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Establishment of a Screening System for Selection of siRNA Target Sites Directed against Hepatitis B Virus Surface Gene

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Abstract To study the inhibitory effects of plasmid-derived small interfering RNA (siRNA) and synthetic siRNA on the expression of the hepatitis B virus surface (HBs) gene, three plasmid-derived siRNAs and one synthetic siRNA that complement the coding region of the HBs gene were prepared. The HBs expression plasmid pHBs-EGFP was also constructed. HeLa cells were co-transfected with pHBs-EGFP and the above siRNAs. The HBs mRNA quantities were measured by reverse-transcription PCR, and the level of HBs-EGFP fusion protein was quantified by fluorescent microscope. The concentrations of the hepatitis B virus surface antigen (HBsAg) derived from the culture supernatant of transfected HepG2.2.15 cells were measured by an enzyme-linked immunosorbent assay (ELISA) kit. The results showed that the three plasmid-derived siRNAs and the synthetic siRNA can effectively reduce the quantities of HBs mRNA and protein. The plasmid-derived siRNA psiRNA1 was found to be the most effective inhibitor of HBs expression. It can inhibit HBs-EGFP expression by 63.3% and suppress HBs mRNA by 75.6%. To further substantiate the above observations, psiRNA1 was transfected into HepG2.2.15 cells (an HBV secreting cell line). The transfections resulted in almost complete blockage of HBsAg production, whereas control vector-transfected cells secreted high levels of HBsAg 7 days post-transfection. In conclusion, our data suggests that RNA interference (RNAi) is an efficient approach for reducing the level of HBs transcripts and proteins and for suppressing HBsAg production.

Key words RNA interference (RNAi); small interference RNA (siRNA); hepatitis B virus surface gene (HBs gene)

Hepatitis B is a major health problem worldwide. It is estimated that more than two billion people are infected globally and 350 million are chronically infected carriers, approximately 15%–25% of whom will develop chronic lifelong liver diseases, often with devastating consequences [1]. The viral replication itself does not appear to be cytotoxic. The severe liver damage has been attributed to the host immune response to viral antigens. The hepatitis B virus surface antigen (HBsAg) belongs to a group of major antigens that induce a host immune response, and it is also involved in HBV virion formation, budding and entry into hepatocytes [2]. In addition, HBsAg may play an important role in HBV-related hepato-carcinogenesis [3,4].

RNA interference (RNAi) is the process by which double-stranded RNA directs the sequence-specific degradation of mRNA in animal and plant cells. RNAi-mediated inhibition of gene expression is a strategy that is widely used not only for studying gene regulation and revealing gene function, but also for targeting disease states, especially cancer and viral infection [5–7]. In mammalian cells, RNAi can be triggered by 21–23 nucleotide duplexes of small interfering RNA (siRNA). These siRNAs are further

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incorporated into an RNA-induced silencing complex (RISC), and the protein-RNA effector nuclease complex recognizes and destroys the target mRNA [8,9]. There are several different methods for producing siRNA: (1) chemical synthesis; (2) plasmid derivation; (3) PCR cassette; and (4) *in vitro* transcription [10–12].

In an attempt to explore the therapeutic potential of siRNA in the treatment of chronic HBV infection, the inhibitory effects of siRNA on the HBs gene were studied. Both the chemically synthesized and plasmid-derived siRNAs targeting the coding region of the HBs gene were prepared. The transfection experiments indicated the feasibility of siRNA-mediated gene therapy for the treatment of chronic HBV infection.

Materials and Methods

Cell culture and transfection

Human hepatoblastoma HepG2.2.15 cells that stably express HBV and human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fatal calf serum (Hyclone).

Twenty-four hours before transfection, 2×10^5 cells were plated into a 12-well culture plate. To detect the expression of HBs-EGFP fused protein, a sterilized cover slip was placed in advance. When the plated HeLa cells were 50%–70% confluent, co-transfection was performed using 0.1 μg of pHBs-EGFP and 1 μg of psiRNA-HBs or the control psiRNA per dish (0.1 μg of pHBs-EGFP and 25 nM, 50 nM and 100 nM synthetic siRNA-HBs). psiRNA1 (1 μg) was transfected into the HepG2.2.15 cells. The transfection reagent used was Lipofectamine™ 2000 (Invitrogen).

