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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

RNA Interference-Mediated Control of Hepatitis B Virus and Emergence of Resistant Mutant

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See editorial on page 789.

Background & Aims: **Present therapy for chronic hepatitis B attains control only in limited proportions. Small interfering RNA (siRNA) offers a new tool with potential therapeutic applications for hepatitis B virus (HBV). Given the importance of sequence identity in the effectiveness of siRNA and the heterogeneity of HBV sequences among different isolates, a short hairpin RNA (shRNA)-expressing plasmid, pSuper/HBVS1, was developed to target a region conserved among major HBV genotypes and assess its effectiveness control of HBV.** ***Methods:*** **HBV replication-competent plasmid was cotransfected with pSuper/HBVS1 to HuH-7 cells or to mice. The levels of viral proteins, RNA, and DNA were examined in transfected cells and animals. The effects of pSuper/HBVS1 on clinical isolates with genotypes B and C were also determined.** ***Results:*** **pSuper/HBVS1 significantly decreased levels of viral proteins, RNA, and DNA for HBV genotype A in cell culture and in mice. Comparable suppressive effects were observed on clinical isolates of genotypes B and C. A clone with a silent mutation in the target region was identified from a patient with genotype C. This mutant revealed diminished sensitivity to pSuper/HBVS1 and could be selected out in the presence of pSuper/HBVS1 in cell culture.** ***Conclusions:*** **These findings indicated that shRNA could suppress HBV expression and replication for genotypes A, B, and C, promising an advance in treatment of HBV. However, the emergence of resistant mutants in HBV quasispecies should be considered.**

With an estimated 300 million chronic carriers, hepatitis B virus (HBV) infection remains one of the most prevalent chronic viral infections of humans.[1] Chronic infections cause serious consequences, including cirrhosis and hepatocellular carcinoma,[2] responsible for at least 1 million deaths annually worldwide.[1] Thus, control of HBV infection by prevention or treatment is imperative.

A preventive vaccine is available to block HBV infection successfully; however, therapeutic intervention is the only option for chronic HBV carriers to delay or prevent the progression to life-threatening, end-stage liver diseases. Immune modulators (ie, interferon α [IFN-α]) and nucleoside analogues (ie, lamivudine) are 2 approved mainstream treatments for chronic hepatitis B. However, both therapies achieve only limited response, and none of them can eradicate the virus effectively.[3] Other strategies, including antisense RNA and DNA constructs, hammerhead ribozyme, and dominant negative HBV core proteins, are still under development.[4,5] Therefore, the quest for potent antiviral medications to treat chronic hepatitis B continues.

Recently, it was found that double-stranded RNA (dsRNA) can trigger a sequence-specific, gene-silencing process called *RNA interference* (RNAi).[6] RNAi is a very specific and potent mechanism to silence gene expression through processing of double-stranded RNA into 21–26 nucleotide (nt), short interfering RNA (siRNA). In plants, it serves as a host-defense mechanism against viruses and transposable elements (reviewed in Waterhouse et al[7]). After the discovery that RNAi pathways also exist in mammalian cells and that the use of siRNA can avoid cell death induced by dsRNA longer than 30 nucleotides,[8] siRNA has emerged as a novel therapeutic approach in the fight against human viral infections. This study therefore examined whether siRNA can in-

Abbreviations used in this paper: RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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hibit HBV replication and expression and exhibit the potential to combat chronic hepatitis B.

During the course of this work, a growing number of studies have shown that siRNA can be effective against several pathogenic viruses, including poliovirus, HIV, HCV replicons, influenza virus, SARS coronavirus, and others.[9,10] Several studies employing siRNA or short hairpin RNA (shRNA) to inhibit HBV[11–18] also revealed that RNA interference indeed has the potential to treat viral infections, including HBV. However, variation of sequences among different viral isolates in clinical settings and emergence of resistant mutants constitute potential problems hindering the efficacy of siRNA. This work identified a target site for siRNA that is conserved among HBV genotypes A to G. A plasmid expressing shRNA targeted this site significantly inhibited the steps in HBV replication that occur in cultured cells and in mice. The problem of resistant mutants was also explored in this study.

Materials and Methods

Plasmids

Plasmid pHBV48 consists of a greater than genome-length HBV fragment (subtype adw, genotype A) that can initiate HBV replication after being transfected into hepatoma cell lines.[19]

To clone the full-length HBV genome from clinical samples, a method described by Gunther et al was used.[20] After amplication, PCR products were purified and cloned into the vector yT&A (Yeastern Biotech Co., Shijr, Taiwan).

shRNA were cloned downstream of the human H1 promoter in the vector pSuper as described.[21] The target sites for siRNA were chosen on the basis of their conservation among the major HBV genotypes. The selected sequences were submitted to a BLAST search to avoid targeting to the human genome. We used the primers 5′-GATCCCCCTGGATCCTGCGCGGGACGTTCAAGAGACGTCCCGCGCAGGATCCAGTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAACTGGATCCTGCGCGGGACGTCTCTTGAACGTCCCGCGCAGGATCCAGGGG-3′ for pSuper/HBVX1; 5′-GATCCCCGGTATGTTGCCCGTTTGTCTTCAAGAGAGACAAACGGGCAACATACCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGGTATGTTGCCCGTTTGTCTCTCTTGAAGACAAACGGGCAACATACCGGG-3′ for pSuper/HBVS1. Relative positions of shRNA targets on HBV are shown in Figure 1. For injection into mice, endotoxin-free plasmid DNA was prepared by the EndoFree Plasmid Kit (Qiagene, Hilden, Germany).

