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Identification of PB2 Mutations Responsible for the Efficient Replication of H5N1 Influenza Viruses in Human Lung Epithelial Cells

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ABSTRACT

Highly pathogenic H5N1 avian influenza viruses have caused outbreaks among poultry worldwide, resulting in sporadic infections in humans with approximately 60% mortality. However, efficient transmission of H5N1 viruses among humans has yet to occur, suggesting that further adaptation of H5N1 viruses to humans is required for their efficient transmission among humans. The viral determinants for efficient replication in humans are currently poorly understood. Here, we report that the polymerase PB2 protein of an H5N1 influenza virus isolated from a human in Vietnam (A/Vietnam/UT36285/2010, virus 36285) increased the growth ability of an avian H5N1 virus (A/wild bird/Anhui/82/2005, virus Wb/AH82) in human lung epithelial A549 cells (however, the reassortant virus did not replicate more efficiently than human 36285 virus). Furthermore, we demonstrate that the amino acid residues at positions 249, 309, and 339 of the PB2 protein from this human isolate were responsible for its efficient replication in A549 cells. PB2 residues 249G and 339M, which are found in the human H5N1 virus, are rare in H5N1 viruses from both human and avian sources. Interestingly, PB2-249G is found in over 30% of human seasonal H3N2 viruses, which suggests that H5N1 viruses may replicate well in human cells when they acquire this mutation. Our data are of value to H5N1 virus surveillance.

IMPORTANCE

Highly pathogenic H5N1 avian influenza viruses must acquire mutations to overcome the species barrier between avian species and humans. When H5N1 viruses replicate in human respiratory cells, they can acquire amino acid mutations that allow them to adapt to humans through continuous selective pressure. Several amino acid mutations have been shown to be advantageous for virus adaptation to mammalian hosts. Here, we found that amino acid changes at positions 249, 309, and 339 of PB2 contribute to efficient replication of avian H5N1 viruses in human lung cells. These findings are beneficial for evaluating the pandemic risk of circulating avian viruses and for further functional analysis of PB2.

Highly pathogenic H5N1 influenza A viruses continue to circulate among avian species. As a result, sporadic avian-to-human transmission has occurred and the threat of a pandemic has persisted. The first human case caused by a highly pathogenic H5N1 influenza A virus was reported in Hong Kong in 1997 (1, 2). Although the mass culling ordered by the Hong Kong government temporarily ended the outbreak, a second outbreak of H5N1 viruses occurred in 2003. With the spread of H5N1 viruses among migrating birds and poultry, these viruses quickly spread beyond East Asia (3). This spread of H5N1 viruses has been accompanied by increasing reports of cases of avian-to-human transmission. As of January 2015, the total number of confirmed human cases of highly pathogenic H5N1 influenza A virus infection had reached 694, with 402 deaths (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/) (4, 5). However, the human cases of H5N1 infection have been limited mainly to individuals in close contact with infected poultry, and reports of human-to-human transmission have been extremely rare (6, 7). For an H5N1 virus to overcome the host barrier, the following conditions must be met: efficient transmission via droplets, efficient replication in the host, and immune vulnerability of the infected populace. Consequently, viruses harboring mutations that promote efficient transmission and replication in humans could lead to a pandemic.

A number of viral determinants have been identified that contribute to the adaptation of avian influenza viruses to mammalian hosts (8–28), and several large-scale comparative analyses of proteins from avian and human viruses have been performed to cat-

Received 15 November 2014 Accepted 15 January 2015

Accepted manuscript posted online 21 January 2015

Citation Yamaji R, Yamada S, Le MQ, Li C, Chen H, Qurnianingsih E, Nidom CA, Ito M, Sakai-Tagawa Y, Kawaoka Y. 2015. Identification of PB2 mutations responsible for the efficient replication of H5N1 influenza viruses in human lung epithelial cells. J Virol 89:3947–3956. doi:10.1128/JVI.03328-14.

Editor: T. S. Dermody

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doi:10.1128/JVI.03328-14

alog the amino acids that are conserved in each host species (29–31). The PB2, PB1, and PA subunits of the polymerase complex play a major role in virus pathogenicity and efficient viral growth in mammals. PB2 is particularly important in overcoming species barriers. For example, lysine at residue 627 (627K) of PB2 enhances the polymerase activity, replication efficiency, and virulence of H5N1 viruses in mammals (8–10). In addition, arginine at residue 591, which is located close to the amino acid at position 627 of PB2 in the three-dimensional structure of the protein, compensates for the lack of lysine at residue 627 and confers efficient viral replication to pandemic H1N1 viruses in mammals (11). Other amino acid substitutions, such as 701N, 271A, and 158G, in PB2 have also been identified as important markers that confer a replicative advantage and high pathogenicity to influenza viruses in mammals (12–15). Recently, we showed that the combination of amino acid substitutions 147T, 339T, and 588T in PB2 confers a high polymerase activity to avian viruses in human cells (28). It is not clear, however, whether we have identified all of the possible amino acid changes that are needed for efficient growth of H5N1 viruses in mammals.

In this study, we attempted to identify amino acids that support the efficient growth of H5N1 viruses in human lung cells. The accumulation of information on adaptive mutations to humans helps us to prepare for an unpredictable but potential H5N1 pandemic and to assess the risk of future isolates with pandemic potential. Here, we attempted to identify new molecular determinants associated with the efficient growth of a human-isolated H5N1 virus in human lung cells by generating reassortant and mutant viruses in the genetic background of an avian H5N1 virus (with low replicative ability in human lung cells) and a human H5N1 virus (with high replicative ability in human lung cells).