Plasmid construction and siRNA preparation

Human genomic DNA was extracted from the HepG2.2.15 cell with the Nucleic acid purification kit MagExtractor Genome (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The purified human genomic DNA was used as a template to amplify the HBs gene. The forward primer was 5′-GCATAAGCTTATGGAGAACATCACATCAGGA-3′, and the reverse primer was5′-GCATGGTACCAATGTATACCCAAAGACAAAAGA-3′. The underlined nucleotides in the primers indicate the recognition sites for *Hin*dIII and *Kpn*I, respectively. The PCR product was ligated into the pGEM-T vector (Promega), and the sequencing result showed that the cloned fragment was 681 bp. The correctly constructed pGEM-T vector was digested by *Hin*dIII and *Kpn*I, and the smaller digested fragment was subcloned into the *Hin*dIII-*Kpn*I site of pEGFP-N3. The GFP-fused HBs protein expression vector pHBs-EGFP was produced in this construct.

The design criteria for siRNA target selection were as follows: (1) secondary structure of the target mRNA was followed; (2) GC content of siRNAs was 40%–55%; (3) BLAST search was used to filter non-specific sites; and (4) sequences that had polyA (>3mer) and polyT (>3mer) were filtered to avoid premature termination of transcription. psiRNA-hH1neo was purchased from Invivogen (San Diego, USA). On the basis of the above criteria, we identified three sense target sequences, which were the specific 21 nt sequences from the ayw strain of the HepG2.2.15 cell genome (nt 161–181, nt 332–352 and nt 397–417, respectively). A hairpin (sense sequence+loop+antisense sequence) was required to be constructed as the siRNA expression vector psiRNA-HBs. The three psiRNA-HBs vectors were named psiRNA1, psiRNA2 and psiRNA3. The synthetic siRNA contained the same target sequence as psiRNA2. The local secondary structures of HBs mRNA at the regions targeted by the siRNAs and the sequences of the above siRNAs are shown in **Fig. 1** and **Table 1**. siRNAs were synthesized by the State Key Laboratory of Molecular Biology (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China), and then annealed at 95 °C for 1 min in an annealing buffer (pH 7.4) containing 2 mM $MgAC_2$, followed by incubation at 37 °C for 1 h.

Reverse transcription PCR analysis

The total RNA was prepared with Trizol reagent (Invitrogen) and digested by RQ1 RNase-free DNase (Promega) at 37 °C for 30 min. The reverse transcription was performed with a reverse transcription kit (Promega). The primers 5′-GCATAAGCTTATGGAGAACATCACATCAGGA-3′and 5′-GCATGGTACCAATGTATACCCAAAGACAAAAGA-3′ were used to amplify 681 bp transcripts of the HBs gene, and the primers 5′-GTGCCACCAGACAGCACTGTGTTG-3′ and 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′ were used to amplify 202 bp transcripts of the β-actin gene. Using 1 μg of cDNA as the template, the PCR process for the HBs and β-actin genes consisted of one cycle at 94 °C for 4 min, 31 cycles at 94 °C for 30 s, 31 cycles at 57 °C for 30 s, 31 cycles at 72 °C for 1 min and one cycle at 72 °C for 10 min. The PCR products were subjected to electrophoresis in 1% agarose gel and visualized by

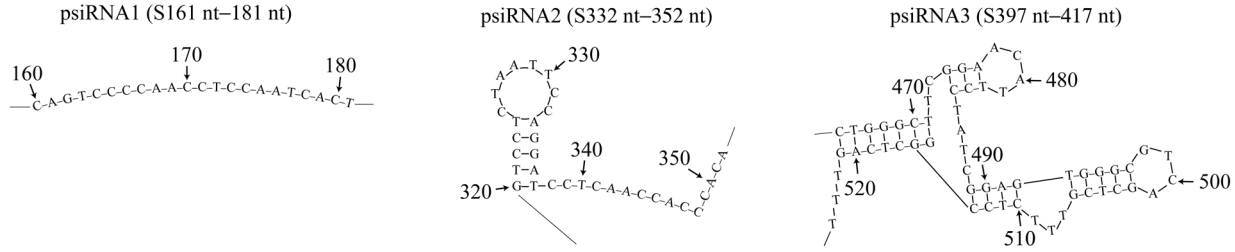

Fig. 1 Local secondary structures of HBs mRNA and siRNA sequences

Local secondary structures of HBs mRNA at the regions targeted by psiRNA1, psiRNA2 and psiRNA3.

