Inhibition of Hepatitis B Virus Replication by *in vitro* Synthesized RNA

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Human hepatitis B virus (HBV) is a pathogen related to the development of liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). However, the efficient methods to suppress HBV replication have not been developed yet. Therefore, we have used RNA interference (RNAi) as a potential tool for the suppression of HBV replication. Here, we designed a 21 nt small intefering dsRNA (siRNA) against hepatitis B virus X (HBx) RNA with 3′ overhanging ends derived from T7 promoter. It has been reported that HBV X protein plays an important role in HBV gene expression and viral replication. The suppression of HBx gene expression by the 21 nt siRNA was investigated by Northern blot analysis and chloramphenicol acetyl transferase (CAT) assay. The level of HBx mRNA was decreased by siRNA in a dose-dependent manner. We also found that the 21 nt siRNA inhibited the HBV replication in hepatocellular carcinoma cell.

Key Words : Hepatitis B virus, HBx RNA, siRNA, HBV replication, New therapeutic agent

Introduction

HBV is the most important human pathogen associated with liver diseases including hepatitis, cirrhosis, and hepatocellular carcinoma.[1] HBV chronically infects about 400 million individuals worldwide and 10% of HBV infected people have chronic hapatitis which causes 2 million deaths each year due to cirrhosis or primary liver cancer. However, the efficient methods to suppress HBV replication are not developed yet.

HBV has a partially double strand DNA genome of 3.2 kb and replicates its DNA genome by reverse transcription of pregenomic RNA. HBV genome contains four overlapping open reading frames encoding the viral surface protein, core protein, polymerase, and X protein.

HBx is a promiscuous multifunctional protein associated with viral replication[2] and development of HCC in patients who are chronically infected with hepatitis B virus.[3] HBx is a transcriptional transactivator of various viral and cellular promoters including all the HBV promoters, SV40 promoter, HIV LTR, c-myc and c-jun promoters.[4-6] Since HBx does not bind DNA directly, HBx transactivates various promoters by interaction with other cellular proteins. HBx activates the signal transduction pathways including protein kinase C[7] and Ras-Raf-MAP kinase[8] which affect several cellular processes including proliferation and differentiation. HBx also interacts with p53 protein[9] and inhibits the activity of p53.[10] HBx can sensitize p53 mediated apoptosis[11] and enhance the apoptosis induced by TNF-α[12] and UV irradiation.[13] The oncogenic potential of HBx is suggested by the observation of HCC in HBx transgenic mice[14] and the oncogenic transformation of cells expressing HBx in culture.[15] Therefore the specific repression of HBx gene expression may be a good strategy to block the viral replication and the progression of liver diseases caused by HBV infection.

RNAi is a sequence specific post transcriptional gene silencing in which long mRNA is rapidly degraded by siRNA that is homologous to the mRNA.[16-18] RNAi is a multistep pathway associated with RNA endonuclease. By RNA endonuclease complex, long dsRNA is cleaved into 19 nt to 23 nt siRNAs which induce sequence specific degradation of target mRNA by RNA-nuclease complex.[19]

At first RNAi had become a valuable experimental tool for investigating gene function in *Caenorhabditis elegans*.[20] Although in most mammalian cells long dsRNA provokes a non specific cytotoxic response,[21] it has been shown that 21-22 nt siRNAs specifically suppress the expression of target genes without nonspecific cellular response.[22] It was also reported that 21-22 nt siRNAs do not activate the dsRNA-inducible interferon system in mammalian cells. Thus, RNAi using siRNA could become not only a major tool for reverse genetics,[23] but also a valuable therapeutic method in mammalian system.[24] Once the mechanism of RNAi is better understood, it may become a powerful technology to apply in any biological systems.

To suppress the HBx gene expression, we designed and tested siRNA derived from the T7 promoter against HBx mRNA. Using the 21 nt siRNA targeting HBx mRNA, the suppression of HBx gene expression was investigated by Northern blot analysis and CAT assay. The level of HBx mRNA was repressed specifically by siRNA in a dose-dependent manner. The level of repression of transactivation activity of HBx by siRNA is much higher than that by antisense single-stranded RNA. We also found that the 21 nt siRNA inhibited the HBV replication in hepatocellular carcinoma cell suggesting that siRNA against HBx RNA may be a new therapeutic agent for chronic hepatitis patients.

