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Specific inhibition of gene expression and transactivation functions of hepatitis B virus X protein and c-*myc* by small interfering RNAs

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Abstract With a view to developing therapeutic strategies against hepatocellular carcinoma (HCC), we have recently shown that co-expression of c-*myc* and the X protein of hepatitis B virus (HBx) resulted in the development of HCC in the X-*myc* transgenic mice [Lakhtakia et al., J. Gastroenterol. Hepatol. 18 (2003) 80–91]. We now show in cell culture-based studies that small interfering RNA (siRNA) corresponding to HBx and c-*myc* can regulate expression and transactivation of the target genes. Expression vectors for small hairpin RNAs (shRNAs) against two different regions each of the HBx and c-*myc* open reading frames were constructed and their regulatory effects were investigated in COS-1 cells. A dose-dependent specific inhibition in the expression levels of HBx and c-*myc* was observed with individual shRNAs. Further, the recombinantly expressed shRNAs also blocked the transactivation functions of their cognate genes. Though each shRNA worked at a different efficiency, the inhibitory effects with two different shRNAs were cumulative. These results appear promising for developing a siRNA-based therapy for HCC.

Key words: Hepatitis B virus X protein; Transactivation; RNA interference; Hepatocellular carcinoma; Chloramphenicol acetyltransferase assay

1. Introduction

RNA interference (RNAi) is a ubiquitous mechanism of gene regulation in a wide variety of organisms, including animals, plants, fungi and protists, that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids [1,2]. It also appears to play a key role in cellular programming of gene expression and development [3]. The process of RNAi involves generation of 21–23-nt double-stranded RNA (dsRNA) termed small interfering RNA (siRNA) and subsequent degradation of target mRNAs (reviewed in [4]). The siRNAs are associated with regulating the levels of proteins. The dsRNA molecules are cleaved by the RNase III-like enzyme 'Dicer' to generate siRNAs that in turn direct the recognition and degradation of homologous mRNAs by a multiprotein complex called RNA-induced silencing complex [5]. Direct introduction of dsRNA into mammalian cells may not produce Dicer-mediated siRNAs as efficiently as in insect cells and therefore may fail to induce RNAi [6]. Nevertheless, the requirement for Dicer in generation of siRNAs can be experimentally bypassed using either synthetic 21-nucleotide siRNA duplexes [7] or small hairpin RNAs (shRNA) transcribed in vitro or in vivo from DNA templates [8,9]. Such siRNAs have been shown to inhibit expression of transfected as well as endogenous genes in several mammalian cells [10]. As siRNAs can trigger sequence-specific RNAi in cells, it has generated considerable interest in annotating the function of genes in different living systems without actually destroying or mutating the genetic material [11–13]. Besides, RNAi seems to hold promise for treating many infectious diseases and cancers including hepatocellular carcinoma (HCC) [14–17].

Recently, we have developed a transgenic mouse model of HCC using a bicistronic recombinant X-*myc* [18]. Co-expression of c-*myc* and the X protein of hepatitis B virus (HBx) resulted in the development of liver-specific tumors in the X-*myc* mice [19,20]. To develop a therapeutic strategy for liver cancer based on gene silencing, we have studied the effect of siRNAs on the expression of cognate genes and their transactivation functions in cell culture. Our results with siRNAs against two different regions of HBx and c-*myc* show that the gene silencing was highly specific for its target. We have also observed additive siRNA effects when more than one shRNA corresponding to different regions of the same gene was used in the experiment.

2. Materials and methods

2.1. siRNA expression vectors

Signature siRNA sequences corresponding to two different functional domains of HBx (X-D and X-E) and the mouse c-*myc* (M-T and M-Z) were selected for the chemical synthesis of oligonucleotides (Sigma Genosys, USA). The 5′ ends of these oligonucleotides were phosphorylated by T4 polynucleotide kinase, annealed in pairs and cloned directly into the pSilencer 1.0-U6 expression vector (Ambion, USA).

Expression vectors for the native HBx (X0) have been described [21]. The mouse c-*myc* expression vector (pSG5-*myc*) was constructed by amplifying the 1.3-kb open reading frame region using reverse transcription polymerase chain reaction and cloning into the pSG5 expression vector (Stratagene, USA) as an *Eco*RI fragment. Details of the RSV-CAT reporter construct having the long terminal repeat of Rous sarcoma virus (−500 to +51) can be found elsewhere [22].

