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Antisense regulation of expression and transactivation functions of the tumorigenic HBx and c-myc genes ☆

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Abstract

Earlier we have shown that the X-*myc* transgenic mice develop hepatocellular carcinoma (HCC) due to co-expression of c-Myc and HBx protein of hepatitis B virus [R. Lakhtakia, V. Kumar, H. Reddi, M. Mathur, S. Dattagupta, S.K. Panda, Hepatocellular carcinoma in a hepatitis B 'x' transgenic mouse model: a sequential pathological evaluation. J. Gastroenterol. Hepatol. 18 (2003) 80–91]. With the aim to develop therapeutic strategies for HCC, we constructed several mono- and bicistronic antisense recombinants against HBx and *c-myc* genes to regulate their expression as well as transactivation function in a human hepatoma cell line. A dose-dependent inhibition in the expression levels of HBx and *c-Myc* was observed with monocistronic constructs. Likewise, the bicistronic recombinants also blocked the expression as well as transactivation functions of cognate genes with equal efficacy. Further, expression of the constituent genes from the X-*myc* transgene could also be inhibited by these antisense constructs in cell culture. Thus, our study points towards clinical implications of antisense regulation of tumor-promoting genes in the management of HCC.

Keywords: Antisense; Bicistronic; CAT assay; Hepatocellular carcinoma; c-Myc; Transactivation; X protein

Antisense or antigene therapy of diseases that are due to aberrant gene expression is an exciting possibility. It involves regulation of gene expression using either single-stranded RNA or DNA complementary to a target RNA [1,2]. It works on the principle of base pairing with the coding DNA strand or RNA transcript resulting in interference at the levels of transcription, stability of transcripts, and/or translation of mRNA. Antisense transcription was recognized as a naturally occurring mechanism in prokaryotes [3] and later reported for some eukaryotic genes like *Drosophila* dopadecarboxylase, chicken myosin heavy chain, and mouse dihydropholate reductase [4–6]. Recent analysis of human and mouse transcriptomes has suggested that antisense transcription is widespread in the mammalian genome and could have an important role in the regulation of gene expression [7,8]. In the recent past, antisense agents like antisense oligodeoxynucleotides (ASO), ribozymes, and double-stranded RNAs have been widely used to elicit potent targeted degradation of complementary RNA sequences and block the expression of specific genes in cell culture, animal models, and in clinical trails for several diseases including cancer [9–12]. Although these agents provide a powerful tool for targeted inhibition of gene expression, there are some valid concerns with regard to low bioavailability and ensuring specificity otherwise leading to off-target and bystander effects [9]. Besides, due to multigenic alterations in tumors, the use of ASOs as single agents does not seem to be effective in the treatment of malignancies, suggesting the need for developing new antisense therapeutics.

☆ *Abbreviations:* ASO, antisense oligodeoxynucleotides; CAT, chloramphenicol acetyl transferase; HBV, hepatitis B virus; HBx, X protein of HBV; HCC, hepatocellular carcinoma; RSV-LTR, long terminal repeat of Rous sarcoma virus; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; siRNA, small interfering RNA.

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With a view to develop therapeutic strategy for liver cancer based on antisense gene therapy, we developed a series of mono- and bicistronic antisense recombinants driven by homologous or heterologous promoter elements. These recombinants are directed against the constituent genes of a bicistronic construct 'X-*myc*' that we have shown earlier to induce hepatocellular carcinoma (HCC) in transgenic mice [13]. The X-*myc* mice develop liver-specific tumors due to co-expression of the X protein of hepatitis B virus (HBx) and c-Myc [14]. Here we report the relative efficacies of these antisense recombinants in regulating the expression and transactivation functions of HBx and c-Myc in cell culture.