Experimental Section

Plasmids and cells. pRSV/X contains the HBx open

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reading frame in HindIII/XbaI sites of the eukaryotic expression vector pRc/RSV.[25] HBV infectious plasmid pcHBV2 contains two copies of HBV genome concatenated in pcDNA3. Human hepatoma HepG2 cell, human liver Chang cell, and HBV particle producing 2.2.15 cells were used for the experiments.

Target design and siRNA synthesis. The HBx target region of siRNA (nt 294-nt 318) was selected by analyzing the secondary structures of HBx mRNA by RNA mfold program (Rensselar Polytechnic Institute). To produce 21 nt siRNA with 2 bp of 3′ overhang ends by T7 RNA polymerase reaction, template DNA oligonucleotides containing T7 promoter sequence in front of each strand of HBx target region were synthesized. The sequence of T7 promoter oligonucleotide is 5′-TAATACGACTCACTATA-3′, sense RNA template is 5′-GACTCCAGCGATGTCAACGATAT-AGTGAGTCGTATTA-3′, and antisense RNA template is 5′-GGTCGTTGACATCGCTGAGAGTATAGTGAGTCGTATT A-3′. To prepare siRNA template for T7 polymerase reaction, the oligonucleotides of T7 promoter and each strand of HBx target were denatured by heating at 95 °C for 2 min and annealed slowly at room temperature. *In vitro* transcription reaction was carried out in a final volume of 200 pmol of each annealed template oligonucleotide, 1 X transcription buffer, 10 mM DTT, 20 U of ribonuclease inhibitor, 1.25 mM dNTPs, DEPC-water, and 20U of T7 RNA polymerase at 37 °C followed by RNase free DNase I digestion. The RNA transcripts were extracted with equal volume of phenol/chloroform, precipitated with ethanol, washed in 70% ethanol, and dissolved in DEPC-water. Sense RNA and antisense RNA were denatured in 10 mM Tris-HCl buffer containing 20 mM NaCl and annealed at room temperature. For control siRNA, 21 nt siRNA against herpes simplex virus type 1 ICP27 mRNA was designed.

The northern blot analysis of HBx RNA. HepG2 cells and Chang cells were plated at the density of 7.5×10^5 cells per 60-mm culture dish. pRSV/X and siRNA were transfected 24 hr after plating by lipofectin (GIBCO BRL). Cells were washed twice with phosphate-buffered saline (PBS) 7 hr after transfection and cells were fed with normal media. 48 hr after transfection, RNAs were prepared from cells with RNAzolB solution (Tel-Test Inc.). The RNAs were extracted with equal volume of phenol/chloroform, precipitated with ethanol, and dissolved in DEPC-water. The total RNAs were separated by electrophoresis in 1% formaldehyde-agarose gel and transferred to nylon membrane (Amersham). The membrane was prehybridized and hybridized using [^{32}P]-labeled HBx specific DNA probe prepared by random-primer labeling kit (Stratagene). The membrane was analyzed by autoradiography and quantified by instant imager.

CAT assay. HepG2 cells were cotransfected with reporter pSV2CAT plasmid, pRSV/X, and siRNA by calcium phosphate method. Cells were harvested 48 hr after transfection and cell extracts were prepared for CAT assay. CAT activities were detected by thin-layer chromatographic separation of [^{14}C] chloramphenicol from its acetylated derivatives and quantified by instant imager.

