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siRNA Pool Targeting Different Sites of Human Hepatitis B Surface Antigen Efficiently Inhibits HBV Infection

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Abstract

The main objective was to determine whether a pool of siRNAs targeting different regions of hepatitis B virus surface antigen (HBsAg) efficiently inhibits HBV infection. siRNAs targeting different regions of HBsAg were transfected into HBV-producing HepG2.2.15 cells and at 72h post transfection, the culture medium was collected for ELISA to determine HBsAg, while total RNA was isolated from the cells for real-time PCR. Three siRNA sequences which efficiently inhibited HBV infection were converted into small hairpin RNAs (shRNAs) and then cloned into a single plasmid psiSTRIKE™ driven by a single U6 promoter. These shRNA expressing plasmids were tested for HBsAg gene silencing in HepG2.2.15 cells. A pool of siRNAs targeting HBsAg efficiently inhibited HBV replication and antigen expression when transfected into HepG2.2.15 cells, compared to the use of single siRNA. Similarly, the plasmid encoding three different shRNAs driven by a single U6 promoter was more effective in silencing HBsAg at DNA, mRNA and protein levels compared to the plasmid encoding single shRNA. No apoptotic change was observed in the cells when the plasmid was transfected at a dose of 0.5-2μg/1×10^6cells after complex formation with Lipofectamine LTX™. Furthermore, transfection with siRNA or shRNA did not increase IFN-γ release, suggesting no induction of interferon response. In conclusion, a pool of chemically synthesized siRNAs as well as the shRNA expression plasmid encoding multiple shRNAs targeting different regions of HBsAg showed high gene silencing in HepG2.2.15 cells.

Keywords

Hepatitis B virus; siRNA; shRNA; HBV replication; HBsAg expression

Introduction

Infection with hepatitis B virus (HBV) is a global health problem, with over 350 million HBV carriers worldwide. Chronic infection by HBV may lead to the development of liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] Although patients with chronic HBV infection have been treated with interferons and nucleoside analogs, available therapies are only partially effective against this viral infection. The low efficacy and undesirable side-effects of interferons and the occurrence of nucleoside analogs resistant HBV mutations remain major obstacles to anti-HBV therapy.[4-6] Liver injury induced by HBV infection is not due to virus replication or cytopathic effects on the infected cells, but due to the immune response against cells expressed viral antigens, especially hepatitis B virus surface antigen (HBsAg).[7] In

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addition, differences in the extent of HBV infection are determined by variations in the immune responses and expression of the HBV antigens in the liver tissue, which reflects the replicative status and the disease activity of chronic hepatitis B.

RNA interference (RNAi) is an evolutionarily conserved process by which double-stranded small interfering RNA (siRNA) induces sequence-specific, post-transcriptional gene silencing of homologous genes in many eukaryotes.[8-12] RNAi usually involves processing of precursor double-stranded RNA (dsRNA) by Dicer to form siRNA duplexes of 19-23 base pairs.[13] The siRNA is then incorporated into the RNA-induced silencing complex (RISC), where one of the strands is selected and act as a guide to target degradation of complementary cytoplasmic RNA.

RNAi has shown promise in inhibiting chronic HBV infection. Many researchers have demonstrated that specific and efficient siRNA can achieve silencing of HBV genes,[14,15] which have prompted the development of RNA-based antiviral agents. As substrates for Dicer, siRNA pool targeting different regions of a target genome has been shown to be more effective in gene silencing than a single siRNA duplex.[16] Therefore, the use of a single plasmid encoding multiple shRNA to target a range of sequences found in different target regions or viral genotypes will be of great interest. In the present study, we screened chemically synthesized siRNAs targeting different regions of HBsAg and then selected three potent siRNAs for converting into small hairpin RNAs (shRNAs) to clone into a plasmid vector driven by a single U6 promoter. We then determined silencing efficiency of different shRNA expression plasmids at DNA, mRNA and protein levels. We demonstrate that plasmid encoding three different shRNAs against HBsAg is more effective than the plasmid encoding single shRNA, with little induction of interferon response.

Materials and Methods

Materials

psiSTRIKE™ vector kit was purchased from Promega corporation (Madison, WI). Antibiotic G418 was purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) (fraction V, purity > 98%) was purchased from USB corporation (Cleveland, OH). Dulbecco's modified eagle's medium (DMEM), penicillin G (5000Unit/ml), Trypsin-EDTA, Trizol, DNase I, Lipofectamine LTX™, Lipofectamine 2000, PureLink™ Viral RNA/DNA Mini Kit, Monoclonal mouse anti-HBsAg and Alexa Fluor 488 goat anti-mouse IgG were purchased from Invitrogen Life Technologies (Carlsbad, CA). Restriction enzymes (PstI, EcoRI, ClaI, BsrGI, SgfI and BglII) were purchased from New England Biolabs (Ipswich, MA). SYBR Green-1 dye universal master mix and Multiscript reverse transcriptase were purchased from Applied Biosystems Inc. (Foster, CA). HBsAg ELISA kits were from Abazyme Company (Needham, MA). siRNAs and primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

siRNA Design and Synthesis

siRNAs targeting different regions of human hepatitis B virus surface antigen (HBsAg) (Gene Bank Accession# NM_U95551) were designed and their sequences are listed in Table 1. These siRNAs are of 19-21nt and 2-nt deoxynucleotide overhangs at the 3′-end and target conserved S region of the ayw HBV genome. These siRNAs were designed according to the guide provided by Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html) and Invitrogen (https://rnaidesigner.invitrogen.com/rnaiexpress/design.do). The sequence specificity of all siRNAs was checked by BLAST search (www.ncbi.nlm.nih.gov). All siRNAs were synthesized by Integrated DNA Technologies, Inc (Coralville, IA).

