Regulation of tumorigenic HBx and *c-myc* expression by RNA interference

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Certificate

This is to certify that the research work embodied in this thesis entitled "**Regulation of tumorigenic HBx and c-myc expression by RNA interference**" has been carried out by Le Hung at Virology Group, under my direct supervision for the degree of Doctor of Philosophy. This work is original and no part of this thesis has been submitted for any other degree or diploma to any other University.

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Publications

1. Hung L. and Kumar V., **2004**. Specific inhibition of gene expression and transactivation functions of hepatitis B virus X protein and c-*myc* by small interfering RNAs. *FEBS Letters* 560: 210 - 214.
2. Hung L. and Kumar V., **2004**. Inhibition of hepatitis B virus HBx and murine c-*myc* genes by antisense recombinants. (under communicated).

Abbreviations

bp	Base pairs
CAT	Chloramphenicol Acetyl Transferase Assay
CMV	Cytomegalovirus
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acid
FBS	Fetal Bovine Serum
Fig.	Figure
h	hour
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B "e" antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	X protein of hepatitis B virus
HCC	Hepatocellular carcinoma
IFN	Interferon
Ig	Immunoglobulin
kDa	kilo Dalton
min	minute
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
MOPS	3-[N-morpholino]-2-hydroxypropanesulfonic acid

NaNs	None antibiotic None serum
nm	nanometer
OD	Optical Density
PAGE	Polyacrilamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PMSF	Phenylmethyl Sulfonyl Fluoride
RIPA	Radioimmunoprecipitation Assay
RNA	Ribose Nucleic Acid
RNase	Ribonuclease
rpm	Revolutions Per Minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SSC	Sodium Saline Citrate
SSPE	Sodium Saline Phosphate EDTA
SV40	Simian Virus 40
TBE	Tris borate EDTA
TBS	Tris Buffered Saline
TBS/T	Tris Buffered Saline / Tween-20
TE	Tris/EDTA
TLC	Thin Layer Chromatography
U	Unit(s)
UV	Ultraviolet
v/v	volume / volume
w/v	weight / volume
X0	HBx expression vector
Xp	X promoter
µg	microgram(s)
µl	micro liter(s)

1. Introduction

Hepatitis B virus (HBV) is one of the causative agents of chronic and acute hepatitis and is associated with the development of liver cirrhosis and primary hepatocellular carcinoma (HCC). The World Health Organization estimated that 400 millions people were chronically infected with HBV virus in the year 2000 (Lee, 1997). HCC is one of the highest relative risks known for a human cancer (Beasley, 1982). Approximately, one million people suffering from HBV-related chronic hepatitis or HCC die per year. The viral X protein (HBx) best characterized as a pleiotropic transactivator has gained attention due to its presumptive role in oncogenesis (Caselmann, 1996). Further, the c-*myc* proto-oncogene has also been found to be selectively activated in the hepatic tumors of related hosts like woodchuck (Hsu *et al*, 1988), ground squirrel (Transy et al., 1992) as well as in human HCCs (Peng et al., 1993). Therefore, c-*myc* appears to be an important host factor in creating a milieu permissive for proliferative signaling.

Though the vaccine for treatment of HBV has been used for over 20 years now as a preventive measure (Mahoney and Kane, 1999), there was a clear need for safe and effective HBV vaccines and strategies for prevention of transmission from mother to new born children. Besides vaccination is not a treatment for established infection, and up to 5% of immuno-competent recipients, especially the adults fail to make an antibody response (Arrand *et al.*, 1998). Moreover, the high cost of the vaccination program has impeded its introduction in many poorer countries where HBV infections are prevalent, afflicting 5 to 20% of the population. Interferon alpha and nucleoside analogues have been added to the method for treatment of chronic viral hepatitis. However, attempts to treat the chronic infections had only limited success, long-term response rates are in the range of only 20 - 30%. Interferon is expensive and has significant immunopathologic side effects; disadvantage of

lamivudine are that the long-term durability of responses appears to be less than with α-interferon and that prolonged therapy is often needed and is associated with a high rate of viral resistance (Lok *et al.*, 2001).

Such a scenario demanded the advent of a therapeutic technique which in not only easy to implement but also long lasting. Antisense oligonucleotides were first applied in cell culture more than 20 years ago (Zamecnik and Stephenson, 1978) and has been used for drug development for a number of diseases, as "Vitravene" treats a condition called cytomegalovirus (CMV) retinitis in people with Acquired Immunodeficiency Syndrome (AIDS) (Hogrefe, 1999). Small interference RNA (siRNA) was discovered few years back but it seems to hold a bright future after it was observed that RNA interference (RNAi) can be used in mammalian cells to silence targeted genes (Elbashir *et al.*, 2001). Double-stranded DNA (dsDNA) and RNA (dsRNA) can silence the expression of genes that are homologous to one of the strands within the duplex. Antisense and siRNAs offer two main advantages over other gene therapy approaches: Speed and Specificity (Baker *et al.,* 2001; Bennett and Cowsert, 1999; Bennett, 1998). Both antisense and siRNAs can bind to the specific RNA coding for its cognate protein, leading to its degradation hence affecting the product accumulation. With their promise of high specificity and low toxicity, it is believed that RNAi will lead to a revolution in cancer and other therapeutics (Offensperger *et al.*, 1995; Dachs *et al.*, 1997).

In a transgenic mouse model of HCC developed earlier in our laboratory, it was observed that co-expression of HBx and cellular oncogene c-*myc* results in early development of liver tumors (Singh *et al.*, 2000). Therefore, the X-*myc* mouse appears to be an excellent model for developing cancer therapeutic strategies and HCC in particular. It was proposed to develop antisense recombinants and siRNA

constructs to regulate the expression of HBx and c-*myc* that may interfere with the process of tumor development. Through this work, an attempt has been made to understand the regulation of tumorigenic HBx and c-*myc* expression by RNAi. Results presented in this thesis should be helpful not only in gaining a better insight into the molecular mechanism(s) involved in the HBV-induced liver cancer, but also generating valuable information on oncogenic co-operativity between HBx and c-*myc* and promises of gene therapy by RNAi.

2. Objectives

The proposed work will include the following three objectives:

1. Construction of antisense recombinants corresponding to the HBx and c-myc open reading frames.
2. Development of the recombinant siRNA constructs to regulate the expression of HBx and c-myc.
3. Evaluation the efficiency of the antisense and siRNA recombinants in cell culture.

3. Review of literature

3.1. VIRAL HEPATITIS

Viral hepatitis represents a global problem due to its pandemic nature. Hepatitis refers to inflammation of the liver. The liver inflammation can be triggered under various conditions such as viral infections, bacterial infections, drugs, alcohol, chemicals, and autoimmune diseases. But when medical professionals speak of hepatitis, they are usually referring to liver inflammation caused by viruses. These specific hepatitis viruses have been labeled A, B, C, D, E, G, TTV and SEN-V. The most common are typical flu- like symptoms including spoil of appetite, nausea, fever, weakness, jaundice (a yellow appearance to the skin and white portion of the eyes), and abdominal pain. While many patients recover from viral hepatitis without any specific treatment, some patients with hepatitis B and C develop chronic hepatitis. Patients with hepatitis A do not develop chronic infections. Those patients who develop chronic hepatitis B or C can be infected for many years and have a life long increased risk of developing liver cirrhosis and/or liver cancer. These differences are reflected at the route of entry of each virus and may influence its tissue distribution as well the nature of the antiviral host response, both of which may influence the tendency of each virus to become persistent.

3.2. HEPATITIS B VIRUS (HBV)

HBV, one of several hepatitis viruses, accounts for a large portion of the disease burden worldwide with upwards of 400 million chronic carriers of the disease (Lee, 1997). The HBV is the aetiological agent of both acute and chronic viral hepatitis. Three the prevalent modes of HBV transmission are through a mixing of blood products, through sexual contact, and perinatally, from mother to foetus (Ranger-Roger *et al.*, 2002; Barrett *et al.*, 1992).

HBV was first identified in 1965 by Blumberg (Blumberg *et al.*, 1965), is the prototype member of the family of *Hepadnaviridae*, a family of small DNA viruses that persistently infect liver cells. The hepatocyte is the only confirmed site of replication for all members of this virus family. HBV shares 70% sequence homology with mammalian hepadnaviruses discovered in woodchuck (WHV) (Summers *et al.*, 1978) and with ground squirrel (GSHV) (Marion *et al.*, 1980). Human HBV is also capable of infecting gorilla (GHBV), chimpanzee (ChHBV), baboons and other great apes (Seeger and Mason, 2000). More distantly related viruses have been found in Peking duck (DHBV) (Mason *et al.*, 1980), and grey heron (HHBV) (Sprengel *et al*, 1988). These viruses share a structure of enveloped virions containing 3 to 3.3 kb of relaxed circular, partially duplex DNA and virion-associated DNA-dependent polymerases that can repair the gap in the virion DNA template and have reverse transcriptase activities.

3.2.1. Epidemiology

All persons who are hepatitis B surface (HBsAg) positive are potentially infectious. The world can be divided into three areas where the prevalence of chronic HBV infection is: high ($\geq$ 8% of the population is HBsAg-positive), intermediate (2%-7% of the population is HBsAg-positive), and low (< 2% of the population is HBsAg-positive) (Mahoney and Kane, 1999). Approximately 45% of the global populations live in areas with a high prevalence of chronic HBV infection; another 43% in areas with a moderate prevalence; and 12% in areas with a low prevalence (Hollinger and Liang, 2001). In high prevalence areas, the lifetime risk of HBV infection is > 60%, and most infections are acquired at birth or during early childhood when the risk of developing chronic infection is greatest. In these areas, because most infections in children are asymptomatic, very little acute disease

related to HBV occurs. but rates of chronic liver disease and liver cancer in adults are very high. In moderate prevalence areas, the lifetime risk of being infected is 20%-60% and infections occur in all age groups (Mahoney and Kane, 1999). Acute disease related to HBV is common in these areas because many infections occur in adolescents and adults; however, the high rates of chronic infection are maintained mostly by infections occurring in infants and children. In low prevalence areas, the lifetime risk of infection is <20%. Most HBV infections in these areas occur in adults in relatively well defined risk groups (Alter, 2003). The map below outlines these areas.

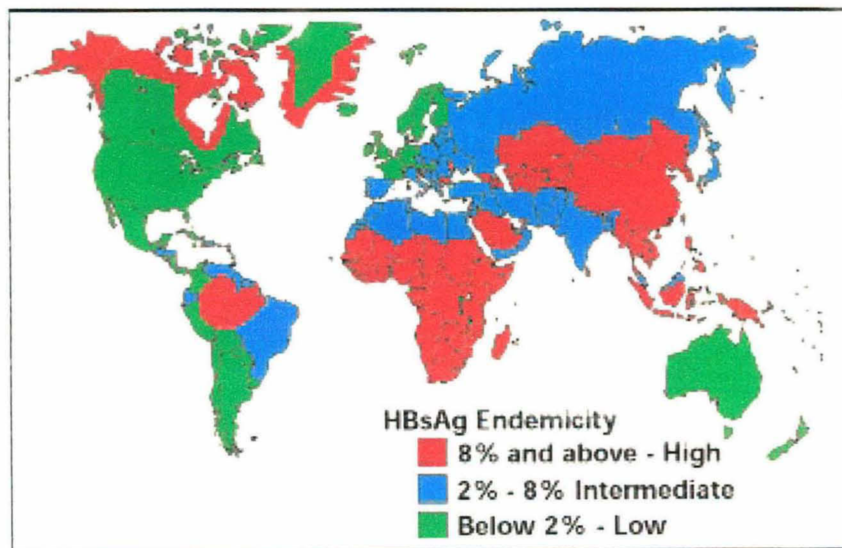


Fig. 1: Global distribution of HBV infection (WHO, 2001).

One of the reasons for chronic HBV infections is that the virus causes chronic noncytocidal infections of hepatocytes. Hepatocytes continuously shed virus into the blood stream, ensuring that 100% of the hepatocyte population is infected. Also, hepatocytes are normally long-lived, with half-lives estimated at 6 - 12 months or longer (Seeger and Mason, 2000). The combination of a long-lived, usually non-dividing host cell and a stable virus-host cell interaction virtually ensures the

persistence of infection in the absence of a robust host immune response (Nowak *et al.*, 1996).

Infection with HBV can be either acute or chronic. For acute infections, the incubation period of the virus varies from 6 weeks to 6 months, and only 33 to 50% of children and adults are symptomatic. An infection is classified as chronic when the individual has HBsAg present in his or her serum for more than 6 months (Hollinger and Liang, 2001). Chronic carriers are at higher risk for developing cirrhosis of the liver (chronic liver disease) or liver cancer (HCC) (Beasley, 1988).

3.2.2. Life cycle

HBV must first attach specifically onto a cell capable of supporting its replication. The liver is the most effective cell type for replicating HBV. As illustrated in Fig. 2, the HBV virion binds to surface of the hepatocyte through the receptors (Vanlandschoot *et al.*, 2002). Hepatotropism in viral infection is controlled by receptor specificity and also is manifested at the level of gene expression but the mechanism of HBsAg binding to a specific receptor to enter cells has not been established yet.

Viral nucleocapsids enter the cell and translocate to the nucleus, where the viral genome is delivered. In the nucleus, through an as yet unknown mechanism, the virus converts its partially double-stranded genome into covalently closed circular DNA (cccDNA), it's presence in hepatocytes indicates a successful initiation of infection (Ruiz-Opazo *et al.*, 1982). cccDNA is the template for transcription of genomic and sub-genomic viral mRNAs that are 3.5 kb, 2.4 kb, 2.1 kb and 0.7 kb in length (Chisari and Ferrari 1995; Mahoney and Kane, 1999). These transcripts are polyadenylated and transported to the cytoplasm, where they are translated into the viral nucleocapsid and precore antigen (C, preC), polymerase (P), envelope and transcriptional transactivating protein (X).

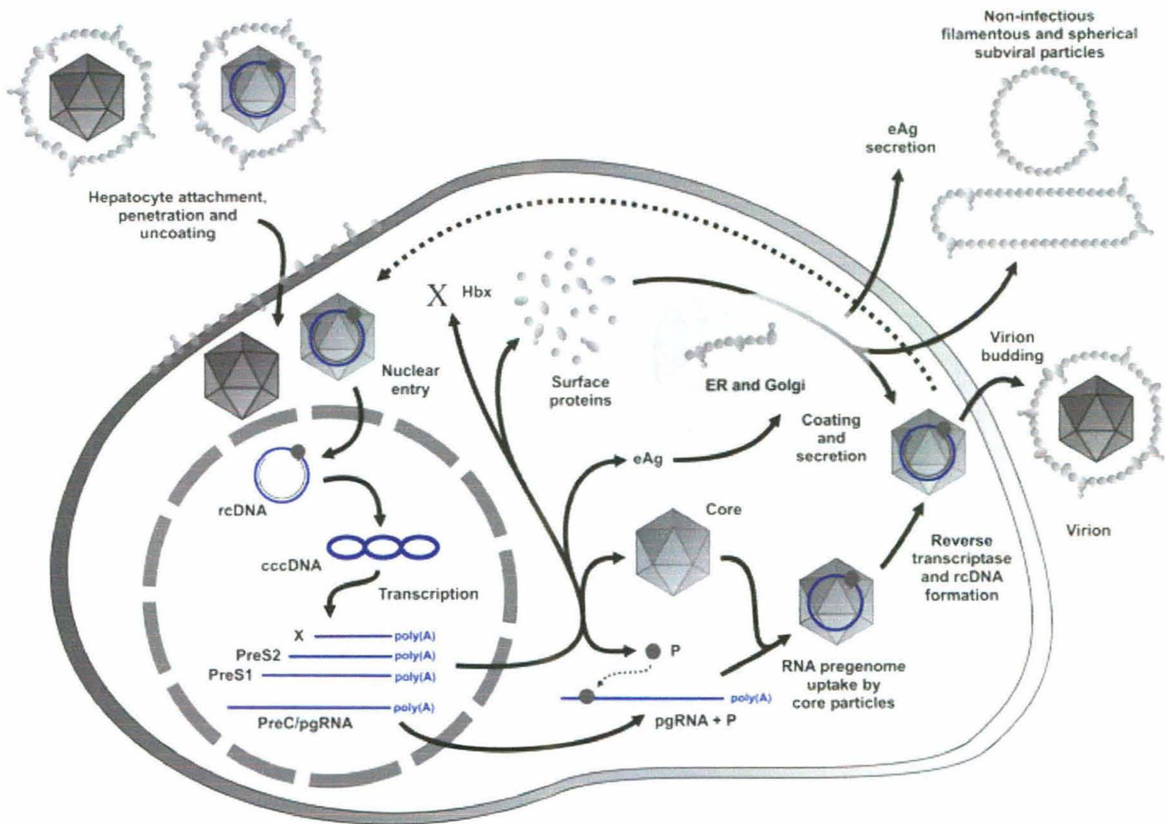


Fig. 2: A schematic illustration of the HBV infection and replicative cycle within the hepatocyte. Infectious virions attach to a cellular receptor(s) and uncoat, releasing nucleocapsids that migrate to the cell nucleus. The partially double-stranded DNA genome is converted to cccDNA, which is the template for the transcription of four viral transcripts. Translation occur following transcript export to the cytoplasm. The pgRNA interacts with two gene products, P and C, to form immature, RNA packaged, nucleocapsids. The pgRNA is reverse transcribed into DNA by P. The DNA genome can be either redelivered to the nucleus, or nucleocapsids can be coated by surface glycoproteins (in the Golgi and endoplasmic reticulum) before being exported as enveloped virions.

The pre-core polypeptide is transported into the endoplasmic reticulum (ER), where it's secreted as pre-core antigen (HBeAg). The 3.5 kb species is packaged together with HBV polymerase and a protein kinase into core particles where it serves as a template for reverse transcription of antisense strand DNA (Mahoney and Kane, 1999).

The X protein is known to interact with different transcription factors in the nucleus to stimulate signaling from the cytoplasm (Murakami, 1999).

The envelope proteins (S) insert themselves as integral membrane proteins into the lipid membrane of the endoplasmic reticulum (Mahoney and Kane, 1999).

The HBV polymerase is a multifunctional enzyme. The products of the P gene are involved in multiple functions of the viral life cycle, including a priming activity to initiate minus-strand DNA synthesis; a polymerase activity that synthesizes DNA by using either RNA or DNA templates; a nuclease activity which degrades the RNA strand of RNA-DNA hybrids, and the packaging of the RNA pre-genome into nucleocapsides (Nassal and Schaller, 1996).

3.2.3. Immunopathogenesis

The infection of HBV can result in both acute and chronic hepatitis (Chisari and Ferrari, 1995). Acute hepatitis represents a transient infection which generally runs a course of one to six months, including an asymptomatic incubation period. During this period, the immune system is either unable or not activated to gain control of the infection, and the entire hepatocyte population of the liver can get infected. Both cellular and humoral immune responses to HBV-encoded antigens are responsible for viral clearance (Chisari and Ferrari, 1995). While the humoral antibody response to viral envelope antigen contributes to the clearance of circulating virus particles, the cellular immune response to the envelope, nucleocapsid, and polymerase antigens eliminates virus infected cells.

The risk of developing a persistent or chronic infection has positive correlation with age and immunocompetence. Chronic HBV infection has been defined as carriage of HBsAg for at least 6 months (Hyams, 1995). The acute form of the disease often resolves spontaneously after a 4 - 8 week illness. Most patients recover without significant consequences and without recurrence. However, 5 to 10% still have the virus persisting in the body of adult patients and much higher in children: 70 - 90% of infants infected in their first few years of life become chronic carriers of HBV (Nakamoto and Kaneko, 2003).

Chronic hepatitis B is a prolonged infection with persistent serum levels of HBsAg, anti-HBcAg and the absence of an anti-HBsAg antibody response (Nakamoto and Kaneko, 2003). In cirrhosis, liver cells die and are progressively replaced with fibrotic tissue leading to nodule formation. The internal structure of the liver is deranged leading to the obstruction of blood flow and decrease in liver function. This damage is caused by recurrent immune responses stimulated by the presence of the virus. The complete infectious virion consists an outer lipoprotein envelope which is made up of the HBsAg, and an internal core or nucleocapsid (HBcAg) surrounding the viral genome. The HBsAg contains a group determinant called "*a*", and subtype determinants termed *d*, *y*, *w* and *r*. Hence, the HBsAg can be subtyped into four major subtypes, *adr*, *adw*, *ayw* and *ayr* (Norder *et al.*, 1992).

3.2.4. Molecular Biology

The HBV genome of 3.2 kb makes it the smallest DNA virus known to man (Robinson *et al.*, 1974). The DNA is circular, partially double-stranded, containing a longer (-) strand which is the template for the synthesis of the viral mRNA transcripts and a shorter (+) strand (Pasek *et al.*, 1979). The viral genome encodes proteins from four overlapping transcription open-reading frames (ORF), as shown in Fig. 3.

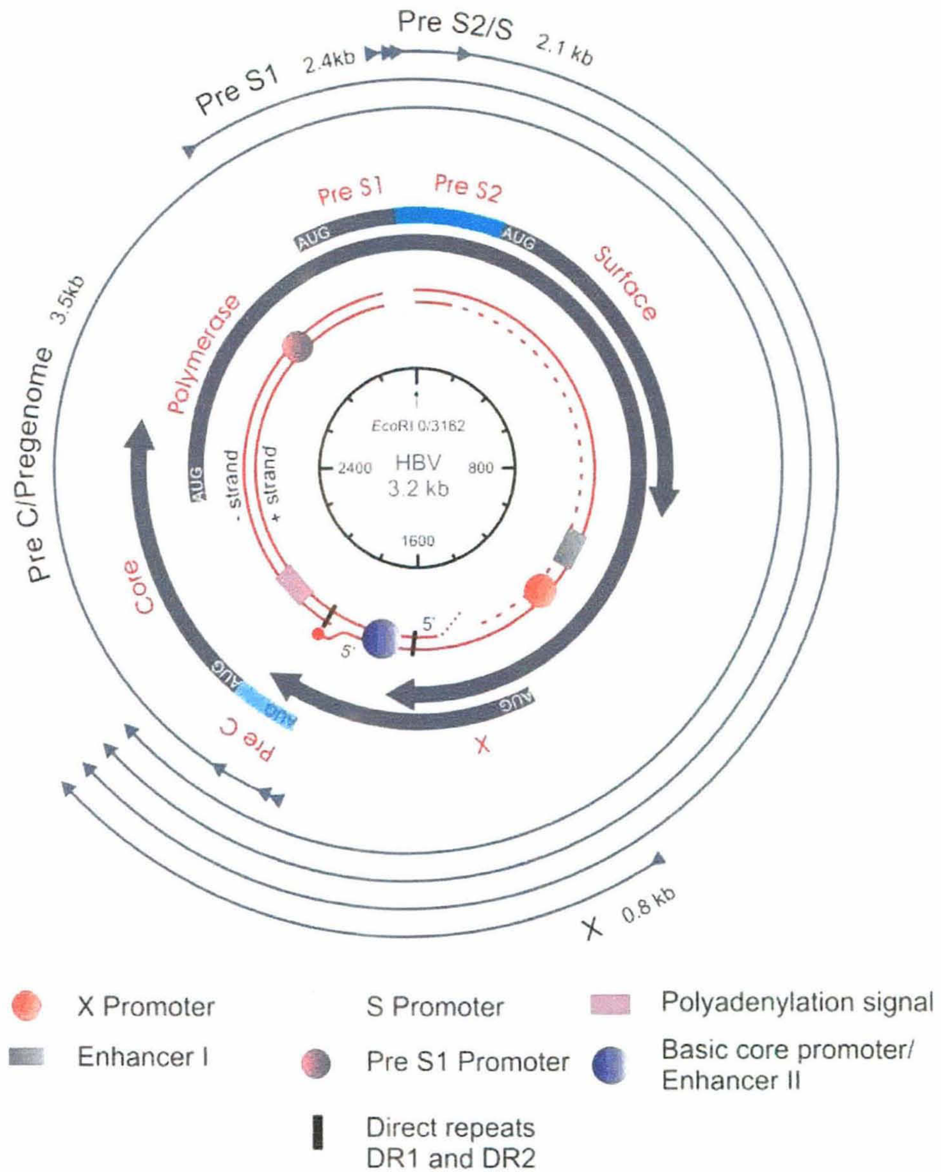


Fig. 3: Transcriptional and translational organization of the hepatitis B virus genome (strain ayw). Co-ordinates of the genome are given relative to the single EcoRI restriction site. Partially double stranded HBV DNA comprises + and – strands with cohesive complementary 5′ ends. The cis-elements that regulate HBV transcription are represented by the circular and rectangular symbols. The positions of direct repeats DR1 and DR2 are indicated as black rectangles. Immediately surrounding arrows (thick) indicate the viral open reading frames (with initiation codons) that encompass the entire genome. Four outer arrows, that give the 5′ to 3′ polarity, indicate the HBV transcripts. Multiple arrowheads at the 5′ ends of the PreC/Pregenome and PreS2/S transcripts indicate heterogeneous transcription start sites. The common 3′ end of all the HBV transcripts is depicted by the identical termination site and sequences that overlap with the HBx transcript.

These ORFs encode the pre-S/S gene coding for the envelope protein, the C gene for the core protein, the P gene for the DNA polymerase/reverse transcriptase, and the X gene coding for a small protein X that functions a pleiotropic transactivator. The shorter strand is of variable length, lacking 15% to 50% of the complementary nucleotide sequence. Cloning and sequencing of the HBV genome demonstrate that different immunologic subtypes show differences in restriction endonuclease sequences (Seeger and Mason, 2000; Tiollais *et al.*, 1985).

Using viral DNA template four major sub-genomic viral mRNAs are produced as described earlier. These polyadenylated RNAs are co-linear with the viral genome and complementary to the minus-strand DNA. They share a common termination site in the core gene that is downstream of a variant polyadenylation signal, although their 5' ends are rather heterogenous (Russnak and Ganem, 1990).

3.2.4.1. Surface antigen (S)

The surface proteins have many functions, including attachment and penetration of the virus into hepatocytes at the beginning of the infection process. It is also the primary target for neutralizing antibodies. There are three alternative translation products of the surface (S) gene including pre-surface 1 (pre-S1), pre-surface 2 (pre-S2) and the surface (S) antigen protein (Brown *et al.*, 1992). These are produced from a single ORF through alternative transcription and translation mechanisms and 2.4 & 2.1 Kb mRNA species are utilized for their expression. The S region codes 226 amino acids for the major viral surface antigen called the S protein (24 kDa). Two other proteins share the C terminal S domain, but different by length and structure of their N-terminal (pre-S) extensions. The large (L) protein (39 kDa) is encoded by pre-S1 region, the pre-S2 region and the S region; the middle (M) protein (31 kDa) contain the pre-S2 and the S region only (Seeger and Mason, 2000; Tiollais *et al.*, 1985).