Cell Culture and Transfection

HuH-7 cells were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle medium, supplemented with 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel) and

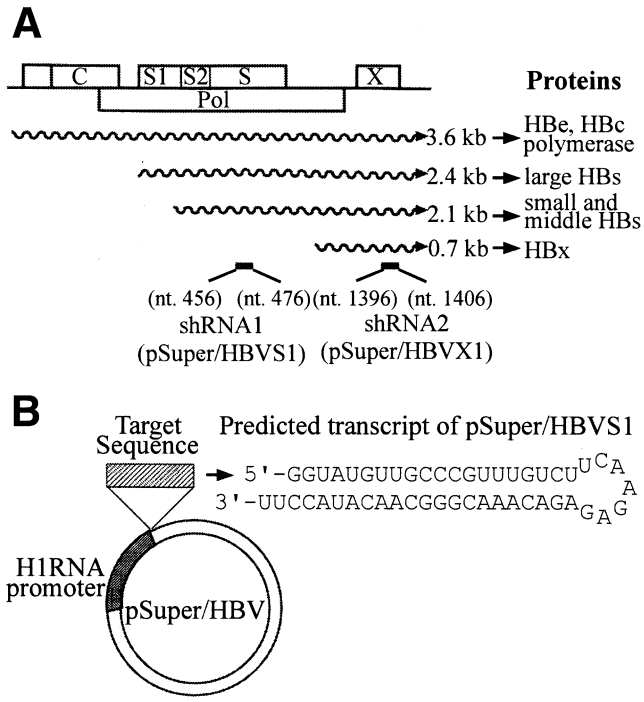

Figure 1. (*A*) Schematic representation of HBV genome and 4 major transcripts and their protein products. The circular HBV genome is presented as a linear form. The coding regions for core, surface, polymerase, and X proteins are displayed and designated as *C*, *S*, *Pol*, and *X*, respectively. The relative locations of the target sites of shRNAs are also depicted. (*B*) Diagrams of vector for generating shRNA and the predicted structure of shRNA from pSuper/HBVS1.

2% L-glutamine. The cells were plated at a density of 9×10^5 cells per 60-mm dish or 4.5×10^5 cells per well in 6-well plates 24 hours prior to transfection. Transfecion of cells was performed with lipofectamine 2000 (Invitrogen Life Technologies, CA) following the user guidelines. To assay the replication of HBV genome from clinical isolates, we cleaved the PCR product or HBV monomer cloned in pyT&A with *Sap*I prior to transfection, as described previously.[22] After transfection, the supernatant was collected for the evaluation of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and the cells were processed for the detection of viral DNA and RNA by Southern and Northern blotting, respectively.

Possible adverse effects of pSuper/HBV S1 on cells were evaluated by the morphology, growth rate, non-HBV protein expression, and cytotoxicity of transfected cells. Cytotoxicity was determined by MTS assay with CellTiter96 Aqueous 1 solution proliferation assay following the instructions of the manufacturer (Promega, Madison, WI). Expression level of one cellular protein, α-fetoprotein, was determined by tumor markers AFP kit of AXSYM systems (Abbott, GmbH Diagnostica, Wiesbaden, Germany).

Hydrodynamic Injection of Plasmid to Mice

For the in vivo experiments, we used 6- to 8-week-old C57BL/6 mice. Five μg pHBV48, 2.5 μg pSEAP2-Control

(BD Biosciences, Palo Alto, CA), and 20 μg pSuper or pSuper-derived plasmid DNA were injected into the tail vein of mice in a volume of phosphate-buffered saline (PBS) equivalent to 8% of the mouse body weight.[23,24] The total volume was delivered within 5 seconds. The secreted alkaline phosphatase (SEAP) level in the mouse serum was measured by BD Great EscAPe SEAP chemiluminescence detection kit (BD Biosciences, Palo Alto, CA) and used to normalize transfection efficiency. The mice sera were assayed for HBsAg, HBeAg, or HBV DNA content at 1, 4, 7, and 11 days after injection. The livers of mice were preserved in optimal cutting temperature (OCT) for immunohistochemical analysis.

All mouse experiments were carried out according to the guidelines established by the Institutional Animal Care and Use Committee at the National Taiwan University College of Medicine.

HBsAg and HBeAg Assays and Immunohistochemistry

Levels of HBsAg and HBeAg in the media of the transfected cells, and in the sera of the treated mice, were determined using the AXSYM systems kit (Abbott, GmbH Diagnostica). HBV core protein (HBc) was visualized by immunohistochemical staining of tissues embedded in OCT by rabbit anti-HBc antibodies and Envision + System, HRP (DAB) (DAKO Corp., Carpinteria, CA). The liver sections were also stained with hematoxylin.

