

Small Interfering RNA Inhibits Hepatitis B Virus Replication in Mice

Hilla Giladi,¹ Mali Ketzinel-Gilad,¹ Ludmila Rivkin,¹ Yaakov Felig,² Ofer Nussbaum,³ and Eithan Galun^{1,*}

¹Goldyne Savad Institute of Gene Therapy and ²Department of Pathology, Hadassah Hebrew University Medical Center, Jerusalem 91120, Israel
³XTL Biopharmaceuticals, Rehovot, 76100 Israel

*To whom correspondence and reprint requests should be addressed. Fax: 972-2-6430982. E-mail: galun@md2.huji.ac.il.

Current therapies for chronic hepatitis B virus (HBV) infection are limited in their effect on viral gene expression and replication. Recent reports have shown that RNA interference can be induced in mammalian cells by short interfering RNA duplexes (siRNA). Here we studied the effects of an HBV-specific 21-bp siRNA targeted to the surface antigen region (HBsAg), where three major viral mRNAs overlap, on HBV gene expression and replication both in a cell culture system and in a mouse model for HBV replication. Transfection of siRNA into HepG2.2.15 cells, which constitutively produce HBV particles, caused a significant reduction in viral RNA production that was accompanied by a >80% drop in the secretion of viral HBsAg and HBeAg into the medium. The effect of RNAi was tested *in vivo* in a mouse model that we have developed for HBV infection, which entails hydrodynamic injection of a plasmid bearing the HBV genome into tail veins of mice. Injection of the HBV plasmid induces viral replication and generation of HBV viral particles detectable in the mouse sera. Co-injection of the HBV plasmid together with siRNA caused a significant inhibition in the level of viral transcripts, viral antigens, and viral DNA detected in the livers and sera of the treated mice relative to control animals. Results suggest that siRNA is capable of inhibiting HBV replication *in vivo* and thus may constitute a new therapeutic strategy for HBV infection.

Key Words: RNAi, HBV, hydrodynamic injection, animal model, antiviral therapy

INTRODUCTION

Hepatitis B virus (HBV) causes both acute transient and chronic infection of the human liver, and despite the availability of a vaccine, hepatitis B remains a major health problem in many countries. The number of HBV carriers worldwide is estimated to be 350 million. Chronic HBV infection, which can lead to the development of liver cirrhosis and hepatocellular carcinoma [1,2], is currently treated with interferon [3] or nucleoside analogs such as Lamivudine, Entecavir, and Adefovir dipivoxil [4–6]. The success rate of these treatments, however, is low and frequently reinfections occur. In this paper we describe a potential alternative approach to inhibit HBV infection and replication, RNA interference (RNAi).

RNAi is a natural process by which double-stranded RNA directs sequence-specific silencing of homologous genes. This process is evolutionarily conserved and is found in a wide range of eukaryotic organisms (see for reviews [7,8]). Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21- to 23-nucleotide duplexes of RNA

(siRNA) [9,10] or, alternatively, by transcription of an expression construct [11], opening up possibilities for controlling replicative processes of pathogens.

This discovery prompted the use of RNAi for specifically inhibiting gene expression and replication of infectious viruses. The replication of a growing number of human pathogenic viruses in cell culture was shown to be inhibited by RNAi, including poliovirus, HIV-1, HCV replicons, and influenza virus [12–19]. Recently it was reported that RNAi can also induce transcriptional silencing and replication of hepatitis B virus [20,21].

The study of HBV replication is hampered by the lack of simple evaluative systems. To study the effect of RNAi on HBV replication in a cell culture model, we used the HepG2.2.15 cell line, a derivative of the human HepG2 hepatoma cell line that has been stably transformed with several copies of the HBV genome. This line supports viral gene expression and viral replication and serves as an *in vitro* model for HBV replication [22]. *In vivo* studies of HBV replication have also suffered from the paucity of animal models, and so far, only chimpanzees appear to support

