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Human RNA Polymerase I-Driven Reverse Genetics for Influenza A Virus in Canine Cells[▽]

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We have established a human RNA polymerase I (pol I)-driven influenza virus reverse genetics (RG) system in the Madin-Darby canine kidney 33016-PF cell line, which is approved for influenza vaccine manufacture. RNA pol I polymerases are generally active only in cells of species closely related to the species of origin of the polymerases. Nevertheless, we show that a nonendogenous RNA pol I promoter drives efficient rescue of influenza A viruses in a canine cell line. Application of this system allows efficient generation of virus strains and presents an alternative approach for influenza vaccine production.

Reverse genetics (RG) technology makes it possible to generate influenza virus entirely from cloned plasmid DNA by cotransfection of appropriate cells, with plasmids carrying the eight influenza genome segments. One commonly used system employs eight bidirectional transcription constructs, each of which uses an RNA polymerase I (pol I) promoter to synthesize negative-sense viral RNA and an RNA pol II promoter to transcribe positive-sense mRNA from one viral cDNA template. This eight-plasmid RG system efficiently generates influenza A virus in human embryonic kidney 293T cells (7) and, to a limited extent, in African green monkey kidney (Vero) cells (13). 293T cells, however, are not currently approved for human vaccine production. Vero cells can be used to manufacture human vaccines, but efficient rescue in these cells is traditionally hampered by their low plasmid transfection efficiency and their low productivity for many influenza strains (5, 13). However, recent advances in RG systems utilizing reduced numbers of plasmids have led to improved virus rescue efficiencies in these cells (11). Nevertheless, the ability to rescue influenza virus in another approved cell substrate that supports efficient influenza virus production is useful for applying RG technology to human vaccine production.

Madin-Darby canine kidney (MDCK) cells support the efficient growth of influenza viruses and are used to produce seasonal and pandemic influenza vaccines. It has been reported that although an RG system driven by the human RNA pol I promoter supports influenza virus rescue in primate cells, it does not support rescue in canine cells due to the host species specificity of RNA pol I (6, 9, 17). Therefore, an alternative 8-plasmid RG system that uses the canine RNA pol I promoter sequence was developed for influenza virus rescue in MDCK cells (9, 17).

The MDCK subline 33016-PF (15) grows efficiently in suspension in serum-free medium and is suitable for influenza vaccine manufacture (16). We have demonstrated that MDCK 33016-PF can be used to generate viruses from an 8-plasmid RG system. These findings could lead to more efficient generation of seed viruses for human vaccine manufacture.

To determine if the canine RNA pol I promoter drives efficient expression in MDCK 33016-PF cells, we cloned a 1,810-bp DNA fragment containing the promoter from MDCK cells (17) and inserted it into a previously described virus-inducible reporter plasmid (8). This reporter plasmid contains the RNA pol I promoter/terminator cassette, with the open reading frame of the firefly luciferase or green fluorescent protein (GFP) gene inserted between the 3′ and 5′ noncoding regions of the influenza A/Udorn (H3N2) nucleoprotein (NP) segment. This plasmid allows reporter gene activity to be detected only in the presence of the influenza A virus polymerase complex, which can be provided either by virus infection or by cotransfection with four additional plasmids that express the NP protein and polymerase subunits PA, PB1, and PB2. This virus-inducible reporter plasmid system allows us to measure reporter gene activity as a surrogate for viral RNA synthesis under the control of the canine pol I promoter. For comparison, we also tested a similar virus-inducible reporter plasmid under the control of the human pol I promoter, which has been reported to be transcriptionally inactive in MDCK cells (9).

We conducted luciferase reporter assays with two canine cell lines (MDCK 33016-PF and MDCK from ATCC) and one human cell line (293T). One microgram of the virus-inducible luciferase plasmid under the control of the canine or human RNA pol I promoter (cPolI-LUC or hPolI-LUC, respectively) was transfected into each cell line using Lipofectamine LTX Plus (Invitrogen), according to the manufacturer's protocol. To drive luciferase expression, cells were either cotransfected with four additional plasmids (1 μg each) that express the viral polymerase complex (A/Udorn [H3N2] PA, PB1, PB2, and NP) or infected with A/Udorn (H3N2) (multiplicity of infection [MOI] = 0.05) at 4 to 6 h posttransfection. For a negative control, we also transfected cells with the luciferase reporter plasmid in the absence of viral polymerase. At 16 to 20 h posttransfection, cells were lysed, and luciferase activities were measured using the Britelite luciferase assay system

