INHIBITION OF HEPATITIS B VIRUS REPLICATION BY RECOMBINANT SMALL INTERFERING RNAs

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Summary. – RNA interference (RNAi) is a biological phenomenon in which introduction of a small, double-stranded interfering RNAs (siRNAs) into a cell causes a specific degradation of homologous single-stranded RNA. siRNA can be delivered into the cell by different approaches including synthetic RNA, *in vitro* transcribed RNA and RNA transcribed from polymerase III-based recombinant vectors. As hepatitis B (HB) represents a worldwide health problem, we attempted to develop a fast and easy approach to generation and screening of specific siRNA-targeted HB virus (HBV) genes. Using PCR amplification, specific siRNA expression cassettes (SECs) were developed and used to generate effective siRNAs against HB virus (HBV) replication and gene expression in mammalian cells. After screening, we identified two SECs that expressed siRNAs which efficiently decreased the level of HBV *pre-c/c* gene expression in transfected Bel-7402 cells by 81.9% and 87.3%, respectively. In addition, the level of HBV DNA was decreased by 83.5% and 85.2% in HepG2 2.2.15 cells, respectively. This study provides (i) a new effective application of RNA interference to study viral gene function and viral replication and (ii) a new tool for the prevention and treatment of human HBV infection.

Key words: Hepatitis B virus; RNA interference; siRNAs; HBV replication; HBV DNA replication

Introduction

HBV infections cause in humans a chronic liver disease, liver cirrhosis and hepatocellular carcinoma, and remains the most common and most serious disease worldwide. Because most current treatments cannot resolve the viral infection, discovery of an alternative therapy is essential. RNAi is a new approach used in the study of gene function and has a potential application to the treatment of viral infections. In eukaryotic cells, it is a natural process by which a dsRNA initiates and directs the degradation of homologous mRNA (Hannon, 2002). This RNA silencing mechanism has been first described in *Caenorhabditis elegans* and *Drosophila melanogaster* (Fire, 1998). It has many similarities to the post-transcriptional gene silencing in plants. A specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21–23 nucleotides long siRNAs (Elbashir, 2001; McCaffrey, 2002) or by the transcription of siRNAs from recombinant DNA vectors by RNA polymerase (SECs) (Brummelkamp, 2002). These findings have open up a brand-new field for the analysis and control of the processes of gene expression, and perhaps pathogen infection either.

The replication of a growing number of human pathogenic viruses in cell culture was shown to be inhibited by RNAi, including poliovirus (Coburn, 2002), HIV-1 (Jacque, 2002; Lee, 2002), Flock house virus (Li, 2002), Rous sarcoma virus (Hu, 2002), Dengue virus (Adelman, 2002), Hepatitis C virus (Kapadia, 2003), influenza viruses (Ge, 2003), Hepatitis B virus (HBV) (Hamasaki, 2003; Marion, 2003; Shlomai, 2003), human papilloma viruses (Jiang, 2002). Recently, it has been reported that RNAi can

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Abbreviations: HB = hepatitis B; HBV = HB virus; siRNAs = small interfering RNAs; RNAi = RNA interference; SECs = specific siRNA expression cassettes

also induce transcriptional silencing of severe acute respiratory syndrome (SARS) coronavirus (He, 2003). In most above studies, synthetic 21 nt-long siRNAs were applied. However, vector-based RNAi techniques in which each vector expresses a unique siRNA that can degrade a specific target have been used more frequently in recent studies.

To date, in general, one of four siRNAs designed is really effective and predicting the effectiveness of a given siRNA in suppressing the expression of its target gene is not reliable. Therefore, a screening of effective and specific siRNAs is necessary. In this study, we developed a relatively fast and easy approach for the generation effective siRNAs that could inhibit HBV replication efficiently.