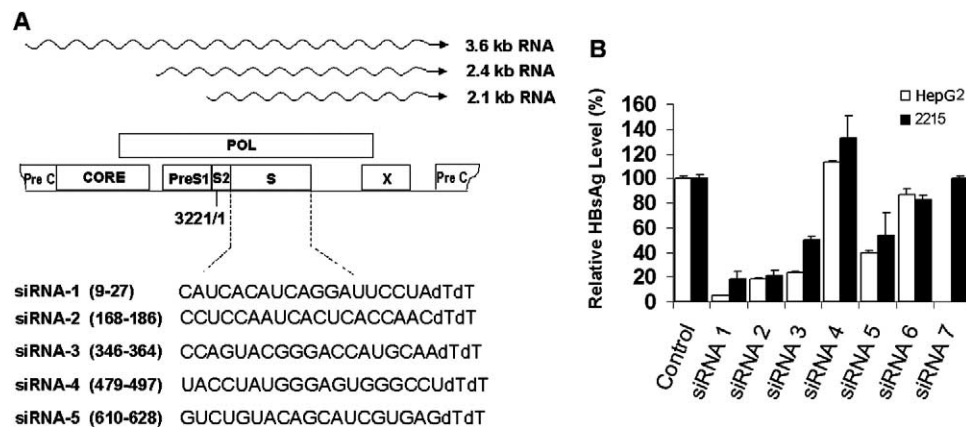


FIG. 1. Inhibition of HBV surface antigen expression in cell culture by different siRNA duplexes. (A) Schematic representation of the HBV genome with the three major transcripts depicted above (wavy lines) and the sequences of the five siRNAs (sense strand) targeted to the small s antigen/polymerase region with the distance from the translational start site of *s* shown in parenthesis. (B) HepG2 cells (5×10^4) were cotransfected with 300 ng plasmid pHBV and 40 nM of each siRNA, and the amount of HBsAg in the culture medium was measured 72 h later and is depicted as a percentage of the HBsAg secreted by cells transfected with the pHBV plasmid alone (control). HepG2.2.15 (2215) cells were transfected solely with 40 nM siRNA, and the amount of HBsAg in the culture medium was measured 48 h posttransfection and is presented as a percentage of the amount secreted by mock-transfected cells (control). siRNA6 and 7 are irrelevant control siRNAs. The data shown are mean values \pm SD based on three independent experiments.

HBV replication. The difficulties in using such large animals at this early stage of investigation, for evaluating the RNAi effect, are obvious. It is possible to use transgenic mice; however, unlike chronic human carriers, in this animal model all the hepatocytes harbor HBV sequences, and HBV replication is generated in part from integrated HBV sequences [23,24]. We (manuscript in preparation) and others [25,26] have been devising a mouse model for studying *in vivo* HBV infection. In our system, Balb/c mice are injected hydrodynamically via the tail vein [27] with the HBV genomic plasmid padwHTD [28], and this results in the production and secretion of HBV-related antigens and replicative intermediates into the serum for over a week. By 10 days, viral particle production subsides, concomitant with the appearance of anti-HBV antibodies.

Here we demonstrate that siRNA directed against the HBV surface antigen region is capable of inhibiting viral gene expression and replication both, in the HepG2.2.15 cell culture system as well as in the mouse model system for HBV replication.

RESULTS

Inhibition of HBV Gene Expression in Cell Culture by siRNA Is Sequence Specific and Dose Dependent

To examine the ability of siRNA to inhibit HBV gene expression we selected as a target the gene for small HBV surface antigen (HBsAg). In this genomic region the major transcripts synthesized during HBV replication overlap and include the pregenomic RNA, which serves both as the template for reverse transcription and the generation of viral DNA and as the mRNA for core and polymerase, and two subgenomic mRNAs, Pre-S/L and S (see for review [29]) (Fig. 1A). We compared five different synthetic 21-bp

siRNA duplexes (siRNA 1 to 5) targeted to different regions of the HBsAg (Fig. 1A) for their ability to suppress expression of HBsAg. To undertake this, we cotransfected human HepG2 hepatoblastoma cells with the HBV molecular clone plasmid padwHTD (pHBV) together with each of the siRNA duplexes and determined levels of HBsAg secreted into the medium 3 days later. In parallel, we transfected HepG2.2.15 cells (2215 henceforth) with the same set of siRNAs and measured the levels of HBsAg in culture supernatants 48 h later. The time points for measuring the effects of siRNA in these cells were chosen following preliminary optimization experiments (not shown). To confirm the specificity of the inhibition, the experiments included as a negative control siRNA-6 directed against hepatitis C viral sequences and siRNA-7 directed against a cellular gene. The results show that all siRNA duplexes (except 4) caused a decrease in HBsAg levels in the medium to varying degrees, in similar patterns in both cell lines (Fig. 1B). The most dramatic inhibition was exhibited by siRNA-1, which is targeted to the region 9 to 27 bases downstream of the translational start site of small S antigen. We chose this siRNA for the following experiments. Inhibition levels caused by the siRNAs were slightly lower in 2215 cells. This could be due to the fact that all 2215 cells secrete HBsAg and thus, inhibition levels are also dependent upon the efficiency of transfection. In cotransfection of HepG2 cells, on the other hand, most cells that received the pHBV plasmid probably received the siRNA as well; hence, the observed level of inhibition was more pronounced.

Unexpectedly, siRNA-4 (located at position 479–497) caused a reproducible (but unexplained) small augmentation in the level of HBsAg secreted into the medium that we did not investigate further. In contrast to the HBV-

