



Biochemical and kinetic analysis of the influenza virus RNA polymerase purified from insect cells

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ABSTRACT

The influenza virus RNA polymerase (RdRp) was purified from insect cells (around 0.2 mg/l). The RdRp catalyzed all the biochemical reactions of influenza virus transcription and replication *in vitro*; dinucleotide ApG and globin mRNA-primed transcription, de novo initiation (replication), and polyadenylation. The optimal Mg concentration, pH and temperature were 8 mM, 8.0 and 25 °C, respectively, which were slightly different from those measured for RdRp of virions. This system is a single-round transcription system. K_m (μ M) were 10.74 ± 0.26 (GTP), 33.22 ± 3.37 (ATP), 28.93 ± 0.48 (CTP) and 22.01 ± 1.48 (UTP), and V_{max} (fmol nucleotide/pmol RdRp/min) were 2.40 ± 0.032 (GTP), 1.95 ± 0.17 (ATP), 2.07 ± 0.17 (CTP), and 1.52 ± 0.38 (UTP), which agreed with high mutation of influenza viruses.

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Introduction

Influenza A virus contains eight single-stranded RNA segments of negative polarity as its genome and RNA polymerase (RdRp) as a virion component [1]. Influenza virus RdRp catalyzes both transcription (the synthesis of plus-strand mRNA containing a host cell-derived cap-1 structure at the 5'-terminus and a poly(A) tail at the 3'-terminus) and replication (the synthesis of full-length plus-strand complementary RNA (cRNA) and the cRNA-dependent synthesis of minus-strand viral RNA (vRNA)) [2]. The viral RdRp also catalyzes polyadenylation at the 3'-termini of mRNA *in vitro* [3] and performs template-dependent capped RNA cleavage and the apparent proofreading of nascent RNA chains [4]. The recombinant influenza virus RdRp purified from insect cells catalyzed the biochemical reactions above mentioned [5,6]. The RdRp purified from influenza virus consists of one part of each of the three subunits, PB1, PB2, and PA [7]. PB1 is the core of the assembly and is involved in polymerase activity [8–11]. PB2 is the cap-binding subunit [12–14], and very recently cap-snatching endonuclease activity was identified in the PA subunit [15,16], although it had been previously assigned to the PB1 subunit [13].

Although influenza virus RdRp was purified by recombinant expression systems, its rigorous enzymatic analysis has been ham-

pered because purification of significant amount of proteins was difficult [5,17]. In the present study, the influenza virus RdRp of the PR8 strain (H1N1) and chimeric RdRp of PR8 and the highly pathogenic avian influenza virus (H5N1) isolated from a Cambodian victim [18] were purified from insect cells. We determined biochemical features and kinetic constants (K_m and V_{max}) of the influenza virus RdRp.

Materials and methods

Cell culture. Sf21AE and TN5 cells were maintained in SF900II (Invitrogen) containing 1% FBS (Invitrogen), 50 U/ml of penicillin (Invitrogen), 50 μ g/ml of streptomycin (Invitrogen), 0.1 mg/ml kanamycin (Invitrogen), and 10 U/ml heparin (Shanghai First Biochemical Pharmaceutical Co.).

Recombinant baculoviruses. The N-terminal 18 \times His tagged PR8PB2 was PCR-amplified from pCPB2 [8] using the primers in Supplementary Table 1 and cloned into the BamHI and NotI sites of pVL1393 (BD Biosciences, Pharmingen), resulting in pVL18 \times His-PR8PB2. PB2, PB1, and PA of influenza A/Cambodia/P0322095/2005 [18] was PCR-amplified using the primers listed in Supplementary Table 1 and cloned into the NotI and BamHI sites of pVL1393, resulting in pVL18 \times His-CampPB2, pVLCampPB1, and pVLCampPA, respectively. The recombinant baculoviruses, Bac18 \times His-PR8PB2, Bac18 \times His-CampPB2_{E627K}, BacCampPB1, and BacCampPA were produced by co-transfection with BaculoGold genome (BD Biosciences, Pharmingen) in Sf21AE cells.