Materials and Methods

Cells and transfection. Two human hepatoma cell lines, Bel-7402 and HepG2.2.2.15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% of heat-inactivated fetal bovine serum at 37°C in 5% CO_2. HepG2.2.2.15 cells were stably transfected with a head-to-tail dimer of genomic HBV (subtype ayw) DNA. The cells were plated at a density of 1–4 x 10^5 cells per well of 24-well or 6-well microplates, reaching an approx. 60% confluency at the time of transfection. The cells were transfected with 0.1 μg of pCMV-Tag-*pre-c/c* or 0.4 μg of pEGFP-N3 (both from Clonetech) together with 0.45 μg or 1.2 μg of a specific SEC, using oligofectaime (Sofast™, Xiamen Sunma Biotechnology Co. Ltd, P.R. China.) according to the manufacturer's instructions. The cells were harvested 48 hrs after the transfection.

Screening of RNAi target sites. Five target sites on HBV genome for RNAi were selected by using an on-line tool from Ambion Co. (http://www.ambion.com/techlib/misc/siRNA_tools.html), namely $HBVE_1$siRNA (5'-GTTCACCTCACCATACTGC-3', nt 2045–2063), $HBVE_2$ siRNA (5'-GGTGTTAATTTGGAAGATC-3', nt 2118–2136), $HBVE_3$ siRNA (5'-GCAACTCTTGTGGTTTCAC-3', nt 2196–2214), $HBVE_4$ siRNA0 (5'-GAAACTACTGTTGTTA GAC-3', nt 2335–2353) and $HBVE_5$siRNA: (5'-GTCTCAATC GCCGCGTCGC-3', nt 2403–2421) (Fig. 1A). These sequences were subjected to a BLAST search for human genome sequences to ensure human genes were not targeted. The sequence of siRNA 5'-GGCTACGTCCAGGAGCGC-3' targeted the green fluorescent protein (GFP) gene used as control (Hamasaki, 2003).

Construction of specific siRNA expression cassettes (SECs). A PCR was performed using a plasmid containing the human U6 promoter as template. The U6 upstream 5'-primer C, complementary to the 35 nts at the 5'-end of the U6 promoter (5'-AATTCCCC-CAGTGGAAAGACGCGCAGGCAAAACGC-3') was used. Two different 3'-primers, namely primer A that contained a 19-nt sense sequence corresponding to the mRNA of *pre-c/c* gene (5'-CAAA AACTGTAAAAAN$_{19}$GGTGTTTCGTCCTTTCCACAAGA-3') and primer B that contained a 19-nt antisense sequence complementary to the primer A (5'-CAAAAACTGTAAAAAN$_{19}$GGTG TTTCGTCCTTTCCACAAGA-3') were employed. Both 3'-primers contained a 57-nt sequence consisting of three parts: a region binding to the U6 promoter, a region containing sequences transcribed into one strand of siRNA, and a U6 terminator sequence. Two PCRs were performed with primer pairs A+C and B+C, respectively and pSilencer-2.1-U_6 (Ambion) as template in 35 cycles as follows: 94°C/1 min, 60°C/1 min and 72°C/1 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and stored at -20°C until use (Fig. 1B). In this way, five different SECs were prepared: $HBVE_1$siRNA, $HBVE_2$siRNA, $HBVE_3$siRNA, $HBVE_4$siRNA and $HBVE_5$siRNAi (Fig. 1B).

Construction of a vector with luciferase gene. The *pre-c/c* gene was cloned into the *Hin*dIII and *Sac*I sites of the pCMV-Tag2A vector (Stratagene) to produce pLucF-*pre-c/c*. Two primers, 5'-GA TATGAGCTCATGCAACTTTTTCACC-3' (sense) and 5'-CTTA TGGTACCACATTGGGAAGCTGGAG-3' (antisense) were used to amplify and clone the *pre-c/c* gene into *Sal*I and *Bgl*II sites of pLucF, a vector carrying the luciferase reporter gene (Fig. 1C).

Luciferase assay. Cells were washed with PBS and lysed with a Luciferase Cell Culture Lysis Reagent (Promega). Ten μl of the cell lysate and 100 μl of a luciferase assay substrate (Promega) were mixed and the light intensity was detected by a luminometer (Turner Design). The assays were performed in triplicate and expressed as means ± SD in % of the vector control.

