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Establishment of Canine RNA Polymerase I-Driven Reverse Genetics for Influenza A Virus: Its Application for H5N1 Vaccine Production▽

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In the event of a new influenza pandemic, vaccines whose antigenicities match those of circulating strains must be rapidly produced. Here, we established an alternative reverse genetics system for influenza virus using the canine polymerase I (PolI) promoter sequence that works efficiently in the Madin-Darby canine kidney cell line, a cell line approved for human vaccine production. Using this system, we were able to generate H5N1 vaccine seed viruses more efficiently than can be achieved with the current system that uses the human PolI promoter in African green monkey Vero cells, thus improving pandemic vaccine production.

H5N1 influenza A viruses continue to cause fatal human infections. The regions where influenza A is epidemic have expanded from Asia to Europe and Africa, raising concerns over a possible pandemic (7). Currently, prepandemic H5N1 vaccines are being stockpiled in many countries. These inactivated vaccines were produced from viruses propagated in embryonated chicken eggs following inoculation of the vaccine seed virus generated by cloned cDNA-based reverse genetics (12-plasmid [3, 14] or 8-plasmid [6] systems) in an African green monkey Vero cell line (9, 15, 20–22) that is approved for human vaccine production (e.g., polio and rabies vaccines [12]). However, the generation of the H5N1 vaccine seed viruses in this cell line is not optimal due to its low plasmid transfection efficiency. In a pandemic situation, vaccines whose antigenicities match those of the circulating strain(s) need to be rapidly produced. Therefore, a more robust reverse genetics system is desirable for pandemic vaccine preparedness. Besides Vero cells, a limited number of other cells are approved for human vaccine production, for example, Madin-Darby canine kidney (MDCK) cells and chicken embryonic fibroblasts (CEF). A modified reverse genetics system that uses the chicken RNA polymerase I (PolI) promoter also supports the generation of influenza virus in CEF (11), with an efficiency of virus generation comparable to that of the human PolI system in Vero cells. MDCK cells also support the efficient growth of influenza virus and are used as a substrate for the production of seasonal influenza vaccines (1, 4, 5). In MDCK cells, however, reverse genetics with the human PolI promoter does not work well, due to the host species specificity of the PolI promoter. Recently, another reverse genetics system with T7 RNA PolII was shown to support influenza virus generation in MDCK cells (2), although the efficiency of virus generation was inconsistent. In the present study, we established an alternative reverse genetics system driven by canine PolI and generated recommended H5N1 vaccine seed viruses in MDCK cells with high efficiency.

Eukaryotic ribosomal DNA consists of well-conserved 18, 5.8, and 28S rRNA genes, clustering head-to-tail repeats (Fig. 1A). The 18S and 28S rRNA genes are separated by intergenic spacer regions (IGS), which contain the PolI promoter and terminator sequences. The PolI promoter region is located next to a 5′ external transcribed spacer (5′ ETS), approximately 3.5 kb upstream of the 18S rRNA gene in the human genome. Although the IGS sequences are not highly conserved among eukaryotes, the sequences around the transcription initiation sites are relatively conserved (Fig. 1B) (18). To identify the canine PolI promoter region, we searched the canine chromosome that contains the 18, 5.8, and 28S rRNA genes in the database of the dog genome (10) (NCBI Dog Genome Resources; http://www.ncbi.nlm.nih.gov/genome/guide/dog/) and found the predicted canine rRNA genes on a chromosome, designated *Canis familiaris* chromosome Un genomic contig, whole genome shotgun sequence (GenBank accession no. NW_878945; hereafter referred to as ChromUN). We then performed a homology search of the PolI transcription initiation site (nucleotide [nt] −8 to +11; +1 is referred to as the transcription initiation site) approximately 3.5 kb upstream of the 18S rRNA gene (5′ end of the predicted 5′ ETS) in ChromUN with the human PolI transcription initiation site by using GENETYX-Win software (Genetyx Corp., Tokyo). Through these analyses, we predicted that the PolI transcription initiation site sequence was positioned from nt 28164 to 28182 on ChromUN (Fig. 1B). We therefore amplified the upstream regions (consisting of 457 or 250 nt) from the predicted transcription initiation site, which most likely contained the canine PolI promoter sequence, by use of a standard PCR using an MDCK cell DNA template and specific primer pairs designed according to the database information (Fig. 1C). The

