JOURNAL OF VIROLOGY, Nov. 2005, p. 14392–14403 Vol. 79, No. 22
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.22.14392–14403.2005

Genomic Analysis of Anti-Hepatitis B Virus (HBV) Activity by Small Interfering RNA and Lamivudine in Stable HBV-Producing Cells

Yong Guo,[1,2,4] Hongyan Guo,[1,2,4] Liang Zhang,[4] Hongying Xie,[4] Xin Zhao,[4] Fangxun Wang,[4] Ze Li,[4] Yahui Wang,[4] Shiliang Ma,[5] Jiaping Tao,[5] Weixing Wang,[6] Yuxiang Zhou,[1,2,3,4,7] Weiping Yang,[4,7] and Jing Cheng[1,2,3,4,7]*

Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China[1]; Medical Systems Biology Research Center, Tsinghua University School of Medicine, Beijing 100084, China[2]; The State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University, Beijing 100084, China[3]; National Engineering Research Center for Beijing Biochip Technology, 18 Life Science Parkway, Changping District, Beijing 102206, China[4]; Analysis Center of Medicine, Pharmacy and Public Health, Peking University, Beijing 100083, China[5]; The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Science, Peking University, Beijing 100083, China[6]; and CapitalBio Corporation, 18 Life Science Parkway, Changping District, Beijing 102206, China[7]

Received 25 May 2005/Accepted 24 August 2005

Hepatitis B virus (HBV) causes acute and chronic hepatitis and hepatocellular carcinoma. Small interfering RNA (siRNA) and lamivudine have been shown to have anti-HBV effects through different mechanisms. However, assessment of the genome-wide effects of siRNA and lamivudine on HBV-producing cell lines has not been reported, which may provide a clue to interrogate the HBV-cell interaction and to evaluate the siRNA's side effect as a potential drug. In the present study, we designed seven siRNAs based on the conserved HBV sequences and tested their effects on the expression of HBV genes following sorting of siRNA-positive cells. Among these seven siRNAs, siRNA-1 and siRNA-7 were found to effectively suppress HBV gene expression. We further addressed the global gene expression changes in stable HBV-producing cells induced by siRNA-1 and siRNA-7 by use of human genome-wide oligonucleotide microarrays. Data from the gene expression profiling indicated that siRNA-1 and siRNA-7 altered the expression of 54 and 499 genes, respectively, in HepG2.2.15 cells, which revealed that different siRNAs had various patterns of gene expression profiles and suggested a complicated influence of siRNAs on host cells. We further observed that 18 of these genes were suppressed by both siRNA-1 and siRNA-7. Interestingly, seven of these genes were originally activated by HBV, which suggested that these seven genes might be involved in the HBV-host cell interaction. Finally, we have compared the effects of siRNA and lamivudine on HBV and host cells, which revealed that siRNA is more effective at inhibiting HBV expression at the mRNA and protein level in vitro, and the gene expression profile of HepG2.2.15 cells treated by lamivudine is totally different from that seen with siRNA.

HBV infection is one of the most prevalent viral infectious diseases in humans. Most primary infections in adults are self-limited, with clearance of virus from blood and liver and the development of lasting immunity to reinfection. However, some primary infections in healthy adults and most in children do not self-resolve but develop into persistent infections, with some of those infected becoming chronic carriers or suffering from chronic HBV infection. Nearly 20% of chronic hepatitis B patents have eventually progressed to liver cirrhosis, and some infections have evolved into hepatocellular carcinoma (9, 23). Current treatments for chronically infected patients are restricted to the use of IFN and nucleotide analogues such as lamivudine; these treatments have met with limited success (9). There is an urgent need to develop more-effective antiviral therapies that can reduce or eliminate the viral infection completely with fewer side effects.

* Corresponding author. Mailing address: Medical Systems Biology Research Center, Tsinghua University School of Medicine, Beijing 100084, People's Republic of China. Phone: (86) 10 62772239. Fax: (86) 10 62773059. E-mail: jcheng@tsinghua.edu.cn.

Progress in RNA interference (RNAi) research in the last 3 years has shed light on the development of new therapeutic drugs. The mechanism of RNAi is based on siRNAs 21 to 23 bp in length, and the siRNA guides an endonuclease complex—termed the RNA-induced silencing complex—to target mRNAs of homologous sequences, resulting in their destruction (13). siRNA as an attractive antiviral agent has been explored previously (1, 7). Recently, several researchers have reported studies examining the effectiveness of siRNA with respect to HBV expression silencing (4, 10, 12, 17, 18, 24–27, 32, 38, 42, 43, 45, 46, 48, 49). However, experiment data on the genome-wide influence of siRNAs on gene expression in HBV-producing cell lines have not been reported, which may provide a clue to interrogate the HBV-cell interaction and to evaluate siRNA's side effects as a potential drug. To better understand the potential value of the use of siRNA to treat chronic HBV infection as a new strategy in the future, it may be interesting to compare the effects of siRNA and lamivudine on the anti-HBV activity and the host cell responses, considering that lamivudine has been a leading drug in the treatment of chronic HBV infection for the past decade (6, 15, 21, 22). Lamivudine is an orally administered nucleoside analog which is highly

effective in inhibiting HBV DNA synthesis. Lamivudine selectively targets the HBV viral reverse transcriptase and directly blocks replication of HBV genome.

In the present study, we developed a method to isolate the siRNA-positive cells by FACS; this homogenous cell population was used for precise assessment of the effects seen with siRNA. We performed genomic gene expression profiling with different siRNA treatments of stable HBV-producing cells (HepG2.2.15) by use of human genome-wide oligonucleotide microarrays. The microarray data revealed that different siRNAs have caused different patterns of gene expression in the host cells. The microarray data further suggested that a set of genes may be involved in the cellular responses for HBV infection. Finally, we compared the anti-HBV effects of siRNA and lamivudine at the mRNA, DNA, and protein level to validate the observations from the gene profiling experiments, which showed that the gene expression profile of HepG2.2.15 treated by lamivudine is totally different from that seen with siRNA.

MATERIALS AND METHODS

Abbreviations. HBV, hepatitis B virus; siRNA, small interfering RNA; IFN, interferon; RNAi, RNA interference; NLS, nuclear localization signal; GFP, green fluorescence protein; FACS, fluorescence-activated cell sorting; HBsAg, HBV surface antigen; CMV, cytomegalovirus; RT-PCR, reverse transcriptase PCR; ELISA, enzyme-linked immunosorbent assay; ISGs, IFN-stimulated genes.

Design and construction of siRNA. Twenty HBV genome sequences from GenBank were aligned and analyzed to identify the conservative regions containing at least 20 continuous nucleotides. The GenBank accession numbers of these genome sequences are as follows: AB076679, AB076678, AY090461, AY090460, AY090459, AY090458, AY090457, AY090456, AY090455, AY090454, AY090453, AY090452, E10905, NC_003977, AB074756, AB074755, AB064316, AB064315, AB064314, and U95551 (the HBV genome in HepG2.2.15 cells). Seven siRNA sequences were designed within the conservative regions from different HBV genes and further analyzed by BLAST to ensure that they did not have significant sequence homology with human genome sequences. The siRNA sequence for luciferase was used as the mock-treatment sequence (8). Selected siRNA sequences were designed to form a hairpin structure when they were transcribed. Those DNA sequences encoding the corresponding siRNAs were cloned into the plasmid pBS/U6 as described by Sui et al. (41). A GFP cassette amplified from the plasmid pCR3.1-Uni (Invitrogen, Carlsbad, CA) was cloned downstream of the siRNA-coding sequence in pBS/U6, under the control of a CMV promoter. A segment of NLS was also inserted in front of GFP; the NLS sequence was 5′-ACCATGCACCGCAGGAAGAAG AAGAGGAGAACC-3′. Plasmids containing NLS-GFP and different siRNAs against HBV cassettes were designated siRNA-1, siRNA-2, siRNA-3, siRNA-4, siRNA-5, siRNA-6, and siRNA-7. The plasmid containing NLS-GFP and siRNA against luciferase was designated siLuci. The plasmid containing only the NLS-GFP cassette was designated pBS/U6. Restriction enzymes used in this study were purchased from New England Biolabs (Beijing, China).

Cell culture and transfection. HepG2.2.15 (36, 37), HepG2, HeLa, and HEK293 cell lines were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. The HepG2.2.15 cells were cultured at 37°C and 5% CO_2 for 20 h to about 60% to 80% confluence in a 100-mm tissue culture dish (BD Biosciences, Bedford, MA) and then transfected with 2 μg of plasmid. The transfection was carried out using Effectene transfection reagent (QIAGEN, Hilden, Germany) per the manufacturer's instructions.

