the PB1-K481H substitution was maintained, and one additional mutation in the PB1 subunit, which resulted in a Tyr-to-Cys change at position 82, was identified (Fig. 2A and B). This amino acid change was caused by an A-to-G transition at the second base of codon 82 (TAT to TGT). We aligned PB1 amino acids 62 to 102 in various strains, as shown in Fig. 2D. PB1 contains a typical right-handed RdRp fold comprising fingers, fingertips, palm, and thumb domains. This region, which contains the Tyr82 residue in the fingers domain, was found to be extremely conserved, suggesting that mutation of PB1-Y82 induced a structural change in PB1 complementing the K481H substitution. Interestingly, Y82C was localized close to the active site, near K481 (Fig. 2C).

To study whether Y82C affected polymerase activity, we constructed expression plasmids encoding the PB1-Y82C single mutant and PB1-Y82C/K481H or -Y82C/K481R double mutant and then carried out minireplicon assays (Fig. 2E). The rate of viral RNA synthesis from PB1-Y82C was nearly equal to that from wild-type PB1. Interestingly, the presence of K481H or K481R together with Y82C increased RNA synthesis activity, and the Y82C/K481H or Y82C/K481R double mutant displayed 10^2 - to 10^3 -fold higher activity than the K481H or K481R single mutant (Fig. 2E, Y82C/K481H mutant, $P = 2.7 \times 10^{-10}$ by Student's *t* test; Y82C/K481R mutant, $P = 1.4 \times 10^{-6}$ by Student's *t* test). These results suggested that the Y82C mutation in PB1 restored the reduced polymerase activity induced by the Lys-to-His or Lys-to-Arg change at position 481.

FIG 2 Reduced polymerase activity of the PB1-K481H mutant is rescued by introduction of the Y82C substitution in PB1. (A) Sequence of the PB1-Y82C region. Virion RNA was isolated, amplified by RT-PCR, and sequenced from PB1 wild-type, PB1-K481H-E2, and PB1-K481H-E3 viruses. The wild-type sequence is TAT (Tyr residue), and the Y82C sequence is TGT (Cys residue). In PB1-K481H-E2 virus, a mixture of A and G was detected at this position. In PB1-K481H-E3 virus, a TGT codon was detected predominantly. (B) Cartoon representations of PB1. PB1-Tyr82 (brown) and PB1-Lys481 (cyan) are indicated in a space-filling Corey-Pauling-Koltun model. Polymerase motifs A, B, C, D, E, and F are represented in red, green, yellow, blue, magenta, and orange, respectively. (C) Positions of Tyr82 and Lys481 in the PB1 subunit in a heterotrimeric polymerase complex. PB2 (red) and PA (green) or PB1 (blue) subunits are shown in a surface representation or ribbon diagram structure, respectively. The vRNA duplex chain is represented in yellow. (D) The Tyr82 residue is conserved in the PB1 viral polymerase subunit. Sequence alignments of regions spanning amino acids 62 to 102 of the indicated viral PB1 subunits are shown. The Tyr82 residue is underlined, and the conserved residues among the indicated strains are marked by asterisks. (E) Effect of PB1-Tyr82 residue substitution on viral RNA polymerase activity. Viral RNA polymerase activity was measured using the minireplicon reporter assay system. The quantitative result is presented as the average and standard deviation of results from at least four independent experiments. Significance was determined using Student's *t* test (*, $P = 2.7 \times 10^{-10}$; **, $P = 1.4 \times 10^{-6}$).

TABLE 1 Mutation frequencies of PR8-PB1 wild-type and PB1-Y82C/K481H viruses

Test virus	Sequenced region (segment 4 amplicon, nt positions)	Total no. of mutations identified	Total no. of nt sequenced	Mutation frequency ^a	Relative fold change ^b
PB1 wild type	26 to 419	284	6,873,825	0.92	1.0
	466 to 869	569	4,304,254		
	913 to 1303	432	3,832,578		
	1356 to 1751	913	8,832,532		
PB1-Y82C/K481H	26 to 419	2,995	7,776,676	5.99	6.5
	466 to 869	2,916	4,226,289		
	913 to 1303	2,375	3,991,675		
	1356 to 1751	6,939	9,408,833		

^aMutation frequency was determined from 10⁴ sequenced nucleotides. Data are represented as the average of four regions.

^bRelative fold change of mutation frequency between PB1 wild type and PB1-Y82C/K481H mutant.

The PB1-Y82C/K481H double mutant has reduced polymerase fidelity. To examine the effect of the PB1-Y82C substitution inserted into the PB1-K481H variant on fidelity, we compared the mutation frequencies of the PB1 wild-type and PB1-Y82C/K481H mutant viruses. We established a system to measure the error rates in the influenza virus HA gene (segment 4) by next-generation sequencing (NGS). We generated a recombinant PR8-PB1-Y82C/K481H double mutant by reverse genetics. After reverse transcription of the full length of segment 4 from purified PB1 wild-type and PB1-Y82C/K481H genomes, the four cDNA regions derived from segment 4 were amplified by PCR. The number of mutations was calculated by NGS of these amplicons, and the mutation frequencies indicated as the average of the four regions are presented in Table 1. The mutation frequency of PB1-Y82C/K481H (5.99 mutations per 10⁴ nucleotides) was 6.5-fold higher than that of the PB1 wild-type strain (0.92 mutations per 10⁴ nucleotides). This result suggested that in addition to inducing functional recovery of polymerase activity in the PB1-K481H strain, PB1-Y82C substitution resulted in low-fidelity influenza virus polymerase. It should be noted that we also constructed a pPol1-PB1 plasmid encoding a combination of the PB1-Y82C and PB1-K481R substitutions to synthesize a reassortant virus; however, the PB1-Y82C/K481R double mutant could not be generated by reverse genetics. This result indicated that the PB1-K481R substitution is nonpermissive for the polymerase function that may act in the process of generation of progeny viral particles.

A Y82C substitution in PB1 polymerase produces a mutator phenotype *in vitro*. We previously reported an influenza virus strain that exhibits a high-fidelity phenotype, PR8-PB1-V43I (45). In that study, we established an *in vitro* limited elongation assay to measure the NTP misincorporation rate. To address the impact of the PB1-Y82C substitution on purified viral polymerase, we used this biochemical assay to evaluate replication fidelity. In the limited elongation assay with a 120-nucleotide (nt) model template (Fig. 3A), RNA synthesis was paused at the first adenine residue on the template and generated a 60-nt RNA product in the absence of UTP (Fig. 3B and C, lanes 1 and 2, 60-nt band). The induction of nucleotide misincorporation by PB1 polymerase on the first adenine during RNA synthesis resulted in the enzyme proceeding to the second adenine residue, thereby generating a 109-nt RNA product (Fig. 3B and C, lanes 1 and 2, 109-nt band). The polymerase fidelity of influenza virus was evaluated by monitoring the synthesis of 109-nt RNA products in this assay.

In the presence of UTP, the rate of synthesis of full-length 120-nt products from the PB1 wild-type viral ribonucleoprotein (vRNP) was approximately equal to that from PB1-Y82C vRNP (Fig. 3C, lanes 3 and 4). This result indicates that the PB1-Y82C substitution did not affect elongation activity in the vRNP during RNA synthesis. In contrast, the amount of 109-nt RNA products synthesized by PB1-Y82C vRNP was higher than that produced by PB1 wild-type vRNP (Fig. 3C, lanes 1 and 2, 109-nt band). The misincorporation efficiency of the PB1-Y82C RNA polymerase was increased by about 2.3-fold compared to that of wild-type PB1 (Fig. 3D, $P = 5.5 \times 10^{-3}$ by Student's *t* test). These results indicate that the purified vRNP carrying the PB1-Y82C substitution

FIG 3 The PB1-Tyr82 residue determines fidelity. (A) A 120-nt model viral RNA template for an *in vitro* limited elongation assay. The adenine residue marked with an asterisk appears as the first templating adenine base during the RNA synthesis. (B) Illustration of limited elongation reaction. In the presence of UTP, purified vRNP generates the full-length 120-nt RNA product. In the absence of UTP, RNA synthesis pauses at the first adenine base (A*). However, the synthesized RNA elongates at a second adenine residue (underlined; nucleotide position 110 from the 3' terminus) by misincorporation of nucleotides at the adenine residue marked with an asterisk. (C) The frequency of misincorporation during RNA synthesis increased following the replacement of Tyr82 by Cys in PB1. An *in vitro* limited elongation assay was carried out with the 120-nt model vRNA and 50 ng of vRNP at 30°C for 1 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of UTP. Reaction products from vRNP catalyzed nucleotide incorporation under the indicated condition. (D) Replication fidelity of the PB1-Y82C polymerase is lower than that of the wild-type enzyme. The amounts of the RNA products derived from the PB1-Y82C mutant were compared with those of the PB1 wild type. Band intensities were determined after subtraction of the background using ImageJ, version 1.4.3.67. Quantitative results are presented as the averages and standard deviations of results from at least three independent experiments. Significance was determined using Student's *t* test (*, $P = 5.5 \times 10^{-3}$).

has a reduced capacity to discriminate between the correct and incorrect nucleotide during the polymerase reaction. Because the concentration of the purified mutated PB1 polymerase in vRNP complex prepared from PB1-Y82C/K481H virions was not sufficient for a limited elongation assay, we could not determine the misincorporation efficiency of PB1-Y82C/K481H in comparison with wild-type PB1 using this *in vitro* assay (see Discussion).