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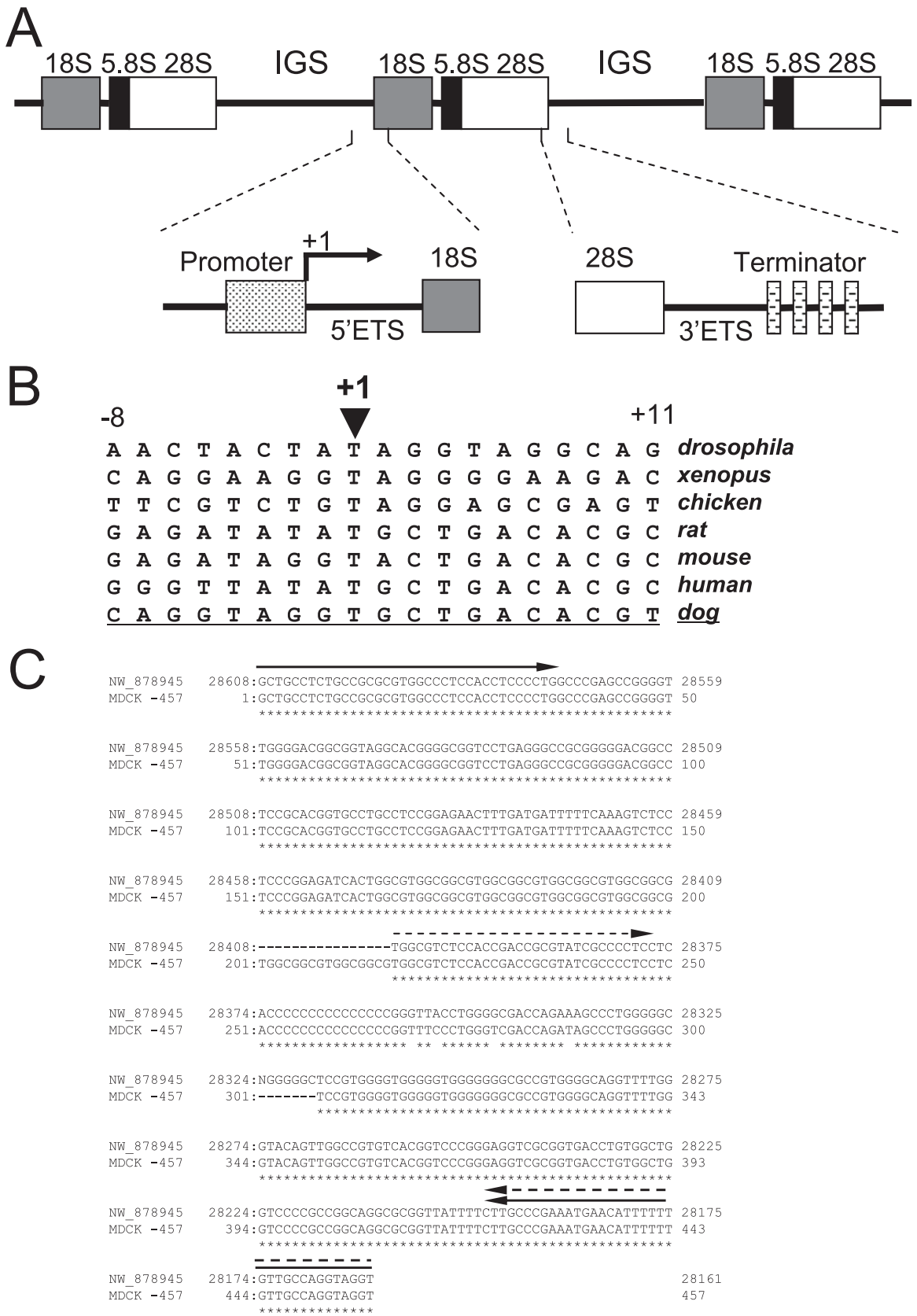

FIG. 1. Cloning of the canine PolI promoter. (A) Molecular map of the canine ribosomal DNA. Head-to-tail repeats of rRNA genes (18, 5.8, and 28S rRNA) are separated by IGS containing the PolI promoter and terminator regions. The PolI promoter region is located directly upstream of the 5′ ETS, and the terminator region is located downstream of the 3′ ETS. The transcription initiation site is indicated as +1. This figure is

