enza A virus infection (23). Therefore, the intracellular induction pathway for type I IFN is also thought to be important for the host defense against influenza A virus infection through the activation of an immunoresponse, especially at the early phase of infection.

Previously, it has been reported that influenza A virus NS1 (nonstructural protein 1) binds to RIG-I and prevents IPS-1 mediated type I IFN production (24–27). On the other hand, an earlier study using a UV-irradiated virus suggested that the influenza viral RNA polymerase is also responsible in the inhibition of type I IFN production (28). A viral RNA polymerase recognizes cap structure at the 5′-end of host mRNA and snatches it as a primer for viral mRNA synthesis (29, 30). Because this function of a viral RNA polymerase causes shutoff of host protein synthesis by decreasing the mature mRNA level, the inhibitory activity of IFN production by the influenza A virus polymerase has been assumed to be dependent on this "cap-snatching" activity. However, there is no direct evidence to support this hypothesis.

In this study, we report that the influenza A virus RNA polymerase performs the function of inhibition of the intracellular induction pathway for type I IFN by binding to IPS-1 independently of its cap-snatching activity. In addition, we show that this inhibitory function is mainly dependent on the PB2 subunit of a viral polymerase, and the expression of *IFNβ* mRNA induced after influenza A virus infection is inhibited by the expression of the PB2 protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The human embryonic kidney cell line HEK293 was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin and grown at 37 °C with 5% CO_2. Transfections were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

Antibodies—The specific antibodies used in this study, rat anti-HA monoclonal antibody (3F10, Roche Applied Science), mouse anti-FLAG M2 monoclonal antibody (Sigma), mouse anti-Myc monoclonal antibody, rabbit anti-IPS-1 polyclonal antibody (Alexis Biochemicals, San Diego, CA), rabbit anti-IRF3 monoclonal antibody (D83B9; Cell Signaling Technology, Beverly, MA), and rabbit anti-phospho-IRF3 (Ser^{396}) monoclonal antibody (4D4G; Cell signaling Technology) were purchased as commercially available products. The information for the specific antibodies raised against influenza A virus PA (clone 58/1), PB1 (clone 11/3), and PB2 (clone 143/3) is described elsewhere (31, 32).

Vector Construction—To construct expression plasmids, the coding regions of PB2 from various strains were amplified from the infectious clones by PCR. A series of the expression vectors for FLAG-tagged deletion mutants of PB2 were constructed by PCR from *PB2* cDNA derived from an influenza virus strain A/Puerto Rico/8/34 (H1N1; PR8). PA, PB1, and NS1 coding regions were then obtained from the total RNA of PR8-infected cells by RT-PCR, and these amplified cDNAs were inserted into the cloning vectors to express epitope-tagged protein designated pcDNA5/FRT/FLAG, pcDNA5/FRT/HA, and pcDNA5/FRT/MYC, respectively. These cloning vectors were generated by an insertion of synthetic oligonucleotide, which encoded the peptide sequence for the epitope tag to pcDNA5/FRT (Invitrogen). The retrovirus vector carrying the *PB2* gene was constructed by an insertion of the fragment encoding full-length PB2 derived from the expression plasmid into pMXs Puro (Cell Biolabs, San Diego, CA). The expression vectors for FLAG- and HA-tagged IPS-1, RIG-I, and MDA5 were described previously (16), and p125 luc, which carries the firefly luciferase gene under the control of an *INFβ* promoter, is described elsewhere (33). pcDNA 3.1(+) TLR3 carried the full-length coding region of the human *TLR3* (Toll-like receptor 3) gene was constructed by RT-PCR. To construct a template plasmid carrying influenza virus matrix (*M*) segment cDNA used for *in vitro* translation, the full-length *M* segment genome cDNA was amplified by RT-PCR and cloned into a pCR blunt II TOPO cloning vector using a Zero blunt II TOPO PCR cloning kit (Invitrogen). pBS HCV 1B IRES, which carries the hepatitis C virus type 1B 5′-untranslated region, was kindly provided by A. Nomoto (University of Tokyo). More detailed vector information is available upon request.

Luciferase Assays—Luciferase activities were quantified with a luminometer (Mtharas LB940; Berthold, Bad Wildbad, Germany) using the Dual-Glo luciferase assay system (Promega, San Luis Obispo, CA) in accordance with the manufacturer's instructions. Single-stranded RNA used in this study to stimulate RIG-I was synthesized by *in vitro* transcription using a MEGAscript T7 kit (Applied Biosystems, Foster City, CA) from the vector carrying the HCV 5′-UTR and influenza virus *M* segment cDNA downstream of the T7 promoter. These RNA were purified by using the MEGAclear kit (Applied Biosystems) according to the manufacturer's protocols. Poly(I-C), the synthetic double-stranded RNA used to stimulate MDA5 and TLR3, was purchased from Sigma. *IFNβ* promoter activities were measured by firefly luciferase activity of the reporter plasmid carrying the human *IFNβ* promoter region, p125 luc, and activations of NF-κB were monitored by pNF-κB-luc (Stratagene, La Jolla, CA), which carries a synthetic promoter containing direct repeats of the NF-κB recognition sequence. The quantified results were normalized with the *Renilla* luciferase activities of the internal control vector (pGL 4.74; Promega).

Immunoprecipitation—To analyze binding of transiently expressed proteins, the expression plasmids indicated in the figures were transfected into HEK293 cells, and the cells were then harvested and lysed by radioimmunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor mixture (Complete Mini; Roche Applied Science). After the cell debris was removed by centrifugation, the lysates were subjected to immunoprecipitation using anti-FLAG M2-agarose (Sigma). To analyze the binding of viral polymerase to the IPS-1 protein in virus-infected cells, the HEK293 cells were infected with the PR8 virus strain (multiplicity of infection = 2). After 8 h, the cells were harvested and were lysed with radioimmunoprecipitation buffer supplemented with the protease inhibitor mixture. The cell lysates were immunoprecipitated with anti-PB2 monoclonal antibody and protein G-Sepharose (GE Healthcare). The resins were washed five times

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

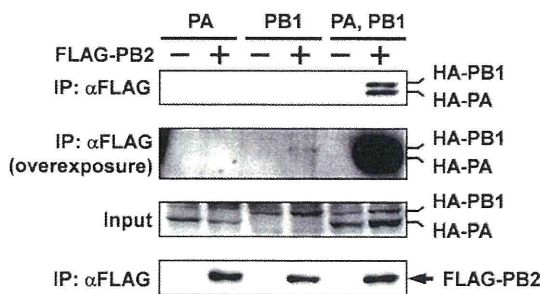


FIGURE 1. **The N-terminal epitope-tagged subunits of influenza A virus RNA polymerase also form the heterotrimer.** Expression vectors for FLAG-tagged PB2 (1.6 μg) and HA-tagged PA and PB1 (1.2 μg each) were transfected into HEK293 cells. After 24 h, the cells were harvested and lysed, and then the whole cell extracts were subjected to the co-immunoprecipitation assay (*IP*) using an anti-FLAG M2-agarose.

with radioimmunoprecipitation buffer, and binding proteins were eluted by boiling for 5 min with 2× SDS-polyacrylamide gel-loading buffer (125 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 4% SDS, 20% glycerol, 0.01% bromphenol blue).

RT-PCR—Quantitative and semiquantitative RT-PCR for endogenously expressed *IFNβ* was carried out as described previously (34). Total cellular RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The isolated RNA was treated with RNase-free DNase I (Roche Applied Science). Subsequently, cDNA was synthesized from the RNA using random hexamer and reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan). Real-time PCRs were performed using SYBR Premix Ex TaqII (Takara, Otsu, Japan) and quantified by the Mx3000P quantitative PCR System (Stratagene).

Virus Infection—HEK293 cells were transfected with 2 μg of expression vector for intact PB2. After 35 h, the cells were infected with influenza virus A/Aichi/2/1968 (H3N2; Aichi) strain (multiplicity of infection = 2), and incubated with DMEM containing 1% BSA and 0.5 μg/ml trypsin for 3 h. Subsequently, the cells were harvested and subjected to RT-PCR and Western blotting analysis.

RESULTS

Inhibition of Activation of Intracellular Induction Pathway for Type I IFN by Influenza A Virus Polymerase—To investigate the function of the influenza virus polymerase complex against the induction of type I IFN, we constructed expression vectors that encode the N-terminal epitope-tagged influenza A virus RNA polymerase subunits PA, PB1, and PB2 derived from the H1N1 subtype influenza virus strain A/Puerto Rico/8/34 (PR8). Initially, we confirmed whether these N-terminal epitope-tagged subunits are able to form a heterotrimeric complex. The expression vectors for FLAG-tagged PB2 and HA-tagged PA and PB1 were transfected into HEK293 cells, and the whole cell extracts prepared from the cells were subjected to immunoprecipitation using anti-FLAG resin. As shown in Fig. 1, the results indicate that when three subunits are expressed together, both HA-tagged PA and PB1 subunits are co-immunoprecipitated with FLAG-tagged PB2 subunit. Previous reports demonstrated that the RNA polymerase subunits of influenza A virus are able to form PA/PB1 and PB1/PB2 heterodimers as intermediates, whereas PA/PB2 is not (35, 36). In agreement with the previous observations, although the signal was relatively weak, HA-tagged PB1 was co-immunoprecipitated with FLAG-tagged PB2, whereas HA-tagged PA was not. These results suggest that these N-terminal epitope-tagged subunits are able to form the heterotrimer of the mature viral polymerases.

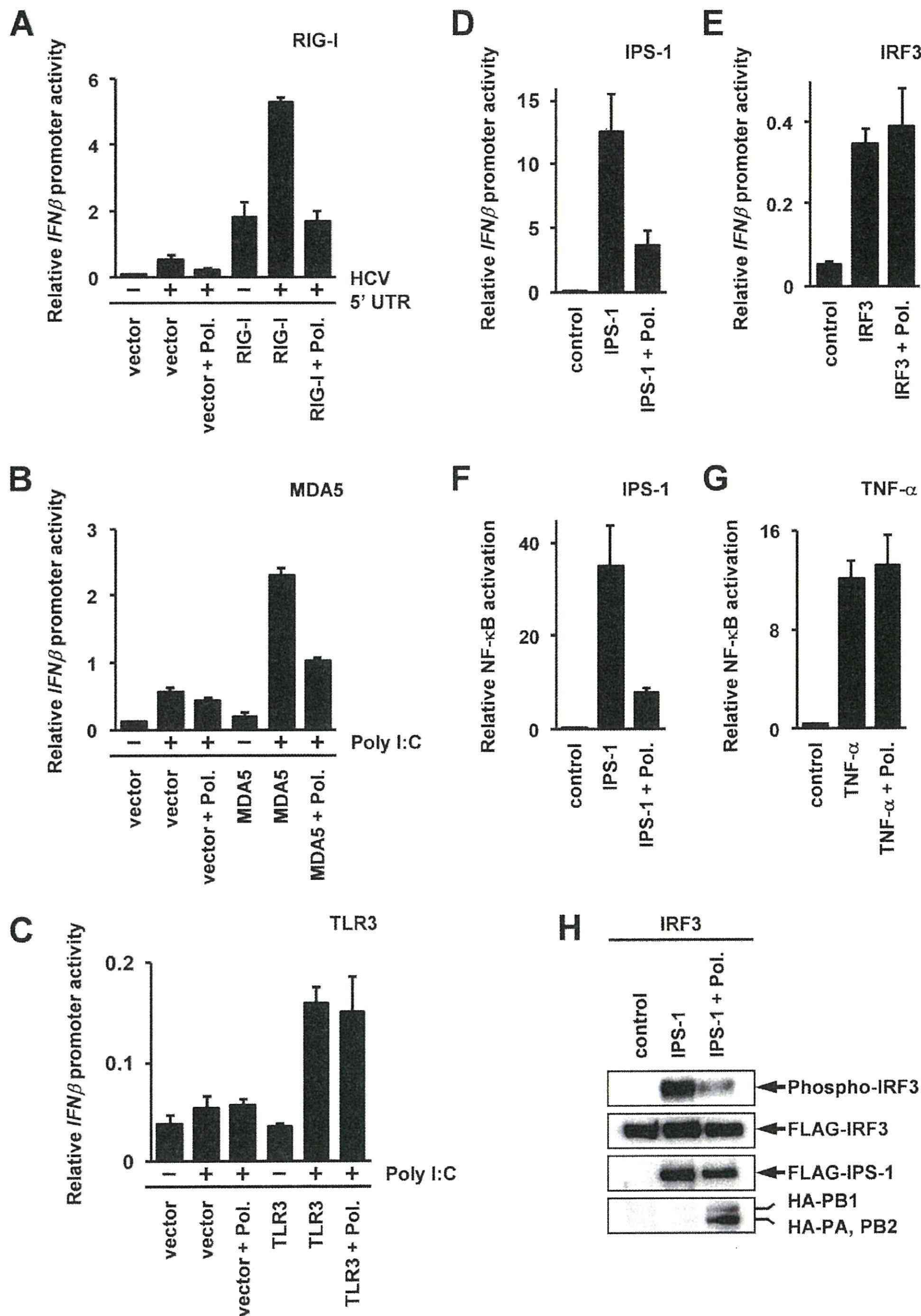
Next, we examined the effect of the expression of these subunits on the activation of the *IFNβ* promoter by stimulation with exogenous RNA by using a reporter gene assay. The results show that activation of *IFNβ* promoter mediated by MDA5 or RIG-I was significantly inhibited by the overexpression of the viral RNA polymerase complex (Fig. 2*A* and 2*B*), whereas TLR3-mediated activation of the *IFN* promoter was not influenced (Fig. 2*C*). Because it has been reported that the conserved flanking sequence lying on viral genomic RNA is required for the activation of the cap-snatching function of the influenza A virus polymerase (37–39), it was assumed that stimulation by both HCV 5′-UTR and poly(I-C) could not activate cap-snatching activity. Therefore, we concluded that the inhibitory effect of a viral polymerase on RIG-I-mediated *IFNβ* promoter activation was independent of its cap-snatching function. This conclusion is also supported by the finding that the expression of a viral polymerase did not inhibit TLR3-mediated *IFNβ* promoter activation.