HBeAg assay. The level of HBeAg in media from cultures of transfected cells was determined by using a diagnostic kit for HBe antigen ELISA according to the manufacturer's instructions (Shanghai Kehua Biotech Co., Ltd., Shanghai, P.R. China). The results from 50 μl of the media in triplicate were normalized to the number of cells per well.

RNA isolation and RT-PCR. Total RNA was extracted from transfected cells by Trizol reagent according to the manufacturer's instructions (Invitrogen). The RT step was performed with total RNA as template, using random primers reverse transcriptase and other components of reaction mixture provided by TaKaRa Biotechnology Co., Ltd. (Dalian, P.R. China). The PCR step was performed with cDNA using the *pre-c/c*-specific primers 5'-ATCTT CTTGTTGGTTCTTCTGGAC-3' (sense) and 5'-CAAACAGTGG GGGAAAGCC-3' (antisense), the ß-actin-specific primers 5'-CTG GGGCGCCCCAGGCACCA-3'(sense) and 5'-CTCCTTAATGT CACGCACGATTTC-3' (antisense), polymerase and other components of reaction mixture provided by TaKaRa Biotechnology Co., Ltd. (Dalian, P.R. China). The PCR ran in 25 cycles consisting of 94°C/1 min, 60°C/1 min, 72°C/1 min and 72°C/10 mins.

Assay of HBV DNA by real-time PCR. To assay the effectiveness of a siRNA on HBV replication in cells, intracellular HBV DNA was extracted as described earlier (Pugh, 1988). Briefly, 1× 10^5 cells were lysed, pelleted and $MgCl_2$ was added to the supernatant. The HBV DNA not protected by virion core was degraded with DNase I (Invitrogen). The lysates were treated with proteinase K and extracted with phenol/chloroform. HBV DNA was ethanol-precipitated and assayed using a real-time PCR kit (PG BIOTECH, P.R. China). In the assay, performed according to the manufacturer's instructions, the primers P1 (5'-ATCCTGCTGC TATGCCTCATCTT-3', nt 412–434) and

P2 (5'-ACAGTGGGGAAAGCCCTACGAA-3', nt 703–725) and the probe 5'-TGGCTAGTTTACTAGTGCCATTTTG-3' (nt 670–694) were employed. The PCR products were analyzed using a PE Gene Amp 7700 (Perkin Elmer, USA).

