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Research Article

Genome-wide expression profiling of RNA interference of hepatitis B virus gene expression and replication

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Abstract. Small interfering RNA (siRNA) has been used repeatedly to down-regulate viral gene expression and inhibit viral replication in mammalian cells. In this study, we showed that siRNAs specific for two conserved regions within the hepatitis B S antigen (HBsAg) gene can inhibit antigen production in two human liver cell lines which constitutively produce and secrete HBsAg. The inhibitory effect was concentration dependent for both PLC/PRF/5 and 2.2.15 cells. Decreases in the corresponding viral transcript levels were observed. The inhibitory effect was observed within 24 h and was still evident 7 days after the initial treatment with siRNA. A significant reduction in virion production was also observed for the 2.2.15 cells. A critical consideration in this study was the specificity of the siRNA-mediated inhibition. To address this, we first examined the effects on cell growth and viability. These were not affected in either cell line. cDNA microarrays were also used to examine genome-wide changes in gene regulation. No significant off-target gene regulation was observed in either cell line. Our findings thus indicate that siRNA can specifically mediate the down-regulation of viral gene expression leading to a reduction in virion production.

Key words. Short interfering RNA; hepatitis B; expression profiling; cDNA microarray.

RNA interference (RNAi) provides the mechanism by which specific post-transcriptional silencing of gene expression can occur. The process is induced by double-stranded RNA (dsRNA) molecules [1] which are cleaved by the ribonuclease III-like enzyme Dicer into small interfering RNAs (siRNAs) of 21–23 base pairs [2–4]. siRNAs are the effectors which mediate the silencing process by facilitating the degradation of homologous mRNA. This is achieved via the formation of the RNA-induced silencing complex (RISC) [5, 6]. Components of this multiprotein-RNA complex have not been completely identified but have been shown to include the antisense strand of the siRNA, a helicase and an RNA-directed nuclease [5, 7–9].

Following the observation that RNAi can trigger efficient sequence-specific gene silencing in *Caenorhabditis elegans* [1], this approach has been used to silence gene expression in many systems. In mammalian cells, long dsRNA induces the interferon response which leads to global degradation and inhibition of mRNA translation [10, 11]. However, Elabshir et al. [12] demonstrated that direct introduction of siRNA could mediate specific gene silencing without inducing the interferon response. This has since led to the widespread use of siRNA to mediate sequence-specific post-transcriptional silencing of gene expression. To date, viral RNA and viral mRNA have

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been shown to be among the effective targets of the siRNA approach [13–15].

Hepatitis B virus (HBV) is a member of the hepadnaviridae family. Human HBV is a small-enveloped DNA virus which can cause acute and chronic infection of the liver [16]. Chronic hepatitis B infection is a major health problem, especially in Asia, and patients with chronic hepatitis are at increased risk of developing hepatocellular carcinoma [17]. Sequence-specific strategies such as the antisense approach and the use of ribozymes have been explored as research tools to examine the roles of viral protein, and as inhibitors of viral replication with potential therapeutic applications [18–20]. Recent work has shown that co-transfection of siRNA or short-hairpin RNA (shRNA) targeting HBV RNA and an HBV expression plasmid can indeed inhibit viral gene expression and replication [21–24]. In addition, during the course of this work, two studies have been published showing that siRNA can inhibit viral gene expression in a stable HBV-producing cell line, 2.2.15 [25, 26], and a third shows similar results with HBV-inducible cell lines [27]. However, in all these studies, little was done to examine if there were any non-specific effects on cellular gene expression.

Here, our aim was to examine if siRNA can mediate down-regulation of viral gene expression without causing off-target effects. Culture systems which express the HBV S antigen (HBsAg) or produce virus particles were utilized for this study. The HBV system was chosen because replication and release of HBV do not result in cell lysis. Our results show that one can inhibit hepatitis B surface (HBs) gene expression and reduce virion production in cultured cells using siRNA that targets the HBs sequence. These effects were specific and there was no adverse effect on cell growth. Expression profiling analysis showed no non-specific off-target regulation of gene expression.

Material and methods

Materials

All siRNAs used in this study were synthesized by Qiagen-Xeragon (Germantown, Md.). The QIAamp DNA Blood Mini kit, RNeasy Mini kit, LabelStar Array kit and HiLight Dual Color Kit were purchased from Qiagen (Hilden, Germany). Dulbeccco's modified Eagle's medium (DMEM) and G418 were purchased from Sigma (St. Louis, Mo.). All other cell culture reagents including Opti-MEM I reduced serum medium were obtained from Gibco (Grand Island, N. Y.). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, Calif.), while TransIT TKO transfection reagent was from Mirus (Madison, Wis.). 3,(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium/phenazine ethosulfate (MTS/PES) reagent (supplied as Cell Titer 96 Aqueous One Solution Cell Proliferation Assay), the Access reverse transcription (RT)-PCR system and AMV reverse transcriptase were purchased from Promega (Madison, Wis.). Vectashield mounting medium was obtained from Vector Laboratories (Burlingame, Calif.). The Murex HBsAg Version 3 Kit was obtained from Murex Biotech Limited (Dartford, U. K.). Affinity-purified HBsAg was from Chemicon International (Temecula, Calif.). The TaqMan Universal PCR Mastermix was obtained from Applied Biosystems (Foster City, Calif.). Biotin-16-dUTP and fluorescein-12-dUTP were purchased from Roche (Mannheim, Germany). Primers and probes for real-time PCR were synthesized by Proligo (Singapore). The PeliCheck HBV-DNA-99 reference panel was purchased from VQC International (Alkmaar, The Netherlands).

Cell culture

The PLC/PRF/5 and the 2.2.15 human liver cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acids at 37 °C under 5% CO_2. For the 2.2.15 cell line, 150 µg/ml G418 was added to the medium.

Synthesis of siRNA

Three 21-nt siRNA duplexes with 2-nt dTdT 3′ overhangs were synthesized by Qiagen-Xeragon (fig. 1). Two of the three siRNAs (S1 and S2) were designed to target regions on the viral genome that are conserved in all the different HBV serotypes. S1 and S2 target regions within the HBsAg (ORF) while the last duplex Scr was a control scrambled sequence with the same base composition as S2 (fig. 1). Fluorescein-labeled S2 siRNA was also synthesized. The label was on the 3′ end of the sense strand.