The M protein contains 281 amino acids encode by pre-S2 region and the S gene. It is a glycoprotein present in two forms, gp33 and gp36, depending on the extent of glycosylation (Tiollais *et al.*, 1985). The pre-S2 domain is hydrophilic and appears to reside extracellulary. It also contains an additional glycosylation site. It appears that the pre-S2 protein is neither required for HBV infectivity nor viral particle morphogenesis. Only 10% of the large-protein pre-S2 region at its N-terminal extremity is essential for virion export and that the remaining part, dispensable for viral secretion, is also dispensable for infectivity (Le Seyec *et al.*, 1998).

HBV binds to its receptor on the plasma membrane of human hepatocytes via the pre-S1 domain of the "L" protein as an initial step in HBV infection. When pre-S1 polypeptides are synthesized in the absence of other viral protein, they are not secreted from cells, despite the presence of a complete S domain in the C-terminal half. Moreover, when co-expressed with the S polypeptides at comparable levels, they drastically inhibit the release of S proteins into the medium. This suggests that some feature of the pre-S1 protein inhibits its own export.

The N-terminal of the large surface protein, including the pre-S1 and pre-S2 domains and a portion of the S domain, remains on the cytosolic face of the ER, where it is available for interaction with nucleocapsids containing single-stranded or partially double-stranded HBV DNA (Ryu *et al.*, 2000; Wonderlich and Bruso, 1996).

3.2.4.2. DNA polymerase (P)

The P region of HBV genome codes for a polypeptide that is 832 amino acids in length (90 KDa) and rich in histidine. It possesses both DNA polymerase as well as reverse transcriptase activities. Mutational analysis has shown that it is a bipartite protein, consisting of an N-termninal polymerase domain region has linked to the 5'

end of the minus strand viral DNA. The C-terminal reverses transcriptase and RNase H domains that cleave RNA if it is present in the hybrids of RNA and DNA. Virion DNA polymerase activity is associated with the Dane particle and resides within the nucleocapsid core (Russnak and Ganem, 1990; Seeger and Mason, 2000). The "P" protein is responsible for both DNA- and RNA-dependent DNA polymerase activity during viral DNA replication. It is further endowed with two functional domains: the terminal protein at its amino terminal, and RNase H-like activity at its carboxy-terminal. The terminal protein is found covalently linked to the 5′ end of HBV minus-strand DNA and is important for the packaging of the RNA pregenome as well as acting as a primer for reverse transcription (Lott *et al.*, 2000). The viral polymerase is principally responsible for reverse transcription of preC/pgRNA to produce minus-strand DNA, concomitant degradation of the RNA template, and synthesis of plus-strand DNA. P protein is also required as a structural component for the packaging of preC/pgRNA into immature core particles and may play a role in orchestrating entry into the host cell nucleus for early replication.

It appears that the activity of P protein is dependent on metal ions in particular magnesium ions for reverse transcription to occur and the viral epsilon stem-loop (Urban *et al.*, 1998). The polymerase protein is quite immunogenic during both acute and chronic infection (Chisari and Ferrari, 1995).

3.2.4.3. Core antigen (C)

The C region, which codes for HBcAg, is in the same orientation and reading frame as the HBsAg coding sequence (Aiba, 2003). HBcAg is the most conserved polypeptide among the mammalian hepadnaviruses with 68% homology between HBV and GSHV and 92% between GSHV and WHV (Michael *et al.*, 1999; Tan *et*

al., 2003). The pre-core protein has a signal sequence at the amino-terminus and hence is directed to the ER co-translationally. A significant portion of the pre-core protein then is translocated entirely into the ER lumen, where it is processed and secreted as the *e* antigen (HBeAg). The C-terminal tail of the core protein also functions as a nuclear localization signal to allow import of core protein into the nucleus. Core protein play central role in virus maturation.

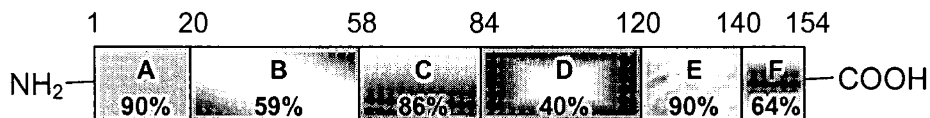
The role of core protein in viral assembly is complex and involves interactions that direct envelopment and secretion. Recent studies have confirmed that core polypeptides can bind DNA as well as RNA (Michael *et al.*, 1999).

3.2.4.4. X protein (HBx)

The existence of `X' gene was finally established in 1985 by the cross reaction of sera from the HBV-infected patients with the recombinant HBx expressed in a mammalian cell line or in *Escherichia coli* (Kay *et al.*, 1985). Although X sequences are found in both genomic and sub-genomic mRNA forms, it is encoded primarily by a 0.7 Kb mRNA species (Guo *et al.*, 1991; Suzuki *et al.*, 1989). The specific function of HBx in natural infection still remains elusive, but its presence is necessary for replication and establishment of viral infection *in vivo* in animals (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Since HBV shows extreme host tropism and cannot be grown easily in heterologous systems, most structure function studies of HBx have been carried out in the human hepatocyte cultures.

The X ORF is present in all mammalian hepadnaviruses (hepatotropic DNA viruses) including HBV (Ganem, 2001). HBx is a small, 17 kDa protein (154 aa) and although poorly immunogenetic, produces antibodies in sera of infected humans and naturally infected animals. A sequence comparison of human HBx with X proteins of other hepadnaviruses including four non-human primates (gorilla,

gibbon, chimpanzee and woolly monkey) and other small mammals like woodchuck, arctic ground squirrel and ground squirrel showed that the HBx is conserved within the group of mammalian hepadnaviruses (Kodama, *et al.*, 1985), and an analysis of codon usage led to the supposition that HBx originally evolves from a cellular gene (Miller and Robinson, 1986). Based on sequence homology, the X protein sequences can be subdivided into six regions (A through F) where the more conserved regions A, C and E are interspaced by the less conserved B, D and F regions.



The amino terminal A (residues 1 to 20) and carboxy terminal E regions (120 to 140) are two most conserved segments of HBx with 90% homology followed by 86% homology in the middle region C (residues 58 to 84). Region D (residues 85 to 119) is the most divergent part (40% homology) while regions B (residues 21 to 57) and F (residues 141 to 154) show a homology 59% and 64%, respectively. There is no evidence for a phylogenetic evolution of HBx from hepadnaviruses of non-human primates.

Hepatitis B virus X protein

Features

- Smallest ORF
- Poly A + mRNA (0.7Kb)
- 154 aa in length
- Present in all mammalian hepadnviruses
- Isoelectric point (pI): 8.3
- Binds TFs & GTFs

Functions

- Phosphorylation and kinase activities
- Transactivation
- Interaction with transcriptonal apparatus and transactivation
- Signal tranduction and transactivation
- Regulation of cell cycle
- Inhibition of protease activity and transactivation

Transforming potential

- Presence & expression in ~ 80% of HCC
- Induces oncogenes (c-*myc*, c-jun/cfos)
- Shortens cell cycle
- Induces liver tumors in transgenic mice

Table 1.

3.2.5. Properties of HBx (see table 1)

3.2.5.1. Functional domains

HBx exerts a pleiotropic effect on common biochemical pathways such as gene transcription, signal transduction, and protein degradation that are ultimately associated with cell transformation and development of HCC. The HBx-induced transcripts fall into three major categories, including genes that encode ribosomal proteins, transcription factors with zinc-finger motifs, and proteins associated with polypeptide degradation pathways (Wu *et al.*, 2001). Approximately 13 amino acids from the C-terminus and at least 50 amino acids of the N-terminus of HBx can be deleted without affecting its transactivation function (Renner *et al.*, 1995; Ritter *et al.*, 1991). Murakami *et al.* have mapped the transactivation domain to residues 52 to 148 and showed a negative regulatory domain in its N-terminal region (Murakami *et al.*, 1994). Kumar *et al* and other authors have showed that the HBx-related transactivation and transgenic studies have been identified in the region 58 to 140 in which residues 120 to 140 are critical for the nuclear transactivation (Kumar *et al.*, 1996, Reddi *et al.*, 2003) while amino acids 58 to 119 are required for signal transduction (Nijhara *et al.*, 2001). More recently, they have identified a strong regulatory function in the highly conserved N-terminal A region (residues 1 to 20) of HBx. Expression of this 20 amino acid region shows a general trans-repression function (Reddi *et al.*, 2003).

The expression of HBx is regulated primary at a transcriptional level, under the control of Enh I, that harbors more than a dozen cis-elements for the ubiquitous and tissue-specific transcriptional factors within approximately 400 bp (Gustin *et al.*, 1993, Seto *et al.*, 1989).

3.2.5.2. Phosphorylation and kinase activities

Schek *et al.* have demonstrated that HBx appears to be phosphorylated in hepatoma cells as evident from its metabolic labeling with [^{32}P]-orthophosphate (Schek *et al.*, 1991) and in insect cells as evident from mass spectrometric analysis (Urban *et al.*, 1997). Further, purified recombinant HBx serves as a substrate for MAP kinase and protein kinase C *in vitro* suggesting a possible posttranslational regulation of this viral protein (Lee *et al.*, 2001).

De-Medina *et al* have shown that HBx is associated with ribo/doxy ATPase activity (de-Medina *et al.*, 1994). A remarkable feature of this activity is that it is DNA-independent and also has tremendous sequence flexibility. Other authors also demonstrated that HBx has GTPase and GTP-ADP nucleoside diphosphate kinase activities. While ATP hydrolysis may be required to regulate the helicase and RNA polymerase II activities (Oelgeschlager, 2002; Qadri *et al.*, 1996; Wang *et al.*, 1992), GTP may be involved in the modulation of signal transduction by HBx (Benn J. and Schneider, 1994), modulation of Ca^{2+} signaling for viral replication (Bouchard *et al.*, 2001, 2003) and nuclear import machinery (Forgues *et al.*, 2001).

3.2.5.3. Role in transactivation

HBx is a transcriptional trans-activator that regulates a wide range of cellular and viral genes transcribed by RNA polymerase II (Caselmann, 1996; Rossner, 1992). HBx not only up-regulates the expression of HBV genes by trans-activating the HBV enhancer but also modifies the environment by trans-activating cellular genes in infected cells to facilitate viral replication (Renner *et al.*, 1995). HBx may have a dual role in transcriptional regulation: cytoplasmic HBx influences the regulation of second messenger systems while nuclear HBx may function at the promoter level. Nuclear HBx may directly interact with the transcription machinery to

facilitate transcription (Benn and Schneider, 1995). The capacity of HBx for nuclear localization may be limited, and most over-expressed HBx was found exclusively in a non-mitochondrial population and a mitochondrial one also (Henkler *et al.*, 2001).

3.2.5.4. Interaction with transcriptional apparatus and transactivation

HBx can interact with a variety of protein: cytoplasmic, nuclear, and those that traffick bween the cytoplasm and the nucleus. Recently, Forgues *et al.*, showed that effect of the nuclear export pathway on the subcellular localization of HBx, they also demonstrated that HBx up-regulates the activity nuclear factor kappa B (NF-κB) by inducing its nuclear translocation in an nuclear export signal dependent manner (Forgues *et al.,* 2001). These results suggest that the nuclear localization of HBx may be under the control of the nuclear export pathway and the sub-cellular localization of HBx may be influenced by the amount of its binding partners.

HBx can act as a regulator to modulate transcriptional factors. The interaction of HBx and factors belonging to the bZip (basic leucine zipper) family has been studied in detail (Barnabas *et al.*, 1997; Maguire *et al.*, 1991; Pflum *et al.*, 2001; William and Andrisani, 1995). HBx increases bZip-DNA stability by increasing the stability of the bZip dimer, as well as increasing the affinity of the dimer for DNA. The region of HBx necessary for CREB, ATF3, and ICERIIγ was mapped within aa 49 - 115 (William and Andrisani, 1995), that for NF-IL6-binding within aa 51 - 150 (Barnabas *et al.*, 1997). The DNA binding specificity of various HBx-bZIP complexes was reported to differ from one to another and from that of the bZIP itself, suggesting that HBx also recognized the composite structure of peptide DNA complex (Perini *et al.*, 1999). HBx region necessary for interaction with the bZip protein is the same as that required for enhancing DNA binding, but that the trans-activation of CREB by HBx seemed to require a larger region of HBx (aa 49 - 140)

(Barnabas and Andrisani, 2000). Recently, some data suggests that the interaction between bZip and HBx is necessary but not sufficient for increased transcriptional potency (Pflum *et al.*, 2001).

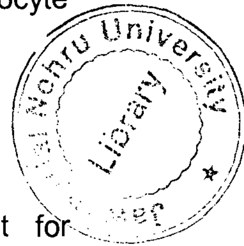
The number of target proteins that directly interact with HBx is increasing, including Smad4 in TNFα signaling (Lara-Pezzi *et al.*,1998), retinoid X receptor in phosphoenol-pyruvate carboxykinase expression (Kong *et al.*, 2000). Protesasome inhibitors prevented the rapid phase of HBx degradation and poly-ubiquitinated forms of HBx were detected (Zang *et al.*, 2000). Proteasome activity can also be inhibited by HBx over-expression. These data suggest that interaction with proteasomes may be an important process for HBx transactivation, although it is possible that proteasome inhibitors may indirectly affect HBx trans-activation.

3.2.5.5. Signal transduction and transactivation

HBx expression is under auto-regulation, and is also regulated by a variety of signaling pathways in inflammation and tumour-promoting responses (de-Medina *et al.*, 1994, Murakami *et al.*, 1994). HBx is now known to stimulate a number of other cell signaling cascades including Ras-Raf-mitogen-activated protein (MAP) kinase (Benn and Schneider, 1994; Tarn *et al.*, 2001), p38 MAP kinase (Tarn *et al.*, 2002). Activation of MAP kinases is necessary for modulating the activity of a wide range of transcriptional factors such as c-jun, c-fos and c-*myc* that in turn regulate cellular growth, differentiation and apoptosis (Hazzalin and Mahadevan, 2002). Constitutive activation of MAP kinase pathway relates to enhanced kinase activity in many tumors and in primary cell culture leading to the transformed phenotypes (Shapiro, 2002). The stimulation of MAP kinase pathway is critical for the activation of AP-1 proteins (Nijhara *et al.*, 2001). The activation of MAP kinase by HBx involves increased GTP uptake and enhanced association among Shc, Grb2 and Sos (Klein

and Schneider, 1997). The binding of HBx to the inhibitory protein IκB, may be one of the mechanisms of release and nuclear translocation of NF-κB for further transactivation (Weil *et al.*, 1999).

Klein and Schneider showed that HBx can also activate the non-receptor tyrosine kinases of the Src family which are upstream activators of Ras GTPases (Klein and Schneider, 1997). Src kinases are constitutively activated in hepatoma cells during the replication of WHV and HBV only in the presence of a functional HBx (Klein *et al.*, 1999). HBx may involve the upstream calcium-dependent PyK2 to activate Src kinases (Bouchard *et al.*, 2001). Src kinases can also activate the JAK/STAT cell signaling pathways that are associated with increased hepatocyte proliferation (Cressman *et al.*, 1995).



3.2.5.6. Anti- and pro-apoptotic effects of HBx

Controlled cell growth and programmed cell death are important for maintaining a normal tissue homeostasis in an organism. The X protein of hepatitis B virus (HBx) is a multifunctional protein that can interact directly with p53 (Elmore *et al.*, 1997), but the inhibition of p53 and the trans-activation are distinct functions of HBx (Lin Y *et al.*, 1997). By associating with p53, HBx can against p53 functions both directly and indirectly. HBx expression was down-regulated by genotoxic stress in a p53-dependent manner. The expression level of p53 seemed to be down-regulated by HBx when HBx direct binding p53. The cis-element of the p53 promoter responsible for the inhibition of HBx was presumed to be the E box, which is the binding site of the bHLH family, including *Myc*, Mad, Max and many transcriptional activators and repressors (Lee and Rho, 2000). These data suggests that HBx and p53 are mutually inhibitory through different mechanism; direct binding and mutual down-regulation at the transcriptional level (Murakami, 2001).

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HBx may also interfere with the nucleotide excision repair (NER) pathway through both p53-dependent and p53-independent mechanisms (Groisman *et al.*, 1999). Because HBx binds to TFIIH-associated proteins, it may interfere with the NER pathway by binding to and altering the activities of helicases necessary for NER activity. This can increase the mutation rate induced by chemical carcinogens, such as aflatoxin B1, during human liver carcinogenesis (Bressac *et al.*, 1991; Jia *et al.*, 1999; Sohn *et al.*, 2000). Further, HBx can significantly inhibit the ability of cells to repair damaged DNA by interacting with XAP-1 (Becker *et al.*, 1998; Jia *et al.*, 1999). The roles of HBx in cell death pathways have been shown in a variety of systems, and the results vary depending on the sources from where the cells were derived, e.g - was transgenic, immortal or cancer-derived cell, and depending on the ecotropic expression of HBx in cells. In apoptotic pathway, HBx can associated with caspase 3 and inhibit caspase 3 activity in rat fibroblasts and human hepatoma cell line (Gottlob *et al.*, 1998). Several indirect role of HBx in pre-apoptotic and apoptotic pathways were proposed. Trans-activation of the NF-κB family by HBx was reported to be related to the resistance of HepG2 cells to anti-Fas killing (Pan *et al.*, 2001). But another studies reported that the sensitization of apoptotic processes by HBx, probably through c-*Myc* activation and stress related MAP kinase pathways (Su and Scheneider, 1997) and HBx exerts anti-apoptotic action through the induction of the NF-κB family, although *Myc* induction by HBx may sensitize cells to killing by HBx plus TNF-α (Su *et al.*, 2001).

The X protein also shows pro-apoptotic effect on liver cells that could be both p53-mediated as well as p53-independent (Elmore *et al.*, 1997; Terradillos *et al.*, 1998). *Myc* protein also appears to sensitize cells to killing by HBx in the presence

of TNFα (Su *et al.*, 2001). The pro-apoptotic action of X protein may have a role in viral spread during the acute phase of HBV infection.

3.2.5.7. Role in hepatocarcinogenesis and co-operation with other oncogenic

Virus - associated tumours comprise approximately 15% of all tumours in human worldwide (Bishop, 1982). Among the virus-associated tumours, approximately 30% are HBV-associated hepatocellular carcinoma, which means 4.5% of all tumours worldwide (Dornburg and Pomerantz, 2000). The mechanisms of oncogenesis by HBV remain obscure. HBV may act non-specifically by stimulating active regeneration and cirrhosis which may be associated with long-term chronicity. HBV carriers have ~ a 100-fold excess risk of developing HCC relative to non-carrier, and the lifetime risk of a chronic HBV carrier developing HCC may be as high as 50% in male (Beasley, 1982). Studies showed that integration of HBV DNA into human DNA were performed initially in the HCC cell line of the patient, who carrier HBsAg (Marion *et al.*, 1980). Also, the presence of integrated HBx genes is a significant proportion of hepatocellular tumours (Zhou *et al.*, 1987). HBx plays a role in the development of HCC because HBx is a more prevalent marker of HBV infection in HCC than the HBV surface and core antigens (Cromlish, 1996).

The first direct evidence of HBx's role in cell transformation/HCC came from a transgenic mouse model in which constitutive expression of the HBx gene under its own regulatory elements led to the development multi-focal areas of altered hepatocytes progressing to benign adenomas and then to malignant carcinomas (Kim *et al.*, 1991; Koike *et al.*, 1994). Madden *et al* demonstrated that HBx expression increased the incidence cancer in transgenic mice treated with diethylnitrosamine (DEN), a hepato-carcinogen, and they also showed that the HBx

transgene augmented mutation frequency, but they could not find any increase in spontaneous mutation in the HBx transgenenic mice (Guo *et al.*, 1991; Madden *et al.*, 2001). Further HBx gene is frequently integrated into the genome of hepatocellular carcinoma cells, inhibition of p53-mediated apoptosis by HBx may give a clonal selective advantage for these cells during the early stages of hepatocarcinogenesis (Huo *et al.*, 2001). Some reports showed that HBx is capable of binding directly to p53 *in vitro* and *in vivo* (Truant *et al.*, 1995; Feitelson *et al.*, 1993; Wang *et al.*, 1994). It has also been reported that p53 is sequestered in the cytoplasm of HBx transgenic mice, leading to its functional inactivation and - as the authors hypothesize - to HCC (Terradillos *et al.*, 1997). These results suggests that HBx alone has no directs carcinogenic role, but that it makes transgenic mice more sensitive to a carcinogenic environment. Selective expression of HBx in the tumor cells could be a reason for chromosomal rearrangement (Livezey *et al.*, 2002; Su *et al.*, 1998).

Terradillos *et al* has demonstrated that HBx alone has no direct pathological effect but it is shown to accelerate tumor development induced by *c-myc*. This data also firmly establish the oncogenic potential of HBx, apparently acting as a tumor promoter (Terradillos *et al.*, 1997).

3.2.6. Transgenic animal models

The first report of direct evidence that HBx might be able to induce transformation is provided by a transgenic mouse model in which constitutive expression of the HBx gene under the control of its own promoter-induced liver tumours at very high frequencies (Kim *et al.*, 1991; Koike *et al.*, 1994). Using a transgenic mouse model where expression of a single HBV gene product, the HBx protein, induces progressive changes in the liver, Ueda *et al.*, showed that tumour

development correlates precisely with p53 binding to HBx in the cytoplasm and complete blockage of p53 entry into the nucleus (Ueda *et al.*, 1995). In other transgenic model, Terradillos showed that HBx alone has no direct pathological effect but it is shown to accelerate tumor development induced by *c-myc*, HBx may contribute to HBV pathogenesis by enhancing apoptotic death in the chronically infected liver (Terradillos *et al.*, 1997, 1998). HBx might trigger an apoptotic process in HBV-infected hepatocytes. HBx expression may contribute to the development of DEN (diethylnitrosamine)-mediated carcinogenesis by promoting the proliferation of altered hepatocytes rather than by directly interfering with the repair of DNA lesions (Madden *et al.*, 2001). But some transgenic model studies of HBx transgenic mice in which no effect of HBx on liver histology was apparent (Madden *et al.*, 2000). In an another hepatitis B 'x' (HBx) transgenic mouse model, some studies have illustrated the interference of cell growth and death because of the collaborative influence of HBx and *c-myc* genes that resulted in the development of hepatocellular carcinoma after a rather short latent period (Kumar *et al.*, 2001; Lakhtakia *et al.*, 2003).

Transgenic animal models will play an increasingly important role in evaluating therapeutic targets and strategies, intended either to treat and eventually eradicate existing cancers or to prevent Hepatitis B virus from developing into HCC disease.

3.3. THERAPEUTICS

HBV infection is a public health problem worldwide, particularly in East Asia. HBV one of the major causative agents of HCC is prevalent in South East Asia and as per projected epidemiological survey will be one of the major reasons for mortality in years ahead. There has been a vast increase in our understanding of the development of liver cancer over last few years, and new treatment modalities based on this knowledge are being tried out. The current therapy for HBV infection

is mostly based on chemical agents (α-Interferon, lamivudine, cytokines ...) and one type of gene therapy - "gene transfer" - implants new, functional genes into cells to alter their function of confer resistance to infection. Gene therapy strategies not only insert new genes into cells, but also attempt to inhibit or repress specific genes already in the cell. Antisense gene therapy targets the transcription and translation of the virus hence preventing the process of protein synthesis. The mechanisms of action of this therapeutics are outlined in Fig. 4.

3.3.1. Interferon (IFN)

Interferon-α was first reported to have beneficial effects in chronic hepatitis B in small uncontrolled studies in the 1970 and in 1992 IFN-α was approved for use in persons with chronic HBV infection (Fried, 1996; Gewirtz *et al.*, 1998). IFN-α has been used treatment for both of HBeAg-positive and negative chronic hepatitis B. Interferon's principal mechanism of action is thought to include both antiviral and immunomodulatory effects (Kuhen and Samuel, 1999).

Most controlled trials of IFN therapy of HBeAg-positive chronic hepatitis B have included only a 1 year follow-up. Studies from North America and Europe reported that 95% - 100% of responders remained HBeAg negative during 5 - 10 years of follow-up and ultimately 30% - 86% of responders lost HBsAg (DeMarco *et al.*, 1999; Lau *et al.*, 1997). In contrast, long-term follow-up of patients in Asian studies showed a lower rate of durable responses, only rare loss of HBsAg, and ultimately a loss of HBeAg in similar proportion of controls as treated patients (Lin *et al.*, 1999). Analysis of results of trials of IFN α in HBeAg-negative chronic hepatitis B showed that 40% - 60% of patients have an on-therapy response to IFN α, but at least half of patients relapse when therapy is stopped, and relapses can occur months and even years after therapy (Papatheodoridis *et al.*, 2000).

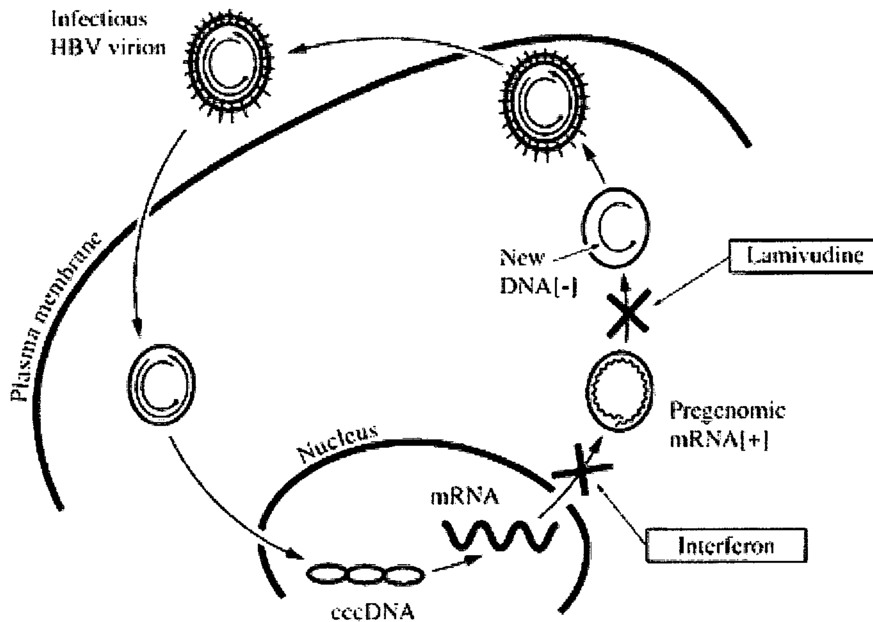


Fig. 4: **Mechanism of hepatitis B virus (*HBV*) replication and the site of action of different treatment methods.** After HBV endocytosis into hepatocytes, the genome is translocated to the nucleus and converted to covalently closed circular DNA (*cccDNA*), which is transcribed and translated to form an RNA intermediate. Translocation of pregenomic RNA can be inhibited by interferon-α, and reverse transcription of the pregenomic RNA by polymerase to HBV DNA can be inhibited by nucleoside analogues. Association of the partially double-stranded DNA with envelope proteins leads to the formation of mature HBV particles that are then released from the hepatocyte. mRNA = messenger RNA.

