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Inhibition of hepatitis B virus X gene expression by 10-23 DNAzymes

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Abstract

The X protein (HBx) of human hepatitis B virus (HBV) is a transcriptional activator protein. The HBx protein plays an important role in viral replication in HBV infected cells and the liver diseases including hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Therefore, the repression of HBx gene expression by 10-23 DNAzymes might be a good way to inhibit HBV replication and counteract HBV-related liver diseases. We designed three 10-23 DNAzymes with different substrate-recognition domains. When each of the 10-23 DNAzymes were cotransfected into human AD293 cells with HBx-EGFP expression plasmid, they could all reduce the level of HBx mRNA as well as the HBx-EGFP protein. These results suggest that the 10-23 DNAzymes might be used for gene therapy of liver diseases caused by HBV.

Keywords: 10-23 DNAzyme; HBV; HBx; Inhibition; Gene expression

1. Introduction

Hepatitis B virus (HBV) is an infectious agent carrying a major health problem. There are approximately 350 million carriers of HBV worldwide and chronic infection is associated with diverse liver diseases including chronic hepatitis, cirrhosis and primary HCC (Ganem and Prince, 2004; Wands, 2004). HBV is a small DNA virus which belongs to *hepadnaviridae*. HBV has a partially double stranded DNA genome of 3.2 kb and replicates its DNA genome by reverse transcription of pregenomic RNA. HBV genome contains four open reading frames (ORF) encoding the viral surface protein (S), core protein (C), polymerase (P) and X protein (Lok, 2005). The 17 kd X protein, also termed HBx, consists of 154 amino acid residues and is conserved among mammalian hepadnaviruses. HBx stimulates not only all the HBV promoters but also several other viral and cellular promoters. The oncogenic potential of HBx is suggested by the observation of HCCs in HBx transgenic mice and the oncogenic transformation of cells expressing HBx in culture. The necessity for X gene expression during the viral life cycle in vivo has been reported (Chen et al., 1993; Zoulim et al., 1994). Therefore, the specific repression of HBx gene expression may be a good strategy to block the progression of liver diseases caused by HBV infection.

RNA cleaving DNAzymes are valuable tools with potential applications in functional genomics and gene therapy. The most prominent representative of this class of catalytically active nucleic acids is the 10-23 DNAzyme, which was obtained by in vitro selection from a combinatorial library (Santoro and Joyce, 1997). This molecule is comprised of a catalytic domain of 15 deoxynucleotides, flanked by two substrate-recognition domains. According to the specific binding between recognition domains and RNA substrate, the selected target RNA is cleaved at specific phosphodiester bond, which is located between an unpaired purine and a paired pyrimidine residue (Santoro and Joyce, 1997, 1998; Joyce, 2001). Different target RNA molecules could be cleaved by changing the deoxynucleotides sequences of recognition domains, which could represent a new approach to inhibit the replication of RNA viruses and to treat infectious diseases caused by RNA viruses (Santoro and Joyce, 1997; Goila and Banerjea, 1998; Zhang et al., 1999; Basu et al., 2000; Sriram and Banerjea, 2000; Unwalla and Banerjea, 2001; Chakraborti and Banerjea, 2003; Dash and Banerjea, 2004; Hou et al., 2005; Schubert et al., 2004; Takahashi et al., 2004; Wo et al., 2005).

HBV is an attractive target for the 10-23 DNAzymes, because the HBV replicative cycle starts from pregenomic RNA. In addi-

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tion, the HBV genome consists of overlapping genes. Thus, anti-HBV 10-23 DNAzymes can cleave multiple viral mRNAs and viral pregenomic RNA at the same time. Here, we designed the 10-23 DNAzymes to cleave target sites at nucleotides AUG in HBx ORF. The activities of 10-23 DNAzymes were analyzed in human AD293 cells.