Design and Construction of shRNA Expression Plasmids

Following screening of different siRNA sequences, three effective sequences and control were chosen for converting into shRNAs against different target regions of HBsAg (Table 2A). To express shRNA of anti-HBsAg, pHBV-S1, pHBV-S2, pHBV-S4 and control vectors were constructed using psiSTRIKE™, which is a linearized plasmid and contains a U6 RNA polymerase promoter.[17] These shRNAs contain 2 complementary oligonucleotides that were annealed to form double-stranded DNA for ligation into psiSTRIKE™ vector corresponding sites under U6 promoter using T4 DNA ligase for 2h at 25°C. Following transformation in Top10 supercompetent cells and amplification in terrific broth media, plasmids were purified using QIAGEN® Plasmid Mini Kit (QIAGEN, Valencia, CA). To construct pool shRNA expression plasmids pHBV-2S and pHBV-3S, a linker (sense strand: 5′-ACC GGA ATT CCG GAT ATC GAT GTA CAG CGG CCG CGA TCG CGA C-3, antisense strand: 5′-TGC AGT CGC GAT CGC GGC CGC TGT ACA TCG ATA TCC GGA ATT C-3′**)** containing multiple restriction enzyme sites was synthesized and inserted into psiSTRIKE™ plasmid, named as pEsiST vector. We used EcoRI and ClaI to digest pEsiST containing multiple restriction enzyme sites, and the sense **(**5′-AAT TCG TGG TGG ACT TCT CTC AAT CTT CCT GTC AAT TGA GAG AAG TCC ACC ACAT-3′) and antisense (5′-CGAT GT GGT GGA CTT CTC TCA ATT GAC AGG AAG ATT GAG AGA AGT CCA CCA CG-3′) strand of shRNA-S1′ were annealed and cloned into EcoRI- ClaI sites of the pEsiST vector, named as pshRNA-S1′ plasmid. We used SgfI and BglII to digest pshRNA-S1′ plasmid, and the sense (5′-CGC GGT ATG TTG CCC GTT TGT CCT TCC TGT CAG ACA AAC GGG CAA CAT ACC **TTTTT** A-3′) and antisense (5′-GATCT AAA AAG GTA TGT TGC CCG TTT GTC TGA CAG GAA GGA CAA ACG GGC AAC ATA CC GCGAT-3′) strand of shRNA-S4′ were annealed and cloned into SgfI-BglII sites of the pshRNA-S1′ plasmid, named as pHBV-2S plasmid, and the end of shRNA-S4′ duplex contains a TTTTT stretch to create the pol III termination signal. Then we used BsrGI and SgfI to digest pHBV-2S plasmid, and the sense (5′-GTACA AAC CTC CAA TCA CTC ACC AAC CTT CCT GTC AGT TGG TGA GTG ATT GGA GGT T GCGAT-3′) and antisense (5′-CGC AAC CTC CAA TCA CTC ACC AAC TGA CAG GAA GGT TGG TGA GTG ATT GGA GGT TT-3′) strand of shRNA-S2′ were annealed and cloned into BsrGI-SgfI sites of the pHBV-2S plasmid, named as pHBV-3S vector plasmid. So two or three shRNA duplexes (Table 2 B) were inserted into the psiSTRIKE™ Vector with a linker to create pHBV-2S and pHBV-3S vector plasmids, respectively (Fig 1).

Cell Culture and Transfection

HepG2.2.15 cell line was kindly provided by Dr. Pamela Norton of the Department of Microbiology and Immunology, Drexel University College of Medicine. HepG2.2.15 cell line was cultured in DMEM supplemented with 4% fetal bovine serum and 330μg/ml antibiotic G418 at 37°C in a humidified incubator with 5% CO_2. Twenty four hours before transfection, cells were trypsinized and seeded in 24-well culture plates at a density of 1×10^5 cells per well. The cells were transfected with 40 and 80nmol/L siRNAs after complex formation with Lipofectamine2000 at 1μg/1μg ratio. In case of shRNA expression plasmids, cells were transfected with shRNA expression vectors at doses of 0.5μg, 1μg, 2μg/well after complex formation with Lipofectamine LTX™ according to the manufacturer's protocol. At 72h post-transfection, the cells were harvested and culture supernatant was collected for further analysis.

