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Polymerase complex with lysine at position 627 of the PB2 of influenza virus A/Hong Kong/483/97 (H5N1) efficiently transcribes and replicates virus genes in mouse cells

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Abstract

Influenza virus A/Hong Kong/483/97 (H5N1) (HK483-K) has the PB2 with lysine at position 627 (PB2-627K) and is highly pathogenic in chickens and mice. On the other hand, the pathogenicity of mutant virus (HK483-E), which was generated by substituting lysine with glutamic acid at the position of the PB2, is lower than that of HK483-K in mice, but is highly pathogenic in chickens. The PB2 is one of the components of heterotrimeric polymerase complex, which plays roles in the transcription and replication of virus genes. Cell-free polymerase assay revealed that intrinsic transcription activity of the polymerase complex with PB2-627K is higher than that of glutamic acid (PB2-627E). In chicken cells, transcription efficiency of the polymerase complex with PB2-627E was not lower than those with PB2-627K, indicating that transcription of virus genes is modulated by some host factors in chicken cells, resulting in high growth. Polymerase complex with PB2-627K efficiently transcribes and replicates virus polymerase genes in mouse cells, leading to high growth of HK483-K compared with that of HK483-E. The results of our experiments clearly suggest that efficient transcription and replication of virus genes by polymerase complex result in the higher pathogenicity in mice.

1. Introduction

Influenza virus belongs to the *Orthomyxoviridae* family and has 8 gene segments of linear, negative-sense, single stranded RNA. Virus polymerase complex, consisting of the PB2, PB1, and PA protein molecules, transcribes and replicates virus genes. In transcription, virus polymerase complex catalyzes not only RNA polymerization but also cleavage of host mRNA to generate the capped RNA fragment as a virus mRNA primer. The cap-binding and cap-dependent endonuclease domains are localized in the PB2 and PA, respectively (Guilligay et al., 2008; Honda et al., 1999; Yuan et al., 2009). The domain with RNA polymerization activity is localized in the PB1 (Li et al., 1998).

H5N1 highly pathogenic avian influenza viruses have been transmitted sporadically from chickens to humans. Fifty percent chicken lethal doses (CLD₅₀) of A/Hong Kong/483/97 (H5N1) (HK483-K) and A/Hong Kong/486/97 (H5N1) (HK486) were 10^{2.3} and 10^{2.4} plaque-forming units (PFU), whereas 50 % mouse lethal doses (MLD₅₀) of these viruses were 10^{0.3} and more than 10^{3.9} PFU, respectively (Gao et al., 1999; Hatta et al., 2001). Experimental infection of mice showed that a mutation at position 627 of the PB2 influences the outcome of pathogenicity in mice (Hatta et al., 2001). An amino acid residue at position 627 in the PB2 is exposed on the surface of the molecule (Tarendeau et al., 2008) and participates in the binding of the PB2 molecule to RNA (Kuzuhara et al., 2009); however, the molecular basis of the pathogenicity of HK483-K, which has the PB2 with lysine at position 627 (PB2-627K), in mice is not well understood.

In the present study, HK483-K and mutant virus HK483-E with substitution of lysine with glutamic acid at position 627 of the PB2 of HK483-K were generated by reverse genetics. Pathogenicity of HK483-K and HK483-E was assessed in chickens and mice by experimental infection. Growth rate, transcription and replication

1 efficiency of virus genes, and the amounts of virus proteins of HK483-K were compared
2 with those of HK483-E in chicken and mouse cells. In addition, intrinsic activity of the
3 polymerase complex derived from virus particles was examined by cell-free polymerase
4 assay.

5 6 **2. Materials and Methods**

7 **2.1 Viruses**

8 HK483-K was provided by Dr. K. F. Shortridge, University of Hong Kong. Each of
9 the PCR products of 8 gene segments of the virus was cloned in pGEM-T vector
10 (Promega, Madison, WI, U.S.A.). Eight gene segments of HK483-K were subcloned
11 into a dual-promoter plasmid, pHW2000 (Hoffmann et al., 2000). To generate mutant
12 virus HK483-E, substituting lysine for glutamic acid at position 627 of the PB2 of
13 HK483-K, the pHW2000 plasmid with a mutant PB2 gene segment was constructed by
14 site-directed mutagenesis with primers, pHW2000 plasmid with the PB2 gene segment
15 of HK483-K, and the QuikChange Site-Directed Mutagenesis Kit (Stratagene,
16 Heidelberg, Germany). Reverse genetic viruses of HK483-K and HK483-E were
17 generated by 8 plasmids of HK483-K and a plasmid with a mutant PB2 gene segment.
18 The pHW2000 plasmid with the HA gene segment of A/Puerto Rico/8/34 (H1N1) (PR8)
19 was provided by Drs. E. Hoffmann and R. Weddy, St. Jude Children's Research Hospital.
20 In order to obtain the nucleocapsids of HK483-K and HK483-E, PR8/HK483-K and
21 PR8/HK483-E were generated by replacing the HA gene segment of HK483-K or
22 HK483-E with that of PR8. The virus titers of HK483-K and HK483-E were
23 determined by plaque assay in Madin-Darby Canine Kidney (MDCK) cells.

