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獸醫學博士 學位論文

Novel molecular determinants related with mammalian pathogenicity in influenza A virus

인플루엔자 A 바이러스의 포유류 병원성 관련 신규 유전자 표지 분석

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이 충 용

Novel molecular determinants related with mammalian pathogenicity in influenza A virus

By

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A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology

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Department of Veterinary Medicine
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Abstract

Novel molecular determinants related with mammalian pathogenicity in influenza A virus

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Influenza A virus (IAV) is an RNA virus belonging to family *Orthomyxoviridae*, which contains negative sense, single-stranded, segmented RNA genomes. IAV is further categorized into

subtypes by surface glycoproteins, HA and NA proteins. Considering variety in subtypes of IAVs, migratory waterfowl has been suspected to be served as reservoirs for IAVs. The avian-origin influenza A virus (AIV) have been occasionally transmitted to human, and some of these viruses were successfully adapted, causing severe influenza pandemics. Nonetheless, the understanding of AIV transmission to mammals has not been well elucidated. In this study, I have generated several genetic reassortants of AIVs using reverse genetics techniques and found the novel pathogenic determinants in AIVs contributing to adapt AIVs to mammals and their role in viral transmission and pathogenicity in mammals.

The polymerase complex of IAV is a heterotrimer composed of PB2, PB1, and PA proteins. Given their role in the viral replication and transcription, the polymerase complex, especially PB2 protein, have been considered as one of the important host determinant factors. The polymerase complex of the low-pathogenic avian influenza viruses [A/chicken/Korea/KBNP-0028/2000 (H9N2)] (0028) and [A/chicken/Korea/01310/2001 (H9N2)] (01310) has previously been characterized, and novel amino acid residues present in PB2 and PA proteins that likely contribute to pathogenicity toward mammals have been identified. For the analysis of these novel molecular determinants in PB2 protein, I firstly generated the recombinant PR8 virus carrying the mutated 01310 PB2, and found that key amino acid mutations (I66M, I109V and I133V, collectively referred to as MVV) of 01310 PB2 increase the replication efficiency of recombinant

PR8 virus carrying the mutated PB2 in both avian and mammalian hosts. The MVV mutations caused no weight loss in mice, but they did allow replication in infected lungs, and the viruses acquired fatal mammalian pathogenic mutations such as Q591R/K, E627K, or D701N in the infected lungs. The MVV mutations are located at the interfaces of the trimer and are predicted to increase the strength of this structure. These findings suggest that gaining MVV mutations might be the first step for AIV to acquire mammalian pathogenicity. Next, for evaluating the novel pathogenic determinants in PA proteins, I generated recombinant PR8 viruses containing the 0028-PA gene with a single amino acid substitution and test their pathogenicity and replication ability. A substitution from glutamate to glycine at position 684 (E684G) significantly increased viral replication in mammalian cells and mortality in mice, with significantly increased interferon β expression. Thus, the E684G mutation in the PA gene may play an important role in viral pathogenicity in mice by increasing viral replication and the host immune responses. In addition to the coding sequences of the viral genome, the noncoding sequences could also affect the viral pathogenicity. Hoffmann's 8-plasmid reverse genetics vector system introduced mutations at position 4, C4 to U4, of the 3′ ends of NA and M vRNAs of wild-type A/PR/8/34. For evaluating the effect of C4 to U4 mutation on viral pathogenicity, I generated 4 recombinant viruses with C4 in the NA and/or M vRNAs and rgPR8 by using reverse genetics and compared their pathobiological traits. The mutant viruses showed lower replication efficiency than rgPR8 due to the low

transcription levels of NA and/or M genes. Furthermore, C4 in the NA and/or M vRNAs induced lower PR8 virus pathogenicity in BALB/c mice. The results suggest that the constellation of C4 and U4 among vRNAs may be one of the multigenic determinants of IAV pathogenicity. These novel pathogenic determinants found in this study will broaden the understanding of host jumping mechanism of AIVs, and these determinants may contribute to the surveillance and prevention of next pandemic threats.

Keyword: *influenza A virus, reverse genetics, polymerase activity, molecular determinants, pathogenicity*

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Abbreviations

293T	Human embryonic kidney cells
A2M	Alpha 2 macroglobulin
AIV	Avian influenza virus
ANOVA	Analysis of variance
BSA	Bovine serum albumin
cRNA	Complementary RNA
DMEM	Dulbecco's modified Eagle medium
Dpi	Day post-inoculation
ECE	Embryonated chicken egg
EDE	Embryonated duck egg
EID50	50% chicken embryo infectious dose
HA	Hemagglutinin
HI	Hemagglutination inhibition
Hpi	Hour post-inoculation
HRP	Horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IAV	Influenza A virus
IFN	interferon
IRD	Influenza research database
KCTC	Korean Collection for type cultures
LOF	Loss-of-function
LPAIV	Low-pathogenic avian influenza virus
M	Matrix
MDCK cells	Madin-Darby canine kidney cells
MLD50	50% mouse lethal dose
MOI	Multiplicity of infection
mRNA	Messenger RNA
MxA	Interferon-induced dynamin-like GTPase
NA	Neuraminidase
NCR	Noncoding region

NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non-coding protein
PA	Polymerase acidic protein
PB1	Polymerase basic protein 12
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pdm09	2009 pandemic H1N1 influenza virus
PK-15	Porcine kidney cells
PL	PDZ ligand domain
PR8	A/Puerto Rico/8/34
RBS	Receptor-binding site
rg	Reverse genetics
SA	Sialic acid
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sia-a2,3-Gal	Salic acids linked to galactose by a2,3-linkages
SNUIBC	Seoul National University Institutional Biosafety Committee
SP-D	Surfactant protein-D
TCID50	50% tissue culture infectious dose
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
vRNA	Viral genomic RNA
vRNP	Viral ribonucleoprotein

General Introduction

Influenza A viruses (IAVs) belong to family *Orthomyxoviridae*, which have single-stranded, negative sense, and segmented RNA genomes. They are divided into subtypes by two immuno-dominant surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Since 1918 Spanish flu, H1N1, H2N2, and H3N2 subtypes of IAVs have caused influenza pandemics in human (Chang, 1969, Mulder and Masurel, 1958, Dowdle, 1984). Among them, H1N1 and H3N2 IAVs have been continually causing a lot of problems in human health as seasonal flu (Jeffery and David, 2006, Edwin, 2006). Considering the genetic diversity of avian influenza viruses (AIVs) and the origin of all influenza pandemics, aquatic birds are presumed to serve as the main reservoir of IAVs (Edwin, 2006). Therefore, the exact risk evaluations of AIVs causing mammalian transmission may be one of the most important actions not only to evaluate the pathogenicity potential of IAVs in human but also to prevent the next pandemic threat.

IAV genome consists of eight RNA segments, which are PB2, PB1, PA, HA, NP, NA, M, and NS segments. HA and NA proteins play crucial roles in invasion and budding in host cells, respectively. HA is a major protective antigen of IAV, and it has evolved to both escape host immune responses and adapt to new hosts by multiple amino acid substitutions (Herve et al., 2015, Matrosovich et al., 2000, Kim et al., 2013). The polymerase of AIVs is a heterotrimer composed of

PB1, PB2 and PA. In this structure, PA and PB2 bind to the N- and C-termini of PB1, respectively (Detjen et al., 1987, Gonzalez et al., 1996). These three subunits are involved in the host range, tissue tropism and mammalian pathogenicity of AIV (Detjen et al., 1987, Hatta et al., 2001, Massin et al., 2001, Snyder et al., 1987, Subbarao et al., 1993). Diverse mutations in the polymerase subunits that determine the mammalian pathogenicity of AIV have been reported, and E627K in PB2 is considered a key mutation (Hatta et al., 2001, Subbarao et al., 1993, Zhang et al., 2014, E.Kanta Subbarao, 1993). Amino acid position 627 on PB2 is located in the C-terminal RNA-binding domain, and the E627K mutation is known to increase both RNA binding and polymerase activity, increasing viral replication efficiency at 33°C, the approximate temperature of the human upper respiratory tract (Kuzuhara et al., 2009, Steel et al., 2009). The alternative mutations such as 591K/R or 701N are also important to mammalian replication and pathogenicity (Sediri et al., 2015, Liu et al., 2012). This mutation is known to contribute to increasing the viral replication at lower temperature (33 to 35°C) (Massin et al., 2001). Several other important residues PB2-158G, -199S, 253N, -256G, and -271A also likely affect the viral polymerase activity in mammals (Bussey et al., 2010, Zhou et al., 2011, Mok et al., 2011, Manzoor et al., 2009). Although these mutations play important roles in the viral pathogenicity in mammals, the minimal essential mutations for avian influenza virus to replicate in mammalian hosts were not well elucidated. Previously, I identified two avian origin PB2 genes with different degrees of pathogenicity in the mammal (Kim et al.,

2014). In chapter 1, I identified the minimal essential mutations for adaptation of AIVs to mammals by comparing the difference in amino acids between two avian PB2 genes and evaluated their effects on viral replication and pathogenicity in both avian and mammalian hosts.

PA also plays crucial roles in the life cycle and host adaptation of IAVs (Hu and Liu, 2015). To date, various mutations of PA increasing mammalian pathogenicity of AIVs have been reported, and they could be categorized by location in different functional motifs and domains (M21I, F35L, A36T, T97I, E133G, K142N, P224S, L295P, C241Y, L336M, K351E, K356R, S421I, T552S, Q556R, K615N, S616P, and F666L) (Song et al., 2009, Sun et al., 2014, Brown et al., 2001, Nam et al., 2011, Kim et al., 2010, Seyer et al., 2012, Gabriel et al., 2005a, Mehle et al., 2012, Bussey et al., 2011, Xu et al., 2016a, Yamaji et al., 2015, Zhu et al., 2012). PA can be cleaved into N-terminal (1-257, PA-N) and C-terminal domains (277-716, PA-C) by tryptic proteolysis (Hara et al., 2006). Cap-snatching endonuclease and protease are located in the PA-N (Yuan et al., 2009, Hara et al., 2006). The PA-N is also associated with the IRF3-binding site and two nuclear localization signals (NLS I and NLS II) composed of 124-139 and 186-247 amino acid residues, respectively (Yi et al., 2017, Nieto et al., 1994). The PA-C contains 350-355 loop conferring mammalian pathogenicity and two RNA polymerase II binding sites, which are composed to K635 and R638 for site 1, and K289, R454 for site 2 (Xu et al., 2016a, Pflug et al., 2017). In addition, amino acids in terminal regions of PA-C interact with 15 amino acids of N-terminus of PB1 (He et al., 2008, Obayashi et al.,

2008). Therefore, mutations arisen in the vicinity of the functional motifs and domains may affect viral polymerase activity as well as interactions with host factors. In chapter 2, I investigated the effects of candidate mammalian pathogenic PA mutations (T129I, G351E, M628V, and E684G) which had been identified by comparing non-pathogenic PA gene of A/chicken/KBNP-0028/2000 (H9N2) (0028) with other pathogenic AIVs on mammalian pathogenicity (Kim et al., 2014). I generated PR8-derived recombinant viruses carrying mutated 0028 PA genes with a single amino acid substitution, but recombinant viruses carried the additional single mutation of NP. To date, a direct interaction between PA and NP has not been well elucidated. However, considering that the binding of mutant NS1 to CPSF30 was stabilized by cognate PA and NP, there are expected to have unidentified interaction between them (Kuo and Krug, 2009). Therefore, I expanded the study to unravel the effects of PA as well as NP mutations on polymerase activity, replication efficiency and pathogenicity in mammalian hosts.

The noncoding regions (NCRs) of the 3′ and 5′ ends of viral RNA (vRNA) of IAV form a ‘corkscrew’-like structure and function as promoters for the transcription of messenger (mRNA), complementary RNA, and viral genomic RNA (vRNA) (McCauley and Mahy, 1983, Desselberger et al., 1980, Flick and Hobom, 1999). The promoter function is reported to be localized to 12 conserved nucleotides at the 3′end of vRNA, and nucleotides 9–11 were shown to be crucial for promoter activity (Seong and Brownlee, 1992). Mutations at positions 11 and 12 of the 3′ and 5′

ends of neuraminidase (NA) vRNA of influenza A/WSN/33 reduced the NA mRNA and protein levels, as well as the virus titer, and resulted in attenuated phenotypes in mice (Solorzano et al., 2000, Fodor et al., 1998). Therefore, the NCRs of the 3′ and 5′ ends of vRNA may have an important role in viral pathogenicity as well as viral gene expression. During analysis of the sequences of NCRs, I observed different combinations of U4 and C4 in NCRs of wtPR8 and rgPR8. The polymerases (PB1, PB2, and PA), NA, and M vRNAs of wtPR8 possessed C4 rather than U4, but those of rgPR8 acquired U4 mutations in NCRs of the NA and M vRNAs (Hoffmann et al., 2002, Hoffmann et al., 2001, de Wit et al., 2004). Therefore, in chapter 3, I generated 4 recombinant viruses with C4 in the NA and/or M vRNAs and a rgPR8-like constellation of C4 and U4 by undertaking reverse genetics. I then compared their replication efficiency in ECEs, transcription levels of vRNA and mRNA of the NA and/or M genome segments in MDCK cells, and their pathogenicity in mice,

H5N1 AIVs have been occasionally transmitted to human since 1990, and recent human infectious H7N9 viruses have suffered a great deal of damage on humans through genetic reassortment of AIVs in the birds. As the globalization accelerates, there is a high possibility that a wider variety of AIVs will spread to humans in the upcoming future. Therefore, it is important to uncover the novel pathogenic mutations acquired in the avian species for the surveillance and preventing the risk of the next influenza pandemic. In this thesis, I evaluated the effect of several

pathogenic mutations in avian origin PB2 and PA proteins on viral replication and pathogenicity to mice, and I found that the promoter variation in the 3' end of viral genomes may be one of the pathogenic factors by regulating viral transcription and replication.

Literature review

1. Introduction

Influenza A viruses (IAVs) are classified in the family *orthomyxoviridae*, and it has a single stranded negative sense RNA genome. IAV has eight RNA segments (PB2, PB1, PA, HA, NP, NA, M, and NS) coding for 11 proteins (PB2, PB1, PB1-F2, PA, HA, NP, NA, M, M2, NS1, and NEP) (Table L.1). Recently, novel viral protein, such as PA-X (expressed by ribosomal frameshifting) and N40 (expressed by leaky ribosomal scanning process) have been identified, and those proteins play important roles in the interaction with host immune response (Jagger et al., 2012, Akkina et al., 1991, Rodriguez-Frandsen et al., 2015, Wise et al., 2009).

IAVs are further classified into subtypes based on their surface immune-dominant protein, HA and NA protein. 16 HA (H1-H16) subtype and 9 NA (N1-N9) subtype are known, and novel subtypes, H17N10 and H18N11, have been discovered in the bat (Tong et al., 2013, Tong et al., 2012, Fouchier et al., 2005). The replication cycle in the host cell of AIVs have been reviewed by various investigators (Figure 1) (Watanabe et al., 2010, Medina and Garcia-Sastre, 2011, Manz et al., 2013b). Firstly, the surface protein, HA attached to host cell receptors containing either terminal a-2,3-linked or a-2,3-linked sialic acid (a-2,6-SA or a-2,3-SA) moieties, and it enters by receptor-

mediated endocytosis. After cleavage of HA by the cellular protease, the viral envelope and the endosomal membrane are fused, and the viral ribonucleoproteins (vRNPs) are released into the cytoplasm. vRNPs are transported into the nucleus, where the RNA-dependent RNA polymerase transcribes and replicates the viral genomes. the transcribed viral mRNAs are exported to the cytoplasm for translation. The viral protein needed in replication and transcription are transported back to the nucleus. After the formation of whole virus particles, the virus is released from the host cells by the neuraminidase activity, which destroys the sialic acid of the cellular membrane binding with viral glycoproteins.

It is generally agreed that aquatic birds are served as major reservoirs for IAVs (Webster et al., 1992a). Although most AIVs cause no or mild clinical signs in aquatic birds, they can be occasionally transmitted to territorial birds or mammals. Most cases just caused a mild cough or transient infection, but some of the viruses accidentally could be adapted and get the transmissibility (Taubenberger et al., 2001, Garten et al., 2009, Dowdle, 1984). There are two ways in which IAVs can adapt to new hosts; antigenic drift, antigenic shift. Antigenic drift generally means the point mutations, which have been accumulated during viral replication. The specific mutations in HA or NA protein could allow the virus to escape host immunity, and that is why the seasonal flu vaccine strains have been renewed annually. Meanwhile, if two different IAVs are infected simultaneously on one host, they could exchange their eight segmented genomes. That phenomenon is called an

antigenic shift, also known as genetic reassortment. Antigenic shift can introduce a new virus subtype previously not circulating in human. By using these two ways adapting virus to the new hosts, several IAVs originated from avian influenza virus occasionally caused human infection.

The 1918 Spanish H1N1 influenza pandemic killed almost 50 million people worldwide, which has been known as the deadliest single event recorded in recent human history (Jeffery and David, 2006, Schrauwen and Fouchier, 2014). The reason why the 1918 Spanish flu was so fatal did not well elucidated, it was speculated that the 1918 Spanish flu was originated from avian influenza virus with or without adaptive stages in an intermediate host, and it is the likely ancestor of four of the human and swine H1N1 and H3N2 influenza virus lineage, as well as H2N2 lineage (Reid et al., 2004, Jeffery and David, 2006, Schrauwen and Fouchier, 2014, Dowdle, 1984).

The Asian influenza pandemic virus caused by H2N2 IAVs (Mulder and Masurel, 1958). Because the most of young people did not have the experience about the pandemic, with the exception of people >70 years of age, the Asian flu caused as many as two million deaths globally (Hattwick et al., 1976). The PB1, HA, and NA genes of Asian flu derived from H2N2 avian influenza virus, and the remaining genes came from the circulating H1N1 virus prior to 1957 (Schrauwen and Fouchier, 2014). The Hong Kong influenza pandemic emerged in 1968 and caused an estimated one million people deaths worldwide (Chang, 1969). This virus occurred by the reassortment between HA and PB1 gene of avian H3 influenza virus and the other genes from H2N2

IAVs. This virus was likely to less pathogenic than previous pandemics, possibly as a result of prior antibodies against IAVs. The reintroduction of H1N1 IAV caused severe damage on human public, called H1N1 'Russian flu'. This pandemic was genetically similar to viruses that circulated prior to 1950 without definitely genetic evolution, suggesting that it might be accidentally released from a laboratory (Nakajima et al., 1978).

The 2009 pandemic H1N1 influenza (pdm09) was the first pandemics occurred in the 21st century (Garten et al., 2009, Girard et al., 2010). It firstly emerged in Mexico, after which it spread around the world in only a few months. Similar to avian flu, the people over 65 years of age, who experienced IAVs circulating before 1957, showed mild clinical signs because of the cross-protective immunity. The pdm09 virus outbreak was occurred by the recombination between a triple reassortant virus combining avian, human and swine influenza virus (PB2, PB1, PA, HA, NP, and NS genes) and Eurasian swine influenza virus lineage (NA and M genes) (Garten et al., 2009, Smith et al., 2009, Cohen, 2009, Igarashi et al., 2010). The fact that this virus originated from swine influenza virus supports the hypothesis that pig plays an important role in the transmission of avian origin virus to human, serving as a mixing vessel (Smith et al., 2009, Cohen, 2009).

This review will discuss several subtypes of IAVs not previously circulating in human populations. Fortunately, these new viruses did not acquire the driving force for sustainable transmission until now. Nonetheless, if viruses get the transmissibility to human population, they

would pose a serious human health threat. Therefore, these viruses need constant monitoring and pathogenic evaluation. Next, the important molecular determinants for acquiring pathogenicity in mammals will be described for effective evaluation of the transmissibility risk of next pandemic candidate viruses.

Table L.1. RNA segments and proteins of Influenza A virus

RNA segment	Length (nucleotides)	Proteins Encoded	Protein Size (aa)	Protein Function
1	2341	PB2	759	Component of RNA polymerase; cap recognition
2	2341	PB1	757	Component of RNA polymerase; RNA dependent RNA polymerase
3	2233	PA	716	Component of RNA polymerase; endonuclease activity, protease
4	1778	HA	566	Surface glycoprotein; receptor binding; fusion activity; antigenic determinant
5	1565	NP	498	Nucleocapsid protein; RNA binding; RNA synthesis;
6	1413	NA	454	Surface glycoprotein; neuraminidase activity; dissociation of virus aggregates
7	1027	M1	252	Matrix protein; RNP nuclear export, assembly and budding
		M2	97	Membrane protein, ion channel activity
8	890	NS1	230	Down-regulates host cell mRNA processing; sequesters dsRNA and reduces interferon response
		NEP	121	RNP nuclear export; regulation of RNA synthesis

Edited from Nicholas H. Acheson, 2007

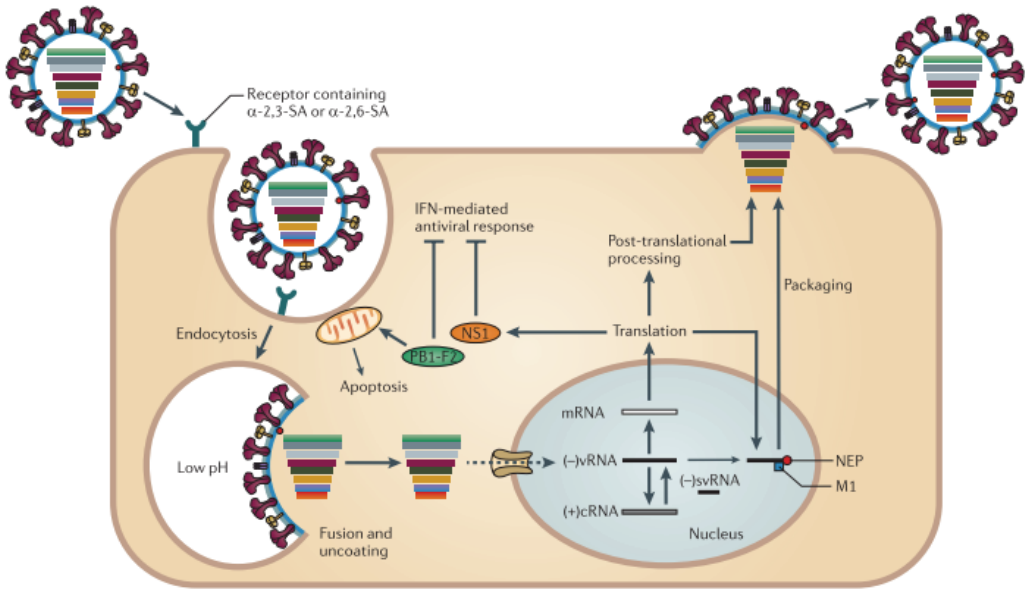

Figure L.1. Replication of influenza A virus.

Adapted from Medina, R.A. and Garcia-Sastre, A., 2011

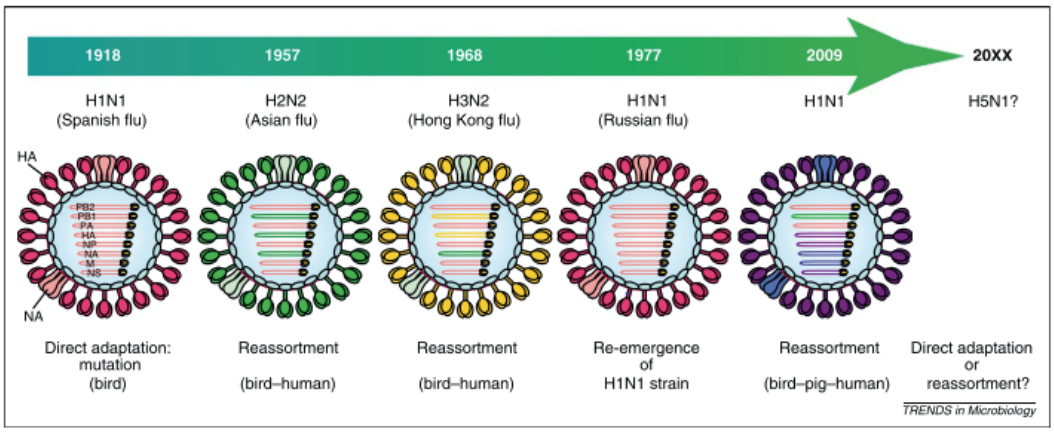

Figure L.2. Timeline and evolution of pandemic influenza viruses. Pandemics in the 20th century were caused by infection by an avian influenza virus (1918), an avian–human reassortment virus containing avian HA, NA and PB1 (1957), and an avian–human reassortment virus containing avian HA and PB1 (1968), with the other gene segments from the circulating human virus. Re-emergence of the H1N1 strain that circulated in the 1950s led to a limited pandemic in 1977. The latest pandemic influenza (2009) was caused by an avian–swine–human reassortment producing a virus containing PB2 and PA from an avian virus, PB1 from a human virus, and the other gene segments from two distinct lineages of swine viruses. Future pandemic strains could arise through any of the mechanisms: adaptation by accumulation of mutation(s), and genetic reassortment and re-emergence of a virus that has not infected humans for a generation. Common names of the past pandemics and the virus origins are shown in parentheses at the top and bottom, respectively.

