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BRIEF REPORT

The mouse Pol I terminator is more efficient than the hepatitis delta virus ribozyme in generating influenza-virus-like RNAs with precise 3′ ends in a plasmid-only-based virus rescue system

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Abstract Reverse genetics systems for generating recombinant influenza viruses are based on two different mechanisms for obtaining the 3′ end of the viral RNA: one uses the self-cleaving hepatitis delta virus ribozyme (HDVR), and the other uses the murine RNA polymerase I (Pol I) terminator. In this study, we employed EGFP and Renilla luciferase reporter constructs to compare the efficiency of both methods. Our results indicate that the murine Pol I terminator was more efficient than the HDVR, which will be helpful in choosing an influenza virus rescue system, as well as in establishing other RNA virus rescue systems.

Influenza A viruses contain eight segments of single-stranded RNA of negative polarity. They belong to the genus *Influenzavirus A*, family *Orthomyxoviridae*. As the causative agents of influenza, they pose a significant public-health threat. The generation of influenza A viruses completely from plasmid DNA, so-called reverse genetics, has significantly simplified the process of obtaining various recombinant influenza viruses [2, 6, 13]. Reverse genetics also contributes to our understanding of the basic molecular mechanisms of influenza virus replication. For example, recovery of the remote 1918 Spanish influenza A virus from synthesized DNA made it possible to study its virulence [17]. The packaging of eight segments into mature virions has been demonstrated to occur in a selective manner rather than in a random manner, with specific packaging sequences for each genomic segment being precisely located [3, 4, 9, 11, 12, 18]. The generation of vaccine seed virus is greatly simplified by producing reassortants containing the hemagglutinin (HA) and neuraminidase (NA) genes of currently circulating influenza virus strains in a background of the genome of an influenza virus that is well adapted to growth in vitro [19].

Initially, rescue systems contained at least 12 plasmids, eight of which were for the transcription of viral RNA (vRNA) and four of which were for the expression of PB2, PB1, PA and NP proteins. These four proteins constituted the RNA polymerase complex [2, 13]. The human RNA polymerase I promoter (huPol I) was used to generate the vRNA 5′ ends. To generate the 3′ ends of the RNA, one system adopted the human hepatitis delta virus ribozyme (HDVR) [2], while another used the murine Pol I terminator (mTer) [13]. Subsequently, mTer was employed in most of the advanced versions of influenza virus rescue systems requiring fewer plasmids [6, 14, 15]. Wit et al. [20] obtained a higher titer of recombinant virus when using mTer as a terminator as opposed to using HDVR. The difference in rescue efficiency might be due to the different mechanisms of generating the vRNA 3′ end. As the influenza virus rescue system can potentially be applied to

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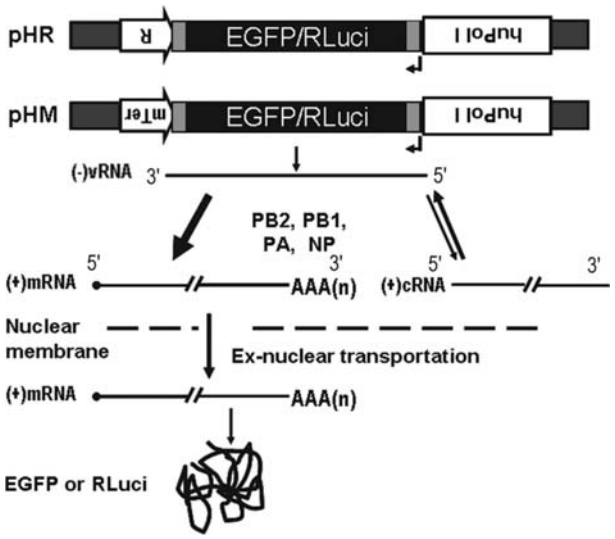