IFN's have significant side effects, including flu-like symptoms; fever; myalgia; mild bone marrow suppression; thyroid abnormalities in 2% to 5% of patients; and psychiatric side effects, such as depression, in approximately 15% of patients (Renault *et al.*, 1987). Also IFN is not effective in all patients. Despite patient selection, only 30% to 40% have achieved sustained responses. Clinical trials of IFN have been unsuccessful for patients infected with pre-core mutant strains of HBV (Brunetto *et al.*, 1993).

Because the overall rate of beneficial response to IFN α is not satisfactory and re-treatment is rarely helpful, so other agents such as prednisone, interferon gamma, thymosin, didanosine, levamisole, and acyclovir have been used against hepatitis B (Hoofnagle and Lau 1997). Other chemotherapeutic agents which hold promise for the treatment of patients with chronic hepatitis B are several new nucleoside analogues, such as famciclovir, lamivudine, lobucavir. These drugs were developed as therapy for other viral infections and later found to have activity against HBV both *in vitro* and *in vivo* (Doong *et al.*, 1991). Lamivudine has been evaluated most extensively.

3.3.2. Lamivudine

Lamivudine is the first nucleoside analogue used in chronic HBV infection (Josefson, 1998) and is the only nucleoside analogue that has been studied in long-term clinical trials. Lamivudine competitively inhibits viral reverse transcriptase and terminates pro-viral DNA chain extension (Cammack *et al.*, 1992; Schalm *et al.*, 1995). Unlike IFN, lamivudine and the other newer nucleoside analogues, such as lobucavir, famciclovir and adefovir, do not affect the host immune response. Lamivudine decreases HBV replication by approximately 3 to 4 log copies in most patients (Honkoop *et al.*, 1998). It induces a more rapid pattern of response than

interferon: Levels of HBV DNA showed a median reduction of 97% after 2 weeks and 98% by 1 year. In the doses used for chronic HBV infection, lamivudine has an excellent safety profile (Leung *et al.*, 1998). However, drug resistance has been noted in the form of mutation at the YMDD locus in patients receiving long-term therapy with lamivudine (Allen *et al.*, 1998; Niesters *et al.*, 1998). Because lamivudine-resistant mutants do not replicate as well as wild-type virus (Melegari *et al.*, 1998), wild-type virus quickly replaces the mutated virus if treatment is discontinued. Thus, it is a difficult proposition to stop lamivudine treatment because of the risk for disease exacerbation (Chayama *et al.*, 1998; Malik and Lee, 2000) and hence uneconomical for a vast majority population.

Other drug as mentioned above can be used as combination therapy but none have lasting beneficial effects on HBV DNA levels or disease activity, and many are toxic or poorly tolerated. Additionally, the cost for treatment chronic HBV infections is fairly expensive.

3.3.3. Antisense oligonucleotides (ASO's)

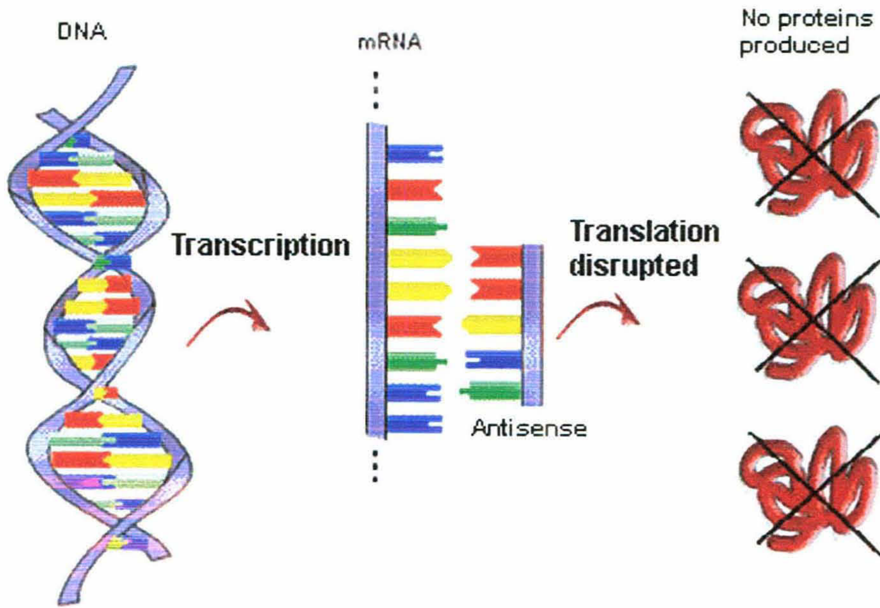
In the past two decades, a lot of ASO's have been developed and tested in preclinical and clinical studies against a wide range of target genes. Many of them have shown *in vitro* reduction in the target gene expression and promising activity against a variety of tumors.

Antisense therapy works on the principle of inhibition of translation as well as down-regulation of target transcripts (Paterson *et al.*, 1977; Stephenson and Zamecnik, 1978; Mizuno *et al.*, 1984; Rosenberg *et al.*, 1985) (Fig. 5A). However, because of the multigenic alterations of tumors, the use of ASOs as single agents does not seem to be effective in the treatment of malignancies. Goodarzi *et al.* has demonstrated the effect of a series of antisense oligo deoxyribonucleotide

[oligo(dN)] ~ 15-mer on the expression of the surface antigen (HBsAg) gene of human hepatitis B virus (HBV). They showed that oligo(dN) directed at the cap site of mRNA and regions of the translational initiation site of the HBsAg gene was highly specific to the HBsAg gene and appeared to be at the level of translation (Goodarzi *et al.*, 1990). Also using antisense oligodeoxy-nucleotides in primary duck hepatocyte cultures *in vitro* as well as in DHBV-infected Pekin ducks *in vivo*, Offensperger *et al.* showed that most effective antisense oligo-deoxynucleotide was directed against the 5' region of the pre-S gene and resulted in a complete inhibition of viral replication and gene expression *in vitro* and *in vivo* (Offensperger *et al.*, 1993). Since then, hundreds of other oligos have been successfully targeted to HBV genome and have showed that the antisense phosphorothioate oligo-deoxynucleotides can inhibit HBV replication in the human hepatoma cell line, oligonucleotides directed against the 5'-region of the HBV surface antigen (HBsAg) gene (S gene), the preS1 open reading frame, and the HBV core antigen (HBcAg) gene (C gene) were effective at depressing virus production, while molecules targeting the HBV e antigen (HBeAg), open reading frame and the HBV polymerase gene were little effective (Korba and Gerin, 1995; Moriya *et al.*, 1996; Nakazono *et al.*, 1996; Yao *et al.*, 1995, 1996). These experiments showed that antisense phosphorothioate oligonucleotides can substantially block viral gene expression and viral replication after transfection of HBV DNA.

Less commonly used, but triplex-forming-oligonucleotides (TFOs) which directly binds to double-stranded DNA are also seem to have promising application. Some authors showed that TFOs can inhibit transcription both *in vitro* and *in vivo* (Bailey *et al.*, 1998; Cooney *et al.*, 1988; Durland *et al.*, 1991; Duval-Valentin *et al.*, 1992; Ing *et al.*, 1993). The idea used in this technique is to block RNA polymerase transit, occupy the binding sites of transcription factors, or induce sequence specific damage to DNA.

A



B

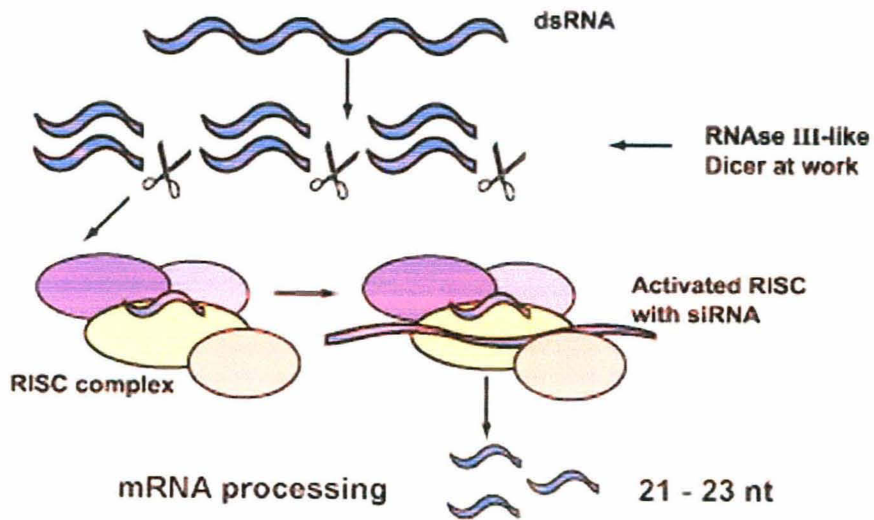


Fig. 5: Mechanisms of RNA interference

A. Antisense

B. siRNA

3.3.4. Ribozyme

An alternative to targeting with oligo-deoxynucleotides is the utilization of hammerhead ribozymes. Ribozymes are small catalytic RNA molecules that can cleave targeted RNA in a sequence-specific manner. Hairpin ribozymes have been shown to have the potential to disrupt HBV replication by targeting the pre-genomic RNA as well as specific mRNAs encoding the HBV surface antigen (HBsAg), the polymerase and the X protein (Weinberg *et al.*, 2000). Two of three ribozyme tested were capable of cleaving their respective RNA substrates and it can reduce the level of viral particle production. X protein also was objective for ribozyme (Kim *et al.*, 1999; Weinberg *et al.*, 2000). Some ribozymes have been found to be effective in inhibiting the expression of functional HBx protein and decrease HBV mRNA encoding surface antigen (Passman *et al.*, 2000).

3.3.5. RNA interference

Posttranscriptional gene silencing (PTGS) is a nucleotide sequence-specific defense mechanism that can target both cellular and viral mRNAs. A natural manifestation of PTGS is the RNA-mediated defense induced in virus-infected cells (Ratcliff *et al.*, 1997). The sequence-specific described class of small (20 - 23 nt) RNA molecules. They are produced from larger precursor transcripts of 90 - 100 nt, which have a hairpin structure (Hutvagner *et al.*, 2001). They bind to mRNAs that have complementary sequences, and thus they can regulate gene expression by modulating transcript stability or translation of the target mRNA (Fig. 5B).

Recently, the discovery of RNA interference was demonstrated the potent and role of double-stranded RNA (Fire *et al.*, 1998). RNAi promises as a new approach to treat viral-induced diseases, including viral-induced cancer as hepatocellular carcinoma. Unlike siRNAs, functional small-hairpin RNAs (shRNAs) can be

expressed *in vivo* from DNA templates using RNA polymerase III promoter (Paddison *et al.*, 2002; Tuschl, 2002).

The discovery that dsRNA can be used for RNA interference in certain invertebrates and plants have allowed the scientists thinks about its application in mammalian cells. In plant and drosophila cells, siRNAs are generated by dicer, an endonuclease (Bernstein *et al.*, 2001) that cleaves long dsRNA molecules into fragments of 21 to 23 nt (Elbashir *et al.*, 2001b; Zamore *et al.*, 2000). These siRNAs associate with helicase and nuclease molecules to form a large complex, termed "RNA-induced silencing complex", which unwinds siRNA and directs precise, sequence-specific degradation of mRNA.

Based on specific effectiveness in silencing endogenous genes, some authors had concentrated studies inhibition of hepatitis B virus expression and replication by siRNA to establish a new small animal model and new treatment options can be studied easily. They showed that siRNA can reduce the process hepatitis B virus transcription and protein in cell culture, as well as in the viral replicate forms (Hamasaki *et al.*, 2003; Konishi *et al.*, 2003; McCaffrey *et al.*, 2003; Shlomai and Shaul, 2003). Recently, some experiments *in vivo* found that siRNA can led to a significant inhibition in the level of viral transcripts, viral antigens and replication (Chen *et al.*, 2003; Giladi *et al.*, 2003; Klein *et al.*, 2003).

siRNA approach have advantage over antisense in that lower concentrations of the same are required to achieve levels of knockdown that are comparable to antisense reagents. Also, siRNAs can be expressed intracellularly from RNA polymerase III promoter (Miyagishi and Taira, 2002; Paul *et al.*, 2002; Thompson, 2002), that enables the production of stably expressing siRNA cell lines with sustained knockdown of a target and the potential to produce transgenic animals.

3.3.6. Vaccine

In order to combat the life-threatening effects of hepatitis B infection, hepatitis B vaccines have been developed in 1970s. Individuals who recover from acute hepatitis B infection produce antibodies (anti HBs) to HBsAg and have lifelong immunity to re-infection. First generation vaccines, comprising HBsAg from the donated plasma of hepatitis B carriers, are still in use in some country. This vaccine will prevent perinatal transmission from viraemic mothers to their infants, especially if the first dose is given within 24 h of birth. However, protective efficacy rates vary widely (from 70% to 90%). Plasma-derived vaccines remain an option, particularly in areas where carrier rates are high, providing that safety and quality control measures are adhered strictly.

The recombinant vaccine for hepatitis B was developed using expression systems for HBsAg in yeast (*Saccharomyces cerevisiae*) and mammalian (Chinese hamster ovary, CHO) cells. Yeast systems give rather higher yields and are used more commonly. Recombinant plasmids are based on the self-replicating element, the 2 μ circle, and an expression cassette comprising the surface gene driven by a strong yeast promoter (GAL1 or PHO5) and yeast transcriptional termination sequences. Most plasmid constructs encode only the 226 aa major surface protein N-terminal and C-terminal but inclusion of pre-S2 and pre-S1 sequences is possible. HBsAg synthesized in mammalian cells is secreted into the culture fluid as 22 nm particles which resemble those found in the circulation of carrier. Yeast-derived vaccines which lack pre-S components are roughly equivalent to plasma-derived vaccines in terms of immunogenicity and protective efficacy (Arrand *et al.*, 1998).

As stated earlier, up to 5% of immunocompetent individuals fail to respond to a full course of vaccine. Immunological studies in mice suggest that the immune

responses to the major surface protein and pre-S domains are regulated separately and pre-S2 epitopes may augment the response to the major surface protein (Milich and Leroux-Roels, 2003).

Alongside the efforts to develop preventive vaccine, there are a range of approaches under investigation for treatment of cancers. Gene therapy is one such new therapeutic strategy against HBV infection, involving the transmission of "gene" drugs into liver cells by specific delivery systems and methods including antisense oligonucleotides (ASOs), ribozymes and RNA interference.

RNA molecules are attractive because they form more stable duplexes with their mRNA targets. In theory, this might lead to more efficient antisense effects. Nevertheless, because of the stability issues discussed above, antisense RNAs and ribozymes are typically expressed inside the cell from a vector designed for this purpose. This is problematic for all of the reasons that are now widely appreciated, including efficiency of transfection, expression, host cell range, and vector persistence (Verma and Somia, 1997). Despite these concerns, expressing antisense RNA or ribozymes from a vector is often the only practical approach one can take when long-term presence of the antisense sequence is desired, ie, when attempting to target an mRNA that encodes an abundant and long-lived protein. It should be noted that antisense RNA, especially in the form of ribozymes, has been delivered to cells externally (Castanotto *et al.*, 1997). For this approach, the RNA molecules must be protected, eg, by stabilizing the phosphodiester bonds, and by packaging the material in liposomes (Gewirtz *et al.*, 1998).

4. Materials & Methods

4.1. MATERIALS

4.1.1. Reagents and Enzymes

All reagents and enzymes used were of molecular biology grade and were supplied by the following companies: Armersham (UK), Boheringer Mannheim (Germany), Clontech (USA), Invitrogen (Netherlands), Merck (India), New England Biolabs (UK), Promega (USA), Pharmacia (USA), Serva (Germany), Sigma (USA).

4.1.2. Antibodies:

Monoclonal antibody against HBx (B-8/2/8) was produced in our laboratory (Kumar *et al.*, 1998). Other antibodies were procured through commercial sources: goat anti-mouse IgG-HRP (200 μg/ml), c-*myc* (C8), mouse monoclonal IgG_{2a} (200 μg/ml) (both Santa Cruz Biotechnology). Protein A Sepharose (Amersham Pharmacia Biotech).

4.1.3. Plasmids:

- pSG5 (Stratagene, USA)
- pIRES1-neo (Clontech, USA)
- pEGFP1-RSV (kind gift from Dr. Ravi Mahalingam, USA)
- pPolyIII (Lathe *et al.*, 1987).
- p*Silencer*™ 1.0-U6 siRNA (Ambion)
- pGEM-T (Promega)
- pCMV-neo: the IRES fragment of pIRES1-neo was removed by digestion with *Eco*RV & *Sma*I and re-ligated vector to generate this vector.
- pSG5-X0 (HBx expression vector) (Kumar *et al.*, 1996)
- pSG5-*myc*: expression vector for the murine c-*myc* cDNA
- SV40-pc-*myc*: expression vector of HBV precore-murine c-myc fusion protein

4.1.4. Antisense constructs

The following three antisense constructs were developed and cloned either in pCMV-neo or in pSG5 expression vectors.

4.1.4.1. ***A1**: pCMV-neo - Xp-anti-X0:* contains the promoter of HBx gene (Xp) and the X open reading frame (X0).

4.1.4.2. ***A2**: pCMV-neo - SV40-anti-X0:* contains the SV40 promoter and the X0.

*4.1.4.3.**A3**: pSG5-anti-myc:* contains murine *c-myc* cDNA in the antisense orientaion

4.1.5. shRNA constructs

The following four constructs were developed and cloned in pSilencer vector:

4.1.5.1. **X-D**: shRNA for D domain of the X open reading frame (X0)

4.1.5.2. **X-E**: shRNA for E domain of the X open reading frame (X0)

4.1.5.3. **M-T**: shRNA for transactivation domain of the *c-myc*

4.1.5.4. **M-Z**: shRNA for Leucine-zipper domain of the *c-myc*

Hairpin siRNAs were designed as Ambion protocol and Tusch's ruler.

4.1.6. Cell lines:

- COS-1: SV40 transformed African Green monkey kidney. (ATCC CRL 1650).
- Huh-7: Human hepatoma cell line (Nakabayashi *et al.*, 1982)

4.1.7. Bacterial strains:

- *E. coli* cells (DH5α)

4.1.8. Kits:

- GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia, USA)
- Phototope®-HRP Western Blot Detection System, with Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology, USA).

- Nick Translation Kit (Promega, USA).

- Trizol Reagent (Invitrogen, USA).

4.1.9. Radioactivity:

- [γ–^{32}P]-ATP (6000 Ci/mmol)

- [α–^{32}P]-dCTP (3000 Ci/mmol)

- [^{35}S] Protein labeling mix (1175.0 Ci/mmol)

- [^{14}C] Chloramphenicol

4.1.10. X-ray film

- Kodak BioMax MR film (USA)

4.2. METHODS

4.2.1. Competent cells and transformation

Appropriate *E. coli* cells (DH5α) were streaked onto LB-Agar plates and incubated overnight at 37°C to get bacterial colonies. A single colony was inoculated into 5 ml of LB-broth and shaken at 200 rpm overnight at 37°C to get saturated starter culture. Finally, 1 ml of this culture was transferred into 100 ml of LB medium and shaken at 200 rpm, 37°C until O.D_{600} nm reach 0.6. The cells were chilled on ice for 30 min and aseptically transferred to ice-cold 50 ml poly-propylene tubes. The cells were recovered by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the cells re-suspended in 25 ml chilled 50 mM calcium chloride. After incubation on ice for 30 min, the cells were centrifuged again 10 min at 4°C. The pellet was re-suspended in 2 ml of 50 mM calcium chloride. Competent cells were either used immediately or stored at -70°C with 20% glycerol.

Transformation was carried out with 100 ng of DNA using 100µl of competent cells. The cells were incubated in microfuge tubes on ice for 30 min. The samples

were heat-shocked by placing tubes at 42°C for 90 second and transferred immediately on ice for 5 min. LB-medium (800 μl) was added to each tube and incubate further at 37°C for 45 - 60 min. Finally, the samples were centrifuged at RT for 5 min at 5000 rpm, and the pellet was re-suspended in 100μl of LB-medium and spread onto LB-Agar plates containing appropriate antibiotics pre-warmed at 37°C and incubated overnight at 37°C.

4.2.2. PCR amplification of DNA fragments

Each PCR reaction was setup as follows:

✶	Template DNA (100ng/μl)	1.0μl
✶	Forward Primer (5') (10pmol/μl)	10.0μl
✶	Reverse Primer (3') (10pmol/μl)	10.0μl
✶	Taq Polymerase (5 units/μl)	0.5μl
✶	Taq polymerase Buffer (10X)	5.0μl
✶	dNTP mix (10mM each)	4.0μl
✶	Sterile Distilled Water	19.5μl
	Total	50.0 μl

4.2.2.1. Template used for amplification

The template used for experiments was HBV genome or shRNA cloning.

4.2.2.2. Primers used for amplification

For: *X promoter (Xp)*

*Bam*HI *Hind*III

GT1a (FORWARD): 5'- CG**G GAT CCA AGC TT**C CTG TTA ACA GGC C -3'

*Eco*RI

GT2a (REVERSE): 5'- G**GA ATT C**AC GAG AGG ACG ACA G -3'

For: *X0*

HBx17 (FORWARD): 5'- GCT GCT AGG CTG TAC TGC -3'

HBx30 (REVERSE): 5'- TGC AGA GGT GAA GCG AAG -3'

For: *pre-core (pc)*

*Bgl*II

GT4 (FORWARD): 5'- GA**A GAT CT**A TGC AAC TTT TTC ACC TC -3'

*Bam*HI

GT5 (REVERSE): 5'- CG**G GAT CC**C CAA AGC CAC CCA AG -3'

For: *shRNA,* both shRNA cloning using T3 primer as reverse primer:

T3: 5'- ATT AAC CCT CAC TAA AG -3'

- Gene-specific forward primer:

For shRNA (X-D): D11_HBx: 5'-TGT CAA CGA CCG ACC TTG AGG -3'

For: *shRNA (X-E):* E11_HBx: 5'-GGT CTT TGT ATT AGG AGG CTG T -3'

For: *shRNA (M-T):* TAD11_myc: 5'-GAA CAT CAT CAT CCA GGA CTG -3'

For: *shRNA (M-Z):* L-zipper11_myc: 5'- ACT CGA ACA GCT TCG AAA CTC T -3'

4.2.2.3. Purification of the PCR amplified DNA fragment:

The PCR products were digested with appropriate enzymes; incubated at 37°C for 2 h. After inactivating enzymes at 65°C for 10 min, each sample was purified using GFX™ PCR DNA and Gel Band Purification Kit as per manufacturer's protocol and cloned in corresponding vectors.

4.2.3. Preparation of plasmid DNA

4.2.3.1. Small scale (Mini) preparation

DNA was isolated as described in Sambrook & Russell (2001). Aliquots (10μl) were used for restriction analysis, otherwise stored at –20°C until further use.

4.2.3.2. Large scale (Ultrapure) preparation by CsCl density gradient centrifugation

DNA was isolated according to Sambrook & Russell (2001). Aliquots (2μl) were used for estimating the concentration of DNA and finally diluted to have 1μg/μl for experimental use. Aliquots (100μl) of each sample were stored at 4°C otherwise stored at –20°C after ethanol precipitation until further needed.

4.2.3.3. Buffers / solutions

TE: - 10 mM Tris - HCl (pH 8.0)
- 1 mM EDTA

4.2.4. Purification of DNA fragments

After 2 - 3 h of restriction digestion, the DNA samples were electrophoretically separated in agarose gel (~ 1%). Fragments with sticky and blunt ends were purified by protocol include with GFX™ PCR DNA and Gel Band Purification Kit. After that, the samples were dephosphorylated as described in section 4.2.6 (below), purified again and used for ligation.

4.2.5. Vector dephosphorylation

All the linearized vector DNA samples with blunt or sticky ends were dephosphorylated prior to ligation with DNA fragment of choice. The reaction was set up as described in Sambrook & Russell (2001). After incubated for 1 h at 37°C, the enzyme was inactivated at 65°C for 15 min and purified by GFX column.

4.2.6. Mammalian cell transfection

4.2.6.1. Cell splitting and seeding

Monkey kidney cells (COS-1) or human hepatoma cells (Huh-7) were maintained in complete medium containing Dulbecco's Modified Eagle's Medium

(DMEM) with 10% Fetal Bovine Serum (FBS), penicillin (100U/ml) and 100 μg/ml streptomycin. Cells were incubated at 37°C in humidified incubator with 10% CO_2.

The flask of cells to be trypsinised was washed once with sterile PBS and incubated with 1 ml of Trypsin/EDTA (0.25%/0.03% in pBS pH 7.2) at 37°C for 1 - 2 min until cells started detaching from the surface. After adding complete medium (5 ml), cells were spun at 1000 rpm for 5 min. The supernatant was decanted and the cell pellet was re-suspended in a small volume of medium depending on the seeding density. Cells were plated with 6 - 8 x10^5 COS1 cells or 5 - 6 x 10^5 for Huh7 cells in 60 mm plate had already 2.5 ml complete medium and pre-warmed at 37°C. Cells were transfected at about 40 - 60% confluency.

4.2.6.2. Transient transfection

The plasmid DNA samples in sterile microfuge tubes were diluted as required in 100 μl antibiotic and serum free DMEM medium (NaNs). In parallel set of tubes, 10 μl of Lipofectin or Lipofectamine Reagent was also diluted to 100 μl NaNs medium. The samples were allowed to stand for around 30 - 45 min at room temperature. After that, two solutions were mixed gently and incubated further for 15 min at room temperature. Meanwhile the cells were washed with sterile PBS and once with 2 ml NaNs medium. For transfection, 1.8 ml NaNs medium were added to each plate and the Liposome-bound DNA samples (200 μl) were added to respective plates. After incubation for 5 - 6 h at 37°C in a humidified incubator with 10% CO_2, the medium was removed and replaced with 3 ml of complete medium and incubated further for 48h. Cells were harvested for expression or activity assays at 48 h post-transfection.

4.2.7. Metabolic labelling and Immunoprecipitation

4.2.7.1. Labelling of cells:

About 36 h post-transfection, cells were washed once with 3 ml of PBS and incubated in methionine and cysteine - free medium DMEM for 1h at 37°C. After that, medium was aspirated and replaced with 1 ml methionine & cysteine-free medium and 100 μCi/ml ^{35}S of methionine and cysteine and incubated further for 4 - 6 h at 37°C. The radioactive medium was removed carefully with pasteur pipette, and the cells were washed three times with chilled PBS. Chilled RIPA buffer (0.8 ml) was added to each dish and incubated at 4°C for 30 min. The adherent cells were removed with the help of a rubber policeman. The cell lysate were transferred to 1.5 ml microfuge tubes and the cellular debris was removed by centrifugation at 13,000 rpm at 4°C for 15 min. The supernatant was transferred to fresh tube and kept at -70°C till further analysis.

