博士論文

論文題目

Identification of amino acid mutations that support the adaptation of highly pathogenic H5N1 influenza A viruses to humans

(H5N1 高病原性鳥インフルエンザの

ヒトへの適応に関するアミノ酸変異の同定)

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ABBRIVIATIONS

MDCK	**Madin-Darby canine kidney cells**
MEM	**minimal essential medium**
DMEM	**Dulbecco's modified Eagle medium**
FBS	**fetal bovine serum**
BSA	**bovine serum albumin**
Wb/AH82	**A/wild bird/Anhui/82/2005**
Ck/TY31	**A/chicken/Vietnam/TY31/2005**
Ck/UT3091	**A/chicken/Central Java/UT3091/2005**
36285	**A/Vietnam/UT36285/2010**
36282	**A/Vietnam/UT36282/2010**
36236	**A/Vietnam/UT36236/2010**
31676	**A/Vietnam/HN31676DH/2009**
31641	**A/Vietnam/UT31641Ⅱ/2008**
31604	**A/Vietnam/UT31604Ⅰ/2009**
36250	**A/Vietnam/UT36250Ⅰ/2010**
MLD_{50}	**dose lethal to 50% of mice**
PFU	**plaque-forming units**
MOI	**multiplicity of infection**

Preface

Influenza viruses are single-stranded RNA viruses that belong to the family Orthomyxoviridae; three types (A, B, and C) have been identified. Influenza A viruses are classified into subtypes determined by the hemagglutinin (HA) protein they use for host-cell entry and by the neuraminidase (NA) protein they use for the release of progeny viruses. At present, 18 HA (H1-H18) and 11NA (N1-N11) subtypes have been reported (Fig. 1).

The natural reservoir of influenza A virus is considered to be aquatic birds because almost every subtype of virus has been isolated from them. They circulate among aquatic birds with no or only mild symptoms. The viruses replicate in the bird's intestine and are then shed in feces to infect other birds. They can occasionally infect terrestrial poultry, such as chickens or quail, or mammalian hosts, including humans, pigs, livestock, and seals. Most of these infections result in only limited spread, with infection being restricted to the initial recipient or a small number of close contacts.

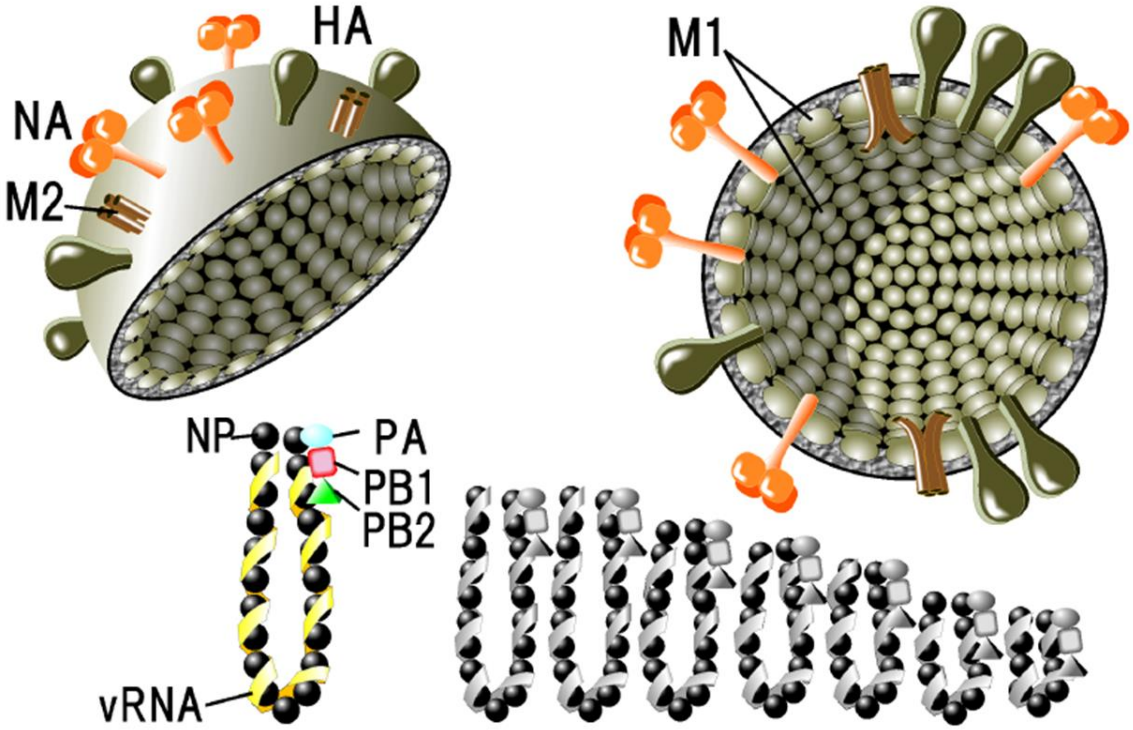

Fig. 1: The structure of influenza virus

The genome of influenza A viruses is composed of eight segments of negative-sense single-stranded RNA within a viral envelope that is coated with matrix protein (M1). The viral RNA segments are associated with nucleoprotein and bound by the polymerase complex. The envelope is covered with two types of spikes, composed of hemagglutinin (HA), and neuraminidase (NA). Influenza A virus is classified according to the 18 antigenically distinct types of hemagglutinin (HA) and 11 antigenically distinct types of neuraminidase (NA).

On the basis of their pathogenicity in terrestrial poultry, avian influenza viruses can be classified as either highly or low pathogenic avian influenza viruses. Low pathogenic avian influenza viruses are not lethal and are commonly isolated from many species of wild birds. The recurring infection of terrestrial poultry by low pathogenic avian influenza viruses is thought to promote the evolution of the low pathogenic avian influenza viruses into highly pathogenic avian influenza viruses. Highly pathogenic avian influenza viruses cause severe illness and a mortality rate of nearly 100%. All highly pathogenic avian influenza viruses isolated to date belong to the H5 and H7 subtypes, although not all viruses of these subtypes cause highly pathogenic. H5N1 highly pathogenic influenza A viruses have continued to circulate among avian species, with accompanying sporadic avian-to-human transmission. Yet, the human cases of H5N1 infection have been limited mainly to individuals in close contact with infected poultry (1,2). For their efficient transmission among humans, H5N1 viruses require further adaptation. For example, efficient interactions between viral components and host cellular factors are thought to be essential in order to break through host-specific barriers. For the virus to enter the host cell, the first hurdle it must overcome is receptor specificity. Although human influenza A viruses preferentially bind to receptors with sialic acid linked to galactose by an α2,6 linkage (Sia-α2,6Gal), avian influenza A viruses prefer receptors with Sia-α2,3Gal (3-6) (Fig. 2). This is one of the main reasons why human viruses do not replicate well in birds, and similarly, avian viruses grow poorly in humans. A number of studies have

shown that certain amino acid substitutions in the HA protein enable avian influenza viruses to bind to receptors with Sia-α2,6Gal. Moreover, H5N1 influenza viruses bearing amino acid mutations that permit binding to receptors with Sia-α2,6Gal have been isolated (7-9).

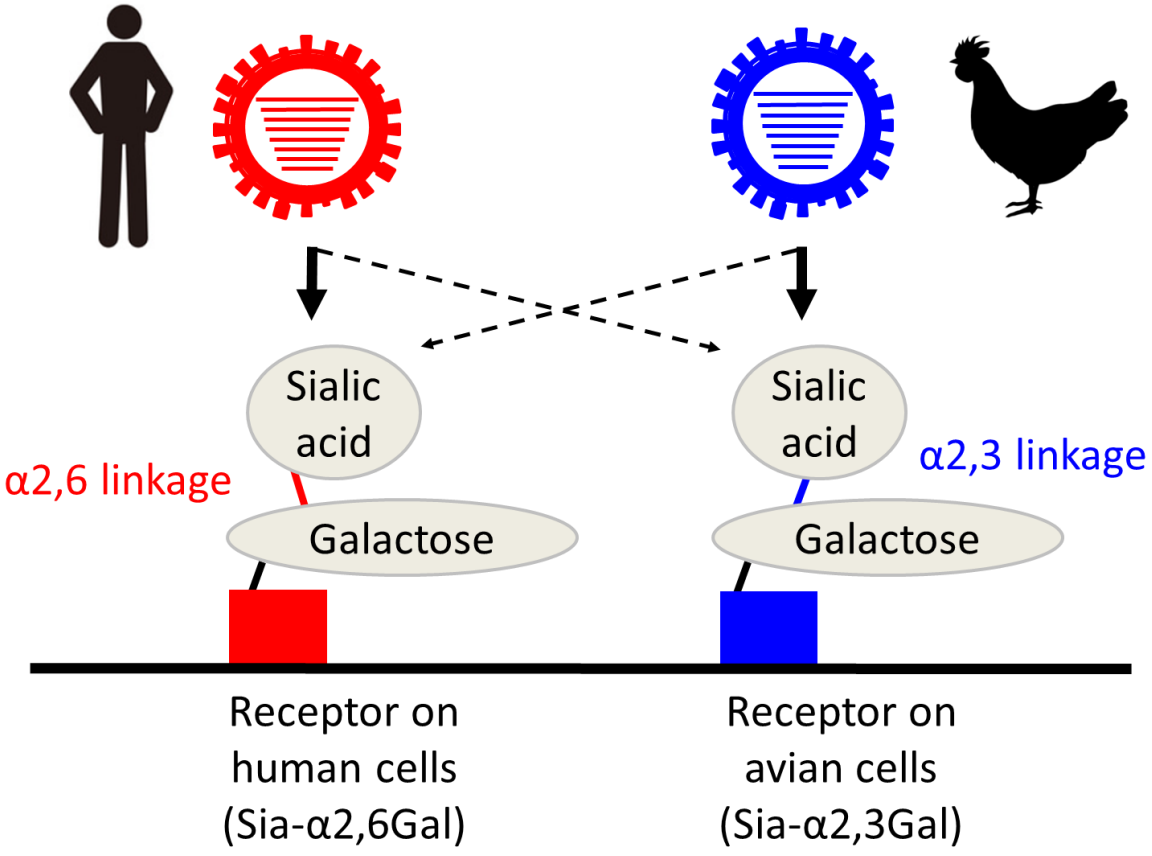

Fig. 2: Receptor binding specificity of influenza A virus

Avian and human influenza A viruses typically have different sialic acid binding preferences: human influenza A viruses preferentially bind to Sia-α2,6Gal receptors, whereas avian influenza A viruses prefer Sia-α2,3Gal receptors.

Several amino acid residues in the polymerase complex, which plays a central role during the transcription and replication of the virus in the host cell nucleus, are also important determinants of host range specificity. One of the best-known amino acid substitutions that support the adaptation of the H5N1 viruses to humans is lysine (K) at residue 627 of PB2. Several studies have shown that the PB2 E627K mutation elevates viral replication capability at the low temperature that is found in the human upper respiratory tract (10-12). Therefore, efficient replication in the human upper respiratory tract is probably a foundation for the adaptation of avian H5N1 influenza viruses to humans and for efficient person-to-person virus transmission.

H5N1 viruses have already been isolated that possess mutations known to increase binding to receptors with Sia-α2,6Gal (13-15); moreover, in Egypt, some of these viruses also possess the PB2-627K mutation that confers efficient replication in mammals (16,17). However, no H5N1 virus that can transmit efficiently in humans has yet been isolated from the natural world. This indicates that additional amino acid substitutions are needed for adaptation to humans. Consequently, we need to identify other mechanisms that play some role in adaptation to humans.

Over the course of human history, smallpox and rinderpest are the only infectious diseases that have been declared eradicated. The unique trait of smallpox was that its host was

exclusively human, allowing the successful development of a vaccine that could induce permanent immunity; a similar scenario played out with rinderpest and its exclusive host, the even-toed ungulate. In contrast, influenza A virus infection is a zoonosis and a large reservoir exists, which prevents us from eliminating influenza viruses from our world. Furthermore, we cannot predict when the next pandemic virus will emerge. We also cannot predict when an H5N1 virus will change to a virus with the ability to adapt to humans; we do know that if that happens, it could cause a pandemic.

Therefore, one of our key missions is to identify and monitor adaptive mutations to humans. This process will enable us to prepare for a potential H5N1 pandemic and to evaluate the risk associated with future isolates. Worldwide studies will help control the spread of influenza virus infection among humans and minimize the loss of human lives and the lives of other animals. In my study, I assessed which amino acid mutations are responsible for the replicative efficiency of H5N1 viruses in human lung cells by comparing two H5N1 viruses isolated from a human and a bird, respectively.

Abstract

Highly pathogenic H5N1 influenza A viruses have caused outbreaks among poultry worldwide, resulting in sporadic infections in humans with about a 60% mortality rate. 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. Viral determinants for efficient replication in humans are currently poorly understood. Here, I identify amino acid mutations that facilitate the replicative ability of H5N1 viruses in human lung cells. The polymerase PB2 protein of an H5N1 influenza virus isolated from a human in Vietnam (A/Vietnam/UT36285/2010, 36285) increased the growth ability of an avian H5N1 virus (A/wild bird/Anhui/82/2005, Wb/AH82) in human lung epithelial A549 cells. Furthermore, I demonstrate that 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. I also found that the PA of 36285 increased the growth ability of an avian H5N1 virus (A/chicken/Vietnam/TY31/2005, Ck/TY31) in A549 cells. Five PA amino acid substitutions (V44I, V127A, C241Y, A343T, and I573V) enhanced the virus growth capability in A549 cells. In addition, these substitutions dramatically increased virus pathogenicity in mice, suggesting that these five PA mutations are responsible for adaptation to mammalian hosts. My data are of value for assessing the pandemic risk of isolates and further our understanding of the mechanism of H5N1 virus adaptation to mammalian hosts.

Introduction

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. As of October 2014, the total number of human cases of highly pathogenic H5N1 influenza A virus infection had reached over 600, with a mortality rate of greater than 60% (393 deaths out of 668 confirmed cases according to http://www.who.int/) (21,22) (Fig. 3). The first human case caused by a highly pathogenic H5N1 influenza A virus was reported in Hong Kong in 1997 (18,19). 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 expansion of the range of birds infected by the H5N1 viruses, these viruses quickly spread beyond East Asia (20). The H5N1 viruses are classified into "clades" on the basis of their HA gene sequence homology. The H5 NA gene has evolved into numerous clades and sub-clades of the second, third, and fourth order, mostly within clades 1 and 2. H5N1 viruses of different HA clades and sub-clades are circulating in different regions of the world, where they continue to evolve and give rise to new, unique sub-clades (Fig. 4).The spread of H5N1 viruses has been accompanied by increasing reports of cases of avian-to-human transmission. However, the human cases of H5N1 infection have been confined mainly to individuals in close contact with infected poultry, and occurrence of human-to-human transmission has been extremely rare (1,2). The H5N1 virus can break

through the host species barrier when the following conditions are met: efficient transmission via air or droplet, efficient replication in respiratory cells, and immune vulnerability of the infected populace. Therefore, viruses with mutations that facilitate efficient transmission and replication in humans could cause a pandemic. Such adaptive mutations facilitate the efficient replication of H5N1 viruses in human cells (Fig. 5).