Table 1 Sequences of the vector-derived siRNAs and chemically synthetic siRNA

Name	Sequence	Target fragment
psiRNA1	5′-TCCCAGAACATCACATCAGGATTCCCCACCGGAATCCTGATGTGATGTTCTT-3′	nt 161–181
	5′-CAAAAAGAACATCACATCAGGATTCCGGTGGGGAATCCTGATGTGATGTTCT-3′	
psiRNA2	5′-TCCCATCACTCACCAACCTCCTGTCCCACCGACAGGAGGTTGGTGAGTGATT-3′	nt 332–352
	5′-CAAAAATCACTCACCAACCTCCTGTCGGTGGGACAGGAGGTTGGTGAGTGAT-3′	
psiRNA3	5′-TCCCATCATCTTCCTCTTCATCCTGCCACCCAGGATGAAGAGGAAGATGATT-3′	nt 397–417
	5′-CAAAAATCATCTTCCTCTTCATCCTGGGTGGCAGGATGAAGAGGAAGATGAT-3′	
siRNA-HBs	5′-UCACUCACCAACCUCCUGUTT-3′	nt 332–350
	5′-ACAGGAGGUUGGUGAGUGATT-3′	

ethidium bromide staining.

Fluorescent microscopy

Forty-eight hours after transfection, the cells were washed twice with PBS (0.01 M) and fixed with 4% paraformaldehyde for 30 min at 4 °C. Cover slips were then mounted directly onto a glass slide with a tiny drop of 50% glycerol in PBS. Fluorescent images were captured by Olympus BX-50 fluorescent microscope with the use of a 10× objective. The fields were randomly chosen under phase contrast fluorescence microscopy. Randomly chosen fields from different cultures in the same experiment were photographed using the same exposure time. The proportion of HeLa cells expressing HBs-EGFP fusion proteins was calculated by counting the number of green fluorescent cells and the total number of cells in the same field under the inverted fluorescent microscope.

Analysis of HBsAg concentrations by ELISA

The concentrations of HBsAg derived from the culture supernatant of transfected HepG2.2.15 cells were measured by enzyme-linked immunosorbent assay (ELISA) kit (Sino-American Biotechnology Company) according to the manufacturer's instructions. A substrate buffer (20 μl) was added to each well. The absorbance at 450 nm (A_{450}) was determined after incubation at 37 °C for 30 min.

Statistical analysis

All experiments were performed three times. The data were presented in the mean±SD format and compared by one-way ANOVA, and a P value of less than 0.05 was considered statistically significant. The analysis was conducted using the SPSS 11.0 software.

Results

Construction of psiRNA-HBs and pHBs-EGFP

The agarose gel analysis showed that the HBs gene was amplified from hepatoblastoma HepG2.2.15 cell genomic DNA. The restriction digestion analysis and DNA sequencing indicated that the plasmids psiRNA1,

psiRNA2, psiRNA3 and pHBs-EGFP were successfully constructed.

Inhibitory effects of siRNAs on HBs transcripts

To test the inhibitory effects of siRNAs on HBs gene expression, three plasmid-derived siRNAs (psiRNA1, psiRNA2 and psiRNA3) and one synthetic siRNA were constructed. The inhibitory effects of the three plasmid-derived siRNAs were determined by quantifying the level of HBs transcripts at 48 h after co-transfection. The HBs transcript quantities were normalized by the β-actin mRNA. The mean level of HBs/β-actin was 0.80±0.11 in the control group, while the mean levels of HBs/β-actin were 0.19±0.05, 0.34±0.08, 0.54±0.13 in the psiRNA1-, psiRNA2- and psiRNA3-treated groups, respectively [**Fig. 2(A,B)**]. There were significant differences among the four groups (P<0.05). psiRNA1 inhibited the HBs mRNA most effectively (75.6%). psiRNA2 and psiRNA3 inhibited the HBs mRNA by 56.8% and 36.4%, respectively.

