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VACCINES AND ANTIVIRAL AGENTS

Generation of a Genetically Stable High-Fidelity Influenza Vaccine Strain

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ABSTRACT Vaccination is considered the most effective preventive means for influenza control. The development of a master virus with high growth and genetic stability, which may be used for the preparation of vaccine viruses by gene reassortment, is crucial for the enhancement of vaccine performance and efficiency of production. Here, we describe the generation of a high-fidelity and high-growth influenza vaccine master virus strain with a single V43I amino acid change in the PB1 polymerase of the high-growth A/Puerto Rico/8/1934 (PR8) master virus. The PB1-V43I mutation was introduced to increase replication fidelity in order to design an H1N1 vaccine strain with a low error rate. The PR8-PB1-V43I virus exhibited good replication compared with that of the parent PR8 virus. In order to compare the efficiency of egg adaptation and the occurrence of gene mutations leading to antigenic alterations, we constructed 6:2 genetic reassortant viruses between the A(H1N1)pdm09 and the PR8-PB1-V43I viruses; hemagglutinin (HA) and neuraminidase (NA) were from the A(H1N1)pdm09 virus, and the other genes were from the PR8 virus. Mutations responsible for egg adaptation mutations occurred in the HA of the PB1-V43I reassortant virus during serial egg passages; however, in contrast, antigenic mutations were introduced into the HA gene of the 6:2 reassortant virus possessing the wild-type PB1. This study shows that the mutant PR8 virus possessing the PB1 polymerase with the V43I substitution may be utilized as a master virus for the generation of high-growth vaccine viruses with high polymerase fidelity, low error rates of gene replication, and reduced antigenic diversity during virus propagation in eggs for vaccine production.

IMPORTANCE Vaccination represents the most effective prophylactic option against influenza. The threat of emergence of influenza pandemics necessitates the ability to generate vaccine viruses rapidly. However, as the influenza virus exhibits a high mutation rate, vaccines must be updated to ensure a good match of the HA and NA antigens between the vaccine and the circulating strain. Here, we generated a genetically stable master virus of the A/Puerto Rico/8/1934 (H1N1) backbone encoding an engineered high-fidelity viral polymerase. Importantly, following the application of the high-fidelity PR8 backbone, no mutation resulting in antigenic change was introduced into the HA gene during propagation of the A(H1N1)pdm09 candidate vaccine virus. The low error rate of the present vaccine virus should decrease the risk of generating mutant viruses with increased virulence. Therefore, our findings are expected to be useful for the development of prepandemic vaccines and live attenuated vaccines with higher safety than that of the present candidate vaccines.

KEYWORDS RNA polymerases, fidelity, influenza, influenza vaccines, reverse genetic analysis

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Vaccination is considered the most effective tool for the prevention of morbidity and mortality caused by influenza epidemics and pandemics. Generally, inactivated vaccines are utilized for influenza prophylaxis (1). These are prepared from a vaccine seed virus that is grown in embryonated chicken eggs and purified from the allantoic fluid. The influenza vaccine virus strain is updated every year to provide coverage for the circulating virus strains. Seed viruses must be antigenically similar to the circulating viruses and proliferate efficiently within eggs. However, viruses isolated from patients with influenza, including the novel H1N1 virus (2), exhibit poor growth in chicken eggs. In addition, the high pathogenicity of avian H5N1 viruses poses a challenge for vaccine production (3). The circulating H5N1 viruses also grow poorly and/or exhibit high virulence to chicken eggs, resulting in low virus yields. Therefore, manufacturing processes require the selection of high-growth (HG) reassortant viruses that retain the antigenicity of the original virus isolate. Alternatively, suitable viruses may be selected by multiple passages of the seed virus for vaccine production. Such methods are time-consuming, and it is difficult to predict whether they would be competent for the production of the appropriate vaccine seed virus.

In order to overcome this problem, reverse genetics systems have been developed for the generation of reassortant influenza viruses (4–6). The main antigenic epitopes of the influenza A virus are located on the hemagglutinin (HA) and neuraminidase (NA) transmembrane glycoproteins. The genome segments encoding the HA and NA of currently circulating strains are cloned into rescue plasmids. These plasmids, which are under the control of the polymerase I (PolI) promoter, are introduced into a mammalian cell line, together with plasmids carrying the remaining six genome segments. The reassortant virus is then reconstituted using plasmids expressing the influenza virus polymerase subunits, PB1, PB2, and PA, and nucleoprotein (NP). The reconstituted vaccine strain containing the HA- and NA-encoding genes is designated a 6:2 reassortant virus, as it comprises six genome segments from a high-growth master virus and two genome segments (HA and NA) from the circulating wild-type virus. A high-growth virus, A/Puerto Rico/8/1934 (PR8), has been developed as the backbone master virus. The WHO has approved the use of this reassortant virus for pandemic influenza vaccine production (7). In order to improve antigen yields of the reassortant vaccine virus in eggs further, the vaccine seed virus may require egg adaptation, which involves multiple passages in eggs (2, 8). The egg adaptation procedure is generally applied to any low-producing vaccine candidate strain. However, viral replication during the egg adaptation process frequently results in gene mutations, which may alter the antigenic properties of the HA and NA proteins derived from the wild-type viruses of concern (9). Optimal vaccine production requires that seed viruses exhibit high growth in eggs, without significant changes in their antigenic properties.

RNA viruses exhibit the highest mutation rates (10); therefore, amino acid changes occur frequently in the influenza virus proteins as a result of mutations during the egg adaptation process. The molecular basis of the high mutation frequency of RNA viruses, such as poliovirus and the influenza virus, lies in the high error rates of viral RNA-dependent RNA polymerases that lack efficient proofreading capabilities; these rates are estimated to be as high as 10^{-3} to 10^{-5} errors per nucleotide site per round of viral replication. The high mutation rates generate considerable viral genome diversity, thereby promoting rapid evolution through selection of viruses with higher fitness, and enable RNA viruses to adapt to new host cells rapidly. The increase in the fidelity of viral RNA (vRNA) polymerase has been shown to reduce the pathogenicity of poliovirus, chikungunya virus, West Nile virus, enterovirus 71, and coxsackievirus B3, leading to an attenuated virus phenotype, and to restrict quasispecies complexity (11–16). The development of an attenuated poliovirus vaccine, in which the viral RNA polymerase was engineered to exhibit enhanced fidelity, has been reported previously (17, 18). It was shown that the high-fidelity poliovirus vaccine strain was genetically stable and exhibited an impaired ability to revert to a pathogenic wild-type phenotype. In animal models, such viruses showed a decreased ability to spread through the infected organisms, suggesting a reduced risk of spread of the live viruses into the environment.

