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Active recombinant Reverse Transcriptase Domain of human Hepatitis B Virus Polymerase

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Abstract

Hepatitis B virus polymerase plays a critical role during HBV life cycle, and polymerase/reverse transcriptase (RT) activities are critical for HBV-pol during viral replication. To investigate RT domain of human HBV polymerase, a 5' end Polyhistidine tagged RT DNA (304-693 amino acids) of HBV-pol was successfully expressed in Escherichia coli. Recombinant RT was purified in native condition employing Ni-NTA affinity column. Purified RT showed a stable reverse transcriptase activity and a much stronger DNA polymerase activity, compared to RT expressed in rabbit reticulocyte lysate coupled transcriptase-translation system. We present a new simplified way of obtaining active RT protein using the Escherichia coli expression and Reticulocyte lysate system. The purified RT was a stable protein and showed a low selective polymerase activity. Computer modeling results also indicated that RT domain banded to nucleotide substrate in a loose mode.

Key words: Hepatitis B Virus, polymerase, reverse transcriptase, detergent

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Introduction

Hepatitis B virus (HBV) infection is a major global public health problem. It is estimated that approximate 1/3 world population has been infected by HBV, and over 350 million are chronically infected[1,2]. Patients with chronic HBV infection carry a great risk of developing severe liver diseases such as cirrhosis and hepatocyte cellular carcinoma, and these diseases result in a million mortalities annually. HBV is a member of the *hepadnaviridae* family. Despite containing a DNA genome, HBV replicates via a reverse transcription process, using the polymerase encode by its own gene. HBV polymerase (HBV-Pol) consists of three functional domains: (from N-terminal to C-terminal) terminal protein (TP) domain, polymerase/reverse transcriptase domain (RT), and RNase H (RH). TP domain is separated from other two domains by a spacer sequence. During HBV genome replication, HBV progenomic RNA (pgRNA) acts as the template for minus strand of HBV genome DNA replication. The replication is triggered by the protein-priming of TP domain, and then the minus strand DNA synthesis is initiated following the pgRNA degraded by RH domain of HBV-Pol. The plus strand of HBV genome is synthesized following the nascent minus strand DNA as template.

It is suggested that replication of HBV genome indicated that protein-priming activity, RNase H activity and the two critical activities: RNA-dependent DNA polymerase activity (RDDPa) and DNA-dependent DNA polymerase activity (DDDPa) should be presence in HBV-Pol harmoniously. Although HBV-Pol which is responsible to RDDPa and DDDPa was documented[3], and then the TP domain and RH domain of human HBV-Pol, which are responsible for protein-priming activity and RNase H activity respectively, have been proved for decades[4-6]. However, it is still ambiguous whatever, RDDPa and DDDPa are encoded by a specific domain of HBV-Pol or cooperative of the domain of HBV-Pol. Based on the homology of the RT domain of HBV-Pol with known reverse transcriptase, it is reasonable to believe that the RT domain confer-RDDPa. The difficulties to acquire active recombinant HBV-Pol or RT domain have hampered the characterization of biochemical study of human HBV-Pol. Some groups attempted to express certain domains of human HBV-Pol separately for biochemical study or employ duck HBV polymerase (dHBV-Pol) as research model[7,8]. Expressed TP and RT could form highly stable complex that was active in nucleotide synthesis priming and reverse transcription within insect cells[4]. RDDPa was detected with in insect cells which supplied molecular chaperon such as HSP90 for HBV RT stabilization, specific binding and possible protein folding assistance. However, activities of human HBV RT expressed in rabbit reticulocyte lysate expression system were inves-

tigated, and that recombinant HBV-RT exhibited DDDPa lack of RDDPa [9]. Recombinant HBV-RT synthesized in *Pichia methanolica* also showed DDDPa only [10]. Although biochemical research of other domains of human HBV-Pol has been well reported, activities of RT domain of human HBV still need further investigation.

Nowadays, most of approved medications on Chronic Hepatitis B infection (CHB) are nucleotide reverse transcriptase inhibitors (NRTIs) such as Lamivudine, Adefovir Dipivoxil, Entecavir, Telbivudine and Clevudine, all these NRTIs are targeted on HBV polymerase (HBV-Pol)[1,11]. The spatial relationships between primer-template/reverse transcriptase complex and NRTIs has been well developed [12,13]. To further understand the spatial interaction between RT and template-primer substrate complex, a molecular modeling study was also conducted employing an existed RT domain modeling template which conserved motifs and key amino acid residues are coincident with HBV-RT [12]. We have previously described that functional intact human HBV-Pol has been expressed in *E. coli* without co-expression molecules or in the presence of certain helper chaperons. In this work, stable RDDPa and DDDPa of recombinant HBV-RT expressed in both prokaryotic and eukaryotic expression systems were observed. In terms of modeling result, DDDPa and RDDPa should be presence in human HBV-RT harmoniously, and this result also was coincident with the activity characteristics of recombinant HBV-RT.