A point mutation in the PB1-Tyr82 residue influences virus growth and polymerase activity. We hypothesized that Y82 plays an important role in modulating intrinsic influenza virus RNA polymerase fidelity. To test this, we generated recombinant viruses and expression plasmids in which Y82 was replaced with each of the other amino acids, and we assessed viral growth, RNA polymerase activity, and replication fidelity of the mutant in comparison with the wild-type virus. First, propagation of each mutant virus in chicken eggs was examined by plaque assay. Before virus titration, virus

TABLE 2 Characterization of PR8-PB1-Y82 mutant viruses

Test virus	No. of PFU ($\log_{10}$) ^a at:		Polymerase activity		Relative fold change of mutation frequency ^f
	34°C	37°C	Relative fold change ($\log_{10}$) ^d	<i>P</i> ^e	
PB1 wild type (Y82)	5.0	4.7	5.00		1.00
PB1-Y82C	4.3	4.0	4.92	3.7×10^{-2}	1.91
PB1-Y82G	3.7	2.5	4.46	2.2×10^{-3}	2.15
PB1-Y82A	3.3	2.0	4.81	1.7×10^{-3}	1.63
PB1-Y82V	4.4	3.3	4.74	1.6×10^{-4}	2.32
PB1-Y82I	5.4	5.4	4.62	3.4×10^{-4}	2.49
PB1-Y82L	2.7	ND ^c	4.69	4.6×10^{-3}	1.13
PB1-Y82M	3.4	ND	4.81	1.3×10^{-4}	0.90
PB1-Y82F	3.7	ND	3.84	5.9×10^{-4}	1.83
PB1-Y82T	3.9	2.7	4.83	6.4×10^{-3}	1.47
PB1-Y82S	3.5	2.7	4.73	9.4×10^{-3}	1.39
PB1-Y82N	3.7	2.7	4.88	1.6×10^{-4}	2.89
PB1-Y82Q	2.4	1.6	3.94	5.0×10^{-4}	0.92
PB1-Y82H	3.9	3.0	4.86	8.2×10^{-4}	1.46
PB1-Y82K	3.3	3.0	4.45	2.4×10^{-3}	1.14
PB1-Y82R	3.3	ND	4.68	2.6×10^{-4}	1.90
PB1-Y82W	NT ^b	NT	0.98	5.1×10^{-5}	NT
PB1-Y82P	NT	NT	2.49	3.5×10^{-8}	NT
PB1-Y82D	NT	NT	2.24	3.5×10^{-8}	NT
PB1-Y82E	NT	NT	1.12	3.5×10^{-8}	NT

^aThe numbers of PFU were determined using prepared virus particles at the same HA titer, 8 HA/50 μ l.

^bNT, not tested.

^cND, not detected.

^dValues were calculated using data shown in Fig. 4B.

^e*P* values were calculated using the *t* test, in which we compared the polymerase activity for a particular mutation with the polymerase activity obtained using the PB1 wild-type virus.

^fValues were calculated using data shown in Table 3.

particles harvested from eggs were adjusted to the same HA titer (8 HA/50 μ l) by the HA assay. The numbers of PFU of the Y82 variants are shown in Table 2. Of the 19 variants, 15 were viable at 34°C. This high number of viable variants indicates that position 82 has structural plasticity and can tolerate a wide range of substitutions. In contrast, the Glu(E), Asp(D), Trp(W), Pro(P) variants were not recoverable. In addition, the PB1-Y82L(Leu), -Y82M(Met), -Y82F(Phe), and -Y82R(Arg) variants did not generate plaques at 37°C; therefore, these substitutions might induce temperature sensitivity of replication.

Next, we tested the effect of Tyr82 residue substitution on viral polymerase activity by using the minireplicon assay (Fig. 4). We constructed PB1 expression plasmids for mammalian cells and confirmed these mutant proteins by Western blot analysis (Fig. 4A). The RNA synthesis rates of the PB1 wild-type strain and Y82 mutants are shown in Fig. 4B and Table 2 (see “relative fold change”). The viral RNA polymerase activities of the mutants with Y82P, Y82W, Y82D, and Y82E substitutions were significantly reduced (Table 2, Y82P, Y82D, and Y82E mutants, $P = 3.5 \times 10^{-8}$ by Student's *t* test; Y82W mutant, $P = 5.1 \times 10^{-5}$ by Student's *t* test), whereas activities were reduced by only about 10-fold in the other, viable Y82 mutants. These data indicated that generation of Y82P, Y82W, Y82D, and Y82E mutant viruses was not observed because these mutated PB1 polymerases induced a significant decrease in RNA synthesis activity for viral proliferation. The polymerase activities of viable Y82 variants, including PB1-Y82C, were nearly the same as or were slightly lower than that of wild-type PB1.

The Tyr82 residue of PB1 is crucial for fidelity control. To compare the mutation frequencies of the PB1-Y82 variants with their wild-type counterpart in the viral replication cycle, we determined the error rates in the HA gene of influenza virus by use of NGS analysis (Table 2, see “relative fold change of mutation frequency,” and Table 3). We sequenced the four regions of the HA gene of each PB1-Y82 variant to calculate the error rates by reading more than 3,000,000 nt per region. The mutation frequency in PB1-Y82C virus was 1.91-fold higher than that in PB1 wild-type virus. Interestingly,

FIG 4 Importance of the PB1-Tyr82 residue in viral RNA synthesis. (A) Western blotting of PB1-Y82 mutants in 293T cells. Transfected PB1 proteins were confirmed with anti-PB1 polyclonal antibody, and equal sample loading was verified with anti- β -actin antibody (bottom). (B) Effect of Tyr82 amino acid substitution on viral RNA polymerase activity using a reporter assay system for the influenza virus genome replication. The 293T cells were transfected with pRL-SV40, pHH-vNS-Luc, pCAGGS-PB2, pCAGGS-PA, pCAGGS-NP, and either pCAGGS-PB1 wild type or pCAGGS-PB1-Y82 mutants. Cells were incubated at 37°C for 24 h, harvested, and subsequently assayed for luciferase activity. The quantitative result is presented as the average and standard deviation of results from at least three independent experiments.

PB1-Y82N(Asn), -Y82I, -Y82V, and -Y82G(Gly) variants had >2-fold-higher mutation frequencies than the wild type (2.89-, 2.49-, 2.32-, and 2.15-fold, respectively). On the other hand, the Y82M, Y82Q(Gln), Y82L, and Y82K(Lys) variants had nearly the same mutation frequencies (0.90-fold to 1.14-fold) as the wild type, which is the natural mutation spectrum of influenza virus. However, the other viable Y82 variants [Y82S(Ser), Y82H, Y82T(Thr), Y82A(Ala), Y82F, and Y82R] produced low-fidelity polymerase (1.39-fold to 1.90-fold compared to the wild-type virus). Together, these data suggested that Y82 in PB1 has a critical role in determining replication fidelity.

The PB1-Y82C mutation confers increased RNA mutagen sensitivity. To further confirm the decrease in polymerase fidelity in PB1-Y82C virus, we evaluated the effect of lethal mutagenesis by using two nucleotide analogs, ribavirin and 5-azacytidine, which are mutagenic for a number of RNA viruses, including influenza A virus (48). PB1 wild-type and PB1-Y82C viruses were compared in terms of plaque formation in the presence of RNA mutagens, because plaque development requires multiple cycles of infection (Fig. 5). Madin-Darby canine kidney (MDCK) cells were infected with 30 PFU per well, and plaque assays were carried out in the absence and presence of ribavirin or 5-azacytidine. Interestingly, PB1-Y82C virus produced a mixed population in the absence of RNA mutagens, as plaques varied considerably in size (Fig. 5A, 0 μ M ribavirin and 0 μ M 5-azacytidine). This result suggested that the mutation rate in the genome segment determining viral proliferation efficiency was increased by low-fidelity PB1-Y82C polymerase. The genetic diversity in PB1-Y82C virus was associated with reduced pathogenicity in the infected host (see Fig. 6).

