Expression of RNase H of human hepatitis B virus polymerase in Escherichia coli

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Abstract
AIM: To amplify HBV-RNase H gene fragment and expression of RNase H for further use in the studies of HBV associated liver diseases.

METHODS: The encoding gene of HBV-RNase H was separately amplified for the first half and second half (H1 and H2) by PCR from full length HBV gene and cloned into pT7Blue-T vector. Clones were first screened by digestion with XbaI and Hind III enzyme for the correct size, and analyzed further by DNA sequencing. The RNase H1 and H2 fragments isolated from XbaI and Hind III digestion products of pT7 Blue-RNase H plasmid were ligated to the GSTag expressing vectors separately, and expressed in E.coli BL21. The expressed proteins were checked by PAGE gel and Western blot.

RESULTS: Both H1 and H2 nucleotide sequeces consisting of known genes and proteins, in correct size, were further confirmed by Western blot to be the GST and RNase H1 or H2 fusion proteins.

CONCLUSION: The successful cloning and expression of HBV-RNase H will contribute to further research and application in HBV-associated diseases.

INTRODUCTION
Human hepatitis B virus (HBV) infection has a wide range of clinical outcomes, from self-limited silent or acute infection to fulminant hepatitis. It has been estimated that over 300 million cases of chronic HBV infection exist globally[1]. In China, nearly 100 million people have a persistent infection with HBV, who are at risk of developing chronic hepatitis leading to liver cirrhosis and hepatocellular carcinoma[2-6]. Significant advances have been made during the last few years in the treatment of chronic hepatitis B[7-23]. Several new antiviral agents have been shown to be safe and effective in inhibiting HBV replication[10-20]. However, it has remained refractory to the treatment since not all patients respond properly and still there is no breakthrough results in therapeutic vaccine[21-23]. The increase of chronic hepatitis and hepatocellular carcinoma associated with the HBV infection has become a worldwide medical problem.

HBV replication is accomplished by its own polymerase. Hepadnavirus polymerases are multifunctional enzymes that play critical roles during the viral life cycle[24]. Ribonuclease H (RNase H), the HBV RNaseH domain of HBV polymerase, is one of the four domains (Terminal, Spacer, Reverse Transcriptase and RNase H) encoded by HBV polymerase gene. With 480 bp in full length and encoding a 16ku protein which is responsible for degrading RNA from RNA-DNA intermediate, HBV-RNase H plays a pivotal role in the HBV life cycle. Although the rest of HBV encoded antigen rather than RNase H has been studied in detail[23-30], there is less paper about HBV-RNase H which is intimately related with HBV replication. To explore the potential use of HBV-RNase H in the diagnosis and treatment of HBV associated liver diseases, we cloned and expressed RNase H of the HBV polymerase.

MATERIALS AND METHODS

Materials
pTKHH2 plasmid containing the full length HBV genomic DNA of subtype adw2 was kindly provided by Dr. Lingxun Duan in Thomas Jefferson University (Philadelphia PA). The primers with restriction enzyme site XbaI at 5’ end and Hind III at 3’ end used to amplify HBV-RNase H gene was synthesized in Gibco Inc. pT7Blue cloning vector, DH5α competent cells, GSTag expression vector and anti-GST antibody were products of Novagen Inc.

Plasmid construction[29,32]

Two pairs of primers were used in PCR reactions to amplify the first half (H1 1-240) and the second half (H2 241-480) fragments of RNase H gene from pTKHH2 plasmid (P1: 5’ - TTCTAGACCGCCAGGTCTGTGCAAGTGT-3’; P2: 5’ - AAGGCTAGCATGCGAGGCAGCACAAATCGTAAT-3’ for H1; and P3: 5’ - TTCTAGACA TTCTGCCCGGAGGCAGCTCCTTGT-3’; P4: 5’ - AAGCTTAGTGGGCTTGTCCTCTGTCCCAAGAC-3’ for H2). The amplified H1 and H2 fragments were purified on a 15 g/L low-melting agarose gel, utilizing the PCR purification system (Promega Inc). The purified PCR products were directly ligated into the pT7 Blue-T vector respectively. After transformation of the Escherichia coli strain DH5α, recombinants were selected on x-Gal plates. Ten white colonies were selected for minipreparation and the insert evaluation was by enzyme digestion and DNA sequencing. The plasmids with proper inserts were recut with XbaI and HindIII enzyme and ligated into pGSTag vector. The pGSTag containing H1 or H2 fragment were transformed into BL21 Escherichia coli strain and propagated in LB medium.