Materials and Methods

Computer modeling

A homologous model of reverse transcriptase (protein Data Bank accession no. 1RTD.pdb) as modeling templates was constructed, which covered amino acid residues 319 to 700, comprising the entire RT domain of HBV polymerase. The HBV RT was modeled in a conformation similar to that of RT domain of HIV polymerases, with the nucleic acid substrate-binding cleft defined by structural elements from the fingers, palm, and thumb sub-domains. A spatial relationship between RT domain and substrate was investigated briefly.

Plasmid construction

Liver tissue was obtained from a chronic HBsAg carrier who developed hepatocellular carcinoma (HCC) and underwent surgical resection (Hospital of Chonbuk National University, Jeonju, South Korea). Briefly, the tumor tissues were dissected and immediately cut into small pieces and stored in liquid nitrogen until use. Cellular DNA was isolated from the tissue by SDS-protease K digestion and phenol-chloroform extraction, as described previously (Sambrook). The HBV-RT sequence [spanning 1005-2079 bps , 304-693 amino acids] (GenBank accession number AF286594) was amplified with PrimeSTAR^{TM} high fidelity polymerase using RTNotIF01: GTT GCG GCC GCT TAA TGG ACT ACT GCC TCA CC and RTBstBIR01: GAA TTC GAA AAT TCC TGA CCG TTG CCG GGC for RT fragments. The vectors were designed as shown in Figure 2 (Fig-2).

For expression of HBV-Pol in E.coli system, the plasmid pTrcHis-A-RT was constructed as showed in Fig-2a. The RT frame was under the control of Trc promoter and Lac operator. His-tag and Xpress antigen were fused at 5'end of RT domain sequence. For expression of HBV-Pol in rabbit reticulocyte lysate system, the plasmid pT7-Pol was constructed as showed in Fig-2b. The HBV-Pol frame was driven by T7-promoter.

The pTrcHis-A-RT and pT7-RT vectors were constructed by the following procedure. RT domain of HBV-Pol full-length sequence was cloned into NotI and BstBI sites of the two expression vector pTrcHis-A (Invitrogen, US). RT domain of HBV-Pol full-length sequence was cloned into the NotI and BstBI sites of the two expression vectors, pTrcHis-A (Invitrogen, US) and pT7 (Promega, US). For expression of HBV-RT in the E.coli system, the plasmid pTrcHis-A-RT was constructed as shown in Fig-2-1. The rhHBV-RT expression was under the control of a Trc promoter and a Lac operator. His-tag and Xpress antigens were fused at the 5' end of the HBV-RT sequence. For expression of rhHBV-RT in a rabbit reticulocyte lysate system, the plasmid pT7-RT was constructed as shown in Fig-1-2. The rhHBV-R expression was driven by the T7-promoter

***E.coli* transformation**

Plasmid pTrcHis-A-R was chemically transformed in competent DH5α (*F-,φ80dlacZΔM15,Δ(lacZYA-argF) U169,deoR,recA1,endA1,hsdR17(rk-,mk+),phoA,supE44,λ-,thi-1,gyrA96,relA1*) cells following the manual supplied by Real Biotech Corporation (South Korea).

Expression and purification of histidine-tagged RT domain

The supernatant was transferred to Ni-NTA resin column (bed volume: 2ml) which had been equilibrated with 16ml equilibration buffer (same components of previous lysis buffer without lysozyme). Resin and lysate supernatant was mixed thoroughly but gently for 60min at 4℃. The resin was washed with 8ml washing buffer (50mM Phosphate buffer, pH8.0; 0.5mM NaCl; 1%NP-40; 10mM Imidazole; 20mM 2-mercaptoethanol; 1X Roche protein inhibitor cocktail) for 6-8 times. The RT fractions were eluted with 6ml Elution buffer (50mM Phosphate buffer, pH8.0; 0.5mM NaCl; 1%NP-40; 50,100 250mM Imidazole respectively; 1X Roche protein inhibitor cocktail, dithiothreitol stock solution added in harvest tube first to achieve final concentration to 5mM). RT Elution fractions were harvested and stored at -70℃.