The quantitative analysis of HBV replication. HepG2 cells (1×10^6 cells per 100-mm dish) were cotransfected with HBV infectious plasmid pcHBV2 and siRNA by calcium phosphate method. Cells were washed with cold PBS 5 days after transfection and resuspended in 1ml of lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40) at 37 °C for 15 min. Cell lysates were centrifuged at 12,000 g for 30 min to remove nuclei and other insoluble materials. The supernatant was adjusted to 10 mM $Mg(OAc)_2$ and 20 U/mL DNase1, and incubated at 37 °C for 2 hr. After DNase1 treatment, the reaction mixture was centrifuged at 12,000 g for 5 min. The supernatant was overlayered onto sucrose buffer (20% sucrose in 20 mM Tris-HCl pH 8.0, 150 mM NaCl) and ultracentrifuged for 45,000 rpm at 20 °C for 3 hr. The virus pellet was resuspended in protease digestion buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS) containing 1 mg/mL protease K and incubated at 37 °C for 20 hr. After protease reaction, the mixture was extracted twice with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. Purified viral DNAs were separated on 1% agarose gels and transferred to nylon membranes. The membrane was hybridized to the [^{32}P]-labeled HBV 3.2 kb genomic DNA probe and analyzed by autoradiography.

Results

Design and synthesis of siRNA against HBx RNA. Since HBx protein plays important roles in HBV replication and hepatocellular carcinogenesis, the specific suppression of HBx gene expression is valuable in developing a new strategy to treat hepatitis and HCC progression. Therefore we decided to design and develop a siRNA that could specifically inhibit the HBx gene expression. To select the target region of siRNA against HBx RNA, the secondary structure of HBx RNA was predicted by mfold program. The putative target region was determined on the base of accessibility toward siRNA and the stability of structure.[26] The selected target site is located between nt 296 and nt 318 in HBx open reading frame. The target site of siRNA is located within stem regions in all the possible HBx RNA secondary structures and it is expected that siRNA can hybridize the target site easily.

To make DNA templates of 21 nt siRNA *in vitro*, the 17 bp oligonucleotide of T7 promoter is annealed to each 38 bp oligonucleotide containing the complementary sequence of T7 promoter in front of the target HBx sequence (Figure 1). By *in vitro* T7 RNA polymerase reaction, two complementary RNA strands were transcribed from two different DNA templates. The transcribed RNAs are 21 nt single stranded RNAs containing 19 bp HBx sequences that are complementary with each other and 2 bp protruding sequence at 3′ ends. Both sense RNA and antisense RNA were annealed to make siRNA against HBx RNA.

The siRNA could specifically block the HBx gene expression. RNAi interferes a gene expression post-

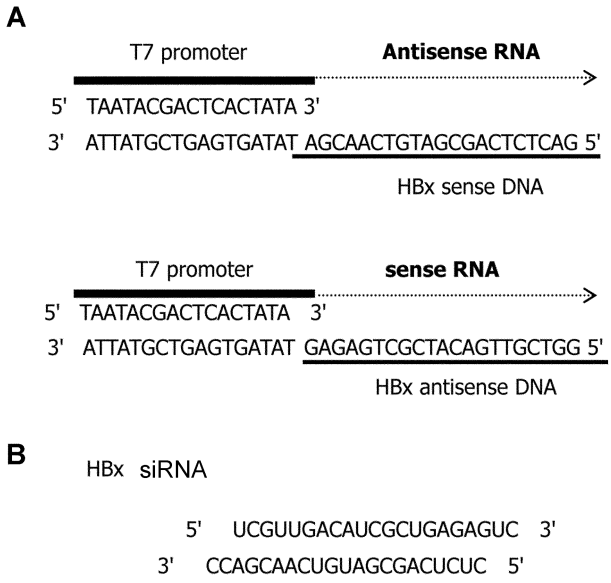

Figure 1. Schematic representation of the siRNA against HBx mRNA. A: DNA oligonucleotide template for HBx siRNA consists of T7 promoter and the sequence of target gene. B: HBx siRNA was prepared by annealing of each RNA strand synthesized by *in vitro* transcription reaction with T7 RNA polymerase.

