

# Mapping of a Region of the PA-X Protein of Influenza A Virus That Is Important for Its Shutoff Activity

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**Influenza A virus PA-X comprises an N-terminal PA endonuclease domain and a C-terminal PA-X-specific domain. PA-X reduces host and viral mRNA accumulation via its endonuclease function. Here, we found that the N-terminal 15 amino acids, particularly six basic amino acids, in the C-terminal PA-X-specific region are important for PA-X shutoff activity. These six basic amino acids enabled a PA deletion mutant to suppress protein expression at a level comparable to that of wild-type PA-X.**

PA-X was found to be expressed from the PA segment of influenza A viruses as an alternative product (1). It consists of an N-terminal endonuclease domain of PA (191 amino acids) and a C-terminal PA-X-specific domain (61 amino acids), which is encoded by an overlapping open reading frame generated by a ribosomal frameshift (1, 2). PA-X decreases host and viral mRNA accumulation via its shutoff activity, which is dependent on its endonuclease activity (1, 3). Although PA-X is not essential for viral replication *in vitro*, it modulates host responses *in vivo* (1, 4–7).

PA has lower shutoff activity than PA-X, although it has the N-terminal endonuclease domain (1, 3, 8–12), suggesting that the C-terminal PA-X-specific region is also important for the shutoff activity. To examine this possibility, we constructed two C-terminal deletion mutant forms of PA [A/WSN/33(H1N1)] (Fig. 1A). PA, PA-X, PA\_191, or PA\_252 was expressed in 293 cells together with *Renilla* luciferase by means of plasmid transfection with Trans IT-293 (Mirus). *Renilla* luciferase activities were measured with the *Renilla*-Glo luciferase assay system (Promega) at 24 h posttransfection (Fig. 1B). *Renilla* luciferase activities were suppressed by PA (0.6-log-fold), PA-X (2.0-log-fold), PA\_191 (1.3-log-fold), and PA\_252 (0.7-log-fold). To confirm these results, we analyzed the levels of expression of *Renilla* luciferase and the PA-X and PA variants by Western blotting with an anti-*Renilla* luciferase polyclonal antibody (Promega) (Fig. 1C) and an anti-PA monoclonal antibody, clone 55/2 (13) (Fig. 1D). *Renilla* luciferase was detected in the mock lane but not in the PA-X lane. In the PA, PA\_191, and PA\_252 lanes, the *Renilla* luciferase expression levels were lower than in the mock lane (43.6, 53.7, and 52.7%, respectively). PA-X was barely detected because of self-suppression, whereas PA, PA\_191, and PA\_252 were expressed to similar extents. These results indicate that the shutoff activity of PA\_191 and PA\_252 is lower than that of wild-type PA-X, suggesting that the C-terminal PA-X-specific region is important for PA-X shutoff activity.

To pinpoint the amino acid residues in the C-terminal PA-X-specific region that are critical for PA-X activity, we prepared a series of C-terminal deletion mutant forms of PA-X (Fig. 2A) and examined their shutoff activities in the luciferase assay (Fig. 2B). Compared with wild-type PA-X (which exhibited 1.8-log-fold less than the mock sample), PA-X\_X10, PA-X\_X5, and PA\_191 showed significantly lower shutoff activity (1.6-, 1.1-, and 1.1-log-

fold less than the mock sample, respectively), whereas the PA-X\_X15 shutoff activity was similar to that of wild-type PA-X. These results indicate that the N-terminal 15 amino acids in the C-terminal PA-X-specific region are important for PA-X shutoff activity.

Next, we compared the N-terminal portion of the PA-X-specific 15 amino acids with that of PA (Fig. 3A). We found a characteristic difference: six basic amino acids (lysine [K] and arginine [R]) in PA-X were acidic amino acids (glutamic acid [E]) in PA. To evaluate the significance of these six basic amino acids for PA-X shutoff activity, we constructed mutant PA-X proteins in which we replaced these six basic amino acids (K and R) with E (PA-X\_6E), aspartic acid (D) (PA-X\_6D), alanine (A) (PA-X\_6A), R (PA-X\_6R), K (PA-X\_6K), or R and K (PA-X\_KR) (Fig. 3B) and examined their shutoff activities (Fig. 3C). PA-X\_6E, PA-X\_6D, and PA-X\_6A showed greater *Renilla* luciferase activities than wild-type PA-X, whereas the *Renilla* luciferase activities of PA-X\_6R, PA-X\_6K, and PA-X\_KR were similar to that of wild-type PA-X. The trend of the levels of expression of wild-type and mutant PA-X (Fig. 3D) and the corresponding *Renilla* luciferase level (Fig. 3E) was similar, with the exception of PA-X\_6A; although PA-X\_6A suppressed *Renilla* luciferase and its own expression when analyzed by Western blotting (Fig. 3D and E), in the luciferase assay, the suppression of *Renilla* luciferase activity by PA-X\_6A (Fig. 3C) was limited for an unknown reason. The six amino acid mutations with D or E affected the mobility of the mutant PA-X proteins (Fig. 3D). To identify the amino acid essential for PA-X shutoff activity, we introduced a single point mutation (K or R to E) at each of the six basic amino acids. The

