PB1-F2 Amino Acids Regulate Influenza A Viral Polymerase Activity

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Abstract: In 2001, the 11th influenza A viral protein PB1-F2 was detected and found to be encoded by an alternative open reading frame in the PB1 polymerase gene. PB1-F2 has several unique functions, including roles in promoting apoptosis, increasing inflammation, and regulating viral polymerase activity. This study focused on a single PB1-F2 function: regulation of polymerase activity. We constructed a minigenome system to determine the influence of PB1-F2 amino acid (aa) mutations on polymerase activity. We examined four types of aa mutations: three species-specific aa mutations and one mutation that alters pathogenicity in mice. We discovered that an arginine (R) residue at aa position 29 is highly conserved in avian-derived virus strains. Introducing this mutation into mammalian strain A/WSN/33 (H1N1) led to a marked increase in polymerase activity in mammalian cells.These findings suggest that as PB1-F2 in H5N1 viruses regulates viral polymerase activity, it could be targeted for control of avian influenza infection and drug discovery.

Keywords: Influenza A virus, PB1-F2, polymerase activity, minigenome system.

1. INTRODUCTION

Influenza is a zoonotic disease with significant impact in both humans and animals [1]. It was previously discovered that the influenza A virus (IAV) has eight segmented, negative-stranded RNAs encoding ten viral proteins: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), nucleoprotein (NP), matrix proteins (M1, M2), non-structural protein (NS1), non-structural protein 2/nuclear exporting protein (NS2/NEP), and the surface glycoproteins, namely, hemagglutinin (HA), and neuraminidase (NA) [2]. In 2001, the 11th IAV protein PB1-F2 was discovered [3]; it is encoded by the +1 reading frame of the PB1 gene segment. PB1-F2 polypeptide size varies by strain or subtype, ranging from 52 to 101 amino acids (aa) in human IAVs, 52 to 90 aa in avian IAVs, and 27 to 90 aa in swine IAVs [4]. The biological functions of PB1-F2 also vary with host cell type and/or virus strain (e.g., mitochondrial inner membrane-associated and pro-apoptotic function [5-7], immune response modulator [8], type I interferon inhibitor [9], and pathogenicity modulator in mice [10]). Moreover, PB1-F2 directly and indirectly effects the regulation of viral polymerase activity [11-14]. In this study, we focused on the polymerase-enhancing function of PB1-F2 and determined the influence of PB1-F2 aa mutations on polymerase activity.

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2. MATERIALS AND METHODS

Detection of Species-Specific aa in PB1-F2

Human influenza type A viruses are believed to originate in aquatic birds. When compared to those of avian strains, the differences in the cDNA sequences of human H1N1/H3N2 strains reveal the history of the process by which they adapted to humans. We obtained the PB1-F2 sequence data from 5329 strains (human H1N1, 1319 strains; human H3N2 strains, 3330 strains; human H5N1, 148 strains; duck H5N1, 196 strains; chicken H5N1, 336 strains) in the NCBI database and processed their sequence alignment analysis using BioEdit.

Plasmids

To identify the aa of the IAV PB1-F2 protein most important for viral polymerase activity, we designed four types of PB1-F2 expression plasmids, each of which contained one type of aa mutation: pCAGGS-mtPB1F2-D6G, -mtPB1F2-K29R, -mtPB1F2-N66S, and -mtPB1F2-T89I (Table **1**). The mammalian expression plasmid pCAGGS has been previously described [15]. Each mutation was introduced using PCR-based site-directed mutagenesis, with primer pairs containing a point mutation based on the PB1-F2 of the WSN (pCAGGS-wtPB1F2/WSN). To construct the *in vitro* minigenome-system for IAV, we prepared a second set of pCAGGS expression plasmids that encoded WSN-PB2, -PA, -NP, -PB1, and -PB1-F2 knockout PB1 (pCAGGS-PB2/WSN, pCAGGS-PA/WSN, pCAGGS-NP/WSN, pCAGGS-wtPB1/WSN, and pCAGGS-

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Table 1: Mutant PB1-F2 Expression Plasmids

Amino acid No.	6	29	66	89
Avian	D	R	N	I
Human	G	K	N	T
A/WSN/33	D	K	N	T
pCAGGS-mtPB1F2-D6G	G	K	N	T
pCAGGS-mtPB1F2-K29R	D	R	N	T
pCAGGS-mtPB1F2-N66S	D	K	S	T
pCAGGS-mtPB1F2-T89I	D	K	N	I

F2koPB1/WSN), as well as a plasmid that expressed the pseudo-virus RNA (NP/WSN combined with luciferase) by cellular RNA polymerase I (referred to as PoI-NP-Luc) [16], and a control reporter plasmid (referred to as pRL-TK). The F2koPB1 was generated as a PB1-F2 deficient PB1 that contains nonsense mutations for PB1 expression (in-frame AUG codon of ORF(PB1-2) was mutated to ACG; insertion of a stop codon 129 TCA>TGA).