FACS. For mRNA and microarray assays, HepG2.2.15 cells transfected with siRNA-expressing constructs were harvested at 48 or 72 h posttransfection for FACS. For HBV core-associated DNA assay, the cells were harvested after 6 days. Briefly, transfected cells were trypsinized with 0.05% trypsin and 0.02% EDTA and centrifuged at 112 × *g* for 5 min. Then cells were resuspended and passed through a 38.5-μm metal mesh in ice-cold phosphate-buffered saline containing 10% fetal bovine serum at a concentration of approximately 1×10^6 to 5×10^6 cell/ml for sorting. Cells expressing NLS-GFP were sorted by use of FACSDiVa (Becton Dickinson, Franklin Lakes, NJ). Sorting purity analysis was also performed using FACSDiVa. The separated cells were collected in 5-ml Falcon nonpyrogenic tubes (Becton Dickinson) containing 0.5 ml fetal bovine serum. The purified cells were further examined under a Leica MPS60 fluorescence microscope (Solms, Germany).

Lamivudine preparation. The compound of lamivudine was extracted and purified from lamivudine tablets (GlaxoWellcome, United Kingdom). Lamivudine tablets (24 tablets, 100 mg each) were ground and extracted with hot methanol. Silica gel was added to this methanol solution, and the resultant solution was stirred for 30 min. Methanol was removed by evaporation, and the residue was applied to a silica gel column. The desired material was eluted with a 9:1 mixture of chloroform-methanol. The eluate was concentrated and dissolved in methanol-toluene to produce crystals of lamivudine. The yield was 1.61 g. The compound structure was confirmed with mass spectrum and nuclear magnetic resonance, and the purity (>99%) was analyzed by high-pressure liquid chromatography (16). Lamivudine was dissolved in sterile water and stored at −20°C in aliquots to avoid repeated freezing and thawing. Medium containing the indicated drug was prepared freshly before use.

Quantitative assay of HBsAg. For HepG2.2.15 cells treated with siRNA, the FACS-purified cells were centrifuged at 112 × *g* for 5 min and seeded in a six-well plate (Corning, New York, NY) at a density of 1.5×10^5 cells per well. The culture supernatants in the six-well plate were sampled at 24-h intervals up to 120 h after FACS. The expression of HBsAg in culture medium was quantified using a commercial HBsAg assay kit (InTec Products, Xiamen, China).

HepG2.2.15 cells were treated with lamivudine using a modification of a previously described method (19, 20). Briefly, 4×10^4 cells were grown to approximately 80 to 100% confluence in 24 wells and exposed to various concentrations of lamivudine. Medium was changed daily with fresh compounds for 8 days. The culture medium was collected each time for HBsAg assay.

An HBsAg control panel from the National Institute for the Control of Pharmaceutical and Biological Products of China was used to generate the calibration curve for determining the HBsAg concentration. The result was calibrated against the cell number determined using a 1/400-mm^2 hemocytometer (Qiujing Biochemical Reagent & Instrument, Shanghai, China).

Quantitative assay of HBV mRNA. For HepG2.2.15 cells treated with siRNA, the total RNA of the FACS-purified cells was extracted with Trizol (Invitrogen). For HepG2.2.15 cells treated with lamivudine (2 μM), the total RNA of the HepG2.2.15 cells in 6-well plates was extracted with Trizol after lamivudine treatment at 4 or 6 days. A 10-μg volume of total RNA was incubated with 10 U RNase-free DNase I (Invitrogen) at 37°C for 30 min. RNA was further purified with an RNeasy Mini kit (QIAGEN) for subsequent use. The purified total RNAs (500 ng) were reverse transcribed with 200 U M-MLV reverse transcriptase (Invitrogen). Real-time PCRs were performed by employing a DNA Master SYBR green I kit and a LightCycler (both from Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocols. Genes encoding HBsAg and human GAPDH were amplified in parallel. Primers for quantitative real-time PCR were as follows: HBV forward (5′-ATGGAGAACATCACATCAGGA TTCC-3′) and HBV reverse (5′-AAAGAAAACAGAAACCCATATGTAAAT T-3′) for the HBsAg gene, and GAPDH forward (5′-CGGATTTGGTCGTAT TGGG-3′) and GAPDH reverse (5′-TCTCGCTCCTGGAAGATGG-3′) for the GAPDH gene. The results were analyzed using LightCycler software version 3.5 (Roche Diagnostics). The real-time PCR amplification product was analyzed by melting curve analysis and 1.2% agarose gel electrophoresis, respectively. To analyze the relative expression ratio of HBsAg, a mathematical model reported by Pfaffl (30) was employed. Briefly, fourfold serially diluted HepG2.2.15 cell cDNA was amplified for both HBsAg and GAPDH to calculate the amplification efficiency, and the relative expression ratio was determined by the formula $E_{HBsAg}^{(CP1 - CP2)}/E_{GAPDH}^{(CP3 - CP4)}$, where E is real-time PCR efficiency and CP is the real-time PCR crossing point.

Quantitative assay of HBV core-associated DNA. For HepG2.2.15 cells treated with siRNA, intracellular core-associated HBV DNA was extracted from the FACS-purified cells by use of a previously described method (31). For HepG2.2.15 cells treated with lamivudine, the intracellular core-associated HBV DNA of the HepG2.2.15 cells in 24-well plates was extracted after the cells were treated with various concentrations of lamivudine at 8 days. The DNA quality was assessed by 1% agarose gel electrophoresis and was quantified by UV spectrophotometry. An aliquot of purified total DNA was used as the template for real-time PCR. Genes encoding HBsAg and GAPDH were amplified in parallel. The purpose of amplifying GAPDH is to provide an indicator of chromosomal DNA contamination in the presence of the viral DNA template for real-time PCR. Fourfold serially diluted total DNA was amplified for HBsAg to identify the amplification efficiency. The relative expression ratio was determined by the formula $E_{HBsAg}^{(CP1 - CP2)}$.

Microarray analysis. A human genome 70-mer oligonucleotide microarray was obtained from CapitalBio Corporation (Beijing, China). Briefly, a human

TABLE 1. siRNA sequences used in this study

siRNA	siRNA coding sequence
siLuci	GGCGTACGCGGAATACTTCGA
1	GGCTGCTATGCCTCATCTTCT
2	GCCTATGGGAGTGGGCCTCAG
3	GGAAGCCTCCAAGCTGTGCCT
4	GGAAGAAGAACTCCCTCGCCT
5	GGACTCGTGGTGGACTTCTCT
6	GGCCAAGTGTTTGCTGACGCA
7	GGCTCCTCTGCCGATCCATAC