Although subcellular localization of RIG-I or MDA5 is different from that of TLR3, the overall induction pathway for type I IFN by RIG-I and MDA5 is mediated by the same signaling molecules as the pathway mediated by TLR3. These molecules transduce the signals to the same transcription factors, IRF3, IRF7, and NF-κB, mediated by the intermediate signaling molecules, such as TBK1 (TANK-binding kinase 1) and IKKϵ (IκB kinase ϵ) (40, 41). Therefore, our data, derived by using a reporter gene assay (Fig. 2, *A–C*), suggest that the targets of viral RNA polymerase are not these overlapping signaling molecules that inhibit the intracellular induction pathway for type I IFN. From these facts, we thought that there is a possibility that the viral polymerase complex targets to IPS-1 and inhibits its function, because IPS-1 is an adapter molecule that binds RIG-I and MDA5 but not TLR3.

Initially, we investigated the effect of an expression of the influenza A virus polymerase to the *IFNβ* promoter activated by the overexpression of IPS-1. As shown in Fig. 2*D*, the results show that a viral polymerase strongly inhibited *IFNβ* promoter activation induced by the expression of IPS-1. To confirm that the viral polymerase specifically inhibits the IPS-1-mediated signaling pathway, we evaluated the effects of the viral polymerase on the *IFNβ* promoter activation induced by overexpression of IRF3. The results show that activation of the *IFNβ* promoter induced by overexpression of IRF3 was not affected by the expression of the viral polymerase (Fig. 2*E*).

It is known that activation of the *IFNβ* promoter is regulated by IRF3, IRF7, and NF-κB (40, 41), and NF-κB is involved in the induction of a variety of inflammatory cytokines in addition to type I IFN (42). Taking this into consideration, we used a reporter plasmid that carries the luciferase gene under the control of the synthetic NF-κB binding site (pNF-κB-luc; Promega) and evaluated the effect of the viral polymerase expression on IPS-1-induced NF-κB activation. The results show that the viral polymerase significantly inhibited NF-κB activation induced by

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012



Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

FIGURE 2. **Inhibition of intracellular IFN inducing pathways by overexpression of influenza viral polymerase complex.** *A* and *B*, the expression plasmids for each subunit of influenza viral RNA polymerase derived from PR8 strain (200 ng) were transfected with the p125 luc (80 ng) and pGL4.74 (20 ng) reporter constructs together with 100 ng of expression vector for RIG-I (*A*) or MDA5 (*B*) into HEK293 cells. After a 24-h post-transfection incubation period, the cells were stimulated with 800 ng of HCV 5′-UTR (*A*) or poly(I-C) (*B*) by transfection. After an additional 24-h incubation, luciferase activities were measured by luminometer. *C*, PA (300 ng), PB1 (150 ng), PB2 (300 ng), and TLR3 (125 ng) expression plasmids were transfected with the p125 luc (100 ng) and pGL4.74 (25 ng) reporter constructs into HEK293 cells. After 24 h, the cells were treated with poly(I-C) (50 μg/ml) for 12 h, and luciferase activities were measured. *D*, HEK293 cells were transfected with the p125 luc (80 ng) and pGL4.74 (20 ng) reporter constructs together with expression vectors for IPS-1 (50 ng) and each component of influenza virus RNA polymerase (200 ng). After a 24-h period of incubation, luciferase activities were measured. Total amounts of DNA in each transfection were equalized by empty vectors. *E*, the expression vector for the FLAG-tagged IRF3 and each subunit for the viral polymerase were transfected into HEK293 cells with p125 luc and pGL4.74 plasmids. After 24 h, the activation of IFNβ promoter was analyzed by a luciferase assay. *F*, the expression plasmids for FLAG-tagged IPS-1 (50 ng) and each subunit of viral polymerase (250 ng) were transfected into HEK293 cells together with the reporter construct plasmids, pNF-κB luc (50 ng) and pGL4.74 (50 ng). After 24 h, the activation of NF-κB was measured by a luciferase assay. *G*, the expression plasmids for the FLAG-tagged each viral polymerase subunits (250 ng) were transfected with pNF-κB (50 ng) luc and PGL 4.74 (50 ng) into HEK293 cells. After 24 h, the cells were stimulated by 25 ng/ml TNF-α. After an additional 8-h post-stimulation incubation period, activation of NF-κB was analyzed by the luciferase assay. Data represent relative *IFNβ* promoter activities or NF-κB activations, which were normalized with *Renilla* luciferase activities. *Error bars* indicating the S.D. values were calculated from at least three independent experiments. *H*, the expression plasmids for FLAG-tagged IRF3 (0.8 μg) and IPS-1 (40 ng) were transfected with expression vectors for the HA-tagged subunits of influenza viral RNA polymerase (1.0 μg) into HEK293 cells. After 24 h, the cells were harvested, and phosphorylation of IRF3 was analyzed by immunoblotting specific antibody against phospho-IRF3 (Ser[396]).

FIGURE 3. **Binding of each component of influenza virus RNA polymerase to IPS-1.** *A*, HEK293 cells were transfected with expression vectors for HA-tagged PA, PB1, or PB2 (3.5 μg) together with 0.5 μg of FLAG-tagged IPS-1. *B*, FLAG-tagged PA, PB1, PB2, or M (3.5 μg) expression vectors were transfected with HA-tagged IPS-1 expression constructs (0.5 μg) into HEK293 cells. *C*, the expression plasmids for Myc-tagged PB2 (1.5 μg), HA-tagged PA (1.5 μg) and PB1 (0.6 μg) were transfected into HEK293 cells with 0.4 μg of expression plasmid for FLAG-tagged IPS-1. Whole cell extracts from the transfected cells were immunoprecipitated (*IP*) by using FLAG M2-agarose and subjected to Western blotting analysis. *D*, HEK293 cells were infected with influenza A virus PR8 strain (multiplicity of infection = 2). After 8 h, the cells were harvested and lysed, and then the lysates were subjected to immunoprecipitation using a mouse monoclonal antibody against PB2. The antibody that recognizes Ebola ZGP was used for the control. IPS-1 and each subunits of the viral polymerase were detected by using the specific antibodies.

the IPS-1 overexpression (Fig. 2*F*) but did not inhibit the activation induced by TNF-α stimulation (Fig. 2*G*). TNF-α is known to be an inflammatory cytokine belonging to the TNF superfamily and is also known to be a strong NF-κB inducer that activates a signaling pathway distinct from the IPS-1-mediated signaling pathway (43).

Finally, we examined whether IPS-1-mediated activation of IRF3 is inhibited by the expression of the viral RNA polymerase. It has been reported that the Ser^{396} residue of IRF-3 is phosphorylated by the infection of RNA viruses and the transfection of double-stranded RNA (44) and is also phosphorylated by the overexpression of IPS-1 (16). We evaluated the activation of IRF3 by monitoring the phosphorylation level of IRF3 at Ser^{396}, using its specific antibody. As shown in Fig. 2*H*, The results show that the phosphorylation of Ser^{396} in IRF3 was increased by the IPS-1 overexpression and was significantly inhibited by the expression of the viral RNA polymerase. These results suggest that the influenza A virus RNA polymerase has an activity to inhibit internal IFN induction pathway through the inhibition of IPS-1 function.

Inhibition of IPS-1-mediated Induction of Type I IFN through Binding to IPS-1 by Influenza A Virus Polymerase—To reveal the molecular mechanism for the inhibition of the IPS-1-mediated signaling pathway, we initially examined a possibility that viral RNA polymerase inhibits the IPS-1 function through the binding to the IPS-1. To investigate the binding of a viral polymerase with IPS-1, we performed a co-immunoprecipitation assay. IPS-1 with PA, PB1, or PB2 was expressed in HEK293 cells, and the binding of IPS-1 with PA, PB1, or PB2 was analyzed. Each component of the influenza viral polymerase complex was co-immunoprecipitated with IPS-1 (Fig. 3, *A* and *B*). These results indicated that all subunits of a viral RNA polymerase have the ability to bind with IPS-1.

Next, we investigated whether all components of viral polymerase bind to IPS-1 simultaneously. As shown in Fig. 3, *A* and *B*, PA and PB2 were detected to have the same mobility by Western blotting, so it was difficult to distinguish between the two proteins. To resolve this problem, we constructed a Myc-tagged PB2 expression vector. Expression vectors for HA-tagged PA, PB1, Myc-tagged PB2, and FLAG-tagged IPS-1 were transfected into HEK293 cells, and the cell lysates were immunoprecipitated by anti-FLAG resin. The results show that all components of the viral polymerase were co-immunoprecipitated with IPS-1 at the same time (Fig. 3*C*). A viral polymerase forms a stable complex (1, 36, 45), and we confirmed that the N-terminal epitope-tagged polymerase subunits used in this study are also able to form a complex (Fig. 1). From these results, it is likely that a viral polymerase binds to IPS-1 as a complex.

Finally, we investigated whether the viral polymerase binds to IPS-1 in virus-infected cells. The PB2 proteins in the lysates of the influenza virus-infected cells were immunoprecipitated with anti-PB2 monoclonal antibody, and co-immunoprecipitated proteins were analyzed by Western blotting. As shown in Fig. 3*D*, the results indicate that PA and PB1 subunits were largely co-immunoprecipitated with PB2, and IPS-1 was also co-immunoprecipitated together with these proteins. The data suggested that the PB2 subunit binds to IPS-1 in the virus-infected cells and probably makes a complex.

Dependence on PB2 in Inhibition of IPS-1-mediated Signaling Pathway for Induction of IFNβ by Influenza A Virus Polymerase—To investigate which subunit of viral polymerase is responsible for the inhibition of IPS-1 function, we examined the inhibitory activity of each polymerase subunit by using a reporter gene assay. Each polymerase subunit and NS1 were independently expressed in HEK293 cells together with RIG-I, and the cells were stimulated with single-stranded RNA derived from the influenza virus matrix (*M*) segment. The results indicated that *IFNβ* promoter activation by single-stranded RNA was equally inhibited by each subunit of the viral polymerase (Fig. 4*A*).

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

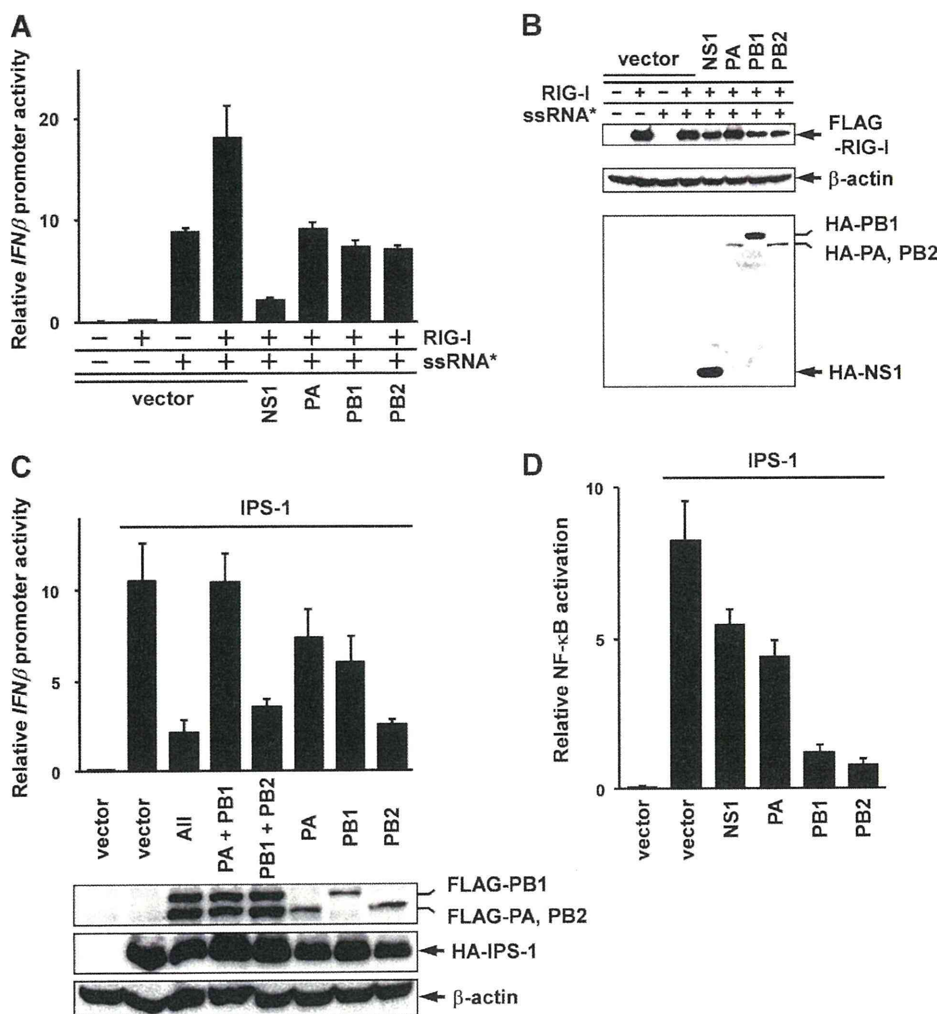


FIGURE 4. **PB2 as a major component for inhibition of IPS-1-mediated IFNβ and NF-κB promoter activation by influenza viral polymerase.** *A*, the expression plasmids for HA-tagged NS1, PA, PB1, and PB2 (600 ng) were transfected with the p125 luc (80 ng) and pGL4.74 (20 ng) reporter constructs together with 100 ng of expression vector for FLAG-tagged RIG-I into HEK293 cells. After a 24-h post-transfection incubation period, the cells were stimulated by the transfection of single-stranded RNA (800 ng) derived from the matrix (*M*) segment of influenza virus PR8 strain. The luciferase activities were measured after a 24-h poststimulation incubation period. *B*, Western blotting analysis of whole cell extracts from the same transfectants. *C*, the expression plasmids for HA-tagged PA (280 ng), PB1 (140 ng), and PB2 (280 ng) were transfected with the expression construct for FLAG-tagged IPS-1 (20 ng) together with p125 luc (64 ng) and pGL 4.74 (16 ng) into HEK293 cells. After 24 h, the luciferase activities were measured, and part of the transfectants were harvested and subjected to Western blotting analysis. *D*, the vectors to express FLAG-tagged NS1, PA, PB1, and PB2 (800 ng) were transfected with the pNF-κB luc (80 ng) and pGL4.74 (20 ng) reporter plasmids together with 50 ng of expression vector for FLAG-tagged IPS-1 into HEK293 cells. After 24 h, the luciferase activities were measured. Total amounts of DNA in each transfection were equalized by empty vectors. Data represent relative *IFNβ* promoter activities, which were normalized with *Renilla* luciferase activities. *Error bars* indicating the S.D. values were calculated from at least three independent experiments. *ssRNA*, single-stranded RNA.