Transfection

The cellular transfection procedure has been described elsewhere [16]. In brief, human embryonic kidney cells (HEK293) in six-well plates (5.0×10^5/well) were maintained in Dulbecco's modified Eagle's medium (D-MEM) with 10% fetal calf serum and antibiotics for 18 h at 37°C with 5% CO_2. Cells were transfected with the mixture of plasmids (Table **2**) by using TransIT-293 and incubated for 24 h at 37°C with 5% CO_2.

Luciferase Assay

Luciferase activity was measured *via* the Dual-Luciferase® Reporter Assay System, (Cat# E1910; Promega, USA). The cells in each well were washed twice with phosphate buffer solution (PBS) before adding 1 mL cell lysis buffer and transferring the lysates to 1.5-mL tubes. After centrifugation at 20000 × *g* for 1 min, the supernatants were transferred to new 1.5-mL tubes. The activities of firefly luciferase and *Renilla* luciferase were sequentially determined using a luminescence microplate reader (Infinite®200; Tecan Japan Co., Ltd, Kanagawa). Three samples were measured for each condition, with statistical analysis performed using Wilcoxon signed-rank test.

3. RESULTS AND DISCUSSION

Previous studies have shown that PB1-F2 of the A/WSN/33 strain (WSN;H1N1) upregulates viral polymerase activity *in vitro* [11]. We tested aa mutations in PB1-F2 in the WSN for their influence on virus polymerase activity *in vitro*.

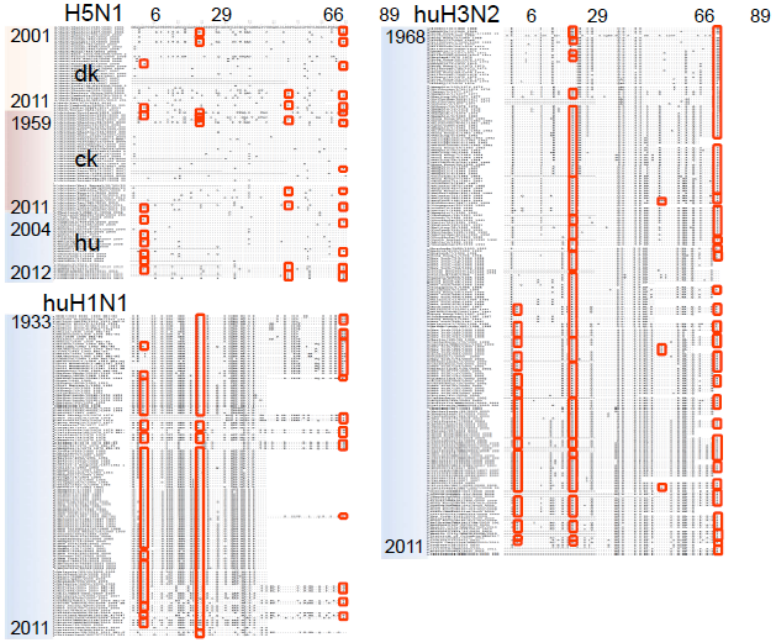
Table 2: Preparation of Transfection Mix

	I	II	III	IV	V	VI
pCAGGS-PB2/WSN	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg
pCAGGS-PA/WSN	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg
pCAGGS-NP/WSN	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg
pCAGGS-F2koPB1/WSN	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg	1.0 μg
pCAGGS-wtPB1F2/WSN	1.0 μg	-	-	-	-	-
pCAGGS-mtPB1F2-D6G*	-	-	2.5 μg	-	-	-
pCAGGS-mtPB1F2-K29R*	-	-	-	2.2 μg	-	-
pCAGGS-mtPB1F2-N66S*	-	-	-	-	2.0 μg	-
pCAGGS-mtPB1F2-T89I*	-	-	-	-	-	1.1 μg
PolI-NP-Luc	0.7 μg	0.7 μg	0.7 μg	0.7 μg	0.7 μg	0.7 μg