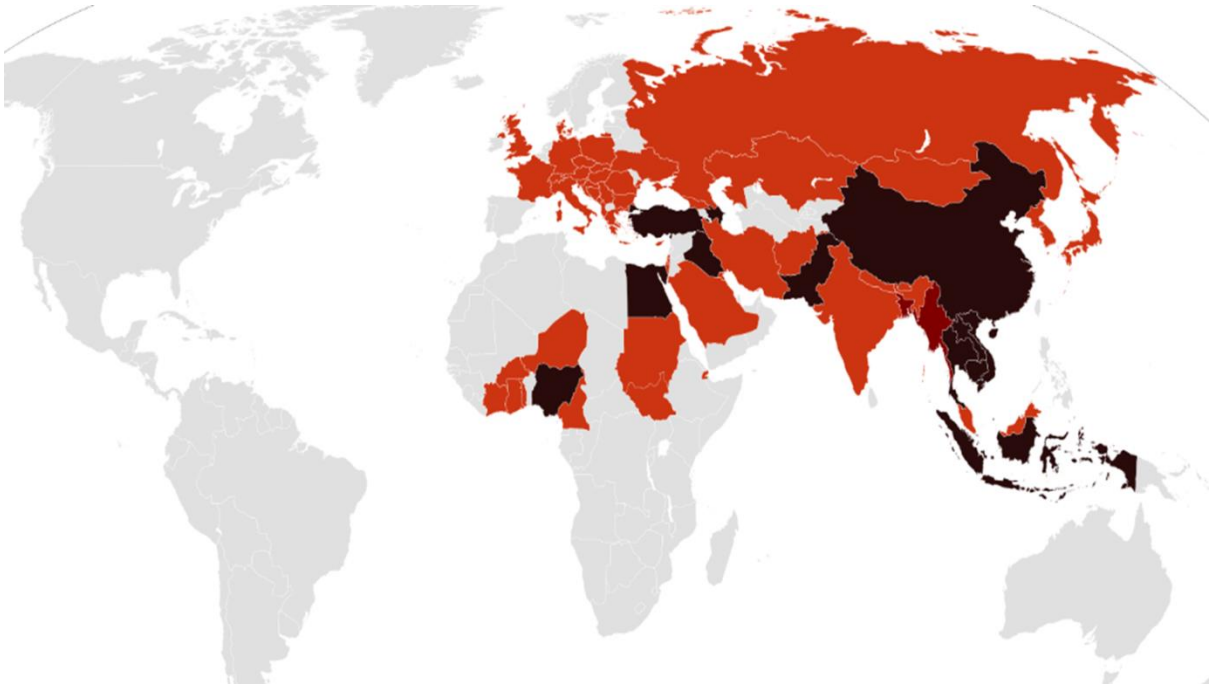

Fig. 3: The global spread of H5N1 viruses.
Since 1997, when the first human infection with an H5N1 virus was reported, the cumulative number of human infections has been increasing. H5N1 viruses have been isolated not only from birds (red), but also from humans (brown).

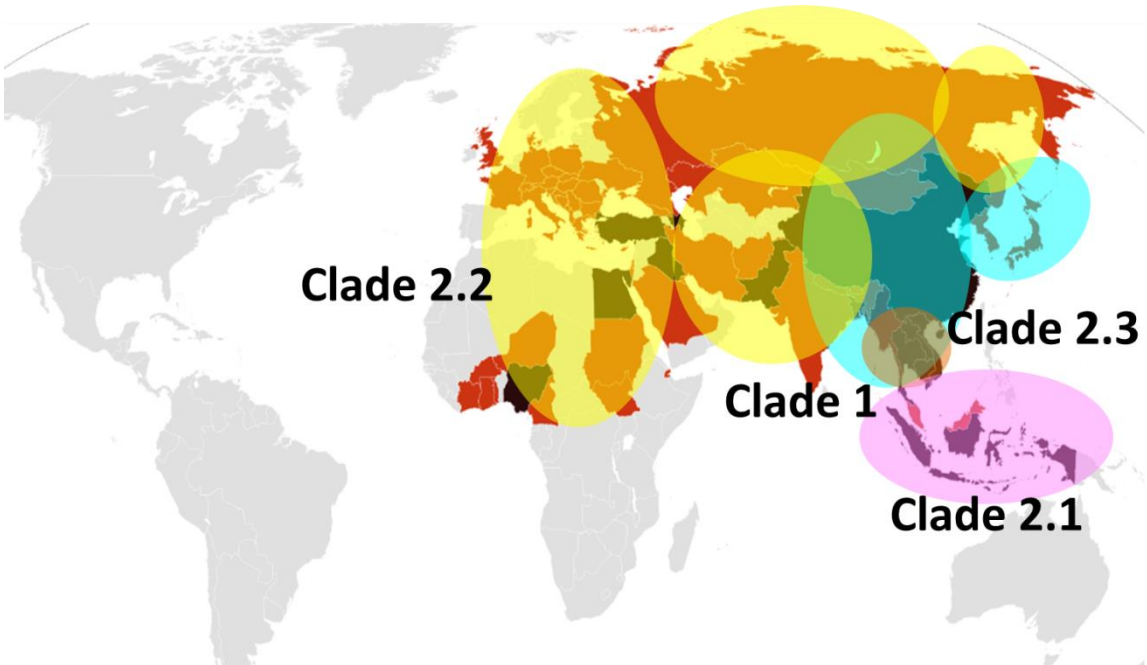

Fig. 4: The global spread of H5N1 viruses and the clades and sub-clades of H5 HA

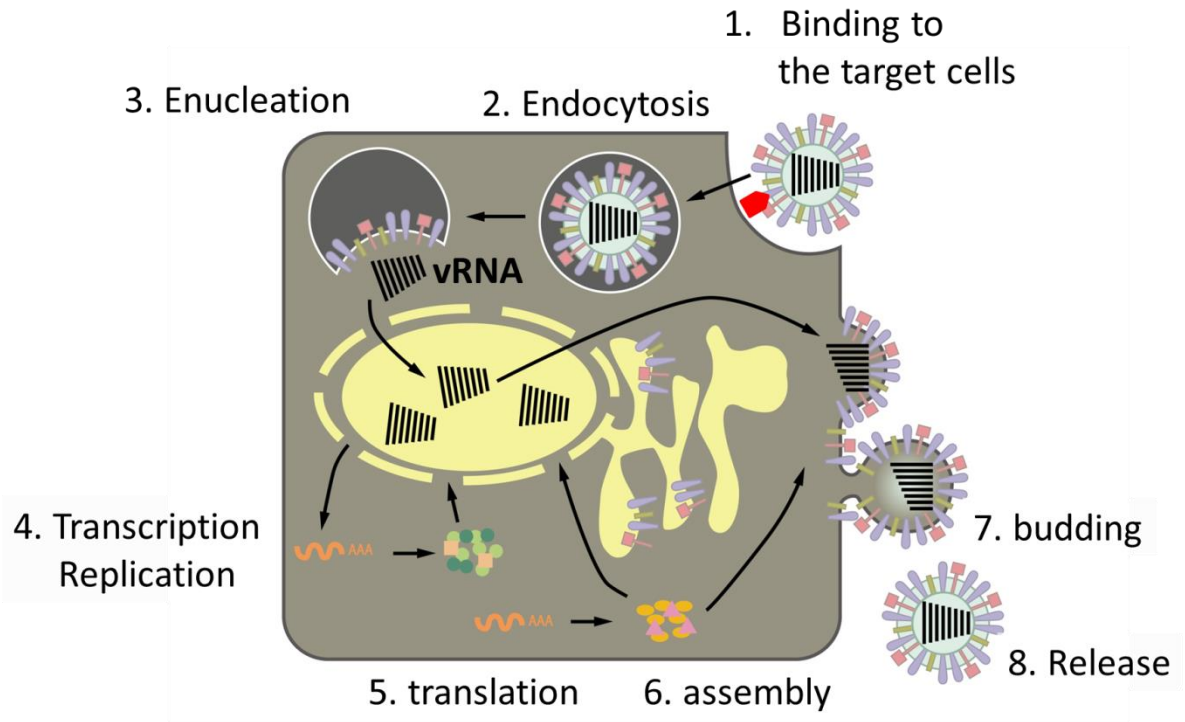

Fig. 5: The influenza A virus replication cycle

The influenza A virus particle binds to the cellular receptor and enters the cell via endocytosis. The viral ribonucleoproteins are released into the cytoplasm by acidification of the endosome. vRNPs are transported into the nucleus by cooperating with host proteins and transcription and replication occur in the nucleus. mRNAs are exported to the cytoplasm for translation. Viral RNAs and ribonucleoprotein complex then travel to the site of assembly. The viral progeny budsfrom the host cell, ready to infect other cells.

Several viral determinants have been identified that contribute to the adaptation of avian influenza viruses to mammalian hosts (10-12,23-52), and several large-scale comparative analyses of proteins from avian and human viruses have been performed to catalog the amino acids that are conserved in each host species (53-55). 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 (K) at residue 627 of PB2 enhances the polymerase activity, replication efficiency, virulence of H5N1 viruses in mammals, and transmissibility between mammals (10-12). Several studies have reported that PB2 627K plays an important role in host adaptation by using different importin isoforms depending on the host (23). However, other theories have been proposed for the mechanism of host adaptation mediated by PB2 627K (24,25). The PB2 701N mutation has also been shown to improve the binding of PB2 to mammalian importin-α isoforms (26-28), leading to increased viral replication in mammalian cells, pathogenicity in mice, and more efficient transmission in guinea pigs (11,29-31). 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 (32).Other amino acid substitutions such as PB2-271A and PB2-158G have also been identified as important markers that confer a replicative advantage and high pathogenicity to

influenza viruses in mammals (33,34). Our laboratory showed that the combination of amino acid substitutions 147T, 339T, and 588T in PB2 confer high polymerase activity to avian viruses in human cells (35). Recently, PB2-K526R has been reported to increase viral replicative capability in mammal cells, particularly in combination with 627K, through an interaction with the nuclear export protein (36). The PB2 protein thus serves as an essential element for virus adaptation to humans.

Furthermore, several amino acid mutations in the PA protein have been shown to enhance H5N1 virus growth in mammalian hosts. They include the PA K142E, I353R, P149S, R266H, L357I, and T515S mutations (37-39). One study showed that PA-K142E, which was found in A/Viet Nam/1203/2004 (VN1203), contributes to high polymerase activity in mammalian cells (37). A comparison of two H5N1 viruses that greatly differ in their pathogenicity in mice demonstrated that PA-I353R contributes to high replication, polymerase activity in mice, and manipulation of the innate response (38). Furthermore, PA-P149S, R266H, L357I, and T515S were reported to increase the polymerase activity of H5N1 virus in 293T cells (39). Many PA amino acid mutations in H1 and H7 subtype viruses have also been shown to play a role in overcoming host species barriers (40-48).

Although many amino acids that contribute to the adaptation of avian influenza viruses to mammalian hosts have been identified, no H5N1 virus that transmits efficiently between individuals has been found. Accordingly, we must assume that more amino acid

mutations are needed for the efficient replication of H5N1 highly pathogenic influenza A viruses in mammals or human cells. In this study, I attempted to identify amino acids that support the efficient growth of H5N1 viruses in human lung cells. The accumulation of information about adaptive mutations to humans helps us to prepare for the potential of an H5N1 pandemic by assessing the risk of future isolates with pandemic potential. Here, I identified new molecular determinants associated with the efficient growth of a human-isolated H5N1 virus in human lung cells, by generating reassortant and mutant viruses derived from avian and human H5N1 viruses that differ in their pathogenicity in mice and replicative ability in human lung cells.

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, I used the following ten H5N1 viruses: A/wild bird/Anhui/82/2005 (Wb/AH82), A/chicken/Vietnam/TY31/2005 (Ck/TY31), A/chicken/Central Java/UT3091/2005 (Ck/UT3091), A/Vietnam/UT36285/2010 (36285), A/Vietnam/UT36282/2010 (36282), A/Vietnam/UT36236/2010 (36236), A/Vietnam/HN31676DH/2009 (31676), A/Vietnam/UT31641 Ⅱ /2008 (31641), A/Vietnam/UT31604 Ⅰ /2009 (31604), and A/Vietnam/UT36250 Ⅰ /2010 (36250). 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% BSA and incubated at 37°C for 48 hours. 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 two-year-old female, onset on April 2, 2010, hospitalized on April 4, 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, RT-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. Mutations in the PB2 gene of Wb/AH82 were generated by PCR amplification of the respective PB2 construct with primers possessing the desired mutations. Mutations in the PA gene of Ck/TY31 were generated by PCR amplification of the respective PA 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 (56). 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 post-infection, 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 dose lethal to 50% of mice (MLD_{50}), 5 mice/group were anesthetized with isoflurane and inoculated intranasally with 10^1 to 10^5 plaque-forming units (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 post-infection. MLD_{50} values were calculated by using the method of Reed & Muench (1938). For virus replication in organs, groups of mice (6 mice/group) were infected intranasally with 10^3 PFU. Three mice in each group were euthanized on days 3 and 6 post-infection, respectively. Organs (brains, lungs, nasal turbinates, duodenums, rectums, liver, kidneys, and spleens) were collected for virus titration by use of plaque assays in MDCK cells. The data shown are the mean virus titers ± standard deviation. 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% bovine serum albumin (BSA) at 33°C and 37°C. DF-1 cells were further incubated in DMEM containing 0.3% bovine serum albumin (BSA) at 39°C. Aliquots of supernatants were harvested at 24-h intervals, 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's *t*-test. Differences in mean maximum body weight loss in mice were also statistically analyzed by using the Student's *t*- test.

CHAPTER Ⅰ

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

Results

Comparison of the growth ability of H5N1 viruses in A549 and DF-1 cells

To identify human adaptive mutations in H5N1 influenza viruses, I first compared the growth properties at a multiplicity of infection (MOI) of 0.0002 in carcinomic human alveolar basal epithelial A549 cells of 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/UT31641 Ⅱ /2008 (31641), A/Vietnam/UT31604 Ⅰ /2009 (31604), and A/Vietnam/UT36250 Ⅰ /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. 6A). Sequence analysis suggested that the PB2 amino acid 627K accounted for the high growth ability of 36250, 36236, 31604, and 31641. The PB2 amino acid 591R likely accounted for the high growth ability of 36282. On the other hand, 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 I focused on the replicative efficiency of 36285. First, I 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. 6B). I next compared the growth property of 36285-RG in A549 cells at 37˚C with that of 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. 6C). Ck/TY31-RG and Wb/AH82-RG grew poorly with maximum titers of less than $10^{4.5}$ PFU/ml (Fig. 6C). Of note, both 36285 and Wb/AH82 belong to the same H5 HA sub-clade 2.3.4 and are genetically closely related. Therefore, I compared 36285 and Wb/AH82 to elucidate the mechanism of H5N1 adaptation to humans.

Table. 1

PB2 amino acid markers advantageous for efficient growth in mammals.

		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

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-colored amino acid residues indicate human-type amino acids that are markers for efficient growth in mammals. *: The PB2 amino acids 147T, 339T, and 588T together enhance the polymerase activity of H5N1 virus (35).

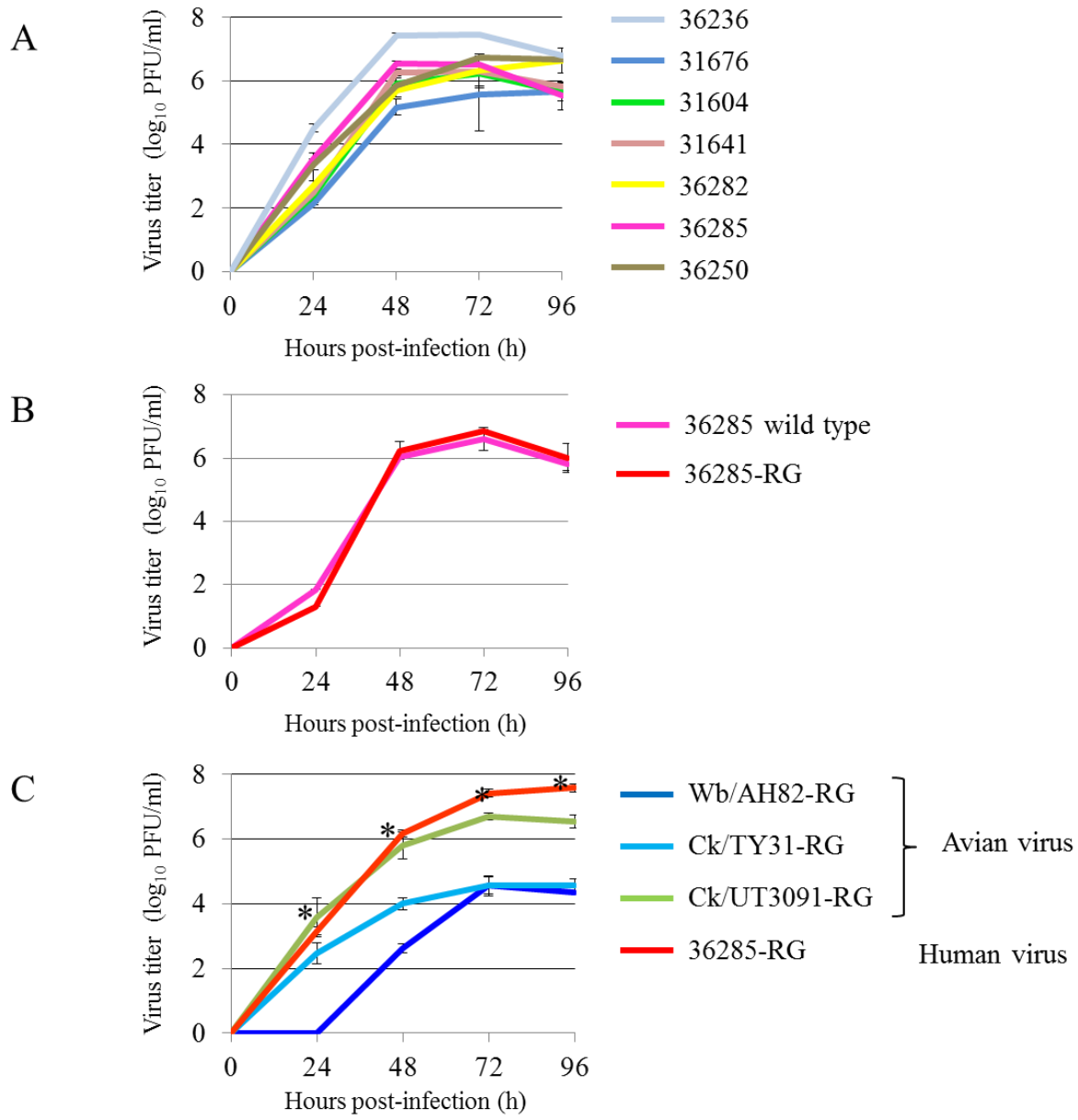

Fig. 6: Comparison of the growth properties of H5N1 viruses isolated from humans and birds

(A) A549 cells were infected with H5N1 human viruses isolated in Vietnam in 2010 at a multiplicity of infection (m.o.i) of 0.0002. Virus release into cell culture supernatant was titrated by plaque assays with MDCK cells at the indicated time points. The standard deviation represents 3 independent experiments.