Adapted from Watanabe et al., 2012

2. The mammalian transmission of avian influenza viruses

2.1. H5Nx viruses

The highly pathogenic avian influenza (HPAI) H5N1 virus has become the major concern of poultry industries since the identification of the strain A/Goose/Guangdong/1/1996 (GsGD) in Guangdong province of China (Xu et al., 1999). The first zoonotic transmission of HPAI H5N1 virus was recorded in Hong Kong in 1997, and it led to 18 individual infections, of whom 6 died (Chan, 2002, Claas et al., 1998b, de Jong et al., 1997). Given that HA gene from human infected H5N1 virus genetically related to that from GsGD H5N1 virus, GsGD H5N1 virus and one or more different avian influenza viruses were suspected to be progenitors of human infected H5N1 virus (Xu et al., 1999). Fortunately, this virus was disappeared by depopulation of live bird market and poultry farms in late 1997. Since 2003, HPAI H5N1 viruses have been widely spread and caused severe damages on poultry industries in numerous countries throughout Asia, Europe, and Africa (Beigel et al., 2005). Due to severe economic losses, the poultry vaccination of HPAI H5N1 virus has been implemented in the poultry farm since 2004 in China (Fan et al., 2015, Swayne et al., 2014). Some of countries have succeeded in reducing HPAI outbreaks in both poultry and human, but without strict bio-security precautions this strategy might increase the risk of human infection (Peyre et al., 2009, Kayali et al., 2016). Furthermore, the poultry vaccination can lead to the diversification

of HPAI virus in order to escape host immune responses, and this diversification may increase the possibility of human infection (Cattoli et al., 2009, Kayali et al., 2016, Fan et al., 2015, Peng et al., 2017, Xu et al., 2016b). Since 2003, 859 patients were reported to be infected with the H5N1 viruses with more than 50% mortality (http://www.who.int/influenza/human_animal_interface/2017_07_25_tableH5N1.pdf).

In April 2014, the novel subtype of H5 viruses, H5N6 virus human infection was firstly identified in China (Pan et al., 2016). In February and July 2015, respectively, the third and fourth human H5N6 infection were reported from Shangrila city, Diqing prefecture, Yunnan province (Xu et al., 2016b). The clinical signs of H5N6 human infection were similar to those of H5N1 infection with fever and coughing (Wang et al., 2014a, Xu et al., 2016b). However, considering the gradual and dynamic evolutions of H5Nx viruses, a novel subtype of H5Nx HPAI viruses may cross the host-specific barrier and pose a serious human health threat.

2.2. H7N9 virus

In 2013 China, the first cases of H7N9 infection in humans occurred (Gao et al., 2013, Li et al., 2014). The infected individuals had severe lower respiratory tract illness, and they had the history of exposure to poultry without epidemiologic relationship (Li et al., 2014). Considering the detection of H7N9 in live poultry markets at a similar time and epidemiological date demonstrate that contact

with poultry or contaminated environments in live bird markets was the most likely source of human infection (Watanabe et al., 2014, Li et al., 2014, Liu et al., 2013, Chen et al., 2013). The novel H7N9 viruses emerged upon the reassortment of at least four avian influenza virus strain without human or other mammalian influenza virus (Wang et al., 2014b, Gao et al., 2013). The HA gene of the H7N9 viruses is closely related to the Eurasian lineage of avian influenza viruses and to that of the avian H7N3 viruses recently isolated from ducks in Eastern China (Liu et al., 2013). The NA gene is genetically related to NA gene of avian H2N9 and/or H11N9 influenza viruses isolated from wild migratory birds. The remaining six internal genes likely originated from two H9N2 sub-lineage circulating in poultry in Eastern China. Interestingly, during transmission to human, H7N9 viruses circulating poultry acquired important mammalian pathogenicity related mutations, such as 591K/R, 627K, and 701N in PB2 and 226L/I in HA. These mutations likely arose during H7N9 virus adaptation to humans (Watanabe et al., 2014, Gao et al., 2013, Chen et al., 2013, Mok et al., 2014). Since 2013, H7N9 influenza virus has caused five infection waves, and the fifth waves, emerging in 2016-2017, led to the highest number of patients exceeding the sum of those in the four preceding ones (Huo et al., 2017, Quan et al., 2018). Leading to the fifth waves of infection, H7N9 viruses have been diversified into at least 36 genotypes by genetic ressortment (Quan et al., 2018, Kim et al., 2016). Among these new variants, a highly pathogenic variant of H7N9 virus firstly emerged in the poultry farms from 2013 low pathogenic ancestor, and this HPAI H7N9 virus also caused human

infections (Quan et al., 2018, Qi et al., 2018, Yang and Liu, 2017). Fortunately, there was no evidence that HPAI H7N9 virus led to higher pathogenicity in human than LPAI H7N9 virus. However, given the continual circulation and the risk of systemic dissemination to multiple organs, HPAI H7N9 virus has to be continuous monitoring and need further investigation (Quan et al., 2018, Zhang et al., 2017, Qi et al., 2018).

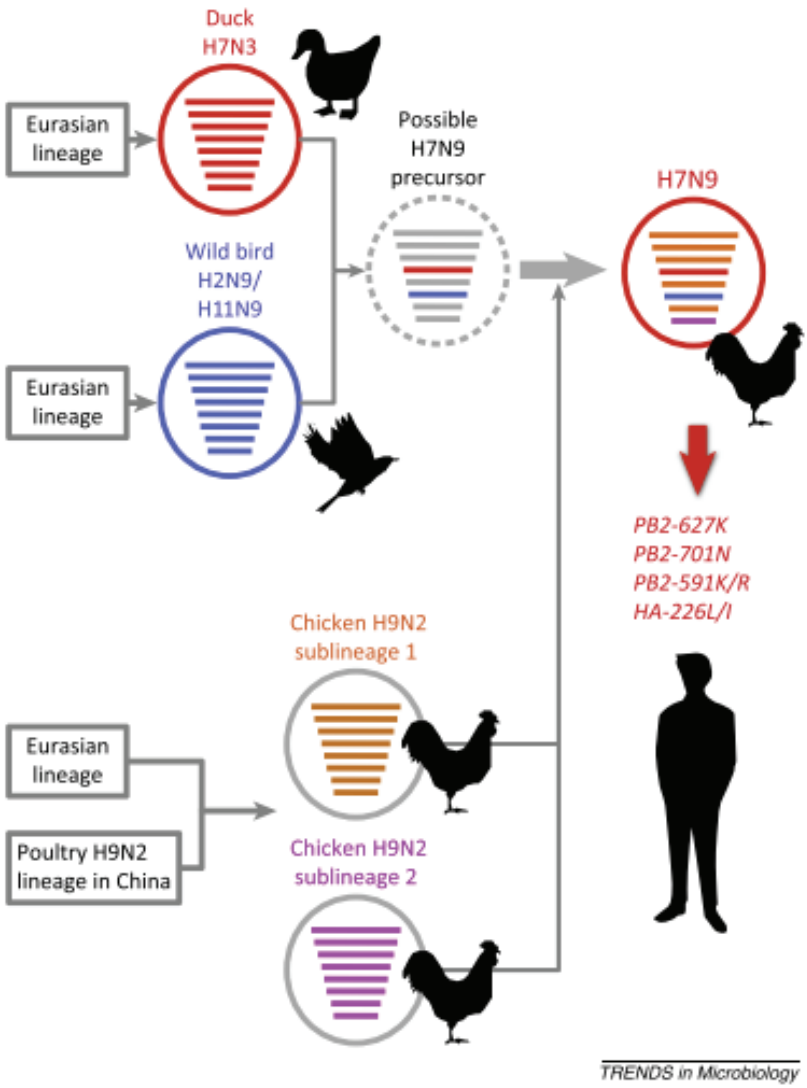

Figure L.3. The genesis of avian influenza A (H7N9) influenza virus. The novel H7N9 viruses likely resulted from the reassortment of at least four avian influenza A virus strains.

Adapted from Watanabe et al., 2014

2.3 Other viruses

In march 1999, zoonotic infection of H9N2 viruses have been firstly reported upon two human contact infection with poultry, although the illness in two children infected with H9N2 was mild and self-limited (Peiris et al., 1999). Importantly, H9N2 viruses have been continually circulating in pig with numerous reassortment events, which potentially increase the risk of human infection (Wang et al., 2016, Peiris et al., 2001, Xu et al., 2004, Cong et al., 2007, Yu et al., 2011). Recent studies suggested that 10 passages of H9N2 in ferrets resulted in the acquisition of ferret adaptation mutations, and this H9N2 mutant acquired airborne transmissibility (Sorrell et al., 2009). Given continuous outbreaks of H9N2 human infection, H9N2 viruses have to be considered as the potential pandemic influenza and further investigation upon the widespread of H9N2 viruses in pig will broaden the understanding of mammalian transmission of AIVs (He et al., 2016, Huang et al., 2015, Khan et al., 2015).

H6 subtype influenza viruses are known as the most abundantly detected subtype and the viruses are likely to have a broader host range (Munster et al., 2007). In 2013, The first human infected case of H6N1 virus was reported in Taiwan (Wei et al., 2013, Yuan et al., 2013). Although there were no further occurrences, H6 viruses have a potential to infect several mammalian hosts without prior adaptation (Gillim-Ross et al., 2008, Wang et al., 2014c). Furthermore, the virus acquired the human-type receptor preference by introducing only a single mutation to HA protein

(de Vries et al., 2017). Taken together, H6 viruses must be considered as one of potential pandemic viruses, and rigorous investigation will be needed for accurate evaluation of their pathogenicity in mammal.

H10 subtype influenza viruses have been occasionally transmitted to human. H10N7 viruses were found to lead to human infection in Egypt in 2004 and in Australia in 2010 (Arzey et al., 2012). More recently, a novel H10N8 viruses caused three human infected cases in 2013-2014 (Chen et al., 2014). H10 viruses were occurred relatively rare, but the risk of human transmission should not be underestimated (Arzey et al., 2012).

3. Adaptive mutations important in host adaptation

Influenza A viruses have eight segmented viral genomes, which are PB2, PB1, PA, HA, NP, NA, M, and NS genes, and specific residues in these segments are important to the replication and pathogenicity of IAVs to mammals.

3.1. HA protein

The envelope of IAVs is embedded with HA and NA glycoproteins, and these two glycoprotein act to the major immune dominant proteins. Among them, the HA protein is important surface glycoprotein binding to host cell receptor, sialic acid receptor, and the protein is essential to viral entry (Chen et al., 1998, Klenk et al., 1975, Rogers and Paulson, 1983). There are numerous genetic factors involving in transmission and host adaptation, but HA protein is the major fundamental determinant of host adaptation (Xiong et al., 2014a, Matrosovich et al., 2000).

The major structural features of receptor binding site are 190 helix (a short helix positioned in 190-195), 130 loop (a loop region that form a proximal boundary), 150 loop (at the right side of the site), 220 loop (at the left side of the site), and a hydrogen-bonded network of well-conserved residues including Tyr98, Trp153, His183, and Tyr195 (Xiong et al., 2014a, Lin et al., 2012). The switch of receptor preference from avian type (a2,3-linkage) to human type (a2,6-linkage) by

mutations in the receptor binding site allowed avian influenza virus to replicate and infect in human or mammalian hosts (Rogers et al., 1983, Connor et al., 1994). Therefore, a lot of studies have focused on the adaptive mutations on the receptor binding site (RBS) of HA protein (Matrosovich et al., 2000, Rogers and Paulson, 1983, Tzarum et al., 2015, Bradley et al., 2011, Gamblin et al., 2004).

The preference to a2,6-linkage sialic acid receptor of H3N2 viruses and H2N2 viruses associates with both cases with Q226L and G228S mutations in the receptor binding site (Matrosovich et al., 2000, Connor et al., 1994). Interestingly, G228S mutation was observed in some of H7N9 human infected viruses, which suggest that the receptor preference shift from avian to human might be critical point for successful adaptation of H7N9 virus to human (Gao et al., 2013, Tharakaraman et al., 2013). Meanwhile, the mutations at position 190 (E to D), and position 225 (G to D) correlate with increased affinity of the viruses for a2,6 sialic acid receptor, which are related with the outbreak of the 1918 pandemic influenza (Matrosovich et al., 2000, Gamblin et al., 2004, Glaser et al., 2005). N186I substitution also induce a dramatic shift in receptor-binding preference to a2,6 sialic acid of H2N2 virus (Matrosovich et al., 2000, Connor et al., 1994).

The balance of HA and NA activity also play a crucial role in effective growth of IAVs in mammalian cells, and it has been presumed that the balance contribute to viral virulence in human (Wagner et al., 2000, Matsuoka et al., 2009, Chen et al., 2012, Benton et al., 2015). The acquisition of additional N-glycosylation in HA and a deleted NA stalk region affect the HA/NA activity,

increasing the replication properties in both mammalian and avian cells, and rising the viral pathogenicity in mice (Wagner et al., 2000, Matsuoka et al., 2009). Moreover, the additional N-glycosylation near the RBS of HA may affect not only escaping host immune response, but also increasing viral virulence to mammals (Zhao et al., 2017, Herve et al., 2015, Hensley et al., 2009, Kosik et al., 2018). Therefore, the additional acquisition of N-glycosylation in HA protein is one of the critical adaptive mutations in viral adaptation and pathogenicity in mammals.

3.2 Polymerase and NP proteins

The polymerase of IAVs is heterotrimer composed of PB1, PB2, and PA. PB1 protein is the catalytic subunit of the RNA polymerase complex directly involved in the RNA synthesis. Contrast to its importance in viral RNA synthesis, adaptive mutations involved in viral pathogenicity and adaptation in mammals have not been well illustrated. T296R mutation in PB1 contributed to the mice virulence of 2009 pandemic H1N1 virus by increasing polymerase activity (Yu et al., 2015). 473V and 598P residues of PB1 protein increase viral polymerase activity, so they may contribute to effective viral replication in mammalian cells (Xu et al., 2012). The PB1-F2 protein, encoded by the +1 reading frame of PB1, is important for viral replication by regulating host anti-viral response, and it has been considered as a new virulence factor of IAVs in host-dependent manner (Lee et al., 2016, Chen et al., 2001, Conenello et al., 2011).

PB2 protein is a cap binding protein, and it has been known as one of important protein in viral adaptation to new host. E627K mutation is one of the most famous adaptive mutation in the PB2 protein (Subbarao et al., 1993). Given that the most of avian influenza viruses acquired the 627K mutation after introducing to mammalian hosts, E627K mutation definitely act as important adaptation factor (Gao et al., 2013, Shinya et al., 2007, Min et al., 2013, Zhang et al., 2014). E627K mutation increase not only viral polymerase activity but also viral replication in mammalian cells (Manzoor et al., 2009, Kim et al., 2010, Subbarao et al., 1993). The role of 627 residue has not been well understood, but it contribute to the cold sensitivity, viral genome importing to the nucleus, and the interaction with the cellular proteins such as Mitochondrial Antiviral Signaling Protein (MAVS) or ANA32A (Graef et al., 2010, Massin et al., 2001, Long et al., 2016, Hudjetz and Gabriel, 2012). Not only E627K mutation, but also Q591K/R and D701N mutations also play important role in viral adaptation to mammals (Sediri et al., 2015, Liu et al., 2012). Especially, G590S/Q591R mutations, called as SR polymorphism, totally substituted E627K mutations in 2009 swine-origin H1N1 influenza virus infection (Mehle and Doudna, 2009). Similar to SR polymorphism, D701N mutation may substitute E627K mutation by enhancing nuclear import of influenza vRNP in mammalian cells (Sediri et al., 2015). Several other important residues PB2-158G, -199S, 253N, -256G, and -271A also likely affect the viral polymerase activity in mammals (Bussey et al., 2010, Zhou et al., 2011, Mok et al., 2011, Manzoor et al., 2009).

PA protein is likely to consist of two domains; the endonuclease domain in the N-terminus and a C-terminal domain that mediate the interaction with PB1 protein (He et al., 2008, Yuan et al., 2009, Obayashi et al., 2008). Among two domains, adaptive mutations for host adaptation in PA gene seem to cluster in the endonuclease-containing N terminus (Sun et al., 2014, Seyer et al., 2012, Bussey et al., 2011). Moreover, The N terminus of PA is important residues in both viral genome promoter binding and regulation of cRNA/vRNA synthesis (Hara et al., 2006). T97I mutation in PA protein increased the virulence of H5N2 AIVs to mice (Song et al., 2009, Nam et al., 2017), and L295P mutation in PA were responsible for the enhanced virulence of mouse-adapted H1N1 variant in mice (Ilyushina et al., 2010). Additional mutations were identified at various positions in PA, including 336M, 552S, and 615N, and in PB1 mutations at positions 473, 598, and 296 were shown to increase viral polymerase activity in mammals (Hu and Liu, 2015, Manz et al., 2013b, Xu et al., 2012, Yu et al., 2015).

NP protein is essential for the formation of vRNP complex by encapsidating the virus genome (Ye et al., 2006). NP protein interact with various viral proteins and host factors for the purpose of RNA transcription, replication and packaging (Gui et al., 2014, Agustin Portela, 2002, Dittmann et al., 2008, Manz et al., 2013a, Nakada et al., 2015). Adaptive mutations such as 100, 283, and 313 exhibited the resistance to murine Mx1 and human MxA, resulting in increased virulence (Manz et al., 2013a). Residues 41V and/or 210D could increase polymerase activity and likely

contributed to replicate at lower temperature (Zhu et al., 2015b). Because the NP genes are relatively well conserved in the most of IAVs, the adaptive mutations of NP have not been well elucidated (Shu et al., 1993). Given its importance on viral replication, the further researches on the role of NP in the adaptation to new hosts will be needed for the understanding of IAVs.

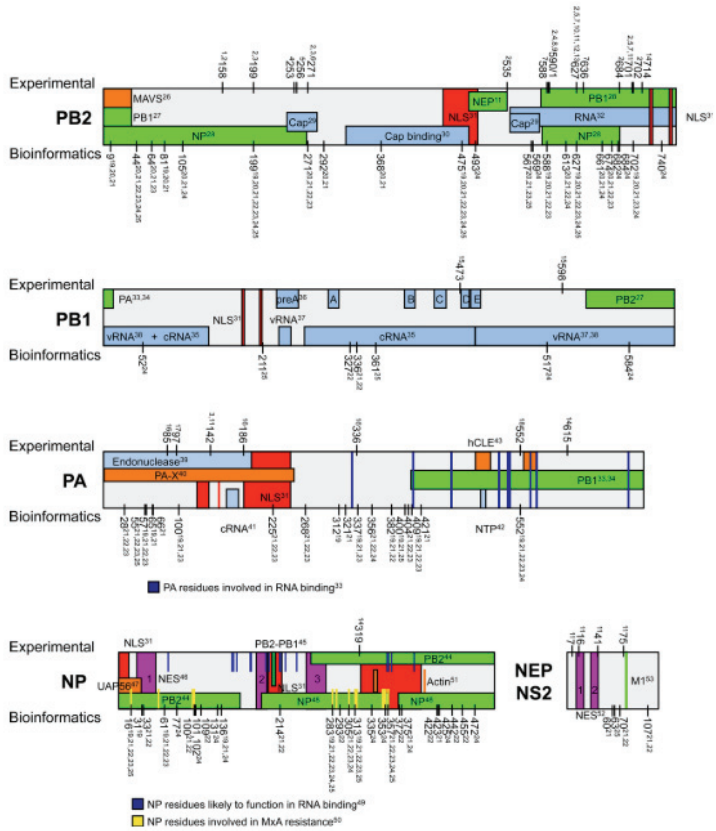

Figure L.4. Described mutations increasing polymerase activity in mammalian cells. Published data were analyzed to screen for predicted host-adaptive amino acids (Bioinformatics) and mutations experimentally shown to increase activity of an avian influenza virus polymerase in the context of mammalian cells or to increase pathogenicity in a viral infection (Experimental). Functional domains are indicated in green (interaction with viral proteins), red (involved in nuclear localization), purple (involved in nuclear export), orange (interaction with cellular proteins), yellow (MxA resistance), and blue (RNA binding).

Adapted from Manz, B., Schwemmle, M., and Brunotte, L., 2013

3.4 The NS1 protein

Recent studies indicated that the NS1 protein, an IFN antagonist, contribute to adapt and transmit avian influenza virus to mammals. The double strand RNA binding domain of NS1 (aa1-73) and CPSF30 binding domain are essential for the inhibition of type I IFN transcription and production (Ramos et al., 2013). D92E mutation in NS1 protein is important for H5N1 viruses to resist the antiviral effects of IFN, which resulted in increased pro-inflammatory cytokine expression (Seo et al., 2002). A PDZ ligand domain (PL) (of the X-S/T-X/V type) present in the C-terminus of NS 1 protein, and it also play an important role in virulence (Obenauer et al., 2006). In the previous study, our researcher found that GSEV-PL motif of NS1 gene attenuated viral pathogenicity in mice, and also demonstrated that amino acid residues at position 139 and 151 in NS1 gene attribute as the mammalian pathogenic factors (Kim et al., 2015). Recently, K55E, K66E, and C133F substitutions in the NS1 protein decreased the interferon beta response by binding to the CPSF30 (Li et al., 2018). These findings demonstrate that the specific amino acid substitution of NS1 protein successfully regulate host dependent immune response by interacting with host immune regulatory factors (Park et al., 2014, Li et al., 2018, Kochs et al., 2007).

3.5. The noncoding regions

The 5'- and 3'-terminal of IAV genomes have 13 and 12 conserved nucleotides,

respectively. These terminal sequences are essential for viral genome transcription and replication (Li and Palese, 1992, Yamanaka et al., 1991, Crow et al., 2004). The explainable model for the terminal sequences were firstly proposed as "pan-handle structure", later as "fork model", and finally the "cock screw model" (Flick and Hobom, 1999, Desselberger et al., 1980, Fodor et al., 1995). The promoter functions have been known to be localized to 12 conserved nucleotides at the 3'end of vRNA, and nucleotides 9-11 were shown to be crucial for the activity (Seong and Brownlee, 1992). The mutations at positions 11 and 12 of the 3' and 5' ends of NA vRNA of A/WSN/33 reduced the neuraminidase mRNA and protein levels, as well as the virus titer, and resulted in attenuated phenotypes in mice (Fodor et al., 1998). The fourth nucleotide at the 3'end of genome has either uracil (U) or cysteine (C), and the substitution from C to U increase the transcription and the replication of viral genome (Lee and Seong, 1998, Jiang et al., 2010). According to recent study, the U to C mutation at fourth nucleotide at the 3'end of of NA and M genes may affect viral pathogenicity in mice by regulating viral transcription and replication (Lee et al., 2017b). These findings suggest that the promoter regions present at the 5'-and 3'-end of viral genome are one of potential molecular virulence markers by affecting the replication and transcription of viral genomes.

4. Conclusions

Since 1918 pandemic H1N1 viruses were firstly reported as the direct transmission of avian origin virus, IAVs have continually posed the human health threat. More recently, in addition to H1,

H2, and H3 viruses, a variety of novel subtypes of avian influenza viruses have caused human infection with high mortality (Chen et al., 2014, Gao et al., 2013, Yuan et al., 2013, de Jong et al., 1997). Therefore, not only the continual surveillance but also the exact evaluation of the risk of transmission to human will help to respond to the next pandemic early. Furthermore, to understand the mechanism of the virulence mutations of IAVs may be applied to the developments of novel anti-viral reagent.

Chapter Ⅰ

The prerequisite of mammalian pathogenicity by influenza A virus with a prototypic avian PB2 gene

Abstract

The polymerase of avian influenza A virus (AIV) is a heterotrimer composed of PB2, PB1 and PA. PB2 plays a role in overcoming the host barrier; however, the genetic prerequisites for avian PB2 to acquire mammalian pathogenic mutations have not been well elucidated. Previously, I identified a prototypic avian PB2 that conferred non-replicative and non-pathogenic traits to a PR8-derived recombinant virus when it was used to infect mice. Here, I demonstrated that key amino acid mutations (I66M, I109V and I133V, collectively referred to as MVV) of this prototypic avian PB2 increase the replication efficiency of recombinant PR8 virus carrying the mutated PB2 in both avian and mammalian hosts. The MVV mutations caused no weight loss in mice, but they did allow replication in infected lungs, and the viruses acquired fatal mammalian pathogenic mutations such as Q591R/K, E627K, or D701N in the infected lungs. The MVV mutations are located at the interfaces of the trimer and are predicted to increase the strength of this structure. Thus, gaining MVV mutations might be the first step for AIV to acquire mammalian pathogenicity. These results provide new insights into the evolution of AIV in birds and mammals.

Keywords**:** *avian influenza A virus, prototypic PB2, polymerase activity, mammalian pathogenicity, first-step mutations*

1.1. Introduction

Aquatic birds are natural reservoirs for influenza A virus (IAV), and 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes for IAV have been identified (Fouchier et al., 2005, Webster et al., 1992b). Recently, the additional subtypes H17N10 and H18N11 were discovered in New World bats, which are considered another reservoir for a diverse pool of IAVs (Pflug et al., 2014, Tong et al., 2013). Pigs have long served as intermediate hosts for the reassortment of avian and mammalian IAVs, but direct transmission of avian IAVs to humans has become a worldwide public health threat (Claas et al., 1998a, Webster et al., 1992b).