Transformed cells were grown in 100 ml of LB broth until the culture reached an OD_{600} of 0.6-0.8. Recombinant protein expression was then induced by treatment of the culture with 1 mM Isopropylthiogalactopyranoside (IPTG, Sigma, USA) for 4 h at 37 ^{o}C with shaking. Induced cells were harvested by centrifugation at 2,000×g for 20 min at 4 ^{o}C. Four volumes of lysis buffer were added to the obtained cell pellet. The lysate was incubated at room temperature for 30 min and then sonicated for 10×10 sec. The samples were cooled down on ice for 5-10 sec between each sonication. The suspension was centrifuged at 20,000×g for 30 min at 4 ^{o}C. The supernatant was transferred to a nickel-based resin (Invitrogen, US) column (bed volume: 2 ml) that had been equilibrated with 16 ml equilibration buffer (same components as the lysis buffer, without lysozyme). The resin and lysate supernatant were mixed thoroughly but gently for 45-60 min at room temperature. The resin was washed with 8 ml washing buffer (50 mM phosphate buffer, pH 8.0; 0.5 mM NaCl; 1% NP-40; 10 mM imidazole; 20 mM 2-mercaptoethanol; 1X Roche protein inhibitor cocktail) 6-8 times. The rhHBV-RT fractions were eluted with 6 ml each of E-50, E-75, and E-100 Elution buffer (50 mM phosphate buffer, pH 8.0; 0.5 mM NaCl; 1% NP-40). Roche protein inhibitor cocktail (1X) and dithiothreitol (5 mM) were added to the harvest tubes previously. The concentration of imidazole in E-50, E-75, and E-100 buffers was 50 mM, 75 mM and100 mM, respectively. RhHBV-RT elution fractions were harvested and stored at -70 ^{o}C. The protein concentrations of the purified protein samples were determined by use of a bicinchoninic acid assay using bovine serum albumin (BSA) as a standard.

SDS-PAGE and Western Blot analysis

Total lysate and other purified protein samples were mixed with electrophoresis sample buffer and were denatured at 95℃ for 5min. Proteins were separated by SDS-PAGE. Gels were stained with Coomassie Blue. For western blot analysis, protein was electrophoretically transferred to a polyvinylidene difluoride (PVDF) blotting membrane, and membrane was treated with first a 1/4,000 dilution of anti-Xpress antibody followed Goat-anti-mouse conjugated alkaline phosphatase (Invitrogen, US).

Expression of RT domain of HBV polymerase in in vitro expression system

The recombinant plasmid pT7-RT was purified with Qiagen Midiprep DNA purification kit (Qiagen , Ger) . *In vitro* transcription and translation reactions were performed using TNT T7 coupled reticulocyte lysate system (Promega, US). 2 μg of the plasmid DNA template was transcribed and the protein was translated in each 50 ml reaction in the presence or absence of 40 mCi of [^{35}-S]-methionine (1000 Ci/mmol) (Amersham, UK) at 30℃ for 75 minutes[9]. The *in vitro* translation reaction was stopped by the addition of 0.1 mg/ml cycloheximide for the polymerase activity assay or SDS sample buffer for checking the efficiency of translation. The *in vitro* translated proteins were separated by 4-12% SDS-PAGE and dried prior to autoradiography.

DNA polymerase activity and reverse transcriptase activity assays

DNA-dependent DNA polymerase activity (DDDP) and reverse transcriptase/RNA dependent DNA polymerase activity (RDDP) were monitored by the synthesis of DNA using poly (dA) · oligo (dT) $_{12–18}$ and poly (rA) · oligo (dT) $_{12–18}$ as template-primer (Amersham Biosciences Corp.), respectively. The standard enzyme reaction (50 ml) contained 50 mM Tris-Cl pH 7.4, 50 mM KCl, 10mM $MgCl_2$, 1 mM DTT, 0.01% Nonidet P-40, 50 ng of homopolymer template (poly (dA) · oligo $(dT)_{12–18}$ for DDDP activity assay and poly (rA) · oligo $(dT)_{12–18}$ for RDDP activity assay), and 2 mCi of [α–^{32}P]dTTP (3000 Ci/mmol), (PerkinElmer, USA). For RDDP activity assay, RNase inhibitor and RNase free water were employed in the reaction [9,14]. Reactions were started by the addition of 0.5 mg of the purified RT or 5μl products from TNT-T7 coupled reticulocyte lysate system into reaction buffer. The endogenous DNA polymerase activity from the reticulocyte lysate was suppressed by the addition of 60 mM aphidicolin and 1mM NEM. After incubation at 37℃ for 75min, reactions were stopped by the addition of 0.2 mg/ml of protease K in the presence of 0.5% SDS. Incubation was then continued for another 20 min, followed by spotting on Whatman DE81 filter paper. Filters were washed 3 times with 0.5 M Na_2HPO_4, once with distilled H_2O[9]. Incorporation of radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Series 2300 liquid scintillation counter.