(B) A549 cells were infected with the H5N1 human viruses 36285-wild type and 36285-RG at a m.o.i of 0.0002. Error bars represent the standard deviation of three independent experiments.

(C) A549 cells were infected with H5N1 human virus 36285-RG and avian viruses at a m.o.i

of 0.0002. Supernatants were titrated by plaque assays with MDCK cells at the indicated time points. The standard deviation represents 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)

In chicken embryo fibroblast DF-1 cells, 36285-RG and Wb/AH82-RG both grew well at 39˚C (Fig. 7A), 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. 7B), demonstrating its potential to replicate in mammalian cells.

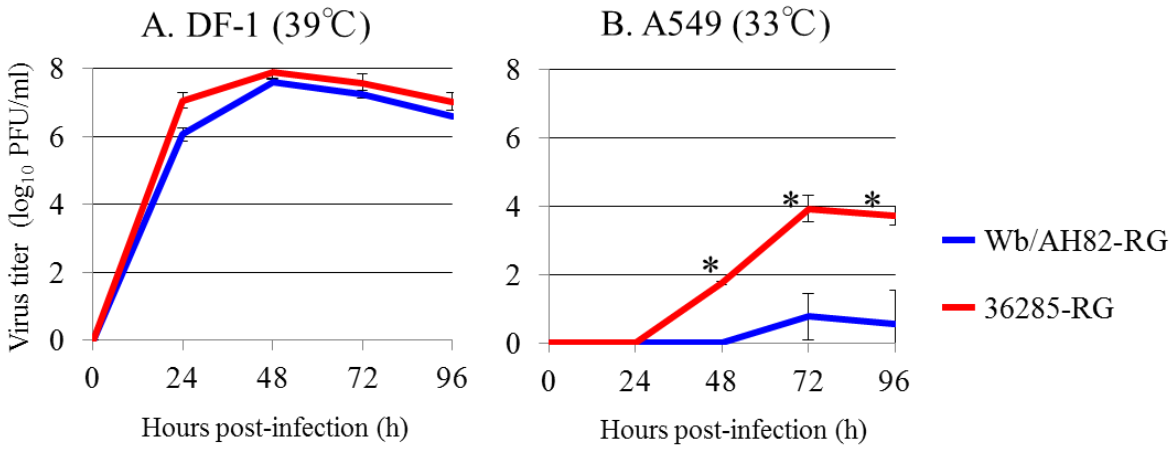

Fig. 7: Comparison of the growth properties of 36285-RG virus and Wb/AH82-RG virus in DF-1 cells and A549 cells.

(A) DF-1 cells were infected with the 36285-RG virus and the Wb/AH82-RG virus at a m.o.i of 0.0002 and cultured at 39°C. The standard deviation represents 3 independent experiments. (B) A549 cells were infected with the 36285-RG virus and the Wb/AH82-RG virus at a m.o.i of 0.0002 and cultured at 33°C. Supernatants were titrated by plaque assays with MDCK cells at the indicated time points. The standard deviation represents 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)

Comparison of the pathogenicity of Wb/AH82-RG and 36285-RG in mice

I 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. On the other hand, inoculation of 10^4 and 10^5 PFU of Wb/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. 8C, 8D).

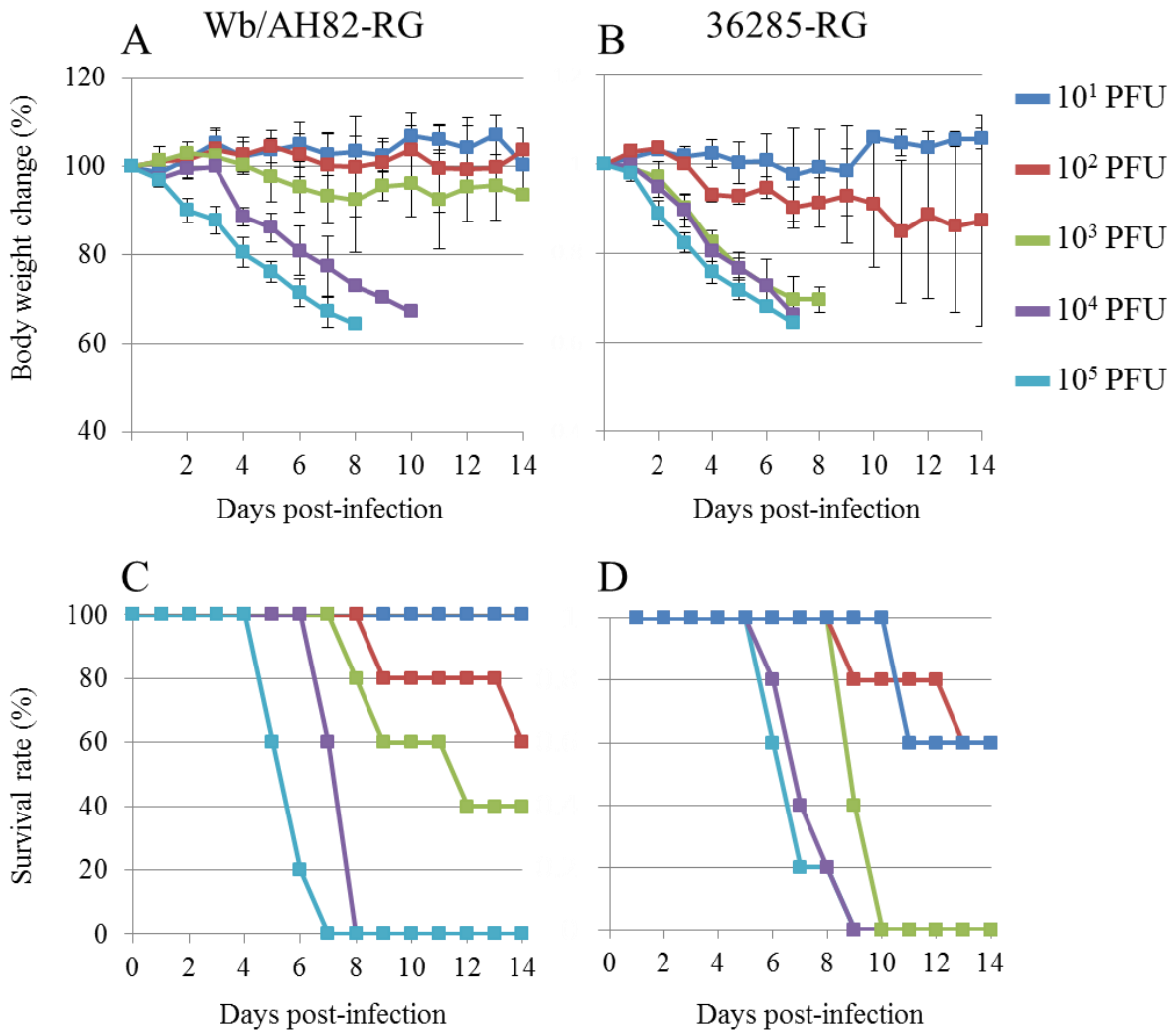

Fig. 8: Comparison of the virulence of 36285 virus and Wb/AH82 virus in mice.
Mice (5 per group) were inoculated with 10^1, 10^2, 10^3, 10^4, or 10^5 PFU of 36285-RG virus or Wb/AH82-RG virus and monitored for weight loss (A, B) and survival (C, D) for 14 days.

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, I generated a series of reassortant viruses (as illustrated in Fig. 9A) 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, PA, and 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. 9B). On the other hand, 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 lesser 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 capability between Wb/AH82-RG and 36285-RG in A549 cells.

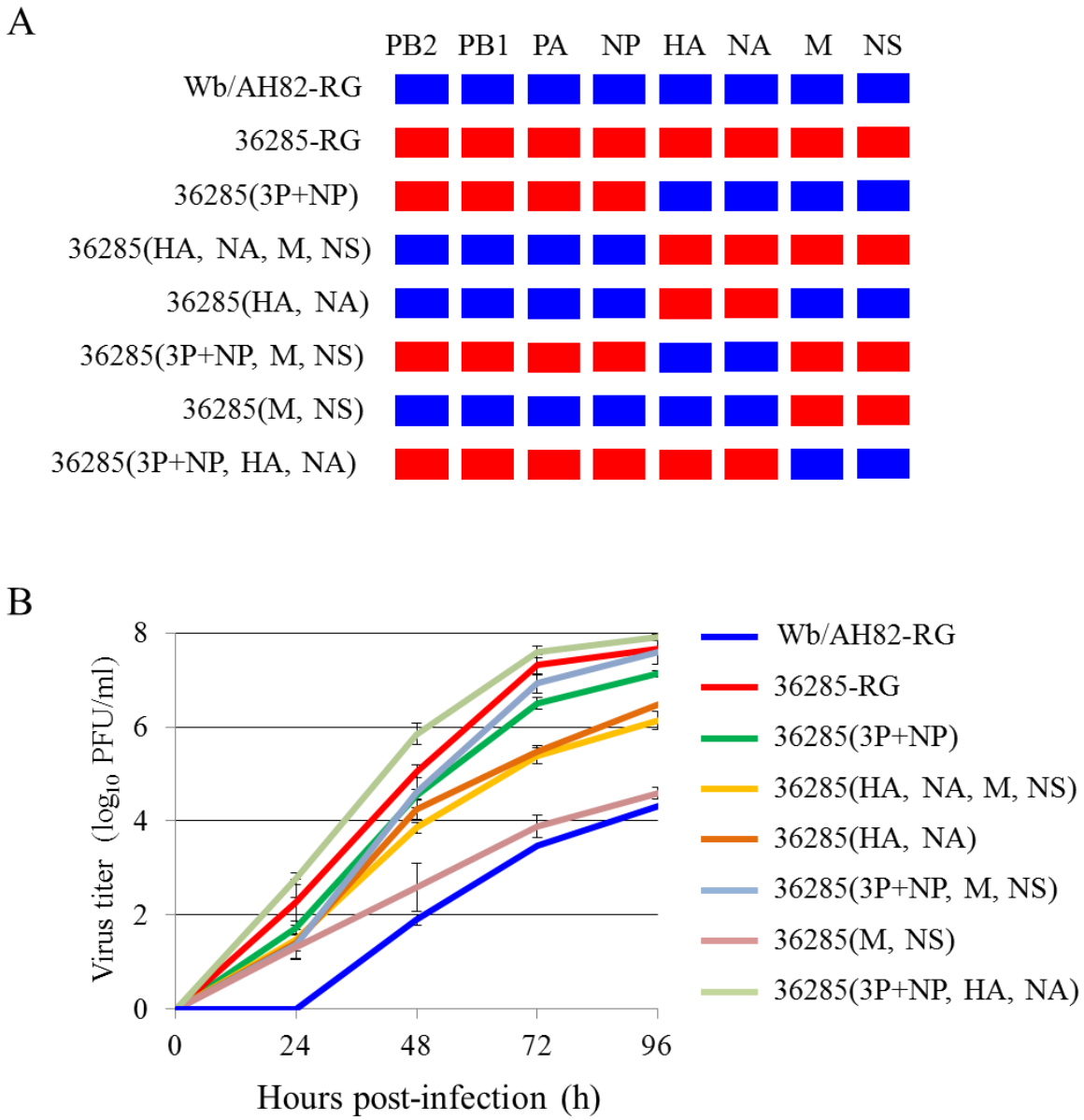

Fig. 10: Comparison of the growth properties of reassortant viruses in A549 cells.

(A) Schematic diagram of reassortant viruses.

(B) A549 cells were infected with the reassortant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

To determine which viral segment(s) among PB2, PB1, PA, and NP contributed to the high replication of 36285-RG, I generated a range of reassortants (Fig. 10A) and compared their growth properties in A549 cells. Only viruses possessing 36285(PB2) grew well (Fig. 10B). By 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 capability between Wb/AH82-RG and 36285-RG in A549 cells.

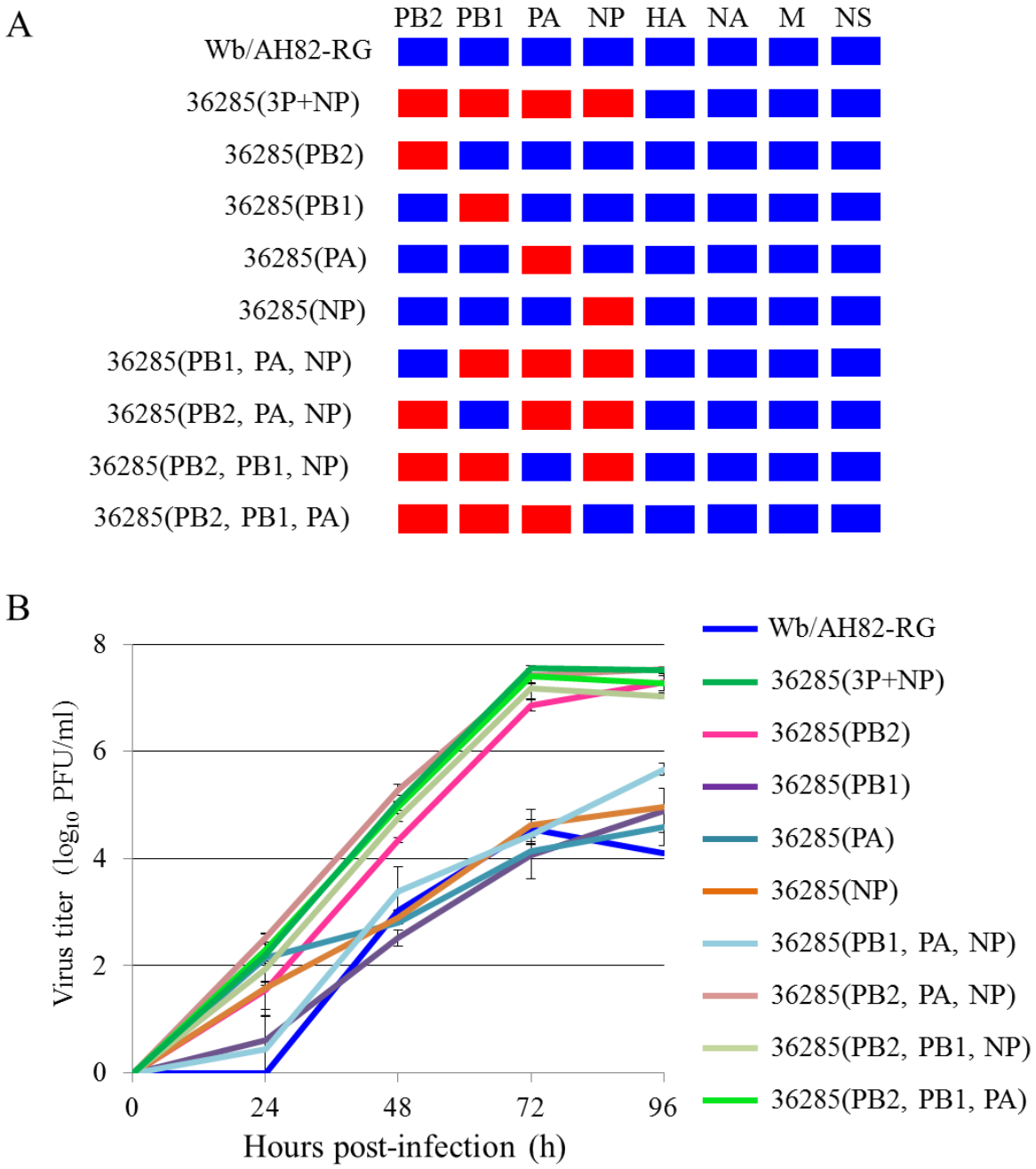

Fig. 10: Comparison of the growth properties of further reassortant viruses in A549 cells.