transcriptionally through mRNA degradation. Thus, to test whether the designed siRNA could suppress HBx gene expression or not, we transfected the HBx expression plasmid pRSV/X along with siRNA into HepG2 cells. Total RNAs were prepared 48 hr after transfection and the amount of HBx mRNA was determined by Northern blot analysis using [^{32}P]-labeled HBx probe. When the various amounts of HBx siRNA were introduced into HepG2 cells transiently, the levels of HBx mRNA expression were dramatically reduced in a dose-dependent manner (Figure 2). In addition, the siRNA against HBx had no interference on endogenous gene expression of glyceraldehyde 3 phosphate dehydrogenase (GAPDH). However, even the large amount of control HSV-1 ICP27 siRNA did not suppress the mRNA expression of HBx at all (Figure 3). Now it is evident that the siRNA against HBx mRNA is strongly suppressing the gene expression of HBx specifically in human hepatoma HepG2 cell line. To test the suppression of HBx expression

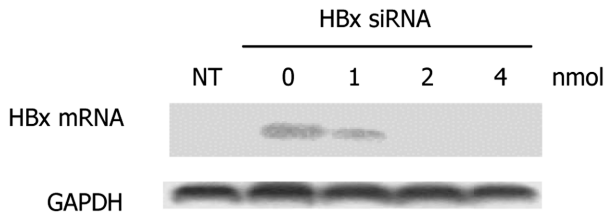

Figure 2. Silencing of HBx gene expression by the HBx siRNA in HepG2 cells. The HBx expression plasmid pRSV/X (5 μg) and various amounts of siRNA (1, 2, 4 nmol) were cotransfected into HepG2 cells. 48 hr after transfection, total RNAs were harvested and analyzed by Northern hybridization using [^{32}P] labeled HBx probe. NT is nontransfected HepG2 cells. The RNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an internal control of Northern blot analysis.

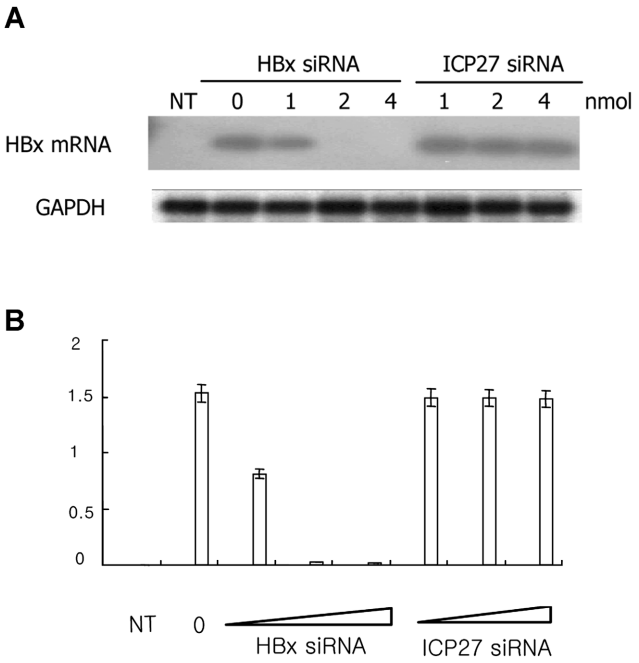

Figure 3. Specific inhibitory effect of the HBx siRNA on HBx gene expression in HepG2 cells. A: The HBx expression plasmid pRSV/X (5 μg) with either HBx siRNA (1, 2, 4 nmol) or ICP27 siRNA (1, 2, 4 nmol) were cotransfected into HepG2 cells. 48 hr after transfection, total RNAs were harvested and analyzed by Northern hybridization using [^{32}P] labeled HBx probes. The RNA of GAPDH is an internal control. B: The levels of HBx RNA were quantitatively evaluated by image analysis software. The data were quantitated from several independent experiments.

by siRNA in a different cell line, the same experiments were carried out in human Chang liver cell that is established from non-malignant tissue. In Chang cell, siRNA also suppressed the HBx gene expression very efficiently as in HepG2 cell (Data not shown).