Fig. 1 Schematic diagram of reporter gene expression. Two forms of reporter plasmid, pHR and pHM, were constructed. EGFP or Renilla luciferase (*RLuci*), flanked by the influenza A virus NP non-coding region, was inserted between the human RNA polymerase I (*huPol I*) and the hepatitis delta virus ribozyme (HDVR, *R*) in pHR or the murine Pol I transcription terminator (*mTer*) in pHM. Minus vRNA [*(−)vRNA*] of EGFP or RLuci is transcribed by huPol I in the nucleus, and the 3′ ends are generated by using HDVR or mTer. In the presence of PB2, PB1, PA and NP, vRNAs are transcribed into positive messenger RNA (*mRNA*) with a cap at the 5′ end and a polyA tail at the 3′ end. In the cytoplasm, mRNAs are translated into EGFP or RLuci proteins. The vRNAs also serve as templates for the synthesis of positive complementary RNA (*cRNA*), and cRNAs serve as templates for vRNAs and vice versa

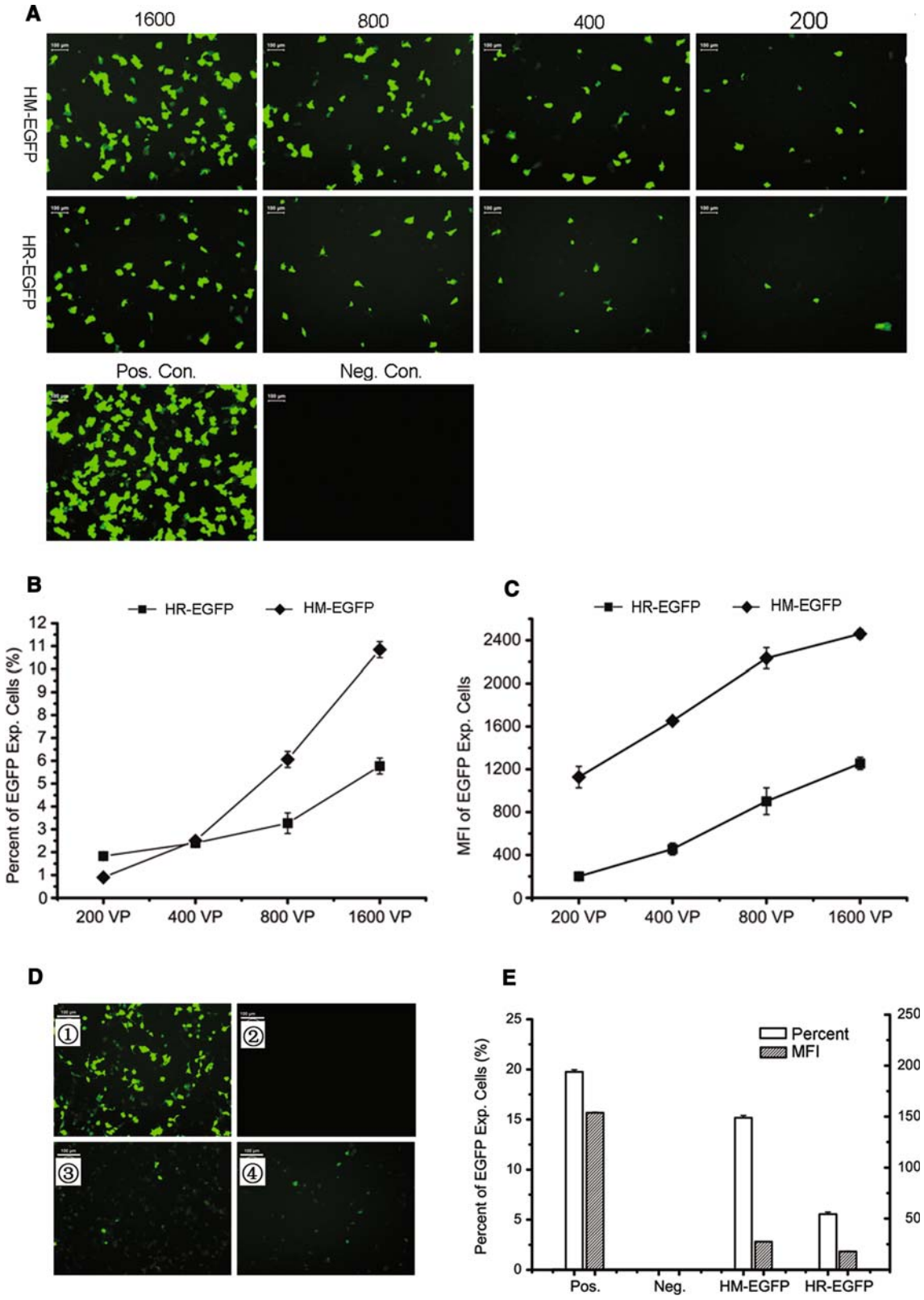
generate other recombinant RNA viruses, such as hepatitis C virus, enteroviruses, etc., a direct efficiency comparison between mTer and HDVR seemed to be warranted.