2. Materials and methods

2.1. Design and synthesis of 10-23 DNAzymes

10-23 DNAzymes named DrzHBVX-7, DrzHBVX-8 and DrzHBVX-9 specific to HBV X gene (gi:2182117) ORF A^{1376}UG by Watson–Crick base pairing were designed and synthesized respectively based on previous reports (Santoro and Joyce, 1997). The 10-23 DNAzyme motif was the 23rd clone from round 10 during the course of 10 rounds of in vitro selection of a general-purpose RNA-cleaving DNA enzyme, accordingly termed as 10-23. The sequence GGCTAGCTACAACGA was a specifically catalytic domain of 10-23 DNAzyme molecule, which was flanked by two substrate-recognition domains of seven to nine deoxynucleotides, respectively. All the DNAzymes were synthesized by Shanghai Sangon Biological Company, China (Fig. 1).

2.2. Construction of HBx-EGFP fusion protein expression plasmid

The HBV X gene encompassing nucleotides 1376–1837 (478 bp) (gi:2182117) was amplified using primers with engineered restriction sites to facilitate the fusion of HBx 'in-frame' to the N′ terminus of EGFP in the pEGFP-N1 vector (Clontech, Mountain View, CA) to construct fusion protein expression plasmid termed pHBx-EGFP, as shown in Fig. 2A. Sense primer (5′-CCCAAGCTTATGGCTGCT-AGGCTGTGCTG-3′) containing *Hin*dIII restriction site and antisense primer (5′-CGGAATTCCGGCAGAGGTG-AAAAAGTTGCA-3′) containing *Eco*RI restriction site were synthesized by Shanghai Sangon Biological Company, China.

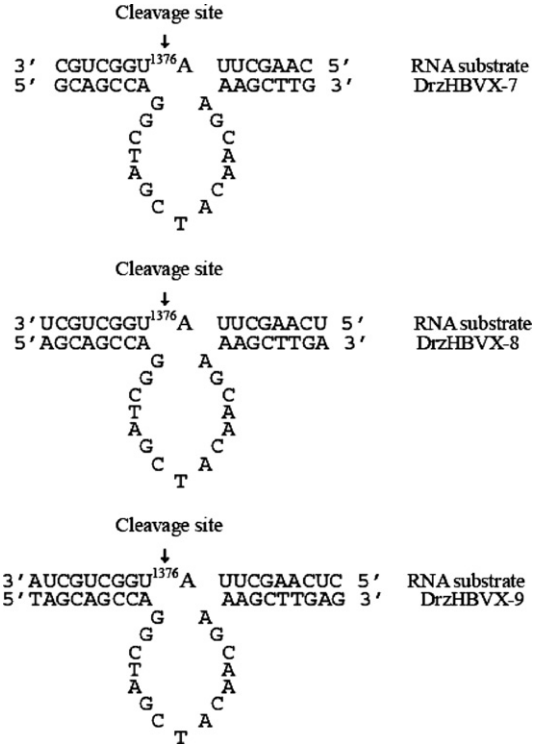

Fig. 1. Sequence of 10-23 DNAzymes and RNA substrates (arrow indicating cleavage site).

The HBx PCR product and pEGFP-N1 DNA were digested with *Hin*dIII and *Eco*RI (Promega, Madison, WI). Products were gel-purified and DNA ligations performed using T4 DNA ligase (Invitrogen, Carlsbad, CA). *E. coli* DH5α was transformed using kanamycin antibiotic selection (50 ng/ml) and bacterial cultures were screened for recombinant clones by restriction enzyme analysis and confirmed by sequencing.

2.3. Cell culture

Human AD293 cells were originally obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 100 ml/l fetal bovine serum (FCS) at 37 °C in a humidified atmosphere containing 50 ml/l CO_2. AD293 cells were seeded into 6-well plates at a density of 5×10^5 cells 24 h prior to transfection. AD293 cells at 80% confluence were prepared for transfection.