Measurement of Particle Size and ζ Potential

Plasmid (0.5μg, 1μg and 2μg) and Lipofectamine LTX™ (1, 2 and 4μl) were diluted separately to a volume of 250μl with a final glucose concentration of 5%w/v, and then the DNA was added to the Lipofectamine LTX™ with mild mixing by pipetting. Complex formation was allowed to proceed for 45 min at room temperature. Lipid/plasmid complexes of different charge ratios were prepared by adjusting the stoichiometry of plasmid and Lipofectamine

LTX™. The particle size and ζ potential of Lipofectamine LTX™/plasmid complexes were determined by dynamic light scattering at 25°C using a Malvern Zetasizer (Southborough, MA) as described by Mahato et al.[18]

Quantification of HBsAg mRNA and Culture Medium HBV DNA by Real-time PCR

HBV virion DNA in culture medium and intracellular HBsAg mRNA levels were determined by real time PCR and real time RT-PCR, respectively after transfection of HepG2.2.15 cells with siRNA or shRNA expression plasmids. Following transfection, the culture medium was collected and total RNA was extracted from the cell pellet with Trizol, and RNA concentration was measured by UV spectrophotometry using Biomate 3 spectrophotometer (ThermoSpectrome, Waltham, MA). Then, 200ng of DNase I-treated total RNA was reverse-transcribed to cDNA by Multiscript reverse transcriptase in a 10μl reaction system containing oligo (dT) final concentration 2.5μM for 10min at 25°C, 30min at 48°C, and 5min at 95°C. Reverse-transcribed cDNA was quantified by real-time PCR using SYBR Green-1 dye universal master mix and HBV primers specific for the S gene: sense strand : 5′-GAT TCC TAG GAC CCC TTC TC-3′, antisense strand : 5′-GGA GGA CAG GAG GTT GGT GA-3′. Because of its invariant expression across tissues and treatments, 18S ribosomal RNA was used as an internal control for quantitative RNA analysis: forward: 5′-CGG CTA CCA CAT CCA AGG AA-3′, reverse: 5′-GCT GGA ATT ACC GCG GCT-3′. Amplification and detection were performed with an ABI 7700 sequence detection system. The real-time PCR conditions were as follows: 50°C, 2min; 95°C, 10min; 95°C, 15s, 54°C, 1min, ×40 cycles;72°C, 5min. Three independent experiments were performed and the amount of reverse transcribed cDNA in treated cultures was expressed as a percentage of the mock-treated controls. Results were analyzed with SDS 2.0 software from Applied Biosystems.

HBV DNA was extracted from 200μl culture medium using a PureLink™ Viral RNA/DNA Mini Kit (Invitrogen Life Technologies Carlsbad, CA). Purified HBV DNA was quantified by real-time PCR using the primers and conditions described above.

Immunofluorescence and Confocal Microscopy

At 72h after transfection of HepG2.2.15 cells with shRNA expression plasmids, the cells were fixed with 3% paraformaldyhyde for 15min, washed three times with Dulbecco's Phosphate Buffered Saline (DPBS), permeabilized with 0.1% Brij98/PBS for 2min, washed two times with DPBS, blocked with 20% goat serum for 30min. Cells were sequentially incubated with monoclonal mouse anti-HBsAg primary antibody and Cy3-goat anti-mouse IgG conjugated secondary antibody at room temperature for 1h, respectively, and mounted on the fixed cell slip. The cells were observed with Bio-Rad 1024 confocal microscope (Bio-Rad, Hercules, CA) at 488nm excitation wavelength.

Results

Effect of siRNA Sequences on Gene Silencing

To examine the ability of siRNA to silence HBV gene expression, we selected HBsAg as a target gene. We compared four different 19-21bp siRNA duplexes targeted to different regions of the HBsAg open reading frame along the HBV genome (Table 1). These siRNA duplexes were transfected into HepG2.2.15 cells either alone or as a pool of all four siRNAs after complex formation with Lipofectamine2000. As shown in Figure 2, HBsAg gene silencing by siRNAs was sequence-specific and dose-dependent. Three siRNAs had caused a significant decrease in HBsAg mRNA levels, while the control siRNA had almost no effect on HBsAg gene expression. The level of HBsAg mRNA was reduced by 40.9, 13.41, 17.4 and 40.16% for siRNA-S1, siRNA-S2, siRNA-S3 and siRNA-S4 treated samples, respectively when transfected at the dose of 40nmol/L. There was significant increase in HBsAg gene silencing

as we increased the siRNA dose to 80nmol/L, being 71.77, 58.79, 54.49 and 84.69% for siRNA-S1, siRNA-S2, siRNA-S3 and siRNA-S4, treated samples, respectively (Fig 2A).

We also determined the level of HBsAg secreted into the culture medium at 3 days post-transfection by ELISA. The levels of HBsAg into the medium were reduced by 39.67, 31.79, 30.55 and 42.29% for siRNA-S1, siRNA-S2, siRNA-S3 and siRNA-S4 treated samples, respectively when transfected at the dose of 40nmol/L. There was significant increase in HBsAg gene silencing, leading significant decrease in HBsAg concentration in the culture medium as we increased siRNA dose to 80nmol/L: 58.72, 55.03, 46.01 and 61.71% for siRNA-S1, siRNA-S2, siRNA-S3 and siRNA-S4 treated samples, respectively (Fig 2B). Among these four siRNA duplexes, siRNA-S4 showed the highest gene silencing ability at both mRNA and protein levels: 84.69% and 61.71% inhibition of HBsAg at mRNA and protein levels when HepG2.2.15 cells were transfected at the dose of 80nmol/L. Other siRNAs resulted in varying degrees of inhibition for HBsAg expression. The control siRNA had no effect on HBsAg levels in the media of the treated cells, indicating that inhibition by the HBsAg targeted siRNAs was sequence specific and due to RNAi. Combination of four siRNAs was more effective in the inhibition of HBsAg mRNA and antigen expression as compared with the use of any one while the final concentration of siRNAs in those treatments were the same.