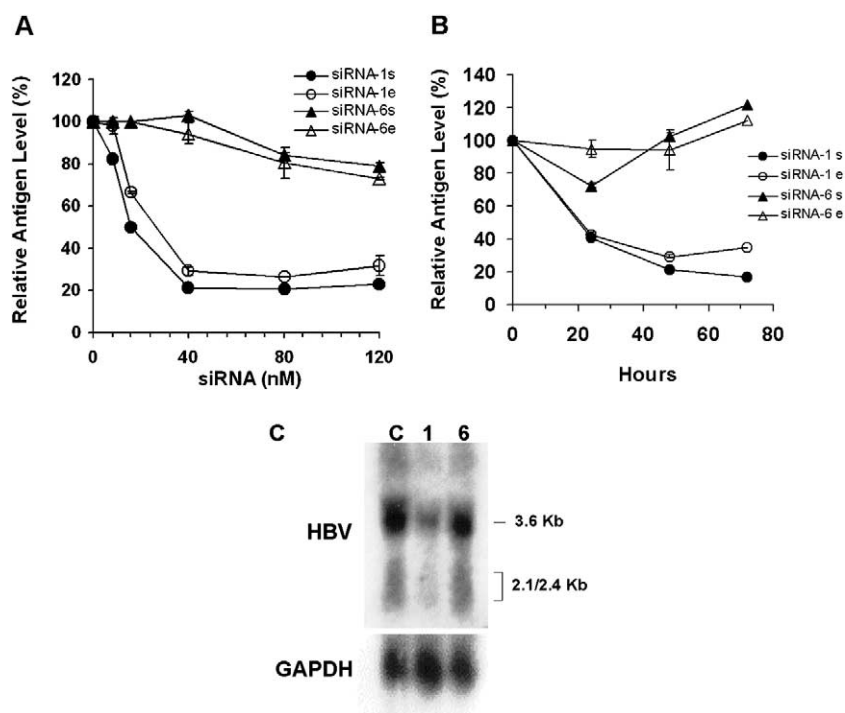


FIG. 2. siRNA-1 interferes with viral antigen and viral mRNA production in cell culture. (A) Dose-dependent inhibition of HBsAg and HBeAg expression. 2215 cells (5×10^4) were transfected by the indicated amounts of the HBV-specific siRNA-1 or the irrelevant siRNA-6 and assayed for the amount of HBsAg and HBeAg secreted into the medium after 48 h. (B) Kinetics of inhibition of HBsAg and HBeAg expression by siRNA. The levels of HBsAg and HBeAg secreted into the medium by 2215 cells transfected with 40 nM siRNA-1 or the negative control siRNA-6 were measured 24, 48, and 72 h posttransfection. The amounts of HBsAg and HBeAg are shown as percentages of the amounts secreted by mock-transfected cells (control). The data represent the mean values \pm SD of two independent experiments performed in duplicate. (C) Specific inhibition of HBV RNA by siRNA. Northern blot analysis performed on total RNA isolated from 2215 cells mock transfected (C) or transfected with 40 nM siRNA-1 (1) or siRNA-6 (6) 48 h posttransfection. The numbers on the right denote the positions of the major HBV transcripts.

specific siRNAs, the control siRNAs (6 and 7) had no effect on HBsAg levels in the media of the treated cells, indicating that inhibition by the HBV-targeted siRNAs was specific and due to RNAi.

To confirm further the specificity of the inhibitory effect of siRNA on HBV gene expression, we measured the level of α -fetoprotein constitutively secreted by HepG2 and HepG2.2.15 cells [30] and found no change between cells transfected with siRNA-1 or siRNA-6 and mock-transfected cells (data not shown). This suggests that general cellular transcription and translation were not affected by the particular siRNA employed.

We conducted a dose-response analysis (Fig. 2A) by transfection of 2215 cells with increasing amounts of siRNA-1 or negative control siRNA-6 and by measuring the amounts of HBsAg and HBeAg secreted into the medium 48 h posttransfection. Secretion of HBeAg to the medium by 2215 cells correlates with HBV viral particles that are produced and, thus, provides a surrogate marker for viral replication [29]. The results show that at concentrations of 40 to 80 nM siRNA per 5×10^4 cells, the highest level of inhibition (fivefold) of both viral antigens was obtained with siRNA-1, whereas siRNA-6 had no effect (Fig. 2A). At higher doses of siRNA, we observed a slight nonspecific inhibitory effect of siRNA-6 on both HBsAg and HBeAg. The observation that siRNA-1 inhibits the level of HBeAg as well as HBsAg secreted by 2215 cells strongly supports the notion that siRNA-1 inhibits viral replication as well as viral gene expression.

Kinetic study results (Fig. 2B) revealed a continuous

increase in the specific inhibition of HBsAg by siRNA-1 from 24 to 72 h after transfection. Inhibition of HBeAg expression was maximal at 48 h and started to decline by 72 h. The effect of siRNA in cells is known to be transient and to last 3 to 4 days [7].

To confirm that the effect of siRNA-1 on HBsAg and HBeAg expression was through the reduction of HBV RNA levels, we performed Northern blot analysis with RNA extracted from 2215 cells 48 h after transfection with siRNA-1 or the control siRNA-6. Results revealed that the level of the HBV 3.6 Kb and the 2.1/2.4 Kb mRNA species was reduced by 60% in siRNA-1-transfected cells compared to siRNA-6 and mock-transfected cells (Fig. 2C). In fact, siRNA-6 displayed no effect on levels of these mRNA species. This observation further supported the notion that inhibition of HBsAg and HBeAg expression in 2215 cells (as described above) was the result of RNA interference mediated by siRNA-1 of the major viral mRNA species. The reduction in the level of pregenomic RNA (3.6 kb) is expected to have an effect on viral DNA production as well, since this RNA provides the template for DNA synthesis.

siRNA-Mediated Inhibition of HBV Gene Expression *in Vivo*

We first selected a siRNA with the strongest *in vivo* inhibitory effect on HBV gene expression. Using the hydrodynamic tail vein injection method, we gave separate groups of mice 15 μ g of pHBV together with 1 nmol of each of the siRNA duplexes (1 to 5). Two days later, we obtained serum samples and tested them for HBsAg levels. In ac-