Materials and methods

DNA recombinants. Expression vectors for native HBx (X0) [15] and mouse *c-myc* (c-Myc) [16] have been described earlier. Details of the RSV-CAT can be found elsewhere [17]. All the sense (X0, c-Myc, and pc-Myc) and antisense (AS1–AS7) expression constructs are shown schematically in Fig. 1. These were constructed using standard molecular biology protocols [18]. pc-Myc was developed by inserting a 105 bp pre-core region of hepatitis B virus at the 5′-end of *c-myc* open reading frame in c-Myc. AS7 was made by cloning the 1.3 kb *Eco*RI fragment of *c-myc* in pSG5 (Stratagene, USA). The remaining six antisense recombinants (AS1–AS6) were assembled in the pCMV-neo backbone. The pCMV-neo vector (4.28 kb) was derived from pIRES1*neo* (5.25 kb; Clontech, Palo Alto) by deleting a 970 bp *Eco*RV–*Sma*I region followed by religation. AS1 was constructed by ligating a PCR amplified 400 bp EnhancerI/X promoter (EnI/Xp) region of hepatitis B virus (HBV) followed by cloning of X0 in antisense orientation (anti-X0). AS2 was created from AS1 by replacing the EnI/Xp region with SV40 early promoter from pSG5. AS3 was assembled by cloning a 255 bp PCR fragment encompassing the enhancer II/core promoter (EnII/Cp) region of HBV followed by cloning a 315 bp trimeric pre-core region of HBV in antisense orientation. AS4 was constructed from AS3 by replacing the EnII/Cp region with a 590 bp long-terminal repeat of Rous Sarcoma virus (RSV-LTR) from RSV-CAT. The bicistronic constructs AS5 and AS6 were derived by assembling together the antisense regions of AS1 and AS3, and AS2 and AS4, respectively, in pCMV-neo.

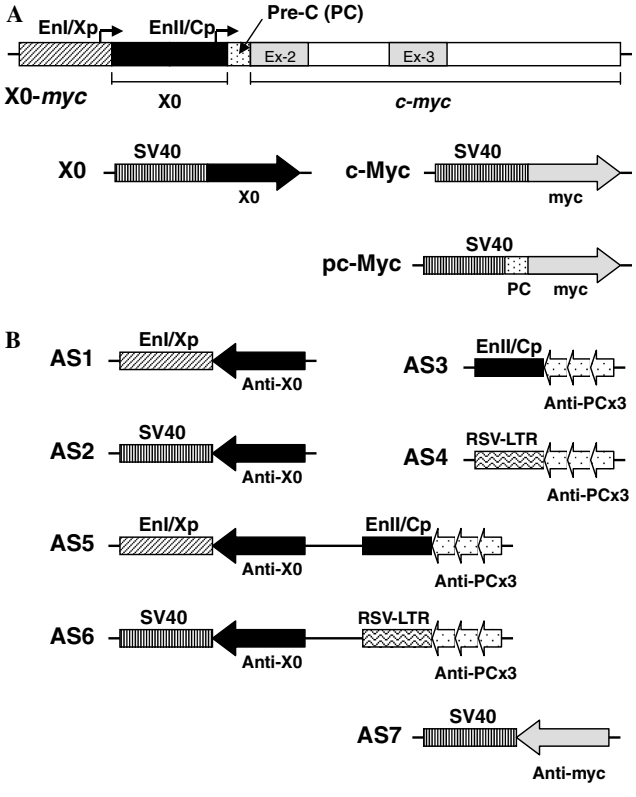

Fig. 1. Schematic representation of the sense and antisense recombinants. (A) Sense constructs X0-*myc*, X0, c-Myc, and pc-Myc. (B) Antisense constructs AS1–AS7. EnI/Xp, X promoter of HBV along with enhancer I; EnII/Cp, core promoter of HBV along with enhancer II; Ex-2 and Ex-3, exons 2 and 3 of the *c-myc* gene, respectively; PC, pre-core of HBV; RSV-LTR, long terminal repeat of Rous sarcoma virus; SV40, SV40 early enhancer promoter; X0, expression vector for wild type HBx.

