

# Involvement of the N-terminal portion of influenza virus RNA polymerase subunit PB1 in nucleotide recognition

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1 **Title:** Involvement of the N-terminal portion of influenza virus RNA polymerase  
2 subunit PB1 in nucleotide recognition

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16

1 **ABSTRACT**

2

3       The influenza virus PB1 protein functions as a catalytic subunit of the viral  
4 RNA-dependent RNA polymerase and contains the highly conserved motifs of  
5 RNA-dependent RNA polymerases together with putative nucleotide-binding sites.  
6 PB1 also binds to viral genomic RNAs and its replicative intermediates through  
7 the promoter regions. The detail function and interplay between functional  
8 domains are not clarified although a part of structures and functions of PB1 have  
9 been clarified. In this study, we analyzed the function of PB1 subunit in the sense  
10 of nucleotide recognition using ribavirin, which is a nucleoside analog and inhibits  
11 viral RNA synthesis of many RNA viruses including influenza virus. We  
12 screened ribavirin-resistant PB1 mutants from randomly mutated PB1 cDNA  
13 library using a mini-replicon assay, and we identified a single mutation at the  
14 amino acid position 27 of PB1 as an important residue for the nucleotide  
15 recognition.

16

17

18 **Keywords:** influenza/ nucleotide recognition/ resistant mutant/ ribavirin

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## 1 **1. Introduction**

2

3           Influenza A virus belongs to the family of *Orthomyxoviridae*. Its genome  
4 consists of eight-segmented and single-stranded RNAs of negative polarity  
5 (vRNA). Each segment is encapsidated by nucleoprotein (NP) and associated with  
6 viral RNA polymerases to form viral ribonucleoprotein (vRNP) complexes. The  
7 vRNP complex is a basic unit for both transcription and replication [1]. The viral  
8 mRNA transcription is initiated using capped oligonucleotide as a primer. The  
9 elongation of mRNA chain proceeds until the viral polymerase reaches oligo U  
10 sequence present near the 5'-terminus of vRNA, and then the poly A tail is added  
11 by the viral RNA polymerase. In the viral genome replication, full-length cRNA  
12 (complementary RNA to vRNA) is generated from vRNA in a primer-independent  
13 manner, and progeny vRNAs are amplified from cRNA by the viral RNA  
14 polymerase. The viral RNA polymerase consists of PB1, PB2, and PA. PB1  
15 functions as a catalytic subunit and the assembly core of the viral RNA  
16 polymerase [2,3,4,5,6,7]. PA is genetically found to be involved in the replication  
17 process and the polymerase assembly [8] and have the endonuclease activity  
18 [9,10,11,12]. PB2 is responsible for the recognition and binding of the cap  
19 structure [1,13,14,15,16].

20           The 14 amino acids residues from the N-terminus of PB1 interact with PA  
21 [4,5,6,7,17,18,19,20], while the C-terminal region of PB1 between amino acid  
22 (a.a.) positions 678-757 interacts with PB2 [4,5,6,21,22]. PB1 contains the motifs

1 highly conserved among RNA-dependent RNA polymerases [2]. There are two  
2 putative nucleotide-binding sites between a.a. positions 179-297 and 458-519  
3 [23,24]. Moreover, the N-terminal (a.a. positions 1-83) and C-terminal (a.a.  
4 positions 494-757) regions of PB1 are suggested to interact with the vRNA  
5 promoter [25]. In addition, the a.a. positions 249-254 of PB1 is important for the  
6 vRNA binding, and Phe251 (when the number indicates the amino acid position)  
7 and Phe254 are essential for this binding [26]. It is also reported that the regions  
8 between a.a. positions 1-139 and 267-493 bind to the cRNA promoter [27].