2.2. Cell culture, DNA transfection and immunoprecipitation

COS-1 cells (ATCC 1650) in 60-mm culture dishes were transiently

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Abbreviations: CAT, chloramphenicol acetyltransferase; HBx, hepatitis B virus X protein; dsRNA, double-stranded RNA; RNAi, RNA interference; shRNA, small hairpin RNA; siRNA, small interfering RNA

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transfected with 1 μg of either HBx or c-*myc* expression vectors using Lipofectin (Gibco-BRL, USA). In parallel, the cells were co-transfected with increasing amounts (0.5, 1 and 2 μg) of the siRNA expression plasmids X-D or X-E (for HBx), and M-T or M-Z (for c-*myc*). After 48 h, the cells were harvested in sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/w SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% bromophenol blue) and the proteins were resolved in a 15% SDS–polyacrylamide gel electrophoresis. After transfer to Hybond ECL nitrocellulose membrane, the blot was first incubated with specific monoclonal antibodies for HBx [21] or c-*myc* (Santa Cruz, USA) followed by incubation with the horseradish peroxidase-conjugated secondary antibody. The protein bands were detected using LumiGLO chemiluminescent reagent (Cell Signaling Technology, USA). Fold expression was estimated by densitometric analysis of the autoradiograms using Kodak digital science 1D (ver. 1.6). The levels of significance were calculated by Student's *t*-test.

2.3. RNA isolation and Northern blot assay

Total RNA was isolated form the transfected COS-1 cells using TRIzol reagent as per the supplier's instructions (Gibco BRL, USA). The samples (20 μg) were resolved in a formaldehyde/agarose gel (1%) and Northern hybridization was performed using ^{32}P-labeled HBx or c-*myc* probes [23]. Fold inhibition in the expression of RNA was determined by densitometric analysis.

2.4. Chloramphenicol acetyltransferase (CAT) assay

COS-1 cells were transiently transfected with the RSV-CAT reporter construct (0.5 μg) and the expression plasmids for either HBx or c-*myc* (1 μg each) as above. For the transactivation inhibition studies, the cells were also co-transfected with increasing amounts (0.5, 1 and 2 μg) of the shRNA expression vectors. After 48 h, the cells were harvested and assayed for CAT activity [22]. Fold CAT inhibition was estimated by densitometric analysis as above and the levels of significance were calculated by Student's *t*-test.

3. Results

3.1. siRNA design and expression vectors

siRNAs for HBx and mouse c-*myc* were designed using Jack Lin's siRNA sequence finder (http://www.ic.sunysb.edu/Stu/shilin/rnai.html) or Qiagen's design by sequence (http://python.penguindreams.net/Xeragon_Order_Entry/jsp/SearchBySequence.jsp). Though several putative sites for siRNA were predicted across the length of both HBx and c-*myc*, we selected two regions of molecule that encode important functional regions. Fig. 1 shows the scheme of domain structures of HBx and c-*myc*, location of the siRNA sites and oligonucleotide sequences of the shRNA inserts. For HBx (Fig. 1A), regions D and E play important roles in signal transduction and transactivation [24,25] and therefore, the shRNAs directed against these regions were termed X-D and X-E respectively. The shRNA constructs directed against the transactivation and leucine zipper domains of c-*myc* [26] were called M-T and M-Z respectively. The lengths of all the sense and antisense deoxyribo-oligonucleotides were 53 and 61 nucleo-