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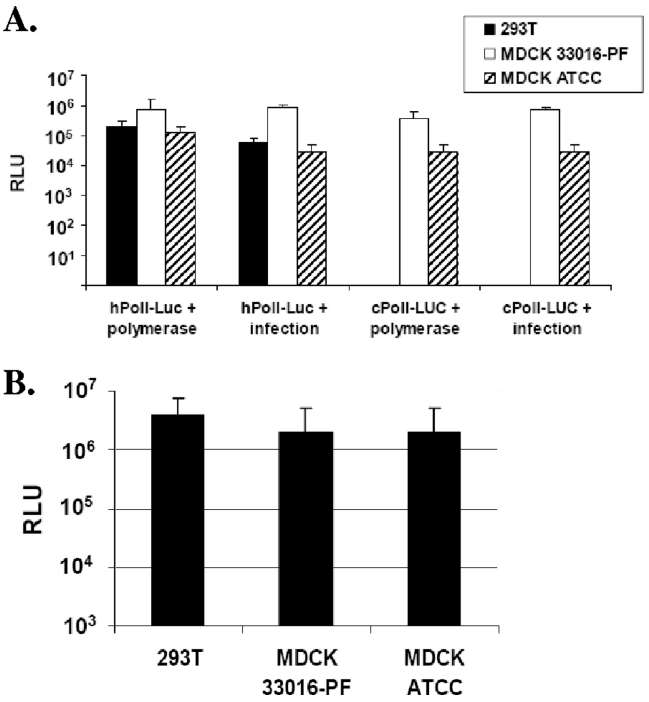

FIG. 1. (A) Comparison of human and canine RNA pol I promoter activity in human and canine cells using a luciferase reporter assay. The influenza polymerase complex was provided by transfected plasmids (+ polymerase) or by influenza virus infection (+ infection). (B) Comparison of transfection efficiency in human and canine cells using a CMV promoter-driven luciferase reporter plasmid. The mean results and standard deviations of triplicate samples from three independent experiments are presented. RLU, relative light units.

(PerkinElmer). Luciferase levels were determined after subtracting background values obtained from transfections with hPolI-LUC- or cPolI-LUC-only negative controls (Fig. 1A).

Both canine cell lines had high levels of luciferase activity when transfected with the canine pol I promoter-driven reporter plasmid in the presence of viral polymerase. The human cell line did not. Surprisingly, both canine cell lines and the human cell line had high levels of luciferase activity when transfected with the human pol I promoter-driven reporter plasmid in the presence of viral polymerase. Results from the luciferase reporter assay were confirmed by comparable results from equivalent experiments using GFP as the reporter gene (8) (data not shown).

Based on published reports of host specificity (1, 6), the human pol I promoter was not expected to function efficiently in canine cells. Nevertheless, these data demonstrate that both the human and canine pol I promoters are active in MDCK cells. Surprisingly, luciferase activity under the control of the human pol I promoter in the MDCK ATCC cell line appeared as strong as that in the 293T cells, whether the reporter gene expression was driven by coinfection or cotransfection of the viral polymerase complex. Human pol I promoter-driven luciferase activity also appeared approximately 10-fold stronger in the MDCK 33016-PF cell line than in 293T cells under the same two experimental conditions. In addition, luciferase activity in MDCK 33016-PF cell lysates was consistently more than 10-fold higher than that in MDCK ATCC cell lysates, regardless of which RNA pol I promoter was used. We note that the luciferase assay is a surrogate for measuring pol I promoter activity, but the level of this activity may be influenced by transfection efficiency, viral polymerase complex expression, and/or luciferase translation and stability. We determined that the discrepancy in apparent pol I promoter activity in this assay was not due to differences in transfection efficiency *per se*, as shown by comparable levels of luciferase activity when each cell type was transfected with a plasmid carrying the luciferase gene under the control of a cytomegalovirus (CMV) promoter (Fig. 1B). To address whether the discrepancy could be due to potential differences in viral polymerase expression in the different cell lines, we studied the expression of three out of the four proteins that make up the viral polymerase complex (NP, PB1, and PB2) by Western blot analysis of cell lysates, following virus infection or transfection of plasmids expressing the polymerase genes. Higher expression of all three proteins was detected in MDCK 33016-PF cell lysates compared to expression of lysates from 293T and MDCK ATCC cells (data not shown). These data suggest that viral proteins that form the polymerase complex are expressed with higher efficiency in MDCK 33016-PF cells, which may contribute to the higher pol I promoter activity observed in these cells in the luciferase assay.