4.2.7.2. Immunoprecipitation

Protein A - sepharose beads was washed twice with RIPA buffer and 20% slurry was prepared with RIPA buffer.

The primary antibody (7.5 μl) was added to the samples and incubated on ice for 1 h. Finally, 100μl of the protein A-Sepharose slurry was added and incubated at 4°C for 1 h on shaker. The beads were collected by centrifugation at 10,000 rpm for 15 sec at 4°C and washed five times, each with 0.5 ml of RIPA buffer and transferred to a fresh tube. After adding 30 μl of 2X SDS sample buffer, equalized amounts of the samples were loaded on SDS-polyacrylamide gel and electrophored at 100 volts till bromo-phenol blue dye front reached the bottom of gel. The gel was immersed in 10% iso-propanol for 15 min and soaked in 0.5M sodium salicylate for 30 min. After drying under vacuum, the gel was exposed using X-ray film.

4.2.7.3. Buffers and solutions

RIPA buffer

- NaCl	150 mM	- Sodium deoxycholate	1%
- Triton X-100	1%	- SDS	0.1%
- Iodoacetamide	5 mM	- Tris-HCl (pH 8.0)	50 mM
- EDTA	1 mM	- Leupeptin	1 μg/ml
- Azide Sodium	4 mM (diluted from Azide sodium 400 mM)		
- PMSF Solution	1 mM (adding before use)		

2X SDS sample loading buffer

- Tris - HCl (pH 6.8)	125 mM
- SDS	4 % (w/v)
- Glycerol	20%
- DTT	100 mM
- Bromophenol blue	0.02 % (w/v)

4.2.8. Trans-activation studies

Forty-eight hours post transfection; the cells were harvested by washing once with PBS and scraped in 1 ml PBS. The cells were collected by spinning at 5,000 rpm for 5 min at 4°C. The pellet was re-suspended in 200 μl of 250 mM Tris-HCl (pH 7.8) and cells lysate was prepared by five repeated cycles of freeze-thawing using liquid nitrogen bath and 37°C water-baths respectively. The cell lysate was clarified by centrifugation at 13,000 rpm, 10 min, 4°C and the supernatant was saved. Protein was estimated with 2μl aliquots using Bradford reagent at 595 nm against blank. Normalized protein amount of each sample was used for CAT assay.

4.2.8.1. Chloramphenicol Acetyl Transferase (CAT) Assay

Normalized protein value of each sample was taken in fresh microfuge tubes and adjusted the volume to 120 µl with 250 mM Tris (pH 7.8). After mixing well, the endogenous deacetylases were inactivated by heating at 65°C for 10 min. After cooling on ice for 5 min, 40 µl of [^{14}C] chloramphenicol mix (1 : 40 v/v in water) and 20 µl of Acetyl Co-enzymeA were added to sample and incubated for 1 h at 37°C. The acetylated chloramphenicol was extracted in 1 ml of ethyl acetate, vortexing well and centrifuging at 13,000 rpm for 10 min at 4°C. The upper organic phase was carefully transferred into fresh microfuge tubes and dried using Speed-vac.

4.2.8.2. Thin Layer Chromatography (TLC)

The above dried samples were dissolved in 20 µl of ethyl acetate by vortexing, and spotted on a silica gel TLC plate at even distances from each other, 2.5 cm from the bottom of the plate. The samples were resolved in a chromatographic chamber containing a solvent mixture (Chloroform:Methanol / 95:5, v/v) up to a distance of 12 cm from the region of spotting. The TLC plate was removed from the chamber and air dried and exposed to X-ray film. The CAT activities were determined by densitometry analysis of the sports using software Kodak Digital Science.

4.2.8.3. Buffers and solutions

PBS (1 litter)

- KCl	0.2 g	Adjust the pH to 7.4 using 1 N HCl or 1 N NaOH
- KH_2PO_4	0.2 g	
- NaCl	8.0 g	
- Na_2HPO_4	1.15 g	

4.2.9. Denatured Salmon Sperm DNA

Salmon sperm DNA (1 g) was dissolved in 100 ml of 0.4 M NaOH and stired over night at RT. The DNA was sheared by sonication and neutralized with glacial acetic acid to bring down pH near 7.0. The debris was removed by centrifuge in 30 min, at 2500-3000 rpm and DNA was precipitated by adding 2 volumes ethanol and kept at -20°C for at least 1 h or over night. DNA pellet was collected by centrifugation at 13,000 rpm for 15 min, washed with 70% ethanol, dried briefly and dissolved in 50 ml 1X TE. The DNA concentrations of samples were determined by absorbance at 260 nm and aliquots were stored at -20°C.

4.2.10. *Western Immuno-blotting*

4.2.10.1. Protein Blotting

Cells were washed with 1X PBS and lysed by adding 1X SDS sample buffer (100 μl per plate of 10 cm^2 plate). Immediately, the cells were scraped off the plate and the extract was transferred to a microfuge tube kept on ice. The samples were sheared by vortexing well for 5 times (each time 10 seconds). Finally, samples were heated to 95 - 100°C for 5 min; cooled on ice and spun for 5 min. Equalized amounts of the samples were loaded on SDS-PAGE gel and electrophored at 100 V until the bromophenol blue front reached the bottom of gel.

4.2.10.2. Transfer of protein from SDS-PAGE to Hybond Nitrocellulose Membrane

The gel was soaked in the protein transfer buffer for 15 min. Assemble the electroblotting cassette and set up the transfer apparatus as follows: Three pieces of Whatman 3MM paper, gel, nitrocellulose membrane, three pieces of Whatman 3MM paper. All of this was placed between the electrodes in the blotting unit. The nitrocellulose membrane must be on the anode side of the gel. Transfer was run at 65 Volts for 45 - 60 min at 4°C.

4.2.10.3. Membrane Blocking and Antibody Incubation

After transfer, the membrane was washed in 25 ml TBS for 5 min and incubated in 25 ml of blocking buffer for 1 h at RT. Then, membrane was washed three times for 5 min each with 15 ml of TBS/T. Finally, membrane was incubated with 5 μl primary antibody in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C, followed by washing three times for 5 min each with 15 ml of TBS/T. Finally, membrane was incubated with 5 μl HRP-conjugated secondary antibody diluted in 10 ml of blocking buffer with gentle agitation for 1 h at RT followed by washing three times for 5 min each with 15 ml of TBS/T.

4.2.10.4. Detection of Proteins

The membrane (7 cm x 9 cm) was incubated with 2.5 ml LumiGLO® solution mix (0.125 ml 20X LumiGLO®, 0.125 ml 20X Peroxide and 2.25 ml Milli-Q water) for 1 min at RT. After that, membrane was drained in excess of developing solution (do not let dry), wrapped in plastic wrap and exposed to X-ray film.

4.2.10.5. Buffers and solutions

1X SDS sample buffer

- Tris-HCl pH 6.8	62.5 mM
- SDS	2 % (w/v)
- Glycerol	10 %
- DTT	50 mM
- Bromophenol blue	0.01 % (w/v)

Protein transfer buffer

- 25 mM Tris base
- 0.2 M glycine
- 20 % methanol

} pH 8.5

Tris buffered saline (TBS) 10X (1 litter)

- Tris base 24.2 g
- NaCl 80 g
- Adjust pH to 7.6 with HCl (use at 1X)

Blocking buffer

- 1X TBS
- 0.1 % Tween-20 (v/v)
- 5 % nonfat dry milk (w/v)

Wash buffer (TBS/T)

- 1X TBS
- 0.1 % Tween-20 (v/v)

4.2.11. *Radiolabelling of oligo (phosphorylation)*

The P^{32}-labeled oligo was prepared by 5' end labeling with T4 kinase in the presence of [γ^{32}P]-ATP as described in Sambrook & Russell (2001). The sample was incubated at 37°C for 30 min and the reaction was stopped by adding 5 µl of a 1% SDS/100 mM EDTA solution to stop the kinase reaction mix. The labeled oligonucleotide probe was purified by spin column or Urea-PAGE gel.

4.2.12. Radioactive labelling of DNA

X0, *c-myc*, pre-core were used as templates for incorporation of labeled dNTPs into duplex DNA. The reaction was set up as described by manufacturer's protocols for Nick Translation Kit. The reaction was incubated at 15°C for 60 min, and stopped by adding 5 µl of the Nick Translation Stop Mix. Finally, the probe was purified by Sephadex G-50 spin columns.

4.2.13. Transcription in vitro (probe for detection of siRNA)

4.2.13.1. Template preparation

X0 and *myc* PCR products were cloned separate in pGEM-T. pGEM-T-X0 and pGEM-T-*myc* were used as template for transcription in vitro reaction. pGEM-T-X0 and pGEM-T-*myc* were digested with *Nde*I and dephosphorylated to linear. The reaction was setup as follow:

- Transcription Optimized 5X Buffer 4.0μl
- DTT, 100mM 2.0μl
- Recombinant RNasin Ribonuclease Inhibitor 20u
- rdNTP (ATP, GTP and UTP 2.5mM each) 4.0μl
- 100 mM CTP 2.4μl
- linearized template DNA (1mg/ml) 1.0μl
- [α^{32}]CTP (400Ci/ mmol, 10mCi/ml) 5.0μl
- T7 RNA Polymerase (20u/μl) 1.0μl
- Add Nuclease-Free water to final volume 20.0μl

4.2.13.2. Transcription in vitro

The reaction was incubated at 37°C for 60 min, followed by adding RNase-Free DNase to a concentration 1 u/μg of template DNA and incubated at 37°C for 30 min more. After that, the reaction was added with 1 volume of acid (pH 4.5) phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 1 min and centrifuged at 12,000 rpm for 2 min. The upper aqueous phase was transferred to a fresh tube and added 1 volume of chloroform:isoamyl alcohol (24:1), vortexed for 1 min and centrifuged at 12,000 rpm for 2 min. The upper aqueous phase was transferred to a

fresh tube, followed by adding 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of 100% ethanol, mixed and placed at -70°C for 1 h then centrifuged at 12,000 rpm for 5 min. The supernatant was removed carefully and the pellet was washed with 1 ml of 70% ethanol and dried under speed-vac. The RNA sample was re-suspended in 20 µl of TE and added 300 µl of carbonate buffer (200 mM) and incubated at 60°C for as long as it took to reduce the probe to an average size of 50 nucleotides. The formula to calculate the time was used as follow:

$$t = (Li - Lf)/(k.Li.Lf), \text{ where:}$$

t = time in minutes

Li = initial length of probe in kb

Lf = final length of probe in kb (i.e 0.05 in our case)

k = rate constant = 0.11 kb. min^{-1}.

For X0 and myc probes, it took around 3 h for the hydrolysis and stopped by adding 20 µl of 3M NaOAc (pH 5) to the reaction and kept in -20°C till use.

4.2.14. Annealing of shRNA

4.2.14.1. Preparation siRNA insert

The dried oligonucleotide was dissolved at a convenient concentration as 100 µM, in DEPC-treated water. Each RNA oligo was aliquot and diluted separately to a concentration of 50 µM. 30 µl solutions each RNA oligo (sense and antisense) were mixed with 15 µl of 5X annealing buffer. Final volume was 75 µl and the final concentration of siRNA duplex was 20 µM. The solution was incubated for 2 - 3 min in a water bath at 90 - 95°C, and allowed cooling to room temperature (below 30°C). It took 45 - 60 min. After that, shRNA was used directly in a ligation reaction or stored at -20°C until further use.

4.2.14.2. Ligating and select annealed hairpin siRNA insert into pSilencer

The pSilencer 1.0-U6 vector was linearized by digestion with *Apa*I and *Eco*RI restriction enzymes. Hairpin siRNA insert was ligated with vector in corresponding sites. The ligation reaction was set up with 10 μl as follow:

- Double-stranded siRNA (10 ng) 1 μl
- Linearized vector (100 ng) 1 μl
- 10X T4 DNA ligase buffer 1 μl
- T4 DNA ligase (5 U/μl) 1 μl
- Nuclease-free water 6 μl

The ligation reaction was incubated at 16°C over night and transformed in *E. coli* DH5α competent cell.

4.2.14.3. Buffers and solutions

Annealing buffer 5X (1 litter)

- Potassium acetate 500 mM
- HEPES-KOH (pH 7.4) 150 mM
- Magnesium actate 10 mM

4.2.15. RNA extraction

Forty-eight hours post-transfection, the cells were washed once with 5 ml of chilled PBS. After adding 1 ml of TRIzol and the cell were lysed by passing several times through a pipette. The RNA was extracted as per protocol included with TRIzol Reagent. The RNA samples were re-dissolved in 100% formamide and stored at -70°C until use. RNA was quantitated in 2μl aliquots by taking absorbance $O.D_{260 / 280}$.

4.2.16. Extraction of low molecular weight RNA

The RNA was extracted as above and dissolved in water - DEPC treated. PEG (MW = 8000) and NaCl was added to a final concentration of 5% and 0.5M respectively and placed on ice for 30 min. The samples were spun at 10,000 rpm for 10 min to collect supernatant in fresh tube. RNA was precipitated by adding three volumes of absolute ethanol to the supernatant and placed at -20°C for at least 2 h. RNA pellet was obtained by centrifugation at 10,000 rpm for 10 min and dissolved in formamide for Northern blot assays.

4.2.17. Northern hybridization

4.2.17.1. Preparation and running of Agarose/Formaldehyde gel

Agarose/Formaldehyde gel was prepared as described in Sambrook & Russell (2001). The gel was pre-run for 10 min in MOPS 1X buffer prior to loading the samples. RNA samples were prepared by mixing 1 part RNA sample with 2 parts RNA sample buffer up to a total volume of 30 μl. The samples were heated at 65°C for 5 min, cooled to RT and 2 μl of RNA loading buffer were added. The samples were loaded and gel was run at 4 - 5 Volts/cm until the bromophenol blue migrated to 2/3 of the gel.

4.2.17.2. Transfer of RNA to membrane

The gel was soaked in DEPC-treated water for several times to remove the formaldehyde followed by soaking in 20X SSC for 45 min. The transfer of RNA was done on Nylon membrane (N+, Hybond Amersham) for over night at RT. After the transfer was complete, the membrane was removed and washed in 5X SSC for 5 min at RT and UV-crosslinked at 120 miliJoules/cm^2 for 1 min.

4.2.17.3. Hybridization

The membrane was incubated first in pre-hybridization solution at 42°C for 4 h followed by the hybridization solution after adding appropriate amounts of the denatured, labelled probe. Hybridization was done at 42°C over night followed by washing with 200 ml of stringency wash solution I two times for 5 min each at RT. 200 ml of stringency wash solution II pre-warmed to 50°C was added to washing 2 times for 5 min each. Finally, the blots was washed with 200 ml of 2X SSC two times for 10 min each at RT with gentle shaking to remove excess SDS and blot was wrapped in plastic wrap and exposed using X-ray film.

4.2.17.4. Buffers and solutions

Pre-hybridization / Hybridization

- 50 %	deionized formamide
- 5X	SSPE
- 2X	Denhardt's Reagent
- 0.1 %	SDS
- 100 μg/ml	denatured salmon sperm DNA

MOPS 5X buffer (2 litter)

- 0.2 M	MOPS	Adjust the pH to 7.0 with 10 N NaOH
- 0.05 M	sodium acetate	
- 0.005 M	EDTA, pH 8.0	

RNA sample buffer

- 10.0 ml	deionized formamide
- 3.5 ml	37% formaldehyde
- 2.0 ml	MOPS 5X buffer

RNA loading buffer

- 50 %	glycerol
- 1 mM	EDTA
- 0.4 %	bromophenol blue

SSPE 20X (1000 ml)

- 175.3 g	NaCl	Adjust pH to 7.4 with 10 N NaOH
- 27.6 g	$NaH_2PO_4.H_2O$	
- 7.4 g	EDTA, disodium salt	

SSC 20X (500 ml)

- 87.7 g	NaCl	Adjust pH to 7.2 with 10 N NaOH
- 44.1 g	sodium citrate	

Denhardt's Reagent (100 ml)

- 1 g	Ficoll (Type 400)
- 1 g	Polyvinylpyrrolidone
- 1 g	Bovine serum albumin (fraction V)

Wash solution I

- 2X	SSC
- 0.1%	SDS

Wash solution II

- 0.1X	SSC
- 0.1 %	SDS

4.2.18. Preparation and running Urea- Acrylamide gel for low molecular weight RNA

Urea - Acrylamide gel was prepared with composition as follow: 20% polyacrylamide (19:1); 7M urea; 0.5X TBE. The samples RNA were heated to 65°C for 5 min and placed on ice followed by adding 1/3 volume of 4X loading solution. The gel was run in 0.5X TBE at 100 - 500 volts until bromophenol blue reached the bottom of gel (usually 2-6 h). The ^{32}P kinased DNA oligos (see 4.2.12) was used as markers. After gel run was done, the RNA was transferred to blot by using a BioRad "wet" electroblotter. Finally, the membrane was placed in 20X SSC for 30 min and dried thoroughly followed by UV-crosslinked at 120 mJoules for 2 min.

4.2.18.1. Hybridization

The membrane was incubated first in pre-hybridization solution at 42°C for 4 h followed by the hybridization solution after adding appropriate amounts of the denatured, labelled in vitro transcribe RNA probe (see 4.2.14). Hybridization was done at 42°C over night followed by washing with 100 ml of stringency wash solution I (2X SSC, 0.2% SDS) 2 times for 5 min each at RT. 100 ml of stringency wash solution II (0.1X SSC, 0,1% SDS) pre-warmed to 50°C was added to washing 2 times for 5 min each. Finally, the blots was washed with 100 ml of 2X SSC 2 times for 10 min at RT with gentle shaking to remove excess SDS and blot was wrapped in plastic wrap and exposed using X-ray film.

4.2.18.2. Buffers and solutions

4X loading solution

- 2X	TBE,
- 40%	sucrose
- 0.1%	bromophenol blue

Pre-hybridization / Hybridization

- 50 %	deionized formamide
- 0.3 M	NaCl
- 50 mM	$NaHPO_4/NaH_2PO_4$ (pH 7.0)
- 5X	Denhardt's Reagent
- 7 %	SDS
- 100 μg/ml	denatured salmon sperm DNA

5. Results

5.1. VERIFICATION OF EXPRESSION VECTORS AND ANTISENSE RECOMBINANTS

5.1.1. Verification of expression vectors

5.1.1.1. pCMV-neo

The plasmid pIRES1 - neo (~5.3 Kb) was digested with *Sma*I and *Eco*RV to remove the IRES fragment (~ 1 Kb) and re-ligated to create pCMV- neo (Fig. 6).

5.1.1.2. pSG5-myc

The complete murine c-*myc* cDNA (1.3 Kb) was excised from pGEM-T - *myc* vector (gifted N. Kalra) by *Eco*RI digestion and cloned in the sense orientation in the pSG5 vector. The orientation of the clones was verified by digestion with *Xba*I/*Eco*RV enzymes (Fig. 10A, C).

5.1.1.3. SV40-pc-myc:

Since c-*myc* was expressed a pre-core (pc) fusion protein under the control of Cp by X-*myc* bicistronic construct (Lakhtakia et al., 2003), a SV40-pc-*myc* expression vector was constructed by cloning an inframe pre-core fragment (105 bp) of HBcAg in SV40-*myc* vector (gifted N. Kalra) at *Bam*HI and *Bgl*II sites. The orientation of the clone was checked by digestion with *Bam*HI/*Sal*I enzyme (Fig. 11).

5.1.2. Verification of antisense recombinants

5.1.2.1. SV40-antiX0

The pSG5 - X0 plasmid was digested with *Eco*RI and the 470 bp X0 fragment was cloned in antisense orientation in plasmid pSG5. Orientation of the insert was verified by digesting the clones with *Bam*HI (Fig. 7B, C). The antisense clones released a fragment of 30 bp (lane 3), while the one in sense orientation released a 440 bp fragment (lane 4).

5.1.2.2. pCMV-neo - Xp-anti-X0 **(A1)**

The promoter of HBx gene (Xp) was PCR amplified using HBV genomic template and GT1a (5'-CGG GAT CCA AGC TTC CTG TTA ACA GGC-3') & GT2a (5'-GGA ATT CAC GAG AGG ACG ACA G-3') oligonucleotide primers. The 420 bp PCR product was digested with *Eco*RI and *Bam*HI and cloned at the corresponding sites in pPolyIII vector (Lathe *et al.*, 1987). Then, the *Eco*RI fragment of X0 was cloned in antisense orientation downstream of Xp. A 275 bp *Xba*I - *Xho*I fragment from pIRES1-neo carrying the polyadenylation signal was also cloned 3' to the anti-X0 region. Finally, the *HBx* gene was placed under the control of the X promoter (Xp) and was followed by poly A signal (Fig. 8A). The entire Xp - anti-X0 cassette (~ 1.25Kb) was excised by *Xho*I digestion and cloned into pCMV-neo vector at *Xho*I site (Fig. 8B). The cloning and orientation of clones were verified by digestion with *Xho*I and *Hind*III (Fig. 8C) that released 1.25 Kb (lane 2) and 1.14 Kb fragments (lane 1) respectively.

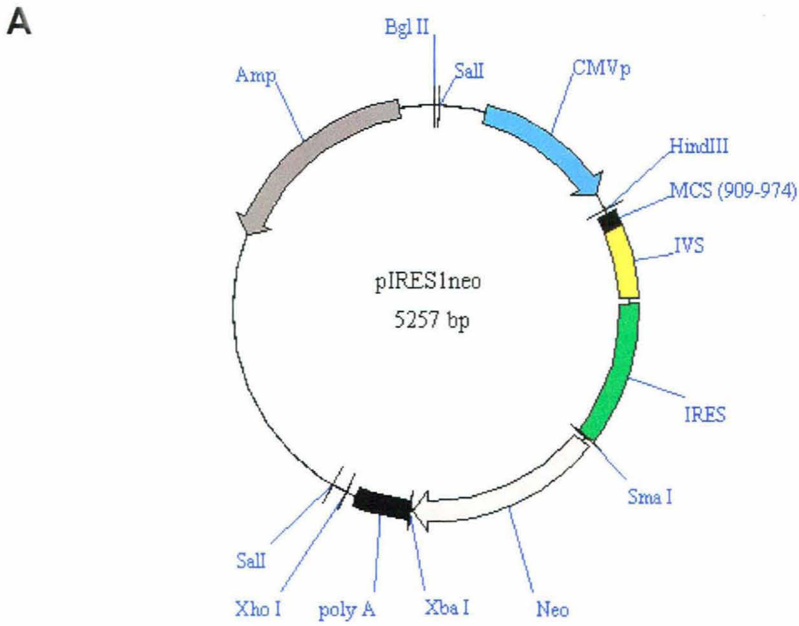
5.1.2.3. pCMV-neo - SV40-antiX0 **(A2)**

The SV40 - anti-X0 construct (Fig. 7B) was digested with *Sal*I and the 1.7 Kb fragment was cloned in pCMV-neo vector at *Xho*I site. Orientation of the pCMV-neo - SV40 -anti-X0 clone was verified by *Bgl*II digestion that released a 2.4 Kb fragment (Fig. 9)

5.1.2.4. pSG5 - anti-myc **(A3)**

The complete murine c-*myc* cDNA (1.3 Kb) was excised from pGEM-T - *myc* vector by *Eco*RI digestion and cloned in the antisense orientation in the pSG5 vector. The orientation was checked by digestion with *Xba*I/*Eco*RV enzymes. (Fig. 10B, C).