9 Ribavirin (1- $\beta$ -D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as  
10 Virazole) is a synthetic purine nucleoside analogue first synthesized by Sidwell  
11 and colleagues in 1972 [28]. It is phosphorylated by cellular adenosine kinases  
12 into ribavirin monophosphate, diphosphate, and triphosphate (RMP, RDP, and  
13 RTP, respectively) [29,30]. Ribavirin inhibits various RNA-dependent RNA  
14 polymerases such as those from influenza virus [31], vesicular stomatitis virus  
15 [32], La Crosse virus [33], Hantaan virus [35], Foot and mouth disease virus [36],  
16 West Nile virus [37], Andes virus [39], and Hepatitis C virus [40]. In contrast,  
17 ribavirin does not inhibit cellular RNA polymerase I, RNA polymerase II, and  
18 poly (A) polymerase [41]. Ribavirin inhibits the inosine monophosphate  
19 dehydrogenase, so that the *de novo* synthesis of purine nucleosides is interrupted  
20 [42]. Further, it is proposed that ribavirin inhibits the RNA capping and RNA  
21 polymerization by virus-encoded enzymes [42]. It is also known that since  
22 ribavirin forms hydrogen bonds with cytidine and uridine, the incorporation of

1 ribavirin into viral genomic RNA induces G to A transition leading to the lethal  
2 mutations [29].

3 In this study, to elucidate functional residues required for the PB1 activity,  
4 we tried to isolate PB1 mutants which are resistant to ribavirin. We found that the  
5 amino acid position 27 of PB1 is important for nucleotide recognition.

6

## 1 **2. Materials and methods**

2

### 3 *2.1. Biological materials*

4

5 Monolayer cultures of 293T and MDCK cells were maintained at 37°C in  
6 Dulbecco's Modified Eagle Medium (DMEM) and minimal essential medium  
7 (MEM) (Nissui), respectively, supplemented with 10% fetal bovine serum  
8 (Bovogen). Influenza virus strain A/WSN/33 was prepared as previously  
9 described [8]. Ribavirin (Sigma) was dissolved in water to make stock of 100 µM.

10

### 11 *2.2. PBI random mutagenesis*

12

13 For construction of a mammalian expression vector for PB1 containing  
14 random mutations, we used a PCR-based cloning strategy in the presence of  
15 MnCl<sub>2</sub>. cDNA corresponding to the full-length PB1 was amplified with specific  
16 primers 5'-CCCCAAGCTTGCCGCCACCATGGATGTCAATCCGACCTT-3'  
17 and 5'-CATGCGGCCGCCTATTTTTGCCGTCTGAGCTCTT-3'. The PCR  
18 product was then cloned into the *Hind* III and *Not* I sites of pEGFP-N1 and  
19 replaced *EGFP* gene with mutated *PBI* cDNA. The mutation rate of the plasmid  
20 library was confirmed by sequencing randomly selected 20 clones using specific  
21 primer 5'-GGAAGGCTCATAGACTTCCTTA-3', which is corresponding to the  
22 nucleotide position from 560 to 1050 of segment 2. The plasmid library was then

1 used to analyze the influenza virus RNA polymerase activity in a mini-replicon  
2 assay system.

3

### 4 *2.3. Mini-replicon assay system*

5

6 293T cells were transfected with plasmids for the expression of viral  
7 proteins, PB1 (wild-type or mutants), PB2, PA, and NP, and a plasmid for the  
8 expression of artificial influenza virus genome containing either *EGFP* gene (for  
9 screening) or the *firefly luciferase* gene (for luciferase assay) of negative polarity,  
10 which is synthesized in cells by the human DNA-dependent RNA polymerase I  
11 [43]. The mRNAs encoding either *EGFP* or *luciferase* genes are transcribed in a  
12 viral RNA polymerase-dependent manner. For the screening, ribavirin was added  
13 (0 or 75  $\mu$ M) after 3 hours post transfection (hpt), and the fluorescence of EGFP  
14 was observed at 15 hpt. For the luciferase assay, ribavirin was added in the  
15 medium at various concentrations after 3 hpt, incubated at 37°C for 15 h, and then  
16 the luciferase activity was determined using commercially available reagents  
17 (Promega) according to the manufacturer's protocol. The relative luminescence  
18 intensity was measured with a luminometer for 20 sec. A plasmid for the  
19 expression of *Renilla* luciferase driven by the simian virus 40 (SV40) promoter  
20 was used as an internal control for the dual-luciferase assay. As a negative control,  
21 293T cells were transfected with the same plasmids, except for the PB1 expression  
22 plasmid.



