Genetic analyses of the function of PB1 subunit of the influenza virus RNA-dependent RNA polymerase

（インフルエンザウイルス RNA 依存 RNA ポリメラーゼ PB1 サブユニットの遺伝学的機能解析）

２０１３

筑波大学大学院博士課程人間総合科学研究科

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博 士 （医 学） 学 位 論 文

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Chapter I. Introduction

1. General remark

1-1. Significant impact on our life by influenza virus infection

Influenza virus was the first isolated from pigs by Shope and Lewis in 1930 (Shope and Lewis 1931). It is one of the most dangerous viruses which constantly circulate in many animal hosts, such as humans, birds, horses, dogs an pigs. This virus infection at upper respiratory tract of human results in several symptoms, such as fever, chills, muscle aches, fatigue and headaches. Through human history, influenza virus caused epidemics and severe pandemics at several times.

As mentioned above, many influenza pandemics occurred in human history. However, since 19th century, reports and data about influenza pandemics have been more comprehensive and reliable. In 1918, Spanish influenza, which killed 40-50 million people, is the first of the two pandemics involving H1N1 influenza virus (the second being the 2009 influenza pandemic) (Taubenberger et al, 2006). Furthermore, in 1957-1958 and 2009, two influenza pandemics spread in southern China killed about 4 million people (reported by WHO, 2005), and also a recent outbreak of human infections with a new avian influenza A (H7N9) virus, which has involved 132 patients, including 37 deaths in 2013 (reported by WHO, 2013).

1-2. Significance of viral research

Seasonal influenza virus infections in humans cause annual epidemics, leading to millions of human infections worldwide and having significant health and economic burdens; influenza pandemics can also have devastating effects globally, resulting in millions of deaths. Within one year, this virus spread to all the world and caused >18,000 confirmed deaths (Medina et al, 2011). Thus, this virus research plays an important role for protection of public health as well as economic developments globally. Also important to development of new vaccines and anti-influenza drugs.

2. Purpose and summary of this study

PB1 functions as a catalytic subunit of RNA polymerase and contains the highly conserved motifs of RNA-dependent RNA polymerases. Putative nucleotide-binding sites have been mapped adjacent to this region. PB1 also binds to vRNA and cRNA, and the putative promoter binding sites have been mapped. PA and PB2 interact with the N-terminal and C-terminal regions of PB1, respectively. Thus, some structures and functions of PB1 have been clarified. However, the relationship between the structures and functions are not clarified. Therefore, to clarify this relationship based on its structure, I started the functional analysis of PB1 subunit using mutant viruses by determining the replicational and transcriptional activities and using ribavirin by isolation of ribavirin-resistant PB1 mutants.

3. Abbreviations

a a	amino acid
CHX	cycloheximide
cRNA	complementary RNA
DMEM	Dulbecco’s modified Eagle’s medium
FBS	Fetal bovine serum
HA	hemagglutinin
hpi	hour(s) post infection
hpt	hour(s) post transfection
M1	matrix protein 1
M2	matrix protein 2
MEM	Minimum essential medium
MOI	multiplicity of infection
NA	neuraminidase
NP	nucleoprotein
NS1	nonstructural protein 1
NS2	nonstructural protein 2
NLS	nuclear localization signal
PA	polymerase acidic protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
RdRp	RNA-dependent RNA polymerase
RNP	ribonucleoprotein

rpm	round per minute
PFU	plaque formation unit
Pol II	DNA-dependent RNA polymerase II
vRNA	viral RNA, virus genome
vRNP	viral ribonucleoprotein

4. General remarks of influenza virus

4-1. Structure of influenza virus genome and viral proteins

Influenza virus belonging to family of *Orthomyxoviridae* can range from spherical shape with a particle size about 80 to 120 nm in a diameter (Fig. 1A). Influenza viruses can be categorized into 3 types: A, B and C. Influenza A and B viral genomes consist of eight segmented - and single-stranded RNAs of negative polarity (vRNA), while influenza C virus genome consists of seven segmented - and single-stranded RNAs of negative polarity. The three largest RNA segments (segment 1, 2, and 3) encode the three viral RNA-dependent RNA polymerase (RdRp) proteins: polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), and polymerase acidic protein (PA), respectively. The RNA segment for PB1 also encodes non-structural proteins, PB1-F2, a proapoptotic virulence factor (Wise et al, 2009), and PB1-N40, its function is unknown. Except for PB1, these two proteins are not essential for replication. Segment 4, 5, and 6 encode hemagglutinin (HA), nucleoprotein (NP), and neuraminidase (NA), respectively. Segment 7 encodes the M1 matrix protein and the M2 ion-channel protein, segment 8 encodes two nonstructural proteins, NS1A and NS2/NEP (Kalyan Das et al, 2010). The influenza A, B, and C viruses can be distinguished on the basis of antigenic differences between their NP and M proteins. Influenza A viruses are further divided into subtypes based on the antigenic nature of their HA and NA glycoproteins. These spikes are of two types: HA is rod-shaped spikes and NA is mushroom-shaped spikes. The ratio of HA to NA varies but is usually 4:1 to 5:1. The viral matrix protein, M1, is thought

to underlie the lipid bilayer and to associate with the replicated virus genome. M1 is the most abundant virion protein. The HA spike glycoprotein is a homotrimer of noncovalently linked monomers. Each HA polypeptide chain (H3 subtype) consists of an ectodomain of 512 residues, a carboxyl-terminal proximal transmembrane domain of 27 residues, and a cytoplasmic tail of 10 residues. HA exists either in an uncleaved precursor form (HA0) or in a cleaved form consisting of two disulfide-linked chains (HA1, and HA2). The NA integral membrane protein is the second subtype-specific glycoprotein of influenza A and B viruses. The NA is a homotetramer containing a head domain that is enzymatically active and a stalk region that is attached to the membrane. NA is important for removing sialic acids, which function as a viral receptor, from glycoproteins expressing on host cell variation. In addition, influenza pandemics are being caused by the appearance of an emerging strain containing amino acid changes in HA and NA surface proteins. These changes partially overcome pre-existing immunity in humans, and these new strains are largely responsible for seasonal influenza epidemics. The matrix protein M1, which lies inside the lipid envelope and constitutes the most abundant polypeptide in the virion, and the M2 protein, which is a minor component of virions and has an ion chanel activity. The NS1 protein is expressed in large amounts in influenza virus-infected cells, but it has not been detected in virions, hence the designation NS for *non-structural*. NS1 is found in infected cells in the nucleus and is associated with polysomes. The NLS (nuclear localization signal) within NS1 has been mapped, and it has been found that the protein contains two separate signals (residues 34

to 38 and within residues 203 to 237). The NS1 protein also contains a nuclear export signal (NES), a short leucine-rich sequence that mediates the nuclear export of proteins. The NS1 NES has been mapped to residues 138 to 147. The NS2 protein, originally thought to be non-structural, is now known to exist in virions and to form an association with the M1 protein. The subcellular localization of NS2 has been indicated to be nuclear and cytoplasmic (Knipe et al, 2001). The NES on the N-terminus of NS2 is recognized by chromosomal maintenance 1 (CRM1), but this CRM1 binds with RanGTP to an unknown site. The C-terminal hairpin of NS2 in turn associates with the N-terminal nuclear localization signal of the viral matrix protein M1, and the M1 protein binds to the vRNP through a C-terminal interaction with NP. These interactions have led to the description of the vRNP nuclear export complex as a daisy chain CRM1-RanGTP-NS2-M1-vRNP (Paterson, 2012).

The N-terminal region of PB1 interacts with the C-terminal region of PA (Ghanem et al, 2007; Gonzalez et al, 1996; Ohtsu et al, 2002; Perez et al, 1995; Toyoda, et al, 1996; Zurcher et al, 1996), and the crystal structure of this interaction has been resolved (He et al, 2008; Obayashi et al, 2008). The C-terminal region of PB1 between amino acid (a.a.) positions 678-757 interacts with the N-terminal region of PB2 (Gozalez et al, 1996; Ohtsu et al, 2002; Poole et al, 1981; Toyoda et al, 1996), and this interaction was analyzed by crystallography (Sugiyama et al, 2009). PB1 contains the motifs highly conserved among RNA-dependent RNA polymerases (Biswas et al, 1994). There are two putative nucleotide-binding sites between a.a. positions 179-297 and between a.a.

positions 458-519 (Asano et al, 1997; Kolpashchikov et al, 2004). Moreover, the N-terminal (a.a. positions 1-83) and C-terminal (a.a. positions 494-757) regions of PB1 are suggested to bind to the vRNA promoter (Gozalez et al, 1999a). In addition, it is reported that the vRNA binding site of PB1 is located at the a.a. positions 249-254, and phenylalanines at the positions 251 and 254 are essential for this binding (Li et al, 1998). It is also reported that the N-terminal region (a.a. positions 1-139) and a region between a.a. positions 267 and 493 bind to the cRNA promoter (Gozalez et al, 1999b). Recently, novel proteins, PB1-F2 and PB1-N40, produced by splicing were identified (Wise et al, 2009). It is suggested that these are involved in the virulence, but not essential for the replication.

4-2. Structure and function of RNP complex

Each segment of influenza virus is encapsidated by nucleoprotein (NP) and associated with viral RNA polymerases to form viral ribonucleoprotein (vRNP) complexes. The viral RNA polymerase is composed of one molecule each of three viral proteins, PB1, PB2, and PA. PB1 functions as a catalytic subunit and assembly core of the viral RNA polymerase (Biswas et al, 1996; Gozalez et al, 1996; Ohtsu et al, 2002; Zurcher et al, 1996). PA is genetically found to be involved in the replication process and the polymerase assembly (Kawaguchi et al, 2005) and have the endonuclease activity (Dias et al, 2009; Fodor et al, 2002; Hara et al, 2006; Yuan et al, 2009). PB2 is responsible for recognition and binding the cap structure (Fechter et al, 2003; Guilliary et al, 2008; Li et al, 2001; Plotch et al, 1981; Wakai et al, 2011). The vRNP complex is a basic unit for both

transcription and replication (Nagata et al, 2008) (Fig. 2). Both vRNA and cRNA promoters form a specific secondary structure, depicted as “corkscrew model”. This model structure includes the partial duplex regions formed by the 5’- and 3’-terminal sequences of vRNA and cRNA that are partially complementary to each other. These duplexes are conserved in all eight segments and act as *cis*-acting elements for the viral RNA synthesis (Nagata et al, 2008).

4-3. Transcription and replication of influenza virus genome

Replication and transcription of the viral RNA genome occur in the nucleus. Transcription of the viral genome is initiated using as a primer the oligonucleotide containing the cap-1 structure derived from cellular pre mRNAs. The capped oligonucleotide is generated through recognition of the cap structure by PB2 (Nagata et al, 2008) and endonucleolytic cleavage by PA (Dias et al, 2009; Fodor et al, 2002; Hara et al, 2006; Yuan et al, 2009). The elongation of the mRNA chain proceeds until the viral polymerase reaches a polyadenylation signal, which consists of 5-7 uridine residues located near the 5’- terminus of the vRNA. The viral polymerase generates a poly (A) tail at the end of the viral mRNA by stuttering and slipping between the template and nascent elongating RNA chain. It is shown by mutants in the promoter of vRNA that the cleavage of the cap structure and the polyadenylation by the viral polymerase are regulated through the promoter structure. In contrast, the genome replication is primer-independent and generates full-length cRNA from vRNA, and progeny vRNA is in turn copied from the cRNA (Fig. 2) (Nagata et al, 2008).

5. Materials and Methods

5-1. Plasmids

To construct plasmids from which human DNA-dependent RNA polymerase I (Pol I) transcribes mutated vRNAs, I amplified fragments containing mutated segment 2 by PCR using a plasmid containing wild type WSN segments in pHH21 vector (Neumann et al, 1999) as a template with sets of phosphorylated primers (see Table 1). The amplified PCR products containing 5'-PO_4 and 3'-OH were self-ligated using ligation high (TOYOBO). Sequences of plasmids containing these mutated segment 2 were confirmed by sequencing with Dyenamic ET sequencing system (ABI).

5-2. Cell culture

Monolayer cultures of 293T and MDCK cells were maintained at 37^{o}C in Dulbecco's Modified Eagle Medium (DMEM) and minimal essential medium (MEM) (Nissui), respectively, supplemented with 10% fetal bovine serum (Bovogen). Influenza virus strain A/WSN/33 (A/WSN/33) was prepared as previously described (Kawaguchi et al, 2005).

5-3. Antibodies

Rat polyclonal antibodies against PB1, PB2, and PA were prepared as described previously (Kawaguchi et al, 2005). Rabbit polyclonal antibody against PB2 was prepared as described previously (Naito et al, 2007). Mouse monoclonal antibody against β-tubulin was purchased from Sigma.