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In vitro mutagenesis. Mutations 627E → K of CampB2 (CampB2_{E627K}); and the polymerase knock-out mutation [9], 445D → A of CampB1 (CampB1_{D445A}) were introduced using the oligonucleotides in Supplementary Table 1 and pVLCampB2, and pVLCampB1 using the QuickChange II Site-Directed Mutagenesis kit (Stratagene). Each mutation was confirmed by DNA sequencing.

Expression and purification of the influenza virus RNA polymerase. BacPR8PB1 (MOI of 4), BacPR8PA (MOI of 2) [19], and Bac18×His-PR8PB2wt (MOI of 5) were infected into Tn5 cells (10⁶ cells/ml). Four days after infection, the cells were harvested, and nuclei were collected by centrifugation at 5000 rpm for 10 min at 4 °C after disruption in a 2 packed cell volume of hypotonic buffer (10 mM Hepes–NaOH (pH 7.9), 0.1% Triton X-100, and 1 mM PMSF). Nuclei were extracted at 4 °C for 30 min with 5 packed cell volumes of the extraction buffer (500 mM NaCl, 20 mM Hepes–NaOH (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 0.1% mercaptoethanol, 1 tablet/25 ml EDTA-free protease inhibitor cocktail tablets (Roche)). The nuclear extract was cleared by centrifugation in a Beckman 45 Ti rotor at 142,000g for 30 min and applied to ProBond (Invitrogen). The bound RdRp were eluted at 250 mM imidazole after washing of 10 column volumes of 100 mM imidazole, followed by dialysis against BC200 (20 mM Tris–HCl (pH 7.9), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10% glycerol). Chimeric influenza virus RdRp were purified as described above.

Model RNA template. The model RNA template, v84, was transcribed by T7 RNA polymerase as published previously [11].

In vitro transcription. One hundred nanomolar of the RdRp were incubated in the standard transcription condition; 50 mM Tris–HCl (pH 8.0); 8 mM MgCl₂, 150 mM NaCl; 2 mM DTT; 25 µg/ml actinomycin D; 0.5 mM of ATP, CTP, and GTP, 0.05 mM [α -³²P] UTP, 0.1 mM ApG, or 10 µg/ml globin mRNA; 2000 U/ml RNase inhibitor; and 200 nM v84. The transcript was analyzed by 6% PAGE containing 8 M urea, followed by image analysis with Typhoon Trio (GE).

For kinetic analysis of ATP, CTP and GTP, 100 nM of the RdRp of CampB_{E627K}-PR8PB1-PR8PA were incubated in the standard transcription condition with 50, 25, 12.5, 10, 5, 2.5, 2, 1.5, 1, 0.5, 0.25, 0.2, and 0.1 mM of ATP, CTP, or GTP for 1 h. For UTP, the heterotrimer was incubated in 50, 25, 12.5, 10, 5, 2.5, 2, 1.5, 1, 0.5, 0.25, 0.2, and 0.1 mM UTP, 0.5 mM ATP and GTP, and 0.05 mM [α -³²P] CTP. The mean product amounts produced in three independent experiments were plotted on a Lineweaver–Burk plot.

Oligo dT chromatography. ApG-primed transcripts were bound to the oligo dT magnetic beads of PolyAtract mRNA Isolation System III (Promega) in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, washed with 75 mM NaCl buffer and the bound polyadenylated transcripts were eluted with H₂O.