We established an influenza-virus-based replicon system for determining the ability of mTer and HDVR to generate functional vRNA 3′ ends. The pHM plasmid was made by replacing HDVR in the pPol I plasmid [2] (here termed pHR) with synthesized mTer [13] containing a *Sap*I site for inserting an mini-genome. Enhanced green fluorescent protein (EGFP) and Renilla luciferase (RLuci) reporter genes were amplified by using PCR methods in which the 5′ and 3′ non-coding regions of the influenza virus nucleoprotein (NP) were added as described in the literature [10]. EGFP and RLuci were then inserted, by using *Sap*I digestion, to obtain the pHR-EGFP, pHM-EGFP, pHR-RLuci and pHM-RLuci plasmids (Fig. 1). As indicated in Fig. 1, only the influenza-virus-like RNAs (here termed 'vRNAs') of EGFP (vEGFP) and RLuci (vRLuci) that were transcribed from the Pol I promoter were able to serve as templates for transcription of the mature mRNA of EGFP and Rluci with a 5′ cap and a 3′ polyA tail in the presence of PB2, PB1, PA and NP proteins provided *in trans*. Then, mRNAs were transported out of the nucleus for EGFP and

Fig. 2 Comparison of the efficiencies of HDVR and mTer. **a–c** Vero cells were transfected with pHM-EGFP or pHR-EGFP (1 μg for each well in a 24-well plate). Then, cells were infected with Ad5-PB2 + NS, Ad5-PB1 + M, and Ad5-PA + NP at concentrations of 200–1,600 VP/AdV/cell for 1.5 h. Afterwards, the adenovirus inoculum was removed and replaced with fresh media. Thirty-six hours postinfection, EGFP-expressing cells were observed using inverted fluorescence microscopy. **a** Positive control (*Pos. Con.*): pCDNA-4To-EGFP, in which the EGFP gene was inserted at *BamH*I and *EcoR*I sites. Negative control (*Neg. Con.*): pHM-EGFP or pHR-EGFP plasmid only without infection with adenoviruses. These were then analyzed using FACS analysis. The percentage of EGFP-expressing cells was calculated (**b**), and the mean fluorescence intensity (*MFI*) of EGFP-expressing cells is shown (**c**). In **d** and **e**, 293T cells were co-transfected with 0.8 μg pHM-EGFP or pHR-EGFP (③ and ④ in **d**, respectively) and pCAGGs-WSNPB2 (0.8 μg), pCAGGs-WSNPB1 (0.8 μg), pCAGGs-WSNPA (0.8 μg) and pCAGGs-WSNNP (0.8 μg). Thirty-six hours after transfection, cells were observed using inverted fluorescence microscopy (**d**) and were then analyzed using FACS analysis. The percentage of EGFP-expressing cells was calculated, and the mean fluorescence intensity (*MFI*) of EGFP-expressing cells is shown (**e**). As positive and negative controls, respectively, 293T cells were co-transfected with 0.8 μg of pCDNA-4To-EGFP or 0.8 μg of pCDNA-4To and the PB2, PB1, PA and NP plasmids (① and ② in **d**). A representative of at least three independent experiments is shown. *Scale bar* 100 μm

RLuci expression. Meanwhile, vRNAs also served as templates for the synthesis of positive-strand complementary RNAs (cRNA), which served as templates for the synthesis of vRNA, and vice versa.