2.4. HBx-EGFP and DNAzymes cotransfection

Three different 10-23 DNAzymes were cotransfected with pHBx-EGFP containing HBx-EGFP fusion gene into AD293 cells as DNAzyme-treated groups, and pHBx-EGFP with nonsense oligonucleotide (NSON) (5′-GCGAAGCTT-ATGAGCACGAATCCTAAAC-3′) transfected into AD293 cells as control group, respectively. Each group in triplicate was delivered using Lipofectamine 2000 according to the Manufacturer's instructions. Briefly, 2.0 μg of each 10-23 DNAzyme and 2.0 μg pHBx-EGFP were added into 250 μl Opti-MEM I reduced serum medium as DNAzyme-treated group, 2.0 μg nonsense NSON and 2.0 μg pHBx-EGFP added into 250 μl Opti-MEM I reduced serum medium as control group. Ten microlitres of Lipofectamine 2000 was diluted into 250 μl of Opti-MEM I reduced serum medium. DNA solution and Lipofectamine 2000 were mixed gently and incubated for 20 min at room temperature and then applied to the cells. The total volume of plating medium of each well was 2 ml. After 6 h of incubation at 37 in a humidified atmosphere containing 50 ml/l CO_2, the cells were washed twice with phosphate-buffered saline (PBS), and 2 ml of medium supplemented with 10% FBS was added to each plate. About 48 h after transfection, expression of pHBx-EGFP was examined by fluorescent microscopy and flow cytometry analysis, pHBx-EGFP mRNA levels were analyzed by semi-quantitative