However, the expression level of PB1 was higher than that of PA and PB2 (Fig. 4*B*). In addition, the expression level of NS1 was higher than that of PA, PB1, and PB2. However, the inhibitory activity of NS1 on *IFNβ* promoter activation was detected as stronger than that of each polymerase. Therefore, it may be assumed that the inhibitory effect of PB2 on *IFNβ* promoter activation was very strong.

Next, we adjusted the amount of expression vectors for the transfection to equalize the protein expression level of each subunit, and then we tested their inhibitory effect on IPS-1-induced *INFβ* promoter activation. The results show that the relative inhibitory activity of PB2 on *IFNβ* promoter activation depended on the PB1/PB2 heterodimer and the viral polymerase complex (Fig. 4*C*). To confirm this strong inhibitory activity of PB2 against the IPS-1 function, we investigated the inhibitory activities of each viral polymerase component on IPS-1-mediated activation of NF-κB by using a reporter gene assay. The results show that NF-κB activation by overexpression of IPS-1 was also most strongly inhibited by PB2 (Fig. 4*D*). The amounts of transfected expression plasmids for viral polymerase subunits and NS1 were the same in this case. In addition, because NS1 binds to PI3K and activates the NF-κB pathway (46–48), NS1 did not show strong inhibitory activity on IPS-1-mediated NF-κB activation. Taken together, it was demonstrated that the PB2 protein acts mainly to inhibit the function of IPS-1 in the activation of the *IFNβ* promoter and NF-κB.

The Binding Activity of PB2 to IPS-1 Is Mainly Dependent on the N-terminal Region of PB2—Because our results using a reporter gene assay revealed that the PB2 subunit plays a pivotal role in the inhibition of activation of *IFNβ* promoter mediated by IPS-1, we next analyzed the binding region of PB2 to IPS-1 by a co-immunoprecipitation assay using deletion mutants of PB2 for further analysis of the inhibitory mechanism. As shown in Fig. 5*A*, we constructed the expression vectors for a series of FLAG-tagged deletion mutants of PB2 for the analysis. These expression vectors were transfected with that of HA-tagged IPS-1 into HEK293 cells, and then the PB2 mutants were immunoprecipitated using anti-FLAG resin from the whole cell extracts of the cells. The results show that although no deletion mutant of PB2 indicated a complete loss of binding activity to the HA-tagged IPS-1, PB2 ΔN242 and PB2 ΔN482 exhibited significantly weak binding activity to the IPS-1 in comparison with that of wild-type PB2 (Fig. 5*B*). Both of these mutants lack the N-terminal 242-amino acid region of PB2. On the other hand, PB2 ΔC257 which expresses the N-terminal 256-amino acid region of PB2 exhibits binding activity to the IPS-1 as well as that of wild-type PB2. Therefore, the N-terminal 242-amino acid region of PB2 is thought to be mainly responsible for the binding to IPS-1. Comparable results were obtained by the

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

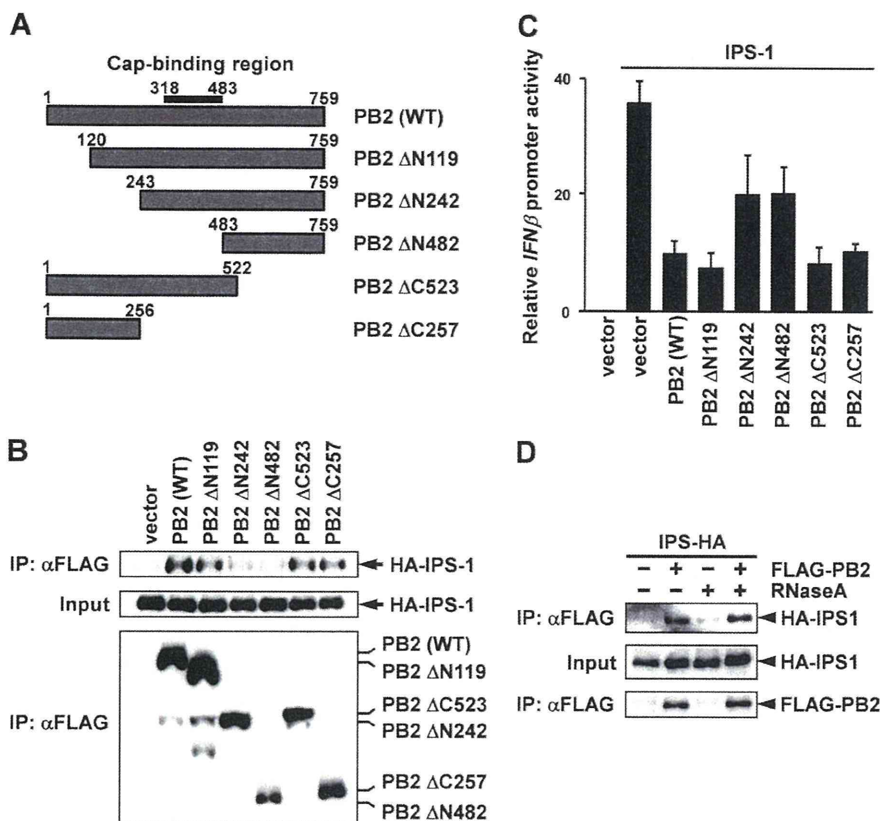


FIGURE 5. **Cap-binding region of PB2 is not required for inhibition of the activation of IFNβ promoter mediated by IPS-1.** *A*, *schematic diagram* of PB2 deletion mutants. The *numbers* indicate the amino acid sequence position of PB2, and the minimal cap-binding domain of PB2 is indicated in the *bold bar* (61). *B*, HEK293 cells were transfected with the expression vectors for the series of FLAG-tagged deletion mutants of PB2 (3.5 μg) together with the expression vector plasmid for HA-tagged IPS-1 (0.5 μg). At 24 h post-transfection, the cells were harvested and subjected to an immunoprecipitation assay (*IP*) as described under "Experimental Procedures." *C*, the expression vectors for the HA-tagged IPS-1 (100 ng) and the series of FLAG-tagged deletion mutants of PB2 (400 ng) were transfected with p125 luc (80 ng) and pGL4.74 (20 ng) into HEK293 cells. After 24 h, luciferase activities in the cells were quantified by the luminometer. *Error bars* indicating the S.D. values were calculated from at least three independent experiments. *D*, expression vectors for HA-tagged IPS-1 (0.4 μg) and FLAG-tagged PB2 (3.6 μg) were transfected into HEK293 cells using FuGENE HD transfection reagent (Roche Applied Science). The cells were harvested at 24 h post-transfection and lysed with radioimmunoprecipitation buffer. The lysates were then separated into two aliquots. One aliquot was treated with RNase A at 4 °C for 2 h, whereas the other aliquot was left untreated. Both aliquots were then subjected to the immunoprecipitation.

reporter gene assay using these mutants. As shown in Fig. 5*C*, the deletion mutants, PB2 ΔN242 and PB2 ΔN482, exhibited significantly weak inhibitory activity against *IFNβ* promoter activation induced by overexpression of IPS-1 in comparison with that of wild-type PB2.

Most of the other viral molecules that are known to be functional for the inhibition of IPS-1-mediated type I IFN production exhibit the inhibitory function through the binding to the viral RNA and inhibit the function of RNA recognition molecules (*e.g.* RIG-I and MDA5) by competition (18–22). Taking this into consideration, we investigated whether the IPS-1 binding activity of PB2 is dependent on the RNA binding activity of PB2. As shown in Fig. 5*D*, the IPS-1 binding activity of the PB2 subunit was not affected by RNase treatment. These results suggest that PB2 binds to IPS-1 in an RNA-binding independent manner, and it is likely that PB2 inhibits the function of IPS-1 through the protein-protein interaction.

Inhibition of IFN Induction by Influenza A Virus Polymerase Is Independent of NS1 Function—It is known that NS1 inhibits the induction of IFN by binding to RIG-I, which is located upstream of IPS-1 and acts as a sensor molecule for viral RNA. To investigate whether the function of viral polymerase in the inhibition of *IFNβ* promoter activation is competitive with that of NS1, we transfected NS1 and each polymerase component at the same time and measured the inhibitory activity by using a reporter gene assay. The results show that inhibition of IPS-1-induced *IFNβ* transcription by the viral polymerase is enhanced by the expression of NS1 (Fig. 6*A*). Therefore, it is indicated that the inhibitory function of the viral polymerase is independent of the NS1 function. Although we transfected fewer expression vectors for NS1 than for each polymerase component in this case, the protein expression levels of NS1 in the cells simultaneously transfected were detected, as were the protein expression levels of polymerase subunits, by Western blotting (Fig. 6*B*). Based on these data, we have concluded that the relative inhibitory activity normalized with the protein expression level did not significantly differ between NS1 and the viral polymerase.

Inhibition of IFNβ Promoter Activation by Suppression of IPS-1 Function by PB2 of Various Strains of Influenza A Virus—In the experiments described thus far, we used viral components derived from the PR8 strain, which belongs to the H1N1 subtype influenza virus. To confirm whether the RNA polymerase subunits of other strains of influenza A virus perform a similar function, we tested the inhibitory effect of influenza A virus PB2 derived from other strains on *IFNβ* promoter activation. Expression vectors for PB2 proteins from H1N1 influenza virus, A/WSN/33 (WSN), and H5N1 highly pathogenic avian influenza virus, A/Hong Kong/483/97 (HK483) and A/Hong Kong/486/97 (HK486), were used for this assay, and the results show that PB2 derived from these strains also inhibited IPS-1-induced *IFNβ* promoter activation (Fig. 6*C*). From these data, it can be assumed that the function of PB2 in the inhibition of *IFNβ* promoter activation is common to various influenza A viruses.

Modulation of Cellular IFNβ Gene Activation Induced by Influenza A Virus by Expression of PB2—To investigate whether the protein expression level of PB2 affects the inhibition of

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

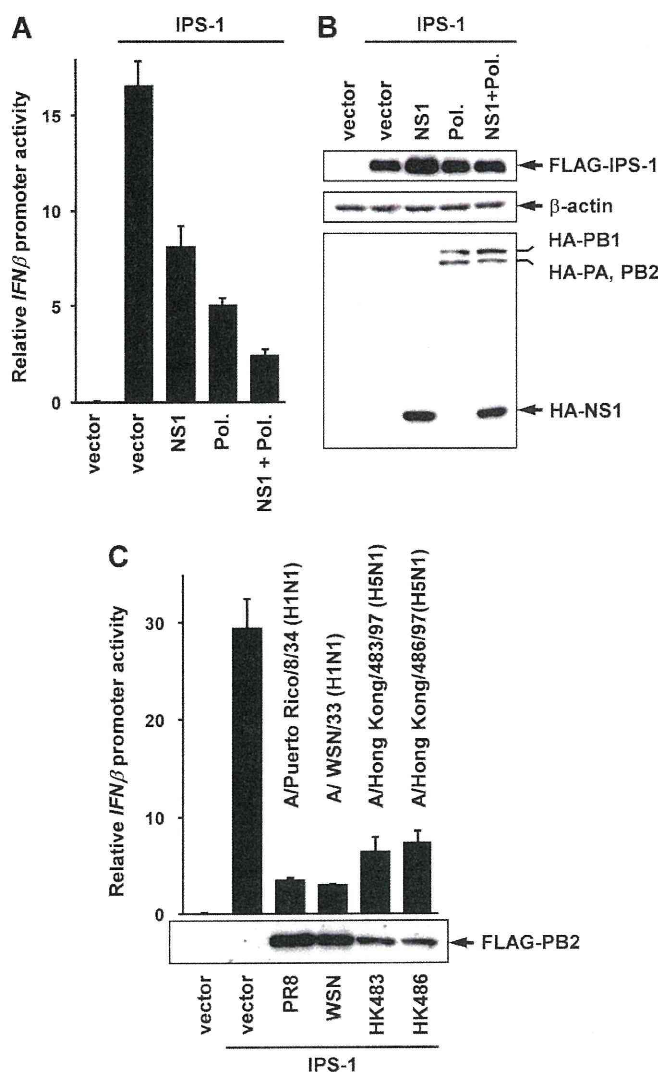


FIGURE 6. **Inhibition of IFN induction by influenza A virus polymerase is not competitive with the NS1 function.** *A*, the expression plasmids for HA-tagged NS1 (100 ng), PA (200 ng), PB1 (100 ng), and PB2 (200 ng) were transfected with the expression construct for FLAG-tagged IPS-1 (20 ng) together with p125 luc (64 ng) and pGL 4.74 (16 ng) into HEK293 cells. After 24 h, the luciferase activities were measured. *B*, Western blotting analysis of whole cell extracts from the same transfectants. *C*, the expression constructs for IPS-1 (20 ng) and PB2 derived from various influenza virus strains indicated in the figure (500 ng) were transfected into HEK293 cells with p125 luc (64 ng) and pGL 4.74 (16 ng). After 24 h, the luciferase activities were measured, and part of the transfectants were harvested and subjected to Western blotting analysis. The total amounts of DNA in each transfection were equalized by empty vectors. The data represent relative *IFNβ* promoter activities, which were normalized with *Renilla* luciferase activities. *Error bars* indicating the S.D. values were calculated from at least three independent experiments.