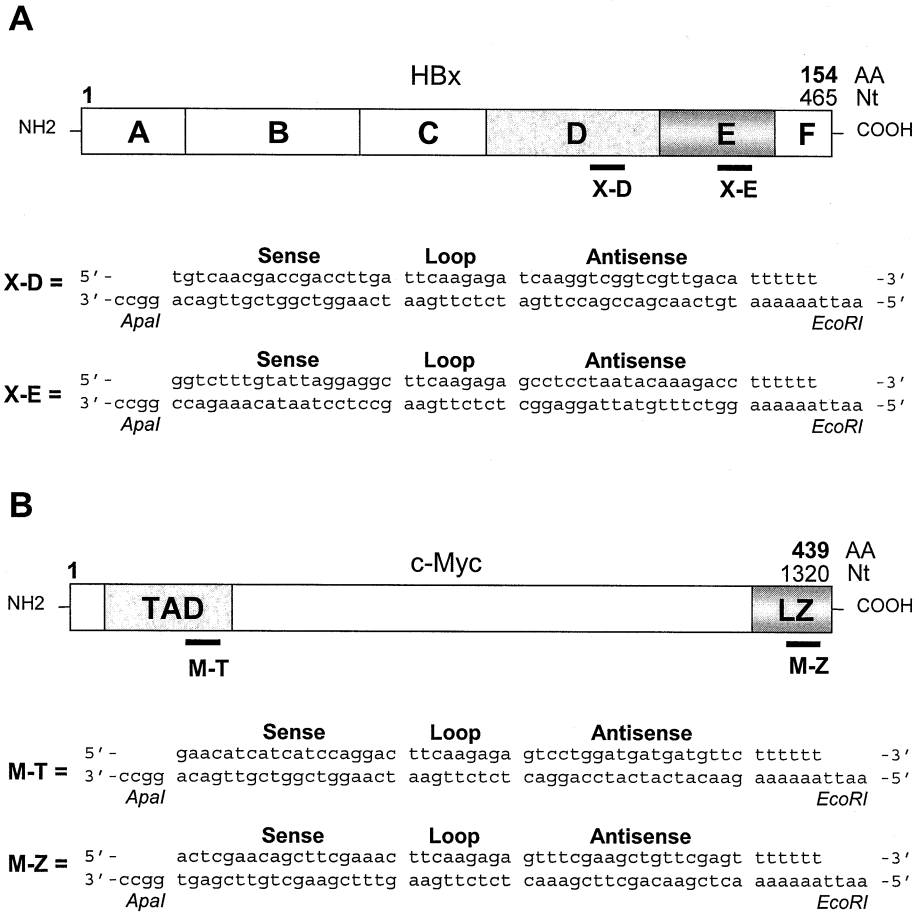

Fig. 1. Design and DNA sequence of the oligonucleotides for the expression of shRNA against HBx and c-*myc*. A: Domain structure of HBx (A through F), location and sequence of shRNA for domains D (X-D) and E (X-E). B: Schematic organization of c-*myc* showing transactivation (TAD) and leucine zipper (LZ) domains, sequences of shRNA specific for TAD (M-T) and LZ domains (M-Z) with their location.

tides respectively. Each oligonucleotide had a small sense, loop and an antisense region so as to express siRNA as a stable hairpin structure [27]. The four sets of shRNA oligonucleotides were cloned between the *Apa*I and *Eco*RI sites of the pSilencer 1.0-U6 plasmid (Ambion) to put them under the transcriptional control of the U6 RNA polymerase III promoter. The dideoxy chain termination method was used to confirm the sequence of each construct.

3.2. Specific inhibition of protein and RNA levels by shRNAs

To investigate the inhibitory action of siRNAs on the levels of target proteins, the expression vectors for HBx and mouse c-*myc* were transfected in COS-1 cells along with increasing amounts of the shRNA plasmids and analyzed by immunoprecipitation. As shown in Fig. 2A, the level of HBx was specifically inhibited by both X-D (lanes 3–5) and X-E shRNA (lanes 6–8). Though X-D was relatively more efficient than X-E in inhibiting the HBx levels, the effect was cumulative in the presence of the two shRNAs (lanes 9–11). The inhibition levels ranged between 1.5- and 3-fold with individual shRNAs. However, the inhibition (~5-fold) was significantly higher ($P < 0.003$) in the presence of both X-D and X-E. The inhibitory effect was specific for HBx as no interference was observed with the *myc*-specific shRNAs M-T and M-Z (lanes 12–14).

Like HBx, both shRNAs against c-*myc* were also effective against the target gene (Fig. 2B). M-T (lanes 3–5) was more effective than M-Z (lanes 6–8) and showed a 5–10-fold inhibition in the expression of c-*myc*. Maximum inhibition was only two-fold at the highest concentration (2 μg) of M-Z (lane 8). The interference was cumulative and the expression of target protein was virtually abolished ($P < 0.008$) in the presence of both M-T and M-Z (lanes 9–11). Further, no interference in the c-*myc* levels was observed in the presence of HBx-specific X-D and X-E (lanes 12–14) showing their specificity for the respective target genes.

To confirm whether the decreased protein levels of HBx and c-*myc* were due to down-regulation of the respective mRNA levels by shRNAs, Northern blot assay was done using total RNA from the transfected cells. As observed with the X protein, a two- to three-fold decrease in the mRNA levels was also observed in the presence of X-D and/or X-E (Fig. 2C). The RNA level was effectively lowered by nearly three-fold in the presence of either X-D or X-E (lanes 2 and 3). The inhibition was cumulative as the RNA levels dropped to ~8-fold in the presence of both X-D and X-E (lane 4). Similarly, the *myc* mRNA levels were also inhibited in the presence of M-T or M-Z (data not shown). Further, accumulation of 21–22-nucleotide RNA species was also observed in the presence of all four shRNAs (data not shown).