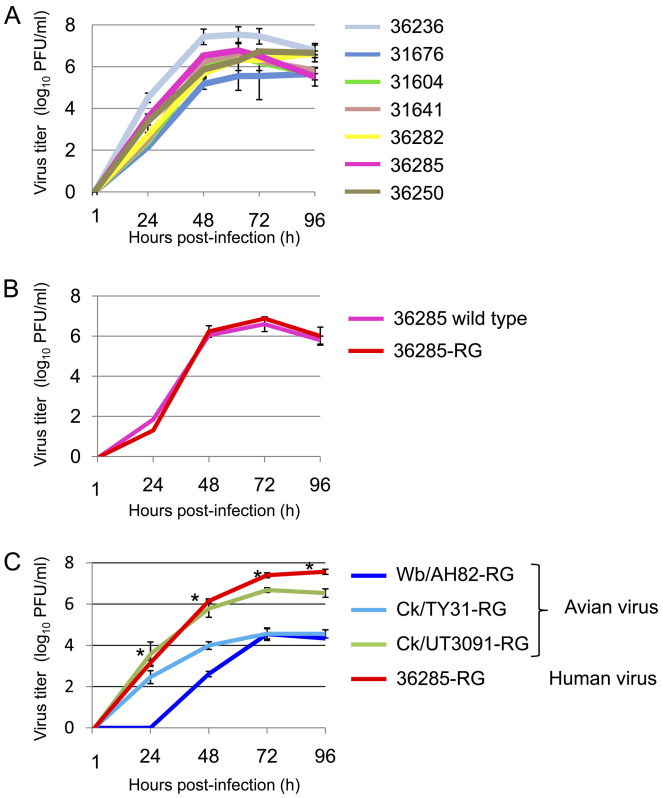

FIG 1 Comparison of the growth properties of H5N1 viruses isolated from humans and birds. (A) A549 cells were infected with H5N1 human viruses isolated in Vietnam in 2010 at a multiplicity of infection (MOI) of 0.0002 and cultured at 37°C. Virus release into cell culture supernatant was titrated by plaque assays with MDCK cells at the indicated time points. Error bars represent the standard deviations of the results of 3 independent experiments. (B) A549 cells were infected with the H5N1 human viruses 36285-wild type and 36285-RG at an MOI of 0.0002 and cultured at 37°C. Error bars represent the standard deviations of the results of 3 independent experiments. (C) A549 cells were infected with H5N1 human virus 36285-RG and avian viruses at an MOI of 0.0002 and cultured at 37°C. Supernatants were titrated by plaque assays with MDCK cells at the indicated time points. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the 36285-RG virus are significantly different from those of the Wb/AH82-RG virus ($P < 0.01$, Student's t test).

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) containing 5% newborn calf serum, vitamins, essential amino acids, and antibiotics. Human embryonic kidney 293T cells, A549 human lung adenocarcinoma epithelial cells, and DF-1 chicken embryo fibroblast cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. MDCK, 293T, and A549 cells were cultured at 37°C with 5% CO_2. DF-1 cells were cultured at 39°C with 5% CO_2 unless otherwise stated.

Viruses. In this study, we used the following 10 H5N1 viruses: A/wild bird/Anhui/82/2005 (virus Wb/AH82), A/chicken/Vietnam/TY31/2005 (virus Ck/TY31) (Genbank accession numbers EU118136.1 [hemagglutinin; HA] and EU118127.1 [neuraminidase; NA]), A/chicken/Central Java/UT3091/2005 (virus Ck/UT3091) (accession numbers GQ122490 to GQ122495, GQ122415, and GQ122395), A/Vietnam/UT36285/2010 (virus 36285), A/Vietnam/UT36282/2010 (virus 36282), A/Vietnam/UT36236/2010 (virus 36236), A/Vietnam/HN31676DH/2009 (virus 31676), A/Vietnam/UT31641II/2008 (virus 31641), A/Vietnam/UT31604I/2009 (virus 31604), and A/Vietnam/ UT36250I/2010 (virus 36250). Newly determined sequences for viral polymerase proteins PB2, PB1, and PA and the HA, nucleoprotein (NP), NA, matrix (M), and nonstructural (NS) proteins were deposited in GenBank. Throat swabs were collected from H5N1 influenza virus-infected patients in northern Vietnam and were sent to the National Institute of Hygiene and Epidemiology in Hanoi, Vietnam. To isolate H5N1 virus, clinical specimens were inoculated to MDCK cells in MEM containing 0.3% bovine serum albumin (BSA) and incubated at 37°C for 48 h. Stock viruses were propagated in MDCK cells at 37°C and stored at −80°C. 36285 was isolated from a patient who exhibited influenza symptoms and recovered (a 2-year-old female, onset on 2 April 2010, hospitalized on 4 April 2010). All experiments with H5N1 viruses were performed in a biosafety level 3 containment laboratory approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan.

Isolation of viral RNA, reverse transcription-PCR, and generation of viruses by use of reverse genetics. Viral RNA was extracted from the supernatants of virus-infected MDCK cells by using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany). Extracted RNA was reverse transcribed with SuperScript III (Invitrogen, Carlsbad, CA) and the universal primers specific for influenza A virus genes to generate cDNA. The resulting products were PCR amplified by using KOD FX DNA polymerase (Toyobo, Osaka, Japan) with specific primers for each virus gene and cloned into the RNA polymerase I plasmid pHH21 (32). Mutations in the PB2 gene of Wb/AH82 were generated by PCR amplification of the respective PB2 construct with primers possessing the desired mutations. Primer sequences are available upon request. All constructs were sequenced to ensure the absence of unwanted mutations. All avian, reassortant, and mutant viruses were generated by use of plasmid-based reverse genetics, as described previously (32). The culture supernatant derived from transfected cells was amplified in DF-1 cells grown in DMEM containing 0.3% bovine serum albumin. At 48 h postinfection,

TABLE 1 PB2 amino acid markers that are advantageous for efficient growth in mammals[a]

		PB2							
		627	271	591	158	701	147*	339*	588*
Proposed human adaptation marker		K	A	R/K	G	N	T	T	T
Human H5N1 isolates	36250	K	T	Q	E	D	T	T	A
	35236	K	T	Q	E	D	T	M	A
	31604	K	T	Q	E	D	T	M	A
	31641	K	T	Q	E	D	T	T	A
	36282	E	T	R	E	D	T	M	A
	36285	E	T	Q	E	D	T	M	A
	31676	E	T	Q	E	D	T	M	A
Avian H5N1 isolates	Wb/AH82	E	T	Q	E	D	I	T	A
	Ck/TY31	E	T	Q	E	D	I	K	A
	Ck/UT3091	E	T	Q	E	D	I	T	A

[a] The amino acid residues listed above have been shown to contribute to efficient virus growth in mammalian cells or to high pathogenicity in mammals. The yellow highlighting indicates human-type amino acids that are markers for efficient growth in mammals. Asterisks mark the PB2 amino acids 147T, 339T, and 588T, which together enhance the polymerase activity of H5N1 virus (28).

the culture supernatant was harvested, clarified, divided into aliquots, and stored at −80°C. The virus titers of all human and avian viruses were determined by using plaque assays in MDCK cells.