24 25 **2.2 Cells**

MDCK cells were maintained in Minimum Essential Medium (MEM; Nissui, Tokyo, Japan) supplemented with 0.3 mg/ml L-glutamine (Wako Chemicals, Tokyo, Japan), 10% calf serum (Sanko Junyaku, Tokyo, Japan), 100 U/ml penicillin G (Meiji Seika, Tokyo, Japan), 0.1 mg/ml streptomycin (Meiji Seika) and 8 µg/ml gentamicin (Merck Sharp and Dohme, Rahway, NJ, U.S.A.). NIH/3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 0.3 mg/ml L-glutamine, 10% fetal calf serum (Cambrex, Walkersville, MD, U.S.A.) and antibiotics. Chicken Embryo Fibroblasts (CEF) cells were prepared from 10-day-old chicken embryos and were cultured in MEM supplemented with L-glutamine, calf serum, and antibiotics.

2.3 Animal experiment

In order to determine the MLD₅₀, 50 µl of serial 10-fold dilution of HK483-K or HK483-E was intranasally inoculated into 6-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan), anesthetized with tiletamine hydrochloride (20 mg/kg) (United States Pharmacopeia, Rockville, MD, U.S.A.), zolazepam hydrochloride (20 mg/kg) (United States Pharmacopeia), and xylazine (20 mg/ml) (Bayer Healthcare, Wuppertal, Germany), and the mice were observed for 2 weeks. In order to determine the CLD₅₀, 100 µl of serial 10-fold dilutions of HK483-K or HK483-E was intranasally inoculated into 4-week-old chickens (Boris brown) and the chickens were observed for 2 weeks. Chickens and mice were housed in a self-contained isolator unit (Tokiwa Kagaku, Tokyo, Japan) at a BSL-3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The experiments were performed according to the guidelines of the institutional animal care and use committee of the Graduate School of Veterinary Medicine (approval number: 11-0087).

2.4 Plaque assay

Serially diluted viruses were inoculated into confluent monolayers of MDCK cells and incubated at 35 °C for 1 hour. Unbound viruses were removed, and the cells were washed with MEM. Cells were then overlaid with MEM containing 1.0 % Bacto agar (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.). After 24 hours of incubation at 35 °C, the cells were stained with 0.005 % neutral red and the plaques counted.

2.5 Virus growth kinetics in cultured cells

HK483-K or HK483-E was inoculated into the confluent monolayer of NIH/3T3 and CEF cells at a multiplicity of infection (MOI) of 0.01. After 1 hour incubation at 37 °C, unbound viruses were washed away and culture medium was added. The cells were incubated at 37 °C and the supernatants were collected at 6, 24, and 48 hours post infection (hpi). Virus titers were quantified by plaque assay.

2.6 Purification of virus nucleocapsids

In order to obtain nucleocapsids of HK483-K and HK483-E, PR8/HK483-K or PR8/HK483E was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. After 48 hours incubation at 35 °C, allantoic fluid was harvested as the virus solution. Virus particles were precipitated by centrifuge centrifugation at 25,000 rpm for 1.5 hours at 4 °C using a Hitachi P27A rotor and were resuspended in phosphate-buffered saline (PBS). Virus particles were purified by centrifugation on a 30 to 45 % sucrose step-wise gradient. Nucleocapsids were obtained according to the protocol (Shimizu et al., 1994). Virus particles were lysed by solubilization buffer

1 containing 200mM HEPES (pH7.9), 300mM NaCl, 10mM MgCl₂, 2% Triton x-100,
2 10mM dithiothreitol (DTT), and 10% glycerol, and incubated at 30 °C for 10 minutes
3 (min). Nucleocapsids were fractionated by centrifugation on a 30 to 60 % glycerol
4 step-wise gradient in the presence of 100mM HEPES (pH7.9), 150mM NaCl, and 1mM
5 DTT. After centrifugation at 45,000 rpm for 4 hours at 4 °C using a Beckman SW55 Ti
6 rotor, fractions of 450 µl were collected from the top of the gradient. Fractionation was
7 assessed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. After mixing
8 fractions 4, 5, and 6, the amount of the NP of the mixture was quantified by SDS-PAGE
9 and CBB staining.

