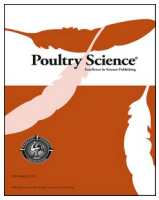
104 (2025) 104570

Contents lists available at ScienceDirect

Poultry Science

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



Research Note: Establishment of vector system harboring duck RNA polymerase I promoter for avian influenza virus

Yunyueng Jang [1], Yoon-Gi Baek [1], Yu-Na Lee, Ra Mi Cha, Yun-Chan Choi, Min-Ji Park, Youn-Jeong Lee, Eun-Kyoung Lee [*]

Avian Influenza Research & Diagnostic Division, Animal and Plant Quarantine Agency, 177 Hyeoksin 8-ro, Gimcheon-si, Gyeongsangbuk-do 39660, Republic of Korea

ARTICLE INFO

Keywords:
Duck RNA polymerase I
Avian influenza
Reverse genetics

ABSTRACT

Reverse genetics (RG) systems are extensively utilized to investigate the characteristics of influenza viruses and develop vaccines, predominantly relying on human RNA polymerase I (pol I). However, the efficiency of RG systems for avian-origin influenza viruses may be compromised due to potential species-specific interactions of RNA pol I. In this study, we reported the polymerase activities of the duck RNA pol I promoter in avian cells and the generation of recombinant avian-derived influenza viruses using a novel vector system containing the duck RNA pol I promoter region to enhance the rescue efficiency of the RG system in avian cells. Initially, we explored a putative duck promoter region and identified the optimal size to improve the existing system. Subsequently, we established an RG system incorporating the duck RNA pol I promoter and compared its rescue efficiency with the human pol I system by generating recombinant influenza viruses in several cell lines. Notably, the 250-bp duck RNA pol I promoter demonstrated effective functionality in avian cells, exhibiting higher polymerase activity in a minigenome assay. The newly constructed RG system was significantly improved, enabling the rescue of influenza viruses in 293T, DF-1, and CCL141 cells. Furthermore, HPAI viruses were successfully rescued in DF-1 cells, a result that had not been achieved in previous experiments. In conclusion, our novel RG system harboring duck RNA pol I offers an additional tool for researching influenza viruses and may facilitate the development of vaccines for poultry.

Introduction

Influenza A virus (IAV), a member of the Orthomyxoviridae family, has a genetic material composed of negative-sense single-stranded RNA divided into eight segments. During its life cycle, IAV enters the host cell through endocytosis and undergoes fusion with the host cell membrane (Jung et al., 2020). Following entry, the viral ribonucleoprotein of the influenza virus is released into the cytoplasm and transported to the host cell nucleus, where it replicates viral RNA (vRNA) and transcribes mRNA (Dou et al., 2018). Eventually, the influenza virus self-assembles using the replicated vRNA and host cell machinery to produce new infectious virions.

Reverse genetics (RG) is a widely used method to investigate the characteristics of influenza viruses and to enhance the efficiency of vaccine production (Neumann et al., 2005; Nogales et al., 2016; Youk et al., 2021). For decades, Research on RG system have primarily relied on RNA polymerase I (pol I). Notably, a significant portion of recombinant influenza studies have utilized human RNA pol I, facilitated by the use of pHW2000 vector system (Hoffmann et al., 2000). However, RNA pol I functions in a species-specific manner, meaning that using different host cells or RNA pol I promoters may not efficiently amplify influenza viruses or their components (Heix et al., 1995). To address these limitations, research has explored the use of RNA pol I promoters from various hosts, including dogs, chickens, horses, and pigs (Massin et al., 2005; Wang et al., 2007; Lu et al., 2016; Wang et al., 2017).

Luciferase assays have frequently been employed to investigate polymerase promoters (Shinagawa et al., 2003; Diebel et al., 2010; Liu et al., 2023). In studies of influenza viruses, the polymerase activity of the viral ribonucleotide protein (RNP) complex is typically measured to assess promoter capacity or the effects of mutations in the RNP complex (Murakami et al., 2008; Suzuki et al., 2014). A reporter plasmid containing the RNA pol I promoter and luciferase gene, flanked by influenza

* Corresponding author.
E-mail address: ensenble@korea.kr (E.-K. Lee).
[1] Yunyueng Jang and Yoon-Gi Baek are equal contributors to this work and designated as co-first authors.

https://doi.org/10.1016/j.psj.2024.104570
Received 20 August 2024; Accepted 21 November 2024
Available online 22 November 2024

virus non-coding regions, is used to measure the activity of the influenza virus RNP complex. This setup allows for an indirect measurement of RNA pol I promoter activity in various cell lines (Li et al., 2009).