5-4. Generation of recombinant viruses

To generate recombinant viruses, reverse genetics system was used as described previously (Neumann et al, 1999). Briefly, 293T cells were transfected with 4 plasmids for expressing viral proteins (PB1, PB2, PA, and NP) and 8 plasmids for expressing a full set of the viral RNA genome. After incubation at 37^{o}C with 5% CO_2 for 48 hours post transfection (hpt), aliquots of cell culture supernatants were used for virus amplification in MDCK cells. At 48 hours post infection (hpi) at 37^{o}C with 5% CO_2, culture fluid was collected, and the virus titer of these recombinant viruses was determined by plaque assays.

5-5. Plaque assay

The plaque assay was performed in 6-well tissue culture plates. The MDCK cells were seeded at 1 x 10^6 cells per well in (Minimum essential Medium Eagle) (Sigma) with 10% FBS before carrying the experiment. After 12h, 50 PFU of influenza virus (WSN) were added to the cell monolayer and the plates incubated at 37^{o}C for 1h with 5% CO_2 before removal of unbound viral particles by aspiration. The cell monolayer was washed once with MEM (Nissui, Tokyo, Japan) just before being overlaid with 1.6% low melting agarose (final concentration, 0.8%) (Type I, Sigma) was mixed with an equal volume of 2x maintenance medium (final concentration, 1x) with or without supplementing compounds, the mixture was maintained at 40^{o}C and dispensed in the wells (3 ml/well). The plates were incubated for 48h at 37^{o}C with 5% CO_2. After 48h, the wells were fixed with ethanol and acetic acid (ratio 1: 1), the agarose gel had

been removed before the monolayers were stained with 0.5% Amido Black solution (0.5 g amido black, acetic acid 10 ml, ethanol 45 ml, and up water to 100 ml) and the plaques were counted.

5-6. RNA analysis

MDCK cells were infected with recombinant viruses at the multiplicity of infection (MOI) of 2.5, and incubated at 37^{o}C with 5% CO_2. At 3, 6, and 9 hpi, total RNAs were isolated by the acid guanidine-phenol-chloroform (AGPC) method.

5-7. Quantitative RT-PCR (qRT-PCR)

To measure the accumulation levels of viral mRNA, cRNA, and vRNA, quantitative RT-PCR was performed. Total RNAs were subjected to reverse transcription using ReverTraAce (TOYOBO) with either (i) oligo $(dT)_{20}$ for synthesizing cDNA from viral mRNA, (ii) 5'-AGTAGAAACAAGGGTATTTTTCTTTA-3', which is complementary to the 3' portion of segment 5 cRNA between nucleotide sequence positions 1540 and 1565 for synthesizing cDNA from cRNA, or (iii) 5'-GACGATGCAACGGCTGGTCTG-3', which corresponds to segment 5 cRNA between nucleotide sequence positions 424 and 444 for synthesizing cDNA form vRNA. The synthesized single-stranded cDNAs were subjected to real-time quantitative PCR analysis (Thermal Cycler Dice real-time system TP800; TaKaRa) with SYBR Premix Ex *Taq* (TaKaRa) and a set of specific primers, 5'-

GACGATGCAACGGCTGGTCTG-3', which corresponds to segment 5 cDNA between nucleotide sequence positions 424 and 444, and 5'-AGCATTGTTCCAACTCCTTT-3', which is complementary to segment 5 cDNA between nucleotide sequence positions 595 and 614. The levels of these RNAs were normalized by the amount of cellular β-actin mRNA measured using specific primers 5'-ATGGGTCAGAAGGATTCCTATGT-3', which corresponds to β-actin cDNA between nucleotide sequence positions 1363 and 1385, and 5'-GGTCATCTTCTCGCGGTT-3', which is complementary to the β-actin cDNA between nucleotide sequence positions 1567 and 1584. Experiments are carried out three independent with standard deviations, and the level of significance was determined by Student's *t* test (unpaired).

5-8. Primary transcription activity of mutant viruses

MDCK cells were infected with mutant viruses at MOI of 2.5 and incubated at 37^{o}C with 5% CO_2 in the presence of 100 μg/ml of cycloheximide (CHX). At 9 hpi, the accumulation levels of viral mRNA and vRNA were measured by qRT-PCR as described above. Experiments are carried out three independent with standard deviations, and the level of significance was determined by Student's *t* test (unpaired).

5-9. Immunoprecipitation

MDCK cells were infected with recombinant viruses at MOI of 1. At 7 hpi, cells were lysed by sonication in a buffer A containing 50 mM Tris-HCl (pH 7.9),

100 mM NaCl, 30 mM KCl, and 0.1% Nonidet P-40. The lysates were subjected to centrifugation at 16,000 x g, at 4^{o}C for 10 min. RNA polymerase complex in the supernatant fraction was subjected to immunoprecipitation with rabbit anti-PB2 antibody bound to protein A sepharose beads (GE Healthcare) at 4^{o}C, for 2 h. The beads were washed 4 times with buffer A and proteins bound to the beads were eluted by boiling them in an SDS-PAGE loading buffer and were subjected to 7.5% SDS-PAGE. To detect each viral RNA polymerase subunit, rat anti-PB1, PB2, and PA antibodies were used for western blotting analysis, respectively.

5-10. PB1 random mutagenesis

For construction of a mammalian expression vector for PB1 containing random mutations, I used a PCR-based cloning strategy with $MnCl_2$. cDNA corresponding to the full-length of PB1 was amplifed with specific primers 5'-CCCCAAGCTTGCCGCCACCATGGATGTCAATCCGACCTT-3' and 5'-CATGCGGCCGCCTATTTTTGCCGTCTGAGCTCTT-3'. The PCR product was then cloned into the Hind III and Not I sites of pEGFP-N1 and replaced with *EGFP* gene with mutated *PB1* cDNA. The composition of the plasmid library was confirmed by sequencing using specific primer 5'-GGAAGGCTCATAGACTTCCTTA-3', which is corresponding to the position from 560 to 1050 nucleotide in segment 2. The plasmid library was then used to analyze the influenza virus RNA polymerase activity in mini-replicon assay system.

5-11. Mini-replicon assay system

293T cells were transfected with plasmids for the expression of viral proteins, PB1 (wt or mutated-type), PB2, PA, and NP, and a plasmid for the expression of artificial influenza virus genome containing either *EGFP* gene (for screening) or the *firely luciferase* gene (for luciferase assay) of negative polarity, which is synthesized in cells by the human DNA-dependent RNA polymerase I [pol I]) (Turan et al, 2004). mRNAs containing *EGFP* and *luciferase* genes are transcibed in a viral RNA polymerase-dependent manner. For the screening, ribavirin was added (0 or 75 μM) after 3 hours post transfection (hpt) and the fluorescence of EGFP was observed at 15 hpt. For luciferase assay, either ribavirin (Sigma) was added in the medium at various concentrations after 3 hpt, and incubated at 37°C for 15 h, or after 12 hpt, different concentrations of methotrexate (MTX) was added, and incubated at 37°C for 10 h, and then the luciferase activity was determined using commercially available reagents (Promega) according to the manufacturer's protocol. The relative luminescence intensity was measured with a luminometer for 20 sec. A plasmid for the expression of Renilla luciferase driven by the simian virus 40 (SV40) promoter was used as an internal control for the dual-luciferase assay. As a negative control, 293T cells were transfected with the same plasmids, except for the omission of the PB1 expression plasmid.

Chapter II. The N-terminal region of influenza virus polymerase PB1 adjacent to the PA binding site is involved in replication but not transcription of the viral genome

1. Purpose

In this study, I have focused my study on the N-terminal region (a.a. residues 1-83) of the putative vRNA and cRNA promoter binding sites of PB1. An alignment analysis of the amino acid sequence has revealved that the a.a. positions 1-50 was highly conserved between influenza A and B viruses, but the amino acid positions 16, 27, and 44 differ between two viruses. To identify the functional importance of these positions in replication and transcription of the viral genome, I generated viruses containing mutations at these positions by reverse genetics, and determined the replicational and transcriptional activities of these mutants.

2. Results

2-1. RNA synthesis of influenza A mutant viruses containing influenza B virus-type amino acid signatures

The promoter sequence of influenza A virus genome forms panhandle like structure (Fodor et al, 1994). These stem-loop structures are important for the interaction of viral polymerase (Leahy et al, 2001). When viral polymerase interacts with the promoter element, it has been reported that the RNA synthesis activity and capping activity are significantly stimulated (Leahy et al, 2002),

suggesting that the enzymatic activities of viral polymerase are regulated by the interaction with promoter sequence. But, the detail of regulatory mechanisms is still unclear. Further, influenza B promoter also forms panhandle like structure similar to that of influenza A virus. However, influenza A polymerase weakly recognize the influenza B promoter (Muster et al, 1991). Thus, it is possible that by comparing the amino acid sequence of the influenza A and B in the promoter binding site, we can find important residues for the promoter recognition and its viral polymerase activity.

The C-terminal region of PB1 contains a putative vRNA promoter binding site, while the N-terminal region of PB1 (1-83 a.a.) contains both putative vRNA and cRNA promoter binding sites and PA binding site (1-15 a.a.) (Fig. 3A). However, except for the PA binding site, the crystal structure of which was determined (He et al, 2008; Obayashi et al, 2008), the function of N-terminal region of PB1 was poorly understood. The a.a. positions from 1 to 50 of PB1 was highly conserved (85%) between influenza A and B viruses but that from 51 to 757 of PB1 was not highly conserved (50-60%). The region between a.a. positions 1 and 50 are highly conserved between influenza A and B viruses, a.a. positions 40 and 48 containing Met and Gln of influenza A and Ile and Glu of influenza B have the same properties (nonpolar and polar), respectivly, except for the PA binding site, amino acids at positions 16, 27, and 44 are different between these viruses (Fig. 3B). To elucidate the functional importance of these amino acids for viral RNA synthesis, I generated influenza A viruses containing Ala at the a.a. position 16, Val at the a.a. position 27, and Ile at the a.a. position 44 by

reverse genetics. These mutated viruses are designated N16A, D27V, and N44I, where capital letters at the first position, numbers at the second position, and capital letters at the third position indicate wild type a.a., a.a. position, and replaced a.a., respectively. I examined the RNA polymerase activity by measuring the accumulation levels of viral mRNA, cRNA, and vRNA at 3 and 6 (Fig. 4), and 9 hpi (Fig. 5) by quantitative reverse transcription-PCR (qRT-PCR). At 9 hpi, the levels of all three type RNAs from D27V were increased compared with those from wild type and N16A virus (2.5-fold), while those from N44I were significantly decreased (about 3-fold) (Fig. 5). Based on the result that these mutations affect the RNA synthesis activity of viral mRNA, cRNA, and vRNA equally, there could be two possibilities: (i) amino acids at the positions 27 and 44 affect cRNA/mRNA synthesis from vRNA through recognition of the promoter on vRNA, but do not affect vRNA synthesis from cRNA through recognition of the promoter on cRNA, or (ii) these mutations affect independently the synthesis of mRNA, cRNA, and vRNA, but the sum of effects leads similar outputs in the synthesis of mRNA, cRNA, and vRNA.

To elucidate whether these mutations affect viral replication (cRNA and vRNA synthesis) and/or transcription (viral mRNA synthesis) activities, I measured the primary transcription activity (Fig. 6A). MDCK cells were infected with mutant viruses and then incubated with the medium containing cycloheximide (CHX), a potent inhibitor of protein synthesis. In the presence of CHX, newly synthesized RNA polymerases are not supplied, and the replication process does not occur (Kawaguchi et al, 2005). I utilized this method to

measure the primary transcription activity that depends just only on incoming vRNP and is not affected by the replication process. At 9 hpi in the presence of CHX, the levels of viral mRNA and vRNA were measured by qRT-PCR, and the transcription activity was represented as a ratio of viral mRNA/vRNA (Fig. 6A). This result shows that the transcription activity is not affected by these mutations, and thereby strongly suggest that these mutations affect the replication activity. If it is hypothesized that the recognition mechanism of the promoter on vRNA for mRNA synthesis would not be different from that for cRNA synthesis, the replication process from cRNA to vRNA could be affected by these mutations. This also leads to the possibility that vRNA and cRNA promoter binding sites may be seperately affected by mutants.

2-2. Uncharged amino acid at the a.a. position 16 is required

To know whether the charateristics for the viral polymerase activity of the amino acid at the position 16 affect viral RNA synthesis or not, I generated N16D (D: position 27) and N16Q (structure of Q is close to that N), in addition to N16A (Fig. 7 and Fig. 8). At 9 hpi, the amount of each viral RNA of N16D was decreased compared with that of wild-type (3.5 fold), while those of N16Q and N16A were not significantly changed compared with wild type (Fig. 8). Negatively charged amino acid may decrease the viral RNA polymerase activity.