11 **2.7 Cell-free polymerase assay**

12 Cell-free RNA synthesis reaction was carried out at 30 °C for 60 min in a final
13 volume of 25 µl containing 50mM HEPES (pH7.9), 50mM KCl, 5mM MgCl₂, 0.5mM
14 each of ATP, CTP, and UTP, 0.025mM GTP, 1.5M DTT, 5µCi of [α -³²P] GTP (3,000
15 Ci/mmol; Perkin-Elmer, Wellesley, MA, U.S.A.), 8U of RNase inhibitor, virus
16 nucleocapsids (50 or 100 ng of the NP equivalents), and 4 µg/µl capped mRNA
17 (Ribonucleic acid, messenger from rabbit globin; Sigma, St. Louis, Mo, U.S.A.) or 0.2mM
18 ApG (Hokkaido System Science, Hokkaido, Japan) (Shimizu et al., 1994). The
19 products were purified by ethanol precipitation and then resuspended in 25 µl
20 RNase-free water. The products were applied to DE81 paper (GE Healthcare Life
21 Science, Buckinghamshire, U.K.), and radioactivity of the product was measured by a
22 liquid scintillation counter LSC-6100 (Hitachi Aloka Medical, Japan, Tokyo, Japan).

24 **2.8 Primer extension assay**

25 HK483-K or HK483-E was inoculated into NIH/3T3 and CEF cells at MOI of 10. After

6 hpi, total cellular RNAs were extracted using TRIzol (Invitrogen). Primer extension assay was performed using radiolabeled probes (Fodor et al., 2002). The targets of the primer extension reactions were located in the 5' terminal of less than 270 nucleotides in each virus RNA. The gene-specific probes against vRNAs and mRNAs were labeled at the 5' end with [γ -³²P] ATP (Perkin-Elmer) and T4 polynucleotide kinase (Invitrogen). Five micrograms of total RNA were mixed with excess of a labeled probe and 0.6M trehalose, and denatured by heating at 94 °C for 5 min. The mixture was cooled on ice and transferred to 42 or 60 °C, and primer extension was performed after addition of 100 U of M-MLV Reverse Transcriptase (Invitrogen) in the reaction buffer provided with the enzyme for 1 hour. The products were separated on 6 % polyacrylamide gels containing 7 M urea in TBE buffer (Invitrogen) and were detected by BAS-2500 bioimage analyzer (Fujifilm, Tokyo, Japan). The products were quantitated by ImageJ software (Rasband, 2010).

2.9 Western blot analysis

HK483-K or HK483-E was inoculated into NIH/3T3 and CEF cells at MOI of 10. After 6 hpi, the cells were washed with PBS and then lysed with 2 % sodium dodecyl sulfate (SDS; Wako Chemicals) and protease inhibitor complete mini (Roche, Basel, Switzerland) in PBS. Sample buffer containing 2 % SDS, 0.05M Tris-HCl (pH6.8), bromophenol blue (Kishida Chemical, Osaka, Japan), and 10 % glycerol (Wako Chemicals) was added to the lysate. After boiling for 5 min, the lysates were loaded onto a 10 % SDS-polyacrylamide gel and electrophoresed. The proteins were transferred onto an Immobilon P membrane (Millipore) by electro-blotting (ATTO, Tokyo, Japan). To detect the PB2, PB1, and PA, anti-PB2 monoclonal antibody (MAb) (143/3), anti-PB1 MAb (17/5), and anti-PA MAb (58/1) were used, respectively (Hatta et

al., 2000a; Hatta et al., 2000b). β -actin or Lamin B1 was detected using anti- β -actin MAb (Applied Biochemical Materials, Richmond, BC, Canada) or anti-Lamin B1 polyclonal antibody (Medical and Biological Laboratories, Nagoya, Japan) as a loading control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was detected by anti-GAPDH MAb (Cell Signaling Technology, Boston, MA, U.S.A.). Horseradish peroxidase (HRP) -conjugated goat anti-mouse IgG (H+L) antibody (BIO-RAD, Hercules, CA, U.S.A.) or HRP-conjugated goat anti-rabbit IgG (H+L) antibody (BIO-RAD) was used as a second antibody. The membrane was developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Milford, MA, U.S.A.). The bands were visualized with Lumi Vision PRO (AISIN SEIKI, Aichi, Japan) and quantified using ImageJ software (Rasband, 2010). The amount of each virus protein was normalized based on the intensity of the band of β -actin or Lamin B1.