Construction of shRNA Expression Vectors

Following screening of different siRNA sequences for HBsAg gene silencing, we selected the three most potent siRNA sequences for converting into shRNA sequences, which contain unique restriction sites at the 5′ and 3′ ends for cloning and a **TTTTT** stretch in the sense to create the pol III terminal signal (Table 2). Then, these shRNA sequences were cloned into psiSTRIKE™ as illustrated in Figure 1. pHBV-S1, pHBV-S2, pHBV-S4, were identified using restriction enzyme digestion assay which yielded two DNA fragments of about 3047bp and 1370bp (results not shown). The pHBV-2S and pHBV-3S vectors were identified by digestion with EcoRI and Bgl II enzymes. The length of products of pHBV-2S and pHBV-3S were 118bp and 163bp, respectively (results not shown). The DNA sequencing result showed the inserted fragment had expected known sequences.

Effect of shRNA expression on HBsAg Secretion

To evaluate the influence of siRNA pool on HBV life cycle, we used psiSTRIKE™ vector to direct the synthesis of multiple siRNAs in HepG2.2.15 cell line. Following cloning, amplification and purification of pHBV-S1, pHBV-S2, pHBV-S4, pHBV-2S and pHBV-3S, we formed lipid/plasmid complexes by mixing equal volumes of Lipofectamine and shRNA expression plasmid, and determined the particle size and ζ potential by a dynamic light scattering technique. These lipid/plasmid complexes had mean particle size of 200~400nm and ζ potential of 20~30 mV. We then determined the effect of shRNA expression plasmids on HBsAg gene silencing after transfection of Lipofectamine/pHBV complexes into HepG2.2.15 cells at doses of 0.5, 1 and 2μg/well. Variable efficacy of HBsAg gene silencing was achieved by each of the sequences (Fig 3). Compared to the control shRNA plasmid transfected group, transfection of HepG2.2.15 cells with the plasmids encoding shRNAs against HBsAg significantly inhibited HBsAg concentrations in the culture medium (Fig 3A). The plasmid encoding three shRNAs (pHBV-3S) decreased HBsAg concentration in the culture supernatant to less than 15% of the control shRNA treated cell level. Compared to the plasmids encoding single shRNA, the plasmids encoding three shRNAs targeting different regions of HBsAg genome were more effective in HBsAg gene silencing.

We also extracted total cellular RNA from transfected HepG2.2.15 cells and subjected to real time RT-PCR. The results confirmed that the plasmid encoding three shRNAs (pHBV-3S)

efficiently reduced HBsAg mRNA concentration by 63.2-87.7% of the control at different concentrations of plasmid when normalized using 18S RNA (Fig 3B).

We also determined HBV virion DNA after extraction from the culture medium by real time PCR. As shown in Fig 3C, the levels of HBV virion DNA significantly decreased when HepG2.2.15 cells were transfected with the plasmid encoding shRNA against HBsAg. There was almost 85% inhibition when the plasmid encoding 3 shRNAs (pHBV-3S) was used for transfection.

Confocal Immunofluorescent Analysis of HBsAg Expression

To further substantiate our observations, HepG2.2.15 cells were transfected with shRNA expression plasmids. At 72h after transfection, cells were immunostained for HBsAg and analyzed by confocal microscopy. The photographs shown in Figure 4 suggest that the intensity of HBsAg staining was the strongest for non-transfected HepG2.2.15 cells, which releases hepatitis B viral particles. Following transfection with shRNA against HBsAg, there was decrease in the intensity of HBsAg staining in the following order: HBV-S1> HBV-S2> HBV-S4> HBV-2S > HBV-3S. The results suggest that HBsAg levels in cells transfected with shRNA plasmids were reduced to different extent compared to the control plasmid. The cells transfected with pHBV-2S and pHBV-3S plasmids showed little fluorescence, suggesting little HBsAg expression.

IFN Response of shRNA Induction

To determine whether shRNA induces interferon (IFN) response pathway, at 72h post-transfection of HepG2.2.15 cells with shRNA expression plasmids, IFN-γ release in the culture supernatants was determined by ELISA. Transfection with shRNA expression plasmids did not increase IFN-γ concentration and IFN-γ levels for all the samples were between 5-6 pg/ml, suggesting no induction of interferon response (data not shown).

Discussion

RNAi is a sequence-specific, post-transcriptional gene silencing mechanism, which is triggered by double-stranded synthetic siRNA or short hairpin RNA (shRNA) expressed intracellularly from a vector.[19,20] Recently, several groups have reported that HBV replication and expression could be inhibited in human hepatoma cell culture and mouse models after direct application of either synthetic siRNAs or endogenously expressed shRNAs.[15,21,22] HBV gene silencing was dependent primarily on siRNA dose and sequences.

