

Inhibition of Hepatitis B Virus Expression and Replication by RNA Interference

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RNA interference (RNAi) is the process of sequence-specific gene silencing, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the target gene. Because it has been shown that RNAi can be accomplished in cultured mammalian cells by introducing small interfering RNAs (siRNAs), much effort has been invested in exploiting this phenomenon for experimental and therapeutic means. In this study, we present a series of experiments showing a significant reduction in hepatitis B virus (HBV) transcripts and proteins in cell culture, as well as in the viral replicative forms, induced by siRNA-producing vectors. The antiviral effect is sequence-specific and does not depend on active viral replication. In conclusion, our data suggest that RNAi may provide a powerful therapeutic tool, acting both on replication-competent and on replication-incompetent HBV. (HEPATOLOGY 2003;37:764-770.)

Hepatitis B virus (HBV) is a 3.2-kb DNA virus, replicating almost exclusively in the liver.¹ Although effective recombinant vaccines are available, HBV infection is still a major global health problem: Each year, acute and chronic HBV infection causes about 1 million deaths. Among the 350 million people with chronic infection, the risk of dying from HBV-related diseases, such as end-stage cirrhosis and hepatocellular carcinoma (HCC) is between 15% to 25%.² Since the early 1990s, chronically infected patients have been treated with recombinant interferons that are effective only in limited cases.³ Recently, nucleoside analogs, which directly affect viral replication by inhibition of its reverse transcriptase activity, were shown to be highly effective in the clearance of HBV-DNA from serum. However, the recurrence of viremia after cessation of therapy and the development of escape mutants with prolonged treatment remain major obstacles in achieving complete cure. Furthermore, nucleoside analogs, such as

3TC- lamivudine, impede viral replication but do not directly promote its eradication.³

RNA interference (RNAi) is the process whereby double-stranded RNA (dsRNA) induces the sequence-specific degradation of homologous messenger RNA (mRNA).⁴ This process is mediated by 21 to 23 nucleotides, called small interfering RNAs (siRNA), cleaved from dsRNA. Although first discovered in *Caenorhabditis elegans*,⁵ it was soon after shown that RNAi can be induced in various mammalian cells by introducing synthetic 21nt siRNAs⁶ to obtain strong and specific suppression (knockdown) of gene expression. Recently, a new vector system called pSUPER (suppression of endogenous RNA), which directs the synthesis of siRNAs and persistently suppresses gene expression in mammalian cells, has been developed.⁷

To evaluate the anti-HBV therapeutic potential of RNAi, we designed 2 pSUPER vectors, each targeted against a distinct 19nt sequence in the HBV genome. We have analyzed the levels of viral proteins and transcripts, as well as the viral replicative forms, in the presence of the constructed pSUPER vectors. We show that RNAi is an efficient approach in reducing the level of HBV transcripts and proteins and in suppression of HBV replication.

Materials and Methods

Cell Culture. Huh7 cells were maintained in Dulbecco's modified Eagle's minimal essential medium as previously described.⁸ Cells were seeded at about 60% confluence 4 to 6 hours before transfection, which was carried out by the CaPi method as previously described.⁸

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RNAi, RNA interference; dsRNA, double-stranded RNA; mRNA, messenger RNA; siRNA, small interfering RNA; pSUPER, suppression of endogenous RNA; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; HBsAg, HBV surface antigen.