Single-round transcription. Single-round transcription assay was performed by addition of 3.13, 6.25, 12.5, 25, 50, 100, 200, and

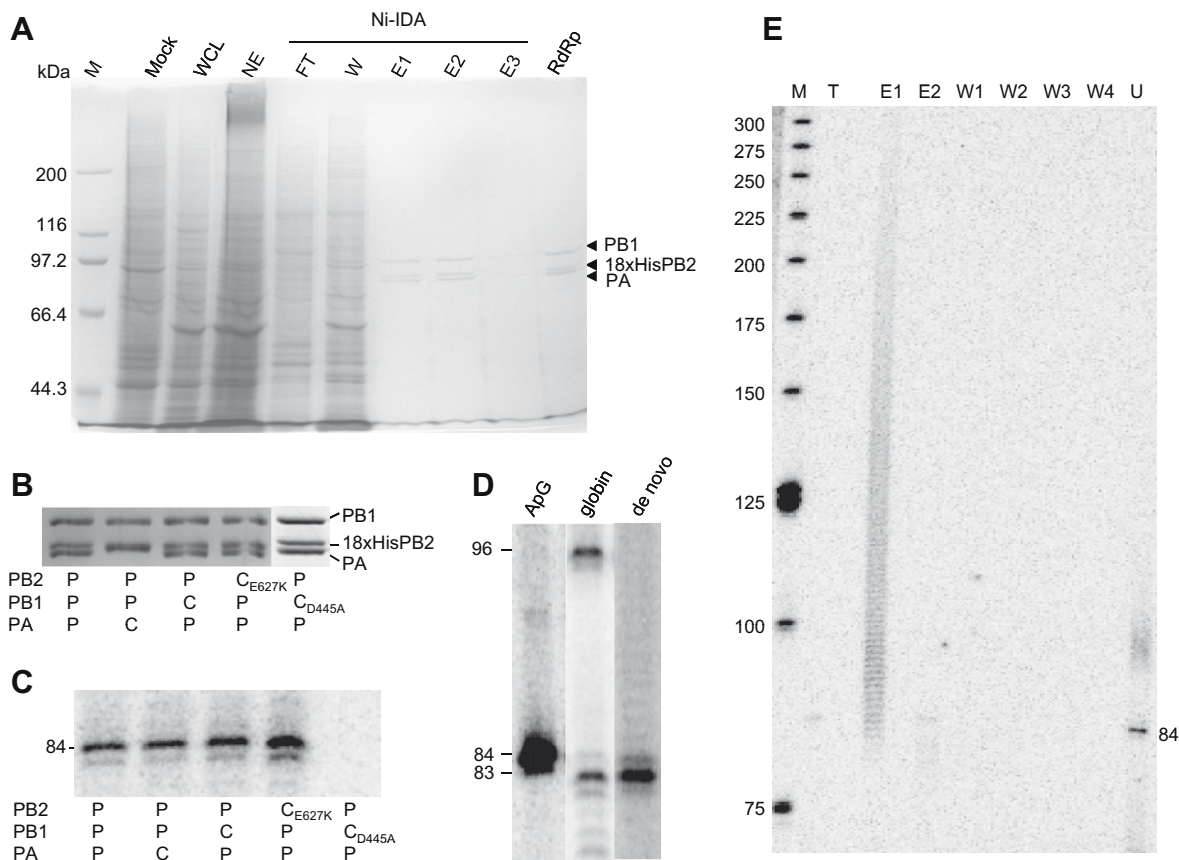


Fig. 1. Purification and *in vitro* transcription of the influenza virus RNA polymerase. (A) SDS–PAGE of the RdRp purification. Ten microliters of each fraction were analyzed by SDS 7.5%–PAGE. M, protein size marker; Mock, mock infected Tn5 cells; WCL BacPB1, Bac18×HisPB2 and BacPA infected Tn5 cells; NE, the infected cell nuclear extract; FT, the flow through fraction from Ni–IDA; W, the 100 mM imidazole fraction; E1–E3, the 250 mM imidazole elution fraction. RdRp, RdRp after dialysis. The position of the marker is indicated on the left. The positions of the RdRp subunits are indicated by arrowheads. (B) The purified chimeric RdRp. Five pmol of each RdRp were analyzed by SDS 7.5%–PAGE. The subunit combination is indicated below the PAGE. The designations of chimeric RdRp are PR8PB2wt–PR8PB1–PR8PA (PPP), PR8PB2–PR8PB1–CamPA (PPC), PR8PB2–CamPB1–PR8PA (PCP), CampB2_{E627K}–PR8PB1–PR8PA (C_{E627K}PP), and PR8PB2–CampB1_{D445A}–PR8PA (PC_{D445A}P), which contains polymerase knock-out mutation of PB1. (C) ApG-primed transcription of chimeric RdRp. The position of the 84 nt RNA is indicated on the left. (D) *In vitro* transcription. Two-hundred nanomolar v84 was transcribed by 100 nM RdRp with ApG (ApG), globin mRNA (globin), or without primers (de novo). The products were analyzed by 6% PAGE containing 8 M urea. The size of RNA is indicated on the left. (E) Oligo dT chromatography. ApG-primed transcript of v84 was fractionated by oligo dT chromatography (see Materials and methods). M: DNA marker, T: total transcript, E1, E2: elution fractions from the oligo dT magnetic beads, W1–W4: wash fractions from the beads, U: unbound fraction of the beads. The position of 84 nt RNA is indicated on the right.

400 µg/ml of heparin together with ApG and nucleotides after the incubation of RdRp and v84 for 30 min at 25 °C. The RdRp was incubated with heparin for additional 2 h.