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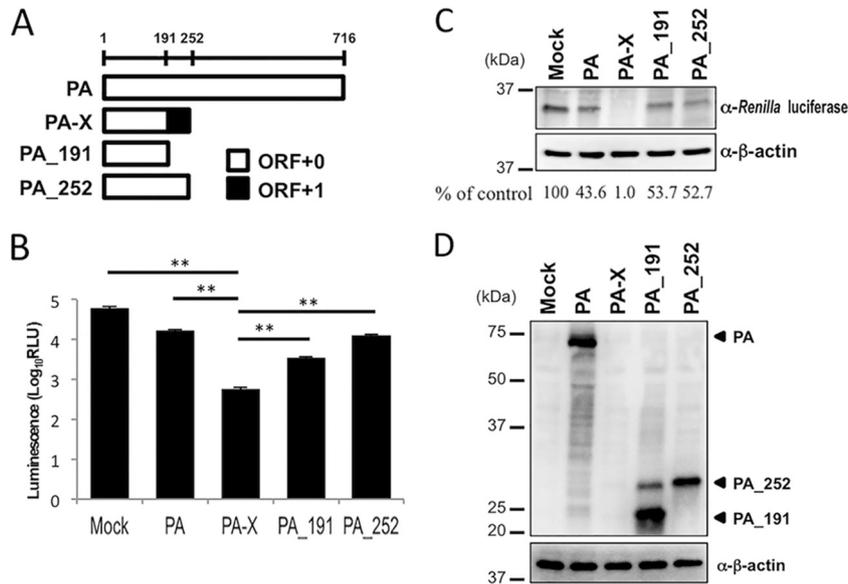
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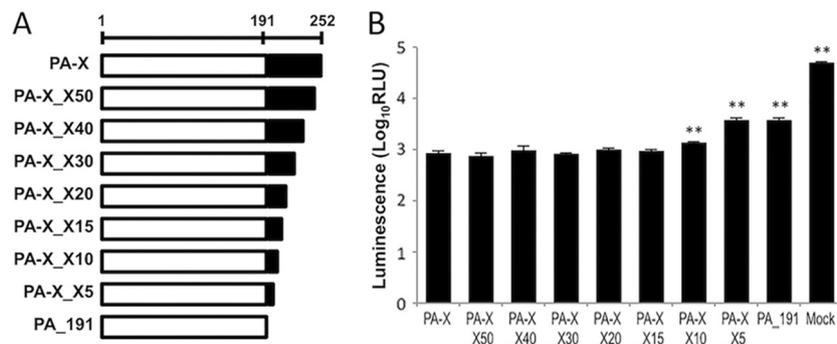
**FIG 1** Comparisons of the shutoff activities of wild-type PA, wild-type PA-X, and C-terminal deletion mutant forms of PA. (A) Schematic representation of PA, PA-X, and C-terminal deletion mutant forms of PA. PA-X is composed of the N-terminal 191 amino acids of PA and a C-terminal PA-X-specific region of 61 amino acids. PA\_191 and PA\_252 contain the N-terminal 191 and 252 amino acids of PA, respectively. (B to D) *Renilla* luciferase activity (B), expression of *Renilla* luciferase (C), and expression of each viral protein (D) in cells transfected with pGL4.74[hRluc/TK] together with an empty plasmid or a plasmid encoding wild-type PA, wild-type PA-X, or deletion mutant forms of PA were evaluated by using a luciferase assay and Western blotting with an anti-*Renilla* luciferase and an anti-PA monoclonal antibody. The luminescence data in panel B are mean values  $\pm$  standard deviations ( $n = 3$ ). \*\*,  $P < 0.01$  (one-way analysis of variance, followed by Bonferroni correction). (C) The intensities of the *Renilla* luciferase and  $\beta$ -actin bands were quantified, and their ratios were calculated. The value of the mock lane was set to 100%. In panels C and D,  $\beta$ -actin served as a loading control. ORF, open reading frame; RLU, relative light units.

mutant proteins produced had similar shutoff activities, all of which were slightly lower than that of wild-type PA-X (data not shown). These data imply that the basic amino acids at these six positions contribute equally to the shutoff activity of PA-X.