IPS-1-mediated *IFNβ* promoter activation, we monitored IPS-1-induced *IFNβ* promoter activities after the expression of various amounts of PB2. The results show that IPS-1-induced *IFNβ* promoter activation was inhibited by the expression of PB2 in a dose-dependent manner (Fig. 7*A*). This finding suggests that the expression level of PB2 regulated the type I interferon production induced by influenza A virus infection.

Next, to confirm that influenza A virus PB2 actually inhibits an endogenously expressed *IFNβ* gene expression mediated by IPS-1, we investigated the cellular *IFNβ* mRNA expression level by using RT-PCR. As shown in Fig. 5*B*, expression of influenza A virus PB2 remarkably repressed the endogenous *IFNβ* expression induced by IPS-1.

Finally, to determine whether the expression level of PB2 actually affects *IFNβ* gene expression in the cells infected by influenza A virus, we investigated the effects of PB2 overexpression on the endogenous *IFNβ* mRNA level after influenza A virus infection by RT-PCR. The results show that the amount of endogenous *INFβ* mRNA expression induced by viral infection was suppressed by overexpression of PB2 (Fig. 7, *C* and *D*), and the expression level of the nucleoprotein (*NP*) gene was not significantly changed by the overexpression of PB2 (Fig. 7*E*). These data suggested that the expression level of PB2 in virus-infected cells is involved in the inhibition of type I IFN response.

DISCUSSION

We show in this report that the influenza A virus RNA polymerase inhibits production of type I IFNs through binding to IPS-1 and suppression of its function. Interestingly, our findings reveal that influenza A virus inhibits the intracellular type I IFN-inducing pathway by additional components that are distinct from NS1, and this indicates the physiological importance of this function in the protection of host cells against influenza A virus infection. The influenza A virus NS1 protein is expressed more abundantly in the virus-infected cells than is the viral polymerase (49, 50). NS1 is known to be a multifunctional protein and is important for the regulation of viral replications and inhibition of host anti-viral response (51). Despite its importance, the mutant virus that lacks the NS1 coding region is able to replicate in interferon-deficient systems (52), and the NS1-deficient virus-infected cells significantly increase the production of type I IFNs. However, as suggested by the previous report using a UV-irradiated virus, type I IFN production was increased by gene disruption by UV irradiation for NS1-deficient virus (28). These findings suggest that the importance of the viral polymerase function in the inhibition of type I IFN production cannot be ignored. Because it is known that the type I IFN production pathway is enhanced in an autocrine manner (33, 53–55), inhibitory activity of the interferon production pathway at the early phase of viral infection might be strongly reflected in the intensity of cellular antiviral responses at the late phase. Therefore, at the early phase of viral infection, at which time an insufficient amount of viral components exist in infected cells, viral polymerase activity to repress type I IFN response might play an important role in escape from host antiviral responses.

As shown in Fig. 4, the PB2 subunit efficiently repressed IPS-1-mediated *IFNβ* promoter activation when it was expressed in isolation. And the results of combinational expression of each polymerase subunit revealed that activation of the *IFNβ* promoter induced by overexpression of IPS-1 was strongly repressed by expression of the PB2-containing complex. Therefore, we have concluded that PB2 performs a central role in this inhibitory function. Interestingly, our results show that the activation of *IFNβ* promoter induced by IPS-1 was not inhibited by the combinational expression of PA and PB1, and the

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

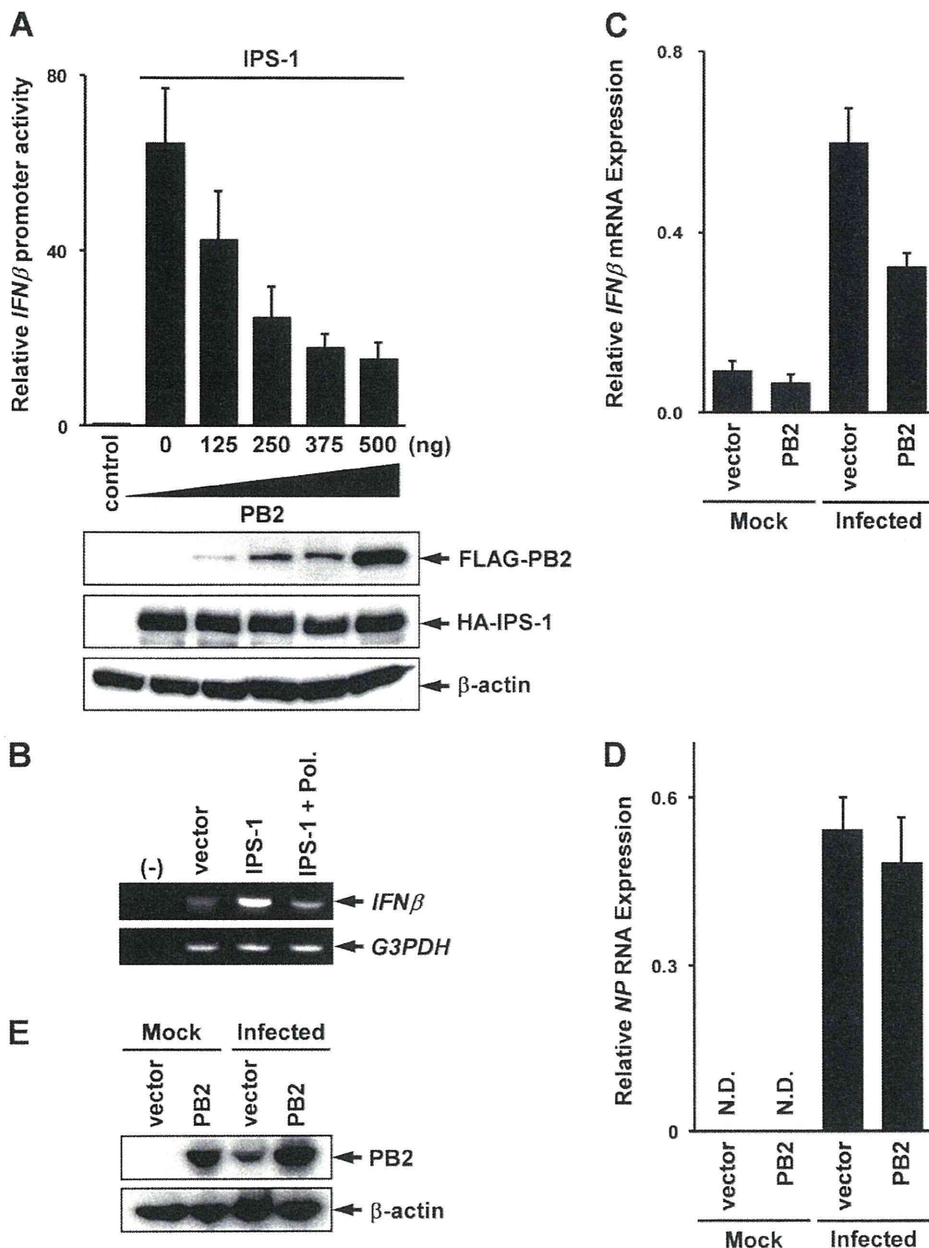


FIGURE 7. **Inhibition of transcriptional activation of cellular IFN*β* gene in influenza virus-infected cells by overexpression of PB2.** *A*, various amounts of PB2 expression vector indicated in the figure were transfected with 25 ng of IPS-1 expression plasmid into HEK293 cells together with p125 luc (80 ng) and pGL4.74 (20 ng). After a 24-h post-transfection incubation period, the luciferase activities were measured by a luminometer. *B*, HEK293 cells were transfected with expression vectors for IPS-1 (0.1 μg), PA (1.5 μg), PB1 (0.7 μg) and PB2 (1.5 μg). After 24 h, the cells were harvested, and the total RNA was isolated from the cells. The expression levels of the endogenous *INFβ* gene were monitored by semiquantitative RT-PCR. The glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene was used for internal control. *C* and *D*, the PB2-expressed cells were infected with influenza A virus Aichi strain (multiplicity of infection = 2). At 3 h postinfection, the expression levels of *IFNβ* mRNA (*C*) and nucleoprotein (*NP*) RNA (*D*) were quantified by real-time RT-PCR. The data are presented as relative amounts of *IFNβ* mRNA, which were normalized with the expression level of *G3PDH* mRNA. *E*, Western blotting analysis of whole cell extracts from the same transfectants using an anti-PB2 monoclonal antibody. The mouse anti-actin monoclonal antibody was used as a loading control. *N.D.*, not detected.

inhibitory activity was apparently weakened compared with its expression in isolation (Fig. 4*C*). A previous report (56) demonstrated that PA and PB1 are mainly localized in cytoplasm when these subunits were expressed alone and are translocated into the nucleus when these subunits formed a heterodimer. These findings suggest that the cytoplasmic localization of PA and PB1 subunits is important for the inhibition of the IPS-1 function. On the other hand, PB2 is mainly observed in the nucleus even when it is expressed alone. However, it has been reported that PB2 carries a mitochondrial targeting sequence at the N terminus and also localizes in mitochondria (57). Because IPS-1 is known to be localized in the mitochondrial outer membrane, it is suggested that the PB2-containing complexes efficiently inhibit the IPS-1 function in this area. From these findings, mitochondrial/cytoplasmic localization of polymerase is thought to be required for the inhibition of IPS-1 function, and the mitochondrial targeting sequence of PB2 is thought to be involved in the inhibitory function of the viral polymerase through the regulation of the subcellular localization.

It was reported that PB2 is a determinant in the difference in pathogenicity to mammalians between influenza virus HK483 and influenza virus HK483, both of which belong to the H5N1 subtype highly pathogenic avian influenza virus (58, 59). However, no significant difference was observed in the inhibitory activity of PB2 derived from these virus strains. Although the inhibitory activity of PB2 on the IPS-1 function does not directly reflect the difference in the pathogenicity between these virus strains, the general expression level of the PB2 protein is influenced by its viral replication efficiency, and highly pathogenic influenza viruses are thought to replicate more efficiently than do low pathogenic viruses in the infected cells. As shown in Fig. 7*A*, PB2 repressed IPS-1-mediated *IFNβ* promoter activation in a dose-dependent manner. In addition, the data shown in Fig. 7, *C–E*, suggest that the expression level of PB2 actually affects the amount of type I IFN production in cells infected by influenza A virus. Therefore, these results might explain part of the reason for the previously reported results, which showed that the production of type I IFN was significantly repressed by highly pathogenic influenza virus infection, such as in the case of the 1918 Spanish flu (60).

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

Molecular biological analysis revealed that the inhibitory action of NS1 on the type I IFN-inducing pathway is dependent on its binding activity to double-stranded RNA (24). On the other hand, the inhibitory mechanism of viral polymerase seemed not to be dependent on its RNA binding activity, at least in the case of PB2, because PB2 could bind to IPS-1 and repress IPS-1-mediated *INFβ* promoter activation, even when its cap-binding region was deleted (61) (Fig. 5, *A–C*). Moreover, PB2 binding activity to IPS-1 was not affected by a treatment with RNase A (Fig. 5*D*). Therefore, it may be suggested that repression of the molecular functions of IPS-1 could be a very important function of the viral polymerase. There is a possibility that IPS-1 is involved in other unknown signaling pathways, as are other adapter molecules, such as MyD88 (myeloid differentiation protein-88), which works not only for the Toll-like receptor-mediated signaling pathway but also for the interleukin-1 receptor signal (7, 8, 62, 63). Recently, our work has shown that IPS-1 is also responsible in the inducement of anoikis, which is known to be an apoptosis induced by a loss of adhesion (64). It may be suggested that a function of IPS-1 is to control cell death that has been induced by various stresses, such as viral infection. Further investigation is required for understanding of the biological importance of the interaction between IPS-1 and a viral polymerase for the pathogenicity of influenza A virus.