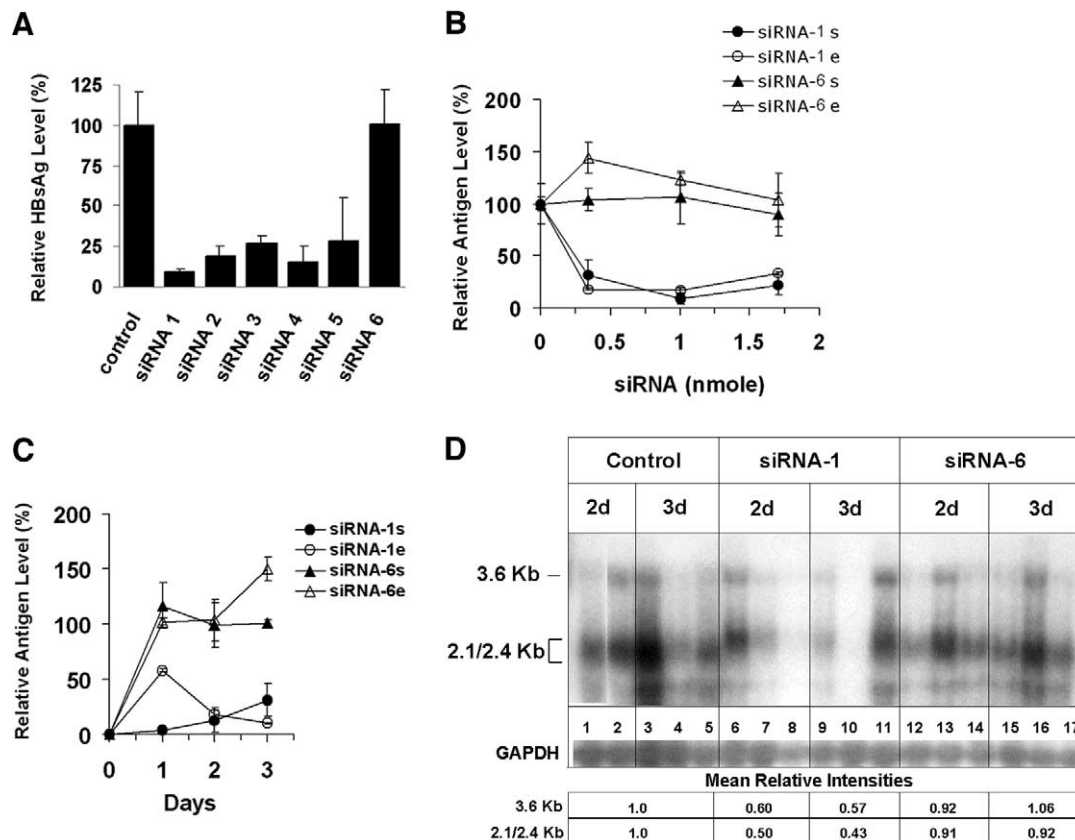


FIG. 3. The effects of siRNA on HBV gene expression in mice. (A) The inhibitory effect of different siRNAs on HBsAg expression *in vivo*. Mice were co-injected with 15 μ g pHBV plasmid DNA (control) or with pHBV together with 1 nmol of the various siRNA duplexes, and the amount of HBsAg in the serum was measured after 48 h. (B) Dose-dependent inhibition of HBV gene expression. Mice were injected with 15 μ g pHBV DNA together with increasing amounts of siRNA-1 or siRNA-6 and assayed for the level of HBsAg and HBeAg antigens after 48 h. (C) Kinetics of the siRNA effect. The amount of HBsAg and HBeAg in the serum was measured at 24, 48, and 72 h after co-injection of mice with 15 μ g pHBV together with 1 nmol siRNA-1 or siRNA-6. Antigen levels are depicted as percentages of the amount detected in the serum of control mice. The data presented are averages \pm SD of at least two independent injection experiments with four to six mice per treatment. (D) The effects of siRNA on HBV mRNA levels in mice livers. Northern blot analyses were performed on total RNA extracted from liver sections of mice injected with 15 μ g pHBV (control) and from mice co-injected with pHBV and 1 nmol siRNA-1 or siRNA-6, 2 and 3 days after injection. Each lane represents an individual mouse. The numbers at the bottom show the mean intensities of the 3.6 and 2.1/2.4 HBV mRNA species relative to the intensities of those in control mice.

cordance with the results of the cell culture experiments siRNA-1, among the other siRNAs, exhibited the strongest inhibitory effect on HBsAg expression (92%) (Fig. 3A). Interestingly, siRNA-4 did not show the stimulatory effect that was seen in the cell culture experiments (Fig. 1B). The negative control siRNA-6 exhibited no effect on HBsAg levels, demonstrating the specificity of the siRNA-1 effect.

To determine dose-response kinetics and optimal concentrations of siRNA for HBV inhibition, we injected mice via the tail vein with 15 μ g of pHBV together with 0.34, 1, or 1.7 nmol of siRNA-1 or the control siRNA-6 and measured the levels of HBsAg and HBeAg in their serum. The results revealed that the most effective inhibitory concentration of siRNA-1 was 1 nmol, which caused 90 and 80% reductions in the levels of HBsAg and HBeAg, respectively, compared to levels measured in sera from mice injected solely with the pHBV plasmid (Fig. 3B). Again, as seen in some of the *in vitro*

experiments, siRNA-6 at a concentration of 1.7 nmol appeared to have a modest antiviral effect, suggesting that at high concentrations, siRNA may have an activity that affects HBV gene expression indirectly.