A



Multi Cloning Sites (MCS):

900

•

CGAGCTCGGATCGATATCTGCGGCCGCGTCGACGGAATTCAGTGGATCCA

*Cla*I *Eco*RV *Not*I *Eco*RI *Bam*HI

950

•

CTAGTAACGGCCGCCAGTGTGCTGGAATTAATTCG

*Bst*XI

B

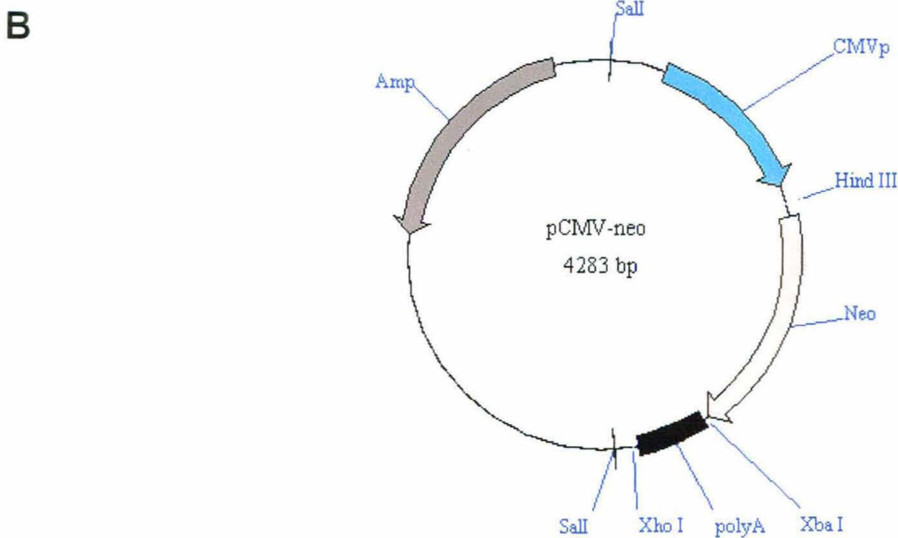


Fig. 6: Construction of pIRES1-neo (A) and pCMV-neo (B)

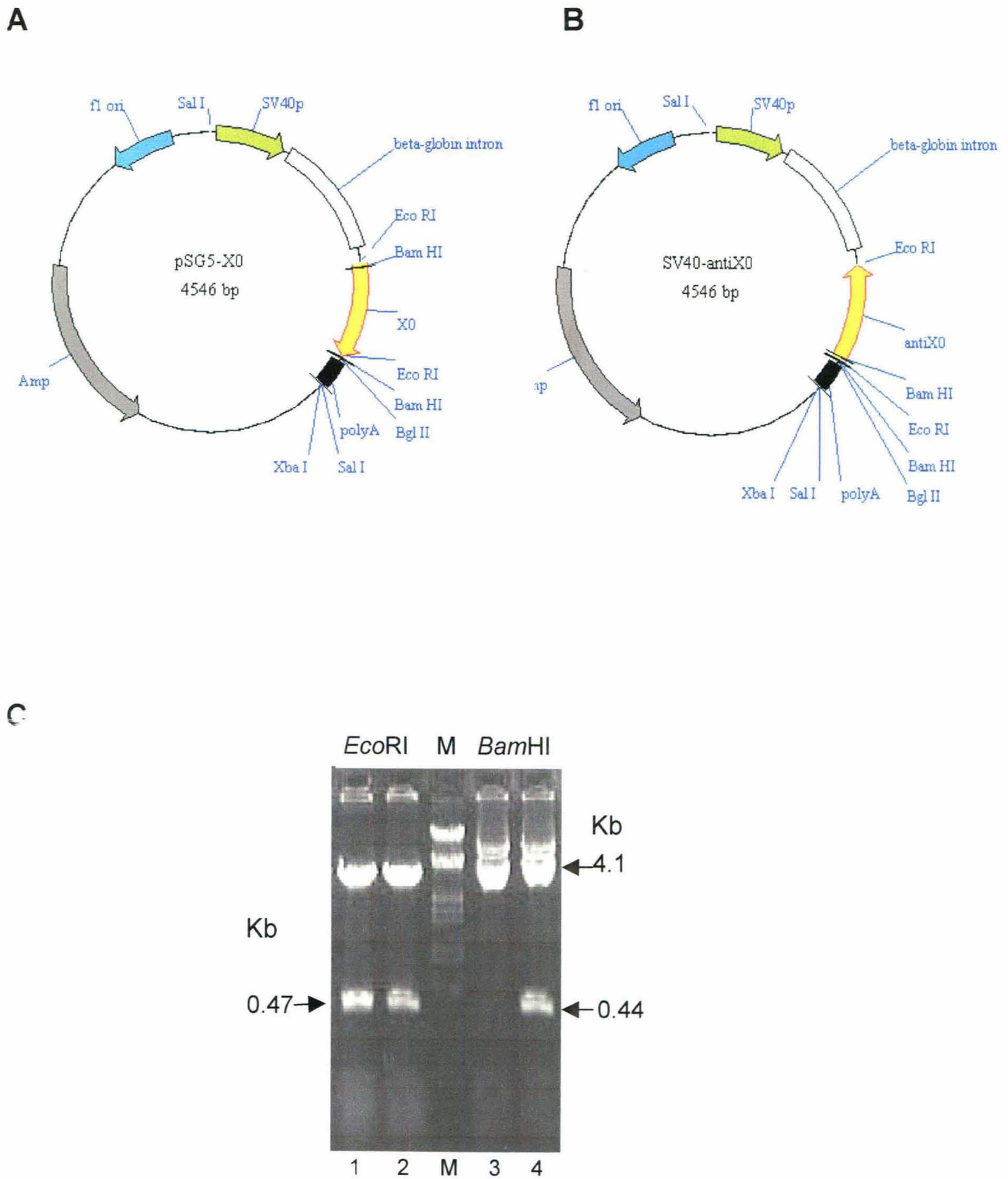
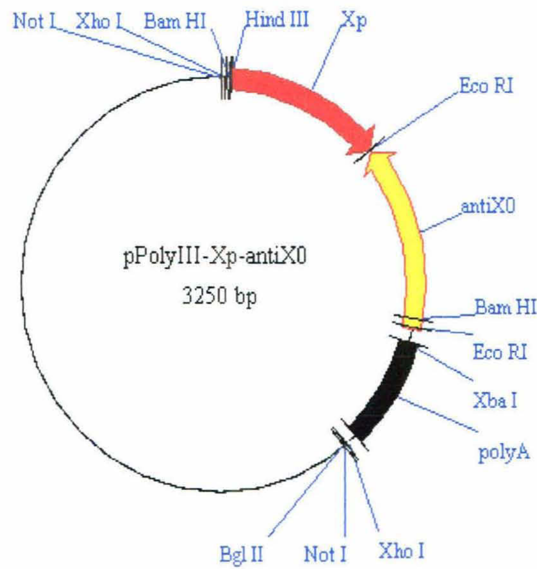
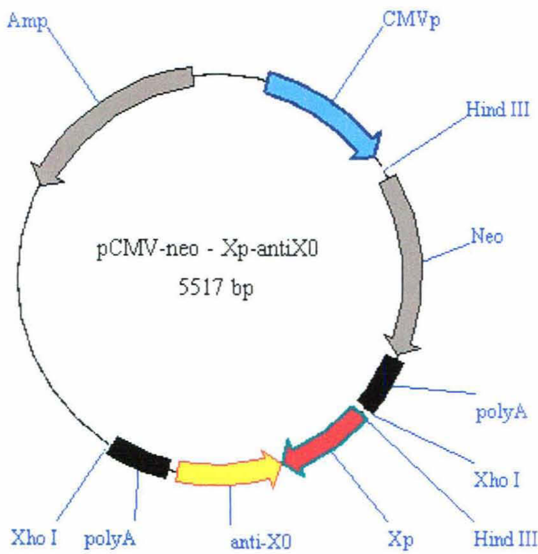


Fig. 7: Construction of the antisense X0 vector. Schematic maps of pSG5-X0 (**A**) and SV40-antiX0 (**B**). The X0 clones were digested separately with *Eco*RI and *Bam*HI and resolved by agarose gel electrophoresis (**C**). M: DNA molecular weight markers.

A



B



C

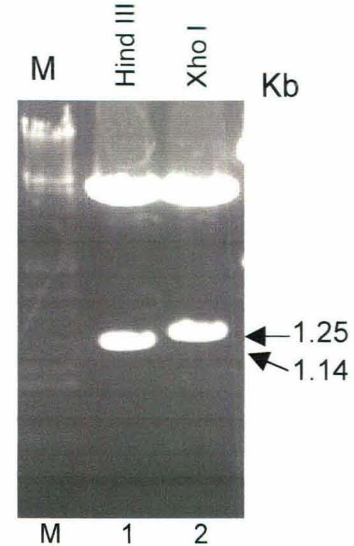
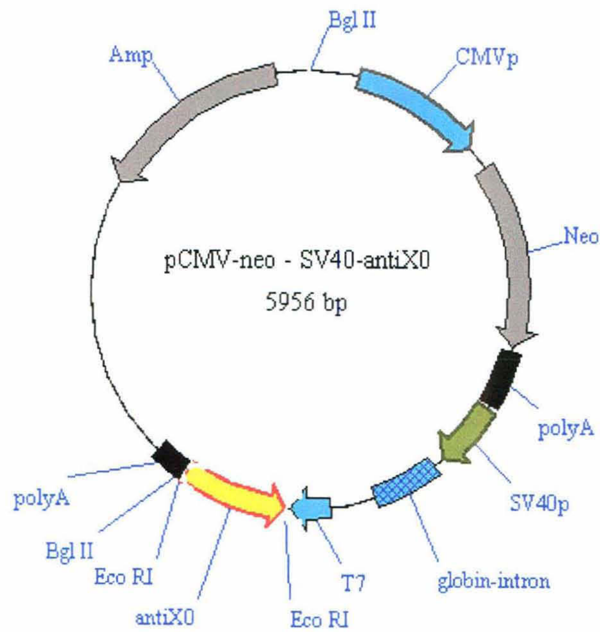


Fig. 8: Construction of the pCMV-neo - Xp-antiX0 vector. Schematic maps of pPolyIII-Xp-antiX0 (**A**) and pCMV-neo - Xp-antiX0 (**B**). To verify the cloning and orientation of Xp - antiX0 cassette, the pCMV-neo - Xp-antiX0 clones were digested separately with XhoI and HindIII and resolved by agarose gel electrophoresis (**C**). M: DNA molecular weigh markers.

A



B

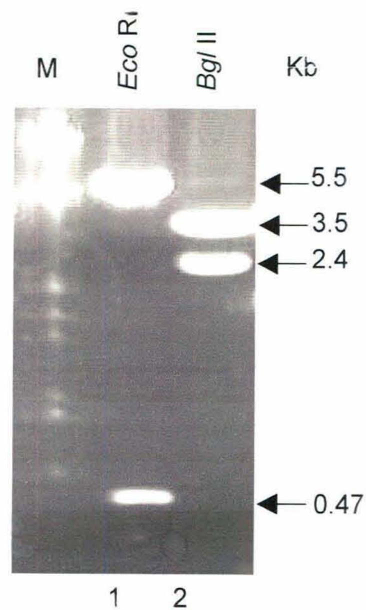
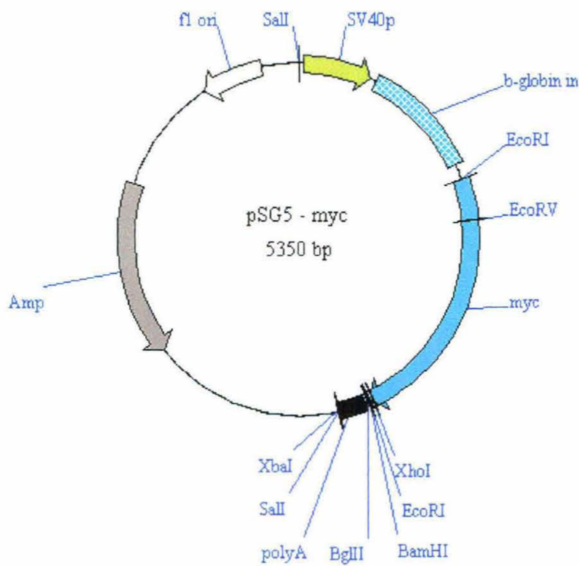
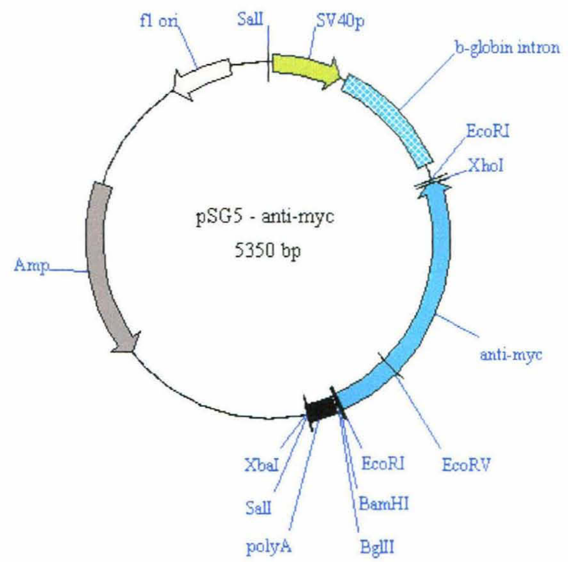


Fig. 9: Construction of the pCMV-neo - SV40-antiX0 vector. **A.** Schematic map; **B.** Agarose gel analysis of the plasmid DNA. To verify the cloning and orientation of SV40 - antiX0 cassette, the pCMV-neo - SV40-antiX0 clones were digested separately with *Eco*RI and *Bgl*II and resolved by agarose gel electrophoresis. M: DNA molecular weigh marker.

A



B



C

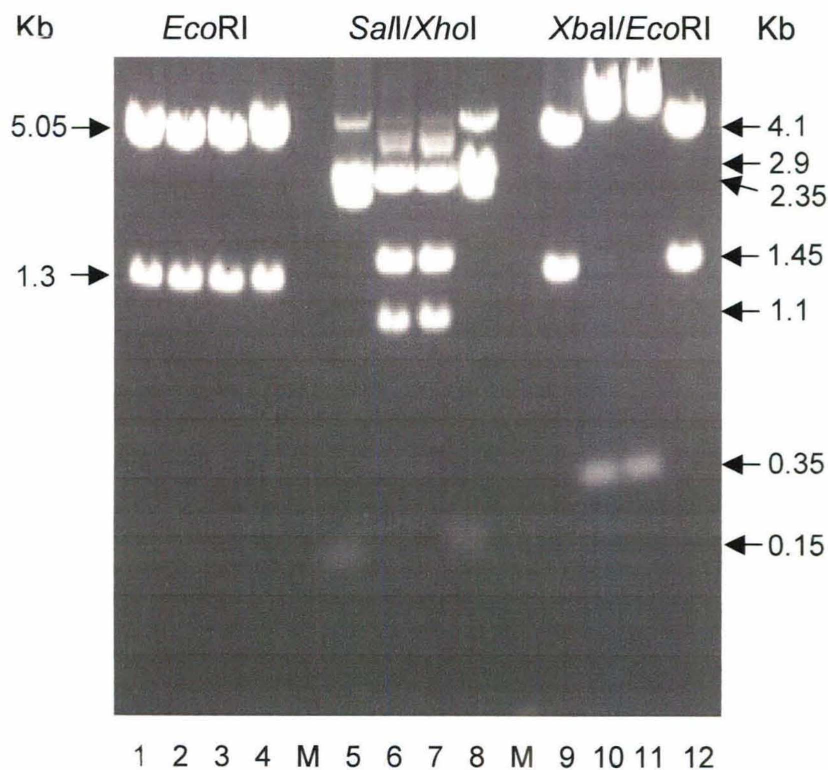
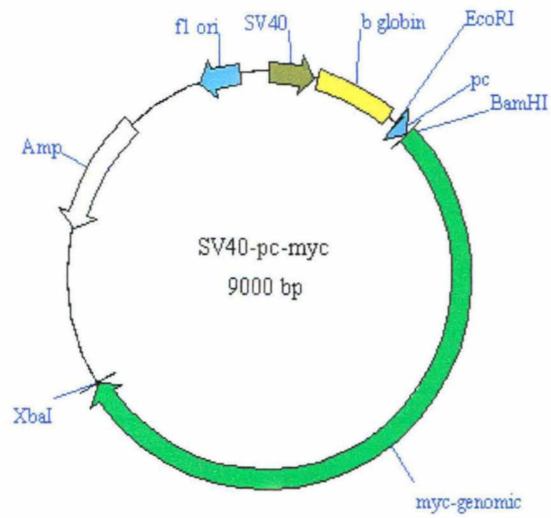


Fig. 10: Construction of the c-*myc* expression vector. **A.** Schematic maps of pSG5 - *myc* and **B**. pSG5-anti-*myc*. **C**. Agarose gel analysis of the clone after digestion with different restriction enzymes. pSG5-*myc* (lane 1, 4, 5, 8, 9, 12); pSG5-anti-*myc* (lane 2, 3, 6, 7, 10, 11); M: DNA molecular weight marker.

A



B

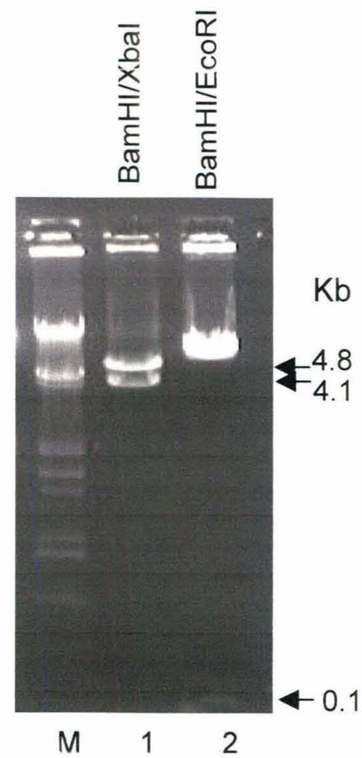


Fig. 11: Construction of the pc-*myc* expression vector. **A.** Schematic maps of pSG5-pc-*myc*, and **B**. Agarose gel analysis of the clone after digestion with different restriction enzymes. M: DNA molecular weight marker.

5.1.3. Specific inhibition of protein by antisense constructs

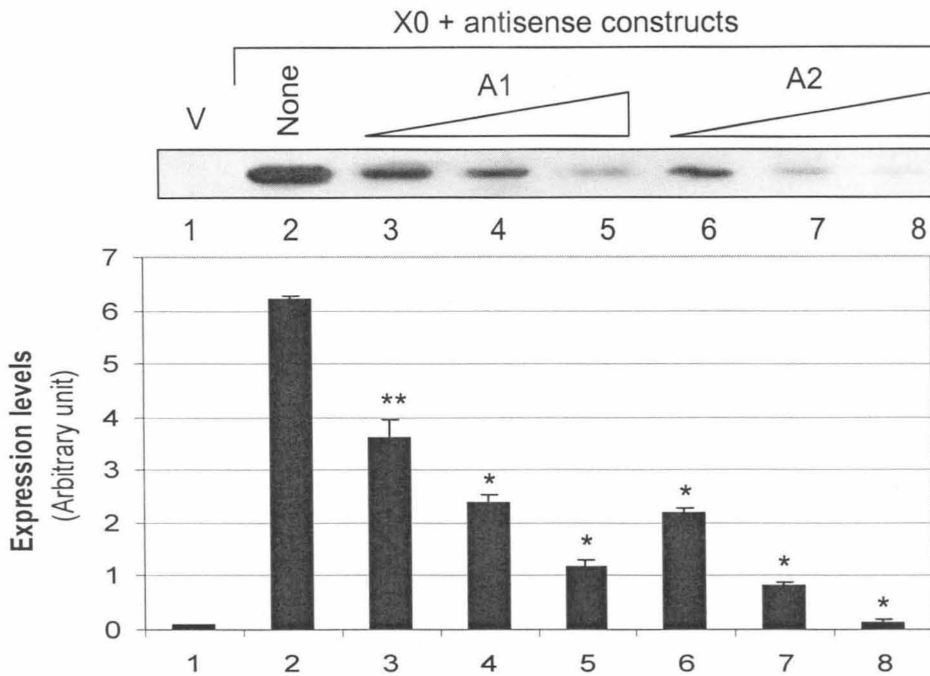
To investigate whether antisense constructs would down regulate the HBx and *c-myc* levels, the corresponding expression vectors were transfected separately in COS-1 and Huh-7 cells without or with increasing amounts of all the six antisense plasmids (A1 through A6) and analyzed by immunoprecipitation. As shown in Fig. 12, the expression of HBx protein was significantly inhibited by the antisense A1 and A2 and the inhibition levels increased with increasing amount of the antisense plasmids. The inhibition with A1 and A2 in COS-1 cells (Fig. 12A) was 82% (lane 5) and 97% (lane 8) respectively. A similar inhibitory trend was also seen in Huh-7 cells (Fig. 12B) where A1 and A2 could inhibit the HBx expression by nearly 62% (lane 5) and 96% (lane 8) respectively. Apparently, A2 was more efficient than A1 for inhibition function.

The regulation of c-*myc* expression was also investigated using anti-*myc* construct. As shown in Fig. 13, c-*myc* expression was significantly inhibited both in COS-1 (A) and Huh-7 cells (B) in the presence of anti-*myc* construct. Interestingly, however, the inhibition was more prominent in Huh-7 cells (~ 6 fold) as compared to that observed in COS-1 cells (3.2 fold).

5.1.4. Specific inhibition of mRNA level by antisense constructs

To confirm whether decreased protein levels of HBx and c-*myc* were due to down-regulation of the respective mRNA levels by antisense constructs, Northern blot assay was done using total RNA from the transfected cells. As shown in Fig. 14, all the two antisense constructs viz A1, A2 that inhibited HBx expression also inhibited the HBx mRNA levels. Further, more inhibition was observed with A2 than A1.

A



B

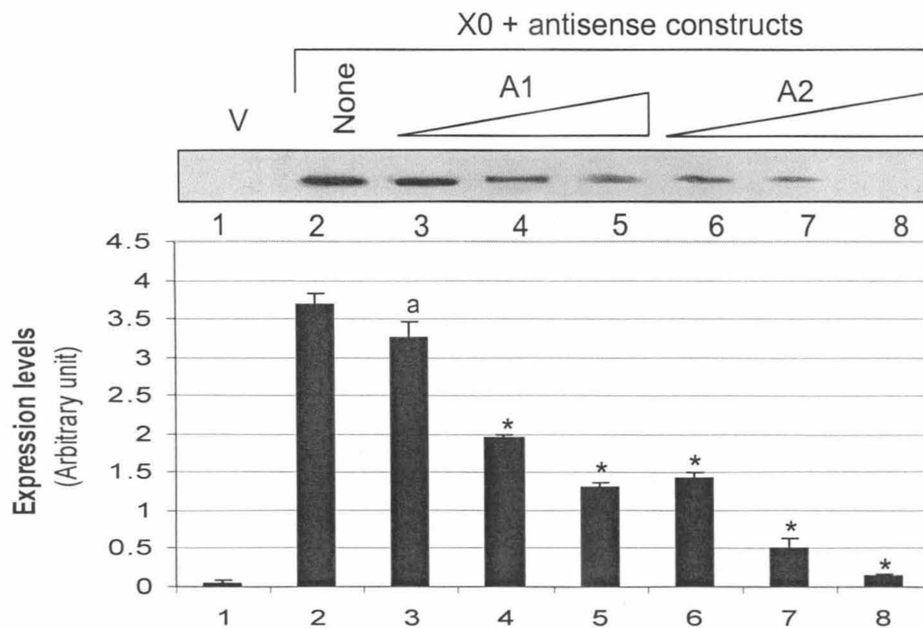


Fig. 12: Inhibition of HBx expression by antisense A1 and A2. X0 (0.5 μg) was transiently cotransfected in COS-1 cells (**A**) or Huh-7 cells (**B**) along with increasing a mounts (0.5, 2 and 5 μg) of antisense DNA A1 and A2. Cell extract were immuno-precipitated using anti-HBx monoclonal antibody and visualized by chemilumi-nescence. The level of expression was analyzed by densitometry. V: pSG5 vector. Level of significance: *, $P < 0.001$; **, $P < 0.01$; a, $P = 0.025$

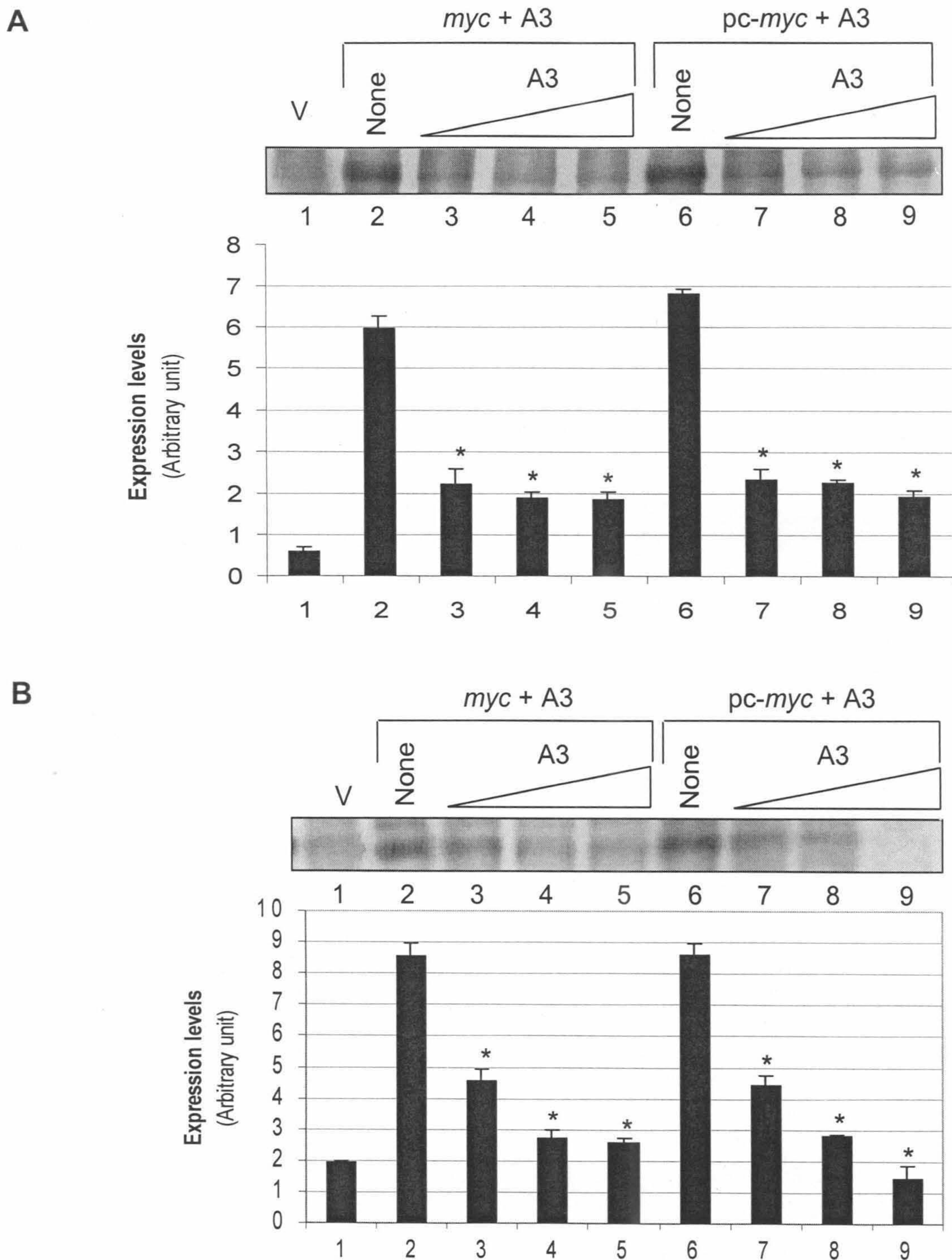


Fig. 13: Inhibition of HBx expression by anti-*myc*. *myc* or pc-*myc* (0.5 μg) was transiently cotransfected in COS-1 cells (**A**) or Huh-7 cells (**B**) along with increasing a mounts (0.5, 2 and 5 μg) of anti-*myc*. Cell extract were immunoprecipitated using anti-*myc* monoclonal antibody and visualized by chemiluminescence. The level of expression was analyzed by densitometry. V: pSG5 vector.