Northern Blot Analysis

Total RNA was extracted from transfected cells with RNA-Bee reagent (TEL-TEST Inc., Freindswood) according to the manufacturer's instructions. Twenty μg RNA were separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). The blot was probed with a DIG-labeled DNA fragment corresponding to nucleotides 1372–1833 of the HBV X sequence. The probe was generated with a PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany).

Southern Blot Analysis

Purification of HBV DNA from intracellular core particles was performed according to the protocol described previously,[25] with minor modifications. Briefly, the cells were lysed in 300 μL isotonic lysis buffer (10 mmol/L Tris-HCl, pH7.5, 1 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Nonidet P-40) per 60-mm dish. The nuclei were removed from the lysates by centrifugation for 10 minutes at 14,000 rpm. The cytoplasmic HBV DNA was purified by digestion of proteins with 200 μg/mL of proteinase K in the presence of 0.5% of sodium dodecyl sulfate at 37°C for overnight, phenol-chloroform extraction, and ethanol precipitation. Purification of HBV DNA from extracellular viral particles was performed following the protocol described in the previous study.[20] One third of the DNA purified was fractionated in a 1% agarose gel, blotted to a nylon membrane, and hybridized with a DIG-labeled DNA fragment covering the entire surface gene.

Genotypic Analysis of the siRNA Target Region of HBV

Viral DNA was isolated from cell-free culture supernatant with the QIAamp viral RNA kit (Qiagene) according to the manufacturer's instructions. Five μL purified viral DNA was used as template for PCR reaction with sense primer HBV+2836 (5′-GGAACAAGAGCTACAGCATG-3′) and antisense primer HBV-695 (5′-AACAAATGGCACTAGTAAAC-3′). PCR products were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taoyuan, Taiwan) and sequenced in an automatic sequencer ABI Model 3700 with primer HBV+56 (5′-CCTGCTGGTGGCTCCAGTTC-3′). For analyzing the sequence of individual clones, PCR products were cloned to the vector pyT&A (Yeastern Biotech Co.).

Results

siRNA Inhibits HBV Gene Expression in Cell Culture

To examine the ability of siRNA to inhibit HBV gene expression, 2 target sequences located on the X (siRNAX1) and surface (siRNAS1) genes of HBV were selected. Because all HBV transcripts overlapped in their C-terminals, multiple viral RNA could be inhibited by a single siRNA (Figure 1). Both siRNA used can target pregenomic RNA that serves as the template for HBV genomic replication and the mRNA for the core and polymerase proteins. These 2 siRNA also target transcripts for HBV surface antigens, the major viral proteins of the envelope. Furthermore, siRNAX1 targets the transcript encoding the X protein. This study employed an H1 promoter-based vector (pSuper) to express the siRNA of interest in the form of shRNA.

To evaluate the effects of HBV siRNA on HBV gene expression, we measured HBsAg and HBeAg levels in the culture media of cells cotransfected with a replication-competent HBV construct (pHBV48) and HBV shRNA-expression plasmids. The present study found that the pSuper/HBVS1 could efficiently suppress the expression of HBsAg and HBeAg for up to 11 days (Figure 2) in culture. The kinetics and efficiency of suppression differs for HBsAg and HBeAg. Maximal suppression for HBeAg occurred around day 2 posttransfection, and the suppression effect decreased thereafter. Suppression of pSuper/HBVS1 on HBsAg expression attained maximum level around days 4 to 6, and maintained a similar level thereafter. Treatment with pSuper/HBVS1 caused approximately 98.2% reduction of HBsAg on day 6 and 62.6% of HBeAg on day 2, respectively, compared