3.3. Regulation of transactivation function by shRNAs

To demonstrate that inhibition of target gene expression was not a direct effect of RNA–protein interaction but rather an effect of the decreased level of the protein, the transactivation property of HBx and c-*myc* was studied in transient transfection assays using the RSV-CAT reporter gene construct. As shown in Fig. 3A, the HBx-mediated transactivation of the RSV long terminal repeat could be inhibited in the presence of both X-D (lanes 2–4) and X-E (lanes 5–7). As observed earlier, X-D was relatively more efficient than X-E in inhibiting the transactivation function of HBx. Further, the effect was cumulative in the presence of the two shRNAs (lanes 8–10) and the transactivation level could be reduced to an undetectable level (lane 10).

Like HBx, the transactivator function of c-*myc* could also be inhibited by the two shRNAs (Fig. 3B). M-T (lanes 2–4) appeared relatively more efficient than M-Z (lanes 5–7) and at a 1:1 molar ratio, no CAT activity could be detected (lanes 3 and 4). The inhibitory effect was cumulative in the presence of both M-T and M-Z (lanes 8–10) and the transactivation level could be inhibited to an undetectable level (lanes 9 and 10).

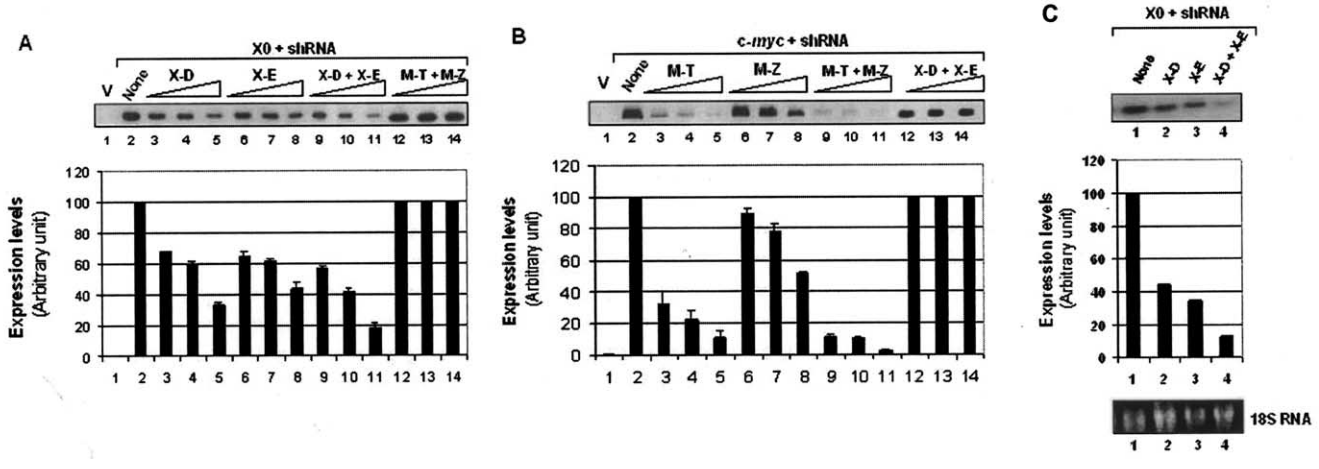

Fig. 2. Inhibition of the expression of HBx and c-*myc* by shRNAs. For monitoring the protein levels, COS-1 cells were co-transfected with expression plasmids (0.5 μg) for HBx (A) or c-*myc* (B) along with increasing amounts (0.5, 1 and 2 μg) of different shRNA constructs. After 48 h, the cell extracts were immunoprecipitated and bands were detected by chemiluminescence. For measuring the mRNA levels (C), cells were co-transfected with HBx (0.5 μg) and the shRNA expression vectors X-D and/or X-E (2 μg each) as indicated. Total RNA was extracted and the expression level was measured by Northern blot assay using ^{32}P-labeled HBx probe. Top bars show immunoprecipitated protein bands (A,B) or RNA bands (C). Densitometric scan was used to give the histograms shown below. Loading control for RNA (18S RNA) is shown at the bottom of C.