To assess whether the effects of psiRNA1 were time-dependent, we detected the level of HBs mRNA at 24 h, 48 h and 72 h after co-transfection of psiRNA1 and pHBs-EGFP in the ratio of 1:10 [**Fig. 2(C,D)**]. The mean levels of HBs/β-actin were 1.28±0.09, 0.80±0.11, 1.10± 0.05 in the control groups, and were 0.75±0.08, 0.19±0.05, 0.69±0.05 in the psiRNA1-treated groups at 24 h, 48 h and 72 h post-transfection, respectively. The difference between the control groups and psiRNA1-treated groups was significant (P<0.05). psiRNA1 reduced the HBs mRNA level most effectively at 48 h post-transfection.

The inhibitory effect of the chemically synthetic siRNA on the expression of HBs mRNA was also tested. The mean level of HBs/β-actin was 0.81±0.077 in the

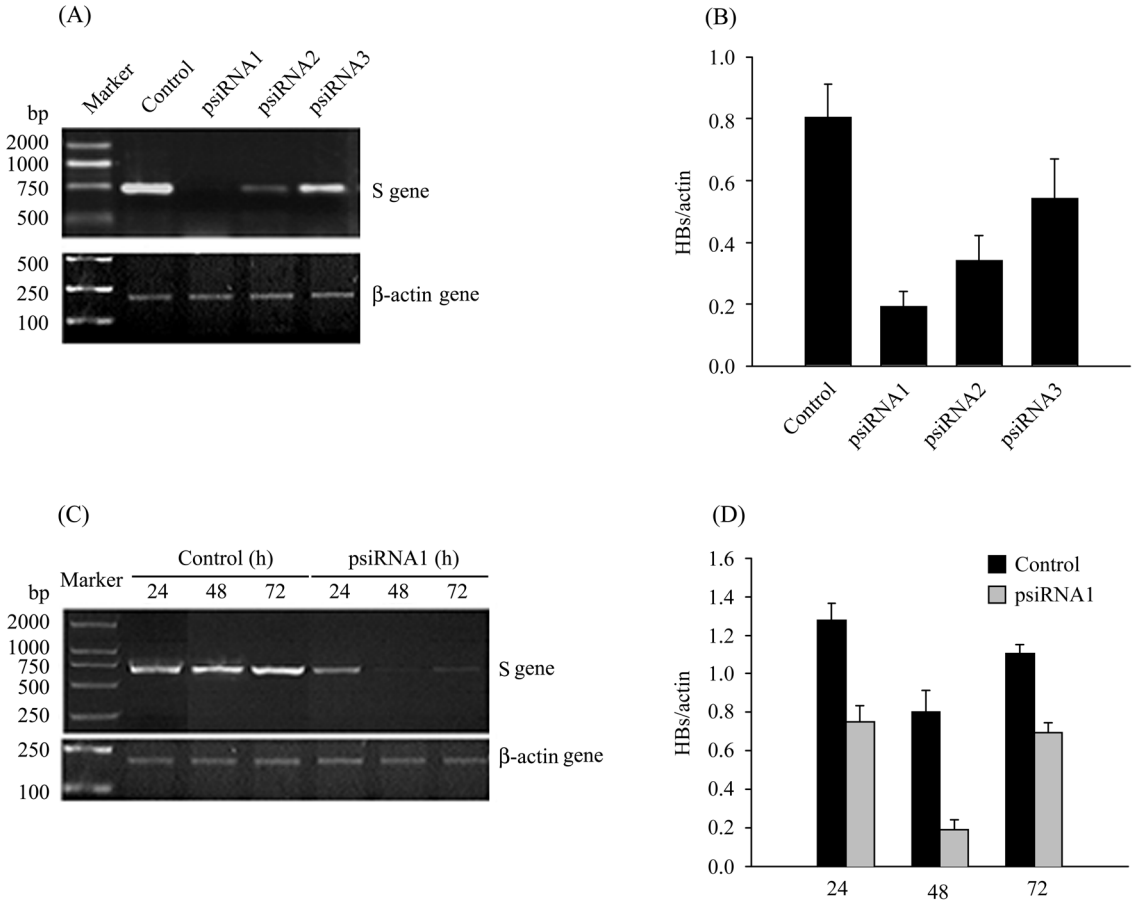

Fig. 2 Suppression of HBs transcripts

(A) Reverse transcription PCR (RT-PCR) analysis of HeLa cells co-transfected with pHBs-EGFP and psiRNA1, psiRNA2 or psiRNA3 at 48 h post-transfection. (B) The mean level of HBs/β-actin was determined based on the results of the RT-PCR. (C) RT-PCR analysis of HeLa cells co-transfected with pHBs-EGFP and psiRNA1 at 24 h, 48 h and 72 h post-transfection. (D) The mean level of HBs/β-actin was determined based on the results of the RT-PCR.

