Gene Regulation by Antisense DNA and Catalytic Nucleic Acids

Thesis Submitted To

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For The Degree of

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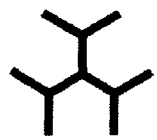

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राष्ट्रीय प्रतिरक्षाविज्ञान संस्थान
NATIONAL INSTITUTE OF IMMUNOLOGY

CERTIFICATE

This is to certify that this thesis entitled **"Gene regulation by Antisense DNA and Catalytic nucleic acids"**, being submitted by Nidhi Gupta embodies the work done by the candidate under my guidance at National Institute of Immunology. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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µCi	micro Curie
µg	microgram
µl	microlitre
µM	micro Molar
Ab	Antibody
APOBEC3G/3F	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G or like-3F
AIDS	Acquired immunodeficiency syndrome
bp	Base pairs
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CaCl	Calcium Chloride
CO_2	Carbon dioxide
cDNA	Complementary DNA
Ci	Curie
cpm	Counts per minute
°C	Degree celsius
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytosine triphosphate
dGTP	Deoxy-guanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTPs	Deoxyribose nucleoside triphosphates
ds	Double stranded
DTT	1, 4-Dithio-DL-threitol
Dz/Dzs	DNA-enzyme/DNA-enzymes
EB	Elution buffer

ECL	Enhanced chemiluminiscence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene gylcol-bis (β-aminoethyl ether) N, N, N′, N′,-tetraacetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum
HBV	Hepatitis B virus
HBx/X	Hepatitis B virus X protein
HCl	Hydro-chloric acid
HEK	Human embryonic kidney cell line
HepG2	Human hepatocellular liver carcinoma cell line
HIV	Human immunodeficiency virus
hrs	Hours
HRP	Horse raddish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
K_{cat}	Catalytic efficiency of an enzyme
kDa	kilo Dalton
K_m	Michaelis-Menten's constant
LB	Luria Bertani
Luc	Luciferase
LTR	Long terminal repeats
mg	milligram
min	minutes
ml	millilitre
mM	milliMolar
mmoles	millimoles
MCS	Multiple cloning site
MgCl	Magnesium Chloride
MOPS	3-[N-morpholino] propane sulphonic acid
mRNA	Messenger ribonucleic acid

NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NC	Nitrocellulose
ng	nanogram
NFW	Nuclease free water
NMR	Nuclear magnetic resonance
nt/nts	Nucleotides
O.D.	Optical density
ODN	Oligodeoxynucleotides
ORF	Open reading frame
PBS	Phosphate buffered saline
PBS-T	Phosphate buffer saline-Tween 20
PCR	Polymerase chain reaction
pmoles	picomoles
rpm	Rotations per minute
rcf	Relative centrifugal force
RNA	Ribonucleic acids
RNAi	RNA interference
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse Transcription-PCR
Rz/Rzs	Ribozyme/Ribozymes
s	small
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SIV	Simian immunodeficiency virus
siRNA	small interfering RNA
ss	Single stranded

SSC	Sodium chloride and sodium citrate
TAE	Tris-EDTA-acetic acid
TCA	Tri-chloro acetic acid
TE	Tris-EDTA
TEMED	N, N, N′, N′-tetramethyl-ethane-1, 2-diamine
Tris	Tris-hydroxy methyl- amino methyl
tRNA	Transfer ribonucleic acid
U	Unit
UNR	Unrelated
UTP	Uridine triphosphate
UTR	Untranslated region
UV	Ultra violet
Vif	Virion infectivity factor
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

The past decade has seen the rapid evolution of small molecule gene-silencing strategies, driven largely by enhanced understanding of gene function in the pathogenesis of disease. Over this time, many genes have been targeted by specifically engineered agents from different classes of nucleic acid-based drugs in experimental models of disease to probe, dissect, and characterize further the complex processes that underpin molecular signaling. Arising from this, a number of molecules have been examined in the setting of clinical trials, and several have recently made the successful transition from the bench to the clinic, heralding an exciting era of gene-specific treatments. This is particularly important because clear inadequacies in present therapies account for significant morbidity, mortality, and cost. The broad umbrella of gene-silencing therapeutics encompasses a range of agents that include ribozymes, DNA enzymes, antisense oligonucleotides, short interfering RNA, decoys, and aptamers, all of which attenuate gene expression by interfering with mRNA or translated protein.

Around the world, studies are being done to evaluate the merit of delivering genes that would be transcribed into short RNA strands. These RNA decoys either bind to regulatory proteins or specific transcript factors and block their functions or bind to mRNA and henceforth, inhibit the translational process. Such mRNA binding RNAs are mainly classified as ribozymes and antisense RNA. Ribozymes are catalytic RNA molecules acting as RNA restriction endonucleases. In nature; these are the only nucleic acids that have enzymatic properties. These include the self-splicing group I and group II introns (A, B), the RNA component of RNase P, which cleaves precursor tRNAs to generate mature tRNAs (Guerrier-Takada et al, 1983), and the various self-cleaving RNAs, including the "hammerhead," "hairpin," HDV (hepatitis delta virus), and VS (*Neurospora* Varkud satellite) motifs (Zaug, et al, 1986; Hampel, et al, 1989). In addition, the RNA component of

the large ribosomal subunit is now recognized to be a ribozyme that catalyzes the peptidyl transferase step of translation (H). Ribosomal RNA is unique among known naturally occurring ribozymes because it contains several modified nucleotides, although it is not clear if these modifications are essential for catalysis. The U2/U6 snRNA complex within the eukaryotic spliceosome also may be a ribozyme (I). These two RNA molecules in isolation catalyze an unusual phosphoryl transfer reaction, but the reaction occurs very slowly and may not reflect the natural catalytic activity of the spliceosome.

Although DNA plays a central role in the storage and transmission of genetic information, its ability to serve in other important biochemical tasks, and particularly to perform catalysis, is severely hampered by a series of intrinsic structural and chemical constraints. DNA side chains are limited in number and diversity, whereas the polynucleotide backbone is negatively charged and highly flexible. All these factors are expected to limit the propensity of DNA to form a compact structure, which is a prerequisite for efficient catalysis (Breaker, 1997).

Furthermore, DNA is endowed with chemical functionalities that are essentially inert under physiological conditions, and it even lacks the 2'-hydroxyl groups that are so important for the structure and function of catalytic RNAs. Despite all these limits, *in vitro* selection approaches have recently produced several DNA enzymes (deoxyribozymes) that efficiently catalyze reactions once thought to belong only to the realm of protein and RNA enzymes (Baum and Silverman, 2008, Chandra and Silverman, 2008). Shortly after the discovery of ribozymes, search was done to look for possible catalytic DNA molecules. DNA in its single stranded form can be made to perform both molecular recognition and catalysis-biochemical operations that were earlier thought to be restricted to macromolecules made up of proteins and RNA. One of the most active areas of catalytic DNA research

has been generation of deoxyribozymes that perform the clinical reaction-RNA cleavage by transesterification. The DNA enzymes can easily be engineered to cleave any target RNA in a sequence-specific and catalytic manner.

Due to occurrence of high mutational rate for viral mRNA transcripts, strategies are being looked forward to design chimera DNA enzymes. These are di-DNA enzymes with two DNA-enzyme in direct tandem with no spacer sequences. These chimera DNA-enzymes target more than one site in target RNA for cleavage.

Some of these *in vitro* evolved nucleic acid enzymes have a functional counterpart among the naturally occurring RNA enzymes, but most catalyze reactions that previously had only been observed among protein enzymes. The chemical diversity and therefore the catalytic potential of nucleic acids are limited compared to that of proteins. Nucleic acids lack a general acid base with a pKa that is near neutral (as in histidine), a primary alkyl amine (as in lysine), a carboxylate (as in aspartate), and a sulfhydryl (as in cysteine). However, the missing functionality can be supplied by employing modified nucleotides or adding a small-molecule cofactor.

Catalytic nucleic acids at times were found to be not very efficient in cleaving long RNA substrates in trans. Use of antisense oligonucleotides (stretches of DNA sequences of around 20 nucleotides that are complimentary to target RNA sequence), upstream and downstream of the cleavage site, along with the ribozyme or DNA enzyme; seems to be an upcoming prospect in the field of nucleic acids as genetic therapeutic agents. Nucleic-acid-based approaches are based on complimentary base pairing of the target RNA with either antisense DNA, catalytic DNA (DNA-enzymes), or catalytic RNA (ribozymes). This allows for specific gene targeting.

Various viruses like HIV-1(family *Retroviridae*), Hepatitis B virus (family *Hepadanviridae*) and mammalian reovirus (family *Reoviridae*) are taken as models to study the modifications of gene expression by usage of antisense oligodeoxynucleotides (ODNs) or ribozymes (Rzs) or DNA-enzymes (Dzs) or small interfering RNAs (siRNAs).

REVIEW OF LITERATURE

The ability of nucleic acids as biological catalysts has become well established in the last few years. Formerly, biochemical dogma dictated that proteins performed all biological chemical reactions, and nucleic acids merely carried the genetic information that encoded the proteins. In particular, more recent significant advances in genomics have led to a substantial shift away from conventional perceptions and dogma to focus on intricate molecular and cellular pathways regulated by an array of key genes. This change has come with the discovery of certain RNA strands that were capable of catalyzing ligation reactions, analogous to that of RNA dependent RNA polymerase. It is at this interface that nucleic acid molecules had emerged as a potent force in further characterizing important molecular pathways and in defining themselves as a sustainable therapeutic class of agent. The ability to selectively attenuate the expression of specifically targeted genes represents an appealing method of therapy and a means of dissecting molecular function.

The use of antisense oligodeoxynucleotides (ODNs) is an approach to study cellular and viral gene functions and to block gene expression in a therapeutic context. Antisense therapy mainly involves delivery into cells of small DNA oligonucleotides (7-30 nt in length), complementary to target RNA. These oligonucleotides specifically hybridize with the target RNA within the cell and interfere with the function of RNA by blocking RNA transport, splicing or translation.

Knowledge of RNAi mechanism in mammalian cell in 2001 brought a storm in the field of drug discovery. During the past few years scientists all over the world are focusing on exploiting the therapeutic potential of RNAi for identifying a new class of therapeutics. Recently this field has converged with reports implicating small regulatory RNAs in the maintenance and pluripotency of stem cells (Stadler and Ruohola-Baker, 2008). RNAi can be developed as an endogenous host defense

mechanism against many infections and diseases. Several studies have demonstrated therapeutic benefits of small interfering RNAs and micro RNAs in animal models (Shrivastava and Shrivastava , 2008).

The various nucleic acids that have catalytic properties are broadly termed as Ribozymes, DNA-enzymes or small interfering RNAs (siRNAs). Also antisense oligodeoxynucleotides (ODNs) are used in order to augment the cleavage efficiency of the various catalytic nucleic acids. All the catalytic nucleic acids and ODNs hybridize with the target RNA based on Watson-Crick base pairing and this provides specificity towards the target RNA.

Ribozymes

The discovery of catalytic RNAs capable of carrying out sequence-specific cleavage reactions has attracted a great deal of attention as a means to selectively down-regulate the expression of target genes especially viral genes. Rz were discovered initially as self-cleaving entities as in ribonuclease P of *Escherichia coli* (Guerrier-Takada et al, 1983) and the intervening sequence of *Tetrahynema thermophila* (Zaug et al, 1986). The two most extensively worked Rz are- hairpin Rz: discovered from the "minus" strand of satellite RNA of tobacco ring spot virus (Hampel et al, 1989) and the other is hammerhead Rz: identified in the virusoid from lucerne transient-streak virus (Bruke, 1994). These two Rzs belong to the class "small Rz" in the classification of Rzs. The other Rzs like ribonuclease P, group I intron Rzs and group II intron Rzs are categorized as "large Rzs".

Rzs offer the potential for the specific inactivation of viral RNA genomes and mRNA products of disease-associated genes that, unlike most therapeutics, does not depend on the structure or function of the proteins that these RNAs encode (Bertrand et al, 1996). A difference in the biochemical requirements of various Rzs is the evidence that Rz can exploit more than one catalytic strategy. Subsequent investigations

confirmed that, despite catalyzing identical chemical reactions, each small Rz motif adopts a unique structure and exploits distinct kinetic and catalytic mechanisms (Buzayan et al, 1986). Enzymatic action by Rzs, is an inherently dynamic process (Munro et al, 2007). Just like protein enzymes, Rzs probably exploit the dynamics of functional groups and domains to guide the catalytic process along a specific reaction coordinate (Hashim and Walter, 2008).

Applications of Ribozymes:

Since the discovery of self-cleavage and ligation activity of the group I intron, the expansion of the research interest in catalytic nucleic acids has provided a valuable non-protein resource for manipulating biomolecules. This arena of catalytic nucleic acids as therapeutic agents has been driven predominantly by the discovery of hammerhead and hairpin Rzs as the latter induce specific RNA cleavage from a very small catalytic domain, allowing delivery either as a transgene expression product or directly as a synthetic oligonucleotide. Recently a number of clinical trials have also seen this gene inactivation technology used directly in humans. Although advances in the development of RNA modifications have improved the biological half-life of synthetic Rzs, their use is restricted by the mechanistic dependence on conserved 2'OH-moieties. RNA performs a wide range of functions in biology including catalysis of chemical reactions. Recently, hammerhead (Hartig and Famulok , 2008) and hairpin (Najafi-Shoushtari and Famulok, 2008) Rzs were used to signal molecular interactions. Another study showed the use of RNase P Rz to inhibit viral gene expression in animals and demonstrated their utility for gene targeting applications *in vivo*. This class of Rz was constructed to target the overlapping mRNA region of two murine cytomegalovirus (MCMV) capsid proteins essential for viral replication, namely the assembly protein (mAP) and M80 (Bai et al, 2008). Clinically, Rzs have been explored therapeutically in several small

trials. The use of hammerhead Rzs targeting a highly conserved portion of 5'-untranslated region of hepatitis C virus (Usman and Blatt, 2002) though showed promising results in phase I and II trials, however, had to be suspended because of toxicological concerns (Peracchi, 2004). Rz have also been evaluated as potential adjuncts in cancer therapy as well. These include the synthetic antiangiogenic ANGIOZYME, which targets the VEGF receptor VEGF R1 (Flt-1) in a variety of solid tumors (Weng et al, 2005), and HERzyme, which targets human epidermal growth factor-2 over expressed in breast and ovarian cell carcinoma (Zinnen et al, 2002). The issue of toxicity could be dealt with either doing certain chemical modifications or reducing the dosage by using facilitators or antisense ODNs (Gupta et al, 2008) and stability can be improved by substituting deoxyribonucleotides for ribonucleotides at non-catalytic bases (Taylor et al, 1992).

Rzs can be delivered either exogenously or endogenously. In exogenous delivery, ribozymal RNA is synthesized *in vitro* and then applied to cells or tissues by using liposomes or some other mechanisms of transportation. The disadvantage of exogenous supply is that RNA is very susceptible to degradation by cellular nucleases. In endogenous delivery, Rz is first cloned in expression vectors under the appropriate promoter like SV40 or CMV and by using delivery reagents like adenoviral vectors or retroviral vectors and lipososmes, is transported to the cells or tissues.

Hairpin Ribozyme:

It performs specific cleavage and ligation reactions required for "minus" strand replication of tobacco ring spot virus. It can function both *in cis* and *in trans* (Bruke, 1994). RNA cleavage by the hairpin Rz requires the 2-amino group of the substrate guanosine immediately 3' of the cleavage site (Chowrira, 1991). A minimal hairpin Rz contains four base paired helices, H1 through H4, and two unpaired loops, A and B,

with the reactive phosphodiester located within loop A. Apart from G11, most of the nucleotide changes are tolerable in the helices but not in the loops, indicating that loops are either involved in tertiary interactions or have direct role in catalysis (Hampel et al, 1990). The shortened form of the hairpin Rz is larger than the minimum hammerhead sequence. This bulky structure is the drawback of this motif since it increases the probability of many more secondary structures other than the main catalytic motif, which in turn leads to a decreased efficiency. The cleavage site for hairpin motif is "N*GUC", where N is any nucleotide and cleavage occurs at the position *.

Hammerhead Ribozyme:

It is the smallest Rz that is composed of approximately 30 nucleotides and is capable of site specific cleavage of a phosphodiester bond (Symons, 1998). The hammerhead Rz motif consists of three base paired helices, connected by two single stranded regions and a loop. The single stranded regions are conserved and possess the catalytic activity. The antisense arms are the "stems I" and "III" and the catalytic consensus sequence flank the "stem II" and loop regions. The catalytic consensus sequence has enzymatic activity to cleave the target RNA. The target sequence for cleavage usually consists of nucleotide sequence "NUX", where N is any base and X can be C, U or A, but not G. Usually, GUC is recommended for "NUX" sequence.

The function of mRNA is dependent on secondary structures composed of stem loops. Rz cleavage site should be located in loop structures in the target mRNAs. By cleavage at "NUX" sequence, Rz can significantly alter the structure of the target mRNA. The hammerhead Rz undergoes a well-defined two stage folding process induced by the sequential binding of two magnesium ions. The first corresponds to the formation of the Rz scaffold, while the second is the formation of the catalytic core of the Rz. The hammerhead Rz is capable of cleaving the

target mRNA *in cis* and *in trans* (Haseloff et al, 1988). In both configurations, the Rz undergoes multiple turnovers and virtually any sequence can be targeted for cleavage.

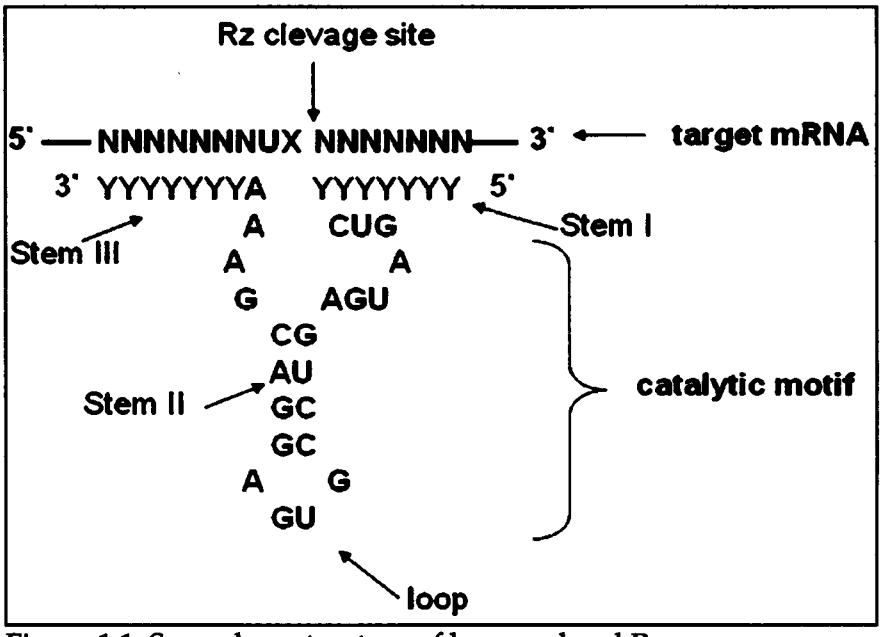

Figure 1.1: Secondary structure of hammerhead Rz

Mechanism of action of hammerhead Ribozyme:

Self cleavage of the hammerhead Rz most likely involves the general acid-base catalysis in which a basic function within the active hammerhead structure accepts the proton from the 2'-hydroxy group and an acidic function provides a proton for the 5'-hydroxy group at the cleavage site (Cech, 1987). The other studies regarding its mechanism suggest a two metal ion mechanism where proton transfer is the rate limiting step (Pontius et al, 1997); and the third mechanism suggested is the formation of a μ-hydroxy bridged magnesium ions cluster that provides hydroxyl ion to activate 2'-hydroxyl nucleophile after a minor and localized conformational change in the RNA (Hermann et al, 1997). A single metal ion mechanism that involves formation of a transition state is also suggested (Murray et al, 2000). The hammerhead mediated cleavage yields fragments that contain a 2', 3'-cyclic phosphate and a 5'-hydroxyl. Much more work has been done to resolve the multistep

pathway of a catalytically active Rz–substrate complex (Liu et al, 2007). Long-residency water molecules play an important role in the dynamic structural communication of local perturbations throughout the catalytic core of a Rz and possibly in general acid–base catalysis (Rhodes et al, 2006).

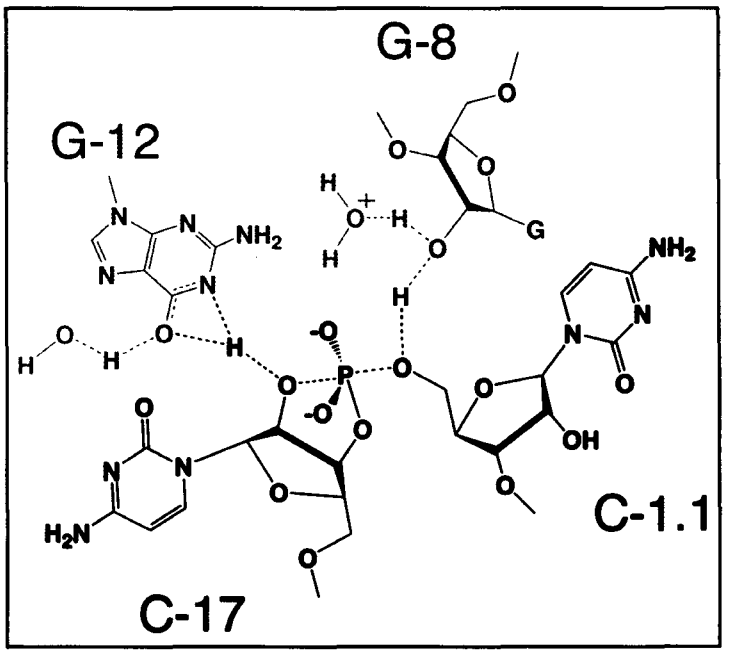

Figure 1.2: Schematic presentation of proposed mechanism for hammerhead Rz catalysis. G-12 is the nucleotide implicated as a general base and G-8 as general acid catalysis. The substrate RNA and water molecules that participate in the reaction play the roles of specific base and specific acid catalysts. The scissile phosphate is depicted as an (unobserved) penta coordinated oxyphosphorane. G-12 can abstract a proton from the 2'-OH of the cleavage-site ribose only if the endocyclic nitrogen N1 becomes deprotonated (as shown). This may happen via simple ionization or through a (rare and transient) tautomerization to the enolic form (as shown). As the 2'-proton is abstracted by G-12, the bond between the 2'O and the phosphorus atom forms, and that between the phosphorus and the 5'O begins to break. As the latter breaks, a negative charge accumulates on the leaving group 5'O. A proton relay may then take place in which the 5'O acquires the 2'-proton from G-8, which is simultaneously replaced with that from an adjacent water molecule or hydronium ion (as shown) (Martrick et al, 2006).

DNA-enzymes

Perhaps the most exciting development in regards to oligonucleotide-based catalyst has been the arrival of RNA-cleaving Dzs or deoxyribozymes (Breaker et al, 1994). The discovery of DNA as an enzyme was unexpected as compared to that of RNA as an enzyme because of the presence of 2'-hydroxyl group in the latter, which was thought to play a central role in Rz function. Dz has never been found in nature. Nevertheless, Santro and Joyce, successfully selected Dzs that catalyze RNA cleavage reaction just like Rzs, with the use of *in vitro* selection procedure. These advances laid the ground work for the discovery of a class of Dz that could cleave RNA molecules in sequence specific manner (Santoro et al, 1997). These molecules exemplified by the 10-23 Dz essentially combine the benefits of highly sequence specific ribonuclease independent RNA destruction, with the relatively stable constitution used in oligodeoxyribonucleotide-based antisense reagents. These new molecules, although rivaling the activity and stability of synthetic Rzs, are limited equally by the inefficient delivery to the intracellular target RNA. These challenges are being met with a multidisciplinary approach with the hope that a greater understanding of each facet of this problem will enable a more optimal utilization of this technology.

Using the technique of *in vitro* selection, individual deoxyribozymes have been identified that catalyze RNA cleavage, RNA ligation (Wang and Silverman, 2003) and a growing range of other chemical reactions. The other bio-chemical reactions carried by DNA enzymes include RNA splicing (Zelin et al, 2006), DNA phosphorylation and adenylation (Wang et al, 2002; Li, et al, 2000), DNA depurination (Hçbartner et al, 2007) and many chemical reactions like Diels-Alder reaction (Chandra and Silverman, 2008). DNA enzymes have been used *in vitro* for applications such as biochemical RNA manipulation and analytical assays for metal ions, small organic compounds,

oligonucleotides, and proteins. Dzs have also been utilized as *in vivo* therapeutic agents to destroy specific mRNA targets (Baum and Silverman, 2008). For *in vitro* applications, DNA is inherently more stable both chemically and biologically than RNA and protein, and this stability can be further enhanced via chemical modifications. Such modifications are readily incorporated, and both modified and unmodified DNA can be produced in large quantities for practical applications.

Joyce's Dzs can be divided into two types:

Type I- Dzs with 8-17 catalytic motif

Type II- Dzs with 10-23 catalytic motif

10-23 DNA-enzyme:

It is the most widely known Dz; named as the 23rd clone identified following 10 cycles of selective amplification. This DNA interacts with its RNA targets using canonical base pairing interactions between the DNA and RNA, and depends on binding of a divalent cation (such as magnesium) for activity (Santoro et al, 1998; He et al, 2002).

The structure of 10-23 Dz is made up of a catalytically active core of 15 near-invariant nucleotides, flanked by substrate binding arms that can be changed to base pair with the sequence of the desired target RNA. 10-23 Dz cleaves the RNA sequence at a phosphodiester bond between a purine and a pyrimidine residue (5'AU 3' most efficiently cleaved). A single nucleotide change in the catalytic motif diminishes its sequence specific cleavage activity; **G14C** completely abolishes the catalytic cleavage activity. These Dzs have high substrate specificity and are able to discriminate the target RNA sequences that differ by a single nucleotide.

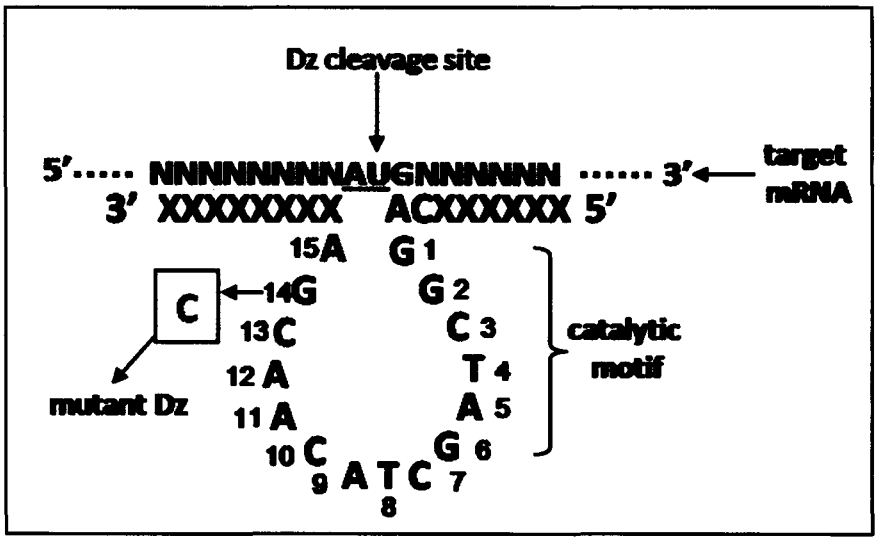

Figure 1.3: Secondary structure of 10-23 Dz

8-17 DNA-enzyme:

This class of Dzs can cleave an RNA sequence at a phosphodiester bond that is located between an A and G residue, in presence of a divalent cation such as magnesium. The catalytic domain consists of 13 nucleotides with a four nucleotide loop adjacent to the cleavage site and a stem loop region that resembles the "stem loop II"region of the hammerhead Rz. The stem loop region in 8-17 Dz is essential for catalysis (Breaker, et al, 1994). This Dz has a special requirement for an 'rG-dT' "wobble" pair located immediately downstream from the target site (Figure 1.4). Substitution with a Watson-Crick pair at this position eliminates catalytic activity.

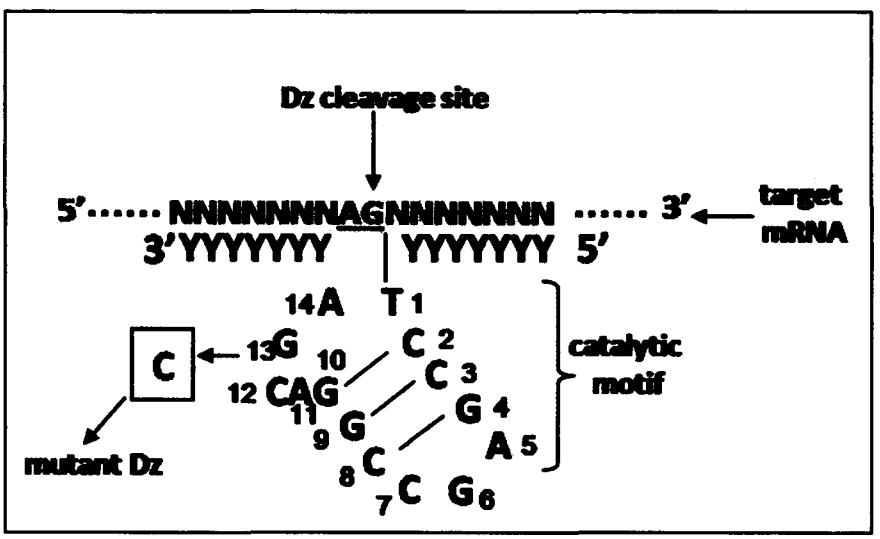

Figure 1.4: Secondary structure of 8-17 Dz

Advantages of DNA-enzymes over Ribozymes:

Dzs exhibit a more rapid rate of catalytic turnover compared with their RNA counterparts. This advantage is due to the relatively fast enzyme product dissociation rates of DNA-RNA heteroduplexes compared with RNA-RNA homoduplexes, which result from subtle conformational differences between the two types of duplexes (He et al, 2002).

Mechanism of action of Dzs:

The catalytic mechanism of RNA-cleaving is thought to be similar to that of hammerhead Rz (Santoro et al, 1997). The putative mechanism involves the divalent metal ion associated deprotonation of the 2'-hydroxyl group adjacent to the cleavage site. This mechanism is favored as it explains the divalent metal ion dependence of many small nucleic acid enzymes as well as the effect that pH has on their reactions.

Also, Dzs with recognition domains of a particular length are more sensitive to mismatches compared with RNA enzymes with guide sequences of the same length, which may allow the avoidance of off-target effects resulting from the cleavage of non-target RNAs (Herschlag et al, 1991).

Applications of Dzs:

Many biological and chemical reactions have found their catalysis applications in Dzs. The list includes RNA cleavage reactions (Geyerand Sen, 1997), RNA ligations (Purtha et al, 2005), DNA phosphorylation (Wang et al, 2002), DNA ligation (Sreedhara et al, 2004), DNA adenylation (Li et al, 2000), oxidative DNA cleavage (Carmi and Breaker, 2001), DNA depurination (Hçbartner et al, 2007) and many more. Dzs have been recently used in many *in vivo* applications against a number of diseases. A brief summary of these applications is shown in table 1.

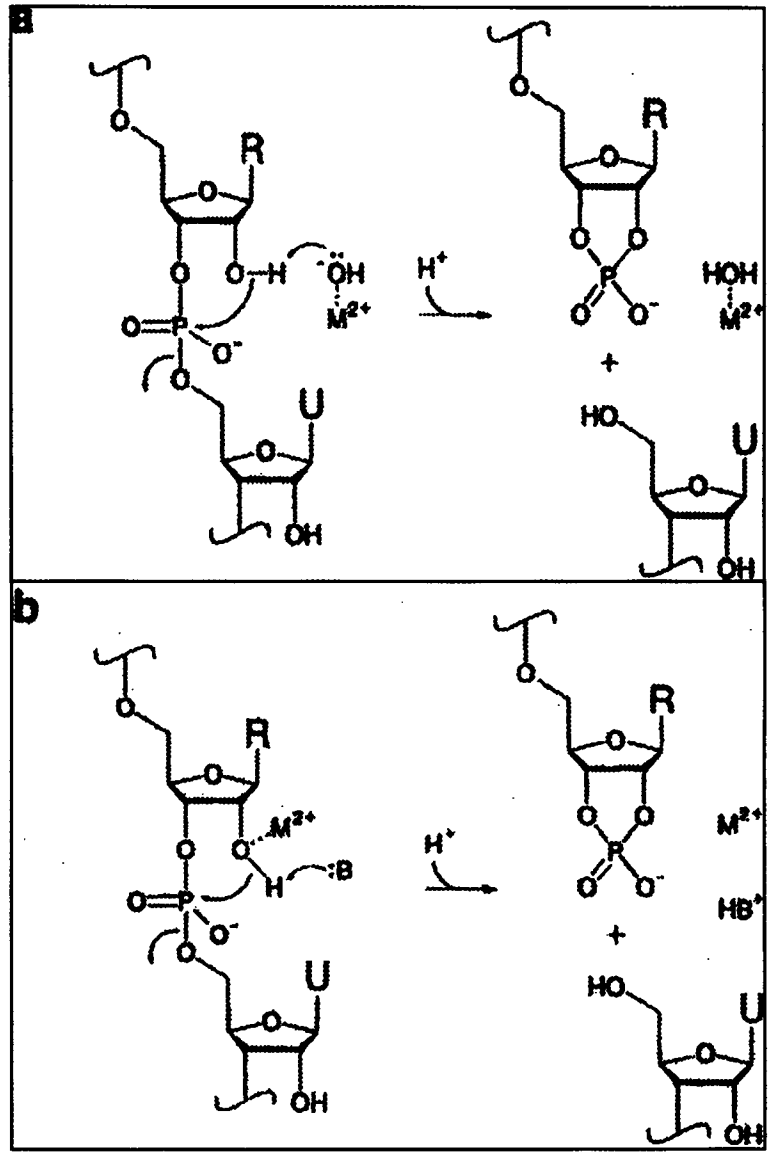

Figure 1.5: Proposed mechanism for RNA hydrolysis by the 10-23 DNA-enzyme. (a) Mg2+ acting as a general base; (b) Mg2+ as a Lewis acid enhancing the acidity of the 2′-hydroxyl group (Santoro et al, 1998).

Table 1: *In Vivo* Applications of Dzs

Gene	Model	Applications	References
c-Jun	Rabbits, rats, mice	Restenosis, neovascularization, inflammation, tumor growth	Fahmy, et al, 2006
Transforming growth factor-β1	Rats	Glomerulonephriti	Isaka, et al, 2004
Xylosyltransferase-1	Mice	Spinal regeneration	Grimpe, et al, 2004
PAI-1	Rats	Myocardial infarction	Xiang, et al, 2004
Tumor necrosis factor-α	Rats	Congestive cardiac failure	Iversen, et al, 2001

Not only the biological and chemical but various physical applications of Dzs have been recently demonstrated. They are now being used extensively in the fields of nanotechnology, logic and gates applications and as sensors. Their usage for amplified biosensing was accomplished by designing aptamer-Dz conjugates that combine recognition units and amplify readout units as in integrated biosensing materials (Willner et al, 2008).

Advantages of Ribozymes over DNA-enzymes:

Many Rzs occur in nature and this helps in learning about the catalytic action and various modifications that could be done for increasing the efficiency of chemically synthesized Rzs. Also, generation of methods for more efficient delivery of Rzs could be thus studied based on their trafficking in the cell. Till date no Dz has been found in nature. A gene for a therapeutic Rz could be inserted into the genome of the host, which would then make the Rz being synthesized continuously; a Dz in contrast has to be synthesized and given as a drug.

Advantages of DNA-enzymes over Ribozymes:

For *in vitro* applications, DNA is inherently more stable both chemically and biologically than RNA and protein, and this stability can be further enhanced via chemical modifications. Such modifications can be readily incorporated. Because of relatively higher stability, Dzs also have potential advantages for *in vivo* therapeutic applications via mRNA degradation. The cost of synthesis of Dzs is much less in comparison to that of Rzs and they are easier to be synthesized.

Antisense oligonucleotides

The usage of nucleic acids as catalytic enzymes to inhibit gene expression requires that Rzs or Dzs efficiently cleave specific sites in

target RNA molecules. However, secondary structure in short oligonucleotide substrates result in an increased k_m and inhibition of assembly of the enzyme-substrate complex. Likewise, Rz cleavage of a long target RNA at sites with known secondary structures is much less efficient than cleavage of short oligonucleotide substrates because of decrease in both the rate of association step (increased k_m) and the chemical step (decreased k_{cat}).The ability of hammerhead Rz to cleave the long target RNA is most strongly correlated with the availability of nucleotides near the cleavage site for base pairing with the Rz (Campbell et al, 1997).

Oligonucleotide effectors (regulators) that bind to both enzyme and substrate were used to regulate the catalytic activity of Rzs & Dzs (Wang et al, 2002).The function of oligonucleotide regulators is to enhance binding between enzyme and substrate and via the three way enzyme-regulator–substrate complex. Goodchild (1992) reported enhancement of Rz catalytic activity by a contiguous oligonucleotide (facilitator) and by 2′-O-methylation. Oligonucleotide facilitators have been used to either enhance (Horn, et al, 1999) or even inhibit catalytic activity of Rzs (Janowsky et al, 1996). Oligonucleotide facilitators have earlier been used to enhance hammerhead Rz mediated cleavage of long RNA substrates with multiple-turnover activity (Jankowsky et al, 1998). These facilitators bind adjacent to catalytic nucleic acid hybridizing sites in the target RNA. The facilitators affect the activity of Rz or Dz by two mechanisms: 1. they facilitate the access of Rz or Dz to the target site by preventing the formation of a stable secondary structure near the target site; and 2. facilitators increase the stability of enzyme-substrate complex by coaxial stacking of the facilitator with the enzyme. The antisense oligonucleotides also work with the similar kind of mechanisms.

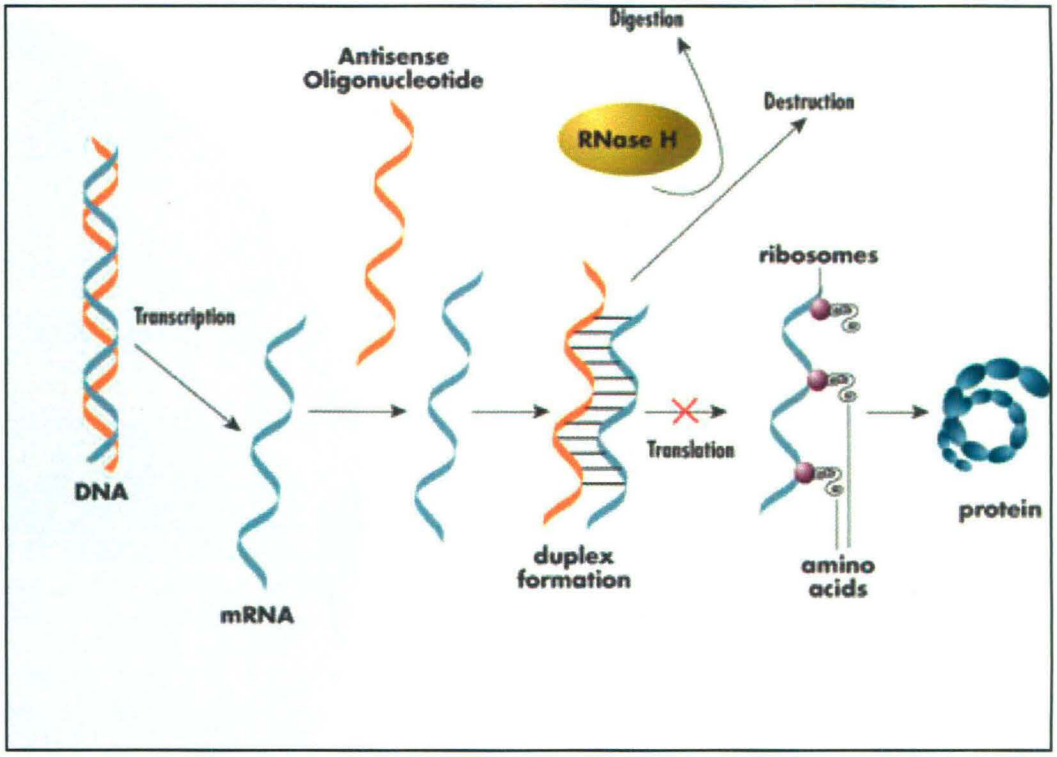

Figure 1.6: Schematic representation of mechanism of action of antisense ODNs

The general belief regarding action of antisense oligonucleotides is that these act to inhibit gene expression by blocking the translation of mRNA or by targeting the RNA for degradation by ribonucleases (RNase H) or if provided, cleavage by Rzs or Dzs, as shown in Figure 1.6. The mRNAs are known to exist in different cellular compartments that include cytoplasm, nucleus or nucleolus. Co-localization of the target RNA with the antisense-based nucleic acids is essential to achieve specific gene suppression. A greater understanding of RNA trafficking pathways will provide the important clues for an improved targeting. A number of clinical studies have used antisense strategies spanning a variety of disease processes, including cancer, cardiovascular disease, inflammation, and infection (Crook, 2004). Antisense approaches for a few disease conditions such as CMV retinitis via intra-vitreal administration (Persidis, 1999), antisense ODN against bcl-2, for treatment of various cancers including chronic lymphocytic leukemia, acute myelocytic leukemia, melanoma, and multiple myeloma, to be

administered via intravenous and subcutaneous routes (Pirollo et al, 2003), have reached various phases in clinical trials.

Specific metabolic pathways in bacteria have documented the existence of entirely RNA-based mechanisms for controlling gene expression (Winkler et al, 2004). Yen and colleagues exploited this information to develop a way of regulating gene expression in mammalian cells. When correctly positioned, the sequences lead to potent inhibition of gene or vector expression, owing to the spontaneous cleavage of the RNA transcript. If a Rz-encoding sequence is placed upstream of the coding region of a gene of interest, the '*cis* construct' is made such that Rz is upstream of the target substrate and as soon as the transcription is initiated, target RNA is cleaved by the Rz (Goila and Banerjea, 2001). It is possible to generate Rz whose *in vitro* cleavage activity is controlled by a specific ligand, either by the 'judicious' linkage of RNA aptamer sequences to specific regions of hammerhead Rz or through the use of *in vitro* evolution technologies (Laising et al, 2004).

Nucleic-acid-based agents use widely varying mechanisms to exert their effects but they all exploit the Watson-Crick base pairing between the two nucleotide chains. It is likely that each strategy would work optimally for a given target.

Small interfering Ribonucleic acids (siRNAs)

The mRNA degradation by RNA interference is probably the most powerful and specific mechanism for gene silencing. Small RNAs function as naturally occurring molecules critical in developmental pathways in plants and animals. This activity is being developed as a potential antiviral therapeutic strategy. Studies *in vitro*, and some *in vivo*, appear to show the feasibility of using RNAi to treat virus infection (Huang, 2008). Therapeutic use of RNAi seems to be promising when directed against viruses that cause localized acute infections in accessible target cells. It was discovered by Fire *et al* that dsRNA is a potent trigger

for RNAi in the nematode *Caenorhabditis elegans* (Fire et al, 1998). This has suggested an approach for efficient induction of gene silencing in eukaryotic organisms, and accelerated the discovery of a unifying mechanism that underlies a host of cellular and developmental pathways. It was first observed in plants in the guise of a mysterious immune response to viral pathogens (Mourrain, 2000). siRNA based therapy has been very promising for many types of cancer especially pancreatic cancer (Huang et al, 2008). Major targets for siRNA therapy include oncogenes and genes that are involved in angiogenesis, metastasis, survival, antiapoptosis and resistance to chemotherapy.

Small interfering RNAs (siRNAs) are a class of 21- to 22-nucleotide double-stranded RNAs (dsRNA) that stimulate posttranscriptional gene silencing through the RNA interference (RNAi) pathway in higher eukaryotes. When a cell encounters a long dsRNA, the specific ribonuclease enzyme, namely Dicer cleaves it into siRNAs. These siRNAs associate with a protein complex known as RNA induced silencing complex (RISC) which recognizes and degrades the sense strand of the siRNA and uses the antisense strand to target genes for silencing, destroying the target mRNA (Zamore et al, 2005). The mechanism of action of siRNA and the role of Dicer enzyme is explained in Figure 1.7. The requirement for Dicer in formation of siRNAs can be bypassed by introducing 21 nucleotide long siRNAs directly into mammalian cells that specifically inhibit gene expression. siRNAs can be expressed from a Pol III promoter in a mammalian cell which allows precise processing of RNAs.

Long dsRNA and miRNA precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. These short dsRNAs are subsequently unwound and assembled into effector complexes, RISCs, which can direct RNA cleavage, mediate translational repression or induce chromatin modification. (Gregory et al, 2004).

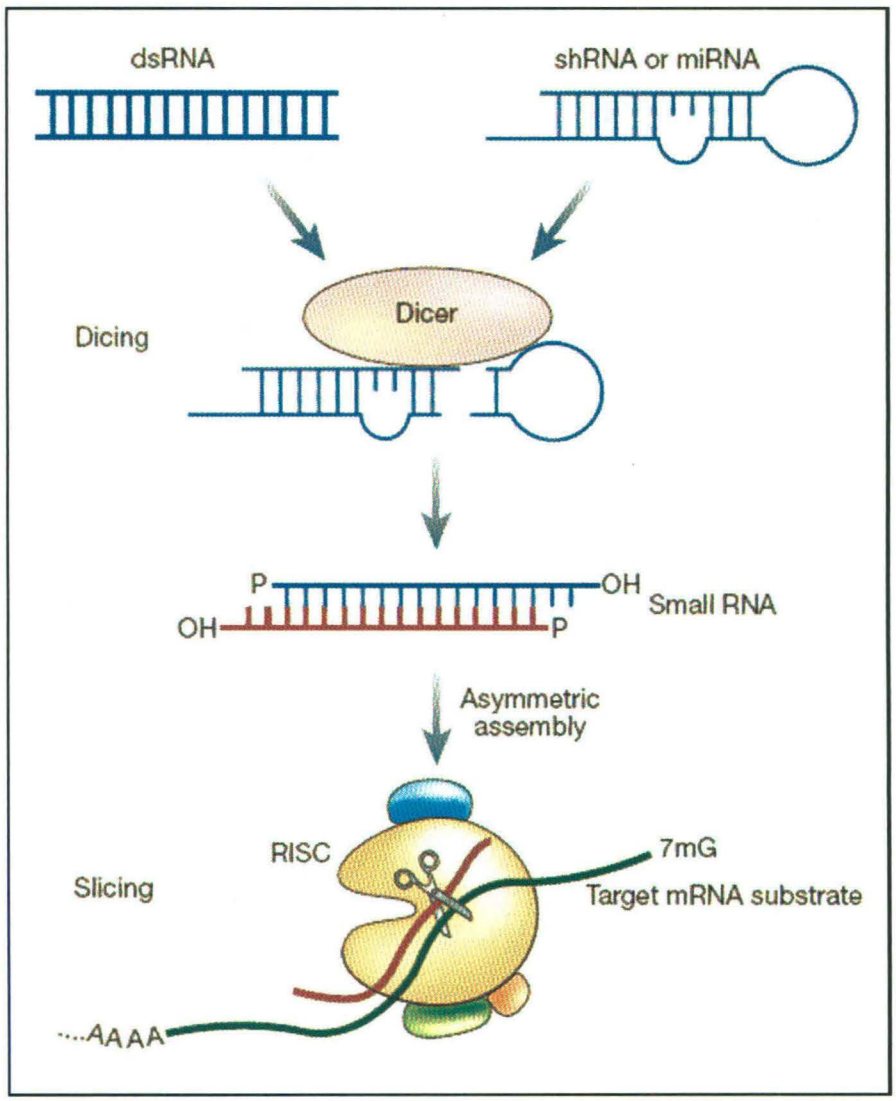

Figure 1.7: RNA silencing pathways in different organisms.

RNAi has begun to produce a paradigm shift in the viral therapeutic field. There is potential for using this technique in the treatment of viral diseases caused by Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Human immunodeficiency virus (HIV). HIV whose lifecycle and pattern of gene expression is well understood, was the first infectious agent targeted by RNAi. RNAi is a very promising strategy that in principle will provide many new targets against HIV infection. The mechanism of sequence compliment utilized by siRNAs against their targets provides a new approach to fight against HIV infection (Singh, 2008). Synthetic siRNAs and expressed shRNAs have been used

to target several early and late HIV-encoded RNAs in cell lines and in primary haematopoietic cells (Jacque et al, 2002; Banerjea et al, 2003). Also various HIV-1 co-receptors like CXCR4 and CCR5 have been targeted (Akkina et al, 2003). But there are drawbacks in targeting cellular HIV cofactors because non-infected cells will inevitably be targeted as well, leading to toxicities. Henceforth, viral targets are required to be included in any successful strategy using RNAi. These targets should be sequences that are highly conserved throughout the various clades to ensure efficacy against all viral strains. Two key challenges in developing RNAi as a therapy are avoiding off-target effects and ensuring efficient delivery.

Catalytic nucleic acids against viruses

Because both DNA and RNA viruses translate their mRNAs to make proteins that assemble and mature into virus particles, they can be targeted by Rz or Dzs or siRNAs to selectively inhibit their gene expression. The required doses of various catalytic nucleic acids can also be reduced with the use of antisense oligodeoxynucleotides along with Rz or Dz. The following two viruses have been taken as models to study and develop catalytic nucleic acids and siRNAs based antiviral strategies:

Hepatitis B virus (HBV)

It is an infectious agent for hepatitis, a major public health problem. It is estimated that more than 350 million individuals are chronically infected with HBV, many of whom would eventually develop severe liver diseases, including liver cirrhosis, and hepatocellular carcinoma (HCC), one of the most common forms of human cancer (Seeger et al, 2007). HBV produces the acute and chronic infections of liver. HBV belongs to family *Hepadnaviridae* and has a

partially double stranded genome of 3.2 kb. The structure of HBV is shown as simplified diagram in Figure 1.8.

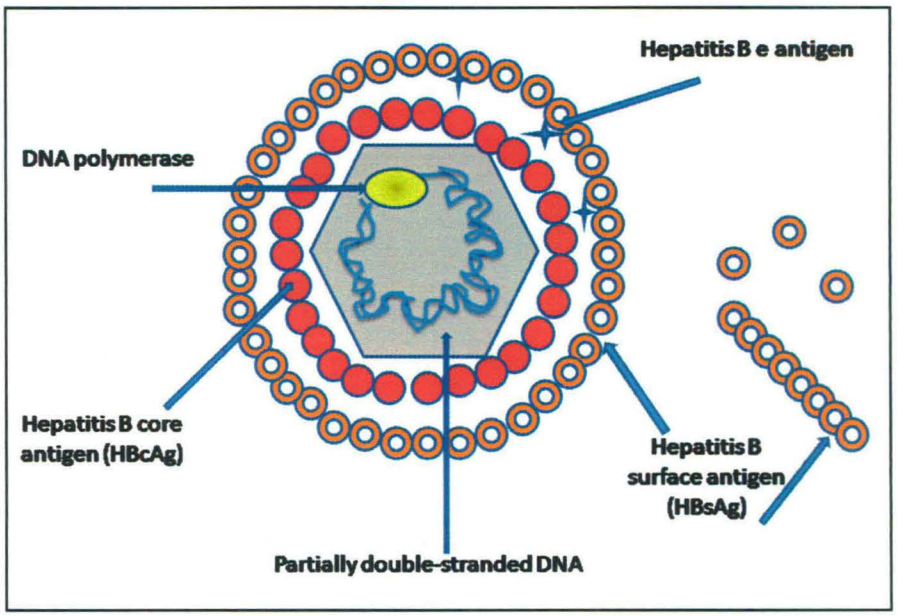

Figure 1.8: Structure of Hepatitis B virus

The virion consists of an outer envelope that is composed mainly of lipids and icosahedral nucleocapsid core that is made up of proteins. The nucleocapsid core encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity. It replicates its DNA genome by reverse transcription of pregenomic RNA (Seeger et al, 2007). The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells.

Organization of HBV:

HBV genome is made of partial double stranded, circular. The longer strand is about 3.2kb and the short one is about 1.7-2.8 kb. It has four open reading frames (ORFs) and encodes four genes, namely, C, X, P and S. The first ORF encodes the various forms of the surface protein and contains three in-frame methionine codons which are used for initiation of translation, as shown in Figure 1.9.

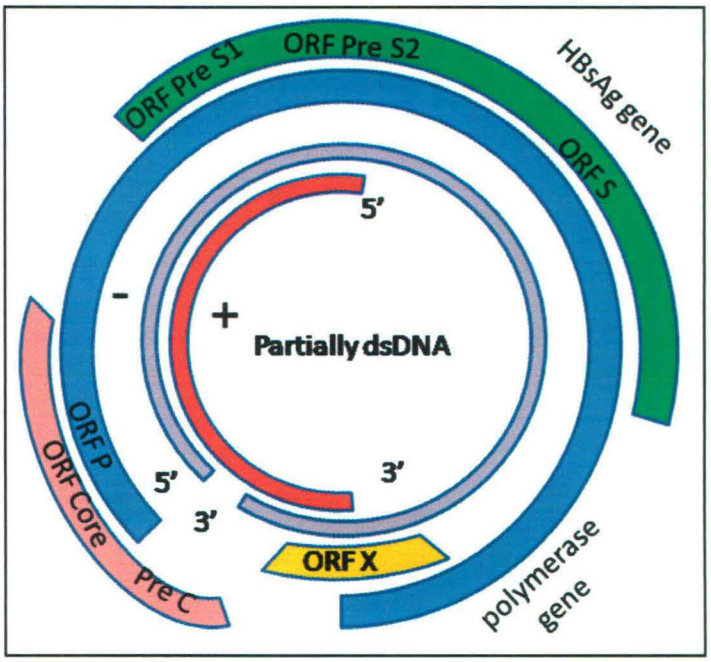

Figure 1.9: Genomic organization of Hepatitis B virus

A second promoter is located upstream of the pre-S1 initiation codon and it directs synthesis of a 2.4 kb mRNA which is translated to yield the large (pre-S1) surface proteins. The "precore" region is highly conserved, has the properties of a signal sequence and is responsible for the secretion of HBeAg. The third ORF is the largest and it overlaps the other three; and encodes the viral polymerase. Recent success in producing recombinant HBV RT protein in *Escherichia coli* has allowed for the development of a defined biochemical reconstitution system that has greatly facilitated efforts to elucidate viral and cellular requirements for RNP formation and protein priming in hepadnaviruses (Hu and Anselmo, 2000; Hu et al, 2002, 2004). The fourth ORF was designated "X" because earlier the function of its small gene product was not known but now "X" has been demonstrated to be a transcriptional transactivator.

Life cycle of HBV:

The life cycle of Hepatitis B virus is complex. It uses reverse transcription as part of its replication process. The virus enters into the

cell by binding to some host cell receptor by endocytotic mediated pathway. The viral genomic DNA is transferred to the cell nucleus by host proteins called chaperones. The partially double stranded viral DNA is then made fully double stranded and transformed into closed circular supercoiled DNA (cccDNA) that serves as a template for transcription of its four viral mRNAs by RNA Polymerase II. The largest mRNA, (which is longer than the viral genome), is used to make the new copies of the genome and to make the capsid core protein and the viral DNA polymerase. These four viral transcripts undergo additional processing and go on to form progeny virions which are released from the cell or returned to the nucleus and re-cycled to produce even more copies. The long mRNA is then transported back to the cytoplasm where the virion P protein synthesizes DNA via its reverse transcriptase activity (Bouchard et al, 2004).

HBx protein of HBV:

The X gene which is the smallest ORF of HBV genome, encodes a multifunctional, 154 amino acids polypeptide, designated as HBx or X-protein. The HBx protein plays an important role in viral replication in HBV infected cells and the liver diseases including hepatitis, cirrhosis and hepatocellular carcinoma (Nguyen et al, 2008). Based on observations that the COOH-terminal truncated HBx was frequently detected in HCC, another recent study has concluded that COOH-terminal truncated HBx plays a critical role in the HCC carcinogenesis via the activation of cell proliferation (Ma et al, 2008). Not only this, HBx promotes genetic instability through formation of defective spindles and subsequent aberrant chromosome segregation (Wen et al, 2008).

HBx can promote cellular transformation by disrupting cell signaling pathways involved in transcription, apoptosis, and cell proliferation (Bouchard et al, 2001; Bouchard and Schneider, 2004). Alternatively, HBx could stimulate viral replication (Leupin et al, 2005)

which may lead to an enhanced immune response against the virus. This will result in the continual destruction and regeneration of hepatocytes due to chronic liver inflammation and thus increase the chance of genetic mutations.

The role of HBx protein in HBV infection and HCC development has not been clearly defined. Concomitant with the development of chronic HBV infection, the levels of HBx is higher and is characteristic in most cases of cirrhosis and primary tumors. During the development of HCC it is the X gene that is most frequently integrated in the hepatocyte chromosomes. HBx is expressed from these integrated fragments, although no other viral proteins are present in most tumor cells. It appears that the HBx protein can favor the clonal expansion of hepatocytes, rather than merely supporting viral replication. In this way, HBx may contribute to initiation of tumor in chronic active hepatitis and cirrhosis (Paterlini et al, 1995). HBV DNA has been reported to be integrated into host chromosomes in tissues of HCC patients or in cell lines derived from HCC (Seeger and Mason, 2000). The expression of HBx in hepatocyte line HL-7702 promoted apoptosis and accumulation of the S phase through the inhibition of DNA repair and checkpoints via post-transcriptional mechanisms (Chen et al, 2008). The study by the group of Zhu suggested that HBx might play an important role in the early stage of HBV-associated hepatocarcinogenesis via induction of hyper-methylation of p16 (INK4A) promoter (Zhu, et al, 2008).

Not only this, HBx also co-localizes with tumor suppressor antigen, p53 and inhibits the regular functions of the latter. High levels of accumulated mutations of p53, including point mutations and chromosomal alterations have been reported in late stages of HCC (Hosono et al, 1993). The frequency of p53 mutations in earlier stages of HBV-related HCC seems to be much lower than those induced by chemical carcinogens. This tendency in HBV-associated HCC suggested that a viral factor may directly counteract p53 function. Several groups

reported that p53-counteracted biological functions of HBx and HBx affected p53-dependent apoptosis (Takada et al, 1997). HBx participates in cellular signal transduction pathways, proliferation, and apoptosis. HBx induces apoptosis by enhancing the translocation of Bax to mitochondria, followed by inducing the loss of mitochondrial membrane potential and release of cytochrome C (Kim et al, 2008). HBx and COX-2 are highly co-expressed in chronic hepatitis, cirrhosis and well-differentiated HCC and HBx has been shown to induce COX-2 expression (Cheng et al, 2004). COX-2 mediates the HBx actions in opposing p53-induced apoptosis and hence provides a survival advantage for preneoplastic or neoplastic hepatocytes in the initiation and progression of HCC (Alfred et al, 2008).

HBx plays a major role in HBV infection and pathogenecity. It is mainly known to activate many heterlogous promoters of the viral and cellular origin. It activates gene expression by two mechanisms: it either directly interacts with the transcriptional machinery or augments RNA synthesis, or it binds to transcription factors and enhances the efficiency of the later for DNA sequence recognition (Williams et al, 1995). HBx can induce telomere shortening by acting as a transcriptional co-repressor of myc-associated zinc finger protein (MAZ) on the human telomerase promoter (Su et al, 2007). HBx is also involved in Ras mediated activation of nuclear transcription machinery (Ganem et al, 2001). It acts on mitochondria or endoplasmic reticulum or on both and efflux calcium signaling in the cytosol which then via PyK2 pathway enhances Ras mediated activation of transcription factors. HBx thus modulates Ca^{2+} signal cascade and various transcription factors. HBx is involved in modulating signal transduction pathways and transcription mediated by CREB that requires the recruitment of the co-activators CREB-binding protein (CBP)/p300. HBx and CBP/p300 synergistically enhanced CREB activity. The expression of HBx increased the recruitment of p300 to the interleukin 8 and proliferating cell nuclear antigen promoters in cells,

and this is associated with increased gene expression. HBx thus might disrupt the cellular regulation, and thus predisposing cells to transformation (Cougot et al, 2007).

The treatment of HBV infection is not yet very effective especially in case of active chronic hepatitis infection, though vaccine is available in order to prevent infection in non-infected individuals. Thus HBx is the most plausible target for nucleic acid-based agents to block the HBV replication. Even a highly truncated version of HBx protein is found to retain its function (Kumar et al, 1996); hence emphasizing the need to develop multitargeted nucleic acid enzymes. Various mono-and di-hammerhead Rz as well as mono- and di-10-23 motif containing Dzs have been successfully designed earlier by Banerjea and coworkers (Goila and Banerjea, 2001 and 2004). The challenge still remains with designing of strategies that might cause more potent suppression of expression of HBx and thus block it functions like transactivation of HIV-1 LTR promoter. These Rz and Dzs either alone or in combination could, in principle, serve as valuable tools to selectively down regulate the expression of the X gene and thereby reduce X gene mediated pathology.

Human Immunodeficiency virus (HIV)

Retroviruses are RNA containing viruses that replicate through a DNA intermediate by virtue of viral coded RNA-dependent DNA polymerase, also called reverse transcriptase. Human Immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are members of the subfamily *Lentivirinae* of the family *Retroviridae* on the basis of genetic, morphological and pathological criteria (Gonda et al, 1986; Haase, 1986). Both HIV-1 and HIV-2 can cause immune deficiency, but HIV-1 infection appears to be more virulent (Marlink et al, 1994). HIV primarily infects

vital cells in the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages and dendritic cells which results in the loss of cell mediated immune response and the body becomes progressively more susceptible to opportunistic infections. Eventually most HIV-infected individuals develop AIDS (Acquired Immunodeficiency Syndrome). 9 out of every 10 HIV infected people, progressively develops AIDS in 5-10 years time period. These individuals mostly die from opportunistic infections or malignancies associated with the progressive failure of the immune system. Estimated number of people living with the infection is 33.2 million and number of deaths by HIV infection in 2007 was 2.1 million. Since the discovery of HIV in 1981, more than 25 million people have died of AIDS. The scenario is much worse in the developing nations especially in sub-Sahara countries where according to current trend estimated set of infected people will be 90 million. HIV-AIDS infection is now pandemic in humans.

Statistics by UNAIDS/WHO in November, 2007, table 2:

	Estimate	**Range**
People living with HIV/AIDS in 2007	33.2 million	30.6-36.1 million
Adults living with HIV/AIDS in 2007	30.8 million	28.2-33.6 million
Women living with HIV/AIDS in 2007	15.4 million	13.9-16.6 million
Children living with HIV/AIDS in 2007	2.5 million	2.2-2.6 million
People newly infected with HIV in 2007	2.5 million	1.8-4.1 million
Adults newly infected with HIV in 2007	2.1 million	1.4-3.6 million
Children newly infected with HIV in 2007	0.42 million	0.35-0.54 million
AIDS deaths in 2007	2.1 million	1.9-2.4 million
Adult AIDS deaths in 2007	1.7 million	1.6-2.1 million
Child AIDS deaths in 2007	0.33 million	0.31-0.38 million

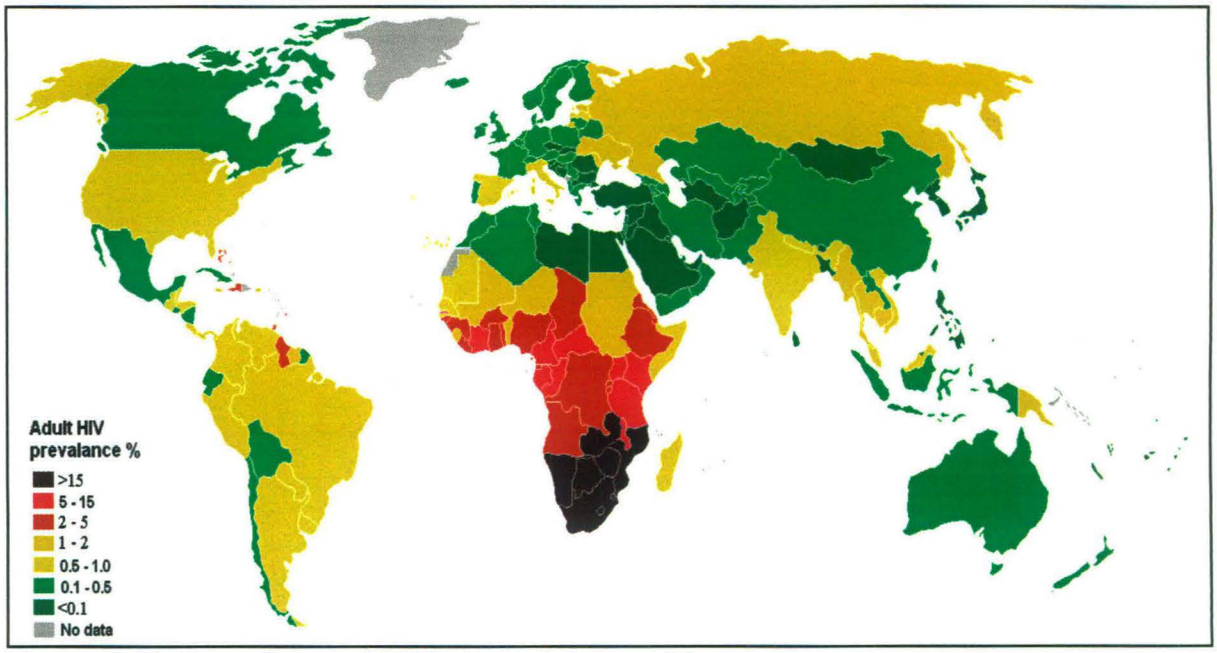

Figure 1.10: Prevalence of HIV around the world

Classification of HIV

HIV differs from many viruses in that it has very high genetic variability. This diversity is a result of its fast replication cycle, with the generation of 10^9 to 10^{10} virions every day, coupled with a high mutation rate of approximately 3×10^{-5} per nucleotide base per cycle of replication and recombinogenic properties of reverse transcriptase (Robertson et al, 1995). HIV is broadly classified into two major subtypes; HIV-1 and HIV-2. HIV 1 includes the most common strains of the virus, and it is further subdivided into three groups called M, N, and O.