Results of a kinetic study revealed that the inhibitory effect of siRNA-1 on HBsAg levels in the serum was strongest (98%) at 1 day after injection and diminished thereafter, reaching 60% inhibition at 3 days after injection (Fig. 3C). HBeAg inhibition displayed different kinetics, lagging behind the effect observed with the surface antigen, starting at 40% inhibition after 1 day and reaching 92% inhibition only at day 3, a time when the effect of siRNA-1 on HBsAg was declining. At 3 days after injection, there was a significant but unexplained increase in levels of HBeAg secreted into the sera of mice injected with the negative control siRNA-6.

To investigate further the effect of siRNA-1 on viral

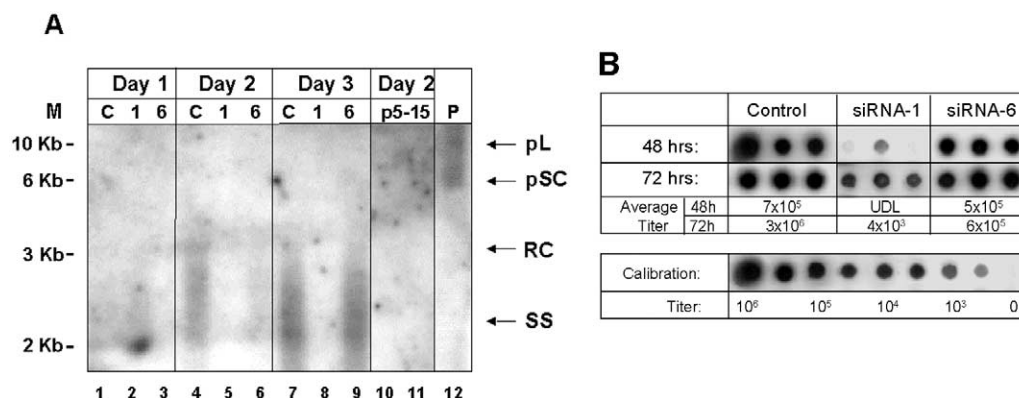


FIG. 4. siRNA inhibits HBV replication in mice. (A) Southern blot analysis performed on DNA extracted from mouse sera taken at 1, 2, or 3 days after injection with 15 μ g pHBV (C) or with pHBV + 1 nmol siRNA-1 (1) or siRNA-6 (6) or injection with the replication-deficient pHBV mutant DNA (p5-15). P, control plasmid DNA; pL and pSC, linear and supercoiled plasmid DNA; RC, relaxed circular viral DNA; SS, single-stranded viral DNA; M, size marker in kb. (B) Semiquantitative PCR/dot-blot analysis. DNA was extracted from mouse sera, amplified by PCR, and analyzed by dot-blot hybridization. Each dot represents the amount of DNA in the serum of a single mouse. The average titer was calculated from a calibration curve obtained with a serum with known titers (see Materials and Methods). UDL, undetected level.

RNA levels in the livers of the injected mice, we performed Northern blot analyses with total RNA extracted from randomized liver sections of mice from each treatment group of the kinetic experiment described above. We observed individual variation between the three mice in each group in HBV mRNA levels (Fig. 3D). Nevertheless, when results were averaged, siRNA-1-treated mice exhibited an overall decrease of approximately 50% in levels of the three major HBV transcripts compared to untreated control mice and mice injected with the irrelevant siRNA-6, supporting the observations (described above) of a sequence-specific effect that could be attributed to RNA interference. Alternatively, the observed differences in the levels of HBV transcripts in the various liver sections might have reflected regional fluctuations in the levels of pHBV. Another possibility is that not all cells were cotransfected with both siRNA and pHBV DNA, explaining why some liver samples of the siRNA-1-treated mice failed to show inhibition (Fig. 3D, lanes 6 and 11). However, as the liver sections were collected in a randomized fashion, it is more likely that the inhibitory effect was due to RNA interference that was caused by siRNA-1.

Inhibition of HBV Replication *in Vivo* by siRNA-1

During HBV replication, the pregenomic RNA is reverse transcribed and the newly synthesized DNA is encapsidated and later secreted as mature virions. To study the effect of siRNA-1 on viral replication, we performed a Southern blot and a semiquantitative PCR/dot-blot analysis with total DNA extracted from the sera of mice treated with pHBV and siRNA, at 1, 2, and 3 days after injection. At 2 days after injection, we detected HBV DNA by Southern blot analysis in sera of mice injected solely with the pHBV plasmid, and its level had increased by day 3 (Fig. 4A, lanes 4 and 7). Migration coincided with the expected sizes of HBV DNA species. The control pHBV

plasmid DNA migrated much slower, with its linear and supercoiled species migrating parallel to the 10 and 6 kb marker DNAs, respectively (lane 12). Co-injection of pHBV with siRNA-1 caused a strong reduction in all HBV DNA species, to undetectable levels, at days 2 and 3 after injection (lanes 5 and 8). At day 1, there was still no HBV DNA detected in the serum in either of the mice sera (lanes 1–3). The negative control siRNA-6 revealed a transient small inhibitory effect that had disappeared by 3 days (lanes 6 and 9).