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Plasmid Constructs. For 1.3× HBV construction, an overlength HBV genome (*adv* strain) of 4,195 bp was produced, harboring a 5' terminus of the unique EcoRV site (nt 1043, considering EcoRI-unique site in the original 3.2kb HBV construct as nt number 1) and a 3' terminus of the unique TaqI site (nt 2017). This EcoRV-TaqI fragment was inserted between the SmaI-AccI unique sites of a pGEM-3Z plasmid, respectively. To create pSUPER plasmids, we used the primers 5'-GATC CCCGGTCTTACATAAGAGGACTTTCAAGAGAAGTCCTCTTATGTAAGACCTTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAGGTCTTACATAAGAGGACTTCTCTTGAAAGTCCTCTTATGTAAGACCGGG-3' (antisense) for pSUPER X; 5'-GATCCCCGATCAGGCAACTATTGTGGTTCAAGAGACCACAATAGTTGCCTGATCTTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAGATCAGGCAACTATTGTGGTCTCTTGAACCACAATAGTTGCTGATCGGG-3' (antisense) for pSUPER core; and 5'-GATCCCCGCCATTCTCTGCTGGGGGGTTCAAAGAGACCCCCAGCAGAGAATGGCTTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAGCCATTCTCTGCTGGGGGGTCTCTTGAACCCCCAGCAGAGATGGCGGG-3' (antisense) for pSUPER core2, each containing a specific 19nt target sequence from the *adv* strain HBV genome (nt 1649 to 1667 for pSUPER X, nt 2191 to 2209 for pSUPER core, and nt 2075 to 2093 for pSUPER core2). Primers were annealed and cloned into BglII-HindIII sites of the pSUPER vector, as described elsewhere.⁷ An HBV X^{mut} was created by introducing 4 silent-point mutations into the X orf of a 1.3× HBV genome, as follows: C→G (nt 1652), A→G (nt 1655), T→C (nt 1658), and G→A (nt 1664). Cloning was performed by polymerase chain reaction, using the following primers: 5'-CACAAATGTGGATATCCTGCC-3' and 5'-GCTCTAGCATTTAGGTGACAC-3' were used as external primers, and 5'-GGTGTTCACAAGAGAACTC-3' (sense) and 5'-GAGTTCTCTTGTGCAACACC-3' (antisense) were used as internal primers. The resulting polymerase chain reaction product was cleaved by EcoRV and HindIII and was ligated to a 1.3× wt HBV cut by the same restriction enzymes. HBV-GFP (green fluorescent protein) plasmid was created by cloning the GFP sequence into BglII-BglII restriction sites in the HBV genome, omitting the Core orf which contains the 19nt sequence recognized by Core siRNA, but leaving the 19nt sequence recognized by X siRNA intact. Enhanced green fluorescent protein (EGFP) expression plasmid is a commercial EGFP plasmid C1 vector (Clontech, Palo Alto, CA). Hemagglutinin-X plasmid was created as previously described.⁹ To create an HA-Core plasmid, a StyI-StyI fragment from HBV genome containing the Core orf was cloned into pCDNA3 plasmid (Invitrogen, Paisley, UK). This plasmid was later cut by XbaI and BamHI, and the core-containing fragment was

cloned into pSG5 plasmid (Stratagene, La Jolla, CA), which contained an HA sequence just upstream to the core-containing cloned fragment.

Isolation and Analysis of Viral RNA. Total RNA was extracted from transfected cells by TRI-Reagent (MRC, Inc., Cincinnati, OH), as previously described.¹⁰ Northern analysis was carried out by agarose-formaldehyde method according to published protocols.¹¹ About 20 μg of RNA per sample was separated on 1% agarose-formaldehyde gel and blotted to a Hybond-N nylon membrane (Amersham, Buckinghamshire, England). HBV transcripts were detected using radioactive probes prepared from the X gene region, labeled by random priming protocol.

Protein Analysis. Proteins were extracted from cells by TRI-Reagent according to the manufacturer's instructions, and subsequently fractionated on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. For Western blot analysis, gels were electroblotted to a nitrocellulose membrane, which was later soaked for 1 hour on a blocking solution (phosphate-buffered saline [PBS] containing 10% nonfat milk and 0.01% vol/vol Tween-20 [Sigma, St. Louis, MO]), and incubated for 1 to 2 hours at room temperature in the presence of either one of the following antibodies: monoclonal mouse anti-HBV core antigen (clone 22, diluted 1:5,000), polyclonal goat anti-HBV surface antigen (HbsAg) (diluted 1:2,000), both antibodies generated as previously described¹²; monoclonal mouse anti-GFP (BabCO, Denver, CO; Covance, diluted 1:10,000), monoclonal mouse anti-HA (BabCO, Covance, diluted 1:2000); or immunoglobulin G of mouse anti-β tubulin (clone no. TUB2.1, Sigma, diluted 1:10,000) antibodies. After incubation, the membrane was washed 3 times, and goat anti-mouse or donkey anti-goat conjugated with horse radish peroxidase (ICN Laboratories, Irvine, CA; diluted 1:10,000) were added and incubation allowed to proceed for an additional 1 hour. Antibody-antigen complexes were visualized by the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL) on radiographic film.