Mouse experiments. Six-week-old female BALB/c mice (Japan SLC) were used for these experiments. To determine the minimum dose lethal to 50% of mice (MLD_{50}), 5 mice/group were anesthetized with isoflurane and inoculated intranasally with 10^1 to 10^5 PFU in a 50-μl volume. The mice were monitored daily for clinical signs of infection and checked for changes in body weight and mortality for 14 days postinfection. MLD_{50} values were calculated by using the method of Reed and Muench (47). All experiments with mice were performed in accordance with the University of Tokyo's Regulations for Animal Care and Use and were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo.

Viral replication assay. Triplicate wells of confluent A549 cells or DF-1 cells were infected with viruses at a multiplicity of infection (MOI) of 0.0002 and incubated for 1 h at 37°C or 39°C, respectively. After the 1-h incubation, A549 cells were further incubated in MEM containing 0.3% BSA at 33°C and 37°C. DF-1 cells were further incubated in DMEM containing 0.3% BSA at 39°C. Aliquots of supernatants were harvested at 1, 24, 48, 72, and 96 h postinfection and frozen at −80°C. Virus titers in the culture supernatants at each time point were determined by plaque assays in MDCK cells.

Statistical analysis. Differences in the virus titers of the supernatants were statistically analyzed by using the Student *t* test. Differences in mean maximum body weight losses in mice were also statistically analyzed by using the Student *t* test.

Nucleotide sequence accession numbers. Sequences for H5N1 viruses were deposited in GenBank as follows: A/wild bird/Anhui/82/2005 (virus Wb/AH82) (accession numbers KP638500 [PB2], KP638559 [PB1], KP638509 [PA], KP638516 [HA], KP638533 [NP], KP638524 [NA], KP638542 [M], and KP638551 [NS]), A/chicken/Vietnam/TY31/2005 (virus Ck/TY31) (accession numbers KP638493 [PB2], KP638558 [PB1], KP638501 [PA], KP638525 [NP], KP638534 [M], and KP638550 [NS]), A/Vietnam/UT36285/2010 (virus 36285) (accession numbers KP638492 [PB2], KP638552 [PB1], KP638507 [PA], KP638560 [HA], KP638530 [NP], KP638523 [NA], KP638539 [M], and KP638547 [NS]), A/Vietnam/UT36282/2010 (virus 36282) (accession numbers KP638494 [PB2], KP638557 [PB1], KP638506 [PA], KP638515 [HA], KP638532 [NP], KP638522 [NA], KP638541 [M], and KP638549 [NS]), A/Vietnam/UT36236/2010 (virus 36236) (accession numbers KP638495 [PB2], KP638556 [PB1], KP638505 [PA], KP638514 [HA], KP638531 [NP], KP638520 [NA], KP638540 [M], and KP638548 [NS]), A/Vietnam/HN31676DH/2009 (virus 31676) (accession numbers KP638496 [PB2], KP638553 [PB1], KP638504 [PA], KP638513 [HA], KP638526 [NP], KP638519 [NA], KP638535 [M], and KP638543 [NS]), A/Vietnam/UT31641II/2008 (virus 31641) (accession numbers KP638497 [PB2], KP638554 [PB1], KP638503 [PA], KP638512 [HA], KP638528 [NP], KP638518 [NA], KP638537 [M], and KP638545 [NS]), A/Vietnam/UT31604I/2009 (virus 31604) (accession numbers KP638498 [PB2], KP638561 [PB1], KP638502 [PA], KP638511 [HA], KP638527 [NP], KP638517 [NA], KP638536 [M], and KP638544 [NS]), and A/Vietnam/UT36250I/2010 (virus 36250) (accession numbers KP638499 [PB2], KP638555 [PB1], KP638508 [PA], KP638510 [HA], KP638529 [NP], KP638521 [NA], KP638538 [M], and KP638546 [NS]).

RESULTS

Comparison of the growth ability of H5N1 viruses in A549 and DF-1 cells. To identify human-adaptive mutations in H5N1 influenza viruses, we first compared the growth properties at a multiplicity of infection (MOI) of 0.0002 in carcinomic human alveolar basal epithelial A549 cells of the following seven H5N1 viruses isolated from humans: A/Vietnam/UT36285/2010 (36285), A/Vietnam/UT36282/2010 (36282), A/Vietnam/UT36236/2010 (36236), A/Vietnam/HN31676DH/2009 (31676), A/Vietnam/UT31641II/2008 (31641), A/Vietnam/UT31604I/2009 (31604),

TABLE 2 Percentages of amino acid identity in 12 proteins of 36285 and Wb/AH82

Protein	% sequence identity
PB2	97.3
PB1	98.4
PB1-F2	87.7
PA	98.4
PA-X	94
NP	98.5
HA	98.4
NA	97.5
M1	100
M2	97.9
NS1	95.1
NEP	96.1

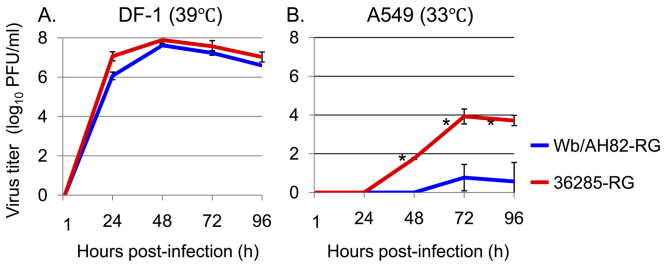

FIG 2 Comparison of the growth properties of 36285-RG virus and Wb/AH82-RG virus in DF-1 cells and A549 cells. (A) DF-1 cells were infected with 36285-RG and Wb/AH82-RG virus at an MOI of 0.0002 and cultured at 39°C. Error bars represent the standard deviations of the results of 3 independent experiments. (B) A549 cells were infected with 36285-RG and Wb/AH82-RG virus at an MOI of 0.0002 and cultured at 33°C. Supernatants were titrated by plaque assays with MDCK cells at the indicated time points. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the 36285-RG virus are significantly different from those of the Wb/AH82-RG virus both at 37°C and at 33°C ($P < 0.01$, Student's *t* test).