Transfection with siRNAs

PLC/PRF/5 cells (5×10^4 cells) were seeded into each well of a 24-well plate and allowed to recover for 24 h.

S1 siRNA
5'-GGUAUGUUGCCCGUUUGUCdTdT-3'
3'-dTdTCCAUACAACGGGCAAACAG-5'

S2 siRNA
5'-CCUCCAAUCACUCACCAAC dTdT-3'
3'-dTdTGGAGGUUAGUGAGUGGUUG-5'

Scr siRNA
5'-CUCAACCUACCAACUCCACdTdT-3'
3'-dTdTGAGUUGGAUGGUUGAGGUG-5'

Figure 1. Sequences of siRNAs used in experiments: S1 and S2 targets sites within the HBsAg open reading frame. Scr serves as a scrambled control and has the same base composition as S2.

Before transfection, the appropriate amount of siRNA was diluted in 150 µl of Opti-MEM I reduced serum medium. In another tube, 3 µl of lipofectamine 2000 was also diluted in 150 µl of Opti-MEM I reduced serum medium. After incubation at room temperature for 5 min, these two solutions were mixed gently, and incubated for 20 min at room temperature to allow the siRNA:lipofectamine 2000 complexes to form. Cells were then rinsed with Opti-MEM I reduced serum medium. Then, 300 µl of siRNA:lipofectamine 2000 complexes was added to each well and mixed gently by rocking the plate back and forth.

2.2.15 cells (2×10^5 cells) were seeded into each well of a 24-well plate and allowed to recover for 24 h. Then, 4 µl of TransIT TKO transfection reagent was diluted in 50 µl of Opti-MEM I reduced serum medium and mixed thoroughly by vortexing. After incubation at room temperature for 10 min, the appropriate amount of siRNA was added and incubated for 20 min at room temperature to allow the siRNA:TransIT TKO transfection reagent complexes to form. Cells were washed with Opti-MEM I reduced serum medium and overlaid with 250 µl Opti-MEM I reduced serum medium. siRNA:TransIT TKO transfection reagent complexes (50 µl) were added to each well and mixed gently by rocking the plate back and forth.

After introducing the siRNA:transfection reagent complexes, the cells were incubated at 37 °C in a CO_2 incubator for 4 h for transfection to take place. Following the transfection process, the transfection reagent was removed and replaced with complete DMEM medium for the PLC/PRF/5 cells or DMEM medium containing 150 µg/ml G418 for the 2.2.15 cells.

Mock transfections were carried out for both cell lines as described above but with the omission of the siRNA. For treatments longer than 24 h, the medium was collected every 24 h and fresh medium was added to each well. At the end of the treatment period, the medium was collected for detection of HBsAg or viral DNA and the cells were used for isolation of total RNA.

To estimate transfection efficiency, cells were grown on glass coverslips and transfected with fluorescein-labeled siRNA as described above. At 1 and 24 h post-transfection, cells were washed twice with phosphate-buffered saline (PBS), and fixed with methanol for 10 min. The cells were then washed again with PBS and a drop of Vectashield mounting medium containing propidium iodide was then added. Cells were observed using a Leica DMLB fluorescent microscope. The percentage of fluorescein-labeled cells in five randomly selected fields from two independent experiments was determined.

Cell viability assay

PLC/PRF/5 cells (5.0×10^3) or 2.2.15 cells (3×10^4) were seeded in each well of a 96-well plate and allowed to recover for 24 h. Transfection and treatment of cells were performed following the methods described above. At 2, 5 and 7 days post-transfection, MTS/PES reagent (20 µl) was added to each well. After incubation at 37 °C for 1 h, the absorbance at 490 nm was measured. All assays were performed in triplicate and as two independent experiments.

Quantitative assay of HBsAg

The amount of HBsAg secreted into the culture medium was determined using the Murex HBsAg Version 3 kit with the protocol described in the manufacturer's manual. Affinity-purified HBsAg was used to generate the standard curve. Results were expressed as percentage of mock transfection which was considered as 100 %.

HBV viral DNA quantitation

Extraction of HBV DNA from the medium of 2.2.15 cell cultures was performed using the QIAamp DNA Blood Mini kit. HBV DNA was extracted from 200 µl of medium following the protocol described by the manufacturer. The DNA was eluted with 50 µl nuclease-free water. HBV DNA (5 µl) was mixed with the HBV genome-specific primers, the probe and the TaqMan Universal PCR Mastermix. The reaction mixture was first incubated at 50 °C for 2 min and 95 °C for 10 min. Following this, the PCR reaction was performed at 95 °C for 15 s and 58 °C for 1 min for 45 cycles. The levels of PCR products were examined with an ABI PRISM 7000 sequencing detection system and analyzed with ABI PRISM 7000SDS software (Applied Biosystems, Foster City, Calif.). Cycle times were determined at a reading of 0.2 fluorescence units. To determine the absolute quantity of viral DNA numbers, a PeliCheck HBV-DNA-99 reference panel with known copy numbers of HBV DNA was used to generate the standard curve. Sequences of the primers and probes are described in table 1 [see ref. 28].

RNA extraction, reverse transcription and PCR

Following treatment with siRNA, total RNA was extracted from the cells using the RNeasy Mini kit. The extraction was carried out according to the manufacturer's instructions.

Four sets of primers were used for RT-PCR. The HBpc primers were 5′ACTGTTCAAGCCTCCAAGCT3′ (forward) and 5′AGGAAAGAAGTCAGAAGGCAAA3′(reverse). This pair of primers was designed to reverse transcribe and amplify the 3.5-kb pre-core and pre-genomic transcripts. The HBps primers were 5′CAAGGTAGGAGCTGGAGCATTC3′ (forward) and 5′GCAGGAG GCGGATTTGC3′ (reverse). The HBps primer pair was designed to reverse transcribe and amplify the 3.5-kb as well as the 2.4-kb transcripts. The GAPDH primers [29] and HBs primers are shown in table 1. The GAPDH primers are specific for the human GAPDH ORF while the HBs primers are specific for the HBsAg ORF and will amplify the 3.5-kb, the 2.4-kb and the 2.1-kb transcripts.

Table 1. Sequences of PCR primers and probes for real-time PCR.