Group M: This is the most common type of HIV, with more than 90% of HIV cases being HIV-1 group M. It is subdivided further into various clades, designated by a letter again. This is further complicated due to changes in the virus during the course of infection, and many cases are given a "circulating recombinant form" or CRF, for example, CRF A/C is a combination of subtypes A and C. Subtype A is common in West Africa (Bobkov et al, 2004) and Subtype B is the dominant form in Europe, the Americas, Japan, Thailand, and Australia. In Southern and Eastern Africa, India and Nepal, subtype C is more prevalent and

subtype D is usually seen in Eastern and Central Africa. Subtype E has never been purified, and is always seen combined with subtype A as CRF A/E (Goudsmit, 1997). Subtypes F, G and H are mainly restricted to various regions of Africa and Subtype J is limited to Central America. Subtype K is limited to the Democratic Republic of Congo and Cameroon.

Group N: This group, discovered in 1998, has only been seen in Cameroon and is extremely rare.

Group O: This strain is not usually seen outside of West-central Africa and is very rare. Like Group N and Group O, HIV-2 has not been widely seen outside of Africa.

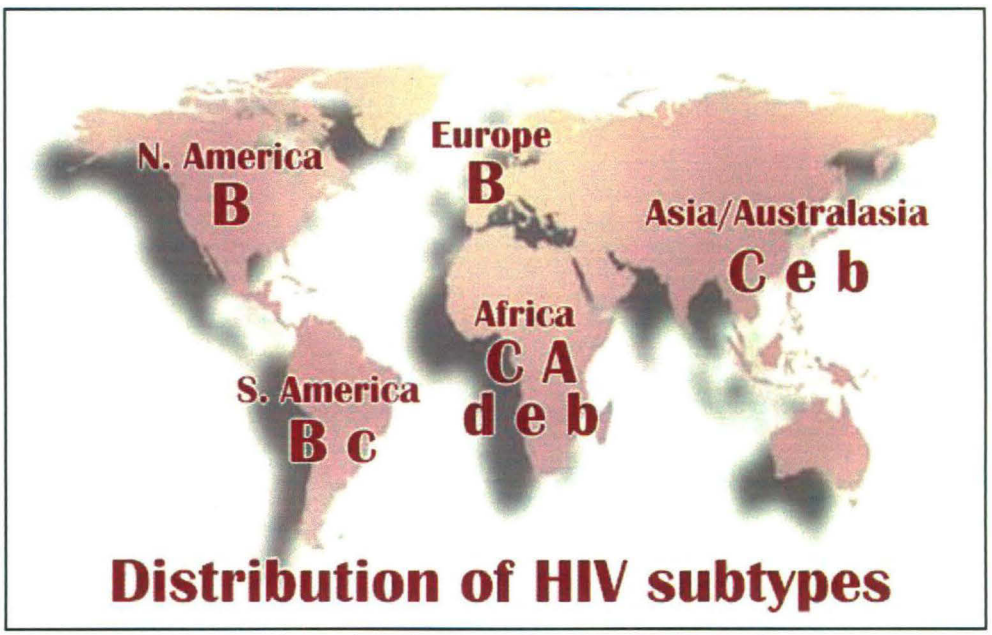

Figure 1.11: Map to show distribution of various HIV-1 subtypes

Role of co-pathogenesis in supporting HIV infections

Co-infections of HIV and other viral or bacterial origins represent a major health crisis around the world. Some of the most common and usually fatal co-infections that occur and help in progression of HIV/AIDS in patients are caused by *Mycobacterium tuberculosis, Hepatitis C virus* and *Hepatitis B virus*.

Mycobacterium tuberculosis: It is the most common opportunistic infection occurring in HIV-infected individuals in resource poor countries and it accelerates HIV-associated morbidity and mortality as well as viral replication (Toossi et al, 2001). AIDS and tuberculosis annually kills more than 3 million people worldwide.

The interaction between HIV-1 and tuberculosis is bi-directional. The hallmark of HIV-1 infection is the progressive decline and dysfunction of CD4 lymphocytes (Fauci, 1996), cells that are critical in containing infection with *M. tuberculosis*. HIV-1 infection confers the greatest known risk for the development of active tuberculosis. The effects of tuberculosis on HIV-1 infection have been observed both at the cellular and clinical levels. Tuberculosis enhances viral replication through mechanisms that involve activation of the cell-mediated immune system through cytokine networks. Peripheral blood invariant Natural killer cells (iNKT) in these two chronic infections show an up-regulated expression of activation markers, suggesting their important role in the immune response and infection (Montoya et al, 2008). Studies have shown increased transcription of the HIV long terminal repeat (LTR) in cultured monocytic cells exposed to either live *M. Tb* or cell wall components (Zhang et al, 1995). Kitaura *et al.* demonstrated that incubation of U1, a chronically HIV infected human promonocytic cell line, with various strains of mycobacteria resulted in enhanced p24 antigen release into the supernatant (Kitaura et al, 2001). Polyfunctional *M. Tb*-specific CD4 and CD8 T cell responses are maintained in the peripheral blood of HIV-1-positive individuals, in the absence of active disease, and the functional capacity of these responses is affected by HIV-1 disease status (Day et al, 2008). The active tuberculosis accelerates the progression of HIV infection and exerts its greatest effect on survival in the early stages of HIV infection, when there is a reserve capacity of the host immune response.

Viral hepatitis

Human immunodeficiency virus type 1 or HIV compromises human immunity mainly by destroying CD4+ T cells, which frequently leads to opportunistic infections and reactivation of latent pathogens. Among the infectious pathogens, many viruses, which include hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis G virus (HGV), transfusion-transmitted virus (TTV) and human T-cell lymphotropic virus type 1 (HTLV-1), share a similar route of transmission and risk factors with HIV. HBV and HCV are the major causes of acute hepatitis, and large number of infected patients' further progress to the chronic diseases such as liver necrosis, cirrhosis and hepatocellular carcinoma and have become an increasingly important cause of morbidity and mortality among HIV infected patients. HIV accelerates HBV and HCV liver disease especially when HIV-associated immunodeficiency progresses. Microbial translocation might be a fundamental mechanism through which HIV accelerates progression of chronic liver disease as HIV-related CD4 (+) lymphocyte depletion was found to be strongly associated with microbial translocation (Balagopal et al, 2008).

Hepatitis C virus:

HCV is an opportunistic infection of HIV disease and is a prominent contributor of morbidity and mortality among HIV infected patients. Liver disease progresses more rapidly in people who are HIV positive and HCV-associated liver damage is more likely to develop in HIV/HCV co-infected people than in those with HCV mono infection (Ragni et al, 2001). The reasons for the accelerated course of HIV/HCV co-infection are mainly related to two principal causes: the persistence of HCV, which depends upon alterations of cell-mediated immunity, and the activation of the immune system towards secretion of proinflammatory and profibrotic cytokines (Bruno et al, 2008). Co-infected people have higher levels of HCV RNA which correlated with

degree of immune suppression. The management of chronic hepatitis C virus infection in patients co-infected with the HIV poses a significant challenge.

Hepatitis B virus:

Due to overlapping routes of transmission, many HIV positive individuals have also been exposed to hepatitis B virus (HBV). Studies suggest that as many as 70%-90% of HIV positive people have incidences of HBV co-infection. Co-infected individuals show an accelerated course of HBV-associated liver disease with faster progression to cirrhosis. HIV and HBV infection share transmission patterns and risk factors. HBV does not significantly affect the course of HIV disease, but HIV does alter the course of HBV. HIV-infected persons are less likely to clear acute HBV infection spontaneously, and HIV/HBV co-infected persons face a higher risk of liver-related death than those infected with either virus alone (Benhamou, 2007).

In the setting of chronic hepatitis B, a persistent state of immune activation has been described in patients with chronic HBV infection that results in upregulating HIV-replication. Moreover, it has been suggested that the X-protein (HBx) super induces ongoing HIV-1 replication and HIV-1 long-term repeated transcription by synergizing with tat protein and with T-cell activation signals (Gomez-Gonzalo et al, 2001). These findings indicate that HBx could contribute to a faster progression to AIDS in HBV/HIV-co-infected individuals.

HIV Structure and Genome:

It is composed of two copies of positive single-stranded RNA that codes for the virus's nine genes enclosed by a conical capsid that is composed of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as reverse transcriptase, proteases,

ribonuclease and integrase. HIV particles surround themselves lipid layer known as the viral envelope (or membrane). Projecting from this are around 72 little spikes, which are formed from the glycoproteins gp120 and gp41, the later anchoring the structure to the viral envelope. Just below the viral envelope is a layer called the matrix, which is made from the protein p17 (Chan et al, 1997) (Figure 1.12).

The RNA genome of HIV consists of at least 7 structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, INS) and nine genes (*gag*, *pol*, and *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*, and *tev*) encoding 19 proteins. Out of these nine genes; *gag*, *pol* and *env* genes encode for the structural proteins while the rest encode for viral regulatory proteins that control the ability of HIV to infect cells, replicate and cause disease (Suzuki and Craigie, 2007).

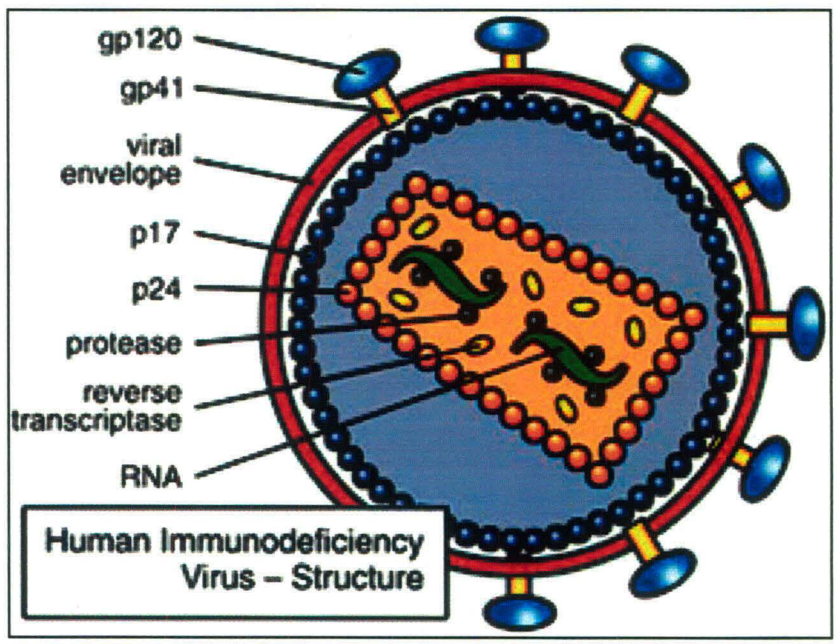

Figure 1.12: Structural organization of HIV

Tat proteins bind with TAR RNA element and thus transactivates the LTR promoter. Rev protein is involved in shuttling RNAs from the nucleus and the cytoplasm by binding to the RRE. Vif protein mediates ubiquatinated degradation of APOBEC3G (a protein of cytidine deaminases that cause G to A hypermutations in viral strand

and thus renders viral inactive or susceptible to action of uracil glycosylases). Vpr arrests cell divsiion at G2/M. Nef protein is involved in down regulation of CD4, the major viral receptor and MHC class I molecules. The Vpu protein influences the release of new virus particles from infected cells (Lin et al, 2005). The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR), Figure 1.13.

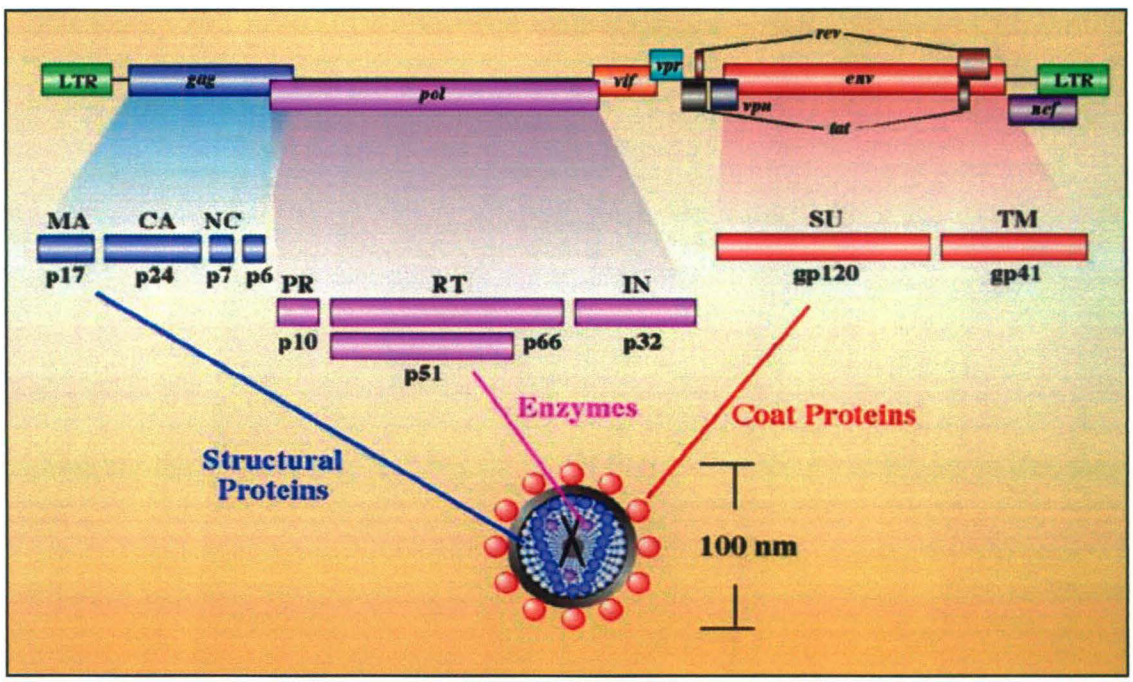

Figure 1.13: Genomic organization of HIV

The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell (Lin et al, 2005). The Psi element (PE) is involved in viral genome packaging and recognized by Gag and Rev proteins. The SLIP element (TTTTTT) is involved in the frameshift in the Gag-Pol reading frame required to make functional Pol (Coakley et al, 2005).

Cis-regulatory elements regulate the expression of HIV-1 genes by several posttranscriptional mechanisms including RNA splicing,

stability, transport, and translation. The *cis*-acting repressive sequence (CRS) or inhibitory/instability sequence (INS) is one such regulatory element (Huffman and Arrigo, 1997) and the mapping of one the CRS i.e, in env region showed that the CRS activity spanned most of the gp120 region with the highest activity within about 1 kb upstream to RRE (Nasioulas et al, 1994).

Life cycle of HIV-1:

HIV can replicate only inside the human cell (Figure 1.14). The process typically begins once HIV virus comes in contact with cell surface proteins CD4 and one of the chemokine receptors. Interaction of the trimeric complex with viral envelope (gp 160 spikes) and CD4 and a chemokine receptor CXCR4 or CCR5 is required for the entry of virus in the CD4+veT cells or macrophages. Viral envelope is made up two portions, gp 120 and gp 41; and gp120 is the domain that is involved in high affinity attachment with CD4. Once gp120 is bound with the CD4 protein, the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor. This allows for a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41 to penetrate the cell membrane (Chan, 1998; Wyatt et al, 1998). The contents of HIV that include viral RNA and various enzymes like reverse transcriptase, integrase, ribonuclease and protease are then released into the cell leaving behind the envelope.

During transport to the nucleus, the viral single strand RNA genome is transcribed into double strand DNA by the viral reverse transcriptase, which is then integrated into a host chromosome with the help of viral integrase enzyme. This process of reverse transcription is highly error prone and during this process various mutations occur in the virus. This integrated viral DNA may then lie dormant, in the latent

stage of HIV infection for a very long duration (Zheng et al, 2005). But when the cell becomes activated, it treats HIV genes in much the same way as human genes. First it converts them into messenger RNA (using human enzymes). Then the messenger RNA is transported outside the nucleus, and is used as a blueprint for producing new HIV proteins and enzymes. To actively produce the virus, presence of certain cellular transcription factors is essential, the most important of which is NF-κB (NF kappa B), which is up regulated when T cells become activated (Hiscott et al, 2001). This means that those cells most likely to be killed by HIV are those currently fighting infection.

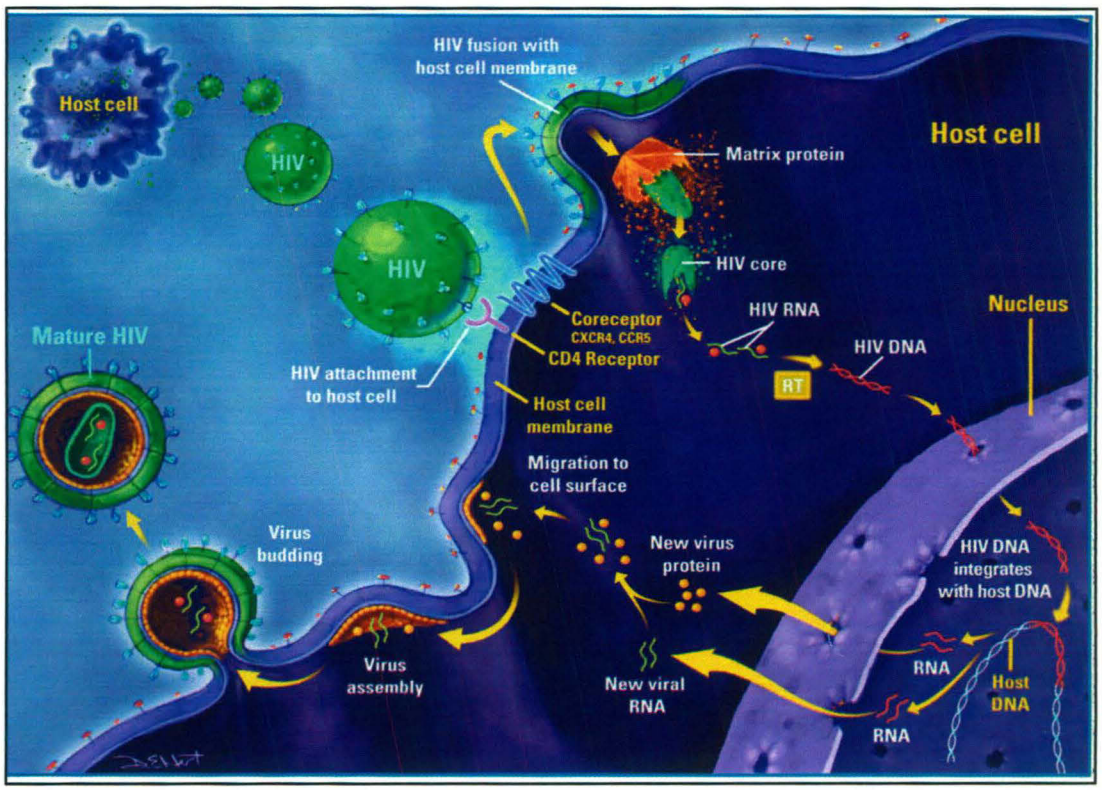

Figure 1.14: HIV-1 life cycle

In this replication process, the integrated provirus is copied to mRNA which is then spliced into smaller fragments of RNA which produce the regulatory proteins Tat (which encourages new virus production) and Rev. With the accumulation of Rev, its concentration increases and this blocks further splicing of the viral mRNA. At this

stage, the structural proteins Gag and Env are also produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles.

The final step of the viral cycle i.e., assembly of new HIV-1 virons, begins at the plasma membrane of the host cell. The Env protein (gp160) is transported from endoplasmic reticulum to the Golgi complex where it is cleaved by protease in to two glycoproteins gp41 and gp120. These are then transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the cellular membrane. The Gag and Gag-Pol proteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the new virion begins to bud from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion. The newly matured HIV particles are ready to infect another cell and begin the replication process all over again. In this way the virus quickly spreads through the human body.

HIV-1 Auxillary proteins:

HIV has nine genes that encode three structural and six regulatory or accessory proteins. Gag, env and pol genes are common to all replication-competent retroviruses and encode structural proteins. Tat, Rev, Nef, Vif, Vpr and Vpu are the viral accessory proteins that are generally not needed for replication and survival, and also are not structural. But they are needed under certain 'stressful' conditions imposed on the virus by its host cells. Various accessory proteins, their role in virus survival; their interacting host partners and the consequences of these interactions are summarized in table 3 (Swanson et al, 2008).

Table 3: Accessory proteins of HIV-1

Virus protein	Function	Host factor	Results of interactions
Vif	Suppresses host antiviral factors, APOBEC3G/F	APOBEC3G/F ElonginC, Cullin5	causes ubiquitinylated degardation of APOBEC3G/F
Vpr	G2/M cell cycle arrest	Nucleoporins CDC25C	Post entry nuclear import Cell cycle arrest
Tat	Potent activator of viral transcription elongation	Cyclin T1 Importin-β	]Promotes viral transcription Nuclear import receptor Induction of apoptosis
Rev	Induces nuclear export of viral RNAs	CRM1/Exportin- β1 Importin-β	Nuclear export receptor, Nuclear import receptor
Vpu	CD4/MHC down regulation; induces virions release	CD4 CD137	Recruits ubiquitin ligase to CD4 resulting in CD4 degradation Blocks virion release
Nef	CD4/MHC down regulation; T-cell activation; blocks apoptosis; pathogenecity determinant	CD4, CD28, MHC-I, MHC-II, TCR-CD3ζ Several kinases Dynamin-2	Connects host surface proteins to clathrin – dependent/independent sorting pathways to regulate trafficking, Roles in signal transduction, blocking apoptosis Enhances viral infectivity

The accessory proteins are often necessary for viral replication and pathogenesis *in vivo* and they carry out many of the essential functions during the viral life cycle. One such important auxillary gene is Tat that encodes 110 amino acids containing small protein which is essential for efficient transcription of viral genes and for viral replication.

Tat potently transactivates LTR-driven transcription, resulting in a remarkable increase of viral gene expression (Zhou and Sharp, 1995). Of the diverse subtypes of HIV-1, subtype-C strains cause a majority of infections worldwide. Tat, being critical for viral infectivity and pathogenesis, may differentially modulate pathogenic properties of the viral subtypes. It has been earlier studied that subtype-C Tat might have a relatively higher ordered and less flexible structure than subtype-B Tat. It has also been demonstrated that subtype-C Tat as a protein was consistently inferior to subtype-B Tat in a variety of biological assays (Siddappa et al, 2006). This study emphasised the need for clade based

studies of various HIV-1 auxillary proteins. There have been reports where clade specific differences in Tat and Vpr activities had been demonstrated (Bano et al, 2007; Mishra et al, 2008).

Another unique HIV-1 accessory protein is Vif. When certain human immune cells-T lymphocytes, monocytes or macrophages, the main reservoirs for HIV-1 *in vivo*, are infected *in vitro* with HIV-1 strains carrying mutant Vif, these strains produce non-infectious viral progeny. By contrast, mutation of other HIV-1 accessory proteins, such as Nef, Vpr and Vpu, leads to reduced but replication competent viruses. This tells about the requirement of Vif by HIV-1 for production of infectious progenies so as to continue with the infection.

Virion Infectivity factor (Vif)

The primary goal of a virus is the replication of its genome in an appropriate host cell and the production of progeny virions for the infection of new target cells. For this, viruses have evolved various mechanisms to avoid recognition and destruction by host immune system. One such mechanism in all lentiviruses with the exception of equine infectious anemia virus is presence of Vif. Vif is required for production of infectious virus particles or virions and thus helps in HIV-1 replication and infection.

The Vif-deficient virions that are produced by primary T cells are non-infectious, and these T cells, and certain other cell lines, are referred to as non-permissive. By contrast, other cell types are termed permissive, because they can produce infectious Vif-deficient virions. Cell-fusion experiments have shown that the non-permissive phenotype is dominant over the permissive phenotype. This also suggests that host factors play a major role in generation of infectious virions. Till date a number of host factors have been identified as possible targets of Vif. The most important among these host factors is CEM15 which is identical to APOBEC3G and is a member of the family cytidine

deaminase (Sheehy et al, 2002). It has recently been shown that Vif-deficient virions produced from human PBMC contain only about 7 (+/-4) copies of APOBEC3G (Xu et al, 2007) yet, these virions are completely non-infectious suggesting that the level of tolerance for virus-associated APOBEC3G is quite low.

The human APOBEC3G (apolipoprotein B messenger-RNA editing enzyme, catalytic polypeptide-like 3G) protein is a single strand DNA deaminase that inhibits the replication of human immunodeficiency virus-1 (HIV-1), other retroviruses and retrotransposons (Bogerd et al, 2006; Cullen, 2006). APOBEC3G is packaged into new viral particles during viral synthesis to combat infectivity. APOBEC3G deaminates deoxycytidine to deoxyuridine of minus single–strand nascent viral DNA during reverse transcription, which results in hypermutation of plus strands DNA. This enzymatic editing of HIV reverse transcripts leads to degradation of deaminated minus-strand DNA (Harris et al, 2003) and blockade of HIV replication (Figure 1.15). To circumvent this protection activity of host cell APOBEC3G, Vif binds to APOBEC3G and mediates its ubiqitinylated proteosomal degradation (Yu et al, 2003). Vif molecules recognize their autologous APOBEC3 proteins through conserved structural features. Various motifs in HIV-1 Vif were identified that were important for both APOBEC3G and APOBEC3F interaction and suppression (Zhiwen et al, 2008).

Latest NMR studies by Kuan-Ming Chen, *et al,* 2008. have described the DNA deaminase domain of APOBEC3G and predicted a model that suggested that R215 and R313 would promote DNA binding, W285 would help to form the hydrophobic active site, and E259 would mediate catalysis (Kuan-Ming et al, 2008). This gives an insight of the interaction of APOBEC3G with viral ssDNA and developing of possible vif-APOBEC3G interaction models.

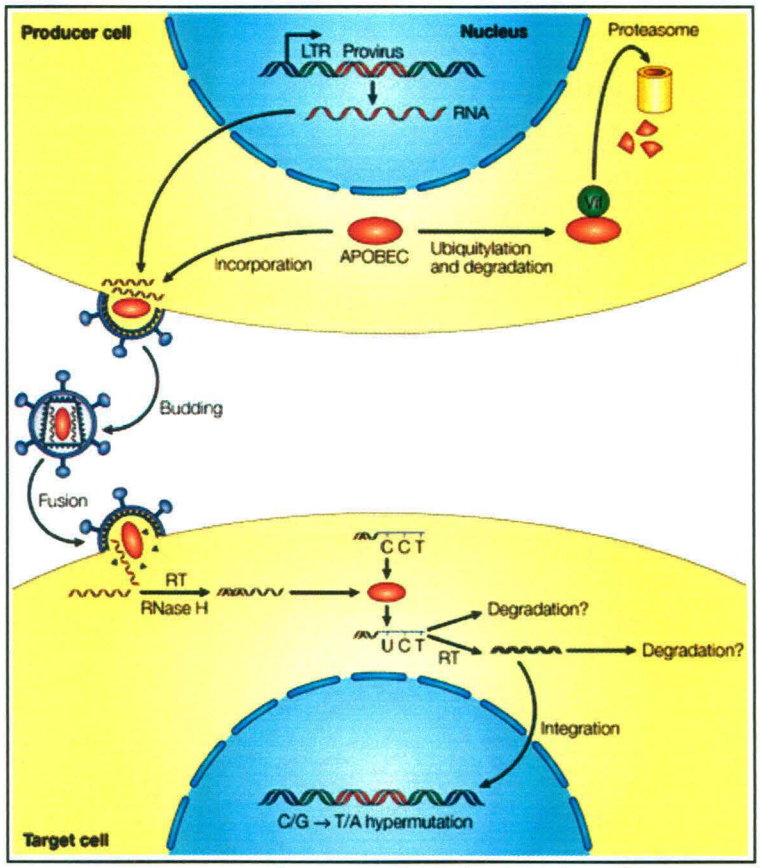

Figure 1.15 Functional aspect of APOBEC3G and HIV-1 vif

Vif itself is a relatively unstable protein with a half-life of ~30 minutes and –like APOBEC3G– is degraded by cellular proteasomes. It is believed that binding of Vif-Cullin-5/elonginB/elonginC/ Rbx1 complexes to APOBEC3G accelerates polyubiquitylation of the deaminase and, as a result, targets APOBEC3G for destruction by the 26S proteasome, Figure 1.17 (Kao et al, 2007)). Strebel and coworkers henceforth, proposed an adapter model for Vif-induced degradation of APOBEC3G and according to this model, (1) Vif is an adaptor molecule with binding sites for APOBEC3G and the Cul5-E3 ligase complex and (2) expression of Vif results in the formation of an APOBEC3G-Vif-E3 ternary complex. (3) This complex formation then triggers poly-ubiquitination of APOBEC3G, (4) resulting in the degradation of APOBEC3G.

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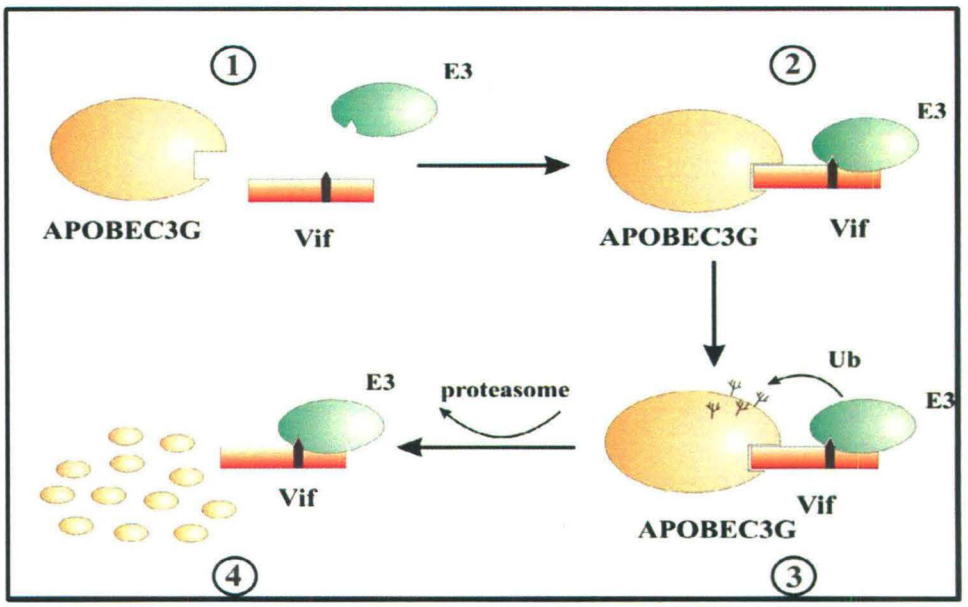

Figure 1.16: Adaptor model for Vif-induced APOBEC3G degradation

It is studied that the turnover rates for the two proteins do not match as well. SLQ and HCCH motifs of Vif are required for APOBEC3G degradation, and neither the deletion nor mutation of these motifs increased the stability of Vif or prevented its polyubiquitination, suggested that Vif is not degraded through the cullin-5 E3 ubiquitin ligase complex. Thus, it could be said that Vif might induce polyubiquitination and degradation of APOBEC3G by recruiting the Cul5-E3 ubiquitin ligase complex; but it seems unlikely that Vif and APOBEC3G are co-degraded in this complex (Goila and Strebel, 2008).

Packaging of Vif into viral particles during productive infection is mediated by an interaction with viral genomic RNA and association with viral nucleoprotein complexes (Karczewski et al, 1996). Indeed, it has been shown that Vif can establish a strong interaction with ribonucleotide homopolymers and synthetic viral RNA *in vitro*. Vif–RNA complexes were also shown to disassemble in the presence of Pr55Gag, suggesting a concerted mechanism for the binding of the two partners (Zhang et al, 2000). RNA-binding properties of Vif could be involved in several functions, such as proper RNA folding and

trafficking in the cytoplasm to enable efficient packaging and prevention of cellular inhibitors, such as APOBEC proteins, from altering HIV-1 RNA.

Therefore, vif gene of HIV-1 is one of the most suitable targets for designing of nucleic acids based antiviral strategies. The human immunodeficiency virus type 1 (HIV-1) affects millions of people worldwide (Hladik and McElrath, 2008). The combination anti-retroviral therapy has dramatically reduced the incidence of AIDS and mortality by suppressing HIV-1 replication. However, a complete cure is not yet possible since the virus latently resides in resting, memory CD4+ lymphocytes in post integrated form. In addition, due to the adverse toxicity of long-term drug remains, development of drug resistant variants and lack of effective vaccines, there is an urgent need to develop alternative therapeutic strategies, including gene therapy approaches. A feature common to many of these intracellular immunization gene therapy strategies is that they are primarily designed to act on various stages of virus replication in a cell that is already infected but would not prevent infection of a naïve susceptible cell. Cells harboring integrated provirus will continue to remain in the host posing the risk of viral activation at a later stage. These potential problems could be overcome by other novel strategies, which would confer target cell protection at the very first viral attachment stage.

The field of nucleic acid therapeutics has evolved considerably with numerous gene targets and methods comprising both naturally occurring and synthetic molecules that have been applied *in vitro* and *in vivo* in a variety of contexts with varying degrees of success. Till date, expressions of many genes involved in viral replication have been suppressed using nucleic acid-based approaches. Inhibition of HIV-1 replication by macrophage-tropic Dzs that were targeted against HIV-1

TAT/Rev RNA was carried out by Banerjea and coworkers (Unwalla et al, 2001a, 2001b). TAT protein is a powerful transcriptional transactivator of viral gene expression and exerts its action via binding to TAR-stem loop structure that is present at the 5′ end of all HIV-1 and HIV-2 transcripts (Gracia-Martinex et al, 1995). This interaction plays a key role in controlling transcription. Two Dzs were identified that exhibited sequence-specific cleavage activities but also brought about significant inhibition of HIV-1 gene expression in primary and chronically infected cells (Chakraborti et al, 2003). These various Rz and Dzs can be exploited for therapeutic purposes against HIV-1.

OBJECTIVES

With the aim of designing and developing catalytic nucleic acid and antisense oligodeoxynucleotides based novel antiviral strategies, the following objectives were thus proposed:

1. To study the possible synergistic action of hammerhead Rz and 10-23 Dzs to achieve more powerful suppression of genes
2. Modulation of activity of hammer head Rz construct by 10-23 Dz
3. To evaluate antisense oligodeoxynucleotide mediated enhancement of catalytic activity of Rz and Dzs
4. To determine and design various Dzs against vif gene of HIV-1 subtype B and Indian isolate subtype C
5. To evaluate efficacy of a novel bi-specific siRNA-Rz (siRNA-Rz) construct against X gene of Hepatitis B virus

CHAPTER II

Synergistic action of hammerhead ribozymes and 10-23 DNA-enzymes on HBx mRNA

Hepatitis B virus (HBV) infection is a worldwide health problem and is one of the major causes of hepatocellular carcinoma (HCC) in the world. The crucial role of HBV in hepatocarcinogenesis remains uncertain and controversial (Zoulim et al, 1994). Hepatitis B virus X gene has been associated with hepatocarcinogenesis because HCC incidences had been reported earlier in animals infected with mammalian hepadnaviruses (Koike et al, 1994). Among these hepadnaviruses, open reading frame-X (X-ORF) is conserved. However, hepatocarcinogenesis has not been found in avian infected with avian hepadnaviruses where X-ORF is absent (Ganem, 1996). HBx, a small protein of 154 amino acids, is a multifunctional regulator that modulates a variety of host processes through directly or indirectly interacting with virus and host factors. (Gomez-Gonzalo et al, 2001; Seeger et al, 2007)

The 'transactivation' function of HBx has been widely studied to elucidate the modulated expression of the HBV genes and host genes that include the genes that are critical or important for cell proliferation, cell cycle progress, acute immune response, protein degradation pathway, genetic stability, cell apoptosis, and also those involved in tissue organization. X protein has been reported to retain a variety of functions in addition to the co-activation function. The interaction between HBx and proteasome subunits is necessary for 'transactivation' of HBx and for efficient HBV replication that may result in suppressing the process of antigen presentation in HBV-infected cells (Bouchard, 2004). HBx also targets nucleotide excision repair (NER) as it directly binds DDB1 and Xeroderma pigmentosa B (XBP) or Xeroderma pigmentosa D (XPD) helicase in general transcription factor IIH (TFIIH) (Lee et al, 1995; Beckar et al, 1998; Hu and Boyer 2006). This

interaction may facilitate hepatocarcinogenesis in the presence of genotoxic stimuli.

Since, X gene is a pleiotropic transactivator and also responsible for increasing the levels of expression of other hepatitis B virus genes, any approach to down regulate specifically the X gene product will be very beneficial to patients suffering from Hepatitis B and its other associated disorders.

The ability of nucleic acids as biological catalysts has become well established in the last few years. Formerly, biochemical dogma dictated that proteins performed all biological chemical reactions, and nucleic acids merely carried the genetic information that encoded the proteins. The various nucleic acids that have catalytic properties are broadly termed as ribozymes and DNA enzymes. These catalytic nucleic acids have been used earlier to suppress the expression of various viral genes, including HBx of Hepatitis B virus (Goila and Banerjea, 2004).

Rzs are catalytic RNA molecules acting as RNA restriction endonucleases. In nature; these are the only nucleic acids that have enzymatic properties. These were discovered initially as self-cleaving entities as in ribonuclease P of *E.coli* (Guerrier-Takada et al, 1983) and the intervening sequence of *Tetrahynema thermophila* (Zaug et al, 1986). The two most extensively worked Rzs are-hairpin ribozyme: discovered from the "minus" strand of satellite RNA of tobacco ring spot virus (Hampel et al, 1989) and the other is hammerhead Rz: identified in the virusoid from Lucerne transient-streak virus (Foster et al, 1987). These two Rzs belong to the class "small ribozymes" in the classification of Rzs. The Rzs have multiple applications in the fields of biology and chemisty. Recently, hammerhead (Hartig and Famulok , 2008) and hairpin (Najafi-Shoushtari and Famulok, 2008) Rzs were used to signal molecular interactions.

Hammerhead ribozyme- It is the smallest Rz that is composed of approximately 30 nucleotides and is capable of site specific cleavage of a phosphodiester bond (Symons et al, 1998; Martrick et al, 2006). The catalytic consensus sequence has enzymatic activity to cleave the target RNA. The target sequence for cleavage usually consists of nucleotide sequence "NUX", where N is any base and X can be C, U or A, but not G. Usually, GUC is recommended for "NUX" sequence.

The structure of mRNA consisits of several single stranded stem-loop structures. Rz cleavage site should be located in loop structures in the target mRNAs. By cleavage at "NUX" sequence, ribozyme can significantly alter the structure of the target mRNA. The hammerhead Rz undergoes a well-defined two stage folding process induced by the sequential binding of two magnesium ions. The first corresponds to the formation of the Rz scaffold, while the second involves the formation of the catalytic core of the ribozyme. The hammerhead ribozyme is capable of cleaving the target mRNA *in cis* and *in trans* (Haseloff et al, 1988). In both configurations, the Rz undergoes multiple turnovers and virtually any sequence can be targeted for cleavage.

Mechanism of action of ribozyme: Self cleavage of the hammerhead Rz most likely involves the general acid-base catalysis in which a basic function within the active hammerhead structure accepts the proton from the 2'-hydroxy group and an acidic function provides a proton for the 5'-hydroxy group at the cleavage site (Liu et al, 2007).

Shortly after the discovery of Rzs, search was undertaken to look for possible catalytic DNA molecules. DNA in its single stranded form can be made to perform both molecular recognition and catalysis-biochemical operations that were earlier thought to be restricted to macromolecules made up of proteins and RNA. One of the most active areas of catalytic DNA research has been generation of deoxyribozymes that perform the RNA

cleavage reaction by transesterification. The DNA enzymes can easily be engineered to cleave any target RNA in a sequence-specific and catalytic manner.

DNA-enzymes- About 15 years ago, a particular strand of deoxyribonucleic acid (DNA) molecule that could act as an enzyme was discovered (Breaker et al, 1994). The discovery of DNA as an enzyme was unexpected as compared to that of RNA as an enzyme because of the presence of 2'-hydroxyl group in the latter, which was thought to play a central role in ribozyme function. These advances laid the ground work for the discovery of a class of Dz that could cleave RNA molecules in a sequence-specific manner (Santoro et al, 1997). Dzs have also been utilized as *in vivo* therapeutic agents to destroy specific mRNA targets (Baum and Silverman, 2008).

Mechanism of action of DNA-enzymes: The catalytic mechanism of RNA-cleaving is thought to be similar to that of hammerhead Rz (He et al, 2002). The putative mechanism involves the divalent metal ion associated deprotonation of the 2'-hydroxyl group adjacent to the cleavage site. This mechanism is favoured as it explains the divalent metal ion dependence of many small nucleic acid enzymes as well as the effect that pH has on their reactions.

10-23 DNA-enzyme- It is the most widely known Dz; named as the 23rd clone identified following 10 cycles of selective amplification. This DNA interacts with its RNA targets using canonical base pairing interactions between the DNA and RNA, and depends on binding of a divalent cation (such as magnesium) for activity (He et al, 2002; Santoro et al, 1998).

The structure of 10-23 Dz is made up of a catalytically active core of 15 near-invariant nucleotides, flanked by substrate binding arms that can be

changed to base pair with the sequence of the desired target RNA. 10-23 Dz cleaves the RNA sequence at a phosphodiester bond between a purine and a pyrimidine residue (5'AU 3' most efficiently cleaved). A single nucleotide change in the catalytic motif diminishes its sequence specific cleavage activity; G14C change in the catalytic motif completely abolishes the catalytic cleavage activity.

The use of Rzs or Dzs has been proposed for selective inhibition of gene expression or as potential antiviral agents. Rzs and Dzs being nucleic acid molecules also lack immunogenicity, thus allowing it to be used *in vivo*. It is also possible to design multiple catalytic nucleic acids against conserved region of viral genome to overcome viral resistance. Because of their simple design and small size, hammerhead Rzs are particularly attractive, and they have been used extensively to down regulate many different eukaryotic genes (Birikh et al, 1997). The effective use of Rzs or Dzs against a rapidly replicating virus presents a challenge. The challenge is even greater with RNA viruses, which continuously undergo mutational changes. Hepatitis B virus replicates through reverse transcription of an RNA intermediate. Multitarget Rzs and Dzs not only maintain the target specificities of the individual catalytic nucleic acid but also significantly raise the overall cleavage efficiency per catalytic RNA molecule. Thus, multitarget approach is likely to increase the chance for a prolonged effectiveness (Paik et al, 1997).

We are interested in studying the possible synergistic effect of Rzs and Dzs in order to knock down more potently the expression of HBx gene of Hepatitis B virus. HBx RNA is the most plausible target for the Rz and Dz to block the HBV replication because the HBx directed catalytic nucleic acids might inhibit expression of all viral genes owing to the presence of HBx ORF region in all HBV RNA transcripts. Another reason for selection of HBx gene

as target is that HBx protein transactivates the transcription from all HBV promoters (Nakatake et al, 1993). Therefore, the inhibition of HBx gene expression may be one of the most efficient ways to block the HBV replication and combinatorial usage of Rzs and Dzs can have more potent effect on suppression of HBx gene at RNA and protein levels.

EXPERIMENTAL PROCEDURES

Plasmids used:

HBx gene (465 bases) of hepatitis B virus (Ohta et al, 1996) obtained as a gift from Dr. Vijay Kumar, ICGEB. New Delhi, was cloned into the *Eco RI* site of the expression vector pSG5 (Stratagene, Texas, U.S.A.) (Figure 2.1) so that the gene was placed under bacteriophage T7 as well as SV40 promoter of pSG5. The former promoter was used to obtain *in vitro* generated transcripts using T7 RNA polymerase, whereas, the SV40 promoter was used to achieve intracellular expression in a mammalian liver specific cell line (Hep G2). The plasmids were purified using plasmid prep columns (Qiagen) for the purpose of transfection into mammalian cells.

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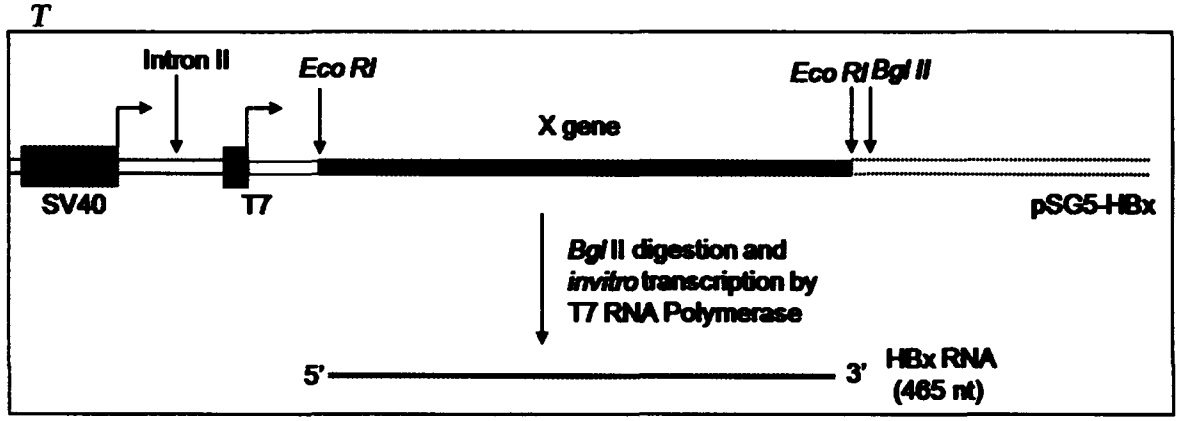

Figure 2.1: Cloning of HBx gene in pSG5

Cleavage site selection for ribozymes and DNA-enzymes:

HBx mRNA sequence was submitted to secondary structure generating software programme, namely, m-FOLD. The various secondary

structures generated by this program were studied for most conserved loop regions and target sites, GUC and AU or GU for Rzs and Dzs respectively (Figure 2.2). The Rz target site chosen was a *'GUC'* at nucleotide position 170 and 200 from the 465 bases long HBx gene for Rz-170 and Rz-200. The nucleotide site with an *'AU'* as target was chosen at position 307 of HBx gene for 10-23 Dz (Dz-307).

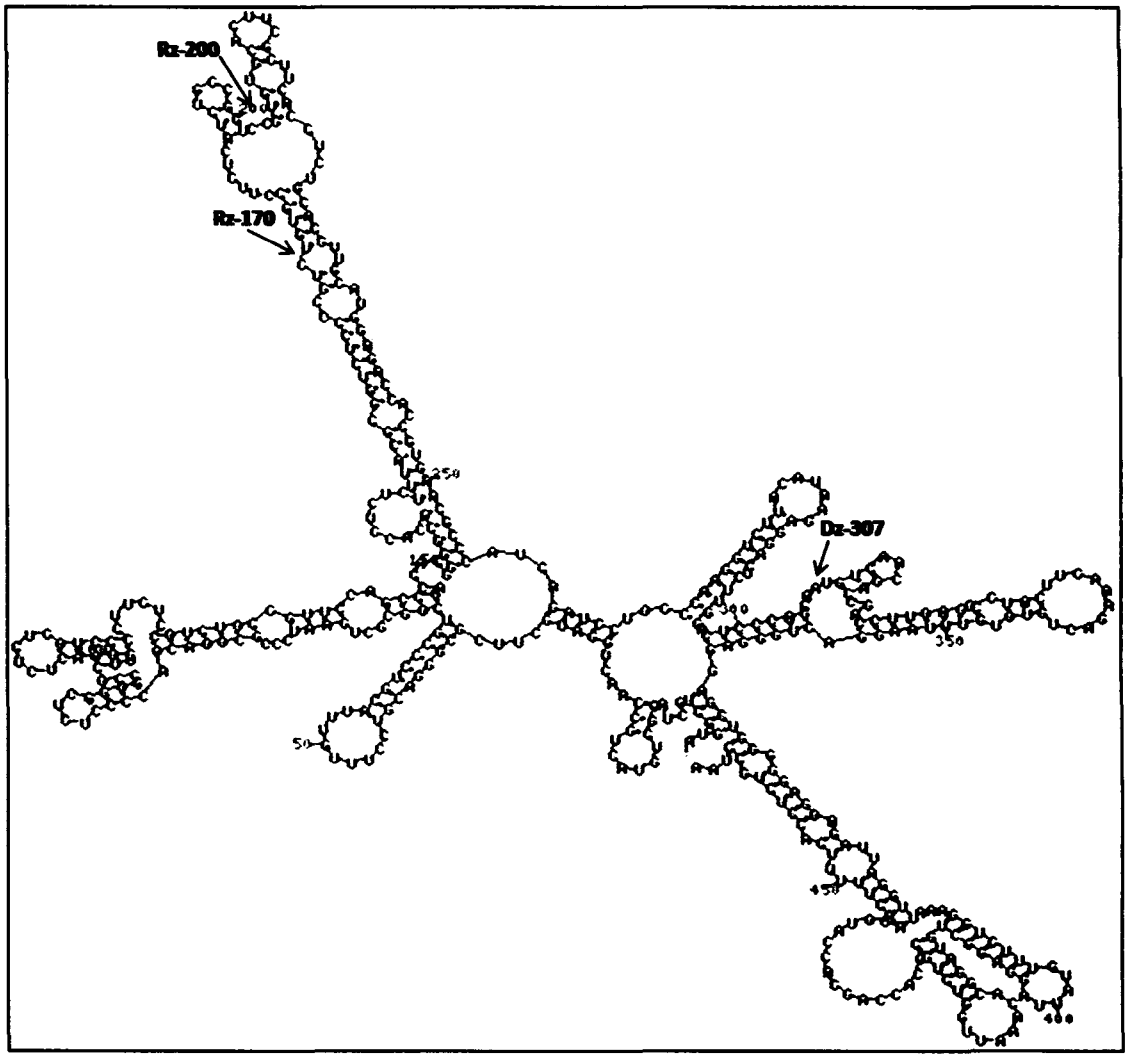

Figure2.2: m-FOLD generated secondary structure of HBx mRNA

Construction of hammerhead ribozymes:

Two hammerhead Rzs, Rz-170 and Rz-200 were designed earlier in the laboratory, using the following strategy. The oligonucleotide encoding

the ribozyme containing the hammerhead catalytic motif flanked on either side by 8 nucleotides complementary to the target RNA was synthesized chemically. Restriction sites (as shown below) were engineered at the 5' and 3' ends to ease cloning into various vectors. The mono-Rz constructs synthesized are shown in Figure 2.3, panels A and B. The oligonucleotides encoding the Rzs were PCR amplified and cloned into a T-tailed vector-pGEM-T-Easy. Then they were cloned into pCDNA3 using the restriction sites, directly under T7 and CMV promoters, the former for *in vitro* transcription and the latter for expression in mammalian cell lines.

Rz-170 (Rz nucleotide)

(1) 5′ GC *GGATCC* GACGGGGA**CTGATGAGTCCGTGAGGACGAA**
ACCGCGTA *GAATTC* GC 3′.

Restriction sites *Bam HI* and *Eco RI* (in italics) were engineered at the 5' and 3' ends of the oligonucleotides respectively with 2 extra bases GC at the terminal ends. The sequence of the catalytic motif of hammerhead ribozyme is shown in bold. 8 bases on either side of this motif provide specificity for the target RNA. Following primers were used to amplify Rz-170 specific DNA oligonucleotides:

(2) Forward primer: 5' GCGGATCCGACGGGGACTG 3'

(3) Reverse primer: 5' GCGAATTCTACGCGGTTTCG 3'

Rz-200 (Rz nucleotide)

(1) 5′ GATC *AAGCTTT* GCACACG **CTGATGAGTCCGTGAGGACGAA**
ACC GGCAG *GGATCC* GATC 3'.

Restriction sites *Hind III* and *Bam H1* were engineered at 5' and 3' ends respectively. The sequence of the catalytic motif of hammerhead ribozyme is shown in bold letters. 8 bases on either side of this motif provide specificity

for the target RNA. Following primers were used to amplify Rz-170 specific DNA oligonucleotides:

(2) Forward primer: 5' GATCAAGCTTTGCACACG 3'

(3) Reverse primer: 5' GATCGGATCCCTGCCGG 3'

PCR conditions have been described in materials and methods. The PCR amplified oligonucleotides were cloned in pGEMT-Easy vector and recombinant plasmids were screened for the insert by restriction digestion. The positive clones were confirmed by sequencing.

Synthesis of 10-23 DNA-enzymes:

The dinucleotide ***'AU'*** at site 307 of HBx gene sequence was chosen as the target site for Dz-307. Oligonucleotides of various lengths were chemically synthesized. 29 nt long Dz-307 was synthesized that had 15 nucleotide conserved 10-23 catalytic motif, flanked by hybridizing arms that were complimentary to seven nucleotide residues on either sides of ***'AU'***. These hybridizing arms provide specificity to the Dz (the A residue is left unpaired and the cleavage is expected to occur after the A residue) (Figure 2.3, panels C and D). The complete sequence of Dz-307 and its mutant variant are shown below in Figure 2.3 (G to C mutation in the catalytic motif of Dz is underlined).

10-23 DNA-enzyme, Dz-307:

5′ *GTTGACA* **GGCTAGCTACAACGA** *TGCTGGG* 3′

Mutant Dz-307:

5′ *GTTGACA* **GGCTAGCTACAAC<u>C</u>A** *TGCTGGG* 3′

The conserved 10-23 catalytic motif is shown in normal letters (panel C) and the G14 to C14 substitution that makes the Dz inactive is shown in bold and underlined (panel D).

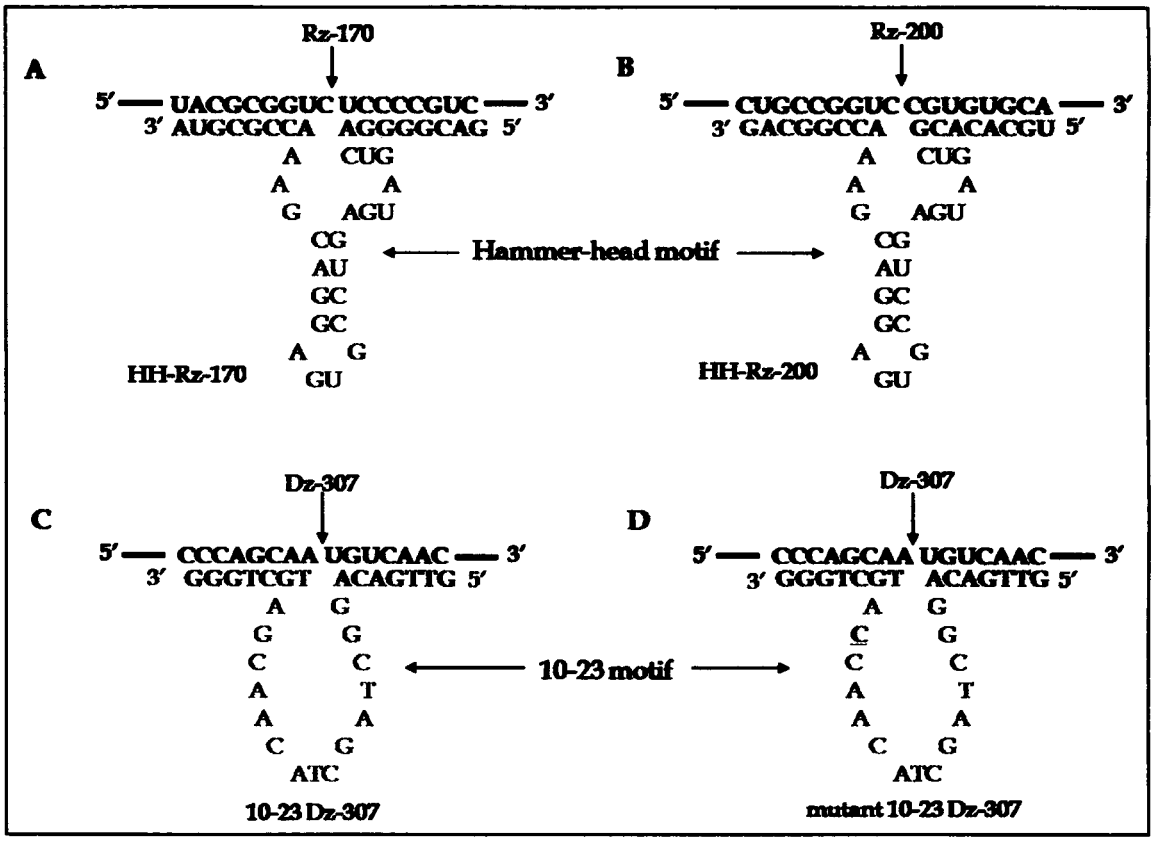

Figure 2.3: Sequences of HH-Rzs and 10-23 Dzs

In vitro transcription of the ribozyme constructs:

All the plasmids were column purified and linearized at the 3′ end of the target gene or the Rz prior to *in vitro* transcription. Plasmid pSG5-HBx was linearized with *Bgl II* restriction enzyme to give full length HBx (465 bases) transcript upon *in vitro* transcription. Plasmid pCDNA-Rz-200 was linearized with *Bam HI* and plasmid pCDNA-Rz-170 with *Eco RI.* Transcription of the linearized plasmid DNA was carried out as described in materials and methods.

In vitro cleavage reaction by ribozyme and DNA-enzyme:

Rz cleavage reaction was carried out by adding equimolar concentration (100 pmoles each) of the ^{32}P UTP labeled target RNA and cold

ribozyme RNA in a reaction buffer containing 50mM Tris-HCl, pH 7.5, in a volume of 10µl. The reaction mixture was briefly heated at 94°C and the cleavage reaction was initiated by adding $MgCl_2$ (10mM, final concentration) at 37°C for 2hrs, referred to as standard conditions. The reaction was stopped using equal volume of the stop buffer (95% Formamide, 0.05% Xylene Cyanol FF, 0.05% Bromophenol Blue, and 20mM EDTA). The samples were denatured at 55°C for 10 min before loading on to the gel. The cleaved products were analyzed by 7M Urea-6% Polyacrylamide gel electrophoresis using sequencing gel apparatus. The vacuum dried gel was exposed to an X-Ray film and the bands were analyzed qualitatively by autoradiography.

Similarly equimolar concentration (100 pmoles each) of labeled substrate RNA and cold Dz were mixed in a 10 µl of reaction mixture (50 mM Tris-HCl, pH 7.5, 10mM $MgCl_2$; standard conditions for cleavage) and incubated for 2hrs at 37°C. The reaction was stopped by adding the stop buffer. The samples were denatured at 55°C for 10 min before loading on to the gel. The cleavage products were resolved by electrophoresis on a 6% polyacrylamide-7M urea gel in Tris-Borate-EDTA buffer.

To study synergistic activity, both Rz and Dz were added to 10µl reaction mixture and the reaction performed under standard cleavage conditions as described earlier.

Intracellular effect of ribozyme and DNA-enzyme on HBx gene expression:

HEK 293 and Hep G2 cell lines were co-transfected with pSG5-HBx plasmid along with pcDNA-Rz with or without 10-23 Dz, using lipofectin as transfecting reagent as per the instructions of the manufacturers. Cells were harvested after 48 hours of transfection using trizol to isolate total RNA and reverse-transcriptase PCR (RT-PCR) was performed to check the effect of catalytic nucleic acids on the expression of HBx RNA level.

Whole cell lysate was prepared as described in methods and materials. 15% SDS-polyacrylamide gel was run to visualize the effect on expression of HBx protein. The western blotting was carried out using polyclonal anti-rabbit HBx antibody (Biovendor, NJ, USA).

RESULTS

In vitro cleavage of HBx transcript in presence of Ribozymes and DNA-enzymes:

Plasmid DNA, pSG5-HBx, encoding HBx gene was linearized by *Bgl II*, which on being subjected to an *in vitro* transcription in the presence of a ^{32}P UTP generated a full-length, labeled HBx transcript of 465 bases. The catalytic activities of the Rzs (Rz-170 & Rz-200) and Dz-307 were analyzed by *in vitro* cleavage assay. The Rz specific RNAs were obtained by linearizing the plasmids encoding them (by *Eco RI* for pcDNA-Rz-170 and by *Bam HI* for pcDNA-Rz-200) and transcribing using T7 RNA polymerase. A schematic representation of the cleavage reaction and the pattern of cleaved fragments that would be generated by the catalytic activities of the Rzs and Dz alone are shown in Figure 2.4, panels A and B. The expected cleavage products obtained by the use of both Rzs (either Rz-170 or Rz-200) and Dz-307 are shown in Figure 2.4, panels C and D.

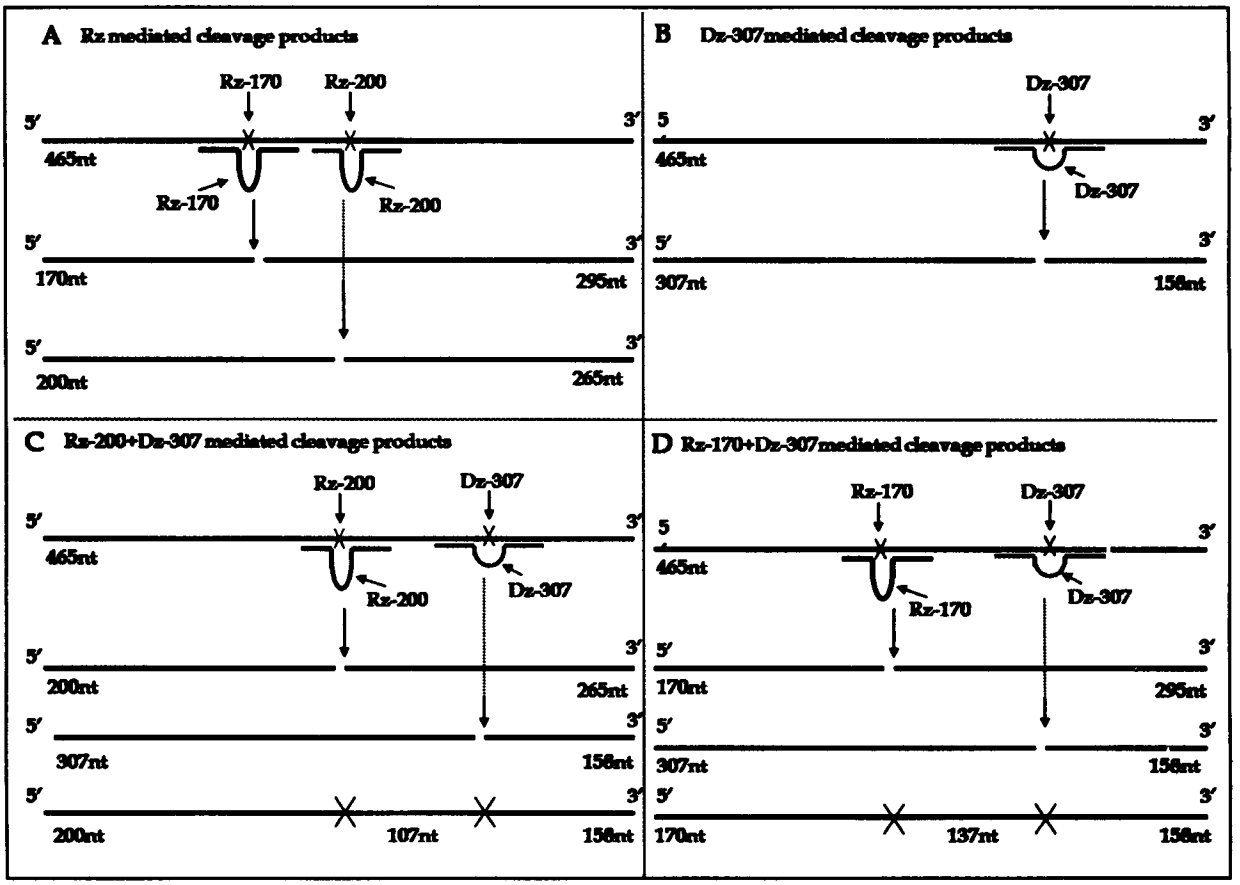

Figure 2.4: Schematic representation of cleavage of HBx by Rz and Dz

In Figure 2.5A, lane 1 corresponds to the control HBx mRNA transcript and lanes 2 and 3 show the cleaved fragments of target RNA in presence of Rz-200 and Rz-170, respectively. Rz-200 cleaved the 465 nucleotides long HBx RNA transcript into two fragments of sizes 265 and 200 nucleotides and Rz-170 generated fragments of sizes 295 and 170 nucleotides. These two lanes also confirmed that both the ribozymes are catalytically active *in vitro*. In lane 4, target RNA was subjected to the activity of Dz-307 and expected fragments of sizes 307 and 158 were observed. The combined effect of either of the Rz and Dz-307 was studied, taking together the two types of catalytic nucleic acids along with the labeled RNA, in the reaction buffer. Both Rzs and Dz-307 were found to efficiently cleave the target HBx

RNA and produced their specific cleavage-fragments as well as the cleaved fragment that was expected when the two nucleic acids worked simultaneously (Figure 2.5A, lanes 5 and 6), i.e. of size 107 in case of Rz-200 and 137 nucleotide with Rz-170. This experiment clearly showed that both Rz and Dz are active under similar reaction conditions when taken together and they maintained their specific cleavage activities.

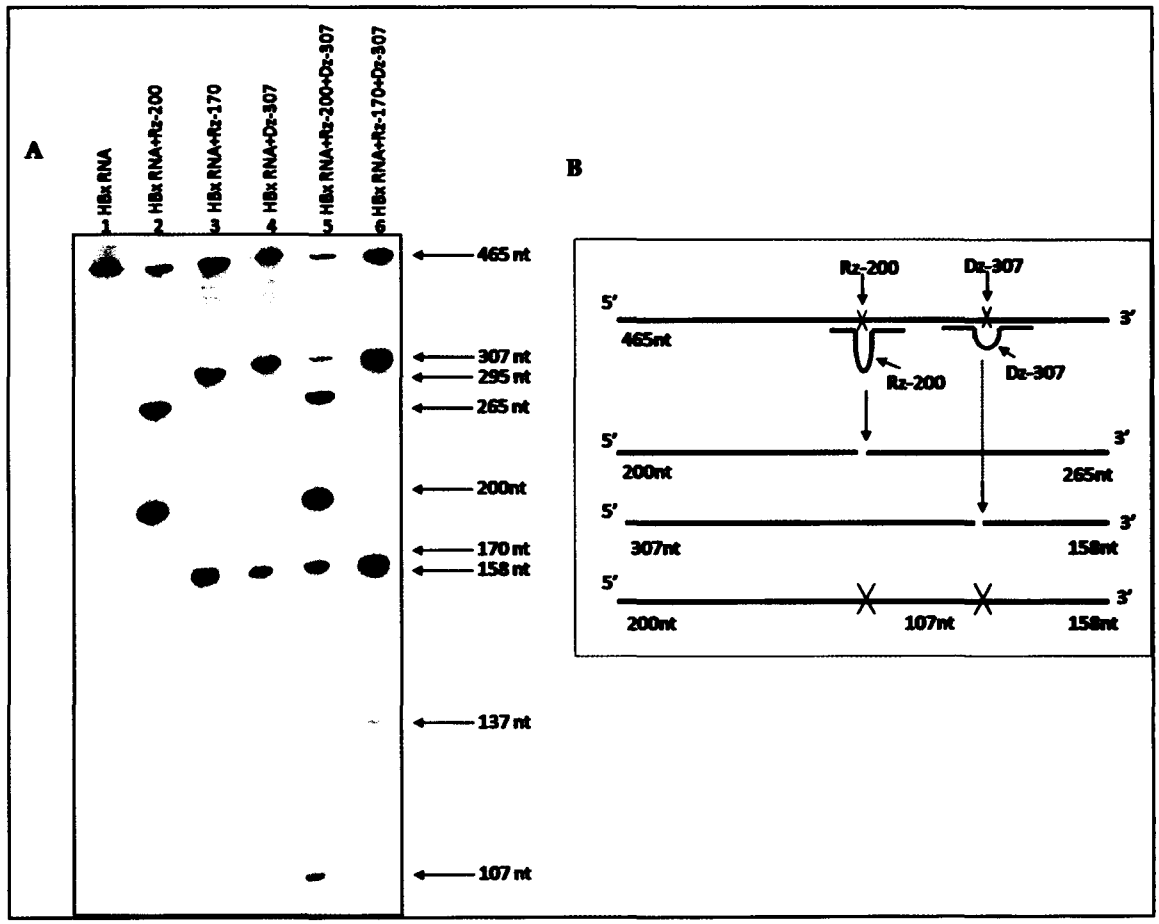

Figure 2.5: *In vitro* cleavage of X RNA by Rzs and Dz together

Concentration dependent action of Rz-170 and Dz-307:

A concentration dependent cleavage reaction was performed for Dz-307 and Rz-170 on labelled HBx mRNA as shown in Figure 2.6A and B. The aim of the study was to find out the minimum concentration at which both Rz and Dz were catalytically active. It is noteworthy that concentration as

small as 5 pmoles for both Rz-170 and Dz-307 (which is 20 folds less than the target RNA) was enough to generate specific cleavage products (Figure 2.6A, lanes 6 and 7). Both Rz-170 and Dz-307 showed evidence of cleavage activity at 50 pmoles concentration onwards, lanes 4 and 5 of Figure 2.6, panel B which increased further at higher conc.