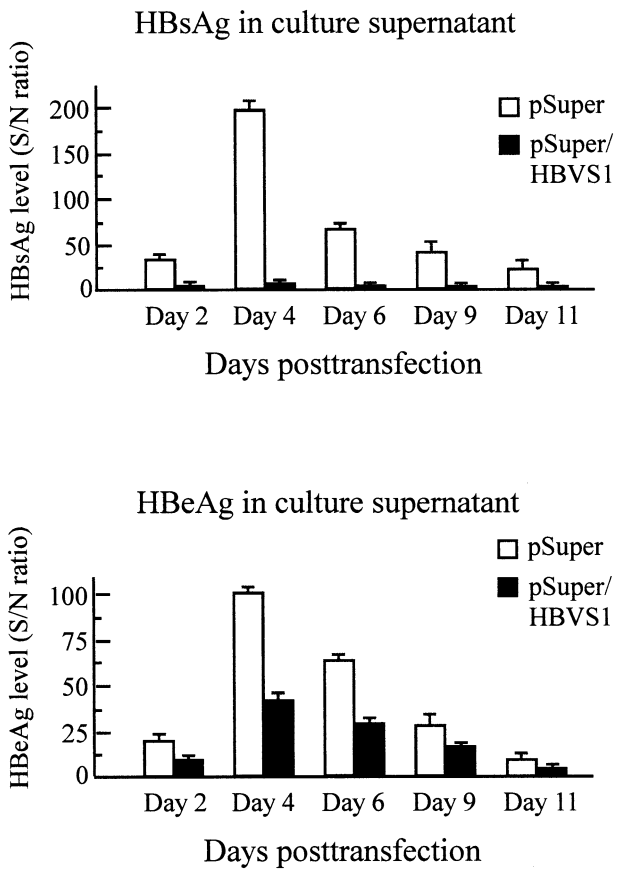

Figure 2. Inhibition of HBV surface and e antigens expression in cell culture by shRNA. HBV replication-competent plasmid, pHBV48, was cotransfected to HuH-7 cells with either vector pSuper or pSuper/HBVS1. Culture media were collected on days 2, 4, 6, 9, and 11 posttransfection, and the levels of HBsAg and HBeAg were determined. The levels of HBsAg and HBeAg were expressed as signal to noise ratio (S/N). The values shown are the average of 3 experiments (means ± standard deviation).

with the empty-vector control (Figure 2). Conversely, pSuper/HBVX1 showed no significant suppressive effect on HBV protein expression (data not shown).

HBV RNA levels in cells were examined by Northern blotting. Total cellular RNA derived from transfected cells was hybridized with a probe-spanning X gene to hybridize equally to all HBV transcripts. The RNA levels of 3.6 kb, 2.4 kb, and 2.1 kb were significantly suppressed by pSuper/HBVS1, compared with those in cells cotransfected with pHBV48 and pSuper vector alone (Figure 3*A*). Consistent with the results of secreted viral proteins in culture supernatant, pSuper/HBVX1 did not show a suppressive effect on HBV transcripts on the RNA level either. These results indicated that the suppressive effect of siRNA on HBV expression on the RNA level is comparable with that on the protein level. We thus used the expression levels of viral antigens as a read-out system for viral RNA expression in further studies.

siRNA Inhibits HBV Replication in Cell Culture

To examine whether shRNA could also suppress HBV replication, we used Southern blotting to detect HBV DNA. As shown in Figure 3*B*, pSuper/HBVS1 can also suppress HBV replication. This was reflected by reduced HBV replication intermediates in transfected cells and HBV DNA in viral particles secreted in culture media. The decreased HBV replication and expression in pSuper/HBVS1-transfected cells was not due to nonspecific cell toxicity. The morphology of cells transfected with pSuper/HBVS1 did not differ markedly from those of pSuper-transfected or untransfected cells. MTS proliferation assay for pSuper/HBVS1 and pSuper-transfected cells showed comparable proliferation results (data not shown). Furthermore, expression levels of cellular RNA or protein were unaffected by the presence of pSuper/HBVS1, including GAPDH RNA (Figure 3*A*), as well as the secreted α-fetoprotein (AFP) expressed by HuH-7 cells (data not shown). The results indicated that the decrease in HBV replication and expression in these cells is not due to a nonspecific cell toxicity problem but is due to a specific suppressive effect of pSuper/HBVS1 on HBV.

siRNA Inhibits HBV Gene Expression in Mice

Because pSuper/HBVS1 showed substantial inhibition on HBV expression and replication in culture, this shRNA expression plasmid was further used to examine

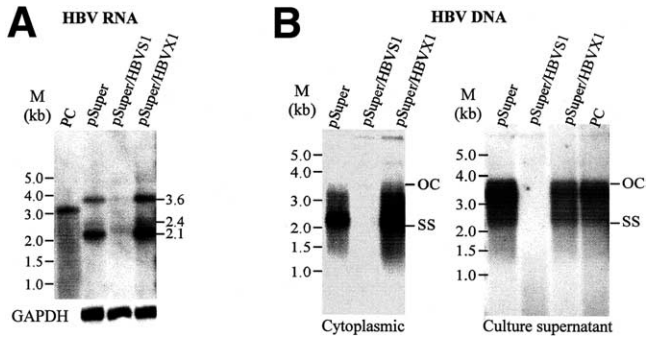

Figure 3. Effects of shRNA on HBV RNA expression and DNA replication. (*A*) Northern blot analysis of HBV transcripts from HuH-7 cells cotransfected with pHBV48 and shRNA-expressing plasmids as indicated. *PC* represents positive control and is a 200 pg, 3.2-kb HBV DNA fragment. The same blot was hybridized with a glyceraldehydes phosphate dehydrogenase (GAPDH) DNA probe as an internal loading control. This blot represents the results of 4 similar experiments. (*B*) Southern blot analysis of HBV replicative intermediates in cytoplasm or virion-associated HBV DNA in culture supernatant of HuH-7 cells transfected with pHBV48 and shRNA-expressing plasmids. *PC* represents DNA samples from HuH-7 cells transfected with pHBV48 alone. This blot represents the results of 2 similar experiments.