Target sequence	Primer	Sequence (5′–3′)
HBV DNA [28]	HBV Forward	CCGTCTGTGCCTTCTCATCTG
	HBV Reverse	AGTCCAAGAGT(C/T)CTCTTATG(C/T)AAGACCTT
	HBV Probe	CCGTGTGCACTTCGCTTCACCTCTGC
HBs	HBs Forward	TGTTGACAAGAATCCTCACAATACC
	HBs Reverse	GGTTGGTGAGTGATTGGAGGTT
	HBs Probe	CAGAGTCTAGACTCGTGGTGGACTTCTCTCAATT
GAPDH [29]	GAPDH Forward	GAAGGTGAAGGTCGGAGTC
	GAPDH Reverse	GAAGATGGTGATGGGATTTC
	GAPDH Probe	CAAGCTTCCCGTTCTCAGCC

The isolated RNA was first subjected to RT-PCR using the Access RT-PCR system (Promega). A typical 25-µl RT-PCR reaction contained 0.5 µg RNA, 0.1 mM dNTPs, 0.5 µM of the forward and corresponding reverse primers, 0.5 mM $MgSO_4$, and 0.05 units/µl of AMV reverse transcriptase and 0.05 units/µl of T*fl* DNA polymerase. The reverse transcription was carried out at 48 °C for 45 min followed by denaturation at 94 °C for 2 min. Following reverse transcription, the cDNA was subjected directly to PCR. Each cycle consisted of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 1 min. The product was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The number of PCR cycles used was the lowest needed to produce a product which could be visualized on the gel.

To quantify the changes in transcript levels, reverse transciption followed by real-time PCR was also carried out. Reverse transcription was carried out in a 50-µl reaction mixture containing 1 µg total RNA and HBs reverse PCR primer (table 1), at 42 °C for 90 min with AMV reverse transcriptase. Then, 5 µl of reverse transcription product was mixed with the HBs PCR primers, the HBs probe and the TaqMan Universal PCR Mastermix. The real-time PCR process was as described in the previous section. Cycle times were normalized to that of GAPDH. Sequences of the primers and probes are shown in table 1.

Microarray procedures and data analysis

Global gene expression patterns were analyzed using the Stanford Human cDNA Microarrays (SFGF001) containing approximately 43,000 elements representing the human genome, printed on Corning UltraGAPS-coated slides (Corning, Acton, Mass.). The following combinations of RNA were used on two slides each:

1) 2.2.15 cells: mock transfection (reference) versus S2 treatment (test)
2) 2.2.15 cells: mock transfection (reference) versus Scr treatment (test)
3) PLC/PRF/5 cells: mock transfection (reference) versus S2 treatment (test)
4) PLC/PRF/5 cells: mock transfection (reference) versus Scr treatment (test)

One microgram of each reference RNA was labeled with biotin-16-dUTP and 1 µg of each test sample with fluorescein-12-dUTP. The resultant labeled cDNAs were purified using the LabelStar Array kit following the manufacturer's instructions. The microarray slides were hybridized with the paired samples overnight at 42 °C in Corning hybridization chambers (Corning, Acton, Mass.). Post-hybridization washing, blocking and detection were carried out using the HiLight Dual Color Kit. Detection of the signals on a HiLight Reader GSD-501 (Qiagen) was by resonance light scattering (RLS), a technology based on the optical light-scattering properties of nano-sized metal colloidal particles [30–32]. The biotinylated reference cDNAs were detected with gold particles coated with anti-biotin antibodies and the fluorescein-labeled test cDNAs were detected with silver particles coated with anti-fluorescein antibodies.

The scanned slides were saved as .tiff files, imported into and analyzed using GenePix Pro4.1 software (Axon Instruments, Union City, Calif.), and loaded onto the Stanford Microarray Database for further analysis [33]. The data filters for the GenePix result sets were: regression correlation >0.6; hybridization signals at least fivefold greater than the local background fluorescence; genes and arrays for which technically adequate data were obtained in at least 80% of experiments; and a cutoff of genes whose R/G normalized ratio is <0.66- or >1.5-fold for at least one of the comparisons. These filtered genes were then clustered by Pearson correlation (non-centered metric) and organized by hierarchical clustering [34].

Results

Selection and transfection efficiency of HBV-specific siRNA

To determine whether RNAi could be used to down-regulate HBV gene expression effectively, two siRNAs (S1 and S2) targeting the HBsAg ORF were designed.

HBsAg is not only a component of the viral envelope but is also present in spherical and tubular particles. These particles do not contain viral DNA but are secreted from cells harboring HBV DNA. Selection of the target sites was carried out as described by Harborth et al. [35]. All target sites were preceded by 5′AA nucleotides and the target sites were conserved in all HBV serotypes. A 21-nt duplex with scrambled sequence (Scr) was included as the control.

Fluorescein-labeled S2 duplexes were used to examine the transfection efficiency of siRNA into the cell lines used in this study. At 1 h post-transfection with 0.2 µM labeled S2, the percentage of fluorescein-labeled PLC/PRF/5 cells was >95% while that of 2.2.15 cells was 74 ± 9%. The fluorescein signal was localized mainly outside the nucleus (fig. 2). Similar percentages of fluorescein-labeled cells were also observed at 24 h post-transfection (data not shown).

Inhibition of HBsAg expression and reduction in virion production

Two cell lines, PLC/PRF/5 and 2.2.15, were used to assess the effectiveness of the S1 and S2 siRNAs. The PLC/PRF/5 cells produce only the spherical and tubular particles while 2.2.15 cells produce the particles and viruses. The inhibitory effects of S1 and S2 siRNA treatments on HBsAg expression in both cell lines were evident at 48 h after treatment with concentrations of 0.01 up to 0.2 µM (fig. 3). The scrambled siRNA, Scr, had no significant effect at concentrations up to 0.2 µM but decreased HBsAg expression was observed at 0.4 µM (fig. 3). Thus, in subsequent experiments, 0.2 µM of siRNA was used.