HBV Replication Intermediates Assay. Analysis of HBV replication intermediates was performed as previously described.¹³ Briefly, 5 days after transfection with HBV expressing plasmids, cells underwent cytoplasmic extraction in the presence of deoxyribonuclease (DNase)I to get rid of the transfected DNA. Then the lysates were treated with proteinase-K and, after phenol extraction, the encapsulated viral DNA was ethanol precipitated. Total DNA was fractionated on a 1.5% agarose-Tris-acetate/EDTA electrophoresis buffer gel, followed by denaturation and southern blotting to a Hybond N nylon membrane (Amersham). Viral DNA was detected by hybridization

with a ^{32}P random primed HBV probe (Roche, Mannheim, Germany).

Immunofluorescence and Microscopy. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 25 minutes at room temperature, washed with PBS containing 0.2% Tween 20, and blocked with fetal calf serum (Gibco, Karlsruhe, Germany) containing skim milk (10% vol/vol) and Tween 20 (0.2% vol/vol) for 45 minutes. Cells were then incubated with polyclonal rabbit anti-core antibodies, generated by repeated injections of bacterially expressed purified core proteins as previously described¹² (diluted 1:75 in PBS containing 0.2% Tween 20 and 10% milk), for 1 hour, washed 6 times for 5 minutes with PBS containing 0.2% Tween 20, and incubated with rhodamine red X-conjugated anti-rabbit antibodies (Jackson Immunoresearch Laboratories, Westgrove, PA; diluted 1:125) for 40 minutes. Finally, cells were washed 6 times, and cover slips were mounted in Aqua-polymount mounting solution (Polysciences, Warrington, PA). Microscopic images were obtained with a Bio-Rad MRC-1024 confocal system (Bio-Rad, Hercules, CA), using an argon-krypton mixed gas laser and mounted on a Zeiss Axiovert microscope (Zeiss, Jena, Germany).

Quantitative Analysis of Viral Proteins, Transcripts, and Replication Activity. To quantify HBV protein level, transcription, and replication, the radioactively probed membranes were visualized and quantified by PhosphorImager (FujiFilm Medical Systems, Stamford, CT) using Image Gauge V3.41 software (FujiFilm Medical Systems). The signal intensity of the control vector was considered as 100%.

Results

To evaluate the influence of RNAi on HBV life cycle, we used a newly introduced pSUPER vector, which directs the synthesis of siRNAs in mammalian cells.⁷ Two pSUPER vectors, one directed against a 19nt sequence in the X ORF (nt 1649 to 1667) and the other directed against a 19nt sequence in the core ORF (nt 2191 to 2209) were synthesized. The obtained plasmids, named pSUPER X and pSUPER core, respectively, are expected to produce specific siRNAs that target the HBV transcripts (Fig. 1). Huh7 cells were cotransfected with either X expression plasmid (HA-X vector) or core expression plasmid (HA-core vector) together with pSUPER X or pSUPER core, respectively. In each experiment, the reciprocal siRNA-producing plasmid was used as a negative control. A Western blot analysis performed 72 hours after transfection revealed a significant reduction, as compared with the control, in the levels of X and Core proteins in the presence of pSUPER X and pSUPER core, respec-

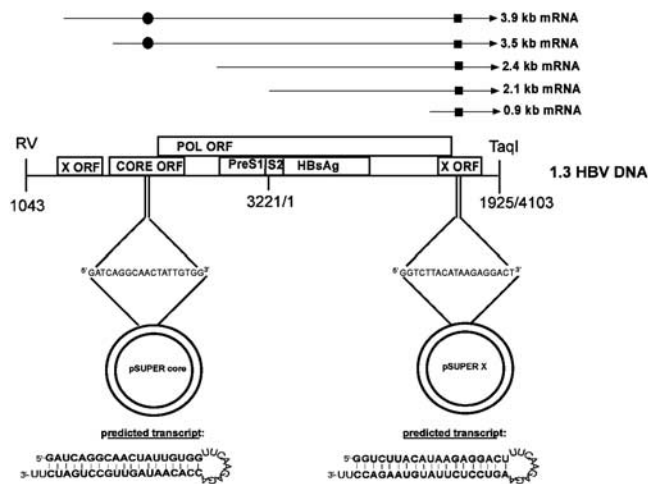


Fig. 1. Construction of pSUPER X and pSUPER core vectors. Two 64-nt primers were synthesized, each containing a 19-nt sequence (in the sense and antisense forms) from a different region of the HBV genome, as indicated. Each primer was cloned into a pSUPER vector as described in Materials and Methods. Notice that the pSUPER X vector is expected to target all the viral transcripts (black squares), whereas the pSUPER core vector targets only the long viral transcripts (black circles). Restriction sites of EcoRV and TaqI restriction enzymes in a 1.3× overlapped HBV genome, used in our transfection experiments, are indicated.