The humoral (antibody) response to epitopes on HBsAg that envelops the virus is responsible for protective immunity against HBV infection and clearance of HBV from the body.[23-25] As HBsAg-specific immunity response is poor, it is widely accepted that the HBsAg-specific immunity response supports anti-HBsAg production.[24] Chronic HBV infection is associated with HBsAg expression, and almost all the HBV-associated hepatocellular carcinoma (HCC) harbored chromosomally integrated viral DNA.[26,27] Although HBxAg has been implicated in the process of HCC, the viral DNA-integrated genome is commonly found partial or complete deletion of HBx gene.[28,29] Moreover, the development of HCC is preceded by chronic liver injury and inflammation. Studies on HBsAg transgenic mice revealed that a severe, prolonged hepatocellular injury, regenerative hyperplasia, and a secondary inflammation preceded HCC.[30-32] Therefore, strategies for treating chronic HBV infection should not be restricted to inhibition of virus replication, and suppression of HBsAg expression should also be considered.

The design of effective siRNAs has been a major obstacle in siRNA-mediated gene silencing, as there is great variability among siRNAs, in terms of their effectiveness. There are often more

than one target regions of a given gene, which can be efficacious in gene silencing. To determine whether we can use RNAi technology to treat HBV induced diseases caused by aberrant or persistent expression of HBsAg, we screened four chemically synthesized siRNA duplexes targeting 256, 322, 423 and 458nt start sites of HBsAg (Table 1). As shown in Figure 2, these siRNA duplexes could significantly inhibit HBsAg synthesis to different extents after transfection into HepG2.2.15 cells. Among four siRNAs we tested, siRNA-S4 was found to be the most potent in inhibiting HBsAg expression *in vitro*. Compared with a single siRNA duplex, the highest level of HBsAg gene silencing was achieved when we used a pool of three siRNA duplexes targeting different region of HBsAg (Figure 2). The rest three siRNAs also inhibited HBsAg to different extent.[21,33] The difference in inhibition among various siRNA may be attributed to the change of affinity between cellular siRNA binding protein and the secondary and/or tertiary structures of target mRNA participating in the induction of RNAi activities.[34]

Since we achieved high levels of HBsAg gene silencing when we used a pool of chemically synthesized siRNAs, we postulated that multiple shRNAs cloned into a single U6 promoter would produce a pool of siRNAs after cleavage of dsRNA by Dicer, leading to significant increase in the intracellular siRNA levels. To prove this hypothesis, we constructed plasmid vectors encoding a pool of two or three shRNAs driven by a single U6 promoter. As shown in Figures 3 and 4, very high levels of HBsAg gene silencing were achieved when we used these plasmids, as confirmed by ELISA, real time PCR and immunofluoresence assays (Figures 3 and 4). mRNA molecules in cells may also have a repairing mechanism like DNA while it is cleaved by biological, chemical, or physical forces. shRNA pool can cleave multiple sites in mRNA target region and may achieve greater gene silencing than a single shRNA. Similar results were also reported in the literature using large dsRNA and long shRNA.[33,35] While we and these authors achieved high gene silencing compared to single shRNA, our strategy is somewhat different from theirs. The long shRNA comprises 62 bp with multiple G:U wobble pairings to facilitate cleavage into multiple siRNAs by DICER.[36] With large dsRNA, formation of siRNAs is likely to proceed with decreased precision and may contribute to off-target effects, since large dsRNA usually traverses the endosomal compartments and interacts with Toll-like receptors. In contrast, we cloned multiple shRNAs together by keeping poly(T) at the end (Figure 1). Moreover, our expressed shRNA is unlikely to have the same immunostimulatory properties as synthetic siRNA, since shRNA transcribed within cells does not typically traverse the endosomal compartments.

In the present study, we studied the silencing effect of siRNA pool targeting three different sites of HBV S gene region, but silencing of other gene regions (C, P, X) of HBV genome at the same time has a great potential for inhibiting HBV infection. This was confirmed in a study which showed that transfecting cells with a panel of siRNAs produced ex vivo using recombinant Dicer is a useful means of attaining reliable silencing.[37] Our strategy to produce several siRNAs from a single plasmid encoding multiple shRNAs (Figure 1) is somewhat similar to long shRNA (lhRNAs)[36] and should improve the probability of assembling effective silencing complexes.

Chemically synthesized siRNA duplex may induce unintended programmed cell death due to stimulation of several inflammatory cytokines and nonspecific gene silencing. Therefore, we measured the relative levels of IFN-γ in HepG2.2.15 cells that were transfected with plasmids encoding shRNAs against HBsAg. We did not find any induction of interferon response, as the IFN-γ levels for all the samples were between 5-6 pg/ml (data not shown). This is in the good agreement with the literature where expressed hairpins have been shown to evade activation of the interferon response more efficiently than exogenous synthetic RNA.[38] Weinberg et al. (2007) also reported no evidence of interferon response induction when transfected Huh7 cells with long hairpin RNA targeting HBx together with HBV target plasmid.