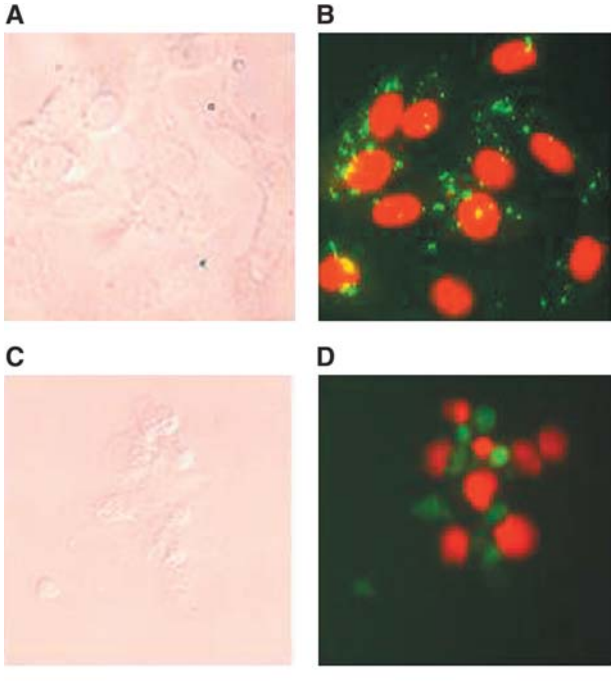

Figure 2. Transfection with fluorescein-labeled siRNA (green) in PLC/PRF/5 and 2.2.15 cells. Bright-field (*A*) and fluorescence image (*B*) of PLC/PRF/5 cells. Bright-field (*C*) and fluorescence image (*D*) of 2.2.15 cells. The nucleus was stained with propidium iodide (red).

At 48 h following the treatment of PLC/PRF/5 with 0.2 µM of S1 or S2, there was a significant decrease in HBsAg secretion into the culture medium. A corresponding decrease in transcripts encoding HBsAg was also observed for RNA isolated from the PLC/PRF/5 cells (fig. 4A). Reduced HBsAg secretion was similarly observed for the 2.2.15 cells. Four viral transcripts with the HBsAg ORF are present in the 2.2.15 cells. These include the 3.5-kb pre-core RNA, the 3.5-kb pre-genomic RNA, the 2.4-kb pre-S RNA and the 2.1-kb HBs RNA. Treatment with S1 and S2 resulted in decreased levels of the 2.4-kb and 2.1-kb transcripts but had little effect on the 3.5-kb transcripts (fig. 5). Real time RT-PCR analysis showed a decrease of 54.9 ± 9.0% and 74.8 ± 4.2% in transcripts encoding HBsAg following transfection by S1 and S2, respectively (fig. 4B).

As HBsAg is an essential component of the viral envelope, we next addressed the question of whether S1 and S2 siRNAs affected virion production. Viral DNA content in the medium of treated 2.2.15 cells was analyzed. Treatment with S1 resulted in a 56.7 ± 2.8% decrease in viral DNA while treatment with S2 led to a 68.0 ± 1.9% reduction (fig. 6). Treatment with the scrambled siRNA, Scr, did not result in any reduction.

The time-course of the effects of the siRNAs was also examined. A significant reduction in HBsAg secretion from PLC/PRF/5 cells was observed a day after siRNA transfection with maximal inhibition of 95.7 ± 0.8% for S1 and 96.3 ± 0.3% for S2 at 2 days. At 7 days post-transfection, significant inhibition was still observed (fig. 7A). Similar observations were also obtained for the 2.2.15 cells (fig. 7B). Maximal reduction of HBsAg secretion was observed at 2 days following siRNA transfection. The decrease was 59.6 ± 7.1% and 71.8 ± 3.3% for S1- and S2-transfected cells, respectively. The S1 and S2 siRNAs as well as the scrambled duplex, Scr, did not affect the cell growth and viability of either cell lines (table 2).

Expression profiling

Human cDNA microarrays containing approximately 43,000 elements were used to examine the global gene expression patterns of cells after transfection with siRNAs. Gene expression profiling comparisons between mock-transfected and cells transfected with 0.2 µM S2 siRNA or 0.2 µM Scr siRNA were carried out in duplicate. The profiling was carried out with both PLC/PRF/5 and 2.2.15 cells. The magnitude of the changes in expression of all genes examined was small. Most changes were less than twofold.