Chemicals and radioisotopes. Actinomycin D, globin mRNA, heparin, Hepes, MOPS, and Tris were obtained from Sigma; the nucleotides were from GE; [α - 32 P] UTP and [α - 32 P] CTP were from New England Nuclear; and ApG, human placental RNase inhibitor, markers, and other enzymes were from Takara.

Results

Biochemical characterization of the influenza virus RdRp

The influenza virus PR8 strain RdRp (around 0.2 mg/l cell culture) was purified from TN5 cells by affinity purification using 18×His sequences tagged onto the N-terminus of the PB2 subunit (Fig. 1A and B).

The optimal transcription conditions of the influenza virus RdRp for ApG-primed transcription were 50 mM Tris–HCl (Hepes–NaCl) (pH 8.0), 150 mM NaCl, 8 mM MgCl₂, 0.1 mM ApG, 2 mM DTT, 25 µg/ml actinomycin D, 0.5 mM of ATP, CTP, and GTP, 0.05 mM [α - 32 P] UTP, 2000 U/ml RNase inhibitor, 200 nM v84, and 100 nM RdRp in 50 µl at 25 °C. In the optimal transcription conditions, the polymerase knock-out mutant (PR8PB2–CampB1_{D445A}–PR8PA) did not show activity (Fig. 1B and C), which validated our *in vitro* system [9]. The RdRp transcribed 84 nt RNA with ApG, 96 nt RNA with globin mRNA and 83 nt RNA without primer from v84 (Fig. 1D). In our experimental system the 14th G from the cap-1 structure (m⁷GmACACTTGCTTTTG) of rabbit globin mRNA (GenBank; M10843) was assigned as the initiation G [20]. The polyadenylated transcripts were analyzed using oligo dT chromatography. ApG-primed transcripts were applied onto oligo dT chromatography. Polyadenylated RNA which ranged from 84 to 300 nt were detected by oligo dT chromatography (Fig. 1E, lane E1).

The biochemical characterization was done for ApG-primed transcription. First, MgCl₂ and MnCl₂ requirement was tested (Fig. 2A). No activity was detected up to 6 mM MgCl₂. The highest activity was obtained at 8 mM MgCl₂. At higher MgCl₂ concentrations, polymerase activity decreased gradually. A similar depen-

Table 1

Kinetic constants of influenza virus and HCV JFH1 RNA polymerases.