It is important to use a combination of several effective shRNAs against HBV S, C, P X genes to achieve a complete inhibition of HBV replication and gene expression. Further investigation is warranted to advance towards this goal.

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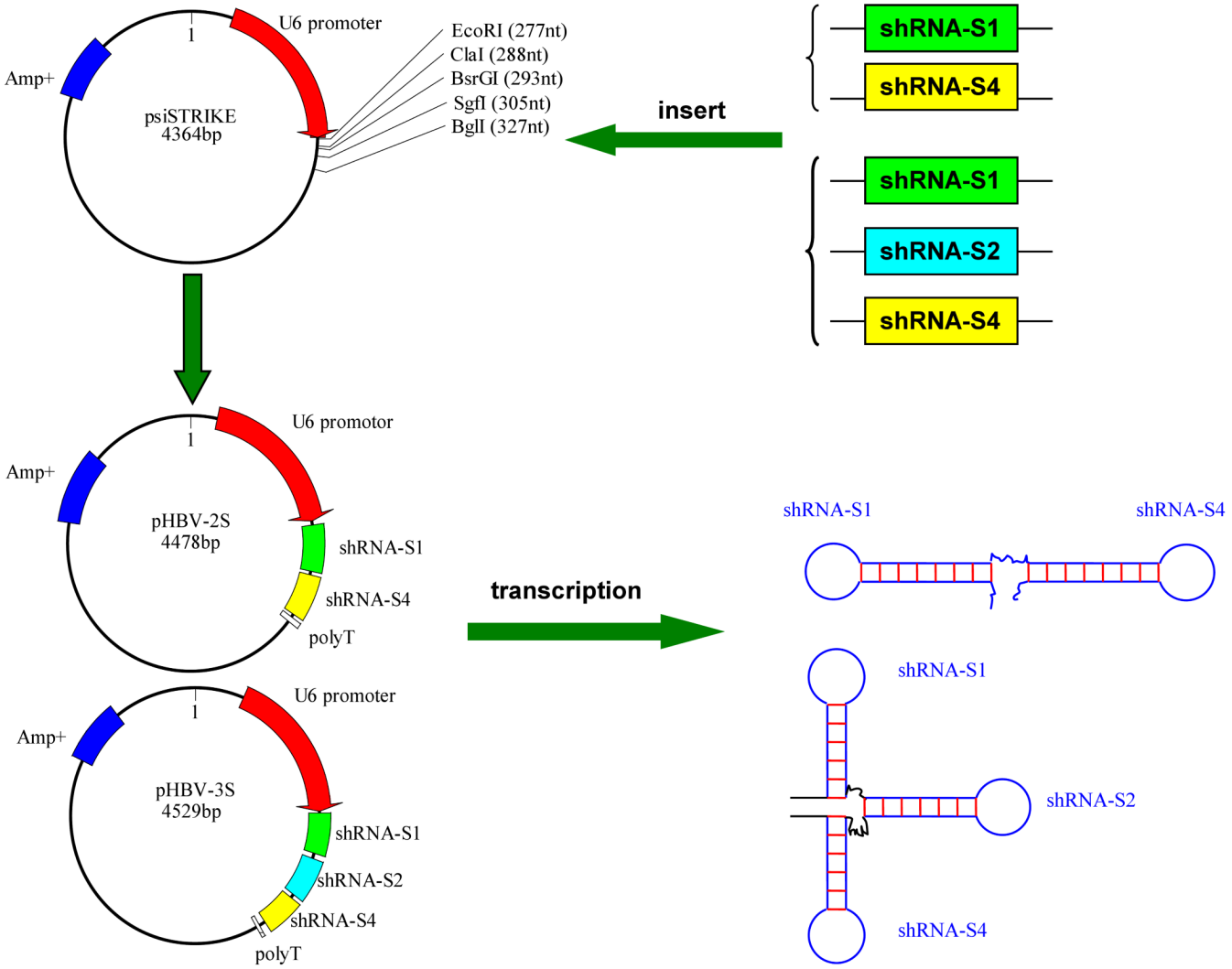

Figure 1.

Construction of a shRNA expression vector encoding multiple siRNAs driven by a single U6 promoter. Three oligonucleotides containing a hairpin sequence, terminator sequence, and overhanging sequence as restriction sites were annealed and cloned into a psiSTRIKE™ vector.