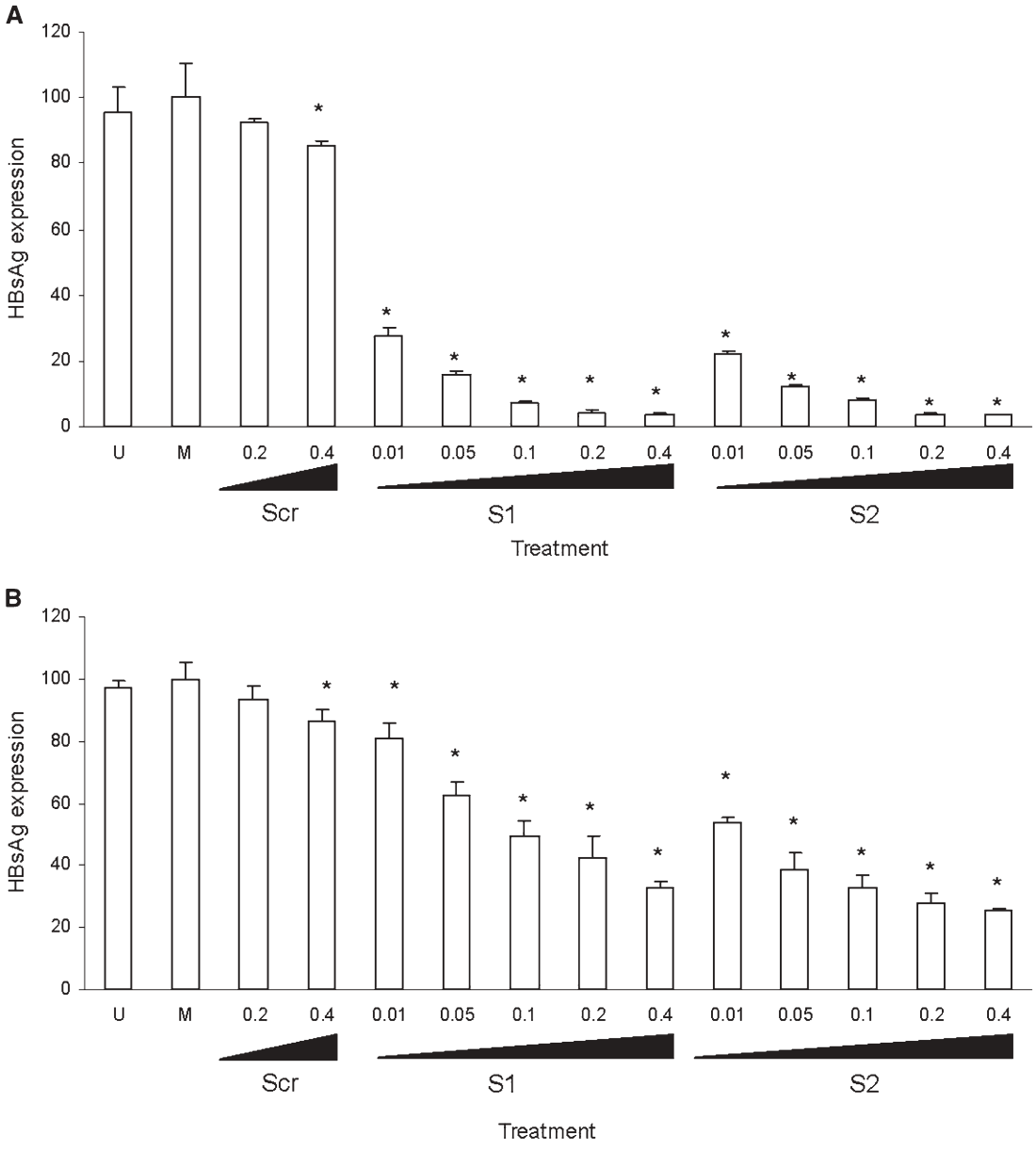

Figure 3. Dose-dependent effects of siRNA on HBsAg expression in PLC/PRF/5 cells (*A*) and 2.2.15 cells (*B*). Cells were left untreated (U), or mock transfected with the transfection agent (M), or transfected with 0.2 or 0.4 μM Scr, or transfected with 0.01–0.4 μM S1, or with 0.01–0.4 μM S2. HBsAg concentrations in the culture medium were determined 48 h after transfection. The HBsAg concentration in mock-transfected samples was 27.2 ± 3.0 ng/ml for PLC/PRF/5 cells and 14.0 ± 0.8 ng/ml for 2.2.15 cells. These were set as 100% for the data in (*A*) and (*B*) respectively. Data shown represent the mean ± SD (n = 3). *$p < 0.01$, ANOVA analysis, for comparison between M and the other treatments.

A total of 49 genes were either up- or down-regulated by at least 1.5-fold for both cell lines (table 3). Of these, treatment of 2.2.15 cells with S2 siRNA and Scr siRNA resulted in 9 and 12 genes, respectively, being differentially regulated by at least twofold. Only 2 of the genes (cytochrome P450 2A6 and a mitochondrial EST of unknown function) were common for both treatments. Treatment of PLC/PRF/5 cells with S2 siRNA and Scr siRNA resulted in 3 and 7 genes, respectively, being differentially regulated by at least twofold, and only 1 gene (ubiquitin-activating enzyme E1-domain containing 1 protein) was common for both treatments. In addition, a comparison of the differentially regulated genes in both cell lines showed that there were only 3 common genes (of unknown functions). None of the genes that were differentially regulated in this study coincides with the 14 genes that were commonly regulated by a series of siRNAs targeted against Rb1, AKT1 and Plk1 [36]. These results indicate that there was no significant off-target gene regulation, and similar to the previous report by Chi

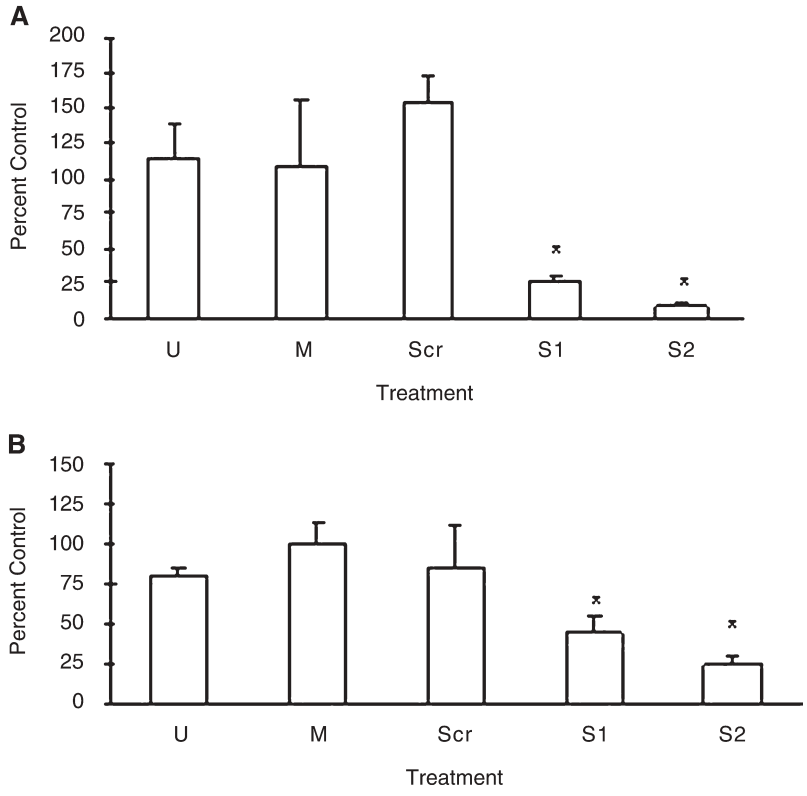

Figure 4. Effect of siRNA on HBsAg RNA in PLC/PRF/5 cells (*A*) and 2.2.15 cells (*B*). Cells were left untreated (U), or mock transfected with the transfection agent (M), or transfected with 0.2 µM Scr, 0.2 µM S1 or 0.2 µM S2. Total RNA was isolated 48 h after transfection. Following reverse-transcription, real-time PCR with primers to the HBsAg ORF was carried out. Results were normalized with GAPDH. The value for the mock-transfected cells was set as 100%. Data shown represent mean ± SD (n = 3). *$p < 0.01$, ANOVA analysis, for comparison between M and the other treatments.