In this study, we established a vector system containing the duck RNA pol I promoter specifically for the avian influenza virus (AIV). We investigated the duck RNA pol I promoter and assessed its activity at various lengths to optimize pol I promoter performance in avian cell lines using luciferase assays. Notably, we constructed a novel vector system using the duck RNA pol I promoter to overcome the limitations of existing vector systems, enabling the efficient rescue of avian-origin influenza viruses.

Materials and methods

Cell and viruses

Human embryonic kidney cells (293T), duck embryo fibroblast (CCL-141) and chicken fibroblast cells (DF-1) were maintained in Dulbecco`s modified Eagle`s medium (DMEM, Gibco, USA), Eagle's minimum essential medium (EMEM, ATCC, USA) containing 10% fetal bovine serum (FBS, Biowest, USA) and 1% antibiotics (penicillin, streptomycin, amphotericin B) (Gibco, USA). A/Puerto Rico/8/1934(H1N1) (PR8) and three H5N8 HPAI viruses, A/broiler duck/Korea/H1731/2014(H5N8) (H1731), A/mallard/Korea/H2102/2015(H5N8) (H2102), and A/mallard/Korea/H1924-6/2016(H5N8) (H1924) were propagated in the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated eggs at 37 °C for 72 h. Subsequently, hemagglutination-positive allantoic fluid was collected from the eggs. All experimental procedures involving potential contact with high pathogenicity avian influenza (HPAI) viruses were conducted in a biosafety level 3 facility approved by the Korean government.

Exploration of duck Pol I promoter

To identify the duck RNA pol I promoter region, we searched the duck chromosome that contains the 18, 5.8 and 28S ribosomal RNA genes in the database of the duck genome (ZJU1.0, duck genome Resources of National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/datasets/genome/GCF_015476345.1/) and found the intergenic spacer (IGS) region between 18S and 28S ribosomal RNA gene sequences on the chromosome (GenBank accession no. NW_024010378.1). Subsequently, the RNA Pol I transcription initiation site (nucleotide [nt] -8 to -11; +1 is referred to as the transcription initiation site) and promoter sequences located in upstream region of 18s rRNA gene were identified and compared with those of other species.

Minigenome assay

To evaluate the promoter activity of the duck RNA pol I, the ORFs of vRNP complex genes of A/PR/8/34 were cloned into pCDNA3.1 vector. The pCDNA3.1-PB2, pCDNA3.1-PB1, pCDNA3.1-PA, and pCDNA3.1-NP plasmids of A/PR/8/34 were constructed by amplifying the coding sequence using PCR, respectively. Each plasmid was obtained by using infusion-HD Cloning Kit (Takara, Japan).

To construct the luciferase reporter plasmid, the putative RNA Pol I promoter of duck and an antisense firefly luciferase coding sequence flanked by the 5′ and 3′ noncoding regions (NCRs) were synthesized based on pUC57 vector (Macrogen, Korea). Luciferase reporter plasmids generating luciferase transcripts consisted of the duck RNA pol I promoters of different lengths (nt −1 to −1000, −750 −500, −250 [DK polI 1000, DK polI 750, DK polI 500 and DK polI 250, respectively]). DF-1 and CCL-141, cultured to 90% confluent in 6-well plates, were transiently co-transfected with the artificial RNP complex plasmids using Lipofectamine3000 (Invitrogen, USA). To investigate transfection efficacy, cells were also transfected with pGL4.73 (Promega, USA)