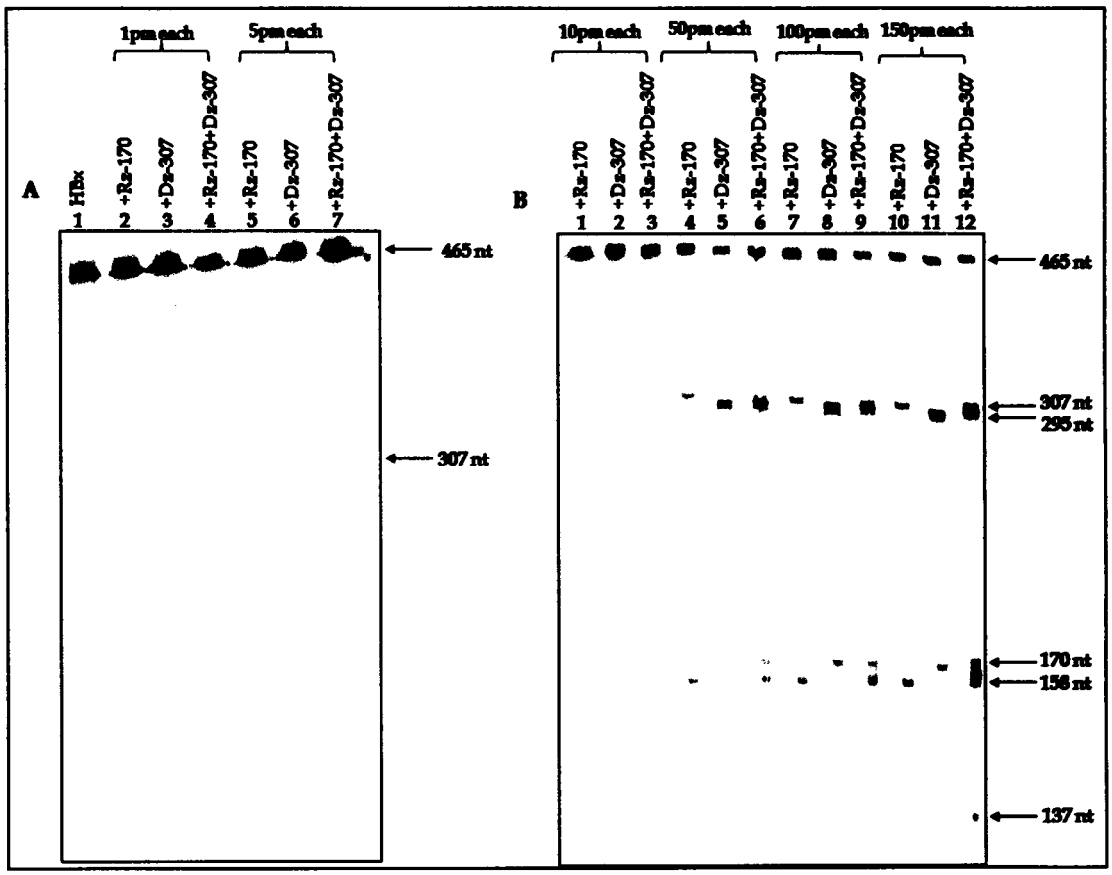

Figure 2.6: *In vitro* cleavage to know about working conc. of Rz-170 & Dz-307

Also, the effect of varying conc. of Dz-307 in presence of constant amounts of Rz-170 (50 pmoles) on the cleavage of HBx mRNA was studied. The cleaved products in the presence of Rz-170 (50 pmoles) alone as in lane 2 in panels A and B, were enhanced by the addition of varying concentration of Dz-307, as shown in Figure 2.7A (lane 3- 5 pmoles, lane 4-10 pmoles and

lane 5- 50 pmoles. There was no enhanced cleavage of the target HBx RNA by Rz-170 in the presence of mutant Dz-307 (Figure 2.7, panel B).

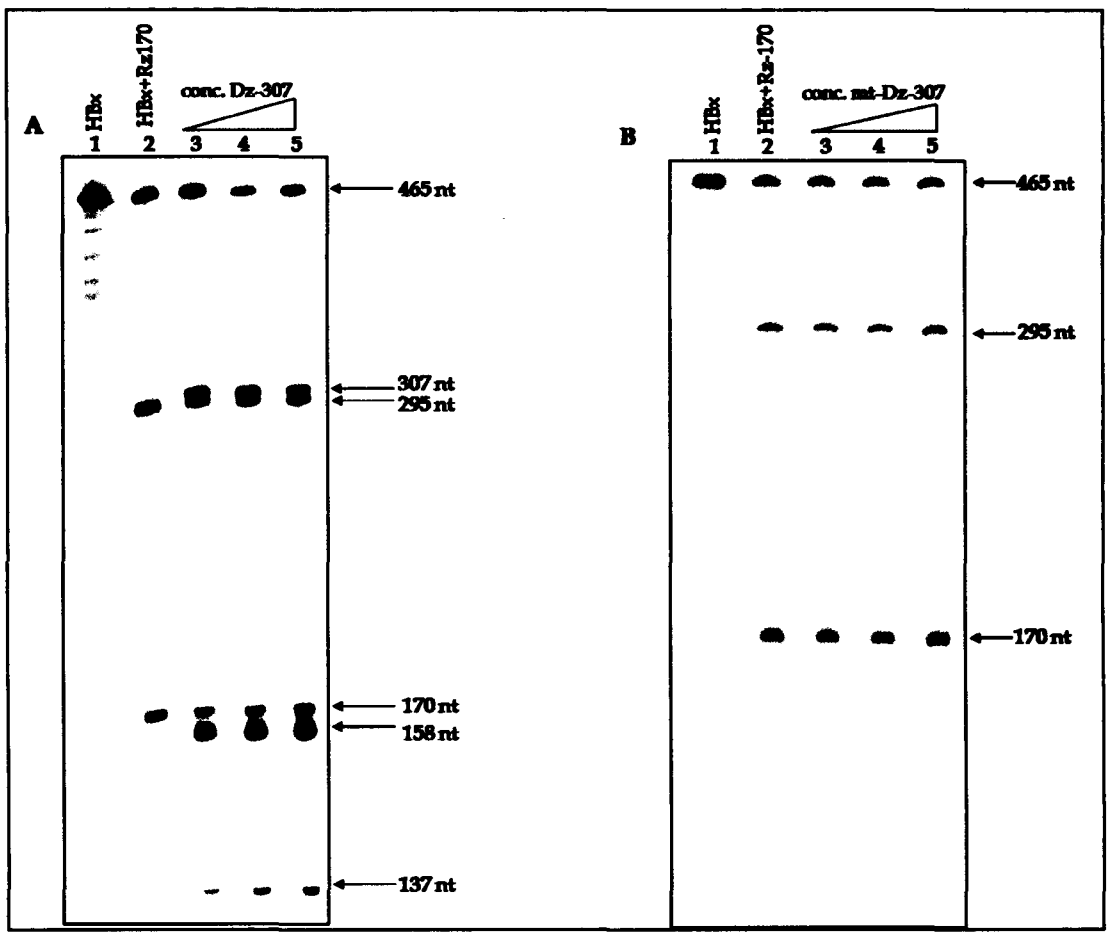

Figure 2.7: In vitro cleavage of X RNA by Rz-170 & wt Dz-307 or mt-Dz-307

Similarly, *in vitro* cleavage reaction was performed with Rz-200 and Dz-307 against the target HBx mRNA. In Figure 2.8A, the concentration of Rz-200 was kept constant as 50 pmoles (lane 2) and variable concentrations of Dz-307 was used (Figure 2.8A, lane 4- 5 pmoles, lane 5- 10 pmoles and lane 6- 50 pmoles). In lane 3, 50 pmoles of Dz-307 was taken alone as positive control. There was augmented cleavage of the target RNA when Rz-200 and Dz-307 were taken in combination and this augmentation in cleavage

efficiency was dose dependent as judged by the intensity of the 107 nts long cleaved fragment.

Also, effect of concentration gradient of Rz-200 RNA; 10 pmoles, 50 pmoles and 100 pmoles (Figure 2.8, panel B, lanes 4, 5 and 6) along with constant Dz (50 pmoles) was studied *in vitro*. In lane 3, only Rz-200 (100 pmoles) was added to cleave the target RNA. The effect of combination of Dz-307 and Rz-200 was similar to that of dose dependent study of Dz-307 along with a fixed concentration of Rz-200. The input target RNA was significantly reduced when Dz-307 and Rz-200 were used in combination as compared to when taken individually.

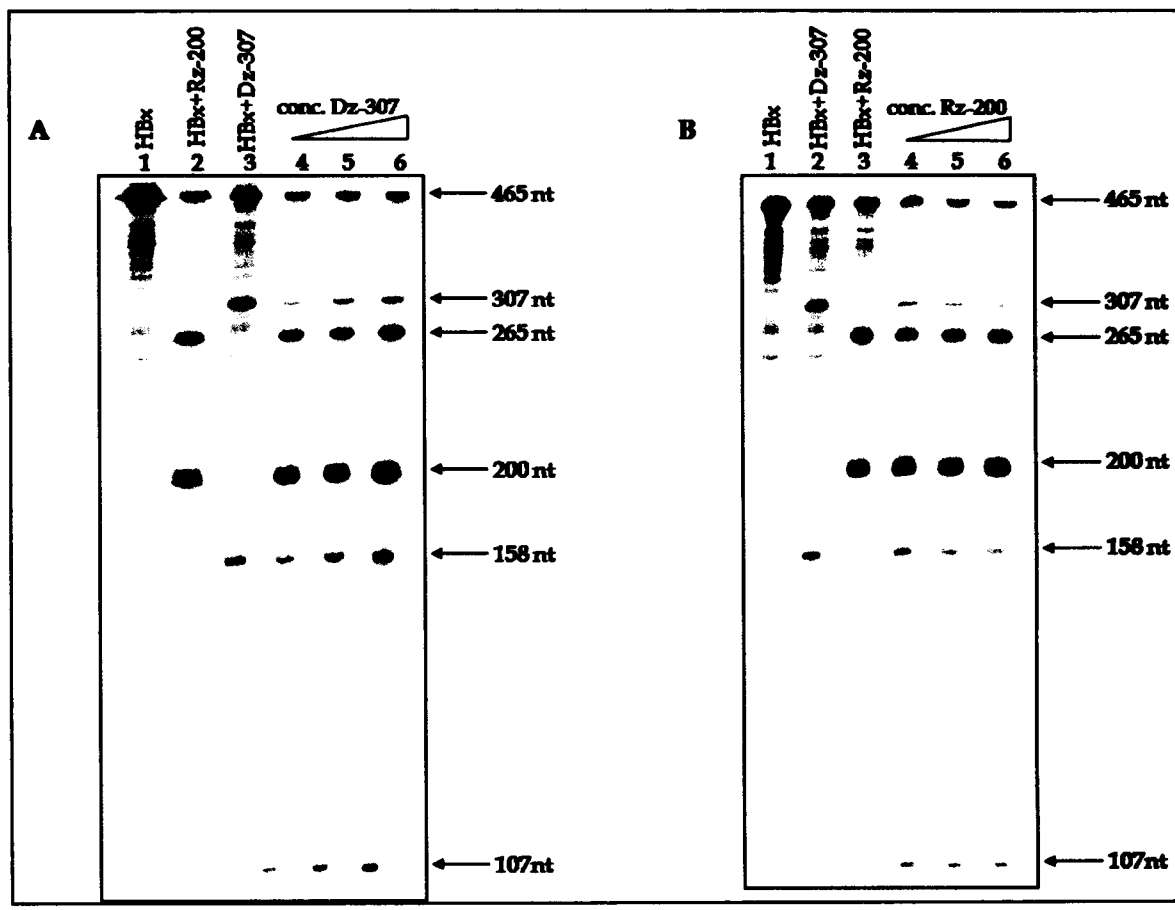

Figure 2.8: Effect of Rz or Dz on cleavage efficiency of the other when one is kept constant

Synergistic action of Rz-170 and Dz-307 on in vitro cleavage of HBx mRNA:

The nature of the combination of Rz and Dz on the cleavage of target RNA was studied where 100 pmoles of either Dz-307 (Figure 2.9A, lane 2) or Rz-170 (Figure 2.9A, lane 3) was used to cleave HBx RNA. When combined together in one reaction buffer, only 50 pmoles of each of Rz-170 and Dz-307 were taken together, so that the final concentration of catalytic nucleic acids was 100 pmoles. This *in vitro* cleavage of HBx mRNA clearly showed that more enhanced cleavage of the target RNA occurred when the two catalytic nucleic acids were combined (lane 4). The quantitative analysis also confirmed the synergistic effect of the combination of the Rz and Dz (Figure 2.9B).

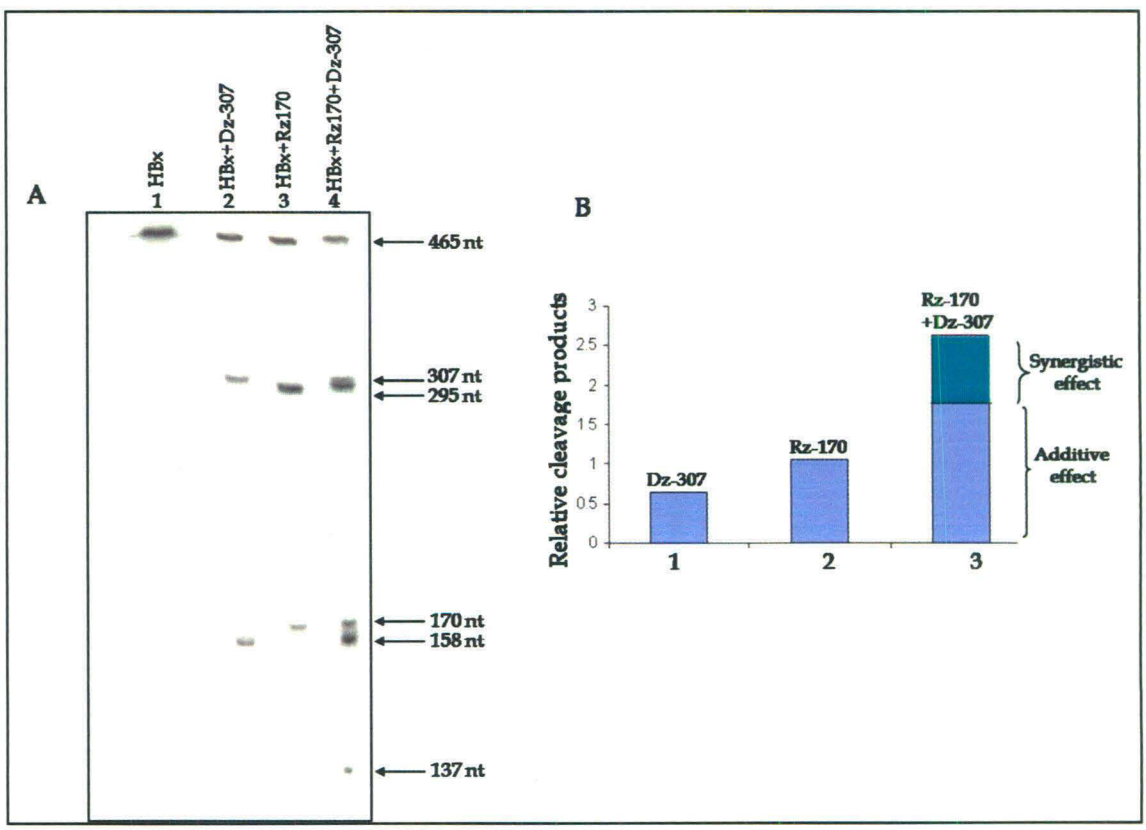

Figure 2.9: Synergistic activity of Rz-170 & Dz-307 on X RNA

Effect of Rz-170 and Dz-307 on expression of HBx mRNA in Hep G2, liver-specific cell line:

Liver specific Hep G2 cell line was co-transfected with plasmids pSG5-HBx and Rz170-pcDNA or with pSG5-HBx and Dz-307, using lipofectin as the transfecting reagent. To observe the combined effect of Rzs and Dz, the two were taken together in equimolar concentration and co-transfected with the HBx gene encoding plasmid. Cells were harvested 48 hours after transfection and total RNA was isolated as mentioned in methods and materials. Reverse-transcriptase PCR was performed using HBx gene specific primer to estimate the levels of the target RNA. As shown in Figure 2.10, lanes 6 and 7, in the presence of Dz-307 and Rz-170, the expression of HBx was significantly reduced in comparison to when Dz-307 or Rz-170 was used alone (lanes 3 and 5, respectively).

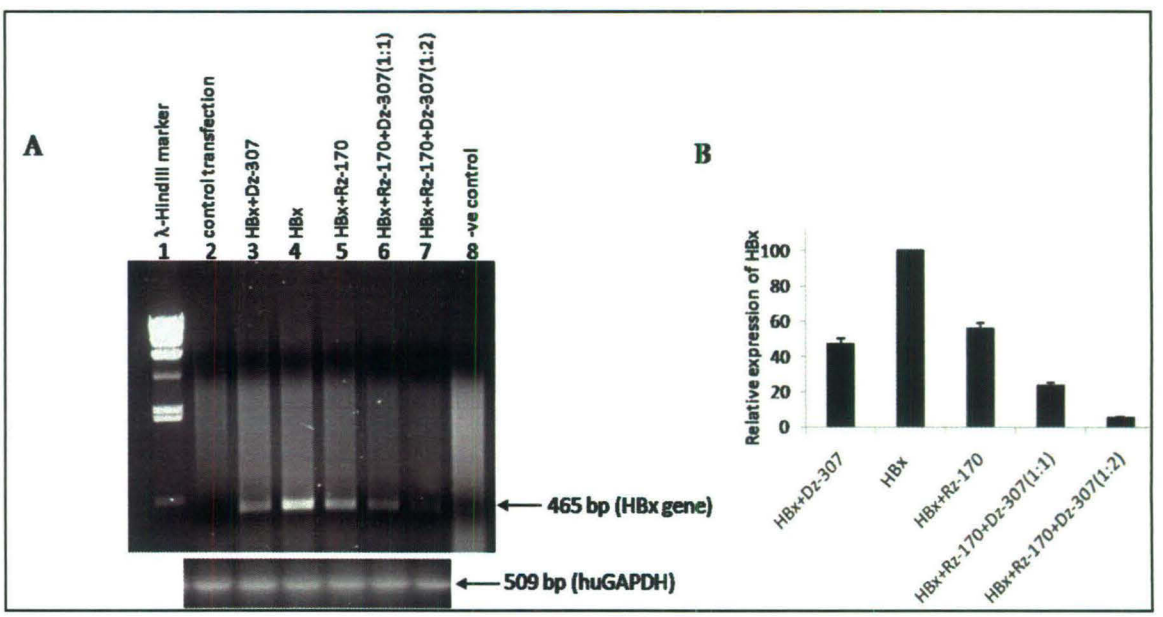

Figure 2.10: RT-PCR assay to study synergistic action of Rz & Dz on intracellular expression of HBx RNA

Western blot analysis to assess the combined effect of Rz-170 and Dz-307 on HBx-protein:

Hep G2 cell line was co-transfected with Rz-170 and Dz-307 along with HBx encoding plasmid, to check the synergistic effect of both the nucleic acids on the expression of HBx at protein levels. Cells were processed after 48 hours of transfection with 1% triton-X 100 lysis buffer and cellular lysate was prepared as described in methods and materials. Equal concentration of total protein was loaded on 15% SDS-PAGE and blot was probed with polyclonal HBx antibody. As clear from Figure 2.11, lane 6, in the presence of both the ribozymes and DNA-enzyme, the expression of HBx protein was almost completely abolished. Also a very significant reduction in protein levels was observed with either Dz-307 (lane 4) or Rz-170 (lane 5) when compared with cells that received HBx encoding plasmid DNA alone (lane 3). No reduction in control protein (actin) was observed. Cells alone did not show any specific band (lane 1). To ensure uniform transfection efficiency, a reporter plasmid pSV-β gal was used with every transfection.

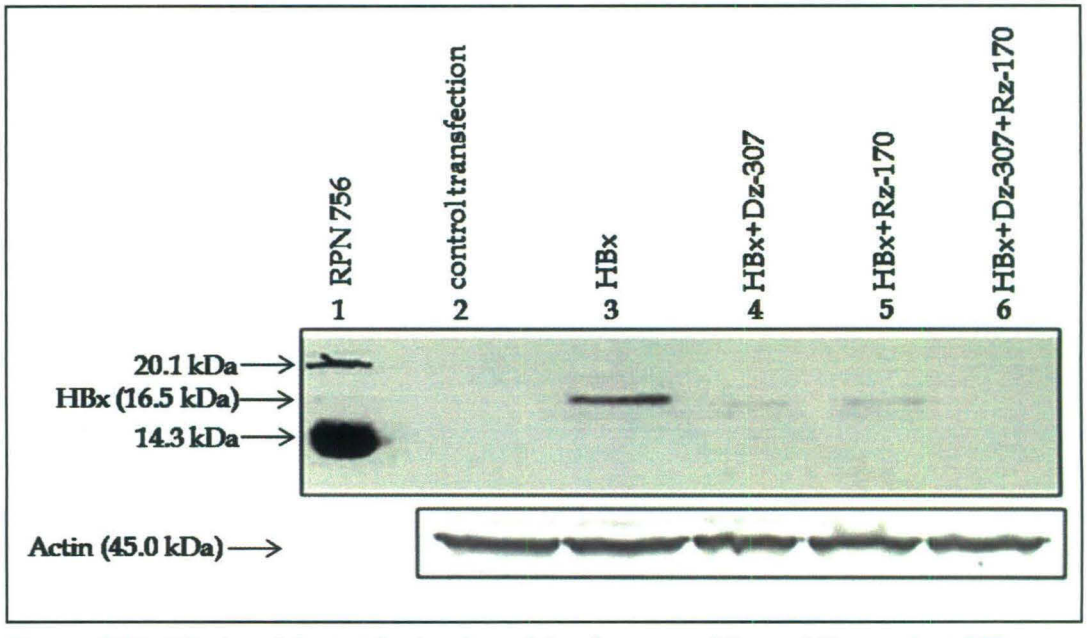

Figure 2.11: Western blot analysis of combined usage of Rz and Dz against HBx

DISCUSSION

The use of catalytic nucleic acids, namely Rzs and Dzs, provide a unique approach towards achieving selective destruction of the target RNAs by cleaving the latter in a more sequence-specific manner. Because both DNA and RNA viruses translate their mRNAs to make proteins that assemble and mature into virus particles, they can be targeted by ribozymes and DNA-enzymes to selectively inhibit their gene expression. Nucleic-acid-based agents use widely varying mechanisms to exert their effects but they all exploit the Watson-Crick base pairing between the two nucleotide chains. It is likely that each strategy would work optimally for a given target.

The most extensively used Rz is hammerhead Rz that is used for suppressing the expression of viral as well as cellular genes. Dzs have an added advantage over Rzs, as they exhibit a more rapid rate of catalytic turnover compared to their RNA counterparts. But at same time, a gene for a therapeutic Rz could be inserted into the genome of the host, which would then make the Rz continuously whereas, a Dz in contrast has to be synthesized and given as a drug. But the two when used together can have augmented effect on the cleavage of the target RNA, without interfering with the catalytic activities of each other.

Inhibition of HBx expression by using either Rzs or Dz has been earlier reported to down-regulate HBV transcription and replication. Efficient hammerhead Rz mediated cleavage of the structured hepatitis B virus encapsidation signal *in vitro* and in cell extracts has been observed (Beck and Nassal, 1995). Hairpin Rzs have also been designed against HBV pregenomic RNA (Welch et al, 1997).

The ribozyme Rz-170 and DNA-enzyme Dz-307 individually were earlier tested for their sequence specific cleavage activities and their ability to interfere with intracellular expression of HBx RNA. But this is the first study

conducted to see if both Rz and Dz could work efficiently in one reaction condition and the kind of effect the combined nucleic acids would have on the cleavage of the target RNA. The expression of HBx RNA was more potently reduced when they were used in combination as compared to when used alone. Also the effect was not only additive but synergistic for the cleavage efficiencies of both the Rzs and Dzs.

Since the Rz and Dz individually target their specific sites, in the conserved region of the X-gene but using a combination of Rz and Dz, more than one site in the HBx gene, was targeted. This is a useful strategy as viruses have high mutational rate. Both the Rz-170 and Dz-307, cleaved the X RNA efficiently under standard conditions of cleavage, yielding specific cleavage products. For the *in vitro* cleavage, when the two were taken together, more augmented cleavage of the target RNA was observed. This could be due to the opening of the target RNA secondary structure and thus more molecules of Rzs or Dzs were able to access the target sites. The other possibility could be sequential cleavage of the target RNA by the Rz and the Dz.

It was earlier shown that 10-23 Dzs lose their ability to cleave by changing one nucleotide in the catalytic core (Santro and Joyce, 1997). We, therefore, engineered the same point mutation (G to C) at position 14 in the catalytic motif of Dz. This Dz, as expected, did not possess any cleavage activity and served as a control for evaluating the combinatorial effect of Dz-307 and Rz-170, both *in vitro* as well as intracellularly.

Rzs and Dzs when taken together could be used to target RNA at multiple sites. This is especially important if the target gene is known to accumulate mutations. Most RNA viruses are known to mutate primarily because they lack the proof reading mechanisms and for those situations this kind of approach would be more appropriate. The multitarget approach constitutes an important feature in our strategy in delaying the appearance of

escape mutants. Two different mutations at the same time have to occur in the target gene to escape the action of the two catalytic nucleic acids, which could be a rare event.

Approximately 2-3 folds more decrease in the levels of HBx RNA was observed when both the Rz and Dz were used as compared to when either Rz or Dz was used alone. In fact, almost entire X protein expression was knocked off when Rz-170 and Dz-307 were used together, which was not the case when equimolar concentration of either Rz-170 or Dz-307 was used, separately.

In conclusion, the results presented here strongly suggest that the Rzs and Dzs possess interesting sequence specific cleavage activities and were able to interfere synergistically with the functional expression of X gene of Hepatitis B virus in a liver specific cell line, HepG2. The usage of Rzs and Dzs in combination could, in principle, serve as valuable tool to selectively down regulate more potently the expression of X gene, a sequence that is conserved in mammalian hepadnaviruses and found in all HBV transcripts. Thus the combined approach may have therapeutic potential.

CHAPTER III

10-23 DNA-enzyme to modulate the activity of any Hammerhead Ribozyme

Ribozymes (Rzs) are short catalytic RNA molecules that possess the ability to cleave the target RNA in a sequence-specific manner. The finding that RNA can perform enzyme-like functions in cells overturned the view that biological catalysis is the exclusive realm of protein enzymes. The discovery of Rzs by Cech and Altman (Cech et al, 1981; Kruger et al, 1982; Guerrier-Takada et al, 1983) changed fundamentally our view of the function of RNA in chemistry, biology and medicine. RNA traditionally has been viewed as a passive molecule that only carries information or provides structure. The work of Cech that led to finding of certain RNA sequences which can mediate their own cleavage in the complete absence of any protein strongly suggests that RNAs could act as regulatory molecules (Cech, 1987). It is now clear that RNA can act as an enzyme, catalyzing not only RNA splicing and cleavage but also perform diverse array of other reactions. The ability of catalytic RNA to facilitate chemical reactions previously associated only with protein enzymes has redefined the role RNA may have played in early evolution (Cech, 1993), and it may play in therapeutic approaches for the treatment of human diseases (Marschall et al, 1994; Christofferson and Marr, 1995).

RNA based antiviral approaches have been used extensively against HIV-1 (for review see Akkina et al, 2003). Recent studies in bacteria have clearly established the exclusive role of RNA-based mechanisms that govern gene expression at the level of transcription and translation. Messenger RNAs containing unique Rz sequences whose self-cleavage could be modulated by binding of small molecules (Winkler et al, 2002 and 2004; Mandal and Breaker, 2004; Cech, 2004) have been described earlier. An analogous RNA-based gene regulation system was earlier designed for

mammalian cells by incorporating sequences encoding self-cleaving RNA motifs into the transcriptional unit of the gene (Yen et al, 2004).

On the other hand, Dzs, as originally described by Santoro and Joyce, 1997), are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner (Banerjea et al, 2004; Joyce, 2004). Over the years several kinds of Dzs with unique catalytic motifs have been described but Dz possessing the 10-23 catalytic motif has been exploited extensively by several investigators (Banerjea et al, 2004; Baum and Silverman, 2008; Chandra and Silverman, 2008). This Dz possesses a conserved 15 nucleotide long 10-23 catalytic motif and is able to cleave any target RNA between purine and pyrimidine under *in vitro* and *in vivo* conditions. Their ability to cleave any target RNA has been exploited for knocking down gene expression against variety of target genes including Hepatitis B virus X-gene (Goila and Banerjea, 2001) and HIV-1 genes (Chakraborti and Banerjea, 2003; Dash and Banerjea, 2004) with varying outcomes.

There is a need to find out alternatives to metabolites that can interfere with the actions of Rzs that work either in *trans* or in *cis*-configuration with respect to its target gene. Never before, a Dz has been used as a tool to regulate the activity of any other catalytic nucleic acid. The current study therefore, deals with designing of a 10-23 Dz that targets the **'GU'** site in the catalytic motif of hammerhead Rz and cleaves the Rz RNA in a sequence-specific manner. Earlier in our laboratory, we had identified two potent hammerhead Rzs (Rz-170 and 200) against the X gene of hepatitis B virus that not only cleaved the target RNA in a sequence-specific and catalytic manner but also interfered significantly with known intracellular functions of the X gene (Goila and Banerjea, 2004). In order to modulate the cleavage activity of Rz-200, we have, exploited the sequence-specific cleavage activity of a 10-23

motif containing Dz. These short catalytic Dzs show almost similar potential and kinetic properties described for hammerhead Rz with respect to cleaving a substrate RNA (Santoro and Joyce, 1997) and therefore, they are ideally suited to modulate the expression of a target RNA (Rz-200) in a sequence-specific manner.

The following approach was used to cleave the Rz-200 RNA *in vitro,* using the reaction buffer conditions similar to that for any 10-23 Dz (Goila and Banerjea, 2001). Also the effect of the Dz on the intracellular expression of Rz-200 was studied. HBx mRNA was the target RNA for Rz-200 as described earlier. Therefore, the possible interference of the Dz with intracellular cleavage activity of Rz-200 on its target RNA, i.e, HBx RNA was undertaken.

Since this DNA-enzyme is targeted against the conserved catalytic region of hammerhead motif, it can potentially act against all such hammer head Rzs can potentially modulate gene expression. This approach is significantly more beneficial to other contemporary approaches that involve use of excess amounts target specific aptamers or other inducer molecules that could be toxic to mammalian cells.

EXPERIMENTAL PROCEDURES

Cloning of Rz-200 and HBx gene and in vitro transcription:

Rz-200 was cloned in mammalian expression vector pcDNA3 (Invitrogen), under CMV and T7 promoters. The former promoter was used for intracellular expression of Rz and the latter was used for obtaining *in vitro* transcripts using the T7 RNA polymerase (Figure 3.1, panel A). For similar reasons, in a *cis*-construct, the entire HBx gene of HBV is placed under CMV

and T7 promoters followed by Rz-200 encoding sequence (Figure 3.1, panel B) in plasmid pcDNA3 (Goila and Banerjea, 2004).

Rz-200-pcDNA3 was linearized with *Bam HI* and *in vitro* transcribed using T7 Riboprobe kit (Promega) as described in methods and materials to generate 58 nts long transcript.

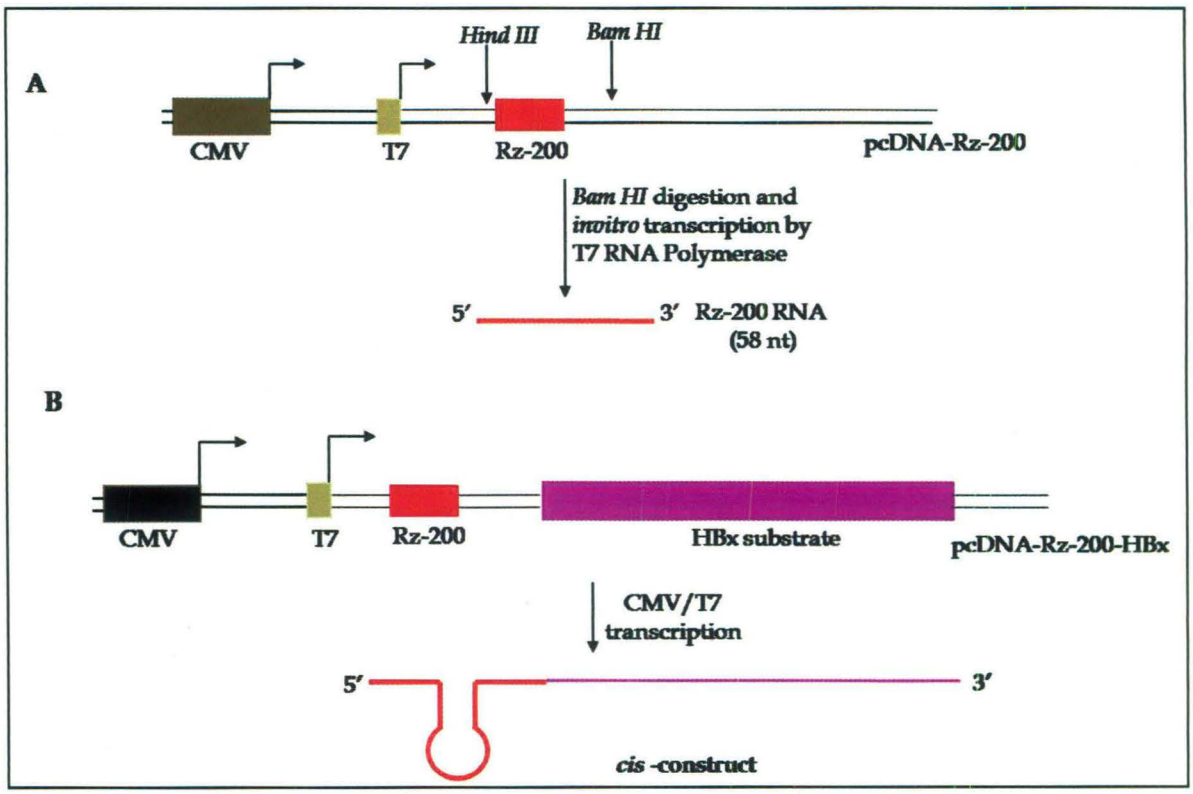

Figure 3.1: Cloning of Rz-200 and *cis*-contruct Rz-200-HBx

Construction of 10-23 catalytic motif containing Dz against the hammerhead catalytic motif of HBx-Rz-200

We focused on targeting the catalytic motif of the hammerhead Rz because this would enable, in principle, to modulate the cleavage activities of hammerhead Rzs in general. A 10-23 catalytic motif containing Dz was designed and it was targeted against the **'GU'** nucleotides in the catalytic

motif of hammer head Rz (10-23 Dz cleaves the target RNA between any purine–pyrimidine pair) (Figure 3.2). The position of the Rz cleavage in the target RNA is shown in a box. The specificity is maintained by 7 to 8 nts long hybridizing arms (red in colour) that were made complementary to the target RNA (hammerhead Rz). The sequence of 10-23 Dz is given below, with the conserved catalytic core in bold and italics.

Sequence of 10-23 Dz:

5′ GTCCTCA***AGGCTAGCTACAACGA***AGGACTCA 3′

Inactive mutant-Dz with same specificity was also synthesized that possessed a single G14 to C mutation in the 10-23 catalytic motif (Figure 3.2, shown by an arrow). The sequence is shown below with G14 to C transition in the catalytic core, underlined.

Sequence of mutant 10-23 Dz:

5′ GTCCTC***AGGCTAGCTACAAC<u>C</u>A***AGGACTCA 3′

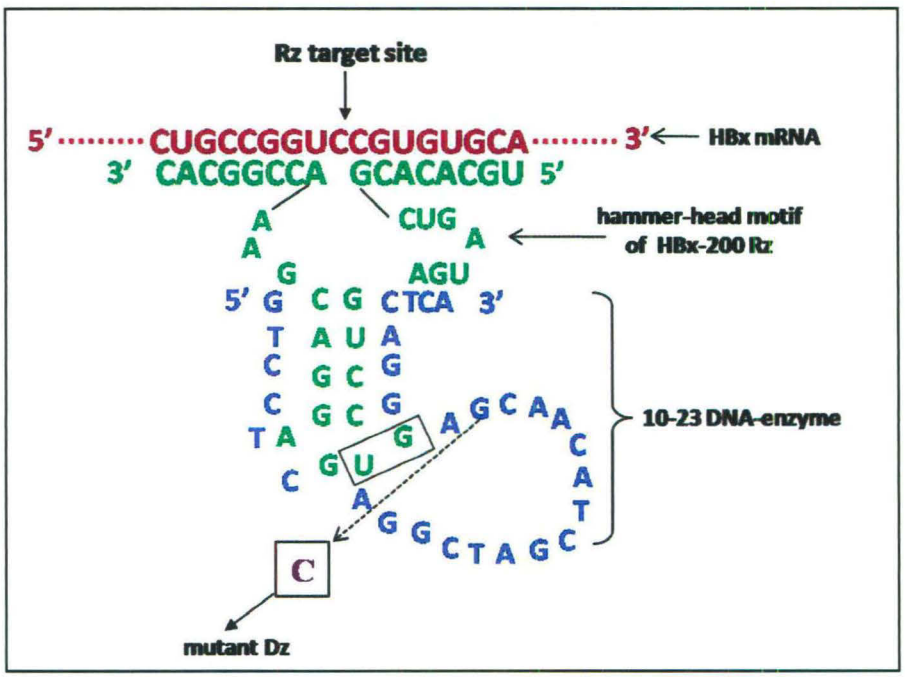

Figure 3.2: Designing of 10-23 Dz against hammerhead Rz

Sequence-specific cleavage of Rz-200-target RNA with 10-23 DNA-enzyme

Uniformly labeled Rz RNA transcript is mixed with equimolar amounts (50 pmoles each) of cold Dz in 10µl reaction buffer containing 50mM Tris-HCl, pH 7.5, in the presence of 10mM $MgCl_2$ (standard conditions). The cleavage reactions were carried out for 1 h at 37^{0}C and the cleaved products were resolved by electrophoresis on a 6% polyacrylamide- 7M urea gel as described in methods and materials. In this case, after mixing equimolar amounts, the reaction mixture was briefly heated at 55^{0}C for 2 min before proceeding further.

Effect of 10-23 Dz on the intracellular expression of Rz-200

1X10^6 HEK 293 cells were seeded 24 hrs prior to transfection in a 6 well plate and grown to 80% confluency. 1µg of plasmid pcDNA-Rz-200 was then either transfected alone or in combination with 10-23 Dz (or mutant 10-23 Dz), using lipofectin, as per the instructions of the manufacturers and incubated for 48 hrs at 37^{0}C in a 5% CO_2 incubator. Total RNA was isolated using trizol, as described in methods and materials and 1µg of total RNA was used to carry out reverse-transcriptase PCR, using primers specific for Rz-200. The sequence of the primers used:

Forward primer:

5′ GAT CAA GCT TTG CAC ACG CTG ATG 3′

Reverse primer:

5′ GAT CGG ATC CCT GCC GGT TTC G 3′

Intracellular modulation of expression of cis-HBx-Rz-200 by 10-23 Dz

Mammalian liver specific cells (Hep G2) were grown to 80% confluency using DMEM+10% FBS in a 6 well plate. They were transfected with plasmid pcDNA-HBx-Rz-200, that transcribes simultaneously both the target RNA

i.e., HBx transcript of HBV and Rz-200 RNA, that cleaved the HBx RNA, as soon as the latter was transcribed, thus efficiently suppressing the expression of HBx gene; in the absence or presence of Dz using lipofectin (Invitrogen) as the transfection reagent. Total RNA was isolated using trizol (Invitrogen) and equivalent amounts of RNA (1μg) was used to estimate the levels of full-length HBx RNA (465 bases) by RT-PCR techniques using the primers specific for HBx gene. The sequence of primers used for HBx:

Forward primer sequence

5′ ATg gCT gCT Agg CTg TAC TgC CAA CTg gAT CCT TCg Cgg gAC g 3′

Reverse primer sequence

5′ TTA ggC AgA ggT gAA AAA gTT gCA Tgg TgC Tgg TgC gCA gAC CAA TTT g 3′

The control (509 nts long human glyceraldehydes-phospho-dehydrogenase– huGAPDH) RNA was simultaneously estimated using the following primer sets:

Forward: 5′ ACC ACC ATG GAG AAG GCT GG 3′

Reverse: 5′ CTC AGT GTA GCC CAG GAT GC 3′

Western blot analysis

Whole cell lysates were prepared from 1 x 10^6 cells 48 hrs after transfection as described in methods and materials using 1% triton X-100 and estimated by BCA reagent. Equal concentration of cell lysates were loaded and resolved on 15% PAGE and transferred to nitrocellulose membrane. The membrane was then subjected to Western blot analysis using mouse generated HBx-specific monoclonal antibody (B/8/2/8) as the primary

antibody (kind gift from Dr. Vijay Kumar, ICGEB, New Delhi) and HRP-conjugated goat anti Ig G as secondary antibody. The expression of the X-protein was visualized using by DAB (Sigma).

RESULTS

Identification and characterization of 10-23 DNA-enzyme that cleaves hammer head Rz-200 in the conserved catalytic motif

The cleavage potential of 10-23 Dz was used to modulate the activity of hammerhead Rz (Rz-200). For this purpose, **'GU'** di-nucleotide (boxed) located at the single stranded-loop region of the hammerhead catalytic motif of the Rz (Figure 3.2) was targeted. ^{32}P labeled Rz-200 RNA (58 nts long) was prepared by *in vitro* transcription as described earlier. When equimolar amounts (50 pmoles each) of 58 nts long labeled Rz (substrate RNA) (Figure 3.3, panel A, lane 1) was subjected to cleavage by Dz at 37^0C, two cleaved RNA fragments of equal size (29 nts long) were observed (lane 2). When Rz-RNA and Dz mixture was subjected to brief denaturation (heated at 55^0C for 2 minutes) prior to cleavage, increased amounts of cleaved products were observed (lane 3). We conclude that 10-23 catalytic motif containing Dz possesses sequence specific cleavage activity against Rz-200.

The effect of increasing amounts of $MgCl_2$ in the cleavage buffer was then studied and the results are shown in Figure 3.3, panel B. Lane 1 is control Rz-200 transcript. In the presence of increasing amounts of $MgCl_2$ (lane 2: 2mM; lane 3: 5mM; lane 4: 10mM; and lane 5: 100mM), dose dependent increase in the 29 nts long cleaved RNA, was observed as judged by the intensity of the 29 nts long cleaved fragment.

Mutant 10-23 Dz failed to cleave Rz RNA. Wild-type Dz (Figure 3.3, panel C, lane 1) and mutant Dz (lane 2) were allowed to react with Rz-200

RNA (Figure 3.3, panel A, lane 1) under identical cleavage conditions as described above. The wild-type Dz cleaved the target RNA effectively but this activity was completely abolished with mutant Dz.

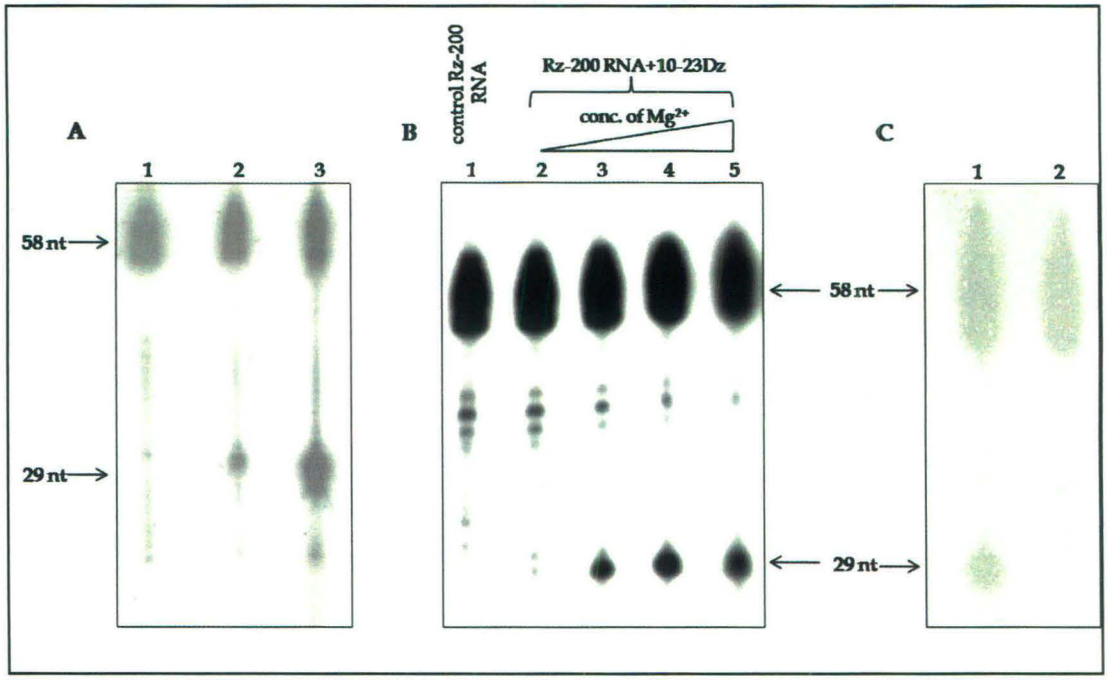

Figure 3.3: *In vitro* analysis of 10-23 Dz against Rz

Effect of 10-23 Dz on the intracellular expression of Rz-200

The bio-efficacy of the wild type 10-23 Dz and its mutant form on the intracellular expression of Rz-200 was then checked by transient transfections in HEK 293 cell lines. Cells were transfected either with plasmid pcDNA-Rz-200 alone (2 µg) or with wt or mutant 10-23 Dzs (2 µg) and total RNA was isolated using trizol and target RNA and control RNA were estimated simultaneously by RT-PCR technique (Figure 3.4). When wild type 10-23 Dz was co-transfected with Rz coding plasmid (panel A, lane 3), there was more than 60% reduction in the expression of Rz-200 as compared to the cells that received only pcDNA-Rz-200 (lane 4) or when mutant 10-23 was co-

transfected (lane 2). The expression of housekeeping gene (huGAPDH) in all the corresponding lanes remained essentially unchanged. We conclude that wild type 10-23 Dz was effective in reducing the intracellular expression of the target RNA, i.e., of Rz-200.

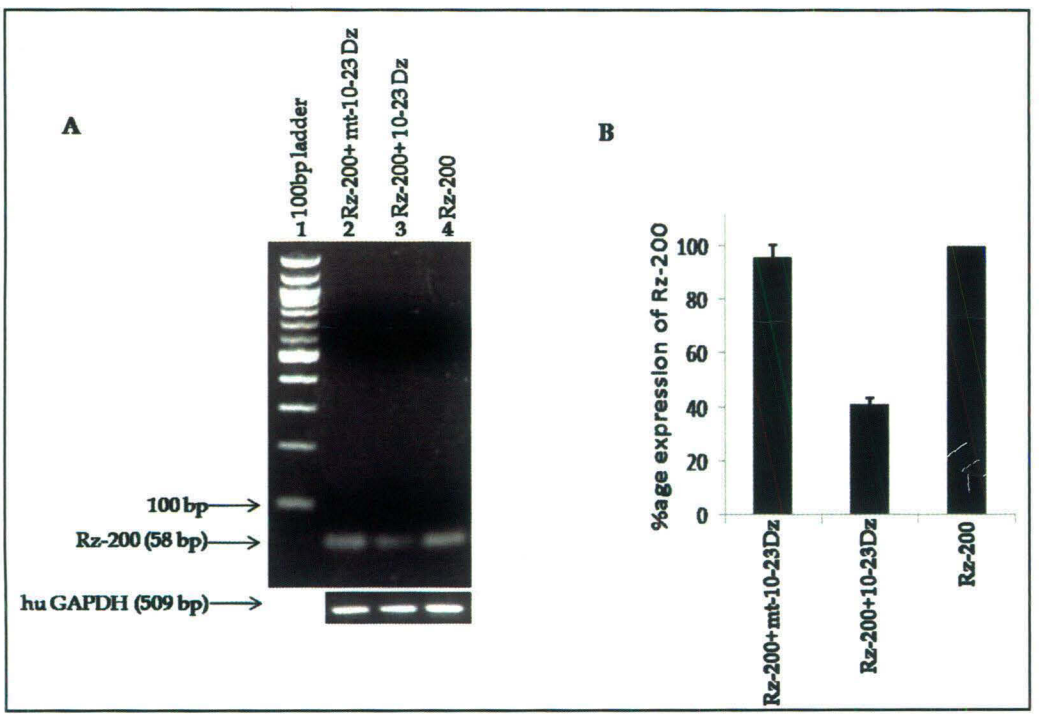

Figure 3.4: RT-PCR analysis for intracellular expression of Rz-200

Modulation of intracellular activity of cis-acting Rz-200 with 10-23 DNA-enzyme:

RT-PCR for estimating HBx target RNA

Earlier study by Yen et al, 2004, suggested that Rz self-cleavage could efficiently occur in a variety of mammalian cells including few primary cells. Transient transfection studies were carried out by co-transfecting *cis*-Rz-200 construct with increasing amounts of 10-23 Dz in Hep G2 cells (1 $X10^6$ cells in a 6 well plate) for 48 hours and analyzed simultaneously for the amount of

HBx specific RNA by RT-PCR. Hep G2 cells, when transfected with 1µg of pSG5-HBx (plasmid that encodes HBx) showed prominent band specific for HBx RNA (Figure 3.5A, lane 6 which served as positive control). The level of expression of HBx was lower when 1 µg *cis*-construct plasmid was transfected (Figure 3.5A, lane 2). When the same amount of *cis*-construct was co-transfected in 1:1 ratio (1µg each) with Dz, almost 1.5 fold more HBx RNA was observed (lane 3). When increased amounts of Dz (1.5µg in lane 4; 2µg in lane 5) were co-transfected along with the same amount of *c*is-construct (1µg), increased amounts of HBx RNA was observed. Approximately 4 fold more HBx RNA was observed in lane 5 compared to lane 2. This is expected as the Dz cleaved the Rz portion of the *cis*-construct (that consists of Rz and substrate) in a dose-dependent manner leaving the substrate intact. This effect is specific because the levels of the control RNA (huGADPH) remained the same in all the corresponding lanes. Lane 7 is a transfection control i.e., transfection with pcDNA3 alone and lane 1 represents negative control in which PCR was performed in the absence of RT. We conclude that by using Dz that specifically cleaved the Rz in either *trans* or *cis*-construct; it is possible to modulate the expression of target gene in a Dz-dose dependent manner.

Western Blot analysis

In cells that were transfected with the *cis*-construct, a reduction in the levels of HBx protein is expected due to specific cleavage of HBx RNA by Rz-200. Upon co-transfection of *cis*-construct and Dz into cells, increased amounts of X protein is expected as the Dz would specifically cleave the Rz portion of RNA. The results of such an experiment are shown in Figure 3.5, panel B. Lane 1 shows the synthesis of X protein when 2 µg of HBx encoding plasmid pSG5-HBx was transfected. Lane 2 shows reduced expression of X

protein when *cis*-construct alone was transfected (because of the action of Rz-200). A dose-dependent increase in the levels of X protein was observed when same amounts of *cis*-construct was co-transfected with increasing amounts of Dz (lane 3- 2 µg, lane 4- 3 µg). We conclude from this study that intracellular self-cleavage of Rz could be controlled by using Dz. Equivalent amount of whole cell lysate was loaded in each lane. Cell lysates were simultaneously tested for the extent of control protein (actin).

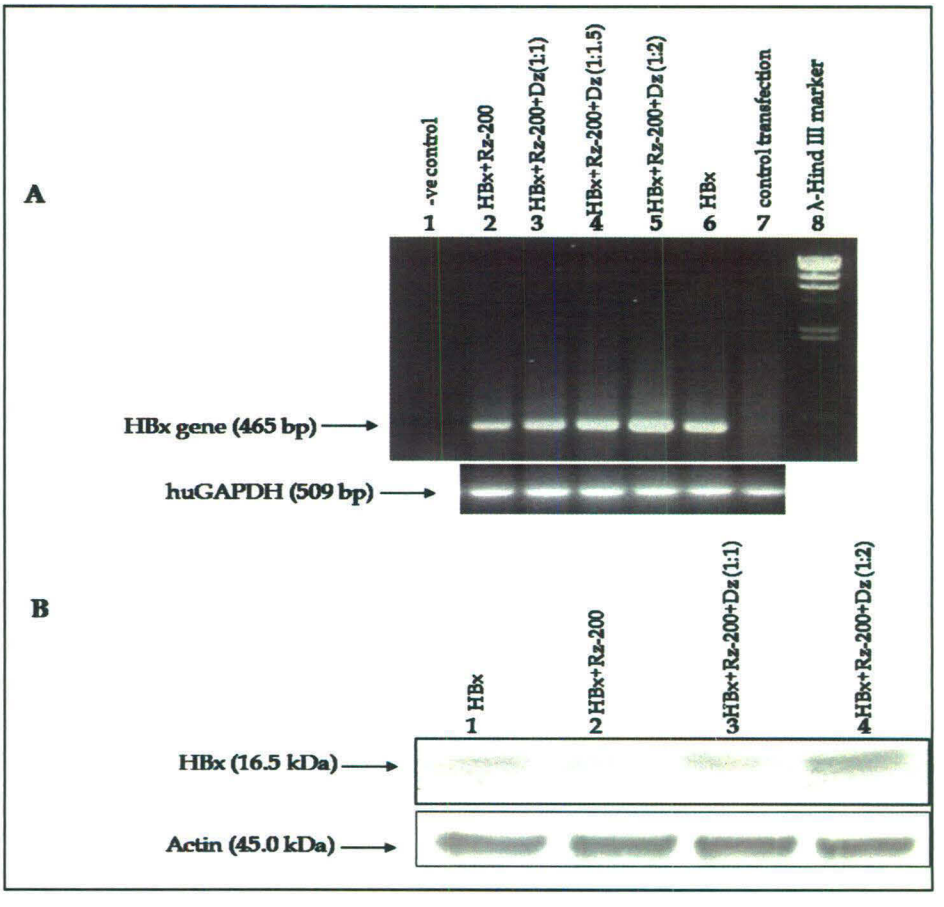

Figure 3.5: Modulation of intracellular effect of Rz-200 on its target HBx by 10-23 Dz

DISCUSSION

The study reported here provides an important 'proof of principle' for mammalian gene regulation approaches that exploits simultaneously the sequence-specific cleavage potentials of catalytic RNA and catalytic DNA molecules that can be designed to cleave any target RNA. Since, 10-23 Dz is targeted against the conserved catalytic portion of hammerhead Rz, it is likely to be effective against any hammerhead Rzs with varying target specificity. Since Rzs and Dzs possess potential to cleave the target RNA intracellularly, the combinations of these two approaches could be exploited to regulate the mammalian gene expression in a controlled manner. As compared to other commonly used approaches for modulating gene expression, the Rz-Dz based system described here is unique in several ways and may be ideal for many experimental applications.

The Dz cleaved the target Rz RNA not only in *in vitro* reaction buffer conditions but also showed effective reduction in the intracellular expression of Rz-200, when the two were co-transfected in HEK 293 cells. Also this Dz has shown potent effect on the suppression of activity of the *cis* configured Rz-200. The *cis*-acting ribozymes cleave their target sequence rapidly, almost concomitantly with the generation of cleaved products. Dzs are ideal candidates that can rival Rzs both in terms of their specificity and catalytic efficiency. Thus attenuation of Rz activity was shown by Dz in a dose dependent manner.

Therefore, a novel combination of transposable regulatory system that could be engineered for any target gene has been designed. If specific Rz sequences are embedded close to a target gene, it should be possible to monitor the function during different developmental stages in specialized tissues by supplying measured amounts of DNA-enzymes. Since the cleavage of the target RNA in *cis*- constructs is almost concurrent with the

transcription reaction, Dzs have to hybridize as soon as the Rz sequence is transcribed for optimal effects. Alternatively the transcription unit consisting of substrate and Rz could be placed under an inducible promoter and Dz can then be applied after the promoter is activated (Habu et al, 2001).

The ability to manipulate gene expression could be of great experimental and therapeutic importance as well. In summary, this study has shown that Dz with a 10-23 catalytic motif targeted against the catalytic region of a hammerhead Rz could be exploited very efficiently in modulating *in vitro* and intracellular target gene expression.

CHAPTER IV

Evaluation of Antisense oligodeoxynucleotides and 10-23 Dz mediated Cleavage efficiency of hammerhead Rz and 10-23 Dz

Ribozymes are catalytic RNA molecules that possess the ability to cleave the target RNA in a sequence-specific manner and control splicing reactions (Cech, 1987; Haseloff and Gerlach, 1998). In nature; these are the only nucleic acids that have enzymatic properties. Although several types of catalytic motifs have been described, the hammerhead Rz being the smallest and with minimal target sequence requirement, has been exploited extensively for variety of purposes including therapeutic applications (Akkina et al, 2003; Kurreck, 2003; Banerjea et al, 2004; Schubert and Kurreck, 2006). The target sequence for cleavage by hammerhead Rz usually consists of nucleotide sequence "NUX", where N is any base and X can be C, U or A, but not G. Usually, GUC is the most favorable cleavage site for hammerhead Rz. By cleavage at "NUX" sequence, Rz can significantly alter the structure of the target mRNA.

On the other hand, DNA-enzymes, as originally described by Santoro and Joyce, 1997), are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner (for reviews see references Banerjea et al, 2004; Joyce, 2004; Silverman, 2005). Over the years several kinds of Dzs with unique catalytic motifs have been described but Dz possessing the 10-23 catalytic motif has been exploited extensively by several investigators (Banerjea et al, 2004; Baum and Silverman, 2008). The structure of 10-23 Dz is made up of a catalytically active core of 15 near-invariant nucleotides, flanked by substrate binding arms that can be changed to base pair with the sequence of the desired target RNA. 10-23 Dz is able to cleave the target RNA sequence at a phosphodiester bond between a purine and a pyrimidine residue (5'AU 3' most efficiently cleaved) under *in vitro* and *in vivo* conditions. A single nucleotide change in

the catalytic motif diminishes its sequence specific cleavage activity; G14C completely abolishes the catalytic cleavage activity of the Dz.

The ability of 10-23 Dz to cleave any target RNA has been exploited for knocking down gene expression against variety of target genes including HIV-1 genes (Banerjea et al, 2004; Chakraborti and Banerjea, 2003; Dash and Banerjea, 2004) with varying outcomes. In some instances very efficient intracellular gene inhibition was observed with 10-23 Dz (Ackerman et al, 2005). It has been reported earlier that 10-23 Dz was more effective in cleaving the full-length CCR5 (HIV-1 coreceptor) RNA compared to a hammerhead Rz (Goila and Banerjea, 1998). Kurreck and others had carried out comparative study between hammerhead Rzs and Dzs that were targeted against the same sequences and concluded that some sequences were cleaved better by Rzs and some by Dzs (Kurreck et al, 2002).

There are several other nucleic-acid based approaches that have been used for specific inhibition of target genes. The catalytic Rzs, aptamers, antisense DNA or RNA or small interfering RNAs (siRNAs) have been used for inhibiting the expression of foreign genes including HIV-1 replication (Akkina et al, 2003). Physiologically relevant RNAs are usually long and consist of multiple stem-loop structures and up to 90% putatitive cleavage sites were earlier shown to be in inaccessible to either Rz or Dz mediated cleavage (Cairns et al, 1999). Earlier, unwinding activity of an RNA helicase was used to increase the cleavage potential of Rzs (Wadhwa et al, 2003). Collectively, all these studies suggest that not all the target sites are available for cleavage by a single kind of catalytic nucleic-acid molecule most probably because the secondary and tertiary structures in the target RNA prevent optimal Watson-Crick base pairing with Rzs or Dzs. Introduction of locked nucleic-acids in antisense design resulted in enhancing its stability (Grunweller et al, 2003) and when incorporated in the substrate recognition arms of a Dz, it improved the catalytic efficiency (Schubert et al, 2004).

Earlier few nucleic-acid based approaches were used to modulate the cleavage activity of the Dzs and Rzs with moderate success but none of them were tested for bio-efficacy. Oligonucleotide effectors (regulators) that bind to both enzyme and substrate were used to regulate the catalytic activity of Rzs & Dzs (Wang and Sen, 2001; Wang et al, 2002). The efficiency of these regulators depended upon the successful formation of a branched three way junction, which could be a potential problem if the same target region has extensive secondary structures. Whether oligodeoxynucleotides (ODNs) that hybridize specifically to the target RNA alone could modulate *in vitro* and *in vivo* the catalytic activity of Rzs or Dzs have not been earlier attempted. Goodchild (1992) reported enhancement of ribozyme catalytic activity by a contiguous oligonucleotide (facilitator) and by 2'-O-methylation. Oligonucleotide facilitators have been used to either enhance (Horn and Schwenzer, 1999) or even inhibit catalytic activity of Rzs (Janowsky and Schwenzer, 1996). Oligonucleotide facilitators have earlier been used to enhance hammerhead ribozyme mediated cleavage of long RNA substrates with multiple-turnover activity (Jankowsky and Schwenzer, 1998). The main reason of this effect was enhancement of association step due to RNA unfolding (or melting) mediated by the facilitators. Although all the above approaches resulted in enhanced cleavage of the target RNA under *in vitro* conditions, it was not known if combinations of Rzs or Dzs with facilitators could be exploited to down regulate intracellular levels of the target RNAs.

The present study deals with the identification and characterization of those ODNs and Dzs that significantly enhanced the Rz and Dz mediated cleavage of full-length HBx RNA of hepatitis B virus (HBV) on one hand and inhibit intracellular expression of the target RNA in a liver specific cell line, HepG2 on the other. The Hepatitis B virus X protein (HBx) acts as a powerful transactivator for several genes including HBV genes and is known to be

strongly associated with hepatocellular carcinoma (Robinson, 1994; Gomez-Gonzalo et al, 2001; Seeger et al, 2007). These results are potentially important for therapeutic purposes for the treatment of HBV infections against which there are no effective antiviral treatment available.

EXPERIMENTAL PROCEDURES

Cloning of HBx gene and in vitro transcription

The entire HBx gene of HBV is placed in plasmid pSG5 (Stratagene, Texas, U.S.A.) under SV40 and T7 promoters (Figure 4.1 A). The former promoter is used for intracellular expression and the latter was used for obtaining *in vitro* transcripts using T7 RNA Polymerase (Banerjea and Joklik, 1990). After linearization with *Bgl II* at the 3'-end, HBx RNA of length 465 nts was transcribed using T7 RNA Polymersae.

Construction and cloning of HBx-Rz-170 targeted against X gene

Rz-170 has been constructed and described earlier by Goila and Banerjea, 2004. It possessed the hammerhead motif and was targeted against the **'GUC'** sequence. *Eco R*1 and *Bam H*1 restriction sites were engineered at the ends of the hybridizing arms of the Rz which facilitated cloning it into pcDNA3 expression vector such that the Rz was placed under T7 and CMV promoter.

In vitro cleavage of target RNA with Rz-170

In vitro transcription of the linearized plasmid DNA was carried out in the presence of labeled P^{32} UTP using T7 RNA polymerase. The cleavage reaction was initiated by adding equimolar amounts (100 pmoles each) of the labeled target RNA and unlabeled Rz RNA in a reaction buffer containing 50mM Tris-HCl, pH 7.5, in a volume of 10 µl and the cleavage reaction was

initiated by adding $MgCl_2$ (final concentration 10mM) at 37°C for 2 hours. The cleaved RNA fragments were resolved on 7M urea-6% PAGE and analyzed by autoradiography.

Antisense oligodeoxynucleotides and DNA-enzymes

All the 20 nts long antisense oligonucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys. The conserved 15 nts long (5′ GGCTAGCTACAACGA 3′) 10-23 catalytic motif was flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. The construction of Dz-237 that cleaved the X RNA specifically into two fragments has been earlier described in detail (Goila and Banerjea, 2001) (Figure 4.1 C). Two 10-23 Dzs, namely Dz-155, and the wild-type and mutant versions of Dz-192 were also assembled using 10-23 catalytic motif (Figure 4.2B). The mutant Dz-192 possessed a single nucleotide substitution (G14 to C) in the 10-23 catalytic motif, shown in bold and underlined, in the table below.