Recently, it has been reported that an influenza virus encoding high-fidelity RNA polymerase was generated by screening ribavirin-resistant H3N2 viruses and that the single amino acid substitution of V43I in the PB1 subunit increased selectivity to a guanosine RNA substrate in H3N2 and H5N1 viruses (19). Ribavirin is a guanosine nucleotide analog that exerts pleiotropic effects on the repression of viral proliferation (20). The inhibition of cellular IMP dehydrogenase activity by ribavirin treatment, which results in a decrease in intracellular GTP levels, reduces viral protein translation and RNA replication. Furthermore, ribavirin triphosphate is directly incorporated into the viral genome during RNA synthesis, resulting in the induction of transition mutations. Ribavirin has been used to select high-fidelity variants of the *Picornaviridae* and *Togaviridae* families (21, 22). In the case of influenza H3N2 and H5N1 viruses, the PB1-V43I mutation has been found to reduce the binding affinity of PB1 for ribavirin and lead to increased selectivity for nucleotide triphosphates (NTPs), thereby restricting viral genetic diversity and reducing lethality and neurovirulence (19). This phenomenon has also been observed in infection by high-fidelity poliovirus (16–18).

Based on these findings, we hypothesized that applying a high-fidelity polymerase to a vaccine master virus would be a rational strategy for reduction of the frequency of antigenic alterations during vaccine production. Therefore, by introducing the PB1-V43I substitution into the PB1 of the high-growth PR8 master strain, we generated a master influenza A virus vaccine with both a high-growth property and high genetic stability in eggs. Indeed, biochemical assays using purified viral RNA polymerase complexes revealed that the rate of misincorporation was reduced by the PB1-V43I substitution. To further elucidate the effect on antigenic change during egg passaging, we constructed high-fidelity 6:2 reassortant viruses between A(H1N1)pdm09 and the mutant PR8 virus encoding PB1-V43I. As a result, although the reassortant viruses replicated to titers comparable to the titer of PR8 wild-type virus, the PB1-V43I substitution was found to reduce the mutation frequency. Moreover, although amino acid substitutions K153E and N156D were predicted at the possible antigenic Sa site in the HA protein (23, 24), no antigenic change was detected by a hemagglutination inhibition (HAI) test during serial passaging for egg adaptation. Consequently, our findings demonstrated that the high-fidelity viral polymerase is useful for the generation of influenza vaccine viruses which have high genetic stability without inducing antigenic alterations.

RESULTS

Effect of substitution of the PB1 Val-43 residue on viral polymerase activity. We first tested whether a V43I substitution in the PB1 subunit of the wild-type H1N1 PR8 strain affected its viral polymerase activity. The N-terminal amino acid sequences of the PB1 subunit from various strains were aligned, as shown in Fig. 1A. The viral RNA polymerase complex is a heterotrimer consisting of the PB1, PB2, and PA subunits. PB1, which binds to the viral genome promoter region and contains the conserved motifs characteristic of RNA polymerases, functions as a catalytic subunit for the sequential addition of nucleotides to the nascent RNA chains (25). This region, which contains the Val-43 residue, was found to be extremely conserved, suggesting that the V43I substitution in PR8-PB1 does not markedly affect the function and structure of the polymerase. Viral polymerase activity was evaluated using a minireplicon system in mammalian cells (26). The level of viral RNA synthesis from PR8-PB1-V43I virus was reduced to 45% of that in the wild-type PR8-PB1 virus (Fig. 1B). Previous studies have shown that the V43I mutation in H3N2 and H5N1 viruses reduced the polymerase activity by approximately 40% compared to that of wild-type PB1 virus and indicated that the generation of H3N2 and H5N1 recombinant PB1-V43I viruses is possible (19). Similarly, viral RNA polymerase activity in viruses with mutant PB1s with V43A, V43L, and V43T substitutions was reduced, whereas PB1 activity was almost lost following a V43M or V43S substitution (Fig. 1B). In order to maintain the polymerase activity, permissible amino acid substitutions at the Val-43 residue are extremely limited. Overall, these data suggested that the V43I mutation can be applied to the high-growth PR8 backbone master virus to improve replication fidelity.

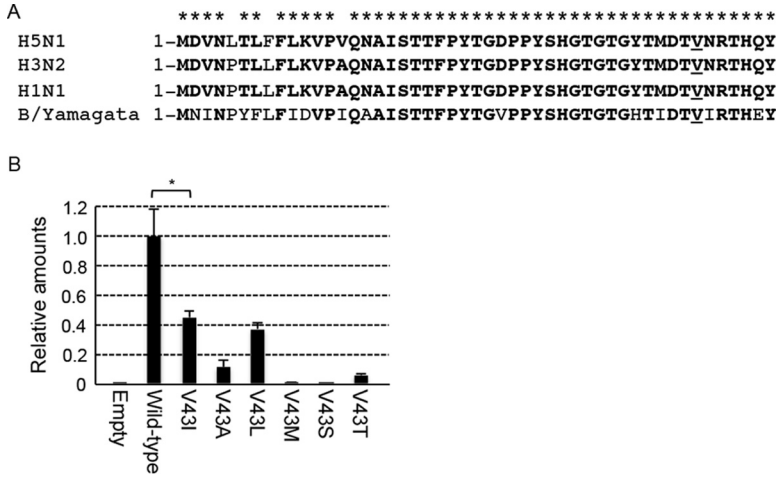

FIG 1 The importance of PB1 Val-43 residue in viral RNA synthesis. (A) The Val-43 residue is conserved in the PB1 viral polymerase subunit. Sequence alignments of N-terminal regions from the indicated H5N1, H3N2, H1N1, and B/Yamagata PB1 subunits are shown. The conserved residues are indicated in bold, and the conserved residues among influenza A viruses are indicated by asterisks. The valine 43 residue is underlined. (B) The effect of Val-43 amino acid substitution on viral RNA polymerase activity using a reporter assay system for the influenza virus genome replication. 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-V43-mutant. At 20 h posttransfection, cells were harvested and assayed for luciferase activity. The luciferase activity was normalized relative to the *Renilla* luciferase activity. Quantitative results are presented as the averages with the standard deviations from at least four independent experiments. Significance was determined using Student's *t* test (*, $P < 0.005$).