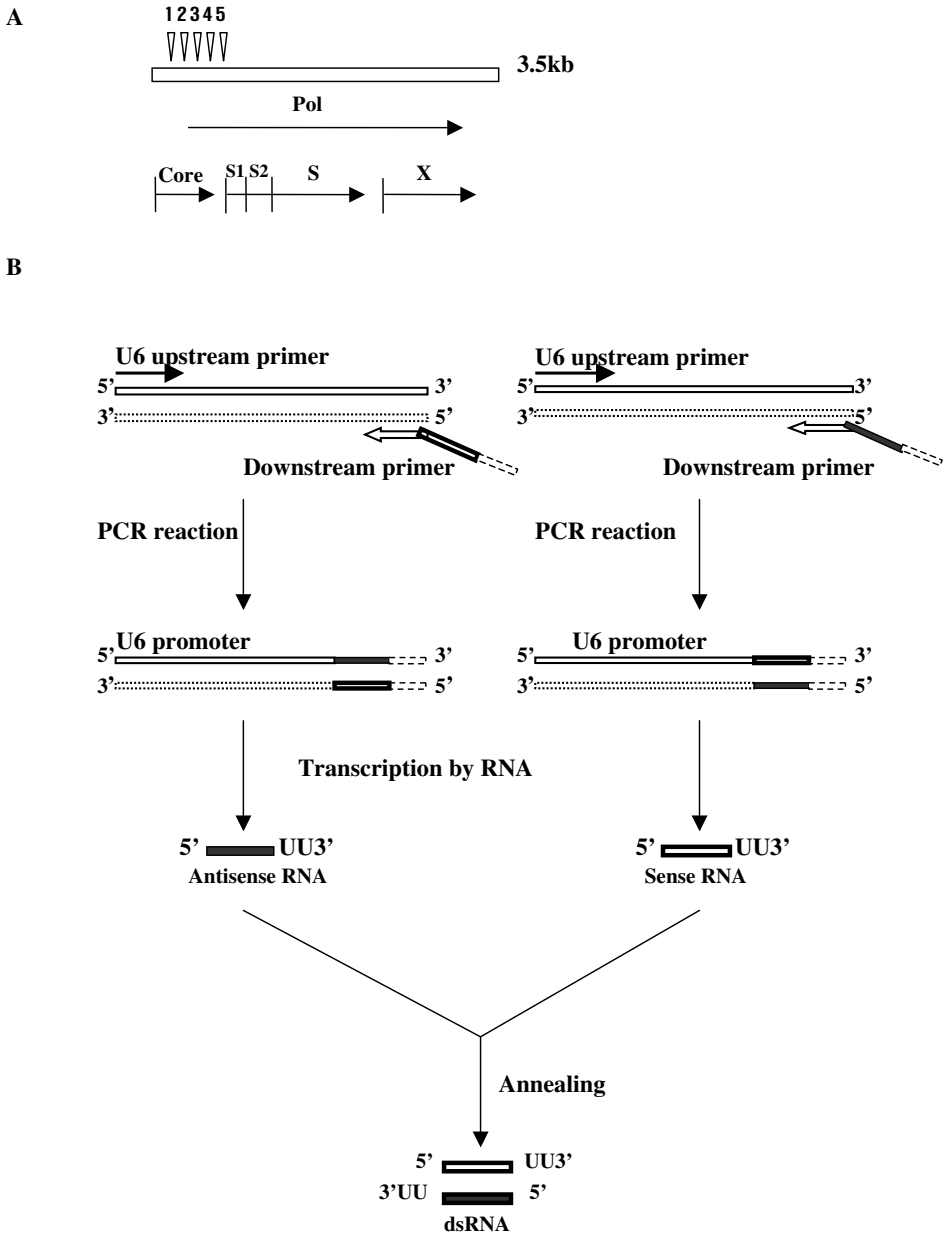

Fig. 1

siRNA target sites on HBV genome, construction of SECs, expression of siRNAs and the fusion gene in the reporter vector

A. Locations of the RNAi target sites 1–5 (arrows) on HBV genome and the structure of pre-genomic HBV RNA. siRNA target sites: 1 = $HBVE_1$siRNA; 2 = $HBVE_2$siRNA; 3 = $HBVE_3$siRNA; 4 = $HBVE_4$siRNA; 5 = $HBVE_5$siRNA. Pol = polymerase; core = HBcAg; S1 = large pre-S antigen; S2 = middle pre-S antigen; S = HBsAg; X = HBx gene. B. Construction of SECs and expression of siRNAs. C. The fusion HBV *pre-c/c*-luciferase gene with CMV promoter in the reporter vector.