TABLE 3 Mutation frequencies of PR8-PB1-Y82 mutant viruses

Test virus	Sequenced region (segment 4 amplicon, nt positions)	Total no. of mutations identified	Total no. of nt sequenced	Mutation frequency ^a
PB1 wild type	26 to 419	1,681	8,935,383	1.84
	466 to 869	1,446	6,601,399	
	913 to 1303	815	4,096,188	
	1356 to 1751	1,409	9,458,554	
PB1-Y82C	26 to 419	2,965	9,948,793	3.51
	466 to 869	2,417	5,865,019	
	913 to 1303	1,802	3,736,532	
	1356 to 1751	3,267	10,220,942	
PB1-Y82S	26 to 419	1,665	9,205,864	2.55
	466 to 869	2,649	7,000,875	
	913 to 1303	1,301	3,964,404	
	1356 to 1751	1,942	9,418,270	
PB1-Y82T	26 to 419	2,003	8,074,409	2.70
	466 to 869	1,719	5,250,826	
	913 to 1303	1,162	3,426,564	
	1356 to 1751	2,090	9,095,173	
PB1-Y82N	26 to 419	3,684	7,515,081	5.31
	466 to 869	6,211	7,621,486	
	913 to 1303	2,092	4,167,245	
	1356 to 1751	3,502	9,847,934	
PB1-Y82Q	26 to 419	1,753	8,912,916	1.70
	466 to 869	1,452	6,505,329	
	913 to 1303	810	3,938,870	
	1356 to 1751	762	8,692,651	
PB1-Y82A	26 to 419	3,035	7,988,904	2.99
	466 to 869	2,574	6,423,709	
	913 to 1303	2,644	12,552,895	
	1356 to 1751	2,325	8,384,252	
PB1-Y82F	26 to 419	3,402	9,107,807	3.36
	466 to 869	2,352	7,231,284	
	913 to 1303	1,303	3,874,216	
	1356 to 1751	3,010	9,715,247	
PB1-Y82G	26 to 419	2,769	8,373,151	3.95
	466 to 869	2,563	6,351,766	
	913 to 1303	1,593	3,435,708	
	1356 to 1751	3,797	9,005,891	
PB1-Y82M	26 to 419	587	7,879,262	1.66
	466 to 869	1,608	6,393,115	
	913 to 1303	852	3,568,862	
	1356 to 1751	1,360	8,646,325	
PB1-Y82V	26 to 419	2,752	8,249,897	4.27
	466 to 869	3,253	5,963,536	
	913 to 1303	1,806	3,455,665	
	1356 to 1751	3,778	9,496,514	
PB1-Y82L	26 to 419	977	6,003,792	2.08
	466 to 869	1,513	5,381,578	
	913 to 1303	822	2,792,109	
	1356 to 1751	1,636	9,586,678	
PB1-Y82I	26 to 419	3,181	8,060,102	4.58
	466 to 869	3,899	6,701,220	
	913 to 1303	1,484	3,415,656	
	1356 to 1751	4,338	10,023,025	

(Continued on next page)

TABLE 3 (Continued)

Test virus	Sequenced region (segment 4 amplicon, nt positions)	Total no. of mutations identified	Total no. of nt sequenced	Mutation frequency ^a
PB1-Y82K	26 to 419	1,270	7,930,078	2.09
	466 to 869	1,367	6,261,324	
	913 to 1303	695	3,104,193	
	1356 to 1751	2,208	9,184,942	
PB1-Y82R	26 to 419	2,262	7,816,413	3.50
	466 to 869	1,941	5,967,641	
	913 to 1303	1,195	3,235,418	
	1356 to 1751	3,415	8,162,932	
PB1-Y82H	26 to 419	1,287	6,962,985	2.69
	466 to 869	2,652	6,672,573	
	913 to 1303	965	3,381,587	
	1356 to 1751	2,279	9,710,336	

^aMutation frequency was determined from 10⁴ sequenced nucleotides. Data are represented as the average of four regions.

In the presence of either of the RNA mutagens, plaque formation ratios were reduced in both PB1 wild-type and PB1-Y82C viruses (Fig. 5A and B). The PB1-Y82C virus produced significantly fewer plaques when exposed to a mutagen than did the PB1 wild-type virus (Fig. 5B, 10 μ M ribavirin, $P = 2.5 \times 10^{-6}$ by Student's t test; 20 μ M ribavirin, $P = 1.6 \times 10^{-5}$ by Student's t test; 10 μ M 5-azacytidine, $P = 8.4 \times 10^{-6}$ by Student's t test; 20 μ M 5-azacytidine, $P = 8.4 \times 10^{-4}$ by Student's t test). These results suggested that the PB1-Y82C mutation introduced in influenza virus confers increased sensitivity to RNA mutagens, which is consistent with findings in previous studies on fidelity variants (15, 16).

The mutator strain of influenza virus is attenuated in eggs. We hypothesized that an increased mutation frequency would have a negative effect on virus proliferation. To test this hypothesis, we infected embryonated chicken eggs with PB1 wild-type and PB1-Y82C viruses and determined the 50% egg infective doses (EID₅₀). The

FIG 5 Effect of PB1-Y82C mutation on ribavirin and 5-azacytidine sensitivities. (A) Plaque assay of PB1-wt and PB1-Y82C viruses in MDCK cells in the presence of ribavirin (left) and 5-azacytidine (right). MDCK cells were infected with 30 PFU per well, and an agar overlay containing 0 μ M, 10 μ M, or 20 μ M ribavirin or 5-azacytidine was added. Cells were incubated at 34°C for 4 days and then stained with amido black 10B. (B) PB1-Y82C virus is more susceptible to RNA mutagens than PB1 wild-type virus. The numbers of virus plaques per well in panel A were plotted (left, ribavirin; right, 5-azacytidine). Quantitative data are presented as the average and standard deviation of results from at least eight independent experiments. Significance was determined using Student's t test (*, $P = 2.5 \times 10^{-6}$; **, $P = 1.6 \times 10^{-5}$; ***, $P = 8.4 \times 10^{-6}$; ****, $P = 8.4 \times 10^{-4}$).

FIG 6 PB1-Y82C virus is attenuated in embryonated chicken eggs. (A) Mutation frequencies of NS gene and EID₅₀ titer. The EID₅₀ was determined using prepared virus particles at the same HA titer (8 HA/50 μ l) between PB1-wt and PB1-Y82C. One EID₅₀ unit is the amount of virus particle that infects 50% of inoculated eggs. The NS gene (nucleotides 63 to 365) of the viruses isolated from embryonated chicken eggs were subjected to NGS analysis. (B) Frequencies of nucleotide substitutions in the NS gene of PB1 wild-type and PB1-Y82C viruses. Values are expressed as the total number of mutations (PB1-wt, 311; PB1-Y82C, 785) and the percentage of total mutations for base composition. The transversion substitutions are indicated in bold. (C) Ratio of transversion and transition mutations. The values were calculated using panel B data.

EID₅₀ value of the PB1-Y82C virus was 10 times lower than that of PB1 wild-type virus (Fig. 6A), indicating that the mutator variant has a lower infectivity than wild-type virus in eggs.

Our data showed that the influenza virus mutator strain PB1-Y82C has nearly normal RNA synthesis activity (Fig. 3 and Table 2) but lower specific infectivity than the wild-type virus in MDCK cells and in eggs. We assumed that a noninfectious progeny particle and/or an attenuated mutant virus would be generated during replication cycles of the PB1-Y82C variant because unnatural mutations would be accumulated by the mutator polymerase. To compare the mutation type bias between wild-type PB1 and PB1-Y82C, amplicons of the nonstructural (NS) gene region were sequenced and the mutation types were analyzed. Mori et al. reported that the frequency of missense mutations in the NS gene is higher than that in other regions of the influenza virus genome (49). NS1 protein is not essential for virus production (50); therefore, this gene is more tolerant to mutations inducing amino acid changes. The mutation frequencies in the NS gene generated by the PB1 wild-type and PB1-Y82C viruses were 3.89×10^{-5} and 1.37×10^{-4} substitutions per nucleotide, respectively (Fig. 6A). Thus, the NS gene mutation efficiency in PB1-Y82C virus was elevated by 3.5-fold relative to that in the PB1 wild-type virus. Comparing the mutation profiles (Fig. 6B and C), mutations were mainly transitions (PB1 wild type, 83%; PB1-Y82C, 60%). Interestingly, transversions were increased from 17% to 40% by introducing the Y82C substitution in PB1. PB1 wild-type virus showed only 0.3% C-to-G transversion, whereas PB1-Y82C exhibited 16.3% C-to-G transversion. These results suggested that the reduction in infectious virus titer induced by PB1-Y82C was caused by the accumulation of missense mutations.