The PB1 Val-43 residue controls NTP misincorporation during RNA synthesis. Using an *in vitro* limited elongation assay, we showed that the Val-43 residue is a determinant of fidelity. Polymerases pause at template sequences at which limited nucleotides are to be incorporated; this pausing triggers the incorporation of an incorrect nucleotide. This process, termed misincorporation, generates mutations in newly synthesized DNA or RNA. Polymerase fidelity was evaluated by detection of misincorporation products generated from the model viral RNA template in the UTP-limited system (Fig. 2). The purified influenza viral RNA polymerase binds to the model RNA genome; this results in elongation as the template sequence contains the 5′- and 3′-terminal sequences of segment 1 (27). In the limited elongation assay with a model 120-nt template (Fig. 2A, upper), the viral RNA polymerase proceeded up to the first adenine residue on the template and generated a 60-nt (60 nucleotide [nt]) RNA product in the absence of UTP (Fig. 2B, lanes 1 and 2, 60-nt band). The induction of nucleotide misincorporation by the viral polymerase on the first adenine during RNA synthesis resulted in the enzyme proceeding to the second adenine residue, thereby generating 109-nt RNA products (Fig. 2B, lanes 1 and 2, 109-nt band). The fidelity of the influenza viral RNA polymerase may be evaluated by monitoring the synthesis of 109-nt RNA products in this system.

In the presence of UTP, the level of synthesis of 120-nt products from the PB1 wild-type viral RNP (vRNP) was approximately equal to that from PB1-V43I vRNP (Fig. 2B, lanes 3 and 4, and C). These results imply that the PB1-V43I substitution did not affect elongation activity in vRNP during RNA synthesis. In contrast, the amount of 109-nt RNA products synthesized by PB1-V43I vRNP was lower than that of the PB1 wild-type vRNP (Fig. 2B, lanes 1 and 2, 109-nt band). Further, the PB1-V43I RNA polymerase exhibited about a 1.5-fold enhancement in fidelity when 60 ng of vRNP was used (Fig. 2C, middle panel). These results indicate that the frequency of misincorporation during RNA synthesis decreased following the replacement of Val-43 by Ile in PB1 polymerase. Therefore, our data represent biochemical evidence that the PB1-V43I substitution in the H1N1 strain is associated with enhanced fidelity.

Generation of reassortant viruses between A(H1N1)pdm09 and PR8 viruses PB1 wild type or PB1-V43I. In order to compare the RNA polymerase activity and

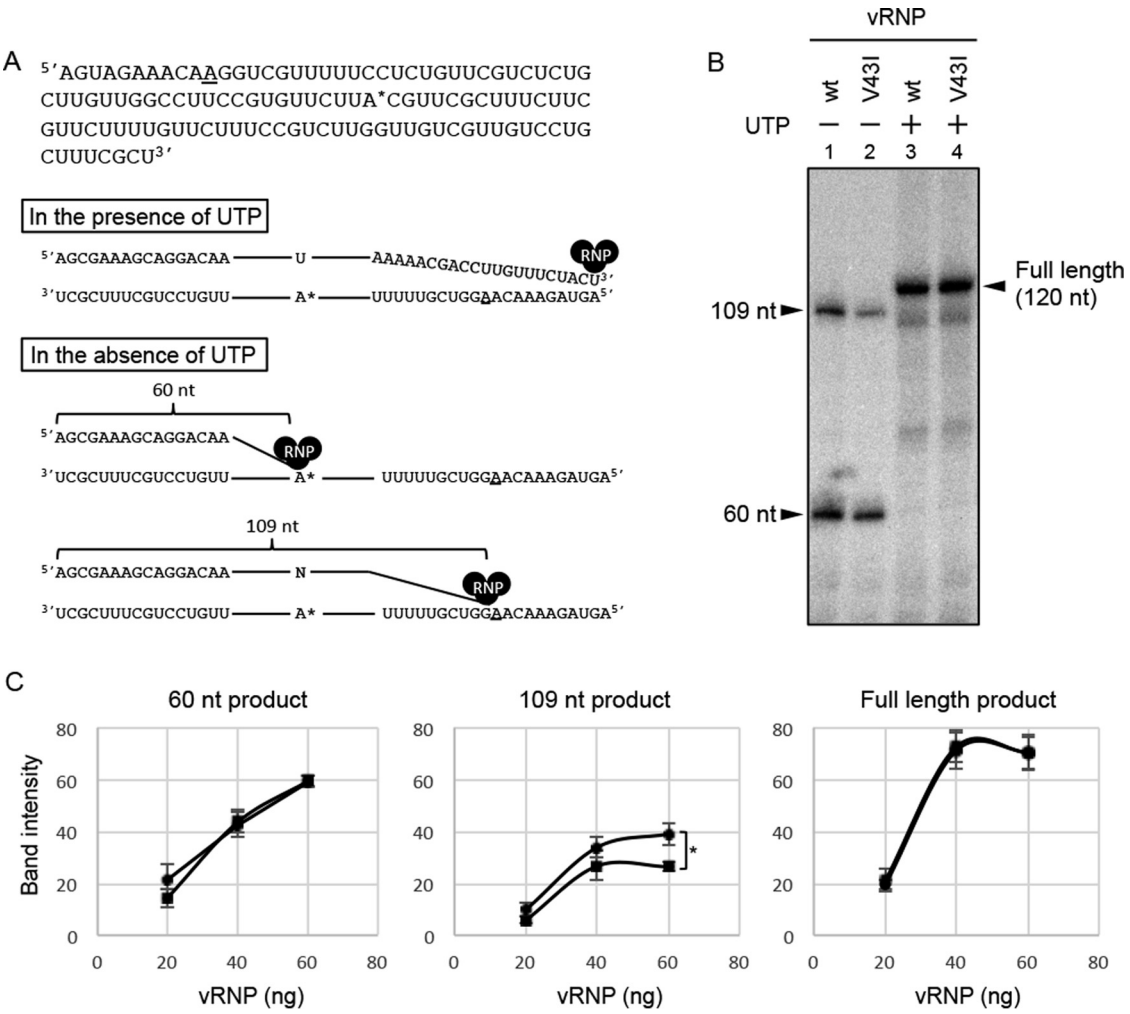

FIG 2 PB1-Val-43 residue is determinant of fidelity. (A) Illustration of an *in vitro* limited elongation assay using a 120-nt model viral RNA template. In the presence of UTP, viral polymerase generates the full-length RNA product (120 nt). In the absence of UTP, RNA synthesis was paused at the first adenine residue at nucleotide position 61 from the 3′ terminus. The synthesized RNA was elongated at a second adenine residue (nucleotide position 110 from the 3′ terminus) by misincorporation of nucleotides at the first adenine residue. The first and the second adenines from the 3′ terminus are indicated by an asterisk and underlining, respectively. (B) Reaction products from vRNP-catalyzed nucleotide incorporation under the indicated condition. A limited elongation assay was carried out with the 120-nt model vRNA and 40 ng of vRNP at 37°C for 1 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of UTP. (C) PB1-V43I polymerase is more faithful than the wild-type enzyme. A limited elongation assay was carried out under the same conditions as for the experiment shown in panel B using 20 ng, 40 ng, and 60 ng of vRNP. The amounts of the RNA products of 60 nt, 109 nt, and 120 nt derived from the PB1-V43I mutant were compared with those of the PB1 wild type. Band intensities (shown as arbitrary units) were determined after subtraction of the background using ImageJ, version 1.4.3.67, image analysis software. Quantitative results are presented as the averages with the standard deviations from at least three independent experiments. Significance was determined using Student's *t* test (*, $P < 0.05$). Circles, vRNP of PB1 wild type; squares, vRNP of PB1-V43I. wt, wild type.