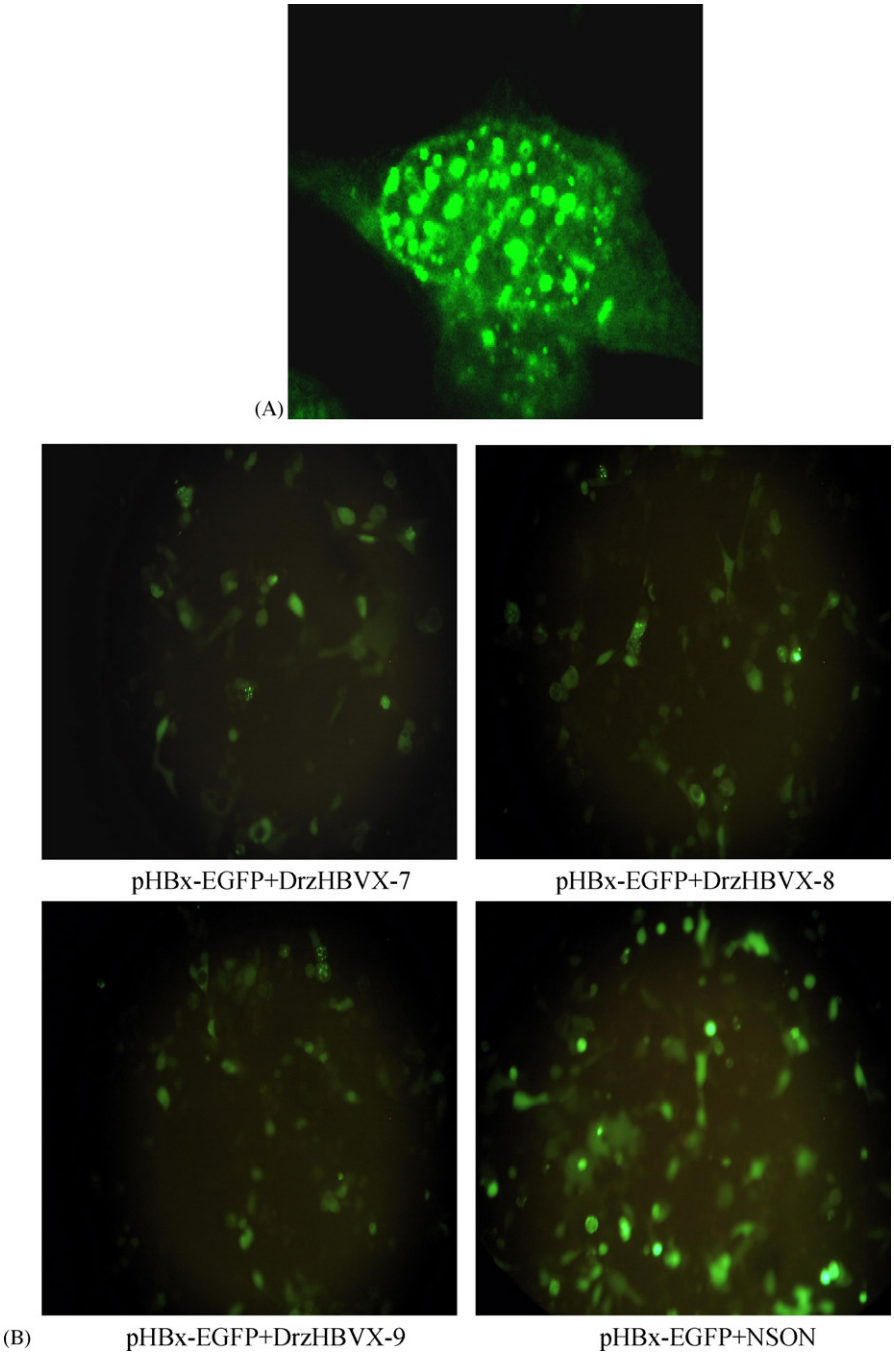

Fig. 2. (A) HBx-EGFP fusion protein was expressed in AD293 cells. (B) Expression of HBx-EGFP in AD293 cells observed by fluorescent microscopy.

RT-PCR (reverse transcription polymerase chain reaction), respectively.

2.5. Expression of EGFP observed by fluorescent microscopy

At 48 h after transfection, the cells were washed twice with PBS. Fluorescent images were captured by a DM-IRB inverted fluorescent microscope (Leica, Germany) with the use of a 10× objective. Randomly chosen fields were photographed using the same exposure time.

2.6. Expression of EGFP analyzed by flow cytometry

For flow cytometry analysis of pHBx-EGFP expression, the cells were harvested at 48 h after transfection and digested with 0.25% trypsin, washed with PBS twice, and then resuspended in PBS to measure the fluorescence using a EPICS-XL flow cytometer (Beckman-Coulter, Fullerton, CA) with filters (emission, 507 nm; excitation, 488 nm). Samples (approximately 10^6 cells each) were counted and analyzed with Coulter System II software, using nontransfected AD293 cells as control. The values were calculated as the percentage (α) of the

cell population that exceeded the fluorescence intensity of the control cells and the mean fluorescence intensity (MFI) of this population. The total fluorescence intensity (TFI) was calculated according to the following formula: TFI = 10,000 × MFI × α. Relative fluorescence expression rate of each cotransfection group was analyzed compared with the control group accordingly.

2.7. RNA extraction

Total RNA from the cells was extracted with TRIZOL Reagent (Invitrogen) according to the Manufacturer's instructions. Briefly, lyse cells directly in 6-well plates by adding 500 μl of TRIZOL Reagent to each well, and passing the cell lysate several times through a pipette. Add 200 μl of chloroform per 500 μl of TRIZOL Reagent, 12,000 × *g* for 15 min at 4 °C. Transfer the aqueous phase to a fresh tube, add 500 μl of isopropyl alcohol per tube, 12,000 × *g* for 10 min at 4 °C. Wash the RNA pellet with 75% ethanol, centrifuge at 7500 × *g* for 5 min at 4 °C. Briefly air-dry the RNA pellet, applying 30 μl RNase-free water to dissolve RNA and store at −20 °C for subsequent RT-PCR.

2.8. Semi-quantitative RT-PCR analysis

pHBx-EGFP mRNA levels were analyzed by semi-quantitative RT-PCR using Super-ScriptTM one-step RT-PCR with Platinum® Taq kit. The 50 μl reaction mixture contained 25 μl of 2 × Reaction Mix, 20 μl total RNA extracted with TRIZOL Reagent described above, 0.5 μmol/l each primer including primers for EGFP (sense: 5′-CGGATGGTGAGCAAGGGCGAG-3′; antisense: 5′-GGCTTACTTGTACAGCTCGTC-3′) and primers for β-actin (sense: 5′-CGCCGCGCTCGTCGTCGACA-3′; antisense: 5′-GTCACGCACGATTTCCCGCT-3′), 1 μl RT/Platinum® Taq Mix and 17 μl ddH_2O. Following 52 °C 30 min for cDNA synthesis, the PCR amplifications were performed in a PTC-200 peltier thermal cycler (MJ Research, Watertown, MA) under the following conditions: After an initial denaturation for 2 min at 94 °C, samples were subjected to 35 cycles of amplification (94 °C, 15 s; 55 °C, 30 s; 72 °C, 1 min), followed by a final extension of 5 min at 72 °C. After 1.5% agrose electrophoresis and scanning, all RT-PCR product bands were analyzed with the help of a IS-1000 digital phophorimager (Alpha Innotech, San Leandro, CA) using the software Gel Pro analyzer 32 and relative HBx-EGFP mRNA expression was estimated by normalization with β-actin.