The polymerase of AIV is a heterotrimer composed of PB1, PB2 and PA. In this structure, PA and PB2 bind to the N- and C-termini of PB1, respectively (Detjen et al., 1987, Gonzalez et al., 1996). PB1 functions as an RNA-dependent RNA polymerase, and PA cleaves the cap containing 10-13 nucleotides from host pre-mRNA, which is then captured by PB2 (Guilligay et al., 2008, Poch et al., 1989). These three subunits determine the host range, tissue tropism and mammalian pathogenicity of AIV (Detjen et al., 1987, Hatta et al., 2001, Massin et al., 2001, Snyder et al., 1987, Subbarao et al., 1993). Diverse mutations in the polymerase subunits that determine the mammalian pathogenicity of AIV have been reported, and E627K in PB2 is considered a key mutation (Hatta et

al., 2001, Subbarao et al., 1993, Zhang et al., 2014, E.Kanta Subbarao, 1993). Amino acid position 627 on PB2 is located in the C-terminal RNA-binding domain, and the E627K mutation is known to increase both RNA binding and polymerase activity, increasing viral replication efficiency at 33° C, the approximate temperature of the human upper respiratory tract (Kuzuhara et al., 2009, Steel et al., 2009). Furthermore, the E627K mutation may increase the mammalian pathogenicity of AIV by promoting a stronger interaction with mammalian importin-α isoforms and enhancing the NP-PB2 interaction in mammalian cells (Tarendeau et al., 2008, Gabriel et al., 2011, Labadie et al., 2007, Rameix-Welti et al., 2009).

I previously identified avian polymerase genes with different degrees of mammalian pathogenicity. A prototypic PB2 gene of an H9N2 low-pathogenic AIV (LPAIV) strain, A/chicken/Korea/01310/2001 (H9N2) (01310), was not replicative and non-pathogenic (no body weight loss) in BALB/c mice after inoculation of 7+1 PR8-derived recombinant virus. Furthermore, the PB2 gene of the H9N2 LPAIV strain A/Korea/KBNP-0028/2000 (H9N2) (0028) was replicative but non-pathogenic in BALB/c mice, and I identified candidate amino acids related to the replication of 0028 PB2 in mouse lungs by comparing the amino acid sequences of PB2 proteins (Kim et al., 2014). Importantly, the identification of key amino acids in 0028 PB2 that confer replicative ability, but not pathogenicity, may improve understanding of the first-step mutations that occur in prototypic 01310 PB2 to facilitate the acquisition of fatal mammalian

pathogenicity. Here, I identified key mutations (I66M, I109V and I133V, collectively referred to as "MVV") that increase replication efficiency in both avian and mammalian hosts and are predicted to increase the structural integrity of the trimeric polymerase. These MVV mutations are essential prerequisites for the subsequent acquisition of fatal mammalian pathogenic mutations. Collectively, these results provide important insights into the first evolutionary step taken by AIV to acquire pathogenicity in mammals.

1.2. Materials and Methods

Viruses, eggs, and cells

This study used A/PR/8/34 virus and A/chicken/Korea/01310/2001 (H9N2) (01310), a strain used for an inactivated oil emulsion vaccine in Korea. To synthesize recombinant PR8 virus (rPR8), a Hoffmann vector system was used as described previously (Hoffmann et al., 2000, Hoffmann et al., 2002). Recombinant PR8 viruses were generated and passaged three times in 10-day-old SPF embryonated chicken eggs (ECEs) (Charles River Laboratories, North Franklin, USA) and then used in experiments. Additionally, 293T, MDCK, and A549 cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea), and PK-15 cell was acquired from American Type Culture Collection (ATCC, VA, USA). 293T, MDCK, and PK-15 cells were maintained in DMEM supplemented with 10% FBS (Life Technologies Co., CA, USA), and A549 cell was maintained in DMEM/F12 supplemented with 10% FBS.

Cloning of the PB2 gene and site-directed mutagenesis of 01310 PB2 genes

Each viral segment was cloned into Hoffmann's bi-directional transcription vector pHW2000 (Hoffmann et al., 2000). The insert sequence was confirmed by sequencing with primers cmv-SF (5'-TAAGCAGAGCTCTCTGGCTA-3') and bGH-SR (5'-TGGTGGCGTTTTTGGGGA

-CA-3'). Site-directed mutagenesis of specific amino acid substitutions in PB2 genes from the 01310 virus and PA genes from the 0028 virus were implemented using a Muta-direct Site Directed Mutagenesis Kit (iNtRON, Korea) as per the manufacturer's protocol.

Mini-genome assay

To evaluate the polymerase activity of each mutated virus, I constructed pHW-NP-Luc plasmids, which have pHW2000 backbones containing the untranslated region of the PR8 NP gene inserted in the antisense direction between the RNA polymerase Ⅰ promoter and the terminator of the vector. The firefly luciferase gene from the pGL-3 vector was inserted between the NP gene 5' and 3' non-coding regions. 293T cells in 12-well plates were co-transfected with 0.1 ㎍ each of pHW-NP-Luc and mutated 01310 PB2 and PR8 PB1, PA and NP genes. Additionally, 0.1 ㎍ of the *Renilla* luciferase plasmid pRL-TK (Promega, USA) was also co-transfected, which served as an internal control to normalize variations in transfection efficiency and sample processing. Then, 24 hours after transfection, luminescence was assessed using a Dual-Glo Luciferase Assay System (Promega, USA) in accordance with the manufacturer's instructions on a TECAN Infinite200 pro machine (Tecan Benelux bv, Giessen, Netherlands). All results shown are the average from triplicate experiments, and the standard deviation was calculated.

Rescue of mutant viruses

PB2, PA, and NP plasmids containing mutations of interesting amino acids and 7 genome segments of PR8 were transfected into 293T cells by transfecting Hoffmann's eight reverse genetics plasmids as described previously with some modifications (Hoffmann et al., 2000, Hoffmann et al., 2002). Briefly, bi-directional pHW2000 vectors containing PB1, PA, HA, NP, M, and NS of the PR8 virus and 01310 virus PB2 containing mutations in amino acids of interest were transfected with 300 ng of each plasmid using Lipofectamine 2000 and Plus reagents (Life Technologies Co., CA, USA). After overnight incubation, 1 ml of Opti-MEM (Life Technologies Co., CA, USA) and 0.5 mg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA) were added. After 24 h, the culture medium was harvested, and 200 μl of the medium was injected into 10-day-old SPF ECEs via the allantoic cavity. Three days after inoculation, the allantoic fluid was harvested and checked for virus growth via HA assay using 1% (v/v) chicken red blood cells (RBCs) according to the WHO Manual on Animal Influenza Diagnosis and Surveillance. All mutant viruses were confirmed by RT-PCR and sequencing.

Titration of viruses

Each mutant virus was inoculated into five 10-day-old SPF ECEs for virus isolation. To

estimate virus titre, each virus was serially diluted from 10^{-1} to 10^{-9} in 10-fold increments, and each dilution was injected into five 10-day-old SPF ECEs as well as inoculated onto MDCK cells. The 50% chicken embryo infectious dose (EID_{50}) and 50% tissue culture infectious dose ($TCID_{50}$) were calculated using the Spearman-Karber method.

Comparative replication efficiency in mammalian cells

To evaluate the replication efficiency of each virus, MDCK (2×10^4/ml), PK-15 (2.5×10^4/ml), and A549 (2×10^4/ml) cells were seeded in 96-well plates (100 ㎕/well). After 24 hours, confluent cells were washed twice with phosphate-buffered saline (PBS). Mutant viruses at 10^7 EID_{50}/0.1 ml were serially diluted from 10^{-1} to 10^{-8} in 10-fold increments, and 200 ㎕ of each dilution was inoculated into each well with DMEM supplemented with 1% bovine serum albumin (BSA) (fraction V) (Roche, Basel, Switzerland), 20 mM HEPES, antibiotic-antimycotic (Gibco, CA, USA), and 1 μg/ml (for MDCK cells), 0.5 μg/ml (for PK-15 cells), and 0.25 μg/ml (for A549 cells) TPCK-treated trypsin (Sigma-Aldrich, USA). The supernatants of the virus-infected cells were collected at 3, 5 and/or 7 dpi, and virus growth efficiency was determined by calculating the $TCID_{50}$ using the Spearman-Karber method. Values are presented as the average of three independent experiments ± s.d.

Animal experiments

Six-week-old female BALB/c mice were purchased from KOATEC (Pyeongtaek, Korea), and a mouse pathogenicity test was carried out by BIoPOA Co (Yongin, Korea) in accordance with national guidelines for the care and use of laboratory animals. To measure the mouse pathogenicity of each mutant virus, five mice were anaesthetized via intraperitoneal injection of 15 mg/kg Zoletil 50 (Virbac, Carros, France) and then intranasally inoculated with 10^6 EID_{50}/50 μl of each virus as described previously (Kim et al., 2014). Negative control (mock) mice were injected with the same volume of sterilized PBS. Mortality and weight loss were measured for 14 days. Mice that lost more than 30% of their original weight were euthanized and recorded as a death. For the measurement of virus replication in the lungs of infected mice, six mice from each group were injected with PBS (mock) or 10^6 EID_{50}/50 μl of mutant virus. The lungs were collected at 3 and 6 dpi and then stored at -70℃ until use. The lungs were ground using a TissueLyzer 2 (Qiagen, Valencia, CA, USA) with 5 mm stainless steel beads and a volume of PBS equal to 10% of the lung weight in suspension. Then, 10 volumes of PBS were mixed with the ground tissues. After centrifugation at 2000 x g for 10 min, the supernatants were used for viral titres, which were measured as described above. To determine whether adaptive mutations were present in the PB2

gene of viruses isolated from infected mouse lung, RNA extraction and RT-PCR were carried out using lung diluents infected with rPB2(01310)-MVV. The PCR products were cloned into a TA cloning vector (RBC, Taiwan). By selecting 10 colonies per specimen and sequencing, the proportions of adaptive mutations were confirmed.

Histopathology

Mice from each group were euthanized on day 6 dpi, and lungs were collected and fixed in 10% phosphate-buffered neutral formalin. One slice from each lung lobe per mouse was pulled, processed routinely, embedded in paraffin, and stained with haematoxylin and eosin for histopathological analyses. Histological changes were evaluated according to the modified methods of McAuley *et al*. (McAuley et al., 2007). Each pulmonary lesion was graded on a scale of 0 to 4. The grading system for histological characterization of the lesions was defined as follows: 0, normal lung; 1, mild infiltration of inflammatory cells around airways and vessels; 2, moderate infiltration of inflammatory cells around airways and vessels and mild leukocyte infiltration of alveolar spaces and interstitium; 3, moderate to severe infiltration of inflammatory cells around airways and vessels, moderate leukocyte infiltration of alveolar spaces and interstitium, mild necrosis, and hyperplasia of airway epithelium; 4, severe infiltration of inflammatory cells around

airways and vessels, severe leukocyte infiltration of alveolar spaces and interstitium, moderate to severe necrosis, and hyperplasia of airway epithelium.

Frequencies of specific amino acids in avian, human, and swine viruses

To evaluate the frequencies of specific amino acids from avian and mammalian hosts, full-length bird (PB2, n=12,561; PB1, n=13,614; PA, n=13,921), pig (PB2, n=3,721; PB1, n=4,047; PA, n=4,164), and human (PB2, n=9,460; PB1, n=17,423; PA, n=17,639) IAV ORF sequences corresponding to the viral polymerase genes were acquired from the NCBI Influenza Virus Resource (http://www.fludb.org). I analysed the PB2, PB1, and PA sequences of several host influenza viruses using complete genome sequences and excluded all pandemic H1N1 sequences in order to eliminate bias. Human influenza viruses were divided into two groups: one group contained the H1, H2 and H3 subtypes (PB2, n=9,161; PB1, n=17,134; PA, n=17,335), and the other group contained the H5, H6, H7, H9, and H10 subtypes (PB2, n=299; PB1, n=289; PA, n=304). Sequences were aligned using CLUSTALW, and amino acid frequencies were compared between avian and mammalian influenza viruses.

Competitive replicative ability of mutant viruses in embryonated chicken and

duck eggs and in MDCK cells

To confirm the host suitability of each mutant virus, 100 times the EID_{50} of mutant virus and control virus were mixed and inoculated into 10-day-old SPF ECEs and 14-day-old EDEs, and MDCK cells were inoculated with virus at an MOI of 1. At 3 dpi, allantoic fluid or cell culture supernatant were harvested and sequenced. By comparing the peaks of the DNA sequence chromatograms, the most abundant codons were estimated.

Molecular modelling

The crystal structure of the A/little yellow-shouldered bat/Guatemala/060/2010(H17N10) polymerase complex (PDB code: 4WSB) was used to locate amino acid residues in the IAV polymerase model with the PyMOL Molecular Graphics System (Version 1.1, DeLano Scientific LLC).

Ethics statement

All mouse experiments were carried out at BioPOA Co. (Yongin, Korea) following a protocol that adhered to the National Institutes of Health's Public Health Service Policy on the

Humane Care and Use of Laboratory Animals. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of BioPOA Co. (BP-2014-0006-2, BP-2016-006-2).

1.3. Results

Identification of key amino acid mutations

Based on previously identified candidate amino acids, I generated mutant 01310 PB2 genes with single amino acid mutations [PB2(01310)-I66M, PB2(01310)-K88R, PB2(01310)-I109V, PB2(01310)-I133V, PB2(01310)-R157K, PB2(01310)-K340R, PB2(01310)-L373I, PB2(01310)-V575M, PB2(01310)-E627K, and PB2(01310)-A674T] and tested their polymerase activity using an in vitro mini-genome assay in the 293T human embryonic kidney cell line (Figure 1.1a). Among the tested mutations, only I133V and E627K significantly increased polymerase activity; these increases were 4- and 80-fold, respectively.

To assess viral growth efficiency in MDCK cells and viral pathogenicity in mice, I generated PR8-derived recombinant viruses containing single amino acid mutations in 01310 PB2. Recombinant viruses with the I133V, L373I or E627K mutations produced higher titres than the parent strain rPB2(01310) ($P < 0.05$) (Figure 1.1b). In murine pathogenicity experiments, most of the mutant viruses, except the rPR8 virus, did not cause body weight loss or mortality during the observation period. Only rPB2(01310)-E627K caused slight body weight loss of up to 4% over 7-10 days post-infection (dpi) ($P < 0.05$) (Figure 1.2a, b). During mouse infectivity screening,

however, I observed detectable growth of mutant viruses containing the I66M, I109V, I133V, E627K or A674T mutations (Table 1.1). The L373I mutation, which increases viral replication efficiency in MDCK cells, was excluded from further analysis because viruses carrying this mutation did not proliferate sufficiently in mouse lung. The E627K mutation has a well-characterized role related to mammalian pathogenicity, whereas the A674T mutation, which is conserved among most human influenza viruses, does not appear to be related to mammalian pathogenicity (Miotto et al., 2008, Bussey et al., 2010). 66M, 109V and 133V are novel mutations that were first characterized in the present study.

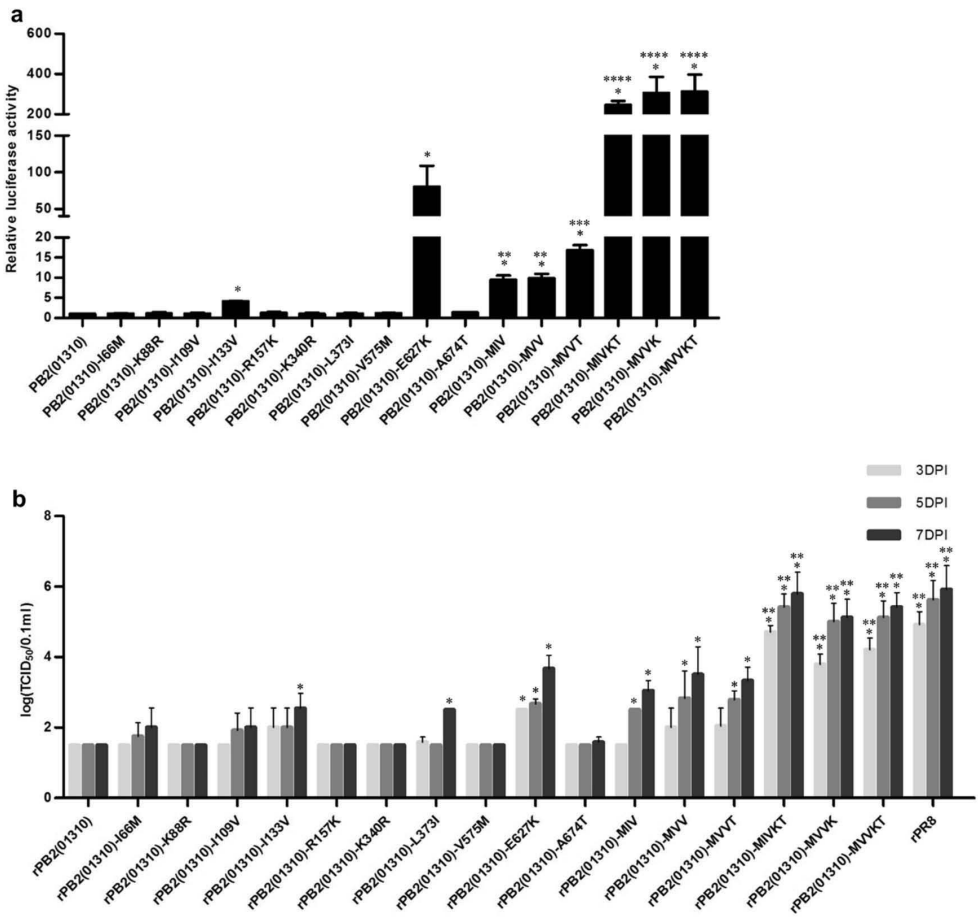

Figure 1.1. Viral polymerase activity and growth kinetics of 01310 PB2 variants.

(a) Viral polymerase activity was measured using mini-genome assays in 293T cells. The data were normalized to the polymerase activity of the wild-type 01310 PB2 gene. Statistical significance was calculated using Student's t-test (compared to PB2(01310), $^{*}P < 0.05$; compared to PB2(01310)-I133V, $^{**}P < 0.05$; compared to PB2(01310)-MVV, $^{***}P < 0.05$). (**b**) Replication

efficiency of 01310 PB2 variants in MDCK cells at 37℃. Wild-type rPB2(01310) and mutant viruses were used to infect MDCK cells at 10^7 EID_{50}/0.1 ml, and the $TCID_{50}$ was determined at 3, 5, and 7 dpi. Statistical significance was analysed by two-way analysis of variance with Bonferroni post-test correction (compared to rPB2(01310), $^{*}P < 0.05$; compared to rPB2(01310)-E627K, $^{**}P < 0.05$). The data presented are the average of three independent experiments ± s.d. from one experiment.

Table 1.1. Comparison of the viral replication efficiencies of 01310 PB2 single amino acid variants in mouse lungs.

Virus	Positive rate*(Virus titre ($logEID_{50}$/0.1 ml))		
	1st experiment	2nd experiment	
	3 dpi	3 dpi	6 dpi
Mock (PBS)	0/3(<0.5)	0/3(<0.5)	0/3(<0.5)
rPB2(01310)	0/3(<0.5)	0/3(<0.5)	0/3(<0.5)
rPB2(01310)-I66M	1/3(2.1)	0/3(<0.5)	0/3(<0.5)
rPB2(01310)-K88R	0/3(<0.5)	nt†	nt
rPB2(01310)-I109V	3/3(1.9)	2/3(1.7)	0/3(<0.5)
rPB2(01310)-I133V	3/3(2.5)	2/3(0.9)	3/3(3.5)
rPB2(01310)-R157K	0/3(<0.5)	nt	nt
rPB2(01310)-K340R	0/3(<0.5)	nt	nt
rPB2(01310)-L373I	1/3(<0.5)‡	nt	nt
rPB2(01310)-V575M	1/3(<0.5)	nt	nt
rPB2(01310)-E627K	3/3(4.1)	3/3(4.9)	3/3(5.3)
rPB2(01310)-A674T	1/3(1.7)	0/3(<0.5)	1/3(2.9)
rPR8	3/3(5.75)	3/3(6.1)	3/3(4.9)

*Number of positive samples/number of mice (virus titre of the pooled lung tissues, log_{10} EID_{50}/0.1ml).

†Not tested

‡Below the limit of assay detection

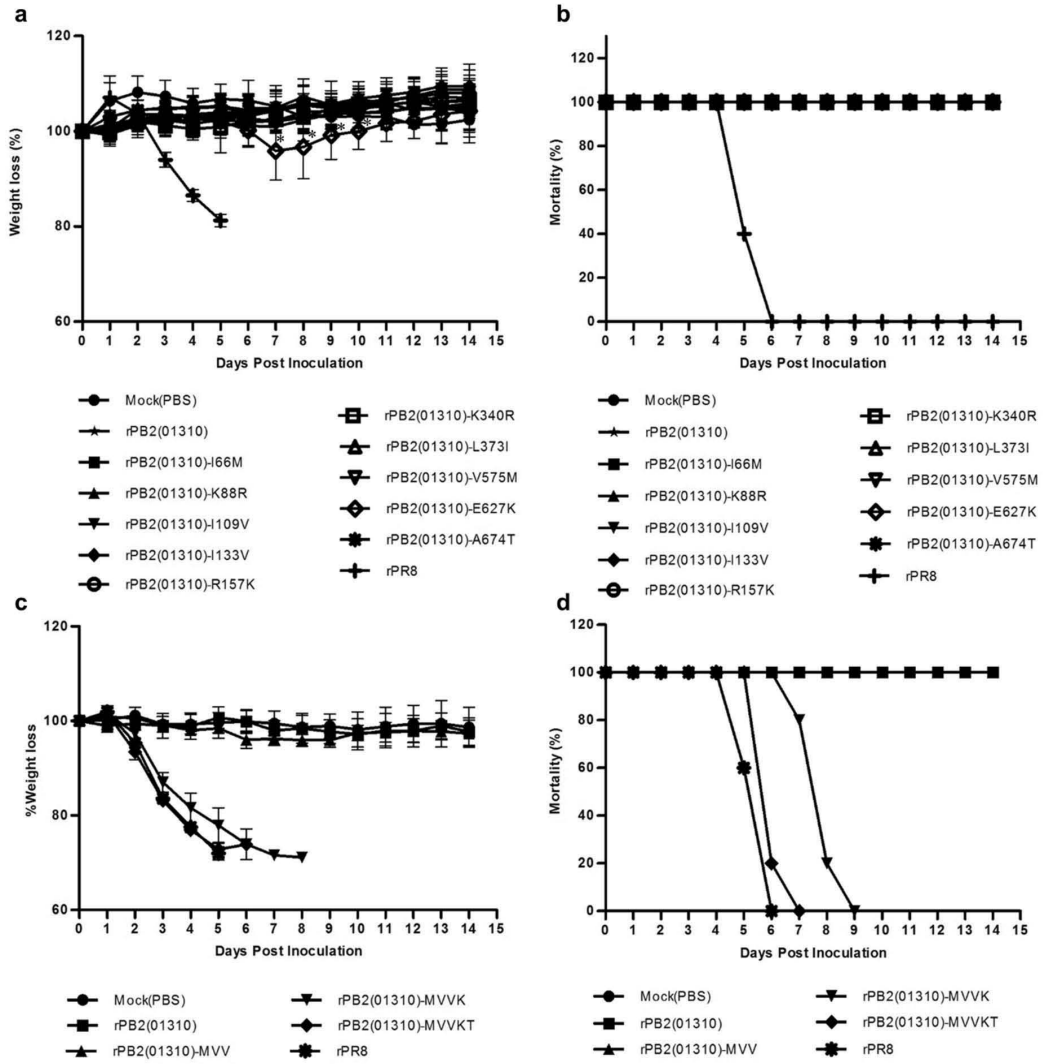

Figure 1.2. Comparison of mouse pathogenicity of 01310 PB2 variants.

The virulence of 01310 PB2 single amino acid variants (**a**,**b**) and multi-amino acid variants (**c**,**d**) was observed by body weight loss (**a,c**) and mortality (**b,d**) of infected mice. Five 6-week-old

BALB/c mice were challenged with 1.0×10^6 EID_{50} of each virus or PBS (mock). Mortality and weight loss were observed for 14 days. The average weight loss ± s.d. was measured by comparing to the initial weight of each mouse. Statistical significance was analysed using Student's t-test (compared to rPB2(01310), $^{*}P < 0.05$).

Frequency of key amino acid mutations

To understand the frequency of each identified mutation (I66M, I109V, I133V, E627K and A674T), I examined IAVs collected from birds, pigs and humans (Table 1.2). The human IAVs were divided into two groups: the first contained the H5, H6, H7, H9 and H10 IAVs (birds to humans), and the second contained the H1, H2 and H3 IAVs (humans), excluding the 2009 pandemic H1N1 virus. Interestingly, most of the IAVs already possessed the 66M, 109V, or 133V mutation, regardless of the host species they were isolated from. However, viruses bearing the 627K and 674T mutations were significantly more common in 'birds to humans' and human IAVs than bird and pig IAVs. The well-known mammalian pathogenic factor, D701N mutation, occurs less frequently than the E627K mutation in the PB2 genes of "human" and "bird to human" IAVs (Table 1.2) (Steel et al., 2009, Zhu et al., 2015a). Thus, I concentrated on the effect of E627K and its synergistic effects with the MVV mutations with regard to mammalian pathogenicity.

Among the combined novel amino acid mutations, the MVV group showed the highest frequencies in birds (92.7%), pigs (85.8%) and humans (birds to humans 98.0%; humans 84.6%). The MIV and IVV groups were the second most frequent in bird (4.2%), human (15.1%), pig (4.6%) and birds to humans (1.0%) IAVs. Furthermore, I calculated the ratios of viruses with or without additional amino acid substitutions related to the mammalian pathogenicity of AIV (9N,

147T, 158G, 192K, 199S, 253N, 271A, 339T, 404L, 526R, 588I/T, 590S, 591K/R, 627K, 674T, 701N, 702R, and 714R) (Subbarao et al., 1993, Gabriel et al., 2005b, Li et al., 2005, Yamada et al., 2010, Mehle and Doudna, 2009, Zhao et al., 2014, Song et al., 2014, Liu et al., 2015, Bussey et al., 2010, Mok et al., 2011, Taft et al., 2015, Zhou et al., 2011, Fan et al., 2014, Kim et al., 2010). The ratios of viruses with additional mammalian pathogenic factors were significantly lower in the MVV group of avian IAVs (25.9%) than in swine or human IAVs (pigs: 99.2%; birds to humans: 83.3%; humans: 100%) ($P < 0.05$). In contrast, the ratios of viruses without any additional mammalian pathogenic factors were significantly higher in the MVV group of avian IAVs (74.1%) than swine or human IAVs (pigs: 0.8%; birds to humans: 16.7%; humans: 0%) ($P < 0.05$).