To demonstrate unequivocally that the DNA detected in the hybridization was newly formed HBV DNA species and not the input plasmid DNA, we added to the Southern blot analysis DNA extracted from sera of mice 2 days after injection with the replication-defective pHBV mutant plasmid, p5-15, carrying a mutation in the polymerase gene, rendering it incapable of supporting viral replication [28]. HBV-specific DNA was not detected in these serum samples (lanes 10 and 11). However, siRNA-1 reduced the serum level of HBsAg by 80% (not shown), indicating that the activity of siRNA-1 does not require viral replication.

We performed semiquantitative PCR/dot-blot analysis using total serum DNA from treated mice. The results revealed a strong anti-replication effect of siRNA-1 resulting in an approximately 5 log reduction in HBV DNA levels to undetectable levels 2 days after injection (Fig. 4B). The inhibitory effect diminished at 3 days but over 100-fold inhibition was still observed. Hence, both analytical methods revealed a dramatic inhibitory effect of siRNA-1 on viral replication for at least 3 days after injection.

Immunohistochemistry Staining for Core and Surface Antigens in the Livers of Treated Mice

We assessed the effect of siRNA on HBV gene expression histologically by immunohistochemical staining for HBsAg and HBeAg in liver sections taken from mice

TABLE 1: HBcAg and HBsAg expression in livers of siRNA treated mice

Treatment:	Control		siRNA-1		siRNA-6	
Hours:	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr
HBsAg*	>0.1	2.76	0	0	>0.1	2.83
HBcAg*	0.56	4.73	0.69	1.37	0.52	5.72

*Percent positive cells out of total hepatocytes counted.

treated in the kinetic experiment described above. At 2 days after co-injection of pHBV and the different siRNAs, the frequencies of cells staining positively for core antigen (HBcAg) were similar in all groups of mice (0.5 to 0.7%), with no inhibitory effect observed in the siRNA-1-treated mice (Table 1). While HBsAg-positive cells were very scarce (>0.1%) at the 2-day time point in the control and siRNA-6 treated mice, they were, however, absent altogether from the livers of siRNA-1-treated mice (Table 1). At day 3 after injection, the frequencies of HBsAg-positive cells increased significantly in the livers of the control groups (to >2.5%) but HBsAg⁺ cells were still not seen at this time point in siRNA-1-treated mice. The number of core-antigen-positive cells also increased significantly in

control and siRNA-6-treated mice (4.7 and 5.7%, respectively) relative to the 2-day time point (Table 1). In contrast, mice treated with siRNA-1 exhibited a small increase in HBcAg-positive cells and, thus, overall had a 70% inhibition compared to control mice (Fig. 5 and Table 1). Core staining was detected mainly in the cytoplasm but in the nucleus as well, whereas HBsAg staining appeared in the cytoplasm (Fig. 5). At day 1 after injection, there was liver damage, caused by the method of injection, which was almost completely repaired by 3 days (data not shown). The results emphasize the significant antiviral activity of the siRNA-1 duplex and its ability to suppress HBV gene expression *in vivo*.

DISCUSSION

We have provided several lines of evidence that the double-stranded RNA duplex siRNA-1 directed against HBV sequences in the s antigen/polymerase region is capable of inhibiting HBV gene expression and viral replication both in HepG2.2.15 cells, which support viral production, and *in vivo* in a mouse model. Inhibition was specific and dose dependent and was sustained for 3 days after administration of the siRNA. Of the five siRNA candidates evaluated, siRNA-1, which targets the region extending from