The inhibitory effect of siRNA on the transactivation function of HBx. Since we identified that the HBx siRNA synthesized *in vitro* could inhibit HBx gene expression, we tested the inhibitory effect of HBx siRNA on the biological function of HBx protein using CAT reporting system. It has been reported that HBx protein is a strong transcriptional transactivator of various kinds of cellular and viral promoters including all the HBV promoters and SV40 early promoter. As we expected, when the reporter plasmid pSV2CAT containing SV40 promoter was transfected into HepG2 cell along with pRSV/X, HBx protein transactivated the CAT expression from SV40 early promoter[27] (Figure 4, lanes 1 and 2). When the siRNAs were introduced, the CAT activities elevated by HBx were significantly reduced suggesting that siRNA could inhibit the transactivation function of HBx protein (Figure 4, lanes 3 and 4). Antisense RNA is another way to suppress the gene expression specifically by blocking the translation of target RNA.[28] To compare the inhibitory efficacy of siRNA with antisense RNA, we transfected antisense RNA against HBx with pRSV/X and tested the transactivation activity of HBx protein. It turned out that antisense RNA exhibited less

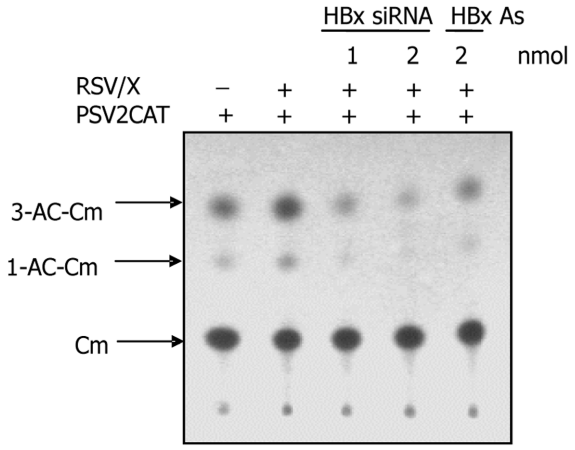

Figure 4. Effects of the HBx siRNA on transactivation activity of HBx in HepG2 cells. The reporter CAT plasmid, pSV2CAT (1 μg), and the HBx expression plasmid RSV/X (2 μg) were cotransfected into HepG2 cells with either the HBx siRNA (1, 2 nmol) or the antisense RNA against HBx (2 nmol). Cm, chloramphenicol; 1-AC, 1-acetyl chloramphenicol; 3-Ac, 3-acetyl chloramphenicol.

inhibitory effect on the transactivation function of HBx than the siRNA against HBx (Figure 4, lane 5). Now, it is very clear that siRNA is a much more powerful method to suppress the specific gene expression than antisense RNA in human liver cells.

The replication of HBV was effectively blocked by siRNA. Since the siRNA could strongly repress the HBx gene expression specifically, we tested the inhibitory effect on HBV viral replication by the siRNA against HBx. The infectious HBV plasmid, pcHBV2 and HBx siRNA were transfected into HepG2 cell and the HBV virus particles were purified from each cell lysate by ultracentrifugation in the sucrose solution. The HBV genomic DNA was prepared from the virus particles and identified by Southern blot analysis using HBV genomic DNA probe (Figure 5). We found that when various amounts of HBx siRNA were introduced into HepG2 cells along with pcHBV2, the yield of virus particles was dramatically reduced (Figure 5). However the siRNA against HSV-1 ICP27 did not reduce the yield of HBV production at all (Figure 5, lanes 7-10). When we introduced either 4 nmol or 8 nmol of HBx siRNA into HepG2 cell, the HBV replications were completely inhibited by HBx siRNA (Figure 5, lanes 5 and 6). These results suggested that the siRNA against HBx mRNA could be used as a new therapeutic agent for the treatment of chronic hepatitis and hepatocellular carcinoma.