REFERENCES

1. Neumann, G., Brownlee, G. G., Fodor, E., and Kawaoka, Y. (2004) *Curr. Top. Microbiol. Immunol.* **283,** 121–143
2. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) *Science* **264,** 1415–1421
3. Platanias, L. C. (2005) *Nat. Rev. Immunol.* **5,** 375–386
4. Arnheiter, H., Skuntz, S., Noteborn, M., Chang, S., and Meier, E. (1990) *Cell* **62,** 51–61
5. Garber, E. A., Chute, H. T., Condra, J. H., Gotlib, L., Colonno, R. J., and Smith, R. G. (1991) *Virology* **180,** 754–762
6. Kolb, E., Laine, E., Strehler, D., and Staeheli, P. (1992) *J. Virol.* **66,** 1709–1716
7. Kirk, P., and Bazan, J. F. (2005) *Immunity* **23,** 347–350
8. Kumar, H., Kawai, T., and Akira, S. (2009) *Biochem. Biophys. Res. Commun.* **388,** 621–625
9. Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004) *Nat. Immunol.* **5,** 730–737
10. Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S., and Randall, R. E. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 17264–17269
11. Meylan, E., Tschopp, J., and Karin, M. (2006) *Nature* **442,** 39–44
12. Takeuchi, O., and Akira, S. (2008) *Curr. Opin. Immunol.* **20,** 17–22
13. Yoneyama, M., and Fujita, T. (2009) *Immunol. Rev.* **227,** 54–65
14. Seth, R. B., Sun, L., Ea, C. K., and Chen, Z. J. (2005) *Cell* **122,** 669–682
15. Xu, L. G., Wang, Y. Y., Han, KJ., Li, L. Y., Zhai, Z., and Shu, H. B. (2005) *Mol. Cell* **19,** 727–740
16. Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O., and Akira, S. (2005) *Nat. Immunol.* **6,** 981–988
17. Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005) *Nature* **437,** 1167–1172
18. Foy, E., Li, K., Sumpter, R., Jr., Loo, Y. M., Johnson, C. L., Wang, C., Fish, P. M., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M., Jr. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 2986–2991
19. Cárdenas, W. B., Loo, Y. M., Gale, M., Jr., Hartman, A. L., Kimberlin, C. R., Martínez-Sobrido, L., Saphire, E. O., and Basler, C. F. (2006) *J. Virol.* **80,** 5168–5178
20. Diamond, M. S. (2009) *PLoS Pathog.* **5,** e1000452
21. Barral, P. M., Morrison, J. M., Drahos, J., Gupta, P., Sarkar, D., Fisher, P. B., and Racaniello, V. R. (2007) *J. Virol.* **81,** 3677–3684
22. Papon, L., Oteiza, A., Imaizumi, T., Kato, H., Brocchi, E., Lawson, T. G., Akira, S., and Mechti, N. (2009) *Virology* **393,** 311–318
23. Kumar, H., Kawai, T., Kato, H., Sato, S., Takahashi, K., Coban, C., Yamamoto, M., Uematsu, S., Ishii, K. J., Takeuchi, O., and Akira, S. (2006) *J. Exp. Med.* **203,** 1795–1803
24. Pichlmair, A., Schulz, O., Tan, C. P., Näslund, T. I., Liljeström, P., Weber, F., and Reis e Sousa, C. (2006) *Science* **314,** 997–1001
25. Mibayashi, M., Martínez-Sobrido, L., Loo, Y. M., Cárdenas, W. B., Gale, M., Jr., and García-Sastre, A. (2007) *J. Virol.* **81,** 514–524
26. Guo, Z., Chen, L. M., Zeng, H., Gomez, J. A., Plowden, J., Fujita, T., Katz, J. M., Donis, R. O., and Sambhara, S. (2007) *Am. J. Respir. Cell Mol. Biol.* **36,** 263–269
27. Opitz, B., Rejaibi, A., Dauber, B., Eckhard, J., Vinzing, M., Schmeck, B., Hippenstiel, S., Suttorp, N., and Wolff, T. (2007) *Cell Microbiol.* **9,** 930–938
28. Marcus, P. I., Rojek, J. M., and Sekellick, M. J. (2005) *J. Virol.* **79,** 2880–2890
29. Hagen, M., Chung, T. D., Butcher, J. A., and Krystal, M. (1994) *J. Virol.* **68,** 1509–1515
30. Li, M. L., Ramirez, B. C., and Krug, R. M. (1998) *EMBO J.* **17,** 5844–5852
31. Hatta, M., Asano, Y., Masunaga, K., Ito, T., Okazaki, K., Toyoda, T., Kawaoka, Y., Ishihama, A., and Kida, H. (2000) *Arch. Virol.* **145,** 895–903
32. Hatta, M., Asano, Y., Masunaga, K., Ito, T., Okazaki, K., Toyoda, T., Kawaoka, Y., Ishihama, A., and Kida, H. (2000) *Arch. Virol.* **145,** 1947–1961
33. Yoneyama, M., Suhara, W., Fukuhara, Y., Sato, M., Ozato, K., and Fujita, T. (1996) *J. Biochem.* **120,** 160–169
34. Opitz, B., Vinzing, M., van Laak, V., Schmeck, B., Heine, G., Günther, S., Preissner, R., Slevogt, H., N'Guessan, P. D., Eitel, J., Goldmann, T., Flieger, A., Suttorp, N., and Hippenstiel, S. (2006) *J. Biol. Chem.* **281,** 36173–36179
35. González, S., Zürcher, T., and Ortín, J. (1996) *Nucleic Acids Res.* **24,** 4456–4463
36. Deng, T., Sharps, J., Fodor, E., and Brownlee, G. G. (2005) *J. Virol.* **79,** 8669–8674
37. Leahy, M. B., Pritlove, D. C., Poon, L. L., and Brownlee, G. G. (2001) *J. Virol.* **75,** 134–142
38. Honda, A., Endo, A., Mizumoto, K., and Ishihama, A. (2001) *J. Biol. Chem.* **276,** 31179–31185
39. Leahy, M. B., Dobbyn, H. C., and Brownlee, G. G. (2001) *J. Virol.* **75,** 7042–7049
40. Bowie, A. G., and Unterholzner, L. (2008) *Nat. Rev. Immunol.* **8,** 911–922
41. Takeuchi, O., and Akira, S. (2009) *Immunol. Rev.* **227,** 75–86
42. Pahl, H. L. (1999) *Oncogene* **18,** 6853–6866
43. Servant, M. J., Grandvaux, N., tenOever, B. R., Duguay, D., Lin, R., and Hiscott, J. (2003) *J. Biol. Chem.* **278,** 9441–9447
44. Bianchi, K., and Meier, P. (2009) *Mol. Cell.* **36,** 736–742
45. Hwang, J. S., Yamada, K., Honda, A., Nakade, K., and Ishihama, A. (2000) *J. Virol.* **74,** 4074–4084
46. Hale, B. G., Jackson, D., Chen, Y. H., Lamb, R. A., and Randall, R. E. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 14194–14199
47. Ehrhardt, C., Wolff, T., Pleschka, S., Planz, O., Beermann, W., Bode, J. G., and Schmolke, M., Ludwig, S. (2007) *J. Virol.* **81,** 3058–3067
48. Pauli, E. K., Schmolke, M., Wolff, T., Viemann, D., Roth, J., Bode, J. G., and Ludwig, S. (2008) *PLoS Pathog.* **4,** e1000196
49. Lazarowitz, S. G., Compans, R. W., and Choppin, P. W. (1971) *Virology* **46,** 830–843
50. Meier-Ewert, H., and Compans, R. W. (1974) *J. Virol.* **14,** 1083–1091
51. Hale, B. G., Randall, R. E., Ortín, J., and Jackson, D. (2008) *J. Gen. Virol.* **89,** 2359–2376
52. García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P., and Muster, T. (1998) *Virology* **252,** 324–330
53. Djeu, J. Y., Stocks, N., Zoon, K., Stanton, G. J., Timonen, T., and Herberman, R. B. (1982) *J. Exp. Med.* **156,** 1222–1234
54. Levy, D. E., Marié, I., Smith, E., and Prakash, A. (2002) *J. Interferon Cytokine Res.* **22,** 87–93
55. Grandvaux, N., tenOever, B. R., Servant, M. J., and Hiscott, J. (2002) *Curr.*

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

Opin. Infect. Dis. **15,** 259–267
56. Huet, S., Avilov, S. V., Ferbitz, L., Daigle, N., Cusack, S., and Ellenberg, J. (2010) *J. Virol.* **84,** 1254–1264
57. Carr, S. M., Carnero, E., García-Sastre, A., Brownlee, G. G., and Fodor, E. (2006) *Virology* **344,** 492–508
58. Hatta, M., Gao, P., Halfmann, P., and Kawaoka, Y. (2001) *Science* **293,** 1840–1842
59. Shinya, K., Hamm, S., Hatta, M., Ito, H., Ito, T., and Kawaoka, Y. (2004) *Virology* **320,** 258–266
60. Kobasa, D., Jones, S. M., Shinya, K., Kash, J. C., Copps, J., Ebihara, H., Hatta, Y., Kim, J. H., Halfmann, P., Hatta, M., Feldmann, F., Alimonti, J. B., Fernando, L., Li, Y., Katze, M. G., Feldmann, H., and Kawaoka, Y. (2007) *Nature* **445,** 319–323
61. Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R. W., Ortin, J., Hart, D. J., and Cusack, S. (2008) *Nat. Struct. Mol. Biol.* **15,** 500–506
62. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, CA., Jr. (1998) *Mol. Cell.* **2,** 253–258
63. Takeuchi, O., and Akira, S. (2002) *Curr. Top. Microbiol. Immunol.* **270,** 155–167
64. Li, H. M., Fujikura, D., Harada, T., Uehara, J., Kawai, T., Akira, S., Reed, J. C., Iwai, A., and Miyazaki, T. (2009) *Cell Death Differ.* **16,** 1615–1621

Downloaded from www.jbc.org at HOKKAIDO DAIGAKU, on March 21, 2012

Virus Genes (2010) 41:351–357
DOI 10.1007/s11262-010-0530-3

Antigenic, genetic, and pathogenic characterization of H5N1 highly pathogenic avian influenza viruses isolated from dead whooper swans (*Cygnus cygnus*) found in northern Japan in 2008

Masatoshi Okamatsu · Tomohisa Tanaka · Naoki Yamamoto · Yoshihiro Sakoda · Takashi Sasaki · Yoshimi Tsuda · Norikazu Isoda · Norihide Kokumai · Ayato Takada · Takashi Umemura · Hiroshi Kida

Received: 17 July 2010 / Accepted: 2 September 2010 / Published online: 17 September 2010
© Springer Science+Business Media, LLC 2010

Abstract In April and May 2008, whooper swans (*Cygnus cygnus*) were found dead in Hokkaido in Japan. In this study, an adult whooper swan found dead beside Lake Saroma was pathologically examined and the identified H5N1 influenza virus isolates were genetically and antigenically analyzed. Pathological findings indicate that the swan died of severe congestive edema in the lungs. Phylogenetic analysis of the HA genes of the isolates revealed that they are the progeny viruses of isolates from poultry and wild birds in China, Russia, Korea, and Hong Kong. Antigenic analyses indicated that the viruses are distinguished from the H5N1 viruses isolated from wild birds and poultry before 2007. The chickens vaccinated with A/duck/Hokkaido/Vac-1/2004 (H5N1) survived for 14 days after challenge with A/whooper swan/Hokkaido/1/2008 (H5N1), although a small amount of the challenge virus was recovered from the tissues of the birds. These findings indicate that H5N1 highly pathogenic avian influenza viruses are circulating in wild birds in addition to domestic poultry in Asia and exhibit antigenic variation that may be due to vaccination.

Keywords H5N1 highly pathogenic avian influenza virus · Whooper swan · Antigenic variation

M. Okamatsu · N. Yamamoto · Y. Sakoda · Y. Tsuda · N. Isoda · H. Kida (✉)
Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060-0818, Japan
e-mail: kida@vetmed.hokudai.ac.jp

T. Tanaka · T. Umemura
Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

T. Sasaki · N. Kokumai
Avian Biologics Department, Kyoto Biken Laboratories, Inc., Uji, Kyoto 611-0041, Japan

A. Takada · H. Kida
Research Center of Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan

Introduction

Since 2003, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have spread to 63 countries in Asia, Europe, and Africa. Japan and some other countries where H5N1 virus infection occurred in poultry flocks were successful in rapid eradication of the infection by an aggressive stamping-out policy [1, 2]. However, the virus still persists in Asian and North African countries. Thousands of migratory birds of several species died due to H5N1 HPAIV infection at Qinghai Lake in China in 2005 [3, 4]. Viruses similar to the Qinghai-virus spread to Asia, Europe, and Africa [5–7], raising concerns that migratory birds may transmit HPAIVs to poultry and even to humans.

The responses to infection with the H5N1 HPAIV vary in different wild water birds. Ducks inoculated with HPAIV survived and showed neurological signs with the replication of the virus in the brain [8, 9]. On the other hand, highly susceptible species such as swans (*Cygnus* spp.) showed high mortality by infection with HPAIV [10]. There are few reports on the pathology of swans naturally infected with H5N1 HPAIV [11, 12].

The long-term endemic of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries. A broadly cross-protective vaccine for antigenic variants of H5N1 viruses may be a useful option as a tool for the control of avian influenza [13]. Previously, we developed avian influenza vaccine prepared

from non-pathogenic avian influenza viruses isolated from migratory ducks [14]. The vaccine conferred protective immunity to suppress the manifestation of disease signs and reduction of virus shed in chickens and monkeys (*Cynomolgus macaques*) against H5N1 viruses isolated in 2004 and 2005 [15, 16].

In this study, a whooper swan found dead beside Lake Saroma was pathologically examined and the H5N1 virus isolate was compared genetically and antigenically with other isolates from swans found dead in Japan in 2008 [20, 21]. An inactivated avian influenza vaccine prepared from A/duck/Hokkaido/Vac-1/2004 (H5N1) [15] was also assessed for its potency to suppress the manifestation of disease signs.

Materials and methods

Viruses

A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/1/08) and A/whooper swan/Hokkaido/2/2008 (H5N1) (Ws/Hok/2/08) were isolated from trachea of whooper swans found dead at Notsuke Peninsula and at Lake Saroma, respectively, in Hokkaido Prefecture, Japan. All viruses used in this study were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 h and stored at −80°C until use.

Sequencing and phylogenetic analysis

Viral RNAs were extracted with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) from allantoic fluids. Nucleotide sequences of all eight gene segments were determined after RT-PCR as described previously [14]. The sequence data were analyzed using GENETYX ver. 9.1 (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis of HA gene was performed using BioEdit ver. 7.0 and MEGA 4 by the neighbor-joining method with 1000 bootstraps. The nucleotide sequences obtained in this study are available from DDBJ/EMBL/GenBank under accession numbers AB436547–AB436554 and AB436899–AB436906.

Intravenous pathogenicity test in chickens

The intravenous pathogenicity test of chickens for influenza viruses was carried out according to the OIE standard method [13]. Briefly, 1/10 dilutions of infectious allantoic fluids containing the viruses were intravenously inoculated into eight 7-week-old chickens. These chickens were housed for 10 days in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan) at a BSL 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Histopathology and immunohistochemistry

An adult male whooper swan found dead beside Lake Saroma on 5th May 2008, was pathologically examined. The tissues of the swan were fixed in 10% formalin in PBS (pH 7.2). Paraffin-embedded sections were processed for hematoxylin and eosin staining and immunohistochemistry. For the detection of influenza virus antigens in the tissues, the sections were incubated with rabbit anti-A/whistling swan/Shimane/499/1983 (H5N3) hyper-immune serum at 1:1000 dilution. Bound antibodies were detected by the peroxidase-labeled streptoavidin–biotin method (Histofine SAB-PO rabbit kit: Nichirei, Tokyo, Japan).