fidelity of the PR8 master viruses with those of the PB1 wild-type and PB1-V43I viruses, we generated reassortant viruses with the V43I substitution in the PB1 segment, using pPol1 plasmids encoding HA and NA cDNAs derived from A(H1N1)pdm09, and compared their biological properties with those of the wild type.

First, propagation of each master virus in chicken eggs was examined via infectivity assay and HA test. HA titers of primary isolates of HG-PR8-PB1-wild type and HG-PR8-PB1-V43I viruses were 4,096 and 2,048 HA units, respectively (Table 1). Infectivity of both master viruses grown in eggs reached the order of 10^8 PFU/ml. These findings showed that the PB1-V43I substitution did not affect the growth of the PR8 master virus in eggs. However, both of the 6:2 reassortant viruses replicated very poorly in eggs, with titers lower than 2 HA units (Table 1). Previous reports have shown that, in order to improve the HA yields in eggs of the conventional vaccine candidate virus between

TABLE 1 Virus growth potential in eggs

Reassortant virus	HA titer	Growth (PFU/ml)
HG-PR8-PB1-wild type	4,096	5.0×10^8
HG-PR8-PB1-V43I	2,048	1.0×10^8
PB1-wt-A(H1N1)pdm09(6:2)	<2	NT[a]
PB1-V43I-A(H1N1)pdm09(6:2)	<2	NT

[a]Not tested.

the A(H1N1)pdm09 virus and HG-PR8 with wild-type PB1, the virus is required to undergo egg adaptation via serial passages in eggs (2, 28). HA titers of both of the egg-passaged 6:2 reassortant viruses increased gradually with the number of passages (Fig. 3). The PB1-V43I-A(H1N1)pdm09(6:2) (abbreviated here as PB1-V43I-6:2) virus gained a high HA titer similar to that of the PB1-wt-A(H1N1)pdm09(6:2) (where wt is wild type; abbreviated here as PB1-wild type-6:2) virus. These results suggest that the mutation(s) responsible for egg adaptation occurred in the HA and/or NA genes of both of the vaccine candidate viruses at similar rates.

PB1-V43I substitution reduces mutational frequencies of the H1N1 vaccine virus. In order to examine the effect of the PB1-V43I substitution on fidelity, we compared the mutational frequency of the PB1-wild type-6:2 and PB1-V43I-6:2 viruses. A system for the measurement of error rates in the nonstructural (NS) gene of influenza virus was established using next-generation sequencing (NGS) analysis (29). The NS genes of PB1-wild type-6:2 and PB1-V43I-6:2 viruses that were passaged in eggs were analyzed (Table 2). In the twice-passaged viruses, the mutational frequency of the PB1 wild-type vaccine (10.6 mutations per 10^5 nucleotides) was 1.1-fold higher than that of the PB1-V43I vaccine (9.5 mutations per 10^5 nucleotides). Furthermore, the mutation rates were found to be elevated to 1.9-fold between PB1 wild-type (28.5 mutations per 10^5 nucleotides) and PB1-V43I (14.7 mutations per 10^5 nucleotides) vaccine viruses, as calculated using each virus that was passaged 12 times.

Amino acid substitutions and antigenic changes following virus passaging in eggs. In order to identify the position at which amino acid substitution occurred in the HA and NA proteins, we analyzed the nucleotide sequences of the HA- and NA-specific reverse transcription-PCR (RT-PCR) products from the viral genome of each egg-adapted virus. The observed amino acid changes are summarized in Table 3. For the PB1-wild type-6:2 virus, which underwent egg passages 12 times, seven amino acid residues were replaced at positions 108 (V108M), 116 (I166M), 153 (K153E), 154 (K154N), 155 (G155E), 156 (N156D), and 223 (Q223R) in the HA protein, and three amino acid residues in the NA protein were altered at positions 40 (L40P), 83 (V83A), and 146

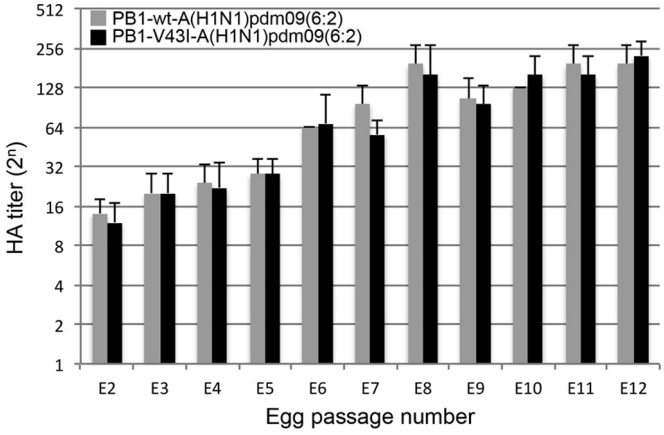

FIG 3 Growth kinetics of 6:2 vaccine viruses in egg passage. HA titers of viruses that were isolated and passaged in eggs ($n = 3$ to 4) were determined from the 1st to 12th passages. Quantitative results are presented as the averages with the standard deviations from at least four independent experiments.

TABLE 2 Mutational frequencies of the PB1-wild type-6:2 and PB1-V43I-6:2 vaccine viruses[a]

Test virus	Passage no. in eggs	No. of mutations	No. of quality-filtered reads	Mutational frequency[b]
PB1-wt-A(H1N1)pdm09(6:2)	2	84 ± 36	2,600 ± 830	10.6 ± 1.7†
	12	176 ± 62	2,038 ± 664	28.5 ± 1.2‡
PB1-V43I-A(H1N1)pdm09(6:2)	2	76 ± 50	2,650 ± 1876	9.5 ± 1.6†
	12	85 ± 33	1,920 ± 603	14.7 ± 1.5‡

[a]With the exception of passage number, values are averages ± standard deviations from three independent experiments. PB1-wild type-6:2, PB1-wt-A(H1N1)pdm09(6:2); PB1-V43I-6:2, PB1-V43I-A(H1N1)pdm09(6:2).
[b]Mutational frequency was determined from 10^5 nucleotides sequenced. The NS gene (nucleotides 63 to 365) of the viruses isolated from embryonated chicken eggs were subjected to NGS analysis. Results for the two vaccine viruses in passages 2 and 12 were compared, and significance was determined by a Student's *t* test. †, $P > 0.5$; ‡, $P < 0.05$.