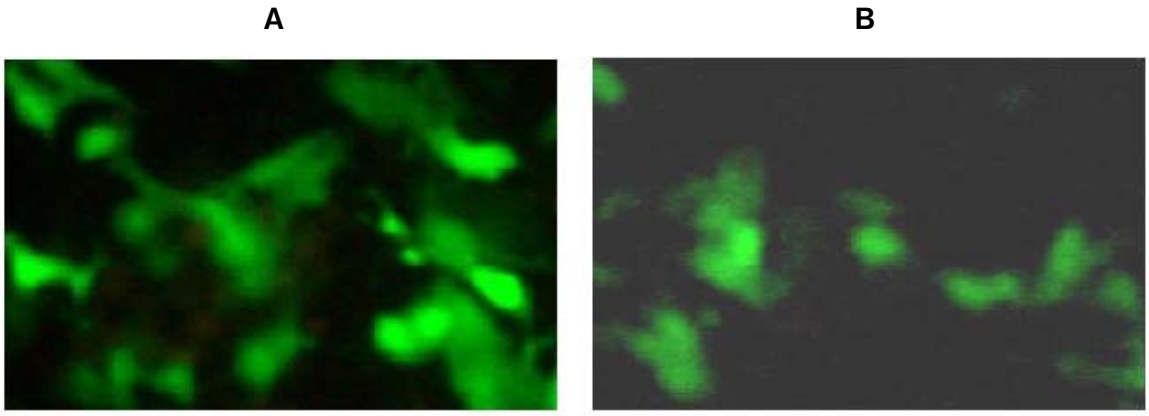

Fig. 2

Inhibition of GFP gene expression in Bel-7402 cells by GFPsiRNA

A. The cells co-transfected with pEGFP-N3 and $HBVE_1$siRNA (control). B. The cells co-transfected with pEGFP-N3 and GFPsiRNA.

Results

Effect of siRNAs on the GFP gene expression

First the effectiveness of the siRNAs generated from SEC in affecting the GFP gene expression was determined. Bel-7402 cells were co-transfected with the reporter vector pEGFP-N3 and the SEC containing the sequence known to knock down the GFP gene (GFPsiRNA). The results showed that the intensity of green fluorescence in the co-transfected cells was significantly reduced (Fig. 2B) compared with that in control cells (the cells co-transfected with pEGFP-N3 and $HBVE_1$siRNA) (Fig. 2A). This result proved that siRNA was generated by the SEC used and these siRNAs reduced the formation of GFP mRNA and GFP protein as well.

Effect of siRNAs on the formation of HBV pre-c/c-luciferase fusion protein

Since the siRNAs generated from a SEC knocked down the GFP gene expression, the next step was to test whether these siRNAs would have the same effect on HBV gene expression in mammalian cells. For this purpose the HBV *pre-c/c* gene was fused in frame with the luciferase gene to generate a reporter vector construct (pLucF-*pre-c/c*) (Fig. 1C). Following expression, the luciferase activity would represent the level of *pre-c/c* gene expression. This vector construct was then used in the screening of siRNAs generated from SECs that specifically targeted the *pre-c/c* gene expression.

Bel-7402 cells were co-transfected with pLucF-*pre-c/c* and each of the five SECs. The cells co-transfected with pLucF-*pre-c/c* and GFPsiRNA served as negative control. The results of luciferase activity assays showed that the luciferase activity in the cells co-transfected with the reporter construct and two SECs ($HBVE_3$siRNA and $HBVE_4$siRNA) was reduced by 84.4% and 82.9%, respectively (Fig. 3).

These results indicated that the siRNAs generated from $HBVE_3$siRNA and $HBVE_4$siRNA were effective in degrading the *pre-c/c*-luciferase fusion mRNA in the cells. On the other hand, the other three SECs ($HBVE_1$siRNA, $HBVE_2$siRNA, and $HBVE_5$siRNAi) had only a slight effect.

Effect of siRNAs on the HBeAg formation

Next, the possible effects of siRNAs on HBeAg formation in Bel-7402 cells and HepG2.2.15 cells carrying the HBV

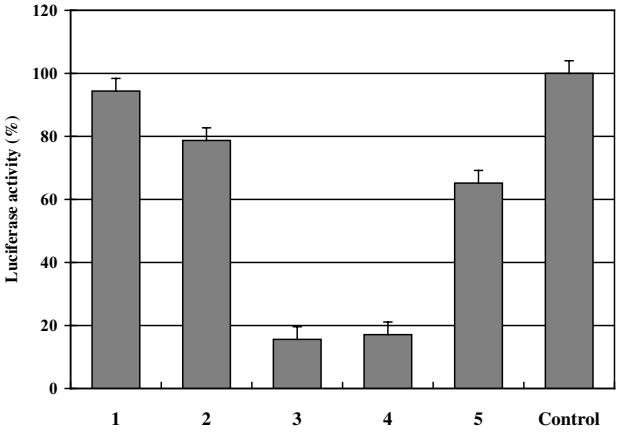

Fig. 3

Inhibition of luciferase activity in Bel-7402 cells by $HBVE_3$siRNA and $HBVE_4$siRNA

The cells were co-transfected with pLucF-*pre-c/c* and each of the five SECs. GFPsiRNA served as control. 1 = $HBVE_1$siRNA; 2 = $HBVE_2$siRNA; 3 = $HBVE_3$siRNA; 4 = $HBVE_4$siRNA; 5 = $HBVE_5$siRNA. The luciferase activity was determined 48 hrs after transfection.