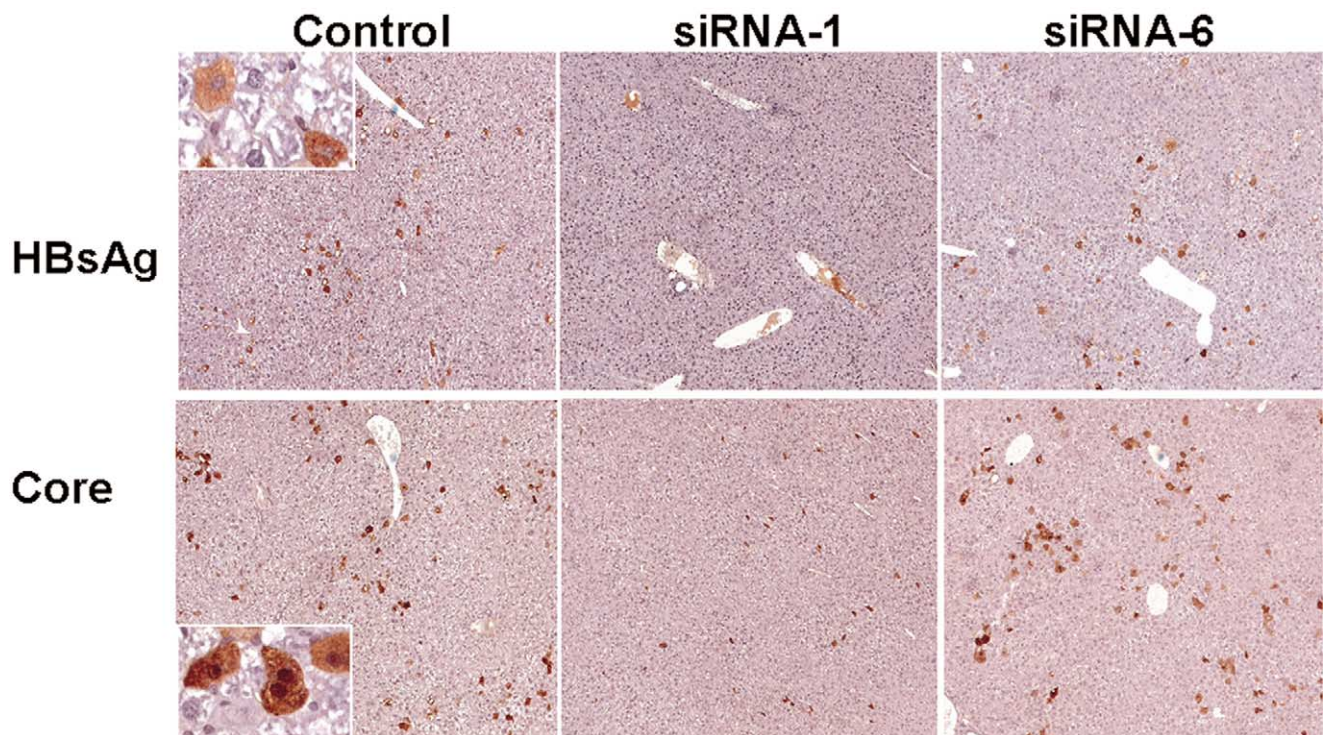


FIG. 5. The effects of siRNA on core and HBsAg expression in mouse liver sections. Liver sections taken from mice injected with pHBV (control), pHBV + siRNA-1, or pHBV + siRNA-6, 3 days after injection, were immunostained for core and HBsAg. Liver sections from two mice of each treatment group were stained and counted (100× original magnification, inserts at 400× original magnification).

7 to 29 bases downstream of the ATG translational start site of the small s antigen, exhibited the highest level of inhibition both *in vitro* and *in vivo*. This region is shared by the major viral transcripts, and predictably, all these RNA species were suppressed by siRNA-1. In addition to inhibiting viral RNA, siRNA-1 had a dramatic effect on viral DNA production, rendering HBV DNA undetectable in sera of treated mice within 2 days after injection. By 3 days there was only a slight increase in serum HBV DNA, but it was still lower by approximately 2 logs relative to control mice. The effect of siRNA-1 on viral DNA production is most likely an indirect one and may be the result of suppression of both the RNA template and the polymerase enzyme, which are essential for viral DNA synthesis.

The immunostaining of liver sections revealed a strong association between the decline in HBV serum marker levels and the reduced number of HBsAg- and core-positive hepatocytes induced by siRNA-1 treatment. This could imply that co-injection of pHBV and siRNA resulted in coordinate transfection of the same hepatocytes. HBsAg and core were not detected in endothelial and Kupffer cells and siRNA silencing was not associated with a specific liver parenchymal zone. siRNA-1 did not cause any significant histological changes or inflammatory infiltrate in liver tissue.

Interference with transgene and endogenous gene expression in mice following administration of siRNA duplexes has been reported by several groups [31,32]. The study described herein shows that RNAi induced by a synthetic siRNA can be used to inhibit replication of a human pathogen in a mammal model system and may be applicable as a therapeutic strategy for naturally occurring HBV infections.

The use of RNAi in the therapy of HBV would be advantageous in that the effect appears to be specific for the targeted gene, minimizing side effects relative to those observed with conventional drug therapies. The ability to use simultaneously siRNAs targeted to different regions of the virus may increase the efficiency of the treatment and, in addition, will prevent the appearance of viral revertants resistant to the treatment. Another possible advantage to siRNA treatment over conventional drugs such as Lamivudine is the fact that inhibition by RNAi does not require viral replication, as we demonstrated with the replication-deficient p5-15 plasmid (Fig. 4A).

However, the interference with HBV replication induced by the use of siRNA duplexes was transient. To eradicate HBV infection, it will be essential to prolong the expression of siRNA and to ensure its delivery to every infected cell in the body, including extrahepatic viral reservoirs. Current methods for prolonging siRNA expression include the use of plasmids that express endogenously the siRNAs. An alternative approach is to express the siRNA from an integrating viral vector and, in that way, to achieve continuous and prolonged expression. A recent article [21] published during the preparation of our

work for publication reports on the anti-HBV activity of a short hairpin RNA (shRNA) homologous to HBV mRNA expressed from a U6 promoter. Their results are similar to ours and suggest that RNAi has anti-HBV potential in humans. There was no overlap between target sites used in both works, but siRNAs or shRNAs targeted to different sites on the HBV genome resulted in varying degrees of inhibition. There are, however, interesting differences between the two reports. The main difference lies in the time interval of the response. siRNA showed a relatively fast effect (1 day after injection), whereas shRNA seemed to require about 4 days for the same inhibitory effect. Furthermore, in our work, we show that in addition to the inhibitory effect on core antigen in the liver, siRNA caused a reduction in the amount of HBsAg-positive stained hepatocytes as well. In the future, if the induction of RNAi will be used as an anti-HBV drug, we believe that there will be room for immediate, short-term, and prolonged treatments and possibly the use of both treatments simultaneously. In some cases there may be an advantage to using siRNA because of safety issues regarding the administration of foreign DNA into a patient.