To confirm that the MDCK cells were of canine origin and free of contamination with human cells, we used previously described multiplex PCR-based assays to verify the species identity of the cultures (4, 14). We extracted DNA from the cell cultures and amplified two mitochondrial genes for cytochrome *c* oxidase subunit I (cox I) and cytochrome *b*, using mixtures of human-specific and canine-specific primer pairs. These mixtures generate size-specific amplicons for each species. The cytochrome *b*-specific primer set amplified the signature canine bands from both MDCK 33016-PF and MDCK ATCC cells and the signature human band from 293T cells (Fig. 2A, lanes 1 to 3). Likewise, the cox I-specific primer set amplified the signature canine bands from both MDCK 33016-PF and MDCK ATCC cells and the signature human band from 293T cells (Fig. 2A, lanes 5 to 7). All amplicons produced were subsequently sequenced to verify that they correspond to the correct human or canine target genes (data not shown). To test the sensitivity of the multiplex PCR, we added human 293T DNA to MDCK ATCC DNA to mimic 1%, 5%, 10%, and 20% contamination of canine cells by human cells (Fig. 2B, lanes 9 to 13). The presence of human cox I DNA was clearly detected when 1% of human DNA was added to the canine DNA (Fig. 2, lane 9). The complete absence of the signature human bands in MDCK 33016-PF and MDCK ATCC cells indicate that the MDCK cell lines are more than 99% pure. Therefore, the activity of the human pol I promoter in MDCK cells is unlikely due to contamination.

To rule out the formal possibility that human cell contamination at a level of less than 1% was responsible for the observed human pol I promoter activity in the canine cells, we tested the species identity of a subset of MDCK 33016-PF cells that had been sorted on the basis of their high levels of human pol I promoter activity. MDCK 33016-PF cells were cotransfected with a plasmid carrying a GFP gene under the control of the human pol I promoter (together with viral polymerase-expressing plasmids). GFP-positive cells were sorted by a flu-

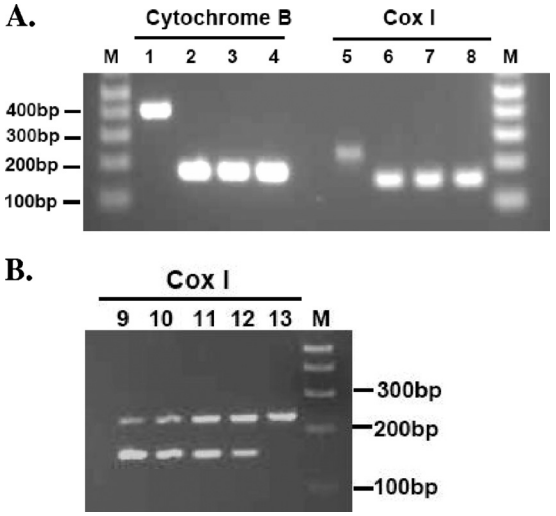

FIG. 2. Multiplex PCR amplification with species-specific primer pairs. (A) Detection of cytochrome *b* and cytochrome *c* oxidase I (cox I) genes from 293T cells (lanes 1 and 5), MDCK 33016-PF cells (lanes 2 and 6), MDCK ATCC cells (lanes 3 and 7), and a sorted subpopulation of MDCK 33016-PF cells that display high human pol I promoter activity (lanes 4 and 8). Expected sizes are 391 bp (human) and 172 bp (canine) for cytochrome *b* and 229 bp (human) and 153 bp (canine) for cox I. (B) Sensitivity to human cell contamination of canine cells. Human 293T DNA was added to canine MDCK ATCC DNA at ratios of 1:99, 5:95, 10:90, 20:80, and 100:0 (lanes 9 to 13, respectively) before performing multiplex PCR with the cox I primer pairs. "M" indicates the molecular weight marker (1-kb Plus DNA Ladder; Invitrogen). PCR products were separated on a 2% agarose gel. In each panel, a representative gel is presented from at least two independent experiments.