tively (Fig. 2A). These results suggest that X and Core siRNAs efficiently and specifically reduce the levels of ectopically expressed HBV proteins.

Next, we asked whether siRNA function is maintained in the context of the whole HBV virus. To this end, Huh7 cells were cotransfected with a plasmid containing 1.3× wt HBV genome, and with either pSUPER X or pSUPER core. A western blot analysis revealed significant reductions of 89% and 63% in the levels of Core protein when HBV was cotransfected with either pSUPER X or pSUPER core, respectively (Fig. 2B). However, whereas transfection of pSUPER X resulted in a reduction of about 60% in the level of the viral surface proteins (HBsAg), transfection with pSUPER core had no effect. This is expected given the fact that siRNA expressed by pSUPER X targets all the viral transcripts, whereas that expressed by pSUPER core exclusively targets the long 3.5-kb and 3.9-kb transcripts (Fig. 1). To rule out a nonspecific effect, such as transfection efficiency, Huh7 cells were cotransfected with wt HBV-DNA, a core expression plasmid (HA-Core) and pSUPER X. Unlike the HBV Core transcript, the vector-based HA-Core transcript does not contain the target sequence of the X siRNA. Therefore, it is expected that, on transfection, both proteins will be produced, but only the core protein expressed by the HBV will be susceptible to siRNA effect. Indeed, the level of the HBV core protein, but not that of HA-core protein, was significantly reduced (Fig. 2C), suggesting a specific effect of the X siRNA.