2.9. Statistical analysis

The data were collected from at least three independent experiments. All statistical analyses were performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL). Descriptive statistics were expressed as mean ± standard deviation. Statistical significance was determined by using Student's *t*-test and one-way ANOVA (LSD) analysis. The *P* value less than 0.05 was considered statistically significant.

Table 1
Mean fluorescence intensity and the percentage of positive cells detected by flow cytometry (means ± S.D.s)

	MFI	α (%)
DNAzyme-treated group		
pHBx-EGFP + DrzHBVX-7	22.3 ± 2.8*	(35.3 ± 1.9)
pHBx-EGFP + DrzHBVX-8	21.4 ± 3.1*	(34.8 ± 2.6)
pHBx-EGFP + DrzHBVX-9	19.8 ± 2.6*	(33.9 ± 2.7)
Control group		
pHBx-EGFP + NSON	45.5 ± 3.7	(46.0 ± 2.2)

DNAzyme-treated group vs. control group.
* $P < 0.05$.

3. Results

3.1. HBx-EGFP fusion expression plasmid construction

Using standard recombinant techniques, HBV X gene with 478 bp was precisely fused with the N′ terminus of EGFP in the pEGFP-N1 vector. Recombinant clones were screened for the correct size of HBx insert by *Hin*dIII and *Eco*RI digestion. Recombinant clones were also confirmed by sequencing (data not shown). When these recombinant plasmids were transfected into AD293 cells, HBx-EGFP fusion protein was expressed, as shown in Fig. 2A. Green fluorescence was mainly located at the cytoplasm or the periphery of nucleus in granules.

3.2. Inhibition of HBx-EGFP expression by 10-23 DNAzymes in AD293 cells

Forty-eight hours after transfection, the expressions of HBx-EGFP in DNAzyme-treated groups were less than those in control groups observed by fluorescent microscopy (Fig. 2B). Mean fluorescence intensity (MFI) and the percentage (α) of the cell positive detected by flow cytometry, as shown in Table 1, demonstrated that MFI of each DNAzyme-treated group was less than that in control group ($P < 0.05$; $P < 0.05$; $P < 0.05$). The relative fluorescence expression rate of each DNAzyme-treated group was 44.1%, 42.3%, 39.1%, respectively (Fig. 3), MFI and relative fluorescence expression rate of each DNAzyme-treated had no significant difference. The results showed that all three

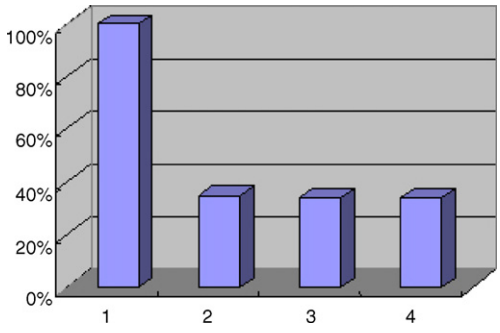

Fig. 3. Relative fluorescence expression rate of each group. (1) Control group (pHBx-EGFP + NSON); (2) DNAzyme-treated group (pHBx-EGFP + DrzHBVX-7); (3) DNAzyme-treated group (pHBx-EGFP + DrzHBVX-8); (4) DNAzyme-treated group (pHBx-EGFP + DrzHBVX-9).