2.10 Nuclear fractionation

Nuclear fraction was obtained from cells infected with HK483-K and HK483-E at 6 hpi. Cells were harvested with trypsin and then incubated with nuclear fractionation buffer containing 10mM HEPES (pH7.9; Wako Chemicals), 1.5mM MgCl₂ (Wako Chemicals), 10mM KCl (Nacalai Tesque, Kyoto, Japan), and 0.05% NP-40 (Nacalai Tesque) for 5 min on ice. After incubation, samples were centrifuged at 350 x *g* for 3min. Pellets were lysed with 2% SDS and complete mini in PBS, and then lysates were analyzed by Western blotting. Fractionation of the nucleus was confirmed by the presence of Lamin B1 protein and the absence of GAPDH protein.

3. Results

3.1 Pathogenicity of HK483-K and HK483-E in chickens and mice

HK483-K and HK483-E were generated by reverse genetics. HK483-E is a mutant virus which has the PB2 substituting lysine with glutamic acid (PB2-627E) at position 627 of HK483-K. To compare the pathogenicity of HK483-K and HK483-E, virus was intranasally inoculated into chickens and mice. The CLD₅₀ of HK483-E was comparable to that of HK483-K, while MLD₅₀ of HK483-K was higher than that of HK483-E (Table 1).

3.2 Growths of HK483-K and HK483-E in chicken and mouse cells

To compare the virus growth of HK483-K and HK483-E in chicken and mouse cells, virus titers were quantitated by plaque assay. Growth rate of HK483-E was comparable to that of HK483-K in chicken cells, while HK483-K replicated more efficiently than HK483-E in mouse cells (Fig. 1).

3.3 Transcription and elongation activities of the polymerase complex with PB2-627K and PB2-627E in cell-free polymerase assay

To investigate the catalytic activity of the virus polymerase complex, virus nucleocapsids were obtained from virus particles by density-gradient centrifugation. High-intensity of the bands corresponding to the PB2, PB1, PA, and NP were detected in fraction 4, 5, and 6 (Fig. 2a). In transcription of virus genes, virus polymerase complex snatches 5' terminal region with a cap structure from host mRNAs as primer. Cap-dependent transcription activity and primer-dependent elongation activity were assessed by cell-free polymerase assay with rabbit globin mRNA as a donor substrate of the cap domain and ApG as a dinucleotide primer, respectively. Transcription activity of the polymerase complex with PB2-627K was higher than that with PB2-627E (Fig. 2b). In contrast, no significant difference was found in the results of the reaction with

ApG (Fig. 2c).

3.4 Transcription and replication of HK483-K and HK483-E in chicken and mouse cells

Transcription efficiency of polymerase complex was examined in chicken and mouse cells infected with HK483-K and HK483-E at 6 hpi. The mRNAs of the PB2, PB1, HA, and M genes in chicken cells infected with HK483-E were more abundant than in those with HK483-K (Fig. 3a). In contrast, the PB2, PB1, PA, NP, and M genes were efficiently transcribed in mouse cells infected with HK483-K compared with those with HK483-E (Fig. 3b). Since virus polymerase complex catalyzes not only the transcription but also the replication of virus genes, the amounts of vRNAs were quantified in chicken and mouse cells infected with each virus. The vRNAs of the PB1 and M genes in chicken cells infected with HK483-E were more abundant than in those with HK483-K, whereas the vRNAs of the PB2 and NP genes of HK483-K were more abundant than in those of HK483-E (Fig. 3c). In mouse cells, the replication efficiencies of the PB2, PB1, PA, NP, and M genes of HK483-K were higher than those of HK483-E (Fig. 3d)

3.5 Translation of virus mRNAs in chicken and mouse cells

Translation products of virus mRNAs upon infection with HK483-K and HK483-E were compared in cell lysates prepared from the cells at 6 hpi. No significant difference was found in the amounts of translation products of virus polymerase mRNAs in chicken cells infected with HK483-K and HK483-E (Fig. 4a), whereas the amounts of polymerase proteins in mouse cells infected with HK483-K were greater than these with HK483-E (Fig. 4b). Since virus polymerase proteins are transported

into the nuclei and polymerase complex transcribes and replicates virus genes (Deng et al., 2005), the accumulation of the PB2, PB1, and PA in the nuclei of cells infected with HK483-K was compared with those infected with HK483-E at 6 hpi. Fractionation of the nuclei was confirmed in the presence of Lamin B1 and in the absence of GAPDH (Fig. 5a). The amounts of the PB2, PB1, and PA in the nuclei of the cells infected with HK483-K were greater than in those infected with HK483-E (Fig. 5b).