Table 1.2. Frequency of residues in PB2 related to mammalian replication of influenza A virus between different hosts.

Host species	Frequency of amino acid residue (%)						Frequency of specific amino acid pattern at residues 66, 109, and 133 (%)						
	66M	109V	133V	627K	674T	701N	MVV*	MIV	IVV	MVI	MII	IVI	IIV
Birds (n=12,561)	97.6†	95.4	99.6	2.8	0.7	0.1	92.7 (74.1‡/25.9§)	4.2 (86.0/14.0)	0.3 (39.5/60.5)	0.4 (3.3/96.7)	0.0	0.0	0.0
Pigs (n=3,721)	87.4	92.1	99.9	8.0	1.7	15.9	85.8 (0.8/99.2)	1.2 (0/100)	4.6 (0/100)	0.0	0.0	0.0	0.0
Birds to humans (H5/H6/H7/H9/H10) (n=299)	99.0	99.3	99.7	41.1	0.3	6.0	98.0 (16.7/83.3)	0.0	1.0 (0/100)	0.3 (0/100)	0.0	0.0	0.0
Humans (H1/H2/H3) (n=9,161)	99.7	84.8	100.0	99.6	97.6	0.2	84.6 (0/100)	15.1 (0/100)	0.1 (0/100)	0.0	0.0	0.0	0.0

*Amino acid residues at positions 66, 109 and 133 in PB2.

†Percentage of viruses containing specific amino acid residues.

‡Percentage of viruses containing no mammalian pathogenicity-related mutations (9N, 147T, 158G, 192K, 199S, 253N, 271A, 339T, 404L, 526R, 588I/T, 590S, 591K/R, 627K, 674T, 701N, 702R, and/or 714R).

§Percentage of viruses containing mammalian pathogenicity-related mutations.

Effects of multiple amino acid mutations

Based on the high frequencies of the MVV and MIV mutations among the examined AIVs, I introduced multiple mutations combined with E627K and A674T into 01310 PB2 (MIV, MVV, MVVT, MIVKT, MVVK, and MVVKT) and performed mini-genome assays. The combination of 66M and 133V (MIV), as well as that of 66M, 109V and 133V (MVV), significantly increased polymerase activity compared to the individual amino acid mutations at 33°C and 37°C (Figures 1a and 3). The combination of MVV with 627K (MVVK) significantly increased polymerase activity compared to the single 627K mutation at 33°C and 37°C (Figures 1.1a and 1.3) ($P < 0.05$). The 674T mutation did not influence polymerase activity; however, in combination with MVV (MVVT), it increased polymerase activity significantly compared to MVV alone.

A panel of recombinant viruses was generated to assess viral replication efficiency in MDCK cells (Figure 1.1b). Viruses containing the MIV [rPB2(01310)-MIV], MVV [rPB2(01310)-MVV] and MVVT [rPB2(01310)-MVVT] mutations showed significantly higher replication efficiency than rPB2(01310). The combinations of 627K or 627K and 674T with MIV (MIVKT) or MVV (MVVK and MVVKT) significantly increased the replication efficiencies of the corresponding recombinant viruses [rPB2(01310)-MIVKT, rPB2(01310)-MVVK and rPB2(01310)-MVVKT] in MDCK cells compared to the 627K mutation alone [rPB2(01310)-

E627K]. Furthermore, rPB2(01310)-MVV showed higher replication efficiency than rPB2(01310) in a porcine kidney cell line, PK-15, and a human lung adenocarcinoma cell line, A549; rPB2(01310)-MVVK showed higher replication efficiency than rPB2(01310)-MVV in PK-15 and A549 cells at 33°C and 37°C (Figure 1.4).

In the murine pathogenicity experiments, rPB2(01310)-MVV successfully proliferated in lung tissue at 3 dpi ($10^{3.4}$ EID_{50}) and 6 dpi ($10^{3.0}$ EID_{50}), whereas rPB2(01310) did not (Table 1.3). Furthermore, compared to rPB2(01310)-E627K and rPB2(01310)-MVV, rPB2(01310)-MVVK and rPB2(01310)-MVVKT efficiently replicated in the lungs ($10^{5.8}$ EID_{50} and $10^{6.0}$ EID_{50}, respectively, at 3 dpi; $10^{4.1}$ EID_{50} and $10^{4.4}$ EID_{50}, respectively, at 6 dpi) and caused severe body weight loss and 100% mortality (Table 1.3 and Figure 1.2c, 2d). The mean time to death after rPB2(01310)-MVVKT inoculation was 6.2 ± 0.45 days, which was shorter than for rPB2(01310)-MVVK and similar to rPR8 virus (8 ± 0.71 days and 5.6 ± 0.55 days, respectively) ($P < 0.05$). Thus, the MIV and MVV mutations were demonstrated to have synergistic effects with 627K and 674T in the present study.

To further evaluate the effects of the MVV mutations, I constructed loss-of-function (LOF) genes using the mouse pathogenic PR8 PB2 gene (Figure 1.5a). The polymerase activities of the LOF mutants possessing the 66I-109I-133I [PB2(PR8)-III], 66I-109I-133I-674A [PB2(PR8)-IIIA],

or 627E [PB2(PR8)-K627E] mutations were significantly lower than that of the PR8 PB2 gene [PB2(PR8)]. Furthermore, the combination of III with 627E or 627E and 674A [PB2(PR8)-IIIE and PB2(PR8)-IIIEA] significantly decreased the polymerase activity compared to PB2(PR8)-III, PB2(PR8)-IIIA and PB2(PR8)-K627E ($P < 0.05$).

I next generated LOF mutant viruses possessing the above-described mutated PR8 PB2 genes and compared their replication efficiency in MDCK cells. The virus titre of rPB2(PR8)-III at 3 dpi was slightly lower than that of rPR8, but it recovered at 5 and 7 dpi (Figure 1.5b). Furthermore, rPB2(PR8)-IIIE and rPB2(PR8)-IIIEA replicated less efficiently than rPR8 and rPB2(PR8)-K627E.

Next, I assessed the pathogenicity of rPB2(PR8)-IIIE, rPB2(PR8)-III and rPB2(PR8)-K627E in mice. rPB2(PR8)-III exhibited high virulence, similar to rPR8 (Figure 1.5c, d). However, the mean time to death for rPB2(PR8)-III was 8.6 ± 0.89 days, which is approximately 2 days longer than that for rPR8 (6.6 ± 0.55 days) ($P < 0.05$). Moreover, the pathogenicity of rPB2(PR8)-IIIE was markedly attenuated; this variant caused no body weight loss and produced much lower virus titres in the lungs of infected mice than rPB2(PR8)-K627E and rPB2(PR8)-III (Table 1.3). These findings suggest that the MVV mutations in the PR8 PB2 protein have a substantial effect on viral polymerase activity and replication efficiency through synergistic cooperation with 627K.

The histopathological pulmonary lesions of mice infected with rPB2(01310), rPB2(01310)-E627K, rPB2(01310)-MVV, rPB2(01310)-MVVK, rPB2(PR8)-III, rPB2(PR8)-IIIE and rPR8 were compared, and the average lesion scores of 5 mice per virus type, except rPB2(PR8)-III (4 mice due to 1 mouse death) and rPR8 (2 mice due to 3 mouse deaths), were calculated (Figure 1.6). The pulmonary lesions in the rPB2(01310)-infected mice were similar to those in the mock group. Although the lesion score for rPB2(01310)-MVV did not significantly differ from that of rPB2(01310), rPB2(01310)-MVV induced mild to moderate pulmonary lesions in some infected mice. The pulmonary lesions induced by rPB2(01310)-E627K and rPB2(01310)-MVVK were characterized by necrotizing bronchiolitis, severe peribronchiolitis and interstitial pneumonia (average lesion scores 3.5 and 4, respectively), but rPB2(01310)-MVVK induced more severe necrotizing bronchiolitis in infected mice. rPR8 and rPB2(PR8)-III infection also caused necrotizing bronchiolitis, severe peribronchiolitis, interstitial pneumonia and bronchiolar epithelial proliferation (average lesion scores 3.5 and 4, respectively). These two viruses induced a similar degree of inflammation, but one rPR8-infected mouse showed more marked bronchiolar epithelial hyperplasia. The rPB2(PR8)-IIIE-infected mice showed less severe inflammation compared to the rPB2(PR8)-III- and rPR8-infected mice.

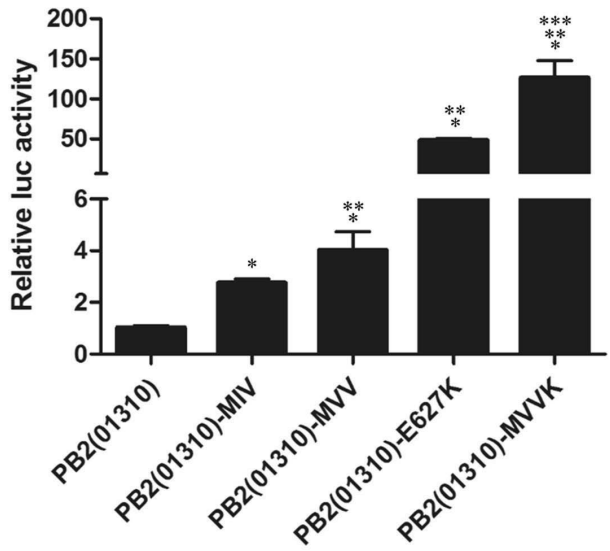

Figure 1.3. Viral polymerase activity of 01310 PB2 variants at 33 ℃.

Viral polymerase activities of 01310 PB2 variants were measured using mini-genome assays in 293T cells at 33°C. The data were normalized to the polymerase activity of the wild-type 01310 PB2 gene. Statistical significance was calculated using Student's t-test (compared to PB2(01310), $^{*}P < 0.05$; compared to PB2(01310)-MIV, $^{**}P < 0.05$; compared to PB2(01310)-E627K, $^{***}P < 0.05$).

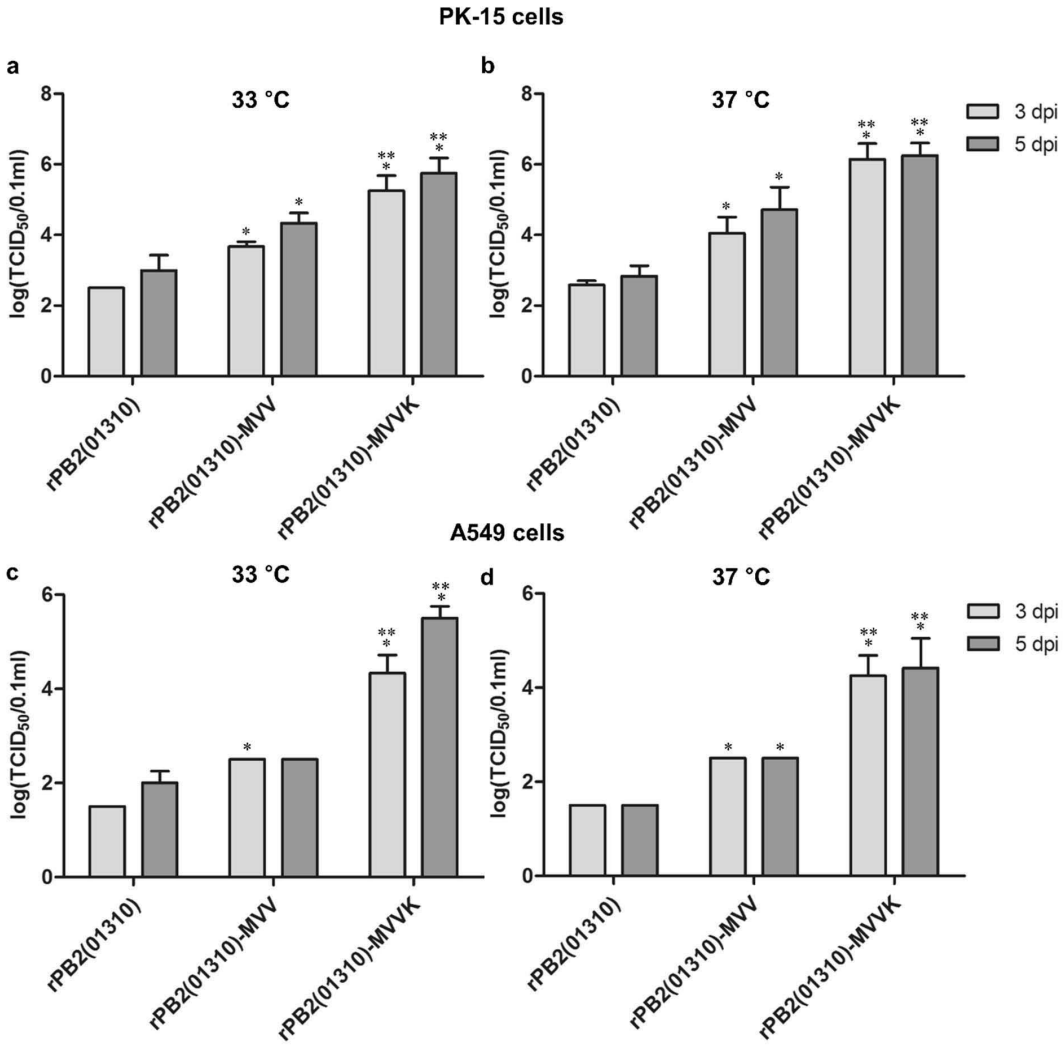

Figure 1.4. Replication efficiency of 01310 PB2 variants in porcine and human cell lines.

Replication efficiency of rPB2(01310), rPB2(01310)-MVV, and rPB2(01310)-MVVK in PK-15 (**a**,**b**) and A549 (**c**,**d**) cells at 33°C (**a**,**c**) and 37°C (**b**,**d**). Wild-type rPB2(01310), rPB2(01310)-MVV, and rPB2(01310)-MVVK were infected to PK-15 and A549 cells at 10^7 EID_{50}/0.1ml, and the $TCID_{50}$ was measured at 3 and 5 dpi. Statistical significance was analysed by one-way analysis of

variance with Bonferroni post-test correction (compared to rPB2(01310), $^{*}P < 0.05$; compared to rPB2(01310)-MVV, $^{**}P < 0.05$). The data presented are the average ± s.d. of three independent experiments.

Table 1.3. Comparison of viral replication of PB2 variants in mouse lungs.

Virus	Mean titre (log EID_{50}/0.1 ml*)	
	Lung	
	3 dpi	6 dpi
Mock (PBS)	<0.5	<0.5
rPB2(01310)	<0.5	<0.5
rPB2(01310)-MVV	3.4 ± 0.5	3.0 ± 1.5
rPB2(01310)-MVVK	5.8 ± 0.3	4.1 ± 0.4
rPB2(01310)-MVVKT	6.0 ± 0.4	4.4 ± 0.4
rPB2(PR8)-III	6.3 ± 0.5	5.0 ± 0.4
rPB2(PR8)-IIIET	1.4 ± 0.7	1.5 ± 0.7
rPR8	6.3 ± 0.2	4.5†
rPB2(PR8)-K627E	4.8 ± 0.7	4.6 ± 0.1

*Six BALB/c mice were inoculated with 10^6 EID_{50} of each virus; three mice were euthanized at 3 and 6 days post inoculation.

† Data from only one mouse due to the death of two mice.

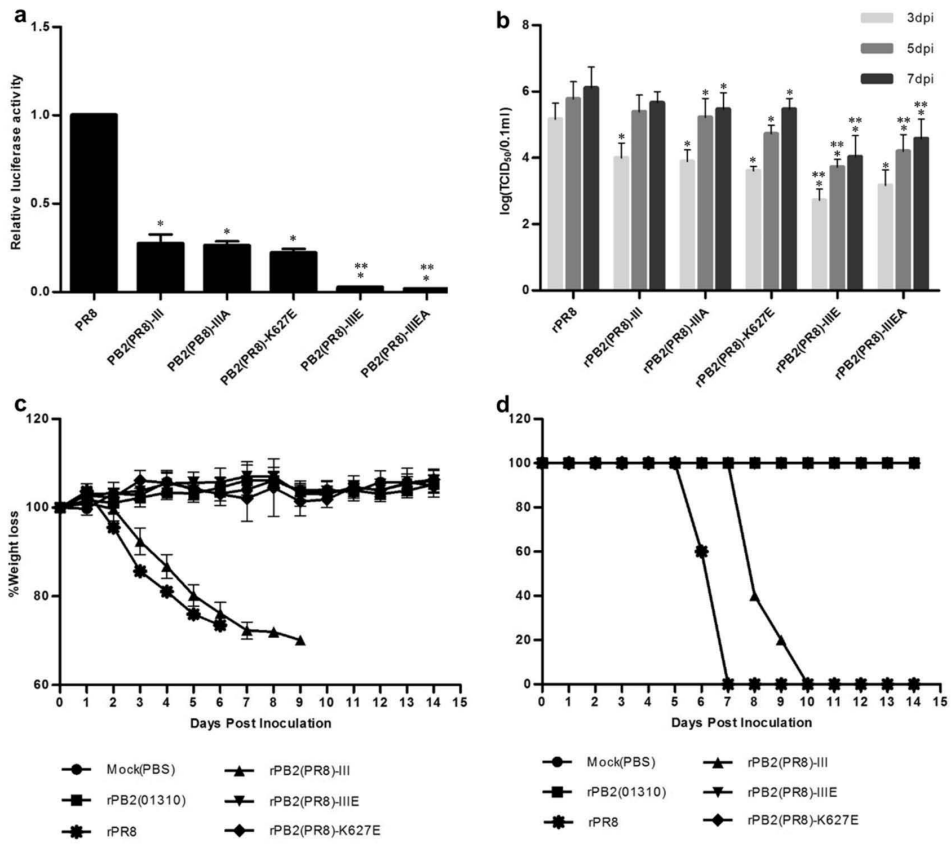

Figure 1.5. Effects of PR8 PB2 variants on mammalian adaptation.

(a) Viral polymerase activity was measured using mini-genome assays in 293T cells. The data were normalized to the polymerase activity of wild-type PR8 PB2. The data presented are the average of three independent experiments ± s.d. from one experiment. Statistical significance was analysed using Student's t-test (compared to the polymerase activity of PR8 PB2 gene, $^{*}P < 0.05$; compared to the polymerase activity of PB2(PR8)-K627E, $^{**}P < 0.05$). (**b**) Replication efficiency of

PR8 PB2 variants in MDCK cells at 37℃. Wild-type PR8 and mutant viruses were used to inoculate MDCK cells at 10^7 EID_{50}/0.1 ml, and the $TCID_{50}$ was determined at 3, 5, and 7 dpi. The data presented are the average of three independent experiments ± s.d. from one experiment. Statistical significance was analysed by two-way analysis of variance with Bonferroni post-test correction (compared to PR8, $^{*}P < 0.05$; compared to rPB2(01310)-K627E, $^{**}P < 0.05$). The virulence of PR8 PB2 variants was observed based on the body weight loss (**c**) and mortality of infected mice (**d**). Five 6-week BALB/c mice were anaesthetized and challenged with 1.0×10^6 EID_{50}/50㎕ of each virus or PBS (mock). Mortality and weight loss were observed for 14 days. The average weight loss ± s.d. was measured by comparing to the initial weight of each mouse.

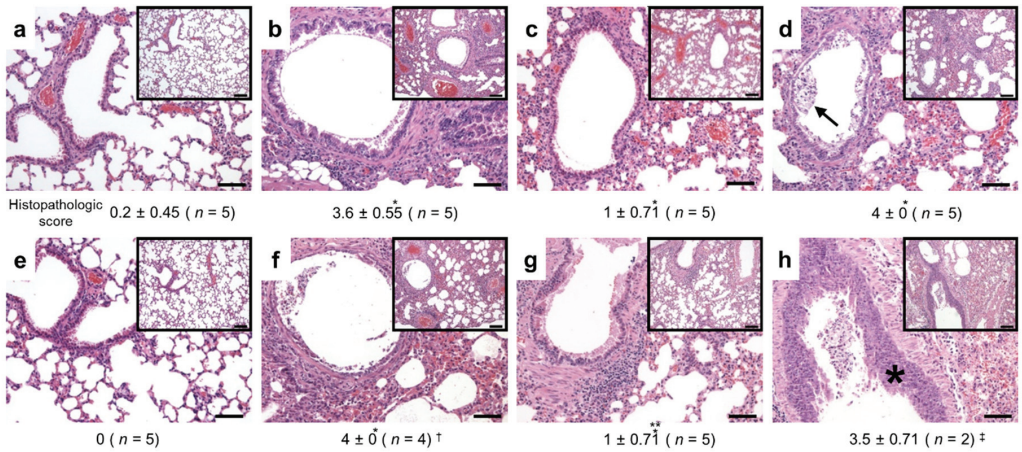

Figure 1.6. Histopathology of the lung tissue of the BALB/c mice infected with PR8 viruses with mutated PB2.

The lung samples of each group of mice inoculated intra-nasally with 10^6 EID_{50}/50 μl of PB2 mutated PR8 viruses or PBS were collected at 6 days post inoculation. rPB2 (01310)-E627K (**b**) and rPB2(01310)-MVVK (**d**) induced more severe inflammation than rPB2(01310) (**a**). **r**PB2(01310)-E627K and rPB2(01310)-MVVK infected mice showed necrotizing bronchiolitis and interstitial pneumonia. In particular, rPB2(01310)-MVVK cause marked bronchiolar epithelial necrosis (arrow). rPB2(E01310)-MVV (**c**) caused mild to moderate peribronchiolitis, but rPB2(01310) infected mice showed no significant histopathological lesions compared to the control mice (mock) (**e**). The pulmonary lesions of rPB2(PR8)-III infected mice (**f**) were similar to those of rPR8 infected mice (**h**), but marked proliferation of bronchiolar epithelium (asterisk) was observed in the rPR8 infected mice. rPB2(PR8)-IIIE (**g**) induced less severe inflammation compared to

rPB2(PR8)-III and rPR8 (H&E, 200 x; Inset = 100 x). The histopathologic score of each group is shown in bottom of each figure. Statistical significance was analysed using the Mann-Whitney test and compared with the histopathologic score of mock $^{*}P < 0.05$ or the histopathologic score of rPR8, $^{**}P < 0.05$,

† 4 mice were measured due to 1 mouse death

‡ 2 mice were measured due to 3 mouse deaths

Acquisition of fatal mammalian mutations

To investigate the effect of the MVV mutations on the acquisition of fatal mammalian adaptive mutations, I performed quasi-species analysis using the lungs of mice infected with rPB2(01310)-MVV at 6 dpi (Table 1.4). Cloned amplicons encoding amino acid residues 590-701 were sequenced, and well-known mammalian pathogenic mutations such as Q591R/K, E627K, and D701N were identified in four out of five lungs. Surprisingly, at least half of the quasi-species of three mice possessed mammalian pathogenic mutations (Q591K, E627K or D701N), and double mutations [E627K (10/10) and Q591R (1/10)] were observed in one mouse.

Table 1.4. Adaptive mutations of rPB2(01310)-MVV in 6 dpi mouse lung.

Residue†	Mouse-A*	Mouse-B	Mouse-C	Mouse-D	Mouse-E
590S	0/10	0/10	0/10	0/10	0/10
591R	0/10	**1/10**	0/10	0/10	0/10
591K	0/10	0/10	**5/10**	0/10	0/10
627K	**2/10**	**10/10**	0/10	0/10	0/10
701N	0/10	0/10	0/10	**8/10**	0/10

*Mouse lungs infected with rPB2(01310)-MVV were collected at 6 days post-inoculation., RT-PCR and cloning procedures were performed, and I then sequenced the indicated numbers of PB2 clones. Mammalian pathogenic factors in 590/591, 627, and 701 residues were found in lungs of mice infected with rPB2(01310)-MVV.

†Widely known important mammalian pathogenicity-associated residues are shown.

Boldface letters indicate PB2 clones with mammalian pathogenicity mutations.

Host selectivity of mutant viruses

To compare the relative replication efficiencies of rPB2(01310) and rPB2(01310)-MVV in mammalian and avian hosts, I mixed equal titres of both viruses and inoculated them into cultured MDCK cells, 10-day-old embryonated chicken eggs (ECEs), and embryonated duck eggs (EDEs). EDEs were used in addition to ECEs to better generalize our results among different avian hosts. Culture media supernatant and allantoic fluid were collected from the ECEs and EDEs and subjected to sequencing analysis, along with the pre-inoculation virus mixture. According to four or five independent experiments, rPB2(01310)-MVV outgrew rPB2(01310) in both mammalian (MDCK cells, 4/4) and avian (ECEs and EDEs, 5/5) hosts after only one passage (Figure 1.7). In the same experimental context, I mixed equal titres of rPB2(01310) and rPB2(01310)-E627K and inoculated MDCK cells, ECEs and EDEs. rPB2(01310)-E627K outgrew rPB2(01310) in MDCK cells (4/4), but rPB2(01310) outgrew rPB2(01310)-E627K in ECEs and EDEs (5/5) after only one passage (Figure 1.6).

