Proteolysis of the Reverse Transcriptase of Hepatitis B Virus by Lon Protease in *E. coli*

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Hepatitis B virus (HBV) polymerase, which possesses the activities of terminal binding, DNA polymerase, reverse transcriptase and RNaseH, has been shown to accomplish viral DNA replication through a pregenomic intermediate. Because the HBV polymerase has not been purified, the expression of HBV polymerase was examined in an *E. coli* expression system that is under the regulation of arabinose operon. The expressed individual domain containing terminal binding protein, polymerase, or RNaseH turned out to be insoluble. The activities of those domains were not able to be recovered by denaturation and renaturation using urea or guanidine-HCl. The expressed reverse transcriptase containing the polymerase and RNaseH domains became extensively degraded, whereas the proteolysis was reduced in a *lon-* mutant. These results indicate that Lon protease proteolyzes the HBV reverse transcriptase expressed in *E. coli*.

The P gene of human hepatitis B virus is the gene for HBV polymerase that possesses the activities of terminal binding, DNA-dependent DNA polymerase, reverse transcriptase, and RNaseH (Radziwill et al., 1990). The replication of this virus has been studied mainly using the duck hepatitis virus as a model. Genomic replication is demonstrated to proceed through the pregenomic RNA, which is produced by transcription of the closed circular viral DNA by host RNA polymerase (Summers and Mason, 1982). This pregenomic RNA serves as a template for minus strand DNA synthesis, which is primed by the terminal binding activity, the N-terminal portion of the polymerase, and is catalyzed by the reverse transcriptase activity. During elongation of the minus strand DNA, the template RNA is degraded by the RNase H activity. Then, the fully synthesized minus strand serves as a template for plus strand DNA synthesis by the DNA polymerase activity of the polymerase. This polymerization is primed by a short RNA, which was presumably produced by the RNase H activity (Lien et al., 1986). However, this predicted replication mechanism has not been biochemically confirmed, mainly because of failure to obtain purified functional HBV polymerase or the domains containing each functional activity in large amount.

In this report, we have attempted to express human

HBV polymerase in each separate functional domains of terminal binding protein, polymerase, and RNase H in *E. coli*. Also, the expression of combined domains as well as whole polypeptide was tried. Here, we showed that the expressed reverse transcriptase containing polymerase and RNase H domains was degraded in *E. coli*, and that Lon protease is one of the responsible proteases for the degradation of the reverse transcriptase domain in *E. coli*.

Materials and Methods

Reagents

Sources were as follows: Nitrocellulose membrane was obtained from Amersham-Pharmacia; peroxidase-labeled goat anti-rabbit IgG and 4-chloronaphthol were from Biorad; restriction and cloning enzymes were purchased from Promega. Unless otherwise indicated, all other reagents were purchased from Sigma.

Bacterial strains and plasmids

The *E. coli* strains MC1061 (Meissner et al., 1987) and DH5α (Hanahan, 1983) were previously described. *E. coli* MC1061 was used for expression of functional domains of HBV polymerase and DH5α for cloning and isolation of plasmid DNAs. The BL21(*lon⁻*) , SG21118 (*clp⁻*), and SG12051(*lon⁻ clp⁻*) mutant strains were previously described (Phillips et al., 1984; Seol et al., 1995). The plasmids pBAD18 (Guzman et al., 1995) and pGEM-7Zf(+) containing replication competent

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Table 1. The primers used for HBV polymerase constructs

Primer	Sequence*	Restriction enzyme
1	CTACAGCTAGCATGCCCCTATCTTATCAAC	*Nhe*I
2	CTGATCTAGATCATTGTTCCCAAGAATATGGT	*Xba*I
3	CTACAGCTAGCGAGGACTGGGGACCCTGT	*Nhe*I
4	CTGATCTAGATCATTGCCGAGCAACGGGGTA	*Xba*I
5	CTACAGCTAGCCGGCCTGGTCTGTGCCAA	*Nhe*I
6	CTGATCTAGATCACGGTGGTCTCCATGCAAC	*Xba*I

* The translational stop codons were described as bold face.

HBV gene (subtype adw) (Blum et al., 1991) were previously described. The PCR product from pET15b containing ribosome-binding sequence (rbs) and hexa-histidine residues at the N-terminal region of the expressed polypeptide were inserted into the *Nhe*I/*Sac*I fragment of the plasmid pBAD18 and was named pBADNH.