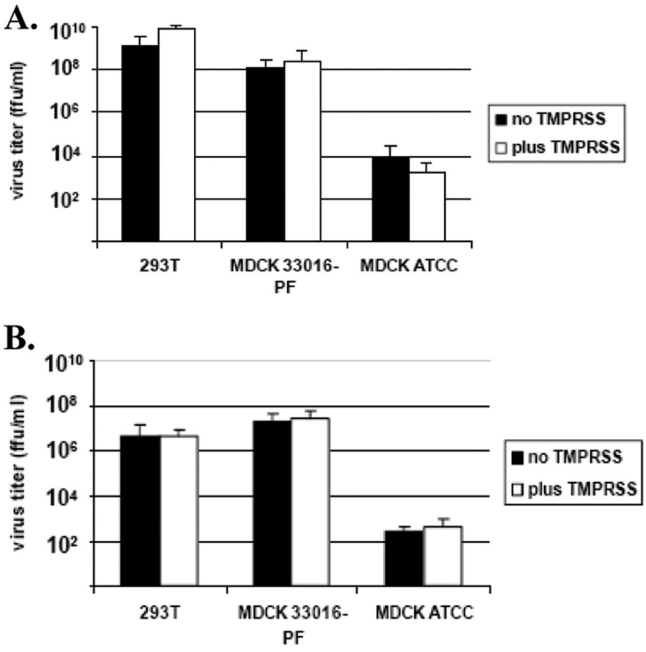

FIG. 3. Rescue of influenza virus by human pol I promoter-based reverse genetics in human and canine cells. (A) Rescue of A/Puerto Rico/8/34 virus in the presence or absence of TMPRSS2 with the postrescue addition of feeder cells. (B) Rescue of A/Puerto Rico/8/34 virus in the presence or absence of TMPRSS2 helper plasmid without any addition of feeder cells. Titers of the indicated viruses from each cell type are expressed as focus-forming units (FFU) per milliliter. The mean results and standard deviations from at least three independent experiments are shown.

orescence-activated cell sorter (FACS), expanded, and analyzed by multiplex PCR. More than 90% of the sorted MDCK 33016-PF cells were positive for GFP fluorescence. Multiplex PCR amplified the canine signature bands for the cytochrome *b* and cox I genes (with the same intensity as bands from the unsorted MDCK 33016-PF cells) but not the human signature bands (Fig. 2A, lanes 4 and 8). These data demonstrate that the cells in the MDCK 33016-PF population that are able to efficiently transcribe genes driven by the human pol I promoter are of canine origin.

This finding prompted us to compare the efficiencies with which the human pol I-driven eight-plasmid RG system (7) could rescue influenza viruses in the three cell types. We first attempted to generate the vaccine-approved strain A/Puerto Rico/8/34 (PR8; H1N1) using an optimized virus rescue protocol. We transfected cells with a set of eight bidirectional transcription plasmids carrying the influenza genome segments (1 μg each), together with the TMPRSS2 helper plasmid, which encodes a serine protease required to cleave hemagglutinin (HA) inside the cells (3). At 24 h posttransfection, media was removed. To amplify the rescued virus more efficiently, fresh feeder MDCK 33016-PF cells in serum-free media plus 0.5 μg/ml TrypZean (Sigma) were added. Cleared medium supernatants were collected at 72 h posttransfection, and virus titers were determined on fresh MDCK 33016-PF cells using a slight modification of previously described focus-forming assays (10, 12).

Using this optimized virus rescue protocol, we were able to rescue the A/Puerto Rico/8/34 virus in each cell type. The human pol I-driven RG system generated average titers of approximately 10^8 focus-forming units (FFU)/ml from MDCK 33016-PF cells (Fig. 3A). Generation of virus in 293T cells was more efficient, with average titers of approximately 10^{10} FFU/ml. Generation of virus in MDCK ATCC cells was much less efficient, with average titers of approximately 10^3 FFU/ml. Using similar methods, we also demonstrated efficient rescue from MDCK 33016-PF cells of two other recombinant viruses, A/WSN/33 and a 6+2 reassortant virus composed of the six A/Puerto Rico/8/34 internal gene segments (PB1, PB2, PA, NP, NS, and M) and the A/WSN/33 HA and NA genome segments (data not shown).