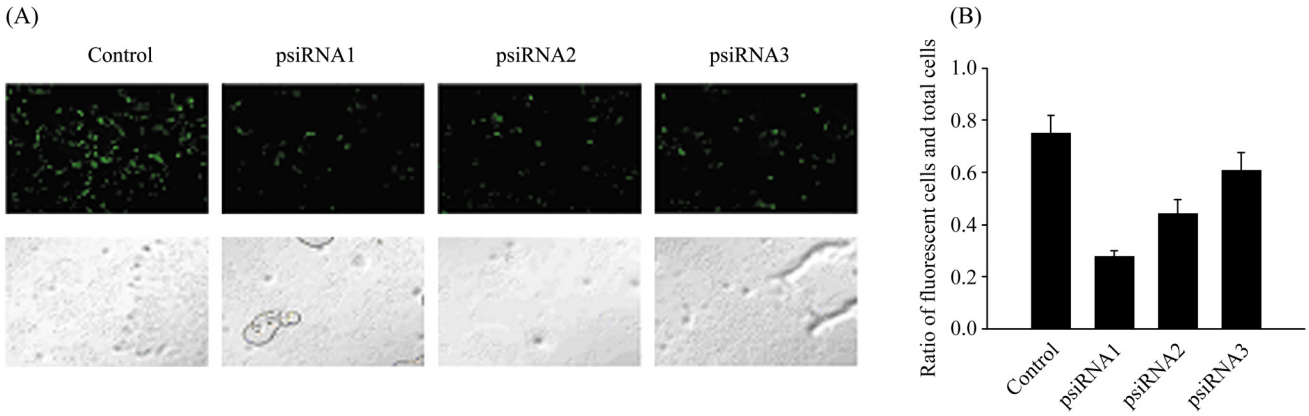

Fig. 3 Suppression of the HBs-EGFP fusion protein

(A) Fluorescent microscope analysis on HeLa cells co-transfected with pEGFP-HBs and psiRNA1, psiRNA2 or psiRNA3 at 48 h post-transfection. Magnification, ×10. (B) The proportion of green fluorescent cells and the total number of cells in the same field were determined based on the results of the fluorescent microscope analysis.

control group, and the mean levels of HBs/β-actin were 0.49±0.030, 0.32±0.19, 0.47±0.53 in the synthetic siRNA-treated groups at concentrations of 25 nM, 50 nM and 100 nM, respectively. There was a significant decrease in the HBs mRNA level in synthetic siRNA-transfected cells (P<0.05). The synthetic siRNA suppressed the HBs mRNA by 61.3% at a concentration of 50 nM.

Inhibitory effects of siRNA on the fused HBs-EGFP protein

The inhibitory effects of siRNA on the HBs protein were evaluated by counting the number of fluorescent cells and the total number of cells using the inverted fluorescent microscope. Both the fluorescence micrographs and the corresponding phase contrast micrographs of cells co-transfected with pHBs-EGFP and psiRNA1, psiRNA2 or psiRNA3 were prepared. **Fig. 3** showed a significant reduction in the number of fluorescent cells in the presence of specific psiRNA1, psiRNA2 and psiRNA3. Based on the results of the fluorescent microscope analysis, psiRNA1, psiRNA2 and psiRNA3 inhibited the HBs-EGFP fusion protein by 63.3%, 41.4% and 18.9%, respectively. These results are consistent with those at the mRNA level. Together, these data showed that the specific siRNA affecting the HBs gene can induce a significant and sequence-specific reduction in the level of HBs protein.

Inhibition of HBsAg expression in HepG2.2.15 cells by psiRNA1

The above results showed that psiRNA1 was the most effective inhibitor of HBs transcripts and proteins in HeLa cells. To further investigate the effect of siRNA on HBsAg expression in hepatoma cells, we used HepG2.2.15 cells, which stably express all HBV antigens, including HBsAg. HBsAg levels produced by the transfected HepG2.2.15 cells were determined using ELISA 3 days, 5 days and 7 days after cell seeding. In the culture supernatant, HBsAg production was almost completely blocked in psiRNA1-transfected cells, whereas high levels of HBsAg persisted in the control vector-transfected group 7 days post-transfection. The maximum suppression of HBsAg production was 85.56% 7 days post-transfection (**Fig. 4**).