2-3. Uncharged amino acid at position 27 enhances the RNA synthesis activity

The amino acid position 27 of PB1 is aspartate, and aspartate is highly conserved among influenza A viruses, except for an H4N8 strain isolated from least sandpiper that contains asparagine (GenBank: ACI90144.1). I generated D27E and D27N in addition to D27V (Fig. 9 and Fig. 10). At 9 hpi, the levels of mRNA, cRNA, and vRNA of D27N and D27V were increased significantly (about 2 to 2.5 fold) compared with those of wild-type and D27E (Fig. 10). These results suggest that uncharged residues at amino acids position 27 may enhance the viral RNA synthesis activity.

2-4. Hydrophilic residues at amino acid position 44 is important for the viral RNA synthesis activity

The amino acid substitution from asparagine to isoleucine at the a.a. position 44 reduced the viral polymerase activity. To clarify the importance of the amino acid position 44, I additionally generated N44D and N44Q viruses in addition to N44I and examined the viral RNA synthesis activity (Fig. 11 and Fig. 12). At 9 hpi, the synthesis level of each viral RNA of N44I was decreased largely, while the amounts of mRNA and cRNA of N44D and N44Q were similar to those of wild type (Fig. 12A and 12B). In addition, the amount of vRNA of N44Q was more than that of wild type (about 2 fold) (Fig. 12C). These results suggest that the amino acid residue at the a.a. position 44 would be a water-soluble characteristic, and especially glutamine at this position stimulates the vRNA

synthesis. It is assumed that asparagine and glutamine at this position have a similar effect/function on the viral RNA synthesis, although the N44Q polymerase may have some cooperative property in RNA binding, subunit interaction, and/or etc. This cooperative property could appear as a function of infection time, namely, the involvement of increasing amounts of viral proteins and/or viral RNAs.

3. Discussion

In this report, to elucidate the function of a putative promoter binding region of PB1, I focused on the N-terminal region of PB1 between a.a. positions of 1-50 (Fig. 3). I have studied on three positions, *i. e.,* a.a. positions 16, 27, and 44, which are not conserved between influenza A and B viruses. The RNA synthesis activity of D27V containing influenza B virus-type a.a. was enhanced compared with that of wild type virus, while that of N44I was decreased (Fig. 5). Based on these, I carried out further mutational analyses. The N44I showed the decreased level of RNA synthesis in three types of viral RNAs, while N44D showed the viral RNA synthesis activity similar to wild type virus. Interestingly, N->Q mutation at the a.a. position 44 increased vRNA synthesis with little effect on viral mRNA and cRNA synthesis. Asparagine, glutamine, and aspartic acid are polar or charged, whereas isoleucine is uncharged. These results suggest that a hydrophilic moiety of amino acids at the position 44 may be important. It is also possible that the length of side chain group of glutamine may stimulate the cRNA promoter binding and increase the vRNA synthesis activity.

On the a.a. position 27 (Fig. 10), D->V and D->N mutations increased the

viral RNA synthesis, while D->E mutation gave no effects. Aspartic acid and glutamic acid are acidic, but asparagine and valine are uncharged amino acids. Furthermore, when the amounts of viral RNAs of D27V were analyzed at various moi (0.5, 2.5, 10, and 25), those of D27V were increased (Fig. 13). Although uncharged amino acid at this position enhances the viral RNA synthesis, molecular evolution has selected negatively charged amino acids. Therefore, it is assumed that charged amino acids at this position, even with low efficiency for the replication, are needed for PB1. Recently, mutational analyses showed that the sequences surrounding the PB1 AUG codon are multifunctional, and contain overlapping signals for translation initiation and for segment specific packaging (Wise et al, 2011). It is assumed that there is some regulatory coupling between replication and packaging and the a.a. position 27 has a role in this hypothetical mechanism.

To confirm whether these mutations affect replication and/or transcription, I analyzed the primary transcription activity in the presence of cycloheximide (Fig. 6), which is not affected by replication processes. The transcription activity of these mutants was similar to that of wild type. This results indicate that these mutations affected the replication activity but not the transcription activity.

These a.a. positions are present within putative vRNA and cRNA promoter binding sites (Gonzalez et al, 1999a; Gonzalez et al, 1999b), and the PB1-RNA interaction could be affected by the presence of PA. PB2 subunit participates in the recognition of vRNA but not cRNA (Gonzalez et al, 1999b). Moreover, as these amino acids are close to the PA binding site, I examined

whether these mutations affect the assembly of the polymerase (Fig. 14). The assembly of PB1 with PA and PB2 was not affected by these mutations. Taken altogether, it is quite likely that amino acid residues at the a.a. positions 27 and 44 are involved in the viral genome replication, possibly via the cRNA promoter recognition with little effects on the transcription activity and the assembly of the RNA polymerase complex.

All eight genomic RNA segments of influenza A virus have 12 and 13 conserved 5'- and 3'-terminal nucleotides, respectively. This conservation, together with two or three segment-specific nucleotides show inverted partial complementarity. Interaction between the 5'- and 3'-end, through base pairing, is required for promoter activity (Crow et al, 2004). Both vRNA and cRNA promoters form a specific secondary structure, depicted as "corkscrew model" (Nagata et al, 2008). The cRNA and vRNA promoter structures are different substantially (Park et al, 2003; Tchatalbachev et al, 2001). Recognition of the vRNA promoter depends on the 5'-arm of the promoter (Flick et al, 1996; Gonzalez et al, 199a; Lee et al, 2002; Tiley et al, 1994). In contrast, binding of the RNA polymerase to the 3'-arm of the vRNA promoter is weak, but this binding is highly improved by the presence of the 5'-arm (Gozalez et al, 1999a; Jung et al, 2006; Tiley et al, 1994). Furthermore, it is suggested that, in order for the viral template RNA to be recognized by the viral polymerase, this RNA has to be encapsidated by the NP (Parvin et al, 1989). Recognition of the cRNA promoter by the polymerase has been shown by the *in vitro* binding of the PB1 subunit with the 5'- and 3'-arms of the cRNA promoter (Gonzalez et al, 1999b). The structural

differences may also account for the preferential encapsidation of vRNPs versus cRNPs (Tchatalbachev et al, 2001). Flexibility within the two uridines of the internal loop of the cRNA promoter required for protein binding in the cRNP complex (Park et al, 2003). Biochemical studies have shown that conformational changes in PB1 of the influenza A virus RNA polymerase lead to the interaction with either vRNA or cRNA (Gonzalez et al, 1999b). Based on previous reports and our findings, the a.a. positions 27 and 44 may affect the PB1 structure, resulting in affecting PB1 binding activity to the 3'-arm of the cRNA promoter. Thus, I would propose that these positions in PB1 is important for the replication activity by recognizing the cRNA promoter.

Chapter III. Asparagine at position 27 in PB1 is responsible for ribavirin resistance

1. Purpose

Ribavirin (1-β-D-ribofusanosyl-1,2,4-triazole-3-carboxamide, also known as Virazole) is a synthetic purine nucleoside analogue first synthesized by Sidwell and colleagues in 1972 (Sidwell et al, 1972). It is phosphorylated by a cellular adenosine kinase into ribavirin monophosphate, diphosphate, and triphosphate (RMP, RDP, and RTP respectively) (Dixit et al, 2006; Feld et al, 2005) and has been shown to exhibit the antiviral activity against many distinct viruses *in vitro* as well as *in vivo* via indirect (inosine monophosphate dehydrogenase inhibition, immunomodulatory effects) and direct (interference with RNA capping, polymerase inhibition, lethal mutagenesis) mechanisms (Graci et al, 2006). Ribavirin inhibits viral RNA polymerases such as those of influenza virus (Eriksson et al, 1977), VSV (vesicular stomatitis virus) (Tolzis et al, 1988), La Crosse virus (Cassidy et al, 1989), Reo virus (Rankin et al, 1989), Hantaan virus (Sun et al, 2007) FMDV (Foot and Mouth Disease Virus) (Gu et al, 2006), human immunodeficiency virus (Fernandez et al, 1990), West Nile virus (Jordan et al, 2000), LCMV (lymphocytic choriomeningitis virus) (Moreno et al, 2011), Andes virus (Safronetz et al, 2011), and HCV (Hepatitis virus) replication (Magg et al, 2001). While, ribavirin does not inhibit RNA polymerase I, RNA polymerase II, or poly (A) polymerase (Muller et al, 1977).

Ribavirin is known to increase the error in the viral genome during viral

replication by being incorporated in place of guanosine in nascent viral RNA strands (Dixit et al, 2006). The *in vitro* incorporation studies suggested that ribavirin should induce transition mutations. This incorporation will induce G→A transition if incorporated into genomic RNA (Cameron et al, 2001; Crotty et al, 2000), ribavirin also causes chain termination in HCV and polio viruses (Maag et al, 2001). Since the influenza virus RNA polymerase replicates its genome with a low fidelity (Aggarwal et al, 2010), in the presence of ribavirin, the replication defect and error catastrophe are found by mis-incorporation of the ribavirin (Crotty et al, 2001; Gu et al, 2006; Severson et al, 2003).

In this study, to determine the catalytic active site of PB1, resistant mutant of PB1 against ribavirin was identified. Ribavirin inhibits viral RNA synthesis as an analog of both adenine and guanine, and causes mutations in viral genome influenza viruses. Isolation of the ribavirin-resistant PB1 mutants are useful to know the mechanism of nucleotide recognition by the viral RNA polymerase.

2. Results

2-1. Screening of ribavirin-resistant PB1 mutant

To make PB1 mutant library, random mutagenesis was examined by PCR with 0.1 mM of Mn^{2+} and 1.5 mM of Mg^{2+} as described in materials and methods. To know the mutation rate of this library, I transformed the library into *E.coli* DH5α high competent cells and got 3 x 10^4 colonies. To evaluate the mutation rate, plasmids were isolated from 20 colonies and sequenced at the position from 560-1055 of segment 2. The results of sequencing showed that mutation was

introduced in 4.7 mutation/ 2274 nucleotides of segment 2 (approximately 1-2 aa/PB1). With this result, I started screening ribavirin-resistant PB1 from the mutant library (Fig. 15A). At first, this library was divided into 10 groups, and mini-replicon assay was performed in the presence of ribavirin (Fig. 15A and B). The fluorescence of EGFP was checked at 15 hpt. Based on the fluorescence of each group, I chose 1 group which showed the highest fluorescence among 10 groups followed by dividing into additional 10 groups and performing mini-replicon assay again. After 5 time repetitions of this step, D27N mutant of PB1 was isolated (Fig. 16A).

2-2. Characterization of D27N mutant

To confirm the ribavirin resistance, I carried out the mini-replicon assay with EGFP reporter gene (Fig. 16B). The ratio of EGFP fluorescence on ribavirin-treated cells of D27N mutant (69%) was higher than that of wild-type (24%). This result indicates that D27N mutation in PB1 contributes to ribavirin resistance. To quantitatively measure the viral polymerase activity, luciferase assay was carried out in the presence of various concentrations of ribavirin. The luciferase activity of D27N mutant was higher than that of wild type (Fig. 16C). IC_{50} of ribavirin for D27N mutant was about 18 μM, while that for wild-type was around 10 μM (Fig. 16C). Furthermore, the expression level of PB1 was confirmed by western blot analysis. The expression level of D27N mutant PB1 were similar to that of wild-type and not affected by the addition of ribavirin (Fig. 16D). To further analyze this mutant, we used methotrexate (MTX). MTX is an inhibitor for purine

biosynthesis, resulting in decrease of purine concentration in cell. The mini-replicon assays were performed in the presence of various concentration of MTX. The viral polymerase activity of D27N was significantly remained even in the presence of MTX compared with that of wild type (Fig. 17).

3. Discussion

PB1 functions as a catalytic subunit of viral RNA polymerase (Biswas et al, 1996; Gozalez et al, 1996; Ohtsu et al, 2002; Zurcher et al, 1996) and contains the highly conserved motifs of RNA-dependent RNA polymerases (Biswas et al, 1994). Putative nucleotide-binding sites have been mapped adjacent to this region (Asano et al, 1997; Kolpashchikov et al, 2004). PB1 also binds to vRNA and cRNA, and the putative promoter binding sites have been mapped (Gozalez et al, 1999a). PA and PB2 interact with the N-terminal and C-terminal regions of PB1, respectively (Ghanem et al, 2007; Gonzalez et al, 1996; Ohtsu et al, 2002; Perez et al, 1995; Toyoda, et al, 1996; Zurcher et al, 1996; Poole et al, 1981). Thus, some structures and functions of PB1 have been clarified (He et al, 2008; Obayashi et al, 2008; Sugiyama et al, 2009). However, the relationship between the structure and function are not clarified. Therefore, to clarify this relationship, I started the functional analysis of PB1 subunit using ribavirin. Ribavirin inhibits viral RNA synthesis as an analog of both adenine and guanine, and causes mutations in RNA dependent replication in RNA viruses as well as influenza viruses. To isolate a ribavirin-resistant PB1 mutant(s), I screened this resistance from the PB1 mutant library by the mini-replicon assay using *EGFP* gene (Fig.