(N146K). In contrast, only three amino acid changes were found in the PB1-V43I-6:2 virus, at positions 153 (K153E), 156 (N156D), and 223 (Q223R) on the HA protein. Substitution at residue 223 (Q223R) of HA has been observed to occur during the propagation of viruses in eggs (28, 30); in the present study, this mutation also appeared in both PB1-wild type-6:2 and PB1-V43I-6:2 viruses during egg adaptation.

L40P, V83A, and N146K substitutions in the NA were observed only with the PB1-wild type-6:2 virus following prolonged passaging. Mutant A(H1N1)pdm09 viruses with an amino acid substitution in NA at the Leu-40 or Val-83 residue were isolated in the 2013-2014 influenza season (31). There is currently no evidence that these substitutions exert specific functional effects. The N146 residue is the predicted site of N-linked glycosylation in all NA subtypes (32), alteration of which can affect NA activity in N1 and N8 subtypes (33, 34). The N146K mutation in NA of A(H1N1)pdm09 might affect substrate preference and/or virus proliferation.

Furthermore, these data suggested that the total number of amino acid substitutions introduced into the HA and NA proteins of A(H1N1)pdm09 (Table 3) was nearly consistent with the 2-fold difference in error rates observed between the PB1 wild-type and PB1-V43I vaccine viruses (Tables 2 and 3).

Next, in order to confirm the occurrence of antigenic changes following egg adaptation, we performed an HAI assay using antisera from postinfection guinea pigs (Table 4). The HAI titer of the 6:2 reassortant viruses passaged twice in eggs was 640 units each. The HAI titers of PB1-V43I-6:2 viruses that were passaged 12 times were the same as those of the viruses passaged twice. In contrast, the HAI titer of the PB1-wild type-6:2 virus was reduced from 640 to 160 following 12 passages in 3 of the 4 groups. Tables 3 and 4 show that the K154N mutation in group 3 and the G155E mutation in groups 1 and 2 were accompanied by low HAI titers. Several studies have attributed the low HAI titer to the amino acid substitutions at positions 154 and 155 of the HA protein (28, 30). On the other hand, the amino acid changes K153E and N156D, which occurred

TABLE 3 Amino acid substitutions in egg-passaged viruses

Reassortant virus	Expt no.[a]	Substitution(s) in: HA[b]	NA
PB1-wt-A(H1N1)pdm09(6:2)	1	I116M, G155E, N156D, Q223R	L40P, V83A
	2	K153E, G155E, Q223R	N146K
	3	K154N, Q223R	None
	4	V108M, Q223R	None
PB1-V43I-A(H1N1)pdm09(6:2)	1	N156D, Q223R	None
	2	K153E, Q223R	None
	3	Q223R	None
	4	Q223R	None

[a]Data represent results from four independent virus serial passages performed with 3 to 4 eggs per group.
[b]The amino acid number denotes the position in the sequence of the H1 subtype. Residues 154 and 155 were involved in a change in antigenicity. The Q223R substitution is required for egg adaptation.

TABLE 4 HAI assay of vaccine viruses with guinea pig antiserum

Test antigen	Expt no.[a]	Passage no. in eggs	HAI titer[b]
PB1-wt-A(H1N1)pdm09(6:2)	1	2	640
		12	**160**
	2	2	640
		12	**160**
	3	2	640
		12	**160**
	4	2	640
		12	640
PB1-V43I-A(H1N1)pdm09(6:2)	1	2	640
		12	640
	2	2	640
		12	640
	3	2	640
		12	640
	4	2	640
		12	640

[a]Data represent results from four independent virus serial passages performed with 3 to 4 eggs per group.
[b]Titers were determined using guinea pig antiserum against A(H1N1)pdm09. Boldface indicates a 4-fold difference in values from those of viruses passaged twice in eggs.

in both 6:2 viruses, were located at the antigenic epitope Sa site (23, 24). However, the antigenic properties of the virus were indistinguishable from those of the original A(H1N1)pdm09 virus, as indicated by HAI testing using guinea pig antiserum. This finding indicated that the amino acid change in HA during egg adaptation, but not the mutation in the antigenic site, was introduced into the PB1-V43I-6:2 virus during egg passaging. However, the mutation in the antigenic site was introduced into the HA protein of the PB1-wild type-6:2 virus. The egg-adapted A(H1N1)pdm09 vaccine retained its immunogenicity as a result of the PB1-V43I substitution; this finding was attributed to the increased fidelity of RNA synthesis exhibited by the PB1-V43I virus relative to that of the PR8 wild type. These data suggest that the reduction of mutational frequencies during genome replication of the PB1-V43I vaccine virus leads to the introduction of egg adaptation mutations but not to antigenic changes.

DISCUSSION

In this study, we generated a high-fidelity PR8-based vaccine master virus in which the viral RNA polymerase exhibited a lower mutation rate. Val-43 was replaced by Ile in the PB1 subunit of the present vaccine; the PB1-V43I substitution is known to increase selectivity to nucleotides in H3N2 and H5N1 viruses, resulting in increased fidelity of viral polymerase and reduction in genetic diversity of the viral population (19). The Val-43 residue and amino acid sequences of the N-terminal region of the PB1 subunit are highly conserved between influenza A virus strains. It was therefore expected that the introduction of the PB1-V43I single mutation would enhance the polymerase fidelity, thereby reducing antigenic alterations of influenza vaccine viruses during egg adaptation/propagation processes.