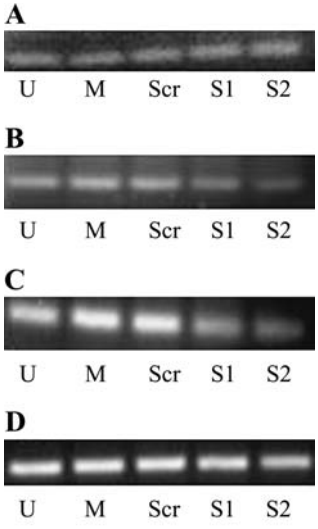

Figure 5. RT-PCR analysis of HBV transcripts in 2.2.15 cells. The RT-PCR products for the 3.5-kb pre-core/pre-genomic transcripts (*A*), both the 2.4-kb pre-S1 and the 3.5-kb transcripts (*B*), the 3.5-kb, 2.4-kb and 2.1-kb transcripts (*C*) and the GAPDH RNA (*D*) are shown for untreated cells (U), cells treated with transfection agent only (M), cells treated with 0.2 µM Scr siRNA, 0.2 µM S1 siRNA and 0.2 µM S2 siRNA.

et al. [37], there were also no consistent response patterns following treatment with siRNA duplexes.

cDNA microarray analysis had been previously employed to examine HBV-induced changes in cellular gene expression. The majority of the genes investigated differed only slightly in HBV-producing cells. However, there were several genes which showed changes of up to about five- to sixfold [38, 39]. In this study, attempts were made to examine if siRNA treatment would reverse the previously described changes. However, from our microarray analysis, no reversal was observed. This may be due to the fact that the siRNA treatment of 2.2.15 cells did not result in complete inhibition and many of the previously documented changes were small in magnitude, and thus any reversal in expression was not detected. In addition, the expression of viral genes in 2.2.15 cells is generally low until several days post-confluence [40]. For certain cellular genes, there is an inverse correlation with HBV RNA levels [41]. Thus, the lack of reversal in cellular gene expression may also be due to the use of pre-confluent cells in this study.

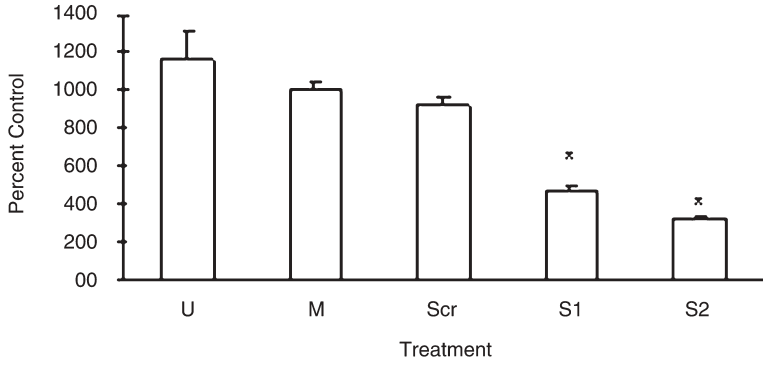

Figure 6. Analysis of HBV DNA levels in culture medium of 2.2.15 cells. Cells were left untreated (U), or mock transfected with the transfection agent (M), or transfected with 0.2 µM Scr, 0.2 µM S1 or 0.2 µM S2. HBV DNA levels were determined 48 h after transfection. $1.0 \times 10^6 \pm 4.6 \times 10^4$ copies/ml were present in the mock-transfected medium and this was set as 100%. Data shown represent the mean ± SD (n = 3). * $p < 0.01$, ANOVA analysis, for comparison between M and the other treatments.

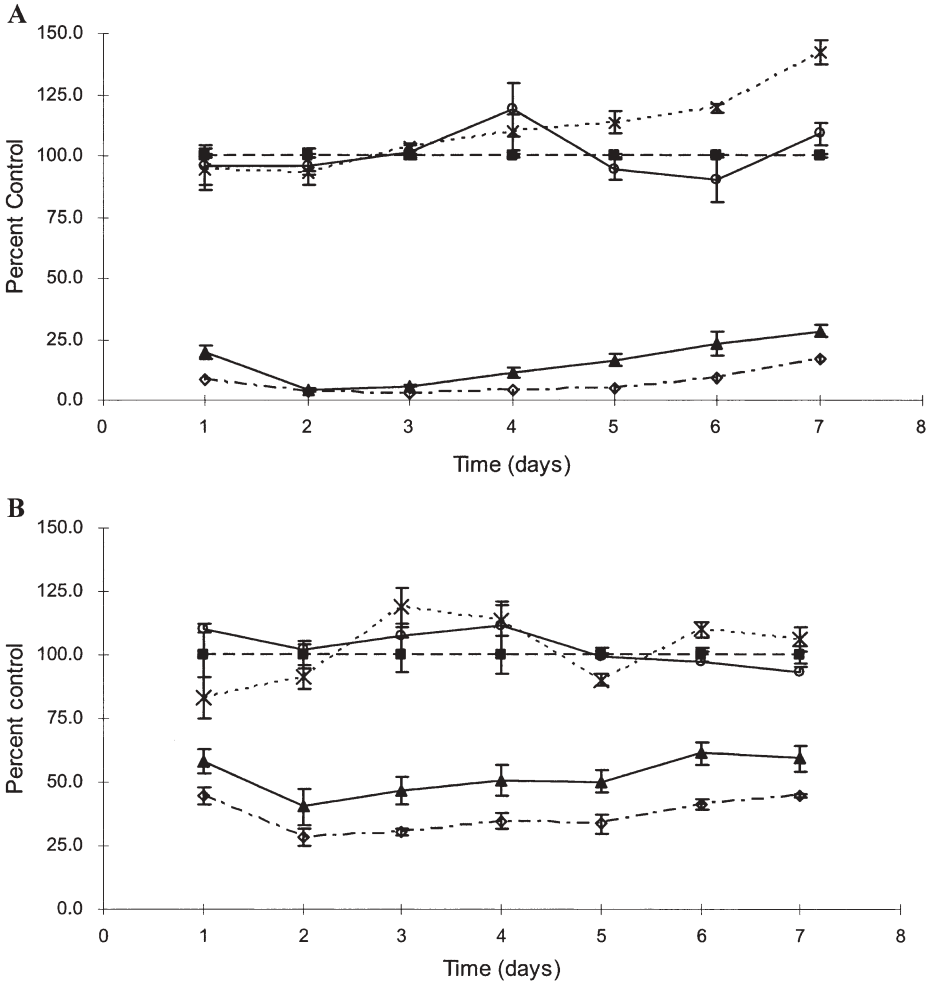

Figure 7. Time-course of HBsAg inhibition by siRNA in PLC/PRF/5 cells (*A*) and 2.2.15 cells (*B*). Cells were left untreated (circles), or mock transfected with the transfection agent (squares) or transfected with 0.2 µM Scr (crosses), or 0.2 µM S1 (triangles) or 0.2 µM S2 (diamonds). Culture medium was collected every 24 h and fresh medium was introduced. HBsAg levels in the culture are presented as the percentage of that in mock-transfected controls. Data shown represent the mean ± SD (n = 3). All data points for S1 and S2 were significantly different from mock- and Scr-transfected cells ($p < 0.01$, ANOVA analysis).