and A/Vietnam/UT36250I/2010 (36250). All seven human H5N1 viruses grew well in A549 cells at 37°C, with maximum titers of over 10^6 PFU/ml, except for 31676, which grew to slightly lower titers (Fig. 1A). Sequence analysis revealed that 36250, 36236, 31604, and 31641 possess lysine at position 627 of the PB2 protein (PB2-627K), whereas 36282 possesses PB2-591R. However, viruses 31676 and 36285 do not have any known PB2 markers that could account for the efficient replication in mammals (Table 1). These findings suggest that the human H5N1 viruses 31676 and 36285 may have unreported amino acids that enhance their viral growth ability in A549 cells. Therefore, in this study, we focused on the replicative efficiency of 36285. First, we generated the 36285 virus by use of reverse genetics (36285-RG) and confirmed that 36285-RG grew as well as wild-type 36285 in A549 cells (Fig. 1B). We next compared the growth property of 36285-RG in A549 cells at 37°C with that of the following three avian H5N1 viruses generated by use of reverse genetics: A/wild bird/Anhui/82/2005 (Wb/AH82-RG), A/chicken/Vietnam/TY31/2005 (Ck/TY31-RG), and A/chicken/Central Java/UT3091/2005 (Ck/UT3091-RG) (Fig. 1C). Ck/TY31-RG and Wb/AH82-RG grew poorly, with maximum titers of less than $10^{4.5}$ PFU/ml (Fig. 1C). Of note, both 36285 and Wb/AH82 belong to the same H5 HA subclade 2.3.4, and they are genetically closely related (Table 2). Therefore, we compared 36285 and Wb/AH82 to elucidate the mechanism of H5N1 adaptation to humans. In chicken embryo fibroblast DF-1 cells, 36285-RG and Wb/AH82-RG both grew well at 39°C (Fig. 2A), demonstrating their potential to replicate in avian cells; in contrast, 36285-RG grew much better than Wb/AH82-RG in A549 cells at 33°C (Fig. 2B), demonstrating its potential to replicate in mammalian cells. We compared the growth capability of H5N1 viruses in A549 cells at both 37°C and 33°C because in humans, the temperature in the lungs is 37°C and that in the upper airway is 33°C. However, the body temperature of birds is 39°C; therefore, we compared the growth capability of H5N1 viruses in chicken DF-1 cells only at 39°C.

Comparison of the pathogenicity of Wb/AH82-RG and 36285-RG in mice. We next examined the pathogenicity of Wb/AH82-RG and 36285-RG in mice. Inoculation of 10^3, 10^4, and 10^5 PFU of 36285-RG virus resulted in 100% mortality, and a 40% fatality rate was observed in the mice that received 10^1 and 10^2 PFU of virus. In contrast, inoculation of 10^4 and 10^5 PFU of Wb/

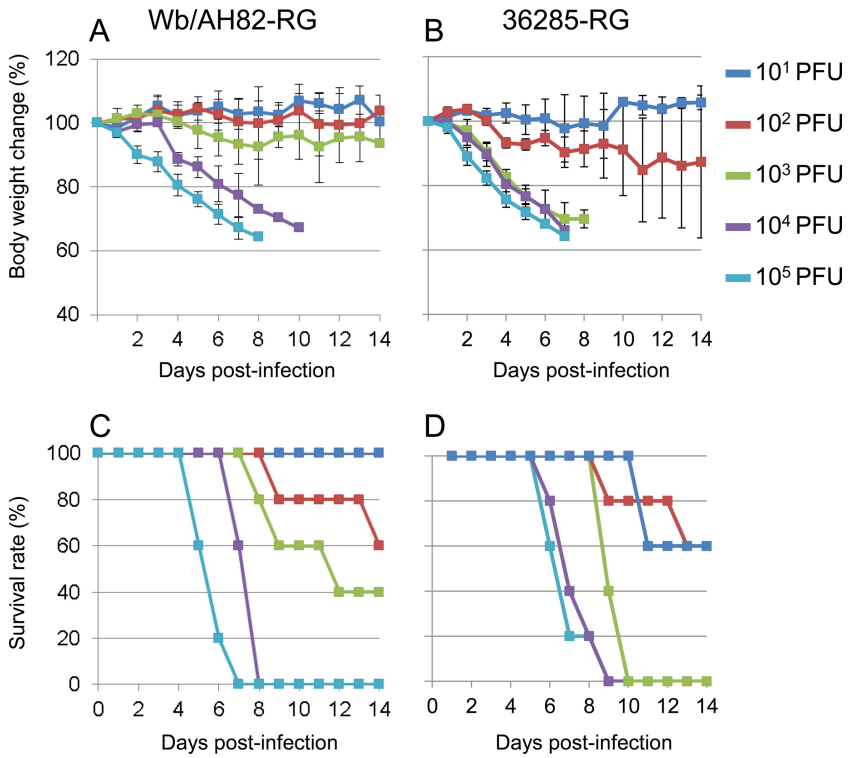

FIG 3 Comparison of the virulence of 36285 virus and Wb/AH82 virus in mice. Mice (5 per group) were inoculated with 10^1, 10^2, 10^3, 10^4, or 10^5 PFU of 36285-RG or Wb/AH82-RG virus and monitored for weight loss (A, B) and survival (C, D) for 14 days.