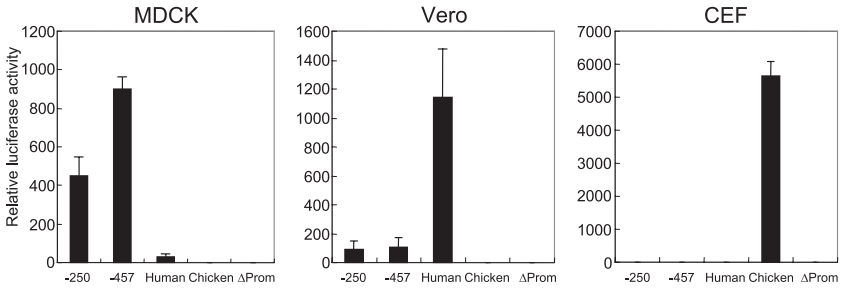

FIG. 2. RNA PolI promoter activities in MDCK, Vero cells, and CEF measured by the expression of a luciferase reporter gene. MDCK, Vero cells, and CEF were transfected with reporter plasmids encoding a firefly luciferase gene inserted between the 3′ and 5′ noncoding regions of the NP segment of A/Puerto Rico/8/34 (PR8) under the control of the canine PolI promoter [pPolIC250-NP(0)Fluc(0) (−250) or pPolIC457-NP(0)Fluc(0) (−457)], the human PolI promoter [pPolI-NP(0)Fluc(0) (human)], or the chicken PolI promoter [pPolGG-NP(0)Fluc(0) (chicken)], or without a PolI promoter [pΔPolIprom-NP(0)Fluc(0) (ΔProm)], together with the four plasmids that express PB2, PB1, PA, and NP from PR8. At 12 h after transfection, cells were subjected to the dual-luciferase assay (Promega). PolI promoter activities, represented as ratios of firefly luciferase to *Renilla* luciferase (as an internal control), are shown. The data presented are the means ± standard deviations of triplicate samples.

PCR products were then cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced. The cloned sequence possessed 94.2% homology with the corresponding region of the ChromUN sequence (Fig. 1C).

To determine whether our cloned regions functioned as a canine PolI promoter in MDCK cells, we examined the synthesis of viral RNA under the control of this region. To this end, we prepared a plasmid that contained viral RNA downstream of the predicted canine PolI promoter region; the human PolI promoter sequence (nt −1 to −425) of pHH21 (14) was replaced with the predicted canine PolI promoter sequences (nt −1 to −250 or nt −1 to −457, designated plasmids pPolIC250 and pPolIC457, respectively). For comparison, we also cloned the chicken PolI promoter region from chicken genomic DNA (11) and constructed pPolIGG, which synthesizes viral RNA. Finally, a series of reporter plasmids, in which the open reading frame of the firefly luciferase gene was inserted between the 3′ and 5′ noncoding regions of the nucleoprotein (NP) segment of A/Puerto Rico/8/34 (PR8; H1N1) [NP(0)Fluc(0)], was prepared with pHH21, pPolIC250, pPolIC 457, and pPolIGG and designated pPolI-NP(0)Fluc(0), pPolIC 250-NP(0)Fluc(0), pPolIC457-NP(0)Fluc(0), and pPolIGG-NP(0)Fluc(0), respectively. For a negative control, a plasmid lacking the PolI promoter region [pΔPolIprom-NP(0)Fluc(0)] was also prepared. For the luciferase reporter assay, each of the plasmids was cotransfected with PB2-, PB1-, PA-, and NP-expressing plasmids (16) into MDCK, Vero cells, or CEF. After 12 h of transfection, cells were harvested and lysed, and their luciferase activities measured and standardized against the activity of *Renilla* luciferase as an internal control by using a dual-luciferase assay kit (Promega) (Fig. 2). MDCK cell lysates transfected with pPolIC250-NP(0)Fluc(0) or pPolIC 457-NP(0)Fluc(0) exhibited >10-fold-higher luciferase activities than those transfected with pPolI-NP(0)Fluc(0) or pPolIGG-NP(0)Fluc(0) ($P < 0.02$; Student's t test). By contrast, the lysates of Vero and CEF cells transfected with pPolIC250-NP(0)Fluc(0) or pPolIC457-NP(0)Fluc(0) exhibited significantly lower luciferase activities than those transfected with pPolI-NP(0)Fluc(0) ($P < 0.02$) or pPolIGG-NP(0)Fluc(0) ($P < 0.002$), respectively. None of the cell lysates transfected with the control pΔPolIprom-NP(0)Fluc(0) showed any detectable luciferase activities. These data demonstrated that the region we cloned contained the functional canine PolI promoter.