Table 4.1: The sequences of various ODNs and Dzs

Oligo	Sequence 5′-------- 3′
ODN 1	CCA CGG GGC GCA CCT CTC TT
ODN 2	GCC CTT TCA TCT GCC GGT CC
ODN 3	CAG ACG GAG AAG GGG ACG AG
Dz-155	AAG AGA GGG GCT AGC TAC AAC GAG CGC CCC
Dz-192	CGG CAG AGG CTA GCT ACA ACG AGA AGG C
mt-Dz-192	CGG CAG AGG CTA GCT ACA AC**C** AGA AGG C

10-23 Dz mediated cleavage of HBx mRNA

Equivalent amounts of unlabeled Dz and labeled substrate RNA (100 pmoles each) were allowed to interact in a final volume of 10 µl in a buffer

containing 50mM Tris-HCl, pH 7.5 in presence of 10mM $MgCl_2$ (standard conditions) as described in methods and materials, for 2 hours at 37°C in the absence or presence of indicated amounts of ODNs. The cleaved products were resolved on a 7M urea-6% PAGE and analyzed by autoradiography.

Intracellular inhibition of the expression of target RNA by Rz-170 and ODNs

The intracellular decrease in HBx-specific RNA after co-transfection in Hep G2 cells with substrate encoding plasmid (pSG5-HBx) and Rz-170 in the presence or absence of ODNs were monitored by RT-PCR based assays. Several dilutions were initially made to determine the linear range for PCR-amplified products. Total RNA was isolated using trizol reagent (Invitrogen) and 1µg of it was used for estimating the levels of full-length HBx RNA using HBx-1 and HBx-2 primers. Another set of reaction was put up for estimating the levels of the house keeping gene, human glyceraldehyde-phosphodehydrogenase (huGAPDH) that was used as the loading control.

The following primers were used for estimating the intracellular levels of full-length HBx RNA.

(1) Forward (HBx-1):

5′ TTAGGCAGAGGTGAAAAAGTTGCATGGTGCTGG 3′

(2) Reverse (HBx-2):

5′ TGGCTGCTAGGCTGTACTGCCAACTGGATCCTTCG 3′

Western Blot analysis to see the effect of ODNs or Dzs along with Rz-170

1 x 10^6 Hep G2 cells were grown to 80% confluency in a six well plate. They were transfected with either Rz-170 (1µg) alone or in the presence of increasing concentrations of ODN 1 and 2 in a final volume of 1ml for 48

hours along with 1μg of HBx gene encoding plasmid, pSG5-HBx, using lipofectin (Invitrogen) as the trasfection reagent. Cell lysates were prepared and equal amount of protein was loaded in each lane, after estimating the protein content by BCA reagent (Pierce). The protein was then transferred on to a nitrocellulose membrane, as described in methodology. Rabbit raised polyclonal antibody to HBx (Biovendor, NJ, USA) was used as a primary antibody in 1:500 dilution in PBS, pH 7.2 containing 0.1% Tween-20. The amount of pSG5-HBx plasmid was kept constant at 1μg in each experimental lane. The absolute amounts of DNA introduced in each well was made equivalent by adding unrelated DNAs.

Inhibition of X protein mediated HIV-1 LTR activation with Rz and ODNs or Dzs

HEK 293 or Hep G2 cells were seeded in a 24 well plate, 24hrs prior to transfection. The cells were then co-transfected with 100 ng of pBS-LTR-B-Luciferase plasmid (henceforth referred to as pLTR-B–the Luciferase reporter gene is placed downstream of the HIV-1 LTR-B promoter) (obtained from AIDS Research and Reference Reagent Program of NIH, MD, USA) in the presence of indicated amounts of ODNs or Dzs in wells that received 100 ng of pSG5-HBx plasmid DNA and constant amount of Rz-170 encoding DNA (100 ng of Rz-170-pcDNA). Lipofectin (Invitrogen) was used as the transfection reagent and cell lysates were prepared 24 hours later using 1X reporter lysis buffer (Promega). The extent of Luciferase activity was determined according to the manufacturer's instructions (Promega).

RESULTS

In vitro synthesis of full-length HBx RNA by T7 RNA polymerase

Plasmid pSG5-HBx (Figure 4.1 A) was linearized with *Bgl* II restriction enzyme and when subjected to *in vitro* transcription using T7 RNA polymerase, a 465nt long full-length HBx transcript is expected using the Riboprobe transcription kit (Promega) in the presence of ^{32}P UTP. Transcripts were subjected to gel analysis and dried gels were exposed to X-ray.

Rz-170 mediated cleavage of HBx RNA in the presence of upstream and downstream antisense oligodeoxynucleotides

Entire sequence of the Rz-170 along with the target RNA is shown in Figure 4.1 B. The target sequence was **'GUC'** located at nucleotide position 170 in the HBx gene. The 22 nts long hammerhead motif was flanked with eight nucleotides long hybridizing arms on either side of the target sequence that were made complementary to the target RNA to provide specificity. When an equimolar amount (100 pmoles each) of Rz-170 was used for cleaving a full-length HBx RNA (465 nts), specific RNA fragments (170 and 295 bases long) are expected (Figure 4.1 B).

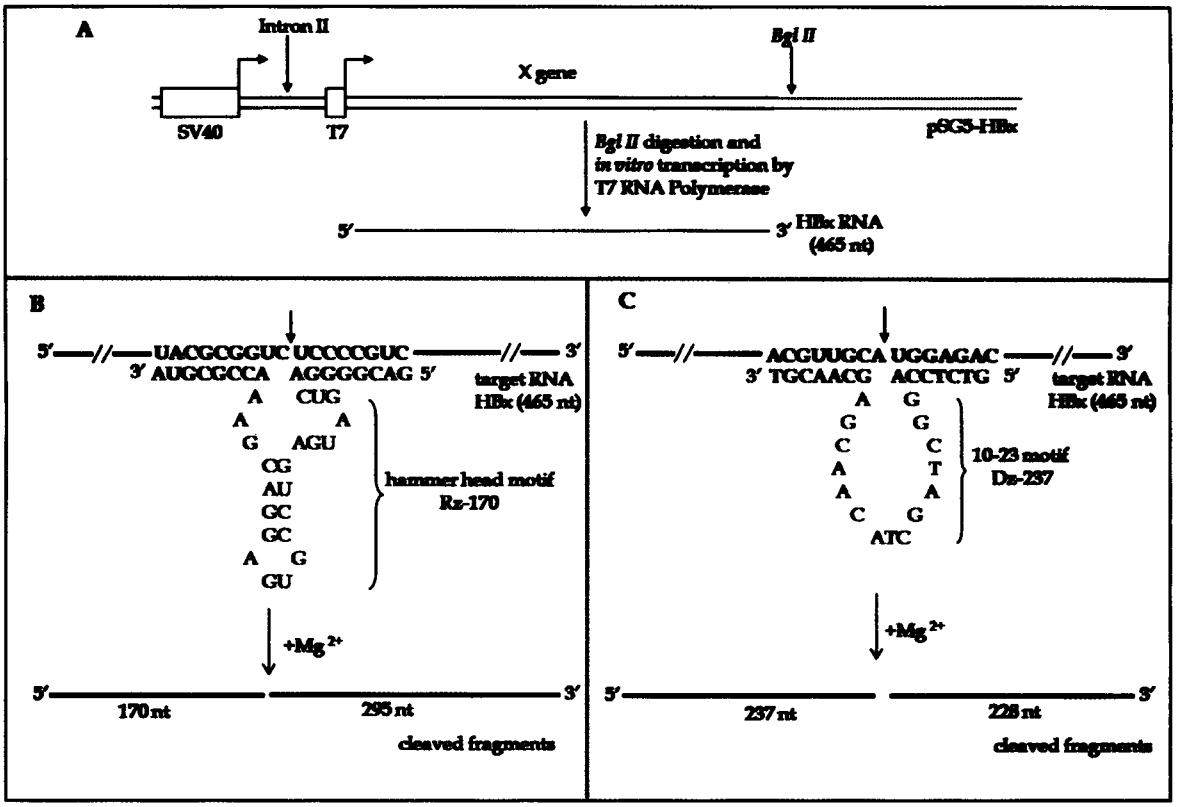

Figure 4.1: Cloning and cleavage pattern of HBx RNA by Rz-170 and Dz-237

In order to increase the Rz-170 mediated cleaved products, 3 antisense ODNs, each 20 nts long, were synthesized. Two of these ODNs (ODN 1 and 2) were designed to hybridize immediately adjacent to the two hybridizing arms of the hammerhead Rz-170 (Figure 4.2A). Another ODN (ODN 3) was designed that was 33 nts upstream from the Rz-170 cleavage site (Figure 4.2, panel A). In the similar manner, two 10-23 catalytic motif containing Dzs were synthesized immediately upstream and downstream of the Rz-170. Exact sequence of these Dzs along with the mutant (disabled Dz-192) is shown in Figure 4.2, panel B.

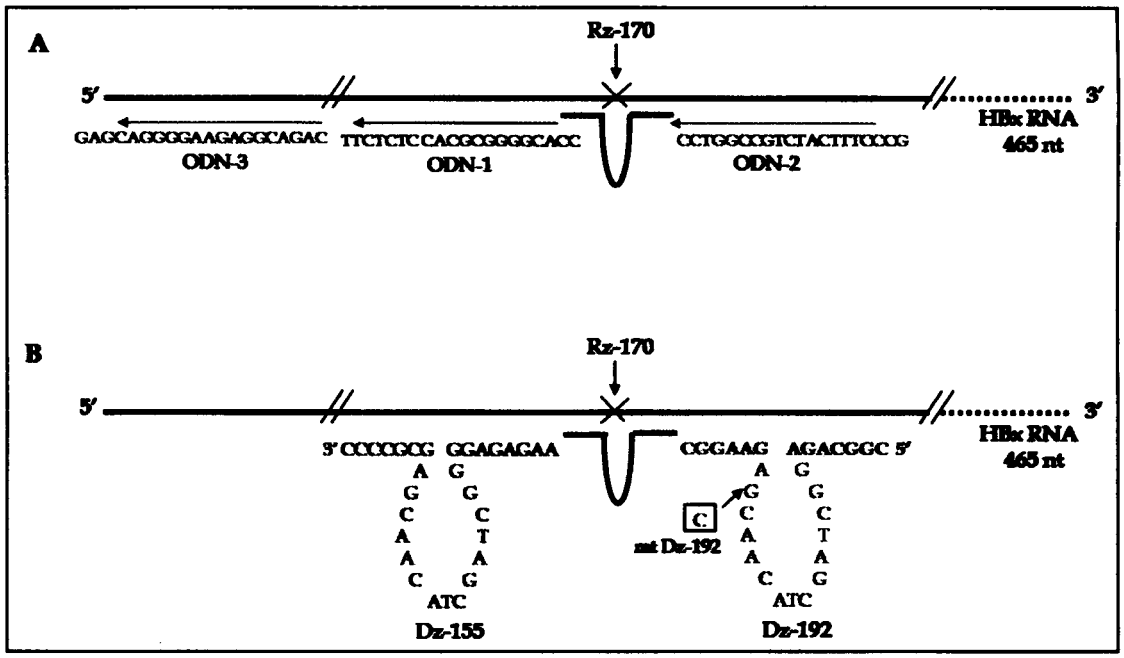

Figure 4.2: Schematic representation of ODNs and 10-23Dzs, designed adjacent to Rz-170

Rz-170 mediated cleavage reaction was performed in the presence of varying amounts of each of the two ODNs (ODNs 1 and 2) individually (10 pmoles, 50 pmoles and 100 pmoles) and the results are shown in Figure 4.3. Lane 1 shows the synthesis of uniformly labeled full-length (465 nts long) HBx RNA. When equimolar amounts (100 pmoles each) of Rz and labeled substrate RNA were used for cleavage under standard conditions two specifically cleaved fragments (295 and 170nt long RNA fragments) were obtained (lane 2). When the same cleavage reaction was carried out in the presence of 10 pmoles (lane 3); 50 pmoles (lane 4); and 100 pmoles (lane 5) of ODN 1, a dose-dependent decrease in the input substrate RNA was observed. Approximately 4-fold decrease was observed in the input substrate RNA when lane 5 was compared with lane 2. In the similar manner, the ODN 2 also caused a dose-dependent decrease in the substrate RNA when similar amounts were used (10 pmoles –lane 6; 50 pmoles –lane 7; 100 pmoles –lane 8). There was almost complete disappearance of the input substrate

RNA in lane 8. Thus it could be concluded that ODNs that hybridized immediately upstream and downstream of Rz-170 were able to significantly augment the Rz-170 mediated cleavage and that ODN 2 was more effective than ODN 1.

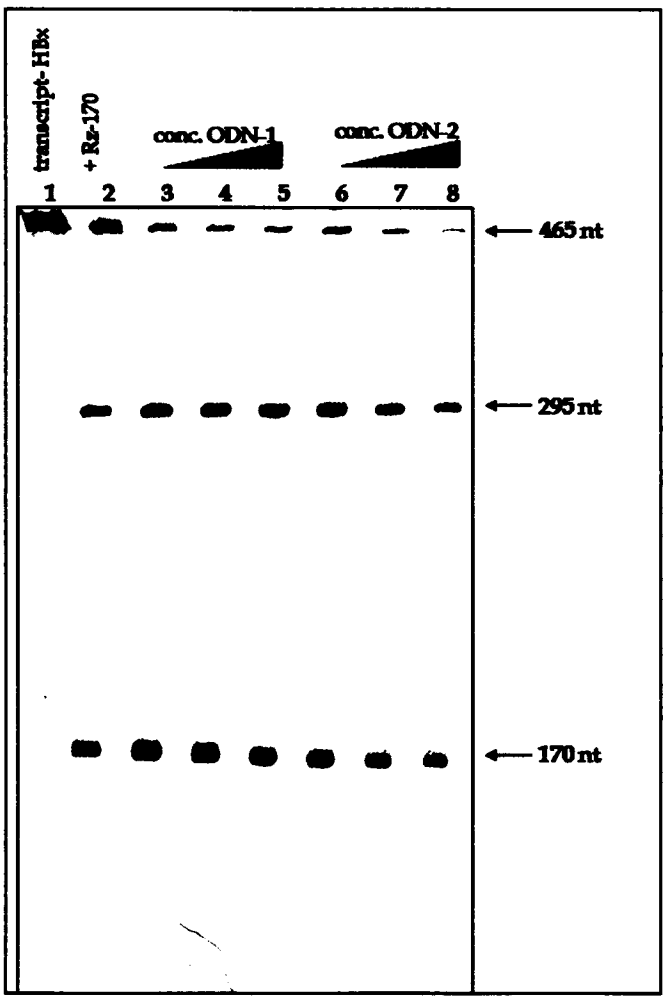

Figure 4.3: Cleavage of HBx RNA by Rz-170 in presence of ODNs 1 and 2

Antisense ODN 3 targeted to hybridize 33nt away from the Rz-170 cleavage site fails to augment its cleavage

The nature of the experiment is same as described in Figure 4.3. Briefly, Rz-170 mediated cleavage of the target RNA (Figure 4.4, lane 1) was studied

in the presence of increasing amounts of ODN 3 (Figure 4.2 A) and the results are shown in Figure 4.4. Lane 2 exhibits the cleavage obtained with equimolar amounts (100 pmoles) of labeled substrate and Rz-170. When increasing amounts of ODN 3 (10 pmoles –lane 3; 50 pmoles –lane 4; 100 pmoles –lane 5) were included in the cleavage reaction, the extent of cleavage remained unchanged. We conclude that ODN that hybridized 33 nts upstream from the Rz-170 cleavage site failed to augment Rz-170 mediated cleavage.

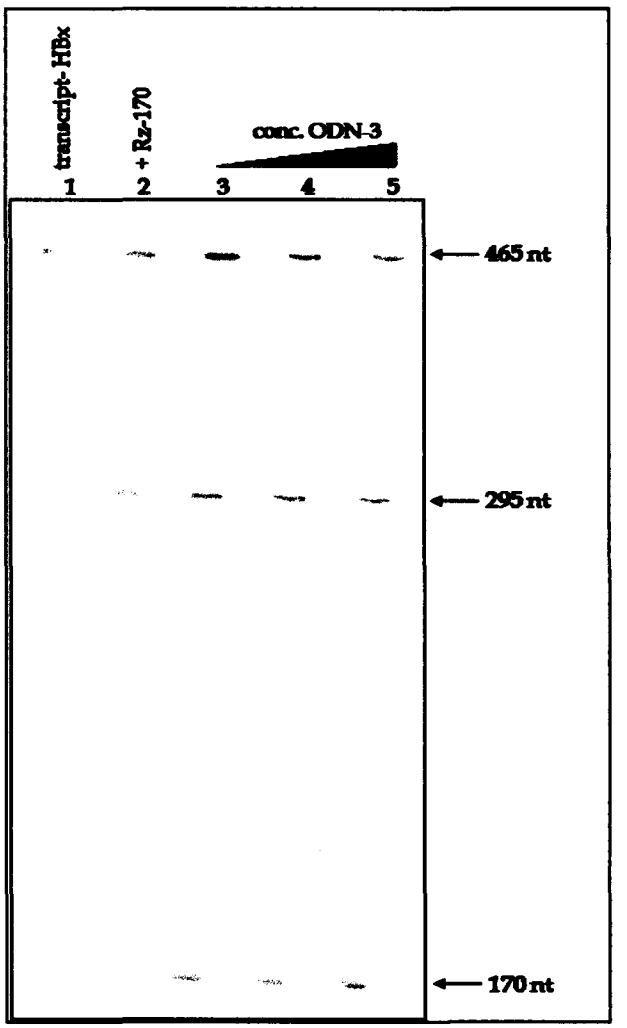

Figure 4.4: Effect of ODN 3 on cleavage activity of Rz-170

Only downstream Dz (Dz-192) significantly enhanced Rz-170 mediated cleavage

Since significant enhancement of Rz-170 mediated cleavage products by the two ODNs (1 and 2) that hybridized immediately upstream and downstream to the hybridizing arms of the Rz was observed, it would be interesting to find out if the two Dzs (Dz-155 and Dz-192) that were targeted to hybridize immediately upstream and downstream of the Rz-170 target site (Figure 4.2 B) could also enhance the Rz-170 mediated cleavage. The various cleavage products (including partially cleaved products) that are expected due to the combined actions of Rz and Dz-155; and Rz and Dz-192 are shown schematically in Figure 4.5. The reaction conditions were same as described before. In Figure 4.6, lane 1 shows the labeled full-length *in vitro* synthesized HBx RNA as described earlier. Lane 2 shows sequence-specific cleavage of the target RNA when equimolar amounts of Rz were used. In presence of increasing amounts of Dz-155 (lane 3- 10 pmoles; lane 4– 50 pmoles; lane 5– 100 pmoles), no significant reduction in the amounts of input substrate RNA was observed. On the other hand when the cleavage reaction was carried out in presence of increasing amounts of Dz-192 (lane 6– 10 pmoles; lane 7– 50 pmoles and lane 8- 100 pmoles), a dose-dependent decrease in the input substrate RNA was observed with almost complete disappearance at 50 and 100 pmoles of Dz-192. From this experiment we concluded that only the downstream Dz-192 was able to significantly enhance the Rz-170 mediated cleavage.

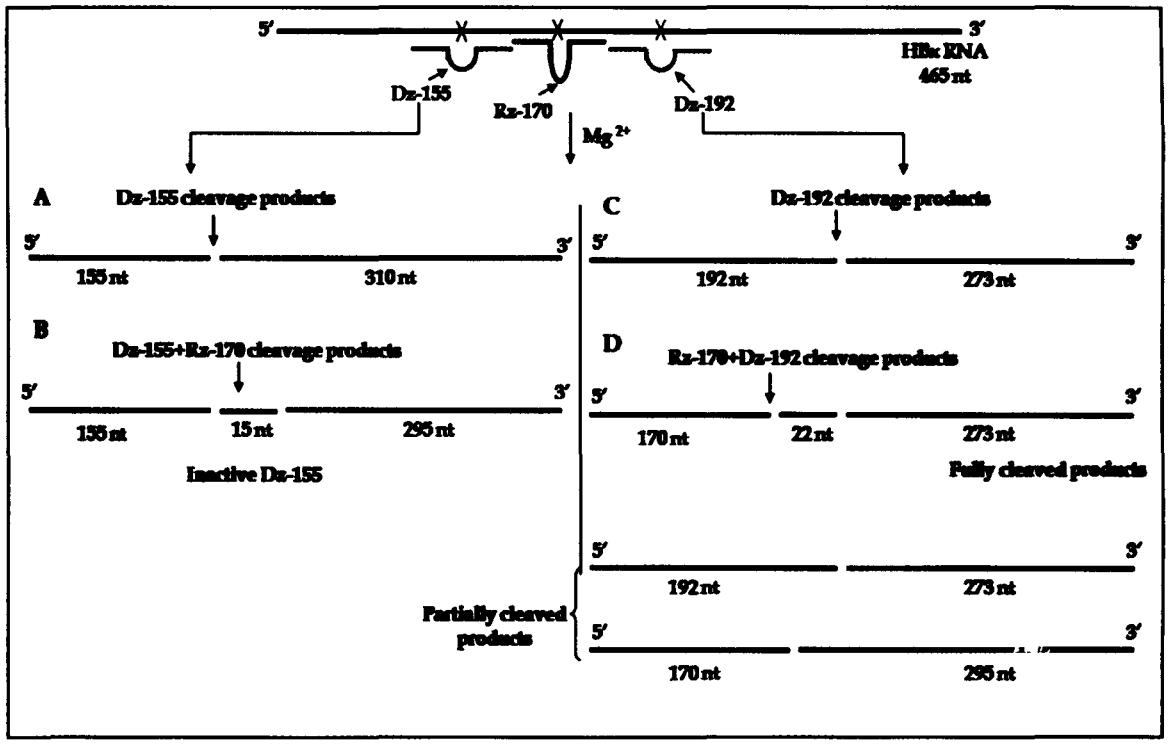

Figure 4.5: Representation of cleavage pattern of Dzs along with Rz-170

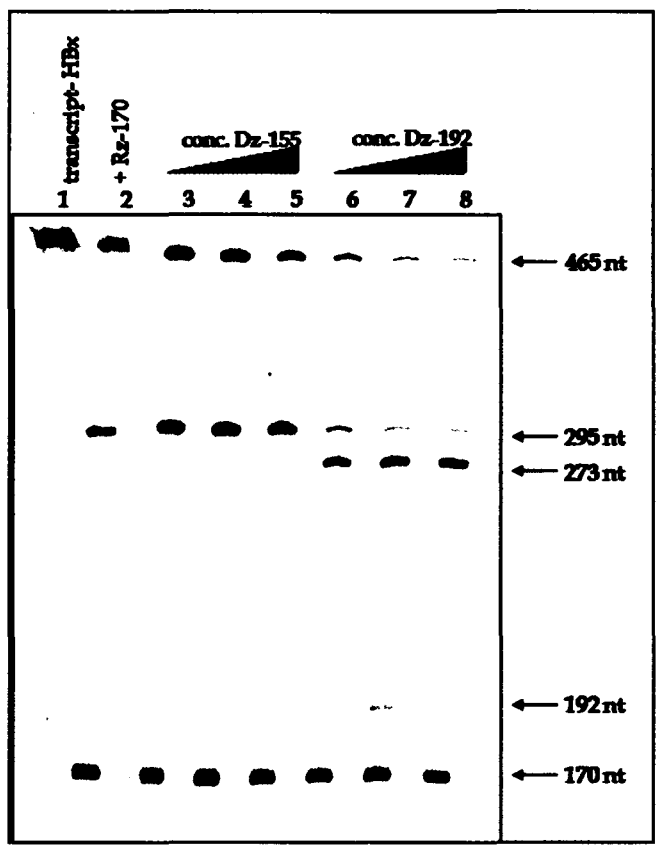

Figure 4.6: *In vitro* cleavage of HBx by Rz-170 and 10-23 Dzs

Wild-type and mutant Dz-192 augment Rz-170 mediated cleavage

The purpose of this experiment was to compare augmentation of the Rz-170 mediated cleavage in the presence of the wild-type and mutant-Dz-192 (disabled). As reported earlier, this mutant Dz (Goila and Banerjea, 2001), failed to cleave the target RNA completely and served as an important antisense control for the wild-type Dz. Exactly same amounts of wild-type (Dz-192) and mutant-Dz-192 were added to the cleavage reaction which contained equimolar amounts of Rz-170 and substrate RNA. The results of this experiment are shown in Figure 4.7, lane 1 corresponds to the HBx transcript and when cleavage reaction was carried out with Rz-170 alone (lane 2), two specific fragments were observed as described before. When the Rz-170 mediated cleavage reaction was carried out in the presence of increasing amounts of Dz-192 (lane 3- 1 pmoles; lane 4- 10 pmoles and lane 5- 100 pmoles), a dose-dependent decrease in the substrate RNA was observed along with multiple cleaved fragments. These fragments match the predicted pattern of cleavage described earlier (Figure 4.5, panels C and D). The same experiment was carried out with increasing amounts of mutant-Dz-192 under identical conditions of cleavage (lane 6- 1 pmoles; lane 7- 10 pmoles and lane 8- 100 pmoles). In this case also a dose-dependent decrease in the input substrate RNA was observed. In both cases, up to 8–10 fold decrease in the input labeled substrate RNA was observed in the presence of highest amounts of Dz used (compare the extent of uncleaved input substrate RNA in lanes 1, 5 and 8). However, as the mutant-Dz is catalytically inactive, no additional cleaved products were observed. The extent of reduction in the input RNA with either wild-type (wt) or mutant-Dz in corresponding lanes was very similar. This indicated that the reduction in the input substrate RNA is mainly due to antisense effect and not because of catalytic activity of the Dzs. We conclude that both wild-type and mutant Dz-192 increased the Rz-170 mediated cleavage products to similar extent.

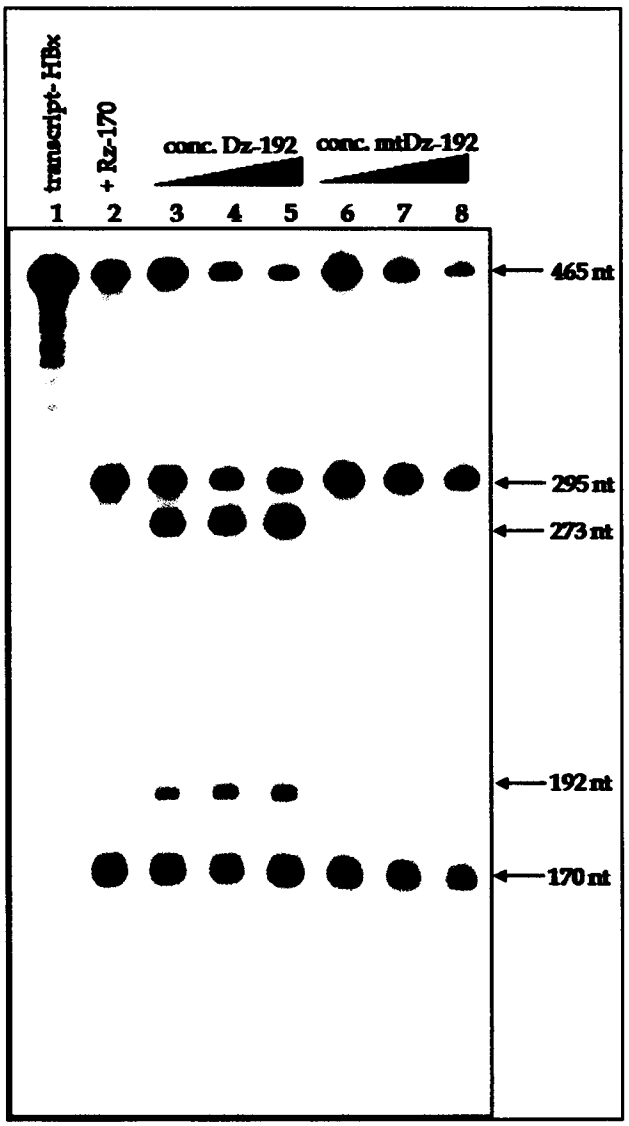

Figure 4.7: *In vitro* cleavage of HBx RNA by Rz-170 with wt-Dz-192 and mt-Dz-192

HBx-Dz-237 mediated cleavage is enhanced moderately by upstream but powerfully by downstream ODN

All the earlier experiments described above were designed to increase the cleavage of Rz-170 and the study was performed to investigate if the earlier identified Dz against HBx RNA, Dz-237 (Goila and Banerjea, 2001), could also be modulated by using 20 nts long ODNs designed to hybridize either upstream or downstream of Dz-237 (Figure 4.8). The

cleavage results of this experiment are shown in Figure 4.9. Lane 1 is the input substrate HBx RNA and lane 2 shows the Dz-237 mediated specific cleavage products (237 and 228 nts long RNA fragments). The substrate HBx RNA was incubated with Dz-237 in the presence of increasing amounts of upstream ODN (ODN 4) (lane 3: 10 pmoles; lane 4: 100 pmoles; lane 5: 200 pmoles). Two fold decrease in the amount of uncleaved substrate RNA was observed with 200 pmoles of ODN 4 (compare lane 5 with lane 2). When the same reaction was carried out in the presence of increasing amounts of downstream ODN (ODN 5) (lane 6: 10 pmoles; lane 7: 100 pmoles; and lane 8: 200 pmoles), significant reduction in the substrate RNA was observed (> 20 fold reduction) with almost complete disappearance in the presence of 200 pmoles of ODN 5, in lane 8. We could therefore conclude that downstream ODN is significantly more effective in enhancing the Dz-237 mediated cleavage reaction.

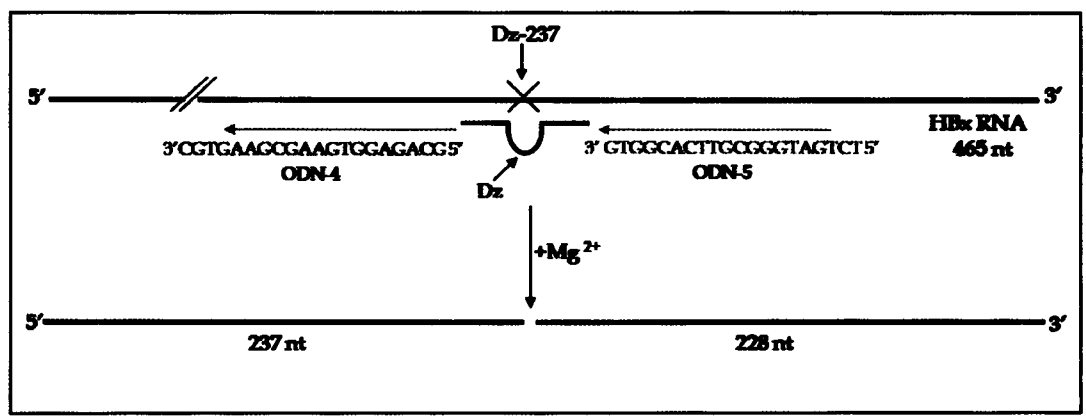

Figure 4.8: Designing of ODNs for Dz-237

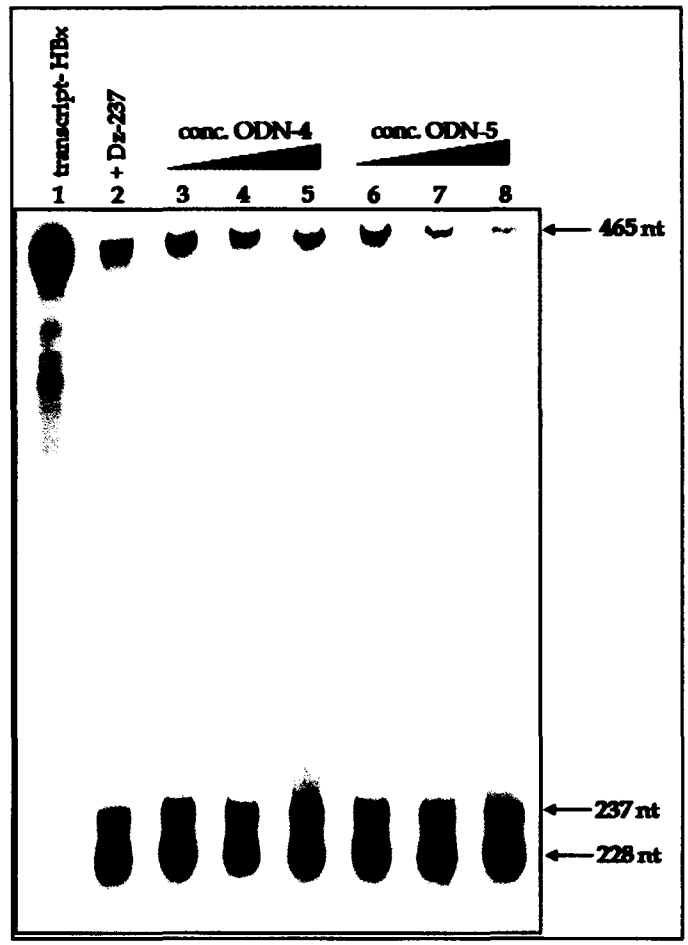

Figure 4.9: *In vitro* cleavage of HBx by Dz-237 along with ODNs 4 and 5

Rz-170 and ODNs treatment results in potent intracellular reduction of target RNA

Hep G2 cells were co-transfected with 1µg each of pSG5-HBx plasmid and Rz-170 construct for 48 hours using 10 µl of Lipofectin (Invitrogen) as the transfection reagent. This dose was predetermined to give about 10% intracellular reduction in the level of target RNA (Figure 4.10, lane 4) when compared with cells transfected with pSG5-HBx plasmid alone, along with equal amounts of unrelated Rz (lane 3). Total RNA was isolated using trizol (Invitrogen) and the target RNA (HBx) and control RNA (huGAPDH) were

estimated simultaneously by RT-PCR. When 4μg of ODN 2 was added to the Rz-170 construct and pSG5-HBx-DNA mixture (lane 5), approximately 70% reduction was observed (compare lane 5 with lane 3). When the same amount of ODN 1 was used, about 20% reduction was observed (lane 6). When the reduced amount of ODN 1 and 2 (2 μg each) were used, about 50% reduction in the target RNA was observed (compare lane 7 with lane 3). RT-PCR carried out with cells transfected with vector plasmid only (lane 2) is the control. The extent of house-keeping gene (huGAPDH) in all the corresponding lanes remained essentially unchanged. From this experiment it was concluded that Rz-170 in combination with downstream ODN was more effective in causing decrease in the levels of intracellular target RNA.

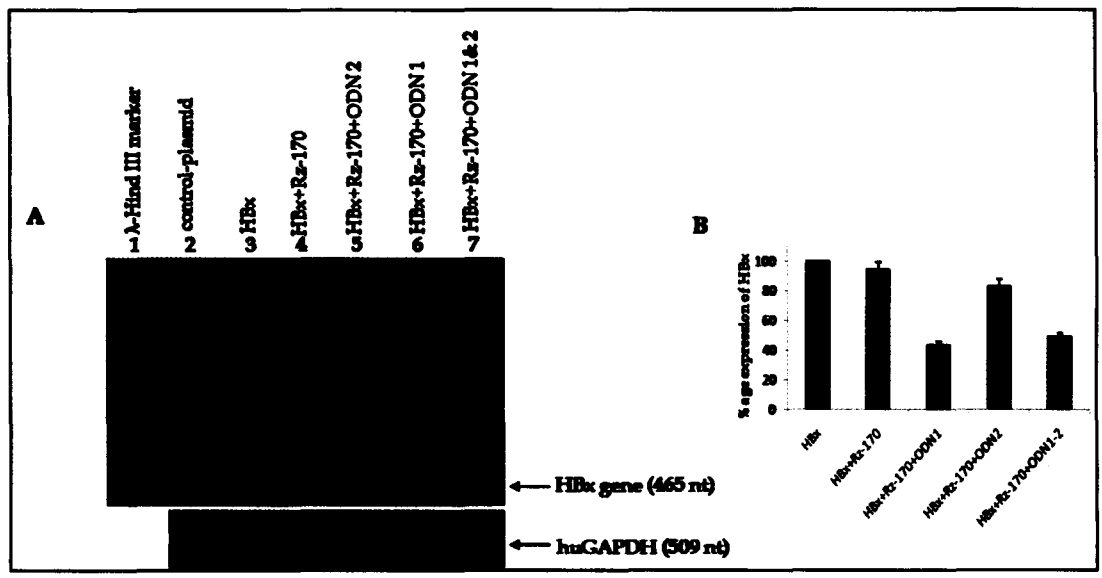

Figure 4.10: Suppression of HBx expression by Rz-170 and ODNs 1 and 2

Western blot analysis

Hep G2 cells co-transfected with Rz-170 and ODNs were processed for total protein and subjected to western blot analysis as described in methods and materials; and the results are shown in Figure 4.11. Lane 1 represents control cells transfected with pcDNA3 only. A prominent immune-reactive HBx protein was observed (lane 2) when transfected with

1µg each of pSG5-HBx plasmid DNA. About 2-fold reduction in the expression of HBx protein band was observed when cells were co-transfected with 1µg each of pSG5-HBx plasmid DNA and pcDNA-Rz-170 plasmid (lane 3). When ODN 1 and 2 (4µg in one ml) were also included, a complete knock down of the HBx protein was observed (lanes 4 and 5). Equal amount of total protein was loaded (as determined by colorimetric reaction with BCA reagent). We concluded that both the ODNs in combination with Rz-170 caused complete intracellular reduction in the levels of HBx protein.

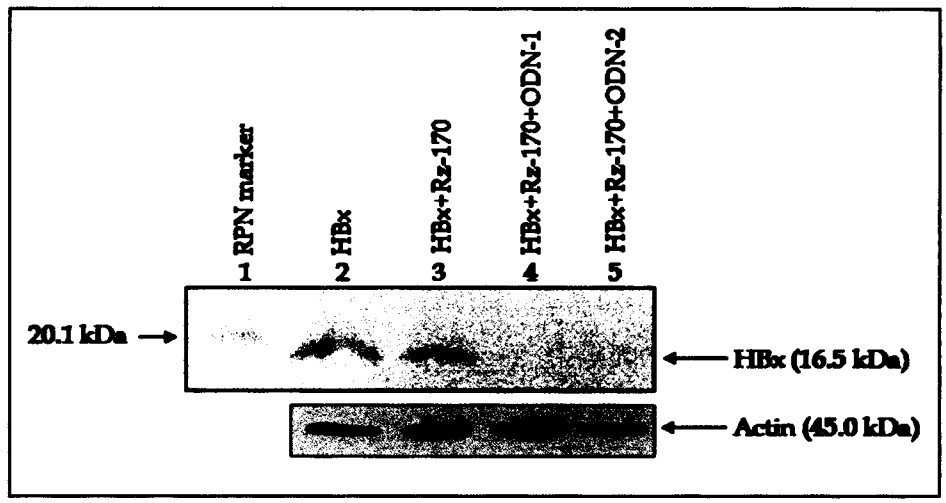

Figure 4.11: HBx protein suppression by Rz-170 along with ODNs 1 and 2

Potent knock down of HBx mediated HIV-1 LTR reporter gene activation by Rz-170 and antisense oligodeoxynucleotides (ODNs 1 and 2) or 10-23 Dz

Hep G2 cells were co-transfected with pSG5-HBx and p-LTR-B plasmid DNA (100 ng each in 200 µl for 1X 10^4 cells) along with indicated amounts of ODNs or Dz and the reporter gene activity (luciferase) (mean +/- SD) obtained from three independent experiments are shown in Figure 4.12. As expected HBx encoding DNA showed significant LTR-promoter activity (compare lane 4 with lane 3). This activity diminished more than 2.4 folds when 100 ng of Rz-170 was added (lane 5). Upon addition of ODN 2, it dropped 8.4 folds (lane 6), with ODN 1 about 5.8 folds (lane 7) and in

combination of both about 8.4-fold (lane 8). Control cells and pSG5-HBx treated cells showed no significant reporter gene activity as expected (lanes 1 and 2). Thus, additional 3-4 folds decrease (when compared with Rz-170 alone) in HBx gene mediated reporter gene activity was observed with ODN 2. More than 17-fold reduction in reporter gene activity was observed with Dz-192 under identical conditions (lane 9) and about 12.6-fold reduction with the mutant version of Dz-192 (lane 10). Therefore, it could be concluded that ODNs (1 and 2), wild-type and mutant Dz-192 treated cells showed impressive functional inhibition of HBx protein. Also, as expected Dz-192 (lane 11) and mutant Dz-192 (lane 12) independently, showed significant reduction in reporter gene activity.

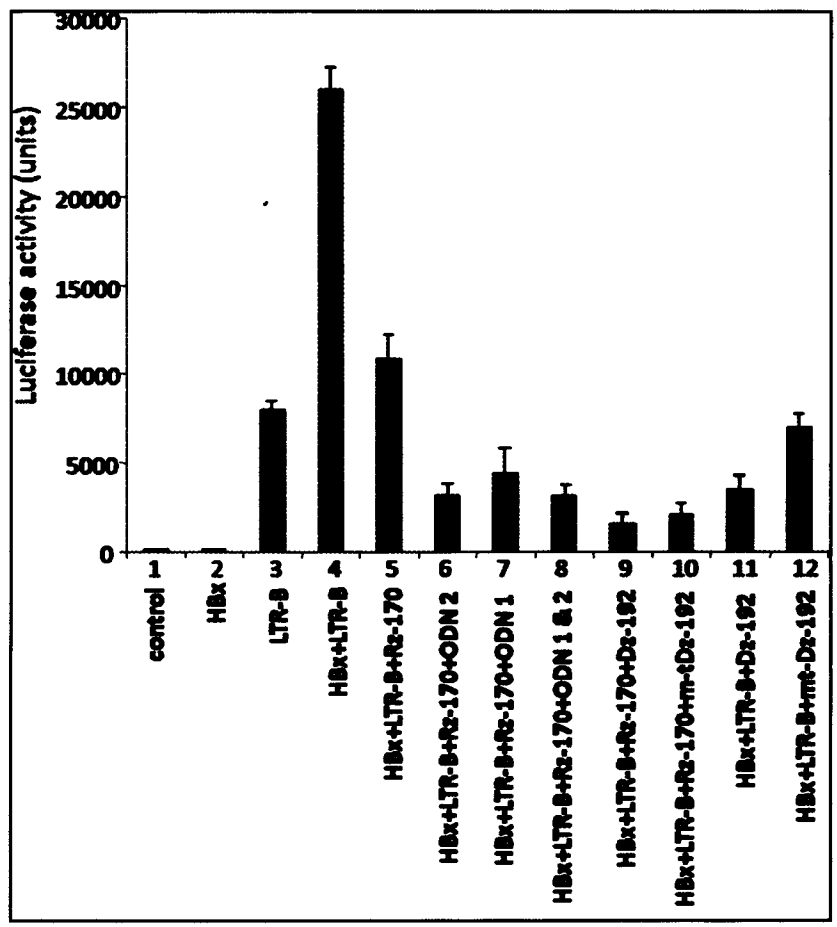

Figure 4.12: Effect of ODNs and Dzs with Rz-170 on HBx activity on HIV-1 LTR-B promoter activation

DISCUSSION

Efficient cleavage of a target RNA by Rz or Dz is important for several reasons including biochemical and gene therapy approaches. Although there are several potential Dz or Rz target sites in a target RNA, most of them are cleaved poorly or not at all when tested on longer and physiologically relevant RNAs. In this study we have tried to develop a strategy for the possible usage of antisense ODNs or 10-23 Dzs which are designed adjacent to active Rz or another Dz for the purpose of achieving more potent cleavage of the target RNA. It was found that Dzs and ODNs that were targeted to hybridize immediately adjacent to the Rz or Dz target sites were able to significantly augment Rz or Dz mediated cleavage activity in dose-dependent manner and that the downstream Dz or ODN usually were more efficacious than the upstream Dz or ODN. In both the cases (Rz and ODNs or Dz and ODNs) almost complete cleavage of the target RNA was achieved under defined set of conditions. Almost 100% *in vitro* cleavage of the target RNA with Rz or Dz in the presence of specific ODNs or Dz was achieved. Also, potent intracellular reduction of the target RNA in a mammalian liver-specific cell line was achieved in the present study. It must be emphasized that all the previous studies that employed either Rz or Dz alone, complete *in vitro* disappearance of the input substrate RNA has never been achieved under standard conditions of cleavage. This data assumes more significance because ODNs and Dzs are currently being exploited for therapeutic purposes (Schubert and Kurreck, 2006; Banerjea et al, 2004). The most likely explanation for enhanced cleavage by Rz or Dz with specific ODNs lies in their ability to melt the secondary structure near the Rz and Dz target sites which facilitated efficient hybridization between Rz-170 or Dz-237 with HBx RNA. This ODN mediated enhancement of cleavage was not observed with ODN that hybridized 33 nts upstream of the target RNA. This

is most probably because long RNAs are known to exist in separate stem-loop structures or independent domains.

The upstream Dz-155 not only failed to increase the cleavage activity of Rz-170 but also was catalytically inactive as no cleavage products were seen. It is likely that Dz-155 failed to hybridize with the target RNA because of the secondary structure constraints. In this connection it is important to note that the ability of ODNs to successfully hybridize to the target RNA (as measured by RNAse H based assay) is essential but not sufficient for affording catalytic activity to Dz (Kurreck et al, 2002). It is noteworthy that the hybridization arms of the Dz or Rz span only 14 or 16 nt (with one gap in the design) in the target RNA whereas the ODNs are uninterrupted 20nt long, which is likely to melt secondary structures better. The advantage of using downstream Dz would obviously be its ability to cleave the target RNA in a catalytic manner. A comparative Rz-170 mediated study between wild-type and mutant Dz-192, suggests that the enhancement of Rz-170 cleavage is largely due to the antisense effects.

In summary, by this study it was shown that both Rz and Dz mediated cleavage of the full-length HBx RNA can be powerfully augmented by specific ODNs and 10-23 Dzs. They not only enhanced the Rz & Dz mediated *in vitro* cleavage products but also reduced very significantly the intracellular target gene expression in a mammalian liver specific cell line– Hep G2. The intracellular effectiveness of these ODNs or Dzs could be substantially improved by using chemically modified (stabilized) nucleotides (Kurreck et al, 2002). These observations are of immediate therapeutic importance and should be applicable to down regulate other target genes also.

CHAPTER V

Usage of Antisense Oligodeoxynucleotide and Catalytic Nucleic Acids to knock out expression of Vif gene of HIV-1 Subtypes B and C and their functional characterization

Viruses have evolved several unique devices to protect themselves from host genes involved with innate immunity. In addition to structural and non-structural proteins essential for replication, lentiviral genomes encode multiple proteins that modulate virus fitness and infectivity *in vivo*. HIV-1, a complex lentivirus, that belongs to family retrovirus; encodes six 'viral accessory' proteins in addition to normal structural and regulatory proteins. One such accessory gene of HIV-1 is virion infectivity factor (vif) that encodes a 23 kDa basic protein. The *vif* open reading frame is present in all lentiviruses except equine infectious anemia virus and is required for viral replication and pathogenicity in vivo (Simon et al, 1998). In the absence of Vif, HIV-1 virions that are produced from non-permissive primary T lymphocytes and certain T cell lines are defective and cannot initiate productive infection. The mechanism of action of vif has still not been fully defined, yet it is clearly important for infectivity (Cullen, 2006). Recently, Vif is shown to neutralize the DNA-editing enzymes (DEE), namely cytidine deaminases of APOBEC family of proteins, by promoting their degradation by proteasomal degradation pathway (Conticello et al, 2003). It has recently been shown that Vif-deficient virions produced from human PBMC contain only about 7 (+/-4) copies of APOBEC3G (Xu et al, 2007) yet, these virions are completely non-infectious suggesting that the level of tolerance for virus-associated APOBEC3G is quite low. APOBEC3G inhibits the replication of human immunodeficiency virus-1 (HIV-1), other retroviruses and retrotransposons (Bogerd et al, 2006; Cullen, 2006). Vif molecules recognize their autologous APOBEC3 proteins through conserved structural features (Zhiwen et al, 2008). Vif facilitates proteosomal degradation of APOBEC3G

(A3G). Consequently Vif may be a new target for antiretroviral drug developments.

The ability of nucleic acids as biological catalysts has become well established in the last few years. Inhibition of HIV-1 replication by macrophage-tropic Dzs that were targeted against HIV-1 TAT/Rev RNA was earlier reported by Banerjea and coworkers (Unwalla and Banerjea, 2001). Catalytic nucleic acid-defined approaches based on complementary base pairing of the target RNA with either antisense DNA, or catalytic DNA (Dz) can therefore, be used to potently knock down the expression of vif of HIV-1 subtype B and Indian isolate subtype C. Dzs are short DNA molecules with catalytic activity. One of the most extensively worked Dz is with 10-23 catalytic motif containing Dz. 10-23 Dz cleaves the RNA sequence at a phosphodiester bond between a purine and a pyrimidine residue *in vitro* as well as *in vivo*. A single nucleotide change in the catalytic motif diminishes its sequence specific cleavage activity; G14C completely abolishes the catalytic cleavage activity.

Due to occurrence of high mutational rate for viral mRNA transcripts, strategies are being developed to design chimeric Dzs. These are di-Dzs with two Dzs joined in direct tandem with no spacer sequences. The chimeric Dz targets more than one site of target RNA for cleavage while maintaining the specificity of the Dzs at the same time.

Catalytic nucleic acids at sometimes were not very efficient in cleaving long RNA substrates *in trans*. Use of antisense oligonucleotides (stretches of DNA sequences of around 20 nucleotides that are complimentary to target RNA sequence), upstream and downstream of the cleavage site, along with the Rz or Dz seems to be a promising complimentary approach in the field of nucleic acids as genetic therapeutic agents. Oligonucleotides are short single-

stranded segments of DNA that upon cellular internalization can selectively inhibit the expression of a single protein. For antisense applications, oligonucleotides interact and form a duplex with the mRNA or the pre-mRNA and inhibit their translation or processing, consequently inhibiting protein biosynthesis (Crooke, 1999). Antisense target recognition is facilitated by Watson-Crick base pairing between the oligonucleotide and the target mRNA.

In the present study, identification and characterization of those Dzs and ODNs that significantly augments Rz or Dz mediated cleavage of full-length vif RNA of HIV-1 and inhibition of intracellular expression of the target RNA in mammalian cell line, HEK 293, was carried out. We found out that 10-23 Dz and chimeric Dz knocked down the expression of vif more potently and were able to prevent the degradation of A3G by vif. These results are potentially important for therapeutic purposes on one hand and allows us to understand the action of vif with respect to A3G degradation and its involvement in the infection on the other.

EXPERIMENTAL PROCEDURES

Cells and plasmid DNAs:

HEK 293 and 293-APOBEC3G-HA (obtained from the National Institutes of Health, Bethesda, Maryland, U.S.A.) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Plasmids pNL4-3 and 93In905 were obtained from the National Institutes of Health (Bethesda, Maryland, U.S.A.) AIDS Repository and grown according to standard procedures. Recombinant plasmid DNAs

encoding the vif gene were purified and transfected with Lipofectin reagent (Invitrogen), as described in materials and methods.

Cloning of vif gene of subtypes B and C of HIV-1 and in vitro transcription:

Vif gene was PCR amplified from pNL4-3 and 93In905 templates, for cloning subtypes B and C, respectively in *Bam HI* and *Xho I* restriction sites of pcDNA3.1 vector (Invitrogen). This places vif gene under T7 and CMV promoter (Figure 5.1). The former promoter could be used to generate ^{32}P labeled target transcript using T7 RNA Polymerase and CMV promoter could be used for expression of vif gene in mammalian cell lines.

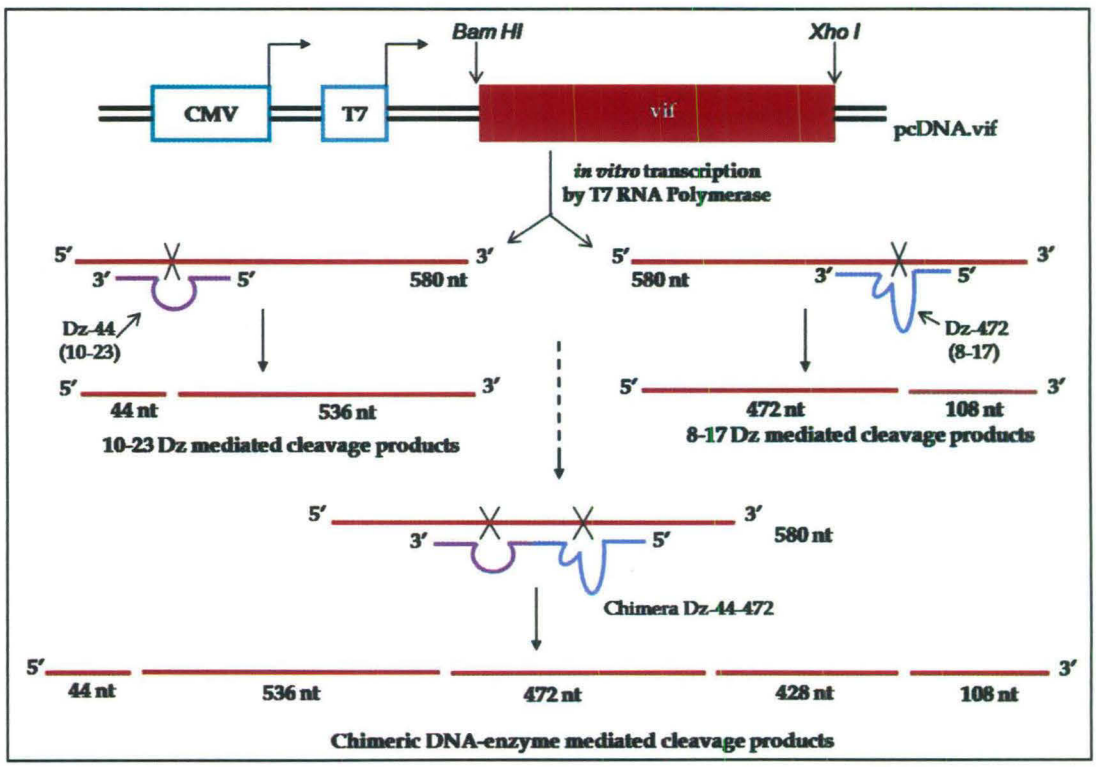

Figure 5.1: Schematic representation of cleavage of vif

Construction of mono-Dzs and chimeric Dz constructs against target vif RNA:

Vif gene was subjected to m-Fold programme to generate secondary structures and screened for the conserved loop regions with **'AU'** or **'AG'** di-nucleotides as target sites (Figure 5.2). The cleavage sites selected for designing of Dzs are shown in Figure 5.3. Dz-472, with 8-17 catalytic motif was targeted against the **'AG'** di-nucleotide sequence at position 472 of vif gene while Dz-44 with a 10–23 catalytic motif was targeted to cleave between A and U at position 44. A mutant 10-23 Dz with a single G14 to C substitution in the catalytic motif served as a negative control. We had earlier shown that this mutation completely abrogates the cleavage activity of the 10-23 Dz (Goila and Banerjea, 2001).

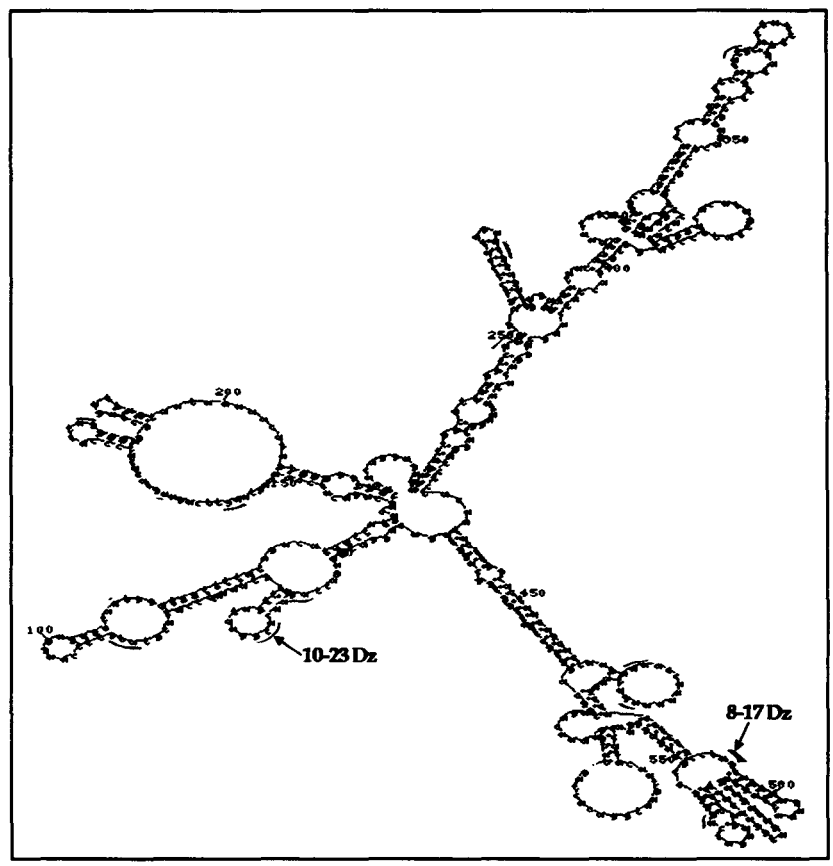

Figure 5.2: m-FOLD predicted secondary structure of vif RNA

The chimeric Dz was designed by joining the two Dzs, together in tandem without any spacer nucleotide in between the hybridizing arms of the Dzs, as shown in Figure 5.3B. The various mutants of chimeric Dz were also designed (Figure 5.3B).

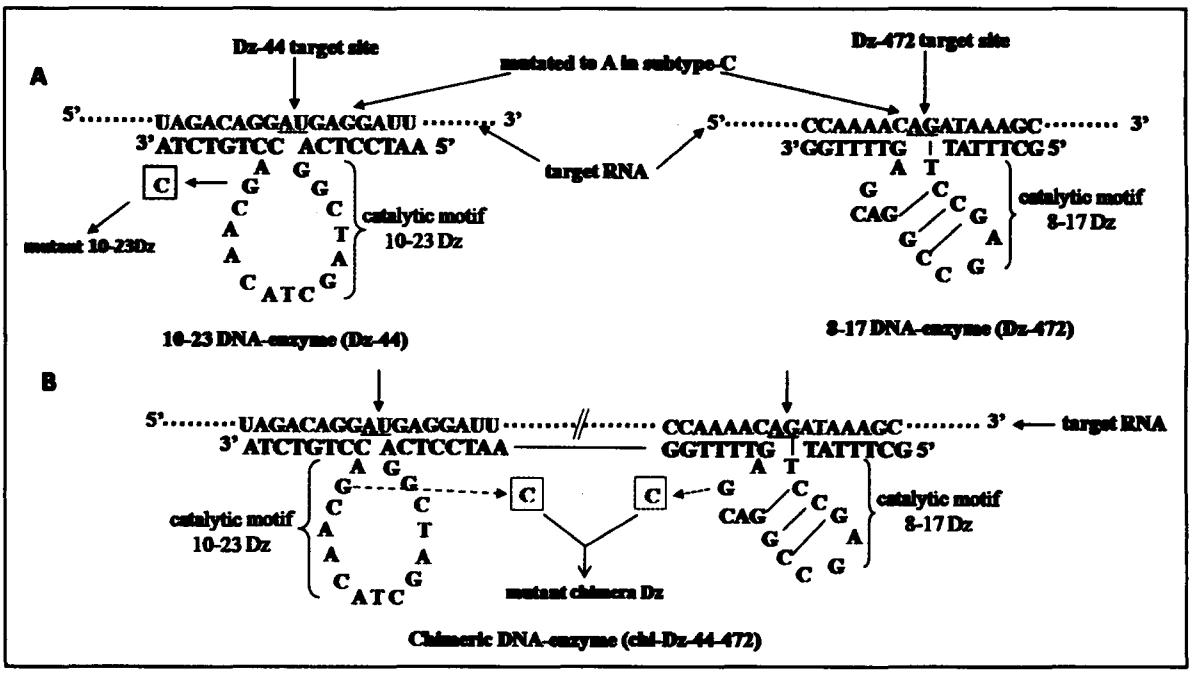

Figure 5.3: Target sites of various Dzs of vif

Primers and Dzs:

All primers or oligonucleotides were synthesized chemically and obtained from Sigma Genosys (The Woodlands, TX). The primers for PCR amplification of vif gene are listed below and restriction sites, *Bam HI* was engineered at the 5′ end and *Xho I* at the 3′ end of vif gene using forward and reverse primers, shown in bold and *italics*.

Forward primer: 5' GC***GGATCC***ATGGAAAACAGATGGCAGG 3'

Reverse primer: 5' GC***CTCGAG***CTAGTGTCCATTCATTGTATGG 3'

Dz-472 with the conserved 13-nucleotide-long 8-17 catalytic motif (5' CCGAGCCGGACGA 3') and Dz-44 with 15 nucleotide long (5' GGCTAGCTACAACGA 3') 10-23 catalytic motif were flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. Dz-44-472, the chimeric Dz was designed by joining the Dzs-44 and 472, in tandem.

Antisense ODN that is 20 nts long with sequence (5' TTTTATTAATGCTGCTAGTG 3') was designed immediately upstream Dz-472 (Figure 5.9, boxed).

Dz-44C and chimeric Dz-44-472 C were designed against vif gene of subtype C, by introducing required changes in the hybridizing arms of the Dzs.

Dz-mediated cleavage:

Equivalent amounts of unlabeled Dz and labeled substrate RNA (100 pmoles each) were allowed to interact in a final volume of 10 µl in a buffer containing 50 mM Tris-HCl pH 7.5 in the presence of 10 mM $MgCl_2$ (standard conditions) as described earlier (Santoro and Joyce 1997) for 2 hours at 37°C. The cleaved products were resolved on 7M urea-6% PAGE and analyzed by autoradiography.

To check the effect of antisense ODN, reaction was set in the similar manner with Dz-472 in presence and absence of indicated amounts of ODN.

Construction of vif B/C chimeric genes

We aimed at making two different vif-B/C chimeric genes. In one construct we fused the N-terminal half from vif-B and C-terminal half of vif-C while the second construct possesses the opposite orientation i.e., N-terminal half from vif-C and C-terminal half from vif-B (Figure 5.4, panels C and D).

For precise fusion of two regions from vif-B and C, fusion PCR technique was used. In this technique, top and bottom fusion primers are designed to amplify the specific regions from two different templates such that the amplified product has the required regions of both templates which can be fused together as one product. In order to fuse 1-290 bases of vif-B and 291-580 bases of vif-C PCR products, the following set of primers was used.

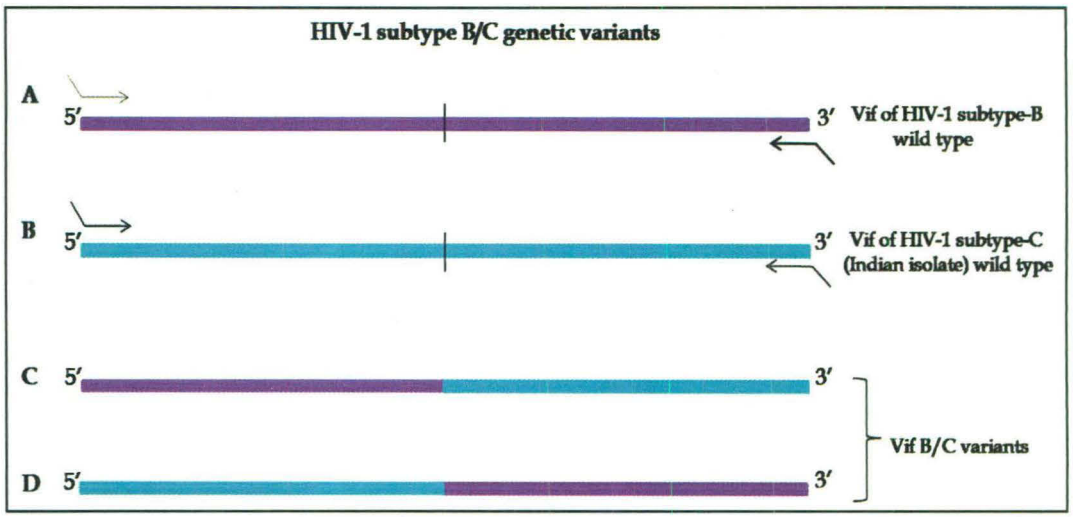

Figure 5.4: vif-B/C genetic variants

Top fusion primer for vif B/C

5′ ggA ggA AAA AgA gAT ATA gCA CAC AAA TAg AAC CTg gCC Tgg CAg

ACC AgC 3′

Bottom fusion primer for vif B/C

5′ gCT ggT CTg CCA ggC CAg gTT CTA TTT gTg TgC TAT ATC TCT TTT TCC

TCC 3′

The following set of primers was used for fusion of 1-290 bases of vif of subtype C and 291-580 bases of subtype B-vif

Top fusion primer for vif C/B

5′ ggA gAT TgA gAA gAT ATA ACA CAC AAg TAg ACC CTg ACC TAg CAg ACC AAC 3′

Bottom fusion primer for B/C

5′ gTT ggT CTg CTA ggT CAg ggT CTA CTT gTg TgT TAT ATC TTC TCA ATC TCC 3'

For final amplification of fusion gene product, forward and reverse primers used for vif amplification and cloning, were used.

***Inhibition of gene HIV-1 expression assayed by reverse transcriptase-polymerase chain reaction*:**

In order to assess the possible effect of various Dzs on the expression of vif B and C RNA, HEK-293 cells were transfected with 1 µg vif-B-pcDNA3 or vif-C-pcDNA3 in presence or absence of Dzs, using lipofectamine 2000 (Invitrogen) as per the instructions of the manufactures. Total RNA was isolated using trizol reagent and subjected to RT-PCR for estimating the vif-B/C specific RNA (580 nts) and the control human-glyceraldehyde-phosphodehydrogenase (huGAPDH) RNA using the improm RT-PCR system (Promega).

***Inhibition of vif expression by western blot analysis*:**

1 x 10^6 HEK 293 or 293-APOBEC3G-HA cells were grown to 80% confluency in a six well plate. They were transfected with vif encoding plasmids (1µg) alone or in the presence of various Dzs in a final volume of 1ml for 48 hours, using lipofectin. Cell lysates were prepared and equivalent amounts of proteins were loaded in each lane after estimating protein concentration using BCA reagent. Protein was resolved on 15% SDS-PAGE

and transferred on to a nitrocellulose membrane. Rabbit polyclonal antiserum for vif-B (obtained from NIH) was used as a primary antibody (1:1,000 dilution in PBS pH 7.2 containing 0.1% Tween 20). The amount of pcDNA-vif-B DNA was kept constant in each experimental lane at 1µg. The absolute amounts of DNA introduced in each well was made equivalent by adding unrelated DNAs.