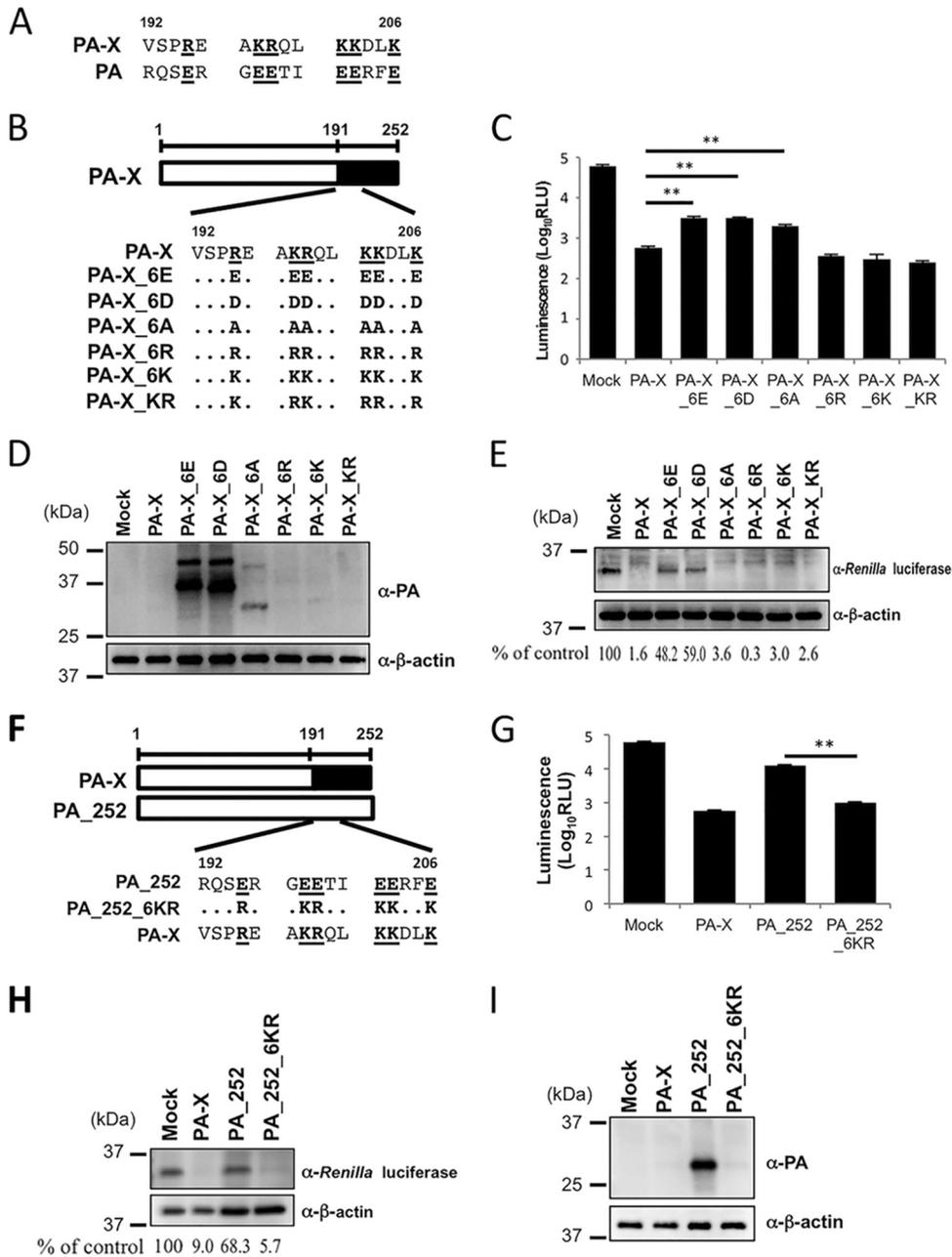
To confirm the importance of these six basic amino acids, we replaced all six E residues in PA\_252 with K or R (PA\_252\_6KR) (Fig. 3F) and assessed the shutoff activity (Fig. 3G). PA\_252\_6KR showed high shutoff activity at a level comparable to that of wild-type PA-X. When we assessed the levels of expression of *Renilla* luciferase and mutant and wild-type PA-X and related viral proteins (Fig. 3H and I), the results were similar to those obtained in

the luciferase assay (Fig. 3G). These results demonstrate that the six basic amino acids at these positions are essential for the shutoff activity of PA-X.