	Influenza ApG-primed transcription	HCV JFH1 ^a de novo transcription
K _m (GTP) (µM)	10.74 ± 0.26	87.01 ± 7.24
K _m (ATP) (µM)	33.22 ± 3.37	4.69 ± 0.24
K _m (CTP) (µM)	28.93 ± 0.48	1.71 ± 0.31
K _m (UTP) (µM)	22.01 ± 1.48	7.48 ± 0.99
V _{max} (GTP) (fmol GTP/pmol RdRp/min)	2.40 ± 0.032	2.45 ± 0.21
V _{max} (ATP) (fmol ATP/pmol RdRp/min)	1.95 ± 0.17	0.65 ± 0.01
V _{max} (CTP) (mol CTP/pmol RdRp/min)	2.07 ± 0.17	0.14 ± 0.01
V _{max} (UTP) (fmol UTP/pmol RdRp/min)	1.52 ± 0.38	0.64 ± 0.04

^a K_m and V_{max} of HCV JFH1 RdRp are modified from Weng et al. [25].

dency curve was obtained with MnCl₂. Polymerase activity was not detected with 8 mM of CaCl₂, CdCl₂, CuCl₂, or NiCl₂ (data not shown). Next, the monovalent ion dependency was tested (Fig. 2B). The highest activity was obtained at 300 mM sodium monoglutamate. Polymerase activity was higher in NaCl and KCl. In NaCl, the highest activity was obtained at 150 mM. In the following experiments, 150 mM NaCl was used.

pH dependency was tested at pH 5 and 6 (50 mM MOPS–NaOH), 7, 7.5, 8, 8.5, and 9 (50 mM Tris–HCl), and 10 (50 mM CAPS–NaOH) (Fig. 2C). The highest activity was obtained at pH 8. No polymerase activity was detected at pH 5, at which the influenza RNP was exposed due to an uncoating process [21]. Activity (33.6%) was detected at pH 10. Polymerase activity was similar in Tris and Hepes buffer at pH 7–8.5 (data not shown). The highest polymerase activity was obtained at 25 °C (Fig. 2D). At 4 and 42 °C, the polymerase activity was 28.7% and 13.4% of that seen at 25 °C, respectively. No polymerase activity was detected at 0 °C. ApG (0.1 mM) showed 85.9% activity of that of 0.5 mM (Fig. 2E). The transcription products accumulated to a maximum at 3 h and maintained this level up to 7 h (Fig. 2F).

The recovery of subunits of PR8 RdRp varied with purifications, and the subunit ratio of PR8 RdRp was 1:2:2 (PB2:PB1:PA) (Fig. 1B).