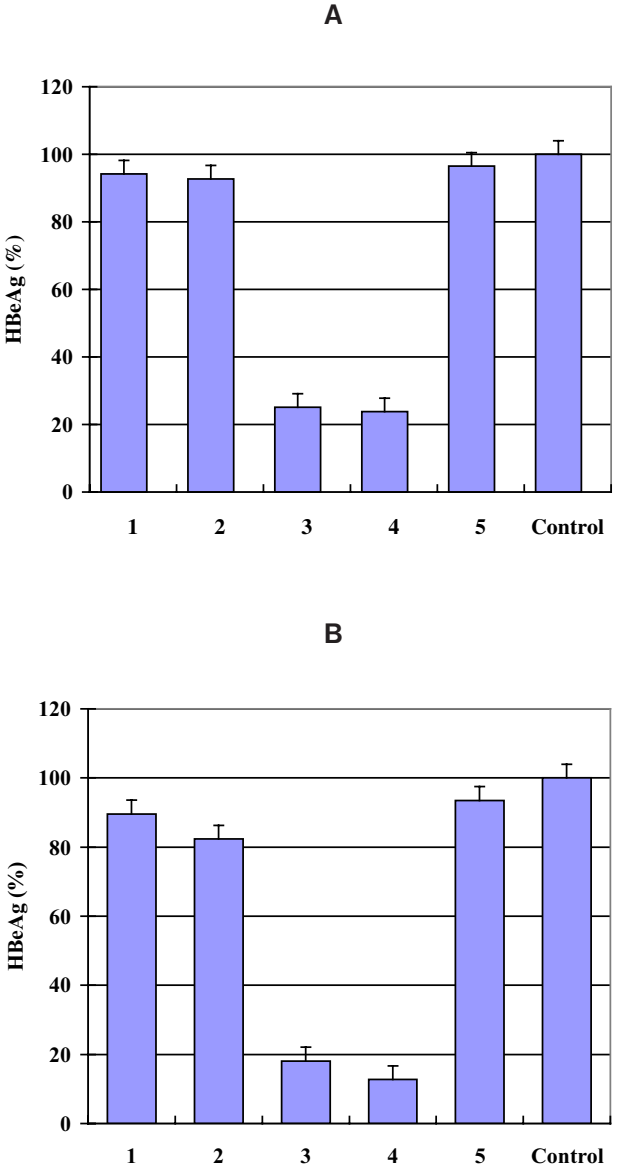

Fig. 4

Inhibition of HBeAg expression by HBVE$_3$siRNA and HBVE$_4$siRNA

1 = HBVE$_1$siRNA; 2 = HBVE$_2$siRNA; 3 = HBVE$_3$siRNA; 4 = HBVE$_4$siRNA; 5 = HBVE$_5$siRNA. A. Bel-7402 cells co-transfected with pCMV-Tag2A-*pre-c/c* and each of the five SECs. B. HepG 2.2.15 cells transfected with each of the five SECs. GFPsiRNA served as control in both A and B.