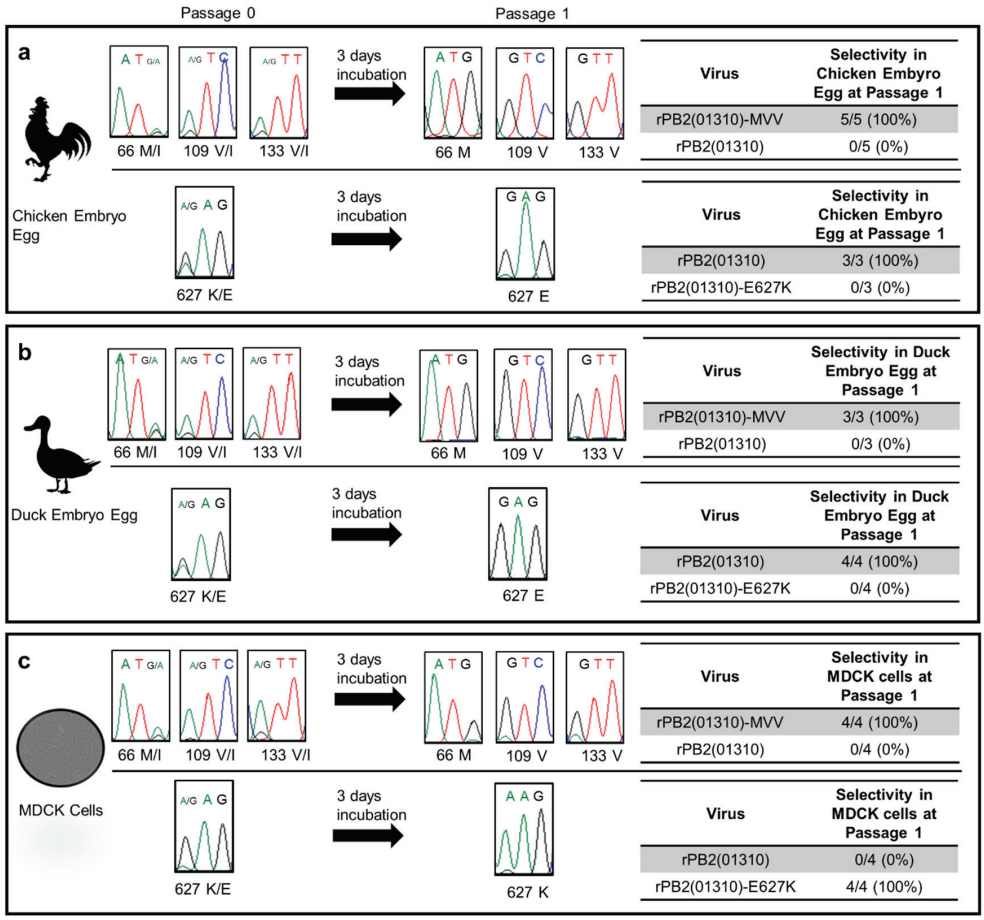

Figure 1.7. Comparison of the growth rate of each mutant virus against control virus in mixed cultures.

Mixtures (1:1) of wild-type rPB2(01310) and rPB2(01310)-MVV (top) or rPB2(01310) and rPB2(01310)-E627K (bottom) were inoculated in ECEs (**a**), EDEs (**b**) and MDCK cells (**c**). At 3 dpi, the allantoic fluid of the embryonated eggs and the cell culture supernatants of the MDCK cells were collected, and the PB2 gene was amplified by RT-PCR and sequenced. The codon for 66I is

ATA, 109I is ATC, 133I is ATT, and 627E is GAG; for the PB2 variants, the codon for 66M is ATG, 109V is GTC, 133V is GTT, and 627K is AAG. The DNA sequence chromatograms shown correspond to one sample from each host.

Structure-function relationship

To predict the structure-function relationship of I66M, I109V and I133V, I located each amino acid residue in a 3D structure of the PB1, PB2 and PA trimer from a bat IAV (Pflug et al., 2014). Interestingly, all the residues at the 66th, 109th, and 133rd positions were predicted to be located at the interfaces of the polymerase complex (Figure 1.8). The 66th and 109th residues were located close to 628N, 629N and 630P as well as 613W and 621R of the PB1 protein. The 133rd residue was located close to 429P, 430I and 433I of PA. I compared the neighbouring amino acid residues of bat IAV PB1 and PA with those of 01310 and PR8 PB1 and PA and found that most (except 621 and 628 of PB1) were conserved. Residues 621 and 628 of 01310 and PR8 are glutamine and leucine, respectively. By analysing the frequencies of the neighbouring amino acid residues in PB1 and PA, it was demonstrated that the 613W, 629N, 628L/M, and 630P residues of most IAVs are located in PB1 regardless of host species, but position 621 in PB1 carries different amino acids depending on the host species (Table 1.5). Residues 429P, 430I and 433I in PA were conserved among most avian and mammalian IAVs (434P, 435I and 438I), but the precise locations differed due to a 5-amino-acid deletion in bat PA. Considering that the neighbouring amino acids are conserved, except for residue 621 in PB1, these residues may form key interactions with 66M, 109V, and 133V. In other words, the I66M, I109V and I133V mutations may affect the structural integrity of the polymerase complex.

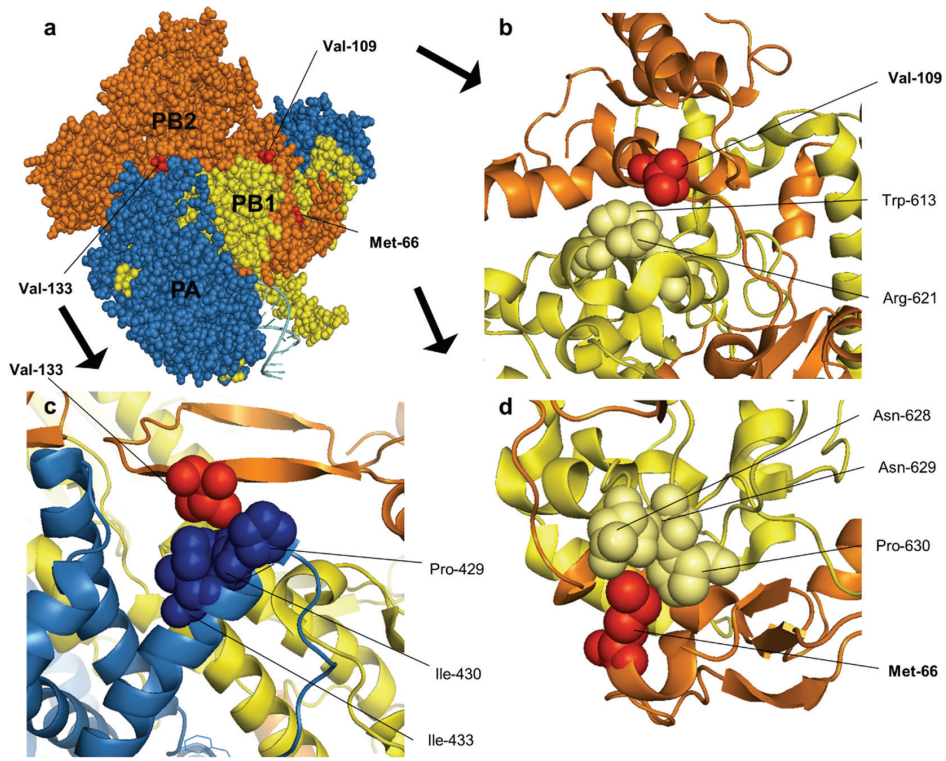

Figure 1.8. Locations of residues 66, 109, and 133 near the PB1-PB2 and PA-PB2 interfaces in the polymerase complex model.

The crystal structure of the A/little yellow-shoulder bat/Guatemala/060/2010 (H17N10) polymerase complex (PDB code: 4WSB) was used to locate amino acid residues in IAV. The analysis was performed using the program PYMOL (W. L. Delane; http://www.pymol.org). (a) The locations of the 66,109, and 133 residues in the polymerase complex. The interface of PB1-PB2 with the residues 109V (b) and 66M (d). The interface of PA-PB2 with the 133V (c) residue. Each

subunit of the polymerase complex is shown in a different colour.

Table 1.5. Frequencies of neighbouring amino acid residues of 66, 109 and 133 amino acid residues of PB2.

Segment	Residue	Host species			
		Birds	Pigs	Humans (H5/H6/H7/H9/H10)	Humans (H1/H2/H3)
PB1	613	W (99.99)	W (99.93)	W (100)	W (100)
		Q (95.86)	Q (10.06)		Q (12.12)
	621	R (2.55)	R (89.30)	Q (100)	R (87.86)
		K (1.58)	K (0.64)		K (0.01)
	628	L (88.70)	L (98.20)	L (68.86)	L (99.93)
		M (10.77)	M (0.57)	M (30.80)	M (0.01)
	629	N (99.99)	N (99.95)	N (100)	N (99.99)
	630	P (99.99)	P (100)	P (100)	P (99.99)
PA	429 (434) *	P (99.97)	P (100)	P (100)	P (99.99)
	430 (435)	I (99.94)	I (99.76)	I (100)	I (99.99)
	433 (438)	I (99.62)	I (98.49)	I (99.67)	I (99.79)

*numbering based on PA of IAVs from birds, pigs and humans.

1.4. Discussion

To date, various amino acid changes in avian IAVs related to mammalian replication and pathogenicity have been reported, and PB2 is one of the most important genes for overcoming host barrier defences (Fan et al., 2014, Schrauwen and Fouchier, 2014). In the present study, I identified the novel amino acid mutations I66M, I109V and I133V, which increased polymerase activity and replication efficiency in mammalian and avian hosts as well as pathogenicity in mice both independently and in combination. The E627K mutation was previously reported to play a key role in the mammalian pathogenicity of avian IAVs (Zhang et al., 2014, E.Kanta Subbarao, 1993, Kim et al., 2010, Hatta et al., 2001). The single E627K mutation in the prototypic 01310 PB2 increased polymerase activity and replication efficiency more effectively than the MVV mutations, but it did not cause apparent body weight loss or mortality in mice (Fig. 1a, b and Fig. 2a, b). However, the combination of E627K with the MVV mutations was sufficient to cause severe body weight loss and mortality (Fig. 2c, d). Although the MDT of the LOF mutant rPB2(PR8)-III was prolonged, the mutant retained sufficient pathogenicity to cause 100% mortality in mice. However, the K627E mutation eliminated the pathogenicity of the LOF mutants rPB2(PR8)-K627E and rPB2(PR8)-IIIE in mice (Fig. 3c, d). Thus, the E627K mutation has more important implications than the MVV

mutations, but the MVV mutations are present at a much higher frequency than E627K in avian, swine and human IAVs and therefore may represent the first-step mutations acquired by the prototypic PB2. Based on the growth competition between MVV and III recombinant viruses, the MVV mutations may be required for efficient replication in avian as well as mammalian hosts. Therefore, the very low frequencies of the intermediate mutants MIV, MVI, IVV, MII, IVI, and IIV and the prototype III in IAVs may imply that the evolution of polymerase activity in birds is approaching 'evolutionary stasis', raising a question about the presence of other hosts of ancient IAVs and their transmission to modern birds and mammals (Table 2) (Webster et al., 1992b).

The significantly lower frequencies of MVV and MIV with additional mammalian pathogenicity mutations in birds compared to pigs and humans may reflect negative selection in birds, but the significantly higher frequencies of MVV and MIV with additional mammalian pathogenicity mutations in pigs and humans may reflect positive selection (Table 2). The different selectivity of the 627E and 627K mutations in avian and mammalian hosts, respectively, has been a matter of conflict (Shinya et al., 2004, Long et al., 2013, Bortz et al., 2011, Chin et al., 2014, Hudjetz and Gabriel, 2012, Mok et al., 2014). In addition, the 627K mutation increased the mammalian pathogenicity of most H5N1 IAVs, but it did not increase the pathogenicity of other IAVs, including equine and some swine IAVs, some H5N1 viruses, and pandemic H1N1 viruses (Gao et al., 1999, Schnitzler and Schnitzler, 2009, Shinya et al., 2007, Hatta et al., 2001). The growth competition

experiment clearly demonstrated that viruses harbouring 627E grew more rapidly than those harbouring 627K in avian hosts and that those harbouring 627K grew more rapidly than those harbouring 627E in mammalian cells (Fig. 4). Thus, the conflicting results of previous reports may be caused by different backgrounds of additional mammalian pathogenicity mutations (Foeglein et al., 2011). Therefore, the prototypic 01310 PB2 may be useful for comparing the effects of mammalian pathogenicity mutations on viral growth efficiency in different hosts and pathogenicity in mammals.

Our quasi-species study revealed that the MVV mutations were the minimum requirement for the acquisition of additional mammalian pathogenicity mutations. Acquisition of additional E627K, D701N or Q591K/R mutations in 4 out of 5 mice during the first mammalian infection was unexpected, but it also supports the fact that the MVV mutations are the minimum essential predisposing mutations to acquire mutations for mammalian pathogenicity. When I compared the mammalian pathogenicity mutation patterns in PB2 from pig and human IAVs, it was found that multiple mutations in different combinations had been accumulated in PB2 (data not shown). These different accumulation patterns may be the result of growth competition in natural hosts. Therefore, tracking of accumulated mutations in PB2 using the prototypic 01310 PB2 may provide information on the effect of single or accumulated mutations on mammalian pathogenicity, providing some insight into the evolutionary steps taken by PB2 during adaptation to mammals

under competition between IAVs.

According to computational structure-function analysis, the 66th, 109th and 133rd residues were predicted to be located at the interfaces of the polymerase complex of a bat IAV (Figure 1.8) (Pflug et al., 2014). The conserved amino acids in PB1 and PA neighbouring the residues at positions 66, 109 and 133 of PB2 supports the importance of the I66M, I109V and I133V mutations. The position 66 isoleucine and methionine have hydrophobic side chains, but methionine may interact more strongly with neighbouring 628L and 630P. Because the side chain of isoleucine is bulkier than that of valine, 109V and 133V may fit better into the interface of PB1 and PA, which may affect the structural integrity of the polymerase complex and thereby increase polymerase activity. Therefore, the MVV mutations are not host-specific but rather universal mutations for avian and mammalian host adaptation. The question of why the polymerase evolved to obtain greater structural integrity remains to be answered. However, a recent report indicated that oviraptorids, ancient relatives of birds in the Cretaceous period, had a lower body temperature than modern birds and mammals, which could have necessitated a more structurally stable complex (Eagle et al., 2015).

In conclusion, the MVV mutations in PB2 have an important role in IAV replication. These mutations not only affect the structural integrity of the polymerase complex but are also an essential

prerequisite for the subsequent acquisition of mammalian pathogenic mutations. Our results raise questions surrounding how the prototypic PB2 was transmitted from ancient hosts, with lower body temperatures, to modern birds, with higher body temperature. In addition to improving understanding of the molecular steps taken by IAV to acquire mammalian pathogenicity, the prototypic PB2 may be useful as a template for the grafting of certain mutations to compare their effects on mammalian pathogenicity.

Chapter Ⅱ

Novel mutations in avian PA in combination with an adaptive mutation in PR8 NP exacerbate the virulence of PR8-derived recombinant influenza A viruses in mice

Abstract

The polymerase complex of the low-pathogenic avian influenza virus [A/chicken/Korea/KBNP-0028/2000 (H9N2)] (0028) has previously been characterized, and novel amino acid residues present in the polymerase acidic protein (PA) that likely contribute to pathogenicity toward mammals have been identified. In the present study, our aims were to generate A/Puerto Rico/8/34 (PR8)-derived recombinant viruses containing the *0028-PA* gene with a single amino acid mutation and to test their pathogenicity and replication ability. I found that the recombinant viruses acquired additional single mutations in the nucleoprotein (NP). Because the additional mutations in NP did not affect viral pathogenicity, but rather attenuated viral replication and polymerase activity, the incompatibility of the avian *PA* gene within the PR8 backbone may have induced an adaptive mutation in *NP*. To minimize the differences due to NP mutations, I generated 0028-PA mutants with an E375G mutation, not affecting viral replication and pathogenicity, in the *NP* gene. The PR8-PA(0028)-E684G mutant showed significantly higher viral replication in mammalian cells as compared to PR8-PA(0028) and led to 100% mortality in mice, with significantly increased interferon β expression. Thus, the E684G mutation in the *PA* gene may play an important role in viral pathogenicity in mice by increasing viral replication and the host immune response.

Keywords: *avian influenza virus; PA; NP; IFN-β; pathogenicity*

2.1. Introduction

Influenza A virus (IAV) has been continually circulating in aquatic birds, and its transmission to mammals has long been considered a worldwide public health threat (Webster et al., 1992b). Genetic rearrangement in IAVs has occasionally led to the establishment of novel lineages that are capable of causing pandemic influenza virus infections (Scholtissek et al., 1978). In addition to genetic rearrangements, single amino acid substitutions in viral genomes can lead to changes in host preference, replicative efficiency, and pathogenicity of IAVs (Connor et al., 1994, Subbarao et al., 1993).

The IAV genome is divided into eight segments, and the evolutionary pathway of each genome segment in terms of pathogenicity to mammals is different. Previously, I reported the existence of prototypic nonstructural (*NS*) and polymerase genes that are derived from nonpathogenic avian IAVs (AIVs), and next identified novel mutations in nonstructural protein 1 (NS1) and polymerase basic protein 2 (PB2), which are involved in the pathogenicity to mammals (Kim et al., 2015, Kim et al., 2014, Lee et al., 2017a). The mutations identified are shared by most AIVs and represent the minimum requirement for efficient replication in mammalian hosts (Kim et al., 2015, Kim et al., 2014, Lee et al., 2017a).

Polymerase acidic protein (PA) performs crucial functions in the life cycle and host

adaptation of IAVs (Hu and Liu, 2015). To date, various mutations in PA that increase the pathogenicity of AIVs toward mammals have been reported, and these mutations can be categorized according to their location in different functional motifs and domains (M21I, F35L, V44I, T97I, V127A, K142N, P224S, C241Y, L336M, A343T, K356R, S421I, T552S, I573V, and S616P,) (Song et al., 2009, Sun et al., 2014, Kim et al., 2010, Seyer et al., 2012, Mehle et al., 2012, Bussey et al., 2011, Xu et al., 2016a, Yamaji et al., 2015, Sakabe et al., 2011). PA can be cleaved into N-terminal (1-257, PA-N) and C-terminal domains (277-716, PA-C) by tryptic proteolysis (Hara et al., 2006). The cap-snatching endonuclease and protease are located in the PA-N domain (Yuan et al., 2009, Hara et al., 2006). The PA-N domain also contains an IRF3-binding site and two nuclear localization signals (NLS I and NLS II) composed of amino acid residues 124–139 and 186–247 (Yi et al., 2017, Nieto et al., 1994). The PA-C domain also contains a loop at amino acid positions 350–355 that can affect pathogenicity toward mammals, and two RNA polymerase II–binding sites, which are composed of K635 and R638 for site 1, and K289 and R454 for site 2 (Xu et al., 2016a, Lukarska et al., 2017). In addition, amino acid residues in the terminal regions of PA-C interact with the 15 amino acid residues at the N terminus of polymerase basic protein 1 (PB1) (He et al., 2008, Obayashi et al., 2008). Therefore, mutations arising in the vicinity of these functional motifs and domains may affect viral polymerase activity as well as the interaction with host factors.

In the present study, I investigated the effects of potential viral pathogenic PA mutations (T129I, G351E, M628V, and E684G) identified by comparing the nonpathogenic *PA* gene from A/chicken/Korea/KBNP-0028/2000 (H9N2) (0028) with that of other AIVs possessing mammalian pathogenicity (Kim et al., 2014). I generated PR8-derived recombinant viruses carrying these parent and mutated *0028 PA* genes with a single amino acid substitution. Of note, these recombinant viruses carried an additional single mutation in the *NP* gene. Among the different NP mutations, I selected a neutral mutation (E375G), which did not affect viral replication and pathogenicity to mice, and successfully generated recombinant viruses possessing single mutations in *0028-PA* in concert with the *E375G NP* mutation. By comparing polymerase activity, replication efficiency in mammalian hosts, and pathogenicity toward mice, I found that the E684G mutation in PA increases viral replication efficiency and pathogenicity in mice along with a significantly high induction of IFN-β expression.

2.2. Materials & Methods

Viruses, eggs, and cells

The *PA* gene from A/chicken/KBNP-0028/2000 (H9N2) (0028) and other genes from A/Puerto Rico/8/34 (H1N1) (PR8) were used to rescue the recombinant PR8 virus using a bidirectional reverse genetics system (Hoffmann et al., 2000). Rescued viruses were propagated three times in 10-day-old Specific Pathogen-Free (SPF) embryonated chicken eggs (ECEs) (Charles River, Wilmington, CA, USA). To estimate the virus titer, each virus was serially diluted from 10^{-1} to 10^{-9} in 10-fold increments, and each dilution was injected into five 10-day-old SPF ECEs and inoculated into MDCK cells. The 50% chicken embryo infectious dose (EID_{50}) and 50% tissue culture infectious dose ($TCID_{50}$) were calculated by the Spearman–Karber method. MDCK and 293T cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). MDCK and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (Life Technologies Co., Carlsbad, CA, USA).

Construction of reverse-genetics plasmids

The *0028 PA* gene was cloned into Hoffmann's bidirectional transcription vector pHW2000 (Hoffmann et al., 2000). The insert sequence was confirmed by sequencing with the

primers cmv-SF (5′-TAAGCAGAGCTCTCTGGCTA-3′) and bGH-SR (5′-TGGTGGCGTTTTTGGGGACA-3′). Site-directed mutagenesis involving specific amino acid (codon) substitutions in the *PA* genes from the 0028 viruses was achieved by means of the Muta-direct Site-Directed Mutagenesis Kit (iNtRON, Daejeon, Korea). The primers for the site-directed mutagenesis are listed in Table 2.1. PA or NP plasmids containing changes in the specific amino acids of interest, and seven genome segments of PR8, were transfected into 293T cells using Hoffmann's eight reverse-genetics plasmids, as previously described (Hoffmann et al., 2000, Lee et al., 2017a). All mutant viruses were confirmed by RT-PCR and sequencing.

Table 2.1. Primers used in this study

Primer name	Sequence (5' -3')	Usage
S66G-F	ATTGTAGAATCGGGCGATCCAAATGCATTA	Mutagenesis of 0028-PA
S66G-R	TAATGCATTTGGATCGCCCGATTCTACAAT	Mutagenesis of 0028-PA
M90V-F	CGATGGCCTGGACGGTGGTGAATAGTATATGC	Mutagenesis of 0028-PA
M90V-R	GCATATACTATTCACCACCGTCCAGGCCATCG	Mutagenesis of 0028-PA
T129I-F	GGAGAGAAGTTCACATCTACTATCTGGAAAAAGCC	Mutagenesis of 0028-PA
T129I-R	GGCTTTTTCCAGATAGTAGATGTGAACTTCTCTCC	Mutagenesis of 0028-PA
L275P-F	GATTACCTGATGGACCTCCCTGCTCCCAACGATC	Mutagenesis of 0028-PA
L275P-R	GATCGTTGGGAGCAGGGAGGTCCATCAGGTAATC	Mutagenesis of 0028-PA
G351E-F	CTTCAAGATATTGAAAATGAGGAGAAAATCCC	Mutagenesis of 0028-PA
G351E-R	GGGATTTTCTCCTCATTTTCAATATCTTGAAG	Mutagenesis of 0028-PA
S409N-F	GCAAGCTGGATCCAGAATGAATTCAACAAGGCG	Mutagenesis of 0028-PA
S409N-R	CGCCTTGTTGAATTCATTCTGGATCCAGCTTGC	Mutagenesis of 0028-PA

I432V-F	CGAAATTGGAGAGGACGTTGCTCCGATTGAGCAC	Mutagenesis of 0028-PA
I432V-R	GTGCTCAATCGGAGCAACGTCCTCTCCAATTTCG	Mutagenesis of 0028-PA
S553A-F	GCTCCTACGGACTGCTATAGGCCAGGTGTCAAGG	Mutagenesis of 0028-PA
S553A-R	CCTTGACACCTGGCCTATAGCAGTCCGTAGGAGC	Mutagenesis of 0028-PA
M628V-F	GAGTCACCCAAAGGAGTGGAAGAAGGTTCCATTGG	Mutagenesis of 0028-PA
M628V-R	CCAATGGAACCTTCTTCCACTCCTTTGGGTGACTC	Mutagenesis of 0028-PA
E684G-F	GGGACCTTCGATCTTGGAGGGCTATATGAAGC	Mutagenesis of 0028-PA
E684G-R	GCTTCATATAGCCCTCCAAGATCGAAGGTCCC	Mutagenesis of 0028-PA
K48R-F	CCAAATGTGCACCGAACTCAGACTCAGTGATTATG	Mutagenesis of PR8-NP
K48R-R	CATAATCACTGAGTCTGAGTTCGGTGCACATTTGG	Mutagenesis of PR8-NP
N101K-F	CCTATATACAGGAGAGTAAAAGGAAAGTGGATGAG	Mutagenesis of PR8-NP
N101K-F	CTCATCCACTTTCCTTTTACTCTCCTGTATATAGG	Mutagenesis of PR8-NP
G102R-F	CCTATATACAGGAGAGTAAACAGAAAGTGGATGAG	Mutagenesis of PR8-NP
G102R-R	CTCATCCACTTTCTGTTTACTCTCCTGTATATAGG	Mutagenesis of PR8-NP
E375G-F	GGAGACTATGGGATCAAGTACACTTGAACTGAG	Mutagenesis of PR8-NP
E375G-R	CTCAGTTCAAGTGTACTTGATCCCATAGTCTCC	Mutagenesis of PR8-NP

Mini-genome assay

To evaluate the polymerase activity of the PA mutants, I used a viral mini-genome assay based on luciferase activity, as described elsewhere (Lee et al., 2017a). Briefly, 293T cells in 12-well plates were cotransfected with 100 ng of pHW-NP-Luc and mutant 0028 PA and/or PR8 NP with *PR8 PB1*, *PB2* genes. Additionally, 100 ng of the *Renilla* luciferase plasmid pRL-TK (Promega, USA) was cotransfected, which served as an internal control to normalize variations in transfection efficiency and sample processing. After that, at 24 h after transfection, luminescence was assessed by means of a Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) on a TECAN Infinite200 pro machine according to the manufacturer's instructions (Tecan Benelux, Giessen, Netherlands). The results are reported as the average from triplicate experiments, and the standard deviation (SD) was calculated.