genome oligonucleotide set (version 2.0) consisting of 5′ amino acid-modified 70-mer probes and representing 21,329 well-characterized *Homo sapiens* genes was purchased from QIAGEN and printed on amino-silaned glass slides. Arrays were fabricated using an OmniGrid microarrayer (Genomic Instrumentation Services, San Carlos, CA). Fluorescent dye (Cy5 and Cy3-dCTP; Amersham Pharmacia Biotech, Piscataway, NJ)-labeled DNA was produced through Eberwine's linear RNA amplification method (44) and subsequent enzymatic reaction. In detail, double-stranded cDNA containing T7 RNA polymerase promoter sequence (5′-AAACG ACGGC CAGTG AATTG T AATA CGACT CACTA TAGGC GC-3′) was synthesized with 5 μg of total RNA by use of a cDNA synthesis system kit according to the protocol recommended by the manufacturer (TaKaRa, Dalian, China). A T7-(oligo)dT primer (5′-AAACG ACGGC CAGTG AATTG TAATA CGACT CACTA TAGGC GC TT TTT TTT TTT TTT TTT -3′) was used instead of the poly(T) primer provided in the kit. The synthesized double-stranded cDNA was purified with a PCR purification kit (QIAGEN), and the final cDNA was eluted in 60 μl of elution buffer. Half of the double-stranded cDNA product was concentrated by vacuuming to 8 μl and was subjected to an in vitro transcription reaction in a 20-μl reaction system by use of a T7 RiboMAX Express large-scale RNA production system (Promega, Madison, WI). The reaction was continued for 3 h at 37°C, and the amplified RNA was purified with an RNeasy Mini kit (QIAGEN). cDNA was fluorescently labeled with Klenow enzyme after reverse transcription. Briefly, 2 μg of amplified RNA was mixed with 2 μg of random hexamer, denatured at 70°C for 5 min, and cooled on ice. Then, 4 μl of first-strand synthesis buffer, 2 μl of 0.1 M dithiothreitol, 1 μl 10 mM deoxynucleoside triphosphate, and 1.5 μl SuperScript II (Invitrogen) were added. Tubes were incubated at 25°C for 10 min and then at 42°C for 60 min. The products were purified using a PCR purification kit (QIAGEN) and vacuumed down to 10 μl. The cDNA was mixed with 2 μg random nonamer, denatured at 95°C for 3 min, and immediately cooled on ice. The deoxynucleoside triphosphates and Cy5-dCTP or Cy3-dCTP were added at a final concentration of 120 μM each dATP, dGTP, and dTTP and 60 μM dCTP and 40 μM Cy5-dCTP (or Cy3-dCTP), respectively. Klenow enzyme (1 μl) from Takara was added, and the reaction was performed at 37°C for 60 min. The labeled DNA was purified with a PCR purification kit (QIAGEN) and then resuspended in elution buffer and quantified by UV spectrophotometry. Labeled control and test samples were quantitatively adjusted based on the efficiency of Cy5-dCTP or Cy3-dCTP incorporation and mixed into 30 μl hybridization solution (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.2% sodium dodecyl sulfate, 25% formamide, and 5× Denhart's solution). DNA in hybridization solution was denatured at 95°C for 3 min before loading onto a microarray. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% sodium dodecyl sulfate, 2× SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature). Arrays were scanned with a ScanArray Express scanner (Parckard Bioscience, Kanata, OT), and the obtained images were analyzed with GenePix Pro 4.0 (Axon Instruments, Foster City, CA). A space- and intensity-dependent normalization based on a LOWESS program (47) was employed. For each test and control sample, two hybridizations were performed by using a reverse fluorescence strategy. Only genes whose alteration tendency was consistent (both above twofold) in both microarrays were selected as differentially expressed genes. The Affymetrix's short oligonucleotide (25-mer) HG U133A 2.0 array (Affymetrix, Santa Clara, CA) analyses were performed per the manufacturer's instructions. To confirm the microarray results, 17 representative genes were analyzed by real-time PCR.

Sequence alignment. The sequences of all down-regulated genes from siRNA versus pBS/U6 microarray profiling were aligned with the coding sequences of siRNAs to find the identical sequences. The Needleman-Wunsch global alignment algorithm (28) from the EMBOSS software package (ftp://emboss.open-bio.org/pub/EMBOSS) was adapted here. The parameters gap-open and gap-extend of the algorithm were set to 100.0 and 10.0 to produce a gap-free alignment. The data for identical base pairs for each alignment were summarized. In addition, we developed a program to automate the above-described process. The mRNA sequences were downloaded from GenBank. Additional details and the program are available from the authors upon request.

Additional microarray information. The description of this microarray study followed the Minimum Information About a Microarray Experiment guidelines (2). The original data from this study are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo); the accession number is GSE3049. The detailed protocols for RNA isolation, amplification, labeling, and hybridization can be provided by the authors upon request.

RESULTS

Anti-HBV siRNA sequences and constructs. To evaluate the influence of siRNA against HBV in HepG2.2.15 cells, seven coding sequences for siRNA were designed based on the

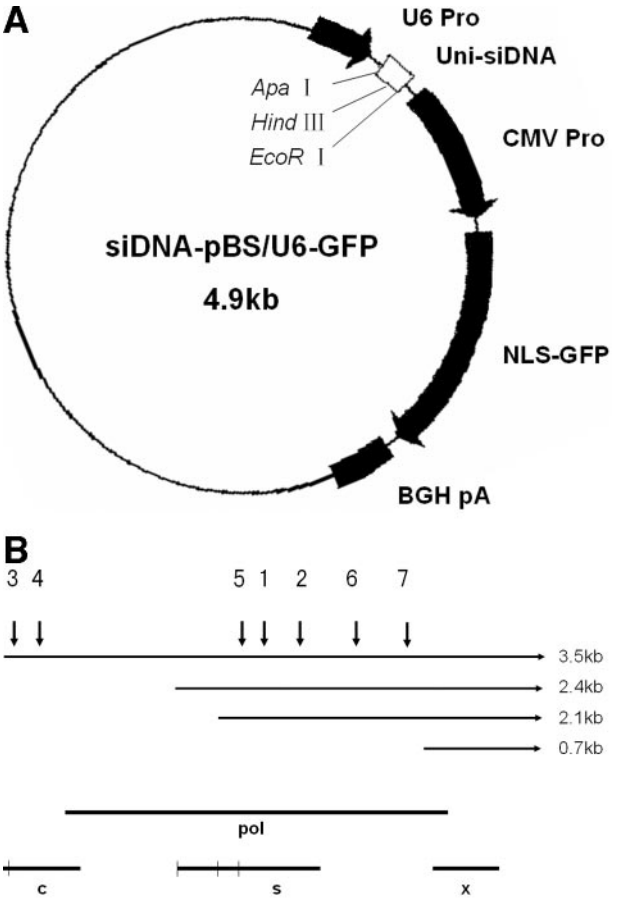

FIG. 1. Vector information and target sites. (A) Plasmid constructed for siRNA and NLS-GFP expression. The 22-nucleotide inverted repeats, separated by a 6-nucleotide linker (HindIII restriction site), were inserted between ApaI and EcoRI sites. The U6 promoter directed the synthesis of hairpin siRNA transcripts. The CMV promoter directed the synthesis of NLS-GFP. The U6 promoter (U6 pro), site of sequence for siRNA synthesis (Uni-siDNA), CMV promoter (CMV pro), green fluorescent protein with a nuclear localization signal (NLS-GFP), and BGH polyadenylation (BGH-pA) are indicated here. (B) Location of siRNA target sites. Downward arrows indicate the location of siRNA target sites within the four HBV transcripts. The HBV open reading frames shown in the bottom row were aligned with the HBV mRNAs. Pol represents polymerase; C, HBcAg; S, HBsAg; and X, X gene. The numbers above the arrows indicate the target site of each siRNA.