(A) Schematic diagram of reassortant viruses.

(B) A549 cells were infected with the reassortant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

A single G-to-D mutation at 309 and the double mutations E249G and T339M enhance the replication of Wb/AH82-RG.

Sequence comparison of the PB2 proteins of Wb/AH82 and 36285 revealed twenty-one amino acid differences (Table 2). To identify the specific changes that give rise to efficient viral replication, I generated mutant viruses possessing a chimeric PB2 protein (Fig. 11A); 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.

Table. 2

Amino acid differences in PB2 between Wb/AH82 and 36285

PB2 position	64	89	108	109	147	249	309	339	355	368	390	461	467
Wb/AH82	T	L	A	I	I	E	G	T	R	R	D	I	L
36285	I	V	T	V	T	G	D	M	K	Q	N	V	M

PB2 position	473	477	478	483	495	560	658	684
Wb/AH82	M	R	V	V	I	V	Y	T
36285	I	G	I	M	V	L	H	A

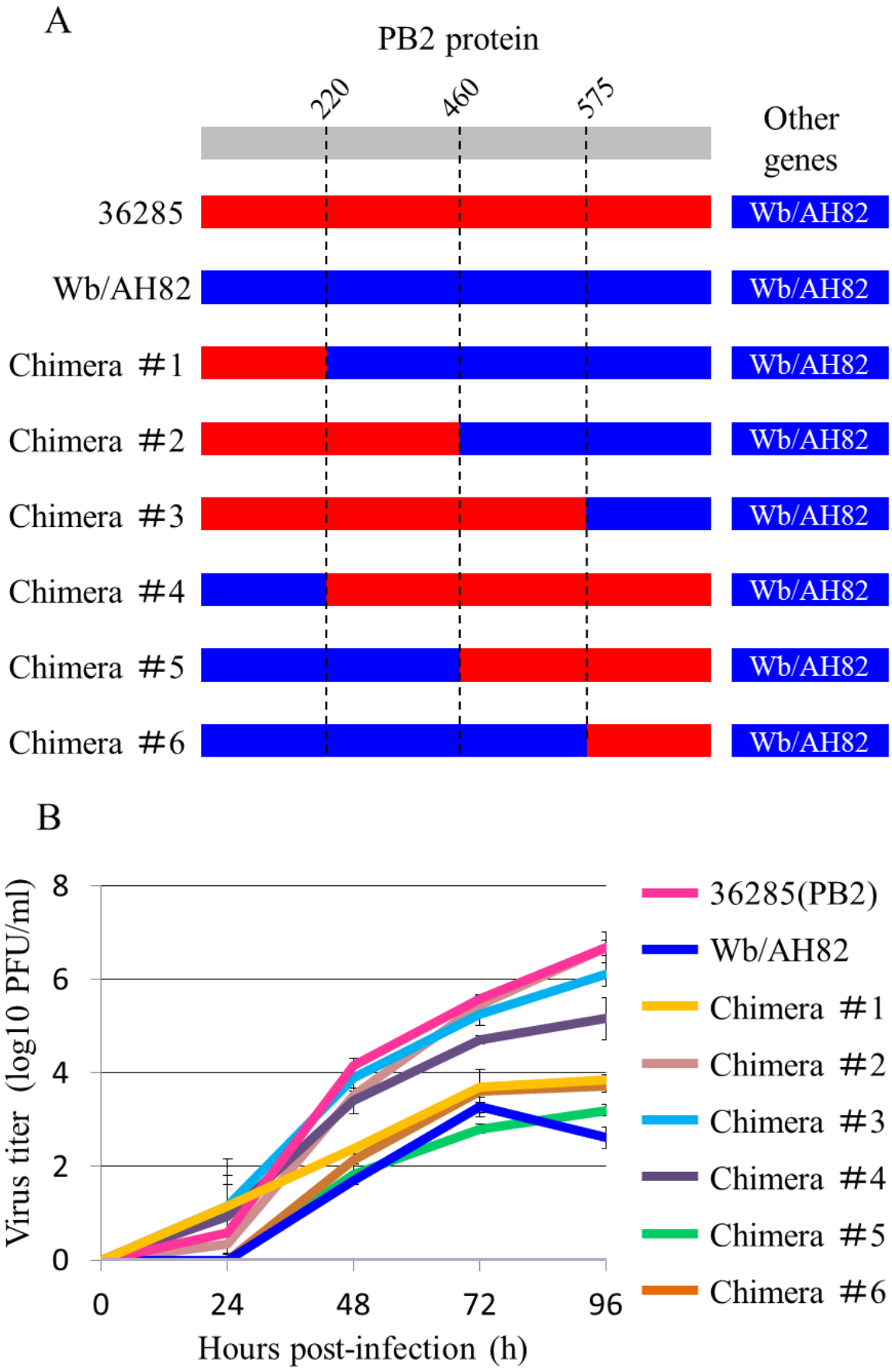
A
PB2 protein
220
460
575
Other genes
36285
Wb/AH82
Wb/AH82
Chimera #1
Chimera #2
Chimera #3
Chimera #4
Chimera #5
Chimera #6
Wb/AH82
Wb/AH82
Wb/AH82
Wb/AH82
Wb/AH82
Wb/AH82
B
Virus titer (log10 PFU/ml)
0
2
4
6
8
0
24
48
72
96
Hours post-infection (h)
36285(PB2)
Wb/AH82
Chimera #1
Chimera #2
Chimera #3
Chimera #4
Chimera #5
Chimera #6

Fig. 11: Comparison of the growth properties of PB2 chimeric viruses in A549 cells.

(A) Schematic diagram of chimeric PB2 mutants.

(B) A549 cells were infected with the mutant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

To further define which amino acid residues enhance the viral replication, I generated viruses with additional chimeric PB2 proteins (Fig. 12A). The mutant viruses possessing chimera 2, 7, 8, and 11 grew well, whereas the growth of the viruses with chimera 1, 9, and 10 was lower compared with 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. 12B).

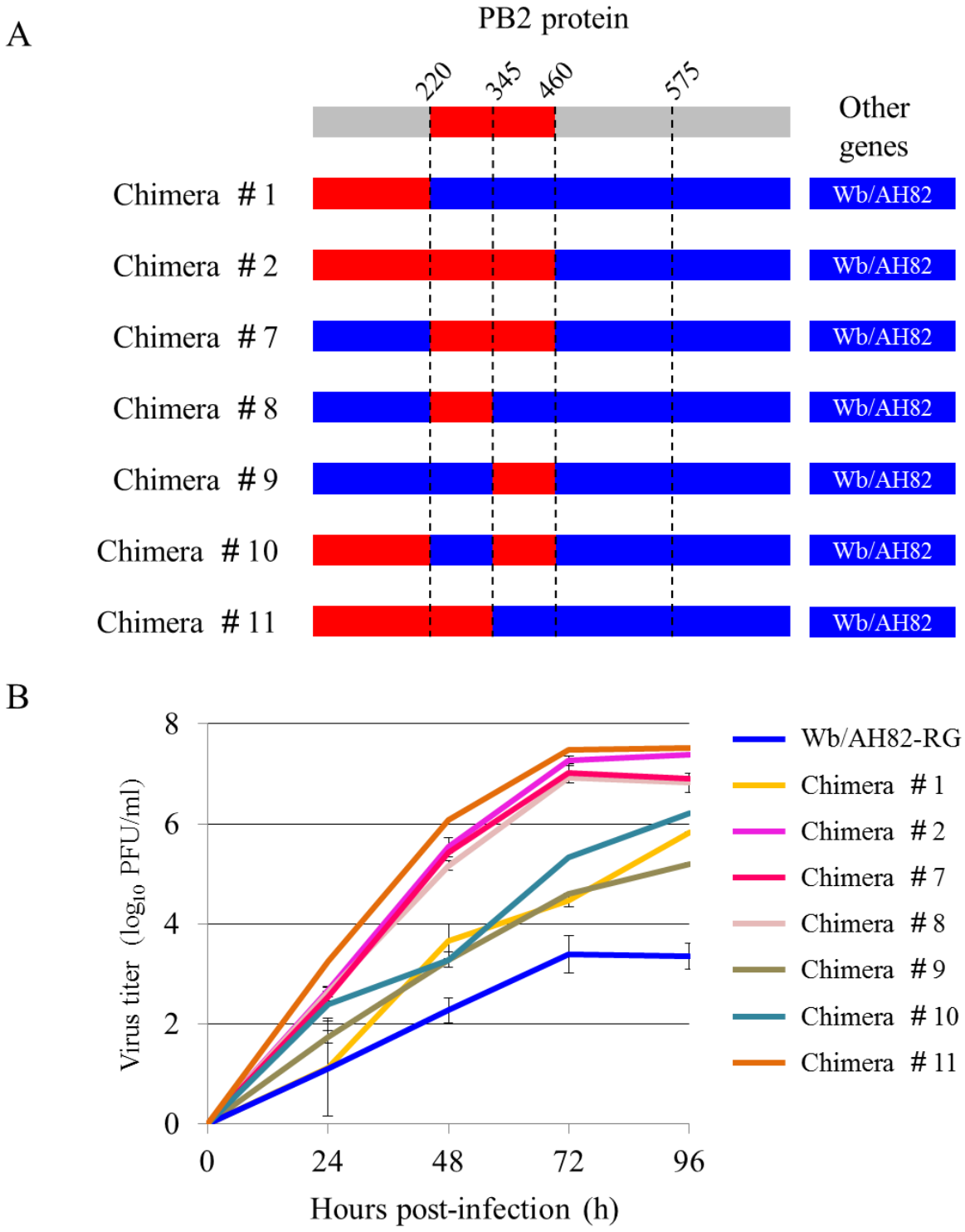

Fig. 12: Comparison of the growth properties of additional PB2 chimeric viruses in A549 cells.

(A) Schematic diagram of chimeric PB2 mutants.

(B) A549 cells were infected with the mutant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

I 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. I singly or in combination introduced the three residues of 249G, 309D, and 339M into the PB2 of Wb/AH82 (Fig. 13A) 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 Wb/AH82-RG (Fig. 13B, 13C). Although the single introduction of PB2-249G, -PB2-339M, or -PB2-309D enhanced the growth capability of Wb/AH82-RG, PB2-339M was least effective (Fig. 8B). All mutants that possessed combinations of two of the PB2 mutations tested grew better than Wb/AH82-RG by more than $10^{1.5}$-fold (Fig. 13C). These results indicate that PB2-249G, PB2-339M, and PB2-309D support efficient viral growth in A549 cells.

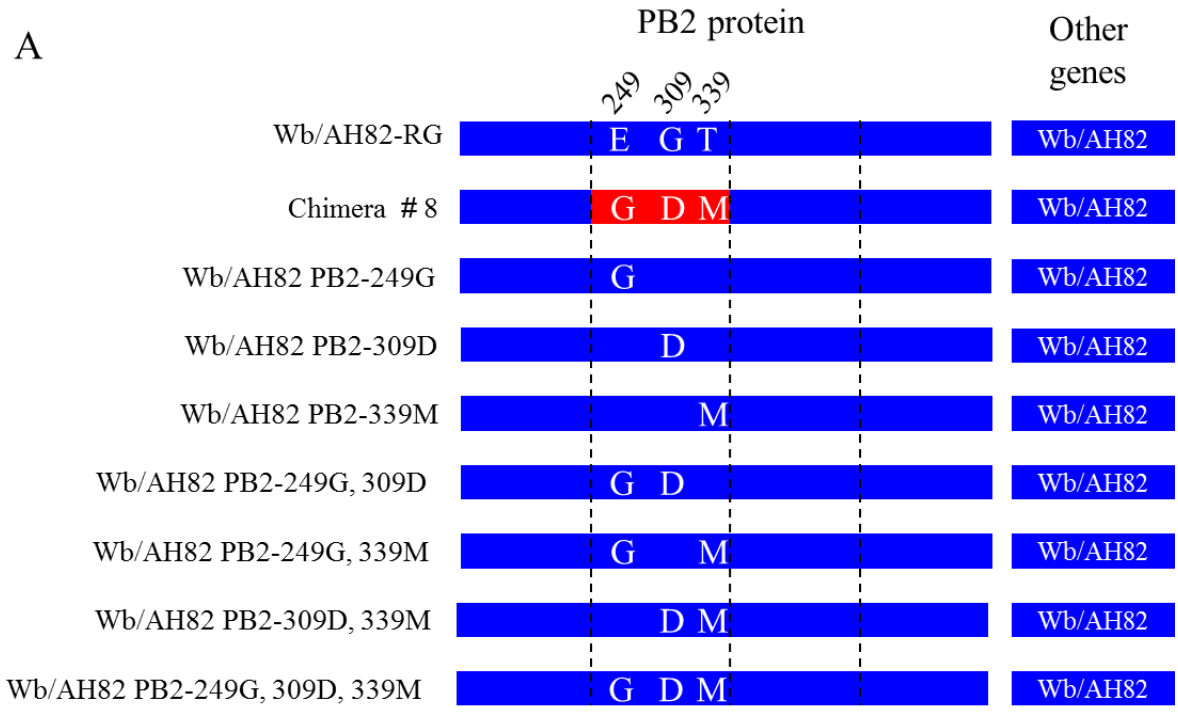

Fig. 13: Comparison of the growth properties of single, double, and triple-amino acid PB2 mutant viruses in A549 cells.

(A) Schematic diagram of single, double, and triple-amino acid PB2 mutants.