Viral growth kinetics

To measure these kinetics, MDCK cells were seeded in 12-well plates. After 24 h, the confluent cells were washed twice with phosphate-buffered saline (PBS). Next, mutant viruses at multiplicity of infection (MOI) of 1.0 were inoculated into each well with DMEM supplemented with 1% of bovine serum albumin (BSA, fraction V) (Roche, Basel, Switzerland), 20 mM HEPES,

an antibiotic-antimycotic solution (Life Technologies Co., Carlsbad, CA, USA), and 1 μg/mL TPCK-treated trypsin (Sigma-Aldrich, Saint Louis, MO, USA). Supernatants from the virus-infected cells were collected at 12, 24, 48, and 72 h postinoculation (hpi), and the viral titers were measured by $TCID_{50}$. The values are presented as the average of three independent experiments ± SD.

Mouse experiment

Six-week-old female BALB/c mice (KOATEC, Pyeongtaek, Korea) were used for the analysis of pathogenicity in mice. Five mice were anesthetized by intraperitoneal injection with 15 mg/kg Zoletil 50 (Virbac, Carros, France), after which the mice were inoculated intranasally with 10^6 EID_{50}/50 μL of one of the viruses, as previously described (Kim et al., 2014). Negative control (mock) mice were injected with the same volume of sterilized PBS. Mortality and weight loss were measured for 10 days. Mice that lost more than 25% of their original weight were euthanized and recorded as deaths. To measure virus replication in the lungs of infected mice, three mice from each group were injected with PBS (mock) or 10^6 EID_{50}/50 μL of a mutant virus. The lungs were collected at 3 days postinoculation (dpi) and then stored at −70°C until further use. The lungs were ground up using a TissueLyzer 2 (Qiagen, Valencia, CA, USA) with 5 mm stainless steel beads in a suspension with a volume of PBS equal to 10% of the lung weight. Next, 10 volumes of PBS were

mixed with the ground tissues. After centrifugation at 2000 × *g* for 10 min, the supernatants were subjected to determination of viral titers, which were measured via the $TCID_{50}$ method in MDCK cells. Lung supernatants at 3 dpi were used to measure the levels of interferon beta (IFN-β) by means of the VeriKine IFN-β ELISA kit (PBL Assay Science, Piscataway, NJ, USA). All mouse experiments were conducted at BioPOA Co. (Yongin, Korea) following a protocol that adhered to the National Institutes of Health's Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of BioPOA Co. (BP-2014-0004-2, BP-2016-006-2).

Frequency of specific amino acids in PA and NP between different subtypes of IAVs

To evaluate the frequency of specific amino acids in the PA and NP genes from different subtypes of IAVs from birds, pigs, and humans, full-length IAV ORF sequences from H1N1 (PA, n = 11,136; NP, n = 12,209), H3N2 (PA, n = 11,099; NP, n = 15,152), H5N1 (PA, n = 2,015; NP, n = 2,000), H7N9 (PA, n = 493; NP, n = 505), and H9N2 (PA, n = 1,256; NP, n = 1,313) were acquired from the NCBI influenza virus resource (http://www.fludb.org), and human H7N9 IAVs ORF sequences (PA, n = 884; NP, n = 626) were obtained from GISAID (http://platform.gisaid.org/). All the sequences were grouped by host species, and human H1N1 IAVs were subdivided into seasonal H1N1 and 2009 pandemic H1N1. The sequences were aligned in CLUSTALW, and the frequencies of specific amino acids was compared. The human H3N2 IAV dataset includes sequences from

1968 to 2017, while the human H7N9 IAV dataset includes sequences from February 2013 to June 2017.

2.3. Results

Generation of PR8-derived recombinant viruses with a single amino acid mutation in 0028-PA

Via three independent transfection experiments, five PR8-derived recombinant viruses carrying single amino acid substitutions in 0028-PA were generated (Table 2.2). When the viral genomes of the successfully rescued recombinant viruses were sequenced, a single missense mutation in the NP gene was unexpectedly found in each recombinant virus (PA(0028)/NP-G102R, PA(0028)-T129I/NP-E375G, PA(0028)-G351E/NP-E375G, PA(0028)-M628V/NP-K48R, and PA(0028)-E684G/NP-N101K). These NP mutations were located in the vicinity of the reported mutations related to both "MxA restriction escape" and viral fitness in the body domain (Figure 2.1) (Manz et al., 2013a, Gotz et al., 2016).

Table 2.2. Generation of PR8-derived recombinant virus containing mutated 0028-PA

Recombinant virus	Amino acid residues in PA			Virus rescue	Adaptive mutations in NP	EID50/ml (log_{10})
	Location	0028	01310/PR8[a]			
PA(0028)/NP-G102R	-	-	-	Yes	G102R	8.0 ± 0.30
PA(0028)-S66G	66	S	G	No	-	-
PA(0028)-M90V	90	M	V	No	-	-
PA(0028)-T129I/NP-E375G	129	T	I	Yes	E375G	8.7 ± 0.35
PA(0028)-L275P	275	L	P	No	-	
PA(0028)-G351E/NP-E375G	351	G	E	Yes	E375G	8.0 ± 0.23
PA(0028)-S409N	409	S	N	No	-	-
PA(0028)-I432V	432	I	V	No	-	-
PA(0028)-S553A	553	S	A	No	-	-
PA(0028)-M628V/NP-K48R	628	M	V	Yes	K48R	8.3 ± 0.72
PA(0028)-E684G/NP-N101K	684	E	G	Yes	N101K	8.8 ± 0.31

[a] [A/Chicken/Korea/01310/2001 (H9N2)] (01310)/ [A/Puerto Rico/8/1934] (PR8)

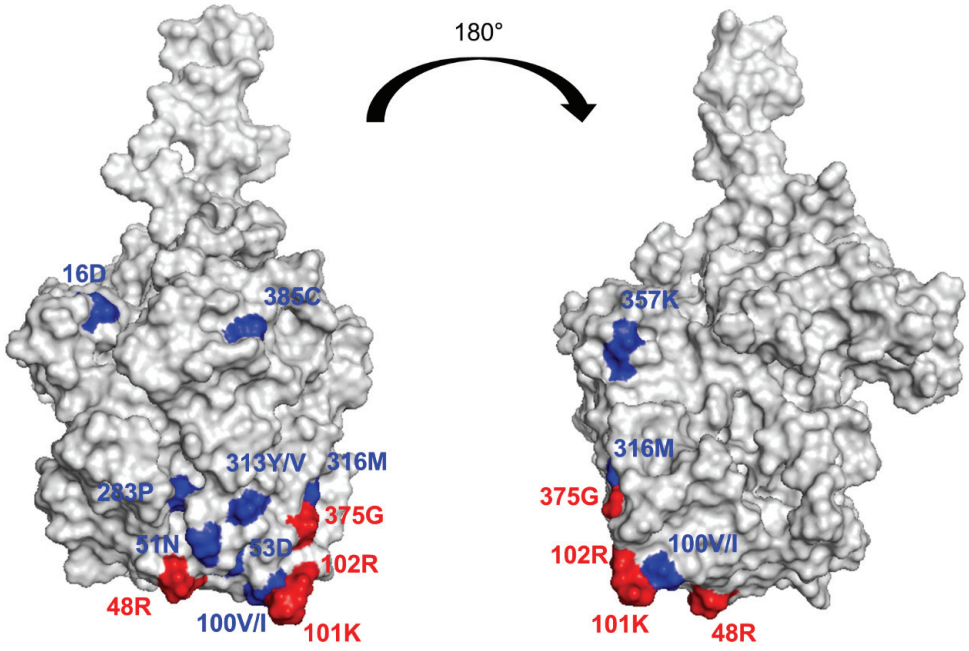

Figure 2.1. Structural model of the PR8 nucleoprotein.

The NP protein structure from PR8 was predicted by homology modeling using I-TASSER and analyzed using Pymol. The sites of the NP mutations generated in the PA recombinant viruses are shown in red, and the major amino acids related to MxA escape are shown in blue.

The effect of PA and NP mutations on polymerase activity

To evaluate the effect of the single amino acid mutations in 0028-PA on viral polymerase activity, a mini-genome assay was performed using *PB2*, *PB1*, and *NP* from PR8 and the mutated 0028-PA. The polymerase activity of each 0028-PA mutant was not significantly different from that of the parent 0028-PA strain when the *PR8 NP* gene was cotransfected (Figure 2.2). The single amino acid mutations (NP-K48R, NP-N101K, NP-G102R, and NP-E375G) in the *NP* gene did not affect *in vitro* polymerase activity in combination with the *PR8 PA* gene. Nonetheless, all combinations of mutated *0028-PA* genes in combination with the mutated *PR8 NP* genes showed lower polymerase activity than that of the PR8 NP gene (Figure 2.2).

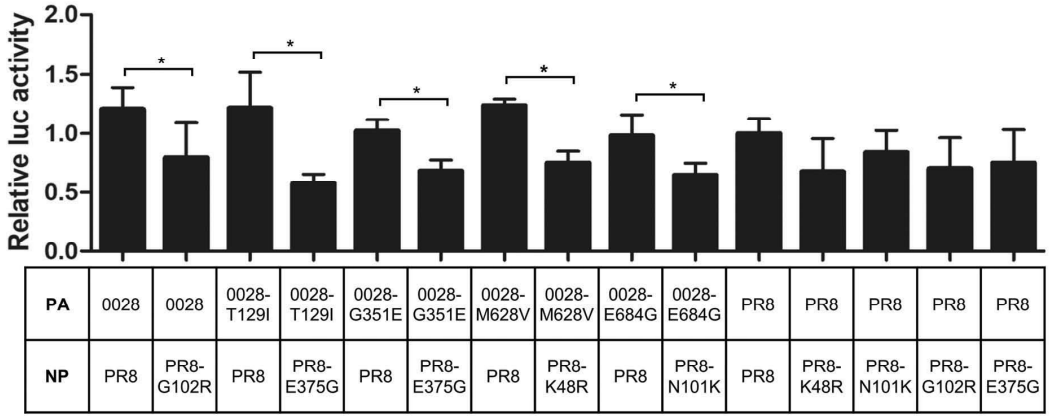

Figure 2.2. The effects of mutation in 0028-PA and PR8-NP on viral polymerase activity.

The effects of mutations in 0028-PA and PR8-NP on *in vitro* polymerase activity were measured in 293T cells at 37 °C. A total of 100 ng of PB2 and PB1 of PR8; 100 ng of NP from PR8 or mutated PR8 NP; 100 ng of PA from PR8 or mutated 0028 PA; 100 ng of pHW-NP-Luc; and 100 ng of pRL-TK were cotransfected into 293T cells. Luminescence was assessed by means of the Dual-Glo Luciferase Assay System. Statistical significance was analyzed by Student's *t* test ($^{*}P < 0.05$). The data are presented as the average of three independent experiments ± SD.

The effect of single mutations in PR8-NP on viral replication and pathogenicity in mice

To understand the role of the single mutations in PR8-NP on viral replication and pathogenicity, I generated mutated recombinant PR8 (rPR8) viruses with a single amino acid change in NP (rPR8-NP-K48R, rPR8-NP-N101K, rPR8-NP-G102R, and rPR8-NP-E375G), and confirmed that all the NP-mutated PR8 viruses did not have any adaptive mutations in their viral genomes. Among the mutated rPR8 viruses, rPR8-NP-K48R mutant had a lower replication efficiency compared to rPR8 at 72 hpi, and in addition, rPR8-NP-N101K had lower replication efficiency at both 48 and 72 hpi (Figure 2.3). Notably, rPR8-NP-G102R had significantly lower replication efficiency at 12, 24, 48, and 72 hpi in MDCK cells as compared to rPR8 (Figure 2.3). Nevertheless, all of the mutated rPR8 viruses led to severe weight loss, and resulted in 100% mortality (Figure 2.4).

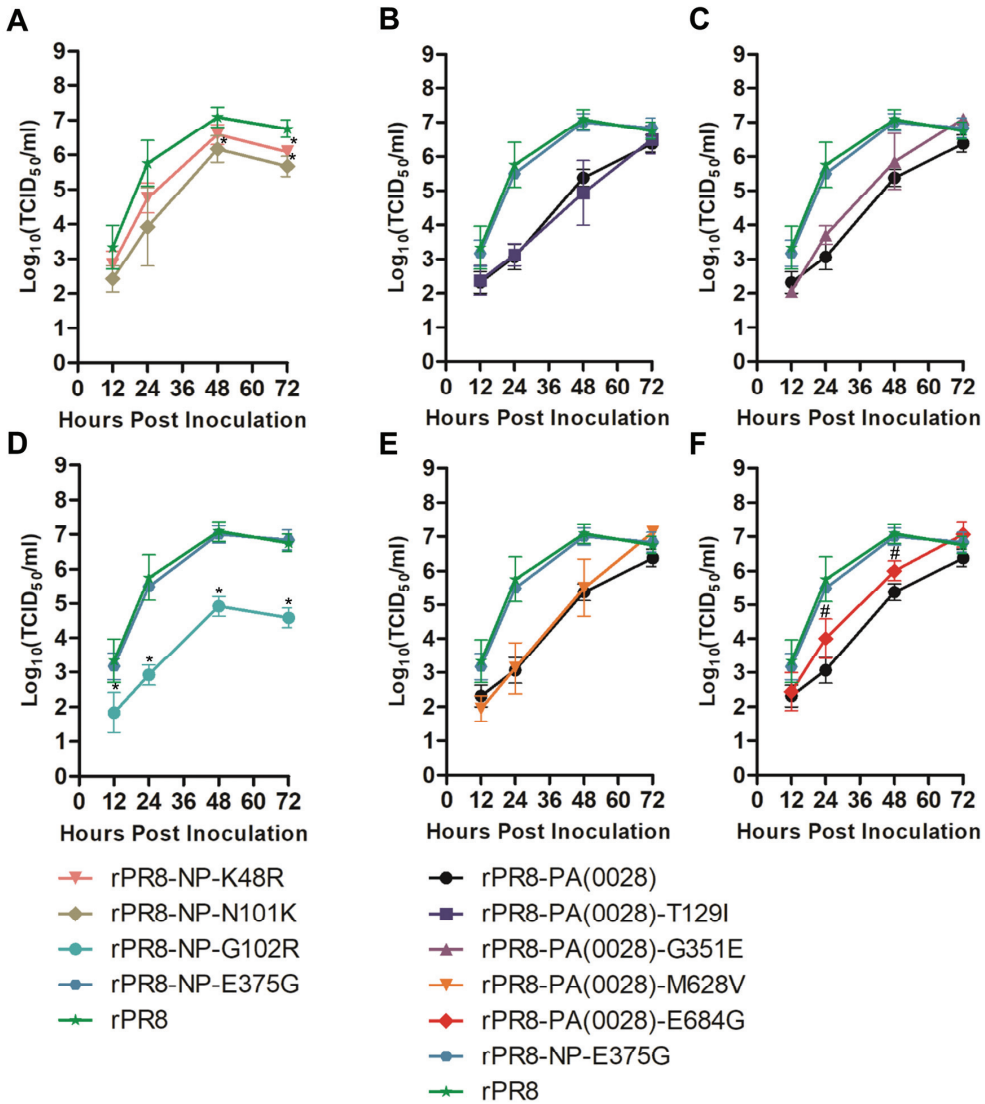

Figure 2.3. The effects of NP and PA mutations on viral replication in MDCK cells.

NP mutants and PA mutants were inoculated into MDCK cells at a MOI of 1.0. At 12, 24, 48, and 72 h postinoculation, the cell supernatants were collected, and viral titers were measured using $TCID_{50}$. Statistical significance was analyzed by Student's *t* test (compared to rPR8, $^{*}P < 0.05$; compared to rPR8-PA(0028), $^{\#}P < 0.05$). The data are presented as the

average of three independent experiments ± SD.

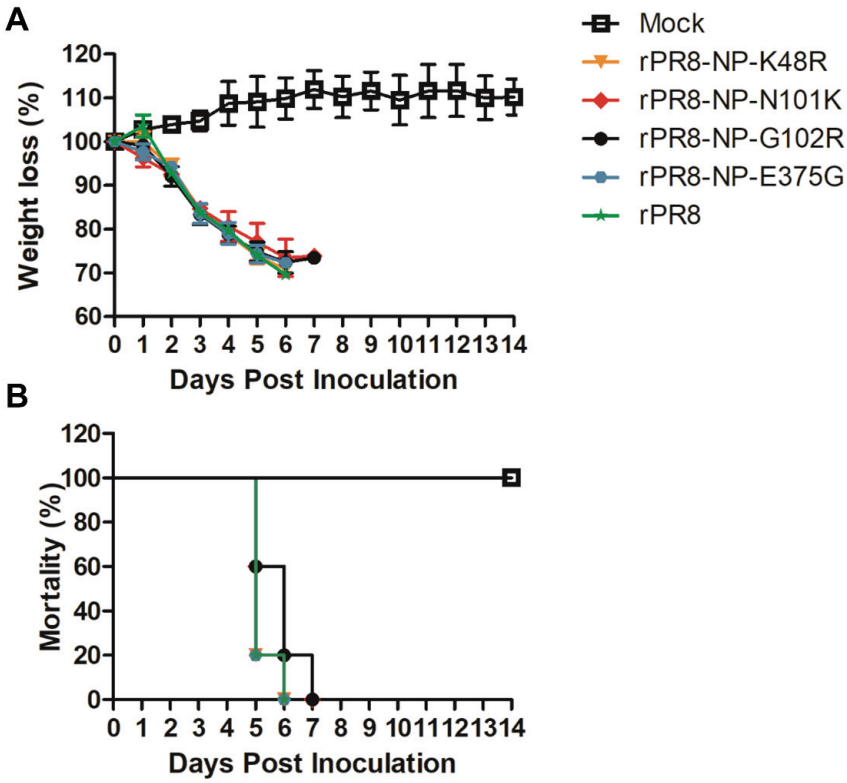

Figure 2.4. The effects of NP mutations on pathogenicity to mice.

Five 6-week-old female BALB/c mice were inoculated with 10^6 EID_{50} of the NP mutants. (A) Body weight loss and (B) mortality were observed at 14 days after infection.

Generation and characterization of new PR8-derived 0028-PA recombinant viruses with an E375G mutation in PR8-NP

To rule out the effect of different NP mutations, I generated new PR8-derived 0028-PA recombinant viruses also carrying the E375G mutation in the *NP* gene (rPR8-PA(0028), rPR8-PA(0028)-T129I, rPR8-PA(0028)-G351E, rPR8-PA(0028)-M628V, and rPR8-PA(0028)-E684G) (Table 2.2). Although residue 375 in NP has been implicated in the virulence of the 2009 pdmH1N1 virus, the E375G mutation was selected because it did not affect either viral replication or pathogenicity in mice (Figures 2.3 and 2.4) (Sakabe et al., 2011). None of the recombinant viruses had any additional mutations in their viral genomes. Among the mutant viruses generated, rPR8-PA(0028)-E684G had a significantly higher viral titer in ECEs and MDCK cells compared to rPR8-PA(0028) (Table 2.2). With respect to the growth kinetics of the mutant viruses in MDCK cells, the T129I, G351E, and M628V mutations did not increase viral replication, whereas rPR8-PA(0028)-E684G had significantly higher replication efficiency in MDCK cells compared to rPR8-PA(0028) at 24 and 48 hpi (Figure 2.3). I then compared the pathogenicity of these recombinant viruses in mice (Figure 2.5). In contrast to the mock (PBS) group, all mutant viruses induced body weight loss with different degrees of severity in mice. rPR8-PA(0028) and rPR8-PA(0028)-T129I led to slight body weight loss during the observation period, but these mutations did not induce mortality. rPR8-PA(0028)-G351E also led to body weight loss but with 20% mortality, whereas rPR8-PA(0028)-M628V

induced a significant weight loss in more than 80% of the mice, with 20% mortality. rPR8-PA(0028)-E684G also showed significantly greater weight loss than rPR8-PA(0028), and caused 100% mortality (Figure 2.5B). All the mutated 0028-PA recombinant viruses had significantly higher viral replication efficiency in the lungs of infected mice as compared to rPR8-PA(0028); in particular, rPR8-PA(0028)-E684G had the highest replication efficiency compared with that of all the other 0028-PA recombinant mutant viruses (Figure 2.5C). ELISA measurement of the levels of secreted IFN-β in the lungs of infected mice at 3 dpi revealed that rPR8-PA(0028)-E684G induced a higher level of IFN-β expression as compared to rPR8-PA(0028) (Figure 2.5D).

Table 2.2. Generation of PR8-derived recombinant virus containing a mutated 0028-PA with the E375G mutation in NP

Recombinant virus	Mutation in 0028-PA	Mutation in PR8-NP	EID_{50}/mL[a] (log_{10})	$TCID_{50}$/mL[b] (log_{10})
rPR8-PA(0028)	-	E375G	7.3 ± 0.12	5.25 ± 0.20
rPR8-PA(0028)-T129I	T129I	E375G	7.7 ± 0.12	5.75 ± 0.20
rPR8-PA(0028)-G351E	G351E	E375G	8.0 ± 0.20	5.83 ± 0.12*
rPR8-PA(0028)-M628V	M628V	E375G	7.6 ± 0.22	5.33 ± 0.12
rPR8-PA(0028)-E684G	E684G	E375G	8.4 ± 0.32*[c]	6.08 ± 0.12*

[a] 50% chicken embryo infectious dose

[b] 50% tissue culture infectious dose

[c] The data are presented as the average of three independent experiments ± s.d. Statistical significance was analyzed by Student's *t* test (compared to rPR8-PA(0028), $^{*}P < 0.05$).

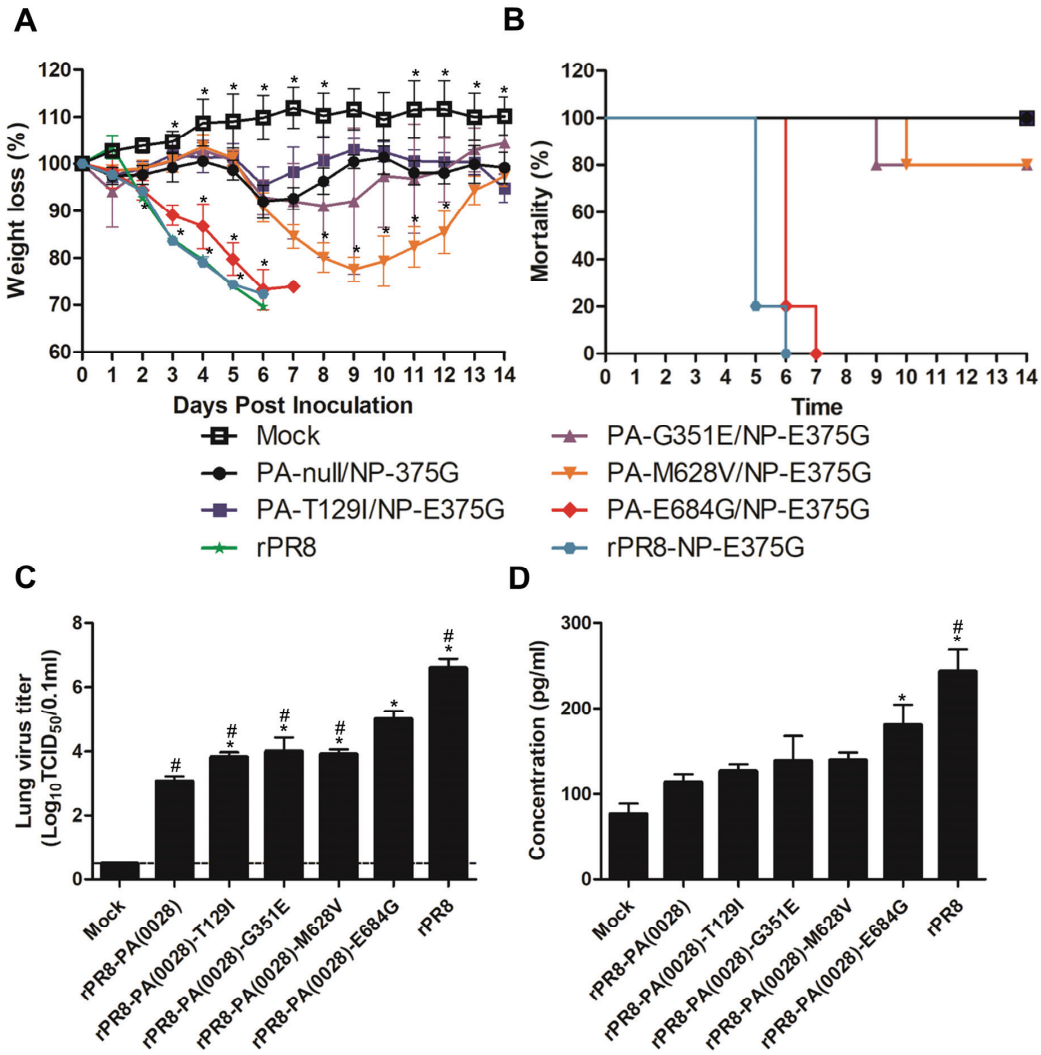

Figure2.5. The effect of PA mutations on pathogenicity to mice.