Level of significance: *, P < 0.001; **, P < 0.01;

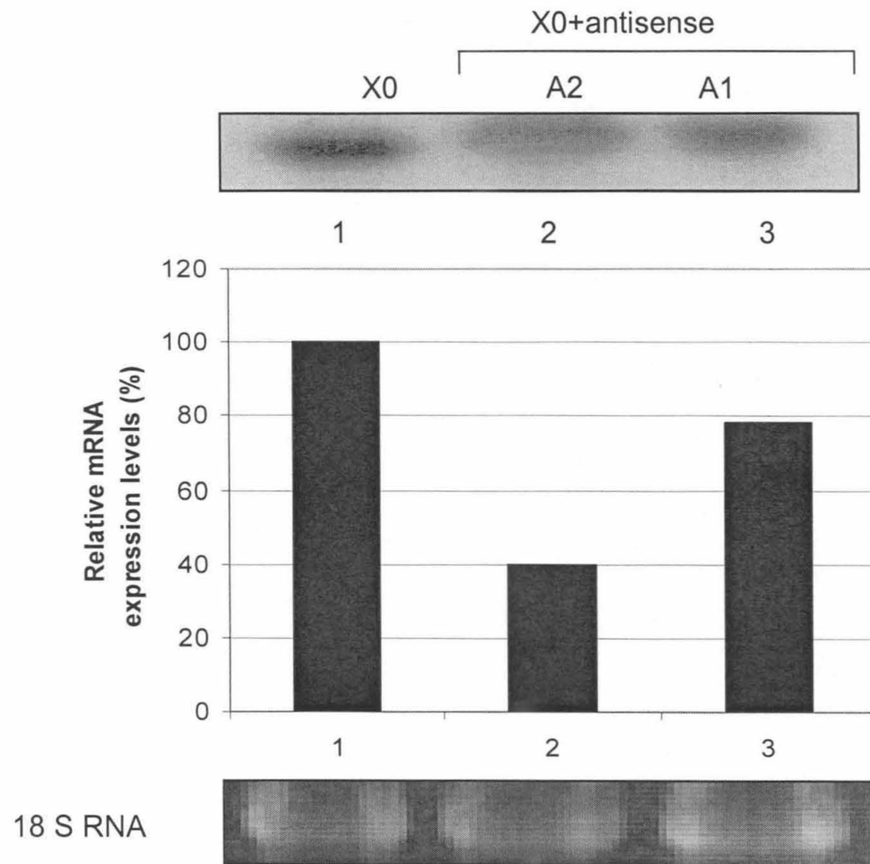


Fig. 14: Inhibition of the HBx mRNA by different antisense constructs. COS-1 cells were transiently co-transfection with X0 and/or antisense constructs (5 μg). Total RNA isolated from the cells was analyzed by Northern hybridization using [^{32}P]-labelled X0 probe. The level of inhibition was determined by densitometry.

5.1.5. Regulation of transactivation function by antisense constructs

To demonstrate that inhibition the target gene expression was not a direct effect of RNA protein interaction but rather an effect of 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. 15, the HBx-dependent transactivation in COS-1 cells was significantly inhibited by antisense constructs A1 and A2 in a dose dependent manner. The transactivation could be inhibited by nearly four folds (lane 5 and 8) with excess of A1 and A2.

A similar inhibitory effect was observed on HBx-CAT in Huh-7 cells (Fig. 16) where A1 and A2 could significantly inhibit transactivation by nearly 7 folds (lane 5) and 10 folds (lane 8) respectively.

Like HBx, the transactivation function of RSV-LTR by c-*myc* and pc-*myc* could also be significantly inhibited by the anti-*myc* in COS-1 cells (Fig. 17) as well as Huh-7 cells (Fig. 18). Again the inhibition was dependent on the amount of anti-*myc* plasmid transfected and the maximum inhibition was around 3 to 4 fold.

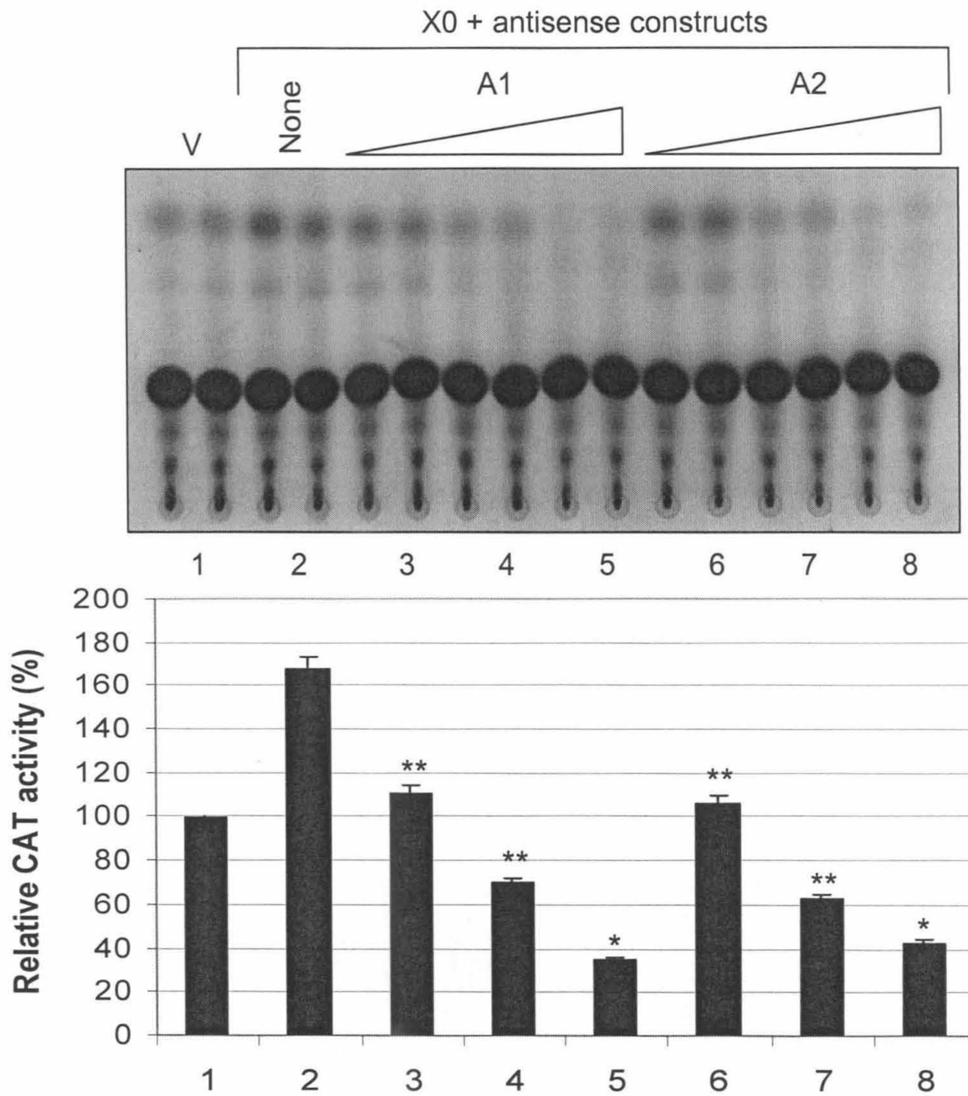


Fig. 15: Inhibition of the transactivation functions of HBx by antisense A1 & A2. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), X0 (0.5 μg) and increasing amounts (0.5, 2 and 5 μg) of different antisense as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector.

Level of significance: *, $P < 0.001$; **, $P < 0.01$.

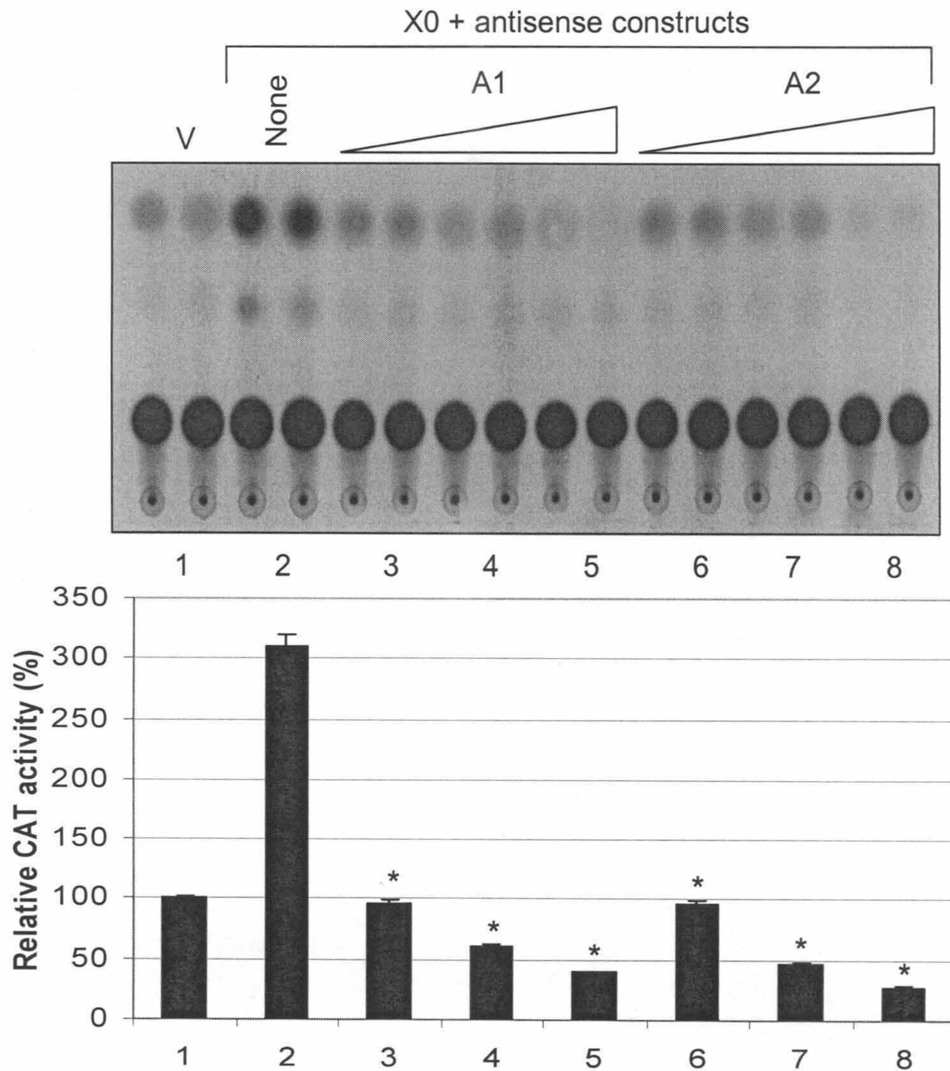


Fig. 16: Inhibition of the transactivation functions of HBx by antisense A1 & A2. Huh-7 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), X0 (0.5 μg) and increasing amounts (0.5, 2 and 5 μg) of different antisense as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector.

Level of significance: *, P < 0.001.

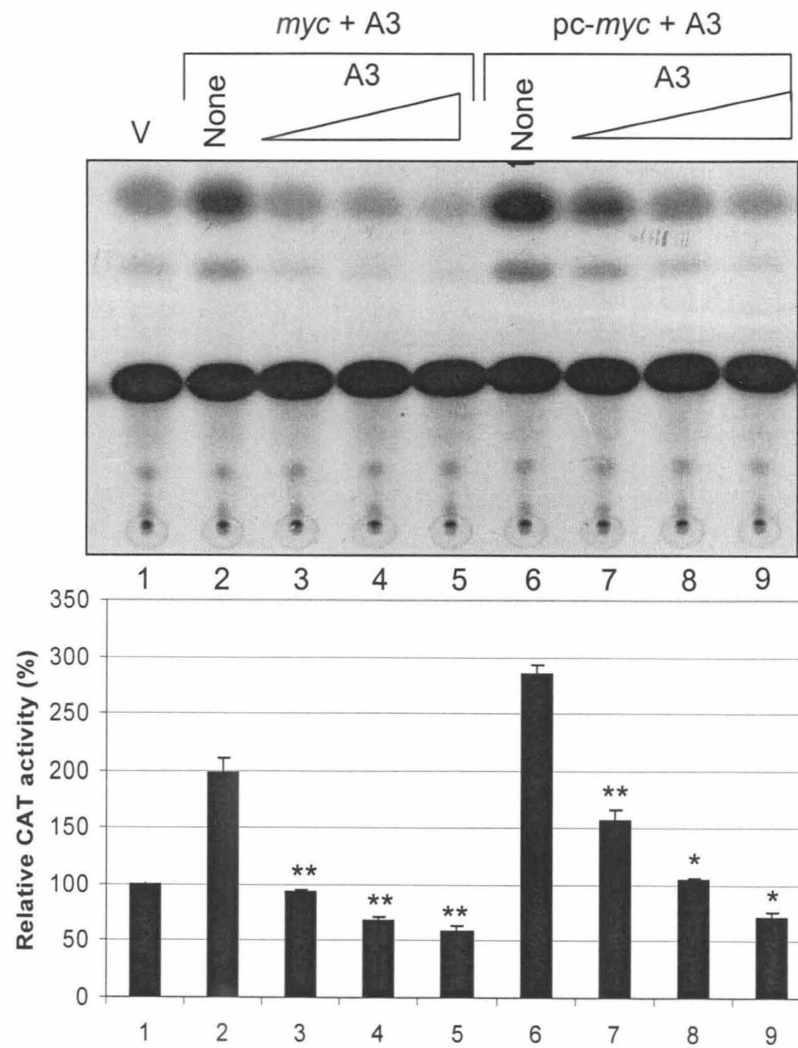


Fig. 17: Inhibition of the transactivation functions of *myc* and pc-*myc* by anti-*myc*. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), *myc* or pc-*myc* (0.5 μg) and increasing amounts (0.5, 2 and 5 μg) of anti-*myc* as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector.

Level of significance: *, $P < 0.001$; **, $P < 0.01$.

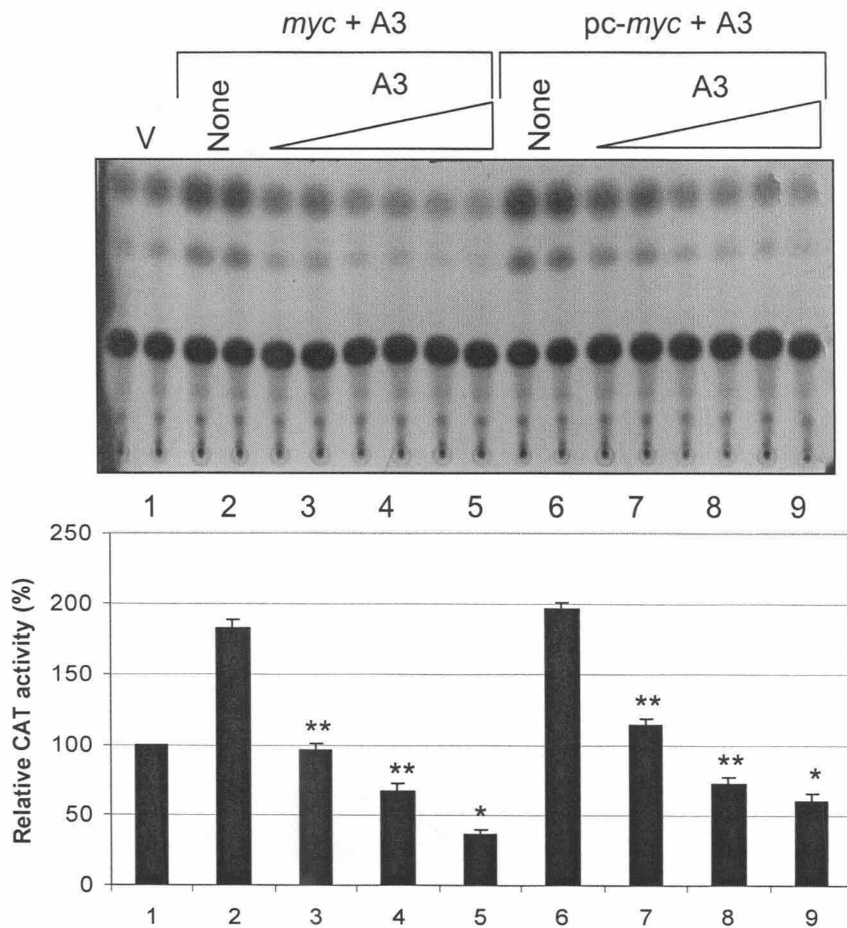


Fig. 18: Inhibition of the transactivation functions of *myc* and pc-*myc* by anti-*myc*. Huh-7 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), *myc* or pc-*myc* (0.5 μg) and increasing amounts (0.5, 2 and 5 μg) of anti-*myc* as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector.

Level of significance: *, $P < 0.001$; **, $P < 0.01$.

5.2. shRNA CONSTRUCTS AND ITS VERIFICATION

5.2.1. Design the shRNA constructs

The pSilencer 1.0 - U6 siRNA expression vector was linearized with restriction enzyme *Eco*RI & *Apa*I to create 3' overhangs and facilitate directional cloning.

- The hairpin siRNA was designed for pSilencer vector as per Ambion protocol depicted in Fig. 25. The oligonucleotides encoding a 19-mer hairpin structure was derived from the mRNA target site. The loop of the hairpin siRNA is located close to the center of the oligo-nucleotides. Near the end of the hairpin siRNA template is a 5 nucleotide poly(T) tract recognized as a termination signal by RNA pol III that will terminate siRNA synthesis. Thus each sense strand is 53 nt in length while the antisense oligonucleotide in 61 nt in length.

DNA sequence of target site

19 nt
5' AA 3'
3' TT 5'

RNA target

5' AA 3'

Hairpin siRNA

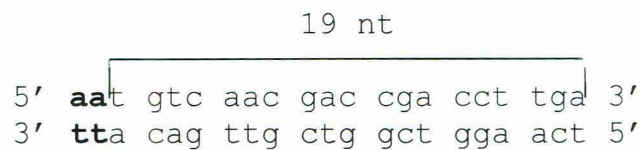
19 nt
5' Sense strand
Antisense strand
3' UUUUUU
U U C A A G A G A
Loop

```
            Sense              Loop       Antisense
    5'-NNNNNNNNNNNNNNNNNNN ttcaagaga NNNNNNNNNNNNNNNNNNN tttttt -3'
3'-ccgg NNNNNNNNNNNNNNNNNNN aagttctct NNNNNNNNNNNNNNNNNNN aaaaaattaa -5'
   ApaI                                                        EcoRI
```

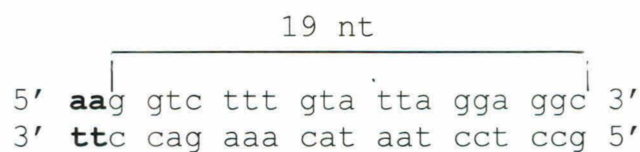
Fig. 19: Hairpin siRNA template design

siRNAs for HBx and mouse *c-myc* were designed using Jack Lin's siRNA sequence under (http://www.ic.sunysb.edu/Stu/shilin/rnai.html) or Qiagen's design by sequence (http://python.penguindreams.net/Xeragon_Order_Entry/jsp/Search-BySequence.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. Figures 20 and 21 shows the scheme of domain structures of HBx and *c-myc* respectively along with the location of the siRNA sites and oligonucleotide sequences of the shRNA inserts. For HBx, regions D and E play important roles in signal transduction and transactivation (Nijhara *et al.*, 2001, Reddi *et al.*, 2003) and therefore, the shRNAs directed against these regions were termed X-D and X-E respectively (Fig. 20). The shRNA constructs directed against the transactivation and leucine zipper domains of *c-myc* (Marcu *et al.*, 1992) were called M-T and M-Z respectively (Fig. 21). Each oligonucleotide had a small sense, loop and an antisense region so as to express siRNA as a stable hairpin structure (Sui *et al.*, 2002). 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 (Fig. 22A). The dideoxy chain termination method was used to confirm the sequence of each construct.

- Sequence of the target site for the D domain of HBx:



- Sequence of the target site for the E domain of HBx:



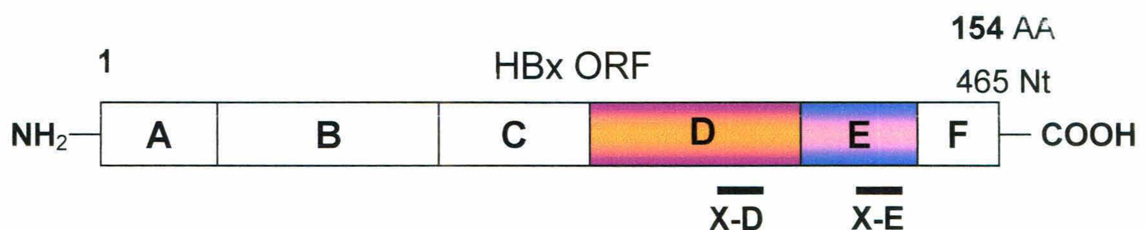
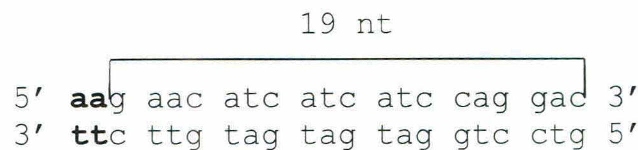
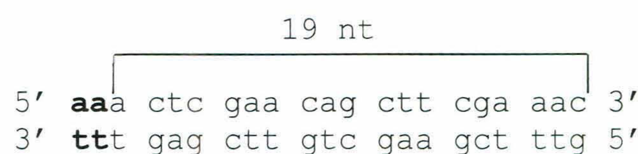


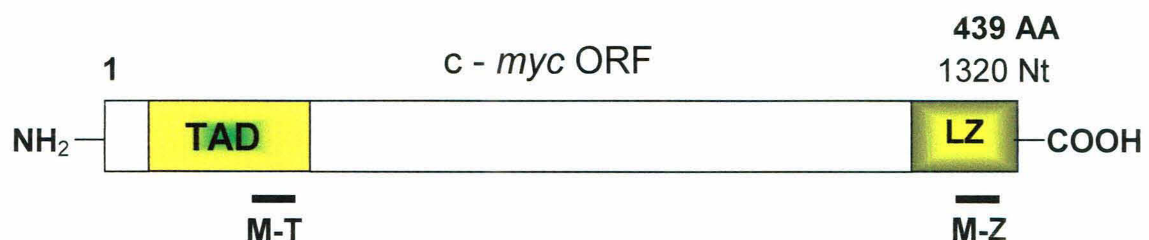
Fig. 20: Design of shRNA against HBx. The putative siRNA sequences in the D and E regions of HBx are shown on top. Domain structure of HBx (A throught F) along with location and sequences of the shRNAs for domains D (X-D) and E (X-E) are shown below.

- Sequence of the target site for the transactivation domain of c-*myc*:



- Sequence of the target site for the Leucine-zipper domain of c-*myc*:





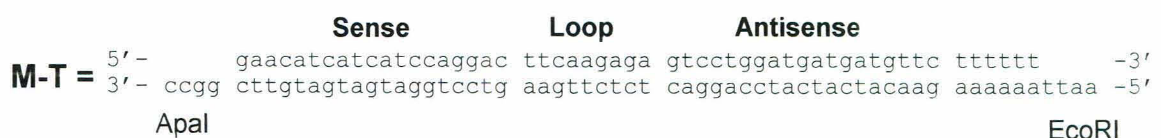


Fig. 21: Design of shRNA against c-*myc*. The putative siRNA sequences in the transactivation (TAD) and leucine zipper (LZ) domains of c-*myc* are shown on top. Domain structure of c-*myc* showing location and sequences of the shRNAs for domains TAD (M-D) and LZ (M-Z) are shown below.

5.2.2. Verification of shRNA constructs

The four shRNA constructs were selected on the basic of *Hind*III digestion (lost in positive clones) (Fig. 22B) and confirmed by digestion with *Eco*RI & *Apa*I or *Kpn*I that released either a ~ 60 bp or 440 bp fragments, respectively (C). The positive clones were further verified by PCR amplification using a gene-specific forward primer and T3 primer (D).

5.2.3. Specific inhibition of protein and RNA levels by shRNAs

To investigate the inhibitory action of siRNA on the levels of target proteins, the expression vectors for HBx and mouse c-*myc* were transfected in COS-1 and Huh-7 cells along with increasing amounts of the shRNA plasmids and analyzed by immunoprecipitation. The results Fig. 23 show that the expression of HBx protein was significantly inhibited by shRNA constructs. The inhibition levels increase proportion with amount of the shRNA. The ratio inhibition of HBx by X-D and X-E on COS-1 cells are 65% and 55%, on Huh-7 cell are 85% and 96%, correspondence. The X-D looks like more efficient than X-E. The effect was accumulative in presence of the two shRNAs, the levels of HBx protein expression only 20% and 3% compare with control on COS-1 and Huh-7 cells, correspondence. No interference in the HBx levels was observed in the presence of c-*myc* - specific M-T and M-Z (lanes 12-14) showing their specificity for respective target genes.

Like HBx, both shRNAs against c-*myc* were also significantly effective against the target gene on COS-1 and Huh-7 cells (Fig. 24). M-T (lanes 3-5) was more effective than M-Z (lanes 6-8) and showed a five to ten fold inhibition in the expression of c-*myc* on COS-1 cells (Fig. 24A). Per contra, M-Z (lanes 6-8) was more effective than M-T (lanes 3-5) and showed a three to six fold inhibition in the expression of c-*myc* on Huh-7 cell (Fig. 24B). The interference was cumulative in

the presence of both M-T and M-Z (lanes 9-11) and the expression of target protein was virtually abolished (>20 fold inhibition). 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 respective target genes on both COS-1 and Huh-7 cells.

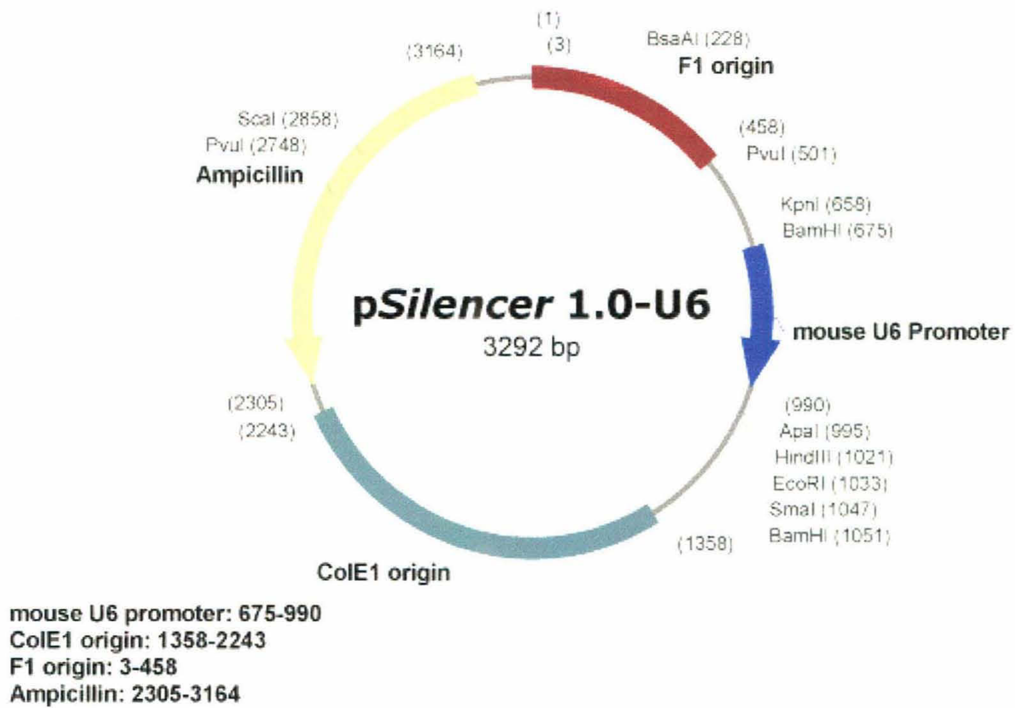
To confirm whether decreased protein levels of HBx and c-*myc* were due to down-regulation of the respective mRNA levels by shRNA, Northern blot assay was done using total RNA from the transfected cells. As observed with the X protein, a two to three fold decrease in the mRNA levels was also observed in the presence of X-D and/or X-E (Fig. 25). The RNA level was effectively lowered by nearly 3 fold in the presence of either X-D or X-E (lane 2, 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). Further, the inhibition by shRNA involved generation of 23 nt dsRNAs in these experiments (Fig. 26).