In conclusion, we show that siRNA could be designed to induce an antiviral effect on HBV replication and gene expression both *in vitro* and *in vivo* and in an animal model. This information should be useful in future studies that will assess the therapeutic potential of siRNAs for treatment of acute and chronic infections by a pathogen.

MATERIALS AND METHODS

Plasmid and siRNA. Plasmid padwHTD (pHBV) consists of a head-to-tail dimer of the wild-type HBV genome (subtype adw) cloned into the *EcoRI* site of pGEM-7 (Promega Corp., Madison, WI) as described [28]. For injection into mice, endotoxin-free plasmid DNA was purified by the EndoFree Plasmid Kit (Qiagen, Germany). Synthetic siRNAs were synthesized by Dharmacon Research (Lafayette, CO) and were obtained as annealed duplexes. Sequences of the siRNAs targeted to HBV are shown in Fig. 1.

Cell culture and transfections. HepG2 and HepG2.2.15 cells (2215) were grown at 37°C in an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% FCS (Biological Industries, Kibutz Beit Haemek, Israel), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2% l-glutamine. For transfection experiments, cells in a 24-well plate (70% confluency) were transfected with Oligofectamine (GIBCO BRL) according to the manufacturer's protocol, in the presence of 600 ng/ml pHBV plasmid DNA and/or 40 nM siRNA. For the dose-response experiment, 2215 cells were transfected with 0, 8, 16, 40, 80, or 120 nM siRNA-1 or siRNA-6. In experiments with HepG2, the level of HBsAg was measured in the medium 72 h after transfection, without any change of medium. In experiments with 2215 cells, after transfection, the medium was removed for analysis every 24 h and the cells were replenished with fresh medium.

siRNA treatment *in vivo*. For all the *in vivo* experiments we used 6- to 7-week-old female Balb/c mice (Harlan Laboratories, Israel). Plasmid DNA and siRNAs were delivered into mice using the hydrodynamic tail vein injection method [27,33], by which 15 µg pHBV plasmid DNA and 1 nmol siRNA dissolved in 1.5 ml PBS were rapidly injected into the tail vein. In the dose-response experiment, mice were co-injected with 15 µg pHBV DNA together with 0, 0.34, 1, or 1.7 nmol siRNA-1 or siRNA-6. The mice were bled, and the serum was separated and assayed for HBsAg, HBeAg, or HBV DNA content at 1, 2, or 3 days after injection. The livers of the mice

were dissected into pieces; part was frozen immediately in liquid nitrogen and part was preserved in formalin for histological analysis.

All mouse experiments were carried out according to the guidelines established by the Institutional Committee on Animal Welfare at the Hebrew University of Jerusalem, Hadassah University Hospital.

HBsAg and HBeAg assays and immunohistochemistry. The 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, Germany). For histology and immunohistochemistry, the formalin-fixed mice livers were embedded in paraffin using an automated device (Tissue-Tek VIP, Sakura). Tissue sections were stained with hematoxylin and eosin. Immunostaining for surface (HBsAg) and core (HBcAg) antigens was performed using an automated staining device (IHC Staining System, Nexes, Ventana) with specific immunohistochemical stains for HBsAg (mouse, clone ZCH16, Code No. 1880023, Zymed) and HBcAg (rabbit, Code No. Bo586, DAKO Corp., Carpinteria, CA).

Northern blot analysis. Total RNA was extracted from 2215 cells (10^6) mock transfected or transfected with 40 nM siRNA, 48 h posttransfection, using 1 ml of the STAT-60 reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. The isolated RNAs were digested with DNase I (DNA-free, Ambion). RNAs (30 μ g) were separated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to a GeneScreenPlus membrane (NEN, Boston, MA). The blots were probed with 32 P-labeled HBV DNA (an EcoRI fragment from pHBV spanning the entire HBV genome) and with a PCR-generated GAPDH fragment labeled by using the random primer labeling mix (Biological Industries). Band intensity levels were measured with the Bio-Image Analyzer BAS1000 (Fuji Photo Film Co., Tokyo, Japan).

Southern blot and semiquantitative PCR analysis. For Southern blot analysis, the sera of three mice from each treatment group were pooled and DNA was extracted using the QIAamp DNA isolation kit (Qiagen). Samples containing the entire amount of DNA isolated were loaded onto a 1% agarose gel, transferred to a GeneScreenPlus membrane, and hybridized with a 32 P-labeled HBV-cDNA probe. Semiquantitative PCR analyses were performed as previously described [34]. DNA was extracted from 100 μ l serum of each mouse, using the DNAzol BD kit (Molecular Research Center, Inc., Cincinnati, OH), and subjected to PCR using primers 5'-CCTCTGCTAATCATCTCTGTACATGTCC-3' and 5'-CCAGAGTTAGCGG-CGAGCGTCTTCTAGAGTTAGAGCCC-3' (HBV coordinates 1828–1857 and 2444–2406, respectively). Fifty microliters of the PCR mixture was dot blotted and hybridized with a 32 P-labeled 424-bp DNA fragment corresponding to nucleotides 1866–2290 of the HBV core sequence, labeled with the Rediprime II DNA labeling kit (Amersham, Buckinghamshire, UK). Spot intensities were measured using the Eagle Eye II System (Stratagene, La Jolla, CA). Quantitation of viral particles in mice sera was determined by comparing these intensities to a standard curve prepared by spiking mice serum with increasing amounts of a human infected serum with a known titer.

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