Results

Molecular Modeling

To understand the spatial interaction relation of RT domain with nucleotide substrate, a three-dimensional homology model of HBV RT domain was developed based on the HIV RT-DNA crystal structure. The most conserved domains based on the sequence alignment between HIV and HBV-RT [12] and the HIV RT-DNA X-ray structure[13] were used to build the HBV RT model. In this study, RT conserved regions surrounding the substrate site was focused. A docked template-primer double stranded DNA was shown in yellow. From the modeling diagram, the side-chains of all residues in docking pocket were closed to a single strand of the substrate, which indicated that hydrogen band would probably form between the side chain and certain single strand of nucleotide substrate.

In this study, the HBV-RT domain was encoded by bases 1005 to 2079 of HBV genome and had 389 amino acids.

The RT domain was expressed in two systems: E.coli and reticulocyte lysate system. Then DNA polymerase activity and reverse transcriptase activity from these two protein products were monitored.

Expression, Induction and Purification of HBV-RT in Escherichia coli

To achieve the high expression level, expression level was examined in various conditions. Appropriate induction time is an important factor to obtain enough recombinant RT domain products. Quantity of expressed protein will be varied in different induction time. After induction optimization, all the induction culture was carried out at 37℃ for 4h. For HBV-RT purification, HBV-RT fractions were eluted with elution buffers (50, 75, 100mM imidazole). In Fig-3a, prominent bands of HBV-RT were detected with SDS-PAGE; analysis of western blot also confirmed the purification results (Fig-3b).In this study, a purification protocol in native condition was applied for RT domain purification. RT protein was eluted with elution buffer with the presence of 50, 100 and 250 mM imidazole step-by step. In Fig-3a, a prominent 50kD band was detected with Coomassie blue staining. Results of western blot also confirmed the purification results (Fig-3b).

Expression RT domain in reticulocyte lysate expression system

Recombinant HBV-RT was also expressed in reticulocyte lysate system as a positive control in activity assay. In terms of previous data, up to 500μg protein will be produced per 50μl reaction (data not shown); therefore 5μl of product from reticulocyte lysate expression reaction was employed in enzyme activity assay.

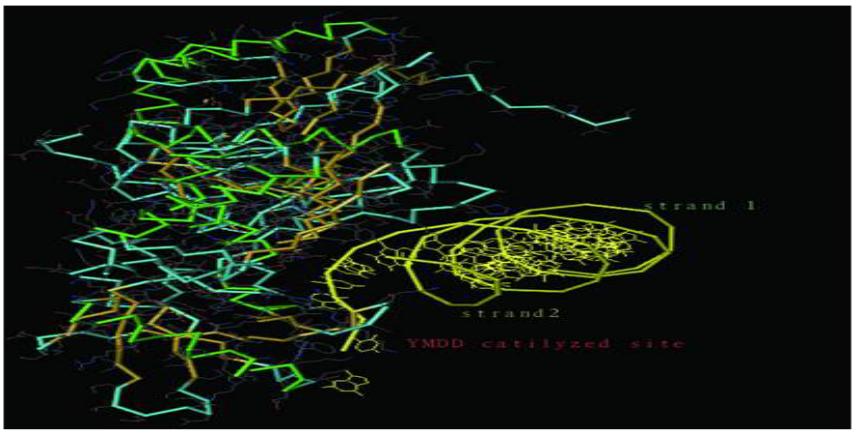

Figure 1. Computer modeling of RT domain. Tube diagram showing the 3D model of the RT domain of HBV Polymerase (residues 304 to 689). *A docked template-primer double stranded DNA is shown in yellow. From the modeling diagram, the side-chains of all residues in docking pocket were closed to one single DNA/RNA strand of the substrate, which indicated that hydrogen bond would probably form between the side chain and certain single strand of substrate.*