4. Discussion

The present results indicate that the effect of lysine at position 627 of the PB2 on virus growth is equivalent to that of glutamic acid in chicken cells (Fig. 1). In addition, HK483-K and HK483-E showed equal high lethality in chickens differently from the case of mice. On the other hand, in mouse cells, the PB2 with lysine at this position contributes to efficient growth of HK483-K (Fig. 1), which is highly pathogenic in mice (Table 1). The results coincide with previous findings (Hatta et al., 2001; Shinya et al., 2004).

Cell-free polymerase assay elucidated that intrinsic transcription activity of polymerase complex with PB2-627K is higher than that with PB2-627E (Fig. 2b). In transcription, the central domain of the PB2 binds to the 5' cap structure of host mRNA and then the 5' terminal of 10-13 nucleotides is cleaved by the PA (Guilligay et al., 2008; Yuan et al., 2009). On the other hand, the domain with RNA polymerization activity is localized in the PB1 (Li et al., 1998). No significant difference was found between elongation activities of polymerases complex with PB2-627K and PB2-627E (Fig. 2c), suggesting that lysine or glutamic acid at position 627 of the PB2 has little effect on the polymerization activity of the PB1. Substitution of lysine with glutamic acid at position 627 of the PB2 results in partial inversion of the positive charge on the surface

1 of the PB2 molecule (Tarendeau et al., 2008). In addition, binding affinities of the PB2
2 with lysine at the position to the 5' capped RNA, non-capped RNA, and vRNA promoter
3 were higher than those with glutamic acid (Kuzuhara et al., 2009). Results of the
4 cell-free polymerase assay and those reports suggest that PB2-627K contributes to the
5 efficient initiation reaction in the transcription of virus genes. Since some host factors
6 packaged in the virus particles exist presumably in cell-free polymerase assay, it is
7 possible that the polymerase is affected by these factors. Therefore, the regulatory
8 mechanism of transcription activity needs to be clarified in the future study.

9 The amounts of virus mRNAs in chicken cells infected with HK483-E were
10 equivalent to or more than those with HK483-K (Fig. 3a), although intrinsic
11 transcription activity of the polymerase complex with PB2-627K is higher than that
12 with PB2-627E (Fig. 2b), suggesting that transcription of virus genes should be actively
13 modulated by host factors in chicken cells. The present findings support that positive
14 factors present in chicken cells enhance the activity of the polymerase complex with
15 PB2-627E (Moncorge et al., 2010). Thus, both HK483-K and HK483-E were highly
16 pathogenic in chickens. On the other hand, virus mRNAs and vRNAs were efficiently
17 synthesized in mouse cells infected with HK483-K compared with those with HK483-E
18 (Fig. 3b and 3d). In addition, large amounts of virus polymerase proteins were
19 accumulated in the nuclei of the cells infected with HK483-K (Fig. 5b). Nucleocapsid
20 with PB2-627K is efficiently transported to nucleus in mouse cells by importin alpha
21 proteins (Hudjetz and Gabriel, 2012). These findings and a report suggest that
22 efficient growth of HK483-K in mouse cells is caused by intrinsic activity of the
23 polymerase complex with PB2-627K in addition to the host factors, resulting in high
24 pathogenicity in mice.

25 Although intrinsic activities of the polymerase complex with PB2-627K were

1 higher than that with PB2-627E, no efficient transcription and replication were shown
2 in the HA, NA, and NS genes in mouse cells (Fig. 3b and d). These findings suggest
3 that transcription and replication of each segment are regulated by cis-element such as
4 enhancer. Regulatory region of RNA synthesis in each segment needs to be clarified in
5 a future study.

6 Since PB2-627K is found in human influenza viruses, whereas PB2-627E is found
7 in avian influenza viruses, the lysine or glutamic acid at position 627 of the PB2 is
8 considered as a factor in the host range of influenza viruses (Subbarao et al., 1993).
9 Efficient transcription and replication of virus genes in mouse cells infected with
10 HK483-K is caused by intrinsic activity of the polymerase complex with PB2-627K,
11 suggesting that viruses with lysine at position 627 of the PB2 are selected in mice. The
12 present findings support that the viruses with the PB2 with lysine at position 627 were
13 selected in mice (Bogs et al., 2011).