Antigenic analysis of the viruses

Hemagglutination-inhibition (HI) test was performed as described by Sever [17]. A panel of monoclonal antibodies to H5 HA of A/duck/Pennsylvania/10218/1984 (H5N2) was used as previously described [14]. Hyper-immune antisera against Ws/Hok/1/08, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04), and A/tern/South Africa/1961 (H5N3) were prepared according to Kida and Yanagawa [18].

Potency test of vaccine efficacy in chickens against Ws/Hok/1/08

The inactivated avian influenza virus Dk/Vac-1/04 vaccine was intramuscularly inoculated to chickens as described previously [15, 19]. Briefly, Dk/Vac-1/04 was inactivated with 0.1% formalin and mixed with oil adjuvant containing anhydromannitol-octadecenoate-ether (AMOE). Eleven four-week-old chickens were intramuscularly immunized and, 3 weeks later, challenged intranasally with a dose 100-fold that of 50% chicken lethal dose (CLD_{50}) of Ws/Hok/1/08. Clinical signs were monitored for 14 days post-challenge (p.c.) and chickens were sacrificed on day 2 and 4 p.c. When chickens died or were sacrificed, tracheal and cloacal swabs and their tissues (trachea, lung, kidney, and colon) were collected. Virus titers were measured by 50% egg infectious dose (EID_{50}).

Results

Pathological findings of the whooper swan

A whooper swan found dead beside Lake Saroma on 5th May 2008 presented as well-nourished with sufficient body fat reserves. Gross lesions were not found except for some

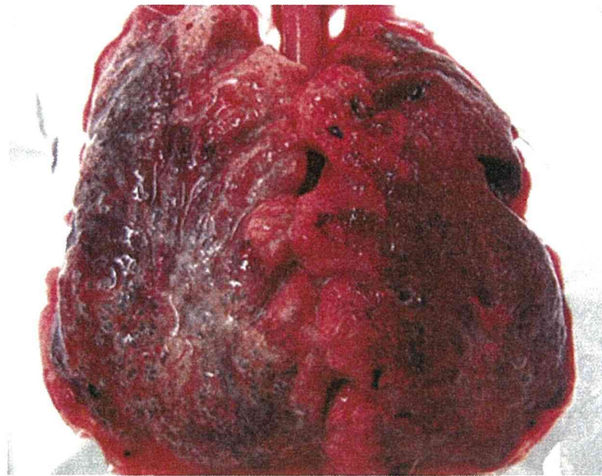


Fig. 1 Gross appearance of the lungs of whooper swan found dead beside Lake Saloma. The lungs show diffuse congestive edema. The pleura is edematously thickened

damage to its head and neck, which may have been due to bites by a wild animal. At necropsy, the swan showed diffuse severe congestive edema of the lungs with thickening of the pleura (Fig. 1). Echymotic hemorrhage was scattered in the pancreas and epicardium. A whooper swan found dead in Notsuke Peninsula on 24th April 2008 was not pathologically examined since the body had already decomposed when it arrived.

The predominant histological lesions were found exclusively in the brain, pancreas, and lungs. In the cerebrum and cerebellum, glial nodules were scattered with spongiform change of the neuropil and with necrosis of nerve and glial cells (Fig. 2a). Small necrotic foci of acinar cells were observed in the pancreas (Fig. 2c). Only a small number of heterophils and macrophages were infiltrated in the cerebral and pancreatic lesions. The lungs were severely congested with diffuse moderate edema of interlobular and peribronchial connective tissues. Small amounts of fibrin and heterophils exuded into parabronchi and infundibula. By the immunohistochemical examination, influenza virus antigens were found in the brain, pancreas, lungs, and trachea. In the cerebrum and cerebellum, nerve and glial cells within and around the glial nodules were stained positive by hyperimmune serum to A/whistling swan/Shimane/499/1983 (H5N3) (Fig. 2b). In the necrotic areas of the pancreas, some necrotic and degenerative acinar cells were stained positive (Fig. 2d). In the lungs and trachea, the antigen was detected in only a few respiratory and mucosal epithelial cells.

Pathogenicity of the isolates in chickens

Ws/Hok/1/08 (H5N1) and Ws/Hok/2/08 (H5N1) were inoculated intravenously into eight 7-week-old chickens, respectively. Within 2 days post-inoculation, all chickens died. This result was consistent with a prediction based on the amino acid sequence at the cleavage site of the HA protein of the isolates in Hokkaido (PQRERRRKR/GLF).

Genetic analysis of virus isolates from whooper swans

To elucidate the genetic relationships of the isolates with other H5N1 influenza virus isolates, all eight gene segments were compared. It was revealed that the all gene segments of the isolates were closely related to each other and to the H5N1 HPAIVs isolated from whooper swans in Akita and Aomori Prefectures in 2008 (more than 99.0% similarity in all genes) [20, 21]. It was also revealed that all isolates found in Japan in 2008 were closely related to those of isolates found in Korea in 2008 (personal communication). Phylogenetic analysis of the HA genes showed that these isolates belonged to Clade 2.3.2 and formed a unique branch with isolates found in Hong Kong in 2007-08 and those found in Russia in 2008 (Fig. 3).

Antigenic characterization of the HA of the isolates

Antigenic analysis of the HA of the isolates with antisera to H5 of influenza viruses and monoclonal antibodies to the HA of A/duck/Pennsylvania/10218/1984 (H5N2) was performed by HI test. The antigenicities of the HA of the isolates in 2008 were similar to each other but different from those of Dk/Vac-1/04, which is the reassortant virus generated from the isolates from fecal samples of wild ducks, and H5N1 HPAIVs isolated from chickens and whooper swans in Asia (Table 1).

Potency of the vaccine against the isolate in chickens

Ws/Hok/1/08 (H5N1) was selected as the challenge strain for the vaccine potency test since the isolates from whooper swans were genetically and antigenically identical. Eleven chickens intramuscularly inoculated with the vaccine prepared from Dk/Vac-1/04 and 3 non-vaccinated chickens were challenged intranasally with Ws/Hok/1/08 on 3 weeks after vaccination. The HI titers of the sera of the vaccinated chickens were 1:128–512 and 1:4–8 with the vaccine strain and with the isolate, respectively. All vaccinated chickens survived without showing any disease signs after challenge, whereas all of the control chickens died within 2 days p.c. Viruses were not recovered from the swabs of any of the vaccinated chickens after challenge. Low titers of infectious virus were recovered from the trachea, lungs, kidneys, and colon of three of the four vaccinated birds on day 2 p.c. (Table 2).

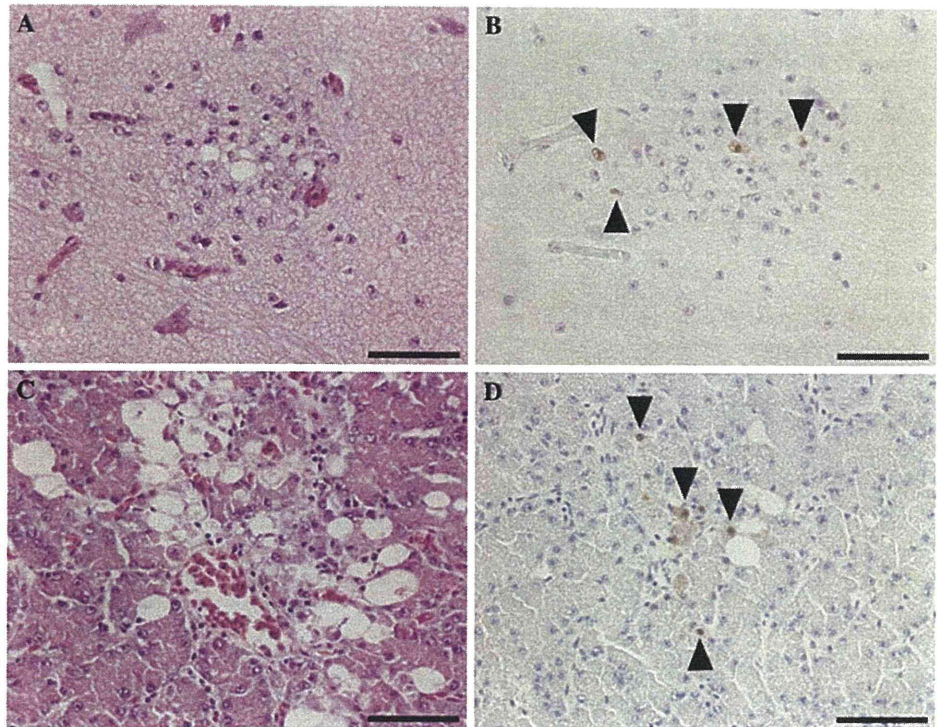


Fig. 2 Histopathological and immunohistochemical findings of the whooper swan. **a** Glial nodule with spongiform change of neuropile. Cerebrum, HE stain. **b** Nerve and glial cells in the glial nodule are positively stained for influenza virus antigen (*arrowheads*). **c** Focal necrosis of acinar cells. Pancreas, HE stain. **d** Necrotic and degenerative acinar cells show positive staining for influenza virus antigen (*arrowheads*). *Bars* = 50 μm

Discussion

In this study, genetic analysis of the H5N1 viruses isolated from the swans in Hokkaido in Japan revealed that they belonged to Clade 2.3.2. The results also indicate that whooper swans were infected with HPAIV though water-borne transmission somewhere in a lake or pond where feral water birds, who were probably infected with HPAIV in Southern China, congregated on the way back to the north in spring. Although viruses belonging to Clade 2.2, Qinghai-like viruses, had been spread in Asia, Europe, and Africa by wild water birds [5–7, 22], the present results indicate that the viruses belonging to Clade 2.3.2, which differ from Qinghai-like viruses, were also spread by wild water birds. Actually, the number of case reports of infections of wild birds with H5N1 HPAIV belonging to Clades 2.3.2 and 2.3.4 have been increasing since 2008 [23].

High mortality in wild water birds infected with HPAIV was not recognized before 2005. However, swans and geese are apparently most commonly infected with the recent H5N1 virus strains [10, 12, 24]. In this study, pathological changes of dead whooper swan with HPAIV were confined to the central nervous system (CNS), pancreas, and lungs. Inflammatory reaction of the wild water birds infected with H5N1 HPAIV was limited. The present findings indicate that the whooper swan died of severe congestive edema of the lungs at an early stage of systemic infection with HPAIV. Neither myocardial necrosis nor influenza virus antigen was found in the heart of the swan. These findings coincide with those of the gross lesions of mute swans and whooper swans that were identified as multifocal pancreatic necrosis, hemorrhage, and lung edema during an outbreak in Germany in 2006 [10].

In the poultry population in Asia, antigenic variants of H5N1 HPAIV have been selected, indicating that these wild birds were infected with the H5N1 viruses prevailing in domestic poultry [25]. Antigenic analysis revealed that the isolates were different from the virus isolates from poultry and wild water birds in Japan, Mongolia, and China including the vaccine strain, Dk/Vac-1/04, that we previously developed [15]. It is suggested that the antigenicity of H5N1 HPAIVs has changed more during circulation in the chicken population since 2007. Given this notion, chickens inoculated with the vaccine that we previously developed were challenged with the present HPAIV isolate. In the challenge study to vaccinated chickens, higher titers of the challenge viruses were recovered from various tissues of the chickens than those from birds challenged with A/chicken/Yamaguchi/7/2004 (H5N1) strain in a previous study [15], although all of the vaccinated chickens were survived for 14 days after the challenge with Ws/Hok/1/08 (H5N1). This may be influenced by an antigenic difference between the vaccine strain and the challenge

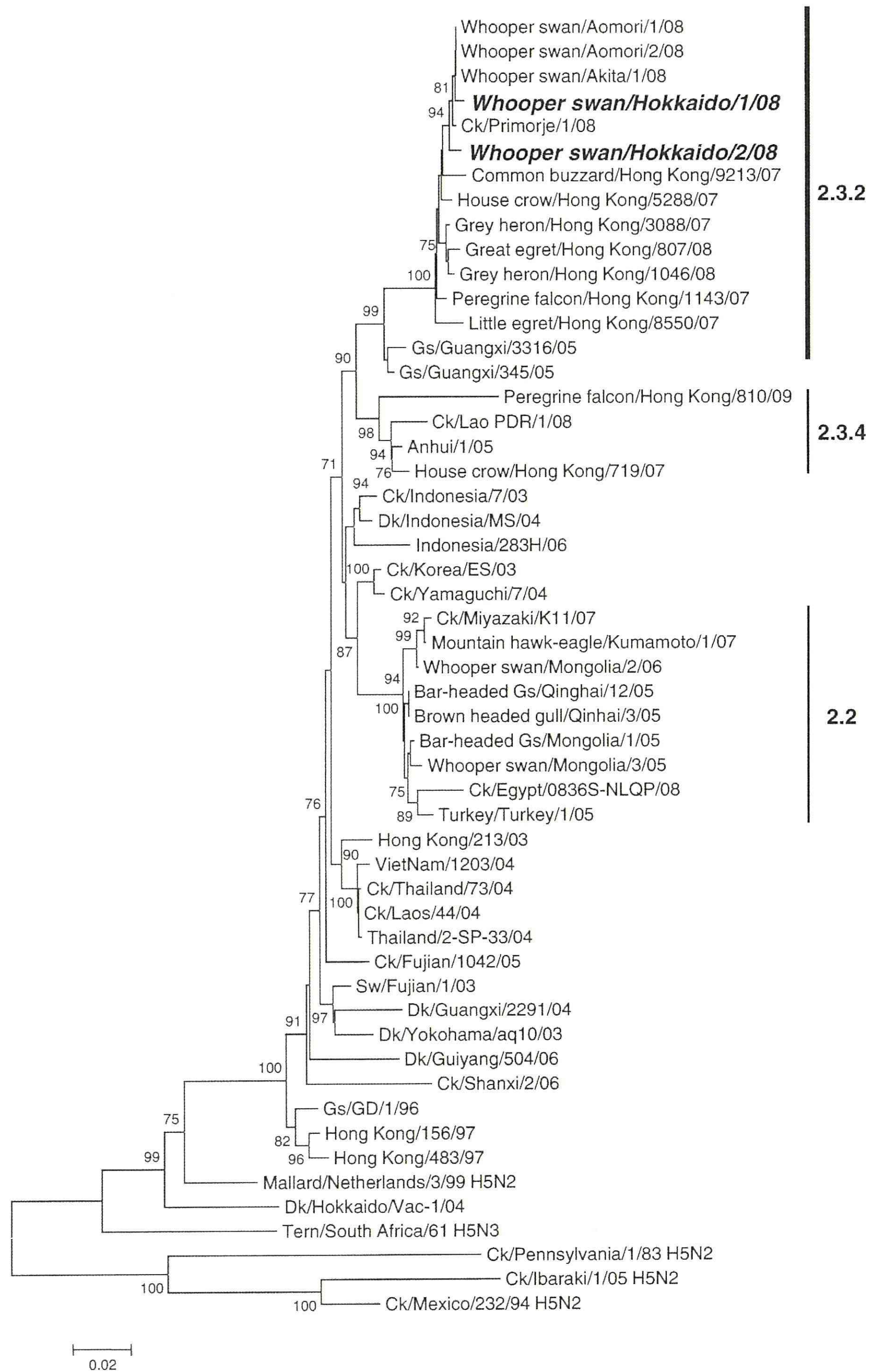


Fig. 3 Phylogenetic tree of the HA genes of H5 influenza viruses. Nucleotide sequences of the HA genes of H5 influenza viruses isolated in the present study (shown in ***bold italic***) and the sequence information of other related viruses were cited from the public database. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. *Numbers* at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. *Ck* chicken, *Gs* goose, *Dk* duck, *Qa* quail, *Sw* swine