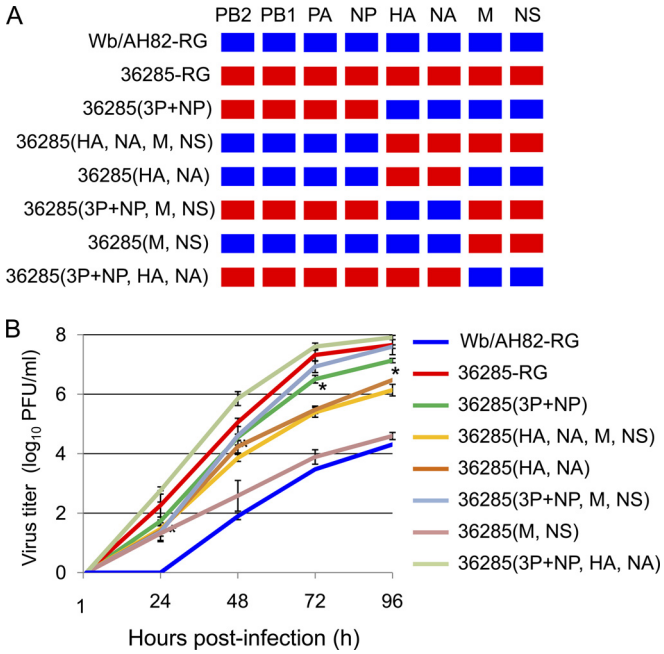

FIG 4 Comparison of the growth properties of reassortant viruses in A549 cells. (A) Schematic diagram of reassortant viruses. The red rectangles represent the genes of Wb/AH82-RG, and the blue ones represent the genes of 36285-RG. (B) A549 cells were infected with the reassortant viruses at an MOI of 0.0002 and cultured at 37°C. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the 36285(3P+NP) virus are significantly different from those of the Wb/AH82-RG virus ($P < 0.01$, Student's *t* test).

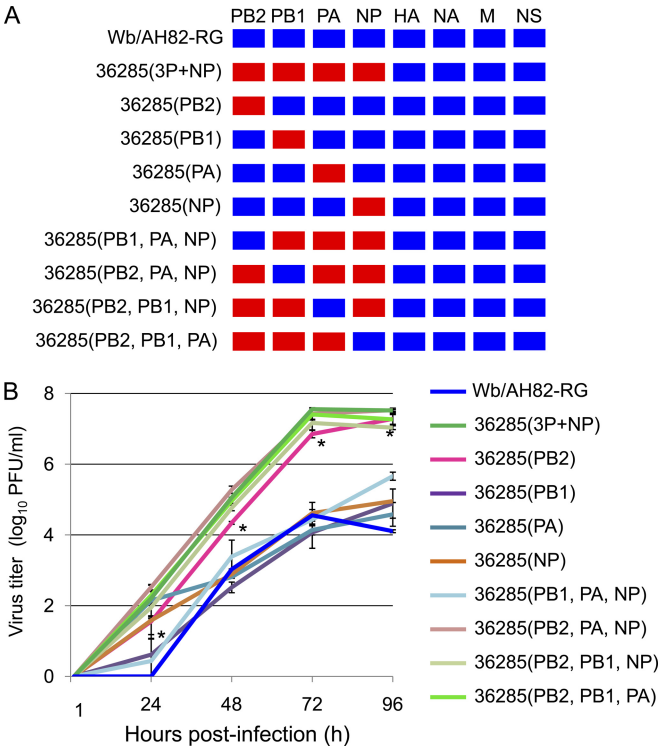

FIG 5 Comparison of the growth properties of further reassortant viruses in A549 cells. (A) Schematic diagram of reassortant viruses. The red rectangles represent the genes of Wb/AH82-RG, and the blue ones represent the genes of 36285-RG. (B) A549 cells were infected with the reassortant viruses at an MOI of 0.0002 and cultured at 37°C. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the 36285(PB2) virus are significantly different from those of the Wb/AH82-RG virus ($P < 0.01$, Student's *t* test).

AH82-RG resulted in 100% mortality and a 60% fatality rate was observed in mice that received 10^2 and 10^3 PFU of virus. The MLD_{50} values for 36285-RG and Wb/AH82-RG were $10^{1.5}$ PFU and $10^{2.7}$ PFU, respectively (Fig. 3C and D). Thus, 36285-RG and Wb/AH82-RG differed in their mouse virulence by only one log MLD_{50} unit.

The PB2 gene segment from 36285 is responsible for its efficient growth in human cells. To elucidate the molecular basis for the replicative difference between Wb/AH82 and 36285 in A549 cells, we generated a series of reassortant viruses (as illustrated in Fig. 4A) and compared their growth properties in A549 cells. The reassortant viruses were named according to the origin of their Wb/AH82 or 36285 genes. For instance, "36285(PB2)" indicates a virus possessing PB2 from 36285 and the rest of its gene segments from Wb/AH82. Three reassortant viruses possessing the PB2, PB1, and PA (3P) plus NP of 36285 [i.e., 36285(3P+NP), 36285(3P+NP, HA, NA), and 36285(3P+NP, M, NS)] were comparable in their growth to 36285-RG; none of these viruses replicated more efficiently than 36285-RG (Fig. 4B). The replicative ability of 36285(M, NS) was similar to that of Wb/AH82-RG, indicating that the M and NS proteins of 36285 do not have a large impact on the difference in the growth capabilities of Wb/AH82-RG and 36285-RG. Of note, the HA and NA of 36285 also contributed, to some extent, to the difference in the growth properties of Wb/AH82-RG and 36285-RG; however, they enhanced viral replication to a much smaller extent than did the polymerase subunits and NP of 36285. These results suggest that the PB2, PB1, PA, and/or NP gene segments of 36285 were the most responsible for the difference in growth capabilities between Wb/AH82-RG and 36285-RG in A549 cells.

To determine which viral segment(s) among PB2, PB1, PA, and NP contributed to the high replication of 36285-RG, we generated a range of reassortants (Fig. 5A) and compared their growth properties in A549 cells. Only viruses possessing 36285(PB2) grew well (Fig. 5B). In contrast, 36285(PB1, PA, NP) grew poorly, similarly to Wb/AH82-RG. These results demonstrate that the PB2 of 36285 makes an important contribution to the difference in growth capabilities between Wb/AH82-RG and 36285-RG in A549 cells.

A single G-to-D mutation at 309 and the double mutation E249G and T339M enhance the replication of Wb/AH82-RG. Sequence comparison of the PB2 proteins of Wb/AH82 and 36285 revealed 21 amino acid differences (Table 3). To identify the specific changes that give rise to efficient viral replication, we generated mutant viruses possessing a chimeric PB2 protein (Fig. 6A); the remaining gene segments were derived from Wb/AH82. In A549 cells, the mutant viruses possessing chimera 1, 5, or 6 grew poorly, whereas those with chimera 2 or 3 grew as well as that with 36285 PB2. These results suggest that residues 221 to 460 contribute most to the difference between 36285-RG and Wb/AH82-RG in terms of growth in A549 cells.