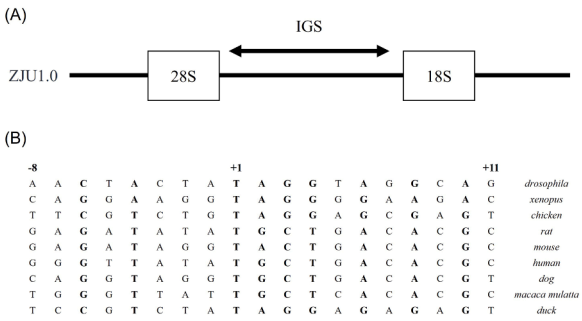


Fig. 1. Exploration of duck RNA polymerase I promoter region. (A) Molecular map of duck genomic DNA fragment from ZJU1.0. The intergenic spacer (IGS) region is highlighted, positioned between 18s and 28s ribosomal RNA on the chromosome. (B) Alignment of the sequence of duck promoter region with those of other species. The 19-nucleotide sequences (-8 to +11) representing the RNA Poly I transcription initiation site located upstream region of 18s rRNA. This sequence alignment encompasses eight species, highlighting conserved nucleotide sequences in bold.

containing Renilla luciferase gene as an internal control. Following transfection, cell was incubated at 37 °C for 48 h post-transfection and then lysed. Luciferase activity was determined by the dual-luciferase assay system (Promega, USA) according to the manufacturer`s instructions. Luciferase activity values were normalized relative to the Renilla luciferase activity.

Reverse genetics

The pDK2022 vector was constructed by replacing the human RNA pol I promoter in the pHW2000 vector with a 250-bp duck pol I promoter to examine whether duck promoter works properly in cells (Supplementary Fig.1). Subsequently, eight segments of A/PR/8/34, H1924, H2102, and H1731 were cloned into the pDK2022 vector and pHW2000 vector for reverse genetic system, respectively.

To generate recombinant viruses set of pHW2000 and pDK2022 plasmids, containing eight segments of each virus as described previously, were transfected into 293T, DF-1 or CCL-141 with 300ng/gene·well using Lipofectamine3000 at 37 °C. At 48 post-infection hours, supernatant of growth media was collected and inoculated into embryonated eggs to propagate the recombinant viruses. Finally, hemagglutination-positive allantoic fluid was harvested from the eggs. The rescued viruses were fully sequenced to ensure no unwanted mutations (Cha et al., 2023).

Statistical analysis

Experimental data were analyzed using unpaired *t*-test by GraphPad software, version 8 (GraphPad Software Inc., SanDiego, CA, USA). Statistical *p*-values less than 0.05 ($p < 0.05$) were considered significant.

Results and discussion

We explored the duck promoter region where RNA pol I transcribes 18S or 28S ribosomal RNA in eukaryotes (Grummt, 2003). RNA pol I recognizes species-specific promoters that contain conserved sequences around transcription initiation sites, although the sequence arrangements vary across species (Song et al., 2013). Using the duck genome obtained from the GenBank database of NCBI, we verified the 18S and 28S rRNA sequences (Fig. 1A). The RNA pol I promoter is located within the intergenic spacer (IGS) region between the 18S and 28S rRNA sequences (Paule et al., 2000). We then compared the 19-nucleotide sequences in the IGS of the duck genome with those of other species known to include promoters and transcription initiation sites (Fig. 1B). By designating the transcription initiation site as +1, we identified