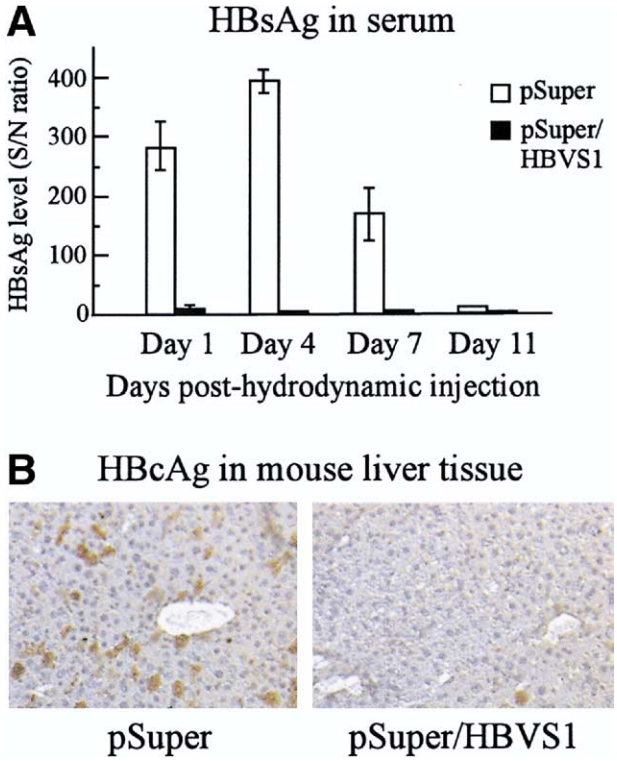

Figure 4. pSuper/HBVS1 suppressed HBV expression in mice. pHBV48 were hydrodynamically injected with pSuper or pSuper/HBVS1 to C57BL/6 mice. There were 3 mice in each treatment group. (*A*) HBsAg levels in sera of mice treated. The level of HBsAg was expressed as signal to noise ratio (S/N). The data represent the mean values ± SD of 3 mice in each treated group. (*B*) Immunohistochemical staining for HBcAg in liver sections embedded in OCT. Representative sections are shown. *Left panel:* liver section from a pSuper-treated mouse. *Right panel:* liver section from a pSuper/HBVS1-treated mouse.

whether it could also display HBV inhibition in vivo. pHBV48 was cotransfected with pSEAP2-Control and pSuper or pSuper/HBVS1 to mouse liver by hydrodynamic injection. The level of alkaline phosphatase in serum was measured to monitor the transfection efficiency of hydrodynamic injection. As shown by studies from the present authors and others,[26] hydrodynamic injection of replication-competent HBV plasmid DNA into mouse resulted in HBV replication in the liver and secretion of viral antigens to the serum. The level of HBsAg in the mouse sera and HBc expression in the mouse liver were used as indicators to evaluate the suppressive effect of pSuper/HBVS1. In concordance with findings from cell culture experiments, the HBsAg expression in mice that received HBV replication-competent construct (pHBV48) was suppressed dramatically by pSuper/HBVS1. Serum HBsAg in mice treated with pSuper/HBVS1 was reduced by over 99% on day 4 when compared with mice without pSuper/HBV1 treatment (Figure 4*A*).

Expression of another important viral protein, the nucleocapsid (HBc), was also inhibited by pSuper/HBVS1. HBV core protein is synthesized in infected or transfected cells from the 3.6-kb pregenomic RNA and is required for viral replication. An immunohistochemical study showed that there were approximately 10% HBc-positive hepatocytes in livers of mice receiving pHBV48 and pSuper (Figure 4*B*, left panel). In contrast, HBc-stained cells were hardly found in livers of mice receiving pHBV48 and pSuper/HBVS1 (Figure 4*B*, right panel). The numbers of stained hepatocytes in sections from pSuper/HBVS1-treated mice was reduced by more than 95%.

pSuper/HBVS1 Suppresses Expression of HBV Clinical Isolates of Different Genotypes

Significant diversities occur in sequences of HBV isolates. Based on sequence divergence, HBV can be classified into at least 8 genotypes.[27] To warrant a widespread application of the potential siRNA therapy, the target sequence chosen for pSuper/HBVS1 was conserved among all major HBV genotypes A–G. pSuper/HBVS1 has been shown to suppress the expression and replication of a laboratory clone of genotype A, pHBV48. Further testing concerned whether or not pSuper/HBVS1 could also inhibit the expression and replication of HBV with different genotypes from clinical isolates. Because genotypes B and C are the most prevalent genotypes in Asia, including Taiwan, this study focused on these 2 genotypes. The effect of pSuper/S1 was examined on a heterogeneous mixture of amplified HBV genomes from clinical samples without cloning. Full-length HBV genome of genotype B or C was amplified from the patient's serum by PCR. The PCR products were digested with *Sap*I and transfected to HuH7 cells with pSuper/HBVS1 or pSuper. A previous study has demonstrated that linear monomeric HBV genomes with *Sap*I ends can initiate a full replication cycle, leading to viral antigen expression.[20] Our results showed that pSuper/HBVS1 could also suppress expression of HBsAg and HBeAg of HBV genotypes B and C from clinical isolates to a similar extent as on a laboratory clone of genotype A (Figure 5).