15A). In addition, to know influenza virus RNA polymerase activity of D27N, luciferae assay was carried out in the presence of various concentrations of ribavirin (Fig. 16C). It was shown that the luciferase activity of this mutant was higher than that of wild type. In addition, the viral polymerase activity of D27N was also resistant to MTX treatment compared with wild type (Fig. 17). It is possible that the nucleotide recognition activity of D27N is higher than that of wild-type.

The Asp amino acid at position 27 of PB1 is conserved over 99.9% of 7,259 sequences of PB1 deposited in the NCBI Influenza Sequence Database. It has been reported that D27 is located at upstream of nucleotide binding site of PB1 but not the catalytic active site. Similarly, the ribavirin-resistant mutant of polio virus has a mutation in a domain not belonging to the catalytic domain of viral RNA polymerase (Pfeiffer et al, 2003). D27N is also present within putative vRNA and cRNA promoter binding sites (Gonzalez et al, 1999a; Gonzalez et al, 1999b) (Fig. 3A). The cRNA and vRNA promoter structures are different substantially (Park et al, 2003; Tchatalbachev et al, 2001). Biochemical studies have shown that conformational changes in PB1 of the influenza A virus RNA polymerase lead to the interaction with either vRNA or cRNA (Gonzalez et al, 1999b). Based on previous reports and our findings, the interaction between asparagine at position 27 in PB1 with RNA promoters may lead to the regulation of RNA polymerase activity and the nucleotide recognition of PB1 subunit. This finding is extended for further study about mechanism of nucleotide recognition of influenza viral RNA polymerase.

Chapter IV. Overview and Prospects

The influenza virus genome forms viral ribonucleoprotein (vRNP) complexes with nucleoprotein and viral RNA polymerases, PB1, PB2, and PA subunits. The vRNP complex catalyzes both replication and transcription reactions (Nagata et al, 2008). PB1 contains the motifs highly conserved among RNA-dependent RNA polymerases (Biswas et al, 1994) and functions as a catalytic subunit of RNA chain elongation (Biswas et al, 1996; Gozalez et al, 1996; Ohtsu et al, 2002; Zurcher et al, 1996). (Ghanem et al, 2007; Gonzalez et al, 1996; Ohtsu et al, 2002; Perez et al, 1995; Toyoda, et al, 1996; Zurcher et al, 1996; Poole et al, 1981). Thus, some structures and functions of PB1 have been clarified (He et al, 2008; Obayashi et al, 2008; Sugiyama et al, 2009). However, the relationship between the structure and function are not clarified. Therefore, to clarify this relationship, I started the functional analysis of PB1 subunit using mutant viruses by determining the catalytic active site of PB1 such as sites for polymerization, nucleotide recognition, and so on, with forward genetics and reverse genetics. For forward genetics, to elucidate the functional domain involved in the nucleotide recognition of PB1, I started to isolate ribavirin-resistant mutants. Ribavirin inhibits viral RNA synthesis and may cause mutations in the genome of influenza virus. I isolated D27N and the viral polymerase activity of D27N was higher than that of wild type in the presence of ribavirin (Fig. 16C). In addition, the viral polymerase activity of D27N was also resistant to MTX treatment compared with wild type (Fig. 17). It is possible that the nucleotide recognition

activity of D27N is higher than that of wild-type.

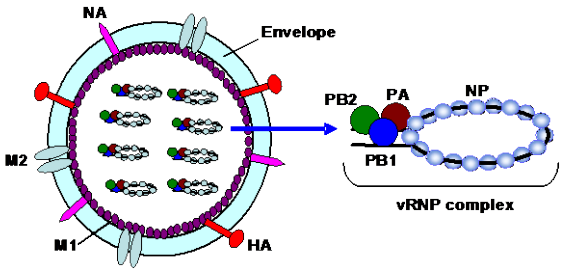
Alignment of amino acid sequences of PB1 subunit showed that this position is not conserved between influenza A and B viruses, this mutation also isolated from H4N8 strain- infected least sandpiper. PB1 subunit of this strain contains asparagine at the position 27 (GenBank: ACI90144.1). In addition, D27N mutant at this position also belongs to cRNA and vRNA promoter binding sites (Fig. 16D). I found that asparagine at position 27 in PB1 is responsible for ribavirin and MTX resistance (Fig. 16C and 17). This finding is extended for further study about mechanism of nucleotide recognition of influenza viral RNA polymerase. For reverse genetics, I analyzed function of PB1 subunit using mutant viruses. The C-terminal region of PB1 between a.a. positions 494-757 contains a putative vRNA promoter binding site, while the N-terminal region of PB1 between a.a. positions 1-83 contains both putative vRNA and cRNA promoter binding sites and PA binding site (Gozalez et al, 1999a) (Fig. 3A). However, except for the PA binding site, the crystal structure and the function of the N-terminal region of PB1 are poorly understood. Here, we have examined the functional structure of the N-terminal region of PB1. The regions between a.a. positions 1-50 are highly conserved between influenza A and B viruses, but amino acids at positions 16, 27, and 44 are different between two viruses (Fig. 3B). To elucidate the functional importance of these amino acids in replication and transcription of the viral genome, I generated viruses containing mutations at these positions by reverse genetics and examined the replication and transcription activities of these mutants. I found that a.a. positions 27 and 44 are responsible for the viral

replication activity but not transcription activity. These positions may affect to the binding between PB1 subunit and cRNA promoter sites, resulting in its catalytic increase or decrease. This findings may be based on for further study about the interaction between PB1 and promoter mutants.

Table 1. Primers for preparation of PB1 mutants

Position	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
N16A	CACAAGCTGCTATAAGCACAACTTTC	CTGGCACTTTTAAGAAAAGTAAAGTCG
N16D	CACAAGATGCTATAAGCACAACTTTC	CTGGCACTTTTAAGAAAAGTAAAGTCG
N16Q	CACAACAAGCTATAAGCACAACTTTC	CTGGCACTTTTAAGAAAAGTAAAGTCG
D27N	CTTATACTGGAAACCCTCCTTAC	GGAAAGTTGTGCTTATAGCATTTTGTGC
D27E	CTTATACTGGAGAGCCTCCTTAC	GGAAAGTTGTGCTTATAGCATTTTGTGC
D27V	CTTATACTGGAGTCCCTCCTTAC	GGAAAGTTGTGCTTATAGCATTTTGTGC
N44I	ATCAGGACACATCAGTACTCAGAAAG	GACAGTATCCATGGTGTATC
N44D	GACAGGACACATCAGTACTCAGAAAG	GACAGTATCCATGGTGTATC
N44Q	CAGAGGACACATCAGTACTCAGAAAG	GACAGTATCCATGGTGTATC

A. Structure of influenza A virus



B. Structure of viral genome

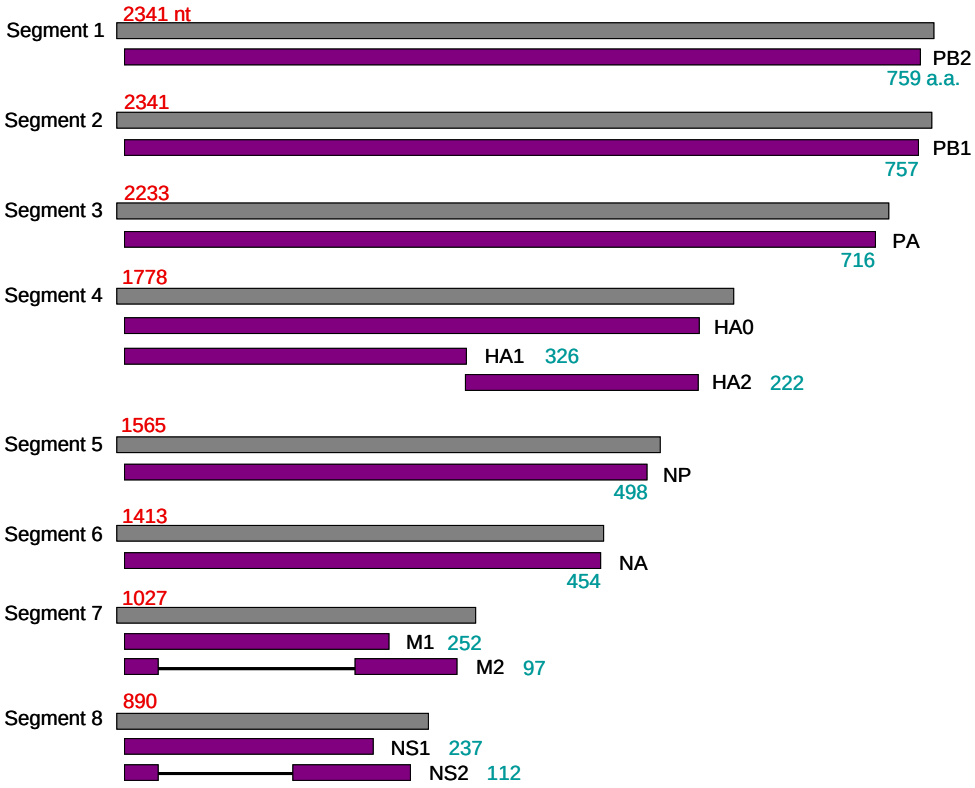


Fig. 1. (A) Structure of influenza A virus. Influenza A virus contains eight segmented and single- and negative-stranded RNAs (vRNA) as its genome. Each segment is encapsidated by NP and associated with the RNA polymerase to form viral ribonucleoprotein (vRNP) complex. The vRNP complex (PB1, PB2, PA, and NP) is a basic unit for both transcription and replication. (B) Structure of viral genome.

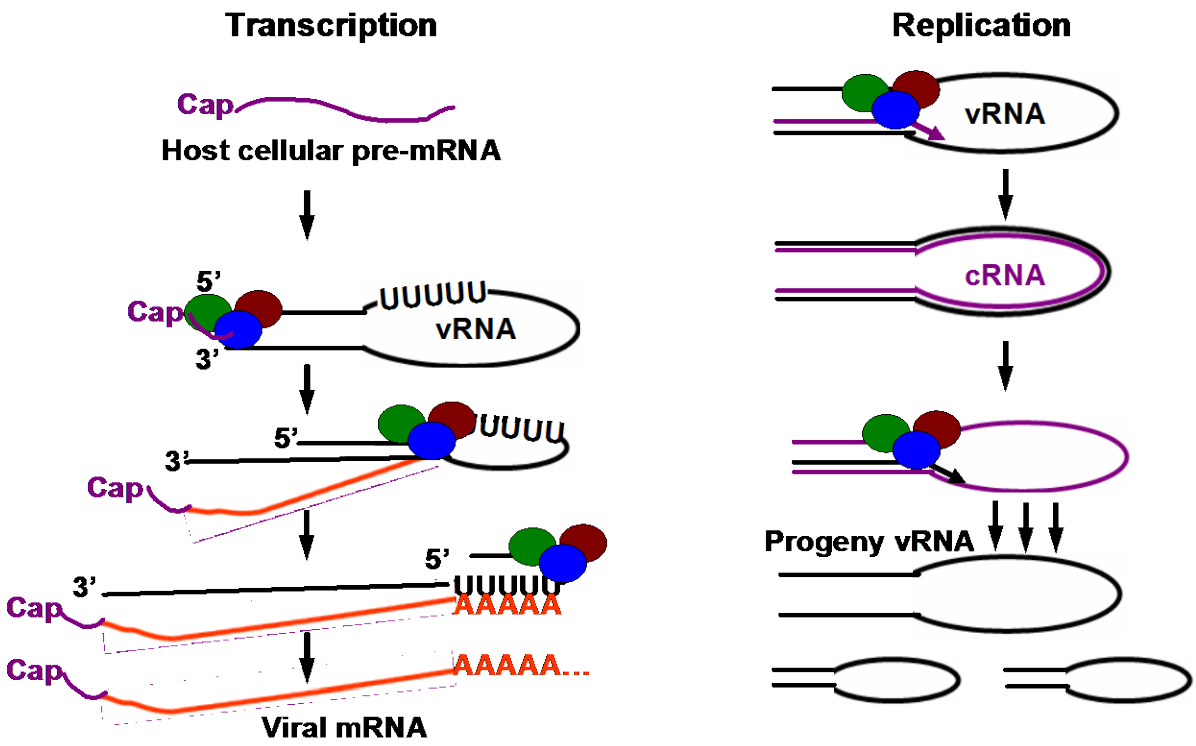
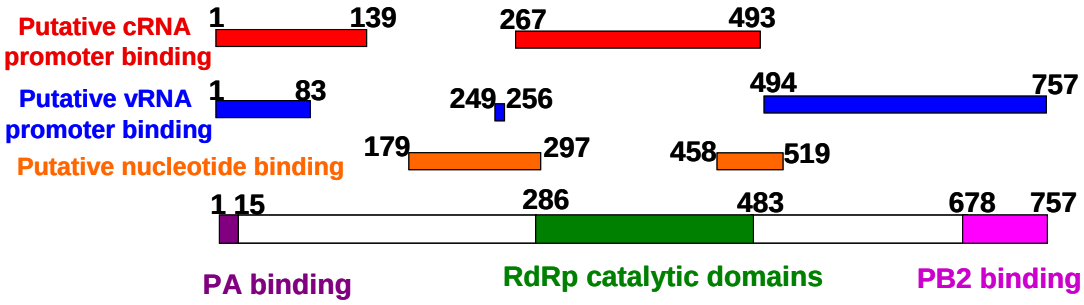


Fig. 2. Transcription and replication of influenza virus. The viral mRNA transcription is initiated using capped oligonucleotide as a primer derived from host cellular pre-mRNA. The elongation of mRNA chain proceeds until the viral polymerase reaches oligo U sequence, and then the polyA tail is added by viral RNA polymerase. In contrast, the viral genome is replicated in a primer-independent manner to generate full-length cRNA from vRNA, and progeny vRNA is copied from the cRNA by the viral polymerase.