Table 2. Effects of siRNAs on the viability of PLC/PRF/5 and 2.2.15 cells.

Treatment	Viability		
	day 2	day 5	day 7
PLC/PRF/5 cells			
Mock transfection	100 ± 4.2	100 ± 3.6	100 ± 1.9
Untreated	102.9 ± 4.4	98.2 ± 4.8	101.5 ± 1.9
0.2 µM Scr	ND	105.4 ± 3.0	109.7 ± 5.3
0.2 µM S1	103.0 ± 7.7	100.4 ± 4.8	108.7 ± 4.2
0.2 µM S2	113.5 ± 12.8	97.6 ± 5.4	108.5 ± 1.6
2.2.15 cells			
Mock transfection	100 ± 10.3	100 ± 9.6	100 ± 6.6
Untreated	113.3 ± 9.3	114.0 ± 4.9	109.0 ± 11.6
0.2 µM Scr	ND	97.0 ± 6.3	102.0 ± 15.0
0.2 µM S1	100.2 ± 10.7	91.7 ± 8.5	97.9 ± 4.5
0.2 µM S2	113.5 ± 12.0	101.0 ± 16.8	96.8 ± 5.0

Cell viability was determined at 2, 5 and 7 days following transfection with siRNAs. This was achieved using the MTS/PES assay and is proportional to the absorbance at 490 nm. The A490 of control cells was set as 100%. Data shown represent the mean ± SD. ND, not determined.

Table 3. Gene expression in 2.2.15 and PLC/PRF/5 cells following treatment with siRNAs.

SUID	NAME	GenBank No.	2.2.15 cells		PLC/PRF/5 cells	
			M-vs-S2	M-vs-SCR	M-vs-S2	M-vs-SCR
Lipid, fatty acid and steroid metabolism						
307401	CYP4F3 || cytochrome P450, family 4, subfamily F, polypeptide 3	AI249090	1.60	1.43	1.54	1.92
330264	CYP2A6 || cytochrome P450, family 2, subfamily A, polypeptide 6	AI343237	**0.42**	**0.43**	0.59	0.55
112881	PCCA || propionyl coenzyme A carboxylase, alpha polypeptide	AA983154	0.62	0.62	**0.45**	0.72
Protein metabolism and modification						
104159	RPL14 || ribosomal protein L14	AI053558	1.13	0.61	0.67	0.97
102992	EEF1A1 || eukaryotic translation elongation factor 1 alpha 1	T89301	0.98	1.36	0.67	0.56
Intracellular protein traffic						
311822	VPS35 || vacuolar protein sorting 35 (yeast)	AA916752	**0.50**	0.73	0.64	0.52
Nucleoside, nucleotide and nucleic acid metabolism						
98713	APOBEC1 || apolipoprotein B mRNA-editing enzyme	AA587939	1.00	0.58	0.57	0.73
117045	SIX3 || sine oculis homeobox homolog 3 (*Drosophila*)	N41052	0.60	**0.31**	0.55	0.52
106655	SOX12 || SRY (sex-determining region Y)-box 12	AA866160	0.54	0.56	0.65	0.57
Unclassified/new ESTs/unknown function						
318884	NDUFV3 || NADH dehydrogenase (ubiquinone) flavoprotein 3, 10 kDa	AI291172	1.44	1.28	1.95	1.80
104774	UBE1DC1 || ubiquitin-activating enzyme E1-domain containing 1	R32014	0.66	0.56	**0.49**	**0.48**
120543	MTMR9 || myotubularin-related protein 9	AA418387	**2.17**	0.85	0.75	0.83
102654	C10orf45 || chromosome 10 open reading frame 45	AA431133	0.71	**0.47**	0.51	**0.40**
104661	C21orf91 || chromosome 21 open reading frame 91	AA400378	**0.50**	0.68	0.64	0.58
99690	DKFZp761I2123 || **hypothetical protein DKFZp761I2123	AA405740	0.57	**0.42**	0.63	**0.45**
117850	DKFZp313M0720 || hypothetical protein DKFZp313M0720	AI054231	1.19	0.62	0.60	0.84
105201	FLJ40919 || hypothetical protein FLJ40919	T92157	0.78	0.74	0.62	0.57
98367	KIAA0114 || KIAA0114 gene product	AI053704	1.14	0.55	0.76	0.54
310463	LOC159090 || similar to hypothetical protein MGC17347	N74901	0.68	**0.42**	0.52	0.62
225970	*Homo sapiens* cDNA FLJ11366 fis, clone HEMBA1000282	AA704270	1.49	1.16	1.44	1.98

Table 3 (continued)