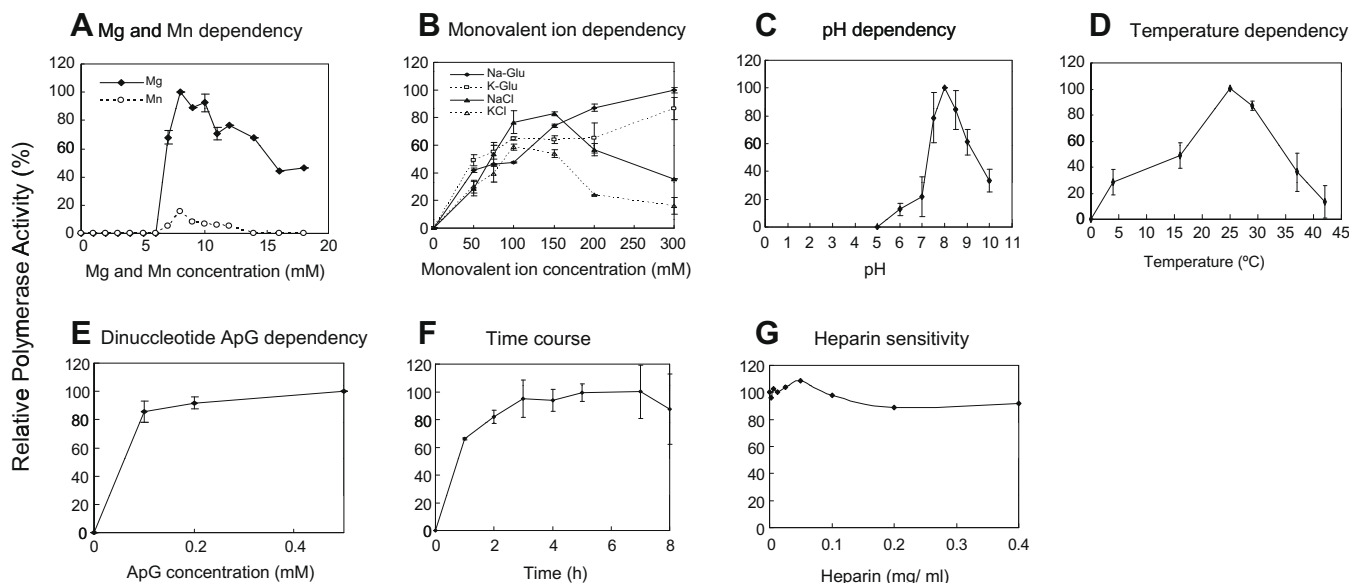


Fig. 2. Biochemical characterization of the influenza virus RNA polymerase. The relative polymerase activities of influenza virus RdRp for MgCl₂ and MnCl₂ concentration (A); NaCl, KCl, monosodium glutamate and monopotassium glutamate concentration (B); pH (C); temperature (D); dinucleotide ApG concentration (E); time course (F); and heparin sensitivity (G) were analyzed (see text). The symbols are indicated in each panel. Mean and standard deviation of the relative polymerase activity were calculated from the autoradiogram of three independent experiments.

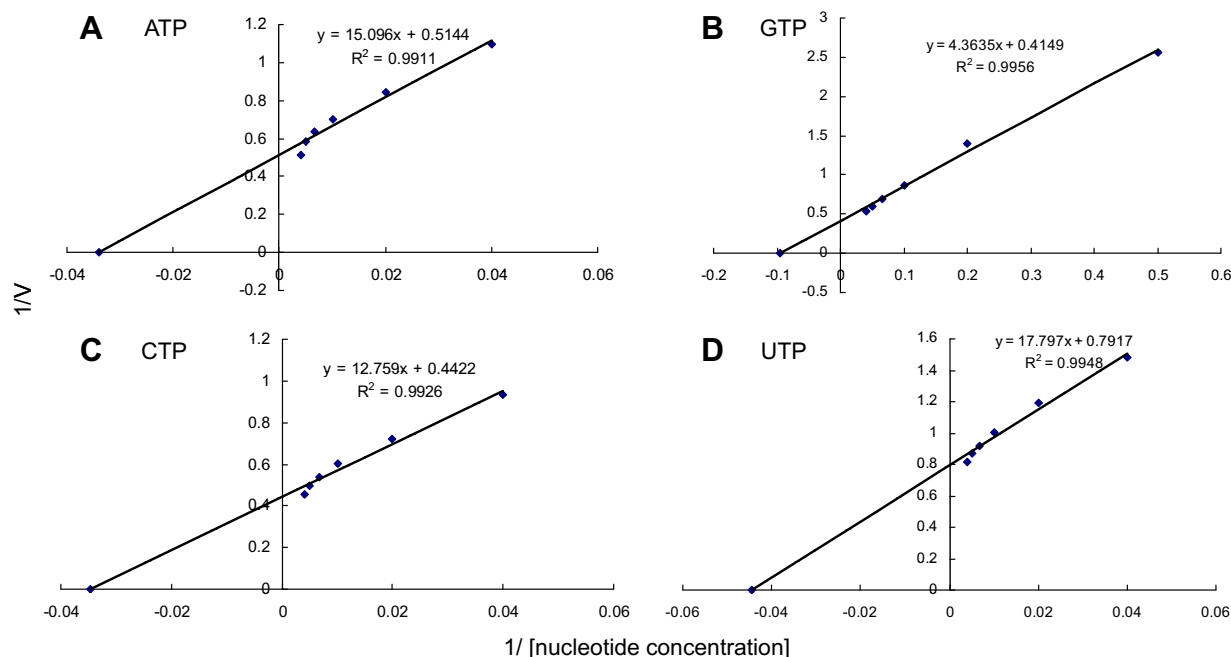


Fig. 3. Lineweaver–Burk plots of the influenza virus RNA polymerase. The mean incorporated NTP amounts for ATP (A), GTP (B), CTP (C), and UTP (D) calculated from three independent experiments were plotted on Lineweaver–Burk plots (see Materials and methods). Equations are indicated in the graph.