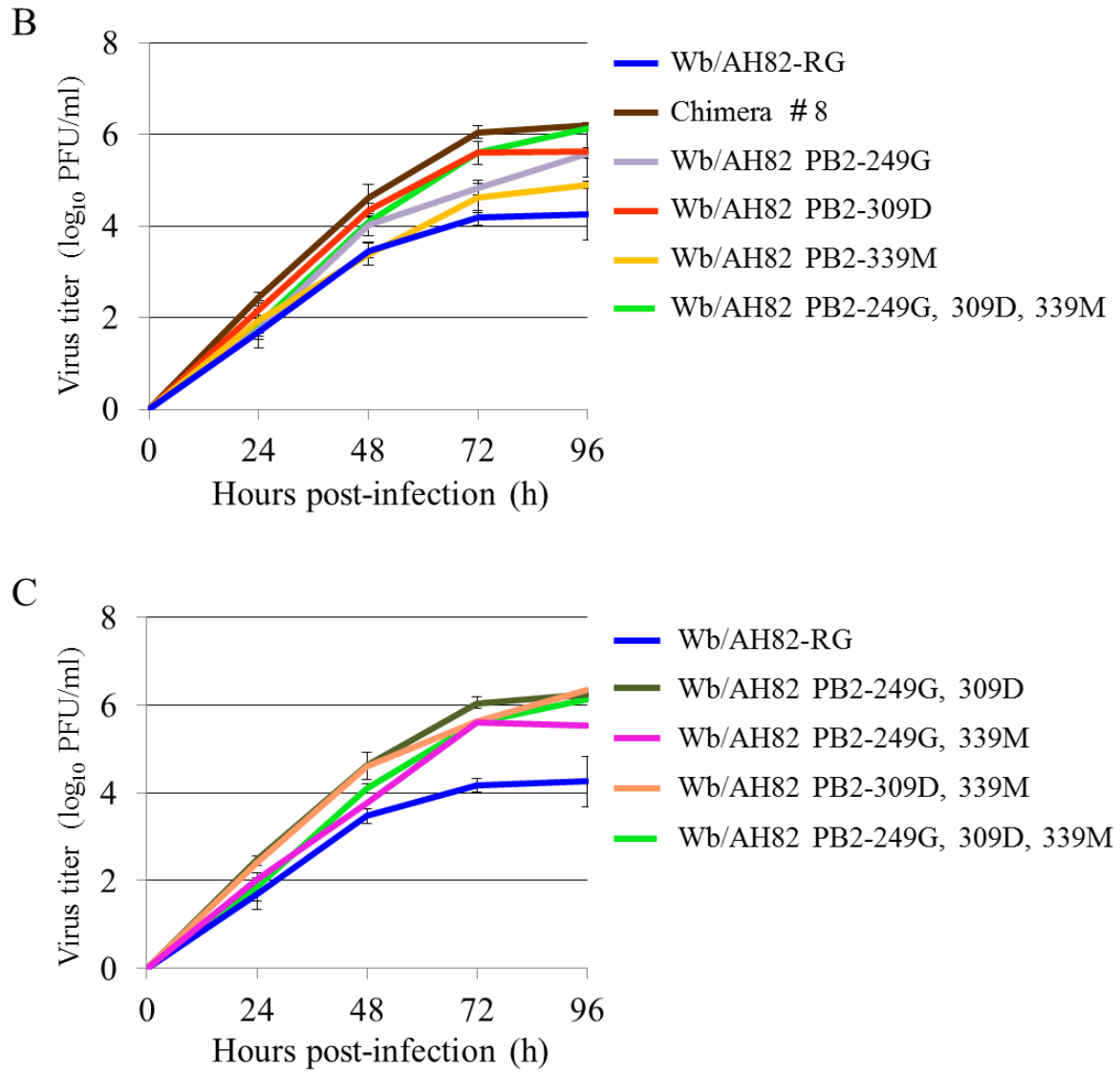

Fig. 13: Comparison of the growth properties of single, double, and triple-amino acid PB2 mutant viruses in A549 cells.

(B, C) A549 cells were infected with the mutant viruses at an m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

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, I collected full-length PB2 sequences of influenza viruses of various subtypes from the Influenza sequence database (ISD), the Influenza Research Database (IRD), and the Global Initiative on Sharing All Influenza Data (GISAID) (Table 3). Almost all of 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. On the other hand, PB2 amino acids 249G and 339M were rare among the avian H5N1 viruses. Intriguingly, no less than 33.1% 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 the human body and increases its fitness for human adaptation.

Table. 3

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

		amino acid residue (%)		
host	subtype	249G	309D	339M
avian	H5N1[a]	1.5 (29/1953)	91.9 (1794/1953)	0.1 (2/1953)
human	H5N1[a]	0.6 (1/181)	94.4 (171/181)	2.2 (4/181)
	seasonal H3N2[a]	33.1 (1314/3968)	99.9 (3965/3968)	0 (0/3968)
	seasonal H1N1[b]	0 (0/932)	65.1 (607/932)	0 (0/932)
	pdm H1N1[b]	0 (0/3939)	99.8 (3932/3939)	0 (0/3939)
swine	H3N2[c]	2 (11/541)	100 (541/541)	0 (0/541)
	pdm H1N1[c]	2 (5/258)	99.6 (257/258)	0 (0/258)
	H1N1 (except for pdm) [c]	1.1 (10/922)	98.4 (908/922)	0 (0/922)

Data cited above are percentages; numbers in parentheses indicate the total number of positive/total number of samples.

[a] Full-length PB2 sequences of avian and human H5N1 viruses, and human seasonal H3N2 viruses from the ISD (Influenza sequence database) were analyzed on July 2013.

[b] Full-length PB2 sequences of human seasonal H1N1 and pdm H1N1 viruses from the GISAID (Global Initiative on Sharing All Influenza Data) were analyzed on July 2013.

[c] Full-length PB2 sequences of all swine viruses from the IRD (Influenza Research Database) were analyzed on July 2014.

Discussion

Several amino acid mutations associated with the adaptation of H5N1 virus to humans have been identified, including 627K (10-12), 591R, 591K (32), 701N (11,29-31), 271A (33), and 158G (34) of PB2. I 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, I 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; I did not generate viruses possessing 'novel' amino acid changes. These data will provide information of value for pandemic preparedness and for use in evaluating the pandemic risk potential of future isolates.

The PB2 protein is a multi-functional protein (Fig. 14) that plays a central role in the initiation of transcription because it is responsible for binding the cap on to host mRNA molecules. NMR and x-ray structure studies have revealed that the C-terminal domain of PB2 has nuclear localization signals, which assist in the nuclear import of the viral nucleoprotein complex by being recognized by human importin ∝5 (28). In addition to binding and interacting with PB1 through the N-terminal of PB2 (57, 58), the PB2 protein also interacts

with PA. However, the exact region of PA that is involved in this interaction has not been defined (59).

PB2-E249G and T339M have an interesting common feature in that they belong to areas identified as ‘cap-binding' sites (60-63) (Fig. 14). 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 10–13 nucleotides by the PA subunit (64-66) (Fig. 15). 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 (63). 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 (61), but another study identified the PB2 amino acid residues 242–282 and 538–577 as important (62), and yet another study suggested that amino acid residues 318–483 were involved (63). The effect of the amino acid 339T *in vivo* and *in vitro* on biologic events is also inconsistent. Our group recently found that 339T has a major impact on the polymerase activity of H5N1 virus jointly with 147T and 588T (35). On the other hand, another paper reported that K339T reduced PB2 cap binding activity and influenza polymerase activity *in vitro*, and also attenuated virulence in mice (67). This 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 (67). In the

current study, I 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 my understanding of PB2 functions.

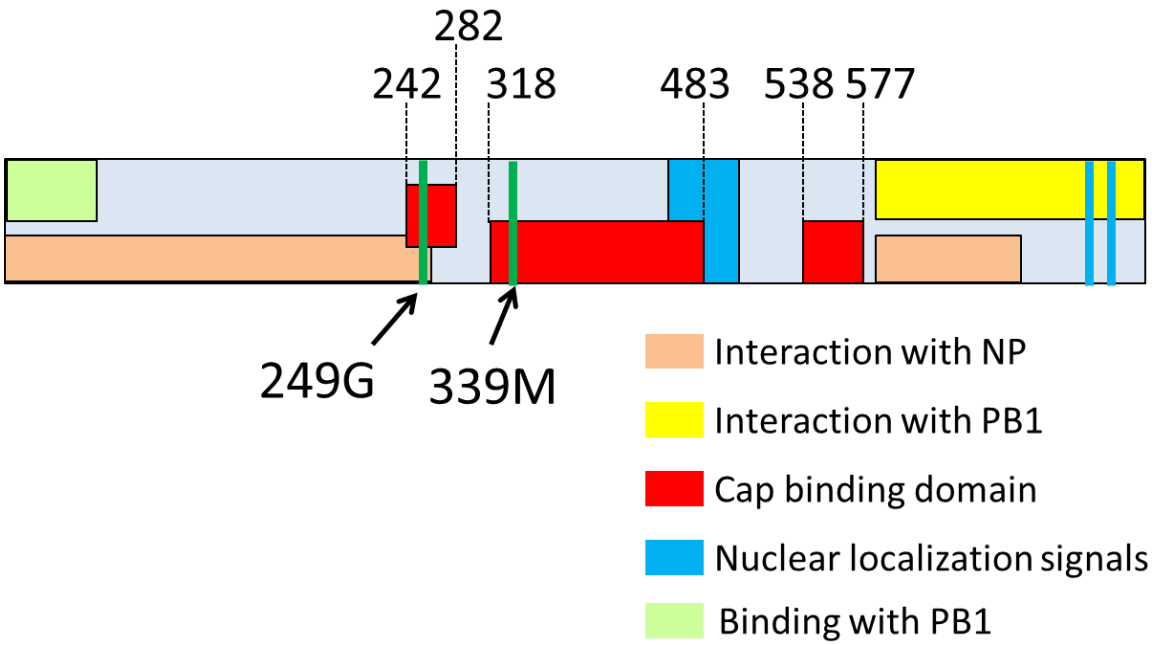

Fig. 14: Schematic representation of the PB2 protein

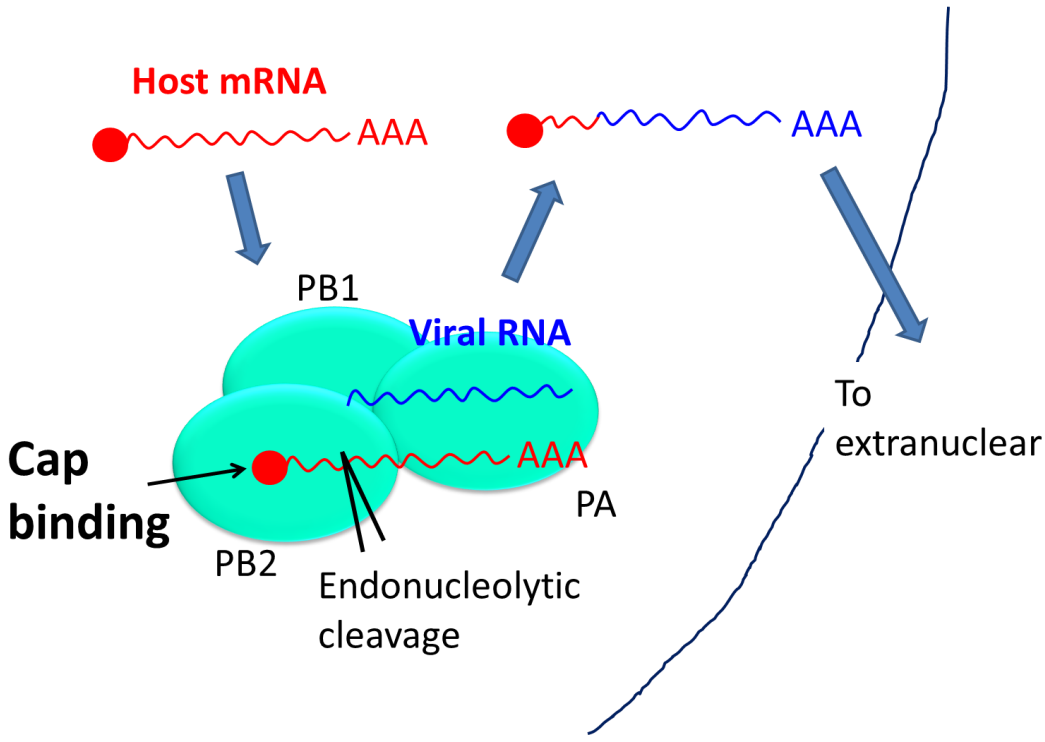

Fig. 15: Cap-snatching mechanism of the influenza virus polymerase.

The PB2 subunit binds to the cap of a host cellular mRNA molecule. The, the endonuclease activity of PA cleaves the cellular mRNA. The viral polymerase then uses the cap as a primer for viral mRNA synthesis.

Human influenza A virus acquired the PB2 amino acid mutation E249G upon mouse adaptation (68), 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 over 30%. 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. 8), whereas the titers of the two viruses in A549 cells differed by more than 3 log units (Fig. 6). The HA and NA genes are involved, to some extent, in the difference in the growth ability 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 (69-72). Humans primarily have sialic acid linked to galactose by an α2,6-linkage (Sia-α2,6Gal) (68-71), whereas mice primarily have Sia-α2,3Gal-type linkages (72). 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 (73). 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, here I found that the PB2 amino acid substitutions E249G, G309D, and T339M enhance the replicative ability of H5N1 virus in A549 cells. My 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.

CHAPTER Ⅱ

Mammalian Adaptive Mutations of the PA Protein of Highly Pathogenic Avian H5N1 Influenza Virus

Results

Comparison of the characteristics of human and avian H5N1 viruses.

Previously, I compared the growth properties in human lung adenocarcinoma epithelial A549 cells of seven H5N1 viruses isolated from humans in Vietnam at 37˚C (submitted). All seven human H5N1 viruses grew efficiently in A549 cells. Of these seven viruses, five contained the PB2 amino acid 627K or 591R, which was responsible for their high replicative capabilities. However, sequence analysis revealed that A/Vietnam/UT36285/2010 (36285) did not have any known PB2 markers of adaptation to mammals (Table 4). Moreover, the virus did not have any markers known to contribute to adaptation to mammals in any of its other segments. These findings suggest that the human H5N1 virus 36285 likely has as yet unreported amino acids that enhance its viral growth capability in A549 cells. Consequently, in this study I focused on the replicative efficiency of 36285. 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. 16).

Table 4

PB2 amino acid markers advantageous for efficient growth in mammals.

		PB2							
		627	271	591	158	701	147*	339*	588*
Proposed human adaptation marker		K	A	R/K	G	N	T	T	T
Human isolate	**36285**	E	T	Q	E	D	T	M	A
Avian isolate	**Ck/TY31**	E	T	Q	E	D	I	K	A

The amino acid residues cited above have been confirmed to particularly contribute to efficient virus growth in mammalian cells or to high pathogenicity in mammals. The yellow-colored amino acid residues show human-type amino acids that are markers for efficient growth in mammals. *: The PB2 amino acids 147T, 339T, and 588T together enhance the polymerase activity of H5N1 virus (35).

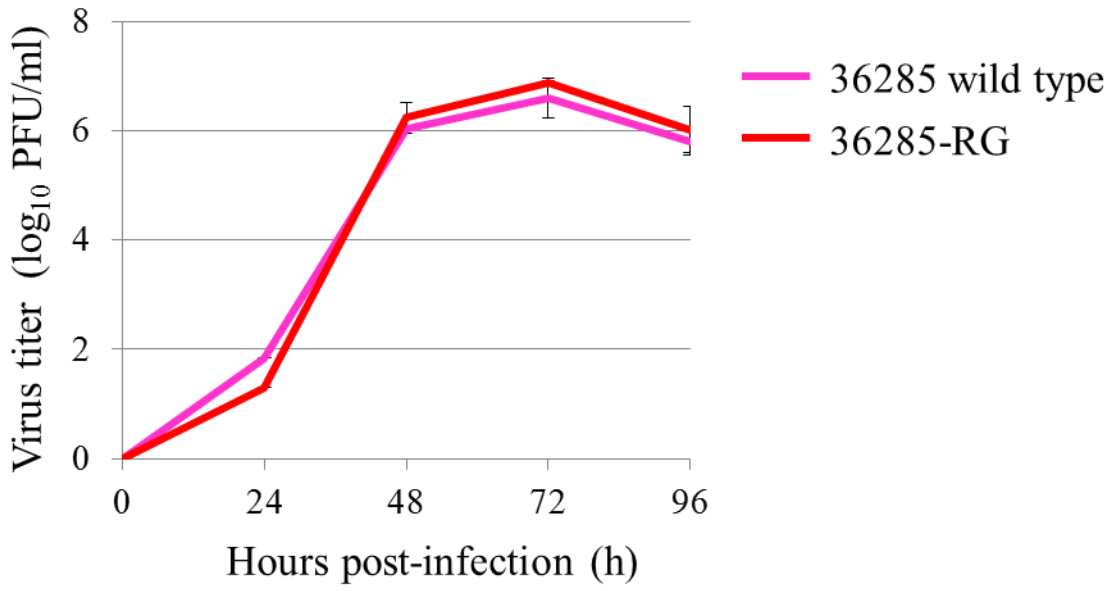

Fig. 16: Comparison of the growth properties of wild-type 36285 and 36285-RG in A549 cells

A549 cells were infected with the wild type of 36285 and 36285-RG at a multiplicity of infection (m.o.i) of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

To compare the biological features of the human virus 36285 with those of an avian H5N1 virus, I needed to select an avian H5N1 virus from our repository. In my previous study, I assessed the growth capabilities in A549 cells of three avian H5N1 viruses that are genetically closely related to 36285. Of these three, I chose Ck/TY31 for my comparative assessment in this study. 36285-RG and Ck/TY31-RG grew similarly well in DF-1 cells (Fig. 17A); however, 36285-RG grew much better than Ck/TY31-RG in A549 cells both at 37°C and at 33°C. (Fig. 17B,C). I therefore decided to determine the amino acid residues responsible for the differences in growth capability between 36285-RG and Ck/TY31-RG to better understand H5N1 virus adaptation to humans.