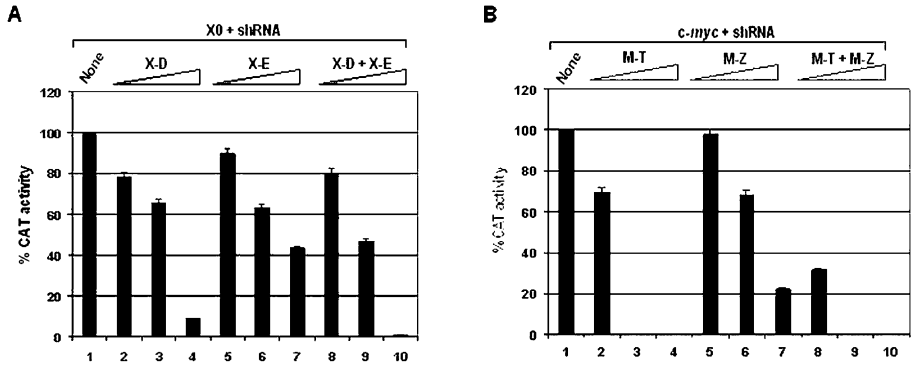

Fig. 3. Inhibition in the transactivation property of HBx and shRNAs. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for HBx (A) or c-*myc* (B) and increasing amounts (0.5, 1 and 2 μg) of different shRNAs as indicated. After 48 h, the cell extracts were assayed for CAT activity. Percent CAT activity is shown as histograms along with the error bars.

As expected, X-D and X-E did not inhibit the *myc*-dependent transactivation, while M-T and M-Z did not interfere with HBx-dependent activity (data not shown).

4. Discussion

Gene expression can be regulated at the post-transcriptional level using a wide range of approaches including antisense RNA, ribozymes and RNA interference. Extensive work has been done to validate the effectiveness of these strategies in cell culture, animal models as well as in limited clinical trials [28–30]. From these studies, it is now clear that the RNAi approach is far more efficient than the antisense RNA and ribozyme strategies [31–33]. The ability to selectively silence mammalian gene expression using siRNA has not only opened new and exciting routes to understand mammalian cell biology in health and disease, but has also given us the ability to selectively regulate the expression of genes that may have detrimental effect(s) on cells including their uncontrolled proliferation. Besides, this has also provided a powerful tool to silence the genes of pathogenic viruses, bacteria, fungi and other agents that may be crucial for survival and/or disease development. More recently, the effectiveness of siRNA has been experimentally demonstrated against some pathogenic viruses such as human immunodeficiency virus-1 [34–36], hepatitis B virus [37,38], hepatitis C virus [39,40], poliovirus [41], rotavirus [42], human papillomavirus [43], Rous sarcoma virus [44], and γ-herpesvirus [45]. Besides, siRNA has also been found effective against trypanosomes [46], plasmodium [47], Fas-mediated fulminant hepatitis in mice [48], cancer growth (HCC) by inhibiting cyclin E overexpression [17], and prion protein accumulation in neuroblastoma cells [49]. Thus, siRNA has tremendous promise as a therapeutic tool for targeted gene silencing. However, its utility will depend on its specificity, i.e. ability to specifically knock down the target gene without interference with the expression or function of other genes or proteins.

With the ultimate aim of developing gene therapy for HCC, we have evaluated the effectiveness of shRNAs on the post-transcriptional regulation of two different transactivator genes – the viral transactivator HBx and the cellular transcription factor c-*myc*. While c-*myc* is known to play a major role in cellular growth and differentiation [50], HBx is essential for viral replication and development of hepatocellular carcinoma [51,52]. Our results from the mammalian cell culture show the effectiveness of the siRNA approach in the regulation of the intracellular levels of HBx and c-*myc*. The effect of the shRNAs used in the present study was highly specific as no cross-interference was observed. Further, the cumulative inhibitory effect of multiple siRNAs against one target gene is likely to be more successful due to the amplification of the RNA-induced silencing complex. The observed differences in the potency of siRNAs targeted to different regions of the same mRNA suggest that target accessibility is an important factor governing the siRNA response. Altogether, these results are encouraging since they suggest that siRNA-based therapeutic agents are likely to have inherent molecular specificity. Though exquisite sequence specificity for the target mRNAs has been questioned recently [53,54] and the activation of interferon responses has also been observed in the presence of siRNAs [55,56], our results on shRNAs for HBx and c-*myc* provide a case-specific example of transcription factors that are known to co-operate in order to induce HCC in the X-*myc* transgenic mouse model [18]. It may be noted that a functional interaction between HBx and c-*myc* appears to be essential for the development of HCC in X-*myc* mice [19] and an analogous situation may be associated with the development of HCC in humans [57]. Thus, our results on the regulation of HBx and c-*myc* by siRNA are encouraging for developing and testing siRNA-based therapeutic agents for HCC.

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