A. A schematic representation of PB1



B. Alignment of amino acid sequences of putative vRNA and cRNA promoters among influenza A and influenza B viruses

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                                 16          27                  44
A/WSN/33(H1N1)          MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYS 50
A/England/67(H2N2)      MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYS 50
A/Hong Kong/68(H3N2)    MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYS 50
A/Narita/98 (H9N2)      MDVNPTLLFLKVPVQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYS 50
B/Aichi/88              MNINPYFLFIDVPIQAAISTTFPYTGVPPYSHGTGTGYTIDTVIRTHEYS 50
B/Alaska/92             MNINPYFLFIDVPIQAAISTTFPYTGVPPYSHGTGTGYTIDTVIRTHEYS 50
B/Argentina/01          MNINPYFLFIDVPIQAAISTTFPYTGVPPYSHGTGTGHTIDTVIRTHEYS 50
B/Hong Kong/93          MNINPYFLFIDVPIQAAISTTFPYTGVPPYSHGTGTGYTIDTVIRTHEYS 50
                        *::** :**:.** * ********** **********:*:*** ***:**
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Fig. 3. Structure of PB1 subunit. (A) A diagrammatic representation of PB1. Red bar, cRNA promoter binding sites ; blue bar, vRNA promoter binding sites ; orange bar, nucleotide binding sites ; pink bar, PB2 binding site ; violet bar, PA binding site, and green bar, RNA dependent-RNA polymerase catalytic domains. (B) Alignment of amino acid sequences of putative RNA binding region common to vRNA and cRNA promoters (1-50 a.a.) among influenza A and influenza B viruses. PB1 sequences of A/WSN/33, A/WSN/1933 (H1N1) ; A/England/67, A/England/10/67 (H2N2) ; A/Hong Kong/68, A/Hong Kong/1/1968 (H3N2) ; A/Narita/98, A/parakeet/Narita/92A/98 (H9N2). These strains are picked up from different clades and periods of PB1 gene phylogenetic tree (33); B/Aichi/88, B/Aichi/5/88 ; B/Alaska/92, B/Alaska/03/1992 ; B/Argentina/01, B/Argentina/132/2001 ; and B/Hong Kong/93, B/Hong Kong/02/1993. Sequences were aligned with CLUSTAL W2. Asterisk (*) indicates positions which have a single, fully conserved residue; colon (:) indicates conservation between groups of strongly similar properties-scoring > 0.5 in the Gonnet PAM 250 matrix, and dot (.) indicates conservation between groups of weakly similar properties-scoring =< 0.5 in the Gonnet PAM 250 matrix.

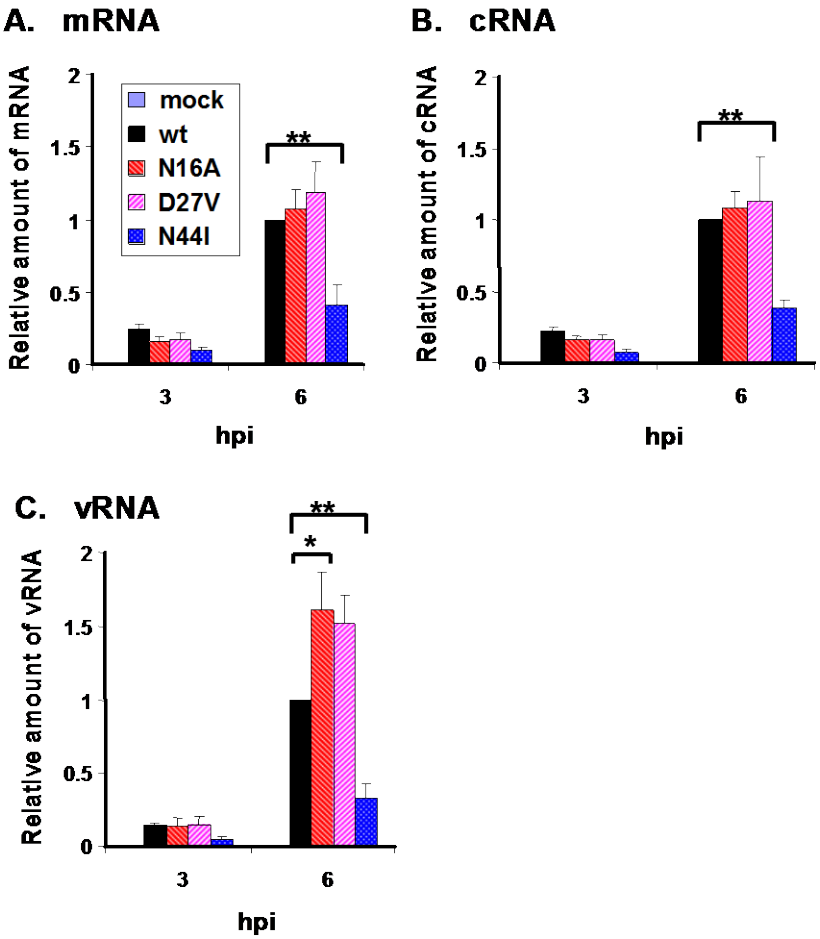


Fig. 4. Comparison of RNA synthesis of mutant influenza A viruses containing amino acids specific for influenza B viruses. The amino acids, N, D, and N at the positions 16, 27, and 44 were mutated to A, V, and I, respectively. MDCK cells were infected with mutant viruses at MOI of 2.5 and incubated at 37^{o}C with 5% CO_2. At 3 and 6 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

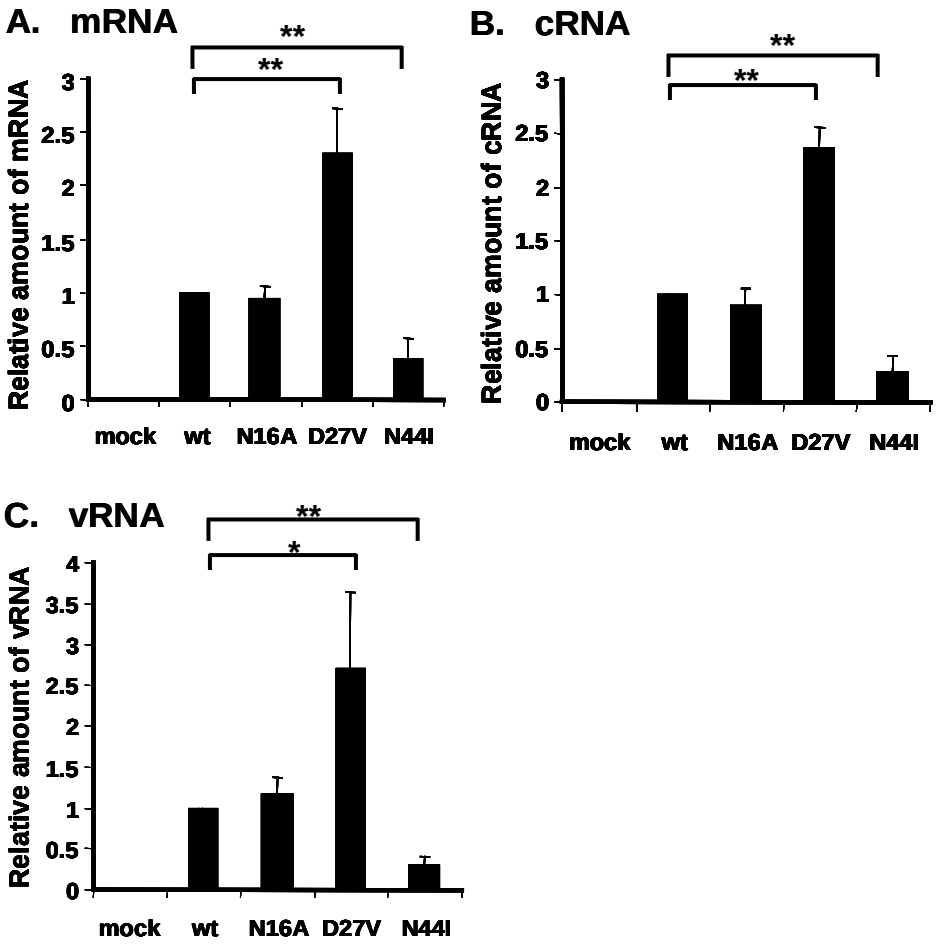


Fig. 5. Comparison of RNA synthesis of mutant influenza A viruses containing amino acids specific for influenza B viruses. The amino acids, N, D, and N at the positions 16, 27, and 44 were mutated to A, V, and I, respectively. MDCK cells were infected with mutant viruses at MOI of 2.5 and incubated at 37^{o}C with 5% CO2. At 9 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

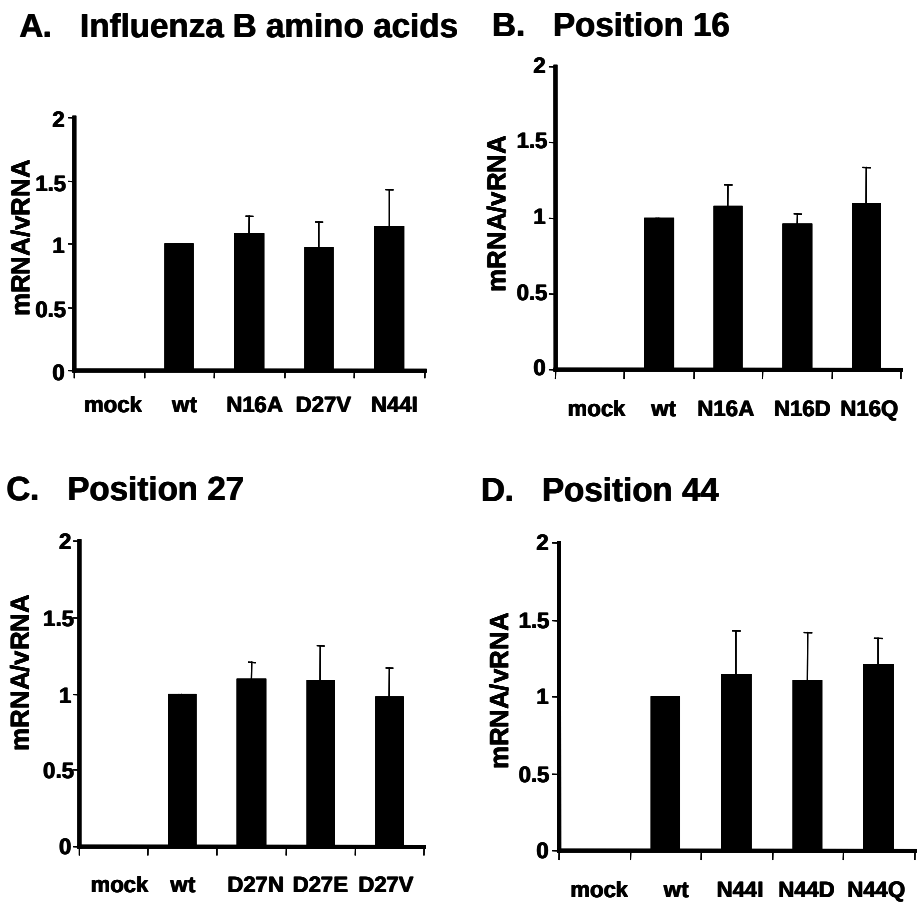


Fig. 6. Primary transcription activity of mutant influenza A viruses containing amino acids specific to influenza B viruses (A), amino acid position 16 (B), amino acid position 27 (C), and amino acid position 44 (D). MDCK cells were infected with mutant viruses at MOI of 2.5 and incubated at 37^{o}C with 5% CO_2 in the presence of 100 μg/ml of cycloheximide. At 9 hpi, the accumulation levels of viral mRNA and vRNA were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. The transcription activity is represented as a ratio of the amount of viral mRNA to that of vRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test.