We constructed the A(H1N1)pdm09 vaccine candidate virus containing the V43I substitution in the PB1 subunit and conducted 12 parallel serial passages in eggs until a high hemagglutination titer was obtained. Deduced amino acid substitutions occurred in both viruses after passages in eggs: seven in HA and three in NA of the PB1 wild-type virus and three in HA and none in NA of the PB1-V43I mutant virus (Table 3). An egg adaptation mutation leading to a Q223R substitution in HA was found to have occurred in the passaged viruses encoding both wild-type PB1 and PB1-V43I. The Q223R mutation in the receptor binding site improves infectivity in egg and in human cells without changing HA antigenicity (28, 35). Interestingly, the K154N and G155E antigenic mutations were introduced into the HA of the PB1 wild-type vaccine virus following passages; however, these mutations were not found in the PB1-V43I vaccine strain (Tables 3 and 4). Our results showed that a single mutation at residue 153 or 156

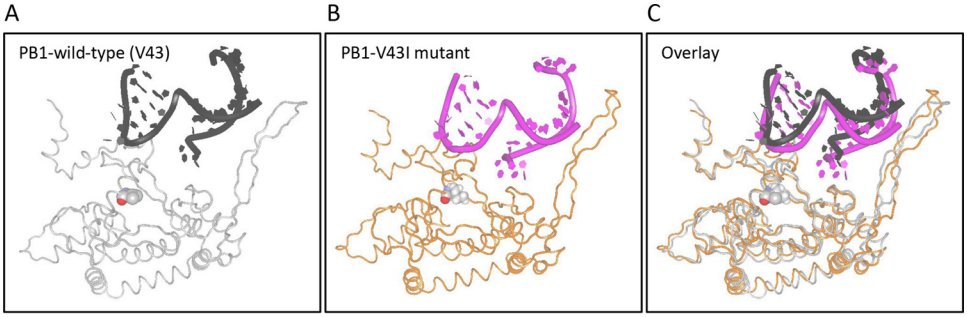

FIG 4 PB1-V43I substitution leads to distortion of viral promoter position. Structures A and B show the homology models, respectively, of the wild-type (peptide is shown in silver) and V43I mutant (peptide is shown in orange) PR8 PB1 polymerase (amino acids 1 to 314). The viral RNAs are shown in black (A) and magenta (B). The Val-43 and the Ile-43 residues are indicated as space-filling Corey-Pauling-Koltun models. An overlay of the structures is shown in panel C.

of the Sa site did affect antigenicity although these mutations were found in both vaccine strains. K153E and N156D may act in coordination with another residue mutation(s) to alter the antigenicity.

In order to confirm the effect of the PB1-V43I single mutation on PR8 polymerase fidelity, the mutational frequencies between PB1 wild-type and PB1-V43I viruses were calculated via NGS analysis and a limited elongation assay (Fig. 2 and Table 2). The vaccine encoding PB1 wild-type virus showed a 1.9-fold higher mutational frequency than the PB1-V43I vaccine after 12 passages in eggs. Furthermore, purified PB1-V43I polymerase showed increased fidelity during RNA synthesis in an *in vitro* biochemical assay. These results imply that the PB1-V43I substitution may be applied to the construction of genetically stable high-fidelity vaccine backbone viruses with reduced frequencies of antigenic mutations during egg adaptation.

All DNA and RNA polymerases share an overall structure that resembles a cupped right hand containing "finger," "palm," and "thumb" domains and catalyze phosphodiester bond formation through a conserved two-metal ion-based mechanism (36, 37). Recently, the crystal structures of the complete heterotrimeric influenza A virus and influenza B virus polymerases bound to the viral RNA promoter have been reported (38, 39). According to these structural data, the Val residue at amino acid position 43 is localized in the PB1 helix α1 domain (amino acids 25 to 49). The PB1 helix α1 is one of component of the finger domain that comprises the putative NTP entrance tunnel site. This helix α1 domain contains a crucial, highly conserved PB1 basic residue, Arg-45, that is involved in NTP binding and nucleotidyltransferase reactions. Our group reported that a single amino acid mutation in the PB1 helix α1 domain induced changes in nucleotide recognition and the viral RNA synthesis reaction (40, 41). To examine the structural effects of V43I substitution, we built a homology model using the PB1 polymerase of bat/Guatemala/060/2010 (H17N10), with which PR8-PB1 shares 79% identity, as a template (38) (Fig. 4A and B). PB1-V43I shares the overall structure with wild-type PB1 (Fig. 4C). On the other hand, V43I leads to a distortion of the viral RNA promoter position compared to that of the PB1 wild-type model. These arrangement changes might produce a high-fidelity polymerase. Our current results suggest that the Val-43 substitution might induce a structural change in the PB1 helix α1 domain, giving rise to a faithful polymerase for nucleotide selection.

For a high-fidelity polymerase enzyme to be suitable for use in a vaccine backbone, the generated seed virus must retain a high growth rate and HA protein yield. Our results indicate that a 1.5- to 1.9-fold-increase in polymerase fidelity did not greatly affect the growth of the PR8 virus. The use of the PB1-V43I virus backbone enabled a reduction in the frequency of occurrence of antigenic mutations during egg adaptation. Our findings should be highly useful for the development of improved influenza vaccines. Although the use of the PB1-V43I virus backbone in A(H1N1)pdm09 vaccine preparation successfully repressed the introduction of antigenic mutations by enabling

a 1.9-fold increase in PB1-V43I polymerase fidelity, the antigenic mutation may occur in a vaccine candidate virus generated from another circulating strain. Therefore, further clarification of the fundamental mechanism underlying nucleotide selection during the catalysis of RNA synthesis is required in order to improve the fidelity of influenza virus polymerase.

In the last decade, the isolation of RNA viruses with altered mutational frequencies has shown that polymerase fidelity may be affected by single amino acid substitutions. In particular, ribavirin-resistant poliovirus with a G64S mutation in its viral polymerase has been shown to exhibit increased replication fidelity (21). Similar results have been observed with regard to a K359R substitution in poliovirus polymerase (18). These engineered viruses with enhanced polymerase fidelity are highly useful for vaccine development as high replication fidelity reduces the frequency of genetic reversion to the pathogenic wild-type phenotype; moreover, reduced genetic variation may lead to viral attenuation (17, 18). Lys-359 of the poliovirus polymerase is reported to be an active-site lysine residue that contributes to the catalytic efficiency of all polymerases, including viral RNA-dependent RNA polymerases (42). RNA polymerase with a K359R substitution has been shown to catalyze nucleotidyl transfer at a 10-fold lower rate than the wild type; however, the fidelity of the former is 5-fold higher. Structural data of the influenza A virus polymerase (38) indicate that the Lys residue at amino acid position 480 or 481 in polymerase motif D of the PB1 subunit represents a highly conserved residue involved in catalytic nucleotidyl transfer. Evaluation of the functional effects of the Lys-to-Arg substitution in the influenza virus PB1 subunit (PB1-K480R or PB1-K481R) may lead to the discovery of a novel polymerase mutant with higher fidelity than the V43I type. Mutational analyses focusing on these Lys residues are currently being performed in our laboratory.