Selection of Resistant Mutant in the Presence of pSuper/HBVS1

Full-length HBV genomes from clinical isolates were also cloned into a vector, and the effect of pSuper/HBVS1 on their expressions was assayed. The results resembled those on PCR products (data not shown). Nevertheless, 1 of 10 clones from a patient with genotype C proved more resistant to pSuper/HBVS1, al-

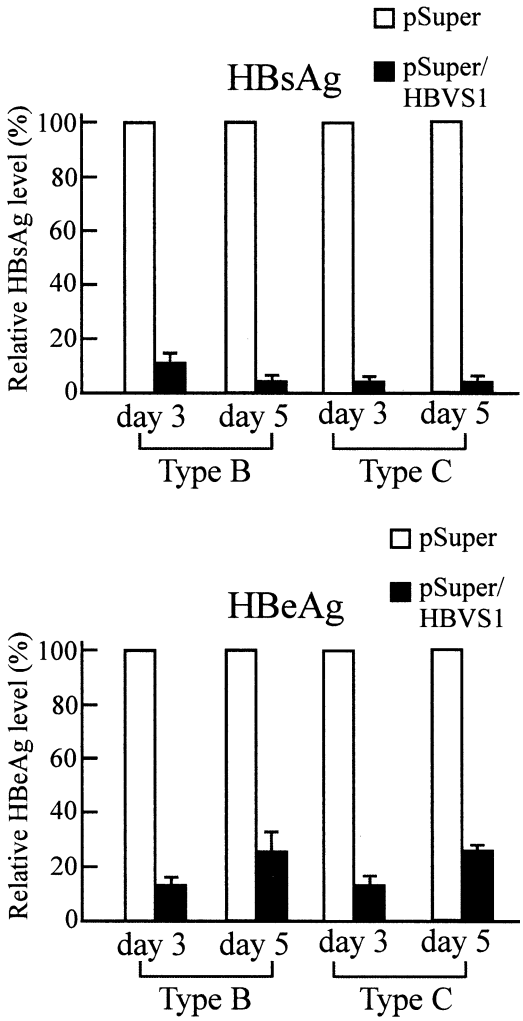

Figure 5. pSuper/HBVS1 inhibits surface and e antigens expression of HBV genotypes B and C. HBV genomic DNA of genotype B or C was extracted from patients' sera and PCR-amplified as described in the Materials and Methods section. Full-genome PCR products were digested with *Sap*I and purified and transfected into HuH-7 cells with pSuper or pSuper/HBVS1. Culture media were collected on day 3 and day 5 posttransfection, and the levels of HBsAg and HBeAg were determined. Levels of HBsAg and HBeAg secreted by pSuper-treated cells were defined as 100%. Values shown are averages of 3 experiments (means ± standard deviation).

though the total PCR product from the same patient showed no obvious resistance to pSuper/HBVS1 treatment. After sequencing, this particular clone (pHBV-CI-m) was found to exhibit a silent mutation in the target sequence of pSuper/HBVS1 (Figure 6*A*). This mutation did not change the amino acid sequence of either the polymerase gene or the surface gene. Neither directly sequenced PCR product nor the other 9 clones from the same patients displayed this nucleotide change within the target sequence of pSuper/HBVS1, indicating that this mutant clone represents only a minor population. The next concern was whether or not this mutant clone would be selected out in the presence of pSuper/HBVS1. Mimicking the heterogeneous population of HBV in patients, pHBV-CI-m was mixed with pHBV-CI-wt at a ratio of 1:9 and transfected to HuH-7 cells in the presence of pSuper/HBVS1 or pSuper. The HBV