## 1 **3. Results**

2

### 3 *3.1. Screening of ribavirin-resistant PB1 mutants*

4

5 To determine the 50% inhibitory concentration (IC<sub>50</sub>) of ribavirin, we carried  
6 out plaque assays with WSN-infected MDCK cells in the presence of various  
7 concentrations of ribavirin (Fig. 1A). Based on the results, we determined that  
8 IC<sub>50</sub> and IC<sub>90</sub> of ribavirin on influenza virus were 20 μM and 75 μM, respectively.

9 To make mutated cDNA library of PB1, random mutagenesis was carried out  
10 by PCR in the presence of 0.1 mM of Mn<sup>2+</sup> and 1.5 mM of Mg<sup>2+</sup> as described in  
11 Materials and methods. To know the mutation frequency of this library, we  
12 transformed the library into *E.coli* DH5α high competent cells and obtained 3 x  
13 10<sup>4</sup> colonies. To evaluate the mutation frequency, plasmids were isolated from 20  
14 independent colonies and sequenced between the nucleotide positions 560 and  
15 1055 of *PB1* gene. The results of sequencing showed that approximately 4.7  
16 mutations were introduced in 2,274 nucleotides of *PB1* gene on average  
17 (approximately 1-2 a.a./PB1 protein). Based on this in hand, we started screening  
18 of ribavirin-resistant PB1 from the mutated cDNA library as shown in Fig. 1B. At  
19 first, this library was divided into 10 groups (Group 1 to 10), and mini-replicon  
20 assays were performed in the presence of ribavirin at IC<sub>90</sub> (Fig. 1C), and thereby  
21 EGFP-positive cells were hardly found in wild-type PB1 transfected cells. In  
22 contrast, in the case of cDNA library-transfected cells, approximately 10 to 30%

1 of EGFP-positive cells were found. Among them, 34% of one of groups, Group 4-  
2 transfected cells were resistant to ribavirin on average. Thus, Group 4 was further  
3 divided into additional 10 groups and subjected to the mini-replicon assays. After  
4 enrichment by 5 time-repetitions of this cycle, we could isolate a single clone  
5 showing the resistance to ribavirin. Even in the presence of IC<sub>90</sub> of ribavirin, 69%  
6 of the isolated clone-transfected cells were EGFP positive (Fig. 2A). By  
7 sequencing of the isolated clone, we found one nucleotide substitution from G to  
8 A at nucleotide position of 103 (where the 5' terminal nucleotide of cRNA is  
9 referred to as nucleotide position 1). This nucleotide change leads to an amino  
10 acid change from Asp to Asn at amino acid position 27 (Fig. 2B).

11

### 12 3.2. Characterization of D27N mutant

13

14 To quantitatively measure the influenza virus RNA polymerase activity, the  
15 mini-replicon assays with the artificial genome containing *luciferase* gene was  
16 carried out in the presence of 12.5, 25, and 50  $\mu$ M of ribavirin, respectively. The  
17 luciferase activity of D27N mutant remained even in the presence of ribavirin  
18 compared with that of wild type. IC<sub>50</sub> of D27N to ribavirin was about 18  $\mu$ M,  
19 while that of wild-type was around 10  $\mu$ M (Fig. 2C). Furthermore, the expression  
20 level of D27N was confirmed by western blot analysis. The expression level of  
21 D27N was unchanged compared with that of wild-type even in the absence or  
22 presence of 50  $\mu$ M ribavirin (Fig. 2D).

1           To further characterize this mutant, we used methotrexate (MTX). MTX is  
2 an inhibitor for purine biosynthesis, resulting in decrease of intracellular purine  
3 concentration. The mini-replicon assays were performed in the presence of  
4 various concentrations of MTX. The viral polymerase activity of D27N was  
5 significantly more than that of wild-type even in the presence of MTX (Fig. 3).