Cell culture, DNA transfection, and Western blotting. Human Hepatoma Huh7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone, USA) and seeded at a density of 0.7 million per 60 mm dish. Transfections were carried out using Lipofectin (Invitrogen, USA) as per manufacturer's protocol using 1 μg of either X0 or c-Myc. For antisense studies, cells were co-transfected with increasing amounts (0.5, 2, and 5 μg) of the antisense constructs AS1, AS2, AS5, AS6 (for HBx), and AS3, AS4, AS5, AS6, AS7 for *c-myc*. After 48 h, the cells were harvested in the SDS buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/w SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) and the proteins were resolved by SDS–PAGE (15%). After transferring to Hybond ECL nitrocellulose membrane, the blot was first incubated with specific monoclonal antibodies for HBx [15] or c-Myc (Santa Cruz, USA) followed by incubation with the HRPO-conjugated secondary antibody. The protein bands were visualized using LumiGLO chemiluminescent reagent (Cell Signaling Technology, USA) and fold expression was measured by densitometric analysis.

Chloramphenicol acetyl transferase assay. Transactivation studies were done in Huh7 cells by transiently transfecting RSV-CAT reporter construct (0.5 μg) along with X0 or c-Myc (1 μg each). For antisense inhibition studies, cells were co-transfected with increasing amounts (0.5, 2, and 5 μg) of different antisense recombinants. After 48 h, the cells were harvested and assayed for chloramphenicol acetyl transferase (CAT) activity [17]. Inhibition in the CAT activity was determined by densitometry.

RNA isolation and Northern blot assay. Total RNA was isolated form the transfected Huh7 cells using TRIzol reagent as per 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 [19]. Inhibition in RNA levels was estimated by densitometric analysis.

Statistical analyses. Statistical analyses were performed using Student's *t* test; *p* values less than 0.05 were considered as statistically significant.

Results

Specific inhibition of protein and RNA levels by antisense recombinants

To investigate the inhibitory action of antisense on the levels of target proteins, the expression vectors for HBx and mouse c-Myc were transfected in Huh7 cells along with increasing amounts of the antisense plasmids and analyzed by immunoprecipitation. As shown in Fig. 2A, the level of HBx was significantly inhibited by antisense constructs AS1 (lanes 3–5) and AS2 (lanes 6–8). Likewise the bicistronic constructs AS5 (lanes 3–5) and AS6 (lanes 6–8) also specifically inhibited HBx levels (2B). Nevertheless, AS2 and AS6 appeared to be relatively more efficient than AS1 and AS5 in inhibiting the HBx expression.

As in the case of HBx, antisense constructs against pc-Myc were equally selective and effective against their