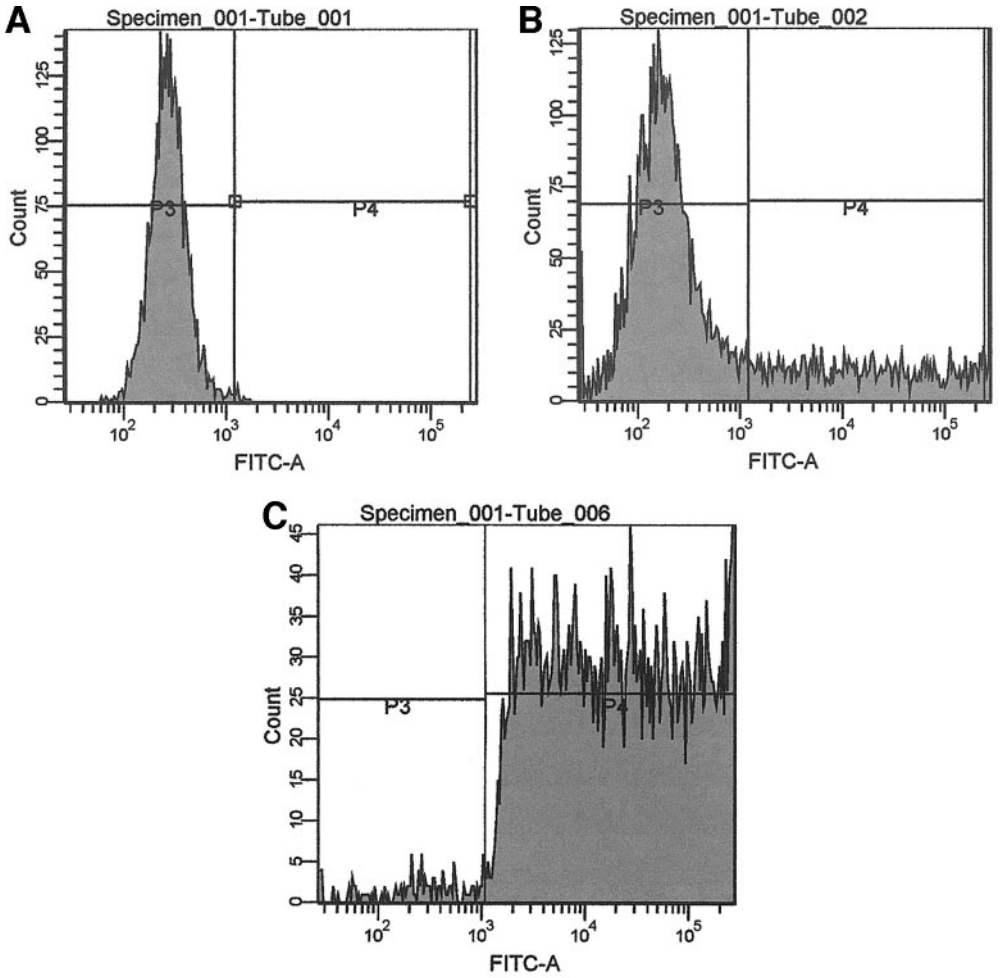

FIG. 2. FACS purification of GFP-positive HepG2.2.15 cells transfected with siRNA-GFP-expressing plasmids. Cells were harvested and separated by FACS at 48 or 72 h posttransfection. For HBV core-associated DNA assay, the cells were harvested after 6 days posttransfection. (A) FACS analysis of HepG2.2.15 cells without plasmid transfection. (B) FACS analysis of HepG2.2.15 cells with siRNA-GFP-expressing plasmid transfection. The presorted population consisted of 80% GFP-negative and 20% GFP-positive cells, meaning that the transfection efficiency was about 20%. (C) Collection and purity analysis of the sorted GFP-positive HepG2.2.15 cells; approximately 95% of the FACS-collected cells expressed NLS-GFP. FITC-A, fluorescein isothiocyanate A.

guidelines described by Sui et al. (41), and all were complementary to different parts of the conserved sequence of the HBV genome. The coding sequences for siRNAs were listed in Table 1. The construct scheme for expressing hairpin siRNA and NLS-GFP is depicted in Fig. 1.

Transfection efficiency and sorting purity. The transfection efficiency is approximately 20 to 30%, as determined by FACS. Approximately 95% of the FACS-collected cells expressed NLS-GFP (Fig. 2).

Real-time PCR efficiencies, reproducibility, and specificity. Real-time PCR efficiencies were calculated from the given slopes in LightCycler software. The corresponding real-time PCR efficiency (E) was calculated according to the following equation: $E = 10^{[-1/\text{slope}]}$. Interrogated transcripts showed high linearity, in the investigated range of from 0.1 to 25 ng cDNA input ($n = 2$) (Pearson correlation coefficient $R > 0.99$). This suggested that the reproducibility of real-time PCR was satisfied.

Specificity of real-time PCR products was documented with high-resolution gel electrophoresis and resulted in a single product with the expected length (HBsAg, 681 bp; GAPDH, 196 bp). In addition, a LightCycler melting curve analysis was performed which resulted in single product-specific melting temperatures as follows: HBsAg, 85.89°C; GAPDH, 84.10°C. No primer-dimers were generated during the applied 45 real-time PCR amplification cycles. These results suggested that the real-time PCR here was very specific.

Effect of siRNAs and lamivudine on HBV expression and replication. HepG2.2.15 cells were used as the blank control, and cells transfected with pBS/U6 or siLuci plasmid were used as the negative control. The results shown in Table 2 and Fig. 3 suggested that siRNA-1 and siRNA-7 could significantly attenuate HBV activity at the mRNA, protein, and DNA level in HepG2.2.15 cells. Lamivudine could significantly attenuate HBV replication, whereas it has little effect on HBV mRNA and HBsAg production in HepG2.2.15 cells.

TABLE 2. The degree of inhibition by siNRA and 2 μM lamivudine of HBV at the mRNA, HBsAg, and DNA level in HepG2.2.15 cells[a]

	% Inhibition (mean ± SD)		
	siRNA-1	siRNA-7	Lamivudine
HBV mRNA[b]	60.94 ± 4.21	86.12 ± 1.59	1.93 ± 8.09
HBsAg[c]	66.66 ± 5.02	88.18 ± 1.2	1.99 ± 8.42
HBV DNA[b]	87.09 ± 0.34	90.42 ± 3.18	93.12 ± 3.81

[a] The FACS-purified HepG2.2.15 cells were used to quantitatively determine the siRNA's inhibitory effects.

[b] The relative expression ratio of mRNA and DNA was evaluated by real-time PCR, and the exact inhibitory rates were calculated by the formula $E_{HBV}^{(CP1 - CP2)}/E_{GAPDH}^{(CP3 - CP4)}$ (E, real-time PCR efficiency; CP1, HBV's crossing point with mock treatment; CP2, HBsAg's crossing point with siRNA or lamivudine treatment; CP3, GAPDH's crossing point with mock treatment; CP4, GAPDH's crossing point with siRNA or lamivudine treatment). This experiment was performed in duplicate. The results were shown as mean ± SD ($n = 2$).

[c] The effect of siRNAs or lamivudine on HBsAg production was determined by ELISA. This experiment was performed in triplicate. The results were shown as mean ± SD ($n = 3$).

Comparison of gene expression signatures of siRNA-1 versus pBS/U6, siRNA-7 versus pBS/U6, and HepG2.2.15 cells versus HepG2 cells. To examine the genome-wide influence of siRNA-1 and siRNA-7 on host cells, microarray analysis was performed for HepG2.2.15 cells transfected with siRNA-1 versus pBS/U6 and siRNA-7 versus pBS/U6. There were 54 genes with a change of magnitude over twofold for siRNA-7 versus pBS/U6, and 499 genes with a change of magnitude over twofold for siRNA-1 versus pBS/U6. There were 18 genes were found down-regulated in both cases. These results indicate that the gene number affected by siRNA-7 is much less than that seen with siRNA-1, and a significant number (18/54) of the genes with a change of magnitude for siRNA-7 versus pBS/U6 also showed the same trends for siRNA-1 versus pBS/U6.

The changed genes in siRNA versus pBS/U6 were correlated with the down-regulation of intracellular HBV protein expression and the nonspecific effect of siRNA itself. The 18 genes changed in common in both cases were especially interesting. Perhaps these genes are the cellular genes responsive to HBV expression. Among the 18 common changed genes, 10 of them were immune response-related genes, and among these 10 genes, 9 (IFIT1, MDA5, STAT1, G1P2, IFITM1, OAS1, G1P3, IFI27, and ISGF3G) were ISGs. In addition, several other genes (e.g., UBE2L6, PPM1B, PLSCR1, SLC25A6, MVP, SYTL2, SWAP2, and AKR1B10) were also down-regulated after the inhibition of HBV expression by RNAi.

To further clarify whether the expression of the 18 genes was related to the expression of HBV, we have also compared gene expression profiles of HepG2.2.15 versus HepG2 cells. HepG2 is the parental cell of HepG2.2.15 without HBV virus. The data showed that among the 18 genes, 7 genes (G1P2, IFITM1, OAS1, BAL, PPM1B, MVP, and SYTL2) were down-regulated in both cases for siRNA versus pBS/U6, whereas they were up-regulated in the case of HepG2.2.15 versus HepG2 cells (as shown in Table 3 and Fig. 4).

Considering that the two siRNAs, i.e., siRNA-1 and siRNA-7, could induce different expression profiles, it is necessary to clarify whether the gene changes, especially those of the 18 genes changed in common, were nonspecific effects of RNAi. We first aligned the sequences of 310 down-regulated