PCR and subcloning

PCR primers for subcloning of functional domain of HBV polymerase into pBADNH were as described in Table 1. PCR products were digested by *Nhe*I/*Xba*I and inserted into *Xho*I/*Xba*I fragment of pBADNH and were confirmed by DNA sequencing (Sambrook et al., 1989).

Expression of HBV polymerase

Cells were grown in 3 ml of L broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 50 μg/ml ampicillin) overnight and then used to inoculate 30 ml of the same medium. The OD600 was monitored, and when it had reached 0.3-0.5, arabinose was added to the final concentration of 0.2%. The cells were grown for the indicated time followed by centrifugation to harvest cells.

SDS-PAGE and Western blotting

SDS-PAGE and western blot analysis using RNase H-anti serum were performed according to the methods previously described (OFarrell, 1975; Burnette, 1981).

Results and Discussion

Expression of HBV polymerase in E. coli

HBV polymerase contains the activities of terminal binding, DNA polymerase, and RNase H (Ganem and Varmus, 1987; Khudyakov et al., 1989). The reverse transcriptase activity is provided by the DNA polymerase and RNase H. Each of those activities resides at the unique locus on a single polypeptide (Fig. 1). Because the purification of active HBV polymerase in sufficient amount has not been achieved except for the RNase H domain (Wei and Peterson, 1996; Lee et al., 1997), individual domains and variable combinations of each domain were overproduced in *E. coli*. The expression vector pBADNH, which is a derivative of pBAD18 containing inducible *araB* promoter (Guzman et al., 1995), was constructed to tag hexa-histidine re-
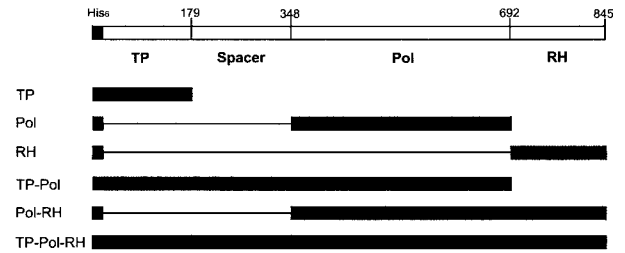

Fig. 1. Domains of HBV polymerase. The HBV polymerase ORF is represented as a rectangular box (Blum et al., 1991). At the amino terminus, the filled rectangular section represents the hexa-histidine and a few plasmid-derived amino acids. The putative domains of terminal binding protein (TP), spacer, polymerase (Pol), and RNaseH (RH) are indicated under the polymerase ORF. Each construct containing individual or combined domains, which are indicated as rectangular boxes, are described.
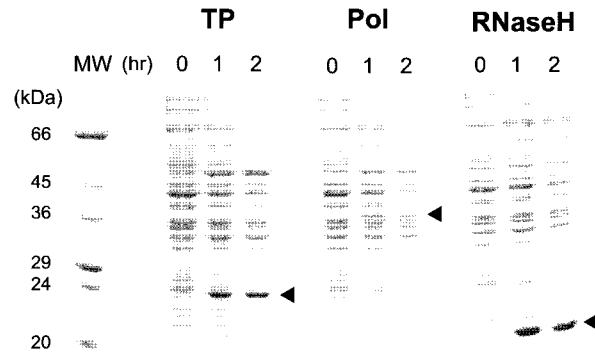
Fig. 2. Expression of the HBV polymerase domains in *E. coli* MC1061. The cells harboring recombinant plasmids that express TP, Pol or RH domain were harvested just before or at the indicated times after the addition of arabinose, boiled for 3 min, and subjected to 14% SDS-polyacrylamide gel elctrophoresis. The expressed TP, Pol, and RH domains are indicated as arrows. Molecular weight markers (MW) were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20 kDa).

sidues at the N-terminal region of expressed polypeptide to facilitate the purification of expressed protein.