We then asked whether these six basic amino acids in the PA-X-specific region were conserved among other influenza virus isolates deposited in the Influenza Research Database in December 2014. PA-X proteins of different viruses had substitutions between K and R at these six positions; however, basic amino acids (K or R) at these six positions were highly conserved among human (99.9% of 3,417 isolates), avian (99.3% of 4,047), swine (96.7% of 1,030), and equine (100% of 6) isolates. This information further sup-



**FIG 2** Importance of the N-terminal 15 amino acids in the PA-X-specific region for the shutoff activity of PA-X. (A) Schematic diagram of the C-terminal deletion mutant forms of PA-X. A series of C-terminal deletion mutant forms of PA-X was constructed to identify the region(s) important for the shutoff activity of PA-X. PA-X\_X50, PA-X\_X40, PA-X\_X30, PA-X\_X20, PA-X\_X15, PA-X\_X10, PA-X\_X5, and PA\_191 possessed 50, 40, 30, 20, 15, 10, 5, and no PA-X-specific amino acids, respectively. (B) Suppression of *Renilla* luciferase activity by each C-terminal deletion mutant form of PA-X in 293 cells. *Renilla* luciferase activity in cells cotransfected with wild-type PA-X, a series of C-terminal deletion mutant proteins, or PA\_191 was measured with a luciferase assay. The luminescence in the each C-terminal deletion mutant sample was compared with that in the wild-type PA-X sample. Luminescence data are mean values  $\pm$  standard deviations ( $n = 3$ ). \*\*,  $P < 0.01$  (one-way analysis of variance, followed by Dunnett's test). RLU, relative light units.