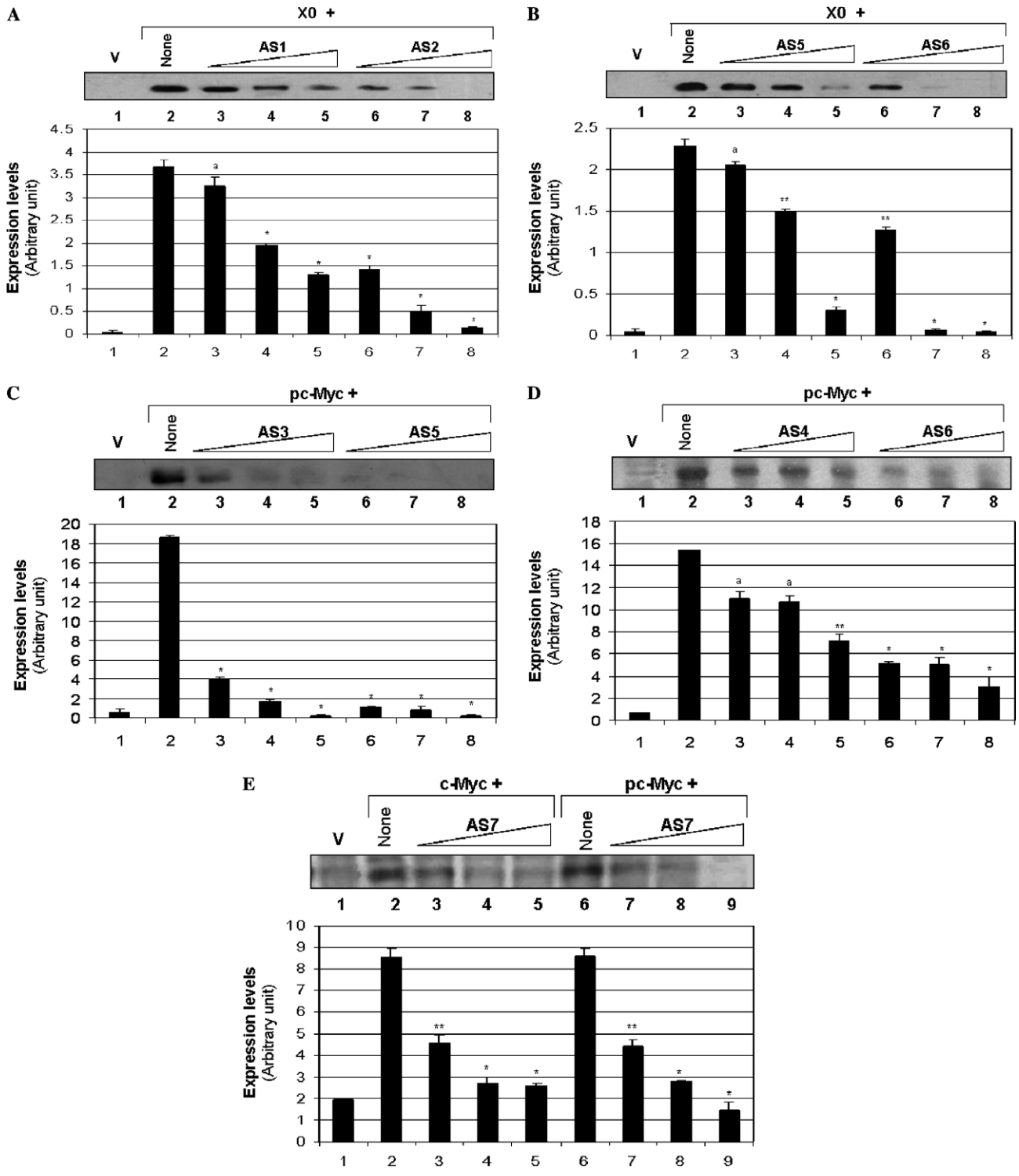

Fig. 2. Inhibition of HBx and c-Myc protein levels by antisense constructs. Huh7 cells were transfected with X0 (0.5 μg) along with increasing amounts (0.5, 2, and 5 μg) of different antisense constructs AS1 or AS2 (A) and AS5 or AS6 (B). Likewise pc-Myc or c-Myc (0.5 μg) were co-transfected with increasing amounts (0.5, 2, and 5 μg) of different antisense constructs AS3 or AS5 (C), AS4 or AS6 (D) and AS7 (E). After 48 h, the cell extracts were immunoprecipitated using either anti-HBx (A,B) or anti-Myc antibodies (C–E) and resolved by SDS–PAGE. The protein bands were visualized by chemiluminescence and inhibition in their levels was statistically validated from three independent observations. $^{*}p < 0.001$; $^{**}p < 0.01$; $^{a}p = 0.025$.

targets. A more prominent inhibition (5- to 10-fold) in the expression of pc-Myc was observed with AS3 and AS5 (Fig. 2C) than with AS4 and AS6 (panel D). As expected, AS7 down-regulated the levels of both c-Myc and pc-Myc to similar levels. Further, Northern blot analysis of the total RNA showed a good correlation with the decreasing levels of both HBx and c-Myc proteins in these samples (data not shown).