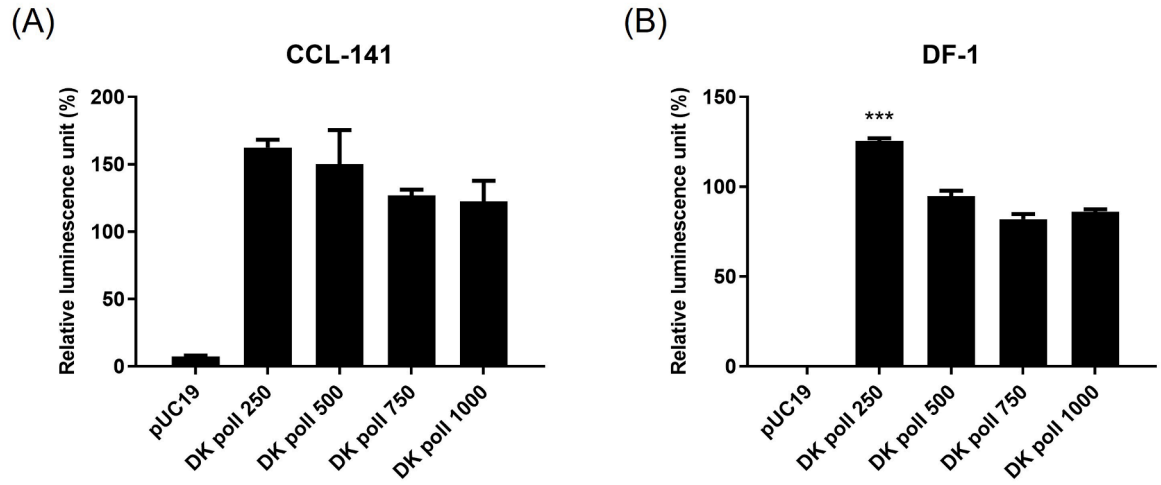


Fig. 2. Luciferase activities of duck RNA polI promoter in avian cells. Plasmids encoding PB2, PB1, PA, and NP proteins derived from influenza virus A/PR/8/34 were transfected into (A) CCL-141 and (B) DF-1 cells, together with luciferase reporter constructs containing different length of the duck RNA poly I promoters (nt −1 to −1000, −750 −500, and −250) and an antisense firefly luciferase coding sequence based on pUC19 vector. Data were normalized relative to the Renilla luciferase activity and represented with ±standard deviation. *** p-value < 0.0001.

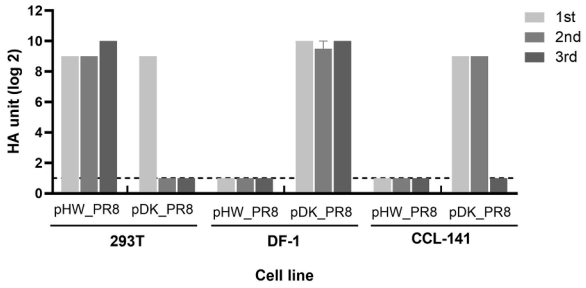


Fig. 3. Comparison of rescue efficiency between the human and duck RNA pol I promoters in 293T, DF-1, and CCl-141 cells. A set of pHW2000 and pDK2022 plasmids containing eight segments of A/PR/8/34 were transfected into 293T, DF-1, and CCL-141. After 48 h post-infection, the growth media supernatant was collected and inoculated into embryonated eggs to propagate the recombinant viruses. Subsequently, allantoic fluid was harvested from the eggs, and the viruses were titered using a hemagglutination assay. Data representing individual rescue trials are presented as mean titers of log2 HA units ±standard deviation.

conserved sequences containing +1 (T), +2 (A or G), +3 (G or C), +4 (G or T), +6 (A or G), +8 (A or G), +10 (A or G), and -4 (A or T), -6 (C or G) (Fig. 1B). Consequently, we identified sequences within the IGS of the duck genome that are likely to constitute the putative duck pol I promoter.

RNA pol I is species-specific and its promoter activities depend on the cell types and the origin of viruses, indicating that promoter activities can be significantly affected by such factors (Heix et al., 1995; Moncorgé et al.,2013; Lu et al., 2022). We performed the luciferase assay to access the promoter activity and identify the optimal length of duck RNA pol I promoter in avian cells. We cloned four different sequence lengths (-1 to -250bp [DK pol I 250], -1 to -500bp [DK pol I 500], -1 to -750bp [DK pol I 750], and -1 to -1000bp [DK pol I 1000]) of the putative duck pol I promoter, starting from the transcription initiation site. We then conducted minigenome assays to determine the promoter activity of each sequence and compared them to a negative control that did not contain the duck promoter in DF-1 and CCL-141 cells. In both avian cell lines, DK pol I 250 exhibited the highest relative luminescence value, with statistically significant differences compared to all other promoter sizes (***p < 0.0001) (Fig. 2). These results suggest that the duck Pol I promoter, inferred from the IGS of the duck genome, functions effectively in avian cells, with the 250-bp promoter being the optimal length for interaction with these cells. Consistent with our findings, previous studies on RNA Pol I promoters in chickens, canines, horses, and pigs have shown that the highest promoter activity occurs when testing sequences from 250 to 500 bp upstream of the transcription initiation site (Wang et al., 2007; Massin et al., 2005; Lu et al., 2016; Wang et al., 2017). Moreover, our research aligns with earlier studies suggesting that RNA Pol I promoters can operate with minimal active units owing to the presence of nearly identical repeated sequence regions.