virus. In poultry, avian influenza viruses have not been under constant immunological selection pressure induced by vaccines. Since vaccine use for poultry has increased in several countries, antigenic variation could occur in H5N1 HPAIV as it did for H5N2 viruses in the 1990s in Mexico [26]. It is strongly emphasized that stamping-out measures without misuse of vaccine is best way in eradication of HPAI. For control of HPAI, continuing surveillance to understand influenza virus infection in birds and mammals and preparation for the diagnosis of influenza virus infection, such as technical training, making antiserum, and sharing information are essential.

Table 1 Antigenic property of influenza viruses isolated in Japan in 2008

Virus	Clade	Polyclonal antiserum (hyper-immune)			Monoclonal antibodies[a]		
		Ws/Hok/1	Dk/Vac-1/04	Tn/SA	A310/39	64/2	25/2
Whooper swan/Hokkaido/1/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Whooper swan/Hokkaido/2/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Whooper swan/Akita/1/08 (H5N1)	2.3.2	1280	20	40	<	<	<
Whooper swan/Akita/2/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Viet Nam/1194/04 (H5N1)	1	80	160	160	40	1280	80
Whooper swan/Mongolia/3/05 (H5N1)	2.2	320	320	160	<	320	320
Whooper swan/Mongolia/2/06 (H5N1)	2.2	80	320	160	<	1280	80
Chicken/Yamaguchi/7/04 (H5N1)	2.5	320	640	320	40	640	160
Duck/Hokkaido/Vac-1/04 (H5N1)	Classical	40	640	160	320	320	40
Tern/South Africa/61 (H5N3)	Classical	40	320	640	<	160	20
Chicken/Ibaraki/1/05 (H5N2)	American	20	80	40	<	<	<

Homologous titer of the antiserum is *underlined*

< = The HI titer was lower than 1:20

[a] Monoclonal antibodies against Dk/Pennsylvania/84 (H5N2)

Table 2 Antibody titers and virus recovery in chickens

	Days p.c.	HI titer (0 dpc)		HI titer (14 dpc)		Virus recovery					
		Dk/Vac-1/04	Ws/Hok/08	Dk/Vac-1/04	Ws/Hok/08	Swabs (log EID_{50}/ml)		Tissues (log EID_{50}/g)			
						Trachea	Cloaca	Trachea	Lung	Kidney	Colon
Vaccinated chickens	2	256	8	NT	NT	–	–	2.0	2.5	2.5	3.5
	2	256	4	NT	NT	–	–	1.8	–	–	2.7
	2	256	8	NT	NT	–	–	–	–	2.5	–
	2	512	8	NT	NT	–	–	–	–	–	–
	4	128	4	NT	NT	–	–	–	3.3	–	–
	4	256	4	NT	NT	–	–	–	–	–	–
	4	256	8	NT	NT	–	–	–	–	–	–
	14	256	4	512	64	NT	NT	NT	NT	NT	NT
	14	256	4	512	64	NT	NT	NT	NT	NT	NT
	14	256	8	512	64	NT	NT	NT	NT	NT	NT
	14	128	4	1024	128	NT	NT	NT	NT	NT	NT
Non-vaccinated chickens	2[a]	<2	<2	NT	NT	4.5	4.3	8.5	7.5	7.3	9.8
	2[a]	<2	<2	NT	NT	6.3	4.8	9.3	7.8	7.3	7.8
	2[a]	<2	<2	NT	NT	6.8	4.5	7.8	7.5	9.5	8.8

– The titer of the virus recovery lower than 0.5 (swabs) or 1.5 (tissues), *NT* not tested

[a] Chicken died

Acknowledgments The authors wish to thank for Hokkaido Government for their cooperation in collecting the samples. This research was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (Start-up) 20890006, 2008. This research was also supported by Global Centers of Excellence Program from Japan Society for the Promotion of Science.

References

1. C.W. Lee, D.L. Suarez, T.M. Tumpey, H.W. Sung, Y.K. Kwon, Y.J. Lee, J.G. Choi, S.J. Joh, M.C. Kim, E.K. Lee, J.M. Park, X. Lu, J.M. Katz, E. Spackman, D.E. Swayne, J.H. Kim, J. Virol. **79**, 3692–3702 (2005)

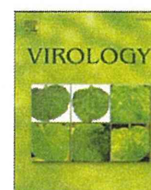
2. M. Mase, K. Tsukamoto, T. Imada, K. Imai, N. Tanimura, K. Nakamura, Y. Yamamoto, T. Hitomi, T. Kira, T. Nakai, M. Kiso, T. Horimoto, Y. Kawaoka, S. Yamaguchi, Virology **332**, 167–176 (2005)
3. H. Chen, G.J. Smith, S.Y. Zhang, K. Qin, J. Wang, K.S. Li, R.G. Webster, J.S. Peiris, Y. Guan, Nature **436**, 191–192 (2005)
4. J. Liu, H. Xiao, F. Lei, Q. Zhu, K. Qin, X.W. Zhang, X.L. Zhang, D. Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, G.F. Gao, Science **309**, 1206 (2005)
5. M.F. Ducatez, C.M. Olinger, A.A. Owoade, S. De Landtsheer, W. Ammerlaan, H.G. Niesters, A.D. Osterhaus, R.A. Fouchier, C.P. Muller, Nature **442**, 37 (2006)
6. G.L. Gall-Recule, F.X. Briand, A. Schmitz, O. Guionie, P. Massin, V. Jestin, Avian Pathol. **37**, 15–23 (2008)
7. S. Weber, T. Harder, E. Starick, M. Beer, O. Werner, B. Hoffmann, T.C. Mettenleiter, E. Mundt, J. Gen. Virol. **88**, 554–558 (2007)
8. N. Kishida, Y. Sakoda, N. Isoda, K. Matsuda, M. Eto, Y. Sunaga, T. Umemura, H. Kida, Arch. Virol. **150**, 1383–1392 (2005)
9. Y. Yamamoto, K. Nakamura, K. Kitagawa, N. Ikenaga, M. Yamada, M. Mase, M. Narita, Avian Dis. **51**, 52–57 (2007)
10. J.P. Teifke, R. Klopfleisch, A. Globig, E. Starick, B. Hoffmann, P.U. Wolf, M. Beer, T.C. Mettenleiter, T.C. Harder, Vet. Pathol. **44**, 137–143 (2007)
11. S. Ogawa, Y. Yamamoto, M. Yamada, M. Mase, K. Nakamura, J. Vet. Med. Sci. **71**, 1377–1380 (2009)
12. N. Palmai, K. Erdelyi, A. Balint, L. Marton, A. Dan, Z. Deim, K. Ursu, B.Z. Londt, I.H. Brown, R. Glavits, Avian Pathol. **36**, 245–249 (2007)
13. OIE (2009), http://wwwoieint/eng/normes/mmanual/A_summry htm
14. K. Soda, H. Ozaki, Y. Sakoda, N. Isoda, Y. Haraguchi, S. Sakabe, N. Kuboki, N. Kishida, A. Takada, H. Kida, Arch. Virol. **153**, 2041–2048 (2008)
15. N. Isoda, Y. Sakoda, N. Kishida, K. Soda, S. Sakabe, R. Sakamoto, T. Imamura, M. Sakaguchi, T. Sasaki, N. Kokumai, T. Ohgitani, K. Saijo, A. Sawata, J. Hagiwara, Z. Lin, H. Kida, Arch. Virol. **153**, 1685–1692 (2008)
16. Y. Itoh, H. Ozaki, H. Tsuchiya, K. Okamoto, R. Torii, Y. Sakoda, Y. Kawaoka, K. Ogasawara, H. Kida, Vaccine **26**, 562–572 (2008)
17. J.L. Sever, J. Immunol. **88**, 320–329 (1962)
18. H. Kida, R. Yanagawa, Zentralbl. Bakteriol. Orig. A **244**, 135–143 (1979)
19. T. Sasaki, N. Isoda, K. Soda, R. Sakamoto, K. Saijo, J. Hagiwara, N. Kokumai, T. Ohgitani, T. Imamura, A. Sawata, Z. Lin, Y. Sakoda, H. Kida, Jpn. J. Vet. Res. **56**, 189–198 (2009)
20. Y. Uchida, M. Mase, K. Yoneda, A. Kimura, T. Obara, S. Kumagai, T. Saito, Y. Yamamoto, K. Nakamura, K. Tsukamoto, S. Yamaguchi, Emerg. Infect. Dis. **14**, 1427–1429 (2008)
21. T. Usui, T. Yamaguchi, H. Ito, H. Ozaki, T. Murase, T. Ito. Virus Genes **39**, 319–323 (2009)
22. I. Monne, T.M. Joannis, A. Fusaro, P. De Benedictis, L.H. Lombin, H. Ularamu, A. Egbuji, P. Solomon, T.U. Obi, G. Cattoli, I. Capua, Emerg. Infect. Dis. **14**, 637–640 (2008)
23. G.J. Smith, D. Vijaykrishna, T.M. Ellis, K.C. Dyrting, Y.H. Leung, J. Bahl, C.W. Wong, H. Kai, M.K. Chow, L. Duan, A.S. Chan, L.J. Zhang, H. Chen, G.S. Luk, J.S. Peiris, Y. Guan, Emerg. Infect. Dis. **15**, 402–407 (2009)
24. T.M. Ellis, R.B. Bousfield, L.A. Bissett, K.C. Dyrting, G.S. Luk, S.T. Tsim, K. Sturm-Ramirez, R.G. Webster, Y. Guan, J.S. Malik Peiris, Avian Pathol. **33**, 492–505 (2004)
25. W.L. Wu, Y. Chen, P. Wang, W. Song, S.Y. Lau, J.M. Rayner, G.J. Smith, R.G. Webster, J.S. Peiris, T. Lin, N. Xia, Y. Guan, H. Chen, J. Virol. **82**, 1798–1807 (2008)
26. C.W. Lee, D.A. Senne, D.L. Suarez, J. Virol. **78**, 8372–8381 (2004)

Virology 406 (2010) 88–94

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory

Yoshihiro Sakoda [a], Sengee Sugar [b], Damdinjav Batchluun [b], Tseren-Ochir Erdene-Ochir [b], Masatoshi Okamatsu [a], Norikazu Isoda [a], Kosuke Soda [a], Hiroki Takakuwa [a], Yoshimi Tsuda [a], Naoki Yamamoto [a], Noriko Kishida [a,c], Keita Matsuno [c], Eri Nakayama [c], Masahiro Kajihara [c], Ayaka Yokoyama [c], Ayato Takada [c], Ruuragchaa Sodnomdarjaa [b], Hiroshi Kida [a,c,*]

[a] *Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*
[b] *State Central Veterinary Laboratory, Zaisan, Ulaanbaatar 210153, Mongolia*
[c] *Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan*

ARTICLE INFO

Article history:
Received 23 March 2010
Returned to author for revision 20 May 2010
Accepted 2 July 2010
Available online 31 July 2010

Keywords:
Avian influenza
H5N1
Surveillance
Migratory waterfowl

ABSTRACT

H5N1 highly pathogenic avian influenza (HPAI) viruses were isolated from dead wild waterfowl at Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes in Mongolia in July 2005, May 2006, May 2009, July 2009, and May 2010, respectively. The isolates in 2005 and 2006 were classified into genetic clade 2.2, and those in 2009 and 2010 into clade 2.3.2. A/whooper swan/Mongolia/6/2009 (H5N1) experimentally infected ducks and replicated systemically with higher mortality than that of the isolates in 2005 and 2006. Intensive surveillance of avian influenza in migratory waterfowl flying from their nesting lakes in Siberia to Mongolia in every autumn indicate that HPAI viruses have not perpetuated at their nesting lakes until 2009. The present results demonstrate that wild waterfowl were sporadically infected with H5N1 HPAI viruses prevailing in domestic poultry in the southern Asia and died in Mongolia on the way back to their northern territory in spring.

© 2010 Elsevier Inc. All rights reserved.