To further define which amino acid residues enhance the viral replication, we generated viruses with additional chimeric PB2

TABLE 3 Amino acid differences in PB2 between Wb/AH82 and 36285

	PB2 sequence position																				
Virus	64	89	108	109	147	249	309	339	355	368	390	461	467	473	477	478	483	495	560	658	684
Wb/AH82	T	L	A	I	I	E	G	T	R	R	D	I	L	M	R	V	V	I	V	Y	T
36285	I	V	T	V	T	G	D	M	K	Q	N	V	M	I	G	I	M	V	L	H	A

proteins (Fig. 7A). The mutant viruses possessing chimeras 2, 7, 8, and 11 grew well, whereas the growth of the viruses with chimeras 1, 9, and 10 was lower than that of the viruses possessing residues 221 to 345 from 36285. These results indicate that residues 221 to 345 from 36285 provide the replicative advantage to 36285-RG (Fig. 7B).

We then tried to determine which amino acids among residues 221 to 345 of PB2 enhanced the growth properties of 36285-RG. There are three amino acid differences in this region between Wb/AH82 and 36285, 249E/G, 309G/D, and 339T/M. We introduced the three residues 249G, 309D, and 339M into the PB2 of Wb/AH82 singly or in combination (Fig. 8A) and generated viruses possessing the mutant Wb/AH82 PB2 gene in the background of the remaining Wb/AH82 genes (note that all mutant Wb/AH82 viruses possess amino acid changes found among circulating influenza viruses). Indeed, the combination of PB2-249G, -309D, and -339M markedly increased the growth capability of Wb/AH82-RG, by around 10^2-fold compared with that of Wb/AH82-RG (Fig. 8B and C). Although the single introduction of PB2-249G, -339M, or -309D enhanced the growth capability of Wb/AH82-RG, PB2-339M was the least effective (Fig. 8B). All mutants that possessed combinations of two of the PB2 mutations

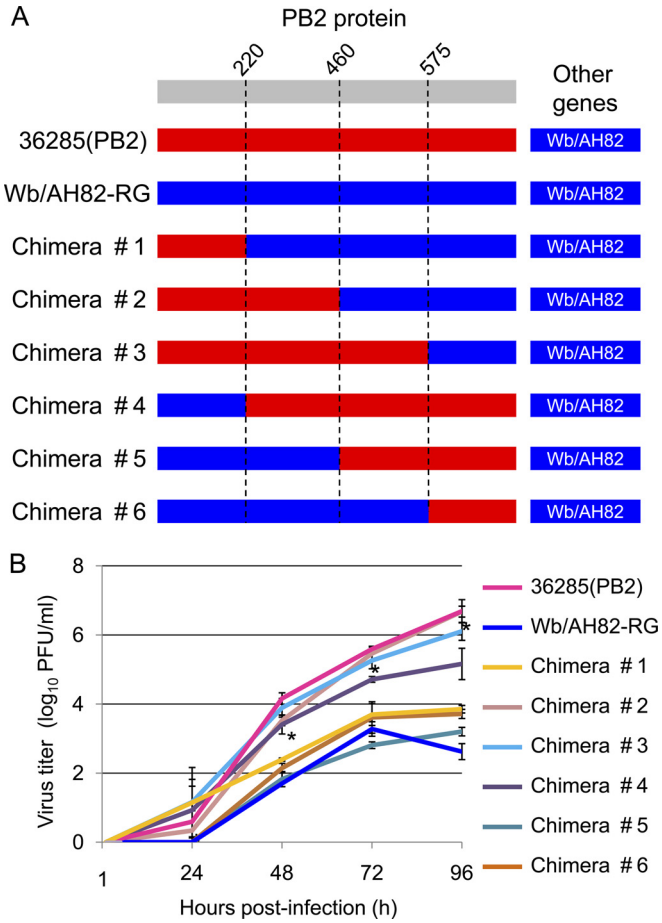

FIG 6 Comparison of the growth properties of PB2 chimeric viruses in A549 cells. (A) Schematic diagram of chimeric PB2 mutants. The red rectangles represent the genes of Wb/AH82-RG, and the blue ones represent the genes of 36285-RG. (B) A549 cells were infected with the mutant viruses at an MOI of 0.0002 and cultured at 37°C. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the chimera 2 virus are significantly different from those of the Wb/AH82-RG virus ($P < 0.01$, Student's *t* test).

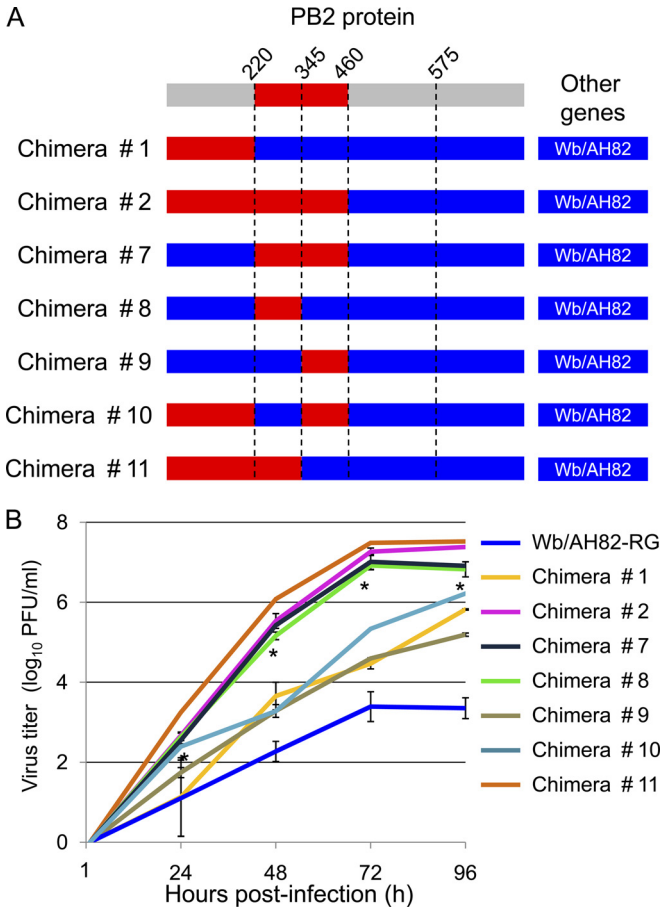

FIG 7 Comparison of the growth properties of additional PB2 chimeric viruses in A549 cells. (A) Schematic diagram of chimeric PB2 mutants. The red rectangles represent the genes of Wb/AH82-RG, and the blue ones represent the genes of 36285-RG. (B) A549 cells were infected with the mutant viruses at an MOI of 0.0002 and cultured at 37°C. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the chimera 8 virus are significantly different from those of the Wb/AH82-RG virus ($P < 0.01$, Student's *t* test).