DISCUSSION

Although a fidelity variant harboring a PB1-Lys481 single mutation was not recoverable, we did isolate a double mutant, PB1-Y82C/K481H, possessing low fidelity

compared to the wild-type enzyme. Further, our data indicated that the single Y82C substitution in PB1 led to a mutator phenotype. To confirm the effect of this mutation on PR8 polymerase fidelity, the mutation frequencies of PB1 wild-type and PB1-Y82C viruses were calculated by NGS analysis and an *in vitro* limited elongation assay. The PB1-Y82C virus showed a 1.9-fold to 3.5-fold higher mutation frequency than the PB1 wild-type virus. Furthermore, purified PB1-Y82C polymerase enzyme demonstrated an increased misincorporation rate during RNA synthesis in an *in vitro* biochemical assay. This finding is in agreement with the fact that most fidelity mutants produce an approximately 2-fold change in diversity compared to wild-type viruses. Although these differences in diversity appear small, these alterations translate to significant attenuation *in vivo* in multiple viruses (13, 14). In addition, we isolated a PB1-Y82C/K481H double mutant virus with markedly reduced fidelity; its mutation frequency was 6.5-fold higher than that of the PB1 wild-type virus. Purified vRNP collected from PB1-Y82C/K481H virions did not reach the polymerase enzyme concentration obtained for PB1-Y82C or PB1 wild type. Therefore, we could not determine the misincorporation frequency in this *in vitro* system. It is possible that PB1-Y82C/K481H strongly altered replicated viral RNA dynamics, producing an immature virus particle and/or a defective interference particle that could not assemble complete vRNP complexes.

The PB1-Y82C mutator virus exhibited no significant genome replication defects in mammalian cell culture but showed marked attenuation in embryonated chicken eggs. This is consistent with the finding that mutator variants of positive-strand RNA virus make more lethally mutagenized viral RNA (13, 14). Further, we showed that the Tyr82 residue of PB1 has an important role in determining intrinsic RNA polymerase fidelity, by replacing this residue with all other amino acids.

Like most polymerases, the influenza virus RNA polymerase can be likened to a right hand, with palm, fingers, fingertips, and thumb domains (46). The catalytic center responsible for template-directed nucleotide addition is located in the PB1 subunit and is formed mainly by the highly conserved polymerase motifs A to F. By comparison with other polymerase structures, key conserved residues for RNA synthesis were identified in the active site of PB1. The conserved Lys481 residue involved in NTP binding is positioned in motif D of the palm domain. Interestingly, Tyr82 is located near motif D and the NTP entrance tunnel. Fidelity-altering mutations are usually found in motifs A and D of the RdRp active site (13). Although position 82 in PB1 may seem distant from the active-site residues, the fingers and/or other polymerase motifs might undergo substantial rearrangement during polymerization by Y82C substitution.

Spontaneous mutator mutants of influenza H3N2 virus (A/Victoria/3/75) have been isolated by fluctuation screening using hemagglutinin-specific monoclonal antibodies (51). The mutation rate of isolated mutator mutants was two to four times higher than that of the wild-type strain; however, the position of the amino acid substitution(s) in viral protein(s) inducing the mutator phenotype was not identified. It is possible that in these H3N2 mutator strains, the Tyr82 residue of PB1 is replaced, because the region containing Tyr82 is extremely conserved (Fig. 2D), assuming that H3N2-PB1-Y82 variants also may produce low-fidelity polymerase to the same extent as H1N1-PB1-Y82 mutant viruses.

High-fidelity polymerase is thought to synthesize the virus genome more slowly because the incorporation process is more accurate than that of wild-type polymerase. In contrast, mutator viruses replicate more quickly than wild-type viruses but introduce many errors during replication. In the current study, the growth rate of mutator virus in cell culture was not significantly decreased. Variants with low-fidelity polymerase are considered to more rapidly accumulate lethal mutations and, therefore, show an attenuated phenotype in the infected host. Mutator variants of poliovirus, coxsackievirus B3, and chikungunya virus reportedly are less fit *in vivo* and fail to reproduce in target organs (17, 32–34). Based on this property, a live attenuated vaccine strategy for coronavirus infection control was examined in a mouse model (52). The low-fidelity coronavirus vaccine strain was stable and did not revert to virulence during long-term persistent infection. Likewise, a live attenuated vaccine strain for Venezuelan equine encephalitis virus based on low-fidelity RdRp has been reported (36).

A live attenuated influenza virus (LAIV) derived from A/Ann Arbor/6/60 (H2N2) was generated by serial passage in primary chicken kidney tissue culture cells, resulting in cold-adapted and temperature-sensitive mutations that resulted in an attenuated phenotype (53). The cold-adapted A/Ann Arbor/6/60 (ca A/AA/6/60)-based LAIV is marketed as FluMist. Derivative vaccines have been produced by generating reassortant viruses that harbor six internal viral RNA segments (PB1, PB2, PA, NP, M, and NS) from the master donor ca A/AA/6/60 strain plus two segments encoding viral membrane proteins (HA and NA) from newly emergent wild-type viruses. Five amino acid mutations in three proteins (PB1, PB2, and NP) play a critical role in the temperature-sensitive phenotype of ca A/AA/6/60 (54, 55). In addition, alteration of internal influenza viral proteins has been employed to develop live attenuated vaccine strains, including NS1 deletion mutants (56, 57), M2 cytoplasmic tail mutants (58), codon pair deoptimized viruses (59–62), and nonreplicating viruses (63–67). The PB1-Y82 variants might be useful for the development of a novel type of live attenuated vaccine strains encoding low-fidelity polymerase. The development of a low-fidelity donor candidate strain with a temperature-sensitive phenotype is currently being examined in our laboratory.

MATERIALS AND METHODS

Molecular modeling. Ribbon diagrams and space-filling representations of the poliovirus and influenza virus polymerases were built utilizing structural data (PDB ID no. 1RA6 and 5M3H) and the MF myPresto software version 3.2.0.33 (FiatLux, Tokyo, Japan).

Cells. 293T and MDCK cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Tokyo, Japan) containing 10% fetal calf serum (Nichirei Biosciences, Tokyo, Japan) and penicillin-streptomycin in an incubator at 37°C with 5% CO₂.

Generation of protein expression plasmids for PB1 mutants. Mutations corresponding to an amino acid substitution at Lys479 (codon, AAG), Lys480 (AAA), and Lys481 (AAG) residues of the PB1 subunit were introduced into a plasmid containing a sequence encoding wild-type PB1 by site-directed mutagenesis. These Lys residue codons in the PB1 gene of the high-growth strain PR8 were changed from AAG or AAA (Lys) to AGA (Arg) or CAC (His) by PCR mutagenesis. To construct the plasmid containing the PB1-K479R coding sequence, two DNA fragments corresponding to the PB1 coding sequence were amplified by PCR using primers PB1-for and K479R-rev or PB1-rev and K479R-for (Table 4) and pPol1-PB1 wild type as the PCR template. The full-length PB1-K479R gene was amplified by PCR using primers PB1-for and PB1-rev. PCR products were digested with KpnI and NotI and cloned into KpnI- and NotI-digested pCAGGS-P7 plasmid. The resultant plasmid was designated pCAGGS-PB1-K479R. Likewise, pCAGGS-PB1-K479H, -PB1-K480R, -PB1-K480H, -PB1-K481R, -PB1-K481H, -PB1-Y82C, -PB1-Y82C/K481H, and -PB1-Y82C/K481R plasmids and the pCAGGS-PB1-Y82 mutant plasmid set were constructed using the primers listed in Table 4.