Introduction

H5N1 highly pathogenic avian influenza (HPAI) virus infections have spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China (Smith et al., 2006; Xu et al., 1999). H5N1 virus infections have become endemic at poultry farms in some countries and cause accidental transmissions to humans, so H5N1 viruses are recognized as the most likely candidate for the next pandemic (Li et al., 2004; Peiris et al., 2007). The widespread presence of H5N1 HPAI viruses in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the transmission of viruses to wild bird populations. Domestic ducks and geese infected with HPAI virus shed progeny viruses in feces at the ponds in the farms, where migratory waterfowl visit. Thus, water-borne transmission easily occurs from domestic waterfowl to migratory waterfowl. In the past, such infections had been restricted to wild birds found dead in the vicinity of infected poultry farms, but there are concerns that infections of wild birds in which HPAI virus has caused mild or no clinical signs (e.g., ducks) could result in spread of the virus over large areas and long distances (Kim et al., 2009). Infections with HPAI viruses in many wild bird species at 2 waterfowl parks in Hong Kong were recorded in 2002 (Ellis et al., 2004) and further, more significant outbreaks in wild waterfowl were found at Lake Qinghai in Western China (Chen et al., 2005). H5N1 HPAI virus infections in poultry and wild birds now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could spread by migratory waterfowl to the west and south, since genetically closely related H5N1 viruses (clade 2.2) were isolated in several countries from 2005 to 2006 (Monne et al., 2008; Salzberg et al., 2007; Starick et al., 2008). From intensive surveillance in China, 2 antigenically distinct virus groups, clade 2.3.2 and clade 2.3.4, were characterized as the dominant isolates in wild birds (Kou et al., 2009; Smith et al., 2009).

A natural reservoir of influenza A virus is wild waterfowl (Kida et al., 1980, 1987; Webster et al., 1978). In previous studies, influenza A viruses of different subtypes were isolated from water of the lakes where migratory waterfowl nest in summer, even in autumn when waterfowl had left for the south for migration, suggesting that influenza A viruses are preserved in frozen lake water each year while

* Corresponding author. Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Sapporo 060-0818, Japan. Fax: +81 11 706 5273.
E-mail address: kida@vetmed.hokudai.ac.jp (H. Kida).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.07.007

Table 1
Identification of H5N1 isolates in Mongolia.

Date of isolation	Place	Isolates[a]	Amino acid sequence of HA cleavage site[b]	Intravenous pathogenicity index in chicken	Database accession no.
July, 2005	Khunt Lake, Bulgan	Bhg/Mongolia/1/05 (H5N1)	GERRRKKR/G	2.95	AB239300–AB239302, AB233319, AB239303–AB239306
	Erkhel Lake, Khuvsugul	Ws/Mongolia/3/05 (H5N1)	GERRRKKR/G	2.90	AB239307–AB239309, AB233320, AB239310–AB239313
May, 2006	Khunt Lake, Bulgan	Ws/Mongolia/2/06 (H5N1)	GERRRKKR/G	2.71	AB264769–AB264770, AB263751–AB263753, AB265202–AB265204
	Erkhel Lake, Khuvsugul	Cg/Mongolia/12/06 (H5N1)	GERRRKKR/G	2.80	AB284321–AB284328
May, 2009	Doityn Tsagaan Lake, Arkhangai	Ws/Mongolia/2/09 (H5N1)	RERRRKR/G	ND[c]	AB517665–AB517666
		Ws/Mongolia/6/09 (H5N1)	RERRRKR/G	2.97	AB520705–AB520712
		Ws/Mongolia/8/09 (H5N1)	RERRRKR/G	ND	AB517667–AB517668
July, 2009	Doroo Lake, Arkhangai	Bhg/Mongolia/X25/09 (H5N1)	RERRRKR/G	ND	AB521999, AB522000
		Bhg/Mongolia/X53/09 (H5N1)	RERRRKR/G	3.00	AB523764–AB523771
		Bhg/Mongolia/X54/09 (H5N1)	RERRRKR/G	ND	AB523366, AB523367
		Rs/Mongolia/X42/09 (H5N1)	RERRRKR/G	ND	AB523756–AB523763
		Rs/Mongolia/X63/09 (H5N1)	RERRRKR/G	ND	AB523368, AB523369
		Cg/Mongolia/X59/09 (H5N1)	RERRRKR/G	ND	AB522001, AB522002
		Cg/Mongolia/X60/09 (H5N1)	RERRRKR/G	ND	AB523772–AB523779
May, 2010	Ganga Lake, Sukhbaatar	Ws/Mongolia/1/10 (H5N1)	RERRRKR/G	3.00	AB569345–AB569352
		Ws/Mongolia/7/10 (H5N1)	RERRRKR/G	ND	AB569353, AB569354
		Ws/Mongolia/11/10 (H5N1)	RERRRKR/G	ND	AB569607, AB569608
		Ws/Mongolia/21/10 (H5N1)	RERRRKR/G	ND	AB569609, AB569610

[a] Abbreviated name of birds of each isolate: Bhg: bar-headed goose, Ws: whooper swan, Cg: common goldeneye, Rs: ruddy shelduck.
[b] A pair of dibasic amino acid residues was underlined.
[c] ND: not determined.

the waterfowl are absent (Ito et al., 1995; Okazaki et al., 2000). To monitor whether these HPAI viruses perpetuate in nature, virological surveillance of avian influenza has been carried out in the lakes in Mongolia where ducks congregate on their migration path from Siberia to the south since 2001.

In July 2005, May 2006, May 2009, July 2009, and May 2010, H5N1 HPAI viruses were isolated from whooper swans and other migratory waterfowl in Mongolia on the way back to their northern territory, although no outbreak was so far reported in poultry in Mongolia. In the present study, influenza A viruses isolated from dead waterfowl and fecal samples in the intensive surveillance of avian influenza in Mongolia were antigenically and genetically characterized. Pathogenicity of the isolated H5N1 viruses in chickens, pigs, and domestic ducks were investigated by experimental infection studies. The present results strongly support the notion that the global surveillance is essential to understand the ecology of influenza viruses for the control of influenza virus infection in birds and mammals.

Results

Isolation and identification of H5N1 HPAI viruses from dead waterfowl

Virus isolation was carried out for tissue samples of dead waterfowl, a bar-headed goose, whooper swan, common goldeneye, and ruddy shelduck, which were found at Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes in 2005, 2006, 2009, and 2010 (Table 1). In July 2005, H5N1 viruses were isolated from tissue homogenates and swab samples of a bar-headed goose and a whooper swan in Khunt and Erkhel Lakes. Similarly, H5N1 viruses were isolated from a whooper swan and a common goldeneye in May 2006 in Khunt and Erkhel Lakes. In May 2009, H5N1 viruses were isolated from 3 whooper swans in Doityn Tsagaan Lake. In late July 2009, H5N1 viruses were also isolated from dead wild birds, 3 bar-headed geese, 2 ruddy shelducks, and 2 common goldeneyes in Doroo Lake. In May 2010, H5N1 viruses were isolated from 4 whooper swans in Ganga Lake. From sequence analysis of the cleavage site of the hemagglutinin (HA), the C-terminus of HA1 had a pair of dibasic amino acid residues, which is a characteristic of HPAI viruses according to the manual of World Organization for Animal Health (OIE, 2009a). Furthermore, representative isolates of each year were highly pathogenic in chickens on intravenous inoculation and IVPIs of each isolate ranged from 2.71 to 3.00 (Table 1). Complete sequences of the HA, neuraminidase (NA), and other segments were deposited in the GenBank/EMBL/DDBJ as accession numbers described in Table 1.

Phylogenetic analysis of H5N1 isolates

The HA genes of H5N1 isolates were analyzed by the neighbor-joining method along with those of other H5 strains containing HPAI viruses recently isolated in the world (Fig. 1). The HA genes of the isolates in 2005 and 2006 were classified into clade 2.2, as Qinghai Lake-type viruses. Isolates from the same year, A/bar-headed goose/Mongolia/1/2005 and A/whooper swan/Mongolia/3/2005 (Ws/Mongolia/3/05), A/whooper swan/Mongolia/2/2006 (Ws/Mongolia/2/06) and A/common goldeneye/Mongolia/12/2006, were closely related and showed the highest homology. The 3 isolates in May 2009, 7 isolates in July 2009, and 4 isolates in May 2010 were classified into clade 2.3.2, the prototype of this clade was isolates from Hong Kong, China, and Vietnam in 2005. A/whooper swan/Mongolia/6/2009 (Ws/Mongolia/6/09) and other 13 isolates were closely related, having high homology with previous isolates from wild birds and chickens in Russia, China, Laos, and Japan.

Pathogenicity of H5N1 influenza viruses in pigs

To assess the pathogenicity of H5N1 isolates in pigs, each of Ws/Mongolia/3/05, Ws/Mongolia/2/06, and Ws/Mongolia/6/09 was inoculated intranasally at $10^{8.0}$ EID_{50} into two 4-week-old SPF pigs. Viruses were recovered from nasal swabs of all pigs infected with each H5N1 virus although apparent clinical signs were not observed in pigs for the 14 days study (Table 2). The periods of virus shedding in the pigs infected with Ws/Mongolia/2/06 were longer than in the pigs infected with Ws/Mongolia/3/05 or Ws/Mongolia/6/09.

Pathogenicity of H5N1 influenza viruses in ducks

To assess the pathogenicity of H5N1 isolates in ducks, each of Ws/Mongolia/3/05, Ws/Mongolia/2/06, and Ws/Mongolia/6/09 was

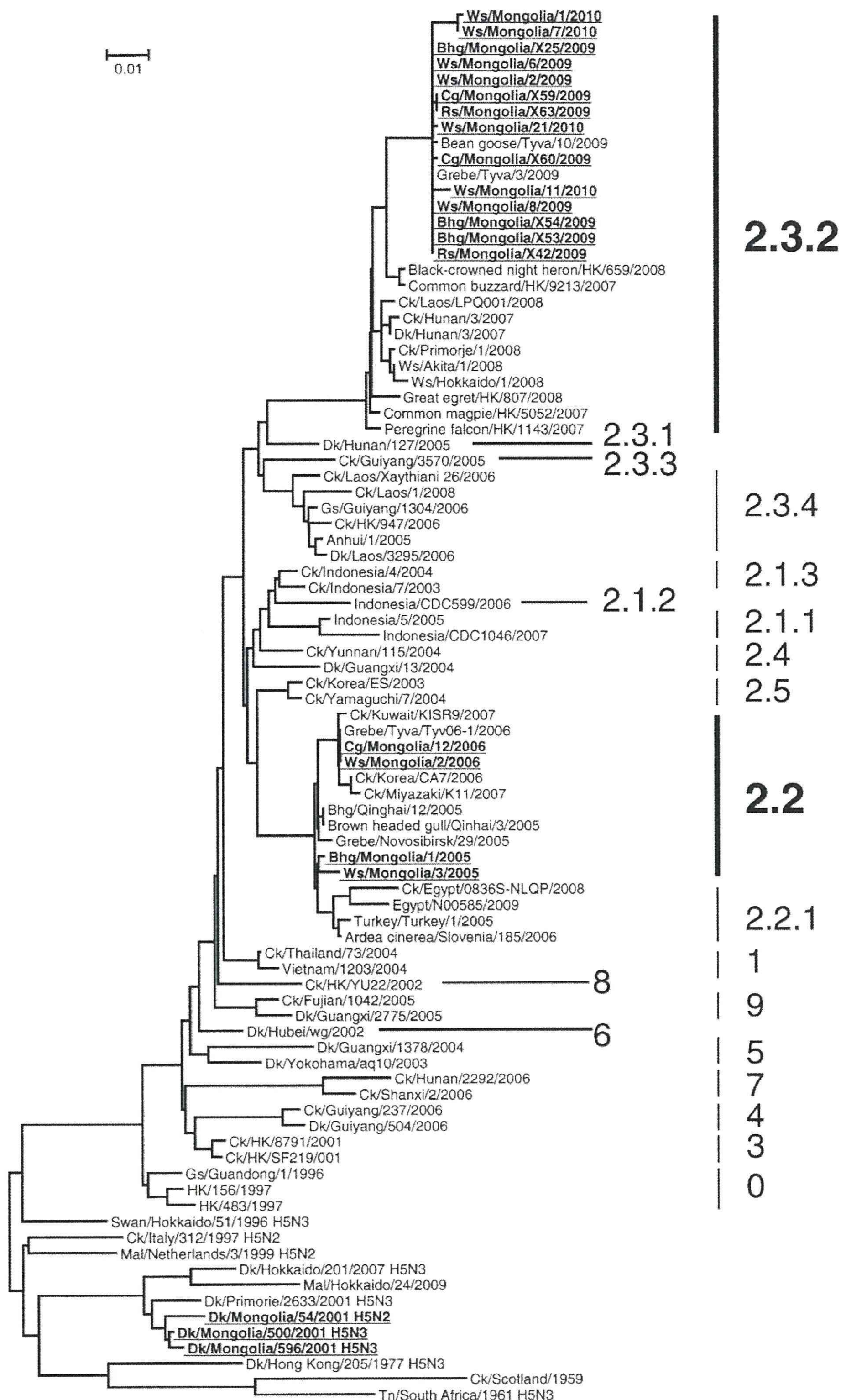


Fig. 1. Phylogenetic trees of the HA genes of H5 influenza viruses. Nucleotide sequences (976 bp) of the HA genes of H5 avian influenza viruses isolated in Mongolia (shown in bold and underlined) and the sequence information of other related viruses were cited from the public database for phylogenetic analysis. The sequence data of Dk/Mongolia/54/01 (H5N2), Dk/Mongolia/500/01 (H5N3), and Dk/Mongolia/596/01 (H5N3) were determined in our previous study (Soda et al., 2008). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Genetic classification (clades 0 to 9) was indicated for recent H5N1 HPAI viruses. HA and NA subtypes were eliminated for the names of H5N1 viruses. Abbreviations: Bhg (bar-headed goose), Ws (whooper swan), Cg (common goldeneye), Rs (ruddy shelduck), Ck (chicken), Dk (duck), Gs (goose), Mal (mallard), Tn (tern), and HK (Hong Kong).