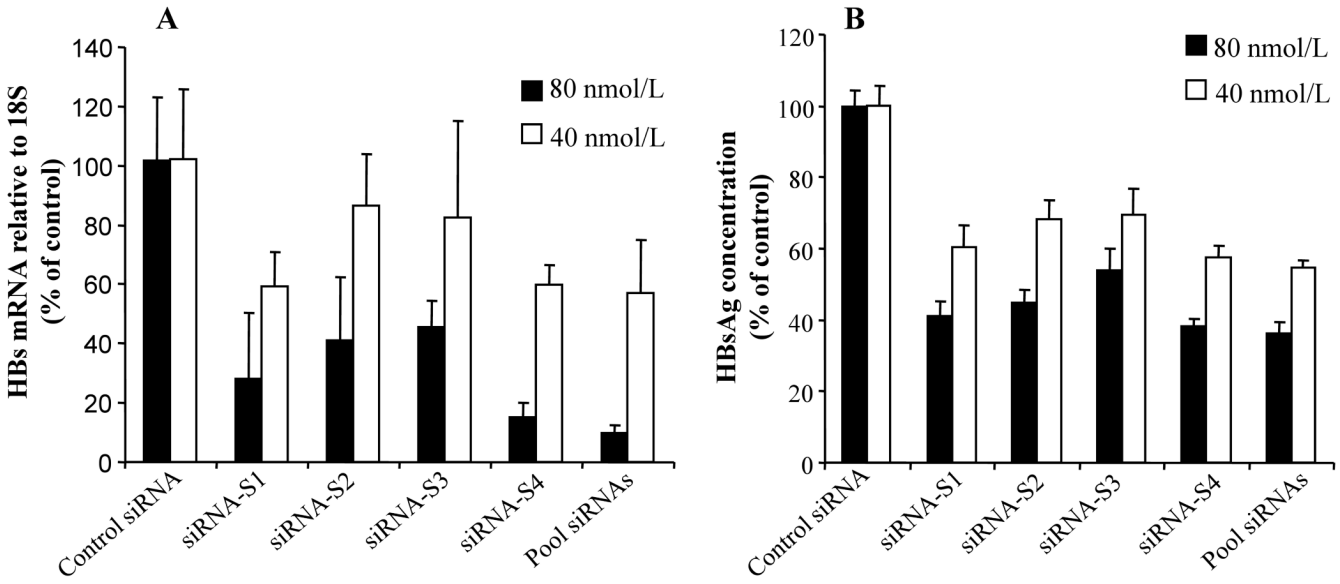

Figure 2.

Inhibition of HBsAg expression after transfection of chemically synthesized siRNAs into HepG2.2.15 cells upon complex formation with Lipofectamine2000. A) mRNA level and B) protein level. The amount at HBsAg mRNA and HBsAg protein levels were determined by real time RT-PCR and ELISA, respectively at 72h post-transfection.

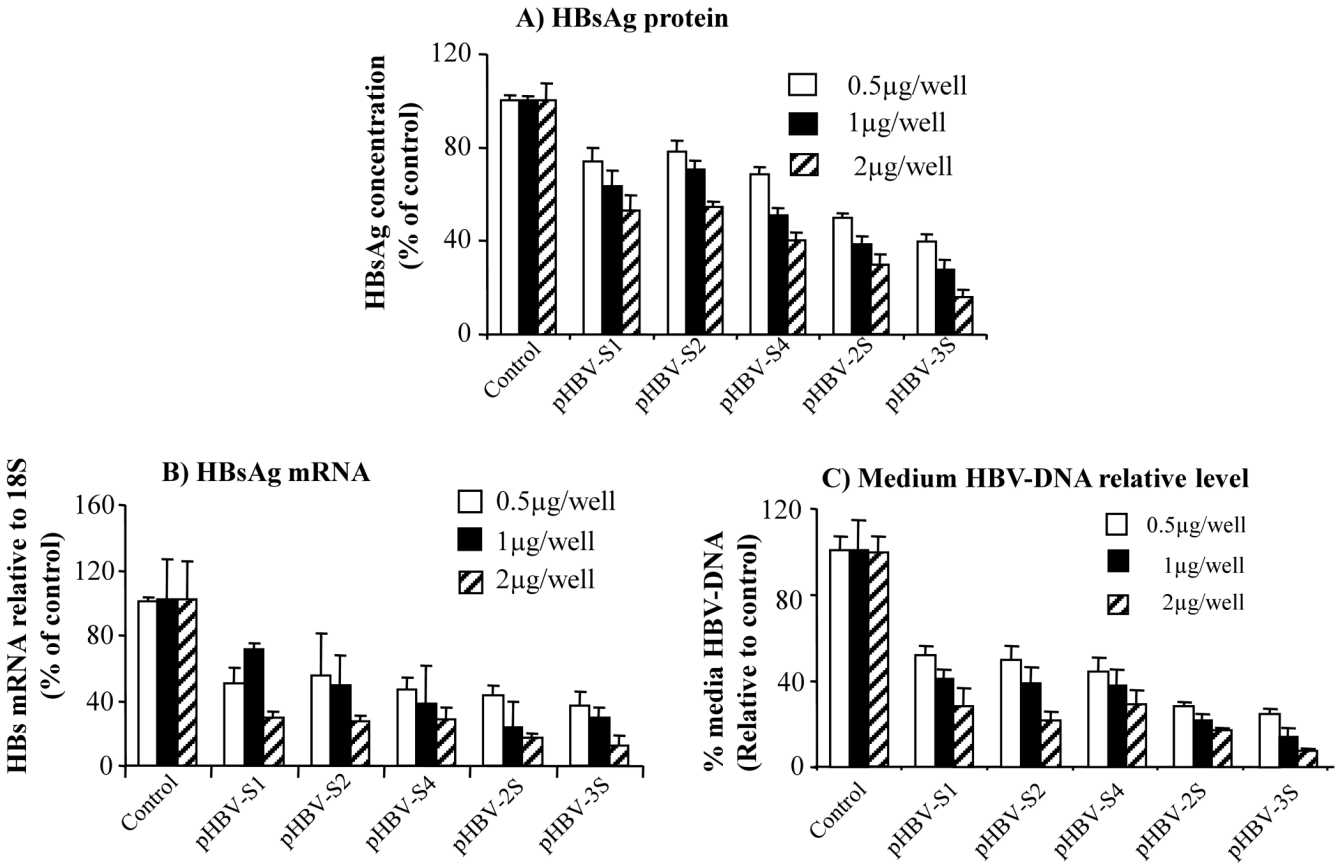

Figure 3.