Figure 2 Structural arrangement of pTrcHis-A-RT and pT7-RT

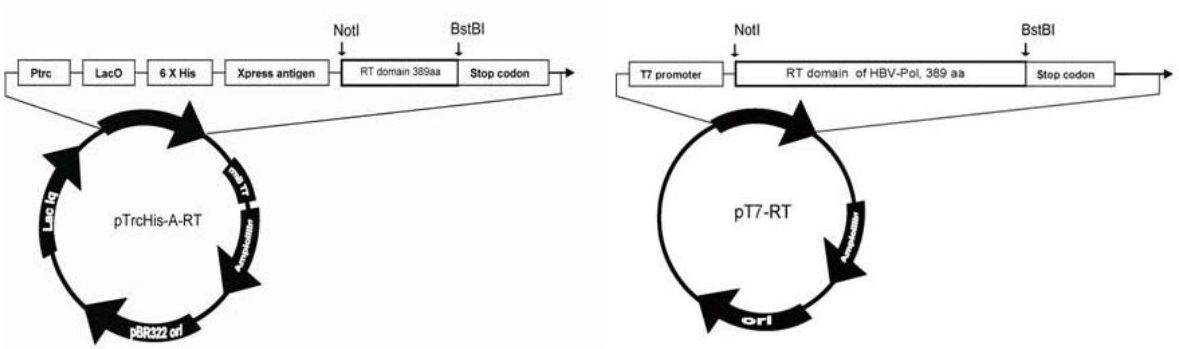

Figure 2. *A:* ***Structural arrangement of pTrcHis-A-RT****. Reverse transcriptase fragments of hepatitis B virus polymerase (HBV-RT) sequence was fused into pTrcHis-A between NotI and BstBI sites under the control of Trc promoter and Lac operator. This polyhistidine tag plays a role in rapid purification using a nickel-based resin. To determine the expression level under different conditions, an Xpress antigen was fused to the 5'-end of the HBV-RT sequence;* ***B: Structure of pT7-RT.*** *HBV-RT sequence was fused to P-T7 between NotI and BstBI sites. HBV-RT sequence was under the control of the T7 promoter for expression in the TNT T7 transcription–translation-coupled rabbit reticulocyte lysate expression system.*

Characterization of purified RT fraction activities and comparison with RT from in vitro expression system

To measure the enzymatic activities of purified RT domain protein, DDDP activity and RDDP activity assays were performed under the reaction conditions described previously. The incorporation of radioactivity into DNA/RNA template-primers in solution was measured with liquid scintillation counter. Enzyme activities comparison between RT from *in vitro* expression system (RT-IV) and purified RT from E.coli expression system (RT-50) was showed Fig-4.

Results from RT-50 showed a very high DDDP activity and a relatively low RDDP activity in these reaction conditions. In a contrary, results from RT-IV showed very low level both in RDDP activity and in DDDP activity. In terms of the results of enzyme activities assay, a phenomenon was observed that RT-50 exhibited high DNA polymerase activities no matter what the template is a DNA strand or a RNA strand. And in RT-IV group, a same phenomenon was also observed. These results indicated that DDDP activity and RDDP activity were harmonious presence in RT domain of human HBV-Pol.

Expression and purification of HBV-RT in E.coli. Expression system

Figure 3. Expression and purification of HBV-RT in E.coli.

Expression and purification of HBV-Pol. Recombinant human HBV-RT was produced in E.coli cells transformed by pTrcHis-A-RT and then purified with nickel-based resin. A detailed explanation of the procedure was presented in Materials and Methods. Protein samples obtained from the purification steps were analyzed by 4-12% SDS-PAGE, and then the gel was stained with Coomassie Blue (Fig. A). The HBV-RT bands were located at the expected molecular mass, approximate 50kDa according to the prestain molecular weight marker. In Fig. B, although HBV-RT bands were also detected in 50mM imidazole (E50-1 and E50-2) washing fractions, mostly part of HBV-RT was eluted by buffer with 100mM (E100) and 250mM imidazole (E250) and E. Purified recombinant human HBV-Pol was also analyzed by immunoblot analysis with an anti-RT antibody (Fig. B). HBV-RT bands were detected in total lysate of induced transformant (TL), but not in 5th washing fraction with 10mM imidazole (W5).