## 1 **4. Discussion**

2

3 PB1 functions as a catalytic subunit of viral RNA polymerase [2,3,4,5,6,7]  
4 and contains the highly conserved motifs of RNA-dependent RNA polymerases  
5 [2]. Putative nucleotide-binding sites have been expected adjacent to the  
6 conserved motifs of RNA-dependent RNA polymerases [23,24]. PB1 also binds  
7 to the vRNA and cRNA promoters [25,27]. In this study, to elucidate the  
8 functional domain of PB1 involved in nucleotide recognition, we isolated  
9 ribavirin-resistant mutants. Ribavirin inhibits the *de novo* synthesis of purine  
10 nucleosides and thus blocks viral RNA synthesis. In addition, it has been  
11 proposed that ribavirin also inhibits directly the viral RNA synthesis. We found  
12 that the viral polymerase activity of D27N was higher than that of wild type in the  
13 presence of ribavirin (Fig. 2C). Further, the viral polymerase activity of D27N  
14 was also resistant to MTX treatment compared with that of wild type (Fig. 3).  
15 These strongly suggest that D27N mutant can polymerize the nascent RNA chains  
16 with the low concentrations of nucleotide. Therefore, it is expected that Asp27 is  
17 involved in the nucleotide recognition.

18 The Asp27 of PB1 is conserved over 99.9% of 7,259 sequences of PB1  
19 deposited in the NCBI Influenza Sequence Database. It has been reported that  
20 Asp27 is located upstream of the nucleotide binding site of PB1 but not in the  
21 catalytic active site. Similarly, the ribavirin-resistant mutant of poliovirus has a  
22 mutation in a domain out of the catalytic active site of the viral polymerase [44].

1 D27N is present within putative vRNA and cRNA promoter binding sites [25,27].  
2 It is shown by mutants in the promoters of vRNA and cRNA that the RNA  
3 synthesis activity, cleavage of the cap structure, and the polyadenylation by viral  
4 polymerase are regulated through the promoter structure [45,46,47]. Further, the  
5 viral polymerase is stabilized by the interaction with its viral promoter [48].  
6 Based on previous reports and our findings, the interaction between Asp27 of PB1  
7 with vRNA and/or cRNA promoters may lead to the regulation of viral  
8 polymerase activity through the nucleotide recognition activity of PB1. This  
9 finding could be useful for further studies about the mechanism of nucleotide  
10 recognition of the influenza viral RNA polymerase.

11

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2

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5

## 1 **References**

2

3 [1] M.-L. Li, P. Rao, R.M. Krug, The active sites of the influenza cap-dependent  
4 endonuclease are on different polymerase subunits, *EMBO J.* 20 (2001) 2078-  
5 2086.

6 [2] S.K. Biswas, D.P. Nayak, Mutational analysis of the conserved motifs of influenza A  
7 virus polymerase basic protein 1, *J. Virol.* 68 (1994) 1819-1826.

8 [3] S.K. Biswas, D.P. Nayak, Influenza virus polymerase basic protein 1 interacts with  
9 influenza virus polymerase basic protein 2 at multiple sites, *J. Virol.* 70 (1996)  
10 6716-6722.

11 [4] S. González, T. Zürcher, J. Ortín, Identification of two separate domains in the  
12 influenza virus PB1 protein involved in the interaction with the PB2 and PA  
13 subunits: a model for the viral RNA polymerase structure, *Nucleic Acids Res.* 24  
14 (1996) 4456-4463.

15 [5] Y. Ohtsu, Y. Honda, T. Toyoda, Fine mapping of the subunit binding sites of  
16 influenza virus RNA polymerase, *International Congress Series* 1219 (2001) 463-  
17 469.

18 [6] T. Toyoda, D.M. Adyshev, M. Kobayashi, A. Iwata, A. Ishihama, Molecular  
19 Assembly of the Influenza Virus RNA Polymerase: Determination of the Subunit-  
20 Subunit Contact Sites, *J. Gen. Virol.* 77 (1996) 2149-2157.

21 [7] T. Zürcher, S. de la Luna, J.J. Sanz-Ezquerro, A. Nieto, J. Ortín, Mutational analysis  
22 of the influenza virus A/Victoria/3/75 PA protein: studies of interaction with PB1  
23 protein and identification of a dominant negative mutant, *J. Gen. Virol.* 77 ( Pt 8)  
24 (1996) 1745-1749.

25 [8] A. Kawaguchi, T. Naito, K. Nagata, Involvement of Influenza Virus PA Subunit in  
26 Assembly of Functional RNA Polymerase Complexes, *J. Virol.* 79 (2005) 732-  
27 744.

28 [9] A. Dias, D. Bouvier, T. Crepin, A.A. McCarthy, D.J. Hart, F. Baudin, S. Cusack,  
29 R.W.H. Ruigrok, The cap-snatching endonuclease of influenza virus polymerase  
30 resides in the PA subunit, *Nature* 458 (2009) 914-918.