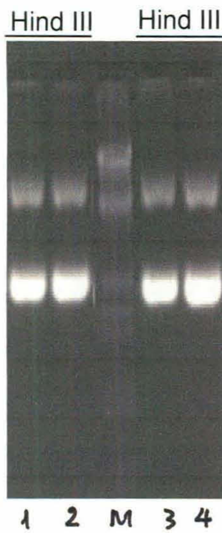
5.2.4. Regulation of transactivation function by shRNAs

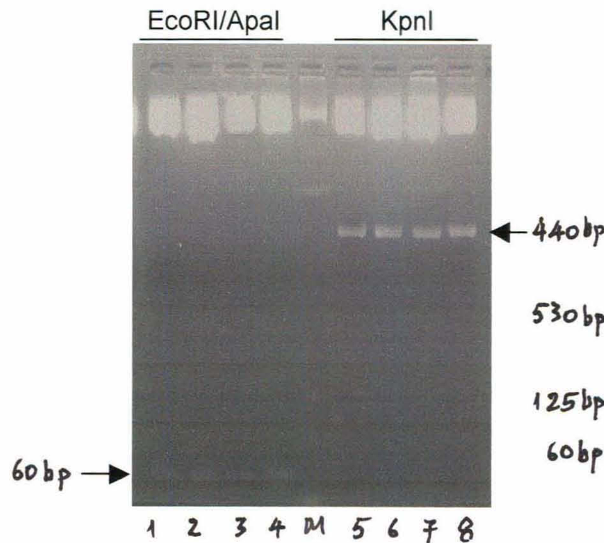
To demonstrate that inhibition the target gene expression was not a direct effect of RNAprotein interaction but rather an effect of decreased level of the protein, the transactivation property of HBx and c-*myc* was studies in transient transfection assays using the RSV-CAT reporter gene construct. As shown in Fig. 27 the HBx-mediated transactivation of RSV-LTR could be significantly inhibited in the presence of both X-D (lanes 3-5) and X-E (lanes 6-8) in COS-1 cells. 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 presence of the two shRNAs (Fig. 28) and the transactivation level could be significantly reduced to a low level (lane 5). A similar pattern in the inhibition of HBx-dependent transactivation was observed in Huh-7 cells (Fig. 29, 30). Maximum inhibition was

nearly 7 fold at the highest concentration (2 μg) of X-D (lane 5). The effect was cumulative in presence of the two shRNAs (Fig. 30) and the transactivation level could be significantly reduced to an undetectable level (lane 5). Most results shown above were highly significant.

Like HBx, the transactivator function of c-*m*yc could also be significantly inhibited by the two shRNAs (Fig. 31). M-T (lanes 3-5) appeared relatively more efficient than M-Z (lanes 6-8) and at 1:1 molar ratio, no CAT activity could be detected (lanes 4, 5). Further, the inhibitory effect was cumulative in presence of both M-T and M-Z (Fig. 32) and the transactivation level could be significantly inhibited to an undetectable level (lane 5). A similar inhibition pattern in c-*myc*-dependent transactivation was observed in Huh-7 cells and most results were highly significant (Fig. 33, 34).







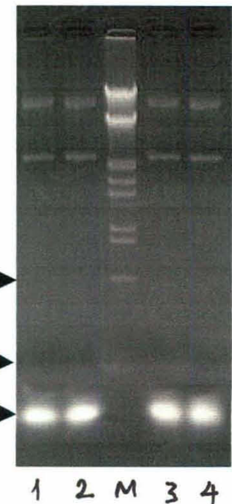


Fig. 22: Construction of the pSilencer vector (A). The clones with insert X-D (lane 1), X-E (lane 2), M-T (lane 3) and M-Z (lane 4) was digested with indicated enzymes and analyzed by agarose gel electrophoresis (B, C) and further confirmed by PCR amplification using a T3 primer and gene-specific forward primer (D) (section 4.2.2.2) . M: DNA molecular weight markers.

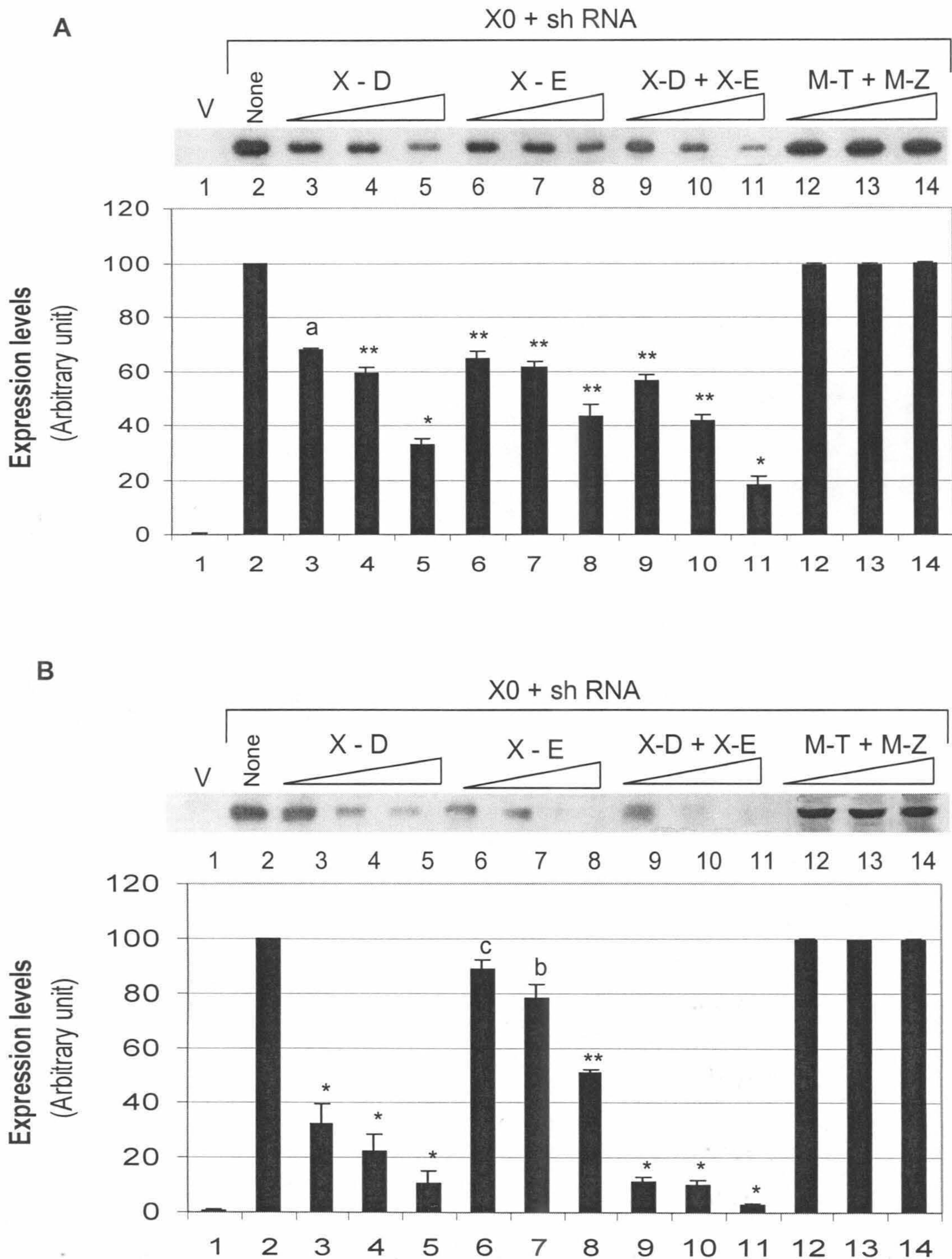


Fig. 23: Inhibition of the expression of HBx by shRNAs. For monitoring the protein levels, Cos-1 cells (A) or Huh7 cells (B) were co-transfected with expression plasmids (0.5 µg) for HBx along with increasing amounts (0.5, 1 and 2 µg) of different shRNA constructs. The cells extracts were immunoprecipitated using anti-HBx antibody and bands were detected by chemiluminescence.

Level of significance: *, $P < 0.001$; **, $P < 0.01$; a, $P = 0.03$; b = 0.04; c = 0.06.

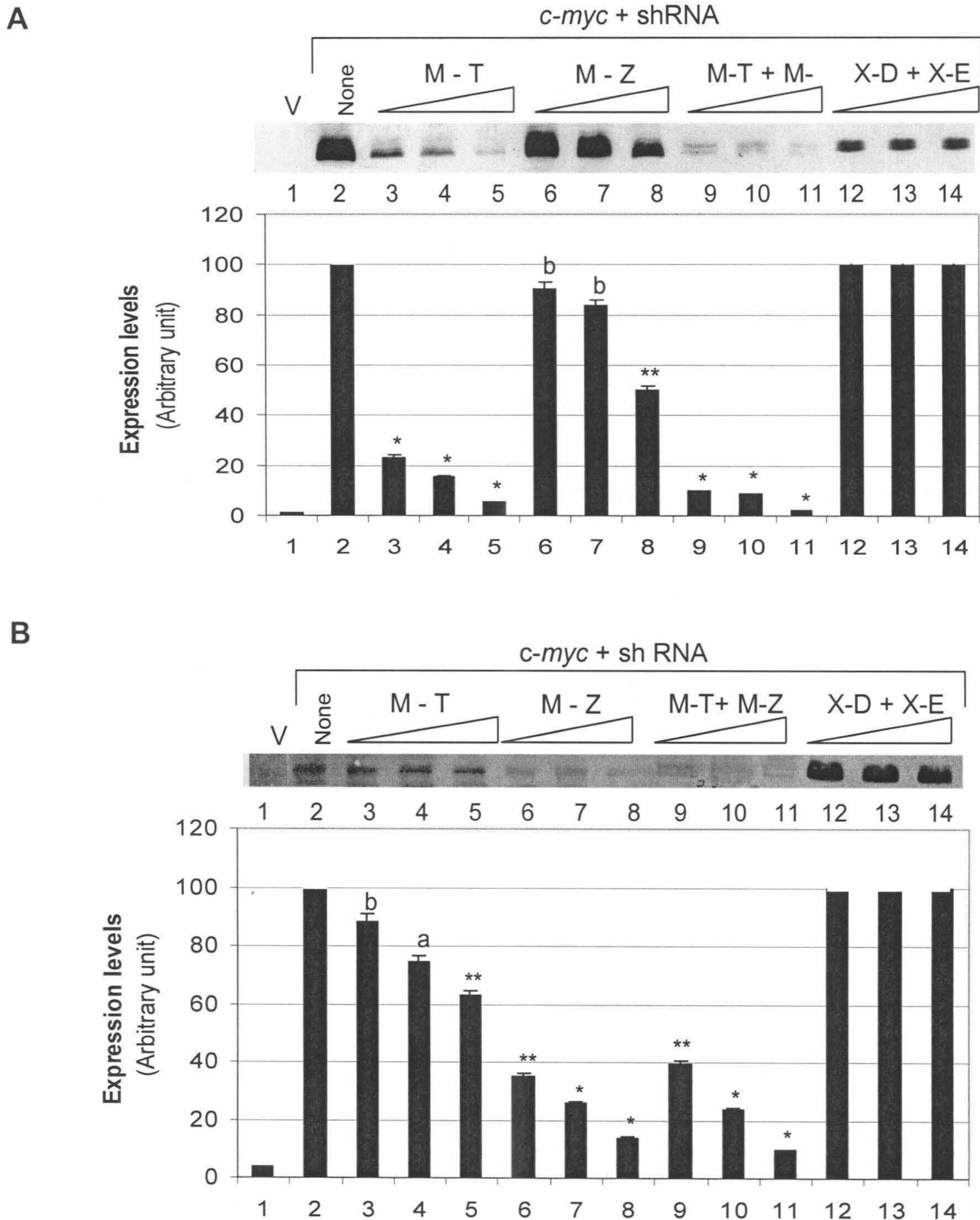


Fig. 24: Inhibition of the expression of c-*myc* by shRNAs. For monitoring the protein levels, COS-1 cells (A) or Huh7 cells (B) were co-transfected with expression plasmids (0.5 µg) for c-*myc* along with increasing amounts (0.5, 1 and 2 µg) of different shRNA constructs. The cells extracts were immunoprecipitated using anti-*myc* antibody and bands were detected by chemiluminescence.

Level of significance: *, $P < 0.001$; **, $P < 0.01$; a, $P = 0.02$; b, $P = 0.06$.

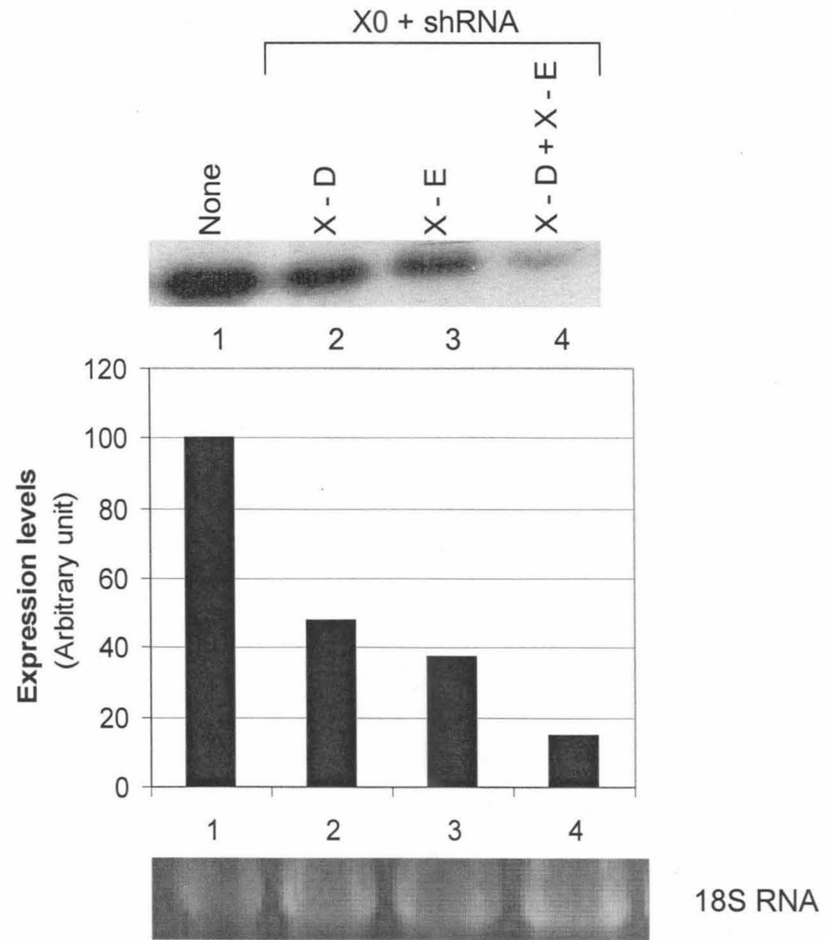


Fig. 25: Inhibition of the HBx mRNA by shRNA X-D and X-E. COS-1 cells were transiently co-transfection with X0 and/or shRNA constructs (2 μg). Total RNA isolated from the cells was analyzed by Northern hybridization using [^{32}P]-labelled X0 probe. The level of inhibition was determined by densitometry.

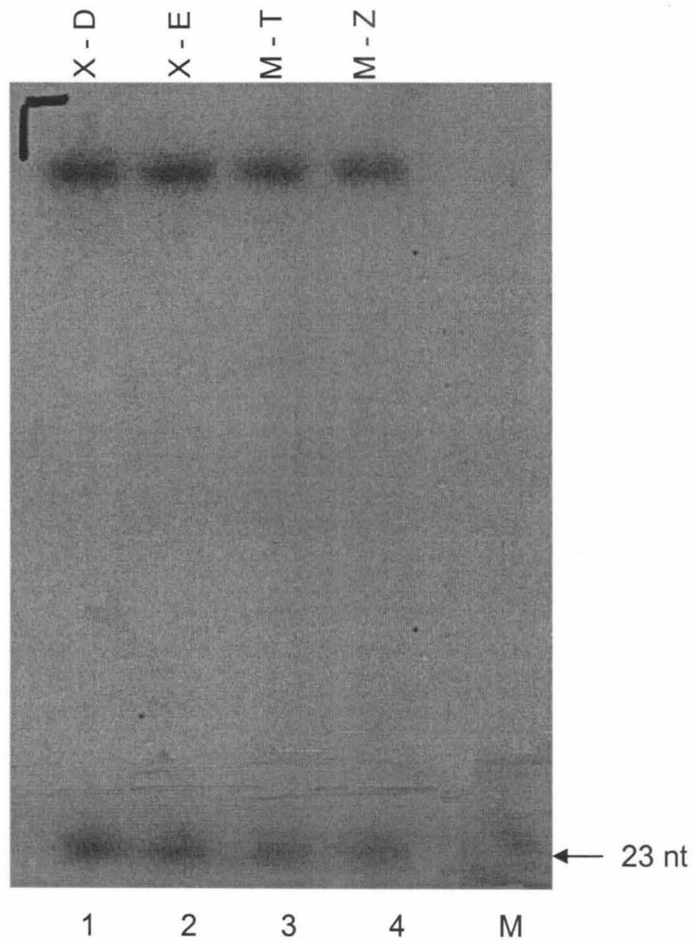


Fig. 26: Generation of 23 nt siRNAs for RNA interference.

COS-1 cells were transfected with the expression vectors for HBx (lanes 1, 2), c-*myc* (lanes 3, 4) along with the shRNA constructs X-D (lane 1), X-E (lane 2), M-T (lane 3) and M-Z (lane 4). Small molecular weight RNA were isolated and resolved by Urea-acrylamide gel electrophoresis, hybridized with X0 and c-*myc* probe (see 4.2.14) and autoradiographed. M, 23 mer oligonucleotide.

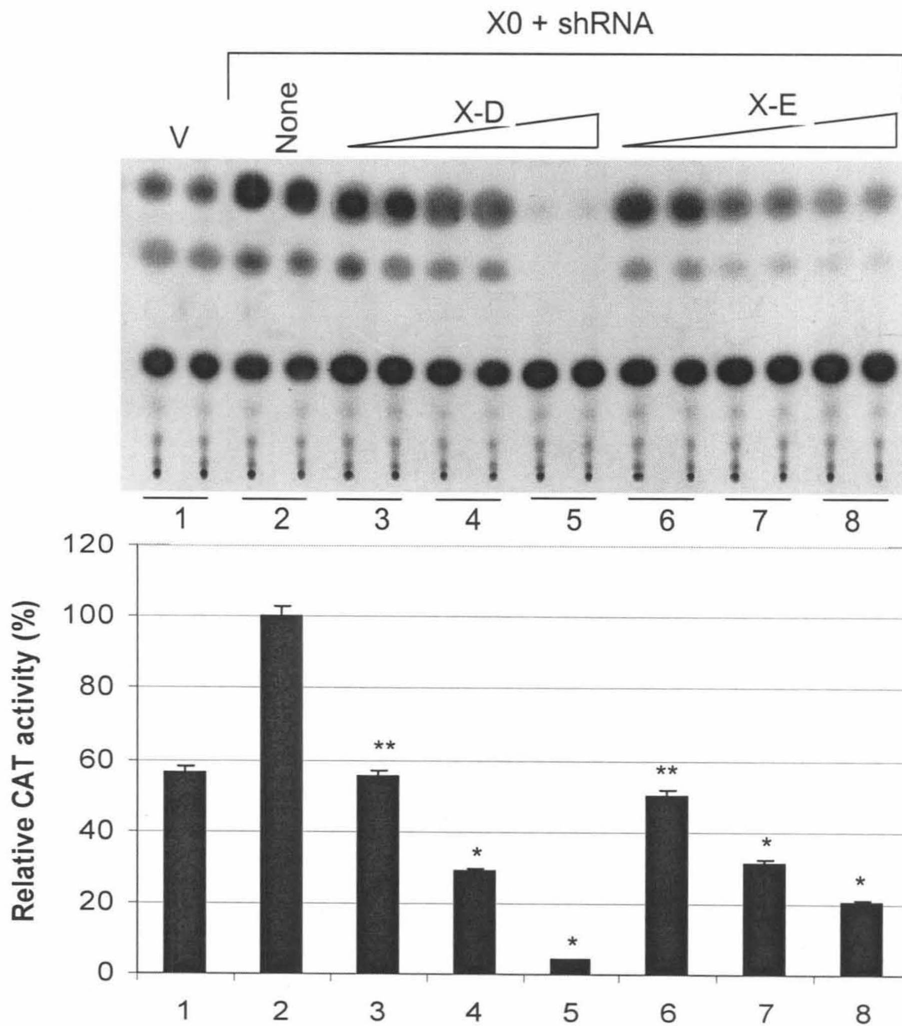


Fig. 27: Inhibition of the transactivation property of HBx by shRNA X-D and X-E. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for HBx and increasing amount (0.5, 1 and 2 μg) of different shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector Level of significance: *, $P < 0.001$; **, $P < 0.01$.

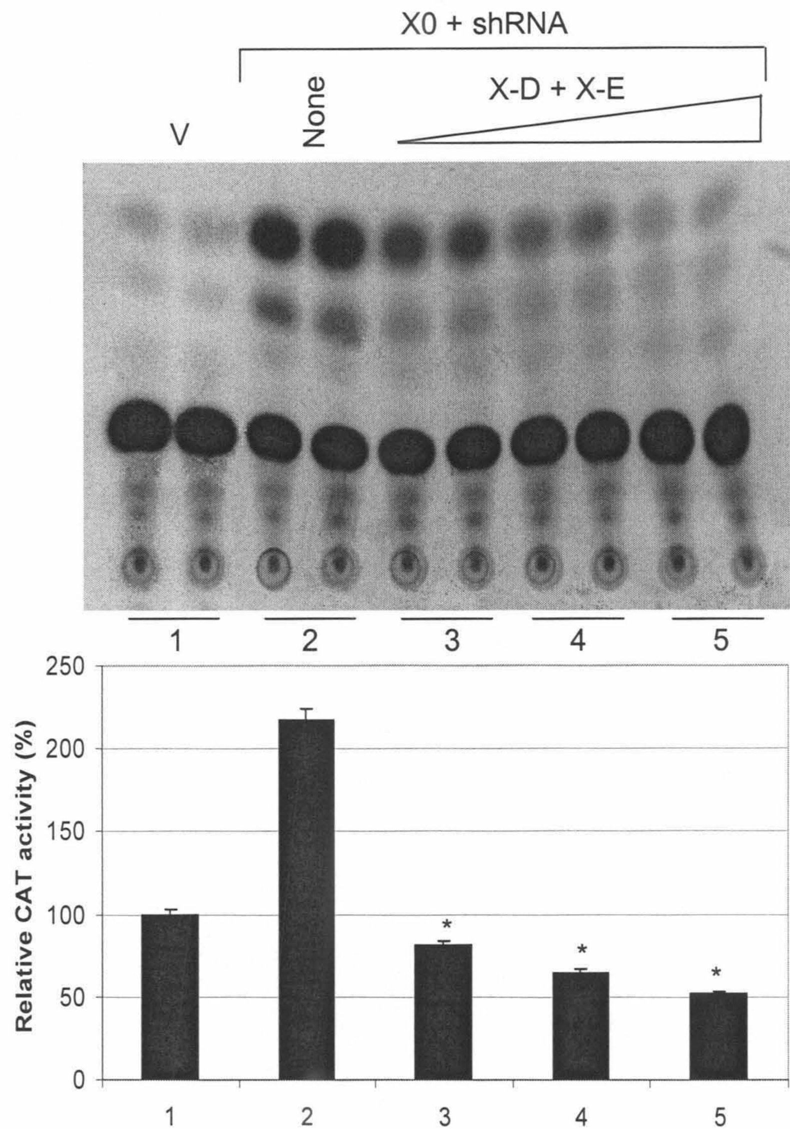


Fig. 28: Inhibition of the transactivation property of HBx and mix shRNA X-D and X-E. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for HBx and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector Level of significance: *, $P < 0.001$.

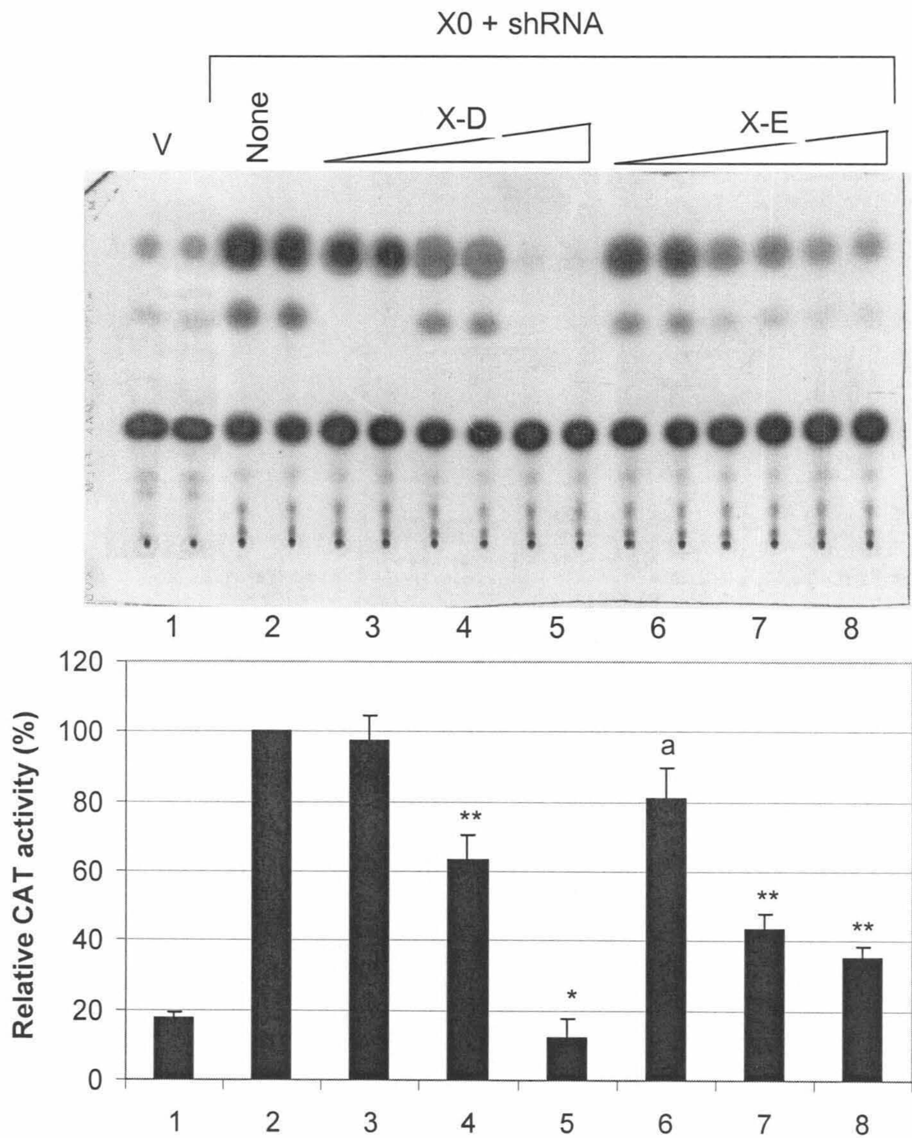


Fig. 29: Inhibition of the transactivation property of HBx and shRNA X-D and X-E. Huh-7 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for HBx and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector Level of significance: *, $P < 0.001$; **, $P < 0.01$; a, $P = 0.02$.