An immediate application of the high-fidelity backbone is in the development of a live attenuated vaccine against seasonal and potential pandemic influenza viruses (8). The live attenuated influenza virus (LAIV) vaccine strategy additionally represents an equally promising means for pandemic vaccination as the use of inactivated vaccines since live viruses induce strong immune responses in unprimed populations, potentially allowing for the use of low doses. LAIV vaccine candidates are currently developed using nonreplicating or temperature-sensitive mutant viruses and have been tested in mice, ferrets, chickens, and African green monkeys, as well as in clinical studies in humans (43–46). It is reported that the LAIV vaccine exhibits cross-protection between various clades of the H5N1 virus and broad protection against homologous and heterosubtypic viruses. However, the disadvantage associated with the use of LAIV vaccines is the risk of generating mutant viruses, which may lead to the emergence of strains with increased virulence. We expect that the use of the high-fidelity backbone virus for LAIV vaccine production should lower this risk, enabling the production of vaccines with a higher degree of safety than that of the present vaccine candidates.

We demonstrated that the high-fidelity master virus approach was useful for retaining antigenicity in egg-based vaccine generation. The egg-based vaccine production system is dependent on a continuous supply of eggs. Therefore, in case of a pandemic, the egg-based system may not meet global vaccine requirements due to the limited availability of eggs. Recently, a novel production system using mammalian cell culture was developed to overcome the limitations of the egg-based system. The use of mammalian cells, e.g., Madin-Darby canine kidney (MDCK) cells and African monkey kidney cells (Vero), for the production of influenza viruses has been evaluated. Recently, Ping et al. reported that a novel high-yield PR8 vaccine backbone strain isolated from random mutant virus libraries led to improvement in influenza vaccine titers in MDCK and Vero cells (47). Our findings may be additionally applied to mammalian cell-based systems for improving the efficiency of vaccine production.

MATERIALS AND METHODS

Cells and viruses. 293T and MDCK cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum (Nichirei Biosciences, Japan) and penicillin-streptomycin at 37°C

TABLE 5 Primers and DNA template used in this study

Primer name	Sequence (5′ to 3′)[a]
PB1-for	ACGACCGGTACCGCCACCACCATGGATGTCAATCCGACCTTACTTTTC
PB1-rev	ATGCATGCGCGGCCGCCTATTTTTGCCGTCTGAGCTCTTCAAT
V43I-fof	CACCATGGATACTATCAACAGGACACATC
V43I-rev	GATGTGTCCTGTTGATAGTATCCATGGTG
V43A-for	CACCATGGATACTGCCAACAGGACACATC
V43A-rev	GATGTGTCCTGTTGGCAGTATCCATGGTG
V43L-for	CACCATGGATACTCTCAACAGGACACATC
V43L-rev	GATGTGTCCTGTTGAGAGTATCCATGGTG
V43 M-for	CACCATGGATACTATGAACAGGACACATC
V43 M-rev	GATGTGTCCTGTTCATAGTATCCATGGTG
V43S-for	CACCATGGATACTAGCAACAGGACACATC
V43S-rev	GATGTGTCCTGTTGCTAGTATCCATGGTG
V43T-for	CACCATGGATACTACCAACAGGACACATC
V43T-rev	GATGTGTCCTGTTGGTAGTATCCATGGTG
Pol1-for	GTGTGTCCTGGGGTTGACCAGA
Pol1-rev	CATCGGTGATGTCGGCGATATAG
Bm-Uni-for	CGTATTCGTCTCAGGGAGCRAAAGCAGG
Bm-Uni-rev	CGATATCGTCTCGTATTAGTAGAAACAAGG
pdm-seg4-for	AGCAAAAGCAGGGGAAAATAAAAGC
pdm seg4-rev	AGTAGAAACAAGGGTGTTTT
pdm-seg6-for	AGCAAAAGCAGGAGTTTAAAATG
pdm-seg6-rev	AGTAGAAACAAGGAGTTTTTT
Seg8-for	CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGTGTGTCAAGCTT TCAGGTAG
Seg8-rev	CTATGCGCCTTGCCAGCCCGCTCAGAGCACTGTAGGTCCATTCTGAT ACAAAGAGG
T7 pro-120 mer DNA	TAATACGACTCACTATTAGTAGAAACAAGGTCGTTTTTCCTCTGTTCG TCTCTGCTTGTTGGCCTTCCGTGTTCTTACGTTCGCTTTCTTCG TTCTTTTGTTCTTTCCGTCTTGGTTGTCGTTGTCCTGCTTTCGCT

[a]Underlining indicates the codon sequence corresponding to amino acid 43 of the PB1 protein.

with 5% CO_2. The influenza A(H1N1)pdm09 virus was isolated in the Okayama Prefecture, Japan, in the 2010-2011 season. The virus was stored in liquid nitrogen until use.

Generation of protein expression plasmid for PB1 mutants. Mutations corresponding to an amino acid substitution at the Val-43 residue of the PB1 subunit were introduced into the plasmid containing the sequence encoding wild-type PB1 by site-directed mutagenesis. The Val-43 residue in the high-growth (HG) PR8 PB1 gene was changed from GTC (Val) to ATC (Ile), GCC (Ala), CTC (Leu), ATG (Met), AGC (Ser), or ACC (Thr) by PCR mutagenesis. To construct the plasmid containing the PB1-V43I coding sequence, two DNA fragments corresponding to the PB1 coding sequence were amplified by PCR using PB1-for and V43I-rev or PB1-rev and V43I-for as primers (Table 5) and pPol1-PB1-wild-type as the PCR template. The full-length PB1-V43I gene was amplified by PCR using PB1-for and PB1-rev as primers. PCR products were digested with KpnI and NotI and cloned into the KpnI- and NotI-digested pCAGGS-P7 plasmid. The resultant plasmid was designated pCAGGS-PB1-V43I. Likewise, pCAGGS-PB1-V43-mutant plasmids (V43A, V43L, V43M, V43S, and V43T) were constructed using primers listed in Table 5.

Minireplicon assay system. 293T cells were transfected with plasmids for the expression of viral proteins, PB1 (pCAGGS-PB1-wild type or pCAGGS-PB1-V43-mutant), PB2, PA, and NP, and with a plasmid (pHH-vNS-Luc) for the expression of the artificial influenza virus genome containing the firefly luciferase gene of negative sense, which is synthesized in cells by the human DNA-dependent RNA polymerase I (PolI) (26). The mRNA expressing the firefly luciferase gene was transcribed in the influenza viral RNA polymerase-dependent manner. The luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The luciferase activity was normalized relative to the *Renilla* luciferase activity using a cotransfected pRL-SV40 (where SV40 is simian virus 40) vector (Promega).