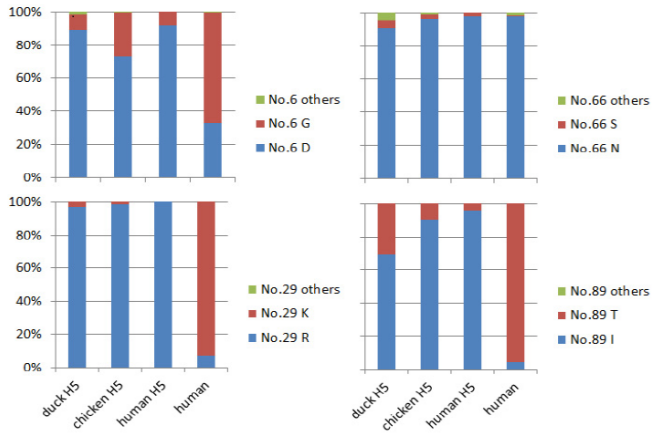
*The values shown were adjusted by a protein expression level comparable to that of the wild type.

First, the conserved species-specific aa of PB1-F2 were studied by referring to the National Center for Biotechnology Information (NCBI) database from 1933 to 2008 (Figures **1a**, **1b**). Three specific aa substitutions in human-derived viruses (D6G, R29K, and I89T) of human H1N1/H3N2 PB1-F2 were identified as differing from those of avian/human H5N1 viruses. The D6G substitution first appeared in the late 1940s and became dominant in the late 1990s; the R29K substitution was typical of human H1N1/H3N2 viruses by the late 1970s, and most human H1N1/H3N2 viruses have exhibited I89T since the 1930s. Human H1N1 viruses possessing the shorter PB1-F2 (58 aa) first appeared in the early 1950s, although the causative N66S substitution, which alters pathogenicity in mice, was not found to be a species-specific substitution in the examined population of field isolates [17, 18].

The secondary structure of full-length PB1-F2 has two short helices at the N terminus and a long C-terminal helix [19]. It is currently believed that the C-



(**a**)



(**b**)

Figure 1: (**a**) Detection of species-specific amino acids (aa) in PB1-F2.
Strains (n = 5329) were obtained from the NCBI database and analyzed using BioEdit. To display the results of protein sequence alignment,we selected three strains from each year (454 strains total: 35 duck H5N1 strains; 41 chicken H5N1 strains, 28 human H5N1 strains, 220 human H3N2 strains, and 130 human H1N1 strains).
(**b**) Comparison of aa ratio at PB1-F2 positions 6, 29, 66, and 89 in each species.
Ratios were determined using the 90-aa-long protein sequences (1200 strains in total: 186 duck H5N1 strains, 310 chicken H5N1 strains, 142 human H5N1 strains, 520 human H3N2 strains, and 42 human H1N1 strains).

terminal long helix of PB1-F2 is necessary for mitochondrial localization [20] and interaction with PB1 [11, 12]. Because no host-specific substitution localized at these helix regions was detected (Figure **2**), we investigated the influence of these PB1-F2 aa mutations on polymerase activity by constructing a minigenome system *in vitro*. As the WSN strain was isolated in 1933, before the emergence of the D6G mutation in the population of human strains, this strain retains the avian-type aa at position 6 in PB1-F2. Because *in vitro* viral polymerase activity studies have been performed using the PB1-F2 from WSN [11], we developed mutants based on the WSN PB1-F2 for consistency. Three species-specific mutations (D6G, K29R, and T89I) and a pathogenicity-related mutation (N66S) were developed (Table **1**). In this minigenome system, overexpression of wild-type (wt) PB1-F2 with the PB1-F2 knockout PB1 reduced viral polymerase activity in comparison to the original PB1 (ratio, 0.67:1). Overexpression of wtPB1-F2 was likely to reduce polymerase activity in mammalian cell lines. To allow comparison with other mutations, the polymerase activity of wtPB1-F2 with the PB1-F2 knockout PB1 (Table **2**) was chosen as a baseline value. The three species-specific mutations and the N66S mutation enhanced polymerase activity by >1.3 fold compared to wtPB1-F2, with a significance value of $P < 0.01$ by Wilcoxon signed-rank test. The species-specific K29R substitution greatly increased polymerase activity (Figure **3**).