Five 6-week-old female BALB/c mice were inoculated with 10^6 EID_{50} of the PA mutants. (A) Body weight loss and (B) mortality were observed at 14 days after infection. (C, D) Three 6-week-old female BALB/c mice were inoculated with 10^6 EID_{50} of the PA mutants. At 3 days postinoculation, the mice were euthanized, (C) the viral titers in the lungs were measured by $TCID_{50}$, and (D) differences in IFN-β expression in the infected mouse lungs

were measured by an ELISA. Statistical significance was analyzed by one-way ANOVA (compared to rPR8-PA(0028), $^{*}P < 0.05$; compared to rPR8-PA(0028)-E684G, $^{\#}P < 0.05$). The data are presented as the average of three independent experiments ± SD.

The distribution and frequency of novel PA and NP mutations among IAVs from different host species

I analyzed the distribution and frequency of each mutation among the different subtypes of IAVs from different host species (Table 2.3). According to the data, 351E and 628V were shared by most of the IAVs compared in this study (not less than 92.8%). Nonetheless, the frequencies of 129I were relatively low in H5N1 (human strains: 38.33% and bird strains: 51.61%), and the frequencies of 684G were also relatively low in H1N1 (pig strains: 78.96%), H7N9 (human strains: 2.49% and bird: 15.42%) and H9N2 (human strains: 25.0% and bird strains: 44.11%). Moreover, most of the early human H3N2 viruses from 1968 did not have the E684G mutation, but instead these viruses acquired a glycine at position 684 during the adaptation period, which has been prevalent during the period from 1970 to 2017 (Figure 2.6A). In contrast, most of the human H7N9 viruses did not carry the E684G mutation (Figure 2.6B). In addition, the frequencies of 48R, 101K, 102R, and 375G in NP were rare in most IAVs, with the exception of 375G in human H3N2 IAVs (80.72% frequency; Table 2.4).

Table 2.3. The frequencies of PA pathogenic factors among the different host species H1N1, H3N2, H5N1, H7N9, and H9N2 isolates from all time

Frequency of	H1N1				H3N2			H5N1		H7N9		H9N2	
PA pathogenic	Human	Swine	2009 pH1N1	Bird	Human	Swine	Bird	Human	Bird	Human	Bird	Human	Bird
factors (%)	(n=1550)	(n=1787)	(n=7324)	(n=475)	(n=9175)	(n=1621)	(n=303)	(n=180)	(n=1,835)	(n=884)	(n=493)	(n=8)	(n=1,240)
129I	99.48	98.10	99.69	99.16	99.86	99.38	96.37	38.33	51.61	99.32	99.19	100	97.58
351E	99.74	99.50	99.75	99.79	99.74	99.32	99.67	98.89	98.58	99.66	100	100	92.82
628V	100	99.50	99.62	99.58	99.93	99.88	100	97.78	98.80	100.00	100	100	99.35
684G	96.45	78.96	99.41	99.79	98.20	93.58	97.03	93.89	97.38	2.49	15.42	25.00	44.11

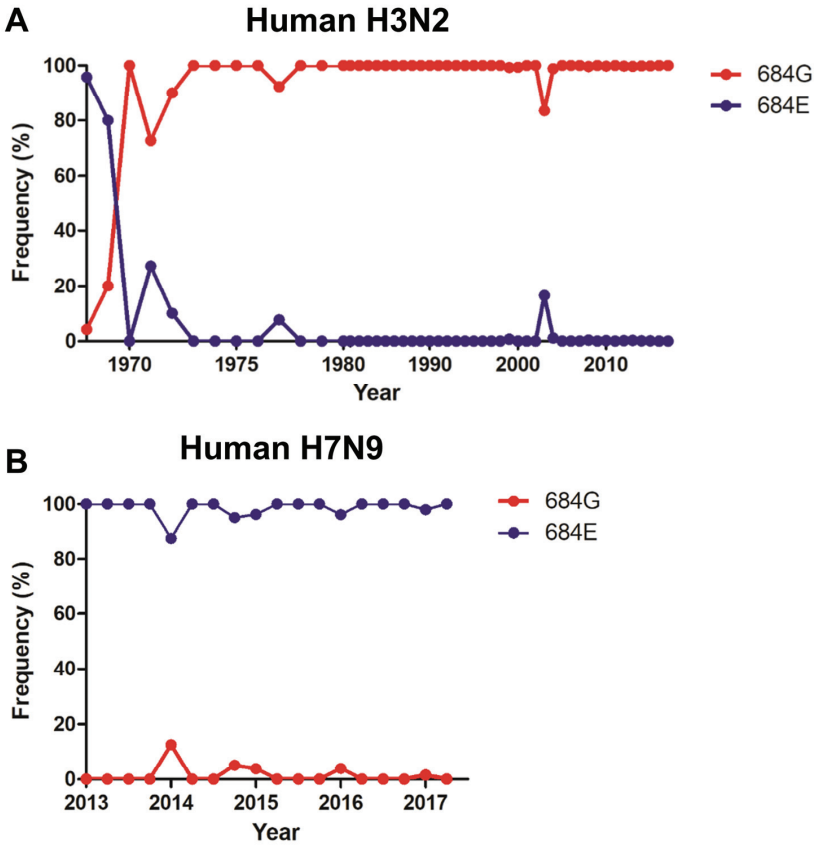

Figure 2.6. The contemporary frequencies of an amino acid at position 684 in human H3N2 and H7N9 PA genes.

(A) Data on 13,209 human H3N2 IAVs were retrieved from the NCBI Influenza virus resource (http://www.fludb.org) and arranged by isolation date from 1968 to 2017. (B) A total of 884 human H7N9 IAVs were acquired from GISAID (http://platform.gisaid.org/) and arranged by isolation date from February 2013 to June 2017. The graph of the rates of 684G and 684E was created in the GraphPad Prism software, version

Table 2.4. The frequencies of NP pathogenic factors among different host species H1N1, H3N2, H5N1, H7N9, and H9N2 isolates

Frequency of amino acid residue (%)	H1N1				H3N2			H5N1		H7N9		H9N2	
	Human (n=1,573)	Swine (n=2,080)	2009 pH1N1 (n=8,086)	Bird (n=470)	Human (n=13,209)	Swine (n=1,650)	Bird (n=293)	Human (n=194)	Bird (n=1,806)	Human (n=626)	Bird (n=505)	Human (n=11)	Bird (n=1,302)
48R	0.00	0.34	0.02	0.85	0.01	0.06	0.00	0.00	0.06	0.00	0.00	0.00	0.00
101K	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
102R	0.95	0.24	0.30	0.21	0.10	0.06	0.00	0.00	0.06	0.00	0.00	0.00	0.00
375G	1.91	0.34	0.04	0.00	80.72	2.00	0.34	0.52	0.17	0.16	0.20	0.00	0.15

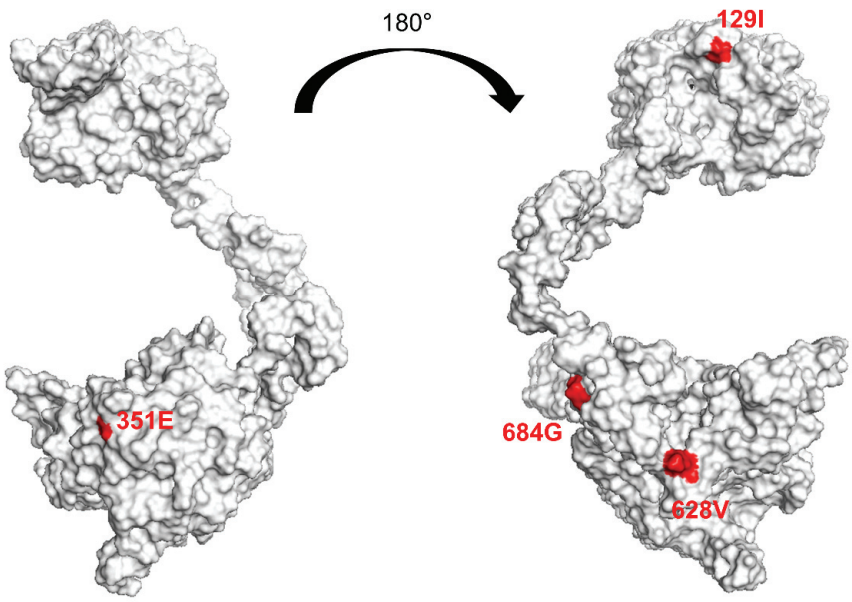

Figure 2.7. Structural model of 0028 PA.

The PA protein structure of 0028 was predicted by homology modeling using I-TASSER and analyzed using Pymol. The sites with PA mutations are shown in red.

2.4. Discussion

The IAV polymerase is a hetero-trimer consisting of subunits PB2, PB1, and PA, all of which interact with not only viral genomes and the NP protein but also with host factors, and IAVs have been evolving to overcome host barriers and to achieve high viral fitness during the host adaptation period (Gabriel et al., 2005b, Graef et al., 2010, Iwai et al., 2010). In this study, I introduced the avian *0028-PA* gene into the human IAV, and the adverse effects of this non-cognate PA gene were examined. The effects of the additional NP mutations on polymerase activity were indistinguishable when these mutations were present in combination with the *PR8 PA* gene. Nevertheless, the NP mutations in *0028-PA* clearly attenuated polymerase activity. Moreover, the K48R, N101K, and G102R mutations decreased viral replication in MDCK cells. The NP protein is an important viral determinant of Mx1 sensitivity, and adaptive mutations in NP have been proposed as one of the key mechanisms by which nonhuman IAVs have adapted to human hosts (Manz et al., 2013a, Gotz et al., 2016). Three residues, namely, 100V, 283P, and 313Y, in the 1918 pandemic H1N1 virus or 53D, 100V and 313V in the 2009 pandemic H1N1, which are all in the body domain of NP, promote “MxA restriction escape” of the IAVs, but they reduce viral fitness by disturbing the intracellular trafficking of the incoming viral ribonucleoprotein complexes (vRNP) (Gotz et al., 2016, Manz et al., 2013a). Given that all the additional mutations in NP

in this study were clustered into the same body domain, the additional mutations might be the results of successful adaptation of the recombinant viruses to the hosts employed in this study (Figure 2.7). In addition, the cognate PA and NP proteins are important for stabilizing the CPSF30-NS1A complex, which is important for suppressing the host IFN antiviral response (Kuo and Krug, 2009). Thus, the incompatibility of the avian PA within the PR8 backbone might have led to adaptive mutations in the *NP* gene to allow for the successful replication of the PA-mutant viruses (Liedmann et al., 2014).

I successfully generated PA-mutated recombinant viruses without any additional mutations using the NP-E375G gene. Among them, rPR8-PA(0028)-E684G had higher viral replication efficiency in ECEs and MDCK cells. In a mouse infection experiment, rPR8-PA(0028)-E684G virus led to severe clinical symptoms and resulted in 100% mortality among the infected mice. Moreover, it induced a higher level of IFN-β expression in the lungs of infected mice. In humans and mice infected with IAVs, there is a close correlation between IFN levels and the virus titer in lungs (McLaren and Potter, 1973, Green et al., 1982). That is, the higher replication capacity of rPR8-PA(0028)-E684G than that of rPR8-PA(0028) caused the higher IFN-β expression (Pearson correlation coefficient of 0.9268; $P < 0.001$). The E684G mutation is located at the end of the PA-C domain, and its participation in viral pathogenicity is not well understood. The C-terminal amino acid residues in the PA domain interact directly with the N-terminal 15 amino acids of PB1, but residue 684 is

located away from the PB1-N terminal interaction site (He et al., 2008, Obayashi et al., 2008). Liedmann et al. have demonstrated that mutations P275L, E351K, and D682N in PR8 PA reduce pathogenicity to mice, likely by decreasing the IFN antagonistic properties in combination with PB1 (Liedmann et al., 2014). Although the exact function of E684G is not clear at present, the fact that the E684G mutation increases viral replication efficiency and pathogenicity in mice may encourage further functional studies.

Mutations I129T, G351E, and M628V in 0028-PA slightly increased the rate of viral replication in the infected murine lungs, with G351E and M628V leading to weight loss and a 20% mortality rate in infected mice. Given the positional relation among the 129I, 351E, 628V, and 684G residues in the 0028-PA protein structure, each mutation may affect different functions (Fig. S2). Currently, however, the precise role of each mutation in the *PA* gene is unclear, but residue 129 is located in NLS I (124-139) and the V127A mutation has been identified as an important contributor to the replication efficiency of the avian H5N1 virus in A549 cells (Yamaji et al., 2015). The amino acid residues in the 350–355 loop confer mammalian tropism and pathogenicity toward mammals. A single K351E mutation has been shown to inhibit the induction of IFN-β and increase pathogenicity in mice (Liedmann et al., 2014). In addition, an H9N2 virus carrying the K356R mutation showed increased nuclear accumulation of PA and polymerase activity, resulting in high virus titers and high pathogenicity to mice (Xu et al., 2016a). Therefore, the G351E mutation, also located in the

350–355 loop, may too contribute to pathogenicity and effective replication in the lungs of mice. Recently, the structure of the binding sites (sites I and II) of PA bound to the C-terminal domain (CTD) of cellular RNA polymerase II has been revealed, showing that K630 and R633 in site I interact with a negatively charged phosphoserine in the CTD peptide (Lukarska et al., 2017). Therefore, further studies examining the effect of the M628V mutation on the binding of PA to RNA pol II may be worthwhile.

The high frequencies of 351E and 628V among the various subtypes of avian and mammalian IAVs suggest that these mutations may be important for the replication of IAVs in both avian and mammalian hosts. By contrast, only 38.33% of H5N1 human isolates possess the 129I residue. With respect to the frequency of the 684G residue, only 2.49% and 25% of H7N9 and H9N2 human isolates carry this residue, respectively. Considering that no sustained human-to-human transmission of H5N1 and H7N9 viruses has occurred until now, the acquisition of all four residues in PA may allow for sustainable transmission of viruses of avian origin to humans. Therefore, further research into the effect of the four PA mutations on viral replication and the pathogenicity of H5N1 and H7N9 viruses will be warranted. Meanwhile, it is noteworthy that the second wave of the 1968 H3N2 Hong Kong pandemic, that occurred from 1969 to 1970, showed higher morbidity and mortality than the first wave did (1968–1969) in spite of their identical origin (Jackson et al., 2010, Webster et al., 1992b). In agreement with its outbreak pattern, H3N2 human IAVs acquired the 684G mutation in 2

years since these strains first spread to humans in 1968 and have remained stable to the present day. The effect of the *PA* gene alone should not be overestimated, and it can be misleading because of the number of sequences per year. Nevertheless, further research into the effects of different PA mutations, including E684G, on the high transmissibility of the second wave may be valuable.

In conclusion, the introduction of an avian *PA* gene into the PR8 virus can induce adaptive mutations in the *NP* gene, probably owing to genetic incompatibility. This link between *PA* and *NP* may imply that cooperation between PA and NP is necessary for the adaptation of AIVs to mammals. Moreover, the novel mutations in PA that are pathogenic to mice may advance the understanding of evolutionary pathways in AIVs required for mammalian adaptation.

Chapter Ⅲ

Effect of the fourth nucleotide at the 3′ end of NA and M viral genomic RNA on the pathogenicity of influenza virus A/PR/8/34

Abstract

Twelve nucleotides located at the 3′ end of viral genomic RNA (vRNA) are conserved among influenza A viruses (IAV) and have a promoter function. Hoffmann's 8-plasmid reverse genetics vector system introduced mutations at position 4, C4 to U4, of the 3′ ends of neuraminidase (NA) and matrix (M) vRNAs of wild-type A/PR/8/34. This resulted in a constellation of C4 and U4 vRNAs coding for low (polymerases) and relatively high (all others) copy number proteins, respectively. U4 has been reported to increase promoter activity in comparison to C4, but the constellation effect on the replication efficiency and pathogenicity of reverse genetics PR8 (rgPR8) has not been fully elucidated. In the present study, I generated 3 recombinant viruses with C4 in the NA and/or M vRNAs and rgPR8 by using reverse genetics and compared their pathobiological traits. The mutant viruses showed lower replication efficiency than rgPR8 due to the low transcription levels of NA and/or M genes. Furthermore, C4 in the NA and/or M vRNAs induced lower PR8 virus pathogenicity in BALB/c mice. The results suggest that the constellation of C4 and U4 among vRNAs may be one of the multigenic determinants of IAV pathogenicity.

Keywords: *influenza virus, pathogenicity, promoter, replication efficiency, reverse genetics*

3.1. Introduction

The noncoding regions (NCRs) of the 3′ and 5′ ends of viral RNA (vRNA) of influenza A virus (IAV) form a 'corkscrew'-like structure and function as promoters for the transcription of messenger (mRNA), complementary RNA, and viral genomic RNA (vRNA) (McCauley and Mahy, 1983, Desselberger et al., 1980, Flick and Hobom, 1999). The promoter function is reported to be localized to 12 conserved nucleotides at the 3′end of vRNA, and nucleotides 9–11 were shown to be crucial for promoter activity (Seong and Brownlee, 1992). Mutations at positions 11 and 12 of the 3′ and 5′ ends of neuraminidase (NA) vRNA of influenza A/WSN/33 reduced the NA mRNA and protein levels, as well as the virus titer, and resulted in attenuated phenotypes in mice (Solorzano et al., 2000, Fodor et al., 1998). Therefore, the NCRs of the 3′ and 5′ ends of vRNA may have an important role in viral pathogenicity as well as viral gene expression.

The nucleotide at position 4 of the 3′ end of NA vRNA has been shown to affect the transcription of vRNA, and a U nucleotide at position 4 (U4) increased the transcription level of NA vRNA above that from a C nucleotide (C4) (Lee and Seong, 1998). Moreover, a U4 to C4 mutation affects viral transcription and replication activity by down-regulating polymerase recognition activity (Jiang et al., 2010). However, various mutant influenza A/PR/8/34 (PR8) viruses possessing different combinations of C4 and U4 did not produce different viral titers

(de Wit et al., 2004). During adaptation in embryonated chicken eggs (ECEs), wild-type (wt) PR8 acquired multiple mutations in coding genes related to increased viral replication efficiency, and the genome segments of a high yield PR8 strain (rgPR8) was used for generation of recombinant vaccine strains by using reverse genetics. During analysis of the sequences of NCRs, I observed different combinations of U4 and C4 in NCRs of wtPR8 and rgPR8. The polymerases (PB1, PB2, and PA), NA, and matrix (M) vRNAs of wtPR8 possessed C4 rather than U4, but those of rgPR8 acquired U4 mutations in NCRs of the NA and M vRNAs (Hoffmann et al., 2002, Hoffmann et al., 2001, de Wit et al., 2004). Although PR8 virus has been widely used to inactivated vaccine backbone strain, the effect of U4 in NCRs of the NA and M vRNAs of rgPR8 have not been fully elucidated (Ping et al., 2015, Hoffmann et al., 2002).

In the present study, I generated 4 recombinant viruses with C4 in the NA and/or M vRNAs and a rgPR8-like constellation of C4 and U4 by undertaking reverse genetics. I then compared their replication efficiency in ECEs, transcription levels of vRNA and mRNA of the NA and/or M genome segments in MDCK cells, and their pathogenicity in mice. Our results indicate that C4 in both the NA and M vRNAs decreased virus replication efficiency in ECEs and pathogenicity in mice. Thus, the position 4 nucleotide in the 3′ end and the constellation of C4 and U4 among vRNAs may be multigenic determinants of pathogenicity. Studies to reveal the profiles of the promoters of IAVs may be valuable in the prediction of potential

pathogenicity.

3.2. Materials and Methods

Viruses, eggs, cells, and plasmids

The rgPR8 virus was generated by using Hoffmann's 8-plasmid reverse genetics vector system and was passaged three times in 10-day-old SPF ECEs (VALO BioMedia, USA) before use. All of the influenza viruses were inoculated in 10-day-old ECEs via the allantoic cavity route, and the eggs were then incubated for 36 to 72 h. After chilling at 4°C overnight, the allantoic fluid was harvested and stored at −70°C until further use. The 293T and MDCK cells were purchased from the American Type Culture Collection (USA) and maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, USA) supplemented with 5% fetal bovine serum (Invitrogen, USA). The 293T cells were used to generate recombinant viruses through the reverse genetic process.

Site-directed mutagenesis

The bidirectional transcription vector, pHW2000, and 8 plasmid vectors with 8 genome segments of PR8 were obtained by following the process described by Hoffmann *et al*. [8]. To understand the effects of C4 in the M and NA vRNAs, I mutated nucleotide sequence T4 of the pHW197-M and pHW196-NA plasmids into C4 by applying site-directed mutagenesis (iNtRON Biotechnology, Korea). The mutated plasmids were named pHW197-M-C4 and pHW196-NA-C4. The mutagenesis primer sets are listed in Table 3.1, and the

constellations of C4 and U4 among the viral genomes of each virus are presented in Table 3.2.

Table 3.1. Primers used in this study

Primer name	Sequence (5' -3′)	Usage
PR8-NAprom-mg-F	TCCGAAGTTGGGGGGGAGCGAAAGCAGGAGTTTAAAATGA	Mutagenesis
PR8-NAprom-mg-R	TCGCTCCCCCCCAACTTCGGA	for NA vRNA-C4
PR8-Mprom-mg-F	TATTCGTCTCAGGGAGCGAAAGCAGGTAGATATTGAAAGATGAG	Mutagenesis
PR8-Mprom-mg-R	TCGCTCCCTGAGACGAATA	for M vRNA-C4
vRNAtag	GGCCGTCATGGTGGCGAAT	Measurement
vRNAtag_PR8seg6_693F	GGCCGTCATGGTGGCGAAT ACTATAATGACTGATGGCCCGAGT	of NA vRNA level
PR8seg6_843R	ACATCACTTTGCCGGTATCAGGGT	
mRNAtag	CCAGATCGTTCGACTCGT	Measurement
mRNAtag-PR8Seg6-dTR	CCAGATCGTTCGAGTCGT TTTTTTTTTTTTTTT TGA ACA AAC TAC	of NA mRNA level
PR8seg6-1318F	TGAATAGTGATACTGTAGATTGGTCT	
vRNAtag	GGCCGTCATGGTGGCGAAT	Measurement
vRNAtag_PR8seg7_511F	GGCCGTCATGGTGGCGAAT TAGGCAAATGGTGACAACAACCAA	of M vRNA level
PR8seg7_624R	GCTGCTTGCTCACTCGATCCAG	
mRNAtag	CCAGATCGTTCGACTCGT	Measurement
mRNAtag_PR8seg7_dTR	CCAGATCGTTCGAGTCGT TTTTTTTTTTTTTTTTAGTAGAAACAA	of M mRNA level
PR8seg7_936F	GAATATCGAAAGGAACAGCAGA	

Table 3.2. Nucleotide sequence of the 3′-noncoding region (NCR) of wild-type (wt) PR8, reverse genetics (rg) PR8, and the recombinant PR8 viruses generated in the present study.

	wtPR8	rgPR8	rPR8-NA-prom	rPR8-M-prom	rPR8-MN-prom
PB1	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu
PB2	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu
PA	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu	3′-ucgCuuu
HA	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu
NP	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu
NA	3′-ucgCuuu	3′-ucgUuuu	3′-ucgCuuu	3′-ucgUuuu	3′-ucgCuuu
M	3′-ucgCuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgCuuu	3′-ucgCuuu
NS	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu	3′-ucgUuuu

Recombinant virus generation by reverse genetics

The rgPR8 strain was generated by transfection using Hoffmann's 8 reverse genetics plasmids as previously described (Hoffmann et al., 2002), with some modifications. The recombinant viruses that possessed C4 in NA, M and both vRNAs were generated by replacing pHW196-NA and/or pHW197-M with pHW196-NA-C4 and/or pHW197-M-C4 and were named rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8), respectively. Briefly, 293T cells were cultured (1×10^6 cells/well in 6-well plates) and transfected with 300 ng of each plasmid by using lipofectamine 2000 and plus reagents (Invitrogen, USA) in a final volume of 1 mL of Opti-MEM (Invitrogen, USA). After overnight incubation, 1 mL of fresh medium and 0.5 mg/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA) were added. After 24 h, the culture medium was harvested and 200 μL were injected into 10-day-old SPF ECEs via the allantoic cavity route. After incubating for 2–3 days, the allantoic fluid was harvested and tested with an HA test using 1% (v/v) chicken red blood cells according to the WHO Manual on Animal Influenza Diagnosis and Surveillance. All experiments were performed after obtaining permission from the Seoul National University Institutional Biosafety Committee (SNUIBC) (approval number: SNUIBC-R150729-1).