- 1 [10] E. Fodor, M. Crow, L.J. Mingay, T. Deng, J. Sharps, P. Fechter, G.G. Brownlee, A  
2 Single Amino Acid Mutation in the PA Subunit of the Influenza Virus RNA  
3 Polymerase Inhibits Endonucleolytic Cleavage of Capped RNAs, *J. Virol.* 76  
4 (2002) 8989-9001.
- 5 [11] K. Hara, F.I. Schmidt, M. Crow, G.G. Brownlee, Amino Acid Residues in the N-  
6 Terminal Region of the PA Subunit of Influenza A Virus RNA Polymerase Play a  
7 Critical Role in Protein Stability, Endonuclease Activity, Cap Binding, and Virion  
8 RNA Promoter Binding, *J. Virol.* 80 (2006) 7789-7798.
- 9 [12] P. Yuan, M. Bartlam, Z. Lou, S. Chen, J. Zhou, X. He, Z. Lv, R. Ge, X. Li, T. Deng,  
10 E. Fodor, Z. Rao, Y. Liu, Crystal structure of an avian influenza polymerase PAN  
11 reveals an endonuclease active site, *Nature* 458 (2009) 909-913.
- 12 [13] P. Fechter, L. Mingay, J. Sharps, A. Chambers, E. Fodor, G.G. Brownlee, Two  
13 Aromatic Residues in the PB2 Subunit of Influenza A RNA Polymerase Are  
14 Crucial for Cap Binding, *J. Biol. Chem.* 278 (2003) 20381-20388.
- 15 [14] D. Guilligay, F. Tarendeau, P. Resa-Infante, R. Coloma, T. Crepin, P. Sehr, J. Lewis,  
16 R.W.H. Ruigrok, J. Ortin, D.J. Hart, S. Cusack, The structural basis for cap  
17 binding by influenza virus polymerase subunit PB2, *Nat. Struct. Mol. Biol.* 15  
18 (2008) 500-506.
- 19 [15] S.J. Plotch, M. Bouloy, I. Ulmanen, R.M. Krug, A unique cap(m<sup>7</sup>GpppXm)-  
20 dependent influenza virion endonuclease cleaves capped RNAs to generate the  
21 primers that initiate viral RNA transcription, *Cell* 23 (1981) 847-858.
- 22 [16] C. Wakai, M. Iwama, K. Mizumoto, K. Nagata, Recognition of Cap Structure by  
23 Influenza B Virus RNA Polymerase Is Less Dependent on the Methyl Residue  
24 than Recognition by Influenza A Virus Polymerase, *J. Virol.* 85 (2011) 7504-7512.
- 25 [17] A. Ghanem, D. Mayer, G. Chase, W. Tegge, R. Frank, G. Kochs, A. Garcia-Sastre,  
26 M. Schwemmle, Peptide-Mediated Interference with Influenza A Virus  
27 Polymerase, *J. Virol.* 81 (2007) 7801-7804.
- 28 [18] D.R. Pérez, R.O. Donis, A 48-amino-acid region of influenza A virus PB1 protein is  
29 sufficient for complex formation with PA, *J. Virol.* 69 (1995) 6932-6939.