Termination efficiencies of HDVR and mTer were determined by using the EGFP reporter gene. Briefly, in a 24-well plate, 85–90% confluent Vero cells, maintained in DMEM (Gibco) with 10% fetal bovine serum (FBS, Hyclone), were transfected with 1 μg of the pHR-EGFP or pHM-EGFP plasmid, which was quantified by using OD260 absorbance, using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After 12 h, the transfected cells were infected with three-first-generation adenovirus recombinants expressing PB2, PB1, PA and NP (Ad5-PB2 + NS, Ad5-PB1 + M and Ad5-PA + NP adenoviruses). The two influenza segmental cDNAs in each adenovirus recombinant were controlled by two independent CMV promoters, and the expression of genes was at the same level. Thirty-six hours after adenovirus infection, more cells expressing EGFP were observed by inverted fluorescence miscopy (Leica) in the pHM-EGFP group than were observed in the pHR-EGFP group (Fig. 2a). FACS analysis confirmed this observation (Fig. 2b, c). Briefly, the samples were collected by using Trypsin EDTA (Gibco) digestion for 5 min. Then, 0.5 ml of complete DMEM medium containing 5% FBS was added to produce a single-cell suspension. After centrifugation at 300×*g* for 5 min at room temperature, the cell pellets were resuspended in 0.5 ml 1× phosphate-buffered saline (PBS). The percentage and mean fluorescence intensity



(MFI) were calculated by using FACS analysis (FACS Calibur, BD), as described in the literature [5]. All statistical analyses were performed by using a two-tailed *t* test. The percentage of EGFP-expressing cells was significantly higher in the pHM-EGFP group than it was in the pHR-EGFP group at concentrations of 800 or 1,600 VP/AdV/

cell (adenovirus viral particles per adenovirus per cell, as three adenoviruses were used) (P-value <0.001) (Fig. 2b). The MFI of EGFP-expressing cells was significantly higher in the pHM-EGFP group than it was in the pHR-EGFP group at all multiplicities of infection (Fig. 2c). This effect was reproduced by co-transfection of nearly confluent 293T cells in a six-well plate with 0.8 μg pHM-EGFP or pHR-EGFP and the four PB2-, PB1-, PA- and NP-expressing plasmids (0.8 μg each). Thirty-six hours after transfection, EGFP proteins were observed (Fig. 2d), and FACS analysis of duplicate wells confirmed that the percentage of EGFP-expressing cells (P-value <0.001) and the MFI of EGFP-expressing cells (P-value <0.001) were significantly higher for the mTer treatments than for the HDVR treatments between the pHM-EGFP and pHR-EGFP groups (Fig. 2e). As Fig. 1 shows, the replication steps consisted of the synthesis of cRNA, which also contributed to an increase in vRNA. Therefore, EGFP expression did not reflect the actual long-term copy number of vRNA in cells that originated from transfected cDNA. However, EGFP was not expressed at high enough levels to be detected in the very early stages, such as at 12 h, and could therefore only give only subjective observational evidence rather than quantitative evidence that mTer was more efficient than HDVR at generating vRNA.