Regulation of transactivation function of HBx and c-Myc by antisense constructs

To establish that inhibition in proteins levels also correlated with its activity, the transactivation function of HBx and c-Myc was analyzed in transient transfection assays using the RSV-CAT reporter gene construct. As shown in Fig. 3A, the HBx-mediated transactivation of RSV-LTR could be inhibited by the antisense constructs AS1 (lanes 3–5) and AS2 (lanes 6–8) in a dose-dependent manner. Similarly, AS5 and AS6 also down-regulated the transactivation function of HBx (3B). The inhibition function of all the four antisense recombinants was comparable.

Like HBx, the Myc-dependent stimulation of RSV-LTR could also be inhibited by myc-specific antisense constructs. Fig. 3C shows that AS3 (lanes 3–5) and AS5 (lanes

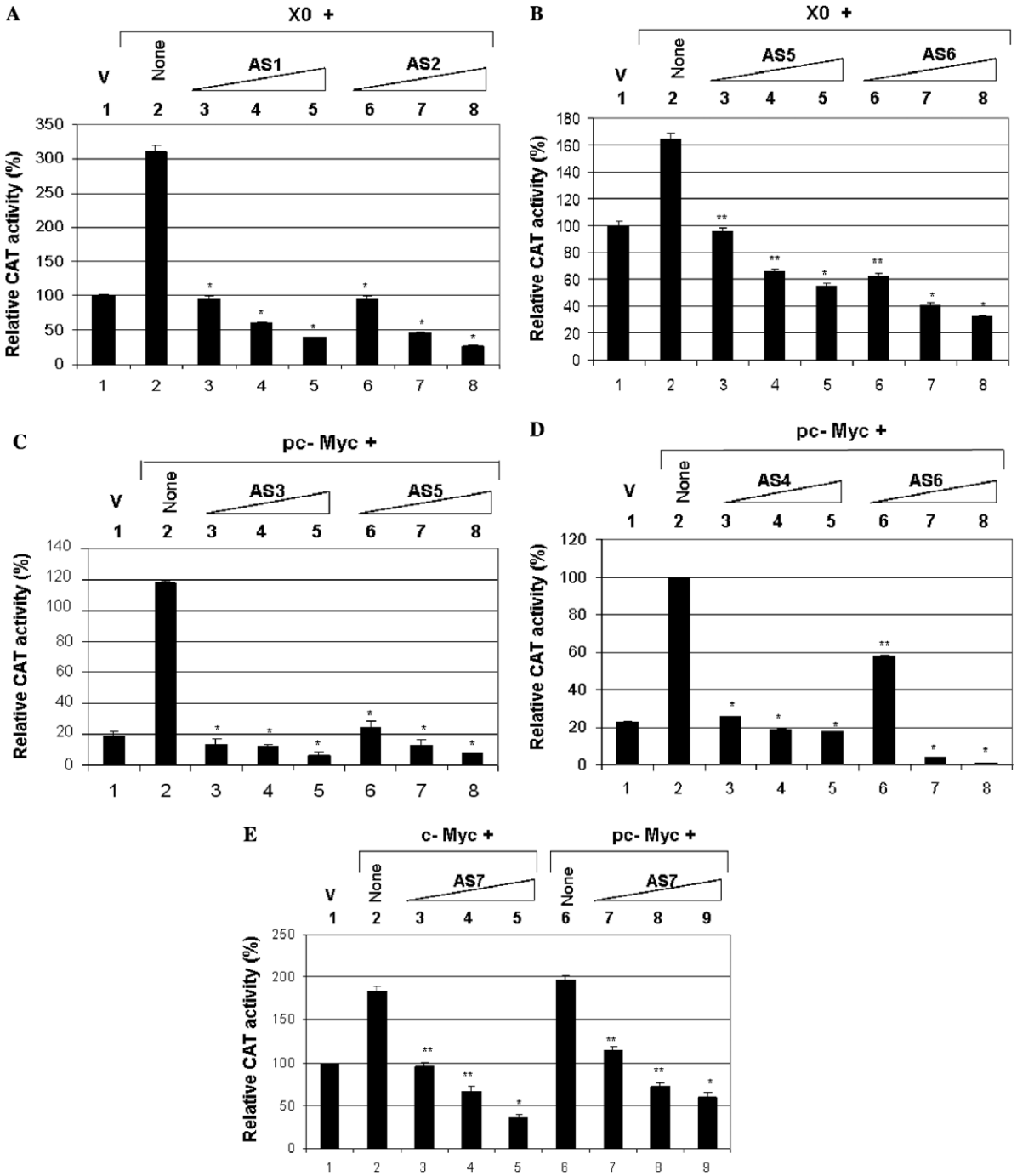

Fig. 3. Inhibition in the transactivation function of HBx and c-Myc by antisense recombinants. The RSV-CAT reporter plasmid (0.5 μg) was co-transfected with X0, pc-Myc or c-Myc (0.5 μg) and increasing amounts (0.5, 2, and 5 μg) of different antisense constructs: AS1 or AS2 (A), AS5 or AS6 (B). AS3 or AS5 (C), AS4 or AS6 (D) and AS7 (E). After 48 h, the cell extracts were assayed for CAT activity and statistically validated from three to five independent observations. $^{*}p < 0.001$; $^{**}p < 0.01$.

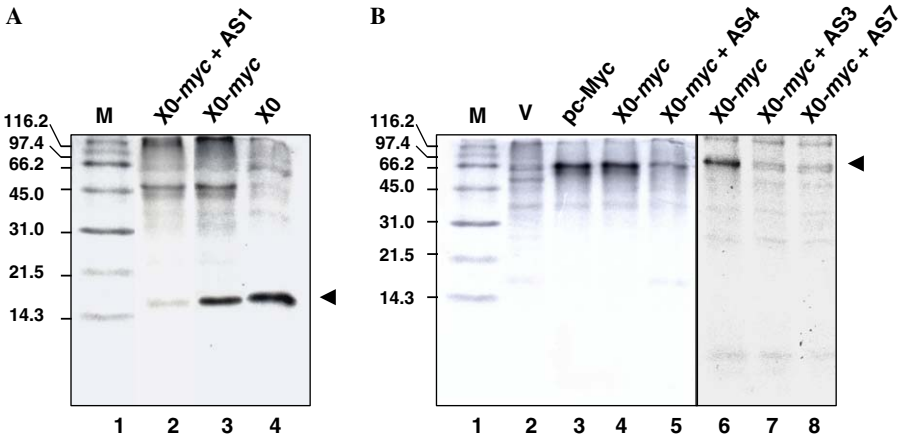