- 1 [19] E. Obayashi, H. Yoshida, F. Kawai, N. Shibayama, A. Kawaguchi, K. Nagata, J.R.  
2 Tame, S.Y. Park, The structural basis for an essential subunit interaction in  
3 influenza virus RNA polymerase, *Nature* 454 (2008) 1127-1131.
- 4 [20] X. He, J. Zhou, M. Bartlam, R. Zhang, J. Ma, Z. Lou, X. Li, J. Li, A. Joachimiak, Z.  
5 Zeng, R. Ge, Z. Rao, Y. Liu, Crystal structure of the polymerase PAC-PB1N  
6 complex from an avian influenza H5N1 virus, *Nature* 454 (2008) 1123-1126.
- 7 [21] E.L. Poole, L. Medcalf, D. Elton, P. Digard, Evidence that the C-terminal PB2-  
8 binding region of the influenza A virus PB1 protein is a discrete  $\alpha$ -helical  
9 domain, *FEBS Lett.* 581 (2007) 5300-5306.
- 10 [22] K. Sugiyama, E. Obayashi, A. Kawaguchi, Y. Suzuki, J.R.H. Tame, K. Nagata, S.-Y.  
11 Park, Structural insight into the essential PB1-PB2 subunit contact of the  
12 influenza virus RNA polymerase, *EMBO J.* 28 (2009) 1803-1811.
- 13 [23] Y. Asano, A. Ishihama, Identification of Two Nucleotide-Binding Domains on the  
14 PB1 Subunit of Influenza Virus RNA Polymerase, *J. Biochem.* 122 (1997) 627-  
15 634.
- 16 [24] D.M. Kolpashchikov, A. Honda, A. Ishihama, Structure-function relationship of the  
17 influenza virus RNA polymerase: primer-binding site on the PB1 subunit,  
18 *Biochemistry* 43 (2004) 5882-5887.
- 19 [25] S. Gonzalez, J. Ortin, Characterization of influenza virus PB1 protein binding to  
20 viral RNA: two separate regions of the protein contribute to the interaction  
21 domain, *J. Virol.* 73 (1999) 631-637.
- 22 [26] M.-L. Li, B.C. Ramirez, R.M. Krug, RNA-dependent activation of primer RNA  
23 production by influenza virus polymerase: different regions of the same protein  
24 subunit constitute the two required RNA-binding sites, *EMBO J.* 17 (1998) 5844-  
25 5852.
- 26 [27] S. Gonzalez, J. Ortin, Distinct regions of influenza virus PB1 polymerase subunit  
27 recognize vRNA and cRNA templates, *EMBO J.* 18 (1999) 3767-3775.
- 28 [28] R.W. Sidwell, J.H. Huffman, L. Khare, G. P., B. Allen, R. Witkowski, J. T., K.  
29 Robins, Broad-Spectrum Antiviral Activity of Virazole: 1- $\beta$ -D-Ribofuranosyl-  
30 1,2,4-triazole-3-carboxamide, *Science* 177 (1972) 705-706.

- 1 [29] N.M. Dixit, A.S. Perelson, The metabolism, pharmacokinetics and mechanisms of  
2 antiviral activity of ribavirin against hepatitis C virus, *Cellular and Molecular Life*  
3 *Sciences CMLS* 63 (2006) 832-842.
- 4 [30] J.J. Feld, J.H. Hoofnagle, Mechanism of action of interferon and ribavirin in  
5 treatment of hepatitis C, *Nature* 436 (2005) 967-972.
- 6 [31] B. Eriksson, E. Helgstrand, N.G. Johansson, A. Larsson, A. Misiorny, J.O. Noren, L.  
7 Philipson, K. Stenberg, G. Stening, S. Stridh, B. Oberg, Inhibition of Influenza  
8 Virus Ribonucleic Acid Polymerase by Ribavirin Triphosphate, *Antimicrob.*  
9 *Agents Chemother.* 11 (1977) 946-951.
- 10 [32] P. Toltzis, K. O'Connell, J.L. Patterson, Effect of phosphorylated ribavirin on  
11 vesicular stomatitis virus transcription, *Antimicrob. Agents Chemother.* 32 (1988)  
12 492-497.
- 13 [33] L.F. Cassidy, J.L. Patterson, Mechanism of La Crosse virus inhibition by ribavirin,  
14 *Antimicrob. Agents Chemother.* 33 (1989) 2009-2011.
- 15 [34] J.T. Rankin Jr, S.B. Eppes, J.B. Antczak, W.K. Joklik, Studies on the mechanism of  
16 the antiviral activity of ribavirin against reovirus, *Virology* 168 (1989) 147-158.
- 17 [35] Y. Sun, D.-H. Chung, Y.-K. Chu, C.B. Jonsson, W.B. Parker, Activity of Ribavirin  
18 against Hantaan Virus Correlates with Production of Ribavirin-5'-Triphosphate,  
19 Not with Inhibition of IMP Dehydrogenase, *Antimicrob. Agents Chemother.* 51  
20 (2007) 84-88.
- 21 [36] C.-y.Z. Chao-jiang Gu, Qian Zhang, Li-li Shi, Yong Li and San-fu Qu An  
22 antiviral mechanism investigated with ribavirin as an RNA virus mutagen  
23 for foot-and-mouth disease virus, *Biochem. Mol. Biol.* 39 (2006) 9-15.
- 24 [37] I. Jordan, T. Briese, N. Fischer, J.Y.-N. Lau, W.I. Lipkin, Ribavirin Inhibits West  
25 Nile Virus Replication and Cytopathic Effect in Neural Cells, *J. Infect. Diseases*  
26 182 (2000) 1214-1217.
- 27 [38] H. Moreno, I. Gallego, N. Sevilla, J.C. de la Torre, E. Domingo, V. Martin,  
28 Ribavirin Can Be Mutagenic for Arenaviruses, *J. Virol.* 85 7246-7255.
- 29 [39] D. Safronetz, E. Haddock, F. Feldmann, H. Ebihara, H. Feldmann, In vitro and in  
30 vivo activity of ribavirin against Andes virus infection, *PLoS One* 6 (2011)  
31 e23560.