The exact termination efficiencies of vRNA by mTer and HDVR were determined by using Renilla luciferase (RLuci), which was chosen for its high sensitivity. Influenza virus RNAs possess conserved 3′ and 5′ ends, and in order to test the hypothesis that the discrepancy in generating the viral 3′ end by mTer and HDVR is due to differences in how the recombinant influenza viruses are generated, we constructed plasmids containing two nucleotide insertions at the 3′ and 5′ ends by using *Sap*I digestion, as described above (Table 1). Vero cells were transfected with 0.25 μg of plasmid per well in a 96-well plate and were then infected with adenoviruses expressing PB2, PB1, PA and NP at concentrations of 800 and 1,600 VP/AdV/cell for 1.5 h. The adenovirus inoculum was then removed and replaced with fresh medium. Samples were collected at 12, 24 and 48 h from duplicate wells. The relative light units (RLUs) of RLuci were measured by using the Dual Glu™ Luciferase assay system (Promega). Briefly, cells were washed with 100 μl 1× PBS and were collected by using cellular passive lysis buffer at 50 μl/well. Then, 50 μl Stop&Glo reagent was added to each well. The RLUs were detected by using a Veritas microplate luminometer. It has been shown that the addition or deletion of two nucleotides at either the 5′ or the 3′ end results in significantly lower RLUs than in the unmodified versions of the genes (Table 1). The ratio of RLUs between pHM-RLuci and pHR-RLuci, both with unmodified ends, showed that mTer might be 3.5 to 4 times more powerful than HDVR in generating vRNA with accurate 3′ ends. It was also observed that the ratio decreased to about 2.5 to 2.8 at 24 and 48 h. The results indicated that the accurate 3′ ends might be crucial for influenza virus cRNA and mRNA generation (Fig. 1). Similar results were observed with another segmented RNA virus, showing that the 3′-consensus sequence of rotavirus mRNA is critical for minus-strand synthesis [1].

Table 1 Efficiency comparison between mTer and HDVR in generating accurate vRNA 3′ ends

Plasmid		RLUs at time P.I.[a]		
		12 h	24 h	48 h
pHM-RLuci	Unmodified	607,260	44,911,650	223,173,314
	vRNA 3′ +AG	263,372	22,951,117	122,161,830
	vRNA 3′ −AG	39,933	90,979	207,201
	vRNA 5′ +AG	251,184	24,021,555	184,390,703
	vRNA 5′ −AG	40,809	74,039	90,592
pHR-RLuci	Unmodified	161,878	17,423,587	80,104,992
	vRNA 3′ +AG	66,908	4,308,839	45,909,291
	vRNA 3′ −AG	43,929	48,239	45,340
	vRNA 5′ +AG	43,421	4,478,112	44,369,341
	vRNA 5′ −AG	23,519	32,405	70,520
Ratio of mTer/HDVR[b]		3.75	2.58	2.79

[a] Time (h) postinfection with adenovirus expressing PB2, PB1, PA and NP. Values are the average of three parallel wells. A representative of at least three independent experiments is shown

[b] Ratio of RLUs (relative light units) of pHM-RLuci with unmodified ends versus pHR-RLuci with unmodified ends

To test whether the two different mechanisms for generating vRNA 3′ ends account for the different efficiencies with which influenza virus recombinants are generated from cDNAs, we constructed two plasmid-based reverse genetics systems, both of which included bidirectional transcription cassettes. The pM system contained the mTer element and the pR system contained the HDVR element (detailed descriptions of the construction of the plasmids used here will be provided on request). Except for two *Sap*I restriction sites used for inserting foreign genes in the backbone, the pM plasmid is similar to the previously used pHW2000 [6], including the CMV-BGH cassette for mRNA transcription and the huPol I-mTer cassette for vRNA transcription. The pR plasmid differed from pM in that it contained HDVR rather than mTer for vRNA generation. We inserted the eight cDNA segments of A/PR/8/

Table 2 Rescue efficiency difference between two influenza virus reverse genetics systems

	Virus yield (EID50)[a]			
	Batch 1	Batch 2	Batch 3	Average
mTer system	72,271	84,912	232,257	129,813
HDVR system	232	106	500	279
mTer/HDVR[b]	312	801	465	526

mTer system represents the pM reverse genetics system, HDVR system represents the pR reverse genetics system. The arrangement of elements in these two bidirectional transcription cassettes are similar to those in the pHW2000 plasmid [ref. 5]