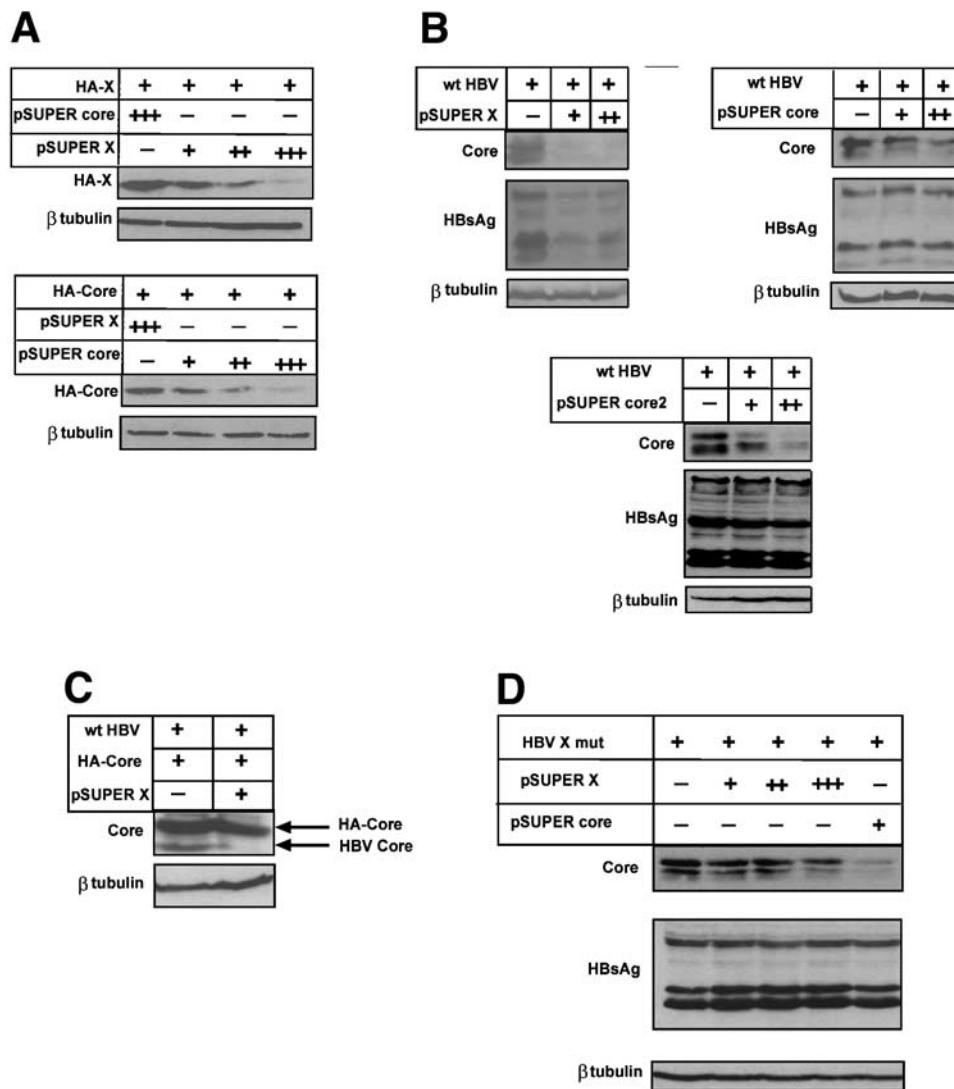


Fig. 2. RNAi results in a sequence-specific reduction of HBV proteins: (A) Huh7 cells were cotransfected with 4 μ g of either HA-X plasmid or HA-core plasmid, together with increasing amounts (4 μ g, 8 μ g, and 15 μ g) of either pSUPER X or pSUPER core plasmids, respectively. In each experiment, the reciprocal pSUPER plasmid (15 μ g) was used as a control. Proteins were analyzed 72 hours after transfection by western blot using an anti-HA antibody in the case of HA-X (**upper panel**), or an anti-core antibody in the case of HA-core (**lower panel**). (B) Huh7 cells were cotransfected with 10 μ g of the 1.3 \times HBV-DNA and increasing amounts (0 μ g, 5 μ g, and 10 μ g) of either pSUPER X (**left panel**), pSUPER core (**right panel**), or pSUPER core2 (**lower panel**) vectors. In each experiment, an empty pSUPER vector was used as a control vector. Proteins were analyzed 72 hours after transfection by Western blot, using anti-core and anti-HBsAg antibodies. (C) Huh7 cells were cotransfected with 10 μ g of 1.3 \times HBV-DNA, 4 μ g of HA-core plasmid, and 15 μ g of either pSUPER X or empty pSUPER vector. Seventy-two hours after transfection, cells were harvested and proteins were analyzed by Western blot using an anti-core antibody. The locations of core and HA-core proteins are indicated. (D) Huh7 cells were cotransfected with 10 μ g of 1.3 \times HBV X^{mut}, as described in Materials and Methods, and with either increasing amounts of pSUPER X (5 μ g, 10 μ g, and 15 μ g) or 15 μ g of pSUPER core. Seventy-two hours after transfection, cells were harvested and proteins were analyzed by Western blot using anti-core and anti-HBsAg antibodies.

The specificity of the siRNA also was addressed by construction and employment of an HBV X^{mut}, which contains 4 silent-point mutations in the 19-nt sequence targeted by X siRNA. A Western blot analysis revealed that neither core nor X protein levels were reduced when HBV X^{mut} was cotransfected with pSUPER X (Fig. 2D), suggesting this mutant had escaped X siRNA effect. However, when HBV X^{mut} was cotransfected with pSUPER core, the level of core protein was significantly reduced,

because the 19-nt sequence in the core orf, targeted by pSUPER core, is intact. Finally, pSUPER X was cotransfected with a 1.3 \times HBV-GFP, in which the core orf was replaced by GFP. In this construct, the GFP protein is translated from the 3.5-kb mRNA that harbors the sequence of X siRNA and, therefore, is expected to be knocked down by pSUPER X. Microscope examination revealed that the amount of the fluorescent cells was not affected when the control EGFP was cotransfected with