Minireplicon reporter assay system. 293T cells were transfected with expression plasmids encoding PB1 (pCAGGS-PB1 wild type or pCAGGS-PB1 mutants), PB2, PA, and NP and a plasmid (pHH-vNS-Luc) for the expression of the artificial influenza virus genome containing firefly luciferase gene in negative sense, which is synthesized in cells by the human DNA-dependent RNA polymerase I (PolI) (68). The mRNA encoding firefly luciferase was transcribed in an influenza viral RNA polymerase-dependent manner. Luciferase activity was determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol and was normalized to *Renilla* luciferase activity encoded by cotransfected pRL-SV40 vector (Promega).

Western blotting. 293T cells were lysed in 20 mM Tris-HCl (pH 7.9), 100 mM NaCl, and 0.1% Triton X-100. After sonication, homogenates were centrifuged at 14,000 × *g* at 4°C for 5 min, and the supernatant fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (pore size, 0.45 μm; Merck Millipore, Burlington, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and probed with anti-PB1 rabbit polyclonal (69) or anti-β-actin rabbit polyclonal (PM053; Medical & Biological Laboratories, Nagoya, Japan) antibody. The membranes were washed with TBS-T and incubated with IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE, USA). After washing with TBS-T, the proteins were detected with the Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Generation of recombinant viruses. To construct the plasmid from which PolI transcribes the PB1-K479R RNA, we amplified two DNA fragments corresponding to the PB1 coding sequence by PCR using primers Pol1-rev and K479R-rev or Pol1-for and K479R-for (Table 4) and pPol1-PB1 wild type as the PCR template. The full-length PB1-K479R gene was amplified by PCR using primers Pol1-rev and Pol1-for (Table 4). The PCR product was digested with Apal and XhoI and cloned into Apal- and XhoI-digested pPol1 plasmid. The resultant plasmid was designated pPol1-PB1-K479R. Likewise, pPol1-PB1-K479H, -PB1-K480R, -PB1-K480H, -PB1-K481R, -PB1-K481H, -PB1-Y82C, -PB1-Y82C/K481H, and -PB1-Y82 mutant plasmids were constructed using primers listed in Table 4. Plasmids were transfected into 293T cells, and recombinant viruses were rescued as described previously (70). Transfected 293T cells were incubated at 34°C in Opti-MEM (Thermo Fisher Scientific, Tokyo, Japan) with 3.5 μg/ml *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA), and the supernatant was

TABLE 4 Primers used in this study

Primer name	Sequence (5' to 3') ^a
PB1-for	ACGACCGGTACCGCCACCACCATGGATGTCAATCCGACCTTACTTTTC
PB1-rev	ATGCATGCGCGCCGCTATTTTTGCCGCTCTGAGCTCTTCAAT
K479R-for	AATCAATATGAGCAGAAAAAGTCTTACAT
K479R-rev	ATGTAAGACTTTTTCTGCTCATATTGATT
K479H-for	AATCAATATGAGCCACAAAAAGTCTTACAT
K479H-rev	ATGTAAGACTTTTTGTGGCTCATATTGATT
K480R-for	CAATATGAGCAAGAGAAAGTCTTACATAAA
K480R-rev	TTTATGTAAGACTTTCTCTTGCTCATATTG
K480H-for	CAATATGAGCAAGCACAAGTCTTACATAAA
K480H-rev	TTTATGTAAGACTTTGTGCTTGCTCATATTG
K481R-for	TATGAGCAAGAAAAGATCTTACATAAACAG
K481R-rev	CTGTTTATGTAAGATCTTTCTTGCTCATA
K481H-for	TATGAGCAAGAAACACTCTTACATAAACAG
K481H-rev	CTGTTTATGTAAGAGTGTCTTGCTCATA
Pol1-for	GTGTGCTCCTGGGTTGACCAGA
Pol1-rev	CATCGGTGATGTCGGCGATATAG
Y82C-for	TGAACCAAGTGGTTGTGCCCAAACAGATT
Y82C-rev	AATCTGTTTGGGCACAACCACTTGTTCA
Y82A-for	TGAACCAAGTGGTGCTGCCCAAACAGATT
Y82A-rev	AATCTGTTTGGGCAGCAACCACTTGTTCA
Y82V-for	TGAACCAAGTGGTGTGCCCAAACAGATT
Y82V-rev	AATCTGTTTGGGCACCAACCACTTGTTCA
Y82I-for	TGAACCAAGTGGTATTGCCCAAACAGATT
Y82I-rev	AATCTGTTTGGGCATACCACTTGTTCA
Y82L-for	TGAACCAAGTGGTCTTGCCCAAACAGATT
Y82L-rev	AATCTGTTTGGGCAGACCACTTGTTCA
Y82M-for	TGAACCAAGTGGTATGCCCAAACAGATT
Y82M-rev	AATCTGTTTGGGCATACCACTTGTTCA
Y82F-for	TGAACCAAGTGGTTTGCCCAAACAGATT
Y82F-rev	AATCTGTTTGGGCAAAACCACTTGTTCA
Y82W-for	TGAACCAAGTGGTTGGGCCAAACAGATT
Y82W-rev	AATCTGTTTGGGCCCAACCACTTGTTCA
Y82S-for	TGAACCAAGTGGTTCTGCCCAAACAGATT
Y82S-rev	AATCTGTTTGGGCAGAACCACTTGTTCA
Y82T-for	TGAACCAAGTGGTACTGCCCAAACAGATT
Y82T-rev	AATCTGTTTGGGCAGTACCACTTGTTCA
Y82N-for	TGAACCAAGTGGTAATGCCCAAACAGATT
Y82N-rev	AATCTGTTTGGGCATTACCACTTGTTCA
Y82Q-for	TGAACCAAGTGGTCAAGCCCAAACAGATT
Y82Q-rev	AATCTGTTTGGGCTTGACCACTTGTTCA
Y82G-for	TGAACCAAGTGGTGGTGCCCAAACAGATT
Y82G-rev	AATCTGTTTGGGCACCACTTGTTCA
Y82P-for	TGAACCAAGTGGTCTGCCCAAACAGATT
Y82P-rev	AATCTGTTTGGGCAGGACCACTTGTTCA
Y82R-for	TGAACCAAGTGGTAGAGCCCAAACAGATT
Y82R-rev	AATCTGTTTGGGCTCTACCACTTGTTCA
Y82H-for	TGAACCAAGTGGTCATGCCCAAACAGATT
Y82H-rev	AATCTGTTTGGGCATGACCACTTGTTCA
Y82K-for	TGAACCAAGTGGTAAAGCCCAAACAGATT
Y82K-rev	AATCTGTTTGGGCTTTACCACTTGTTCA
Y82D-for	TGAACCAAGTGGTGATGCCCAAACAGATT
Y82D-rev	AATCTGTTTGGGCATCACCCTTGTTCA
Y82E-for	TGAACCAAGTGGTGAAGCCCAAACAGATT
Y82E-rev	AATCTGTTTGGGCTTACCACTTGTTCA
Seg4-Region1-for	ACACTTTTCCCTACACGACGCTCTCCGATCT AGCAAAAGCAGGGGAAAAATAAAAC
Seg4-Region1-rev	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT CTTTCTTTGGGAAATATTTGAATCT
Seg4-Region2-for	ACACTTTTCCCTACACGACGCTCTCCGATCT CTCATGGCCCAACCACAACA
Seg4-Region2-rev	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT TGCGTTTGAGGTGATGATGCC
Seg4-Region3-for	ACACTTTTCCCTACACGACGCTCTCCGATCT TCAATGCATGAGTGTAACACGA
Seg4-Region3-rev	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT CCAGAAATCCATCATCAACTTTTTTA
Seg4-Region4-for	ACACTTTTCCCTACACGACGCTCTCCGATCT ACATTGGACATATAATGCAGAATTG
Seg4-Region4-rev	GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT GTAGAAACAAGGGTGTTTTTCT
2nd-for	AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTTTTCCCTACACGACGC
2nd-rev	CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG

^aUnderlining indicates the codon sequence corresponding to amino acid 82 of the PB1 protein. Bold indicates the sequence of segment 4.

harvested 48 h posttransfection. The supernatant was injected into 11-day-old embryonated chicken eggs to amplify the recovered viruses.

HA assay. A hemagglutination (HA) assay was conducted with 0.5% chicken red blood cells (Nippon Bio-Test Laboratories, Tokyo, Japan) by using the standard method (71).