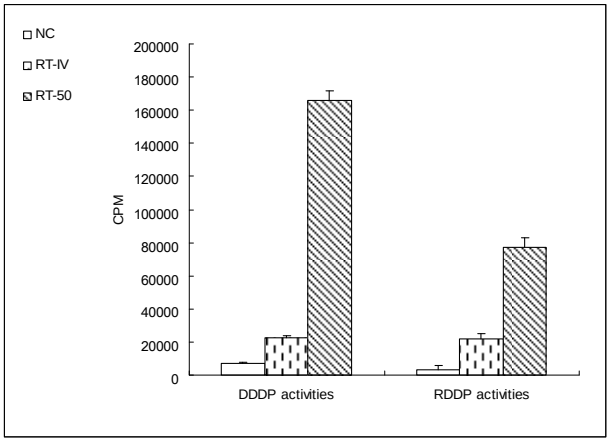

Figure 4. Enzyme activities comparison between RT-IV and RT-E50. *Enzyme activity assay of recombinant HBV-RT. Comparison of enzyme activities between Pol from E.coli expression system (P-E) and Pol from in vitro expression system (P-R).DNA-dependent DNA polymerase activity (DDDPa) and RNA-dependent DNA polymerase activity (RDDPa) of the P-E and P-R were monitored under standard conditions (see Materials and methods). In this assay, all endogenous DDDP activities had been suppressed by 60 mM aphidicolin and 1mM NEM (Fig-4-1). Effects of ATP on DDDP and RDDP activities of purified HBV-Pol.*

Discussion

HBV polymerase plays a crucial role in HBV lifecycle. The activities are highly During HBV genome replication stage, HBV-Pol shows RDDPa and DDDPa in minus strand and plus strand synthesis, respectively. The organization of HBV-Pol has been studied for many decades. Terminal protein (TP) domain triggers minus strand HBV genomic DNA in a protein-priming mechanism. RNase H domain (RH) degrades the RNA template which named progenomic RNA after minus strand of HBV genomic DNA. These two domains play important roles, but they are not the critical domain in DNA replication stage. DDDPa and RDDPa should be presence harmoniously. Therefore we expressed the RT domain of human HBV in eukaryotic expression system and prokaryotic expression system, and then compared the activities of two expressed protein products.

Expression of an active RT domain of human HBV polymerase in heterologous systems has been carried out with limited success. Although recombinant RT had been expressed in different system and purified in various ways, obtaining high purity active RT domain of HBV-Pol (HBV-RT) for biochemical study is still problematic [9,10]. It has been noted that HBV-RT was stable in heterologous expression system and also during purification process. Inclusion body formation of HBV-RT was easily inhibited during purification step using a weak detergent.

To prevent target protein from oxidization and keep cysteine residues (nearly 2% in total amino acid residues) in reduced condition, high concentration reducing agents were employed throughout purification steps.

In terms of results from enzyme activities, HBV-RT showed a low specific DNA polymerase activity. Molecular modeling results also indicated that RT catalyzing site surrounded nucleotide template with only a few potential hydrogen bonds binding to single substrate strand [12]. Considering the function of TP domain, it has lower RDDP activity. In this study, according to the results of activity assay of HBV-RT, a higher DDDP activity was exhibited than its RDDP activity, which mainly because of RNA template-primer substrate which was partially degraded during template-primer binding steps. Human HBV-Pol is critical for HBV genome DNA replication. It showed RDDP activity in minus strand, where as DNA synthesis and DDDP activity in plus strand synthesis. Recent studies have proposed that some chaperones might be needed for RDDP activity at the start point of DHBV replication [15]. The RDDP activity of our DHBV P protein was very weak compared with DDDP activity and the HBV P protein did not have RDDP activity. Most of approved medications for the treatment of chronic hepatitis B such as lamivudine, adefovir dipivoxil, entecavir and telbivudine are targeted for HBV-RT [1]. However, the stable and large scale heterologous expression of functional RT domain of human HBV-Pol in common host such as E.coli or yeast has not successfully purified yet. Employing the approach in this work, a functional RT domain of human HBV-Pol was achieved in E.coli expression system. The availability of this recombinant protein in pure form should facilitate the antibody preparation and detailed analysis of the structure and mechanism of RT domain. Large quantity of functional HBV-RT was also required in high throughout screening assay for potential inhibitors development.

Since the RDDP and DDDP activities of human HBV-Pol represent the critical step in the human HBV life cycle, RT domain is the most important drug target for antiviral agents. In this work, we showed the expression and purification of enzymatically active RT domain from human HBV-Pol by E.coli expression system. It is obvious that large production of functional human HBV-Pol is essential, not only for antibody preparation but also for rational design of the specific inhibitors of human HBV Polymerase.

Acknowledgements

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