14 In conclusion, the present study demonstrated that intrinsic transcription activity
15 of polymerase complex with PB2-627K was higher than that with PB2-627E. In
16 addition, it suggests that the transcription and replication are regulated in each
17 segment. Transcription and replication efficiencies of polymerase complex with
18 PB2-627E are equivalent to or better than those with PB2-627K, resulting in equivalent
19 growth in chicken cells. On the other hand, efficient transcription and replication of
20 virus polymerase genes by polymerase complex with PB2-627K lead to the promotion of
21 polymerase accumulation in the nuclei, resulting in high growth of HK483-K in mouse
22 cells. Thus, although both HK483-K and HK483-E were highly pathogenic in chickens,
23 the pathogenicity of HK483-K was higher than that of HK483-E in mice.

24 25 **5. Acknowledgments**

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FIGURE LEGENDS

Figure 1. Virus growth in chicken and mouse cells. HK483-K or HK483-E was inoculated into CEF and NIH/3T3 cells at MOI=0.01, and the titers in supernatants were determined by plaque assay. The mean value and standard error are shown according to the results of 3 independent experiments. Statistical comparison was performed using the *t* test. *: $p<0.01$.

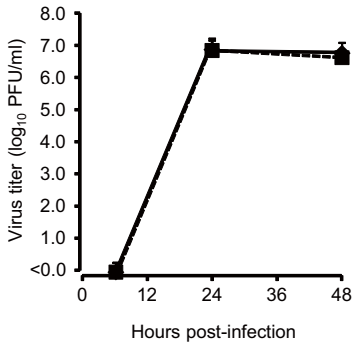
Figure 2. Fractionation of virus proteins and intrinsic transcription and elongation activities of polymerase complex. Virus proteins derived from virus particles were fractionated by density-gradient centrifugation (a). PR8/HK483-K and PR8/HK483-E were generated by replacing the HA gene segment of HK483-K or HK483-E with that of PR8. Nucleocapsids for cell-free polymerase assay were prepared from the fraction 4, 5, and 6. Cell-free RNA synthesis with virus polymerase complex (50 or 100 ng of the NP equivalents) was carried out in the presence of capped mRNA (b) or ApG (c). The mean value and standard error are shown according to the results of 3 independent experiments. Statistical comparison was performed using the *t* test. *: $p<0.05$.

Figure 3. Transcription and replication of virus genes in chicken and mouse cells. The amounts of mRNAs (a and b) and vRNAs (c and d) in CEF and NIH/3T3 cells infected with HK483-K or HK483-E. Total RNAs were extracted from the cells at 6 hpi and each virus RNA in 5 μ g of total RNAs was detected by primer extension assay using gene-specific primers. The mean value and standard error are shown according to the results of 3 independent experiments. Statistical comparison was performed using the *t* test. *: $p<0.05$.

Figure 4. Amounts of virus proteins in chicken and mouse cells. HK483-K or HK483-E was inoculated into CEF (a) and NIH/3T3 (b) cells and total cell lysates were harvested at 6 hpi. Each virus protein was detected by Western blotting. The amounts of virus proteins were normalized by the intensities of the β -actin bands. The mean values and standard error are shown according to the results of 3 independent experiments. Statistical comparison was performed using the *t* test. *: $p<0.05$.

Figure 5. Accumulation of virus polymerase proteins in the nuclei. HK483-K or HK483-E was inoculated into NIH/3T3 cells, and nuclei were obtained from the infected cells at 6 hpi. Lamin B1 and GAPDH were detected by Western blotting (a). “W” indicates whole cell lysate and “N” indicates nucleus lysate. The amounts of virus proteins were normalized by the intensities of the Lamin B1 bands (b). The mean values and standard error are shown according to the results of 3 independent experiments. Statistical comparison was performed using the *t* test. *: $p<0.05$.

CEF



NIH/3T3

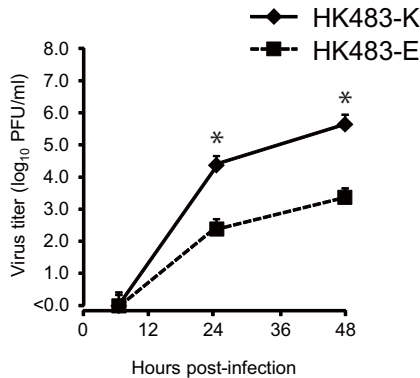


Fig. 1 Yamamoto et al.