Cell-free model virus genome replication. The vRNPs were prepared from purified PR8-PB1 wild-type and PR8-PB1-Y82C viruses, as described previously (72). Micrococcal nuclease-treated vRNPs were prepared by incubation of vRNPs at 25°C for 3 h with 0.83 U micrococcal nuclease (Worthington Biochemical Corp., Lakewood, NJ, USA) per μ l in the presence of 3 mM CaCl_2 . The nuclease reaction was terminated by the addition of EGTA to a final concentration of 3.5 mM, and the micrococcal nuclease-treated vRNPs were used as the enzyme source for the limited elongation assay (45).

Mutation frequency determination by next-generation sequencing. Viral genomic RNA was prepared from PR8-PB1 wild-type and viable PB1-Y82 mutant viruses (including PB1-Y82C/K481H) amplified in 11-day-old embryonated chicken eggs incubated at 34°C. Viral genomic RNA was extracted from virus particles using a QIAamp viral RNA mini kit (Qiagen, Tokyo, Japan). The four regions of cDNA derived from segment 4 were amplified by a first PCR using fusion primers, including adaptors for the MiSeq sequencing system (Illumina, San Diego, CA, USA): Seg4-Region1-for and Seg4-Region1-rev corresponding to segment 4 between nucleotide sequence positions 1 to 25 and 420 to 445, Seg4-Region2-for and Seg4-Region2-rev corresponding to segment 4 between nucleotide sequence positions 446 to 465 and 870 to 890, Seg4-Region3-for and Seg4-Region3-rev corresponding to segment 4 between nucleotide sequence positions 891 to 912 and 1304 to 1329, and Seg4-Region4-for and Seg4-Region4-rev corresponding to segment 4 between nucleotide sequence positions 1330 to 1355 and 1752 to 1774, respectively (Table 4). DNA libraries were amplified by a second PCR using primers 2nd-for and 2nd-rev (Table 4), which include the index tags for discrimination of sequencing samples. DNA libraries were quantitated with Synergy H1 (BioTek, Winooski, VT, USA) and QuantiFluor double-stranded DNA (dsDNA) (Promega, Madison, WI, USA) and were quality confirmed using a fragment analyzer and dsDNA 915 reagent kit (Agilent, Santa Clara, CA, USA). Multiplexed libraries were subjected to cluster generation using a MiSeq reagent kit v3 (600 cycles) on a MiSeq sequencing instrument. The low-quality reads (an average Phred score of <27) were removed by Trimmomatic version 0.36. The processed reads were merged by FLASH version 1.2.11 (73, 74). We filtered the data to identify any minority sequence representing more than 0.3% of the total population. The mutations in each sequence position were counted (Tables 1 and 3). Amplicon generation, next-generation sequencing (NGS) analysis, and calculation of the mutation frequency of the NS gene were carried out as previously reported (Fig. 4) (45, 49).

Virus titration. Virus titer was determined by a plaque assay, as described previously (75, 76). Briefly, 500- μ l aliquots of serial 10-fold dilutions of viruses were inoculated into MDCK cells in a six-well plate. After a 1-h incubation, each well was overlaid with 2.5 ml of agar medium. The number of plaques was counted following amido black 10B (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) staining 3 days after inoculation. The virus titer was calculated as PFU/ml.

EID₅₀ assay. Wild-type PR8-PB1 and PR8-PB1-Y82C viruses were prepared at the same HA titer (8 HA/50 μ l) by HA assay. Each virus was serially diluted from 10^{-1} to 10^{-7} . Six 10- to 11-day-old embryonated chicken eggs were infected with 200 μ l of virus at each dilution. An aliquot of the allantoic fluid was collected from each egg at 48 h postinfection. Each allantoic fluid specimen was subjected to HA assay, and the 50% egg infective dose (EID₅₀) was calculated by the formula of Reed and Muench (77).

RNA mutagens. Ribavirin (R9644; Sigma-Aldrich) and 5-azacytidine (A2385; Sigma-Aldrich) were dissolved in ultrapure water at 16 mM and 30 mM, respectively. Aliquots were stored at -30°C .

Drug treatment of viruses. MDCK cells were seeded in 6-well cell culture plates (Sumitomo Bakelite, Tokyo, Japan) at 5×10^5 cells per well in 2 ml of KBM-220 medium (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). After 24 h of incubation at 37°C, the cells were washed with KBM-220 medium and then incubated with 30 PFU of virus in 1 ml of KBM-220 medium at 34°C for 2 h. After virus adsorption, the cells were washed with KBM-220 medium and then overlaid with agar containing 10 μ M or 20 μ M ribavirin or 5-azacytidine, respectively (15). The plates were incubated at 34°C for 4 days, and then cells were stained with amido black 10B.

Statistical analysis. Pairwise comparisons between means of different groups were performed using Student's *t* test. The data are reported as the sample mean $\pm$ standard deviation, and *P* values are indicated in the text.

Data availability. Raw sequencing data from this experiment in fastq format are available at the Sequence Read Archive under accession number [PRJNA524014](https://www.ncbi.nlm.nih.gov/sra/PRJNA524014).

ACKNOWLEDGMENTS

We thank Y. Kawaoka, University of Wisconsin–Madison, for kindly providing the reverse genetics system of high-growth PR8 virus. We thank Editage (www.editage.jp) for English language editing.

This study was financially supported by JSPS KAKENHI grant number JP17K09170 (T.N.), AMED grant number JP18lm0203008 (T.N.), grants from the Takeda Science Foundation (T.N.) and the Okayama Medical Foundation (T.N.), and Project Research Grants of Kawasaki Medical School (T.N.).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