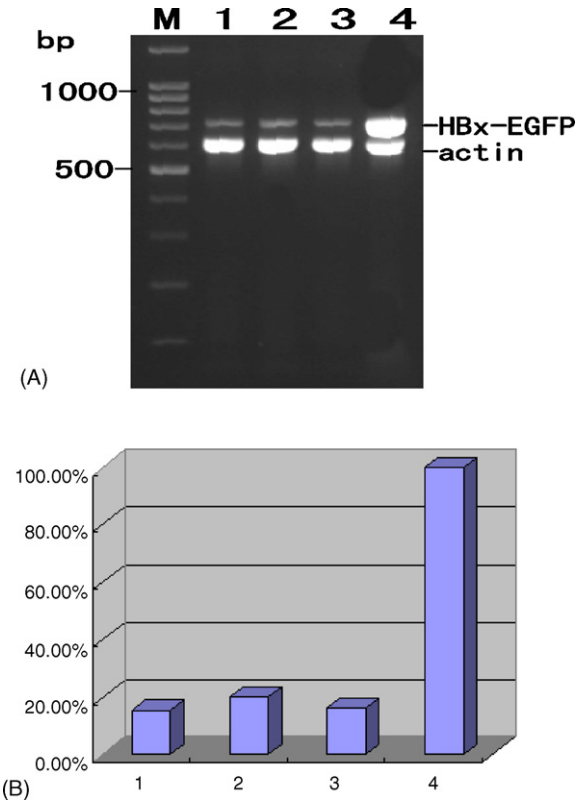

Fig. 4. (A) RT-PCR amplification of HBx-EGFP mRNA from AD293 cells. (B) Relative HBx-EGFP mRNA expression was analyzed by normalization with β-actin. M: DNA marker; (1) DNAzyme-treated group (pHBx-EGFP + DrzHBVX-7); (2) DNAzyme-treated group (pHBx-EGFP + DrzHBVX-8); (3) DNAzyme-treated group (pHBx-EGFP + DrzHBVX-9); (4) control group (pHBx-EGFP + NSON).

DNAzymes inhibited HBV X gene expression in a CMV-driven reporter assay.

3.3. Inhibition of HBx-EGFP-EGFP mRNA expression by 10-23 DNAzymes in AD293 cells

Forty-eight hours after transfection, RT-PCR products of HBx-EGFP mRNA and β-actin mRNA were obtained by One-Step RT-PCR with the size of 720 and 619 bp, respectively. Relative mRNA expression, estimated by normalization with β-actin showed that the expression of HBx-EGFP mRNA in DNAzyme-treated groups was less than that in control group detected by semi-quantitative RT-PCR (15.44%, 20.14% and 16.26%) (Fig. 4). The results showed that all three DNAzymes inhibited HBx gene expression in mRNA levels as well, while all DNAzymes achieving similar levels of HBx gene inhibition.

3.4. Specificity and efficiency of the inhibition effects by 10-23 DNAzymes targeting at HBx

Our above results indicated that 10-23 DNAzymes targeting at HBx could inhibit HBx gene expression in protein levels as well as in mRNA levels. The next questions were whether these inhibition effects were specific and effective. To address the first question, we chose a non-HBV X gene vector, pHCV-UTR/

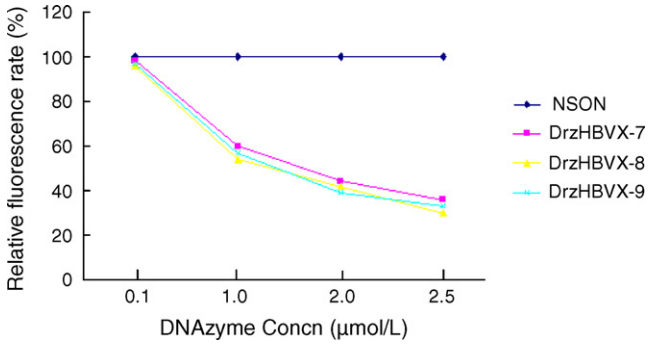

Fig. 5. HBx-EGFP fusion protein stably-expressing HepG2 cells were transfected with various concentrations (0.1, 1.0, 2.0 and 2.5 μmol/l) of 10-23 DNAzymes or NSON for 48 h. Relative fluorescence expression rate were determined 48 h post-transfection.