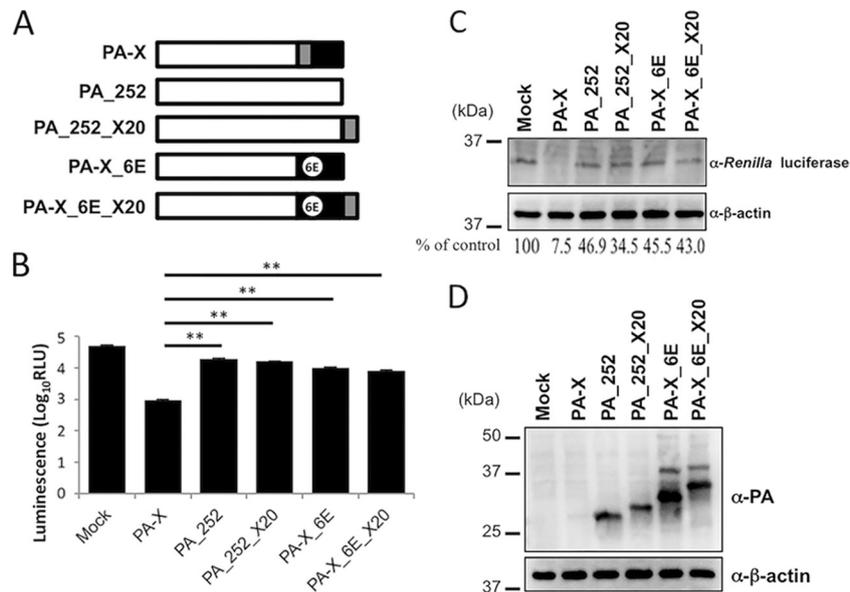


**FIG 3** Identification of amino acids in the PA-X-specific region that are important for the shutoff activity of PA-X. (A) Comparison of the N-terminal PA-X-specific 15 amino acids of PA-X with the identical region of PA. All six basic amino acids (K or R) in PA-X were the acidic amino acid E in PA. The six amino acids are underlined. (B) Substitution of the six basic amino acids in the PA-X-specific region. The six basic amino acids of PA-X were replaced with acidic amino acids (PA-X\_6E or PA-X\_6D), a neutral amino acid (PA-X\_6A), or a basic amino acid (PA-X\_6R, PA-X\_6K, or PA-X\_KR). (C to E) Shutoff activities of mutant PA-X proteins. In 293 cells, *Renilla* luciferase was coexpressed with wild-type PA-X or each mutant PA-X protein. *Renilla* luciferase activity (C) and *Renilla* luciferase (E) and wild-type and mutant PA-X (D) expression levels were evaluated by means of a luciferase assay or Western blotting. (F) Comparison of the N-terminal PA-X-specific 15 amino acids of PA-X with the identical region of PA\_252. Six acidic amino acids in PA\_252 (underlined) were replaced with those of PA-X (PA\_252\_6KR). (G to I) Shutoff activity of PA\_252\_6KR. In 293 cells, *Renilla* luciferase was coexpressed without (mock) or with wild-type PA-X, PA\_252, or PA\_252\_6KR. *Renilla* luciferase activity (G) and levels of expression of *Renilla* luciferase (H) and each viral protein (I) were evaluated by means of a luciferase assay or Western blotting. The luminescence data in panels C and G are mean values  $\pm$  standard deviations ( $n = 3$ ). \*\*,  $P < 0.01$  ( $t$  test, followed by Bonferroni correction). The intensities of the *Renilla* luciferase and  $\beta$ -actin bands were quantified and their ratios were calculated (E and H). The value of the mock lane was set to 100%. In panels D, E, H, and I,  $\beta$ -actin served as a loading control. RLU, relative light units.

ports the concept that these basic amino acids are required for the shutoff activity of PA-X.

To examine whether the N-terminal 20 amino acids in the PA-X-specific region could increase the shutoff activity of PA\_252

or PA-X\_6E, we prepared PA\_252\_X20 and PA-X\_6E\_X20 (Fig. 4A) and evaluated their shutoff activities (Fig. 4B). PA\_252, PA\_252\_X20, PA-X\_6E, and PA-X\_6E\_X20 showed shutoff activity lower than that of wild-type PA-X. We confirmed that the



**FIG 4** Effect of the N-terminal 20 amino acids in the PA-X-specific region on the shutoff activity of PA<sub>252</sub> and PA-X<sub>6E</sub>. (A) Schematic diagram of PA<sub>252</sub>\_X20 and PA-X<sub>6E</sub>\_X20. These proteins contain the N-terminal 20 amino acids of the PA-X-specific region (gray) at the C terminus of PA<sub>252</sub> or PA-X<sub>6E</sub>, respectively. (B to D) Shutoff activities of PA<sub>252</sub>\_X20 and PA-X<sub>6E</sub>\_X20. In 293 cells, *Renilla* luciferase was coexpressed without (mock) or with wild-type PA-X, PA<sub>252</sub>, PA<sub>252</sub>\_X20, PA-X<sub>6E</sub>, or PA-X<sub>6E</sub>\_X20. *Renilla* luciferase activity (B) and the expression of *Renilla* luciferase (C) and each viral protein (D) were evaluated by means of a luciferase assay or Western blotting. (C) The intensities of the *Renilla* luciferase and β-actin bands were quantified, and their ratios were calculated. The value of the mock lane was set to 100%. In panels C and D, β-actin served as a loading control. RLU, relative light units.

levels of expression of *Renilla* luciferase and each viral protein were comparable by Western blotting (Fig. 4C and D). These results indicate that the N-terminal 20 amino acids of the PA-X-specific region contribute to the shutoff activity only when they are located in their original position.

In summary, we found that the N-terminal 15 amino acids, especially six basic amino acids, in the C-terminal PA-X-specific region are required for the full shutoff activity of PA-X. Most influenza A viruses isolated from humans, equines, and avian species encode a PA-X protein that is identical in length to the PA-X protein of the WSN strain, which possesses 61 PA-X-specific amino acids at its C terminus (14). However, most human H1N1pdm and swine viruses possess a stop codon upstream of the stop codon present in other PA-X proteins, leading to a C-terminally truncated PA-X protein that possesses only 41 PA-X-specific amino acids (14). It has previously been reported that the C-terminally truncated PA-X protein has shutoff activity lower than that of full-length PA-X (4). However, our data demonstrate that PA-X proteins with the C-terminal 20 amino acids truncated would have adequate shutoff activity because the N-terminal 15 amino acids in the PA-X-specific region are sufficient for full shutoff activity.

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