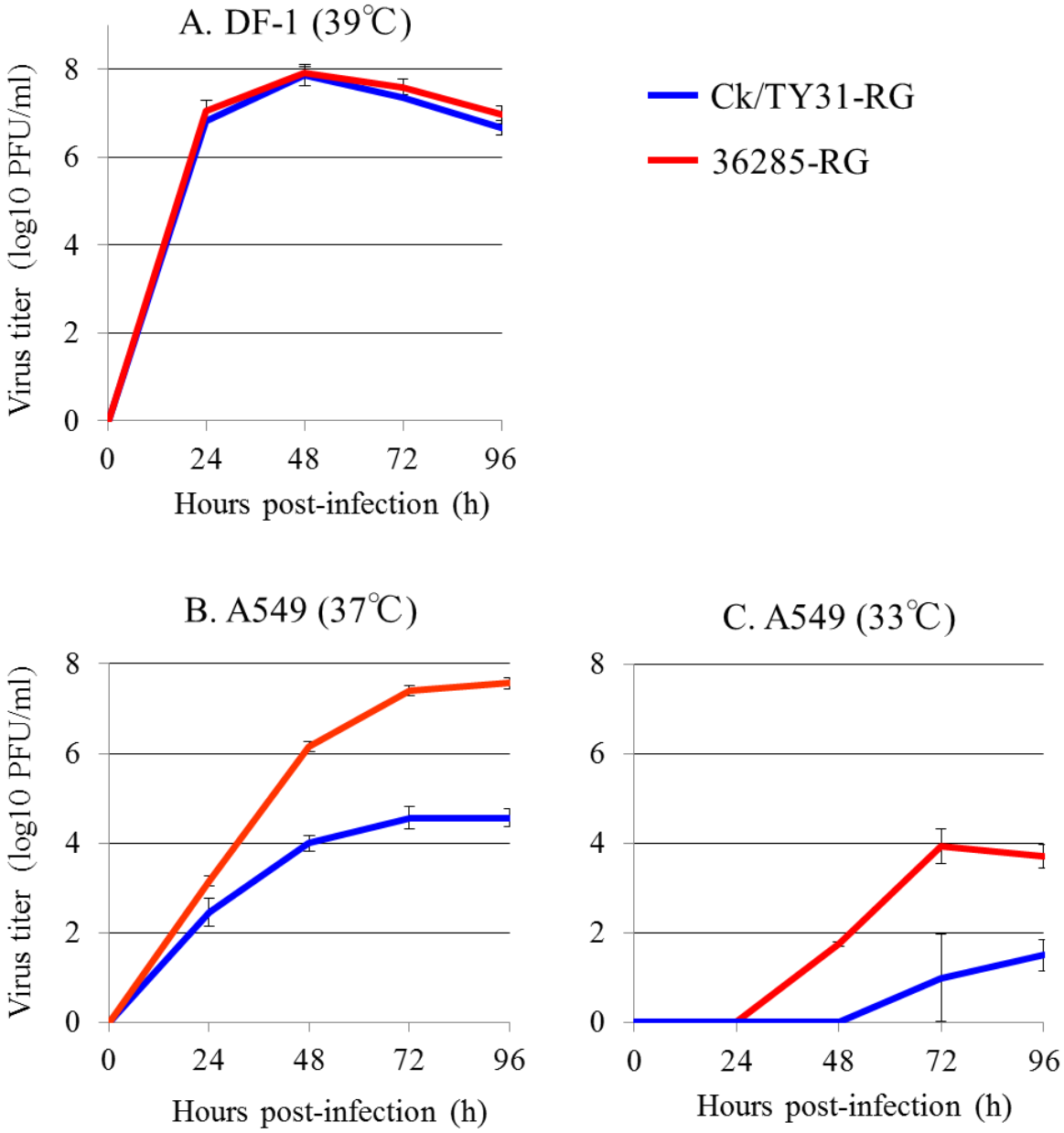

Fig. 17: Comparison of the growth properties of 36285-RG virus and Ck/TY31-RG virus in DF-1 cells and A549 cells.

(A)DF-1 cells were infected with 36285-RG virus and Ck/TY31-RG virus at a m.o.i of 0.0002 and cultured at 39˚C. The standard deviation represents 3 independent experiments.

(B)A549 cells were infected with 36285-RG virus and Ck/TY31-RG virus at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

(C) A549 cells were infected with 36285-RG virus and Ck/TY31-RG virus at a m.o.i of 0.0002 and cultured at 33˚C. The standard deviation represents 3 independent experiments.

Contributions of the PA and NS genes to the efficient growth of 36285 in human cells.

To clarify the molecular basis for the difference in growth capability in A549 cells of Ck/TY31-RG and 36285-RG, I generated a series of reassortant viruses, as illustrated in Fig. 18A, and compared their growth properties in A549 cells at 37°C. The reassortants were named according to the origin of their Ck/TY31 or 36285 genes. For example, "36285(PB2, PB1, PA, NP)" indicates a virus possessing PB2, PB1, PA, and NP from 36285 and the rest of its gene segments from Ck/TY31. Four viruses possessing PB2, PB1, PA, and NP of 36285 [i.e., 36285-RG, 36285(3P and NP), 36285(3P, NP, HA, NA), and 36285(3P, NP, M, NS)], had substantially higher growth ability than that of Ck/TY31 (Fig. 18B). These results indicate that PB2, PB1, PA, or NP of 36285 was responsible for the difference in growth capability between Ck/TY31-RG and 36285-RG in A549 cells. Of note, 36285(M, NS) also grew better (by at least 1.8 log units) than Ck/TY31-RG. Likewise, two viruses possessing the M and NS segments of 36285 [i.e., 36285(3P, M, NS) and 36285(HA, NA, M, NS)] grew better (by at least 1.8 log units) than Ck/TY31-RG. These data show that either M or NS also contributes to the difference in growth capabilities of Ck/TY31-RG and 36285-RG. However, the replicative ability of 36285(HA, NA) was similar to that of Ck/TY31, indicating that the HA and NA of 36285-RG are not responsible for the difference in growth capabilities between Ck/TY31-RG and 36285-RG in A549 cells (Fig. 18B).

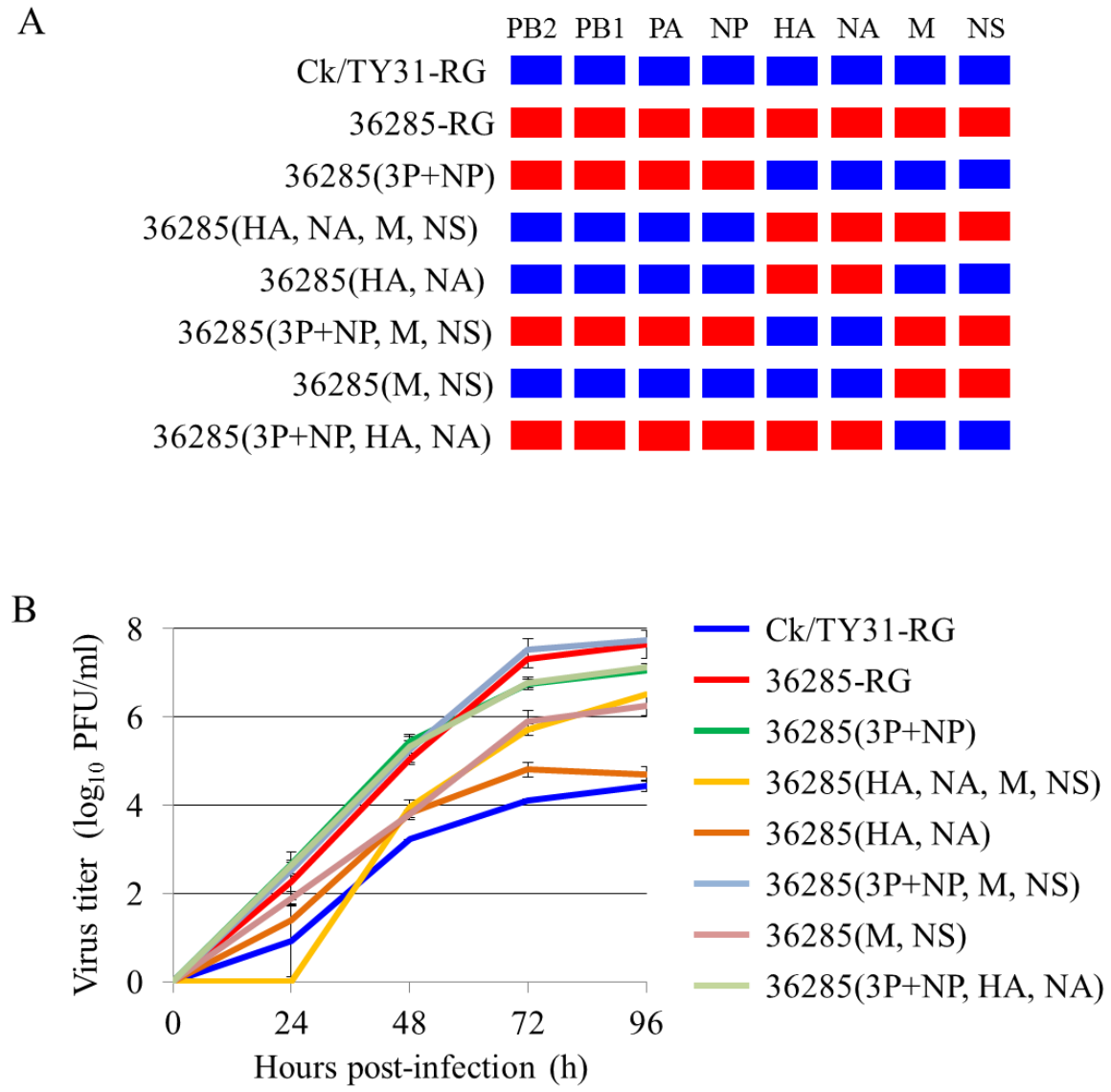

Fig. 18: Comparison of the growth properties of reassortant viruses in A549 cells.

(A) Schematic diagram of reassortant viruses.

(B) A549 cells were infected with the reassortant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

To determine which viral segment(s) among PB2, PB1, PA, and NP contribute to the replicative efficiency of 36285, I generated a series of reassortants (Fig. 19A) and compared their growth properties in A549 cells. 36285(PA) grew better by more than 1 log unit than Ck/TY31-RG, whereas PB2 only slightly enhanced viral replication, and 36285(PB1) and 36285(NP) grew poorly, similarly to Ck/TY31-RG. These results demonstrate that the PA of 36285 makes an important contribution to the difference in growth capability between Ck/TY31-RG and 36285-RG in A549 cells (Fig. 19B).

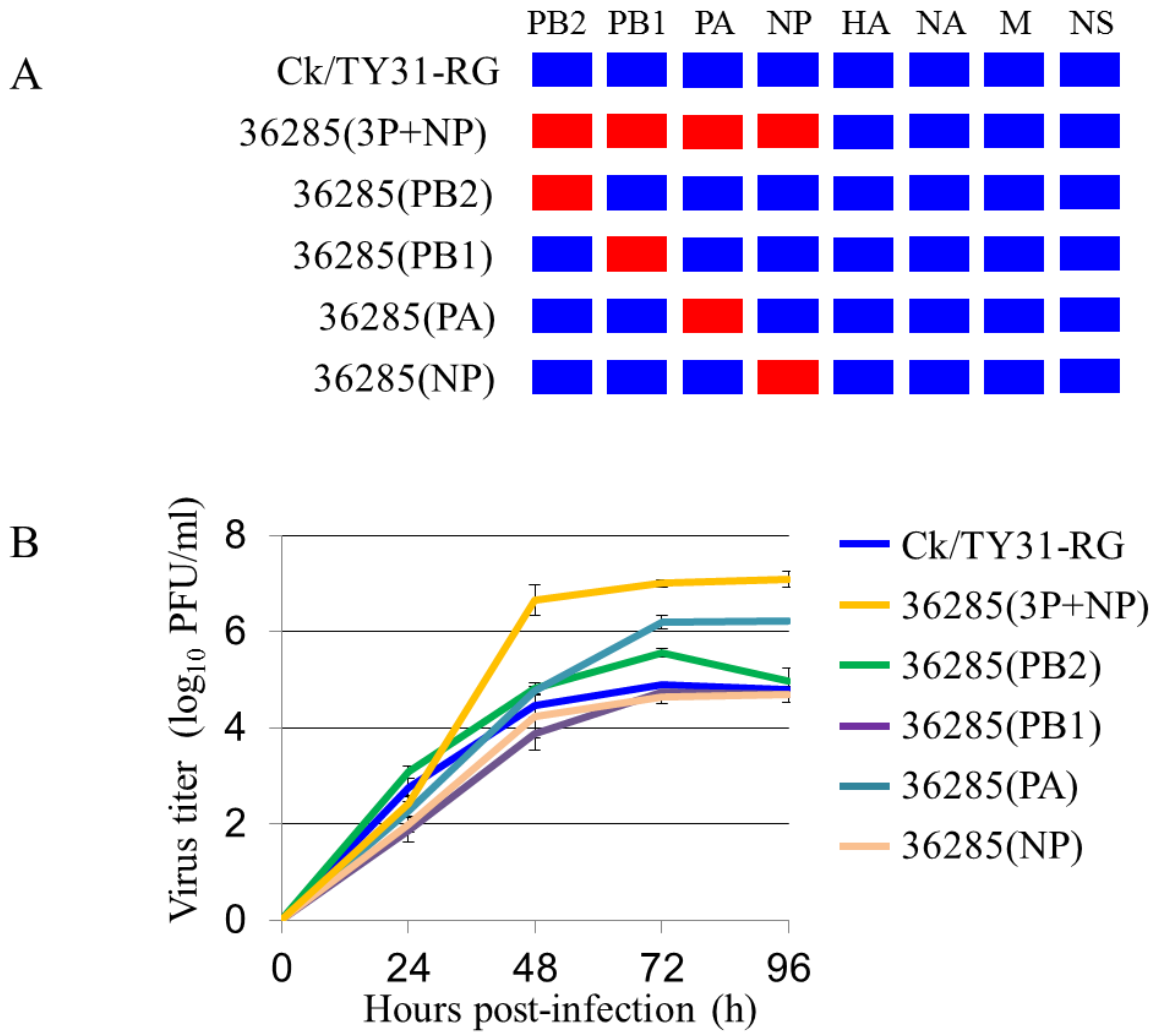

Fig. 19: Comparison of the growth properties of detailed reassortant viruses in A549 cells.

(A) Schematic diagram of reassortant viruses.

(B) A549 cells were infected with the reassortant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

To identify whether the M or NS segment increased the growth capability of Ck/TY31-RG, we created single-gene reassortant viruses and compared their growth properties in A549 cells (Fig. 20A). 36285(NS) grew better than Ck/TY31-RG by about 2 log units. This finding indicates that the NS of 36285 also contributes to the difference in growth capabilities between Ck/TY31-RG and 36285-RG, although it is unclear whether changes in NS1 or NS2, or both, affect growth (Fig. 20B). Taken together, my data show that the PA and NS of 36285 enhance the growth properties of this virus in A549 cells.