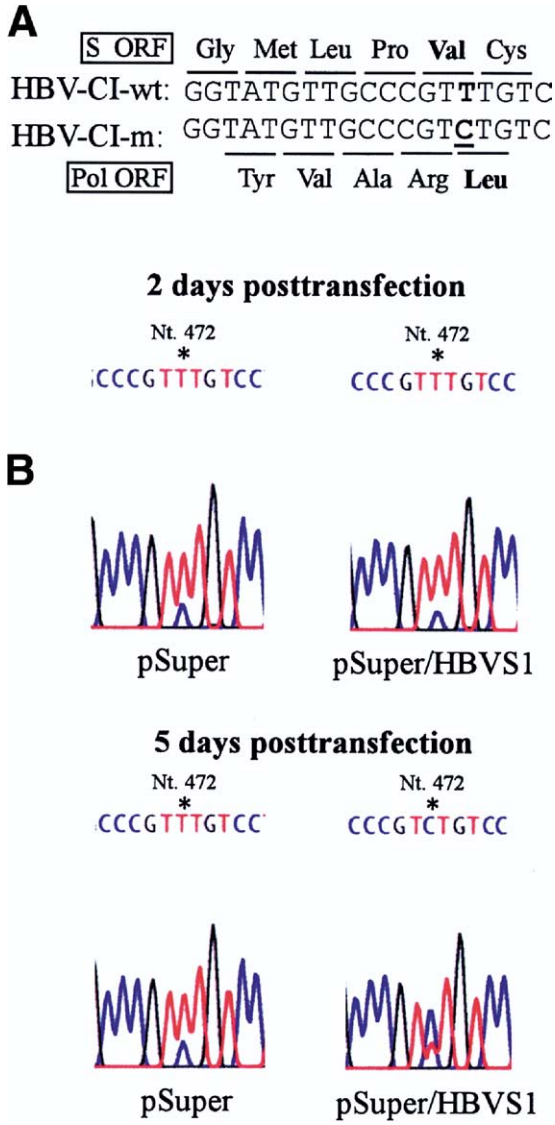

Figure 6. *(A)* Target sequences of pSuper/HBVS1 on HBV-CI-wt and HBV-CI-m. *CI* denotes a genotype C HBV from the patient I, and "*m*" and "*wt*" denote resistant mutant and wild type, respectively. The triplet codons for surface and polymerse genes are illustrated by *lines* located either above (S open reading frame) or below (polymerase open reading frame) the sequence. The mutated nucleotide on the mutant is *underlined*. The nucleotide change from T to C does not change the amino acid sequences of either surface or polymerase genes. Both GTT and GTC encode for valine in the S gene; TTG and CTG encode for leucine in the polymerase gene. (*B*) Sequence electropherogram produced by sequencing PCR products directly. HuH-7 cells were transfected with pHBV-CI-wt and pHBV-CI-m at a 9:1 ratio in the presence of pSuper or pSuper/HBVS1. Viral DNA extracted from virions released from transfected cells on day 2 and day 5 posttransfection were PCR amplified with primers HBV+2836 and HBV-695 then sequenced with HBV+56. *Indicates position of mutated sequence.

Table 1. Summary of the Results of Sequencing Individual Clones After Transfection for 2 Independent Experiments

	Day 2 posttransfection (No. of mutant clones/No. of total clones)	Day 5 posttransfection (No. of mutant clones/No. of total clones)
Experiment 1		
pSuper	2/10 (20%)	3/12 (25%)
pSuper/HBVS1	2/10 (20%)	6/14 (43%)
Experiment 2		
pSuper	Not determined	1/10 (10%)
pSuper/HBVS1	Not determined	3/10 (30%)

NOTE. HuH-7 cells were transfected with clinical full-length clone HBV-CI-wt and HBV-CI-m in a ratio of 9:1 in the presence of pSuper or pSuper/HBVS1. Viral DNA was extracted from culture supernatant at days 2 and 5 posttransfection and amplified by PCR with primers HBV+2836 and HBV-695. PCR products were cloned into the vector pyT&A. Individual clones were randomly picked from different experimental groups and sequenced with primer HBV+56.

genome was purified in the culture supernatants at days 2 and 5 posttransfection. An HBV fragment covering the target sequence of pSuper/HBVS1 was amplified and the PCR products cloned. In the presence of pSuper/HBVS1, the proportion of HBV with mutant sequence was significantly increased at day 5 posttransfection, as reflected by the results from sequencing PCR products directly and at least 10 individual clones for each experiment. In the electropherogram of PCR product sequencing, it was evident that the dominant signal had changed from "T" to "C" for the sample treated with pSuper/HBVS1 at day 5 posttransfection but not at day 2 posttransfection (Figure 6*B*). Results of sequencing individual clones also revealed the increase of the mutant clone (summarized in Table 1). At day 2 posttransfection, ratios of mutant clone were quite similar for samples from cells cotransfected with pSuper or with pSuper/HBVS1 (20%). At day 5 posttransfection, the ratio rose significantly to 43% in the sample treated with pSuper/HBVS1, and the ratio remained at 25% for the sample treated with vector pSuper alone (Table 1). In another independent experiment, the ratios of mutant clones from cells transfected with pSuper/HBVS1 and pSuper were 30% and 10%, respectively, at day 5 posttransfection. All these results strongly implied that siRNA could exert a selection pressure on preexisting resistant mutant(s).

Discussion

With the advantages of being highly specific, potent, and safe for gene silencing, the potential of using siRNA as an antiviral agent has been actively explored in numerous studies. In this work, RNA polymerase III promoter-based vector was used to express shRNA against HBV and clearly demonstrated the strength of this vector in inhibiting HBV expression and replication in both cell culture and in mouse model systems. Furthermore, the shRNA-expressing plasmid used in this study cannot only suppress the expression of laboratory prototype HBV strain but also can suppress expression of HBV from clinical isolates with different genotypes.

During the preparation of this work, several studies documented the anti-HBV activity of RNA silencing by either synthetic siRNA or shRNA-expression plasmids in vitro[11–18] and in vivo.[12,13,18] These results resembled the present investigative outcomes while supporting siRNA as a promising approach of controlling HBV. What makes pSuper/HBVS1 distinctive is that its target site is the only one conserved for 7 HBV major genotypes (A to G) among all siRNA target sequences published so far. Only genotype H contains a nucleotide difference within the pSuper/HBVS1 target sequence. Nucleotide 472 (the same nucleotide in the previously identified pSuper/HBVS1 resistant clone) is G in genotype H rather than T in other genotypes. Nevertheless, genotype H is not common worldwide. Given the high heterogeneity of HBV sequences and the sensitivity of siRNA to the changes of sequences,[28] it is advantageous to design potent siRNA targeting highly conserved regions among various HBV isolates. However, it is very challenging to develop an siRNA targeting highly conserved regions and also being very effective for HBV. In fact, pSuper/HBVS1 is the only one found so far. Conservation of an siRNA target sequence can generalize the utility of siRNA to different HBV genotypes and strains, as illustrated by the suppressive effects of pSuper/HBVS1 on the expression of HBV genotypes A, B, and C in this study. Although the inhibitory effects of pSuper/HBVS1 on other genotypes were not tested because of lack of clinical samples, pSuper/HBVS1 very likely can suppress the expression of other genotypes with the conserved target sequence.