[a] Virus titers were determined by incubation in 10-day-old embryonated eggs and were calculated by the Reed–Muench method

[b] mTer/HDVR represents the ratio differences between the pM reverse genetics system and the pR reverse genetics system

34 (PR8) into both pM and pR using *Sap*I digestion. The plasmids (0.5 μg each) were introduced by cotransfection into a mixture of 293T and Vero cells (ratio = 5:1) in one well of a six-well plate using Lipofectamine 2000. The transfection supernatant was replaced with OptiMEM I containing 1 μg/ml L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone (TPCK)-trypsin (Sigma). Forty-eight hours posttransfection, the supernatants and cells were harvested and diluted serially to inoculate 10-day-old embryonated chicken eggs (four eggs per dilution and 200 μl per egg). The eggs were incubated at 37°C for 2 days, and the allantoic fluids were harvested and subjected to hemagglutination assay to determine the presence of viruses. The virus titers (represented by EID50) in the cell supernatants were calculated by the method of Reed and Muench (Table 2). With PR8 virus as a model virus, the rescue system using mTer was about 500 times more efficient than a rescue system using HDVR in a mixture of 293T and Vero cells (Table 2). Our results are in agreement with an indirect comparison observed by Wit et al. [20]. In their six repeated experiments, PR8 virus could be produced only twice, with equally low virus titers, when using the HDVR-based, 12-plasmid-based system. However, about 10^4 viruses per ml PR8 virus were reproducibly generated as early as 24 h post-transfection when using the mTer terminator. More than 10^7 PR8 viruses were generated if the cell supernatant was collected 72 h after infection [20].

Our results obtained using the EGFP and RLuci reporters in Vero and 293T cells indicate that the efficiency of mTer in generating a precise 3′ end of vRNA was higher than that of HDVR. It is known that mTer and HDVR employ two different mechanisms to generate the RNA 3′ end. The mTer generates accurate 3′ ends in a two-step process that involves an 18-bp DNA sequence element, the so-called Sal box, which can specifically terminate gene transcription by RNA polymerase I (Pol I), followed by a specific upward trimming of ten nucleotides [8]. It has been found that (1) the termination of RNA elongation is entirely directed by the Sal box element, independent of the 5′-and 3′-flanking sequences, and (2) the 3′-end-trimming process requires the participation of cellular RNases. As ribosomal RNA is robustly transcribed in the nucleus, a large number of regulatory elements terminate PoI I transcription and trim pre-rRNA to obtain an accurate 3′ end. Therefore, in the nucleus, vRNA with precise 3′ and 5′ ends can be robustly generated from the Pol I- and mTer-based plasmids. However, the situation might be different for HDVR, which serves as a natural regulatory ribozyme for the generation of progeny hepatitis delta viruses in the cytoplasm [7]. Its maximal self-cleavage activity depends on the participation of suitable metal ions, such as Mg^{2+}, Ca^{2+} and Mn^{2+}, and a slightly alkaline environment (pH 7.3–7.6) [16]. The physical environment of the cytoplasm can provide the appropriate divalent cations and the necessary pH for the optimized functioning of HDVR as HDV virus replicates in the cytoplasm [7]. An in vitro study has indicated that even under optimized conditions, less than 80% of the primary HDVR sequence can be generated [16]. Nevertheless, in the nucleus, where pre-vRNAs are transcribed from plasmids by Pol I, an acid-biased condition might further compromise the optimal functioning of HDVR. Therefore, with a rescue system based on HDVR, less functional vRNA with precise 3′ ends can be generated, and the influenza virus rescue efficiency would be greatly reduced.

In conclusion, we present direct evidence that mTer is more effective than HDVR in the generation of precise 3′ ends of RNA transcribed from cDNA containing the human Pol I promoter. This can explain the lower degree of efficiency of those reverse genetics systems that utilize HDVR as the terminator. Our results will be helpful in studies using different terminators for the construction of plasmid-based systems for the rescue of recombinant influenza virus or other RNA viruses.

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