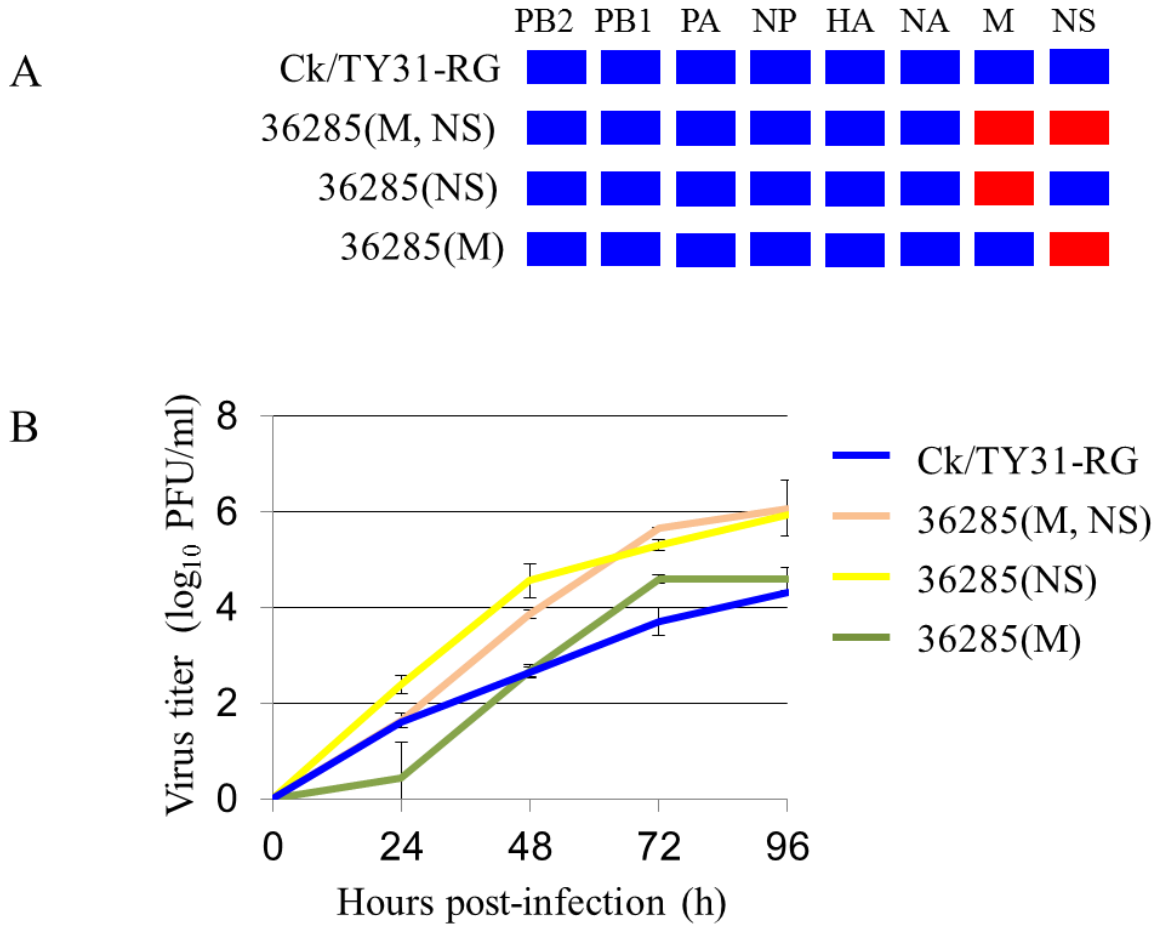

Fig. 20: Comparison of the growth properties of further reassortant viruses in A549 cells.

(A) Schematic diagram of reassortant viruses.

(B) A549 cells were infected with the reassortant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

The PA mutations V44I, V127A, C241Y, A343T, and I573V enhance the growth capability of Ck/TY31 in human cells.

Sequence comparison of the PA proteins of Ck/TY31 and 36285 revealed twenty-five amino acid differences (Table 5). Of these 25 amino acids, 16 of those present in 36285 (shown in yellow in Table 5) were found in the majority of H5N1 avian isolates. I therefore focused on the amino acid residues at the remaining nine positions (Table 6), namley, 44I, 85A, 127A, 231T, 241Y, 305F, 343T, 401K, and 573V. I first singly introduced the nine residues of 44V, 85T, 127V, 231A, 241C, 305Y, 343A, 401R, and 573I that are encoded by Ck/TY31 into the PA of 36285-RG (Fig. 21A). I generated viruses containing Ck/TY31 PB2, PB1, NP, HA, NA, M, and NS and the mutant PA and compared their growth properties in A549 cells. I found that 36285(PA-A85T), 36285(PA-T231A), 36285(PA-F305Y), and 36285(PA-K401R) grew comparably to or slightly better than 36285(PA) at 72 h and 96 h post-infection; none of the differences were statistically significant. These results indicate that the PA amino acids 85A, 231T, 305F and 401K encoded by 36285 do not contribute greatly to the high growth property of 36285 (Fig. 21B). On the other hand, the maximum titers of 36285-RG (PA-I44V), 36285(PA-A127V), 36285(PA-Y241C), 36285(PA-T343A), and 36285(PA-V573I) were at least 3-fold lower than that of 36285(PA). Given these results, I speculated that the PA amino acids at positions 44, 127, 241, 343, and 573 are involved in the difference in growth capability between Ck/TY31-RG and 36285-RG (Fig. 21B).

Table 5

Amino acid differences between the PA of Ck/TY31 and 36285

PA position	44	58	85	101	115	127	129	204	231	241	261	305	343	400	401
36285	I	G	A	D	N	A	I	R	T	Y	M	F	T	P	K
Ck/TY31	V	S	T	E	D	V	T	K	A	C	L	Y	A	S	R

PA position	404	407	554	573	631	648	653	669	712	714
36285	S	I	I	V	S	S	P	V	T	A
Ck/TY31	A	V	V	I	G	N	S	T	A	V

Of the twenty-five amino acid positions shown, the 16 yellow-colored residues were found in the majority of H5N1 avian isolates.

Table 6

PA residues 44, 85, 127, 231, 241, 305, 343, 401, and 573 in viruses isolated from avian, human, and swine sources

Host	Subtype	Amino acid residues (%)									total
		44I	85A	127A	231T	241Y	305F	343T	401K	573V	total
Avian	H5N1 [a]	0	1	0.2	2.2	5.9	0.1	2.4	3.5	0	1932
Human	H5N1 [b]	0.3	9.6	0	5.0	2.6	0.7	4.2	7.6	1.1	260
	Seasonal H3N2 [a]	0	0	0	0	3.1	0	0	0	75.8	3961
	Seasonal H1N1 [c]	0	0.3	0	0.5	98.5	0	0	0	0.3	965
	H1N1 pdm [c]	0	0	0	0	0	0	2.6	0.1	0	3517
Swine	H1 [a]	5	0.9	0.3	0	26.7	0	1.8	1.6	0.3	1179
	H3 [a]	2	3.3	0	0.1	5.5	0	2.6	1	3.1	575
	H9 [a]	0	2.2	0	2.2	6.6	0	6.6	40	0	45
Avian	H1 [a]	0	0	0	0	2.1	0	0	0.2	0	455
	H2 [a]	6	0.2	0	0	0.8	0	0	1.1	0.2	360
	H3 [a]	0	0.5	0.2	0.2	0	0	0.2	0.7	0	1340
	H7 [a]	2	1	0	0.2	0.3	0.2	0.1	0.1	0	820
	H9 [a]	7	2.1	0	0.1	0.5	0	1.6	9.6	0.1	964

Data cited above are percentages; numbers in parentheses indicate the total number of positive/total number of samples.

[a] Full-length PA sequences of all avian viruses, human seasonal H3N2 viruses, and all swine viruses from the ISD (Influenza sequence database) were analyzed on July 2013.

[b] Full-length PA sequences of human H5N1 viruses from the IRD (Influenza Research Database) were analyzed on September 2014.

[c] Full-length PA sequences of human seasonal H1N1 and pdm H1N1 viruses from the GISAID (Global Initiative on Sharing All Influenza Data) were analyzed on July 2013.

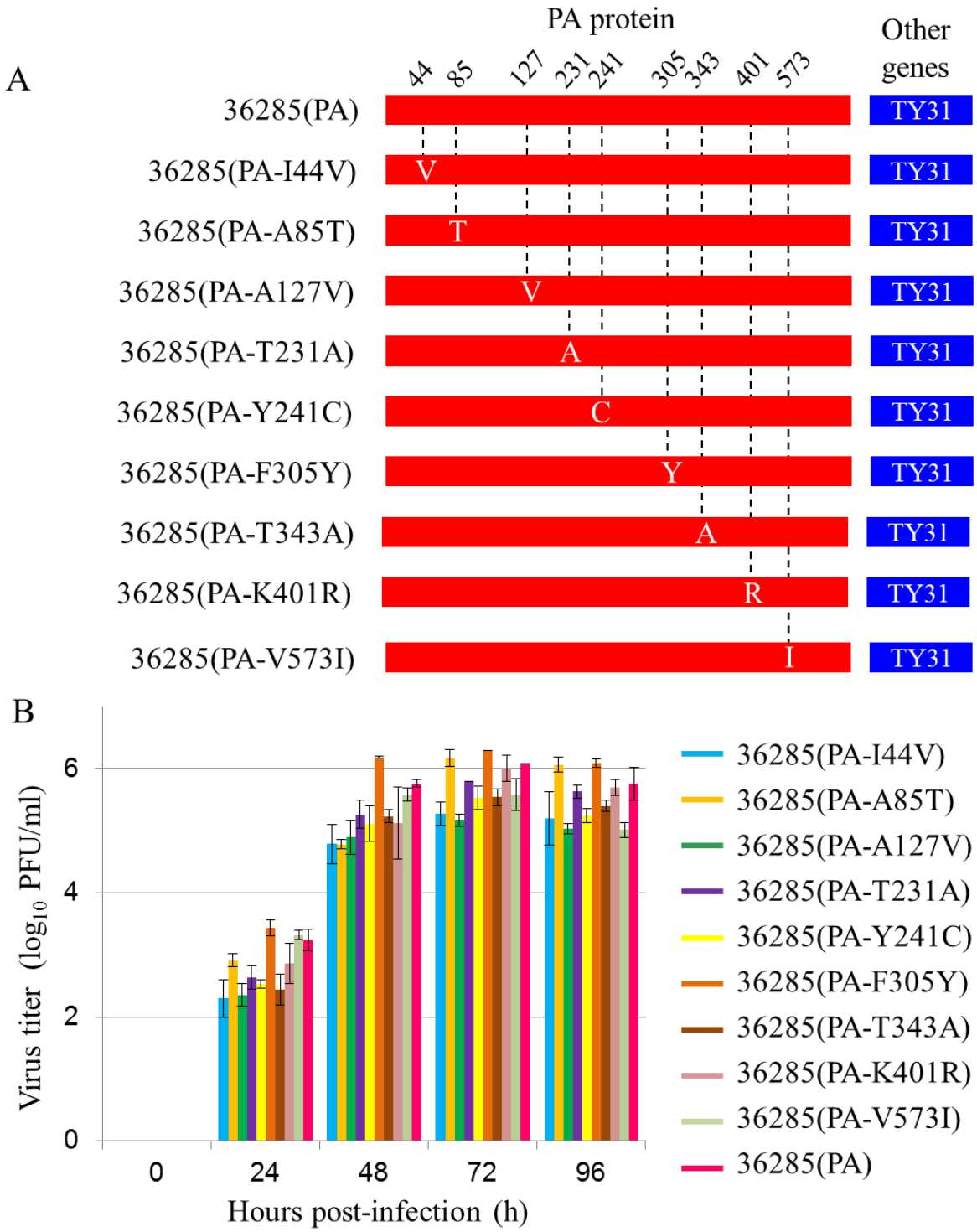

Fig. 21: Comparison of the growth properties of single PA mutant viruses in A549 cells.
(A) Schematic diagram of PA mutants.
(B) A549 cells were infected with the mutant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

To determine whether the five PA residues 44I, 127A, 241Y, 343T, and 573V encoded by 36285 could increase the growth capability of Ck/TY31-RG, I examined the growth of a PA mutant of Ck/TY31 bearing 44I, 127A, 241Y, 343T, and 573V singly or in combination (Fig. 22A). “Ck/TY31-PA 5 mutations” indicates a virus possessing a PA bearing all five amino acid substitutions 44I, 127A, 241Y, 343T, and 573V. All six PA mutant viruses contained other segments from Ck/TY31. I generated the PA mutant viruses and compared their growth kinetics in A549 cells. Ck/TY31-PA 5 mutations grew as well as 36285(PA) until 72 h post-infection (Fig. 22B). The PA amino acids I573V and A343T enhanced the growth capability of Ck/TY31-RG by at least 1 log unit at 96 h post-infection; however, none of the single amino acid substitutions alone enhanced virus replication to the level of 36285 PA. These findings suggest that, in combination, these amino acid substitutions contribute to efficient viral replication in A549 cells (Fig. 22B). Taken together, my findings indicate that the PA amino acids 44I, 127A, 241Y, 343T, and 573V are determinants of the difference in growth capability between Ck/TY31-RG and 36285-RG in A549 cells.

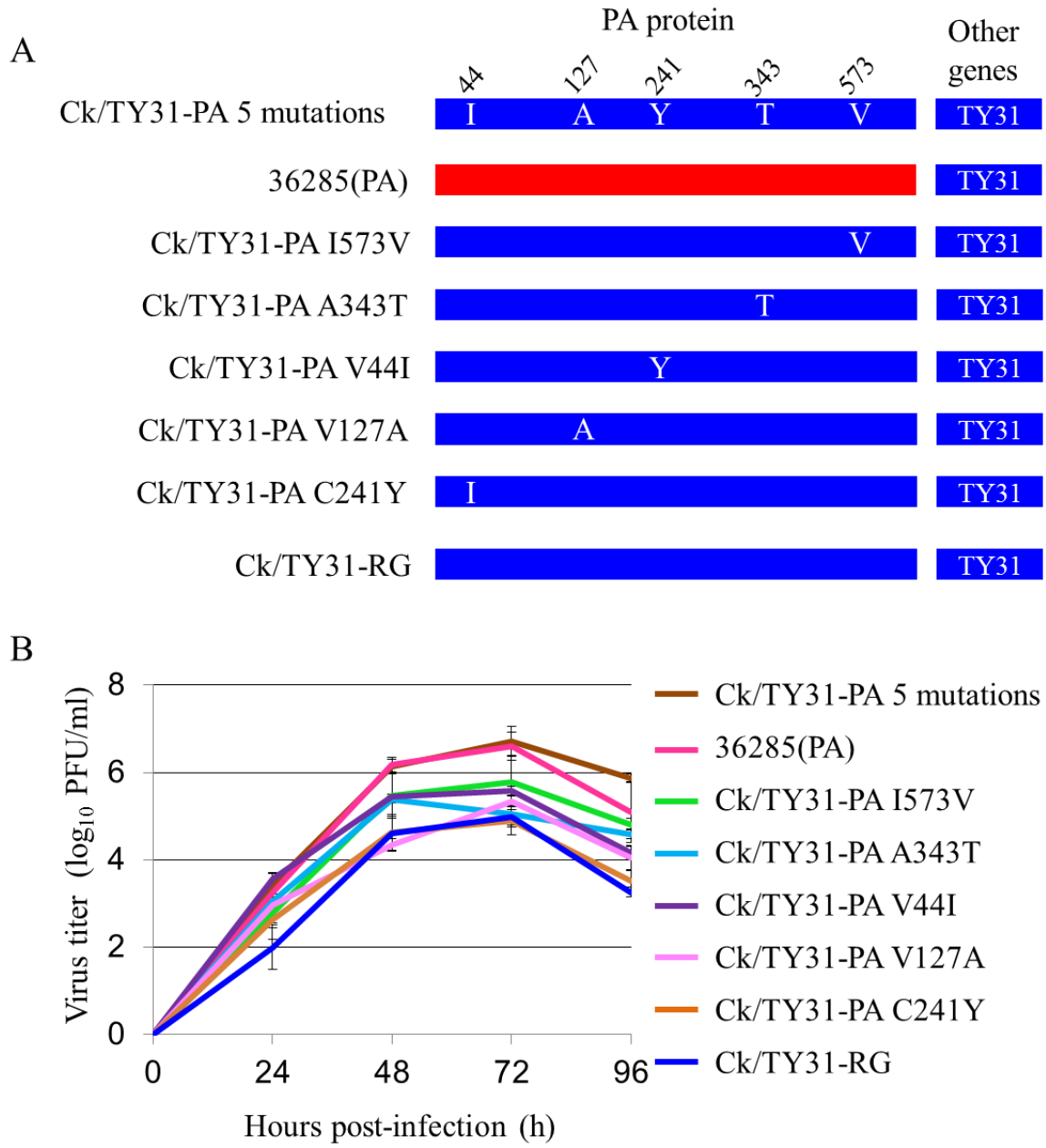

Fig. 22: Comparison of the growth properties of PA avian-to-human mutant viruses in A549 cells.