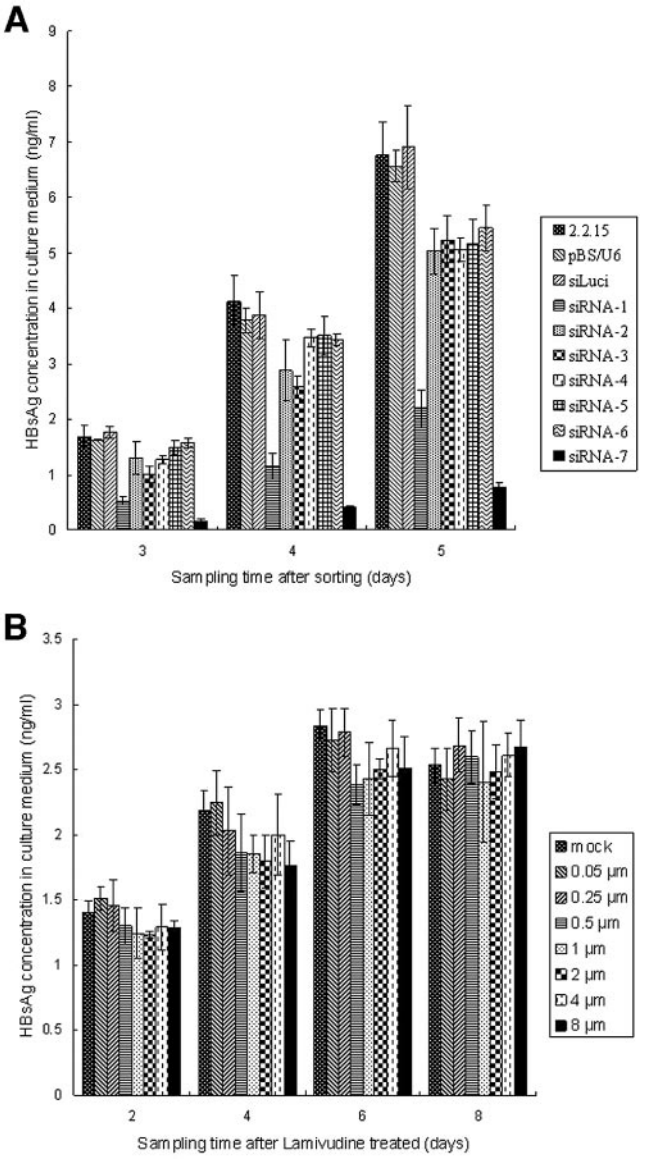

FIG. 3. The effect of siRNAs and lamivudine on HBsAg production. (A) The effect of siRNAs on HBsAg production in cell culture medium. Supernatants were sampled at 24-h intervals until 120 h, and ELISAs were performed. The inhibition rate was determined though ELISA from cells transfected with siRNA-1 or siRNA-7 versus pBS/U6 at 120 h. The inhibition rates were as follows: 66.66 ± 5.02% for siRNA-1 and 88.18 ± 1.2% for siRNA-7; other siRNAs had little effect on HBsAg production. siRNA against luciferase-producing plasmid (siLuci) had no influence on HBsAg production. These experiments were performed in triplicate. The levels of HBsAg in medium were normalized by cell number and are shown as means ± standard deviations (SD); $n = 3$. (B) The effect of lamivudine on HBsAg production in cell culture medium. HepG2.2.15 cells were exposed to various concentrations of lamivudine. Medium was changed daily with fresh test compounds for 8 days. The medium was collected each time for HBsAg assays; lamivudine (0.05 to 8 μM) had little effect on HBsAg production. These experiments were performed in triplicate. The levels of HBsAg in medium were normalized by cell number and are shown as means ± SD; $n = 3$.

TABLE 3. The common genes down-regulated by siRNA-1 and siRNA-7 in HepG2.2.15

HUGO name and gene category[a]	GB accession no.	Fold change					
		siRNA1 vs control in HepG2.2.15 cells	siRNA7 vs control in HepG2.2.15 cells	siRNA6 vs control in HepG2.2.15 cells	siRNA1 vs control in HepG2 cells	siRNA7 vs control in HepG2 cells	HepG2.2.15 cells vs HepG2 cells
Immune response							
IFIT1	NM_001548	0.09	0.17	—[b]	—	—	—
MDA5*	NM_022168	0.11	0.29	—	—	—	—
STAT1	NM_007315	0.15	0.20	—	—	—	—
GIP2	NM_005101	0.15	0.25	—	—	—	2.01
IFITM1	NM_003641	0.21	0.36	—	—	—	9.27
OAS1*	NM_016816	0.26	0.39	—	—	—	2.64
GIP3	NM_022873	0.29	0.42	—	—	—	—
IFI27	NM_005532	0.28	0.42	—	—	—	—
BAL	NM_031458	0.31	0.29	—	—	—	3.02
ISGF3G*	NM_006084	0.47	0.50	—	—	—	—
Metabolism							
MDA5*	NM_022168	0.11	0.29	—	—	—	—
UBE2L6	NM_004223	0.27	0.33	—	—	—	—
OAS1*	NM_016816	0.26	0.38	—	—	—	2.64
PPM1B	AJ271835	0.36	0.47	—	—	—	2.05
ISGF3G*	NM_006084	0.47	0.50	—	—	—	—
Cell growth and maintenance							
PLSCR1	NM_021105	0.12	0.30	—	—	—	—
SLC25A6	BC008737	0.47	0.48	—	—	—	—
Drug resistance							
MVP	NM_017458	0.45	0.49	—	—	—	2.75
Function unknown							
SYTL2	NM_032943	0.35	0.49	—	—	—	31.54
SWAP2	NM_007056	0.47	0.45	—	—	—	—
AKR1B10	NM_020299	0.48	0.41	—	—	—	—

[a] *, genes were classified into two categories.
[b] —, change magnitude was <2; these genes could be considered as exhibiting no change.

genes from siRNA-1 versus pBS/U6 and those of 46 down-regulated genes from siRNA-7 versus pBS/U6 with the coding sequence of siRNA-1 or siRNA-7. We reasoned that if the down-regulated genes, especially the 18 genes changed in common, were silenced off-target by RNAi, the down-regulated genes may have high homology with the coding sequence of siRNA-1 or siRNA-7. It was noted that there are at least five base pair mismatches among the coding sequence of siRNA and the sequences of down-regulated genes (Fig. 5). We then performed microarray analysis for HepG2 cells transfected with siRNA-1 versus pBS/U6 and siRNA-7 versus pBS/U6. In theory, there are no target genes for siRNA-1 and siRNA-7 in HepG2 cells, so the changed genes in HepG2 were not caused by the down-regulation of HBV but by the introduction of siRNA-expressing plasmid. If the 18 common changed genes were nonspecifically down-regulated by siRNAs in HepG2.2.15 cells, they should also be down-regulated by siRNA-1 and siRNA-7 in HepG2 cells. In fact, the 18 genes were not down-regulated by siRNA-1 or siRNA-7 in HepG2 cells. To further identify whether these 18 genes were nonspecifically affected by siRNA-1 and siRNA-7, we have performed microarray analysis using siRNA-6, which had no significant effects on HBV gene expression as a negative control. These 18 genes were not down-regulated by siRNA6 versus pBS/U6 in HepG2.2.15 cells. So it is highly likely that the 18 common changed genes were caused by the down-regulation of HBV in HepG2.2.15 cells (Fig. 4).

Comparison of gene expression signatures of siRNA versus pBS/U6 and lamivudine versus mock-treatment solution. We found that lamivudine (2 μM) can effectively decrease the HBV DNA after 4 to 6 days of treatment. To examine the genome-wide influence of lamivudine on host cells, microarray analysis was performed for HepG2.2.15 cells treated with lamivudine (2 μM) at 6 days. There were 44 genes with a change in magnitude of over twofold for lamivudine treatment versus mock treatment (distilled water). Among the 44 genes, 10 (22%) coded enzymes, 1 (2%) coded enzyme inhibitors, 1 (2%) coded an enzyme activator, and 3 (7%) coded signal pathway proteins, whereas none of them correlated with an immune response, and none of them belonged to ISGs (Fig. 6). These 44 genes were not found in the correlation of siRNA-1 and siRNA-7 versus pBS/U6 results, and none of these 44 genes were correspondingly changed in the profiling of HepG2.2.15 versus HepG2 cells (Fig. 4). To identify the genes that may be nonspecifically affected by lamivudine, the gene profiling experiment was also conducted on HepG2 cells treated with 2 μM lamivudine, and these 44 genes were not affected the same in HepG2 cells. These findings indicated that the gene expression profile of HepG2.2.15 cells treated with lamivudine is totally different from that seen with siRNAs; the results also suggested the decrease in replication of HBV DNA alone cannot alleviate the immune response in HepG2.2.15 cells, since lamivudine does not affect HBV protein synthesis, as documented in our HBsAg quantitative assay.