Viral growth kinetics

Each recombinant virus (10 EID_{50}/200 μL/ECE) was inoculated into eighteen 10-day-

old SPF ECEs, and 3 ECEs were harvested at 8, 12, 16, 24, 32, and 48 h postinoculation. To measure the virus titer at each time interval, the pooled sample was serially diluted 10-fold from 10^{-1} to 10^{-9}, and each dilution was inoculated into MDCK cells. The 50% tissue culture infection dose ($TCID_{50}$/mL) was calculated more than three times by using the Spearman-Karber method (Hamilton et al., 1977).

vRNA and mRNA quantification

To determine the effects of the introduced mutations, the transcription levels of vRNA and mRNA of the M and NA genome segments were measured by performing two-step real-time RT-PCR as previously described (Kawakami et al., 2011, Hu et al., 2016), with some modifications. Briefly, the recombinant viruses were infected to confluent MDCK cells in a 12-well plate at 0.001 multiplicity of infection (MOI) for 1 h and washed twice with PBS. Cells were cultured in maintenance medium [DMEM supplemented with 1% bovine serum albumin (fraction V) (Roche, Switzerland), 20 mM HEPES, antibiotic-antimycotic (Gibco, USA) and 1 μg/mL of TPCK-treated trypsin (Sigma-Aldrich, USA)] at 37°C under humidified 5% CO_2. Cells were harvested after incubation for 6 h, and total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Germany). For the cDNA synthesis, an AmfiRivert cDNA Synthesis Platinum kit (GenDEPOT, USA) was used with the tagged primers (Table 1). Real-time PCR was conducted with SYBR GreenER qPCR SuperMix (Invitrogen, USA) and the specific primer sets (Table 1) by using an ABI StepOne Real-time

PCR machine (Applied Biosystems, USA). Transcription levels were normalized by using transcription levels of cellular GAPDH genes in the infected cells as an internal control. The relative transcription levels of vRNA and mRNA of each recombinant virus were represented by the ratio to those of rgPR8. Three independent experiments were performed.

Pathogenicity in BALB/c mice

Five-week-old female BALB/c mice were purchased from Narabiotech (Korea), and the mouse pathogenicity test was conducted by BioPOA (Korea). The mortality and weight loss of BALB/c mice were measured after intranasal inoculation of rPR8, rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8) as previously described (Kim et al., 2015), with some modifications. Briefly, each recombinant virus was diluted to 10^5 and 10^4 EID_{50}/50 μL, and 5 mice were assigned to receive one dilution of the virus. Mice were anesthetized with Zoletil (15 mg/kg; Virbac, France) and mortality and weight loss were observed every day for 12 days. When the body weight of a mouse had decreased by more than 20%, the mouse was considered moribund and was killed by CO_2 asphyxiation. All procedures performed in studies involving animals were approved by the Institute of Animal Care and Use Committee at BioPOA, Koreas (approval number: BP-2017-001-2). The mouse experiments were carried out in accordance with the protocol of the National Institutes of Health's Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Statistical analyses

The significance of body weight and virus titer changes was evaluated by using one-way analysis-of-variance (ANOVA). The mortality differences observed in pathogenicity testing were assessed by using the Kaplan-Meier method (log-rank test, 95% confidence intervals).

3.3. Results

Growth kinetics of recombinant viruses in ECEs

I generated four recombinant PR8 viruses: a rgPR8-like constellation of C4 and U4 (rgPR8), C4 in NA vRNA (rPR8-NA-prom), C4 in M vRNA (rPR8-M-prom), and a wtPR8-like constellation of C4 and U4 (rPR8-MN-prom) (Table 3.2), and compared their replication efficiency in ECEs. The rgPR8 virus could replicate in ECEs at 8 h postinoculation, and its virus titers were significantly higher than those of recombinant viruses with C4 instead of U4 in the M and/or NA vRNAs at all of the time intervals that were compared ($p < 0.5$) (Figure 3.1).

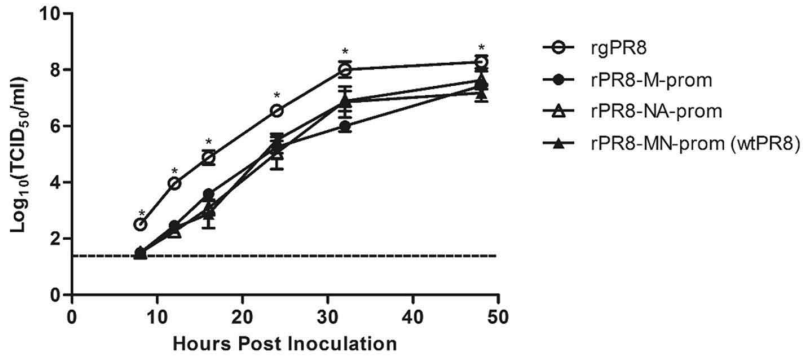

Figure 3.1. Comparison of virus titers of recombinant viruses with different constellation of C4 and U4 in the 3′-end of the noncoding region of the viral genome.

Each recombinant virus (10 EID_{50}/200 uL/ECE) was inoculated into eighteen 10-day-old SPF ECEs, and 3 ECEs were harvested at 8, 12, 16, 24, 32, and 48 h postinoculation. The virus titers were measured by $TCID_{50}$ assay in MDCK cells * represents a significant difference of virus titers between the rgPR8 and the other recombinant viruses ($p < 0.05$).

Comparison of relative vRNA and mRNA transcription levels of recombinant viruses

In order to verify the effects of the U4 to C4 substitutions on viral genome transcriptions, I compared relative vRNA and mRNA transcription levels of the NA and M genome segments of recombinant viruses by using tagged-primer two-step real-time RT-PCR (Figure 3.2). The U4 to C4 substitutions in NA vRNA decreased both vRNA and mRNA transcriptions of the NA genome segment by more than 50%. Similar to the NA results, C4 in M vRNA reduced the vRNA and mRNA transcriptions of the M genome segment. The wtPR8 (rPR8-MN-prom) showed downregulated transcription of both M and NA viral genome segments.

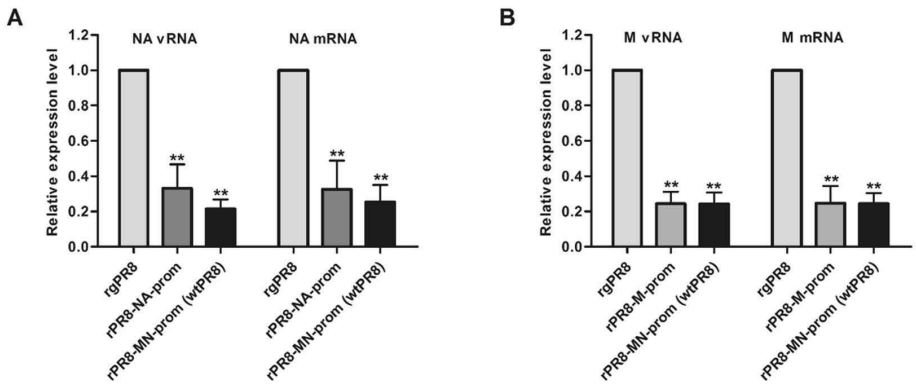

Figure 3.2. Relative transcription levels of vRNA and mRNA of recombinant viruses.

(A) Relative transcription levels of vRNA and mRNA of the NA genome segments. (B) Relative transcription levels of vRNA and mRNA of the M genome segments. MDCK cells were infected by recombinant viruses at 0.001 MOI at 37°C, and cell lysates were harvested at 6 h postinfection. The vRNA and mRNA transcription levels were normalized by the transcription levels of GAPDH gene of the infected cells. The relative transcription levels of vRNA and mRNA of each recombinant virus were represented by the ratio to those of rgPR8. The data presented are the average of three independent experiments. ** represents a significant difference between the rgPR8 and other recombinant viruses ($p < 0.001$).

Comparison of pathogenicity of recombinant viruses in mice

Because the U4 to C4 substitutions affect the viral replication efficiency, I compared the pathogenicity of recombinant viruses with that of rgPR8 in BALB/c mice. The 10^5 EID_{50} inoculation of rPR8-NA-prom, rPR8-M-prom, and wtPR8 (rPR8-MN-prom) led to weight loss less than that of rgPR8, and caused less mortality and a greater mean time to death in mice (Figure 3.3). Furthermore, the inoculation of 10^4 EID_{50} recombinant viruses did not cause any death in contrast to that of 10^4 EID_{50} rgPR8 (Figure 3.3).

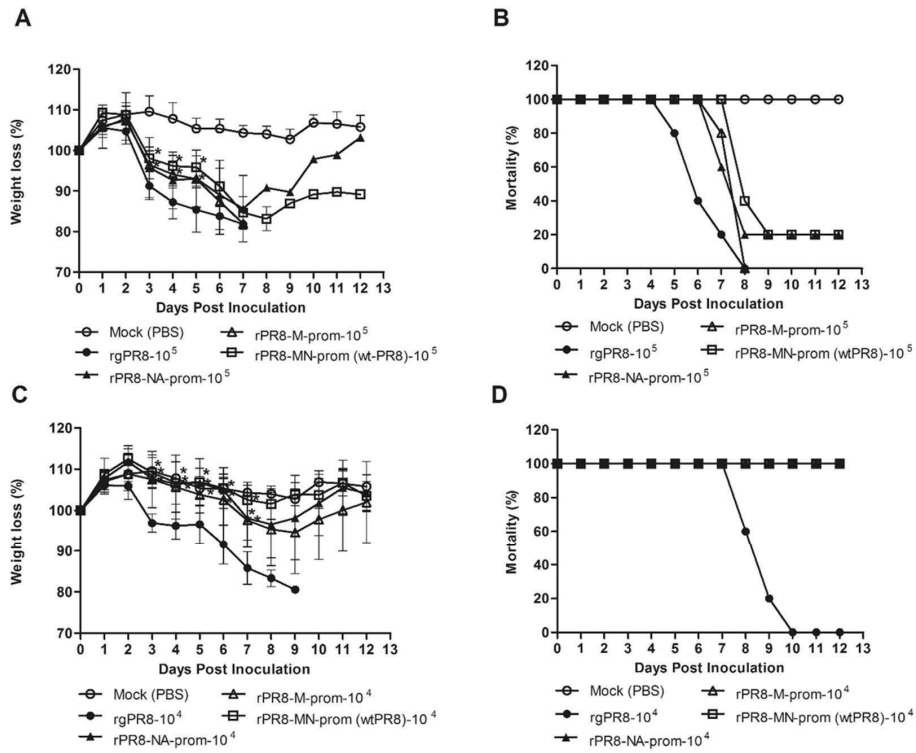

Figure 3.3. Comparison of mouse pathogenicity of recombinant viruses.

The 10^5 and 10^4 EID_{50}/50 µL/mouse recombinant viruses were challenged to five 5-week-old BALB/c mice. Weight loss and mortality were monitored for 12 days. (A) Weight loss and (B) mortality of mice infected by 10^5 EID_{50} of each virus. (C) Weight loss and (D) mortality of mice infected by 10^4 EID_{50} of each virus. * indicates weight loss of recombinant viruses is significantly different from that of rgPR8 ($p < 0.05$).

3.4. Discussion

The conserved, segment-specific NCRs of the 3′ and 5′ ends of the vRNAs of IAVs are related to viral RNA transcription and affect virus replication and pathogenicity (Seong and Brownlee, 1992, Lee and Seong, 1998, Solorzano et al., 2000, Wang and Lee, 2009). The C4 to U4 substitution has been shown to increase the transcription of NA vRNA of WSN/33 by 20-fold, but various combinations of C4 and U4 in vRNAs of PR8 did not affect the virus replication efficiency in 293T cells (Lee and Seong, 1998, Wang and Lee, 2009). In the present study, I showed the virus titers of a rgPR8-like constellation of C4 and U4 (rgPR8) were significantly higher than those of C4 in NA vRNA (rPR8-NA-prom), M vRNA (rPR8-M-prom), and both vRNAs (rPR8-MN-prom) at all time intervals that were compared ($p < 0.05$) (Figure 3.1). Considering that rgPR8 has constellations of U4 in 5 vRNA coding proteins (HA, NP, NA, M, and NS) with relatively high copy numbers and C4 in 3 vRNA coding proteins (PB1, PB2, and PA) with relatively low copy numbers, an optimal constellation of C4 and U4 may result in a harmonized production of viral proteins to form virus particles. Neuraminidase is important for virus budding, and matrix 1 protein is one of the major structural proteins that helps to export RNA and nucleoprotein complex (RNP) to the cytoplasm (Martin and Helenius, 1991, Compans et al., 1969). Therefore, U4 to C4 substitutions in the NA and/or M vRNAs may cause a decrease in the replication efficiency of rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8). The virus titer of a recombinant PR8 virus generated with 8 plasmids with C4 was slightly higher than that of another PR8 virus generated with 8 plasmids with U4. However, the virus titers of 8 types of recombinant PR8 viruses with one C4 genome segment were not improved (de Wit et al., 2004). Thus, balanced production of low copy (PB1, PB2 and PA) and relatively high copy

(HA, NP, NA, M, and NS) number viral protein transcripts by weak (C4) and strong (U4) promoters, respectively, may also be important for efficient virus replication.

Relative vRNA and mRNA transcription levels of the NA and M genome segments of recombinant viruses demonstrated that the U4 to C4 substitutions decreased both vRNA and mRNA transcription of NA and M genome segments (Figure 3.2). The previously reported effects of C4 to U4 substitution on vRNA and mRNA transcription have been inconsistent. Lee and Seong reported U4 downregulated vRNA but upregulated mRNA transcription levels. However, Jiang *et al*. demonstrated C4 decreased both vRNA and mRNA transcription *in vitro* (Lee and Seong, 1998, Jiang et al., 2010). Because strand-specific real-time RT-PCR with tagged primers could measure the number of viral genomes more sensitively than conventional real-time RT-PCR or RNase protection assays, I concluded that U4 to C4 substitutions may reduce not only mRNA but also vRNA transcription of NA and/or M genome segments (Kawakami et al., 2011).

Reduced levels of NA due to decreased promoter activity by mutations at the 3′ and 5′ end NCRs of WSN/33 were reported to attenuate mutant viruses in mice (Solorzano et al., 2000). The effect of the position 4 nucleotide on mice pathogenicity of WSN/33 mutants has been demonstrated, but the tested mutant viruses with all U4 or all C4 were considered too artificial (Jiang et al., 2010). The inoculation of 10^5 and 10^4 EID_{50} doses of rgPR8 to each BALB/c mouse caused 100% mortality (Figure 3.3). The inoculation of 10^5 EID_{50} dose of rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8) caused 80%, 100%, and 80% mortality, respectively, along with greater mean death times. However, 10^4 EID_{50} doses did not cause any mortality (Figure 3.3). The 50% minimal lethal dose (MLD_{50}) of PR8 is slightly lower than the 10^4 EID_{50} dose (Rutigliano et al., 2014). However, C4 in NA and/or M genes of PR8 may attenuate the viral pathogenicity to increase MLD_{50} more than the 10^4

EID_{50} dose. According to our results, rgPR8 was more pathogenic than rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8) in BALB/c mice. Thus, U4 to C4 mutations in NA and/or M vRNA could attenuate viral pathogenicity of PR8 in BALB/c mice, as suggested previously (Jiang et al., 2010).

To date, most full genome sequencing of IAVs has been conducted with primer sets designed on the basis of the conserved nucleotide sequences of the 3′ and 5′ ends of IAVs (Hoffmann et al., 2001). Thus, natural 3′ and 5′ end nucleotide sequences are rarely available among the reports of complete sequences of IAVs. Natural variability of the position 4 nucleotide in 8 vRNAs has been reported, and combinations of C4 and U4 in 8 vRNAs have been more variable than expected (Wang and Lee, 2009, Robertson, 1979, Desselberger et al., 1980). Therefore, sequencing the NCRs of IAVs may reveal important information regarding the variability of promoter profiles among gene segments and virus strains and may provide basic data to predict potential pathogenicity in mice (Wang and Lee, 2009, de Wit et al., 2007)

In conclusion, the position 4 nucleotide of NA and M vRNA of IAVs is important for the effective replication of the PR8 virus in ECEs as well as having importance in virus pathogenicity in mice. Furthermore, an appropriate constellation of weak and strong promoters to low and high copy number protein-coding genome segments is important for virus replication and pathogenicity. Thus, sequence analysis of NCRs of a viral genome may be valuable in elucidating potential pathogenicities of IAVs in mice.

General Conclusion

1918 influenza pandemic, also known as Spanish flu, had been occurred during 1918-1920, which infected 500 million people all around the world with an estimated 20-50 million deaths (Taubenberger et al., 2001). Although it has been debated why and where the virus was transmitted to human, the genomes encoding the HA and NA protein were derived from avian origin IAVs (Taubenberger et al., 2001). As the precursor of all pandemic influenza virus outbreaks in the 20th century, Spanish flu might have had critical pathogenic factors to continually adapt to humans. Therefore, a lot of researches have been focusing on the host jumping mechanism of avian influenza viruses to humans (Manz et al., 2013b, Cauldwell et al., 2014). Q226L substitution in hemagglutinin (HA) abolishes the potential for Gln226 to interact with the avian type sialic acid (Rogers and Paulson, 1983). The mutations including E190D, N186K, G228S in the vicinity of receptor binding site also play important role in a change in the binding preference from avian receptor to human receptor (Glaser et al., 2005, Xiong et al., 2014b, Matrosovich et al., 2000). Amino acid position 627 on PB2 is located in the C-terminal RNA-binding domain, and the E627K mutation is known to increase both RNA binding and polymerase activity, increasing viral replication efficiency at 33°C, the approximate temperature of the human upper respiratory tract (Kuzuhara et al., 2009, Steel et al., 2009). However, because most researches have focused on the mutations in the mammalian host, fewer studies have been conducted on pathogenic mutations acquired in the birds. Therefore, I found the novel pathogenic mutations arisen in the avian hosts and evaluated their role in viral replication, pathogenicity, and host immune responses.

In chapter 1, I found that MVV mutations in PB2 gene successfully converted the non-replicative 01310 PB2 to replicate in mammalian hosts. MVV mutation in PB2 are

prevalent in the most of IAVs, and it was likely to increase *in vitro* replication efficiency of IAVs not only in mammalian species but also in avian species. Therefore, it was predicted to be minimum essential residues for adaptation to mammalian hosts. These findings suggest that the prototypic PB2 gene firstly acquired the MVV mutations for efficient replication, then it has accumulated the mammalian pathogenic factors in a particular way.

In chapter 2, I found that the introduction of the avian-PA gene was likely to lead to the adaptive mutation in the NP gene in order to successful adaptation to a new host. Considering their location in body domain and the influence on both viral replication and host antiviral factors, the adaptive mutation in NP may be related with viral fitness and the interaction with host antiviral responses. I also found that specific mutations in PA could increase the viral replication, virulence, and interferon-beta expression in mice. The frequency analysis of each mutation in PA in several subtypes of IAVs demonstrates that the adaptation pathway of PA to mammalian hosts may have been ongoing.

In chapter 3, I found that the Hoffmann reverse genetic vector system introduced the C4 to U4 mutations, known to contribute to increasing viral genome transcription, in both NA and M genes of PR8 virus. To elucidate the effects of these mutations on viral pathogenicity, I compared the effects of these mutations on viral replication, genome transcription, and viral pathogenicity by using site-directed mutagenesis. The rgPR8 containing U4 in both NA and M genes showed the highest replication efficiency, but the U4 to C4 mutation in NA and/or M genes led to lower viral replication and viral genome transcription. Furthermore, these mutants could attenuate viral pathogenicity of PR8 in mice. Thus, sequence analysis of non-coding regions of the viral genome may be valuable in elucidating potential pathogenicities of IAVs in mice.

To date, a lot of studies have tried to answer the question of what dynamics lead to

host jumping transmission of AIVs, but the exact cause has not yet been revealed. The important genetic markers such as 591R, 627K, and 701N in PB2 protein, 226L and 228S in HA protein play crucial roles in the adaptation and virulence of influenza virus to human (Subbarao et al., 1993, Yamada et al., 2010, Gabriel et al., 2005b, Connor et al., 1994, Matrosovich et al., 2000). However, most markers have been presumed to be acquired in mammalian hosts (Matrosovich et al., 2000). Therefore, to find the pathogenic mutations in AIVs may be valuable for assessing the risk of AIVs transmission to mammals. In this thesis, it was found that the novel pathogenic mutations in AIVs are deeply related with viral replication, pathogenicity, and host immune responses in mammalian hosts. Considering that the novel pathogenic mutations naturally arose in birds, the mutations may broaden the understanding of the host-jumping mechanism of AIVs. Furthermore. the novel pathogenic mutations may be usefully utilized to evaluate the potential of emerging AIVs to jump host specific barrier.

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국 문 초 록

인플루엔자 A 바이러스의 포유류 병원성 관련 신규 유전자 표지 분석

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인플루엔자 A 바이러스 (Influenza A virus, IAV)는 Orthomyxoviridae과에 속하는 RNA 바이러스로 음성 극성의 단일가닥, 분절화 되어 있는 RNA 유전체를 가진다. IAV는 표면 당단백질인 HA와 NA 단백질에 의해서 아형으로 세부적으로 분류된다. 야생철새에서 다양한 아형의 인플루엔자 바이러스가 관찰되는 것을 고려할 때, 현재까지는 야생철새가 조류인플루엔자 바이러스 (avian influenza virus, AIV)의 보유숙주로서의 역할을 한다고 추정된다. AIV는 때때로 사람에게 전파되었으며, 몇몇 바이러스는 성공적으로 적응하여 인플루엔자 대유행을 야기하였다. 그럼에도 불구하고 AIV가 포유류로 전파되는 과정은 아직 잘 규명되지 않았다. 이번 연구에서는 AIV가 포유류에 적응하기 위하여 관여하는 AIV 내에 존재하는 포유류 병원성 관련 신규 유전자 표지들을 밝혀냈으며, 이 표지들이 바이러스의 전파와 병원성에서 어떤 역할을 하는지 밝혀 내었다. polymerase 복합체는 PB2, PB1, 그리

고 PA 단백질로 구성된 heterotrimer이다. 이들 단백질이 바이러스의 복제, 전사에 미치는 영향을 고려했을 때, 이 polymerase 복합체(특히 PB2 단백질)는 바이러스의 숙주 특이성을 결정하는 주요 인자 중 하나일 것으로 추정되고 있다. 이전 연구를 통하여 저병원성 조류인플루엔자 바이러스인 [A/chicken/Korea/KBNP-0028/2000 (H9N2)] (0028) 과 [A/chicken/Korea/01310/2001 (H9N2)] (01310)의 polymerase 복합체가 분석되었으며, 그 중 PB2와 PA 단백질내에 존재하는 포유류 병원성 관련 인자가 규명되었다. 이 포유류 병원성 관련 신규 유전자 표지를 분석하기 위하여, 먼저 01310 바이러스 PB2 유전자 내에 포유류 병원성 관련 신규 유전자 표지들 (I66M, I109V, 그리고 I133V, MVV)을 도입하였고, 이 표지들이 재조합 PR8 바이러스의 조류, 포유류 숙주에서의 증식성을 증가시킬 수 있다는 것을 확인하였다. 이 MVV 돌연변이 바이러스는 마우스에서 체중감소를 일으키지 않았지만 감염된 마우스 폐에서 증식할 수 있었으며, 특히 Q591K/R, E627K, or D701N 과 같은 치명적인 포유류 병원성 돌연변이를 바이러스 유전자 내에 추가적으로 획득하였다. 또한 MVV 돌연변이는 polymerase 단백질들의 trimer경계부분에 존재하였으며 이 구조의 결합력을 증가시킬 것으로 예측되었다. 이런 연구결과들은 MVV 돌연변이의 획득은 조류인플루엔자 바이러스가 포유류 병원성을 획득하는 첫번째 단계일 것이라는 가설을 제시한다. 다음으로, PA 단백질내의 포유류 병원성 관련 신규 유전자 표지를 평가하기 위하여, IAV A/Puerto Rico/8/34 (PR8)을 기반으로 0028-PA 유전자를 도입한 재조합 바이러스에 단일 돌연변이를 적용함으로써 그 돌연변이들의 병원성과 증식성을 평가하였다. 돌연변이 중, 684번 아미노산의 글루타메이트에서 글라이신 돌연변이는 포유류 세포에서의 증식성을 증가시켰으며 감염된 마우스를 100% 폐사시키며 인터페론 베타의 발현을 상당히 증가시켰

다. 그러므로, PA 유전자의 E684G 돌연변이는 바이러스의 증식성과 숙주의 면역 반응을 증가시킴으로써 바이러스의 병원성에 중요한 역할을 할 것으로 추정된다. 바이러스 유전자의 암호화 서열뿐 아니라 비암호화 서열 또한 바이러스의 병원성에 영향을 미칠 수 있다. 호프만의 8개 플라스미드 역유전학 시스템은 야생형 A/PR/8/34 바이러스의 NA, M vRNA의 3'말단 중 4번째 뉴클레오티드의 C4에서 U4로의 돌연변이를 유도한다. 따라서, C4에서 U4로의 돌연변이가 바이러스의 병원성에 미치는 영향을 확인하고자, 역 유전학 (reverse genetic) 시스템을 이용하여 NA와 (또는) M vRNA에 C4를 가지는 3가지 종류의 재조합 PR8 바이러스를 만들었으며, 그들의 병리생물학적 특성을 비교하였다. 이 돌연변이 바이러스들은 NA와 (또는) M 유전자의 낮은 전사수준으로 인하여 rgPR8 바이러스 대비 낮은 증식 효율을 보였다. 게다가 NA와 (또는) M vRNA의 C4는 BALB/c 마우스에서 PR8 바이러스의 낮은 병원성을 유도하였다. 즉, vRNA에서 C4와 U4의 배치는 인플루엔자의 병원성을 결정하는 다중 유전체 인자들 중에 하나일 수 있다는 결론을 얻었다. 이번 논문에서 밝힌 포유류 병원성 관련 신규 유전자 표지들은 인플루엔자 바이러스의 다른 숙주간 전파 과정에 대한 이해를 넓힐 것이며, 특히 이 돌연변이들은 인플루엔자 범유행 위험을 미리 감시하고 예방하는데 기여할 수 있을 것이라 기대한다.

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