Transfection of shRNA expression plasmids into HepG2.2.15 cells. Effect of pHBV-S1, pHBV-S2, pHBV-S4, pHBV-2S and pHBV-3S plasmids on HBsAg gene silencing was determined at A) protein, B) RNA and C) DNA levels by ELISA, real time RT-PCR and real time PCR, respectively at 72h post-transfection.

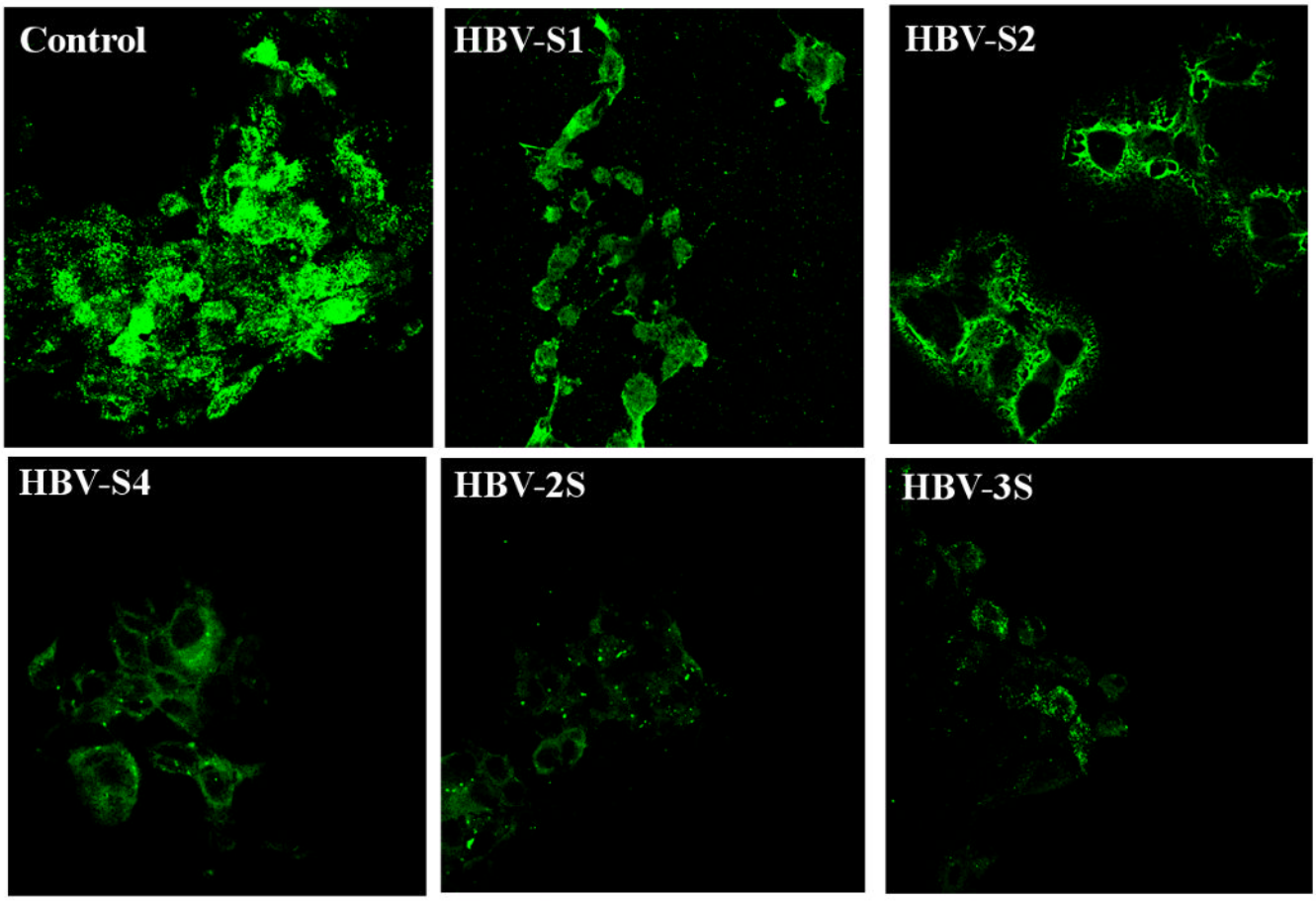

Figure 4.
RNAi effects of plasmid encoding shRNA against HBsAg in HepG2.2.15 cells. The cells were seeded on cover slips in 24-well plates and transfected with 1μg/well of shRNA expression plasmids. At 72h post-transfection, cells were fixed, immunostained for HBsAg protein, and visualized under a confocal microscope. These results show that HBsAg expression levels reduced to different extent in treated cells compared to the control plasmid treated samples.