In order to measure the kinetic constants, we tested the recovery of the chimeric RdRp of PR8 and the Cambodian isolate. The subunit ratio of the chimeric RdRp was 1:1:1 (PB2:PB1:PA) (Fig. 1B). The polymerase activities of ApG-primed transcription of the chimeric RdRp were similar (Fig. 1C).

Finally, single-round transcription was analyzed by addition of heparin (Fig. 2G). Heparin did not affect the amount of the products up to 0.4 mg/ml, which indicated that our RdRp transcribed the template only once.

Kinetic analysis of the influenza virus RdRp

Kinetic constants (K_m and V_{max}) for ApG-primed 84 nt products were measured using CamPB2_{E627K}-PR8PB1-PR8PA and indicated in Table 1 calculated from Lineweaver–Burk plots (Fig. 3). In our system 1.09–1.71 fmol of RNA were transcribed by 1 pmol of RdRp per hour.

Discussion

We established the better purification method of influenza virus RdRp using the recombinant baculovirus expression system. Our RdRp catalyzed ApG and globin mRNA-primed transcription, de novo initiation (replication), and polyadenylation (Fig. 1C–E). Its biochemical features were slightly different from those of the ribonucleoprotein complex (RNP)-assembled RdRp of virions [22,23]. The clearest differences in these optimal biochemical conditions were in the Mg concentration, pH and temperature. Our RdRp preferred alkaline conditions higher than pH 7. The optimal $MgCl_2$ concentration was 8 mM, which was higher than that for RNP RdRp. Our RdRp worked the best at 25 °C, which was lower than RdRp of virions. Our RdRp was resistant to 0.4 mg/ml heparin-treatment, which was different from HCV RdRp [24]. It is likely that our system is a single-round transcription system (Fig. 2G).

The V_{max} of influenza virus RdRp was ten times higher than those of HCV RdRp (Table 1) [25]. The catalytic speed of HIV RT is 1980 min^{−1} for dATP, as calculated from the data of Kati et al. [26]. The V_{max} of poliovirus RdRp is 4.4 for UTP and 16.8 for GTP

in primer-dependent transcription and 3.4 for GTP in de novo transcription [27]. Influenza virus RdRp is much slower than those of HIV RT and poliovirus RdRp.

The K_m of poliovirus RdRp is 40–116 μM for GTP and 61.9 μM for UTP [28]. Influenza virus RdRp had lower affinity for ATP, CTP, and UTP than HCV RdRp, and higher affinity than poliovirus RdRp. There is a consequence of the total lack of replication proof-reading machinery in RdRp of RNA viruses, and influenza A viruses shows high evolutionary rates of 2.3 to 5.7 × 10^{−3} substitutions per site per year [29,30]. The K_m and V_{max} of influenza virus RdRp agreed with its high evolution rate and the apparent proofreading activity [4]. K_m and V_{max} for influenza virus RdRp are still useful values for comparing the biochemical characteristics of RdRp because these parameters were compared from identical experiments [25] although they do not completely fit for the correct simulation of transcription [31].

Host factors and NP are important for controlling the gene expression of influenza virus and its assembly, but not essential for the initiation of transcription and replication in our system [32–34].

Influenza pandemics are unpredictable and uncontrolled. The purified influenza virus RdRp will contribute to the development of new anti-RdRp reagents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2009.11.100](https://doi.org/10.1016/j.bbrc.2009.11.100).

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