To authenticate the canine PolI promoter, we attempted to generate the wild-type PR8 virus in MDCK cells by reverse genetics. The eight viral genes of the PR8 (UW) strain (8) were cloned into the pPolIC250 or pPolIC457 plasmids (pPolIC 250-PB2, -PB1, -PA, -HA, -NP, -NA, -M, and -NS or pPolIC 457-PB2, -PB1, -PA, -HA, -NP, -NA, -M, and -NS, respectively). We then transfected MDCK cells with a set of eight segments of pPolIC250 or pPolIC457 together with plasmids expressing PB2, PB1, PA, and NP, resulting in the generation of virus with titers between 1.3×10^2 and 2.5×10^3 PFU/ml without exogenous trypsin (Table 1). When MDCK cells were transfected with these sets of plasmids in the presence of trypsin, virus titers of more than 5×10^3 PFU/ml were detected. No significant differences in virus yields were observed between sets of pPolIC250 and pPolIC457 plasmids. Because IGS regions contain tandemly arranged enhancer elements for PolI transcription (19), the constructs with nt −1 to −457 may have contained additional enhancer elements compared to the construct with nt −1 to −250, as demonstrated by the data in Fig. 2. However, the region spanning nt −1 to −250 contained a sequence with sufficient PolI promoter activity for virus generation. By contrast, no viruses were detected upon the transfection of MDCK cells with a series of pPolI (human promoter) or pPolIGG (chicken promoter) plasmids (data not

adapted from reference 18 with permission of the publisher. (B) Alignment of the PolI transcription start regions (nt −8 to +11), as predicted by computer analysis, of the canine (underlined) and other species (adapted from reference 11). The transcription initiation site is indicated as +1. (C) Sequences of the canine PolI promoter regions, as predicted by computer analysis. The region (nt −457 to +1) was cloned from the genomic DNA of MDCK cells by using specific primers indicated by the arrows (solid lines for nt −457 to +1 and broken lines for nt −250 to +1). The cloned sequence was aligned with the canine genomic DNA sequence (GenBank accession no. NW_878945).

TABLE 1. Efficiency of PR8 generation in MDCK cells

Expt	Infectivity titer (PFU/ml)[a]			
	pPolIC250 (PR8)[b]		pPolIC457 (PR8)[c]	
	Trypsin−[d]	Trypsin+[e]	Trypsin−	Trypsin+
1	2.5×10^2	1.9×10^4	1.3×10^2	3.0×10^4
2	2.5×10^3	7.8×10^4	1.4×10^2	5.0×10^3
3	4.6×10^2	1.3×10^4	1.5×10^3	1.4×10^4

[a] At 48 h after transfection, virus titers in the supernatant were determined by plaque assays of MDCK cells. The results are from three independent experiments (experiments 1 to 3).
[b] pPolIC250 (PR8) indicates transfection of cells with pPolIC250-PB2, -PB1, -PA, -HA, -NP, -NA, -M, and -NS and pCAGGS-PB2, -PB1, -PA, and -NP.
[c] pPolIC457 (PR8) indicates transfection of cells with pPolIC457-PB2, -PB1, -PA, -HA, -NP, -NA, -M, and -NS and pCAGGS-PB2, -PB1, -PA, and -NP.
[d] Trypsin−, cells were cultured with trypsin-free Opti-Mem.
[e] Trypsin+, cells were cultured with Opti-Mem containing trypsin.

TABLE 2. Efficiency of pPolIC250 (PR8/VN1194) 6:2 reassortant virus generation in MDCK cells

Expt	Infectivity titer (PFU/ml)[a]	
	Trypsin−[b]	Trypsin+[c]
1	1.3×10^2	3.2×10^6
2	1.1×10^2	1.2×10^6
3	1.3×10^2	3.6×10^6

[a] At 48 h after transfection, virus titers in the supernatant were determined by plaque assays of MDCK cells. The results are from three independent experiments (experiments 1 to 3). pPolIC250 (PR8/VN1194) indicates transfection of cells with pPolIC250-PB2, -PB1, -PA, -NP, -M, -NS, -1194HA, and −1194NA and pCAGGS-PB2, -PB1, -PA, and -NP.
[b] Trypsin−, cells were cultured with trypsin-free Opti-Mem.
[c] Trypsin+, cells were cultured with Opti-Mem containing trypsin.