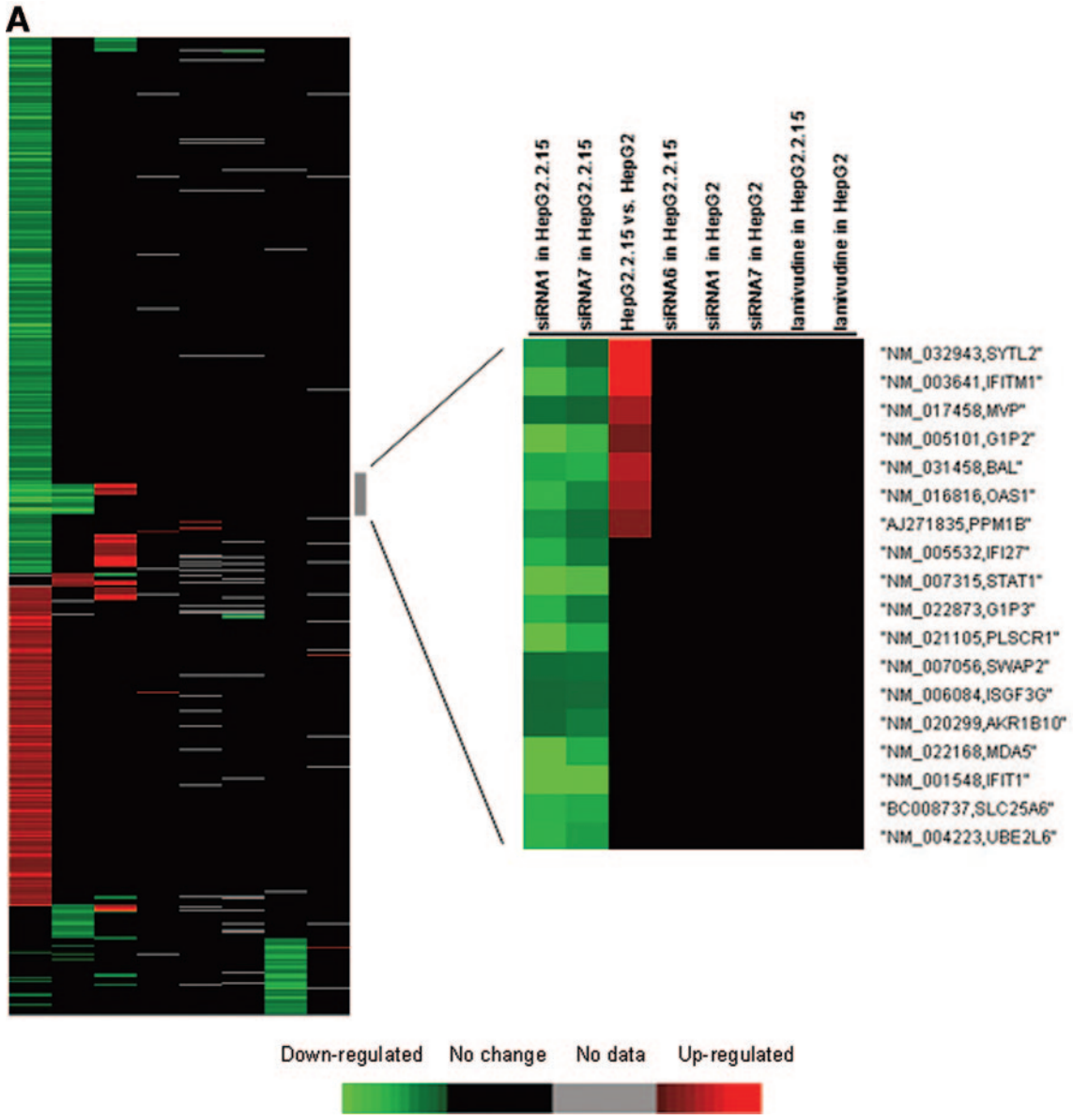

FIG. 4. Expression profiling revealed the genome-wide influence of siRNA and lamivudine on HepG2.2.15 cells. (A) There were 54 genes with a change of magnitude over twofold for siRNA-7 versus pBS/U6 and 499 genes with a change of magnitude over twofold for siRNA-1 versus pBS/U6 in HepG2.2.15 cells. There were 18 genes found down-regulated in both cases; among the 18 genes, 7 genes (G1P2, IFITM1, OAS1, BAL, PPM1B, MVP, and SYTL2) were down-regulated in both cases of siRNA versus pBS/U6 but up-regulated in the case of HepG2.2.15 versus HepG2 cells. The 18 genes were not down-regulated by siRNA-6 in HepG2.2.15 cells, and they were not down-regulated by siRNA-1 and siRNA-7 in HepG2 cells either. (B) There were 44 genes with a change of magnitude over twofold for lamivudine versus mock treatment in HepG2.2.15 cells. None of the 44 genes was also down-regulated in the correlation of siRNA-1 and siRNA-7 in HepG2.2.15 cells. None of the 44 genes was correspondingly up-regulated in the profiling of HepG2.2.15 versus HepG2 cells. None of the 44 genes was down-regulated in the profiling of lamivudine versus mock treatment in HepG2 cells.

The fidelity of microarray profiling. Initially, we compared CapitalBio's long oligonucleotide (Operon 70-mer) microarray with Affymetrix's short oligonucleotide (25-mer) HG U133A 2.0 array. The correlation coefficient (R value) between the two platforms was 0.787 when all 6,754 genes in common were detected using the two platforms, employing the same batch of RNA extracted from HeLa and HEK293 cell lines (Fig. 7). We then further evaluated the fidelity of CapitalBio's long oligonucleotide microarray using RNA from HepG2.2.15 cells transfected with siRNA-7 by conducting a self-to-self comparison. For each test and control sample, two hybridizations were performed by using a reverse fluorescence strategy. The experimental results indicated that the change in magnitude in the self-to-self expression profiling was within twofold, and this suggested that our microarray analysis is accurate enough to determine the values for the changed genes above twofold (Fig. 7). The reproducibility of microarray was evaluated by determining the gene expression results for HepG2.2.15 versus