RESULTS

In vitro synthesis of full-length vif RNA by T7 RNA polymerase

Plasmid pcDNA-vif-B or vif-C (Figure 5.1) was linearized with *XhoI* restriction enzyme and when subjected to *in vitro* transcription using T7 RNA polymerase, a 580 nt long full-length vif-B or vif-C transcript as expected was synthesized in the presence of ^{32}P UTP. Transcripts were resolved on 7M urea-6%PAGE and analyzed by autoradiography.

In vitro cleavage of target RNA by Dzs

The two different catalytic motifs containing Dzs, Dz-44 with 10-23 motif and Dz-472 with 8-17 motif and chimeric Dz with both the 10-23 and 8-17 catalytic motifs, were synthesized. A schematic representation of substrate gene transcription and Dzs cleavage pattern is shown in Figure 5.1. The plasmid encoding the substrate vif gene was linearized with *Xho I* and transcribed *in vitro* using T7 RNA polymerase to obtain a 580 nts long transcript (Figure 5.1).

The uniformly labeled transcript (Figure 5.5, panels A, B and C, lane 1) was then subjected to *in vitro* cleavage by equimolar amounts (100 pmoles) of Dzs in the presence of $MgCl_2$ (10mM). Dz-44 and chimeric Dz-44-472 cleaved

the target RNA very efficiently (>95% cleavage) in a sequence-specific manner (Figure 5.5, panel B, lane 2; and panel C, lane 4) but Dz-472 exhibited very poor *in vitro* cleavage activity (Figure 5.5 A, lane 2). We conclude that 8-17 catalytic motif containing Dz cleaves the target RNA less efficiently than Dzs having the 10-23 catalytic motif. Also the chimeric dz retained the specificity of both the mono-Dzs (Dz-4 and 472).

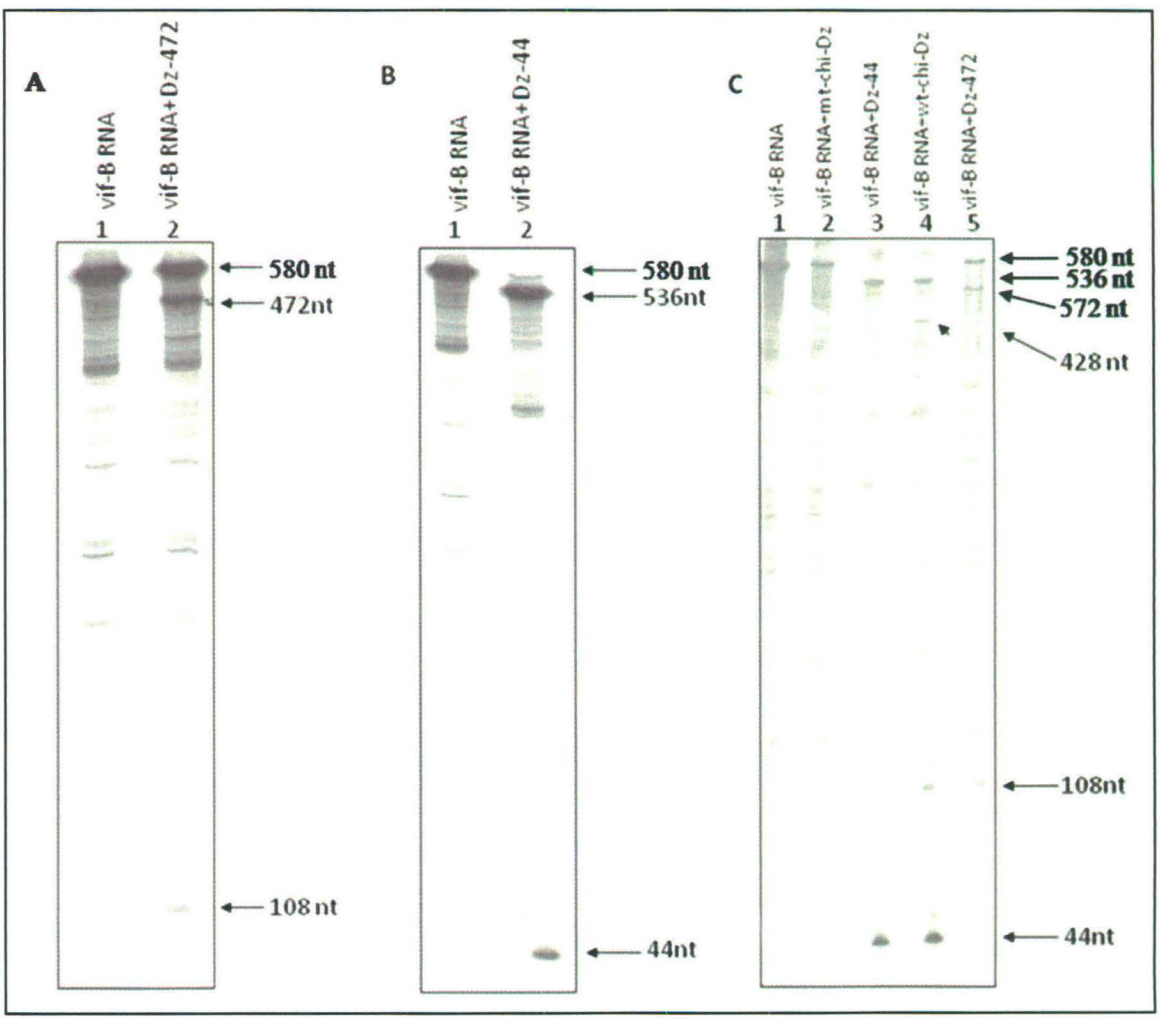

Figure 5.5: *In vitro* cleavage of vif-B RNA by Dzs

Chimeric Dz maintained the catalytic efficiency of both the motifs and generated the products specific for the two Dzs individually. In addition to

these four specific cleaved fragments, chi-Dz-44-472 produced another cleaved fragment of size 428 nts in length which is possible only if the two Dzs have acted simultaneously (Figure 5.6, lane2). As expected mutant-Dz-44 and completely disabled chimeric Dz (both 8-17 and 10-23 catalytic motifs were mutated) failed to exhibit any cleavage activity under similar experiment conditions (lane 4). When one Dz mutated keeping the other one wt, fragments generated from wt Dzs were observed (Figure 5.6, lanes 3 and 5)

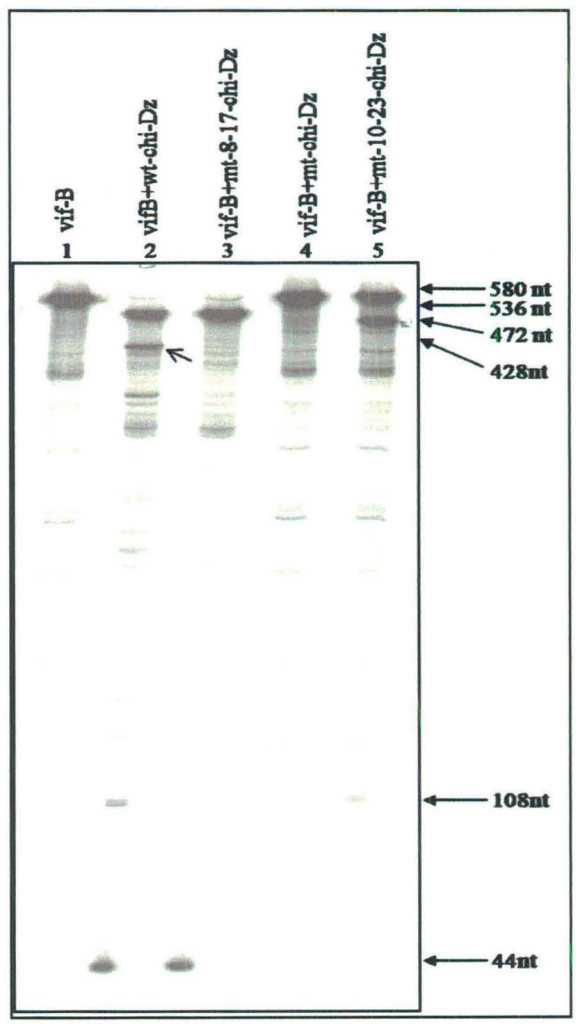

Figure 5.6: *In vitro* cleavage of vif-B RNA by various chimeric Dzs

Effect of $MgCl_2$ on cleavage efficiency of Dz-44:

In presence of increasing concentrations of $MgCl_2$, a dose-dependent increase in the cleavage products could be observed with Dz-44 (Figure 5.7). The concentration of Dz-44 was kept constant throughout at 100 pmoles. The most important observation was that Dz-44 could cleave the target RNA in the presence of 0.1mM $MgCl_2$ in the cleavage buffer (lane 5). The Dz-44 showed significant cleavage activities at micromolar levels of Mg^{2+}. Almost 100% cleavage of the target vif-B RNA was observed by Dz-44, in the presence of 1 to 10mM $MgCl_2$ (lanes 7-10). Since lanes 2-5 do not show the same amount of input substrate RNA as in lane 1, it may be concluded that small amounts of Mg^{2+} are required for the stability of the target RNA.

Effect of $CaCl_2$ on cleavage efficiency of Dz-44:

In presence of increasing concentrations of $CaCl_2$, a dose-dependent increase in the cleavage products could be observed with Dz-44 (Figure 5.8). 100 pmoles of Dz-44 was used and its concentration was kept constant in all the reactions. The cleavage pattern of Dz-44 in presence of Ca^{2+} on target vif-B RNA is very similar to the pattern observed in presence of Mg^{2+}. The Dz showed significant cleavage activities at micromolar levels of Ca^{2+}. Almost 100% cleavage of the target vif-B RNA was observed by Dz-44, in the presence of 10mM $CaCl_2$ and $MgCl_2$ (lanes 10 and 11, respectively). We conclude that Ca^{2+} is just as effective as Mg^{2+} in causing cleavage.

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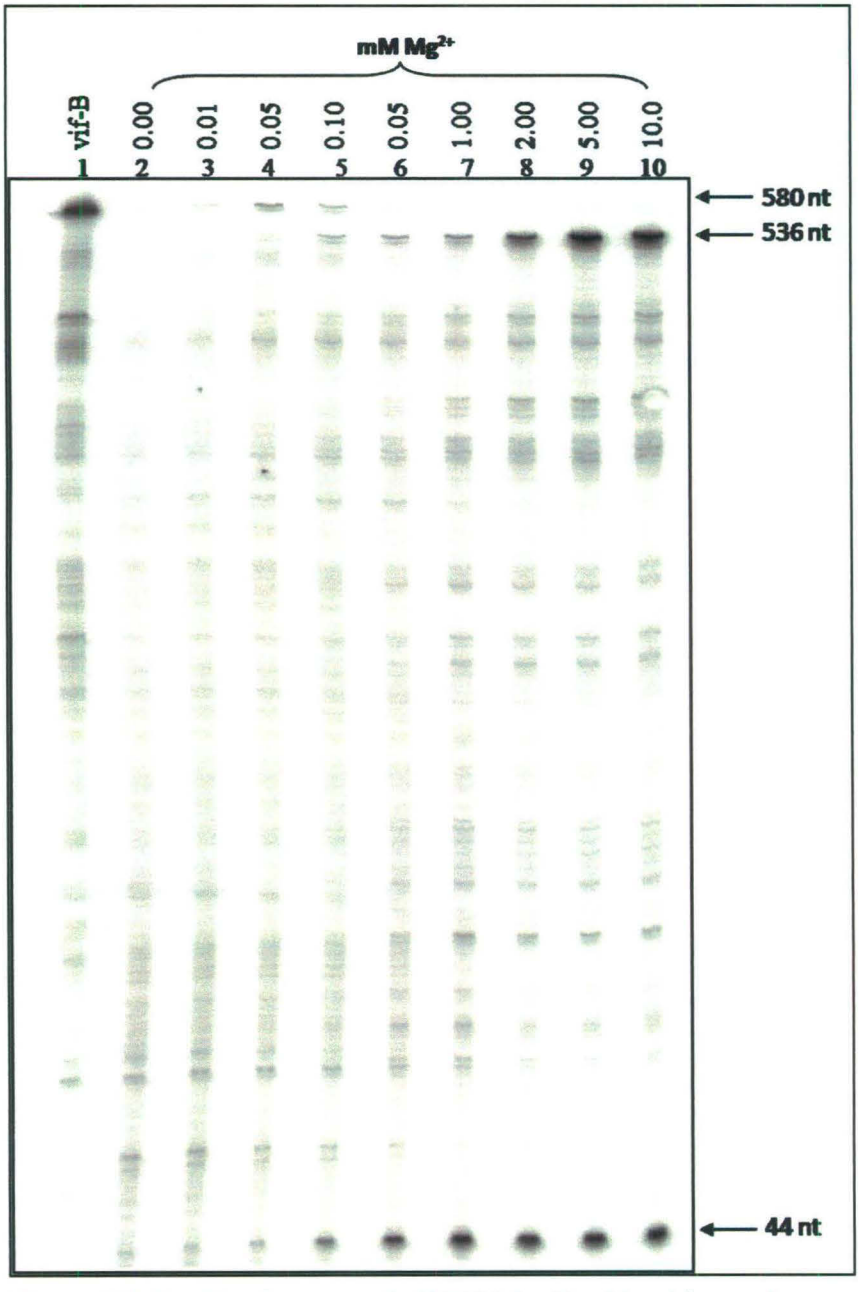


Figure 5.7: *In vitro* cleavage of vif RNA by Dz-44, with varying conc. of Mg^{2+}

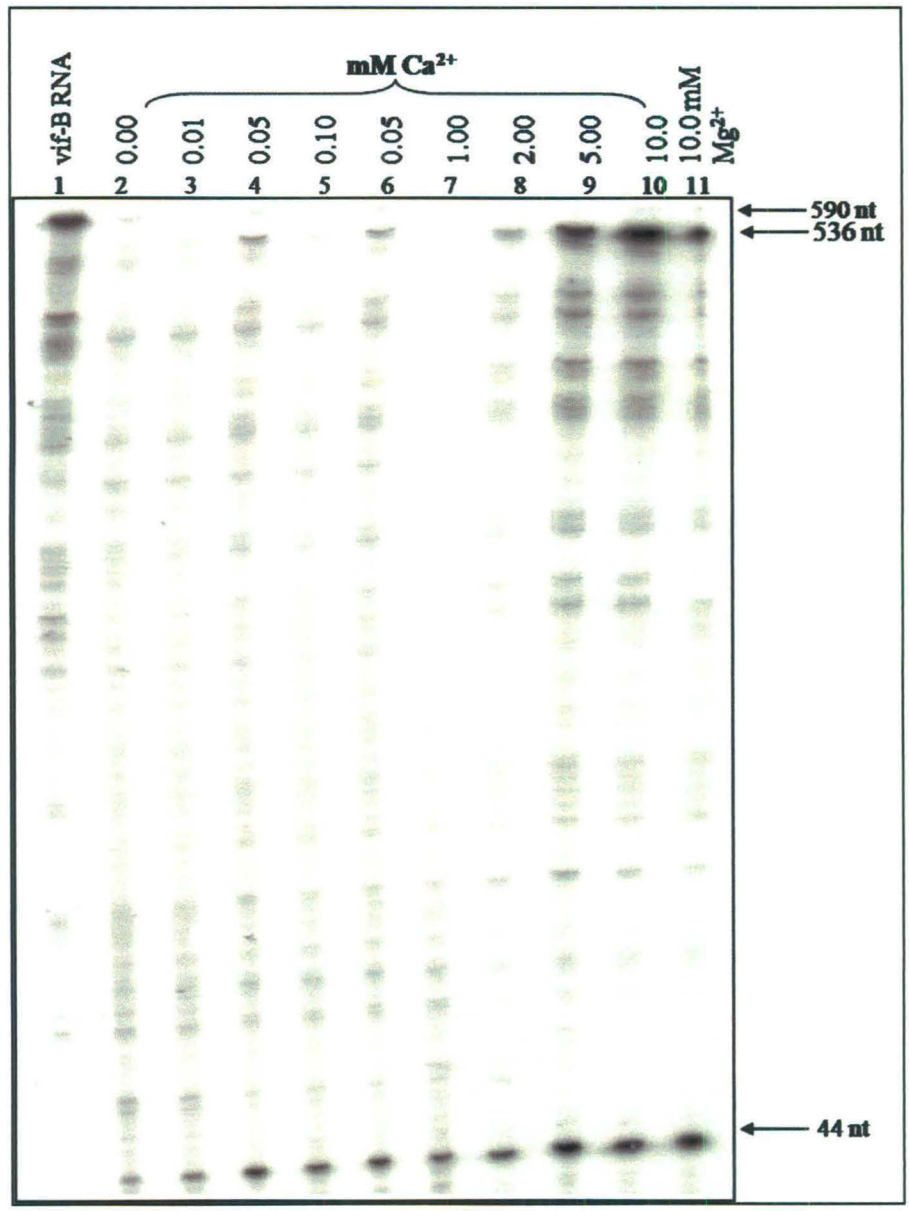


Figure 5.8: In vitro cleavage of vif-B RNA by Dz-44 in varying conc. of Ca2+

Dz-472 mediated cleavage of vif-B RNA in the presence of upstream ODN:

In order to increase the Dz-472 mediated cleaved products, 20nt long antisense ODNs, upstream of the 3' hybidizing arm of Dz-472 was synthesized. Dz-472 mediated cleavage reaction was performed in the presence of indicated amount of ODNs individually and the results are shown in Figure 5.9. Lane 1 shows the synthesis of uniformly labeled full-length (580 nts long) vif-B RNA. When equimolar amount (100 pmoles each) of Dz and labeled substrate RNA were incubated for cleavage under standard conditions, two specifically cleaved fragments (472 and 108 nts long RNA fragments) were obtained (lane 2). When the same cleavage reaction was carried out in the presence of 10 pmoles (lane 3); 50 pmoles (lane 4); and 100 pmoles (lane 5) of ODN increased cleavage products were observed. Approximately, 4 folds decrease was observed in the input substrate RNA. We conclude that ODN that hybridized immediately upstream of Dz-472, was able to significantly augment the Dz-472 mediated cleavage of the substrate RNA as judged by the intensity of 472 and 108 nts RNA band intensity. Also 50 pmoles of ODN produced maximum enhancement of cleavage as higher concentration of the same did not result in any further enhancement of cleavage.

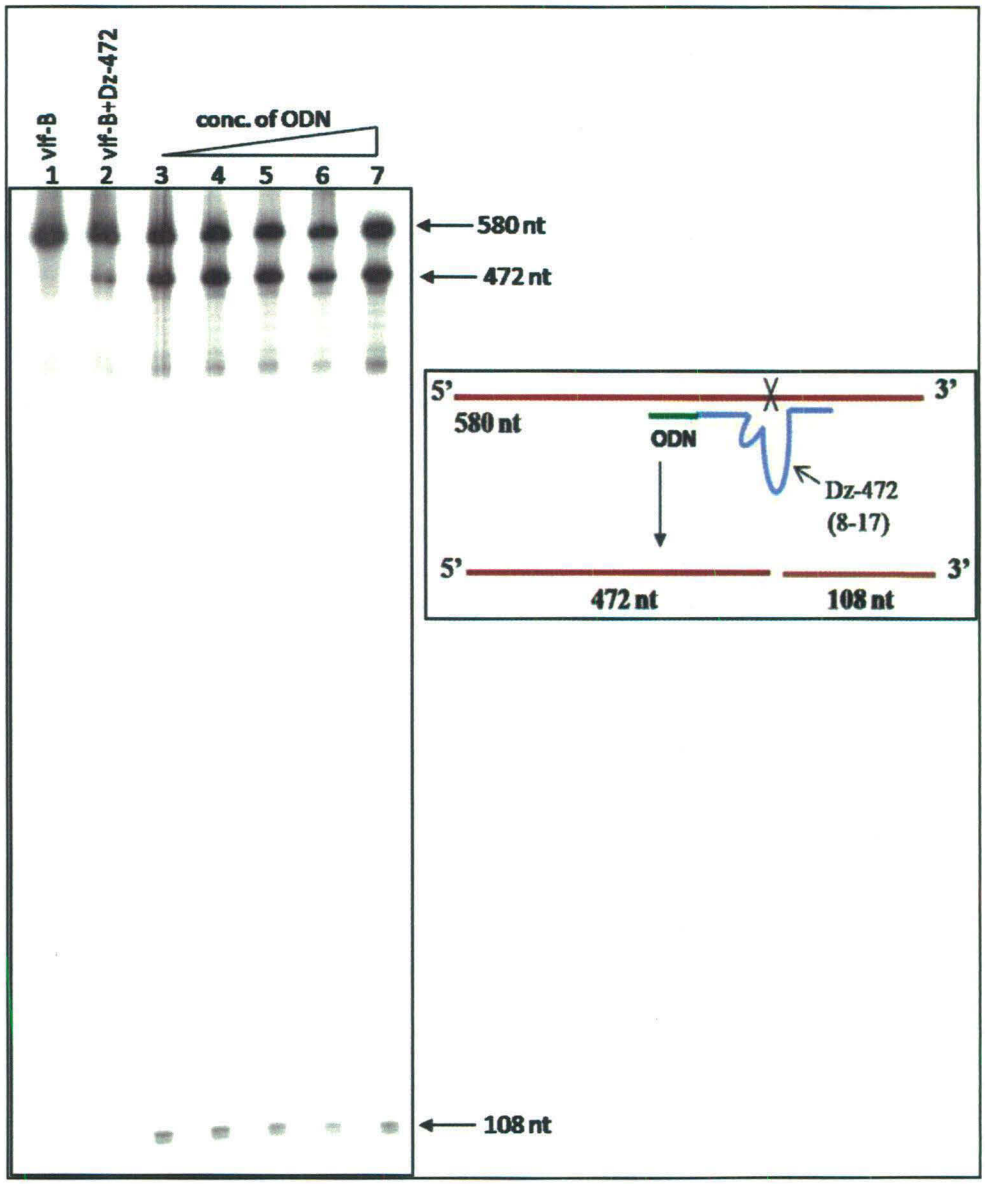


Figure 5.9: *In vitro* augmentation of cleavage activity of Dz-472 by ODN

Comparative cleavage analysis of vif RNAs of subtypes B and C by the Dzs designed for vif-B:

In vitro synthesis of ^{32}P-UTP labeled RNAs of vif-B and vif-C was performed by T7 RNA Polymersae as described in methods and materials. Dzs designed against vif-B were also used to see if they were efficacious against vif-C RNA which has a single mutation in the hybridizing arms when

compared to vif-B RNA (underlined, shown in Figure 5.10). As shown in Figure 5.10, lanes 5 and 6; Dz-472 and Dz-44 cleaved the target vif-C RNA poorly the former being totally inactive. This could be due to improper hybridization of Dzs with the target. But it is remarkable to see that Dz-44 still was able to cleave vif-C RNA (lane 6). Our results show for the first time that some degree of mismatches are tolerated by 10-23 motif containing Dz.

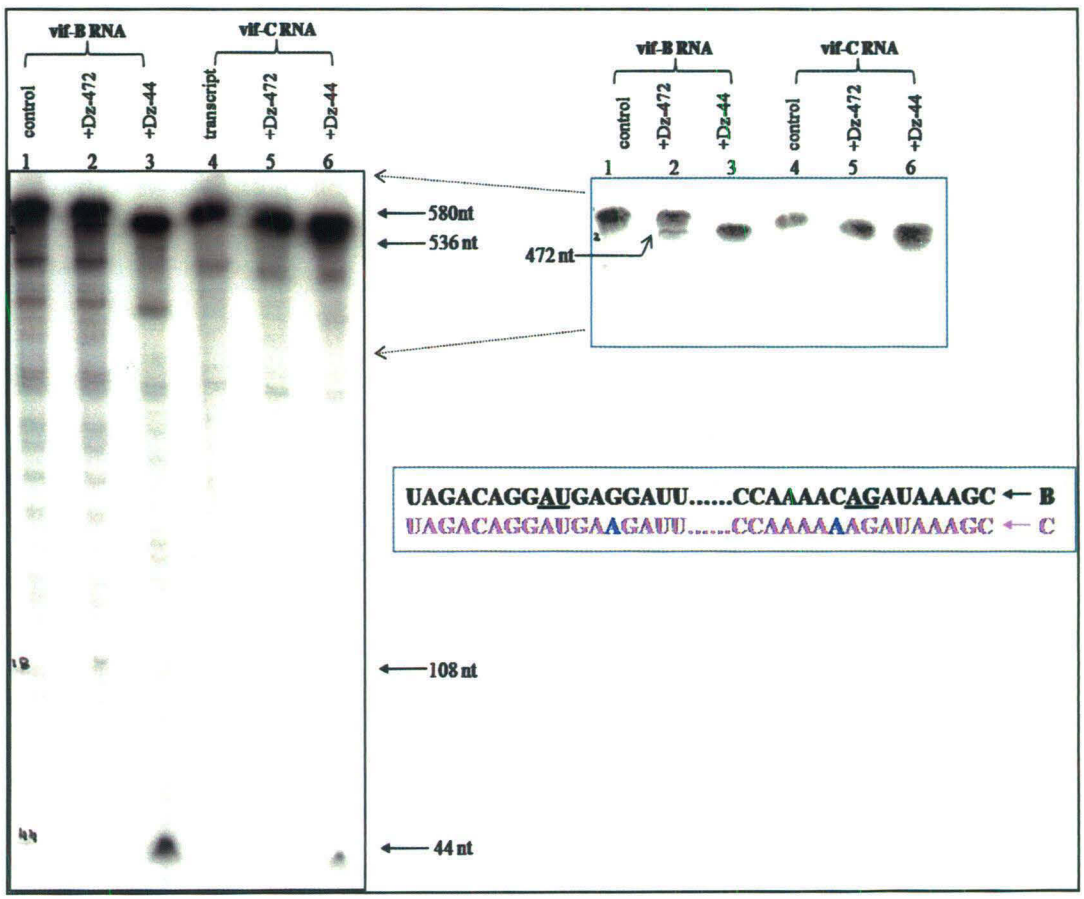


Figure 5.10: Comparative cleavage of vif-B and C RNAs by Dzs for vif-B

Intracellular inhibition of vif-B and vif-C genes expression by Dzs:

Dz-44, Dz-472 and chimeric Dz-44-472 were evaluated for their bio-efficacy after checking their *in vitro* cleavage activity (Figure 5.11). They were co-transfected along with plasmid encoding vif gene of HIV-1 subtype B, i.e. pcDNA3.1-vif-B (1µg for $1X10^6$ HEK-293 cells/well in a 6 well plate) and

incubated for 48 hrs. Total RNA was isolated using trizol reagent and RT-PCR was performed using vif specific primers. It was interesting to note that though Dz-44 was very effective *in vitro* and also showed impressive activity intracellularly (Figure 5.11, panel B compare lane 1 with lanes 3 and 4). But chimeric Dz-44-472 retained its high cleavage efficiency at intracellular levels (Figure 5.11, panel C, lane 4). This could be because Dz-472 was not able to efficiently access the target RNA due to secondary structure of the target RNA (Gupta et al, 2008).

The effect of various mutant versions of chimeric Dz-44-472 were also studied (Figure 5.12). As clear from the Figure 5.12, the wild type chi-Dz-44-472 showed maximum inhibition of the expression of vif-B gene.

In a similar manner, efficiency of Dz-44C and chimeric Dz-44C-472C, specific for vif-C RNA were analyzed (Figure 5.13). In this case also, chimeric Dz retained its higher cleavage potential and successfully suppresses the expression of vif-C RNA (lane 5).

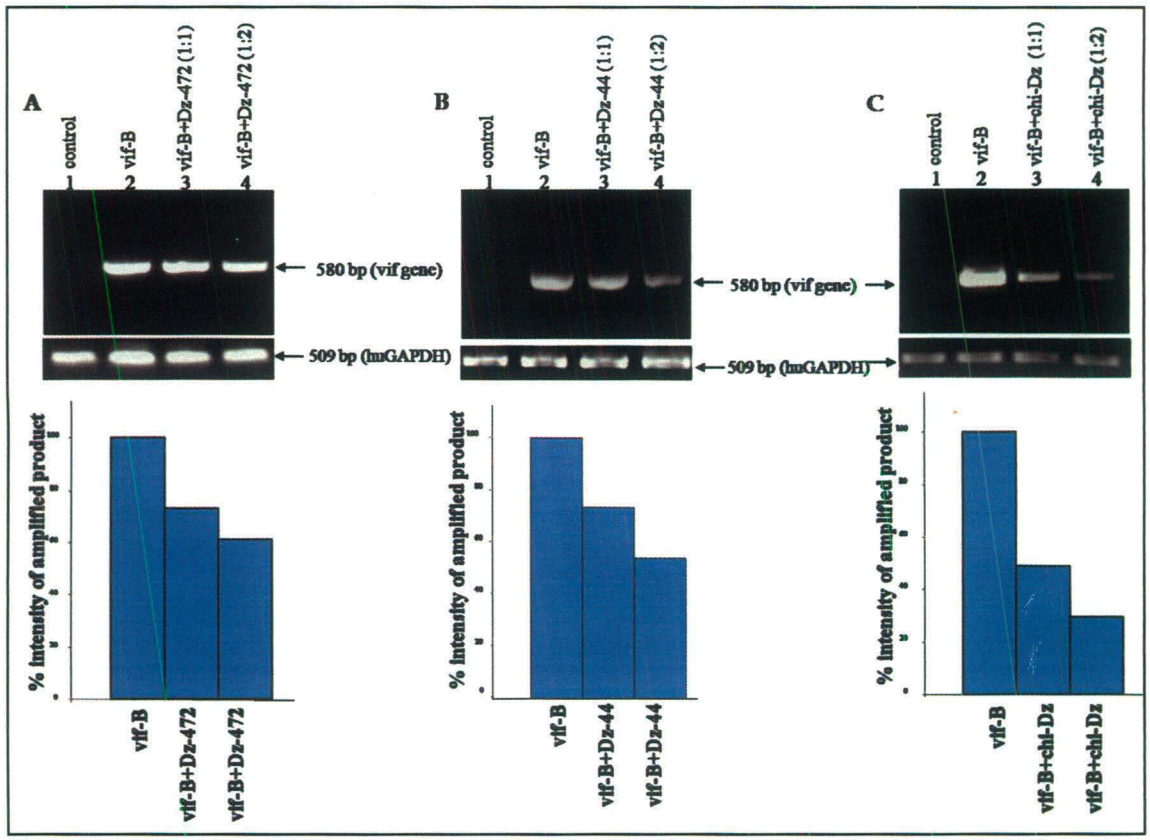


Figure 5.11: RT-PCR assay to check the effect of Dzs on intracellular expression of vif-B

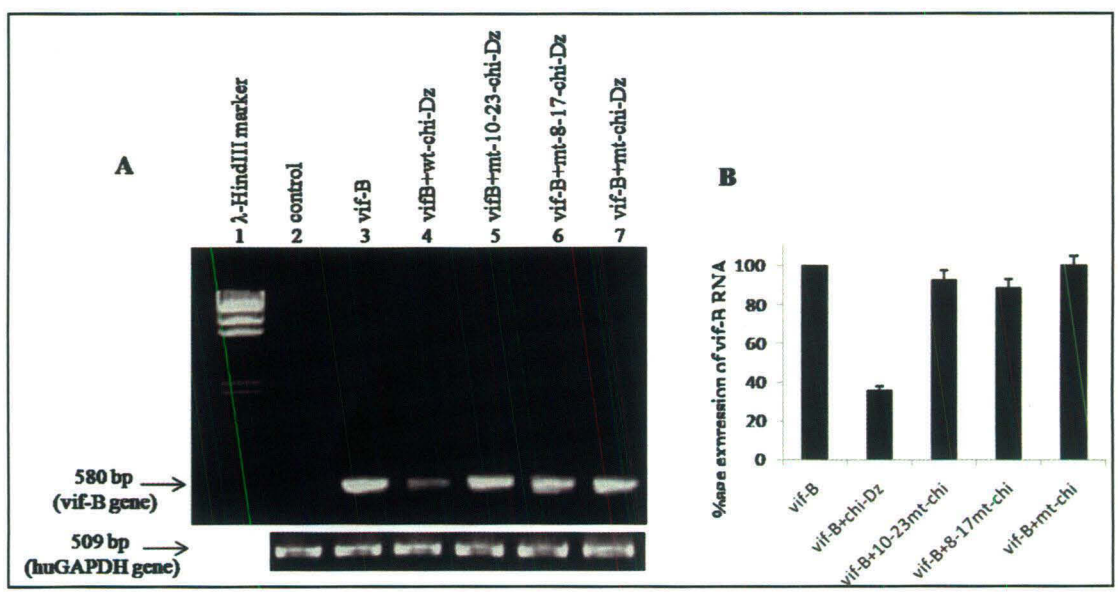


Figure 5.12: Effect of chimeric Dzs on intracellular expression of vif-B

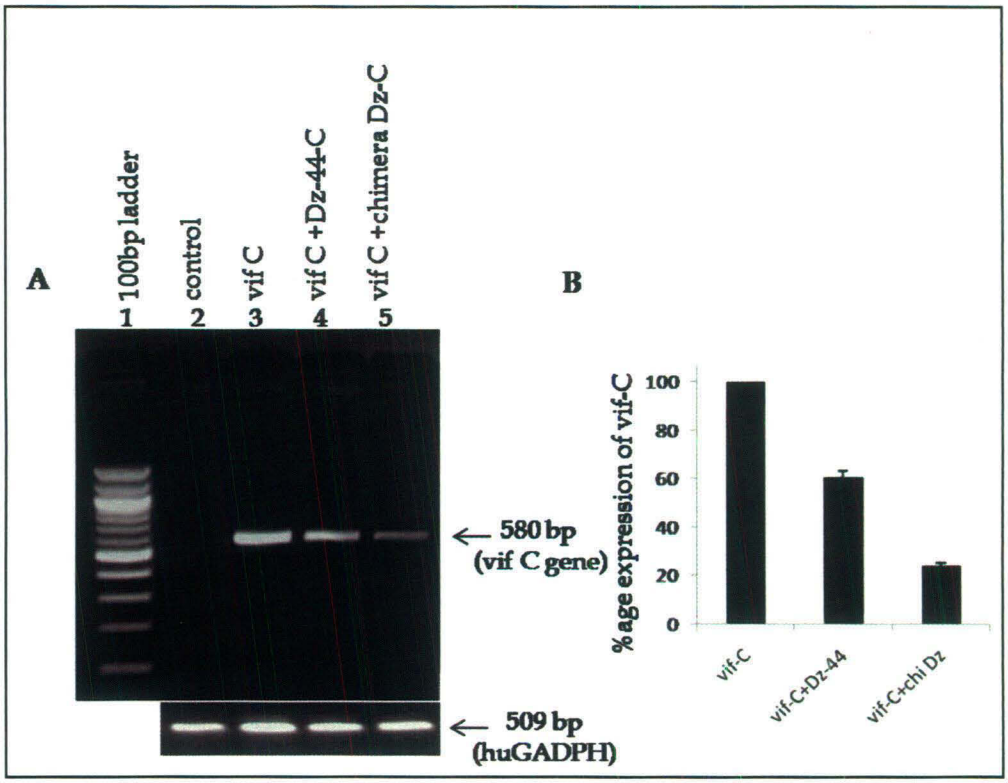


Figure 5.13: Effect Dz-44-C and chimeric-Dz-C on intracellular expression of vif-C

Intracellular inhibition of vif-B by Dz-472 and antisense ODN:

Dz-472 was a weak Dz that led to very poor suppression of vif-B when used in transient transfection studies in HEK 293 cell lines (Figure 5.14, panel A). *In vitro* cleavage analysis by Dz-472 in presence of upstream antisense ODN had showed an enhanced cleavage of the target RNA (Figure 5.8). So, transient transfections were done in HEK 293 cells to see if the presence of antisense ODN could enhance the cleavage efficiency of Dz-472 and thus decrease the expression of vif-B RNA by RT-PCR. As shown in Figure 5.14, in presence of antisense ODN (lanes 6 and 7), the expression of vif-B gene was further suppressed in comparison when Dz-472 was used alone (lane 5).

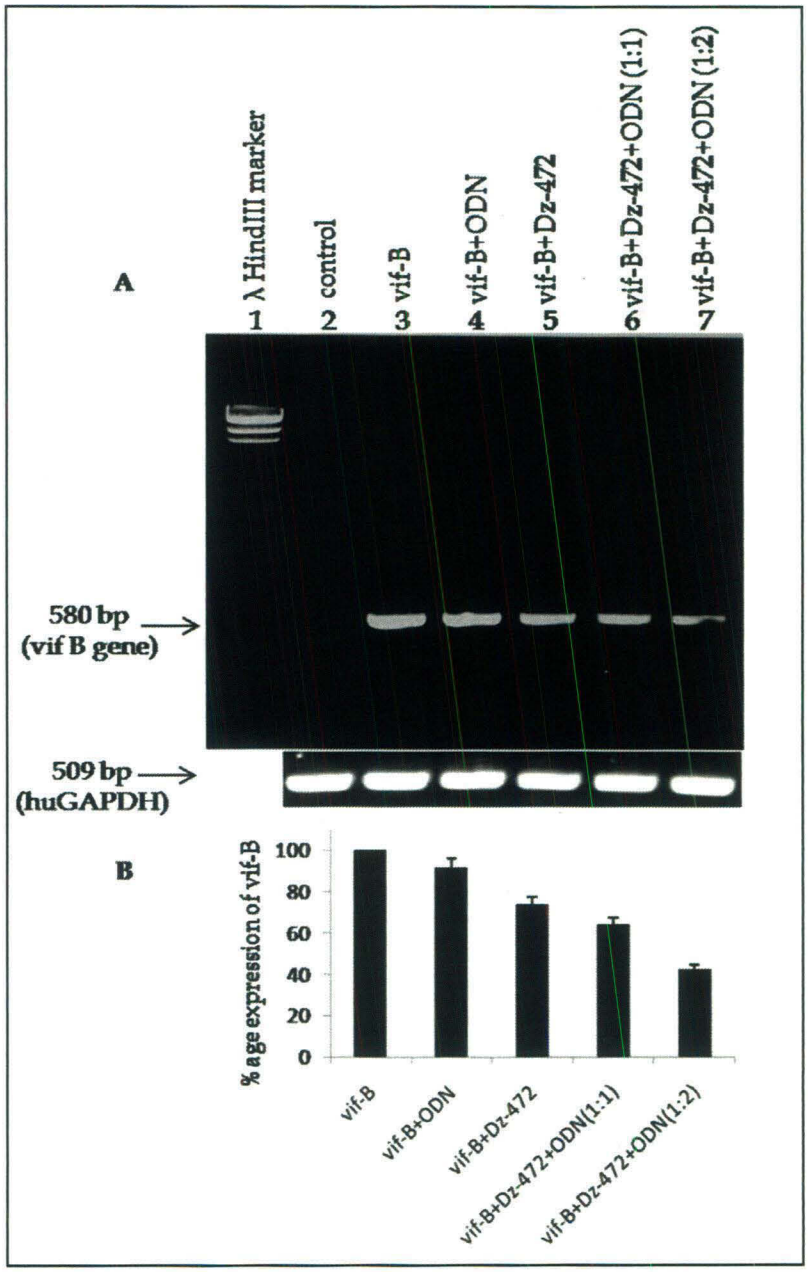


Figure 5.14: Intracellular effect of ODN on activity of Dz-472

Western blot analysis of vif-B protein in presence of various Dzs:

1 X 10^6 HEK 293 cells were grown in a 6 well plate and 1 µg of vif encoding plasmid DNAs along with 2 µg of both Dzs were co-transfected. The cell lysates prepared from equal number of cells were processed for total protein and subjected to western blot analysis as described before and the results are shown in Figure 5.15. Lane 1 represents control cells transfected with vector plasmid only. A prominent immune-reactive vif-B protein was observed (lane 3). About 2 folds reduction in the vif-B protein band was observed (lane 5) when cells were co-transfected with 1µg of pcDNA3-vif-B plasmid DNA and 2.0µg of Dz-44. Dz-44 was more effective than Dz-472 (compare lanes 5 and 4). When chimeric Dz (2.0µg) was used, a complete knock down of the vif-B protein was observed (lane 6). This is not due to unequal amounts of cell lysates that were processed because equivalent amounts of cells were used and same amounts of protein was loaded (as determined by colorimetric reaction with BCA reagent). Therefore, it could be concluded that the chimeric Dz-44-472 caused complete reduction in the levels of vif-B protein.

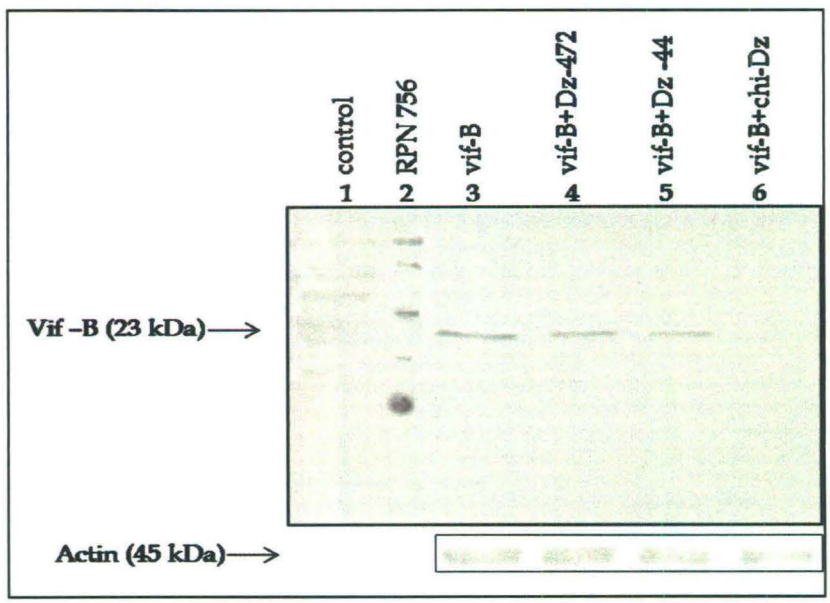


Figure 5.15: Effect of various Dzs on Vif-B protein expression

The various selective mutants were also designed to acheive a controlled suppression of the target RNA. Similarly, by transient transfections in HEK 293, the effect of the various mutants of chimeric Dz was checked on the intracellular expression of vif-B protein (Figure 5.16). Chimeric Dz was more potent (lane 5) as compared to the mutants as indicated (lanes 3, 4 and 6).

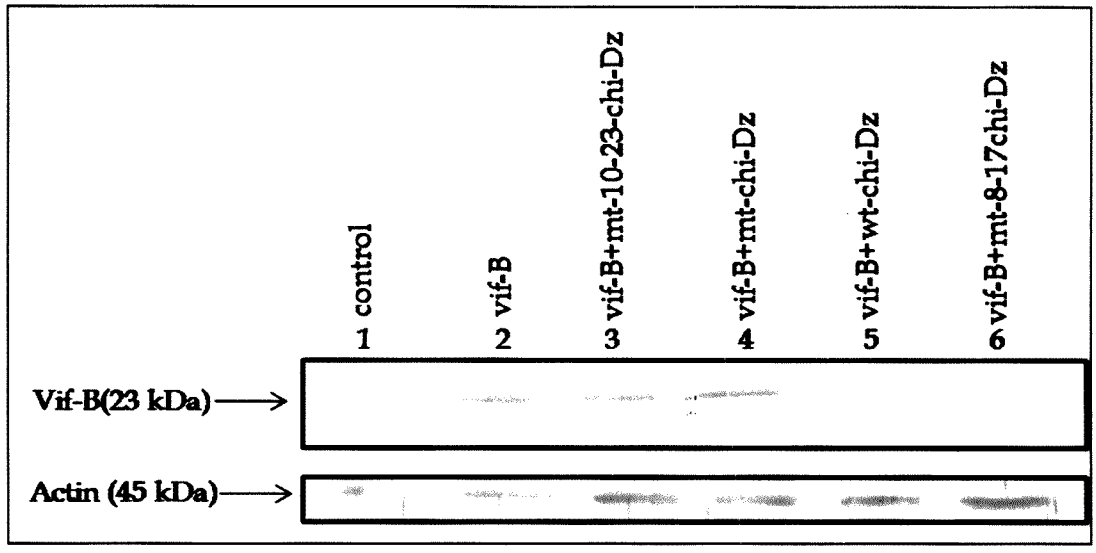


Figure 5.16: Western blot analysis of Vif-B expression in presence of various chimeric Dzs

Effect of vif-B and vif-C on expression of APOBEC3G:

There are lot of genetic sequence variations in the vif of subtype B and Indian isolate: subtype C. This also led to variations in the amino acid sequences of the vif protein encoded by the two subtypes (Figure 5.17). Transient transfections were performed to analyze the effect of these variations on the degradation of APBOBEC3G by vif protein. For this, 1 X 10^6 of 293-APOBEC3G-HA (obtained from NIH) cells were grown in a 6 well plate and 1 µg and 2 µg of each of pcDNA3-vif-B and pcDNA3-vif-C plasmid DNAs were transfected using lipofectin and incubated for 48 hrs. Whole cell lysate was prepared and subjected to western blot analysis. Figure 5.18, lane

1, represents control cells that show the expression of APOBEC3G and in the other lanes reduction in expression of APOBEC3G by vif-B and vif-C, is shown. The expression of APOBEC3G was more reduced in the presence of vif-C than vif-B. Vif-C, from the Indian isolate is significantly more powerful in its ability to neutralize the APOBEC3G activity when compared with subtype-B. This observation is very important from the point of view of HIV-1 mediated pathogenesis.

Vif B and C protein sequences

```
Vif-B   MENRWQVMIVWQVDRMRINTWKRLVKHHMYISRKAKDWFYRHHYESTNPKISSEVHIPLG  60
Vif-C   MENRWQVLIVWQVDRMKIRTWNSLVKHHMYVSRRANGWFYRHHYDSRHPKVSSEVHIPLG  60
        *******:*********:*.**:  *******:**:*:.*******:*  :**:*********

Vif-B   DAKLVITTYWGLHTGERDWHLGQGVSIEWRKKRYSTQVDPDLADQLIHLHYFDCFSESAI  120
Vif-C   EARLVIKTYWGLQTGERDWHLGHGVSIEWRLRRYNTQIEPGLADQLIHMHYFDCFADSAI  120
        :*:***.*****:*********:******* :**.**::*.*******:*******::***

Vif-B   RNTILGRIVSPRCEYQAGHNKVGSLQYLALAALIKPKQIKPPLPSVRKLTEDRWNKPQKT  180
Vif-C   RKAILGHIVIPRCDYQAGHNKVGSLQYLALTALIKPKKIKPPLPSIKKLVEDRWNNPQKI  180
        *::***:**  ***:*******************:******:********::**.*****:***

Vif-B   KGHRGSHTMNGH  192
Vif-C   RGRRGNHTMNGH  192
        :*:**.******

ACCESSION  AF324493 subtype-B
ACCESSION  AF067158 subtype-C
```

Figure 5.17: Comparison of protein sequences for vif-B and C

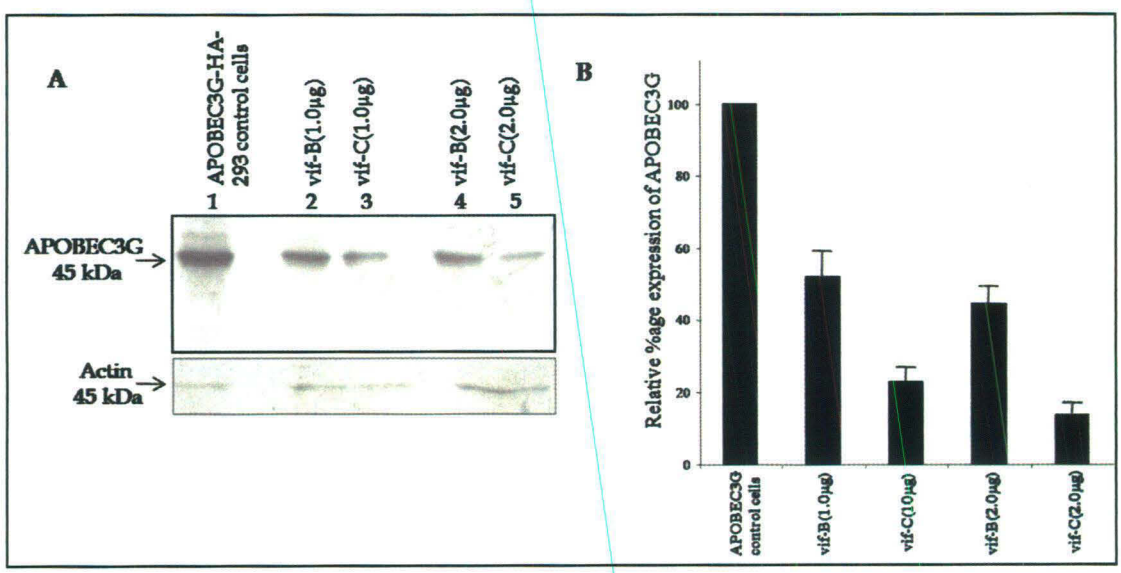


Figure 5.18: Effect of vif-B and C on APOBEC3G expression

Intracellular effect of Dzs on vif-B and on expression of APOBEC3G:

Plasmid pcDNA3-vif-B and Dz were co-transfected in 293-APOBEC3G cells and incubated for 48 hrs. The cell lysates prepared from equal number of cells were processed for total protein and subjected to western blot analysis as described in methods and methodology, and the results are shown in Figure 5.19. Lane 1 represents control cells expressing only A3G and lane 6 represents cells in which reduction in expression of A3G in presence of vif-B has occurred. As clear from lanes 2, 3 and 5 the various Dzs, designed against vif-B, have effect on expression of vif-B; as the expression of A3G is restored but to varying degrees. Dz-472 that has 8-17 catalytic motif did not show much effect on restoring the expression of A3G (lane 5). This correlates with intracellular efficacy of Dzs on vif RNA as was observed earlier (Figure 5.11A). The 10-23 motif containing Dz-44 has comparatively more effect on vif-B expression as approximately 75% of A3G is expression is restored (lane 2). The chimeric Dz-44-472 must have potently knocked off the expression of vif-B because A3G was almost completely restored in the presence of chimeric Dz (lane 3). As expected no significant change in the expression of A3G was not noticed when mutant chimeric Dz-44-472 was co-transfected (lane 4) along with vif-B plasmid (compared lane 4 with lane 6, in the latter only vif-B encoding plasmid was transfected). We conclude that chimeric Dz interfered with the known activity of vif very significantly and all mutant constructs were less effective as expected.

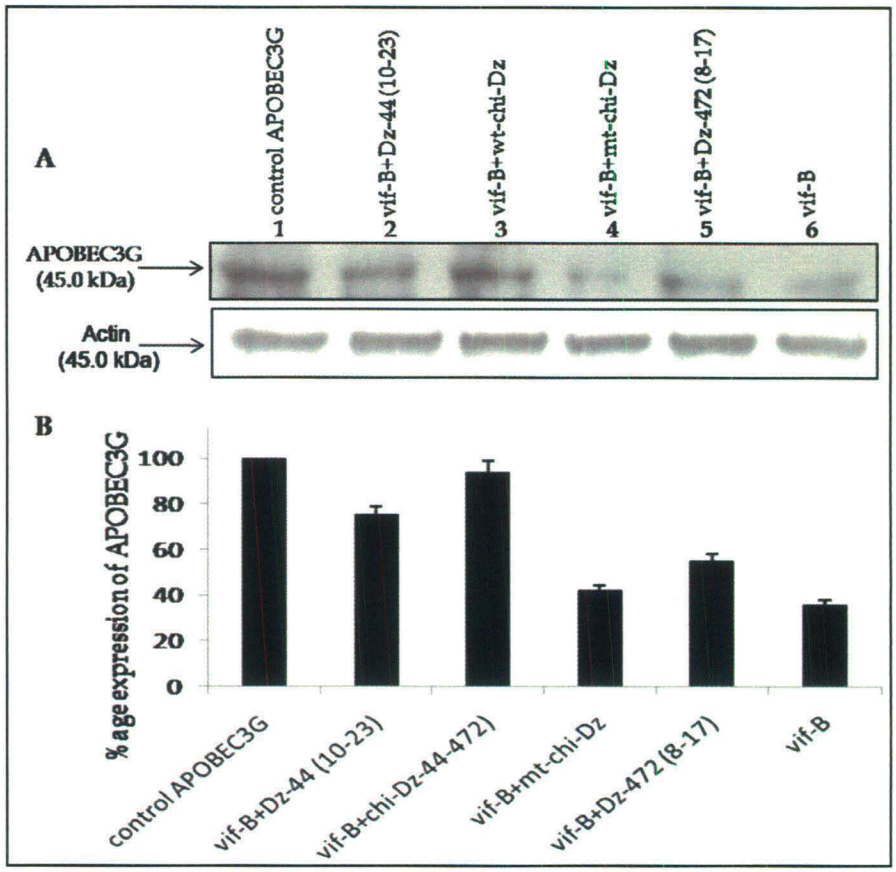


Figure 5.19: Western blot analysis to check the effect of Dzs on Vif-B & hence, on expression of APOBEC3G

Degradation of APOBEC3G by the fusion vif B/C and vif C/B genes:

Initially expression of chimeric vif-B/C and vif-C/B was checked by transfecting plasmid DNAs in which fused genes were cloned in HEK 293 cells. 48 hrs after transfection cell lysates were prepared and probed for vif expression, using polyclonal vif-B antiserum (obtained from NIH). As shown in Figure 5.20, panel A, fused plasmids expressed Vif-B/C (lane 2) and Vif-C/B (lane 3). Vif-C expression was not seen (lane 4) and this could be due to specificity of antiserum for Vif-B (lane 5).

1 X 10^6 293-APOBEC3G cells were plated 24 hrs prior to transfection in a 6 well plate. Plasmids encoding the fused genes of vif-B and vif-C (pcDNA3.1-vif B/C and pcDNA3.1-vif C/B) were transfected in 293-APOBEC3G-HA cells. Whole cell lysate was prepared after 48 hrs of transfection as described in methods and materials and estimated for total protein content by BCA reagent. Equal amount of total protein were loaded on 12% PAGE and then transferred to nitrocellulose membrane. The blot was then probed with rabbit polyclonal anti APOBEC3G antiserum, which was obtained from the AIDS Repository of National Institutes of Health (Bethesda, Maryland, USA).

Vif-C showed more degradative effect on APOBEC3G expression (Figure 5.20, panel B, lane 3) in comparison to that of vif-B (lane 2). Lane 1 is the control cells that express APOBEC3G. Vif B/C (lane 4) caused almost equivalent reduction in the expression of APOBEC3G when compared to vif C (lane 3). The degradation of APOBEC3G by vif C/B (lane 5) was almost equivalent to vif B (lane 2).

We conclude from this experiment that vif C is more potent in degrading APOBEC3G in comparison to vif B and the major determinant for the activity resides in the C-terminal fragment of vif-C (291-580 nts).

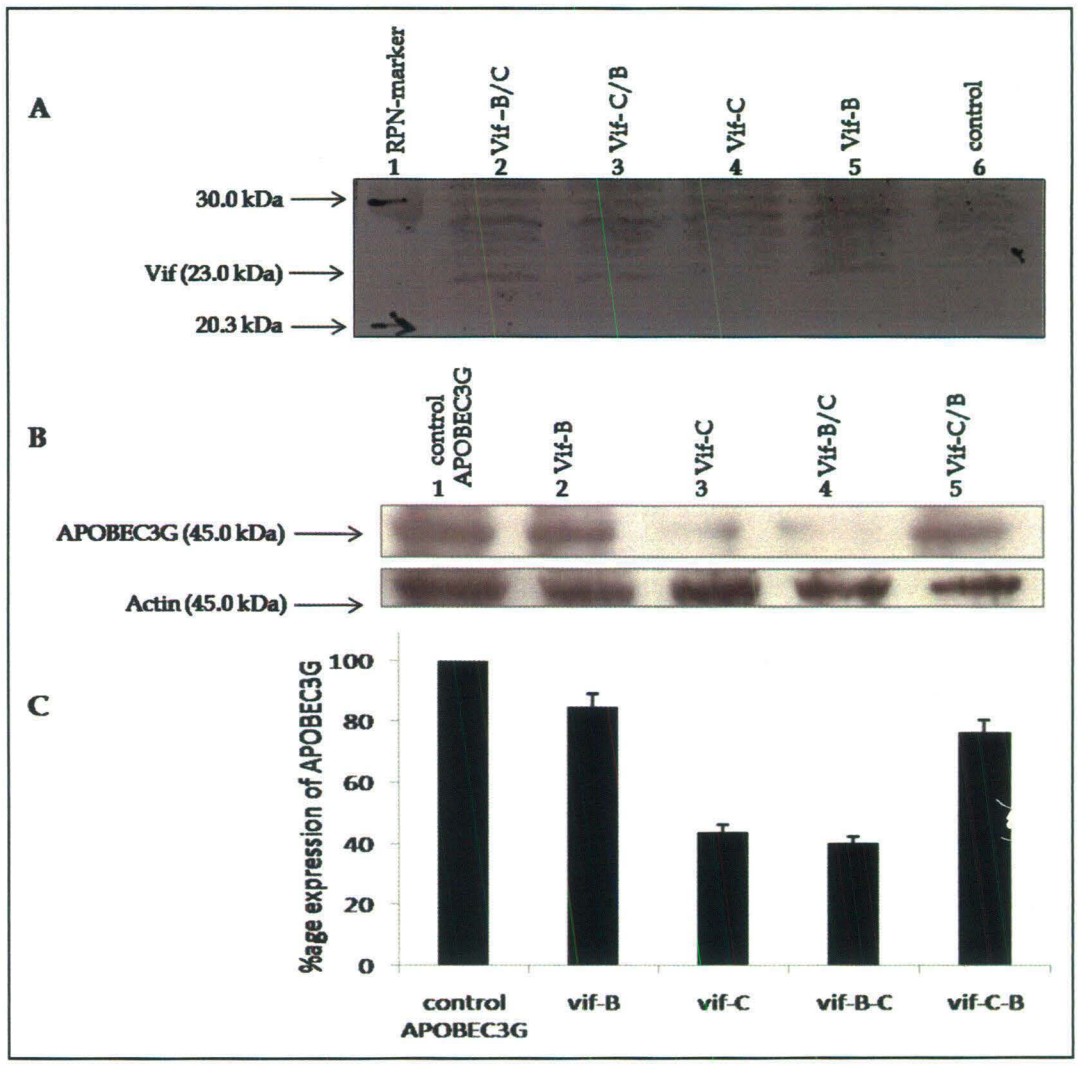


Figure 5.20: Expression of chimeric vif-B/C & C/B and their effect on the expression of APOBEC3G

DISCUSSION

In the present work, we have identified a very potent Dz (Dz-44) which cleaved the target RNA very efficiently. This Dz was targeted against a conserved region of Vif B RNA and possessed a 10-23 catalytic motif. We also designed an 8-17 catalytic motif containing Dz (Dz-472) which showed poor cleavage activity. We clearly show that in presence of specific ODN that was targeted to hybridize immediately upstream of the hybridizing arm of the Dz-472, significant augmentation of cleavage of the target RNA was achieved. Most of the in vitro cleavage activity of the Dzs correlated with their intracellular efficacy.

In order to target more than one region in the Vif RNA, we, for the first time, assembled a di-Dz that consisted of the above two Dzs that were arranged in direct tandem with no spacer sequence. This di-Dz also possessed potent in vitro cleavage activity and intracellular efficacy. Remarkably this Dz retained the cleavage activity of the two mono-Dzs(Dz-44 & 472). Cleavage studies carried out in presence of increasing amounts of Mg++ clearly indicated that catalytic activity increased with higher concentrations of it. The same was true when Mg++ was replaced with Ca++. This property of Mg++ dependent increase of catalytic activity is in common with earlier described several Dzs. Cleavage activity of Dzs in the range of 1 – 2mM $MgCl_2$ is an important property because it is close to physiological concentration.

One remarkable finding that came out of this cleavage study was that small amount of Mg++ was necessary for the stability of Vif RNA. The very fact that Dz-44 could also cleave (albeit poorly) the Vif C RNA, clearly indicated that some mismatches are tolerated in the hybridizing arms of a potent Dz.

We then tested the efficacy of Vif specific Dzs to interfere with the known function of Vif proteins, that is their ability to specifically degrade APOBEC proteins. It is clear from our data that Vif C was more potent in causing the degradation of APOBEC3G when compared with Vif B. This is not entirely surprising because there are several amino acid changes between Vif B and Vif C. Our studies with Vif-B/C amd Vif-C/B chimeras clearly indicated that major determinants for this degradation lies in the C-terminal portion of the Vif gene. Our Dzs interfered with this function very effectively, and here also, the 8-17 containing Dz failed to exert any effect which correlates with its in vitro cleavage activity.

CHAPTER VI

Novel bi-specific siRNA-Ribozyme (siRNA-Rz) constructs against X gene of Hepatitis B virus

RNA interference (RNAi) is a mechanism that inhibits gene expression at the stage of translation or by hindering the transcription of specific genes. RNAi targets include RNA from viruses and transposons (significant for some forms of innate immune response), and also plays a role in regulating development and genome maintenance. Small interfering RNA strands (siRNA) are key to the RNAi process, and have complementary nucleotide sequences to the targeted RNA strand. Specific RNAi pathway proteins are guided by the siRNA to the targeted messenger RNA (mRNA), where they "cleave" the target, breaking it down into smaller portions that can no longer be translated into protein.

The RNA interference pathway is often exploited in experimental biology. Small interfering RNA technology allows one to interfere strongly with the gene expression of variety of genes and is currently favored over any other RNA based approaches as it is known to be effective in the presence of very small amounts (Rossi, 2006). It is an evolutionary conserved mechanism and its role has been identified in many cellular and developmental programs (Scherer and Rossi, 2004; Martim and Caplein, 2007). In this objective, the focus is on the X gene product (HBx) which is known to activate several cellular and viral promoters including the HIV-1 long terminal reporter (LTR) promoter (Robinson, 1994; Gomez-Ganzalo et al, 2001). Although it is strongly associated with the formation of hepatocellular carcinoma (HCC), the exact role of HBx protein in HCC is not understood. It is very likely that the intracellular levels of HBx protein may be critically important for causing cytopathogenesis. Therefore, it is important to develop antiviral approaches that allow one to modulate the expression of the target gene.

Several nucleic acids based antiviral approaches are currently available to achieve gene-specific suppression. They include Rzs, Dzs, antisense RNA or DNA, aptamers and decoy RNAs (Akkina et al, 2003). Catalytic nucleic acids (Rz and Dzs) have both been used extensively to knock out a target gene expression individually and it is possible to obtain both Rz and Dz-specific cleavage under same *in vitro* and intracellular conditions, as studied in the first objective. Furthermore, they acted synergistically inside a mammalian cell and potently knocked down the target gene expression. Very often, the target sequences for Rz, Dz or siRNAs are not accessible due to secondary structures (Cairns et al, 1999; Schubert et al, 2004). It is becoming increasingly clear that some sequences may be more prone to cleavage with either Rz, Dz or siRNAs which needs to be experimentally determined (Kurreck et al, 2002). Earlier constructs with antisense RNA and multimeric Rzs were used against HIV-1 (Ramezani et al, 2006). Very recently siRNA against an HIV-1 gene along with anti-gp120 aptamer was shown to have potent anti-HIV-1 activity (Zhou et al, 2008).

Several mono- (Martinez et al, 2002) and bi-specific siRNA constructs, as originally described by Anderson and Akkina, have been described earlier which targeted CD4, CXCR4, and CCR5 (Anderson et al, 2003). The unique feature of this bi-specific siRNA construct is that the two small-hairpin siRNAs were joined together by an intracellular cleavable linker as originally described (Sioud and Leirdal, 2002) and impressive inhibition of HIV-1 replication was observed. In this study, we designed a construct which combined an earlier described potent hammerhead Rz against the X gene of hepatitis B (Goila and Banerjea, 2004) virus with a small hairpin siRNA against the same gene using the identical short cleavable linker (henceforth called unique bi-specific construct). It is shown in this study that these constructs interfered strongly with the expression of target gene expression both at RNA and protein levels. Furthermore, by disabling either Rz or

siRNA, it was possible to modulate the intracellular gene expression in a controllable manner.

EXPERIMENTAL PROCEDURES

Cloning of HBx gene and in vitro transcription

Plasmid pSG5.HBx encoding the X gene of HBV (Kumar et al, 1996) was a kind gift from Vijay Kumar, ICGEB, New Delhi, India. The entire gene is placed under SV40 and T7 promoters. The former promoter is used for intracellular expression and the latter was used for obtaining *in vitro* transcripts using T7 RNA Polymerase. After linearization with appropriate enzyme at the 3'-end, full-length 465 nucleotides long X RNA was synthesized.