To confirm that the 250-bp promoter verified in avian cells would function correctly in the RG system, we conducted reverse genetics using the PR8 virus. We verified virus production in 293T, CCL-141, and DF-1 cells in triplicate, using either the pHW2000 set or the pDK2022 set of 8 plasmids containing PR8 genes. The reconstructed PR8 virus based on the pDK2022 set (abbreviated as pDK_PR8) was successfully rescued in all cell lines, although the rescue rate varied among them. In contrast, the reconstructed PR8 virus based on the pHW2000 set (abbreviated as pHW_PR8) was only rescued in 293T cells. When transfected into avian cells, pDK_PR8 showed improved rescue efficiency, succeeding in all trials for DF-1 cells and in two out of three trials for CCL-141 cells (Fig. 3).

Thus, our DK pol I 250-based pDK2022 RG system successfully produced the PR8 virus in avian cells, whereas the pHW2000-based PR8 plasmids did not rescue the virus except in 293T cells. These results support previous reports on the species-specific interactions between RNA pol I and RNA pol I promoters. Interestingly, the duck promoter-based pDK2022 RG system was most effective in chicken-derived DF1 cells. The DF-1 cell line may be more amenable to transfection for generating influenza viruses than CCL-141. Rescue efficiency varies across host ranges and could be higher in different host cell lines, leading to more permissive and functional interactions (Lee et al., 2008; Suphaphiphat et al., 2010). For example, when using a canine pol I or human pol I promoter-based RG system in Madin-Darby canine kidney (MDCK) 33016-PF cells, both systems were able to generate adequate virus titers.

In our preliminary studies, we encountered limitations in efficiently rescuing certain avian-origin HPAI strains (H1731, H1924, H2102) using existing systems. To compare the activity of the pDK2022 and pHW2000 systems concerning duck-derived HPAI viruses, we transfected each set of plasmids containing eight segments of three H5N8 HPAI viruses into 293T or DF-1 cells. The pDK2022 RG system efficiently produced avian-origin viruses in the DF1 cell line, demonstrating significantly improved rescue efficiency compared to the pHW2000 RG system. As in previous experimental rescue attempts, the pHW2000 RG

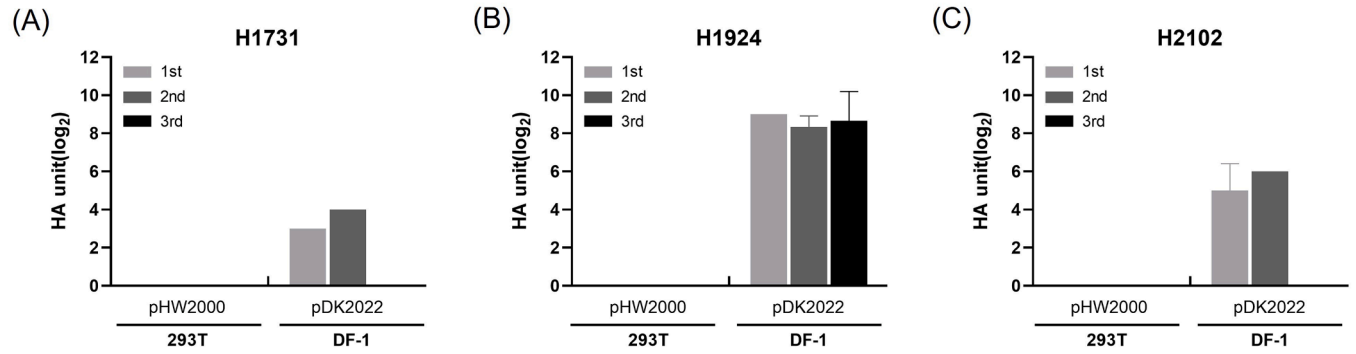


Fig. 4. Rescue efficiency of pHW2000 and pDK2022 systems using avian influenza viruses. A set of pHW2000 and pDK2022 plasmids containing eight segments of (A) H1731, (B) H1924, and (C) H2102 were transfected into 293T and DF-1, respectively. After 48 h post-infection, the growth media supernatant was collected and inoculated into embryonated eggs for the propagation of recombinant viruses. Subsequently, allantoic fluid was harvested from the eggs, and the viruses were titered using a hemagglutination assay. Data representing individual rescue trials are presented as mean titers of log2 HA units ±standard deviation.