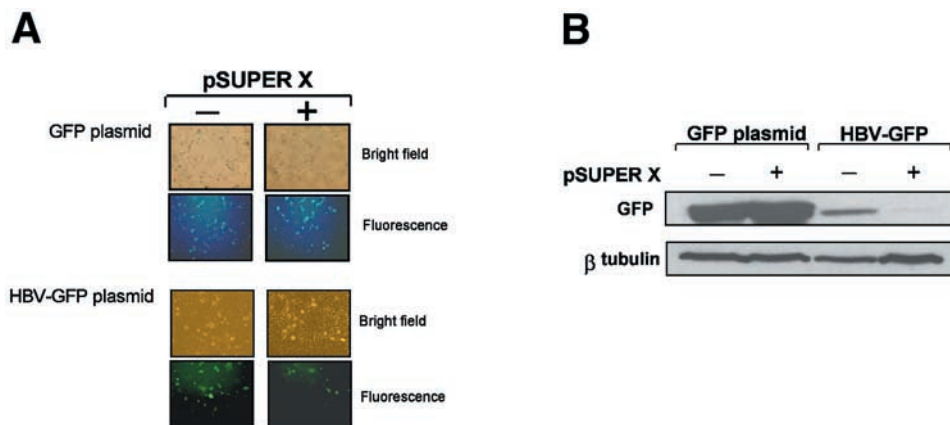


Fig. 3. The effect of pSUPER X on HBV is specific and is not dependent on viral replication. (A) Huh7 cells were cotransfected with 3 μ g of EGFP plasmid and with 7 μ g of either empty pSUPER or pSUPER X plasmids. Alternatively, cells were cotransfected with 6 μ g of HBV-GFP plasmid (see Materials and Methods) and 15 μ g of either an empty pSUPER or pSUPER X plasmids. Cells were visualized 72 hours after transfection with a fluorescence microscope, and representative fields were photographed. (B) The same experiment as (A) was carried out; this time, cells were harvested and proteins were analyzed by Western blot using an anti-GFP antibody.

either pSUPER X or an empty pSUPER vector. However, cotransfection of HBV-GFP with pSUPER X resulted in a significant reduction in the amount of the fluorescent cells (Fig. 3A). A Western blot analysis, using an anti-GFP antibody, confirmed this to be a result of a marked reduction in the level of GFP (Fig. 3B). Altogether, our data show that HBV-specific siRNAs can induce a significant and sequence-specific reduction in the level of HBV proteins.

To further substantiate our observations, we used HepG 2.2.15 cells, which stably express HBV. Cells were cotransfected with a GFP-expressing plasmid to monitor the transfected cells together with either empty pSUPER or pSUPER X vectors. Seventy-two hours after transfection, cells were immunostained for HBV core protein and analyzed by confocal microscopy. Interestingly, core levels in GFP-positive cells were significantly reduced in the presence of pSUPER X (Fig. 4). This was not the case in the control empty pSUPER transfected cells.

To investigate the influence of RNAi on HBV transcripts, Huh7 cells were cotransfected with 1.3 \times HBV-DNA with either pSUPER X or pSUPER core. Northern blot analysis revealed a significant reduction of about 68% in the level of all the viral transcripts when pSUPER X was used (Fig. 5). In contrast, transfection of pSUPER core resulted in only minor reduction of about 13% in the 3.5-kb transcript (Fig. 5), compared with a much more pronounced reduction of about 63% in the corresponding core protein, even though the RNA and proteins were both obtained from the same experiment (Fig. 2B). As expected, the other transcripts were not affected at all. Interestingly, pSUPER core2, containing a 19-nt sequence of the core or from a position located just up-

stream to the original pSUPER core target, was much more efficient in reducing both the viral core protein (Fig. 2B) and the viral pregenomic transcript (Fig. 5) (about 80% reduction in core protein level and about 50% reduction in the level of the 3.5-kb transcript). Overall, these results indicate that RNAi action is targeted mainly at the mRNA levels, even though interference in the translational level can not be ruled out.