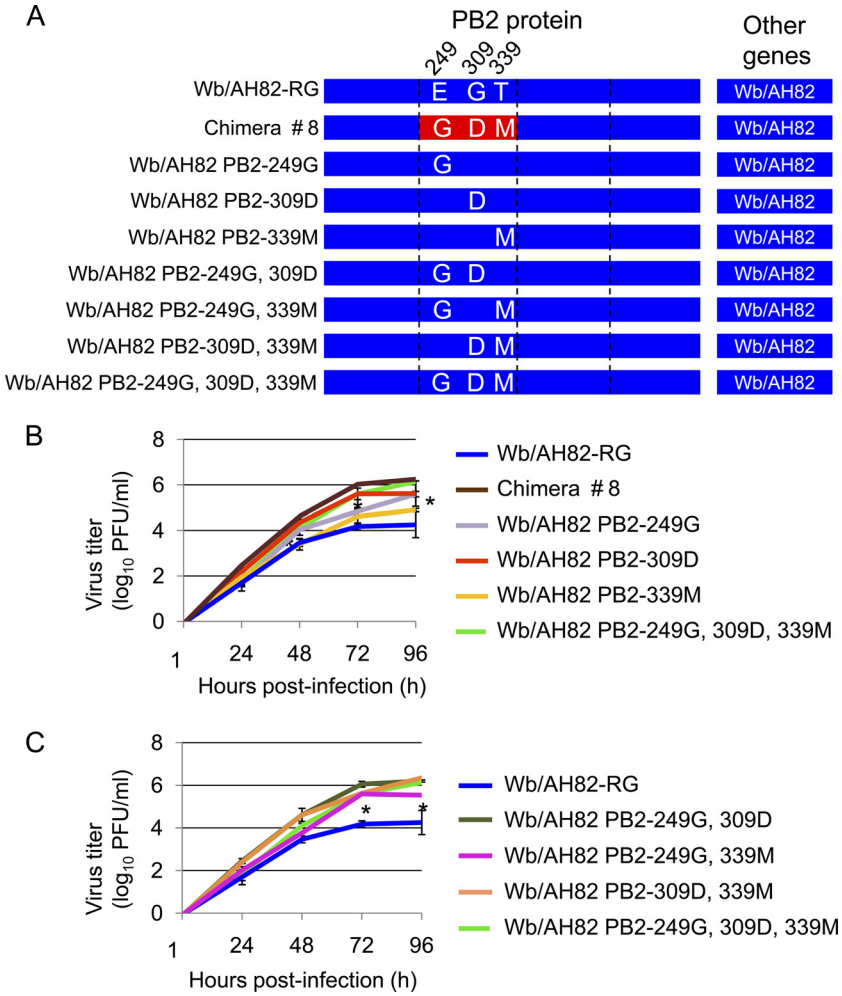

FIG 8 Comparison of the growth properties of single, double, and triple PB2 amino acid mutant viruses in A549 cells. (A) Schematic diagram of single, double, and triple PB2 amino acid mutants. The red rectangles represent the genes of Wb/AH82-RG, and the blue ones represent the genes of 36285-RG. (B) A549 cells were infected with the mutant viruses at an MOI of 0.0002 and cultured at 37°C. Error bars represent the standard deviations of the results of 3 independent experiments. *, The titers of the Wb/AH82 PB2-309D virus are significantly different from those of the Wb/AH82-RG virus at 48, 72, and 96 h postinfection ($P < 0.01$, Student's *t* test). (C) A549 cells were infected with the mutant viruses at an MOI of 0.0002 and cultured at 37°C. *, The titers of the Wb/AH82 PB2-249G, 309D virus are significantly different from those of the Wb/AH82-RG virus at 72 and 96 h postinfection ($P < 0.01$, Student's *t* test).

tested grew better than Wb/AH82-RG by more than $10^{1.5}$-fold (Fig. 8C). These results indicate that PB2-249G, PB2-339M, and PB2-309D support efficient viral growth in A549 cells.

The amino acid mutations E249G and T339M may be associated with the adaptation of H5N1 virus to humans. To further evaluate the potential role of the PB2 amino acid residues 249, 309, and 339 in adaptation to humans, we collected full-length PB2 sequences of influenza viruses of various subtypes from the Influenza Sequence Database (ISD; http://www.fludb.org/brc/home.spg?decorator=influenza) (Table 4). Almost all of the H5N1 viruses contained PB2 amino acid 309D, which is highly conserved among influenza viruses of various subtypes regardless of the host species, implying that this residue does not need to change for a virus to break through the species barrier. In contrast, PB2 amino acids 249G and 339M were rare among the avian H5N1 viruses. Intriguingly, no less than 49.2% of the human seasonal H3N2 viruses had PB2 amino acid 249G. This finding supports the hypothesis that PB2 amino acid 249G is the amino acid mutation that the virus gains during replication in human respiratory cells and which increases its fitness for human adaptation.