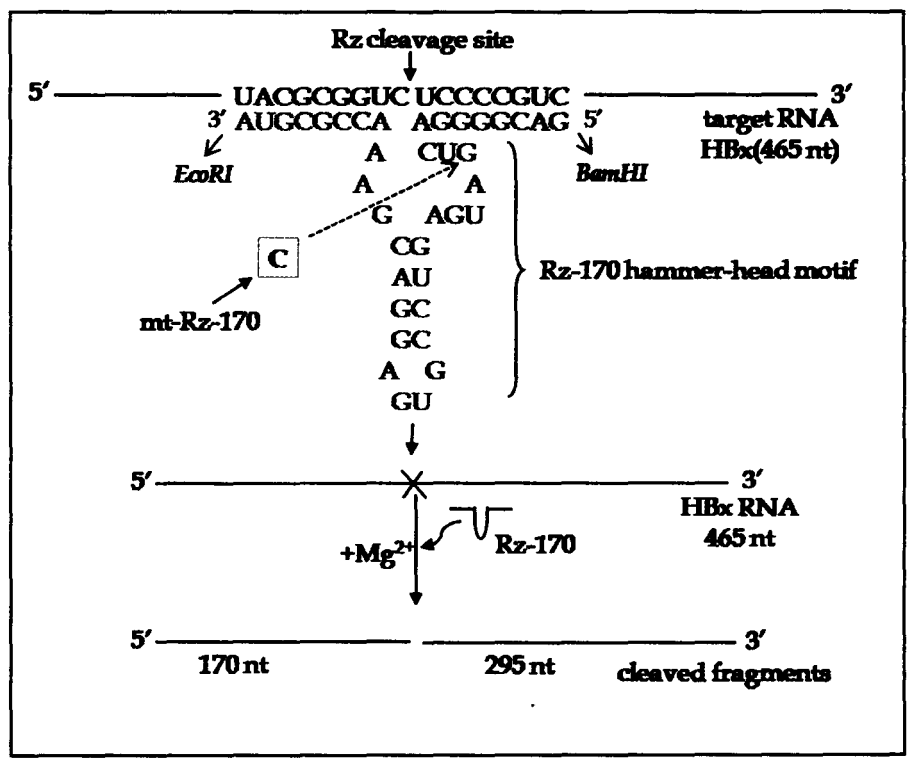


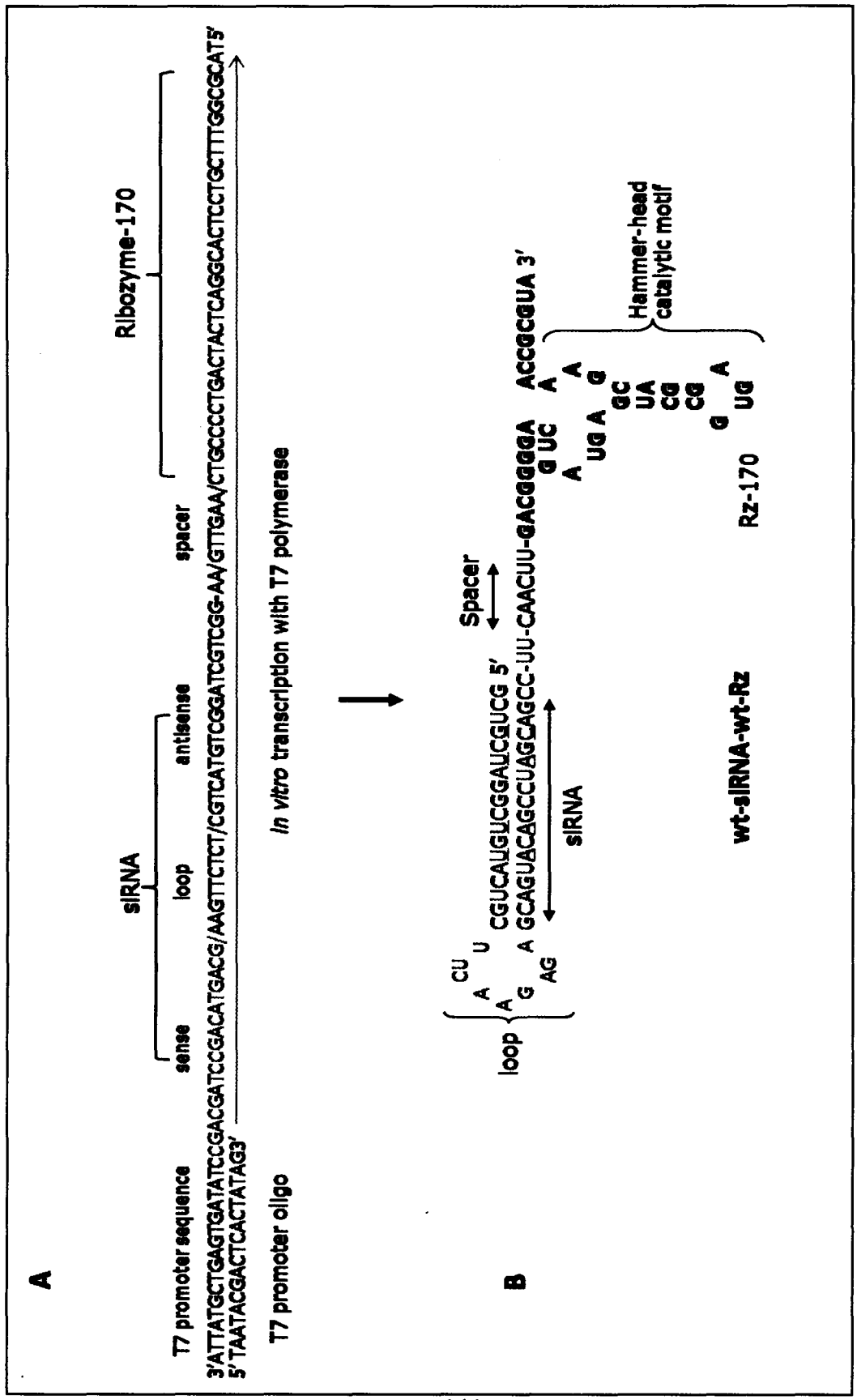
Figure 6.1: Cleavage pattern of HBx mRNA by Rz-170

Construction and cloning of HBx-Rz-170 targeted against X gene

The construction of Rz-170 and its mutant versions (Figure 6.1) has been described in detail previously (Goila and Banerjea, 2004). It possessed the hammerhead motif and targeted against the **'GUC'** sequence. *Eco RI* and *Bam HI* restriction sites were engineered at the ends of the hybridizing arms of the Rz which facilitated cloning it into pcDNA3 expression vector, under T7 and CMV promoter.

Construction of chimeric-si-RNA-Rz construct against HBx gene

For making the bi-specific construct, an oligonucleotide was synthesized that consists of Rz-170, a short spacer (Sioud and Leirdal, 2002) followed by the small hairpin type of siRNA that ends with the complementary sequences for the T7 primer (Figure 6.2, panel A). To this oligonucleotide, the T7 primer was hybridized and the desired RNA was synthesized *in vitro* using T7 RNA Polymerase as described earlier (Donze and Picard, 2002) using the transcription kit (henceforth this is referred to as wt chimeric construct (Figure 6.2, panel B). A four point mutant version of the si-RNA was made which retained the normal sequences of the Rz (Figure 6.2, panel C shown as bold and underlined– henceforth referred to as mt-siRNA-wt-Rz or chimeric mutant -1). In the other construct, siRNA portion matched the wt construct but the Rz was disabled by changing a single nucleotide (G to C, shown by an arrow- henceforth referred to as wt-siRNA-mt-Rz or chimeric mutant-2, panel D) in the hammerhead catalytic motif. Another mutant version was designed in which both the siRNA portion and the Rz-170 were mutated by introducing the required changes in siRNA and Rz sequences, panel E, explains the construction of mt-siRNA-mt-Rz or chimeric mutant-3 (completely disabled). *In vitro* synthesis of these RNAs indicated that they were of correct size.


A
siRNA
Ribozyme-170
T7 promoter sequence
sense
loop
antisense
spacer
3'ATTATGCTGAGTGATATCCGACGATCCGACATGACG/AAGTTCTCT/CGTCATGTCGGATCGTCGG-AA/GTTGAA/CTGCCCCTGACTACTCAGGCACTCCTGCTTTGGCGCAT5'
5'TAATACGACTCACTATAG3'
T7 promoter oligo
In vitro transcription with T7 polymerase
B
loop
CU
A
A
G
U
AG
CGUCAUGUCGGAUCGUCG 5'
A GCAGUACAGCCUAGCAGCC-UU-CAACUU-GACGGGGA ACCGCGUA 3'
Spacer
siRNA
G UC
A
UG A g
gc
UA
cg
cg
g A
UG
wt-siRNA-wt-Rz
Rz-170
Hammer-head
catalytic motif

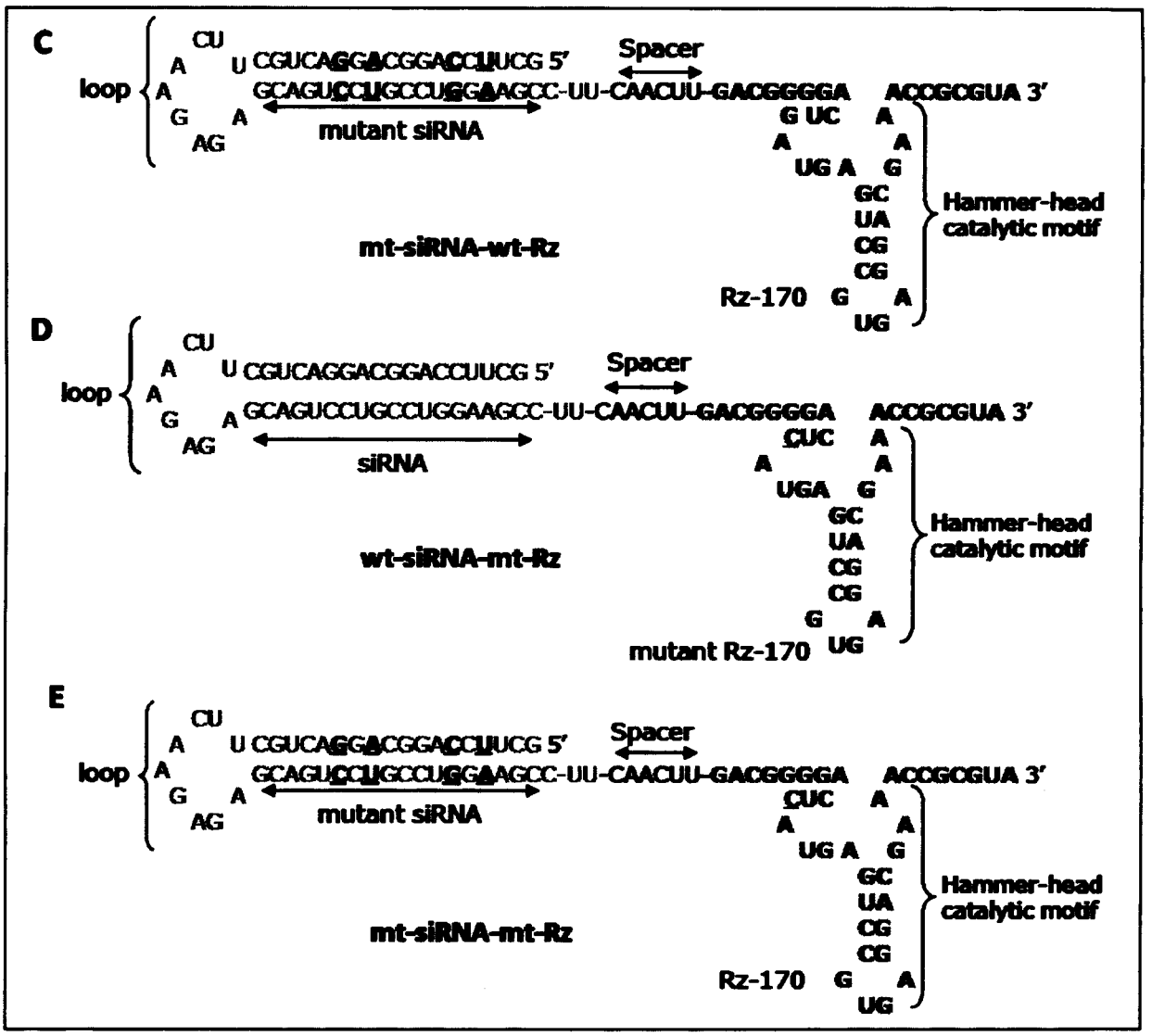


Figure 6.2: Designing of various bi-specific constructs

In vitro cleavage of target RNA with Rz

In vitro transcription of the linearized plasmid DNA was carried out in the presence of labeled ^{32}P UTP using T7 RNA Polymerase. The cleavage reaction was initiated by adding equimolar amounts (100 pmoles each) of the labeled target RNA and unlabeled Rz in a reaction buffer containing 50mM Tris-HCl, pH 7.5, in a volume of 10µl. The reaction mixture was heated

briefly at 94°C and the cleavage reaction was initiated by adding $MgCl_2$ (final concentration 10mM) at 37°C for 2 hours. The cleaved RNA fragments were resolved on 7 M urea-6% PAGE and subjected to autoradiography.

Cleavage of wild type (wt) chimeric siRNA-Rz in the presence of mammalian cell extracts

Cytoplasmic protein extract was prepared from 293 cells. Briefly the cells were washed twice with PBS and lysed at 4°C with lysis buffer (0.2% NP40 in PBS and protease inhibitors). After 20 min of incubation, cell lysate was centrifuged for 5 min at 10,000 rpm and supernatant was collected and used as cytoplasmic protein extract for the cleavage of labeled chimeric RNAs. *In vitro* transcribed siRNA-Rz-170 RNA were obtained from these oligonucleotides and T7 primer using T7 RNA polymerase in presence of α-P^{32} rUTP as described in methods and materials. After transcription, RNA was purified using chloroform and phenol and stored at -70°C. Internally labeled siRNA-Rz-170 RNA was incubated with or without cellular protein extracts for 2minutes at room temperature, treated with phenol/chloroform mixture and analyzed on 15% polyacrlamide gel-7M urea followed by autoradiography.

Intracellular decrease in HBx RNA by wt and mutant chimeric constructs

2 µg of HBx specific chimeric constructs were co-transfected with 1 µg of HBx encoding plasmid DNA and the levels of target RNA was monitored by RT-PCR. All the plasmids were Qiagen column purified and the *in vitro* synthesized RNAs showed no toxicity under the experimental conditions. The cell lystaes were prepared 48 hours after transfection. Several dilutions were initially made to determine the linear range for PCR-amplified products. Total RNA was isolated using trizol (Invitrogen) following the procedure described by the manufacturer and divided into two equal sets.

One set was used for estimating the levels of full-length X RNA using HBx1 and HBx2 primers and the second set was used for estimating the levels of the house keeping gene, human glyceraldehyde phosphodehydrogenase (huGAPDH) as described earlier (Paik et al., 1997). The following primers were used for estimating the intracellular levels of full-length X RNA.

Forward primer (HBx1):

5′ TTAGGCAGAGGTGAAAAAGTTGCATGGTGCTGG 3′

Reverse primer (HBx2):

5′ ATGGCTGCTAGGCTGTACTGCCAACTGGATCCTTCG 3′

Western Blot analysis

1 x 10^6 HepG2 cells were grown to 80% confluency in a six well plate. They were transfected with 1µg of HBx encoding DNA (pSG5-HBx) with 1µg of unrelated RNA or in combination with 1µg of the wt chimeric construct in a final volume of 1ml for 48 hours using lipofectin. Cell lysates were prepared and equal amount of protein was loaded in each lane after estimating total protein by BCA reagent. They were divided into two equal parts; one was used for estimating the levels of HBx protein and the other for the actin protein (control). They were subjected to gel analysis and transferred on to a Nitrocellulose membrane. Rabbit polyclonal antibody to HBx (Biovendor, NS, USA) was used as a primary antibody in 1:500 dilution in PBS pH 7.2 containing 0.1% Tween 20. Polyclonal antibody to actin (Sigma) was used to determine the levels of the control protein.

RESULTS

In vitro synthesis of full-length HBx RNA

Plasmid pSG5-HBx was linearized with *Bgl II* restriction enzyme present immediately downstream of the cloned gene in the multiple cloning site as described earlier (Goila and Banerjea, 2001). *In vitro* transcription was carried out using T7 RNA polymerase based transcription kit. HBx RNA of 465 nts was transcribed (Figure 6.3, lane1).

Construction of Rz-170 and sequence-specific cleavage of HBx RNA

Hammerhead Rz-170 was targeted to cleave **'GUC'** sequence present in the target RNA. The conserved catalytic motif was flanked by 8 nts long hybridizing arms leaving the **'C'** residue unpaired in this design. When the full length target RNA (465 nts long) was subjected to cleavage with Rz-170 in the presence of Mg^{2+}, specific cleavage products (170 and 295 nts long fragments) were expected (Figure 6.1). Specific cleavage of the target RNA is shown in Figure 6.3, lane 2. Lane 1 shows the synthesis of the full-length 465 nts long target RNA (HBx) as expected. Mutant Rz-170 that was created by introducing a single nucleotide change (G to C) in the catalytic motif, failed to show any cleavage as seen in lane 3.

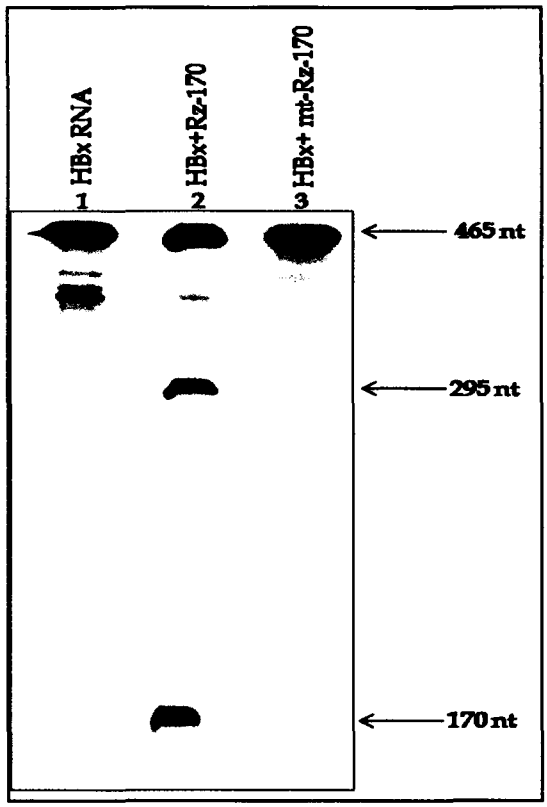


Figure 6.3: *In vitro* cleavage of X RNA by Rz-170

In vitro synthesis of wild type and mutant si-RNA-Rz-170 constructs

The exact sequence of the oligonucleotide that encodes for Rz-170 and siRNA against the HBX gene is shown in Figure 6.2, panel A. It is noteworthy that the 3'-end of this oligonucleotide contains the complementary sequence of the T7 promoter. This allows for rapid *in vitro* synthesis of target RNA in large amounts using T7 RNA Polymerase. Altogether, four constructs were made, namely the wild type chimeric construct with wt-siRNA and wt-Rz-170 (wt-siRNA-wt-Rz-170); mutant-1 with wt-siRNA and mutant Rz-170 i.e., wt-siRNA-mt-Rz-170; mutant-2 with wild type Rz-170 and mutant si-RNA, named as mt-siRNA-wt-Rz-170; and mutant-3 with siRNA and Rz-170 both mutated, mt-siRNA-mt-Rz-170 (Figure 6.2).

In vitro cleavage of bi-specific constructs in the presence of cellular extracts

The transcripts were generated under *in vitro* conditions with equal efficiencies and cleaved by the cellular extracts (CE) at the spacer sequence to generate free siRNA and Rz-170, as shown in Figure 6.4. Lane 1 corresponds to the control wt-siRNA-wt-Rz-170 which was not treated with cellular extract. In lanes 2, 3 and 4, wt-siRNA-wt-Rz-170, mt-siRNA-wt-Rz-170 and wt-siRNA-mt-Rz-170 were respectively loaded after being incubated with cellular extracts for 2 minutes as described in the procedures. The results showed the cleavage of the bi-specific constructs and fragments of expected size (52 and 40 nts) were detected.

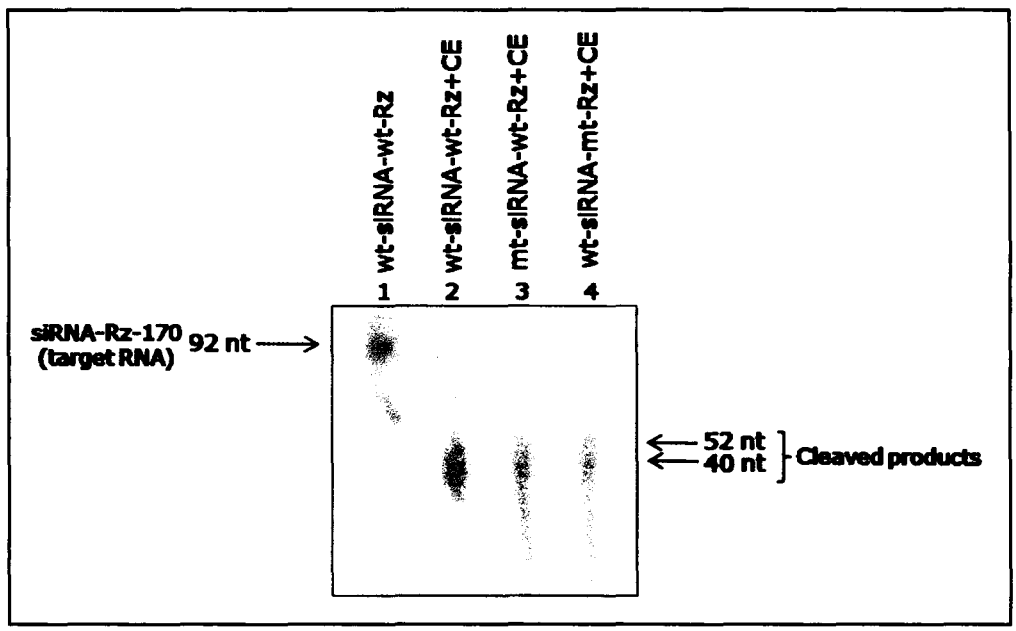


Figure 6.4: Cleavage in presence of cellular extract

In vitro cleavage of HBx RNA by various bi-specific constructs and comparison with Rz-170

The various chimeric constructs were checked to see if they retained the catalytic activity of the Rz when linked to siRNA. For this labeled X RNA was incubated with RNA of the bi-specific constructs in 50 mM Tris-HCl, pH

7.5 and 10 mM $MgCl_2$ at 37°C for 2 hours. The results are shown in Figure 6.5 in which lane 1 is control HBx RNA and in lane 2, X RNA was subjected to the treatment of Rz-170 RNA which resulted in generation of cleaved fragments of sizes 295 and 170 nts. In lanes 3 and 4, HBx RNA was treated with wt-siRNA-wt-Rz-170 and mt-siRNA-wt-Rz-170, respectively. Rz-170 specific cleaved fragments were seen. But when the construct wt-siRNA-mt-Rz-170 RNA was used to cleave X RNA, no Rz-170 specific cleaved fragments were seen. Similar results were obtained when bi-specific construct in which both siRNA and Rz-170 (mt-siRNA-mt-Rz-170) were mutated. No Rz-170 specific cleavage products were seen when HBx RNA was incubated with mt-siRNA-mt-Rz-170 (lane 6). We conclude that all the chimeric constructs that possess active Rz-170 catalytic motif retained the ability to cleave the target RNA.

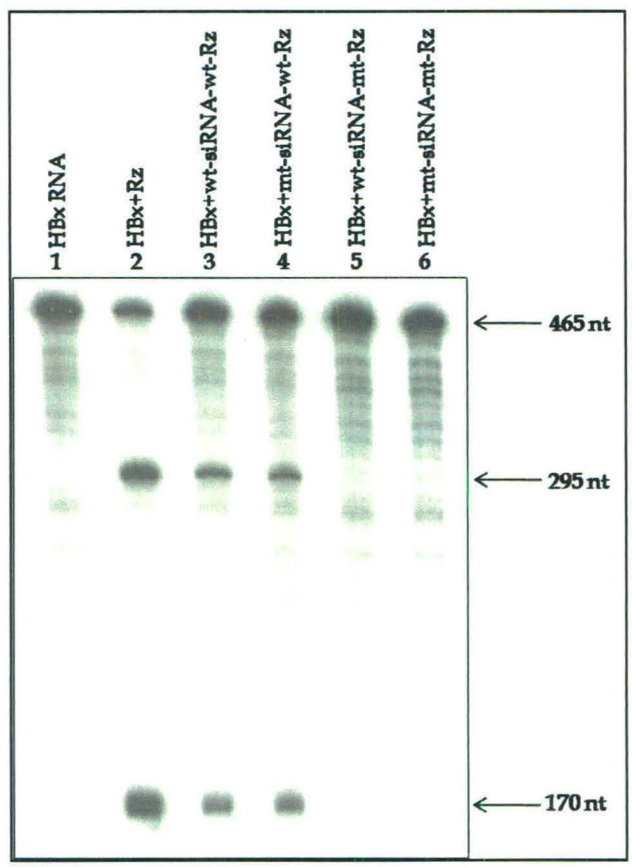


Figure 6.5: In vitro cleavage of HBx RNA by the bi-specific constructs

Modulation of intracellular target gene expression by wild-type and mutant constructs

HEK 293 or HepG2 cells were co-transfected with either 1µg of HBx encoding DNA along with 1µg of unrelated RNA of same length (Figure 6.6 A, lane 2) in the final volume of 1ml or in presence of 1µg of wt-siRNA-wt-Rz (wt chimeric) (lane 1); 1µg each of mt-siRNA-wt-Rz (lane 3) and 1µg of wt-siRNA-mt-Rz (lane 4). RT-PCR was performed using HBx specific primers to check the efficiency of various bi-specific constructs. Prominent HBx specific gene expression was observed in lane 2 as expected. The wt chimeric construct transfected cells showed about 68% reduction (compare lanes 1 & 2). On the contrary, mt-siRNA-wt-Rz (chimeric mutant-1) transfected cells showed about 31% reduction (lane 3) and wt-siRNA-mt-Rz (chimeric mutant-2) transfected cells showed about 61% reduction (lane 4) in the expression of HBx RNA. It could thus be concluded from this experiment that most of the intracellular reduction in the target RNA was due to the siRNA component of the chimeric construct. The levels of the control RNA (huGAPDH) remained same in all the corresponding lanes which suggest that chimeric constructs down regulated the target gene expression in a specific manner.

The effect of Rz-170 RNA alone on the intracellular expression of HBx was then compared with the wt-chimeric construct by RT-PCR. The results are shown in Figure 6.6, panel C. In comparison to Rz-170 alone (lane 3) that could reduce X RNA expression only by 20%, the bi-specific construct worked more potently in reducing the expression of HBx RNA, by more than 65% (lane 5). As expected in presence of mt-siRNA-mt-Rz construct no significant reduction in the expression of HBx RNA was observed (lane 4).

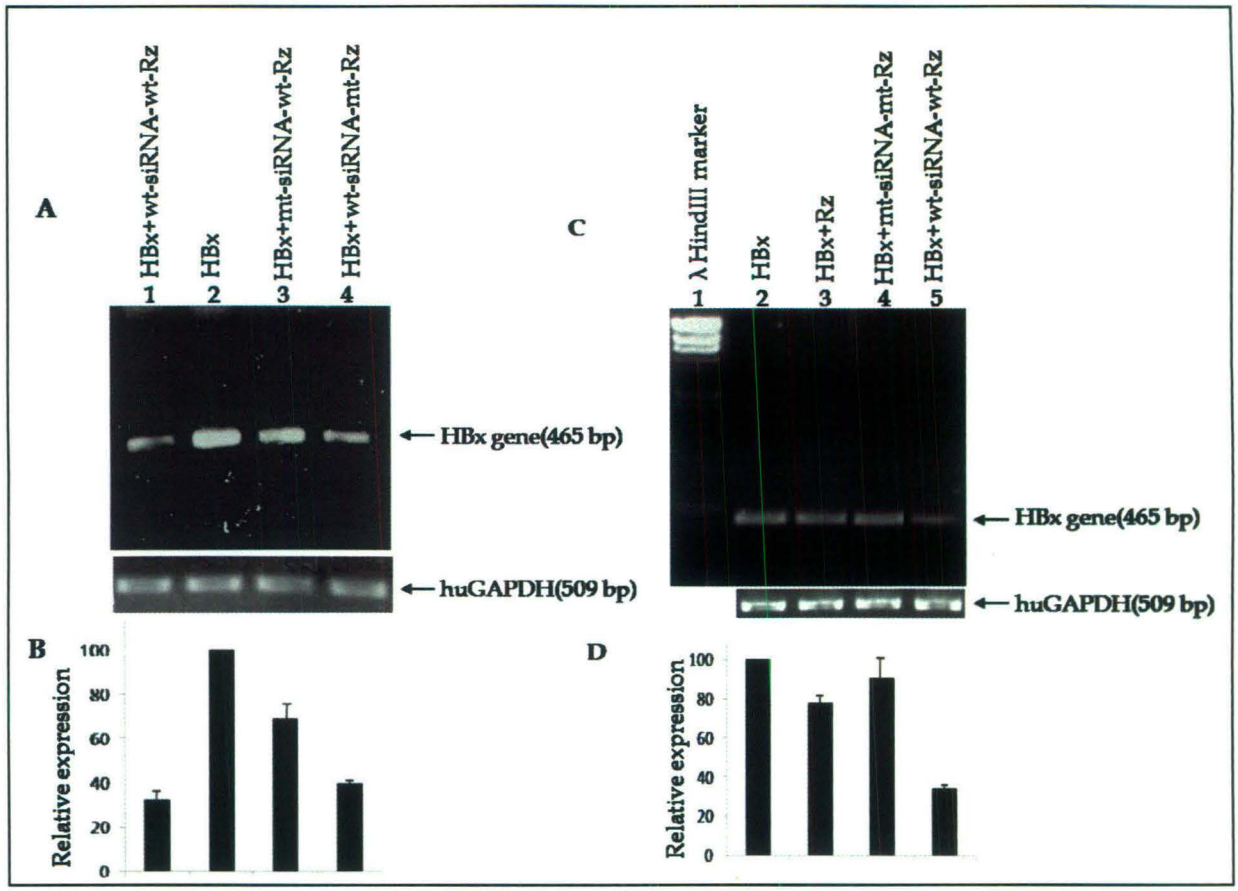


Figure 6.6: Effect of chimeric constructs on intracellular expression of HBx RNA

Northern blot analysis to measure the effect of various chimeras on intracellular expression of HBx RNA

Total RNA was isolated from HEK 293 cell lines after 48 hrs of trasfection with various constructs using trizol. ^{32}P labeled antisense HBx mRNA was *in vitro* transcribed using T7 RNA Polymerase. For this antisense HBx sequence containing plasmid DNA was linearized with *Bam HI* and subjected top transcription. This probe was then used to hybridize with the nylone membrane to which total RNA was transferred. For ensuring the equal loading, the formamide agarose gel was also EtBr stained to for 23s and 18s rRNA (control RNA). As shown in Figure 6.7, lane 1 is only pSG5-HBx transfected cells. In lane 2, wt-siRNA-wt-Rz RNA was co-transfected

along with HBx DNA. In this lane less than 20% HBx mRNA was observed which suggested that the wild type construct was very potent in reducing the intracellular levels of X RNA. Varying degree of reduction in X mRNA was observed when selective mutant chimeras were co-transfected (lanes 3, 4 and 5). These are representative data from separate experiments (mean ± S.D.)

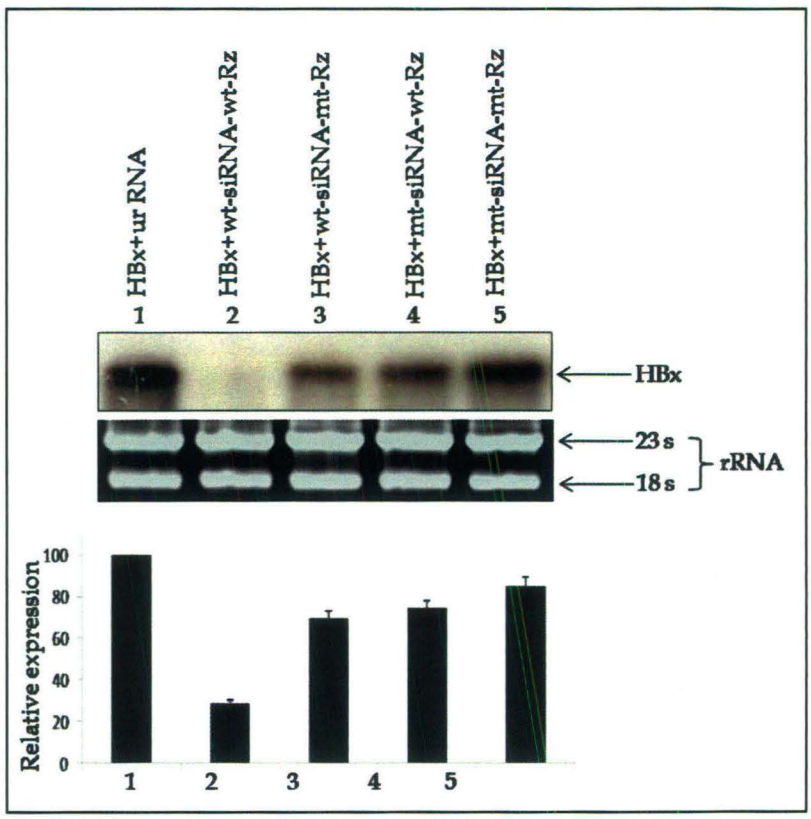


Figure 6.7: Northern blot analysis

Wild-type chimeric construct potently knocks down HBx protein expression in mammalian cell lines

Hep G2 cells when transfected with HBx encoding DNA, a prominent 16.5 kDa immuno-reactive band was observed (Figure 6.8, lane 1) by western blot analysis. Cells that were co-transfected with wt-siRNA-wt-Rz along with equal amounts of HBx encoding DNA (1µg/ml) exhibited

significantly reduced expression (lane 4). Quantitation of the target RNA in corresponding lanes is shown in panel B. Cells that received mt-si-RNAi-wt-Rz reduced the expression by 20% (lane 2) and wt-si-RNA-mt-Rz caused about 45% reduction (lane 3). Chimeric construct mt-si-RNA-mt-Rz showed only about 5% reduction when compared with control (lane 5). This reduction of target gene is specific because the levels of control actin protein in all the corresponding lanes remained constant (representative data from three different experiments).

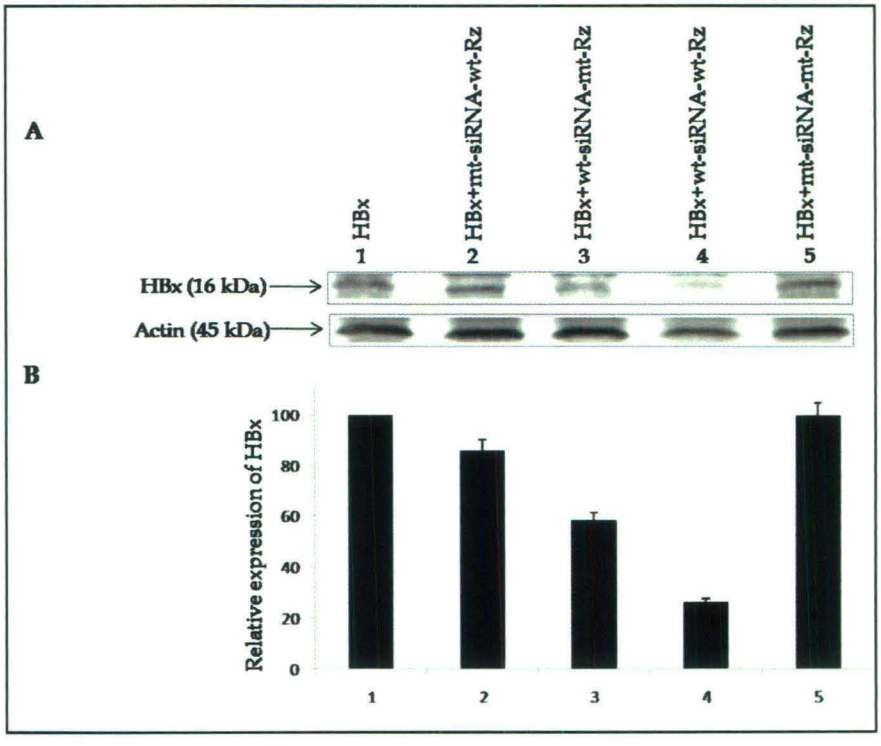


Figure 6.8: Western blot analysis

Wild-type and mutant constructs interfere with the X protein mediated transactivation function in a differential manner

The HBx protein is known to activate a number of cellular promoters including the HIV-1 LTR promoter (Robinson, 1994) and (Gomez-Gonzalo et

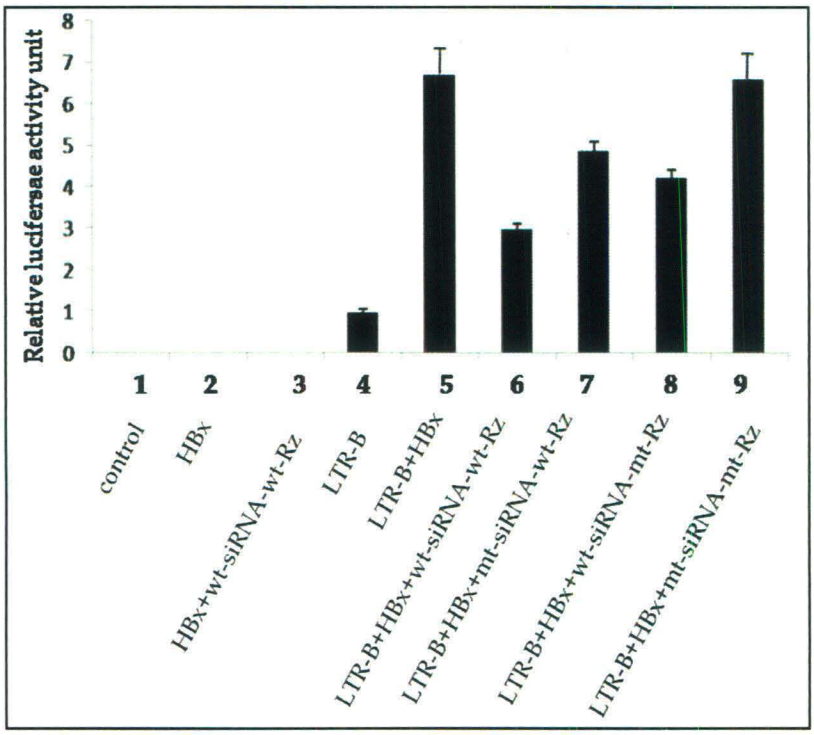


Figure 6.9: Effect of chimeric constructs on HBx transactivation activity

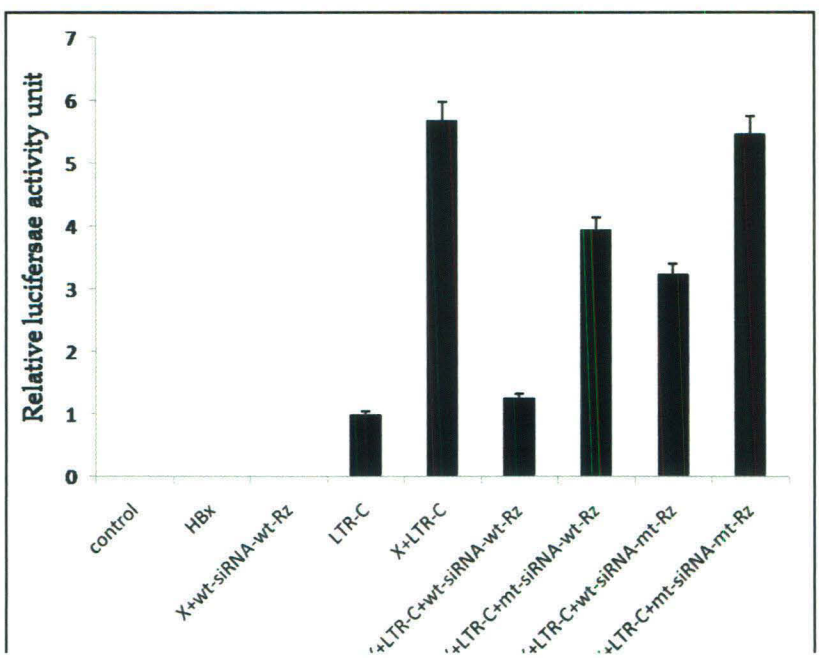


Figure 6.10: Inhibition of transactivation of LTR-C by HBx in presence of chimeric constructs

al., 2001) and in the presence of wt or mutant chimeric construct against the X gene, reduced activation is expected due to either cleavage of the HBx RNA with Rz or selective degradation by siRNA mediated pathway. HEK 293 cells were co-transfected with 100 ng each of HIV-1 LTR driven LTR-B-luciferase reporter plasmid DNA and either wt or mutant chimeric constructs in 1ml of a 6 well plate (1 X 10^6) cells for 48 hours. Thereafter the cells were harvested and luciferase activity was determined as described earlier (Goila and Banerjea, 2001) and according to the manufacturer's direction (Promega). The results are shown in Figure 6.9. The cell control (lane 1) along with HBx encoding DNA (lane 2) and wt-siRNA-wt-Rz (lane 3) showed only background levels of reporter gene activity. The cells that received only the LTR-luc reporter plasmid DNA showed activation as expected (lane 4); in the presence of 100 ng of HBx encoding DNA, a significant increase in luciferase activity was observed (lane 5). In the presence of HBx and wt-chimeric construct along with LTR-Luc DNA, about 4-fold drop was observed (compare lanes 5 and 6). When wt-chimeric construct was replaced with chimeric mutant construct-1, the level of inhibition was only 2-fold (lane 7). Therefore it is concluded that wt-chimeric construct interfered very significantly with the known function of HBx and that this inhibition could be modulated with one of the mutant constructs.

Similarly, the effect of HBx expression on transactivation of HIV-1 LTR-C promoter (HIV-1 subtype C) could also be inhibited to varying degrees in the presence of the various bi-specific chimeric constructs as shown in Figure 6.10

Effect of antisense ODN on efficiency of wt-chimeric construct

20 nts long antisense oligodeoxynucleotide (ODN) was designed immediately downstream the siRNA target site in HBx sequence. For this initially a minimum dose of wt construct was determined such that only 20% reduction in HBx expression was gained. The ODN was then co-transfected along with wt-chimeric RNA (0.2µg) and HBx encoding plasmid DNA (1µg) in HEK 293 cells and were assayed for X gene expression by RT-PCR using HBx specific primers (Figure 6.11). In presence of unrelated ODN and wt-chimeric RNA (lane 5) only 15% reduction was observed when compared with cells that received wt construct RNA alone (lane4). On the contrary in presence of relevant ODN and wt-chimeric RNA more than 50% reduction of X RNA was achieved (lane 6).

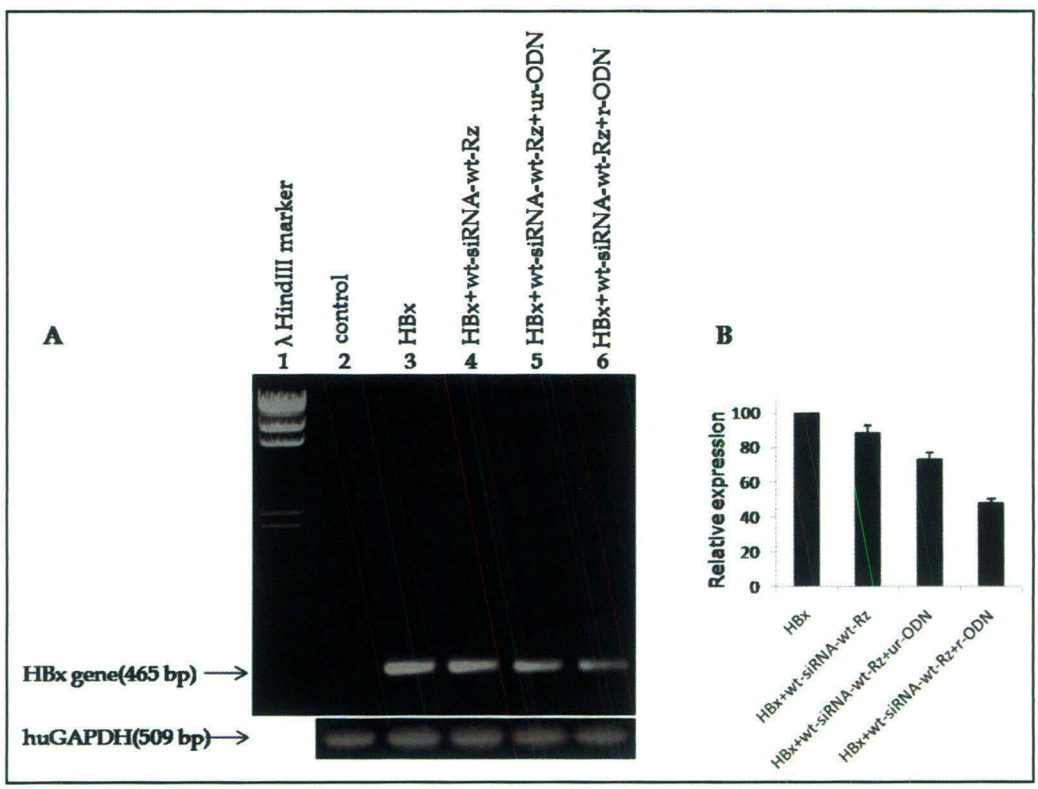


Figure 6.11: RT-PCR to check the effect of ODN on siRNA mediated reduction of X RNA

Similar effects were observed when indicated amounts of ODN and wt-si-RNA-wt-Rz-170 RNA were co-transfected along with plasmids encoding HBx and LTR-B promoter in HEK 293 cell line. In presence of relevant ODN, wild type construct was able to inhibit the activity of HBx protein more potently as shown in Figure 6.12 (compare lanes 7 and 8).

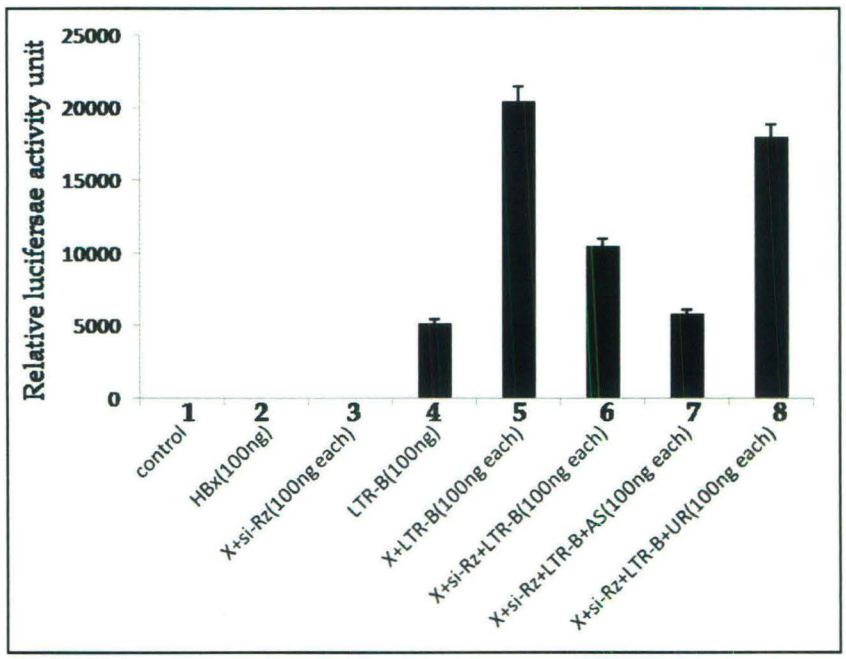


Figure 6.12: Effect of ODN and siRNA on HBx transactivation

Comparative study of wt-siRNA construct and 10-23 Dz

Transient transfections were done in HEK 293 cell line in parallel to compare the efficiency of wild type siRNA and 10-23 Dz that were designed at the same target site of HBx RNA as the siRNA site i.e., at the initiation codon of HBx. The results are shown in Figure 6.13, panel A, where lane 2 represents control cells in which only vector plasmid was added. Lane 3 represents the expression of X RNA and in lanes 4 and 5 co-transfections were done along with either wt-siRNA or with 10-23 Dz respectively. The quantitation of the RT-PCR (panel B) shows that si-RNA is more potent

(almost 2 old more) than 10-23 Dz in suppressing the expression of X RNA under identical experiment conditions.

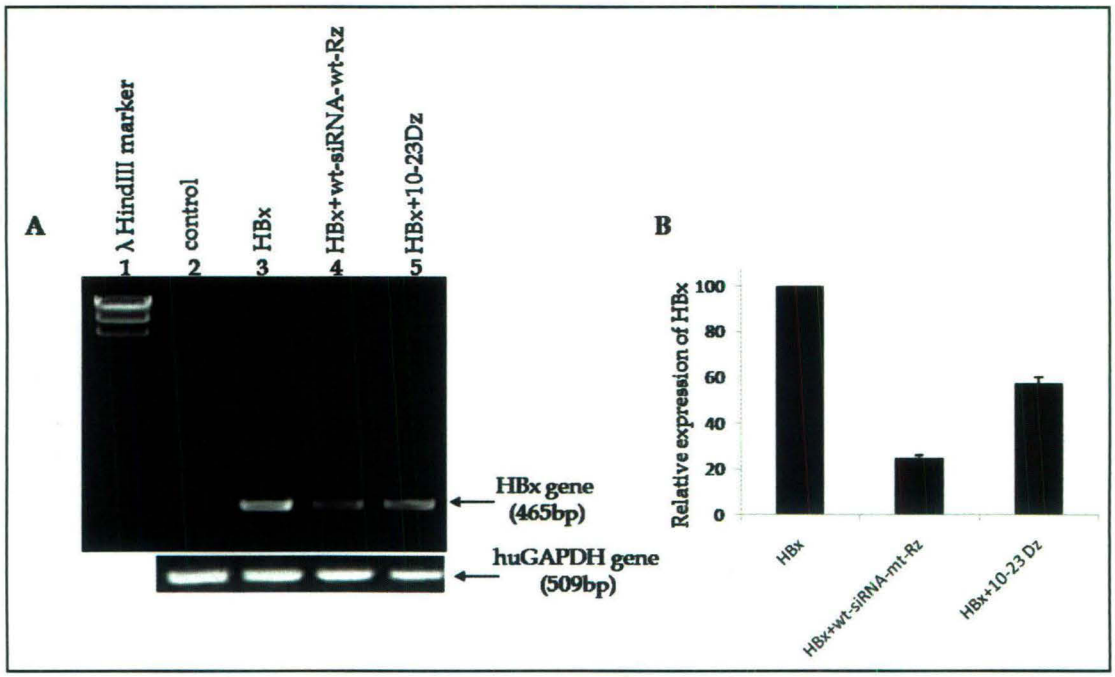


Figure 6.13: Comparison of efficiencies of siRNA and 10-23 Dz

Cloning of wt-siRNA-wt-Rz-170 in pU6+1 vector and its effect on the intracellular expression of X RNA in comparison to bi-specific RNA constructs

The wild type siRNA-wt-Rz-170 construct was cloned in plasmid U6+1 which is a mammalian expression vector containing Pol III promoter which is flanked between *Sal I* and *Xba I* restriction sites. The effect of this cloned construct was then compared with RNA of wild type construct by doing transient transfections in Hep G2 cell line. For this purpose, 24 hrs before 1 X 10^6 cells were plated per well in a 6 well plate. 1µg of pSG5-HBx plasmid DNA was either co-transfected with 1µg of wt-siRNA-wt-Rz-170 RNA (lane 4) or with 1µg of pU6+1 driven wt-siRNA-wt-Rz-170 encoding

plasmid pU6+1 (lane 5) and the target gene was analyzed after 48 hrs by RT-PCR. As shown in Figure 6.14, lane 3, cells transfected with HBx encoding plasmid exhibit a prominent HBx RNA specific amplification. When wt-siRNA-wt-Rz-170 RNA was co-transfected, as expected there was almost 60% reduction in the expression of X RNA (lane 4). In comparison when the cloned wild type construct encoding plasmid DNA was co-transfected (lane 5), the reduction was about 80%. We concluded that wildtype construct which expresses siRNA under Pol III promoter was more efficient than *in vitro* synthesized wt-chimeric RNA in suppressing the intracellular expression of HBx RNA.

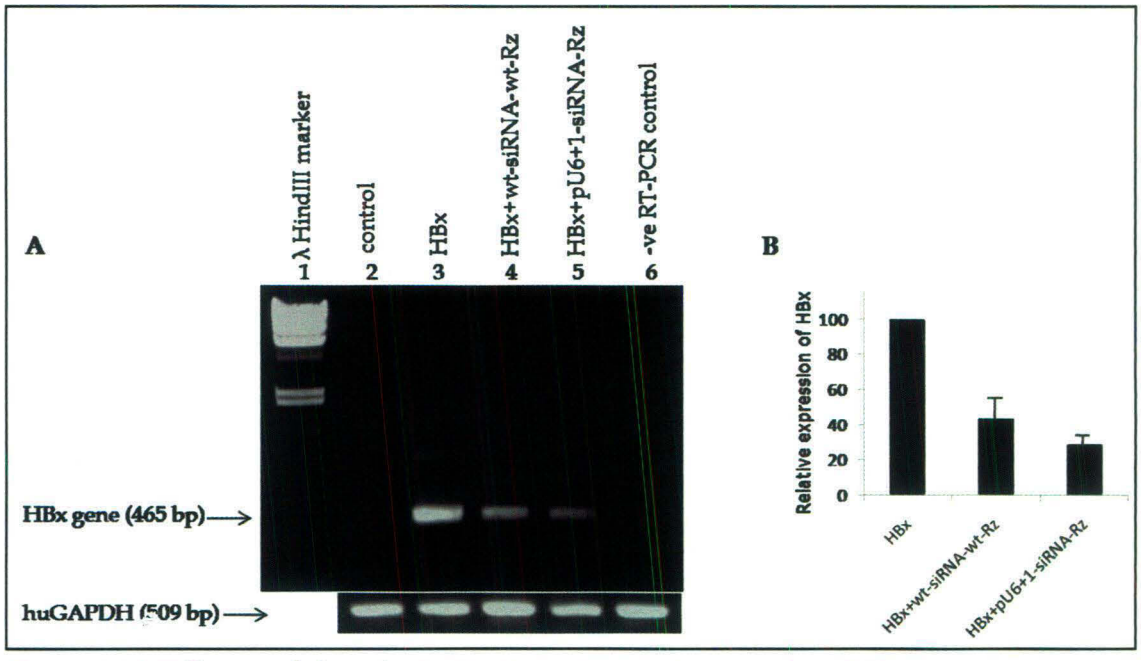


Figure 6.14: Efficacy of cloned wt-siRNA-wt-Rz construct against HBx

DISCUSSION

Sequence-specific cleavage of specific target RNA by short catalytic nucleic acid molecules has been exploited for achieving specific inhibition of gene expression. Most earlier studies have used hammerhead Rz and currently being exploited along with antisense (RNA or DNA) and aptamers with the idea that multiple antiviral approaches can be used simultaneously, either in additive or synergistic manner to inhibit gene expression more powerfully (Akkina et al, 2003; Banerjea, et al, 2004; Joyce, 2004; Schubert and Kurreck, 2006). Studies from our own group (Unwalla et al, 2006), using two different kinds of catalytic nucleic acids (Rzs and 10-23 catalytic Dzs) and others (Goila and Banerjea, 1998; Kurreck et al, 2002) have clearly established that some sites are more accessible to either RNA or DNA based catalytic nucleic acids. Other experiments have shown that both Rz and Dz mediated cleavage of the target RNA can be significantly augmented by allowing specific antisense that hybridized close to the target region. The same argument can be made with respect to siRNAs as most of them show no target RNA reduction and usually requires screening a large number of them.

In the present work, a novel chimeric construct is made with the hope that it would interfere with the intracellular target gene expression more powerfully and by using selectively disabled mutant versions of it, it might be possible to modulate the level of target gene expression. As expected, the wt chimeric construct interfered with the gene expression very powerfully. Usage of selectively disabled chimeric mutants led to the conclusion that siRNA component played a major role towards the inhibition of gene expression. On the contrary, the Rz component contributed less towards the inhibition of gene expression in our constructs. It is noteworthy that the siRNA target site selected have involved the **'AUG'** of the target

RNA which is usually more accessible towards siRNA mediated down regulation. The potency of the approach could also be explained on the basis that once the target RNA is cleaved by either of the two antiviral approaches (siRNA or Rz), it may open up secondary structures at the target sites. This may facilitate more efficient hybridization between the target RNA and Rz or siRNA. Alternatively, the cleaved target RNA fragments are known to become increasingly more susceptible to degradation by nucleases. The potency of the wt chimeric construct was not only established at the target RNA level but a complete knock down of protein production was observed by western blot analysis. These chimeric constructs can be delivered to the desired cell via lentiviral vectors or other vectors for achieving specific gene suppression. Selectively disabled mutant versions may allow modulating the extent of gene suppression and may be exploited for variety of studies, especially for genes that are temporally regulated. Our data also suggested that Pol III mediated expression of chimeric siRNA-Rz was more efficacious in suppression of the target gene and could be exploited for further therapeutic approaches.

SUMMARY & CONCLUSIONS

SUMMARY & CONCLUSIONS

In this study, we have used hammerhead ribozymes, DNA-enzymes, antisense ODNs and siRNA either alone or in combination as our antiviral approach to achieve more potent suppression of the expression of HBx gene of Hepatitis B virus (HBV). We have also designed DNA-enzymes based anti viral strategies targeting HIV-1 vif gene of subtype B and Indian isolate of subtype C. The purpose of the whole work was therefore to look for novel complementary approaches in the field of catalytic nucleic acids so that more potent suppression of the target gene expression could be achieved.

➡ As the first objective, the possibility of using Rz-170 or Rz-200 and Dz-307 simultaneously was explored to cleave the full-length 465 nts long X RNA of hepatitis B virus under identical *in vitro* conditions and efficacy was also monitored at intracellular gene expression level. This combined (Rz + Dz) action resulted in enhancement of cleavage which was more than its individual additive effects and synergistic in nature. These enzymes inhibited intracellular levels of target gene individually in a liver specific cell line, HepG2, and more potently when used in combination.

➡ In order to modulate the activity of any hammerhead Rz in general, the 10-23 Dz was designed such that it targeted ***'GU'*** nucleotides in the catalytic core of the hammerhead Rz. The Dz was found to be active not only under *in vitro* conditions against the Rz-200 RNA but also at the intracellular level where the expression of Rz-200 was efficiently reduced when Dz and Rz-200 were co-transfected in HEk 293 cells. In the second objective, it was therefore, shown that the *in vitro* and *in vivo* catalytic activity of any hammerhead-Rz can be manipulated by the catalytic activity of this generic Dz that can potentially down regulate the activity of any hammerhead Rz.

➡ None of the studies done earlier by either Rzs or Dzs could achieve 100% *in vitro* cleavage of the full-length X RNA. It was reasoned that by melting the secondary structures near the Rz or Dz cleavage site with specific antisense DNA oligonucleotides (ODNs) or 10-23 Dz, it may be possible to achieve this objective. Hammerhead motif containing Rz-170 specific for X RNA was constructed by recombinant techniques and Dz-237 was synthesized using the 10-23 catalytic motif. When specific ODNs or 10-23 Dzs were included in the cleavage reaction with either Rz-170 or Dz-237, increased cleavage was observed in a dose-dependent manner which often resulted in almost complete *in vitro* cleavage of the target RNA as well as potent intracellular reduction of HBx RNA. These findings have important therapeutic implications.

➡ Vif is responsible to counteract host anti viral mechanism. There are a lot of genetic sequence variations in the vif encoded by subtypes B and C. Vif-C was found to be more potent in degrading APOBEC3G in comparison to vif-B. The sequence region from 291-580 of vif-C was found to possess more degrading activity as shown by western blot analysis. This observation has implications for understanding varying pathogenesis observed with different subtypes. We have designed antisense ODNs and various Dzs with different catalytic motifs (10-23 and 8-17 catalytic motifs based Dzs) to study their effect in suppressing the expression of vif gene. Antisense ODNs were used to enhance the cleavage efficiency of 8-17 Dz (Dz-472). Also a chimeric Dz was designed that retained the activity of 10-23 and 8-17 Dzs which not only efficiently knocked down the expression of vif but restored the intracellular expression of APOBEC3G when co-transfected with vif. Thus the Dzs and antisense ODNs can be developed as potential antiviral strategies to suppress vif gene expression.

➡ A multitarget approach is needed for effective gene silencing that combines more than one antiviral strategy. With this in mind, bi-specific chimeric

constructs were designed that consisted of small hairpin siRNA and hammerhead ribozyme (HBx-Rz-170) joined by a short intracellular cleavable linker. The wt-siRNA-Rz construct showed very significant (~68 %) intracellular down regulation in the levels of HBx RNA. The mt-siRNA-wt-Rz caused only 31% intracellular reduction and about 40% reduction in the target RNA was observed when wt-siRNA-mt-Rz was used. This wt chimeric construct also resulted in complete knock down of intracellular HBx protein production. The target gene expression could also be modulated with selectively disabled mutants. The intracellular reduction of target RNA with either wild-type or one of the mutant constructs also interfered to varying degree with the known functions of HBx protein.

MATERIALS & METHODS

MATERIALS & METHODS

MATERIALS REQUIRED:

7.1 Chemicals

Agarose, ampicillin, ammonium acetate, Tris Base, EDTA, SDS, sodium-acetate, potassium-acetate, boric acid, disodium-hydrogen-phosphate, sodium-dihydrogen-phosphate, sodium chloride ethidium bromide, urea, ammonium persulphate, MOPS, glycerol, sodium bicarbonate, Triton X-100, dithiothreitol, magnesium chloride, BSA, IPTG, Orange G, DEPC, Tween-20, acrylamide, calcium chloride, trypsin, EDTA, sodium citrate, bromophenol blue, xylene cyanol FF, were obtained from Sigma-Aldrich Co. (Missouri, U.S.A.). X-gal, NTP and dNTP, sodium chloride, bis-acrylamide, TEMED, PCR buffer and Magnesium chloride for PCR, DNA markers, were from Promega Biotech Co. (Madison, U.S.A.). All other chemicals were at least of analytical grade and were from Qualigens laboratories (Bombay, India) or Merck (New Jersey, U.S.A.) Trizol reagents, DMEM, lipofectin, lipofectamine 2000, antimycotic-antibiotic, gentamicin, RNase-DNase free water were obtained from Invitrogen-GIBCO/BRL (Maryland, U.S.A.). Fetal bovine serum was obtained from Biological Industries (Beit Haemek, Israel). Luria Bertini medium and Luria Miller agar for bacterial culture were obtained from Difco Laboratories (Detroit, U.S.A.). Pre-stained rainbow protein markers, nylon and nitro-cellulose membranes, ECL reagent, all were obtained from Amersham Biosciences (Buckinghamshire, U.K.).

7.2 Kits

Qiaprep spin mini kit and Qiagen plasmid midi kit (West Sussex, U.K.) were used for isolation of DNA. Isolation of DNA fragments from gel was carried out using QiaGel extraction kits or PCR products were purified using nucleotide removal kit from Qiagen (West Sussex, U.K.). PCR core system I

was used to amplify the oligonucleotides (Promega Biotech, Madison, USA). pGEMT-Easy and p-TARGET cloning vectors were also obtained from Promega. *In vitro* transcription was carried out using Riboprobe transcription system (Promega Biotech, Madison, U.S.A.). BCA protein assay kit was obtained from Pierce Biotechnology (Rockford, IL, U.S.A.). Reverse transcription was carried out using ImProm-II™ Reverse Transcriptase kit from Promega. Luciferase activity in the cell extracts was measured using Luciferase assay System (Promega Biotech., U.S.A.).

7.3 Cell lines and Bacterial strains

The cell lines, HEK 293 (human embryonic kidney cells) and HepG2 (human hepatocellular carcinoma) cells were obtained from ATCC. APOBEC3G-HA-293 cell line was obtained from the National Institutes of Health (Bethesda, Maryland, USA) AIDS Repository and grown according to standard procedures. *Ecoli* strains DH5α or XL-Blue were used for DNA cloning.

7.4 Radioisotopes

^{32}P-α-rUTP (3000Ci/mmol) was obtained from Perkin Elmer (California, USA).

7.5 Enzymes

DNA restriction enzymes were purchased from New England Biolabs (Massachusetts, USA) and Promega Corporation, (Madison, U.S.A.). RNase A was obtained from Qiagen (West Sussex, U.K.). DNA ligase, RNA polymerase, RNAsin, Taq DNA polymerase, T7 RNA polymerase, SP6 RNA Polymerase and alkaline phosphatase were obtained from Promega Biotech.

7.6 Oligonucleotides

Oligonucleotides used in this study were chemically synthesized by Sigma- Genosys (The Woodlands, TX, U.S.A.).

METHODS:

7.8 Polymerase chain reaction

The polymerase chain reaction (PCR) was carried out using the PCR Core System I (Promega, U.S.A.). 200ng of template DNA/oligonucleotide and 1μM terminal primers were combined in 25μl reaction volume finally containing 1X Mg free reaction buffer (500mM KCl, 100mM Tris-HCl, pH 9.0, 1.0% Triton X-100), dNTP mix with 0.2mM of each, 1.5mM $MgCl_2$ and 0.625U of Taq DNA Polymerase. 30 thermal reaction cycles from steps 2-4 were repeatedly carried out, in GeneAmp PCR 2400 machine (Perkin Elmer, USA). PCR amplification was analyzed by 1-2 % agarose gel electrophoresis using a 100 bp ladder or λ Hind III marker (Promega, USA).

PCR conditions for amplification of HBx gene of HBV

1. Denaturation- 94°C-5min
2. Denaturation- 94°C-1min
3. Annealing- 42°C-2min
4. Extension- 72°C-2min
5. Final extension-72°C-5min

PCR conditions for amplification of hammerhead-Rz

1. Denaturation- 94°C-5min
2. Denaturation- 94°C-30sec
3. Annealing- 42°C-1min
4. Extension- 72°C-15sec
5. Final extension-72°C-2min

PCR conditions for amplification of vif gene of HIV-1

1. Denaturation- 94°C-5min
2. Denaturation- 94°C-30sec
3. Annealing- 63°C-30sec
4. Extension- 72°C-45sec
5. Final extension-72°C-5min

7.9 Gel elution of DNA fragments:

The plasmid DNAs for cloning, were digested with the respective enzymes, checked on an appropriate percentage of agarose gel along with 100 bp ladder or λ Hind III marker (Promega, USA) and the required fragments were eluted from the gel using the Qiagel Gel Extraction kit (Qiagen, U.K.). According to the manufacturer's directions, the area of the gel containing the DNA fragment was excised using a clean and sharp blade, minimizing the amount of surrounding agarose excised with the fragment. The gel slice was weighed and placed in a microfuge tube. Three volumes of Gel Solubilization Buffer (QG) was added for every one volume of gel. The gel piece was then vortexed and incubated at 50°C for 10 min. The contents were mixed in between, by inverting the tube few times, to ensure gel dissolution. It was then centrifuged at 13,000 rpm for 1min. The flow through in the discard column was carefully removed. Then 500 µl of buffer PB was added to remove the traces of gel and the tube was centrifuged at 13,000 rpm for 1 min. The flow through in the discard column was removed and 750 µl of Wash Buffer (PE) (containing ethanol) was added and the tube was centrifuged at 13,000 rpm for 1 min. The flow through was discarded and another spin at maximum for 2 min was given to remove the traces of wash buffer. The column was then put on a fresh tube and finally, to elute the DNA, 40 µl of Tris-EDTA buffer (TE) or RNase-DNase-free water was added and then centrifuged at 13,000 rpm for 1 min. The flow through in the

eppendorf tube had DNA of interest. The purified DNA fragments were checked on an agarose gel, with an appropriate marker, before setting up the ligation reaction.