As previously mentioned, PB1-F2 possesses diverse *in vivo* functions. The 90-aa-long PB1-F2 isoform contains a C-terminal helical structure and may be a functional protein; it is highly conserved in avian strains. However, PB1-F2 is likely to lose these functions in humans because of evolutionary processes. In fact, human H1N1 strains began to lose their C-terminal helical structure during the late 1950s, and recent studies have suggested that human H3N2 PB1-F2 has no effect on polymerase activity or viral replication [12, 21]. Our study revealed that aa 29R, which is highly conserved in H5N1 viruses, strongly increases polymerase activity. Because PB1-F2 of H5N1 viruses has been shown to influence viral polymerase activity [13], we believe aa 29R in PB1-F2 can be used as an indicator of enhanced viral polymerase activity in mammalian cells.

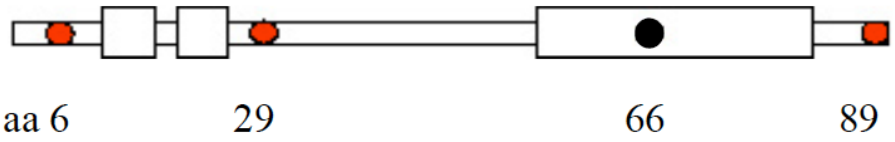


Figure 2: Location of the amino acid mutations on the structure of PB1-F2; each box indicates an α-helix.

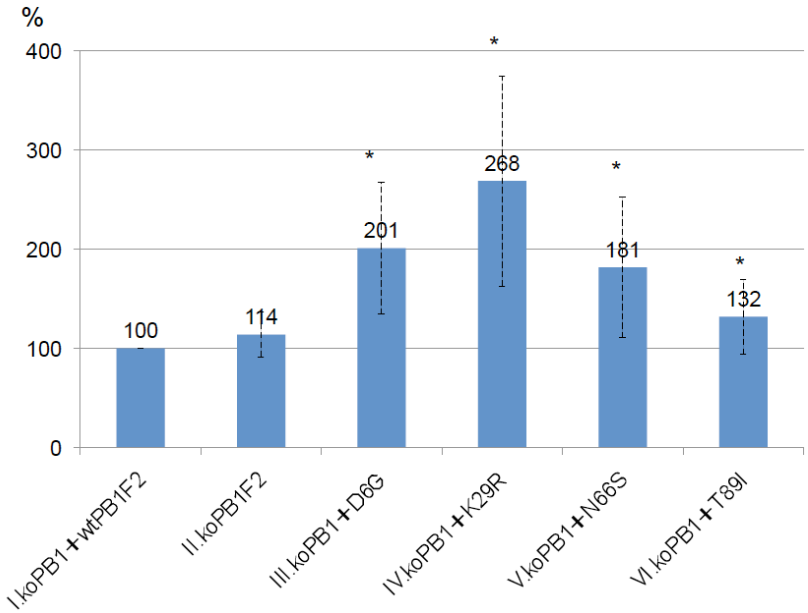


Figure 3: Comparison of viral polymerase activity; viral polymerase activities were indirectly measured by luciferase reporter assay. *Significant at $p \leq 0.01$ by Wilcoxon signed-rank test.

In addition to the development of M2 ion channel blockers and neuraminidase inhibitors, an inhibitor of the influenza virus polymerase is under development [22, 23]. Although our *in vitro* findings were obtained under artificial conditions and require closer inspection, our study suggests inhibition of PB1-F2 function in avian viruses may reduce viral polymerase activity in mammalian cells. Our study may stimulate a shift in attention to PB1-F2 for drug development.

4. CONCLUSIONS

Our study found that avian-type influenza PB1-F2s are potentially important contributors to higher viral polymerase activity in mammalian cells in comparison to human-type PB1-F2s. This finding suggests inhibition of PB1-F2 function in avian viruses might reduce viral polymerase activity in mammalian cells.

AUTHORS' CONTRIBUTIONS

Y.U., M.T., Y.K., M.Y., K.S., and K.S. designed the experiments; Y.U. and M.T. performed the experiments; Y.U., M.T., K.S., and K.S. analyzed the data; Y.U., M.T., Y.K., M.Y., K.S., and K.S. wrote the manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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