DISCUSSION

Several amino acid mutations associated with the adaptation of H5N1 virus to humans have been identified, including 627K (8–10), 591R, 591K (11), 701N (9, 13), 271A (14), and 158G (15) of PB2. We thought that further analysis of H5N1 viruses isolated from humans could identify new mutations needed for their efficient replication in human cells. Even though the 36285 H5N1 virus did not have any amino acid markers that have been previously identified to provide a replicative advantage in mammalian cells, it grew well in human lung cells. By using a viral replication assay, we identified three amino acids in PB2 that are responsible for the replicative efficiency of H5N1 virus in A549 cells. Viruses with these amino acids in PB2 circulate in nature; we did not generate viruses possessing novel amino acid changes. These data

TABLE 4 PB2 residues 249, 309, and 339 in viruses isolated from avian, human, and swine sources

Host	Subtype	% of samples (no. of positive samples/total no. of samples) with indicated amino acid residue		
		249G	309D	339M
Avian	H5N1[a]	0 (0/1,234)	94.0 (1,161/1,234)	1.7 (22/1,234)
Human	H5N1[a]	0 (0/187)	94.1 (176/187)	2.1 (4/187)
	Seasonal H3N2[a]	49.2 (2,617/5,312)	100 (5,312/5,312)	0 (0/5,312)
	Seasonal H1N1[b]	0 (0/1,437)	78.2 (1,125/1,437)	0 (0/1,437)
	Pandemic H1N1[b]	0 (0/4,085)	100 (4,085/4,085)	0 (1/4,085)
Swine	H3N2[c]	1.7 (15/837)	99.4 (832/837)	0.1 (1/837)
	Pandemic H1N1[c]	1.1 (3/261)	99.6 (260/261)	0 (0/261)
	H1N1 (except for pandemic)[c]	0 (0/1,437)	80.3 (1,154/1,437)	0 (0/1,437)

[a] Full-length PB2 sequences of all swine viruses from the IRD (Influenza Research Database) were analyzed in January 2015.

will provide information of value for pandemic preparedness and for use in evaluating the pandemic risk potential of future isolates.

Pflug et al. (33) recently determined the complete crystal structure of the heterotrimeric influenza A polymerase bound to the viral RNA. This structure revealed that all three amino acid substitutions identified in this study (i.e., PB2-E249G, -G309D, and -T339M) are located on the surface of the PB2 protein. This finding supports the concept that these residues likely interact with viral or host proteins, leading to the replicative advantage of the 36285 virus.

PB2-E249G and -T339M have an interesting common feature in that they belong to areas identified as "cap-binding" sites (34–37). Influenza virus has developed a mechanism called "cap-snatching" to steal the cap from the host cellular RNA in order to synthesize viral protein. The polymerase PB2 subunit binds to the 5′ cap of host pre-mRNAs, which are cleaved after 10 to 13 nucleotides by the PA subunit (38–40). An X-ray structure of the cap-binding domain shows that the amino acid at position 339 of PB2 is located at the edge of the cap-binding pocket (37). The precise cap-binding site, however, remains controversial; one study identified the PB2 amino acid residues responsible for RNA cap-binding as PB2-363F and PB2-404F (35), but another study identified the PB2 amino acid residues 242 to 282 and 538 to 577 as important (36), and yet another study suggested that amino acid residues 318 to 483 were involved (37). The effect of the amino acid 339T *in vivo* and *in vitro* on biologic events is also inconsistent. We recently found that 339T has a major impact on the polymerase activity of H5N1 virus jointly with 147T and 588T (28). However, another paper reported that K339T reduced PB2 cap-binding activity and influenza polymerase activity *in vitro* and also attenuated virulence in mice (41). The latter paper suggested that the K339T substitution in PB2 reduced the cap-binding affinity because the side chain of threonine is shorter than that of lysine and threonine is uncharged (41). In the current study, we found that PB2 amino acid T339M helped to facilitate virus growth in A549 cells. Both threonine and methionine are uncharged, but the side chain of methionine is longer than that of threonine. Although the function of the three amino acid mutations identified in this study remains unclear, our findings may help improve the understanding of PB2 functions.

Human influenza A virus acquired the PB2 amino acid mutation E249G upon mouse adaptation (25), suggesting that the amino acid at position 249 probably is involved in some mechanism for adaptation to mammals. Sequence analysis showed that the prevalence of PB2 amino acid 249G in human seasonal H3N2 viruses was almost 50%. Almost all of the seasonal human H3N2 viruses isolated since 2006 have glycine at position 249, implying that some positive pressure for adaptation to humans led to glycine selection over aspartic acid. These data reinforce the notion that PB2 amino acid 249G plays a role in adaptation to humans or mammals.

The MLD_{50} values of 36285-RG virus and Wb/AH82-RG virus in mice differed by one log unit (Fig. 4), whereas the titers of the two viruses in A549 cells differed by more than 3 log units (Fig. 3). The HA and NA genes are involved, to some extent, in the difference in the growth abilities of Wb/AH82-RG and 36285-RG in A549 cells. The pathogenicity in mice could have been influenced by HA, because the dominant types of receptor to influenza viruses that are distributed on bronchi and lungs differ with the host species (42–45). Humans primarily have sialic acid linked to galactose by an α2,6 linkage (Sia-α2,6Gal) (42–44), whereas mice primarily have Sia-α2,3Gal-type linkages (45). One study showed that, on the surface of A549 cells, there are large amounts of Sia-α2,6Gal and a small amount of Sia-α2,3Gal (46). Perhaps the difference between the receptor specificities of the viruses and the receptor types displayed on the lung cells of mice may have influenced the pathogenicity in mice.

In conclusion, in the work presented here, we found that the PB2 amino acid substitutions E249G, G309D, and T339M enhance the replicative ability of H5N1 virus in A549 cells. Our study suggests that these PB2 substitutions could assist H5N1 viruses in adapting to human lung cells. Although the contribution of the PB2 C-terminal domain to virus host range is now well established, that of the middle portion of the PB2 segment remains largely unknown. The full structure of PB2 is needed to better understand its functions and role in host adaptation.

ACKNOWLEDGMENTS

We are grateful to Susan Watson for editing the manuscript. We thank Kiyoko Iwatsuki-Horimoto and Maki Kiso for technical assistance with mouse experiments. We also thank Shufang Fan, Masato Hatta, Gabriele Neumann, Amie J. Eisfeld, and Takeo Gorai for fruitful discussion.

This work was supported by the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by grants-in-aid from the Ministry of Health, Labor and Welfare, Japan, and by the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH).

The authors do not have any competing interests.

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