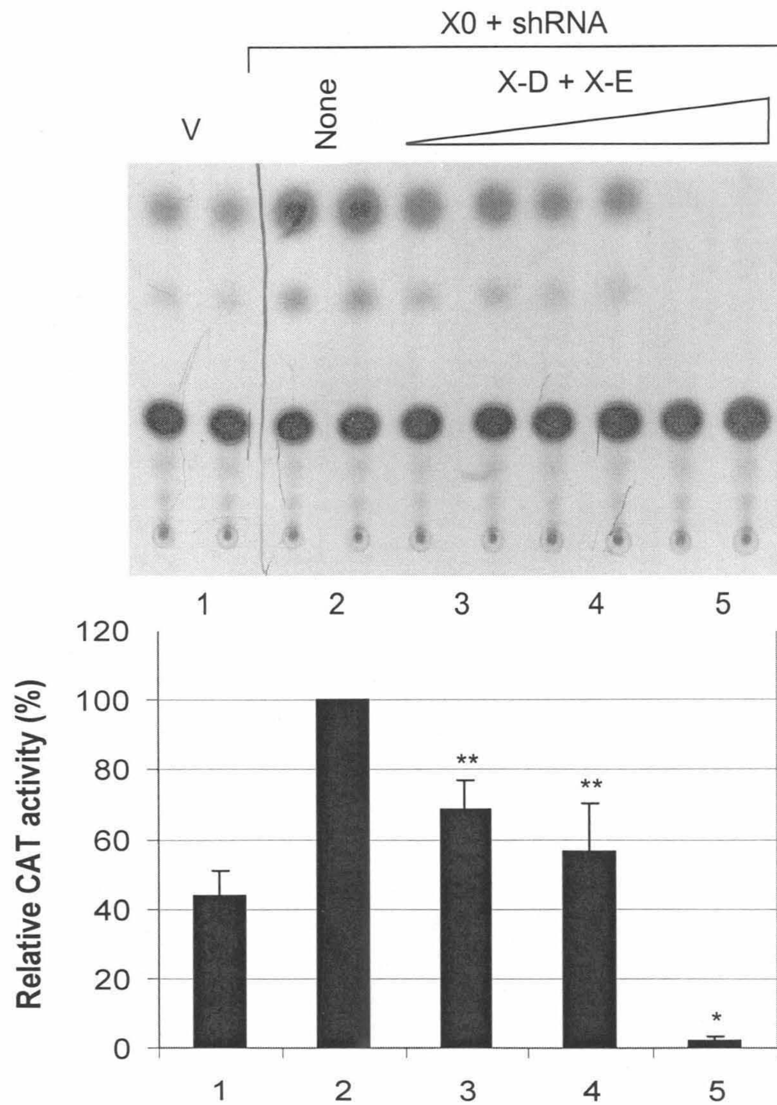


Fig. 30: Inhibition of the transactivation property of HBx and mix shRNA X-D and X-E. Huh-7 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for HBx and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5 vector Level of significance: *, $P < 0.001$; **, $P < 0.01$.

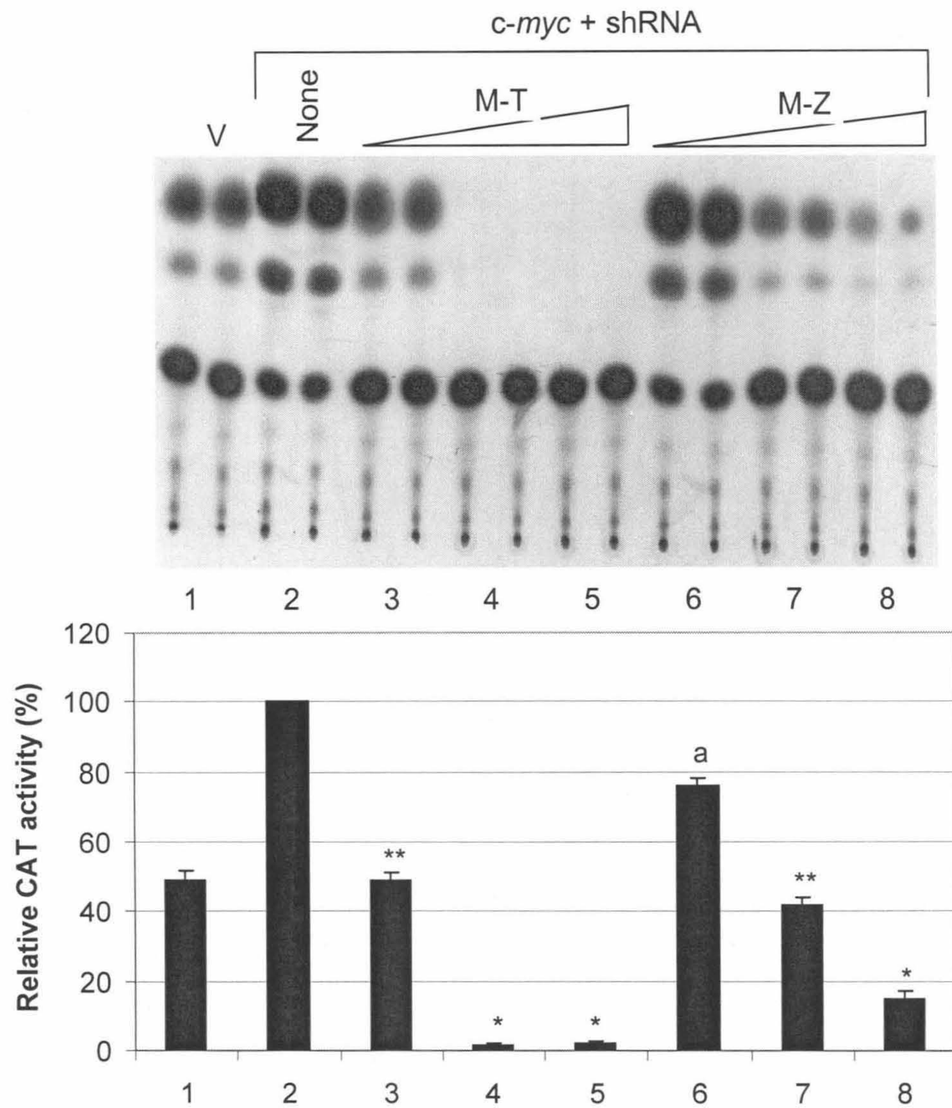


Fig. 31: Inhibition of the transactivation property of c-*myc* and shRNA M-T and M-Z. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for *myc* and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5-*myc* vector.

Level of significance: *, $P < 0.001$; **, $P < 0.01$; a, $P = 0.02$.

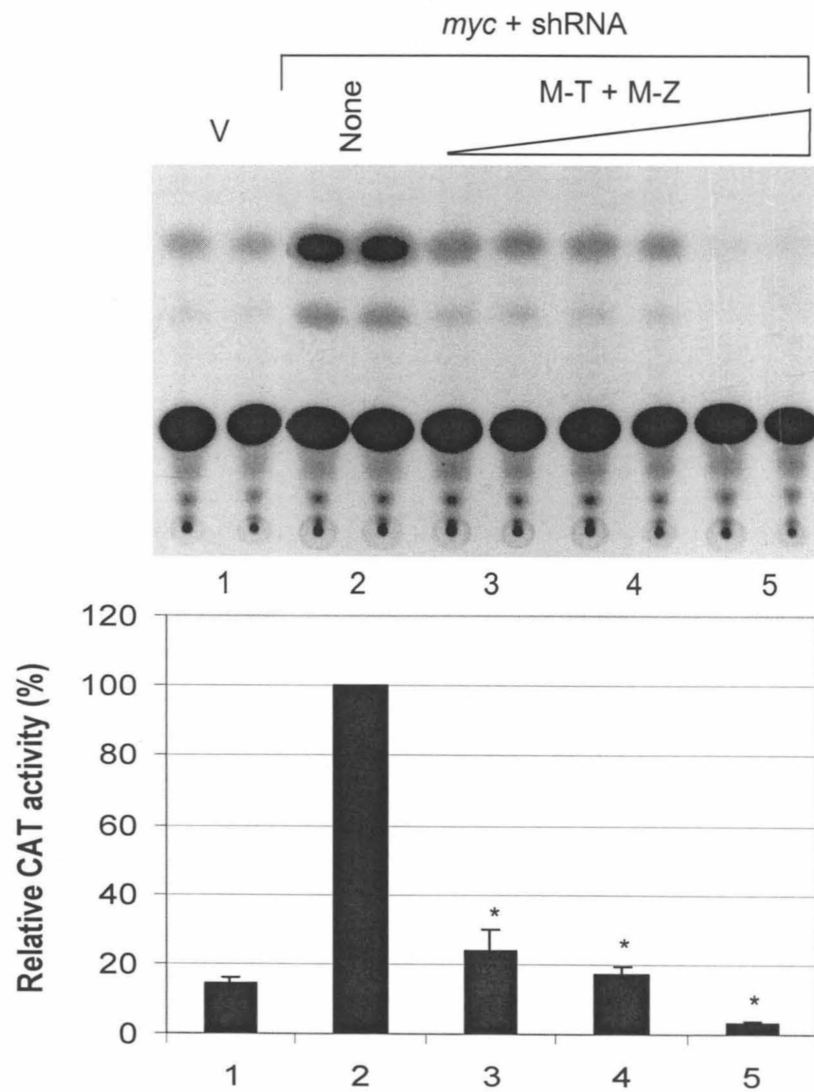


Fig. 32: Inhibition of the transactivation property of c-*myc* and mix shRNA M-T and M-Z. COS-1 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for *myc* and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5-*myc* vector.

Level of significance: *, $P < 0.001$.

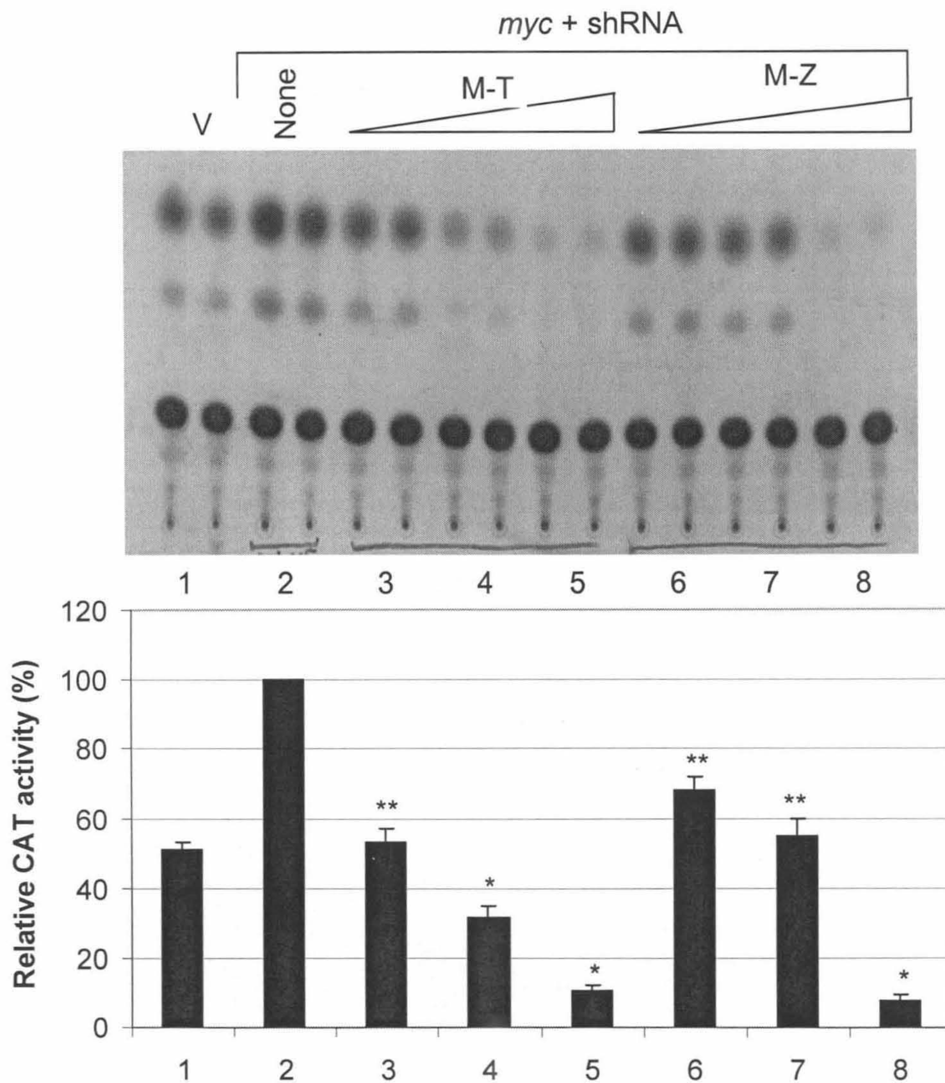


Fig. 33: Inhibition of the transactivation property of c-*myc* and shRNA M-T and M-Z. Huh-7 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for *myc* and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5-*myc* vector.

Level of significance: *, $P < 0.001$; **, $P < 0.01$.

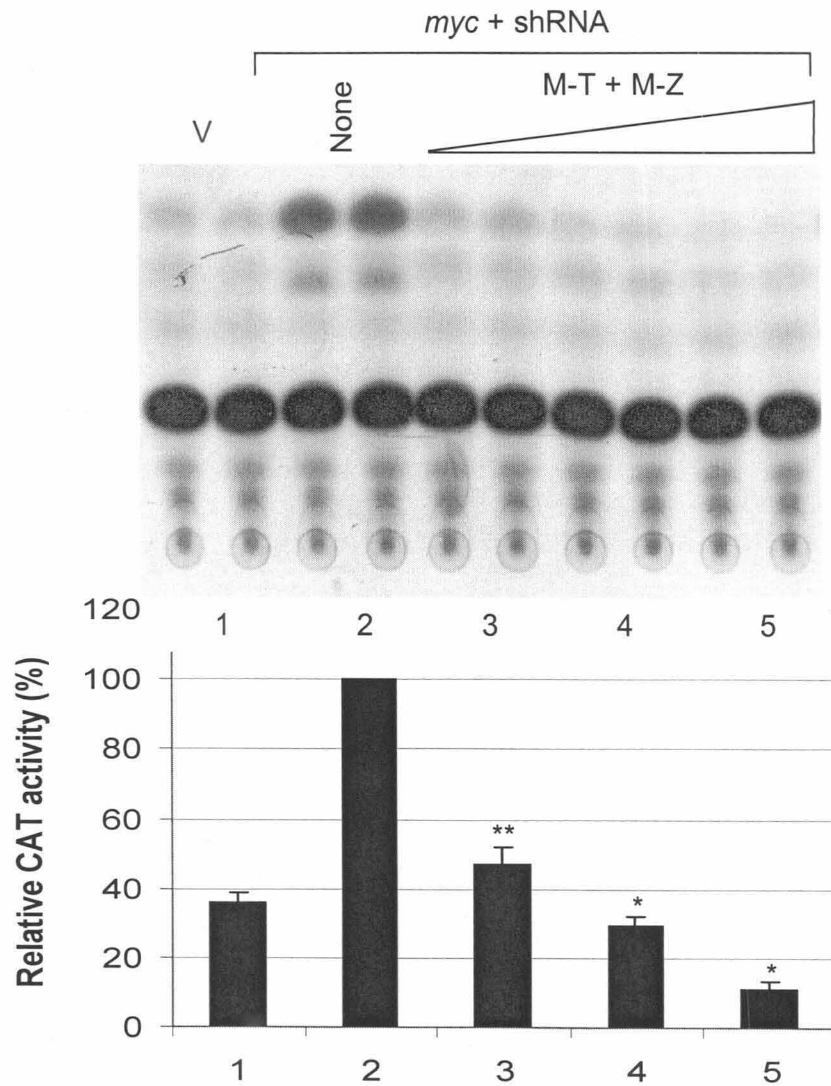


Fig. 34: Inhibition of the transactivation property of c-*myc* and mix shRNA M-T and M-Z. Huh-7 cells were transiently transfected with the reporter plasmid RSV-CAT (0.5 μg), the expression vectors (0.5 μg) for *myc* and increasing amount (0,5, 1 and 2 μg) of both shRNAs as indicated. CAT activity was measured in the cell extracts and values (mean of 3 observations) were determined by densitometry. V: pSG5-*myc* vector

Level of significance: *, $P < 0.001$; **, $P < 0.01$.

6. Discussion

An important goal in treatment for hepatitis B is to prevent the development of HCC for which there is no effective therapy. The integration/expression of the X gene has been observed in majority of the HBV-related HCC (Caselmann 1996; Paterlini *et al.* 1995; Poussin *et al.* 1999; Zhou *et al.* 1987). Further, the oncogenic cooperation of HBx with the *myc* family of genes has been suggested in HCC in the hepadnavirus infected woodchucks (Hsu *et al.* 1988) and ground squirrels (Transy *et al.* 1992). Interestingly, amplification *c-myc* has also been observed in human HCCs (Peng *et al.* 1993). An effective way to control HBV-related HCC would be to interfere with the expression of viral transactivator HBx and cellular transcription factor c-*myc* where c-*myc* is essential for cellular growth and differentiation (Dang 1999) and HBx supports for viral replication (Xu *et al.*, 2002; Cromlish 1996). With the ultimate aim to develop gene therapy for HCC in the X-*myc* transgenic mice (Lakhtakia *et al.*, 2003), cell culture-based study was undertaken to evaluate the efficacy of seven anti-sense and four shRNA constructs on the regulation of constituent transgenes HBx and c-*myc*.

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 (Kurreck, 2003; Lewin and Hauswirth, 2001; Hasuwa *et al.* 2002). In the present study, three antisense recombinants, including two directed against HBx and one against c-*myc*, were developed and carefully analyzed by restriction enzyme digestion. Further, each antisense construct was evaluated for its ability to regulate their cognate gene by transient transfection in the COS-1 monkey kidney cell line and the human hepatoma Huh-7 cells. At least three parameters were monitored in these studies

including RNA (by Northern blot assay) and protein levels (by immunoprecipitation), and the transactivation function of HBx and c-*myc* (by CAT assay). Our results showed that the two antisense recombinants were effective against the expression of HBx protein in both COS-1 as well as Huh-7 cells (Fig. 12). However, the inhibitory effect was more pronounced (>90%) with the antisense construct A2 as compared to A1 suggesting that SV40 promoter was stronger in both cell types than the X gene promoter of HBV. Interestingly, there was only a 50% reduction in the HBx mRNA levels by A2 as against 20% inhibition by A1 (Fig. 14). This result indicated that inhibition in the HBx level was primarily due to blockade at the translation level (antisense effect) and unlikely to be due to generation of siRNAs. Further, the antisense effect also reflected at functional levels. Accordingly, the HBx-dependent transactivation of RSV-LTR was effectively blocked by the antisense constructs A1 and A2 both is COS-1 (Fig. 15) as well as Huh-7 cells (Fig. 16). Attempts have been made in the recent past to regulate the expression of HBx in cell culture and experimental animals using anti-HBx antibody, antisense oligonucleotides as well as ribozymes. With antisense phosphorothioate oligonucleotides directed against the initiation codon of the X gene can prevent the development of pre-neoplastic lesions in transgenic mouse model of HCC (Moriya *et al.* 1996). Some hammerhead ribozymes have also been designed that can inhibit the expression of functional HBx and show anti replicative effect in cell culture (Kim *et al*, 1999; Weinberg *et al.* 2000). However, the potential of ribozymes and antisense oligonucleotides remains to be tested in the treatment of chronic liver disease and HCC. The antisense recombinants used in the present study, are likely to give better results in vivo since these were more effective than antisense oligonucleotides and ribozymes.

The over expression of the *myc* proto-oncogene has been implicated in the pathogenesis of most types of human cancer. Therefore, inactivation of *myc* may be effective in the treatment of neoplasia. Like HBx, we observed that c-*myc* and pc-*myc* could be effectively regulated by antisense *myc* construct A3 both at protein (Fig. 13) as well as transactivation levels (Fig. 17, 18). Though the antisense effect was independent of cell types used, it was relatively more effective in regulating c-*myc* levels in hepatoma cells. Antisense approach has been extensively used in the past to pharmacologically inactivate c-*myc* and therefore, regulate the proliferative capacity of a wide range of cancer cell lines of human origin including promelocytic leukemia cells HL-60 (Wickstrom *et al.*, 1988), breast cancer MCF-7 cells (Carroll *et al.*, 2002; Watson *et al.*, 1991), melanoma cells gastric carcinoma cells SGC7901 (Chen *et al.*, 2001), esophageal cancer EC8712 cells (Zhao *et al.*, 1995), colon cancer cell lines Colo 320 DM, LS174T, SW1116 and SW48 (Li *et al.*, 1995; Yu *et al.*, 1997), small –cell-lung-cancer cells SCLC (Akie *et al.* 2000; van Waardenburg *et al.*, 1997), ovarian cancer cells NIH:OVCAR-3 (Park, 1997), prostate cancer cell lines LNCaP, PC3 and DU145 (Balaji *et al.*, 1997; Steiner *et al.*, 1998) and human hepatoma cells HCC-M, HCC-T, PLC/PRF/5 and HepG2 (Ebinuma *et al.*, 1999, 2001). Further, c-*myc* antisense oligonucleotides have been tested for antitumoral efficacy on melanoma xenografts in nude mice (Leonetti *et al.*, 1996; Pastorino *et al.*, 2003). All these results suggest that inhibition of c-*myc* by targeted antisense therapy could provide an effective approach for the treatment of different cancer at least in an adjuvant setting. Therefore, it would be interesting to study the regulation of HCC by the antisense *myc* recombinant in the X-*myc* mice developed earlier by our laboratory (Kumar *et al.*, 2001).

Recently, RNAi has been used as a powerful antiviral strategy to silence viral-specific genes. From these studies, it is now clear that the RNAi approach is far more efficient than the antisense RNA and ribozyme strategies (Miyagishi *et al.*, 2003; Grunweller *et al.*, 2003; Bertrand *et al.*, 2002). Besides amplification through RISC another important reason could be that siRNA is more resistant to nuclease degradation as compared with antisense oligos, and therefore, have longer therapeutic effects than the antisense approaches. The ability to selectively silence mammalian gene expression using siRNA has opened new and exciting routes to not only understand mammalian cell biology in health and disease, but also has 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 (Jacque *et al.*, 2002; Novina *et al.*, 2002; Qin *et al.*, 2003), hepatitis B virus (Shlomai and Shaul, 2003; McCaffrey *et al.*, 2003), hepatitis C virus (Randall *et al.*, 2003; Kapadia *et al.* 2003), poliovirus (Gitlin *et al.* 2002), rotavirus (Dector *et al.*, 2002), human papillomavirus (Jiang *et al.*, 2002), Rous sarcoma virus (Hu *et al.*, 2002), gamma herpesvirus (Jia and Sun, 2003). Besides, siRNA has also been found effective against trypanosomes (Wang and Englund, 2002), plasmodium (McRobert and McConkey, 2002), Fas-mediated fulminant hepatitis in mice (Song *et al.*, 2003) cancer growth (HCC) by inhibiting cyclin E over expression (Li *et al.*, 2003) and the prion protein accumulation in neuroblastoma cells (Daude *et al.*, 2003). Thus, siRNA has a tremendous promise as a therapeutic tool by targeted

gene silencing. However, its utility will depend on the specificity, i.e., its ability to specifically knockdown the target gene without interference with the expression or function of other genes or proteins. Expression of short hairpin RNAs directed against the X gene appears to induce RNAi response that can dramatically reduce the HBV DNA replication (Shlomai and Shaul, 2003) and secretion levels of surface antigen in cell culture (McCaffrey *et al.*, 2003). Thus, RNAi appears to be an effective therapeutic tool in the management of hepatitis B-associated liver diseases. Our results from the mammalian cell culture show the effectiveness of siRNA approach in the regulation of the intracellular levels of HBx (Fig. 23) and c-*myc* (Fig. 24). The effect of the four shRNAs used in present study was highly specific as no cross interference was observed. As expected, generation of 23 nucleotide species of siRNA was observed in our experiments (Fig. 26) and manifestation of siRNAs appears most likely to be at RNA level (Fig. 25) that ultimately is responsible for down-regulation of cognate proteins (Fig. 23, 24) and inhibition of transactivation function (Fig. 27-34). The cumulative inhibitory effect by multiple siRNAs against one target gene is likely to be more successful due to the amplification of RISC. 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 the siRNA-based therapeutic agents are likely to have inherent molecular specificity. Though exquisite sequence specificity for the target mRNAs has been questioned recently (Jackson *et al.*, 2003; Semizarov *et al.*, 2003) and the activation of interferon responses has also been observed in the presence of siRNAs (Bridge *et al.*, 2003; Sledz *et al.*, 2003), our results on shRNAs for HBx and c-*myc* provide a case specific examples of regulation of transcription

factors that are known to co-operate in the X-*myc* mice to induce HCC (Lakhtakia *et al.*, 2003).

Thus, the above data should be helpful in developing and testing therapeutic strategies for HCC based on RNA interference.

7. Summary

1. Expression vectors for c-*myc* and pc-*myc* were constructed.
2. Three antisense constructs, including two against HBx and one against c-*myc*, were developed.
3. Four shRNA expression vectors - two each against HBx and c-*myc* were made.
4. The RNA interference effects (antisense and siRNA) were investigated in transient transfection assays using COS-1 and Huh-7 cells.
5. The antisense and shRNA recombinants showed a significant inhibition expression of HBx and c-*myc* both at protein as well as RNA levels. Further, the transactivation function of the two transactivation proteins was also blocked.
6. The antisense effect appeared primarily due to blockade at translational level while the siRNA effect was mediated by 23 nucleotide RNA species.

The data presented in the thesis is encouraging for developing and testing therapeutic strategies for HCC based on RNA interference.

8. References

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