Fig. 4. Regulation of the expression of HBx and pc-Myc from the X0-myc transgene by antisense vectors. Huh7 cells were transfected separately with X0, pc-Myc, X0-myc or X0-myc along with AS1, AS3, AS4 or AS7 (1 μg each). After 48 h, the cell extracts were immunoprecipitated either with anti-HBx (A) or anti-myc (B) antibodies, resolved by SDS–PAGE and visualized by chemiluminescence. Arrowheads show the position of HBx (A) and pc-Myc (B). M, protein molecular weight standards; V, vector control.

6–8) significantly inhibited the transactivation function of pc-Myc. Similarly, AS4 and AS6 also inhibited the transactivation function of pc-Myc (Fig. 3D). However, the inhibitory effects were more prominent with AS3 and AS5 than AS4 and AS6. The inhibitory effects of AS7 were comparable for both c-Myc and pc-Myc (Fig. 3E).

Regulation of X0-myc by antisense recombinants

Since the antisense constructs used here showed specific inhibition in the protein levels and transactivation functions of HBx and c-Myc, we further investigated the antisense regulation of bicistronic X0-*myc* (Fig. 1A) that has the potential to induce liver-specific tumors in a transgenic environment [13,14]. As shown in Fig. 4, X0-*myc* expressed both HBx (A, lane 3) and pc-Myc proteins (B, lanes 4 and 6) in hepatoma cells. Co-expression of X0-*myc* and antisense construct AS1 resulted in specific inhibition of HBx (A, lane 2) while AS4 (B, lane 5) AS3 (lane 7) or AS7 (lane 8) specifically inhibited the expression of pc-Myc. Note that the levels of endogenous c-Myc were not affected by AS3 and AS4 but only by AS7 (data not shown).