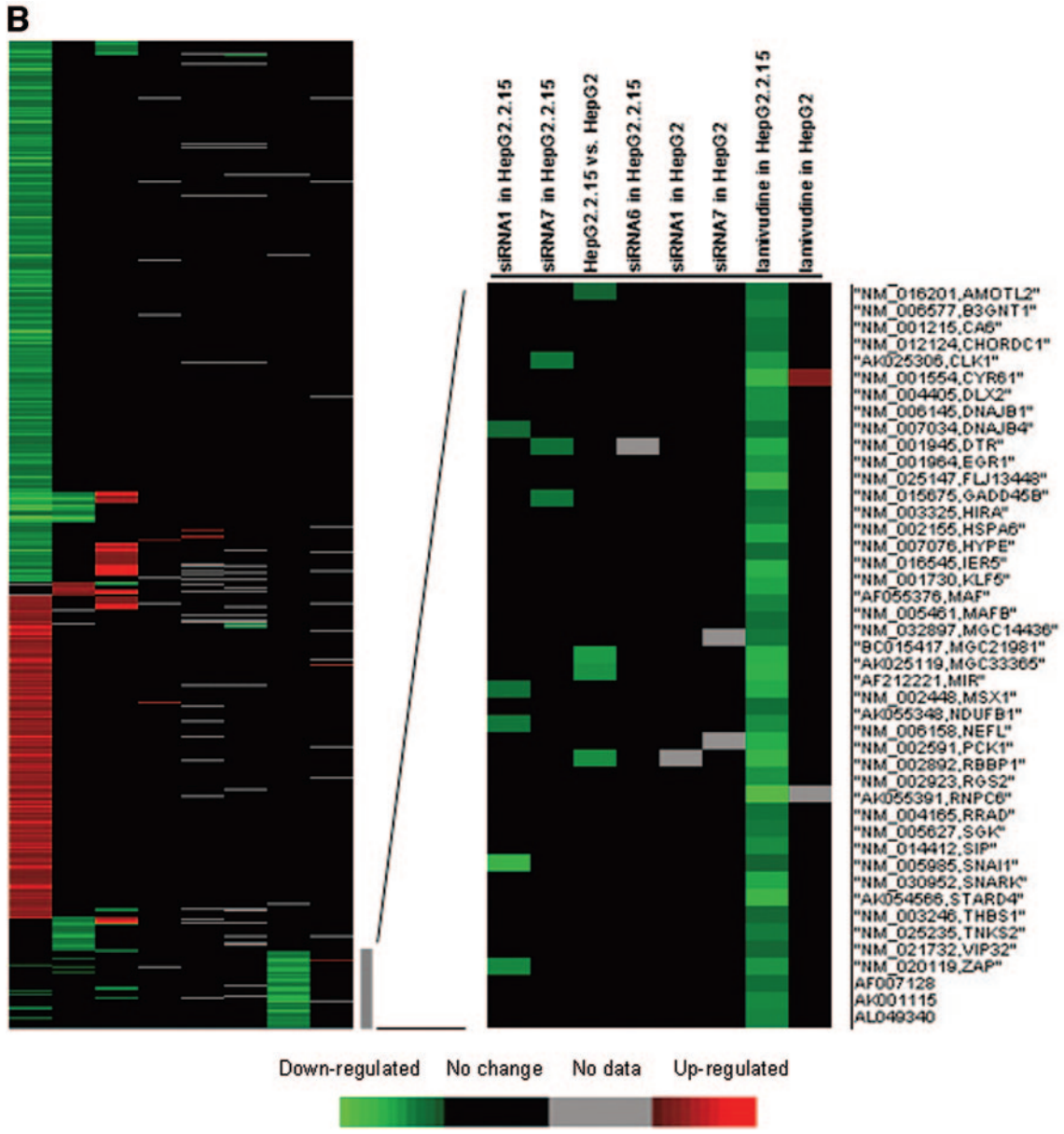

FIG. 4—*Continued.*

HepG2 cells three times independently. For each test and control sample, two hybridizations were performed by using a reverse fluorescence strategy. The Pearson linear correlation coefficient value (R) of two independent experiments was 0.956, which demonstrated a high level of reproducibility between two independent experiments (Fig. 7). To further validate the differential expression of genes obtained by microarray analysis, the gene expression alterations were verified by quantitative RT-PCR. Seventeen genes from the above-described microarray results were selected to compare their changes at the transcription level by use of microarray and quantitative RT-PCR. The results demonstrated a good correlation between the quantities of the changed transcripts measured by both methods (Fig. 7). In the comparison experiment, we chose 17 genes from the microarray data, among which the ratios of only 12 genes were above twofold (the genes were selected from Table 3). The 12 genes were also found to show a change of more than twofold in the real-time PCR experiment. Both microarray data and real-time PCR data showed the down-regulation of these genes; hence, the concordance was satisfied. To identify whether there were "false-negative" results in our microarray study, we selected five genes with a change of less than twofold to perform real-time PCR analysis, and those five genes were also found to exhibit a change of less than twofold in real-time PCR analysis. Among the five genes analyzed using both microarray data and real-time PCR data, three of them were found to have been down-regulated less than twofold. Yet two of them were found with changes in conflict. The reason for this is that the fidelity of our microarray is twofold; therefore, genes with a change of less than

A

Accession No.	HUGOname	Sequence	Identity
	siRNA-1	GGGCTGCTATGCCTCATCTTCT	22/22
BC011775	MGC5466	CTGCTGCTGAGCCTCATCGTCT	8/17
NM_016237	ANAPC5	GGGCTGTTTTGCTGCAGCTTCT	6/17
AF103803	H41	CTGCTATAATGCCTCATCTAAT	11/15
NM_006437	ADPRTL1	ATGTTGAAATTCCTCATCTTCC	10/15
NM_020644	C11ORF15	TGTGTGGTCTTACTCATCTTCT	10/15
NM_004882	CIR	GTCCTCTTCTTCCTCATCTTCC	10/15
NM_017633	C6ORF37	AAGGACCTGGACCTCATCTTCT	11/14
NM_002377	MAS1	ATCATTATATTCCTCATCTTCG	10/14
NM_001609	ACADSB	TGATTCTCATGCCTCATCCTCC	10/14
AF419845	P15RS	GGGCTGCTATGGTTTGAATGGA	11/13

B

Accession No.	HUGOname	Sequence	Identity
	siRNA-7	GGGCTCCTCTGCCGATCCATAC	22/22
NM_006187	OAS3	GGGCTTCCCGGTCTAGCCACAC	5/15
NM_004625	WNT7A	TGGCTGCTCTGCCGACATCCGC	9/14
NM_032943	SYTL2	TGGCTCCTCCACTGAAAACTAC	8/14
AB002308	KIAA0310	TGGCTACCCTGCCGACACCGTC	7/14
BC018130	F2RL1	GCTCTCCTTTGCCGAAGTGTCC	6/14
AB040974	MDS026	TGGGCTTTCTGCTCAGCCATAC	6/14
BC008737	SLC25A6	GGCCTCCTCCACACACACACAC	6/14
NM_003003	SEC14L1	GAGCTCCTCAGTGGTACCTGAA	7/13
NM_007315	STAT1	GGCCTCCTCTCTCAGTTTTTAT	7/13
BC008502	LOC201895	GGGACCCTCTGGTGATAATGGC	6/13

FIG. 5. Sequence alignment of down-regulated genes with siRNA-1 (A) and siRNA-7 (B). Only the top 10 homology genes were listed here. The degree of sequence identity to siRNA-1 or siRNA-7 was indicated as the number of contiguous identical nucleotides/the total number of identical nucleotides. It was noted that there were at least five base pair mismatches among the coding sequence of siRNA and the sequences of down-regulated genes.-

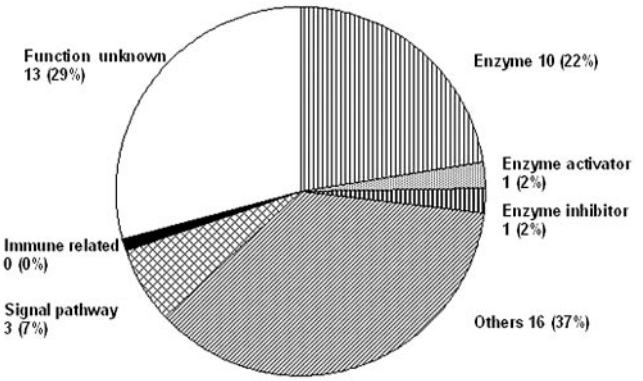

FIG. 6. The results of gene expression profiling of lamivudine versus mock treatment for HepG2.2.15 cells analyzed by gene ontology. Among the 44 genes, 10 (22%) encoded enzymes, 1 (2%) encoded an enzyme inhibitor, 1 (2%) encoded an enzyme activator, and 3 (7%) encoded signal pathway proteins, whereas none of them correlated with immune response.

twofold fell into the "gray zone" of microarray analysis, which means that the calculated up- or down-regulation change of these genes may not be precise, and hence it is reasonable to conclude that parts of these five genes may have some disparities in terms of their regulation changes when the two methodologies were applied.

DISCUSSION

To perform the microarray analysis of siRNA's effect on host cells, it was necessary to collect the homogenous cell population containing siRNA. Since the transfection efficiency of siRNA-producing plasmids is not satisfactory, we developed a novel approach to resolve this problem in the present report. The transfected cells were FACS purified prior to the assay through engineering GFP after siRNA genes in a vector construction; as a result, the siRNA-containing cells after FACS have achieved a level of 95%. This population of purified cells can also be used for quantitative evaluation the siRNA's effect.

The siRNA-1 and -7 versus pBS/U6 microarray profiling in this study revealed that 18 genes were down-regulated. Among the 18 genes, 7 genes were correspondingly up-regulated in the HepG2.2.15 versus HepG2 cells. The expression of the seven genes could be the host response to the expression of HBV. Among the seven genes, G1P2, IFITM1, and OAS1 were ISGs. Many ISGs have been identified, and the functions of some of the ISGs have been described previously, whereas many others still remain uncharacterized (5, 40). In the HBV-infected cells, IFNs are induced by the virus, and in turn the IFNs activate a variety of ISGs. This can further trigger common intracellular antiviral pathways. Among these ISGs, it has been demonstrated that the activity of Janus kinase (Jak), double-stranded RNA-activated protein kinase, and interferon regulatory factor 1 was required for the antiviral effect of IFNs against HBV, but the precise cellular pathway that mediates this antiviral effect has not been elucidated (11, 33). It is possible that these ISGs (G1P2, IFITM1, and OAS1) also mediate the antiviral activity of IFNs against HBV. Although the other four genes (BAL, PPM1B, MVP, and SYTL2) were not ISGs, their expression levels were correlated with the expression of HBV, suggesting that these four genes may be involved in the other yet-undefined anti-HBV pathways in intrahepatic cells. For the HBV biology, the mechanism of RNA-directed DNA synthesis has been well characterized through genetic as well as biochemical studies. In contrast, some events of the viral life cycle, including entry, uncoating, delivery of the viral genome into the cell nucleus, and virion formation, are not well understood (35). Therefore, it should be interesting to explore whether the seven genes have been involved in the life cycle of HBV as cofactors. Theses seven genes require future studies to further define the intracellular molecular events related to the HBV replication. Understanding the cellular anti-HBV mechanism may lead to new therapeutic targets and/or drugs for chronically infected patients. The current report may prove to be an important step towards the discovery of the cellular events that may be involved in the antiviral activities.

In addition to the 18 genes commonly down-regulated in both siRNA-1 and siRNA-7 versus pBS/U6 microarray profiling, there are 36 changed genes in siRNA-7 versus pBS/U6 and 481 changed genes in siRNA-1 versus pBS/U6, respectively. Both siRNA-1 and siRNA-7 were tested against the regions overlapping the viral 3.5-kb, 2.4-kb, and 2.1-kb RNAs, and their anti-HBV efficacies were about the same. However, their global impacts on the host cells differed, suggesting that siRNAs may have broad and complicated effects beyond the selective silencing of HBV genes when introduced into HepG2.2.15 or other cells. Recent reports have also shown that the potential nonspecific effects may be induced by siRNAs (3, 14, 29, 34, 39). Jackson et al. and Saxena et al.