- 1 [40] D. Maag, C. Castro, Z. Hong, C.E. Cameron, Hepatitis C Virus RNA-dependent  
2 RNA Polymerase (NS5B) as a Mediator of the Antiviral Activity of Ribavirin, *J.*  
3 *Biol. Chem.* 276 (2001) 46094-46098.
- 4 [41] W.E. Müller, A. Maidhof, H. Taschner, R.K. Zahn, Virazole (1-beta-D-  
5 ribofuranosyl-1,2,4-triazole-3-carboxamide; a cytostatic agent, *Biochem.*  
6 *Pharmacol.* 26 (1977) 1071-1075.
- 7 [42] J.D. Graci, C.E. Cameron, Mechanisms of action of ribavirin against distinct viruses,  
8 *Rev. Med. Virol.* 16 (2006) 37-48.
- 9 [43] K. Turan, M. Mibayashi, K. Sugiyama, S. Saito, A. Numajiri, K. Nagata, Nuclear  
10 MxA proteins form a complex with influenza virus NP and inhibit the  
11 transcription of the engineered influenza virus genome, *Nucleic Acids Res.* 32  
12 (2004) 643-652.
- 13 [44] J.K. Pfeiffer, K. Kirkegaard, A single mutation in poliovirus RNA-dependent RNA  
14 polymerase confers resistance to mutagenic nucleotide analogs via increased  
15 fidelity, *Proc. Natl. Acad. Sci. USA* 100 (2003) 7289-7294.
- 16 [45] L.L.M. Poon, D.C. Pritlove, E. Fodor, G.G. Brownlee, Direct Evidence that the  
17 Poly(A) Tail of Influenza A Virus mRNA Is Synthesized by Reiterative Copying  
18 of a U Track in the Virion RNA Template, *J. Virol.* 73 (1999) 3473-3476.
- 19 [46] M.B. Leahy, G. Zecchin, G.G. Brownlee, Differential Activation of Influenza A  
20 Virus Endonuclease Activity Is Dependent on Multiple Sequence Differences  
21 between the Virion RNA and cRNA Promoters, *J. Virol.* 76 (2002) 2019-2023.
- 22 [47] M. Crow, T. Deng, M. Addley, G.G. Brownlee, Mutational analysis of the influenza  
23 virus cRNA promoter and identification of nucleotides critical for replication, *J.*  
24 *Virol.* 78 (2004) 6263-6270.
- 25 [48] G.G. Brownlee, J.L. Sharps, The RNA Polymerase of Influenza A Virus Is  
26 Stabilized by Interaction with Its Viral RNA Promoter, *J. Virol.* 76 (2002) 7103-  
27 7113.
- 28
- 29

1 **Figure legends**

2

3 **Fig. 1.** Screening of ribavirin-resistant PB1 mutant. (A) Plaque assays were  
4 carried out with MDCK cells-infected WSN at MOI of  $0.25 \times 10^{-4}$  in the presence  
5 of ribavirin (0, 10, 33, 100, and 300  $\mu\text{M}$ ). The results are averages from three  
6 independent experiments with standard deviations. (B) Assay system for  
7 screening by mini-replicon assay. 293T cells were transfected with plasmids for  
8 the expression of viral proteins, PB1 (wild-type or mutant), PB2, PA, and NP, and  
9 a plasmid for the expression of artificial influenza virus genome containing *EGFP*  
10 gene of negative polarity. (C) At 3 hpt, ribavirin was added (0 or 75  $\mu\text{M}$ ), and the  
11 fluorescence of EGFP was observed at 15 hpt.