C126-EGFP, as control. Different 10-23 DNAzymes with pHCV-UTR/126-EGFP containing HCV-UTR/C126-EGFP fusion gene were cotransfected into AD293 cells. Forty-eight hours after transfection, the results indicated that DrzHBVX-7, DrzHBVX-8 and DrzHBVX-9 all did not inhibit HCV-UTR/C126-EGFP fusion gene expression in protein levels as well as in mRNA levels. In contrast, 10-23 DNAzymes specific to HCV core gene could inhibit HCV-UTR/C126-EGFP fusion gene expression (data not shown), which demonstrated the specificity of the inhibition effects by 10-23 DNAzymes. To address the second question, a concentration range of 0.1–2.5 μmol/l 10-23 DNAzymes were transfected into HBx-EGFP fusion protein stably-expressing $HepG_2$ cells selected by G418, showing a distinct dose-dependent efficiency (Fig. 5). There were no evident cytotoxic effects of these DNAzymes in the range evaluated by 3(4,5-dimethylthiagol-2-yl)-2,5-drphnyl tetragolium brornide (MTT) assays (data not shown). Taken together, the inhibition effects by 10-23 DNAzymes targeting at HBx were specific and effective.

4. Discussion

DNA in biological systems has long been considered a passive molecule carrying genetic information. Single-stranded DNA can also fold into well-defined, sequence-dependent tertiary structures, specifically bind to a variety of target molecules, and exhibit catalytic activities similar to those of ribozymes or protein enzymes (Breaker, 2000, 2004). Catalytic 10-23 DNAzymes have been generated using in vitro selection methodology (Santoro and Joyce, 1997). The 10-23 DNAzymes can cleave effectively between any unpaired purine and pyrimidine of mRNA transcripts. As a result, DNAzymes can be designed specifically to recognize the AU nts of the start codon, which make them promising candidates for potential applications in functional genomics and gene therapy (Achenbach et al., 2004; Dass, 2004; Goodchild, 2004; Joyce, 2004; Mitchell et al., 2004; Peracchi, 2004; Schubert and Kurreck, 2004; Silverman, 2004, 2005; Lu et al., 2005).

We previously demonstrated the inhibitory effects of 10-23 DNAzymes (DrzBS and DrzBC) with substrate-recognition domains of seven deoxynucleotides respectively on the express-

ions of HBV S and C genes in HepG2.2.15 cells (Wo et al., 2005). The expression of HBV S or C genes was dramatically suppressed after the cells had been treated by DrzBS or DrzBC. The concentration for effective inhibition was within 0.1–2.5 μmol/l and the inhibition showed a dose-dependence within this concentration range. The efficiency of inhibiting HBV surface antigen (HBsAg) and e antigen (HBeAg) in HepG2.2.15 cells by DrzBS and DrzBC was higher than that by antisense oligonucleotides for the same target genes.

In this report, we designed three 10-23 DNAzymes, DrzHBVX-7, DrzHBVX-8 and DrzHBVX-9, to cleave target sites at nucleotides 1376 in the HBV X gene ORF. Since fluorescence is easy to see under fluorescent microscope and quantitated by flow cytometry, the EGFP was selected as a report molecule, which has already been used to study ribozyme (Passman et al., 2000; Beger et al., 2001) and RNAi (Nagy et al., 2003; Cao et al., 2004; Qin et al., 2004). When each of the DNAzymes were cotransfected in AD293 cells with HBx-EGFP fusion expression plasmid containing HBx-EGFP fusion gene, DrzHBVX-7, DrzHBVX-8 and DrzHBVX-9 all reduced the HBx-EGFP expression, HBx-EGFP mRNA levels were also reduced dramatically by the DNAzymes. In addition, the specificity and efficiency of the inhibitory effects by these DNAzymes were also demonstrated. All the results suggest that DNAzyme could inhibit HBV X gene expression in vitro, which is useful to develop strategies for gene therapy of liver diseases caused by HBV.

Although RNA cleaving activities of DNAzymes are attractive so much as a controlling tool of genetic expression, which has attracted much attention from biological and medical points of view, their application to cellular system or in vivo is still strictly limited because of their deficient stability against nuclease digestion. The enhancement of the nuclease resistance by mechanisms such as chemical modification with N3′-P5′phosphoramidate (Takahashi et al., 2004), locked nucleic acids (LNA) (Petersen and Wengel, 2003; Fahmy and Khachigian, 2004; Vester et al., 2004), or conjugation with other functional molecules (Takahashi et al., 2004) in combination with an effective delivery system may enable the development DNAzymes as an alternative therapy for the control of chronic HBV infection. However, additional experiments are required to test the efficacy of the HBx-specific DNAzymes characterized in the study on HBV replication.

Acknowledgement

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