(A) Schematic diagram of PA mutant or reassortant viruses.

(B) A549 cells were infected with the mutant viruses at a m.o.i of 0.0002 and cultured at 37˚C. The standard deviation represents 3 independent experiments.

The PA mutations V44I, V127A, C241Y, A343T, and I573V contribute to high virulence in mice.

To evaluate whether there is any difference in the pathogenicity 36285-RG and Ck/TY31-RG in mice, I examined their MLD_{50} values (Fig. 23A-D). Inoculation of mice with 10^3, 10^4, or 10^5 PFU of 36285-RG virus resulted in 100% mortality; the fatality rate was 40% among the mice that received 10^1 and 10^2 PFU of virus. In contrast, inoculation of mice with 10^1 to 10^5 PFU of Ck/TY31-RG resulted in no fatalities. Mice inoculated with 10^5 PFU of Ck/TY31-RG showed no clinical signs or symptoms of diseases, including ruffed fur or hunched posture. There was thus a striking difference in the pathogenicity of Ck/TY31-RG and 36285-RG in mice (MLD_{50} values: > 10^5 PFU and $10^{1.5}$ PFU, respectively) (Fig. 23B, D). Next, to assess the influence of NS and PA mutations on pathogenicity in mice, we determined the MLD_{50} values of 36285(PA), 36285(NS), and TY31-PA 5 mutations. Inoculation of 10^4 and 10^5 PFU of 36285(PA) virus was fatal to mice, and a 75% fatality rate was observed in mice that received 10^5 PFU of 36285(NS). These results indicate that the PA and NS of 36285 independently contribute to virus pathogenicity in mice (Fig. 23F, H). In particular, the five PA mutations V44I, V127A, C241Y, A343T, and I573V enhanced the pathogenicity of Ck/TY31 in mice (Fig. 23J). These results thus demonstrate a correlation between the PA mutations V44I, V127A, C241Y, A343T, and I573V and the adaptation of H5N1 virus to mammalian hosts.

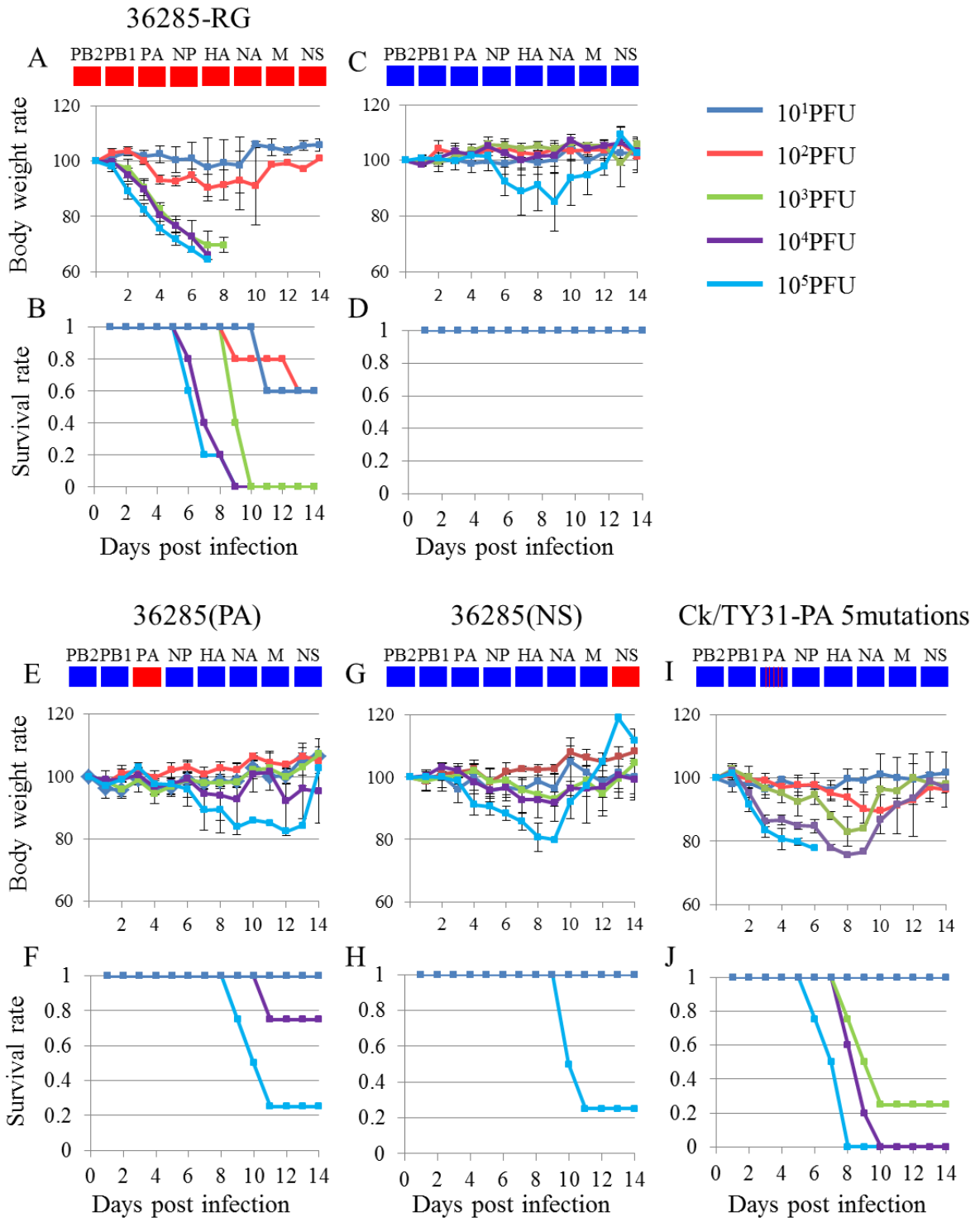

Fig. 23: Comparison of the virulence of reassortant and mutant viruses in mice

Mice (5 per group) were inoculated with 10^1, 10^2, 10^3, 10^4, or 10^5 PFU/body of 36285-RG and Ck/TY31-RG and monitored for weight loss (A and C) and survival (B and D) for 14 days. Mice (4 per group) were inoculated with 10^1, 10^2, 10^3, 10^4, or 10^5 PFU/body of 36285(PA), 36285(NS), and Ck/TY31-PA 5mutations and monitored for weight loss (E, G, and I) and survival (F, H, and J) for 14 days.

The PA mutations V44I, V127A, C241Y, A343T, and I573V increase viral replication in mouse lung tissues.

To examine viral replication in mice, I infected mice with 10^3 PFU of Ck/TY31-RG, 36285-RG, 36285(PA), 35285(NS), and Ck/TY31-PA 5 mutations. Virus titers were examined in various organs including brain, lung, nasal turbinates, duodenum, rectum, liver, kidney, and spleen. Virus was detected in the lung tissue of mice infected with 36285-RG on day 3 post-infection, and systemically on day 6 post-infection. In contrast, virus was not detected in any organs of mice infected with Ck/TY31-RG either on day 3 or day 6 post-infection (Table 7). With respect to the other reassortant and mutant viruses, virus was detected only in lung tissue (Table 7), suggesting that the PA, NS, and group of 5 PA mutations enhance virus growth in the lung tissue of mice. Notably, the five PA mutations V44I, V127A, C241Y, A343T, and I573V markedly enhanced virus growth in the lung tissue of mice on day 3 post-infection, and the virus titer of Ck/TY31-PA 5 mutations was almost the same as that of 36285-RG. This finding suggests that its high replicative ability in lung tissue contributes to the high pathogenicity of Ck/TY31-PA 5 mutations in mice. The differences in replicative ability in mice among the five viruses tested here correlate with their lethality in the mouse model.

Table 7

The replication properties of Ck/TY31, 36285, reassortants, and PA mutants in mice.

Virus name	Days post-infection	Virus titer (mean Log_{10} PFU±SD/g)[a]							
		Brain	Lung	Nasal turbinate	Liver	Spleen	Kidney	Small intestine	Colon
Ck/TY31	3	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
36285	3	-	5.1±0.1	-	-	-	-	-	-
	6	2.4±1.6	6.0±0.1	2.9±0.4	-	0.9±0.8	0.4±0.8	-	-
36285(PA)	3	-	1.0±1.7	-	-	-	-	-	-
	6	-	3.8±1.0	-	-	-	-	-	-
36285(NS)	3	-	3.1±0.8	-	-	-	-	-	-
	6	-	3.9±0.5	-	-	-	-	-	-
Ck/TY31-PA 5 mutations	3	-	4.9±0.2	-	-	-	-	-	-
	6	-	4.3±0.2	-	-	-	-	-	-

[a] Three BALB/c mice for each virus were inoculated intranasally with10^3 PFU of virus in a 50-ul volume; three mice from each group were euthanized on day3 and 6 post-infection for virus titration. The samples were titrated in MDCK cells. The virus titer was calculated as PFU per gram (for tissue) and expressed as log_{10}PFU/gram.

- , titer of < 1.0 log_{10}PFU/ml.

Discussion

In the current study, I found five PA amino acid mutations, namely PA-V44I, PA-V127A, PA-C241Y, PA-A343T, and PA-I573V, that had not previously been reported to contribute to virus virulence in mice and efficient growth in A549 cells. The amino acids at positions 44, 127, and 241 are located in the N-terminal domain of the PA protein. This domain (encompassing residues 1–256) is the major functional portion of the PA protein (Fig. 24). Most importantly, it catalyzes host mRNA cleavage via its endonuclease activity (64-67). Other functions of the PA protein include degradation of viral and host protein via its protease activity (74-76) and supporting nuclear import via nuclear localization signals (NLSs) (77). NLSs are recognized by host proteins, and allow complexes of virus and host proteins to jointly pass through nuclear pore complexes. Two regions of the PA protein have been identified as NLSs: region I (amino acids 124 to 139) and region II (amino acids 186 to 247) (77). The amino acid mutations PA-V127A and PA-C241Y, identified in this study, belong to the nuclear localization signal region (NLS) (77). PA-V127A and PA-C241Y may, therefore, support efficient nuclear import of the viral genome in human cells; a similar mechanism involves the PB2-701 mutation. I did not find any contribution by the PA mutations of either viruses to the polymerase activity, as measured by minigenome assays. Therefore, it remain unclear how PA-V44I, PA-V127A, PA-C241Y, PA-A343T, and PA-I573V affect virus replication in A549 cells and mouse pathogenicity.

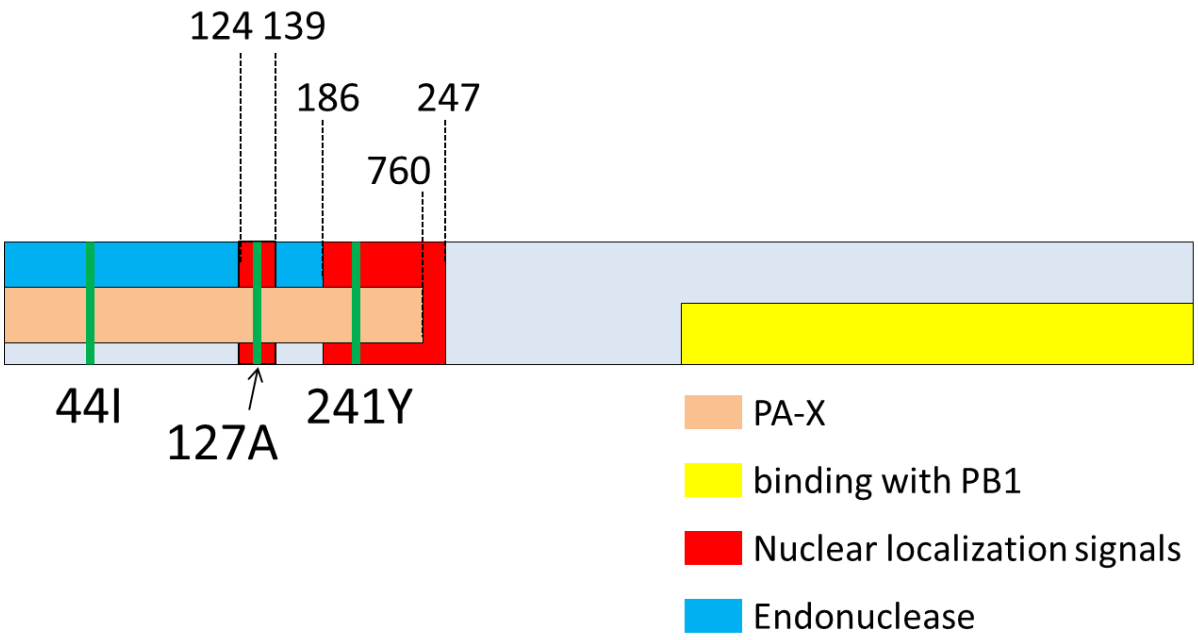

Fig. 24: Schematic representation of the PA protein

Interestingly, sequence analysis showed that the prevalence of PA amino acid 241Y in human seasonal H1N1 viruses was over 90% and that PA-241Y has been highly conserved in human seasonal H1N1 viruses since 1918, in contrast to avian viruses, which encode PA-241C exclusively (Table 3). These data reinforce the belief that the amino acid at position 241 of PA may assist the H5N1 virus in overcoming the hurdle between humans and birds.

The PA segment also encodes, the PA-X protein, which shares the first 182 amino acids with the PA protein, but differs in its C-terminal 70 residues due to a ribosomal frameshift (Fig. 24). Therefore, the nucleotide changes responsible for PA-V44I, PA-V127A, and PA-C241Y also alter the amino acid sequence of PA-X (PA-X-V44I, PA-X-V127A, and PA-X-A241T); hence it is possible that these changes may affect the function of the PA-X protein.

Further sequence analysis demonstrated that almost all of the seasonal human H3N2 viruses isolated since 2000 have valine at position 573 (Table 3). These data imply that PA-573V may be selected by some positive pressure for adaptation to humans and suggest that this amino acid positively affects the growth capability of Ck/TY31-RG, as demonstrated in Fig. 22B.

In conclusion, here I found that five PA amino acid substitutions, namely V44I, V127A, C241Y, A343T, and I573V, additively enhance the replicative ability of an H5N1

virus in A549 cells and enhance its pathogenicity in mice. Although each of these five PA amino acids alone did not significantly enhance virus replication in A549 cells, my data indicate that in combination these five amino acids support efficient virus replication in A549 cells and high virulence in mice. My study thus suggests that these PA substitutions could assist H5N1 viruses in adapting to mammalian hosts. Although the individual roles of these five PA amino acid mutations remain unclear, our findings further our overall understanding of the function of PA.

Concluding Remarks

Although human cases of infection with highly pathogenic H5N1 influenza viruses have not occurred as frequently as in the beginning of the 2000s, the number of human cases has continued to increase. In 2014, several fatal cases of H5N1 virus infection were reported in Cambodia, Vietnam, China, Indonesia, and Egypt successively. In each case, the human infections resulted in only limited spread. However, we cannot predict when and how an H5N1 pandemic will occur. Hence, it is essential that we accumulate and share our expertise so that we are as prepared as possible for such a pandemic.

Here, I identified PB2 and PA amino acid mutations that facilitate the replication of H5N1 virus in human lung cells. In particular, I found that several of these amino acids are highly conserved in human seasonal influenza viruses, suggesting that they may play an important role in the mechanisms for adaptation to humans. These data will aid future surveillance studies and enhance analyses of the functions of the polymerase complex, which are not yet fully understood.

In addition, I demonstrated that the identified mutations have different effects on the biological features of viruses depending on the genetic background of the viruses tested. I compared the growth capability of a human H5N1 virus (36285) with those of two different avian H5N1 viruses: Wb/AH82 in Chapter I, and Ck/TY31 in Chapter II. From the results of

these comparisons, I concluded that distinctive amino acids are responsible for the difference in growth capabilities between the two H5N1 viruses. Some amino acid substitutions have a considerable impact on the biological trait of a virus, but in other viruses, they do not greatly affect that trait. How combinations of amino acid mutations from different segments affect the biological features of viruses is not thoroughly understood. Therefore, further comprehensive analyses of the combinational effects of amino acid mutations in different segments is required to obtain a deeper understanding of the meaning of amino acid mutations that are required to overcome species barriers. Such analyses are essential for the accurate evaluation of the risk of future isolates and the prevention of future pandemics.

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