Cell-free model virus genome replication. The vRNPs (viral ribonucleoproteins) derived from HG-PR8 wild-type and HG-PR8-PB1-V43I viruses were purified from virions as previously described (48) and used as the enzyme source. The limited elongation assay was performed as previously described (49). The 120-nucleotide model vRNA (5′-AGUAGAAACAAGGUCGUUUUUCCUCUGUUCGUCUCUGCUUGUUGG CCUUCCGUGUUCUUACGUUCGCUUUCUUCGUUCUUUUGUUCUUUCCGUCUUGGUUGUCGUUGUCCUGCU UUCGCU-3′) was synthesized by T7 RNA polymerase (TaKaRa) using T7 promoter–120-mer DNA (Table 5) as the template (50). This model vRNA contains the 12-base-long conserved promoter sequence for the viral RNA polymerase at both 3′- and 5′-terminal regions and the subsequent 96-base-long sequence lacking the adenine residues except for nucleotide position 61 from the 3′ terminus. A limited elongation assay was carried out at 37°C for 1 h in a final volume of 25 μl containing 50 mM HEPES-NaOH(pH 7.9), 3 mM $MgCl_2$, 50 mM KCl, 1.5 mM dithiothreitol, 25 μM GTP, 50 μM CTP, 500 μM ATP, 5 μCi of [α-^{32}P]GTP (3,000 Ci/mmol), 8 units of RNase inhibitor (Toyobo), 250 μM ApG dinucleotide, 0.1 pmol of model vRNA, and vRNP (20, 40, and 60 ng of NP equivalents) in the absence or presence of 500 μM UTP. RNA products

were purified by phenol-chloroform extraction, subjected to 6% PAGE with 8 M urea, and visualized by autoradiography (Typhoon 9400; GE Healthcare).

Generation of PR8-PB1-V43I recombinant viruses. To construct the plasmid from which PolI transcribes the PB1-V43I RNA, we amplified two DNA fragments corresponding to the PB1 coding sequence by PCR using Pol1-rev and V43I-rev or Pol1-for and V43I-for as primers (Table 5) and pPol1-PB1-wild-type as the PCR template. The full-length PB1-V43I gene was amplified by PCR using Pol1-rev and Pol1-for as primers (Table 5). PCR product was digested with NheI and XhoI and cloned into the NheI- and XhoI-digested pPol1 plasmid. The resultant plasmid was designated pPol1-PB1-V43I. Plasmids were transfected into 293T cells, and recombinant viruses were rescued as described previously (5). Transfected 293T cells were incubated at 37°C in Opti-MEM (Thermo Fisher Scientific) with 3.5 μg/ml *N*-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma), and the supernatant was harvested at 48 h posttransfection.

Generation of 6:2 reassortant vaccine viruses. The HA and NA genes of the A(H1N1)pdm09 virus were amplified by reverse transcription and PCR using primers specific for the noncoding regions of each gene. The HA or NA cDNAs were amplified by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) with pdm-seg4-for or pdm-seg6-for primers (Table 5). The HA or NA DNA fragments were amplified by PCR using primers pdm-seg4-for and pdm-seg4-rev or pdm-seg6-for and pdm-seg6-rev (Table 5). PCR products were digested with BsmBI and cloned into the BsmBI-digested pPol1 plasmid. To create the 6:2 reassortant viruses, eight pPol1 plasmids [to express viral RNAs encoding HA and NA of A(H1N1)pdm09 and to express PB1 wild type or PB1-V43I and five internal proteins of PR8] were cotransfected with viral protein expression plasmids into 293T cells. The supernatant was collected at 48 h posttransfection and was injected into 11-day-old embryonated chicken eggs to amplify the recovered viruses.

Virus serial passages in eggs. The 6:2 reassortant viruses were diluted 10- to 10^3-fold with phosphate-buffered saline according to each virus titer, and 200 μl of virus suspension was inoculated into eggs. After each passage, from passages 2 to 12 in eggs, viruses with the highest HA titers were pooled to be used in subsequent passages.

Virus titration. Virus titer was determined by a plaque assay as described previously (51, 52). Briefly, 500 μl of aliquots of serial 10-fold dilutions of viruses was inoculated into MDCK cells in a six-well plate. After the plates were incubated for 1 h, each well was overlaid with 2.5 ml of agar medium. The number of plaques was counted following amido black 10B (Wako, Japan) staining 3 days after inoculation. The virus titer was calculated as the number of PFU per milliliter.

Preparation of antisera. A(H1N1)pdm09 virus propagated in MDCK-SIAT cells was concentrated. Subsequently, 4-week-old naive female guinea pigs (Hartley strain; Japan SLC, Japan) were primed and boosted intraperitoneally with the concentrated virus suspension mixed with an adjuvant (TiterMax Gold; CytRx Co.) at 2-week intervals. The antisera were prepared and stored at −80°C until use. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of Kawasaki Medical School prior to initiation of the study.

Hemagglutination assay and HAI assay. A hemagglutination assay and hemagglutination inhibition (HAI) assay were performed with 0.5% chicken red blood cells (CRBC; Nippon Bio-Test Laboratories, Japan) using a standard method (53). Guinea pig antisera raised against viruses at serial passages in eggs were tested by HAI assay.

Mutational frequency determination by NGS. Amplicon generation and next-generation sequencing (NGS) were performed as previously reported (29, 54). Briefly, the cDNA derived from segment 8 was amplified by PCR using the fusion primers, including multiplex identifier tag and adaptor for 454 sequencing, Seg8-for and Seg8-rev corresponding to segment 8 between nucleotide sequence positions 41 to 60 and 366 to 386, respectively (Table 5). PCR products were purified by agarose gel using a Fast Gene gel extraction kit (Nippon Genetics). Amplicons were subjected to ultradeep pyrosequencing using GSJunior (454 Life Sciences, Roche). Mutational frequency was determined as previously reported (29). The output from the GSJunior includes sequence results (FASTA) and quality scores for every sequence position in a read. It is known that the average quality score of a read is inversely proportional to the number of errors in that read (55). To suppress the error associated with NGS, we eliminated the reads having a quality score below 27 at one sequence position. The reads which were passed through the quality filter were aligned by ClustalW as previously described (56), and the number of mutations in each sequence position were counted.

Molecular modeling and dynamics simulation. Models of the PR8-PB1-wild type and the PR8-PB1-V43I mutant were built separately utilizing the structure of the bat influenza A virus polymerase (Protein Data Bank accession number 4WSB), and the software MF myPresto (Medicinally Yielding PRotein Engineering SimulaTOr), version 3.2.0.33 (FiatLux Corporation, Japan), was used to this end. The energy minimization with the myPresto/cosgene program, which is available on the Web (http://presto.protein.osaka-u.ac.jp/myPresto4/index.php?lang=en), was performed as previously described (57).

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