The polypeptides containing each domain of terminal binding, DNA polymerase and RNase H were expressed in *E. coli* (Fig. 2). The expressed proteins exhibited the predicted molecular weights as determined in SDS-polyacrylamide gel. Majority of the expressed polypetides was found in insoluble fractions. The activity of polymerase or RNase H was not detected in insoluble or soluble fractions (data not shown). Because a functional assay was not available for the terminal binding protein, the terminal binding activity could not be detected. Denaturation and renaturation of the insoluble fractions using urea or guanidine-HCl as previously described for RNase H (Wei and Peterson, 1995) failed to recover those activities. Therefore, the polypeptides containing the terminal binding protein and RNase H domain were separately purified using Ni-affinity column and were used for preparation of polyclonal antibodies in rabbits.
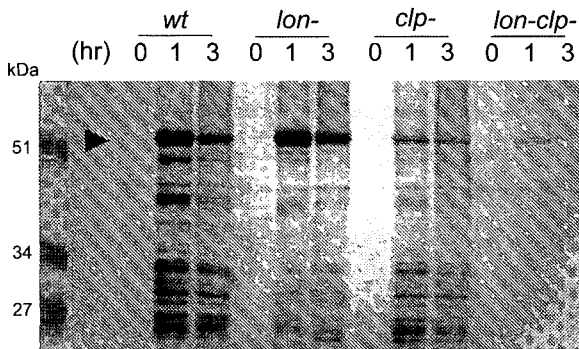
Fig. 3. Proteolysis of HBV reverse transcriptase by Lon proteases. The plasmids containing Pol-RH domain were expressed in MC1061(*lon*⁺), BL21(*lon⁻*), SG21118(*clp⁻*), or SG12051(*lon⁻clp⁻*) and subjected to 12% SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti RNase H-antiserum.

Lon protease degrades the HBV reverse trascriptase expressed in E. coli

The polypeptide containing the domains of terminal binding protein and polymerase (TP-Pol), of reverse transcriptase (Pol-RH), or of terminal binding protein, polymerase and RNase H (TP-Pol-RH) was expressed in *E. coli* MC1061, but those polypeptides were not dectectable in SDS-polyacrylamide gel electrophoresis stained with coomassie brilliant blue (data not shown). Therefore, the expression of the polypeptides was examined using western blot analysis.

The expression of reverse transcriptase (Pol-RH) was induced by the addition of arabinose into culture medium (Fig. 3). The band corresponding to the expected molecular weight of Pol-RH appeared one hour after the induction, which was detected in Western blot analysis using the antiserum raised against RNase H domain. Then, the level of the full length Pol-RH became reduced and the level of smaller sized bands increased, indicating that the expressed Pol-RHs were degraded during the induction. In a Lon protease deletion (*lon⁻*) mutant used as a host for expression, the level of the full size Pol-RH appeared to be greater than that in MC1061 (*lon⁺*) and the level of the degraded products was reduced. The expression of reverse transcriptase in a Clp protease deletion (*clp⁻*) mutant was reduced compared to that in MC1061. The significant proteolysis of Pol-RH was still observable in the *lon⁻* mutant, whereas the proteolytic products were reduced in *lon⁻clp⁻* double mutant. These results indicated that Lon protease participated in the proteolysis of reverse transcriptase expressed in *E. coli*. However, the TP-Pol and TP-Pol-RH polypeptides were barely detected even in *lon⁻* or *lon⁻clp⁻* double mutant (Data not shown).

Production of proteins in heterologous hosts through the use of recombinant DNA technology has recently brought a into focus, the problem that heterologous proteins are more prone to proteolysis (Enfors, 1992; Georgiou, 1995). This protein degradation in all living

cells is essential for eliminating abnormal proteins and for the temporal control of many cellular processes involving short-lived regulators. Most protein degradation in cells is carried out by a few large, energy-dependent proteases. In *E. coli*, Lon and Clp proteases account for up to 80% of protein degradation (Maurizi et al., 1992; Goldberg et al., 1994) with additional contributions from HslUV (also known as ClpYQ) and FtsH (Tomoyasu et al., 1995; Wawrzynow et al., 1995).

The Lon protease is essential for degradation of most abnormal proteins and certain normal short-lived polypeptides, including SulA, an inhibitor of cell division (Gottesman, 1989). Lon protease is a heat shock protease and can be induced when large amounts of abnormal proteins are produced in *E. coli* (Goff and Goldberg, 1985). Therefore, as described above, the majority of expressed HBV polymerase domains formed nonfunctional insoluble inclusion body and might be the target of proteases.

Recently, it was reported that expressed HBV polymerase was stabilized when coexpressed with human chaperone GRP94 in *E. coli* strain BL21(*lon⁻*). (Kim et al., 2000). It is possible that the absence of Lon protease as well as the coexpression of chaperone GRP94 might play a role in the purification of full sized-HBV polymerase.

HBV polymerase is a quite unstable protein, and therefore, expression and purification of the polypeptide in *E. coli* may require the N-terminal fusion of soluble protein such as MBP and GST, and coexpression of a molecular chaperone such as Hsp90 and GRP94 (Cho et al., 2000; Kim et al., 2000). Additionally, introduction of proteases *lon-clp-* mutation to the cell may increase the in vivo level of the full-length polypeptide.

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