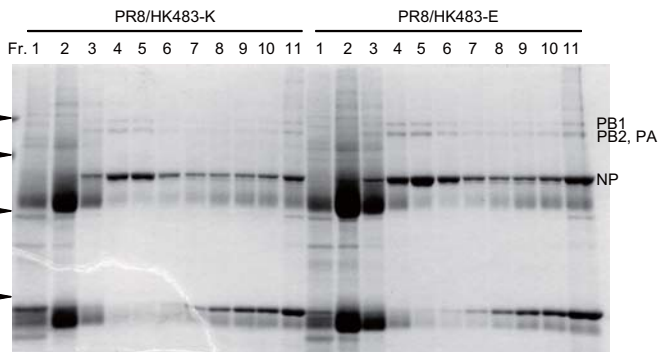
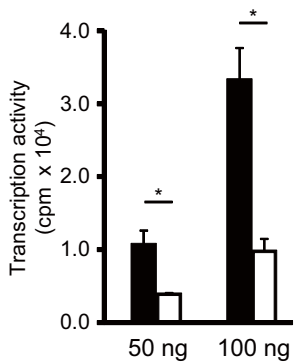
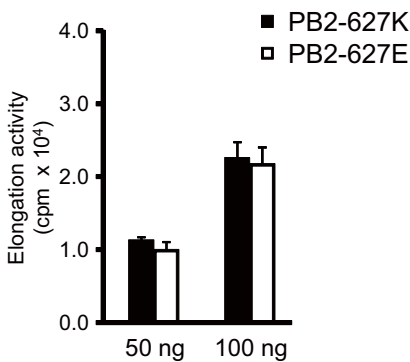
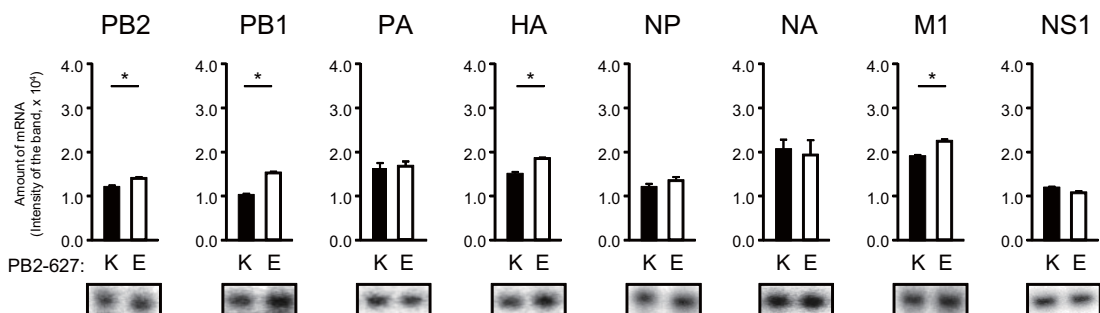
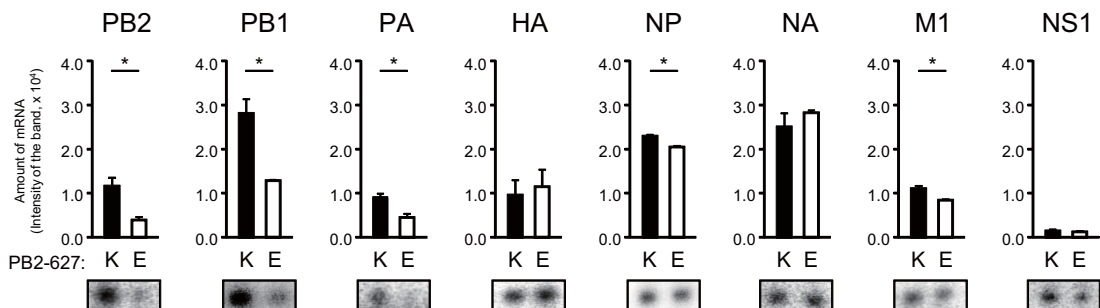
a**b****c**

Fig. 2 Yamamoto et al.