7.10 Ligation and Transformation:

The PCR products were ligated with T-tailed vectors (pGEM-T Easy, pTARGE-T™ from Promega or pcDNA3.1 from Invitrogen). In general, the ligation was carried out at a vector: insert molar ratio of 1:3 or 1:4 in a 10µl reaction volume finally containing 1X T4 DNA ligase buffer (300mM Tris-HCl, pH 7.8, 100mM $MgCl_2$, 100mM DTT, 10mM dATP) and 1U of T4 DNA ligase (Promega, U.S.A.). The reaction mix was incubated at 16°C for 16 hrs (overnight). Following the reaction, the ligated DNA was transformed into Calcium Chloride treated *E.coli*-DH5α or XL-Blue1 competent cells with a high transformation efficiency. The transformed cells were plated on to Luria-Bertani-Agar plates containing 100µg/ml of ampicillin. Additionally, for blue-white screening of the colonies, 20µl of 50mg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) and 10µl of 0.1M IPTG (Isopropyl-β-thiogalactopyranoside) (Promega, U.S.A.) was used. The plated cells were incubated at 37°C for 8-10 hours.

7.11 Plasmid Minipreps:

Each single colony (white colonies, in case of blue-white screening), was inoculated individually in 5ml Luria-Bertani (LB) medium with 100µg/ml of ampicillin. The cultures were grown for 8-10 hours at 37°C with vigorous shaking (~200 rpm). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN, U.K.). According to the manufacturer's directions, the pelleted bacterial cells were resuspended in 250µl of buffer P1 and transferred to a microfuge tube. 250µl of buffer P2 was then added and mixed gently by inverting the tube 4-6 times. Further, 350µl of buffer N3 was

added and mixed by gently inverting the tube 4-6 times. The microfuge tubes were then centrifuged at 13,000 rpm for 10 min. QIAprep spin columns were placed in 2-ml collection tubes and the supernatant was applied to these columns. These were then centrifuged for 1 min and the flow-through was discarded. The Qiaprep columns were then washed by adding 0.75ml of buffer PE and centrifuged for 1 min. The flow-through was discarded and an additional centrifugation was given for another minute to remove traces of the wash buffer. The QIAprep columns were placed in a fresh 1.5ml microfuge tube. Finally, to elute out the DNA, 50µl of buffer EB (10mM Tris-Cl, pH 8.5) or RNase-DNase free water was applied to the center of each column and then centrifuged for 1 min after letting it stand for 1 min. The flow-through contained the DNA of interest.

7.12 Plasmid Midipreps:

For large scale plasmid DNA isolation, the bacterial cells were cultured in 100ml of LB medium with 100µg/ml of ampicillin. The cultures were grown for 8-10 hours at 37°C with vigorous shaking (~200 rpm). Plasmid DNA was isolated using the QIAGEN Plasmid Midi kit (100). Briefly, the bacterial cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 4 ml of the resuspension buffer P1 (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100µg/ml RNase A). 4 ml of lysis buffer P2 (200mM NaOH, 1% SDS) was added, mixed gently by inverting 4-6 times and incubated at room temperature for not more than 5 min. Further 4 ml of chilled neutralization buffer P3 (3.0 M potassium acetate, pH5.5) was added, mixed gently as before and incubated on ice for 10 min. It was then centrifuged at maximum rpm for 30 min at 4°C. The supernatant containing the plasmid DNA was immediately removed and re-centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was now collected in fresh tubes and kept on ice. A QIAGEN-tip 100 was equilibrated

with 4 ml of equilibration buffer QBT (750mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100) and the column was allowed to empty by gravity flow. The supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed thrice with 10ml of wash buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol). The DNA was then eluted with 5 ml of elution buffer QF (1.25M NaCl, 50mM Tris-Cl, pH 8.5, 15% isopropanol). The DNA was precipitated by adding 0.7 volumes of isopropanol to the eluted DNA. It was thoroughly mixed and centrifuged immediately at 13,000 rpm for 30 min at 4°C. The supernatant was carefully decanted. The DNA pellet was washed with 2 ml of 70% ethanol, and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was carefully decanted without disturbing the pellet. The pellet was air dried for 5-10 min and the DNA was dissolved in 200 µl of RNase-DNase free water.

To determine the yield, DNA concentration was determined both by Ultra Violet (UV) Spectrophotometry (DU-65 spectrophotometer, Beckman, U.S.A.) and quantitative analysis on an agarose gel using a UV Transilluminator (UVP, California, U.S.A.). All the putative clones were then screened for the correct recombinant clones by restriction enzyme digestion using appropriate enzymes. The digested samples were checked on an agarose gel along with an appropriate size marker to assess the size of the insert from the putative clones. The clones containing very small fragments were further confirmed by sequencing both strands of the DNA.

7.13 In vitro Transcription:

Plasmids containing the ribozymes or substrates were linearized at their 3′ end with the appropriate enzymes. The linearized DNA was purified using the Qiagen Gel Extraction kit as described before (section 7.9). *In vitro* transcription reaction was then carried out using both T7 or SP6 RNA

polymerase and [α-^{32}P] UTP (specific activity 3000Ci/mmole). The Riboprobe *in vitro* Transcription Systems (Promega) was used to make the *in vitro* transcripts. According to the manufacturer's directions, 0.2-1μg of the linearized DNA template was combined with the following components, in a final volume of 20μl, at room temperature in the following order: 4μl of 5X transcription buffer (200mM Tris-HCl, pH 7.5, 30mM $MgCl_2$, 10mM Spermidine, 50mM NaCl), 2μl of 100mM DTT, 20U of RNasin Ribonuclease inhibitor, 2.5mM each of ATP, GTP and CTP (pH 7.0), 2.4μl of 100μM UTP (pH 7.0), 5μl (50μCi at 10μCi/μl) of [α-^{32}P]UTP and 15-20U of T7 or SP6 RNA Polymerase. For carrying out cold *in vitro* transcription all the four nucleotides (ATP, GTP, CTP, and UTP) were added at 2.5mM concentration and the reaction volume was made up with nuclease free water. The mixture was incubated at 37°C for 60 min. The reaction was stopped using the stop buffer (50mM Tris-Cl, pH 7.5, 5mM EDTA, 25μg tRNA/ml) and chilled on ice. RQ1 RNase- free DNase was added at a concentration of 1U/μg of template DNA and incubated at 37°C for 15 min to remove the DNA template following transcription. The transcripts were then purified by phenol : chloroform : isoamyl alcohol and chloroform : isoamyl alcohol extractions, followed by precipitation with 2.5 volumes of absolute alcohol and 0.5 volumes of 7.5M ammonium acetate and then 0.5 volumes of 1M ammonium acetate to remove the unincorporated nucleotides. After centrifugation for 30 min at 13,000 rpm the supernatant was carefully removed. The pellet was washed with 70% ethanol, vacuum dried and dissolved in 20μl of NFW.

Trichloro acetic acid (TCA) precipitation:

To determine the percentage of incorporation and probe specific activity, 1:10 dilution of the labeled probe was made in NFW. 1μl of this was spotted on to duplicate glass fiber filters (Whatman GF/A, U.S.A.) and air

dried. These were counted directly to determine the total counts. In duplicate tubes, 1µl of the diluted probe was added to 100µg of carrier nucleic acid (tRNA or Herring Sperm DNA) in a total volume of 100µl. To this 500µl of ice-cold 5% TCA was added, mixed thoroughly and incubated on ice for 15-20 min. Glass fiber filters were wet (in duplicate) properly with 5% TCA and then these samples were applied on to them under vacuum. The filters were washed twice with 5ml of chilled 5% TCA and then air dried after rinsing with 2ml of acetone. All the dry filters were inserted into scintillation vials containing scintillation fluid and the counts were taken in a liquid scintillation α-counter (LKB Wallac, 1219 Rackbeta, Sweden). The percentage incorporation, specific activity and the total amount of RNA made was then calculated according to the standard procedures.

$$\% \text{ incorporation} = \frac{\text{Incorporated cpm} \times 100}{\text{Total cpm}}$$

$$\text{Total RNA made (ng)} = \% \text{ incorporation} \times 338$$

$$\text{Specific activity of probe} = \frac{\text{Total cpm incorporated}}{\mu\text{g of RNA synthesized}}$$

Cell culture media and cell lines:

All the cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal bovine serum (FBS) and 1% antibiotic-antimycotic (penicillin, streptromycin and amphotericin B). The cells were maintained at 37°C with 5% CO_2 in a humidified CO_2 incubator (Nuaire-IR Autoflow CO_2 Water-Jacketed incubator).

Transient transfection:

Transfection of cell lines used was carried out using lipofectin reagent (Invitrogen, U.S.A.). In a six well plate 10^5 cells/ well were seeded in 2ml medium supplemented with serum. The cells were incubated in a CO_2

incubator until the cells were 60% confluent. For each transfection, 1-2μg of DNA was diluted in 100 μl serum free media. Also, 10μl of lipofectin reagent was diluted in 100 μl of serum free media and allowed to stand at room temperature for 30-45 minutes. The two solutions were combined, mixed gently and incubated at room temperature for 15 minutes. The cells were washed once with 2ml of serum free medium. For each transfection, 0.8 ml of serum free medium was added to each tube containing lipofectin–DNA complexes. The complex was mixed gently and overlaid onto cells. The plate was incubated for 4-6 hrs in a CO_2 incubator. The medium in each well was replaced with serum containing medium and the cells were further incubated for varying periods of time at 37°C. The concentration of lipofectamine 2000 was used in the ratio 1:2 or 1:3 with DNA. The Rzs and Dzs were either co-transfected with the plasmid DNA of interest or when required to be transfected alone then pBSK+/- was used as carrier plasmid for better transfection efficiency. In order to ensure uniform transfection efficiency a reporter plasmid DNA (pSV-β gal, Promega) was used.

Luciferase assay:

The cells were assayed for Luciferase gene expression using Luciferase Assay kit (Promega, U.S.A.). After transfection, the cells were washed twice with PBS and then lysed by adding reporter lysis buffer provided in the kit. The cell lysate was collected from individual wells in eppendorf tubes, the cells were twice freeze-thawed in liquid N_2 and then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube. 20μl of cell extract was mixed with 100μl of luciferase assay reagent that was kept at room temparature. The activity was determined using a luminometer (Packard lumicount, U.S.A.).

RNA isolation from cell lysates:

After transfection the cells were harvested and RNA was isolated from the cell lysates using Trizol reagent (Invitrogen) and purified according to the manufacturer's directions. Briefly, the cells were lysed directly in the culture dish by adding 1ml of Trizol reagent to each well. The homogenized sample was incubated at room temperature for 5 min to permit complete dissociation of the nucleoprotein complexes. For purifying the RNA, 200µl of chloroform was added, the tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 min. Tubes were centrifuged at 12,000 rcf for 15 min at 4°C. The aqueous phase was collected, mixed with 500µl isopropanol and incubated at room temperature for 10 min. Centrifugation was carried out at 12,000 rcf for 10 min at 4°C. The supernatant was carefully removed and the RNA pellet was washed with 1ml of 70% ethanol by vortexing and then centrifuging at 7500 rcf for 5 min. The pellet was air dried and dissolved in 20µl of NFW.

Reverse transcriptase polymerase chain reaction (RT-PCR):

A series of primers were designed to detect the levels of intact gene of interest or Rz in the cell lysate. The levels of RNA were quantitated by carrying out reverse transcriptase based-PCR using the ImProm-II™ Reverse Transcriptase system (Promega, U.S.A.). 1µg of template RNA and 1µM terminal primers were combined in 5µl reaction volume and the primer/template mix was thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix of volume 15 µl was assembled on ice to contain nuclease-free water, 1X reaction buffer, 1µl reverse transcriptase, 6 mM magnesium chloride, 0.5 mM dNTPs and 1 U ribonuclease inhibitor RNasin. As a final step, the template-primer combination was added to the reaction mix on ice. Following an initial

annealing at 25°C for 5 minutes, the reaction was incubated at 42°C for one hour.

Protein isolation from cell lysates:

After transfection the cells were harvested and protein was isolated from the cell lysates. The cells from each well were pelleted at 2000 rpm for 10 min at 4°C. The supernatant was carefully removed and the pellet was incubated on ice for 1 hr after adding 50µl of lysis buffer (1% triton X100, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1X PI, all in 1X PBS) with intermittent vortexing. The tubes were centrifuged at maximum rpm for 10 min at 4°C and the supernatant, containing the proteins, was collected and stored at –70°C. The purified protein fractions were quantitated using the BCA protein assay kit and the O.D. was taken at 562nm.

Western blot analysis:

The protein samples were diluted with 4X sample buffer which is essentially SDS-reducing buffer (0.5M Tris-Cl, pH 6.8, Glycerol, 10% (w/v) SDS, 2-β-mercaptoethanol, 0.05% (w/v) bromophenol blue). The samples were denatured at 100°C for 10 min and the proteins were resolved on 12-15% SDS-polyacrylamide gel at 25-30mA. For detection, the proteins were transferred on to nitrocellulose (NC) membrane (Hybond-C extra, Amersham, U.K.) at 200mA, for either 1 hr or at 12 mA, 4°C for overnight. After the transfer was over, the NC membrane was washed thrice with PBST (1X PBS with 0.1% Tween 20) and blocked with 2% BSA for 2hrs (in PBST) at room temperature. Primary antibody to HBx/Vif/APOBEC3G-NT raised in rabbit and were diluted to 1:1,000 in PBST. One hour incubation with the primary antibody was followed by three washes with PBST (10 min each) and then 1 hr incubation with 1:1,000 dilution of the secondary antibody (Anti-rabbit IgG (Fc) HRP conjugate) was carried out. The blot was further

subjected to three washes with PBST and two washes with PBS. The blot was developed using the substrate DAB (Sigma, U.S.A.) or with ECL (Amersham Biosciences, U.K.).

Northern Blot Analysis:

Total RNA was isolated from cell lines after 48 hrs of transfection using trizol (Invitrogen, U.S.A.). ^{32}P labeled antisense HBx mRNA was *in vitro* transcribed using T7 RNA Polymerase and Riboprobe kit (Promega, U.S.A.), as described earlier. For generating antisense HBx probe, plasmid DNA was linearized with *Bam HI* and subjected to transcription. Total RNA was quantitated and equal concentration (15-20 µg) was loaded after adding loading dye (50%glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) on 1% formaldehyde-agarose gel and 1X MOPS was used as the running buffer. The gel was then run at 5 V/cm length of the gel. The gel was then treated with 2.5% HCl for 15 min for depurination, 0.4N NaOH for another 15 min and then in 3 M sodium acetate for 15 min, before the transfer was set. Also the nylon membrane prior to transfer was first treated with distilled water for 5 min and then in 0.4 N NaOH for 20 min. The overnight transfer was set up using 20X SSC buffer as the transfer buffer at room temperature. Thereafter, the membrane was cross-linked by UV and then dipped in 2X SSC for 20 min. For pre-hybridization the membrane was soaked in Rapid hybridization buffer (Amersham Biosciences, U.K.) for 2 hr at 65°C in the hybridizing oven. The probe was then added and further incubation for 4 hrs was carried out. Post hybridization the membrane was washed thrice with 6X SSC at 37°C on a shaker. The membrane was then dried on a filter paper and wrapped in a saran wrap. The membrane was then analyzed by autoradiography. For ensuring the equal loading, the formaldehyde-agarose gel was also stained with EtBr for 23s and 18s rRNA.

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APPENDIX

Note: Water treated with a Milli Q water purification system (Millipore Corp, MA, U.S.A.) was used for making all the DNA solutions. DEPC treated or Nuclease free water was used for making RNA solutions.

Freezing media for cell lines

90% FCS

10% DMSO

LB & Agar (per liter)

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

Commercially available LB and agar were used in concentration 25gm/L and 35gm/L, respectively.

10 X MOPS electrophoresis buffer

0.2 M MOPS (pH 7.0)

20 mM sodium acetate

10 mM EDTA (ph 8.0)

1X Phosphate Buffered saline, pH 7.4

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

1X PBS-T, pH 7.4

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

0.1% Tween-20

DNA gel-loading buffer

0.25% (w/v) bromophenlo blue

0.25% (w/v) xylene cyanol

30% (v/v) glycerol in water

10X Formaldehyde gel-loading buffer

0.25% (w/v) bromophenlo blue

0.25% (w/v) xylene cyanol

50% (v/v) glycerol in DEPC treated water

10 mM EDTA (pH 8.0)

RNA gel-loading buffer

95% (v/v) deionized formamide

5 mM EDTA (pH 8.0)

0.25% xylene cyanol FF

0.25% bromophenol blue

0.025% (w/v) SDS

2X SDS gel-loading buffer

100 mM Tris-HCl (pH 6.8)

4% (w/v) SDS

0.2% (w/v) bromophenol blue

20% (v/v) glycerol

200 mM DTT or β-mercaptoethanol

1X SDS-PAGE running buffer (per liter)

250 mM Glycine

0.1% SDS

25 mM Tris-HCl

20X SSC (per liter)

175.3 gm NaCl

88.2 gm sodium citrate

Dissolve in water and adjust pH to 7.0 with 14 N HCl.

10X TE Buffer, pH 7.4

100 mM Tris HCl (pH 7.4)

10 mM EDTA, (pH 8.0)

50X TAE Buffer (per liter)

242 gm Tris HCl

100ml of 0.5M EDTA (pH 8.0)

57.1 ml acetic acid

Volume make up with water.

5X TBE Buffer

54 gm Tris-HCl

27.5 gm Boric acid

20ml of 0.5 M EDTA (pH 8.0)

Transfer Buffer

14.4 Glycine

3.08 Tris-HCl

200 ml methanol and 800 ml water

Trypsin-EDTA

1.0 gm Glucose

1.0 gm Tyrpsin

0.2 gm sodium bicarbonate

0.4 gm EDTA

Make it in 200 ml 1X PBS and filter sterile using 0.22µm filter

PUBLICATIONS

1. **Nidhi Gupta**, Aalia S. Bano, Yogeshwar Sharma, and Akhil C. Banerjea; Potent knock down of the X RNA of hepatitis B by a novel bispecific si-RNA-ribozyme construct and modulation of intracellular target RNA by selectively disabled mutants. **Oligonucleotides. 2008**; 18, 225–234.

2. **Nidhi Gupta**, Aalia S. Bano, Yogeshwar Sharma, Vikas Sood and Akhil C. Banerjea; Potent intracellular knock down of Hepatitis B virus X RNA by catalytic hammerhead ribozymes or DNA-enzymes with antisense DNA-oligonucleotides or 10-23 DNA-enzymes that powerfully augment *in vitro* sequence-specific cleavage activities. **The Open Biotechnology Journal. 2008**; 2, 1-12.

3. Swagata Roy, **Nidhi Gupta**, Nithya S, Tanmoy Mondal, Akhil Chandra Banerjea and Saumitra Das; Sequence specific cleavage of Hepatitis C virus RNA by DNA-enzymes: Inhibition of viral RNA translation and replication. **The Journal of General Virology. 2008**; 89 (Pt 7), 1579-86.

4. **Nidhi Gupta**, Vikas Sood, Aalia Shahr Bano and Akhil C Banerjea; X protein of hepatitis B virus potently activates HIV-1 subtype C LTR promoter: implications of faster spread of HIV-1 subtype C. AIDS. 2007; 21, 1491-92.

5. Aalia Shahr Bano, **Nidhi Gupta**, Vikas Sood and Akhil C Banerjea; Vpr from HIV-1 subtype B and C exhibits significant differences in their ability transactivate LTR mediated gene expression and also in their ability to promote apoptotic DNA ladder formation. AIDS. 2007; 21(13):1832-4.

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DOI: 10.1089/oli.2008.0134

Potent Knockdown of the X RNA of Hepatitis B by a Novel Chimeric siRNA-Ribozyme Construct and Modulation of Intracellular Target RNA by Selectively Disabled Mutants

Nidhi Gupta, Aalia S. Bano, Yogeshwar Sharma, and Akhil C. Banerjea

A multitarget approach is needed for effective gene silencing that combines more than one antiviral strategy. With this in mind, we designed a wild-type (wt) and selectively disabled chimeric mutant (mt) constructs that consisted of small hairpin siRNA joined by a short intracellular cleavable linker to a known hammerhead ribozyme, both targeted against the full-length X RNA of hepatitis B. These chimeric RNAs possessed the ability to cleave the target RNA under *in vitro* conditions and were efficiently processed at the cleavable site. When this wt chimeric RNA construct was introduced into a liver-specific mammalian cell line, HepG2, along with the HBx substrate encoding DNA, very significant (~70%) intracellular downregulation in the levels of target RNA was observed. When the siRNA portion of this chimeric construct was mutated, keeping the ribozyme (Rz) region unchanged, it caused only ~25% intracellular reduction. On the contrary, when only the Rz was made catalytically inactive, about 55% reduction in the target RNA was observed. Construct possessing mt Rz and mt siRNA caused only 10% reduction. This wt chimeric construct also resulted in almost complete knockdown of intracellular HBx protein production, and the mt versions were less effective. The intracellular reduction of target RNA with either wt or mt constructs also interfered with the known functions of HBx protein with varying efficiencies. Thus, in this proof of concept study we show that the levels of the target RNA were reduced potently by the wt chimeric siRNA-Rz construct, which could be modulated with mt versions of the same.

Introduction

SMALL INTERFERING TECHNOLOGY ALLOWS one to interfere strongly with the gene expression of variety of genes and is currently favored over any other RNA-based approaches, as it is known to be effective in the presence of very small amounts (Rossi, 2006). It is an evolutionary conserved mechanism and its role has been identified in many cellular and developmental programs (Scherer and Rossi, 2004; Martim and Caplein, 2007). In the present study we focused our attention on the X gene product (HBx) of hepatitis B virus, which is known to activate several cellular and viral promoters, including the HIV-1 long-terminal reporter (LTR) promoter (Robinson, 1994; Gomez-Ganzalo et al., 2001; Gupta et al., 2007). Although it is strongly associated with the formation of hepatocellular carcinoma (HCC), the exact role in HBx protein is not well understood. It is very likely that the intracellular levels of target protein may be critically important for causing cytopathogenesis in this case, and the same may be true for other diseases/disorders. In order to correlate more meaningfully the extent of pathogenesis with the levels of target gene expression, it is important to device novel approaches that allow the downregulation of target gene expression in a controlled manner. Several nucleic acids–based antiviral approaches are currently available to achieve gene-specific suppression. They include ribozymes (Rzs), DNA enzymes (Dzs), antisense RNA or DNA, aptamers, and decoy RNAs (Akkina et al., 2003; Kurreck, 2003; Joyce, 2004; Banerjea et al., 2005; Schubert and Kurreck, 2006; Bhindi et al., 2007). Although catalytic nucleic acids (Rzs and Dzs) have been used extensively to knock out a target gene expression individually (Unwalla et al., 2006), we showed for the first time that it was possible to obtain both Rz- and Dz-specific cleavage under same *in vitro* conditions, and they acted synergistically to knock down the intracellular target gene expression (Sood et al., 2007; Gupta et al., 2008). Very often Rz, Dz, or siRNAs target sites are not accessible for hybridization with target RNA owing to secondary structures (Cairns et al., 1999; Schubert et al., 2004). It is becoming

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increasingly clear that some sequences may be more prone to cleavage with either Rz, Dz, or siRNAs, which needs to be experimentally determined (Goila and Banerjea 1998; Kurreck et al., 2002). The cleavage of HBx RNA by either Rz or Dz was recently shown by our group to be significantly augmented with oligodeoxynucleotides that were designed to hybridize immediately next to the hybridizing arms of either Rz or Dz (Gupta et al., 2008). Earlier constructs with antisense RNA and multimeric Rzs were used against HIV-1 (Ramezani et al., 2006). Very recently siRNA against HIV-1 gene along with anti-gp120 aptamer was shown to have potent anti-HIV-1 activity (Zhou et al., 2008).

Several mono- (Martinez et al., 2002) and bispecific siRNA constructs were described earlier that targeted *CD4, CXCR4, CCR5* genes (Anderson et al., 2003; Anderson and Akkina, 2005). The unique feature of this bispecific siRNA construct is that the two small-hairpin siRNAs were joined together by an intracellular cleavable linker as originally described by Leirdal and Sioud (2002). Using this approach impressive inhibition of HIV-1 replication was observed. Here, for the first time, we designed and tested constructs that combined an earlier described potent hammerhead ribozyme against the X gene of hepatitis B (Goila and Banerjea, 2004) virus with a small hairpin siRNA using the same short cleavable linker [henceforth called wild-type (wt) chimeric construct]. We show that this and mutant (mt) constructs were processed correctly in the presence of mammalian cell cytoplasmic extracts. The wt construct interfered strongly with the expression of target gene expression both at RNA and protein levels. Furthermore, by selectively inactivating either Rz or siRNA portion of this chimeric construct, it was possible to modulate the intracellular target gene expression.

Materials and Methods

Cloning of HBx gene and in vitro *transcription*

Plasmid pSG5.HBx encoding the X gene of HBV (Kumar et al., 1996) was a kind gift from Vijay Kumar, ICGEB, New Delhi, India. The entire gene was placed under SV40 and T7 promoters. The former promoter was used for intracellular expression and the latter was used for obtaining *in vitro* transcripts using the transcription kit from Promega Biotech., as described earlier (Banerjea and Joklik, 1990). After linearization with appropriate restriction enzyme at the 3'-end, full-length (465 nt) X RNA was synthesized.

Construction and cloning of HBx-Rz-170 targeted against X gene

The construction of Rz-170 (Fig. 1A) has been described by one of us in detail previously (Goila and Banerjea, 2004). It possessed the hammerhead catalytic motif and was targeted against the GUC sequence (C residue is 170th nt in the target RNA; Kumar et al., 1996). *Eco*RI and *Bam*HI restriction sites were engineered at the ends of the hybridizing arms of the Rz that facilitated cloning it into pcDNA3 expression vector (Promega). This placed the expression of Rz under T7 and CMV promoter. Mg++ dependent sequence-specific cleavage of the target RNA is expected in the presence of Rz.

Construction of chimeric siRNA-Rz constructs against HBx gene

The detail methodology for making chimeric constructs has been described by one of us in detail previously (Anderson et al., 2003). In brief, an oligonucleotide was synthesized that consisted of Rz-170, a short spacer (Leirdal and Sioud, 2002) followed by the small hairpin-type siRNA that ends with the complementary sequences for the T7 primer (Fig. 2A). To this oligonucleotide, the T7 primer was hybridized and the desired RNA was synthesized *in vitro* using T7 RNA polymerase as described earlier (Donze and Picard, 2002; Anderson et al., 2003) using the transcription kit provided by Promega Biotech. A four-point mt version of the siRNA was made, which retained the normal sequences of the Rz (Fig. 2B, shown in italics—henceforth referred to as mt-siRNA-wt-Rz construct). The other chimeric construct retained the wt siRNA portion but the Rz was disabled by changing a single nucleotide (G to U—henceforth referred to as wt-siRNA-mt-Rz) in the hammerhead catalytic motif. Finally we made a construct that consisted of mutant siRNA and mutant Rz (mt-siRNA-mt-Rz). *In vitro* synthesis of these RNAs indicated that they were of correct size when compared with RNA control markers (data not shown).

In vitro *cleavage of target RNA with Rz-170 and chimeric constructs*

In vitro transcription of the linearized plasmid DNA encoding X gene was carried out in the presence of labeled UTP using T7 RNA polymerase. The cleavage reaction was initiated by adding equimolar amounts (100 pmoles each) of the labeled target RNA and unlabeled Rz in a reaction buffer containing 50 mM Tris/HCl, pH 7.5, in a volume of 10 μL. The reaction mixture was heated briefly at 94°C and the cleavage reaction was initiated by adding $MgCl_2$ (final concentration 10 mM) at 37°C for 2 hours. The cleaved RNA fragments were subjected to gel analysis as described before (Shahi et al., 2001). The radioactive RNA bands on the X-Ray film were quantitated by densitometry (GS-710 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA, USA).

Cleavage of wt chimeric RNA in the presence of mammalian cell extracts

Cytoplasmic protein extract was prepared from 293 cells. Briefly, the cells were washed twice with PBS and lysed at 4°C with lysing buffer (0.2% NP40 in PBS + protease inhibitors). After 20 minutes of incubation, cell lysate was centrifuged for 5 minutes at 10,000 rpm and the supernatant was collected and used as cytoplasmic protein extract for the cleavage of chimeric RNAs. Uniformly labeled wt-chimeric RNA (50 ng) was incubated in the presence of this extract (20 μg) for 2 minutes and subjected to gel analysis as described before by Leirdal and Sioud (2002).

Intracellular decrease in HBx RNA by WT and chimeric constructs

The intracellular decrease in HBx-specific RNA was studied after cotransfection with substrate encoding

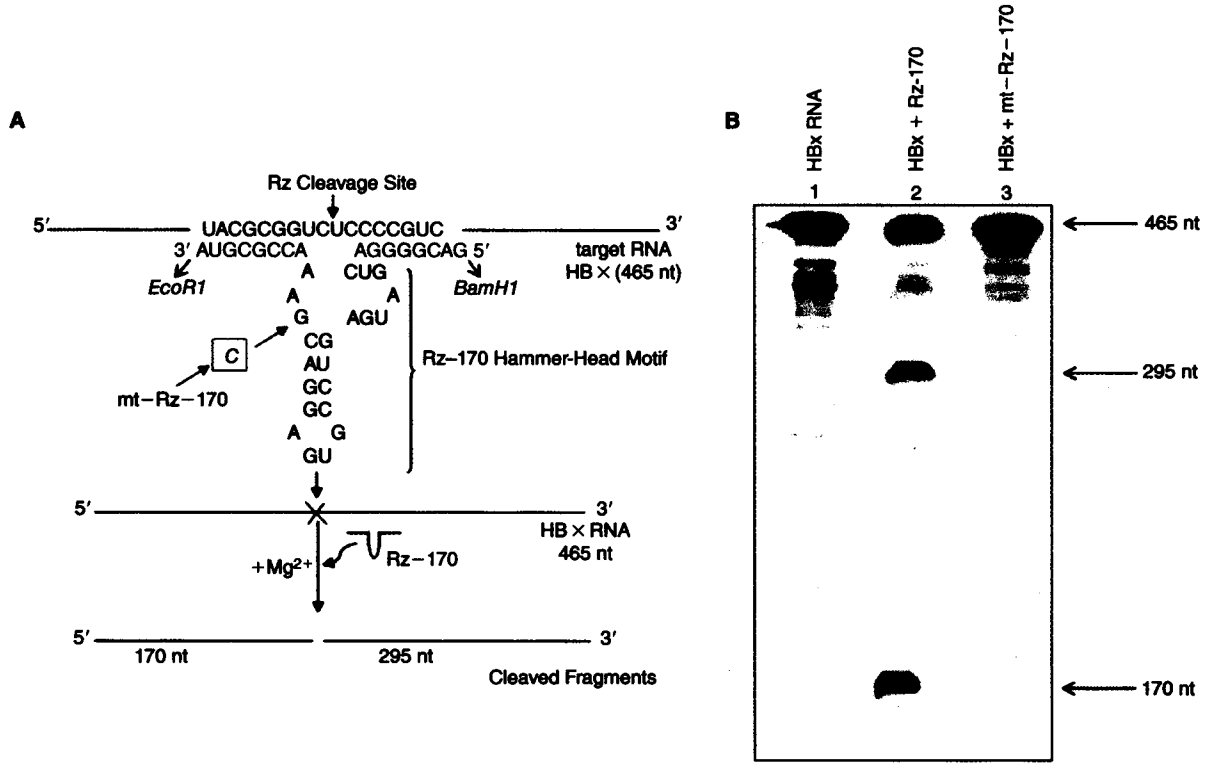

FIG. 1. Construction of Rz-170 and cleavage of HBx RNA: Panel A depicts the sequence of the target RNA that was used to design a hammerhead Rz. Eight nt-long hybridizing arms were synthesized to provide specificity. When the substrate RNA (HBx) is mixed with equimolar amounts of cold Rz-170, specific cleavage products are expected that are 170 and 295 nt long. Panel B: Lane 1 shows the synthesis of 465-nt-long HBx transcript along with some prematurely truncated transcripts, and when mixed with equimolar amounts (100 pmoles) of unlabeled Rz-170, specific cleavage products (295 and 170-nt-long RNA fragments) were observed (lane 2). Mutant Rz-170 failed to show any cleavage activity (lane 3).

DNA (pSG5.HBx) + indicated amounts of chimeric RNA constructs into HepG2 cells, and the levels of target and control RNAs were monitored by RT-PCR based assays. Rz-170 RNA, which was placed under T7 promoter (Goila and Banerjea, 2004), was *in vitro* synthesized using T7 RNA polymerase-based transcription kit and used for comparison with chimeric RNA constructs. Cells that received HBx encoding DNA (control) also received equivalent amounts of unrelated chimeric RNA. To ensure uniform transfection efficiency, we always transfected equal amounts of reporter gene containing plasmid DNA (pSV-β-gal, Promega, WI, USA) using lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and repeatedly obtained about 70–80% transfection efficiency. All the plasmid DNAs were Qiagen column (Qiagen, Hilden, Germany) purified and the *in vitro* synthesized RNAs showed no cellular toxicity under our experimental conditions. The cell lystaes were prepared 48 hours after transfection with liopfectin. Several dilutions were initially made to determine the linear range for PCR-amplified products. Total RNA was isolated using TRIZOL reagent (GIBCO-BRL, MD, USA) following the procedure described by the manufacturer and divided into two equal sets. One set was used for estimating the levels of full-length X RNA using HBx1 and HBx2 primers, and the second set was used for estimating the levels of the housekeeping gene, human glyceraldehyde phosphodehydrogenase (HuGAPDH), as described earlier (Paik et al., 1997). The following primers were used for estimating the intracellular levels of full-length X RNA.

(1) Forward (HBx1): 5′-TTAGGCAGAGGTGAAAAAGTT GCATGGTGCTGG

(2) Reverse (HBx2): 5′- ATGGCTGCTAGGCTGTACTGC CAACTGGATCCTTCG

Northern blot analysis

Total RNA was isolated from HEK 293 cells with Trizol (Invitrogen) that were transfected with 1 μg HBx encoding plasmid DNA (along with 2 μg of unrelated RNA) or HBx + 2 μg of various RNA constructs in 1 mL volume using lipofectamine for 48 hours. RNA was quantitated and equal amounts (20 μg) were loaded in each lane and resolved on a 1% formaldehyde-agarose gel under denaturing conditions. RNA was transferred onto a nylon membrane (Amersham),

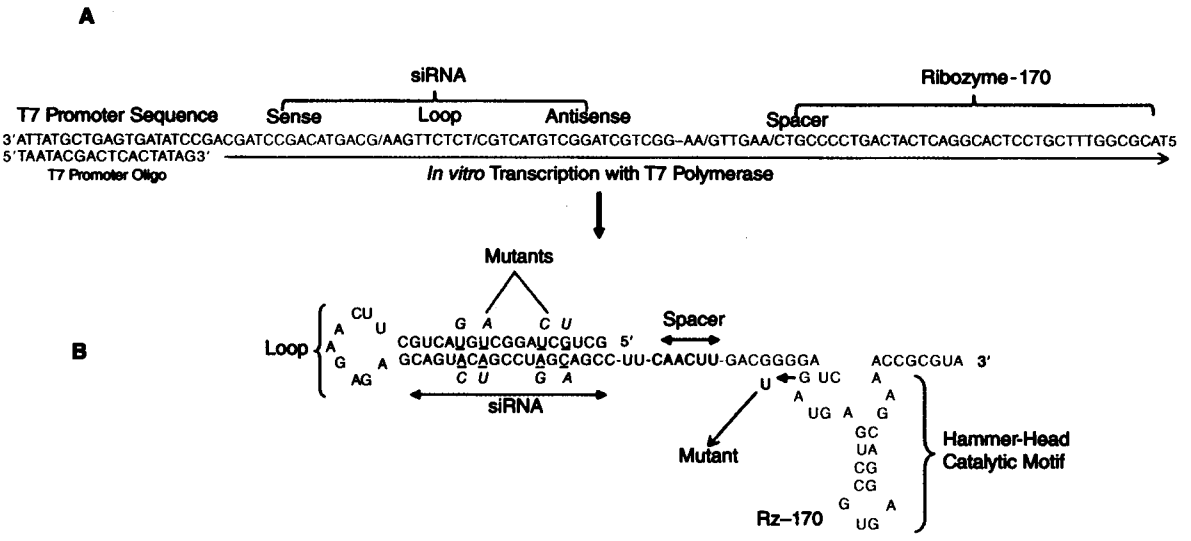

FIG. 2. *In vitro* synthesis of wt and mutant constructs of the chimeric siRNA-Rz RNA: Panel A; The strategy used for the synthesis of this chimeric RNA using T7 RNA polymerase system is same as described by Leirdal and Sioud (2002). In brief, an oligonucleotide is synthesized that encodes the siRNA, the cleavabale linker followed by the hammerhead Rz-170. The chimeric construct is synthesized using T7 primer and T7 RNA polymerase. Panel B: The selectively disabled mutant versions were synthesized that consisted of either mutant siRNA or inactive Rz-170 or both (shown by an arrow—for details, see text).

UVcross-linked, and probed with ^{32}P-labeled HBx antisense probe (full-length), followed by autoradiography.

Western blot analysis

About 1 × 10^6 HepG2 cells were grown to 80% confluency in a six-well plate. They were transfected with 1 μg of HBx encoding DNA (pSG5.HBx) (along with 1 μg of unrelated RNA of similar length) or in combination with 1 μg of the wt chimeric construct in a final volume of 1 mL for 48 hours using lipofectin (Invitrogen). Cell lysates were prepared and equivalent amounts of proteins were loaded in each lane as described earlier (Goila and Banerjea, 2001, 2004). They were divided into two equal parts; one was used for estimating the levels of HBx protein and the other was used for the control actin protein. They were subjected to gel analysis and transferred onto a nitrocellulose membrane. Rabbit polyclonal antibody to HBx (Biovendor, NS, USA) was used as a primary antibody in 1:500 dilution in PBS pH 7.2 containing 0.1% Tween 20. The blot was developed with HRP conjugated secondary anti-rabbit IgG antibody (Santa Cruz, CA, USA) followed by NBT/BCIP treatment (Promega Biotech.). Antibody to actin (mouse-antiactin monoclonal antibody—Calbiochem, San Diego, CA, USA) was used to determine the levels of this control protein.

Results

Sequence-specific cleavage of HBx RNA with Rz-170

When equimolar (100 pmoles each) amounts of the full-length target RNA (465 nt long) were subjected to cleavage with Rz-170 in the presence of Mg++, specific cleavage products (170 and 295 nt long fragments) were observed (Fig. 1B, lane 2). Lane 1 shows the synthesis of the full-length 465 nt long target RNA (HBx) as expected along with minor prematurely truncated transcripts (lane 1). Rz-170, which was created by introducing a single change in the catalytic motif, failed to show any cleavage (lane 3). No cleavage was observed in the absence of Mg++ (data not shown).

In vitro *cleavage of the HBx RNA with chimeric siRNA-Rz constructs*

Equivalent amounts (100 pmoles each) of labeled HBx RNA (Fig. 3, lane 1) were subjected to cleavage with various constructs as indicated on top of each lane. Incubation of labeled HBx RNA with Rz-170 (lane 2) or chimeric constructs containing wt Rz component (lanes 3 and 4) showed specific cleavage products, and as expected, chimeric constructs that possessed mt Rz component failed to cleave the target RNA (lanes 5 and 6). We conclude that chimeric constructs, despite their large size, are still capable of cleaving the target RNA specifically using their Rz component efficiently.

Processing of chimeric constructs with cytoplasmic constructs

Uniformly ^{32}P-labeled 110 nt-long wt chimeric RNA (Fig. 4, lane 1) when treated with equal amounts of cytoplasmic extracts yielded two cleavage products (52 and 40 nt long) (lane 2); lanes 3 and 4 represent the cleavage obtained with mt-siRNA-wt-Rz and wt-siRNA-mt-Rz, respectively. We

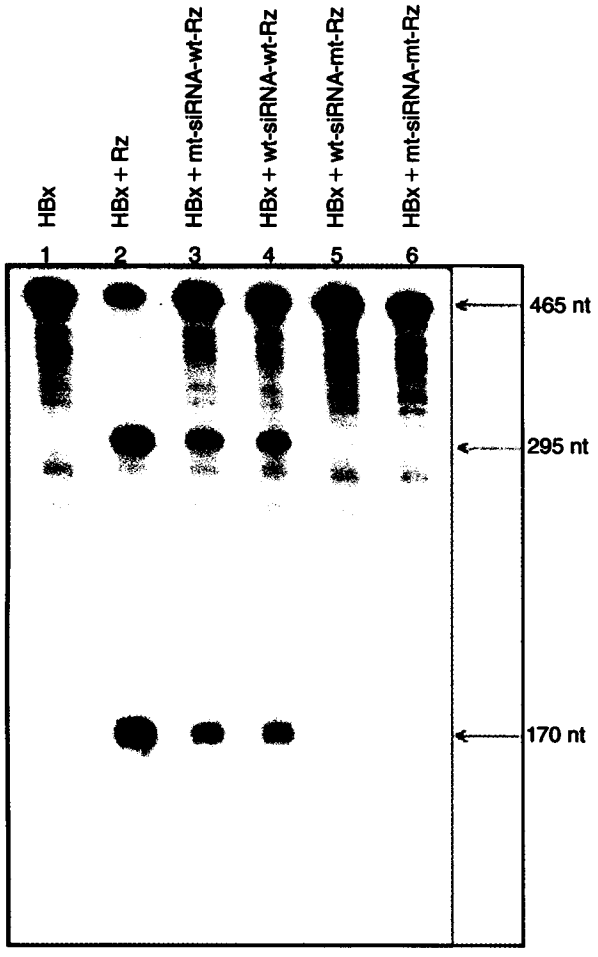

FIG. 3. HBx RNA cleavage by wt and chimeric siRNA-Rz mutants. The nature of the experiment is same as described for Figure 1B. Lane 1 shows the synthesis of full-length HBx RNA (labeled substrate RNA). Specific cleavage products were observed with Rz-170 (lane 2), wt-siRNA-wt-Rz (lane 3), and mt-siRNA-wt-Rz. Chimeric constructs containing inactive Rz, namely wt-siRNA-mt-Rz (lane 5) and mt-siRNA-mt-Rz (lane 6), failed to generate any Rz-specific cleavage products.

conclude that wt and the two mt constructs were processed correctly by cellular nucleases with equal efficiencies.

Modulation of intracellular target gene expression by wild-type and mutant constructs

Either HEK 293 or HepG2 (1×10^6) cells were tranfected with 1 μg of HBx encoding DNA (along with 1 μg of unrelated RNA of same length) in the final volume of 1 mL. A prominent HBx-specific gene expression was observed in these cells (Fig. 5A, lane 2) and taken as 100% for comparison (panel B and D). Wt chimeric construct transfected cells showed about 70 % reduction (compare lanes 1 and 2). Mt siRNA-wt-Rz caused about 30% and siRNA-mt-Rz about 45% (relative intensities of the corresponding lanes are shown in panel B). Cells cotransfected with 1 μg each of Rz-170 RNA + HBx encoding DNA caused only about 30% reduction (Fig. 5C and D, lane 3). When 3 μg of Rz-170 was used for transfection, about 60% reduction was observed (data not shown). On the contrary, 1 μg of mt-siRNA-mt-Rz showed only 10% reduction (lane 4) and 1 μg of wt-siRNA-wt-Rz showed about 70% reduction (lane 5), as observed earlier (Fig. 5A and B, lane 1). The relative intensity of the amplified DNA bands are shown in panels B and D. These are representative results from three independent experiments. We conclude from this experiment that wt construct was significantly more effective in reducing the target gene expression when compared with chimeric mts or Rz-170 alone. The levels of the control RNA (huGAPDH) remained same in all the corresponding lanes, which suggest that chimeric constructs downregulated the target gene expression in a specific manner. PCR carried out in the absence of RT showed no HBx-specific amplification (data not shown). The efficacy of various RNA constructs was determined by Northern blot analysis also, and the results are shown in panel E along with the quantitation of the target RNA (lower panel). Cells transfected with HBx encoding plasmid showed a prominent HBx RNA (lane 1). Wt-siRNA-wt-Rz construct showed 70% reduction (compare lanes 1 and 2). The two selectively disabled mts showed 28–34% reduction (lanes 3 and 4), and the double mt exhibited about 15% reduction (lane 5) (representative of three experiments). The ribosomal RNAs (control) isolated simultaneously showed no changes in all the corresponding lanes.

Inhibition of protein synthesis by chimeric constructs

When HepG2 cells were transfected with HBx encoding DNA (along with an equivalent amount of unrelated RNA), a prominent 16.5 KDa immuno-reactive X protein band was observed (Fig. 6, lane 1) by western blot analysis. In cells that were cotransfected with wt-siRNA-wt-Rz along with equal amounts of HBx encoding DNA (final concentration 1 μg/mL), potent (>70%) knockdown of the expression of X protein was observed (Fig. 6A and B, lane 4, shown by an arrow). Cells that received no DNA (cell control) failed to show any HBx protein-specific band (data not shown). Cells that received mt-siRNAi-wt-Rz reduced the expression by 20% (lane 2) and wt-siRNA-mt-Rz caused about 45% reduction (lane 3). Chimeric construct mt-siRNA-mt-Rz showed only about 5% reduction when compared with control (lane 5). This reduction of target gene is specific because the levels of control actin protein in all the corresponding lanes remained constant (panel B). These are representative data from three independent experiments.

Wild-type and mutant constructs interfere with the X protein-mediated transactivation function in a differential manner

The HBx protein is known to activate a number of cellular promoters, including the HIV-1 LTR promoter (Robinson,

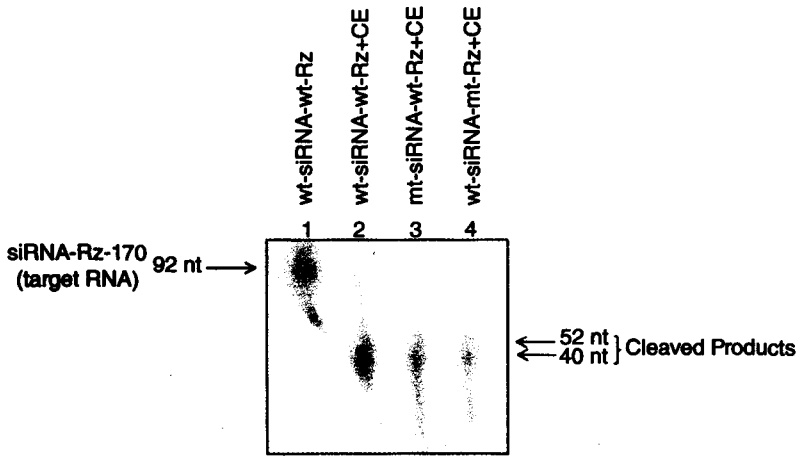

FIG. 4. *In vitro* processing of the wt chimeric RNA with cytoplasmic nucleases: Lane 1 depicts the synthesis of wt chimeric RNA (110 nt long). Specific cleavage products were obtained with wt chimeric construct (lane 2), mt-siRNA-wt-Rz (lane 3), and wt-siRNA-mt-Rz (lane 4) in the presence of cell extracts (CE).

1994; Gomez-Gonzalo et al., 2001), and in the presence of wt or mt chimeric constructs against the X gene, reduced activation is expected because of either cleavage of the HBx RNA with Rz or selective degradation by siRNA-mediated pathway. We cotransfected 100 ng each of HIV-1 LTR-driven LTR-B-luciferase reporter plasmid DNA (reporter DNA) and either wt or mt-chimeric constructs in 1 mL of a six-well plate (1×10^6) of HEK 293 cells for 48 hours, and determined luciferase activity as described earlier (Goila and Banerjea, 2001). Representative results from three independent experiments are shown in Figure 7. The cell control (lane 1), HBx encoding DNA + unrelated RNA (lane 2) and wt construct (lane 3) treated cells showed basal levels of reporter gene activity (all less than 50 units) as expected. Cells that received LTR-β-luciferase reporter DNA (henceforth called Reporter DNA) showed activity that was arbitrarily assigned 1 for comparison (lane 4). HBx + Reporter DNA showed >6.5-fold more activation as expected (lane 5). When the same experiment was carried out in presence of the wt chimeric construct (lane 6) more than twofold drop was observed (compare lanes 6 and 5). On the contrary, the mt constructs caused less than 1.5-fold reduction (lanes 7 and 8). The mt-siRNA-mt-Rz showed almost no reduction in reporter gene activation (compare lanes 5 and 9). We conclude that wt-chimeric construct interfered very significantly with the known function of HBx and that this inhibition can be modulated using mt constructs.

Discussion

In the present work, we made a novel chimeric construct with the idea that it will interfere with the intracellular target gene expression more powerfully, and by using selectively disabled mt versions of it, it may be possible to modulate the level of target gene expression. In order to achieve this objective it was important to establish that this novel unprocessed chimeric RNA constructs with active Rz component possessed the ability to cleave the target RNA and that these chimeric RNAs were processed correctly by nucleases present in the cytoplasmic extracts of a mammalian cell. Our *in vitro* cleavage data with siRNA-Rz chimeric constructs suggest that they possessed the ability to cleave the target RNA specifically. Thus even the unprocessed chimeric constructs may contribute toward intracellular reduction of the target RNA. Furthermore, our *in vitro* data suggests that the wt and two mt constructs were processed by intracellular nucleases with equal efficiency. Thus, the varying efficacy of the wt and the two mt constructs was not due to different *in vitro* processing activities.

It is quite conceivable that the full-length wt chimeric RNA (110 nt long) will acquire additional secondary structures that may decrease the cleavage efficiency. Indeed efficiency of Rz-170 mediated cleavage of HBx RNA was about 10–15% better than any of the chimeric constructs that possessed wt Rz component (Fig. 3). It was earlier observed by several investigators that although Rz may show excellent *in vitro* cleavage activity it may not be effective in causing intracellular reduction of the target RNA. This may be largely due to poor accessibility of the target sites inside the mammalian cells besides other reasons (Schubert et al., 2004). The fact that most of the *in vitro* synthesized wt and mt chimeric RNAs were cleaved specifically in the linker region in less than 2 minutes in the presence of cytoplasmic extracts strongly suggests that the same may be happening inside the mammalian cells also in a rapid manner. As expected, our wt chimeric construct interfered with the target gene expression very powerfully based on our transient transfection data. The selectively disabled chimeric mts allowed us to conclude that siRNA component played a predominant role toward the inhibition of gene expression.

It is noteworthy that we selected the siRNA target site that involved the AUG of the target RNA, which is usually accessible toward siRNA-mediated downregulation. The potency of our approach could also be explained on the basis that once the target RNA is cleaved by either of the two antiviral approaches (siRNA or Rz), it may open up secondary structures at the target sites. This may facilitate more efficient hybridization between the target RNA and Rz or siRNA. Alternatively, the cleaved target RNA fragments are known to become increasingly more susceptible to degradation by nucleases. The potency of our wt chimeric construct was not only established at the target RNA level but

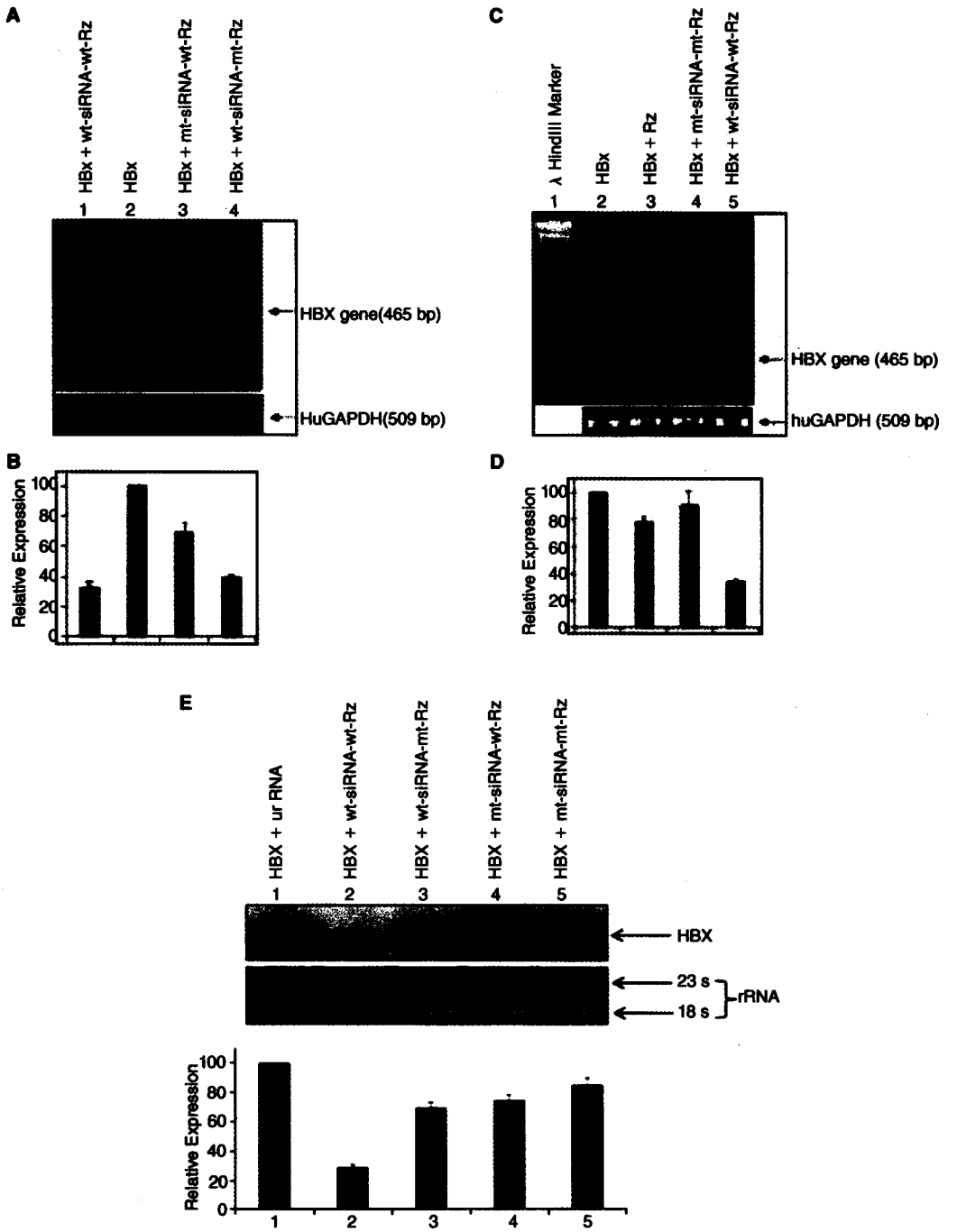

FIG. 5. Inhibition of intracellular target RNA with wt and mutant chimeric constructs: Panel A: Indicated amounts of HBx encoding DNA in presence and absence of wt or selectively inactivated chimeric constructs were transfected into HepG2, and total RNA was isolated as described in the text. Target RNA and control RNA were estimated using RT-PCR based assays. Panel B: The levels of the target RNA was evaluated by quantitating the intensity of the PCR-amplified bands in lane 2 of panel A as 100. Panel C and D: Cells were transfected with Rz-170 RNA (lane 3), with mt-siRNA-mt-Rz (lane 4), and wt-siRNA-wt-Rz (lane 5). Lane 2 is same as lane 2 of Figure 5, panel A. Northern blot analysis (panel E): HEK 293 cells transfected with HBx encoding DNA + unrelated RNA (lane 1); HBx + wt-siRNA-wt-Rz (lane 2); HBx + wt-siRNA-mt-Rz (lane 3); HBx + mt-siRNA-wt-Rz (lane 4); and HBx + mt-siRNA-mt-Rz (lane 5). ^{32}P-labeled full-length HBx gene was used as a probe. Control ribosomal RNA and quantitation of the intensity of the HBx-specific bands are shown in corresponding lanes.

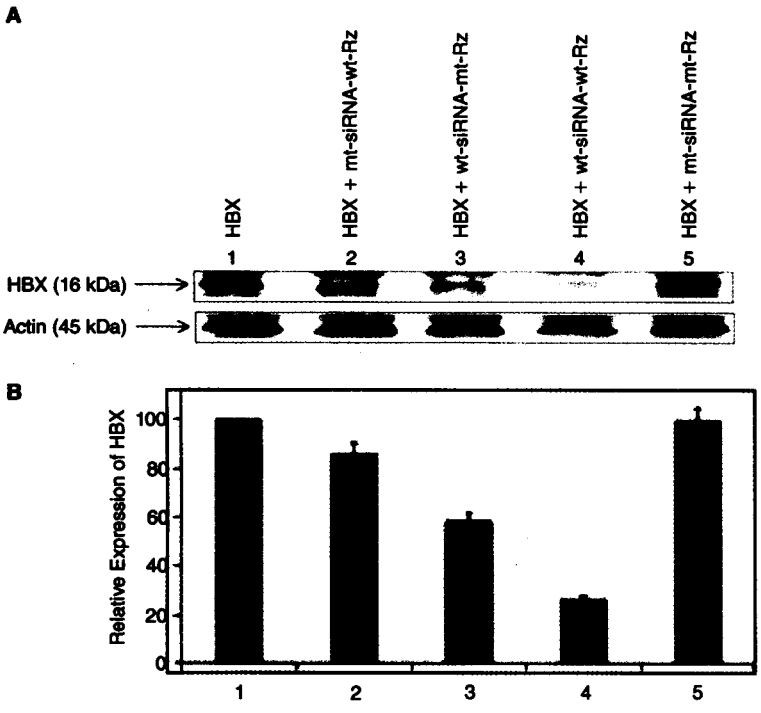

FIG. 6. Potent knockdown of HBx protein synthesis with wt chimeric construct. HBx encoding DNA (along with equal amounts of unrelated RNA) (lane 1) was cotransfected into a mammalian cell with wt chimeric RNA (both at 1 μg/mL) (lane 4), mt-siRNA-wt-Rz (lane 2), wt-siRNA-mt-Rz (lane 3), and mt-siRNA-mt-Rz (lane 5). Equal amounts of proteins were loaded in each lane. Western blot analysis was carried out for estimating the HBx protein and control protein (actin) using specific antiserum as described in the text.

almost a complete knockdown of protein production was observed by western blot analysis. On the other hand the two selectively disabled mts were less effective in causing the downregulation of target gene expression. Completely disabled mt construct (mt-siRNA-mt-Rz) failed to show any significant intracellular reduction of either target RNA or at the protein expression level. Antisense components present in the design of Rz and siRNA in our constructs may account for the residual (5–10%) inhibitory activity present in siRNA-Rz chimeric RNA. These chimeric constructs can be delivered to the desired cell via lentiviral vectors or other vectors for achieving specific gene suppression (Banerjea et al., 2004). Selectively disabled mt versions may allow us to modulate the extent of gene suppression, which can be exploited for variety of studies, especially for genes that are temporally regulated.

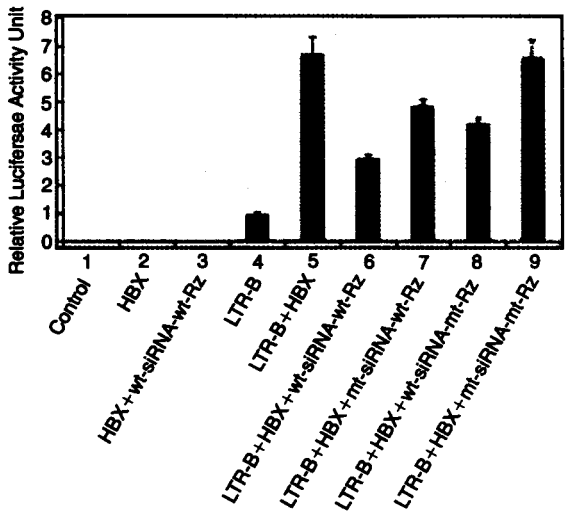

FIG. 7. Functional interference of X protein with chimeric constructs. Indicated amounts of HBx encoding plasmid DNA was cotransfected with HIV-1 LTR-Luciferase reporter gene construct in the presence and absence of wt or mutant constructs. The cell lysates were prepared, and luciferase activity was determined as described earlier (Goila and Banerjea 2001, 2004).

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Potent Intracellular Knock Down of Hepatitis B Virus X RNA by Catalytic Hammerhead Ribozymes or DNA-Enzymes with Antisense DNA-Oligonucleotides or 10-23 DNA-Enzymes that Powerfully Augment *In Vitro* Sequence-Specific Cleavage Activities

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Abstract: Novel antiviral approaches are needed to control Hepatitis B virus infection worldwide. X protein of this virus activates various promoters and is strongly associated with hepatocellular carcinoma. Although several groups, including ours, reported sequence-specific cleavage of X RNA by either ribozymes (Rzs) or DNA-enzymes (Dzs) earlier, but none of these studies reported 100% *in vitro* cleavage of the full-length X RNA. We reasoned that by melting the secondary structures near the Rz/Dz cleavage site with specific antisense DNA oligonucleotides (ODNs) or 10-23 Dz, it may be possible to achieve this objective. Hammerhead motif containing Rz-170 specific for X RNA was constructed by recombinant techniques and Dz-237 was synthesized using the 10-23 catalytic motif. When specific ODNs or 10-23 Dzs were included in the cleavage reaction with either Rz-170 or Dz-237, increased cleavage was observed in a dose-dependent manner which often resulted in almost complete *in vitro* cleavage of the target RNA. Rz-170 in combination with specific ODNs caused potent intracellular reduction of HBx RNA. Thus, the cleavage activity of catalytic nucleic acids (Rzs or Dzs) can be increased significantly by specific ODNs or Dzs and this treatment also results in potent intracellular target RNA reduction. These findings have important therapeutic implications.

INTRODUCTION

Ribozymes (Rzs) are short catalytic RNA molecules that possess the ability to cleave the target RNA in a sequence-specific manner and control splicing reactions (Cech, 1987) [1,2,3,4]. Although several types of catalytic motifs have been described, the hammerhead Rz being the smallest and with minimal target sequence requirement, has been exploited extensively for variety of purposes including therapeutic applications [5,6,7,8]. On the other hand, Deoxyribozymes or DNA-enzymes (Dzs), as originally [9] are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner [10,11,12]. Over the years several kinds of Dzs with unique catalytic motifs have been described but Dz possessing the 10-23 catalytic motif has been exploited extensively by several investigators [11]. This Dz possesses a conserved 15 nucleotide long 10-23 catalytic motif and is able to cleave any target RNA between purine and pyrimidine under *in vitro* and *in vivo* conditions. Their ability to cleave any target RNA has been exploited for knocking down gene expression against variety of target genes including HIV-1 genes [11,13,14] with varying outcomes. In some instances very efficient intracellular gene inhibition was observed with 10-23 Dz [15]. We also reported earlier that 10-23 Dz was more effective in cleaving the full-length CCR5 (HIV-1 coreceptor) RNA compared to a hammerhead ribozyme [16,17] carried out a comparative study between hammerhead ribozymes (Rzs) and Dzs that were targeted against the same sequences and concluded that some sequences were cleaved better by Rzs and some by Dzs. There are several other nucleic-acid based approaches that have been used for specific inhibition of target genes. The catalytic ribozymes, aptamers, antisense DNA or RNA or small interfering RNAs (siRNAs) have been used for inhibiting the expression of foreign genes including HIV-1 replication [8]. Physiologically relevant RNAs are usually long and consist of multiple stem-loop structures and up to 90% putatitive cleavage sites were earlier shown to be in inaccessible to either Rz or Dz mediated cleavage [18]. Earlier, unwinding activity of an RNA helicase was used to increase the cleavage potential of Rzs [19]. Collectively, all these studies suggest that not all the target sites are available for cleavage by a single kind of catalytic nucleic-acid molecule most probably because the secondary and tertiary structures in the target RNA prevent optimal Watson-Crick base pairing with Rzs or Dzs. Introduction of locked nucleic-acids in antisense design resulted in enhancing its stability [20] and when incorporated in the substrate recognition arms of a Dz, it improved the catalytic efficiency [21].

Earlier few nucleic-acid based approaches were used to modulate the cleavage activity of the Dzs and Rzs with moderate success but none of them were tested for bio-efficacy. Oligonucleotide effectors (regulators) that bind to both enzyme and substrate were used to regulate the catalytic activity of Rzs & Dzs [22, 23]. Whether oligodeoxynucleotides (ODNs) that hybridize specifically to the target RNA alone could modulate *in vitro* and *in vivo* the catalytic activity of Rzs or Dzs have not been earlier attempted.[24] reported enhancement of ribozyme catalytic activity by a contiguous oligonucleotide (facilitator) and by 2'-O-methylation. Oligonucleotide facilitators have been used to either enhance

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[25] or even inhibit catalytic activity of Rzs [26]. We recently reported Dz mediated augmentation of *in vitro* cleavage of a truncated HIV-1 Gag gene by specific antisense molecules [27]. Oligonucleotide facilitators have earlier been used to enhance hammerhead ribozyme mediated cleavage of long RNA substrates with multiple-turnover activity [28]. Although all the above approaches resulted in enhanced cleavage of the target RNA under *in vitro* conditions, it was not known if combinations of ribozymes or DNA-enzymes with facilitators could be exploited to down regulate intracellular levels of the target RNAs.

In the present study, we have identified and characterized those ODNs and Dzs that significantly enhanced the Rz and Dz mediated cleavage of full-length X RNA (HBx RNA) of hepatitis B virus (HBV) on one hand and inhibit intracellular expression of the target RNA in a liver specific cell line, HepG2 on the other. The Hepatitis B virus X protein (HBx) acts as a powerful transactivator for several genes including HBV genes and is known to be strongly associated with hepatocellular carcinoma [29, 30]. These results are potentially important for therapeutic purposes for the treatment of HBV infections against which there are no effective antiviral treatment available.

METHODOLOGY

Cloning of HBx Gene and *In Vitro* Transcription

Plasmid pSG5.HBx encoding the X gene of HBV [31] was a kind gift from Vijay Kumar, ICGEB, New Delhi, India. The entire gene is placed under SV40 and T7 promoters (Fig. 1). The former promoter is used for intracellular expression and the latter was used for obtaining *in vitro* transcripts using the transcription kit from Promega Biotech., as described earlier [32]. After linearization with appropriate enzyme at the 3'-end, full-length X RNA will be synthesized.

Construction and Cloning of HBx-Rz-170 Targeted Against X Gene

The construction of Rz-170 (Fig. **2A**) has been described by us in detail previously [33]. It possessed the hammerhead motif and targeted against the GUC sequence. *EcoR*1 and *BamH*1 restriction sites were engineered at the ends of the hybridizing arms of the Rz which facilitated cloning it into pcDNA3 expression vector (Promega). This placed the Rz under T7 and CMV promoter.

In Vitro Cleavage of Target RNA with Rz

In vitro transcription of the linearized plasmid DNA was carried out in the presence of labeled UTP using T7 RNA polymerase. The cleavage reaction was initiated by adding equimolar amounts (100 pmoles each) of the labeled target RNA and unlabeled Rz in a reaction buffer containing 50mM Tris/HCl, pH 7.5, in a volume of 10µl. The reaction mixture was heated briefly at 94°C and the cleavage reaction was initiated by adding $MgCl_2$ (final concentration 10mM) at 37°C for 2 hours. The cleaved RNA fragments were subjected to gel analysis as described before [34]. The radioactive RNA bands on the X-Ray film were quantitated by densitometry (GS-710 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA, USA).