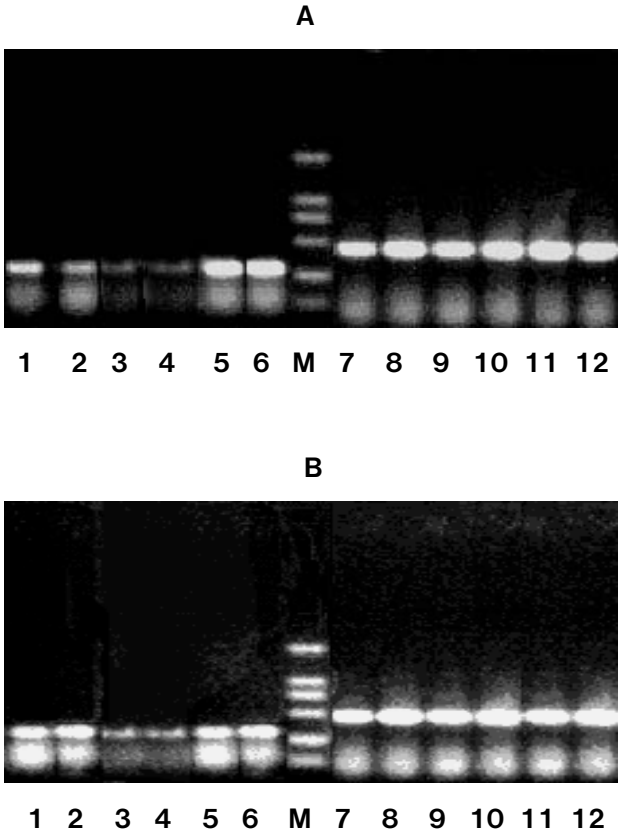

Fig. 5

Inhibition of HBV *pre-c/c* mRNA formation by HBVE$_3$siRNA and HBVE$_4$siRNA

Gel electrophoresis of RT-PCR products. A. Bel-7402 cells co-transfected with pCMV-Tag-*pre-c/c* and each of the five SECs (lanes 1–5). ß-actin controls (lanes 7–11). B. HepG2.2.15 cells transfected with each of the five SECs (lanes 1–5). ß-actin controls (lanes 7–11). GFPsiRNA control (lanes 6 and 12 for both A and B).

genome were tested. The HBeAg concentration in the cell culture media was measured 48 hrs after transfection.

As expected, the HBeAg level in Bel7402 cells co-transfected with pCMV-Tag2A-*pre-c/c* and HBVE$_3$siRNA and HBVE$_4$siRNA was reduced by 81.9% and 87.3%, respectively (Fig. 4A). Similarly, in the HepG2.2.15 cells transfected with HBVE$_3$siRNA and HBVE$_4$siRNA the level of HBeAg was reduced by 74.9% and 76.2%, respectively (Fig. 4B).

Effect of siRNAs on the HBV pre-c/c mRNA formation

As HBVE$_3$siRNA and HBVE$_4$siRNA reduced the formation of HBeAg, it was probable that the primary cause of these effects was a specific degradation of the HBeAg mRNA, namely *pre-c/c* mRNA. Therefore the level of *pre-c/c* mRNA in the Bel-7402 cells co-transfected with pCMV-Tag2A-*pre.c/c* and each of the five SECs and in the HepG2.2.15 cells transfected with the SECs was determined 48 hrs after transfection. As expected, HBVE$_3$siRNA and HBVE$_4$siRNA significantly reduced the level of *pre-c/c* mRNA in both cells, while the other three SECs had no effect (Fig. 5).

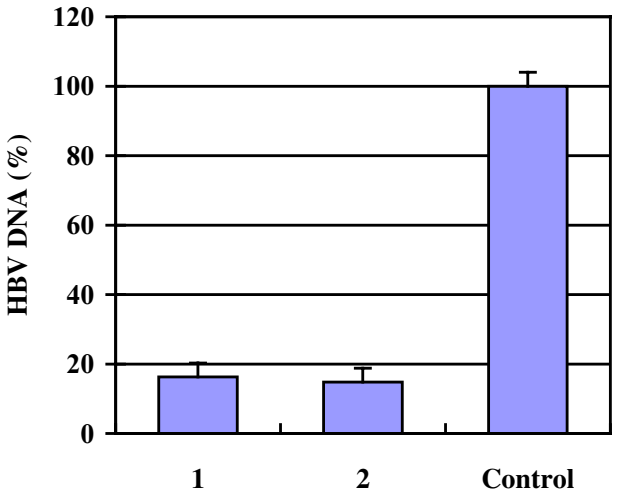

Fig. 6

Inhibition of replication of HBV DNA in HepG2.2.15 cells by $HBVE_3$siRNA and $HBVE_4$siRNA

Real-time PCR. 1 = $HBVE_3$siRNA. 2 = $HBVE_4$siRNA. siRNA targeting the GFP served as control.