Expression[33] and identification of HBV-RNaseH
Picked the recombinant colonies and grew them overnight at 37 °C in 3 mL of LB medium. Removed 1 mL culture and
inoculated into 100 mL of fresh LB medium and grew at 37 °C to an A600 of 0.6. Added 100 mmol/L IPTG to the bacterial culture to a final concentration of 0.3 mmol/L and incubated the culture for an additional 3-4 h. Pelletized the cells by centrifuging at 12 000 g for 3 min and resuspending the bacterial pellet in 3 mL lysis buffer. After the bacteria lysed by ultrasonic machine, the fusion proteins were purified with glutathione Sepharose 4B column and identified by PAGE gel stained with Coomassie blue and further confirmed by Western blot.

RESULTS

Recombinant expression vectors of pGSTag containing Cloned HBV RNase H1 and H2 fragments were identified by restriction enzyme digestion and DNA sequencing and the results showed that the inserted DNA fragment were expected known sequences. The expressed proteins could be seen in Coomassie blue stained PAGE gel with 34 ku band (Figure 1) which were further confirmed by Western blot as GST and HBV RNase H fusion protein.

DISCUSSION

Genome replication of hepadnavirus proceeds by reverse transcription from a viral pregenomic RNA template by a virally encoded polymerase, a polypeptide of 90 to 97 ku[34]. The genome of all hepadnaviruses has the open reading frame, the polymerase gene, and the product or products of this polymerase gene are involved in multiple functions of the viral life cycle. These functions include a priming activity which initiates minus-strand DNA synthesis, a polymerase activity which synthesizes DNA by using either RNA or DNA templates (reverse transcriptase), a nuclease activity which degrades the RNA strand of RNA-DNA hybrids (RNase H), and involvement in packaging the RNA pregenome into nucleocapsids[35].

Molecular genetic studies have revealed that the human HBV polymerase protein, a polypeptide of about 94 ku, which plays a critical role in the HBV life cycle, contains four domains. These are the 5' terminal protein (TP), spacer, RNA reverse transcriptase (RT)/DNA polymerase, and RNase H, respectively, from the amino (N) to carboxy (C) terminus[36-42]. All of the TP and RT and RNase H, rather than spacer protein, play an important role in the process of HBV replication. The RNase H, as it functions in all other retroviruses, can degrade the RNA of DNA-RNA hybrid which plays a role in optimizing the priming of minus-strand DNA synthesis[43]. We had constructed an expression vector containing full length of HBV RNase H DNA and we failed to propagate the transformed bacteria, which might be caused by the toxic effect of RNase H activation as Lee Yi reported[44] earlier. So we redesigned two pairs of primers to clone and expressed with two separate fragments of the RNase H. The successfully expressed RNase H and its function which will be performed in our next experiment will contribute to further generating anti-RNase H antibody producing hybridoma cells for the purpose of HBV related liver diseases gene therapy. It has been confirmed that RNase H can be used as a marker to reflect the low level virus replication without other positive markers[45], so to investigate and analyze the results of anti-HBV RNase H might be able to provide a new marker for the early diagnosis of HBV infection and to assess the effects of clinical therapy of HBV infection diseases.

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Figure 1 PAGE Electrophoresis stained with Coomassie brilliant blue light. 1: Protein MW standards; 2: RNaseH1 protein (34 ku); 3: RNaseH2 protein (34 ku).
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