shown), in agreement with the results of the luciferase assay (Fig. 2). The generation of virus in MDCK cells with the plasmids possessing the canine PolI promoter was robust, with consistent virus generation of more than 10^2 PFU/ml without exogenous trypsin in all experiments ($n = 6$). By contrast, we were able to generate only less than 10 PFU/ml of the virus with plasmids possessing the chicken PolI promoter in CEF or the human PolI promoter in Vero cells, and occasionally we even failed to generate the virus (data not shown), possibly due to the low transfection efficiencies of CEF and Vero cells. Massin et al. (11) reported the generation of PR8 virus by reverse genetics with the chicken PolI promoter in the presence of trypsin, resulting in the virus production of 10^2 to 10^3 PFU/ml on day 3 after transfection in CEF. Less-efficient virus generation by reverse genetics with the human PolI promoter in Vero cells has also been reported; Fodor et al. (3) generated A/WSN/33 (WSN; H1N1) virus with titers of 10 to 20 PFU/ml on day 4 after transfection by using the 12-plasmid system. Because MDCK cells are a suitable substrate for influenza virus replication (4), the canine PolI-driven reverse genetics system in MDCK cells likely supports a higher efficiency of virus generation than do the human PolI and chicken PolI-driven systems in Vero cells and CEF.

We next sought to generate an H5N1 vaccine seed virus, as recommended by the World Health Organization (PR8/H5N1 6:2 reassortant; virus possessing HA and NA genes from an H5N1 virus and the remaining genes from the PR8 [UW] strain [8]), using our canine PolI-driven system. For this purpose, we also cloned HA (with a modified cleavage site; RER RRKKR to RETR) and NA genes, both derived from A/Vietnam/1194/2004 (H5N1; VN1194) (9), into pPolIC250 plasmids (pPolIC250-1194HA and -1194NA, respectively). We selected pPolIC250, not pPolIC457, because there were no significant differences in virus yield between these two pPolICplasmids in our preliminary experiments. These two plasmids were then cotransfected with six PR8 RNA-synthesizing plasmids and four additional plasmids expressing PB2, PB1, PA, and NP into MDCK cells, resulting in the generation of virus with titers of approximately 10^2 PFU/ml in the absence of trypsin and of more than 10^6 PFU/ml in the presence of trypsin (Table 2). By contrast, in Vero cells and in CEF, this PR8/H5N1 reassortant was also generated by transfection with the human PolI or the chicken PolI system, respectively, but to a substantially lesser degree; the virus generation was not consistent, and even when virus was generated, the titers were less than 10 PFU/ml in either system. Taken together, the data lead us to conclude that PR8/H5N1 vaccine seed viruses can be generated more efficiently by canine PolI-driven reverse genetics in MDCK cells than by human or chicken PolI-driven systems in Vero cells or CEF.

The recently described T7 RNA PolII-driven reverse genetics system (2) allows the generation of influenza viruses in MDCK cells. However, it is unlikely that this system will generate vaccine seed viruses efficiently in Vero or MDCK cells, since even in 293T cells with a high transfection efficiency, PR8 virus was poorly generated (<10 50% tissue culture infective doses/ml). Currently, PolI-based reverse genetics is used to produce H5N1 vaccine seed viruses (9, 15, 20–22). Since our results indicate that the canine PolI-based system is more efficient than the human or chicken PolI-based systems, the canine system may be preferable. It would be interesting to directly compare the efficiencies of influenza virus generation between the canine PolI system and the T7 RNA PolII system.

Previously, we established a reverse genetics system with a reduced number of plasmids to overcome the low transfection efficiency of certain cells that are approved for vaccine production (e.g., Vero cells). This system generated WSN virus in Vero cells with high viral yields (10^6 to 10^7 50% tissue culture infective doses/ml) (13). Therefore, a similar strategy could be applied to the canine PolI-driven system, possibly further improving its efficiency in MDCK cells. In addition, an adenoviral vector-mediated reverse genetics system (17) with the canine PolI promoter may also be suitable for influenza vaccine production.

In conclusion, the application of the canine PolI-driven reverse genetics system in MDCK cells would improve the production of seasonal, prepandemic, and pandemic influenza vaccines.

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