Effect of siRNAs on the HBV DNA replication

Next, the possible effect of the SECs on replication of HBV DNA was tested in HepG2.2.15 cells. The level of HBV DNA was determined 2 days after transfection. The results showed that $HBVE_3$siRNA and $HBVE_4$siRNA reduced the level of HBV DNA by 83.7% and 85.2%, respectively, while the other three SECs had no effect (Fig. 6). In other words, the siRNAs, generated from the respective SECs, inhibited the HBV DNA replication.

Discussion

In the last twenty years, HB affects millions of people every year (Kao, 2002). Current therapies including immunomodulators such as interferon-α or nucleoside analogs such as lamivudine cure HB to certain extent, but the sustained virological responses is low (Carreno, 1992; Lai, 1997). siRNAs, a novel potential therapy is now the most hopful alternative strategy.

Different strategies are available for producing siRNAs. (i) Synthetic siRNAs; 21–23 nt-long oligoribonucleotides are chemically synthesized, purified and annealed into duplexes for direct transfection of cultured cells (Elbashir, 2001). (ii) *In vitro* transcribed siRNAs; 21–23 nt-long transcripts can be produced *in vitro* by the transcription driven by T7 RNA polymerase, separated from their DNA templates and annealed into duplexes (Donze, 2002). Compared with synthetic siRNAs, these are less expensive, but their preparation is longer and more laborious. (iii) Processed siRNA mixture; long sense and anti-sense RNA strands transcribed *in vitro* are annealed and then cut into shorter dsRNAs with RNase III or Dicer (Yang, 2002). In contrast to the siRNAs described under (ii), the preparation of these siRNAs is simpler and shorter, but it may activate a non-specific RNA interference with homologous genes. (iv) Polymerase III-based vectors; siRNAs can be effectively transcribed from polymerase III-based recombinant vectors or recombinant viruses (Sui, 2002, Valdes, 2003). The use of these vectors requires cloning the target DNA fragment of interest, screening and generation of transfection-quality DNA. (v) Previously, we have used both single (only 1 site on the vector is expressed) and dual (two sites on the vector are expressed) si RNA treatment to inhibit HBV gene expression and replication (Wu, 2005).

In this report, we describe development of a new system using a PCR-based siRNA expression cassette (SEC) to generate a siRNA in mammalian cells. For this purpose, we designed two 57-nt-long downstream primers, A and B, (both consisting of three regions: a region that binds to the U_6 cassette, a region containing the sequence that is transcribed into siRNA and a U6 terminator sequence) and an upstream primer containing the U_6 promoter. Primer A contained a 19-nt-long sense sequence corresponding to the mRNA target, while primer B contained a complementary, antisense sequence, selected from a different region of the *pre-c/c* gene. Next, we used a two-step PCR to construct SECs, which specifically targeted the HBV *pre-c/c* gene. After transfection of Bel-7402 or HepG 2.2.15 cells with these SECs, siRNAs were generated as expected (data not shown). Using this system, we showed that siRNAs generated from SECs specifically inhibited the expression of GFP gene. We also demonstrated that the expression of HBV *pre-c/c*-luciferase fusion protein was inhibited by siRNAs generated from two specific SECs ($HBVE_3$siRNA and $HBVE_4$siRNA) in transfected Bel-7402 cells. The siRNAs expressed from these two SECs also displayed inhibitory effects on the level of HBV *pre-c/c* protein production and *pre-c/c* gene expression in both transfected Bel-7402 and HepG2.2.15 cells. Moreover, $HBVE_3$siRNA and $HBVE_4$siRNA inhibited the replication of HBV DNA. This approach obviously proved rapid and convenient for the identification of siRNAs optimally targeting the HBV gene expression and replication. The results presented in this study verified this novel approach in which RNAi could be used in the prevention and treatment of HBV infection, which remains the most common and serious disease worldwide.

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