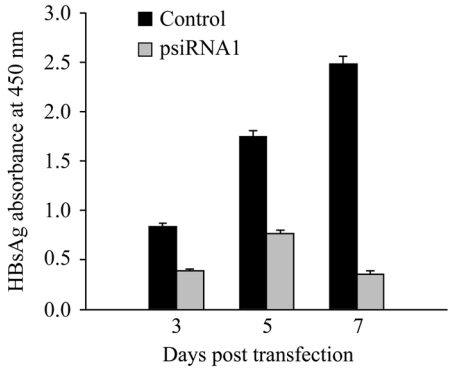

Fig. 4 HBsAg levels of culture supernatant collected from psiRNA1-transfected and control vector-transfected HepG2.2.15 cells

Discussion

HBV infection is still a major health problem worldwide, even though effective vaccines have been available in the past 20 years. Interferon and lamivudine therapies for chronic hepatitis B carriers have been reported to result in long-term remissions in a significant percentage of patients [13,14]. However, these treatments have some drawbacks, including possible serious side effects in the case of interferon therapy, or the recurrence of viremia after cessation of therapy and development of escape mutants after a long period of lamivudine treatment [15]. However, using RNAi as an anti-HBV therapy may have some important advantages. First, specific targeting of the viral transcripts and proteins severely impairs viral replication and promotes the eradication of the virus, hence minimizing undesirable host toxicity. Second, screening for the most effective target sites for RNAi along the viral genome makes it possible to target viral conserved regions, limiting the virus' ability to create escape mutants. The potential to introduce a lot of siRNAs targeted against different sequences simultaneously further limits this ability and makes it possible to treat chronically infected people with diverse circulating HBV genomes. Finally, the ability of siRNA to reduce the quantities of viral mRNA and protein even in the absence of active viral replication makes it a good candidate for use as an adjuvant therapy to lamivudine, which acts only on the replication-competent HBV [16–18].

In this paper, the anti-HBV therapeutic potential of RNAi was described. Two types of siRNA, plasmid-derived siRNA and chemically synthetic siRNA, were prepared. The inhibitory effects of the two types of siRNA were investigated and it was found that both siRNAs can effectively reduce the quantities of HBs mRNA and protein. The gene-silencing effect of siRNA is known to vary strongly with the targeted position of the mRNA [19,20]. Highly effective mRNA target sequences were selected using novel guidelines that were established through an extensive study of the relationship between siRNA sequences and RNAi activity [21–23]. Using the RNA structure, we designed psiRNA1, the target mRNA of which did not bind other nucleotides in the same mRNA molecule, and psiRNA2, the target mRNA of which had a smaller hairpin structure, while a larger hairpin structure was seen in the mRNA targeted by psiRNA3. It was found that psiRNA1 had the highest efficiency and psiRNA3 had the lowest efficiency in suppressing HBs expression. The sequence nt 161 to nt 181 from the ayw strain of the HepG2.2.15 cell genome which was targeted by psiRNA1 was the most effective target site. This is consistent with other studies which propose that the local secondary structure of the target mRNA has a strong effect on the suppression of the gene silencing [24]. This is possible because such a target mRNA may tend to form a hairpin structure by itself and thus cannot be fully open. As a result, the RISC/siRNA complex will be less effective in binding with the target mRNA. The difference in their local secondary structures may affect the gene-silencing efficiencies.

The inhibitory effect of psiRNA1 on HBsAg production in HepG2.2.15 cells was further investigated. As this cell line is naturally infected by HBV, it is a good model for examining the effects of siRNA on HBsAg production. HBsAg production in the culture supernatant of psiRNA1-transfected cells was significantly inhibited in comparison to that in control vector-transfected cells 7 days after transfection. Therefore, psiRNA1 is capable of inhibiting HBsAg production in the hepatoma cell line HepG2.2.15.

In conclusion, using the siRNA approach, specific target sites in the HBV genome can be selected and targeted with ease. In addition, siRNA therapy can be directed at the different levels of viral function, such as transcription or translation. Based on the present data and the advantages of siRNA technology, we believe that siRNA is a potential therapeutic agent against chronic HBV infection.

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