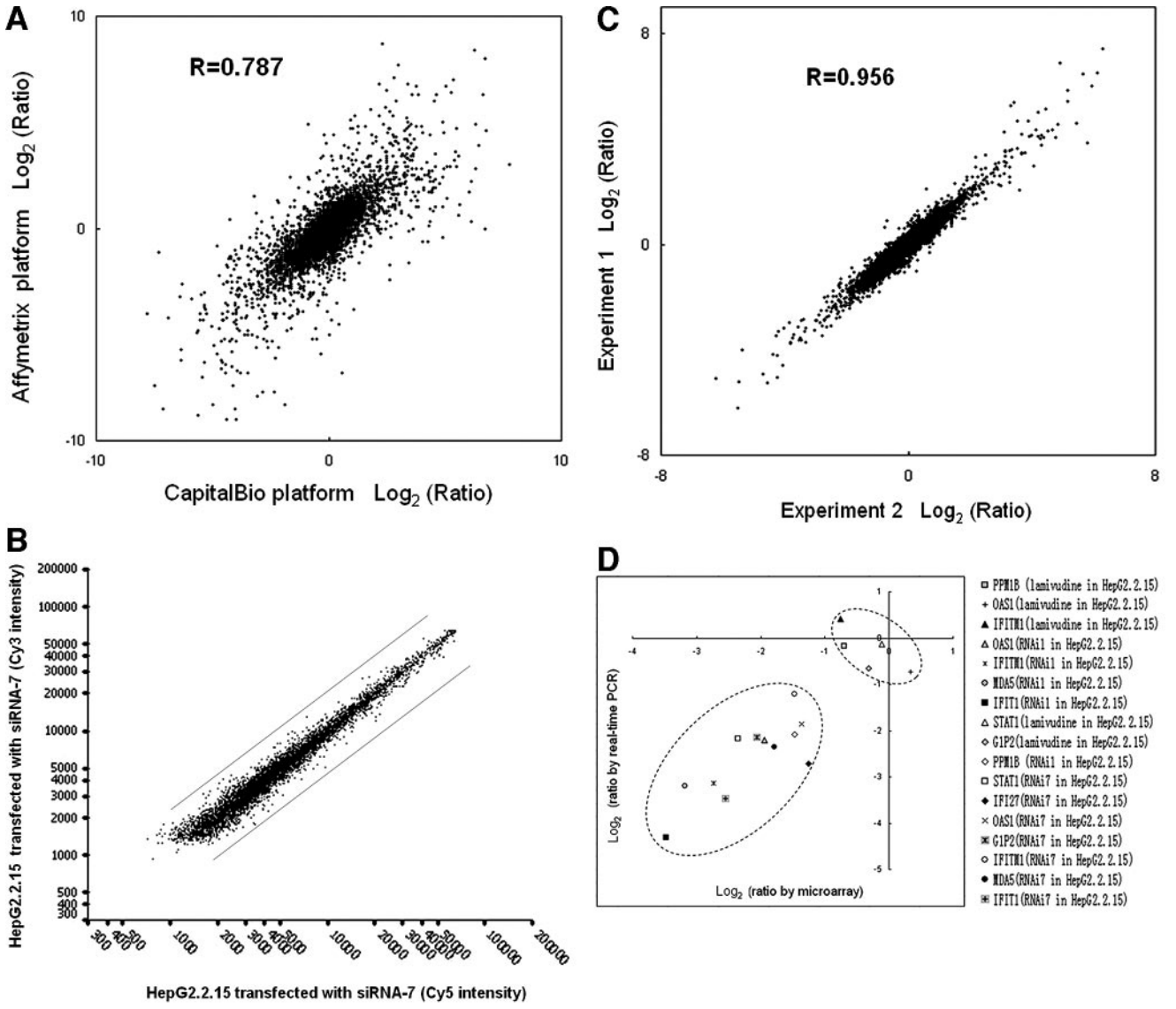

FIG. 7. The fidelity and reproducibility of microarray profiling. (A) The CapitalBio's long oligonucleotide (Operon 70-mer) microarray and Affymetrix's short oligonucleotide (25-mer) HG U133A 2.0 microarray platform were compared, and the correlation coefficient (R value) between the two platforms was 0.787 when the total of 6754 common genes were detected using the two platforms, employing the same batch of RNA extracted from HeLa and HEK293 cell lines. (B) Self-to-self comparison of the gene expression. For each test and control sample, two hybridizations were performed by using a reversal fluorescence strategy. The change of magnitude of self-to-self expression profiling was within twofold, which indicated that a change less than twofold could be considered to represent the noise level of the microarray experiment. The two lines parallel in the graph represent 2- and 0.5-fold changes in expression. (C) The determination of global gene expression of HepG2.2.15 versus HepG2 cells was repeated three times independently. For each test and control sample in one independent experiment, two hybridizations were performed by using a reverse fluorescence strategy. Two sets of the expression ratios were chosen to determine the reproducibility of microarray results. The Pearson linear correlation coefficient value (R) was 0.956. (D) Comparison of expression measurement by quantitative RT-PCR and microarray. The expression changes in the selected 17 genes showed good agreement between the two methods.

found that siRNAs were capable of directly reducing the expression of the nontargeted genes (14, 34); Bridge et al. and Sledz et al. found that the transfection of siRNAs in some cases resulted in global up-regulation of IFN-stimulated genes (3, 39); Persengiev et al. found that siRNAs nonspecifically stimulated or repressed genes involved in diverse cellular functions dependent upon the concentration of siRNAs (29). The nonspecific effects of siRNA are of critical importance for drug development, because the nonspecifically changed genes may produce side effects for the application of siRNA in gene therapy, and this may also suggest that it is important to perform global gene expression assessment through microarray and bioinformatics analysis after the effectiveness of siRNA against the target genes is established.

The gene expression profile of HepG2.2.15 cells treated by lamivudine is totally different from that seen with siRNA. This may be explained by the different anti-HBV effects of siRNA and lamivudine. Both siRNA-1 and siRNA-7 were confirmed

effective in attenuating HBV activity at the mRNA, protein, and DNA level in HepG2.2.15 cells. Lamivudine can significantly attenuate HBV replication, whereas it has little effect on HBV mRNA and HBsAg production in HepG2.2.15 cells. The various levels of HBV protein after siRNA or lamivudine treatment may be responsible for the gene expression of host cells. As for the different anti-HBV effects of siRNA and lamivudine, it is highly reasonable to conclude that it is explained by the different mechanisms of siRNA and lamivudine in HepG2.2.15 cells. Lamivudine selectively targets the viral polymerase and can only decrease the load of HBV core-related DNA. siRNA selectively targets the viral RNA and can decrease the load of HBV mRNA, protein, and DNA. Because of this, the anti-HBV activities of siRNA and lamivudine are different. This difference in anti-HBV activities may significantly affect the treatment of chronic hepatitis patients. For chronic patients, the immune response continues to destroy cells expressing HBV antigens, so the decrease of HBV protein will increase the surviving of liver cells. This will benefit the restoration and maintenance of liver function. In theory, the RNAi strategy may have an advantage over the use of lamivudine for the alleviation of liver inflammation in chronic patients. The global gene expression after the lamivudine treatment also indicates that the decrease of HBV DNA alone without HBV protein cannot alleviate the immune response intracellularly. In fact, further testing with animal models would provide more information about the effectiveness of the siRNA to alleviate the liver inflammation.

In summary, we have systematically evaluated the effects of vector-based hairpin siRNA and lamivudine on HBV expression and replication in HepG2.2.15 cells. Our genomic microarray analyses identified two anti-HBV siRNAs generating divergent gene expression results. The effects of siRNA and lamivudine on anti-HBV activity and the host cell responsiveness were different. The microarray data also suggested that seven genes may be related to HBV infection. These results should provide new insights into the molecular mechanisms for anti-HBV effects of the siRNAs and the HBV-host cell interaction.

ACKNOWLEDGMENTS

This work was supported by the grant of the National Hi-Tech Program of China (No. 2002AA2Z2011).

We are grateful to Yang Shi at Harvard Medical School for providing the pBS/U6 vector. We thank Mark Feitelson at Thomas Jefferson University and Duanqing Pei at Tsinghua University for helpful discussions. We thank Yonghong Ren, Zhiguang Li, Xian Wang, Ailiang Chen, and Rui Zhang for excellent technical assistance. We also thank Qiang Zheng for critically reading the manuscript.

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