Discussion

The ability to selectively regulate mammalian gene expression using antigene therapy has opened new opportunities for the management of health and disease. It has allowed us to regulate the expression of genes that may have detrimental effect(s) on cells including their uncontrolled proliferation, and provided a powerful tool to silence the genes of pathogenic viruses, bacteria, fungi, and other infectious agents. Gene expression can be regulated at the post-transcriptional level using a wide range of approaches including small interfering RNAs (siRNA), ribozymes, and antisense nucleic acids. Effectiveness of these strategies has been validated through extensive research in cell culture, animal models as well as in limited clinical trials [10–12]. Though siRNA approach is considered to be more efficient than the antisense and ribozyme strategies, its exquisite sequence specificity for target mRNAs has been questioned recently [20,21] and the activation of interferon responses has been reported with small interfering RNAs [22,23]. Another major obstacle in the development of such therapeutic agents has been their low bioavailability due to hydrophilic nature. Besides, multigenic alterations in cancers suggest that use of a single agent or approach is unlikely to be effective in the treatment of malignancies. With the aim to develop novel therapeutic strategy for HCC, we have evaluated the effectiveness of several mono- and bicistronic antisense plasmids on the post-transcriptional regulation of two transactivator genes—a viral HBx and a cellular c-myc. While the HBx gene of HBV has been implicated in the development of HCC [24], amplification of the *c-myc* gene has been reported in a variety of human tumors including HCC [25,26]. Besides, the genetic mouse models of HCC with liver-specific co-expression of HBx and *c-myc* genes, develop liver-specific tumors rather aggressively [14,27]. In view of such striking correlation between the experimental and clinical data showing oncogenic co-operativity between HBx and *c-myc* genes leading to HCC, our antisense approach was aimed at regulating the intracellular levels (and functions) of these two genes in hepatoma cells.

We observed that all the seven antisense constructs (AS1–AS7) effectively blocked the expression of cognate genes (HBx or pc-Myc) in a concentration-dependent manner (Fig. 2). The relative efficacies of mono-cistronic antisense constructs for regulating the levels of target proteins were broadly comparable to that of bicistronic constructs suggesting no influence on antisense expression or destabilization due to co-expression. Besides, non-cross inhibition of non-target genes suggested high specificity for cognate genes (data not shown). The target specificity was

further evident from the ability of these antisense constructs to regulate expression from the X0-*myc* transgene (Fig. 4). The levels of HBx and pc-Myc expressed from this transgene were specifically regulated by corresponding antisense recombinants.

Inhibition in the levels of transactivator proteins—HBx and pc-Myc—by different antisense recombinants appeared to directly correlate with their transactivation function. Therefore as expected, stimulation of RSV-LTR by HBx or pc-Myc was specifically and significantly inhibited by respective antisense constructs. Antisense strategy has been used in the past to inhibit HBV replication with the aim to prevent HCC. For example, ASOs directed against the pre-S, surface (S), and core genes of HBV have been shown to be effective in inhibiting viral production from human hepatoma cells [28–31]. Similarly, ASOs for the pre-S region of duck hepatitis virus could inhibit active viral replication in vivo indicating its potential use as antiviral therapeutics [29]. However, none of these studies directly addressed the issue of HCC. The only in vivo study on ASOs directed against the HBx gene has given promising results by preventing the formation of preneoplastic lesions in the liver of HBx transgenic mice [32]. Our cell culture-based study appears equally promising and need to be validated further in vivo like some new anti-neoplastic ASOs [33,34]. In this regard, it will be worthwhile evaluating the efficacies of our antisense constructs on the regulation of tumor development in the X0-*myc* mouse model of HCC developed by us earlier [14]. Such a study would not only be helpful in designing clinical studies in future but also in generating valuable information on the mechanism of oncogenic co-operativity between HBx and c-Myc.

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