system failed to rescue H5N8 HPAI viruses in 293T cells in all trials. Conversely, pDK_H1924 was completely rescued in DF-1 cells, yielding 512 HA units after propagation in SPF-chicken eggs. pDK_H1731 and pDK_H2102 were rescued in two of three trials, although with lower HA units compared to pDK_H1924 (Fig. 4). The HA titers of all rescued viruses were more than 64 HA units in three successive passages with embryonated chicken eggs. Furthermore, the sequences of passaged viruses were identical to parental viruses, indicating that the pDK2022 system stably generates recombinant influenza viruses.

Owing to host restrictions of the human pol I based RG system, we have faced challenges in efficiently propagating avian-adapted viruses in mammalian cells. To address this, we explored a putative duck promoter region and determined the optimal size to improve the existing system by constructing a vector system. Our novel RG system was significantly improved, enabling the rescue of HPAI viruses in DF-1 cells, which was not possible in previous studies. This advancement may facilitate the development of poultry vaccines and provide alternative options for researching avian influenza viruses.

Disclosures

The authors have no conflicts of interest to declare

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We gratefully thank Seo Yoon Hong for excellent technical assistance. This research was supported by the grant from the Animal and Plant Quarantine Agency (B-1543418-2022-23-01) of the Republic of Korea.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104570.

References

Cha, R.M., Lee, Y.N., Park, M.J., Baek, Y.G., Shin, J.I., Jung, C.H., Sagong, M., Heo, G.B., Kang, Y.M., Lee, K.N., Lee, Y.J., Lee, E.K., 2023. Genetic characterization and pathogenesis of H5N1 high pathogenicity Avian influenza virus isolated in South Korea during 2021–2022. Viruses 156, 1403.

Diebel, K.W., Smith, A.L., van Dyk, L.F., 2010. Mature and functional viral miRNAs transcribed from novel RNA polymerase III promoters. RNA 161, 170–185. https://doi.org/10.1261/rna.1873910.

Dou, D., Revol, R., Östbye, H., Wang, H., Daniels, R., 2018. Influenza A virus cell entry, replication, virion assembly and movement. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.01581.

Grummt, I., 2003. Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. Genes Dev. 1714, 1691–1702. https://doi.org/10.1101/gad.1098503R.

Heix, J., Grummt, I., 1995. Species specificity of transcription by RNA polymerase I. Curr. Opin. Genet. Dev. 55, 652–656. https://doi.org/10.1016/0959-437x(95)80035-2.

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. 9711, 6108–6113. https://doi.org/10.1073/pnas.100133697.

Jung, H.E., Lee, H.K., 2020. Host protective immune responses against influenza A virus infection. Viruses 125, 504.

Lee, C.W., Jung, K., Jadhao, S.J., Suarez, D.L., 2008. Evaluation of chicken-origin (DF-1) and quail-origin (QT-6) fibroblast cell lines for replication of avian influenza viruses. J. Virol. Methods 1531, 22–28. https://doi.org/10.1016/j.jviromet.2008.06.019.

Li, O.T., Chan, M.C., Leung, C.S., Chan, R.W., Guan, Y., Nicholls, J.M., Poon, L.L., 2009. Full factorial analysis of mammalian and avian influenza polymerase subunits suggests a role of an efficient polymerase for virus adaptation. PLoS One 45, e5658. https://doi.org/10.1371/journal.pone.0005658.

Liu, L., Madhugiri, R., Saul, V.V., Bacher, S., Kracht, M., Pleschka, S., Schmitz, M.L., 2023. Phosphorylation of the PA subunit of influenza polymerase at Y393 prevents binding of the 5′-termini of RNA and polymerase function. Sci. Rep. 131, 7042. https://doi.org/10.1038/s41598-023-34285-7.

Lu, G., He, D., Wang, Z., Ou, S., Yuan, R., Li, S., 2016. Cloning the horse RNA polymerase I promoter and its application to studying influenza virus polymerase activity. Viruses 86. https://doi.org/10.3390/v8060119.