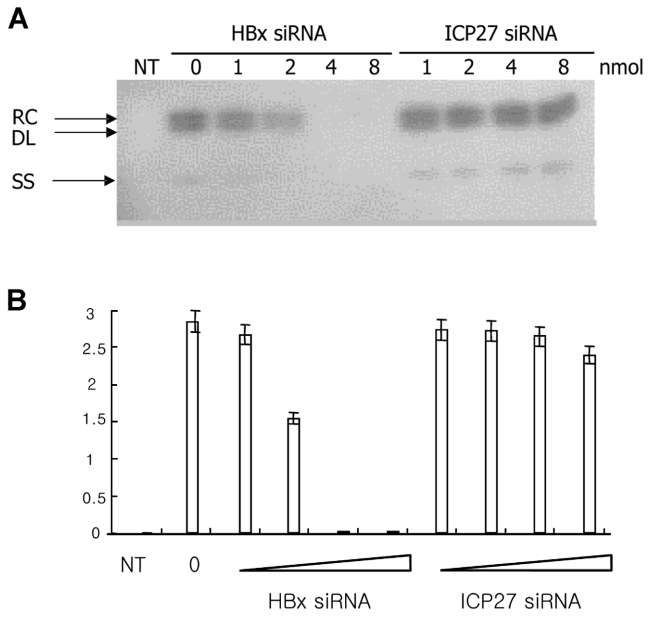

Figure 5. Inhibition of HBV replication by the HBx siRNA. A: The HBV infectious plasmid pcHBV2 (5 μg) with either the HBx siRNA (1, 2, 4, 8 nmol) or the ICP27 siRNA (1, 2, 4, 8 nmol) were introduced into HepG2 cells. 5 days after transfection, cells were harvested and intracellular HBV particles were prepared. The amount of viral genomic DNA in virus particles was identified by Southern blot analysis using [^{32}P]-labeled 3.2 kb HBV genomic DNA. RC: relaxed circular DNA, DL: double stranded linear DNA, SS: single stranded DNA. B: The levels of HBV DNA were quantitated from several independent experiments.

Discussion

In this study we tested the specific effect of siRNA against HBx upon the HBx gene suppression, the transactivation function of HBx and the HBV replication in human liver cells. Since HBx is a multifunctional protein which is essential for the HBV replication and the HBV-associated liver diseases, the specific suppression of HBx gene expression may be a good strategy to block the progression of liver diseases caused by HBV infection.

We designed the template DNAs of siRNA against HBx mRNA derived from the T7 promoter. We produced the 21 nt siRNA containing 2 bp overhang at 3′ end by *in vitro* T7 RNA polymerase reaction. By transient transfection using lipofectin, we could simply introduce siRNA into human liver cells. We found that the HBx gene expression was strongly suppressed specifically by siRNA synthesized *in vitro*. We also tested the effect of siRNA on the transactivational property of HBx using CAT reporting system. HBx siRNA exhibited a stronger suppression of transactivation activity of HBx protein compared with antisense RNA. At first it could be speculated that antisense RNA might suppress the function of target protein more efficiently than siRNA, since antisense RNA inhibits the gene expression by interfering the translational machinery. However it turned out that siRNA is a much better way to inhibit the specific gene expression in human liver cells, suggesting the enzymatic degradation of target RNA associated with siRNA is a very efficient way to repress the specific gene expression. Since HBx protein is important for the viral replication, we carried out the quantitative analysis of inhibitory effect of siRNA upon the HBV production. HBx siRNA reduced the HBV replication severely, suggesting that HBx gene is a good target for the gene therapy of HBV related diseases.

For the chronic hepatitis patients, the high expression of

HBx gene causes more severe liver diseases including cirrhosis and HCC. Accordingly specific suppression of HBx gene expression is inevitable for the treatment of chronic hepatitis. For the development of gene therapy, the safety of agents should be considered in addition to the efficiency.[29] Recently in vivo expression system of siRNA using viral vector is popularly used to repress the specific gene expression.[30] However, a subset of siRNA vectors have disadvantageous limitation due to the insertional mutagenesis in host genome.[31] *In vitro* synthesized siRNA targeting HBx has a tremendous inhibitory effect compared with siRNA vectors in HBV replication.[32,33] Therefore, we should consider using siRNA product synthesized *in vitro* as a reliant therapeutic agent.

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