12

13 **Fig. 2.** Ribavirin-resistance of D27N mutant by mini-replicon assay system. (A)  
14 Mini-replicon assay using *EGFP* gene as a reporter gene. At 3 hpt, ribavirin was  
15 added (0 or 75  $\mu\text{M}$ ), and the fluorescence of EGFP was observed at 15 hpt. (B)  
16 Sequence of ribavirin-resistant PB1 mutant. (C) Mini-replicon assay using  
17 *luciferase* gene as a reporter gene was carried out. At 3 hpt, different  
18 concentrations (0, 12.5, 25, 50, and 100  $\mu\text{M}$ ) of ribavirin were added, and the  
19 luciferase activity was measured at 15 hpt. The vertical axis represents the  
20 percentage of the luciferase activity from ribavirin-treated cells relative to that  
21 from ribavirin-untreated cells. The results are averages from three independent  
22 experiments with standard deviations. (D) Effect of D27N mutation on assembly

1 of PB1 subunit. Mini-replicon assay using *luciferase* gene as a reporter gene was  
2 carried out. At 3 hpt, ribavirin was added (0 or 50  $\mu$ M). At 15 hpt, cells were  
3 lysed, and the lysates were subjected to western blot analysis using anti-PB1  
4 antibody and antibody against  $\beta$ -tubulin.

5

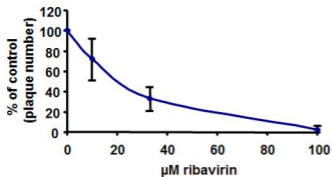
6 **Fig. 3.** Methotrexate-resistance of D27N mutant by mini-replicon assay system.

7 The mini-replicon assay using *luciferase* gene as a reporter gene was carried out.  
8 At 12 hpt, different concentrations (0, 0.3, 1, 3, 10, and 30  $\mu$ M) of methotrexate  
9 were added, and luciferase activity was measured at 22 hpt. The vertical axis  
10 represents the percentage of the luciferase activity from methotrexate-treated cells  
11 relative to that from methotrexate-untreated cells. The results are averages from  
12 three independent experiments with standard deviations.

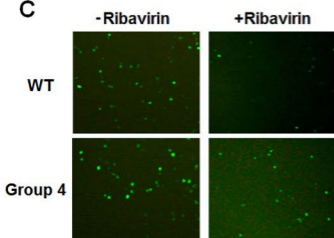
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# Binh, N. T. et al, Figure 1.

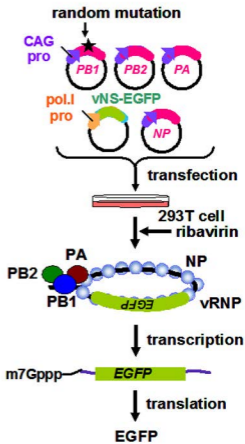
## A



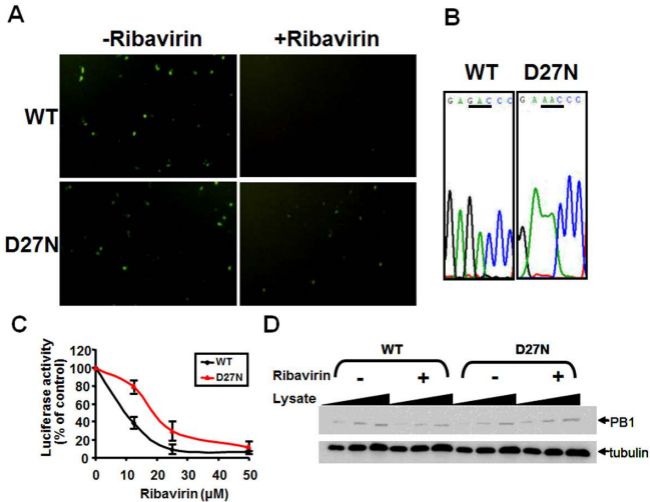
## C



## B



# Binh, N. T. et al, Figure 2.



# Binh, N. T. et al, Figure 3.