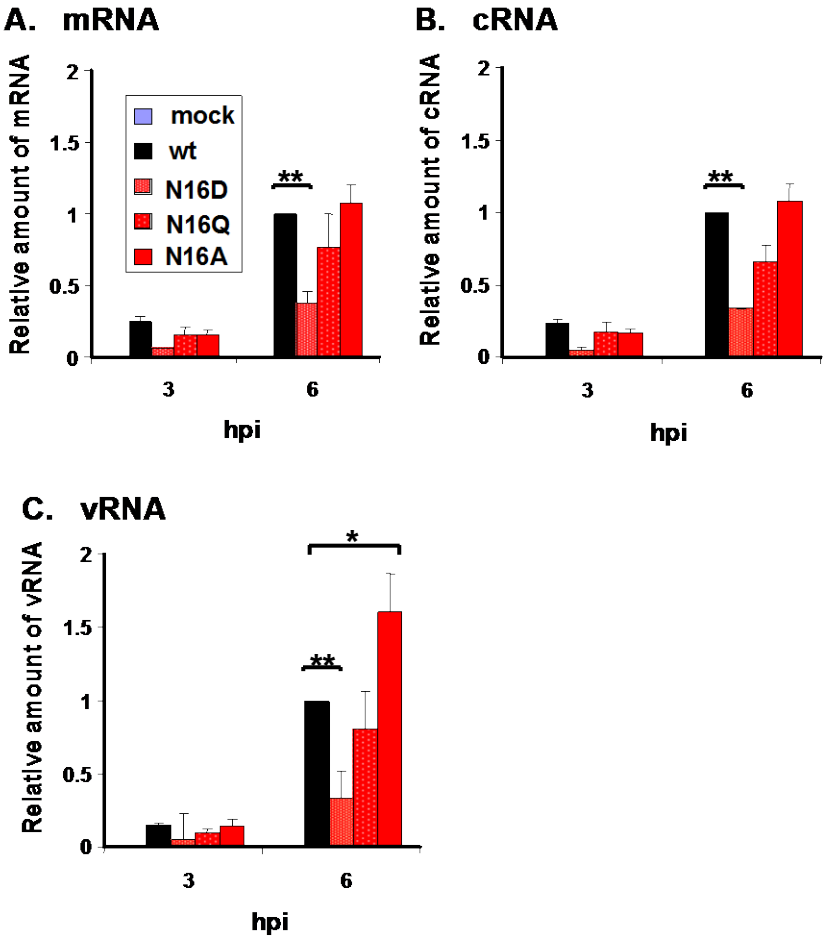


Fig. 7. RNA synthesis of viruses containing mutations at the amino acid position 16. Wild-type virus and mutant viruses containing amino acids N and D, Q, and A, respectively, at the a.a. position 16 were infected into MDCK cells at MOI and incubated at 37^{o}C with 5% CO_2. At 3 and 6 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

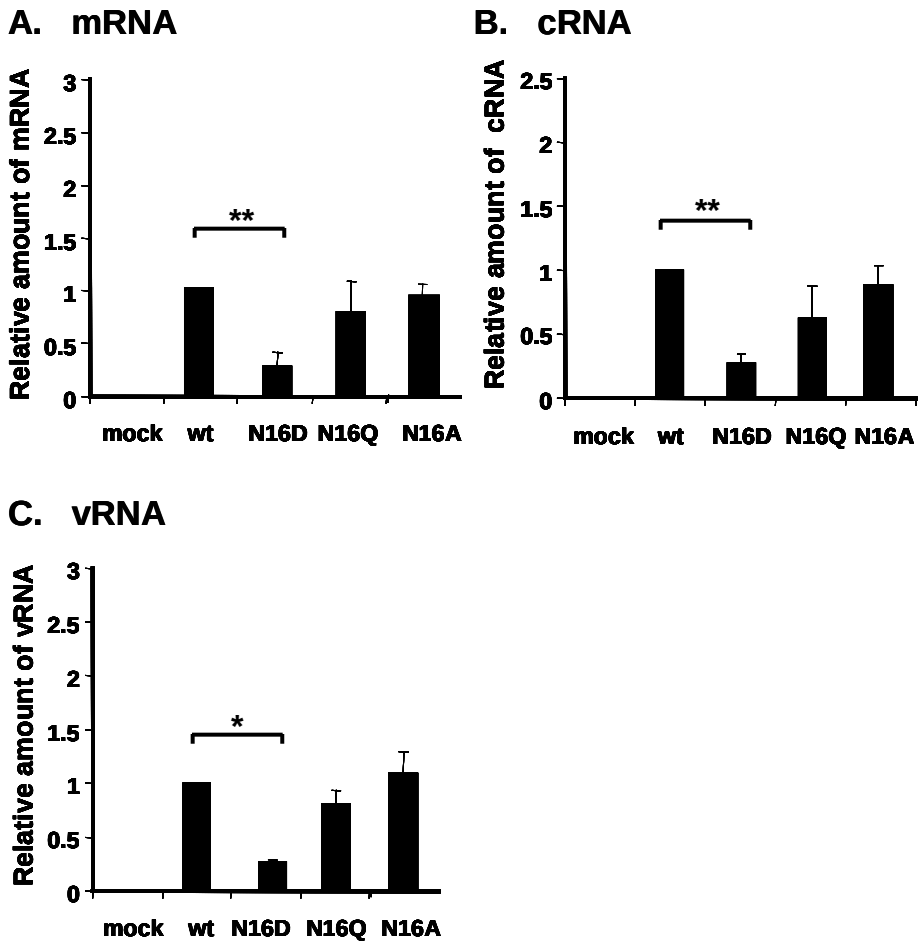


Fig. 8. RNA synthesis of viruses containing mutations at the amino acid position 16. Wild-type virus and mutant viruses containing amino acids N and D, Q, and A, respectively, at the a.a. position 16 were infected into MDCK cells at MOI and incubated at 37^{o}C with 5% CO_2. At 9 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

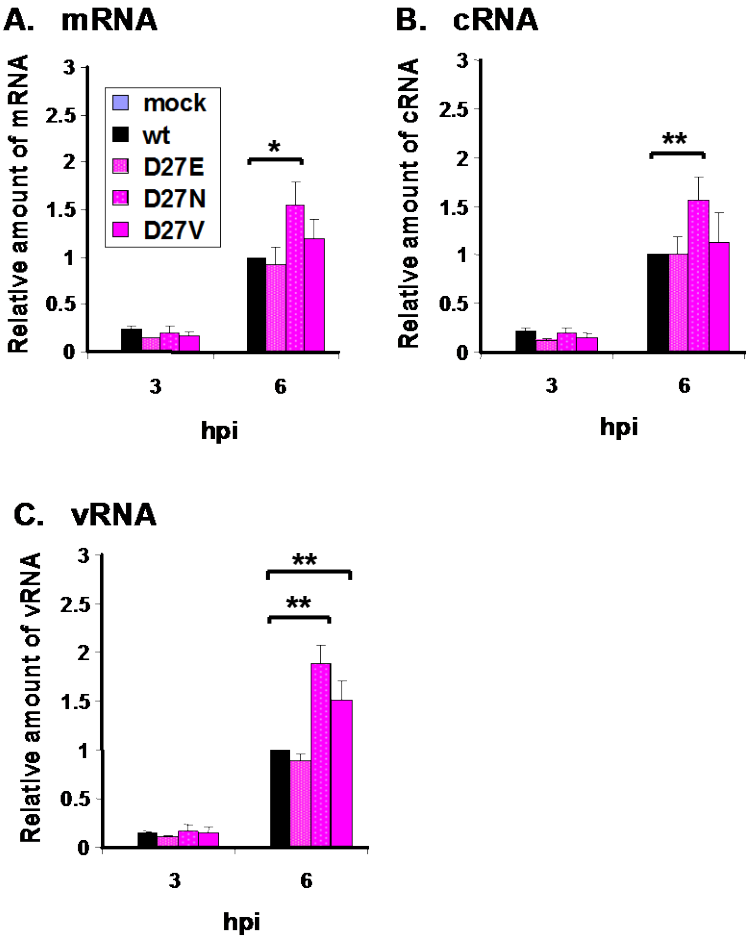


Fig. 9. RNA synthesis of viruses containing mutations at the amino acid position 27. Wild-type virus and mutant viruses containing amino acids D and E, N, and V, respectively, at the a.a. position 27 were infected into MDCK cells at MOI and incubated at 37^{o}C with 5% CO_2. At 3 and 6 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

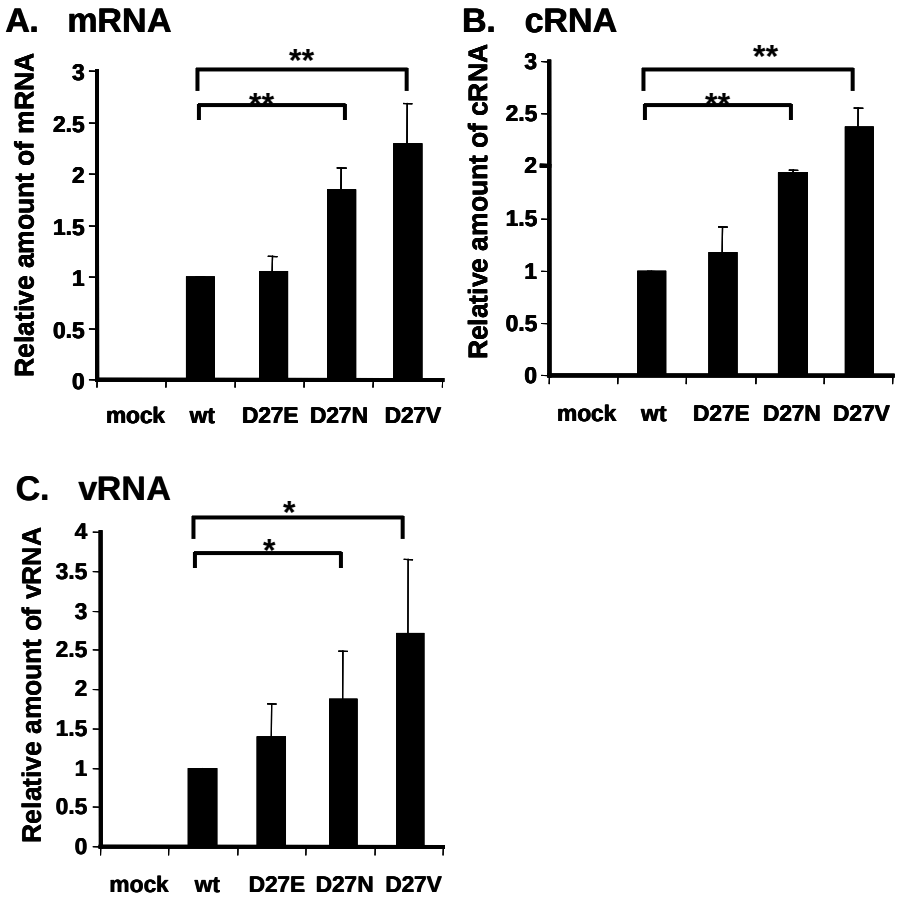


Fig. 10. RNA synthesis of viruses containing mutations at the amino acid position 27. Wild-type virus and mutant viruses containing amino acids D and E, N, and V, respectively, at the a.a. position 27 were infected into MDCK cells at MOI and incubated at 37^{o}C with 5% CO_2. At 9 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

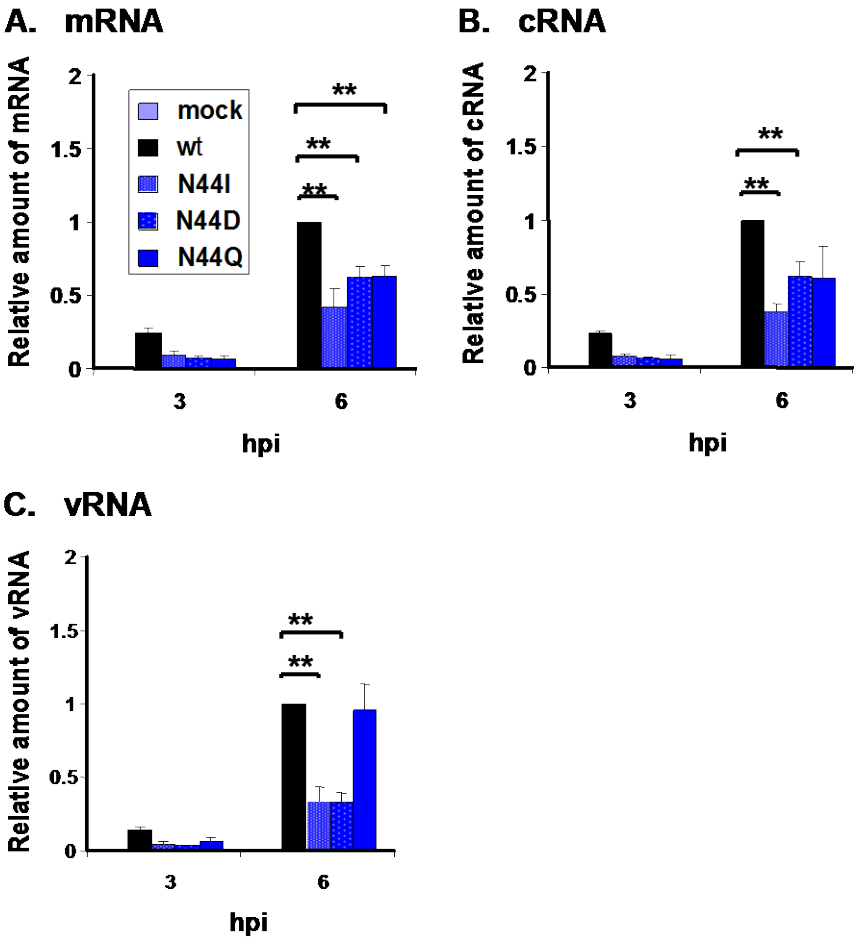


Fig. 11. RNA synthesis of viruses containing mutations at the amino acid position 44. Wild-type virus and mutant viruses containing amino acids N and I, D, and Q, respectively, at the a.a. position 44 were infected into MDCK cells at MOI and incubated at 37^{o}C with 5% CO_2. At 3 and 6 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