Lu, G., Zheng, F., Ou, J., Yin, X., Li, S., 2022. Investigating influenza virus polymerase activity in feline cells based on the influenza virus minigenome replication system driven by the Feline RNA Polymerase I Promoter. Front. immunol. 13. https://doi.org/10.3389/fimmu.2022.827681.

Massin, P., Rodrigues, P., Marasescu, M., van der Werf, S., Naffakh, N., 2005. Cloning of the chicken RNA polymerase I promoter and use for reverse genetics of influenza A viruses in avian cells. J. Virol. 7921, 13811–13816. https://doi.org/10.1128/jvi.79.21.13811-13816.2005.

Moncorgé, O., Long, J.S., Cauldwell, A.V., Zhou, H., Lycett, S.J., Barclay, W.S., 2013. Investigation of influenza virus polymerase activity in pig cells. J. Virol. 871, 384–394. https://doi.org/10.1128/jvi.01633-12.

Murakami, S., Horimoto, T., Yamada, S., Kakugawa, S., Goto, H., Kawaoka, Y., 2008. Establishment of canine RNA polymerase I-driven reverse genetics for influenza A virus: its application for H5N1 vaccine production. J. Virol. 823, 1605–1609. https://doi.org/10.1128/jvi.01876-07.

Neumann, G., Fujii, K., Kino, Y., Kawaoka, Y., 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proc. Natl. Acad. Sci. 10246, 16825–16829. https://doi.org/10.1073/pnas.0505587102.

Nogales, A., Martínez-Sobrido, L., 2016. Reverse genetics approaches for the development of influenza vaccines. Int. J. Mol. Sci. 181. https://doi.org/10.3390/ijms18010020.

Paule, M.R., White, R.J., 2000. Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Res. 286, 1283–1298. https://doi.org/10.1093/nar/28.6.1283.

Shinagawa, T., Ishii, S., 2003. Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. Genes Dev. 1711, 1340–1345. https://doi.org/10.1101/gad.1073003.

Song, M.S., Baek, Y.H., Pascua, P.N.Q., Kwon, H.I., Park, S.J., Kim, E.H., Lim, G.J., Choi, Y.K., 2013. Establishment of Vero cell RNA polymerase I-driven reverse

genetics for Influenza A virus and its application for pandemic (H1N1) 2009 influenza virus vaccine production. J. Gen. Virol. 94, 1230–1235. https://doi.org/10.1099/vir.0.051284-0.

Suphaphiphat, P., Keiner, B., Trusheim, H., Crotta, S., Tuccino, A.B., Zhang, P., Dormitzer, P.R., Mason, P.W., Franti, M., 2010. Human RNA polymerase I-driven reverse genetics for influenza a virus in canine cells. J. Virol. 847, 3721–3725. https://doi.org/10.1128/jvi.01925-09.

Suzuki, Y., Uchida, Y., Tanikawa, T., Maeda, N., Takemae, N., Saito, T., 2014. Amino acid substitutions in PB1 of avian influenza viruses influence pathogenicity and transmissibility in chickens. J. Virol. 8819, 11130–11139. https://doi.org/10.1128/jvi.01564-14.

Wang, K., Huang, Q., Yang, Z., Qi, K., Liu, H., Chen, H., 2017. Alternative reverse genetics system for influenza viruses based on a synthesized swine 45S rRNA promoter. Virus Genes 534, 661–666. https://doi.org/10.1007/s11262-017-1457-8.

Wang, Z., Duke, G.M., 2007. Cloning of the canine RNA polymerase I promoter and establishment of reverse genetics for influenza A and B in MDCK cells. Virol. J. 41, 102. https://doi.org/10.1186/1743-422X-4-102.

Youk, S.S., Leyson, C.M., Seibert, B.A., Jadhao, S., Perez, D.R., Suarez, D.L., Pantin-Jackwood, M.J., 2021. Mutations in PB1, NP, HA, and NA contribute to increased virus fitness of H5N2 highly pathogenic avian influenza virus clade 2.3.4.4 in chickens. J. Virol. 955. https://doi.org/10.1128/jvi.01675-20.