- Drake JW. 1999. The distribution of rates of spontaneous mutation over viruses, prokaryotes, and eukaryotes. *Ann N Y Acad Sci* 870:100–107. <https://doi.org/10.1111/j.1749-6632.1999.tb08870.x>.
- Domingo E, Escarmis C, Sevilla N, Moya A, Elena SF, Quer J, Novella IS, Holland JJ. 1996. Basic concepts in RNA virus evolution. *FASEB J* 10: 859–864. <https://doi.org/10.1096/fasebj.10.8.8666162>.
- Sanjuan R, Domingo-Calap P. 2016. Mechanisms of viral mutation. *Cell Mol Life Sci* 73:4433–4448. <https://doi.org/10.1007/s00018-016-2299-6>.
- Drake JW, Holland JJ. 1999. Mutation rates among RNA viruses. *Proc Natl Acad Sci U S A* 96:13910–13913. <https://doi.org/10.1073/pnas.96.24.13910>.
- Sanjuan R, Nebot MR, Chirico N, Mansky LM, Belshaw R. 2010. Viral mutation rates. *J Virol* 84:9733–9748. <https://doi.org/10.1128/JVI.00694-10>.
- Domingo E, Perales C. 2018. Quasispecies and virus. *Eur Biophys J* 47:443–457. <https://doi.org/10.1007/s00249-018-1282-6>.
- Ojosnegros S, Perales C, Mas A, Domingo E. 2011. Quasispecies as a matter of fact: viruses and beyond. *Virus Res* 162:203–215. <https://doi.org/10.1016/j.virusres.2011.09.018>.
- Lancaster KZ, Pfeiffer JK. 2012. Viral population dynamics and virulence thresholds. *Curr Opin Microbiol* 15:525–530. <https://doi.org/10.1016/j.mib.2012.05.007>.
- Borderia AV, Stapleford KA, Vignuzzi M. 2011. RNA virus population diversity: implications for inter-species transmission. *Curr Opin Virol* 1:643–648. <https://doi.org/10.1016/j.coviro.2011.09.012>.
- Sallie R. 2005. Replicative homeostasis II: influence of polymerase fidelity on RNA virus quasispecies biology: implications for immune recognition, viral autoimmunity and other “virus receptor” diseases. *Virol J* 2:70. <https://doi.org/10.1186/1743-422X-2-70>.
- Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R. 2006. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* 439:344–348. <https://doi.org/10.1038/nature04388>.
- Holland JJ, Domingo E, de la Torre JC, Steinhauer DA. 1990. Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. *J Virol* 64:3960–3962.
- Kautz TF, Forrester NL. 2018. RNA virus fidelity mutants: a useful tool for evolutionary biology or a complex challenge? *Viruses* 10:600. <https://doi.org/10.3390/v10110600>.
- Borderia AV, Rozen-Gagnon K, Vignuzzi M. 2016. Fidelity variants and RNA quasispecies. *Curr Top Microbiol Immunol* 392:303–322. https://doi.org/10.1007/82_2015_483.
- Pfeiffer JK, Kirkegaard K. 2003. A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. *Proc Natl Acad Sci U S A* 100:7289–7294. <https://doi.org/10.1073/pnas.1232294100>.
- Cheung PP, Watson SJ, Choy KT, Fun Sia S, Wong DD, Poon LL, Kellam P, Guan Y, Malik Peiris JS, Yen HL. 2014. Generation and characterization of influenza A viruses with altered polymerase fidelity. *Nat Commun* 5:4794. <https://doi.org/10.1038/ncomms5794>.
- Riemersma KK, Steiner C, Singapur A, Coffey LL. 2018. Chikungunya virus fidelity variants exhibit differential attenuation and population diversity in cell culture and adult mice. *J Virol* 93:e01606-18. <https://doi.org/10.1128/JVI.01606-18>.
- Coffey LL, Beehar Y, Borderia AV, Blanc H, Vignuzzi M. 2011. Arbovirus high fidelity variant loses fitness in mosquitoes and mice. *Proc Natl Acad Sci U S A* 108:16038–16043. <https://doi.org/10.1073/pnas.1111650108>.
- Van Slyke GA, Arnold JJ, Lugo AJ, Griesemer SB, Moustafa IM, Kramer LD, Cameron CE, Ciota AT. 2015. Sequence-specific fidelity alterations associated with West Nile virus attenuation in mosquitoes. *PLoS Pathog* 11:e1005009. <https://doi.org/10.1371/journal.ppat.1005009>.
- Griesemer SB, Kramer LD, Van Slyke GA, Pata JD, Gohara DW, Cameron CE, Ciota AT. 2017. Mutagen resistance and mutation restriction of St. Louis encephalitis virus. *J Gen Virol* 98:201–211. <https://doi.org/10.1099/jgv.0.000682>.
- Meng T, Kwang J. 2014. Attenuation of human enterovirus 71 high-replication-fidelity variants in AG129 mice. *J Virol* 88:5803–5815. <https://doi.org/10.1128/JVI.00289-14>.
- Sadeghipour S, McMinn PC. 2013. A study of the virulence in mice of high copying fidelity variants of human enterovirus 71. *Virus Res* 176: 265–272. <https://doi.org/10.1016/j.virusres.2013.06.019>.
- Sadeghipour S, Bek EJ, McMinn PC. 2013. Ribavirin-resistant mutants of human enterovirus 71 express a high replication fidelity phenotype during growth in cell culture. *J Virol* 87:1759–1769. <https://doi.org/10.1128/JVI.02139-12>.
- Levi LI, Gnadig NF, Beaucourt S, McPherson MJ, Baron B, Arnold JJ, Vignuzzi M. 2010. Fidelity variants of RNA dependent RNA polymerases uncover an indirect, mutagenic activity of amiloride compounds. *PLoS Pathog* 6:e1001163. <https://doi.org/10.1371/journal.ppat.1001163>.
- McDonald S, Block A, Beaucourt S, Moratorio G, Vignuzzi M, Peersen OB. 2016. Design of a genetically stable high fidelity coxsackievirus B3 polymerase that attenuates virus growth in vivo. *J Biol Chem* 291: 13999–14011. <https://doi.org/10.1074/jbc.M116.726596>.
- Zeng J, Wang H, Xie X, Li C, Zhou G, Yang D, Yu L. 2014. Ribavirin-resistant variants of foot-and-mouth disease virus: the effect of restricted quasispecies diversity on viral virulence. *J Virol* 88:4008–4020. <https://doi.org/10.1128/JVI.03594-13>.
- Zeng J, Wang H, Xie X, Yang D, Zhou G, Yu L. 2013. An increased replication fidelity mutant of foot-and-mouth disease virus retains fitness in vitro and virulence in vivo. *Antiviral Res* 100:1–7. <https://doi.org/10.1016/j.antiviral.2013.07.008>.
- Rai DK, Diaz-San Segundo F, Campagnola G, Keith A, Schafer EA, Kloc A, de Los Santos T, Peersen O, Rieder E. 2017. Attenuation of foot-and-mouth disease virus by engineered viral polymerase fidelity. *J Virol* 91:e00081-17. <https://doi.org/10.1128/JVI.00081-17>.
- Tian D, Meng XJ. 2016. Amino acid residues Ala283 and His421 in the RNA-dependent RNA polymerase of porcine reproductive and respiratory syndrome virus play important roles in viral ribavirin sensitivity and quasispecies diversity. *J Gen Virol* 97:53–59. <https://doi.org/10.1099/jgv.0.000316>.
- Arias A, Thorne L, Ghurburrin E, Bailey D, Goodfellow I. 2016. Norovirus polymerase fidelity contributes to viral transmission in vivo. *mSphere* 1:e00279-16. <https://doi.org/10.1128/mSphere.00279-16>.
- Sierra M, Airaksinen A, Gonzalez-Lopez C, Agudo R, Arias A, Domingo E. 2007. Foot-and-mouth disease virus mutant with decreased sensitivity to ribavirin: implications for error catastrophe. *J Virol* 81:2012–2024. <https://doi.org/10.1128/JVI.01606-06>.
- Korboukh VK, Lee CA, Acevedo A, Vignuzzi M, Xiao Y, Arnold JJ, Hempel S, Graci JD, August A, Andino R, Cameron CE. 2014. RNA virus population diversity, an optimum for maximal fitness and virulence. *J Biol Chem* 289:29531–29544. <https://doi.org/10.1074/jbc.M114.592303>.
- Gnadig NF, Beaucourt S, Campagnola G, Borderia AV, Sanz-Ramos M, Gong P, Blanc H, Peersen OB, Vignuzzi M. 2012. Coxsackievirus B3 mutator strains are attenuated in vivo. *Proc Natl Acad Sci U S A* 109: 2294–2303.
- Rozen-Gagnon K, Stapleford KA, Mongelli V, Blanc H, Failloux AB, Saleh MC, Vignuzzi M. 2014. Alphavirus mutator variants present host-specific defects and attenuation in mammalian and insect models. *PLoS Pathog* 10:e1003877. <https://doi.org/10.1371/journal.ppat.1003877>.
- Poirier EZ, Mounce BC, Rozen-Gagnon K, Hooikaas PJ, Stapleford KA, Moratorio G, Vignuzzi M. 2015. Low-fidelity polymerases of alphaviruses recombine at higher rates to overproduce defective interfering particles. *J Virol* 90:2446–2454. <https://doi.org/10.1128/JVI.02921-15>.
- Kautz TF, Guerbois M, Khanipov K, Patterson EI, Langsjoen RM, Yun R, Warmbrod KL, Fofanov Y, Weaver SC, Forrester NL. 2018. Low-fidelity Venezuelan equine encephalitis virus polymerase mutants to improve live-attenuated vaccine safety and efficacy. *Virus Evol* 4:vey004. <https://doi.org/10.1093/ve/vey004>.
- Castro C, Smidansky ED, Arnold JJ, Maksimchuk KR, Moustafa I, Uchida A, Gotte M, Konigsberg W, Cameron CE. 2009. Nucleic acid polymerases use a general acid for nucleotidyl transfer. *Nat Struct Mol Biol* 16: 212–218. <https://doi.org/10.1038/nsmb.1540>.
- Ng KK, Arnold JJ, Cameron CE. 2008. Structure-function relationships among RNA-dependent RNA polymerases. *Curr Top Microbiol Immunol* 320:137–156.
- Ferrer-Orta C, Arias A, Escarmis C, Verdager N. 2006. A comparison of viral RNA-dependent RNA polymerases. *Curr Opin Struct Biol* 16:27–34. <https://doi.org/10.1016/j.sbi.2005.12.002>.
- Castro C, Smidansky E, Maksimchuk KR, Arnold JJ, Korneeva VS, Gotte M, Konigsberg W, Cameron CE. 2007. Two proton transfers in the transition state for nucleotidyl transfer catalyzed by RNA- and DNA-dependent