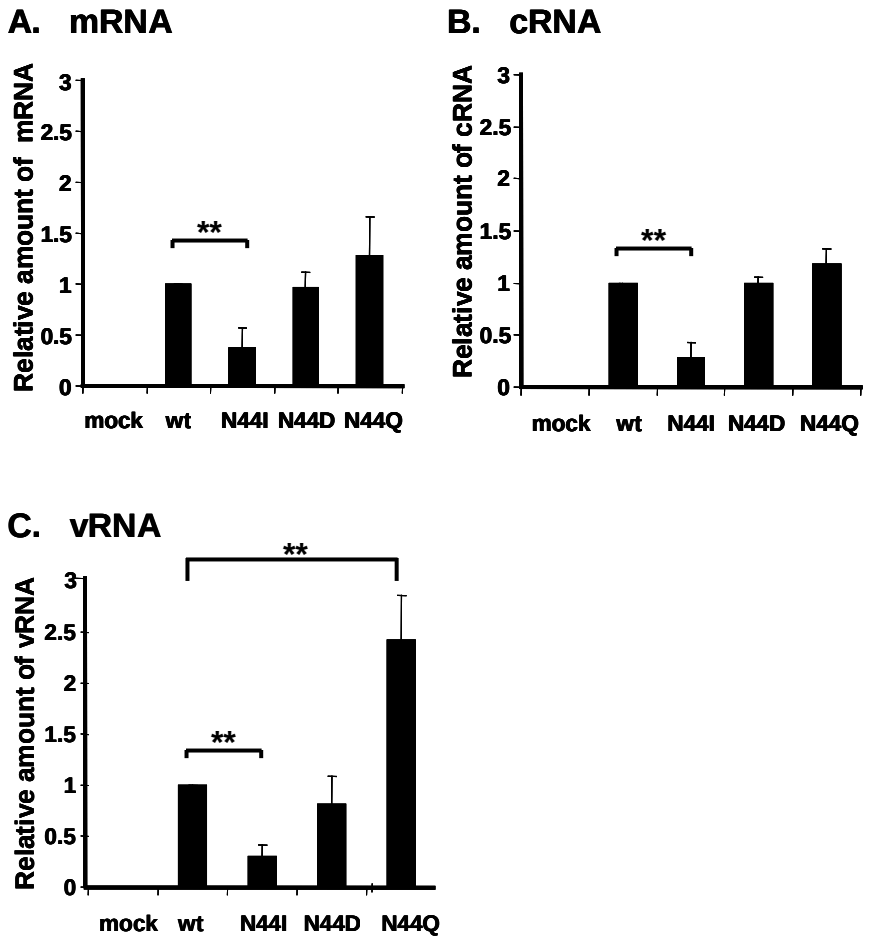


Fig. 12. RNA synthesis of viruses containing mutations at the amino acid position 44. Wild-type virus and mutant viruses containing amino acids N and I, D, and Q, respectively, at the a.a. position 44 were infected into MDCK cells at MOI and incubated at 37^{o}C with 5% CO_2. At 9 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

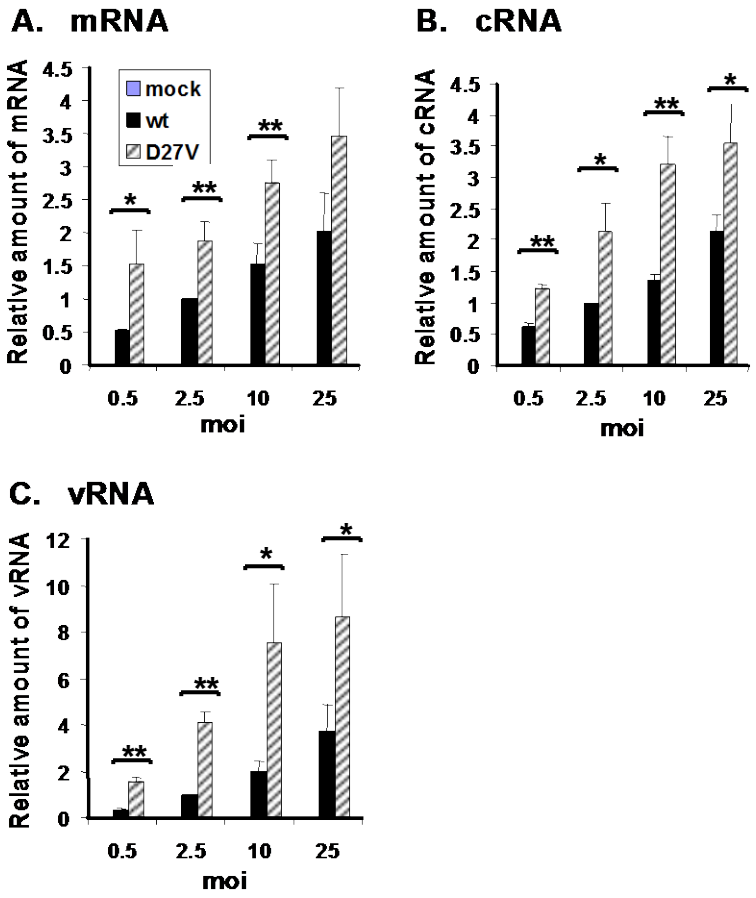


Fig. 13. RNA synthesis of D27V virus at various moi. Wild-type virus and mutant virus containing V at the a.a. position 27 were infected into MDCK cells at various MOI (0.5, 2.5, 10, and 25) and incubated at 37^{o}C with 5% CO_2. At 9 hpi, the accumulation levels of viral mRNA (A), cRNA (B), and vRNA (C) were measured by qPCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations, and the level of significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

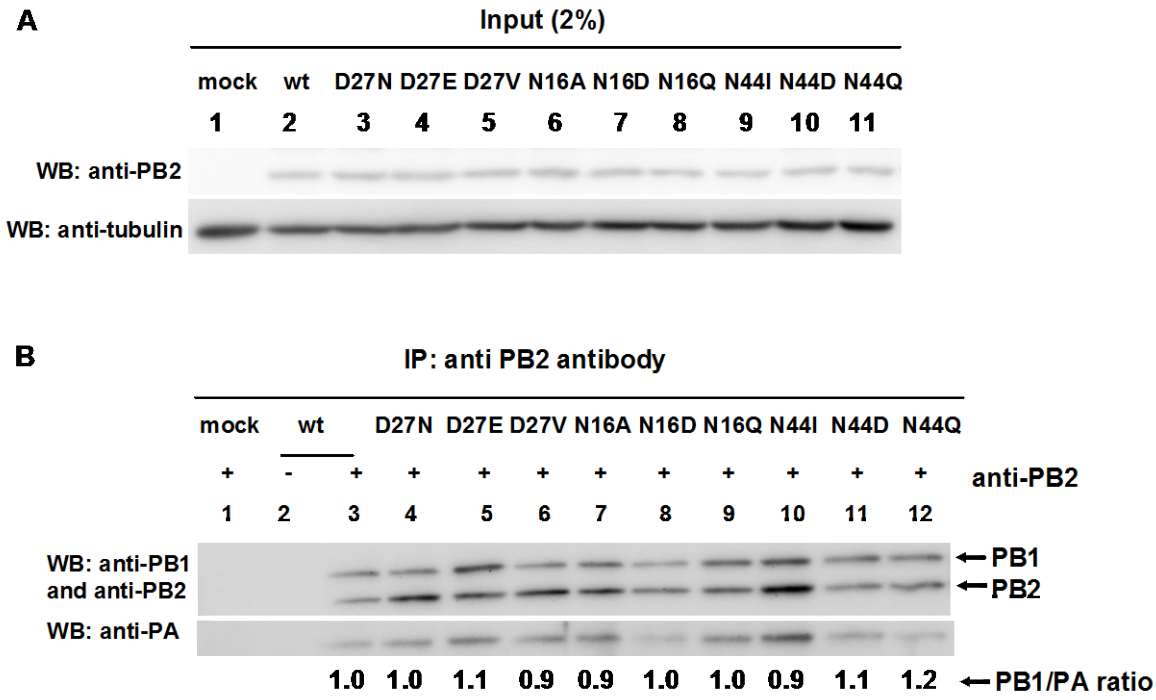
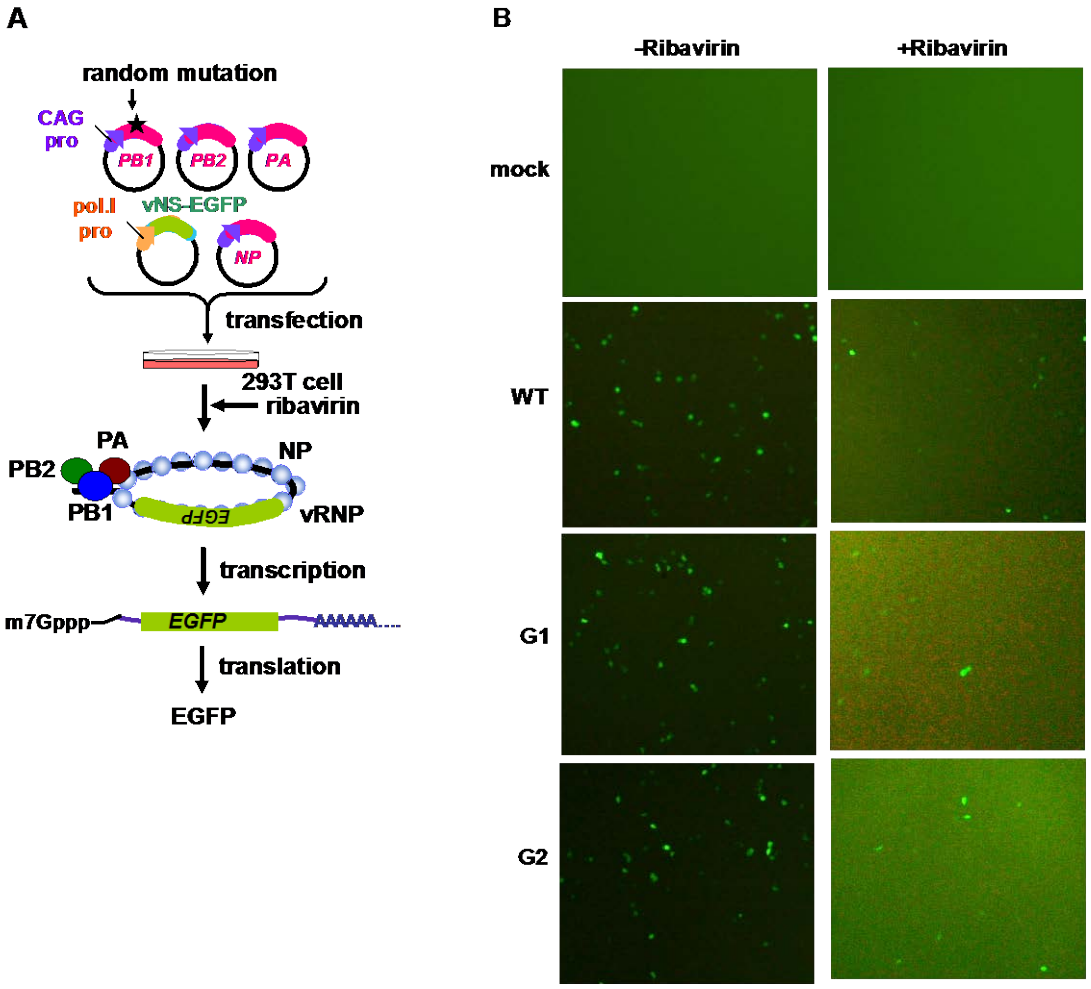


Fig. 14. Effect of mutations on assembly of viral RNA polymerase complexes. MDCK cells were infected with wild type or mutant viruses at MOI of 1 PFU. At 7 hpi, cells were lysed by sonication. RNA polymerase complexes in the supernatant fraction were subjected to immunoprecipitation with rabbit anti-PB2 antibody. Immunoprecipitated proteins were subjected to western blotting analysis. (A) Expression levels of PB2 in infected cells. The amount of PB2 and β-tubulin were determined by western blotting. (B) The interactions of PB2 and PA with PB1 in infected cells. Immunoprecipitation was carried out with (lanes 1 and 3-12) or without (lane 2) anti-PB2. PB1, PB2, and PA in the immunoprecipitated complex were detected by western blotting. The ratio of the amount of PB1 to that of PA is shown under each lane.