Although this and other studies have proved the feasibility of using siRNA to control HBV expression and replication in vivo and in vitro, emergence of resistant mutants may be problematic. Previous studies on using siRNA to control replication of human immunodeficiency virus or poliovirus in culture have noted the emergence of resistant mutants.[29–32] These resistant mutants either emerged de novo with molecularly cloned viral genomes[29,30] or were likely present in the initial virus population and selected out in the presence of siRNA.[32] In this study, an HBV variant with a silent mutation within the target site of pSuper/HBVS1 was found from one HBV chronic carrier. To our knowledge,

this is the first experimental proof that resistant mutant indeed preexists in the quasispecies of HBV patients. Data were also presented supporting the notion that this mutant can be selected out in the presence of siRNA. This finding, together with others, underlines the importance of sequence identity of siRNA to its target sequence to be effective. Combinations of multiple siRNA targeting separate regions of the genome can alleviate the problem of resistant mutants. Use of siRNA targeting functionally indispensable and conserved regions can further minimize this problem, reducing the numbers of siRNA used and therefore the cost. For pSuper/HBVS1, the mutation identified for pHBV-CI-m at nucleotide 472 caused silent mutation for both surface and polymerase genes within this conserved target region. In genotype H, the difference at nucleotide 472 left unchanged the amino acid sequence of HBV surface proteins, causing Leu to Val conservative change in the polymerase reading frame. These findings implied that probably only very few mutations can be tolerated by HBV within pSuper/HBVS1 target sequence. Therefore, preventing the emergence of resistant clones can be simply achieved by including shRNA against the mutant sequence. This problem may also be overcome by combining with other antiviral strategies or siRNA targeting host factors that are dispensable for cell but essential for virus.

Because all HBV transcripts overlap in their C terminals, a single shRNA targeting multiple critical transcripts simultaneously could be designed to maximize the inhibitory potency. In this regard, pSuper/HBVS1 targets all HBV major transcripts except X mRNA. Interestingly, this investigation found that pSuper/HBVS1 suppressed the expression of HBsAg more efficiently than that of HBeAg, although it targeted the same sequence on the transcripts for these 2 proteins. In contrast, the S-target siRNA used by Klein et al resulted in greater suppression of HBeAg than HBsAg.[18] Song et al also noticed that the same siRNA could have varying suppressive effects against human immunodeficiency virus in different cell types.[33] Currently, it is uncertain why siRNA exerts differential suppressive effects on the same target sequence on different RNA molecules or in different cell types. One possible speculation is that the target sequence in different RNA molecules may have different secondary structures or are bound with different proteins and thus make their accessibility to the same siRNA different.

In natural infections, HBV uses covalently closed circular DNA (cccDNA) as the template for transcription. Therefore, to treat chronic HBV infection, the siRNA needs to either sustain in the infected hepatocytes to continuously degrade HBV RNA or to inactivate cccDNA from transcription. Thus far, siRNA-induced gene silencing in mammalian cells seems to be transient because of the likely lack of the RNA silencing-amplification mechanism present in worms and plants.[34] Recently developed lentivirus, retrovirus, and adenovirus-associated virus (AAV)-based shRNA expression vectors may provide a promising approach for long-term expression in mammals, although safety issues would be a concern. The effect of siRNA/shRNA on cccDNA of HBV would not be easy to address in the experimental systems used in the current study. Neither HCC cells transfected with HBV DNA nor mice hydrodynamically injected with HBV DNA have been generally proficient in providing cccDNA templates for pregenomic RNA synthesis.[26,35] Recent advances in inducing DNA methylation by siRNA in human cells[36,37] have shed light on using siRNA to inactivate HBV cccDNA from transcription. Further studies will address the effects of siRNA on HBV cccDNA.

This and other studies have clearly established that siRNA/shRNA could serve as a powerful potential therapeutic tool for treating hepatitis B infection as well as other diseases, although some technical obstacles must be overcome. The quick advances of siRNA technology, including optimization of target sequences, improvement of delivery methods, and expression vectors, would definitely render siRNA more clinically feasible and useful in the future. Furthermore, more knowledge of the working mechanisms of the siRNA-mediated effect on DNA genome and systemic silencing in plants and other organisms (reviewed in Agrawal[38]) would facilitate the development of strategies to induce these activities in mammalian cells. By then, siRNA will have become an even more potent and ideal therapeutic agent for HBV as well as other viral infections.

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