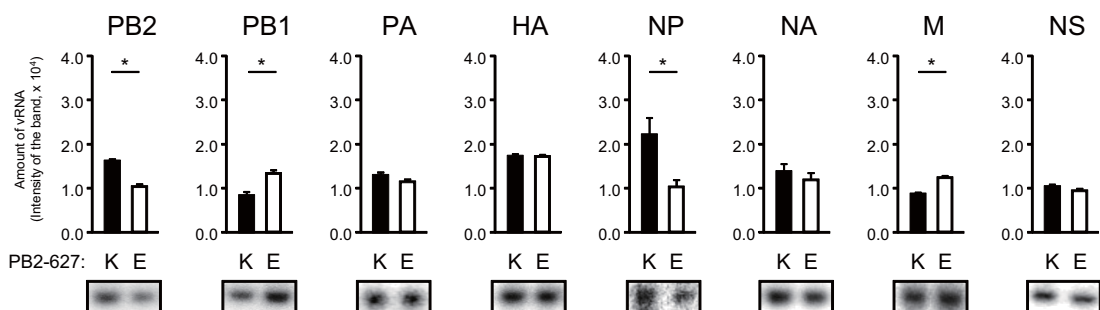
a mRNA [CEF]



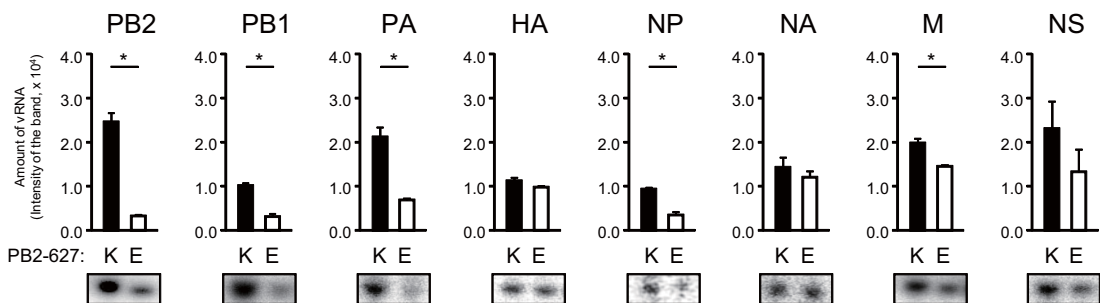
b mRNA [NIH/3T3]



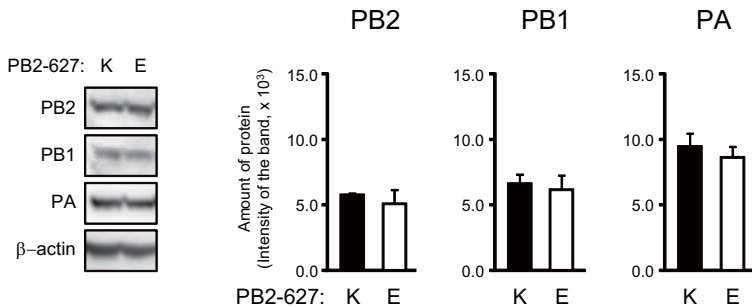
c vRNA [CEF]



d vRNA [NIH/3T3]



a [CEF]



b [NIH/3T3]

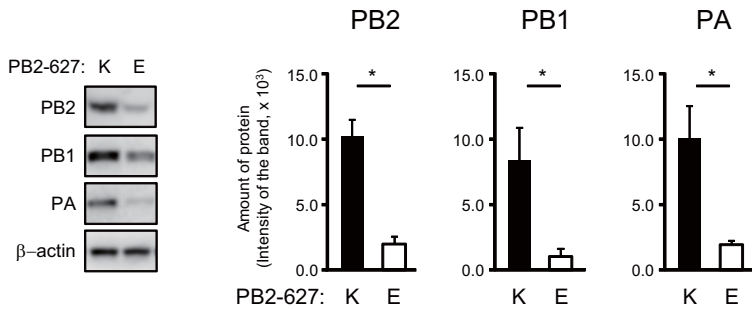
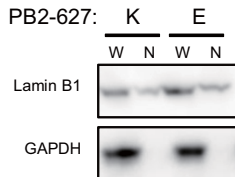


Fig. 4 Yamamoto et al.

a



b

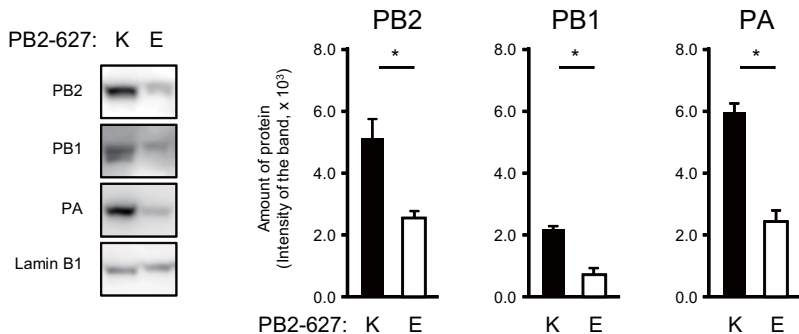


Fig. 5 Yamamoto et al.