Finally, we investigated the influence of RNAi on HBV replication. Huh7 cells were transfected with 1.3 \times wt HBV-DNA and with either pSUPER X or pSUPER core. Analysis of the viral replicative intermediates, per-

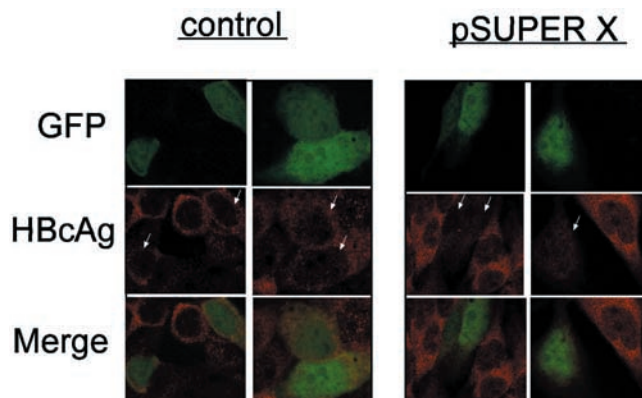


Fig. 4. RNAi effect is maintained in HBV stably transfected cells. HepG 2.2.15 cells, stably expressing HBV, were seeded on cover slips in 12-well plates and transfected with 100 ng of EGFP plasmid together with 2 μ g of either empty pSUPER vector, used as a control, or pSUPER X vector. Seventy-two hours after transfection, cells were fixed, immunostained for HBV core protein, and visualized under a confocal microscope as described in Materials and Methods. Two representative fields from both the control and the pSUPER X experiments are shown. Cells that are GFP-positive are indicated by **arrows** (HBcAg, HBV core antigen).

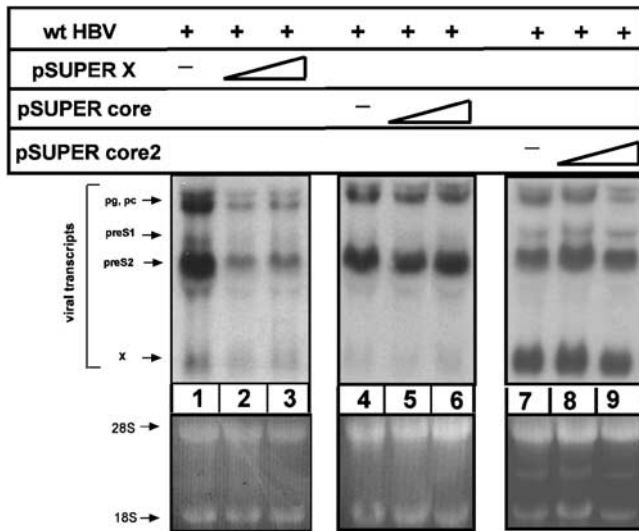


Fig. 5. RNAi in HBV operates, at least partly, at the mRNA level. The same transfection protocol as in Fig. 2B was carried out, using increasing amounts (0 μ g, 5 μ g, and 10 μ g) of either pSUPER X (lanes 1, 2, 3), pSUPER core (lanes 4, 5, 6), or pSUPER core2 (lanes 7, 8, 9). RNA was extracted 72 hours after transfection and analyzed by Northern blot as described in Materials and Methods. The 28s and 18s rRNAs were visualized under ultraviolet light for equal loading control (pg, pre-genomic; pc, precore).

formed 5 days after transfection, showed a dramatic reduction of about 95% in HBV replicative forms when HBV genome was cotransfected with pSUPER X, whereas cotransfection with pSUPER core resulted in only moderate reduction of about 40% in viral replication (Fig. 6). Thus, RNAi is effective in attenuating HBV replication as well.

Discussion

In this study, we show that cotransfection of siRNA-producing vectors, targeted against specific sequences in the HBV genome, results in a significant reduction in the corresponding viral transcripts and proteins. This reduction is highly selective, because only the cognate transcripts and proteins were affected. In addition, RNAi activity is sequence-specific, because introducing point mutations in the target gene abrogated its action. Most importantly, specific siRNAs dramatically inhibit viral replication, as is evident by decreasing levels of all viral replicative forms. Notably, impairing the viral core and polymerase proteins by introducing a GFP sequence into the viral genome does not alter RNAi ability to reduce the expression of viral transcripts, indicating that siRNA activity is not dependent on viral replication.

In this study, we used 3 different anti-HBV siRNAs and all proved effective, but not to the same extent. Comparison of 2 distinct siRNAs designed to target the core orf revealed that targeting different regions even along a

given orf yields different efficacies in gene suppression. Thus, there is room for improvement in searching for the ideal sequence or region in the genome that may yield the optimum activity.