SUID	NAME	GenBank No.	2.2.15 cells		PLC/PRF/5 cells	
			M-vs-S2	M-vs-SCR	M-vs-S2	M-vs-SCR
112373	*Homo sapiens* clone 252 mRNA sequence	AA190764	0.76	0.61	0.68	0.58
313320	*Homo sapiens* transcribed sequences	AI245660	0.63	0.71	0.52	0.93
223940	*Homo sapiens* cDNA FLJ32525 fis, clone SMINT2000060	AA705015	**0.47**	0.71	0.82	0.67
317836	*Homo sapiens* transcribed sequences	AI241280	0.86	**0.47**	0.57	0.61
314256	*Homo sapiens* transcribed sequences	AI371327	0.75	**0.47**	0.53	0.53
312421	*Homo sapiens* cDNA: FLJ22133 fis, clone HEP20529	AI344687	0.93	0.51	0.60	0.69
104905	*Homo sapiens* transcribed sequences	AI053487	0.74	0.57	0.55	0.52
184956	*Homo sapiens* transcribed sequences unknown UG Hs.87530 ESTs	AA281926	0.62	0.56	1.00	0.66
317076	*Homo sapiens* transcribed sequences	AI271427	0.96	1.92	0.69	0.78
331189	*Homo sapiens* similar to gammaglutamyl-transpeptidase 1 precursor	AI344264	**0.42**	0.62	0.58	0.64
433347	mitoch. cont. ESTs	nil	**2.04**	**2.25**	1.47	1.49
433310	mitoch. cont. EST	nil	1.33	1.55	1.87	1.60
433351	mitoch. cont. ESTs	nil	1.69	1.52	1.71	1.46
433300	mitoch. cont. ESTs	nil	**2.14**	1.32	1.64	1.52
433311	mitoch. cont.	nil	1.57	1.40	1.54	1.70
433303	mitoch. cont.	nil	1.06	1.10	1.53	**2.59**
115821	nil	AI054041	0.81	0.71	0.56	0.86
316453	nil	AI333424	**0.47**	0.74	0.68	0.51
308324	nil	AI335863	0.62	0.64	0.52	0.61
106302	nil	AI053735	0.66	0.52	0.57	1.16
110818	nil	AI053443	0.95	0.54	0.80	0.64
116683	nil	AA411391	0.90	**0.48**	0.61	0.83
103556	nil	AI053440	0.60	**0.48**	0.73	**0.47**
102516	nil	N38990	0.91	**0.46**	0.54	0.64
472864	nil	nil	1.86	**2.14**	1.53	1.78
472867	nil	nil	1.26	1.45	**2.22**	1.83
472868	nil	nil	1.50	1.52	1.69	1.62
472862	nil	nil	1.69	1.29	1.13	**2.54**
472866	nil	nil	1.32	1.42	1.40	**2.81**

Comparisons between mock transfection (M) and cells treated with 0.2 μM S2 siRNA or 0.2 μM Scr siRNA at 48 h after transfection with siRNAs were carried out in duplicate. Genes with expression changes that demonstrated coordinate induction or repression by siRNA in the cell lines 2.2.15 or PLC/PRF/5 are shown, grouped together according to their biological functions. Each value is the fold induction in an average of two microarray experiments, with a value <1 indicating gene repression and a value >1 representing gene induction. Values in bold indicate at least a twofold change in gene expression. The Stanford unique identification (SUID) numbers are unique identifying numbers within the Stanford Microarray Database which are specific for arrayed clones or PCR-amplifed regions of genomic DNA. The GenBank numbers, where available, are also indicated.

Discussion

The uniqueness of viral genomes has been exploited for the development of antiviral agents. These approaches include the use of antisense oligonucleotides, ribozymes [18–20] and, more recently, RNAi. The RNAi approach has been used to inhibit expression of viral proteins and to reduce viral titer [13, 14]. Synthetic siRNA duplexes and plasmid-derived shRNA are commonly used to mediate the RNAi effect in mammalian cells. Plasmid-derived shRNA is dependent on cellular transcription and it is difficult to regulate the level of expression. In addition, shRNA contains additional sequences which may contribute to potential non-specific effects. Indeed, shRNA has been shown to trigger an interferon response while the corresponding synthetic siRNA did not [42]. Thus, in this study, we chose to use chemically synthesized siRNAs.

Synthetic siRNAs targeted at two different sites within the HBsAg ORF can specifically inhibit the expression of HBsAg in PLC/PRF/5 cells. This together with a reduction in the corresponding mRNA indicates that this is a true RNAi effect. The siRNA-induced RNA interference also effectively reduced HBsAg expression, in 2.2.15 cells. Reduction in virion production was also observed. This is probably a consequence of reduced HBsAg expression, as both S1 and S2 siRNAs had little effect on the 3.5-kb pre-genomic and pre-core RNA but could effectively reduce the levels of the 2.1-kb/2.4-kb transcripts leading to reduced HBsAg for incorporation into the viral envelope. Although the 3.5-kb RNA has the HBsAg

ORF, there may be secondary structures within the RNA that prevent siRNA-induced cleavage.

The siRNAs were more effective in inhibiting HBsAg production in PLC/PFR/5 cells than in 2.2.15 cells. This is similar to previous observations with minimized ribozymes [43]. The difference in the inhibitory effect is probably due to differences in the uptake of the siRNAs. Transfection of siRNA was more efficient in PLC/PRF/5 cells and this resulted in a >95% reduction in production of HBsAg. In contrast, the transfection efficiency in 2.2.15 cells was only ~74% and this correlated with smaller reductions in HBsAg production.

During the course of this study, two other reports also made similar observations using the same cell line, 2.2.15 [25, 26]. Collectively, our study and these reports indicate that there are multiple sites within the HBV transcripts that can be used as targets for the action of siRNA. Encouraging to note is that in the 2.2.15 system which constitutively produces hepatitis B antigens and virus particles, siRNA can effectively reduce viral gene expression and virion production. siRNA specific for the core gene has also been shown to induce a marked in reduction in HBV replication of both the wild-type and lamivudine-resistant YMDD variant [44]. This augurs well for the potential use of siRNAs as therapeutic agents, although many issues, including the mode of delivery, the stability of the siRNA, and the possibility of non-specific off-target effects, need to be addressed. Unclear from the reported studies is whether the siRNAs targeting the viral mRNA had resulted in any non-specific off-target effects. In this study, cell growth and viability were first examined as parameters to determine if there were any non-specific effects. This was possible with the 2.2.15 cell line used in this study because there is no lytic process following HBV replication. Cell growth and viability were not affected by treatment with the HBV-specific or the scrambled siRNA. Similar observations were also made using another cell line, PLC/PRF/5. Further analysis using cDNA microarrays demonstrated that siRNA-mediated gene silencing was specific and there were few non-specific effects, a finding also observed in two previous studies [36, 37]. In the past year, there have been a series of studies reporting off-target effects [45–48]. However, in these studies [36, 37, 45–48] and our current study, the presence of 21- to 23-nucleotide duplexes lead to no clear single consistent response pattern of gene expression and any observed changes in cellular gene expression may be purely dependent on the sequence of the duplex used. Hence, unlike the general interferon response to long dsRNA molecules, there is no single siRNA-induced response to siRNA duplexes in mammalian cells.

Taken together, these observations indicate that siRNAs can be designed which have few off-target effects. However, care should always be taken to verify that this is indeed so. In addition to demonstrating that there is a reduction in the expression at the mRNA and protein level, other validations such as genome-wide expression profiling may indeed be necessary to confirm that there are no off-target effects.

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