Previous experimental rescue attempts using the standard human pol I-driven eight-plasmid RG system were unable to recover influenza virus from MDCK cells (9, 17). To explore the possibility that the difference between our results and previously published reports is due to the improved rescue efficiency imparted from our inclusion of the TMPRSS2 helper plasmid and/or the subsequent amplification of virus by our addition of feeder cells at 24 h posttransfection, we compared the rescue efficiencies of the A/Puerto Rico/8/34 virus in the presence or absence of TMPRSS2 with and without addition of MDCK 33016-PF feeder cells. We show that A/Puerto Rico/8/34 was efficiently rescued in MDCK 33016-PF cells in all experimental conditions and that virus rescue efficiency in the MDCK 33016-PF cell line was consistently higher than in

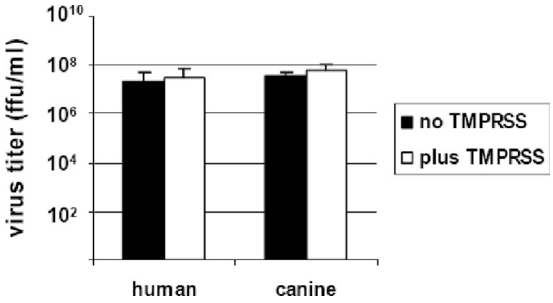

FIG. 4. Rescue of A/Puerto Rico/8/34 influenza virus by human or canine pol I-driven reverse genetics in MDCK 33016-PF cells. Titers are expressed as focus-forming units (FFU) per milliliter. The mean results and standard deviations from three independent experiments are shown.

MDCK ATCC cells (Fig. 3A and B). Addition of the TMPRSS2 helper plasmid and of feeder cells generally improved rescue efficiency in all cell types, but the beneficial effect of feeder cell addition was most significant for virus rescue in 293T cells, which otherwise do not support efficient replication of virus on their own.

We observed a discrepancy between the amount of virus recovery in rescue experiments (Fig. 3) and the level of pol I promoter activity, as judged by the luciferase assay (Fig. 1), especially for MDCK ATCC cells, which displayed levels of human pol I activity comparable to those of MDKC 33016-PF and 293T cells but exhibit much lower efficiency of virus rescue. However, we note that the luciferase assay is an artificial system, so results from this assay do not necessarily correlate with efficiency of virus rescue. One potential reason for this discrepancy is that virus recovery requires the functioning of all eight viral genome segments and not just that of the viral polymerase complex proteins that are required to provide high levels of luciferase activity. Furthermore, virus rescue in the presence of TrypZean (and the plasmid encoding TMPRSS2) allows for multiple replication cycles of virus in the cells. Thus, the higher level of virus growth/replication in MDCK 33016-PF cells relative to MDCK ATCC cells would contribute to the efficiencies in virus recovery in these cells.

To determine if the human pol I-driven RG system can be applicable to future influenza vaccine production strategies, we compared the abilities to recover the A/Puerto Rico/8/34 virus from the MDCK 33016-PF cell line using either the human pol I- or previously established canine pol I-based system in the presence or absence of the TMPRSS2 helper plasmid and without the addition of any feeder cells. Comparable efficiencies in virus rescue between the two systems (Fig. 4) indicate that the human pol I-driven RG system may be used as an alternative approach to the previously established canine pol I-based system for future vaccine production.

The inefficient virus rescue in MDCK ATCC cells is consistent with published reports that the human pol I-driven RG system supports only inefficient virus generation in nonprimate cells (9, 17). However, we have demonstrated that the human pol I-based rescue system can indeed generate adequate virus titers in the MDCK 33016-PF cell line. The mechanism for this difference is currently being investigated. The known mechanism of species specificity for the RNA pol I promoter is due to host accessory proteins and not pol I itself (1, 2, 6). Therefore, it is plausible that MDCK 33016-PF cells may be more permissive to functional interactions between canine transcription factors and species-specific elements on a nonendogenous pol I promoter sequence. Interestingly, MDCK 33016-PF suspension cells and its adherent cell line precursor support higher virus rescue than MDCK ATCC cells (data not shown), which indicates that the difference between MDCK ATCC and MDCK 33016-PF arose at some point during the selection process that occurred prior to adaptation to suspension culture *per se*. The enhanced ability to generate influenza virus in these cells may be a property acquired by or specific to this particular MDCK sublineage. Gene expression profiling of MDCK 33016-PF and MDCK ATCC cells may help to identify potential genetic differences between these two cell lines that affect influenza virus recovery.

In conclusion, although the reported host species specificity of the RNA pol I promoter would suggest that RG systems in MDCK cells require a canine pol I promoter, our reporter gene data indicate that both the human and canine pol I promoters are active in MDCK cells. Furthermore, the human pol I promoter supports efficient RG rescue of influenza A viruses in MDCK 33016-PF cells. The human pol I-driven RG system in MDCK 33016-PF cells is an efficient system that can be used as an alternative approach to generate cell culture-adapted virus strains for future influenza vaccines.

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