- RNA and DNA polymerases. *Proc Natl Acad Sci U S A* 104:4267–4272. <https://doi.org/10.1073/pnas.0608952104>.
41. Yang X, Smidansky ED, Maksimchuk KR, Lum D, Welch JL, Arnold JJ, Cameron CE, Boehr DD. 2012. Motif D of viral RNA-dependent RNA polymerases determines efficiency and fidelity of nucleotide addition. *Structure* 20:1519–1527. <https://doi.org/10.1016/j.str.2012.06.012>.
 42. Te Velthuis AJ, Fodor E. 2016. Influenza virus RNA polymerase: insights into the mechanisms of viral RNA synthesis. *Nat Rev Microbiol* 14: 479–493. <https://doi.org/10.1038/nrmicro.2016.87>.
 43. Stevaert A, Naesens L. 2016. The influenza virus polymerase complex: an update on its structure, functions, and significance for antiviral drug design. *Med Res Rev* 36:1127–1173. <https://doi.org/10.1002/med.21401>.
 44. Weeks SA, Lee CA, Zhao Y, Smidansky ED, August A, Arnold JJ, Cameron CE. 2012. A polymerase mechanism-based strategy for viral attenuation and vaccine development. *J Biol Chem* 287:31618–31622. <https://doi.org/10.1074/jbc.C112.401471>.
 45. Naito T, Mori K, Ushirogawa H, Takizawa N, Nobusawa E, Odagiri T, Tashiro M, Ohniwa RL, Nagata K, Saito M. 2017. Generation of a genetically stable high-fidelity influenza vaccine strain. *J Virol* 91:e01073-16. <https://doi.org/10.1128/JVI.01073-16>.
 46. Pflug A, Guilligay D, Reich S, Cusack S. 2014. Structure of influenza A polymerase bound to the viral RNA promoter. *Nature* 516:355–360. <https://doi.org/10.1038/nature14008>.
 47. Lukarska M, Fournier G, Pflug A, Resa-Infante P, Reich S, Naffakh N, Cusack S. 2017. Structural basis of an essential interaction between influenza polymerase and Pol II CTD. *Nature* 541:117–121. <https://doi.org/10.1038/nature20594>.
 48. Pauly MD, Llaure AS. 2015. Effective lethal mutagenesis of influenza virus by three nucleoside analogs. *J Virol* 89:3584–3597. <https://doi.org/10.1128/JVI.03483-14>.
 49. Mori K, Murano K, Ohniwa RL, Kawaguchi A, Nagata K. 2015. Oseltamivir expands quasispecies of influenza virus through cell-to-cell transmission. *Sci Rep* 5:9163. <https://doi.org/10.1038/srep09163>.
 50. Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252:324–330. <https://doi.org/10.1006/viro.1998.9508>.
 51. Suarez P, Valcarcel J, Ortin J. 1992. Heterogeneity of the mutation rates of influenza A viruses: isolation of mutator mutants. *J Virol* 66:2491–2494.
 52. Graham RL, Becker MM, Eckerle LD, Bolles M, Denison MR, Baric RS. 2012. A live, impaired-fidelity coronavirus vaccine protects in an aged, immunocompromised mouse model of lethal disease. *Nat Med* 18:1820–1826. <https://doi.org/10.1038/nm.2972>.
 53. Maassab HF. 1967. Adaptation and growth characteristics of influenza virus at 25 degrees C. *Nature* 213:612–614. <https://doi.org/10.1038/213612a0>.
 54. Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H. 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology* 306:18–24. [https://doi.org/10.1016/S0042-6822\(02\)00035-1](https://doi.org/10.1016/S0042-6822(02)00035-1).
 55. Jin H, Zhou H, Lu B, Kemble G. 2004. Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. *J Virol* 78:995–998. <https://doi.org/10.1128/jvi.78.2.995-998.2004>.
 56. Zhou B, Li Y, Belser JA, Pearce MB, Schmolke M, Subba AX, Shi Z, Zaki SR, Blau DM, Garcia-Sastre A, Tumpey TM, Wentworth DE. 2010. NS-based live attenuated H1N1 pandemic vaccines protect mice and ferrets. *Vaccine* 28:8015–8025. <https://doi.org/10.1016/j.vaccine.2010.08.106>.
 57. Nogales A, DeDiego ML, Topham DJ, Martinez-Sobrido L. 2016. Rearrangement of influenza virus spliced segments for the development of live-attenuated vaccines. *J Virol* 90:6291–6302. <https://doi.org/10.1128/JVI.00410-16>.
 58. Hatta Y, Hatta M, Bilsel P, Neumann G, Kawaoka Y. 2011. An M2 cytoplasmic tail mutant as a live attenuated influenza vaccine against pandemic (H1N1) 2009 influenza virus. *Vaccine* 29:2308–2312. <https://doi.org/10.1016/j.vaccine.2011.01.023>.
 59. Mueller S, Coleman JR, Papamichail D, Ward CB, Nimnual A, Fletcher B, Skiena S, Wimmer E. 2010. Live attenuated influenza virus vaccines by computer-aided rational design. *Nat Biotechnol* 28:723–726. <https://doi.org/10.1038/nbt.1636>.
 60. Broadbent AJ, Santos CP, Anafu A, Wimmer E, Mueller S, Subbarao K. 2016. Evaluation of the attenuation, immunogenicity, and efficacy of a live virus vaccine generated by codon-pair bias de-optimization of the 2009 pandemic H1N1 influenza virus, in ferrets. *Vaccine* 34:563–570. <https://doi.org/10.1016/j.vaccine.2015.11.054>.
 61. Fan RL, Valkenburg SA, Wong CK, Li OT, Nicholls JM, Rabadan R, Peiris JS, Poon LL. 2015. Generation of live attenuated influenza virus by using codon usage bias. *J Virol* 89:10762–10773. <https://doi.org/10.1128/JVI.01443-15>.
 62. Nogales A, Baker SF, Ortiz-Riano E, Dewhurst S, Topham DJ, Martinez-Sobrido L. 2014. Influenza A virus attenuation by codon deoptimization of the NS gene for vaccine development. *J Virol* 88:10525–10540. <https://doi.org/10.1128/JVI.01565-14>.
 63. Sarawar S, Hatta Y, Watanabe S, Dias P, Neumann G, Kawaoka Y, Bilsel P. 2016. M2SR, a novel live single replication influenza virus vaccine, provides effective heterosubtypic protection in mice. *Vaccine* 34: 5090–5098. <https://doi.org/10.1016/j.vaccine.2016.08.061>.
 64. Si L, Xu H, Zhou X, Zhang Z, Tian Z, Wang Y, Wu Y, Zhang B, Niu Z, Zhang C, Fu G, Xiao S, Xia Q, Zhang L, Zhou D. 2016. Generation of influenza A viruses as live but replication-incompetent virus vaccines. *Science* 354: 1170–1173. <https://doi.org/10.1126/science.aah5869>.
 65. Ui H, Yamayoshi S, Uraki R, Kiso M, Oishi K, Murakami S, Mimori S, Kawaoka Y. 2017. Evaluation of seasonal influenza vaccines for H1N1pdm09 and type B viruses based on a replication-incompetent PB2-KO virus. *Vaccine* 35:1892–1897. <https://doi.org/10.1016/j.vaccine.2017.02.041>.
 66. Hai R, Garcia-Sastre A, Swayne DE, Palese P. 2011. A reassortment-incompetent live attenuated influenza virus vaccine for protection against pandemic virus strains. *J Virol* 85:6832–6843. <https://doi.org/10.1128/JVI.00609-11>.
 67. Baz M, Boonnak K, Paskel M, Santos C, Powell T, Townsend A, Subbarao K. 2015. Nonreplicating influenza A virus vaccines confer broad protection against lethal challenge. *mBio* 6:e01487. <https://doi.org/10.1128/mBio.01487-15>.
 68. Turan K, Mibayashi M, Sugiyama K, Saito S, Numajiri A, Nagata K. 2004. Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome. *Nucleic Acids Res* 32:643–652. <https://doi.org/10.1093/nar/gkh192>.
 69. Naito T, Momose F, Kawaguchi A, Nagata K. 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J Virol* 81:1339–1349. <https://doi.org/10.1128/JVI.01917-06>.
 70. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci U S A* 96:9345–9350. <https://doi.org/10.1073/pnas.96.16.9345>.
 71. World Health Organization. 2011. Manual for the laboratory diagnosis and virological surveillance of influenza. World Health Organization, Geneva, Switzerland.
 72. Shimizu K, Handa H, Nakada S, Nagata K. 1994. Regulation of influenza virus RNA polymerase activity by cellular and viral factors. *Nucleic Acids Res* 22:5047–5053. <https://doi.org/10.1093/nar/22.23.5047>.
 73. Magoc T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27:2957–2963. <https://doi.org/10.1093/bioinformatics/btr507>.
 74. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
 75. Gaush CR, Smith TF. 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. *Appl Microbiol* 16: 588–594.
 76. Tobita K, Sugiura A, Enomoto C, Furuyama M. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med Microbiol Immunol* 162:9–14. <https://doi.org/10.1007/BF02123572>.
 77. Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Hyg* 27:493–497. <https://doi.org/10.1093/oxfordjournals.aje.a118408>.