Here we took advantage of a well-known, but still not completely understood, phenomenon called RNAi, which is the term for a sequence-specific gene silencing in the presence of dsRNA.⁴ Although some studies indicate that RNAi machinery operates at multiple levels,^{14,15} its main action probably is mediated at the posttranscriptional level by rapid destruction of homologous mRNAs.⁵ RNAi was first suggested to be used by plants as a natural antiviral defense mechanism, especially against RNA viruses.¹⁶ Reports about viral proteins that suppress RNA silencing found in plant viruses¹⁷ substantiated this theme. On the other hand, mammalian cells were thought to possess a nonspecific, interferon-mediated antiviral response, induced by viral long (> 35 nt) dsRNA.¹⁸ From the time it became clear that specific RNAi, mediated by 21- to 22-nt siRNAs, is also common to mammals,⁶ a possible role for RNAi as an antiviral mechanism in mammals became a real option. Furthermore, the notion that RNAi can be induced in mammalian cells led to tremendous efforts to take advantage of this unusual phenomenon not only as an excellent experimental tool, but also as a means for therapeutic interventions. The introduction of new vector systems that direct the synthesis of siRNAs, such as the pSUPER system,⁷ provide new impetus for these efforts. Recently, it was shown that RNAi targeted against different regions in HIV genome is effective in attenuating its replication.¹⁹⁻²¹ A strong antiviral effect of RNAi was observed also in the case of polio and human papilloma viruses,^{22,23} making the option of using RNAi as an antiviral weapon much more realistic.

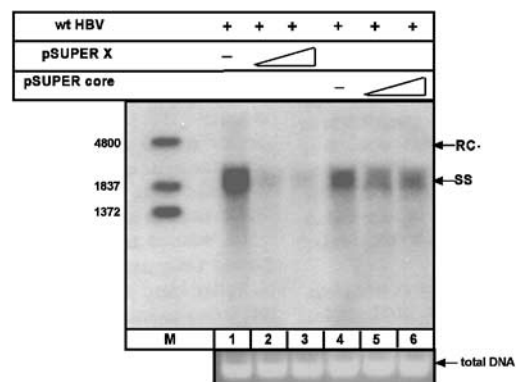


Fig. 6. RNAi efficiently suppresses HBV replication. The same transfection protocol as in Fig. 2B was carried out, using either pSUPER X or pSUPER core. Cells were harvested 5 days after transfection and analyzed for viral replicative intermediates as described in Materials and Methods. Total DNA was visualized with ultraviolet light for equal loading control (M, marker sizes in kb; RC, relaxed circular DNA form; SS, single-stranded DNA form).

HBV infection is still a major health problem, even though effective vaccines have been available for the last 20 years.² Interferon and lamivudine therapy for chronic hepatitis B carriers are reported to result in long-term remissions in a significant percentage of patients^{24,25}; however, these treatments have some drawbacks, including possible serious side effects in the case of interferon or recurrence of viremia after cessation of therapy and development of escape mutants after a long period of lamivudine treatment.²⁶

Therefore, using RNAi as an anti-HBV tool seems to have some important advantages: First, specifically targeting the viral transcripts and proteins severely impairs its replication and promotes its eradication, without activating nonspecific cellular responses, hence minimizing undesirable side effects. In addition, the numerous potential targets for RNAi along the viral genome make it possible to target conserved regions, limiting the viral ability to create escape mutants. The potential to introduce a few siRNAs targeted against different sequences simultaneously further limits this ability and makes it possible to treat chronically infected people with diverse circulating HBV genomes. Moreover, the ability of siRNAs to reduce the levels of viral transcripts and proteins even in the absence of active viral replication makes it a good candidate as an adjuvant therapy to lamivudine, which acts only on the replication-competent HBV.

Even though RNAi therapy shares a major disadvantage with lamivudine therapy, namely, the inability to affect the covalently closed circular DNA pool that resides in the liver cell nuclei of chronically infected people, it seems that its ability to severely affect multiple steps in the viral life cycle is a significant progress. Obviously, the siRNA-encouraging results should be tested in animal models. To this end, developing an efficient delivery method is needed. Methods to be considered include injection of synthetic siRNAs or siRNA expressing vectors into the blood stream²⁷ or directly into the liver. The expected stability of siRNA expression vectors over the synthetic siRNAs may ensure a long enough expression for efficient eradication of the virus. In conclusion, our present results open a new avenue for treating HBV infection, which remains a common and serious disease.

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