A
random mutation
CAG pro
PB1
PB2
PA
pol.I pro
vNS-EGFP
NP
transfection
293T cell
ribavirin
PA
PB2
NP
PB1
EGFP
vRNP
transcription
m7Gppp
EGFP
AAAAAA
translation
EGFP
B
-Ribavirin
+Ribavirin
mock
WT
G1
G2

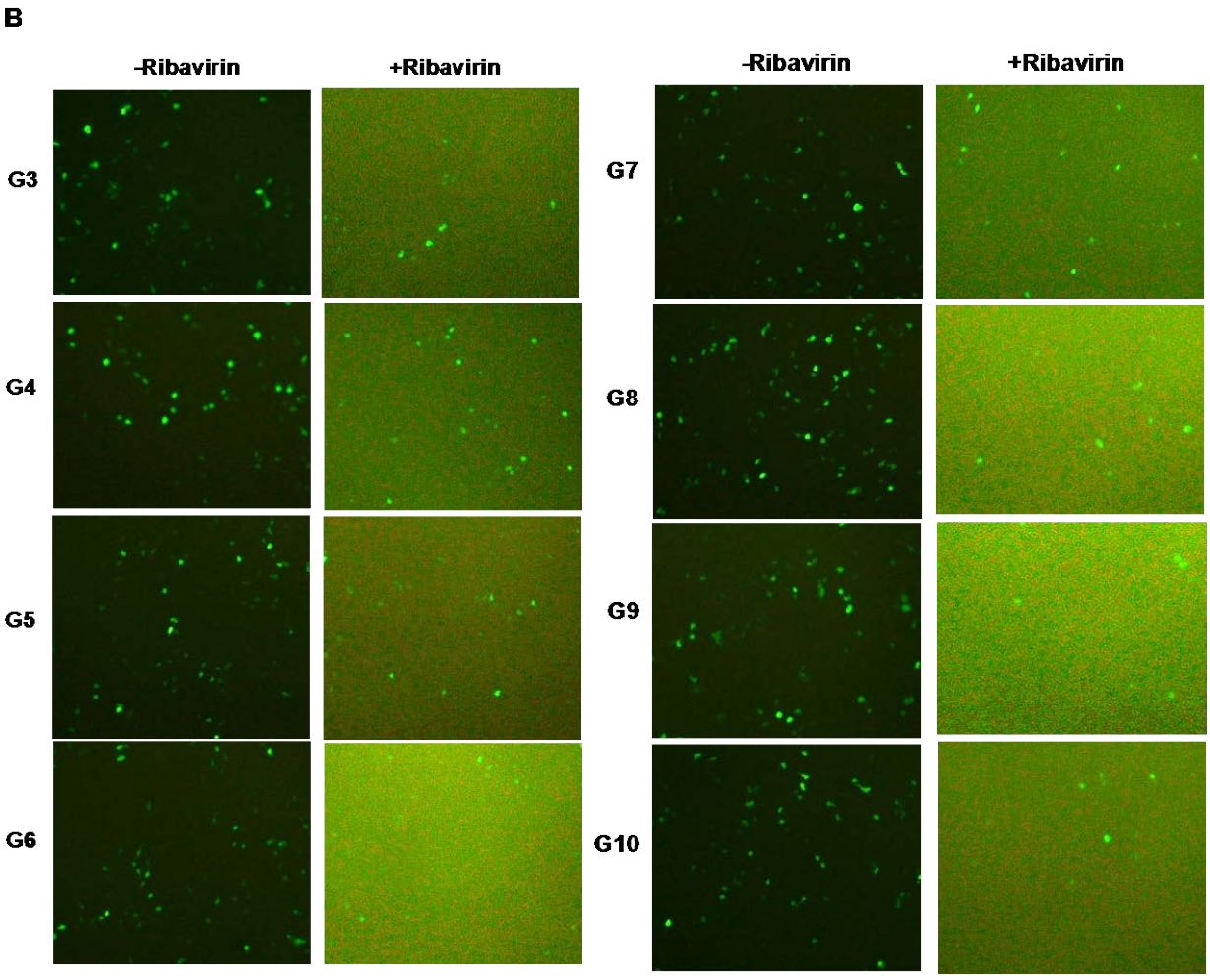

B
-Ribavirin
+Ribavirin
-Ribavirin
+Ribavirin
G3
G4
G5
G6
G7
G8
G9
G10

Fig. 15. Screening of ribavirin-resistant PB1 mutant. (A) Assay system for screening by mini-replicon assay. 293T cells were transfected with plasmids for the expression of viral proteins, PB1 (wt or mutated-type of each group), PB2, PA, and NP, and a plasmid for the expression of artificial influenza virus genome containing *EGFP* gene of negative polarity. (B) EGFP fluorescence in the first screening. After 3 hours of transfection, ribavirin was added (0 or 75 μM) and the fluorescence of EGFP was observed at 15 hpt. G1-G10 indicate each groups divided mutated PB1 library.

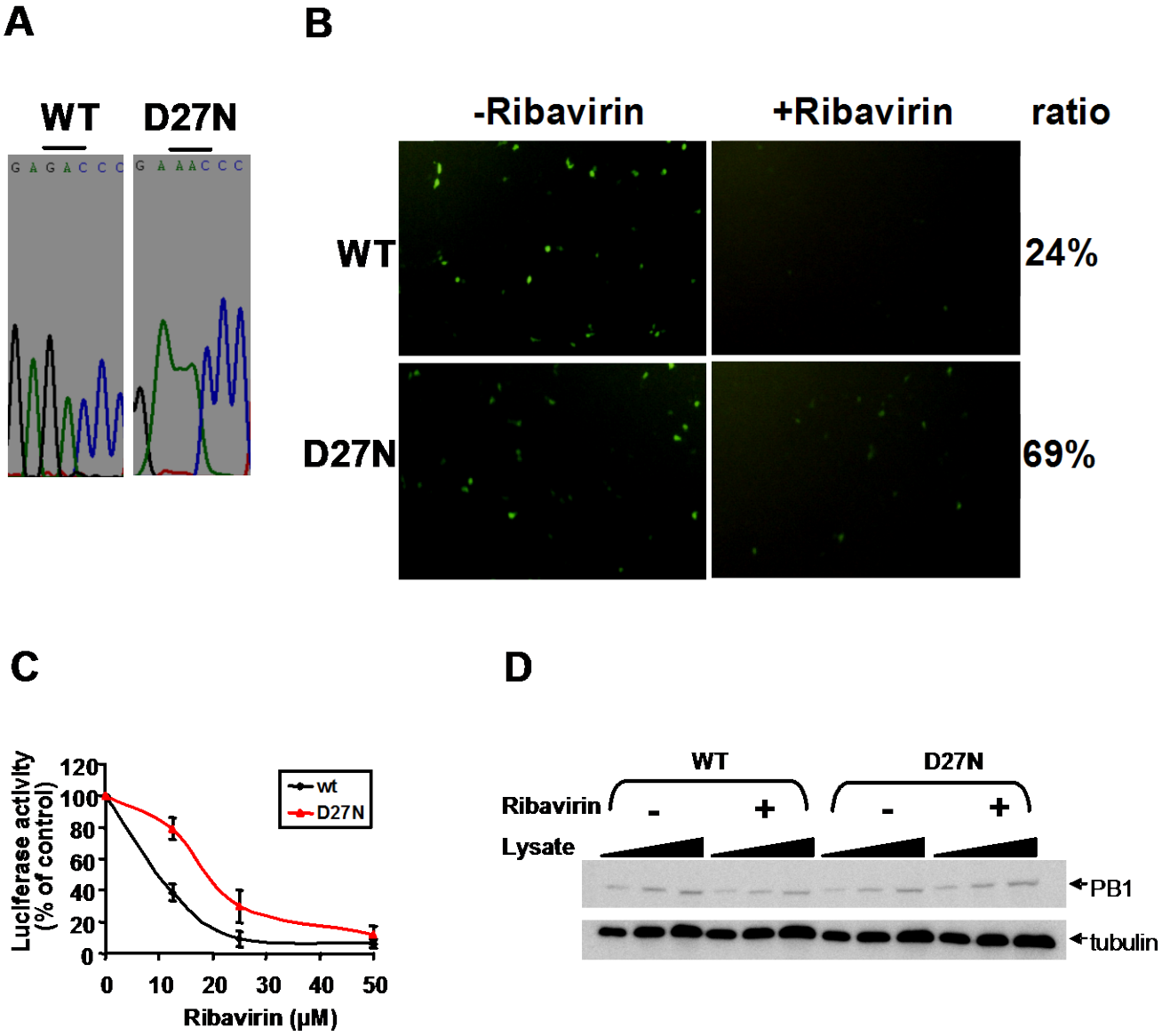

A
WT
D27N
G A G A C C C
G A A A C C C
B
-Ribavirin
+Ribavirin
ratio
WT
24%
D27N
69%
C
Luciferase activity (% of control)
120
100
80
60
40
20
0
wt
D27N
0
10
20
30
40
50
Ribavirin (μM)
D
WT
D27N
Ribavirin
-
+
-
+
Lysate
PB1
tubulin

Fig. 16. Ribavirin-resistance of D27N mutant by mini-replicon assay system. (A) Sequencing of ribavirin-resistant PB1 mutant. (B) Mini-replicon assay using *EGFP* gene as a reporter gene. After 3 hours of transfection, ribavirin was added (0 or 75 μM) and the fluorescence of EGFP was observed at 15 hpt. The ratio of the ribavirin presence and absence in the medium was shown in right side. (C) Mini-replicon assay using *luciferase* gene as a reporter gene was carried out. After 3h of transfection, ribavirin different concentrations 0, 12.5, 25, and 50 were added, and luciferase activity was measured at 15 hpt. % control indicates that the percentage of ribavirin-treated and-untreated cells per ribavirin-untreated cells (D) Effect of D27N mutation on assembly of PB1 subunit. Mini-replicon assay using *luciferase* gene as a reporter gene was carried out. After 3 hours of transfection, ribavirin was added (0 or 50 μM). At 15 hpt, cells were lysed and this lysates were subjected to western blot analysis using anti-PB1 antibody and antibody against β-tubulin.

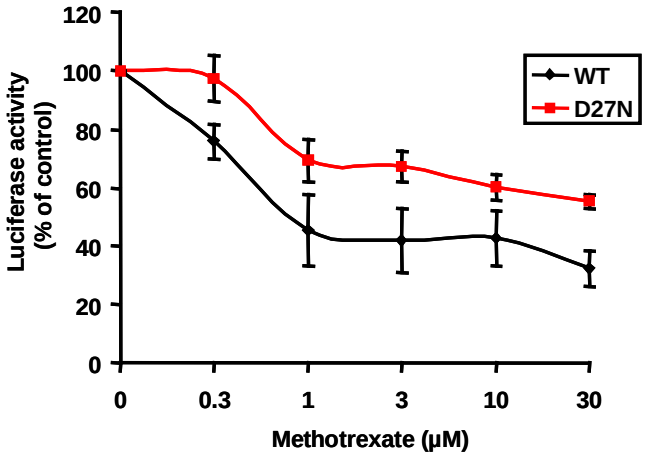


Fig. 17. Methotrexate-resistance of D27N mutant by mini-replicon assay system. Mini-replicon assay using *luciferase* gene as a reporter gene was carried out. At 12 hpt, different concentrations (0, 0.3, 1, 3, 10, and 30 μM) of methotrexate were added, and luciferase activity was measured at 22 hpt. % of control indicates that the percentage of the luciferase activity from methotrexate -treated and -untreated cells per methotrexate -untreated cells.

Acknowledgments

I am greatly indebted to the following people, without whose invaluable help and kindness.

The first person I would like to find some way of expressing my gratitude to Professor Kyosuke Nagata, my research advisor who has provided the best opportunity for me to conduct this thesis.

I would like to gratefully thank Assitant Professor Atsushi Kawaguchi and Ms. Chitose Wakai, who supervise me to complete this thesis.

My sincerely thanks are also extended to all of my laboratory members for their help in running the experiment and their useful recommendations.

I also would like to thank all my Vietnamese friends who study in University of Tsukuba for helping me to adapt to new environment.

Finally, I would like to offer my special thanks for my family.

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