Primers & DNA-Enzyme

All the 20nt long antisense oligonucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys (The Woodlands, TX). The conserved 15 nt long (5'-GGCTAGCTACAACGA) 10-23 catalytic motif was flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. We have earlier described in detail the construction of Dz-237 that cleaved the X RNA specifically into two fragments (Fig. **2B**) [35]. Dz-155, wild-type and mutant versions of Dz-192 were also assembled using 10-23 catalytic motif that possessed a single nucleotide substitution (G to C) in the 10-23 catalytic motif (Fig. **3B**). This change is known to render the Dz catalytically inactive [35].

Dz-Mediated Cleavage

Equivalent amounts of unlabeled Dz and labeled substrate RNA (100 pmoles each) were allowed to interact in a final volume of 10µl in a buffer containing 50mM Tris.HCl, pH 7.5 in presence of 10mM $MgCl_2$ (standard conditions) as described earlier [9] for 2 hours at 37°C in the absence or in the presence of indicated amounts of ODNs. The cleaved products were resolved by gel analysis and cleavage efficiency was determined as described earlier [34, 35].

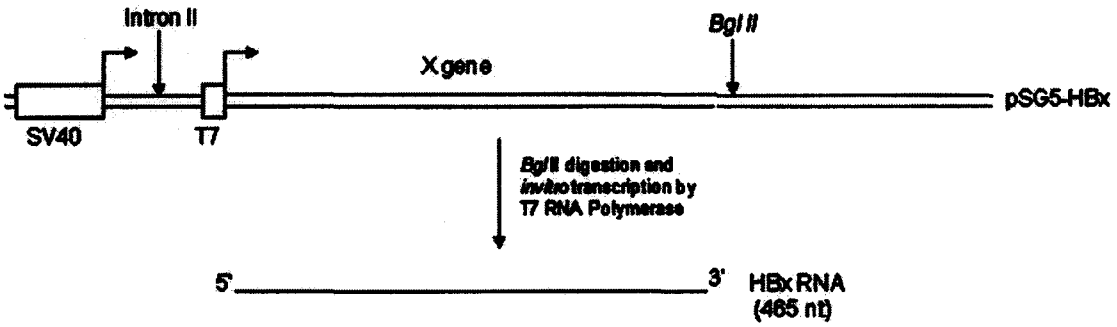


Fig. (1). Full-length HBx encoding DNA and *in vitro* transcription.

Plasmid pSG5.HBx (Kumar et al., 1996) contains the full length X gene of hepatitis B virus. It is placed downstream of the powerful SV40 and T7 promoter of the expression vector pSG5 (Clonetech, Palo, Alto, CA, USA). After linearizing it with *Bgl* II restriction enzyme, a 465nt long transcript is generated by *in vitro* transcription using T7 RNA polymerase.

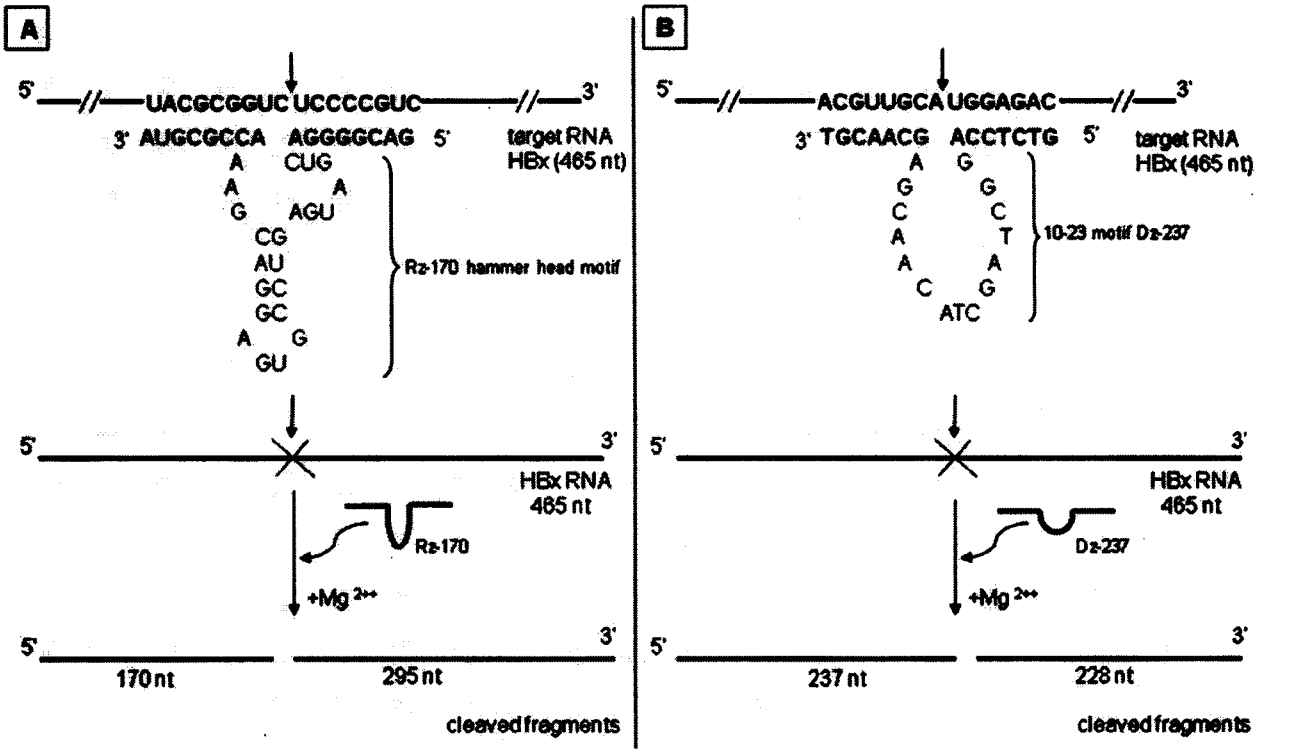


Fig. (2). Sequence of the target RNA against which Rz-170 and Dz-237 were designed are shown along with their specific cleavage products.

For constructing Rz-170, eight bases long hybridizing arms are made complementary to the target RNA to provide specificity (**Panel A**). The hammerhead catalytic motif is same as described before (Shahi et al., 2001). The site of cleavage in the target RNA is shown by an arrow. When the full length HBx RNA is subjected to cleavage by Rz-170 in presence of $MgCl_2$, two specific RNA fragments are expected that are170 and 295nt long. **Panel B:** 10-23 catalytic motif containing Dz-237 was chemically synthesized that targeted AU dinucleotide in the target RNA for cleavage. 7 bases long hybridizing arms were made complementary to the target RNA to provide specificity. The expected cleavage products generated due to the action of Dz are shown.

Intracellular Inhibition of the Expression of Target RNA by Rz + ODNs

The intracellular decrease in HBx-specific RNA after cotransfection into HepG2 cells with substrate encoding DNA (pSG5.HBx) + Rz in the presence or absence of ODNs were monitored by RT-PCR based assays. To ensure uniform transfection efficiency, we always transfected equal amounts of reporter gene containing plasmid (pSV-β-gal, Promega). We repeatedly obtained about 70 – 80% transfection efficiency. All the plasmid DNAs used was Qiagen column purified which showed no toxicity under our experimental conditions. Several dilutions were initially made to determine the linear range for PCR-amplified products. Total RNA was isolated using TRIZOL reagent (GIBCO-BRL) following the procedure described by the manufacturer and divided into two equal sets. One set was used for estimating the levels of full-length X RNA using HBx1 and HBx2 primers and the second set was used for estimating the levels of the house keeping gene, human glyceraldehyde-phosphodehydrogenase (HuGAPDH) as described earlier [36]. The following primers were used for estimating the intracellular levels of full-length X RNA.

(1) Forward (HBx1): 5'-TTAGGCAGAGGTGAAAAAGTT GCATGGTGCTGG .

(2) Reverse(HBx2):5'- TGGCTGCTAGGCTGTACTGCCA ACTGGATCCTTCG

Western Blot Analysis

1 x 10^6 HepG2 cells were grown to 80% confluency in a six well plate. They were transfected with either Rz-170 (1μg /ml) alone or in the presence of increasing concentrations of ODN #1 and # 2 in a final volume of 1ml for 48 hours along with 1μg of X gene encoding DNA, pSG5.HBx, using lipofectin (Invitrogen). Cell lysates were prepared and equivalent amounts of proteins were loaded in each lane as described earlier [35, 33]. They were subjected to gel analysis and transferred on to a nitrocellulose membrane. Rabbit polyclonal antibody to HBx (Biovendor, NJ, USA) was used as a primary antibody in 1:500 dilution in PBS pH 7.2 containing 0.1% Tween 20. For β-actin as control, mouse raised monoclonal antibody (Calbiochem, CA ,USA) was used as primary with 1:10,000 dilution in PBS-T. The amounts of pSG5.HBx DNA was kept constant in each experimental lane at 1μg/ml. The absolute amounts of DNA introduced in each well was made equivalent by adding unrelated DNAs.

Inhibition of X Protein Mediated HIV-1 LTR Activation with Rz and ODNs or Dzs

HEK 293 or HepG2 cells were cotransfected with 100 ng of pBS-LTR-B-Luciferase (henceforth referred to as pLTR-B – the Luciferase reporter gene is placed down stream of the HIV-1 LTR-B promoter) (obtained from AIDS Research and Reference Reagent Program of NIH, MD, USA) plasmid

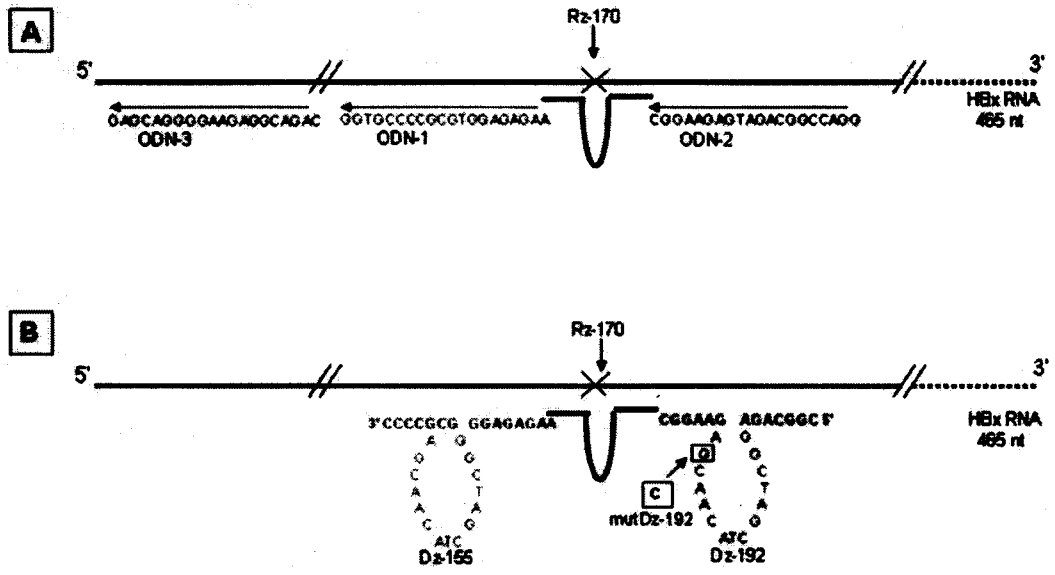


Fig. (3). ODNs and Dzs designed to augment Rz-170 mediated cleavage products.

Panel A: Three 20 nucleotides long antisense oligodeoxynucleotides (ODNs) (#1 to # 3) were synthesized. Two of them (ODN #1 and 2) were targeted to hybridize immediately next to the hybridizing arms of the Rz-170 and their sequences are shown. ODN #3 was 33nt away from the Rz cleavage site and its sequence is also shown. All the oligonucleotides were chemically synthesized and obtained from Sigma Genosys. **Panel B:** Two Dzs (Dz-155 and 192) possessing 10-23 catalytic motif, were synthesized immediately upstream and downstream of Rz-170. A mutant (disabled) version of Dz-192 was synthesized by substituting a C residue in place of G in the catalytic motif.

in the presence of indicated amounts of ODNs or Dzs in wells that received 100ng of pSG5.HBx plasmid DNA that was kept constant in all the wells. Lipofectin was used to introduce the mixture of various DNA combinations and cell lysates were prepared 24 hours later using 1x reporter lysis buffer (Promega). The extent of Luciferase activity was determined according to the manufacturer's instructions (Promega). A control reporter plasmid (pSV-β-gal, Promega) was always included to ensure uniform transfection efficiency.

RESULTS

In Vitro Synthesis of Full-Length HBx RNA by T7 RNA Polymerase

Plasmid pSG5-HBx (Fig. 1) was linearized with *Bgl* II restriction enzyme and when subjected to *in vitro* transcription using T7 RNA polymerase, a 465nt long full-length HBx transcript is expected using the Riboprobe transcription kit (Promega Biotech., WI, USA) in the presence of ^{32}P UTP. Transcripts were subjected to gel analysis and dried gels were exposed to X Ray.

Rz-170 Mediated Cleavage of HBx RNA in the Presence of Upstream and Downstream ODNs

Entire sequence of the Rz-170 along with the target RNA is shown in Fig. (2A). The target sequence was GUC located at nucleotide position 170 in the HBx gene. The 22nt long hammerhead motif was flanked with eight nucleotides long hybridizing arms on either side of the target sequence that were made complementary to the target RNA to provide specificity. When an equimolar amount (100pmoles each) of Rz-170 is used for cleaving a full-length HBx RNA (465nt), specific RNA fragments (170 and 295 bases long) are expected (Fig. 2A). In order to increase the Rz-170 mediated cleaved products, 3 antisense ODNs, each 20nt long, were synthesized. Two of these ODNs (#1 & #2) were designed to hybridize immediately adjacent to the two hybridizing arms of the hammerhead Rz-170. We designed another ODN (ODN#3) that was 33 nt upstream from the Rz-170 cleavage site (Fig. 3, panel A). In the similar manner, 10-23 catalytic motif containing Dzs were synthesized immediately upstream and downstream of the Rz-170. Exact sequence of these Dzs along with the mutant (disabled Dz-192) is shown in Fig. (3, panel B). Rz-170 mediated cleavage reaction was performed in the presence of indicated amount of ODNs individually and the results are shown in Fig. (4A). Lane 1 shows the synthesis of uniformly labeled full-length (465nt long) HBx RNA. When equimolar amounts (100 pmoles each) of Rz and labeled substrate RNA were used for cleavage under standard conditions [7, 12, 31], two specifically cleaved fragments (295 and 170nt long RNA fragments) were obtained (lane 2). When the same cleavage reaction was carried out in the presence of 10pmoles (lane 3); 50pmoles (lane 4); and 100pmoles (lane5) of ODN#1, a dose-dependent decrease in the input substrate RNA was observed. Approximately 4-fold decrease was observed in the input substrate RNA when lane 5 was compared with lane 2, and 2.5 fold reduction when lanes 3 and 4 were compared with lane2 (Fig. 4A). In the similar manner, the ODN#2 also caused a dose-dependent decrease in the substrate RNA when similar amounts were used (10pmoles – lane 6; 50pmoles – lane 7; 100pmoles – lane 8). Note almost complete disappearance of the input substrate RNA in lane 8. We conclude that ODNs that hybridized immediately upstream and downstream of Rz-170, were able to significantly

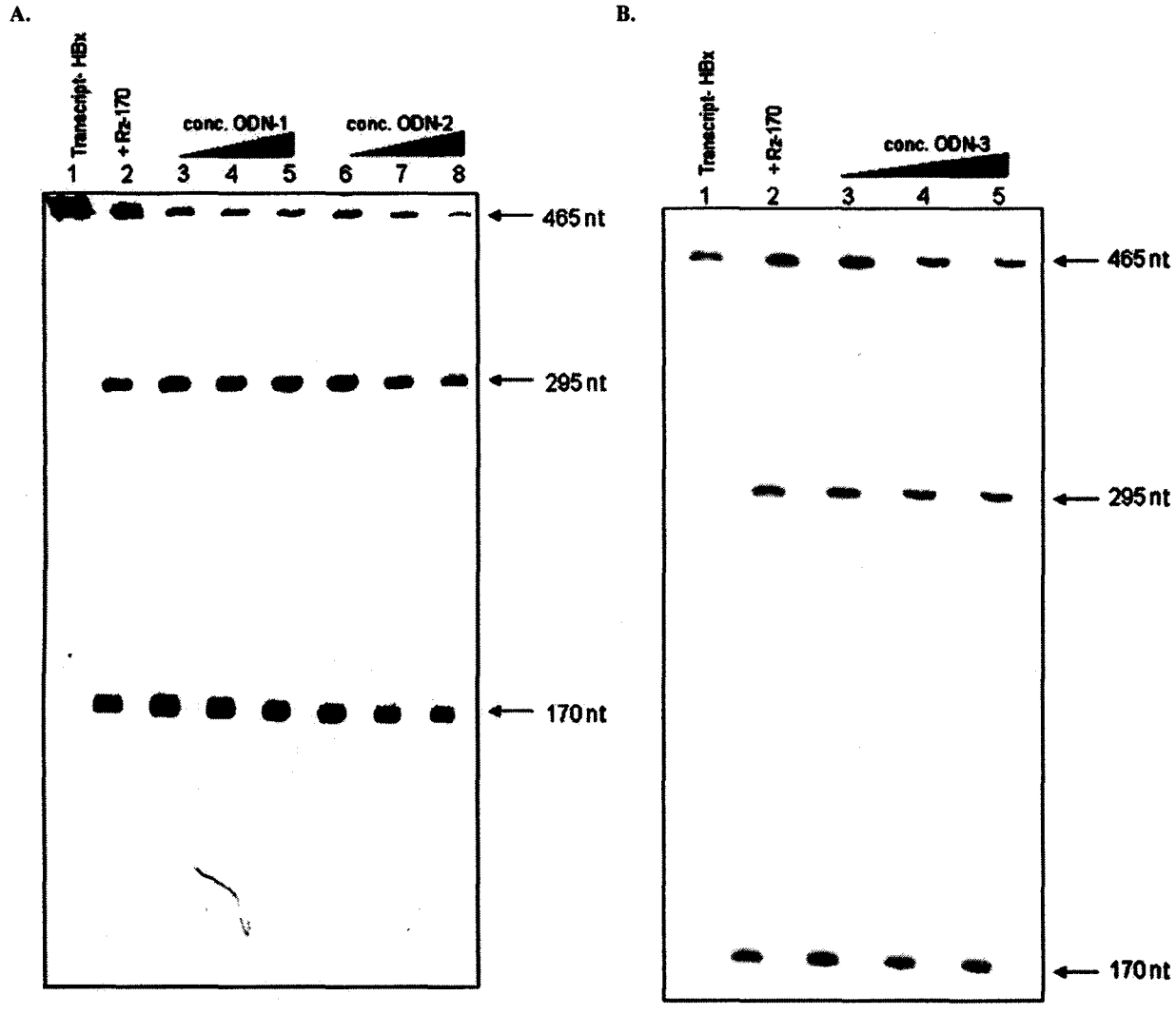


Fig. (4A). Augmentation of Rz-170 mediated cleavage by specific ODNs.

ane 1 shows the synthesis of uniformly labeled 465nt long HBx RNA. When equimolar amounts (100pmoles each) of substrate and Rz-170 were mixed in presence of 10mM $MgCl_2$, specific cleavage products (295 and 170nt long RNA fragments) were observed (lane 2). When the same cleavage reactions were carried out in presence of increased amounts of ODN #1 (10 pmoles – lane 3; 50 pmoles –lane 4; 100 pmoles – lane 5) or ODN #2 (10 pmoles –lane 6; 50 pmoles –lane 7 and 100 pmoles –lane 8), a significant reduction in the input substrate RNA was observed with concomitant increase in the cleavage products.

Fig. (4B). ODN#3 fails to augment Rz-170 mediated cleavage products.

When the same substrate RNA (lane 1) was subjected to Rz-170 mediated cleavage in the presence of increasing amounts of ODN #3 (lane 3 -1pmole; lane 4 -10pmoles; lane 5 – 100pmoles and lane 6 -200pmoles), no significant increase in the cleavage products were observed when compared with Rz-170 mediated cleavage only (lane 2).

augment the Rz-170 mediated cleavage and that ODN#2 was more effective than ODN#1.

Antisense ODN #3 Targeted to Hybridize 33nt Away from the Rz-170 Cleavage Site Fails to Augment its Cleavage

The nature of the experiment is same as described in Fig. (**4A**). Briefly, Rz-170 mediated cleavage of the target RNA (Fig. **4B**, lane 1) was studied in the presence of increasing amounts of ODN#3 (Fig. **3A**) and the results are shown in Fig. (**4B**). Lane 2 exhibits the cleavage obtained with equimolar amounts (100pmoles) of labeled substrate and Rz-170. When increasing amounts of ODN#3 (1pmole – lane 3; 10pmole – lane 4; 100pmoles – lane 5 and 200pmoles – lane 6) were included in the cleavage reaction, the extent of cleavage remained unchanged. We conclude that ODN that hybridized 33nt upstream from the Rz-170 cleavage site failed to augment Rz-170 mediated cleavage.

Only Downstream Dz (Dz-192) Significantly Enhanced Rz-170 Mediated Cleavage

Since we observed significant enhancement of Rz-170 mediated cleavage products by the two ODNs (#1 & #2) that hybridized immediately upstream and downstream to the hybridizing arms of the Rz, we wanted to find out if the two Dzs (Dz-155 and Dz-192) that were designed in a similar manner (Fig. **3B**) could also enhance the Rz-170 mediated cleavage. The various cleavage products (including partially cleaved products) that are expected due to the combined actions of Rz + Dz-155 and Rz + Dz-192 are shown schematically in Fig. (**5**). The reaction conditions were same as described for Fig. (**4A**) and the results are shown in Fig. (**6A**). Lane 1 shows the labeled full-length *in vitro* synthesized HBx RNA as described earlier. Lane 2 shows sequence-specific cleavage of the target RNA when equimolar amounts of Rz were used. In presence of increasing amounts of Dz-155 (lane 3 -10pmole; lane 4 – 50pmoles; lane 5 – 100pmoles), no significant reduction in the amounts of input substrate RNA was observed. Lane 3 and 4 show no decrease but lane 5 shows 1.5 fold decrease when compared with lane 2. On the other hand when the cleavage reaction was carried out in presence of increasing amounts of Dz-192 (lane 6 – 10 pmoles; lane 7 – 50 pmoles and lane 8 - 100pmoles), a dose-dependent decrease in the input substrate RNA was observed with almost complete disappearance at 50 and 100pmoles of Dz-192. Lane6 shows 4 folds, lane7 shows 5 folds and lane 8 shows 5.5 folds decrease when compared with input substrate RNA present in lane2 (Fig. **6A**). We conclude that only the downstream Dz-192 was able to significantly enhance the Rz-170 mediated cleavage.

Wild-Type & Mutant Dz-192 Augment Rz-170 Mediated Cleavage

The purpose of this experiment was to compare augmentation of the Rz-170 mediated cleavage between the wild-type and mutant-Dz-192 (disabled). As reported earlier, this mutant Dz [35] failed to cleave the target RNA completely and served as an important antisense control for the wild-type Dz. Exactly same amounts of wild-type (Dz-192) and mutant-Dz-192 were added to the cleavage reaction which contained equimolar amounts of Rz-170 and Substrate RNA. The results of this experiment are shown in Fig. (**6B**). Lane 1 is the HBx transcript and when cleavage reaction was carried out with Rz-170 alone (lane 2), two specific fragments were seen as described before. When the Rz-170 mediated cleavage reaction was carried out in the presence of increasing amounts of Dz-192 (lane 3 -1pmole; lane 4 -10pmoles and lane 5 -100pmoles), a dose-dependent decrease in the substrate RNA was observed along with multiple cleaved fragments. These fragments match the predicted pattern of cleavage described earlier (Fig. **5**, steps C and D). The same experiment was carried out with increasing amounts of Mutant-Dz-192 under identical conditions of cleavage (lane 6 - 1pmole; lane 7 -10pmoles and lane 8 -100pmoles). In this case also a dose-dependent decrease in the input substrate RNA was observed. In both cases, up to 8 – 10 fold decrease in the input labeled substrate RNA was observed in the presence of highest amounts of Dz used (compare the extent of uncleaved input substrate RNA in lanes 1, 5 and 8). Since this mutant-Dz is catalytically inactive, no additional cleaved products were observed. The extent of reduction in the input RNA with either wild-type (WT) or mutant-Dz in corre-

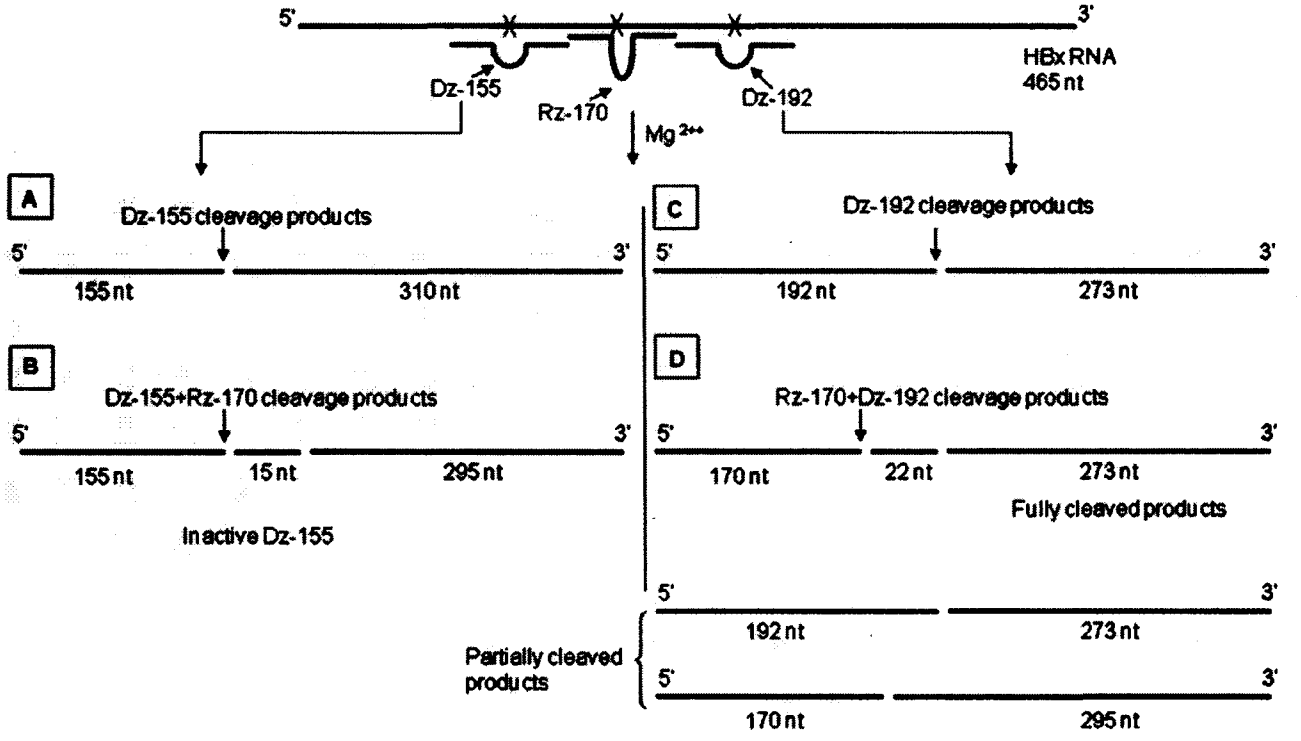


Fig. (5). Modulation of Rz-170 mediated cleavage products with Dzs.

Full-length HBx RNA was used as target RNA for cleavage with Rz-170 in the presence of upstream (Dz-155) and downstream Dz (Dz-192). The expected cleavage pattern generated by cleavage with Rz, Dz and Rz + Dz is shown schematically in Fig. (**6**) (steps marked A to D).

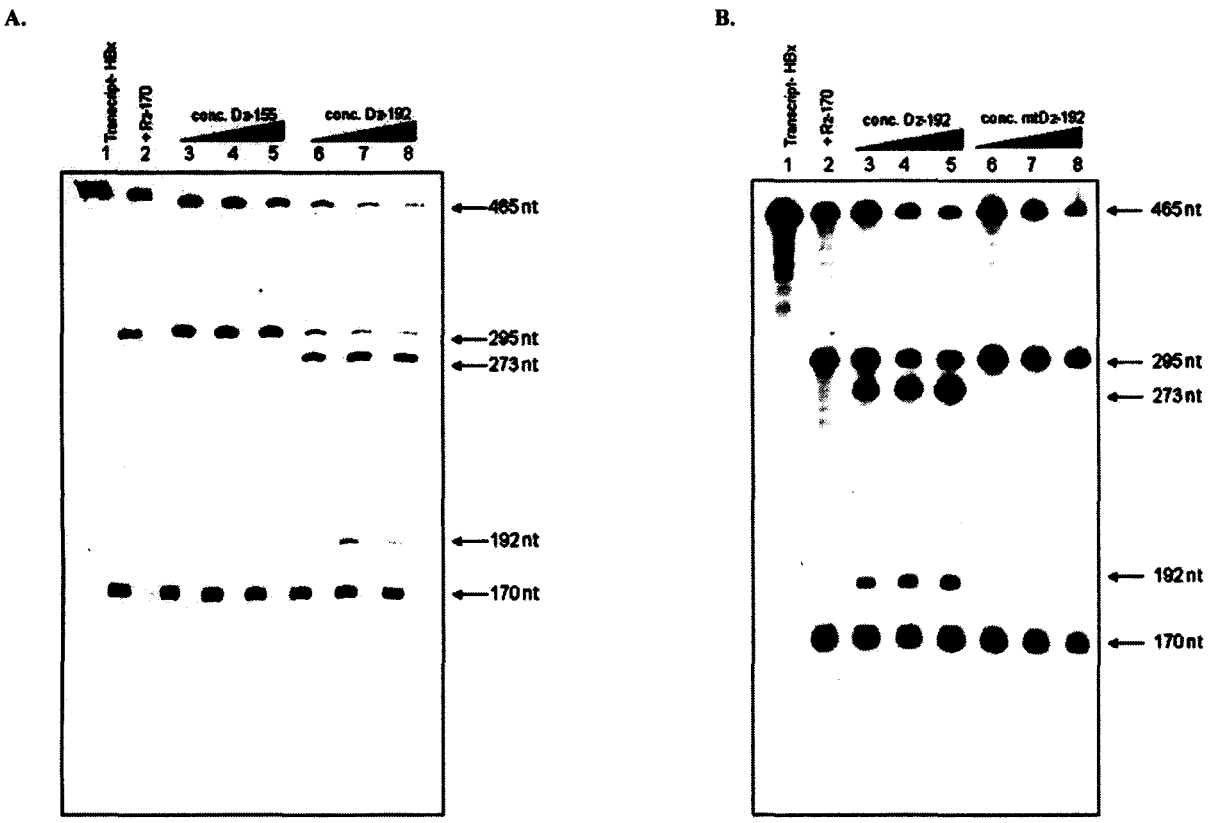


Fig. (6A). Downstream Dz (Dz-192) augments Rz-170 mediated cleavage very significantly.

Rz-170 mediated cleavage reaction was performed in the presence of Dz-155 and 192 in increasing concentrations. Lane 1 shows the HBx RNA (465nt). As expected, Rz-170 reaction generated two specific fragments (lane 2). When increasing concentration of Dz-155 (lane 3 - 10pmoles; lane 4 -50pmoles; lane 5 -100pmoles) was used in the reaction, no significant enhancement of cleavage was observed (lanes 3 -5). On the contrary increasing amounts of Dz-192 (lane 6 -10pmoles; lane 7 -50pmoles and lane 8 -100pmoles) was very effective in cleaving the target RNA (as evident by the reduced amounts of input RNA remaining in lane 7 and 8).

Fig. (6B). Both wild-type and mutant Dz-192 show increased cleavage of the target RNA.

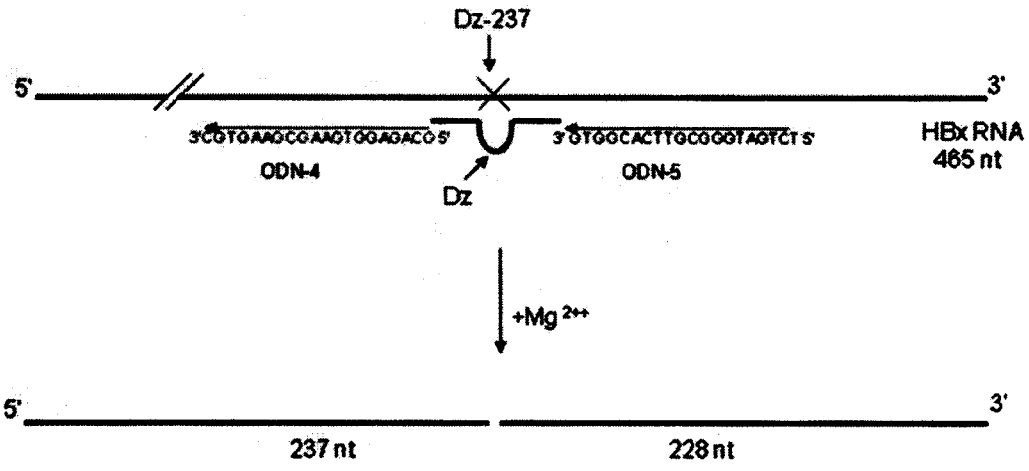
The purpose of this experiment was to compare the ability of catalytically active and inactive Dz-192 in their ability to augment Rz-170 cleavage of the target RNA (lane 1) under exactly identical conditions. Lane 2 depicts the extent of cleavage obtained with Rz-170 alone. When increasing amounts of Dz-192 (lane 3 -1pmole; lane 4 – 10pmoles; lane 5 -100pmoles) was added to the reaction mixture, a dose-dependent increase in the cleavage products was observed that matched the predicted pattern as shown in Fig. (**5D**). In the same manner, in presence of increasing amounts of mutant-Dz-192 (lane 6 – 1pmole; lane 7 -10pmoles; lane 8 -100pmoles), also resulted in dose-dependent cleavage of the substrate RNA.

sponding lanes were very similar. This indicated that the reduction in the input substrate RNA is mainly due to antisense effect and not because of catalytic activity. We conclude that both wild-type and mutant Dz-192 increased the Rz-170 mediated cleavage products to similar extent.

HBx-Dz-237 Mediated Cleavage is Enhanced Moderately by Upstream but Powerfully by Downstream ODN

All the earlier experiments described above were designed to increase the cleavage of Rz-170 and we next wanted to know if the earlier identified Dz against HBx RNA, Dz-237 [35] could also be modulated by using 20nt long upstream or downstream ODNs (Fig. 7A). The cleavage results of this experiment are shown in Fig. (7B). Lane 1 is the input substrate HBx RNA and lane 2 shows the Dz-237 mediated specific cleavage products (237 & 228nt long RNA fragments). In the presence of increasing amounts of upstream ODN (ODN#4) (lane 3 -10pmoles; lane 4 – 100pmoles; lane 5 -200 pmoles), only about 2 fold decrease in the amounts of uncleaved substrate RNA was observed with 200pmoles of ODN# 4 (compare lane 4 with lane 2). When the same reaction was carried out in the presence of increasing amounts of downstream ODN (ODN# 5) (lane 6 - 10 pmoles; lane 7 -100pmoles; and lane 8 -200pmoles), significant reduction in the substrate RNA was observed (> 20 fold reduction) with almost complete disappearance in the presence of 200pmoles of ODN# 5 lane 8. We conclude that downstream ODN is significantly more effective in enhancing the Dz-237 mediated cleavage reaction.

A.



B.

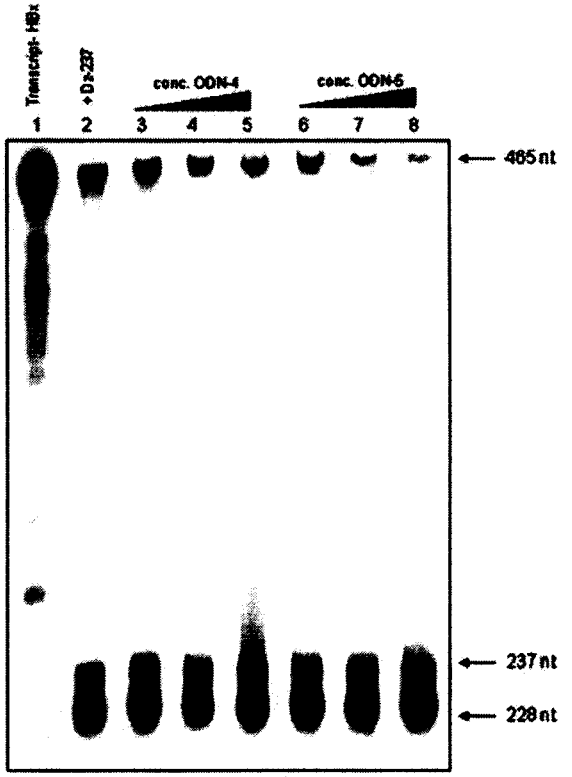


Fig. (7A). ODNs designed for augmenting Dz-237 mediated cleavage.

Dz-237 was used to cleave the target RNA in the presence of either ODN #4 or 5. The sequences of the two ODNs flanking the hybridizing arms of the Dz-237 are also shown along with the expected cleaved fragments. **Panel 7B**: Lane 1 is the substrate RNA and lane 2 exhibits the cleavage products due to Dz-237 cleavage. In the presence of increasing concentrations of ODN#4 (lane 3 – 10pmoles; lane 4 -100pmoles; lane 5 -200pmoles), a dose-dependent decrease in the input substrate RNA was observed. Similar observations were made with ODN# 5 but it was more effective in decreasing the input substrate RNA when used in increasing amounts (lane 6 -10pmoles; lane 7 -100pmoles and lane 8 – 200pmoles).

Rz-170 + ODNs Treatment Results in Potent Intracellular Reduction of Target RNA

HepG2 cells were cotransfected with 1μg each of pSG5.HBx DNA and Rz-170 construct in one ml for 48 hours using 10μl of Lipofectin (Invitrogen). This dose was predetermined to give about 10% intracellular reduction in the levels of target RNA (Fig. **8**, lane 4) when compared with cells transfected with pSG5.HBx DNA alone along with

equal amounts of unrelated Rz (lane 3). Total RNA was isolated using TRIZOL and target RNA and control RNA were estimated simultaneously by RT-PCR technique. When 4μg of ODN #2 was added to the Rz-170 construct + pSG5.HBx.DNA mixture (lane 5), approximately 70% reduction was observed (compare lane 5 with lane 3). When the same amount of ODN #1 was used, about 20% reduction was observed (lane 6). When half the above amount of ODN #1 & #2 (2μg each) were used, about 50% reduction in the target RNA was observed (compare lane 7 with lane 3). RT-PCR carried out with cells only (lane 2) and RT-PCR carried out in the absence of RT (data not shown), as expected, showed no HBx RNA specific amplification. The extent of house keeping gene (HuGAPDH) in all the corresponding lanes remained essentially unchanged. We conclude that Rz-170 + downstream ODN combination was more effective in causing decrease in the levels of intracellular target RNA.

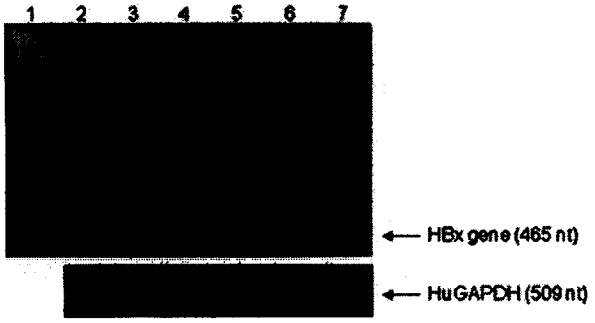


Fig. (8). Intracellular reduction of target RNA in the presence of Rz + ODNs.

Estimation of target RNA (HBx RNA) in HepG2 cells treated with various combinations of Rz and Rz + ODN #1 and #2 by RT-PCR techniques. The levels of full-length X RNA and control RNA (HuGAPDH) were determined simultaneously by RT-PCR as described in materials and methods. Wells that received 1 μg of pSG5.HBx DNA + equivalent amounts of unrelated ODN of similar length, showed a prominent X gene-specific band. This amount was kept constant in all the wells. Cells were cotransfected with 1μg each of Rz-170 + pSG5.HBx DNA (lane 4); pSG5.HBx +1μg Rz + 4μg of ODN #2 (lane 5); pSG5.HBx +1μg Rz + 4μg of ODN #1 (lane 6); pSG5.HBx +1μg Rz + 2μg of ODN #1 + 2μg of ODN #2 (lane 7).

Western Blot Analysis

HepG2 cells cotransfected with Rz + ODNs were processed for total protein and subjected to western blot analysis as described before [35,33] and the results are shown in Fig. (**9**). Lane 1 represents control cells only. A prominent immunoreactive HBx protein was observed (lane 2) when transfected with 1μg each of pSG5.HBx plasmid DNA. About 2-fold reduction in the HBx protein band was observed when cells were cotransfected with 1μg each of pSG5.HBx plasmid DNA and Rz-170 DNA (lane 3). When ODN # 1 and #2 (4μg in one ml) were also included, a complete knock down of the HBx protein was observed (lanes 4 & 5). This is not due to unequal amounts of cell lysates that were analyzed because equivalent amounts of cells containing same amounts of total protein were loaded (as determined by colorimetric reaction with BCA reagent from Pierce). We conclude that both the ODNs in combination with Rz-170 caused complete intracellular reduction in the levels of X protein.

Potent Knock Down of HBx Mediated HIV-1 LTR Reporter Gene Activation by Rz-170 + ODNs or 10-23 Dz

HepG2 cells were cotransfected with pSG5.HBx + p-LTR-B plasmid DNA (100 ng each in one ml for 1X 10^6 cells) along with indicated amounts of ODNs or Dz and the reporter gene activity (luciferase) (mean +/- SD) obtained from three independent experiments are shown in Fig. (**10**). As expectd HBx encoding DNA showed significant LTR-promoter activity (compare lane 4 with lane 3). This activity diminished more than 2.4-fold when 100ng of Rz-170 was added (lane 5). Upon addition of ODN#1, it dropped 8.4 fold (lane 6), with ODN#2 about 5.8 fold (lane 7) and in combination of both about 8.4-fold (lane 8). Control cells and pSG5.HBx treated cells showed no significant reporter gene activity as expected (lanes 1 & 2). Thus, an additional 3 - 4 fold decrease (when compared with Rz-170 alone) in X gene mediated reporter gene activity with ODN #1 is observed. More than 17-fold reduction in reporter gene activity was observed with Dz-192 under identical conditions (lane 9) and about 12.6-fold reduction with the mutant version of Dz-192 (lane 10). We conclude that ODNs (#1 & #2), wild-type and mutant Dz-192 treated cells showed impressive functional inhibition of HBx protein. As expected Dz-192 (lane 11) and mutant Dz-192 (lane 12) showed significant reduction in reporter gene activity.

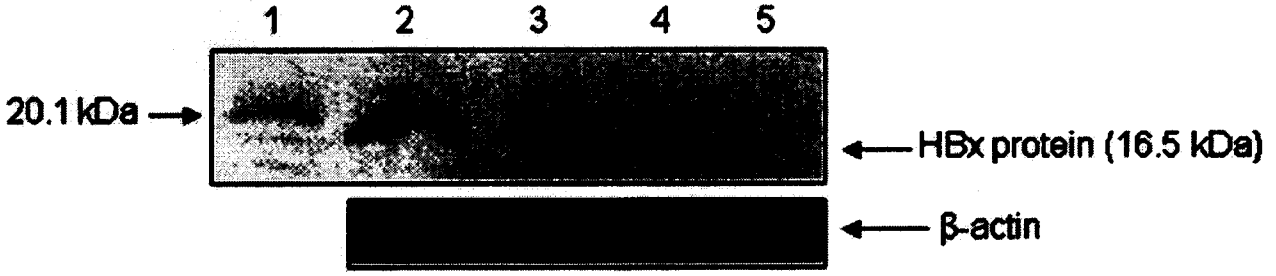


Fig. (9). Intracellular reduction in the levels of X protein by western-blot analysis.

HepG2 cells were transfected with pSG5.HBx plasmid alone (lane 2); cotransfected with pSG5HBx + Rz-170 construct (lane 3); pSG5HBx + Rz-170 construct + ODN #1 (lane 4); pSG5HBx + Rz-170 construct + ODN #2 (lane 5) with indicated amounts (see result section). The total amount of DNA used in each well was kept constant by adding unrelated or unrelated ODNs of similar length. Cell lysates were prepared from equivalent amount of cells and subjected to western blot analysis as described before.

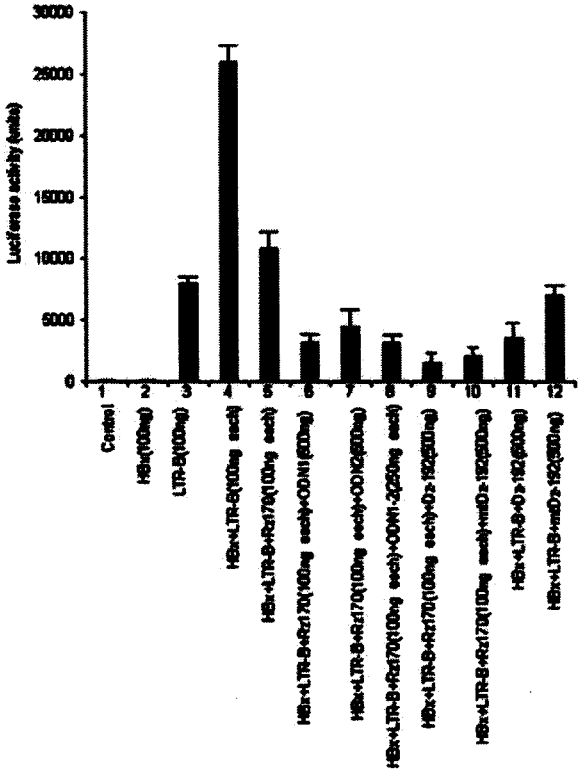


Fig. (10). Inhibition of X protein mediated HIV-1 LTR reporter gene activation with Rz + ODNs.

293 or HepG2 cells were grown to 80% confluency in 24 well plate and then transfected with various combinations of plasmid DNAs as indicated + ODNs or 10-23 Dzs as shown in the bottom of the figure using 2.5μl of Lipofectin (Invitrogen) per well. 24 hours post transfection equivalent amounts of cells were subjected to lysis using 1 x reporter lysis buffer (Promega) and the extent of luciferase activity (light units on Y axis) was determined as described earlier (Goila & Banerjea, 2001).

DISCUSSION

Efficient cleavage of a target RNA by Rz or Dz is important for several reasons including biochemical and gene therapy approaches. Although there are several potential Dz or Rz target sites in a target RNA, most of them are cleaved poorly or not at all when tested on longer and physiologically relevant RNAs. We report that ODNs that were targeted to hybridize directly adjacent to the Rz or Dz target sites were able to significantly augment Rz or Dz mediated cleavage activity in dose-dependent manner and that the downstream Dz or ODNs usually worked better than the upstream Dz. In both the cases (Rz + ODNs or Dz + ODNs) almost complete cleavage of the target RNA was achieved under defined set of conditions. Showing close to 100% *in vitro* cleavage of the target RNA with Rz or Dz in the presence of specific ODNs or Dz on one hand and potent intracellular reduction in a mammalian liver-specific cell line on the other are the major findings of the present work. It must be emphasized that all the previous studies that employed either Rz or Dz alone, complete *in vitro* disappearance of the input substrate RNA was never achieved under standard conditions of cleavage. This data assumes more significance because ODNs and Dzs are currently being exploited for therapeutic purposes [6, 11]. The most likely explanation for enhanced cleavage by Rz or Dz with specific ODNs is their ability to melt the secondary structure near the Rz and Dz target sites which facilitated efficient hybridization between Rz-170 or Dz-237 with HBx RNA. This ODN mediated enhancement of cleavage was not observed with unrelated ODN of similar length (data not shown) or with ODN that hybridized 33nt upstream of the target RNA. This is most probably because long RNAs are known to exist in separate stem-loop structures or independent domains. Results with upstream and downstream (wild-type and mutant Dz-192) are somewhat surprising but not totally unexpected. The upstream Dz-155 not only failed to increase the cleavage activity of Rz-170 but also was catalytically inactive as no cleavage products were seen. It is likely that Dz-155 failed to hybridize with the target RNA because of the secondary structure constraints. In this connection it is important to note that the ability of ODNs to successfully hybridize to the target RNA (as measured by RNAse H based assay) is essential but *not sufficient* for affording catalytic activity to Dz [37]. It is noteworthy that the hybridization arms of the Dz or Rz span only 14 or 16 nt (with one gap in the design) in the target RNA whereas the ODNs are uninterrupted 20nt long, which is likely to melt secondary structures better. The advantage of using downstream Dz would obviously be its ability to cleave the target RNA in a catalytic manner. A comparative Rz-170 mediated study between wild-type and mutant Dz-192, suggests that the enhancement of Rz-170 cleavage is largely due to the antisense effects because the amounts of

input substrate RNA after various treatments remained the same. Similar intracellular level of reduction of target RNA was also observed with Dz-237 + ODN#5 (data not shown).

Although very few studies on enhanced cleavage by nucleic acid approaches have earlier been reported, we report, for the first time, that a combination of Rz-170 and specific ODNs or 10-23 Dz lead to not only complete cleavage of the target RNA but can also cause very substantial intracellular reduction of X RNA. The ability of ODNs to augment *in vitro* cleavage by Rz-170 correlated with their ability to interfere with the intracellular expression of the target gene.

In summary, we show that both Rz and Dz mediated cleavage of the full-length HBx RNA can be powerfully augmented by specific ODNs and 10-23 Dzs. They not only enhanced the Rz & Dz mediated *in vitro* cleavage products, but also reduced very significantly the intracellular target gene expression in a mammalian liver specific cell line – HepG2. The intracellular effectiveness of these ODNs or Dzs could be substantially improved by using chemically modified (stabilized) nucleotide [37]. These observations are of immediate therapeutic importance and should be applicable to down regulating other target genes also.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ODNs	=	Oligodeoxynucleotides
Dz	=	DNA-enzyme
Rz	=	Ribozymes
RT	=	Reverse transcriptase
HBV	=	Hepatitis B virus
LTR	=	Long terminal repeat

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Sequence-specific cleavage of hepatitis C virus RNA by DNAzymes: inhibition of viral RNA translation and replication

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DNAzyme (Dz) molecules have been shown to be highly efficient inhibitors of virus replication. Hepatitis C virus RNA translation is mediated by an internal ribosome entry site (IRES) element located mostly in the 5′ untranslated region (UTR), the mechanism of which is fundamentally different from cap-dependent translation of cellular mRNAs, and thus an attractive target for designing antiviral drugs. Inhibition of HCV IRES-mediated translation has drastic consequences for the replication of viral RNA as well. We have designed several Dzs, targeting different regions of HCV IRES specific for 1b and also sequences conserved across genotypes. The RNA cleavage and translation inhibitory activities of these molecules were tested in a cell-free system and in cell culture using transient transfections. The majority of Dzs efficiently inhibited HCV IRES-mediated translation. However, these Dz molecules did not show significant inhibition of coxsackievirus B3 IRES-mediated translation or cap-dependent translation of reporter gene, showing high level of specificity towards target RNA. Also, Northern blot hybridization analysis showed significant cleavage of HCV IRES by the Dz molecules in Huh7 cells transiently transfected with the HCV–FLuc monocistronic construct. Interestingly, one of the Dzs was more effective against genotype1b, whereas the other showed significant inhibition of viral RNA replication in Huh7 cells harbouring a HCV 2a monocistronic replicon. As expected, mutant-Dz failed to cleave RNA and inhibit HCV RNA translation, showing the specificity of inhibition. Taken together, these findings suggest that the Dz molecule can be used as selective and effective inhibitor of HCV RNA replication, which can be explored further for development of a potent therapeutic agent against HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus, belonging to the family *Flaviviridae*. The viral RNA genome is approximately 9600 nt and encodes a single polyprotein of about 3000 amino acids. The long open reading frame is flanked by 5′ and 3′ untranslated regions (UTRs) that are highly conserved among the majority of HCV genotypes and contain elements that are essential for genome replication (Bartenschlager *et al.*, 2004). The translation initiation of HCV RNA is mediated by the binding of the 40S ribosomal subunit at the internal ribosome entry site (IRES) located mostly in the 5′UTR region. It has been shown that the HCV IRES can directly bind to the 40S ribosomal subunit, even in the absence of any initiation factors, in a manner similar to prokaryotic translation initiation. Subsequently, several canonical and non-canonical *trans*-acting factors facilitate the formation of a functional initiation complex during internal initiation of translation (Hellen & Sarnow, 2001). Since this mechanism is fundamentally different from the ribosome assembly at the 5′ cap-binding complex in cap-dependent translation of host cell mRNA, it serves as an attractive target for antiviral agents (Dasgupta *et al.*, 2004).

HCV causes a multitude of liver diseases in humans, including liver cirrhosis, and often leads to hepatocellular carcinoma if left untreated. Current treatment options involving interferon-α (INF-α) alone or in combination with ribavirin are not very effective. The majority of the patients do not respond well to this therapy because of the short half-life of interferon or degradation of the molecules. Failure to achieve a sustained virological response in majority of the patients has also been shown to be partly due to the varying genotypes of the infecting strain of the virus. HCV has six major genotypes with several subtypes. HCV genotype 1 has been shown to be more resistant to interferon therapy than genotype 3. Genotype 3 was found to be the most prevalent in India, followed by genotype 1 (Gupta *et al.*, 2006). Thus

developing effective antiviral therapeutics using novel approaches is the need of the hour.

Several strategies are being explored to develop antiviral agents against HCV, targeting different viral processes. Recent availability of HCV subgenomic (Lohmann *et al.*, 1999) and full-length (Blight *et al.*, 2002) replicon systems has helped immensely to assay the inhibitory effect of antiviral candidates on HCV genome replication. Earlier, several studies have shown effective inhibition of viral RNA translation when viral enzymes were targeted. Since the translation of genomic RNA is the initial obligatory step, interference with this process will have direct consequence on the viral RNA replication. HCV RNA is translated by recruitment of the ribosome at the IRES element which comprises most of the 5′UTR sequences (except the first 40 nt) and extends to a short stretch of 30–40 nt downstream of the initiator AUG. Since the IRES-mediated translation is distinct from the cap-dependent translation of host cell mRNA, this could be exploited by different approaches to achieve selective inhibition of HCV gene expression.

Currently, nucleic-acid-based antiviral approaches, which include ribozyme (Rz), DNAzyme (Dz), short hairpin RNA (shRNA) and small interfering RNA (siRNA), are being used for inhibiting the gene expression of several target RNAs (Jarczak *et al.*, 2005; Goila & Banerjea, 2004). Among these, catalytic Dzs with 10–23 catalytic motifs are increasingly being exploited over Rzs because they either match or exceed the catalytic efficiencies of the known Rzs. Deoxyribozymes or DNAzymes or DNA-enzymes (Dzs), as originally described (Santoro & Joyce, 1997), are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner (Silverman, 2005, Dash & Banerjea 2004; Joyce, 2004). Dzs are synthetic single-stranded DNA molecules which have three domains: a catalytic domain consisting of 15 nt flanked by two substrate-recognition domains which bind the target RNA through Watson–Crick base pairing. In Dzs a single nucleotide change in the 10–23 catalytic motif completely abrogates the sequence-specific cleavage activity; for example, G14C completely abolishes the catalytic cleavage (Goila & Banerjea, 2001). In some instances efficient inhibition of gene expression was achieved with 10–23 Dz (Ackermann *et al.*, 2005). Based on sequence recognition of the binding arms, Dzs can be synthesized to cleave a target gene in a sequence-specific manner similar to that of Rzs (Asahina *et al.*, 1998; Goila & Banerjea, 1998).

Compared with synthetic Rzs, Dzs are easier to prepare, less sensitive to chemical and enzymic degradation and, more importantly, easier to deliver into cells (Santoro & Joyce, 1997). Over the years, several kinds of Dzs with unique catalytic motifs have been described, but Dzs possessing the 10–23 catalytic motif have been exploited more extensively by several investigators (Banerjea *et al.*, 2004). Various studies suggest that all the target sites are not available for cleavage by a single kind of catalytic nucleic acid molecule, most probably because the secondary and tertiary structures in the target RNA prevent optimal Watson–Crick base pairing with Rzs or Dzs. More than one site is usually selected in the target RNA to get maximum cleavage by catalytic nucleic acids. 10–23 DNA-enzyme cleaves the RNA sequence at a phosphodiester bond between an unpaired purine and a paired pyrimidine residue (5′-AU-3′ most efficiently cleaved). This results in the formation of 5′ and 3′ products, which contain a 2′-3′cyclic phosphate and 5′ hydroxyl terminus, respectively (Santoro & Joyce, 1998).

METHODS

Deoxyribozyme synthesis. All the oligodeoxynucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys. The conserved 15 nt (5′-GGCTAGCTACAACGA-3′) 10–23 catalytic motif was flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. Mutant-Dz was also assembled using a 10–23 catalytic motif that possessed a single nucleotide substitution (G to C) in the 10–23 catalytic motif. This change is known to render the Dz catalytically inactive (Goila & Banerjea, 2001).

We have initially designed five DNA-enzymes, namely Dz88, Dz219, Dz305, Dz327 and Dz336, targeting different regions of HCV 5′UTR IRES (Table 1). Additionally, mutant-Dz219, possessing a point mutation as stated above, was designed, which is termed the 'mutant Dz'. These molecules were specific only for HCV genotype 1b. Later we designed another four 10–23 catalytic motif-containing Dzs, namely Dz161, Dz165, Dz285 and Dz288, that were targeted to cleave 5′UTR regions of all the currently known HCV genotypes (Table 2). The location of cleavage for each Dz is shown by arrows in the predicted 5′-UTR IRES (Fig. 1).

Cell culture and plasmid. Human hepatocellular carcinoma cells (Huh-7 and Huh-7.5 cells) monolayers (Blight *et al.*, 2002) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO_2 atmosphere. For cells supporting the HCV full-length replicon (genotype 1b), 0.8 mg G418 sulfate ml^{-1} (Sigma-Aldrich) was added to the culture medium and for cells bearing the HCV monocistronic replicon (genotype 2a), 25 μg hygromycin B ml^{-1} was added to the culture medium. Replicon 1b carries the 1b genotype HCV 5′UTR followed by a neomycin resistance gene (*neo*), EMCV IRES and NS2–NS5 and the 3′UTR sequence. The replicon 2a carries the 2a genotype HCV 5′UTR followed by a hygromycin resistance gene (*hyg*), a ubiquitin gene (*ubi*) and NS3–NS5 and the 3′UTR. The HCV–FLuc monocistronic plasmid construct pCD (HCV–IRES–FLuc) construct carrying HCV IRES (nt 18–383) was

Table 1. Sequence of DNAzymes for subtype 1b

The mutated residue in the mutant mutDz-219 is highlighted in bold.

DNAzyme	Sequence 5′–3′
Dz88	AACGCCAGGCTAGCTACAACGAGGCTAGAC
Dz305	GCAAGCAGGCTAGCTACAACGACCTATCAG
Dz219	CCAGGCAGGCTAGCTACAACGATGAGCGG
Dz327	CTACGAGAGGCTAGCTACAACGACTCCCGG
Dz336	TGCACGGGGCTAGCTACAACGACTACGAG
mutDz219	CCAGGCAGGCTAGCTACAACCATGAGCGG

Table 2. Sequence of DNAzymes for HCV IRES based on conserved regions

DNAzyme	Sequence 5′–3′
Dz161	GTACTCAGGCTAGCTACAACGACGGTTCC
Dz165	CGGTGTAGGCTAGCTACAACGATCACCGG
Dz285	CAGTACCAGGCTAGCTACAACGAAAGGCCT
Dz288	GGCAGTAGGCTAGCTACAACGACACAAGG

cloned upstream of the gene for the firefly luciferase (Pudi *et al.*, 2003). The pCDFLuc construct contains the luciferase reporter gene and the coxsackievirus B3 (CVB3)–FLuc monocistronic plasmid construct (pCDCVB3-IRES-FLuc) contains the CVB3 5′UTR cloned upstream of the firefly luciferase reporter gene.

***In vitro* cleavage of target RNA with Dz.** *In vitro* run off transcripts of HCV IRES RNA (387 nt) was made from linearized HCV–FLuc monocistronic construct DNA (containing nt 18–383 of HCV IRES) using the T7 RNA polymerase (Promega) in the presence of [α-^{32}P]UTP, following the manufacturer's protocol. The extra nucleotides in the labelled transcript (387 nt) came from the region between the T7 promoter and upstream of the HCV sequence (cloned in polylinker). Equimolar amounts of unlabelled Dz and labelled substrate RNA (100 pmol each) were allowed to interact in a final volume of 10 µl in a buffer containing 50 mM Tris.HCl, pH 7.5 and 10 mM $MgCl_2$ (standard conditions) as described earlier (Santoro & Joyce, 1997) for 2 h at 37 °C. The cleaved products were resolved by electrophoresis and cleavage efficiency was determined as described earlier (Goila & Banerjea, 2001).

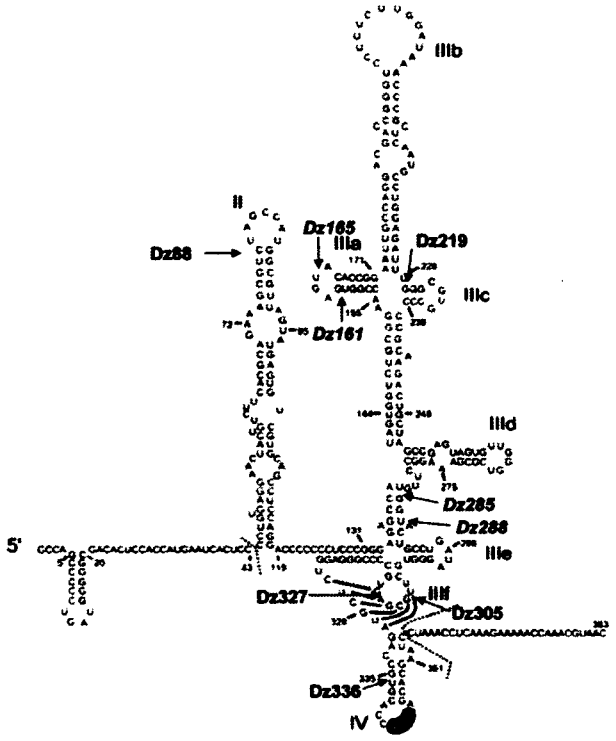

Fig. 1. Schematic diagram of HCV IRES (adopted from Brown *et al.*, 1992), showing the Dz targets. The numbers in bold are specific for genotype 1b and those in italics are conserved for all major genotypes.

Transfections and reporter assay. Monolayers (60–70% confluent) of Huh7 cells in 35 mm dishes were co-transfected with HCV monocistronic plasmid pCDHCV-FLuc or pCDCVB3-FLuc or pCDFLuc. Dzs and pSV-β-gal plasmid were used for normalizing transfection efficiency using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection the cells were harvested using passive lysis buffer (Promega) and FLuc activity was analysed using a luciferase assay system (Promega) in a TD 20/20 luminometer (Turner Designs).

Semiquantitative RT-PCR. HCV full-length and subgenomic replicon-bearing cells were transfected with 0.4 and 0.8 µM Dzs and, 24 h post-transfection, total cellular RNAs were extracted using TRI-reagent (Sigma-Aldrich). Semiquantitative RT-PCR was performed for the HCV-IRES positive strand and actin as described earlier (Dhar *et al.*, 2007). In brief, 5 µg total RNA was reverse transcribed with the HCV 5′UTR and actin primers by annealing at 65 °C and extending at 42 °C for 50 min. After cDNA was synthesized, PCR reaction was performed using both 5′ and 3′ primers specific for HCV 5′UTR to amplify and quantify HCV RNA. The PCR products were run in 1% agarose gel and densitometric analysis was done using MultiGauge software (Fujifilm) and the values were expressed as ratio of HCV IRES to actin.

Northern blot analysis. Total cellular RNA (20 µg) was isolated from Huh7 cells transfected with HCV–FLuc monocistronic constructs with or without Dzs and resolved on formaldehyde-agarose gel (0.8%) under denaturing conditions. RNA were transferred and cross-linked to a nylon membrane (Sigma-Aldrich) and probed with a [α-^{32}P]-labelled firefly luciferase antisense probe, followed by autoradiography. Densitometric analysis was done and the ratio of HCV-IRES to 18S rRNA was expressed graphically.

RESULTS

Sequence-specific cleavage of HCV IRES RNA by various DNAzymes

Since the mechanism of HCV IRES-mediated translation is novel and fundamentally different from cap-dependent translation of host cell mRNA, we have designed several Dzs to target the IRES element for selective inhibition of HCV RNA translation. Furthermore, the sequence-specific cleavage in this region will consequently block viral RNA replication and therefore we designed a number of Dzs that were targeted to the predicted single-stranded loop regions within the HCV IRES element (Fig. 1, Tables 1 and 2). In order to evaluate the cleavage efficiency of the Dzs, *in vitro* cleavage reaction were performed. Out of five Dz molecules only three, Dz219, Dz305, and Dz327, have shown significant cleavage activity *in vitro* in cell-free conditions (Fig. 2a and 2b). Interestingly, the three active Dzs were found to cleave the target RNA in a sequence-specific manner with varying efficiencies (Fig. 2b, lanes 3, 4 and 5). Dz219 showed maximum cleavage activity under standard

in vitro cleavage conditions (Fig. 2b). However, Dz88 and Dz336 failed to show detectable cleavage activity.

Further, to investigate the inhibitory effect of Dzs that would target conserved sequences in all six major genotypes of HCV, several conserved Dz molecules (including Dz161, Dz165, Dz285 and Dz288) were synthesized (Table 1 and Fig. 2a). For this purpose, HCV 5′UTR sequences of all six genotypes were aligned using the CLUSTAL W program (data not shown). The designing of Dzs was based on the secondary structures of RNA for all the strains, obtained by the mfold program (data not shown). All four conserved Dzs were first tested for RNA cleavage activity *in vitro* as described before. Among those four Dz molecules, three, Dz161, Dz285 and Dz288, were found to cleave the target sequence specifically, the last two being more efficient (Fig. 2c). Dz165 did not show significant cleavage activity.

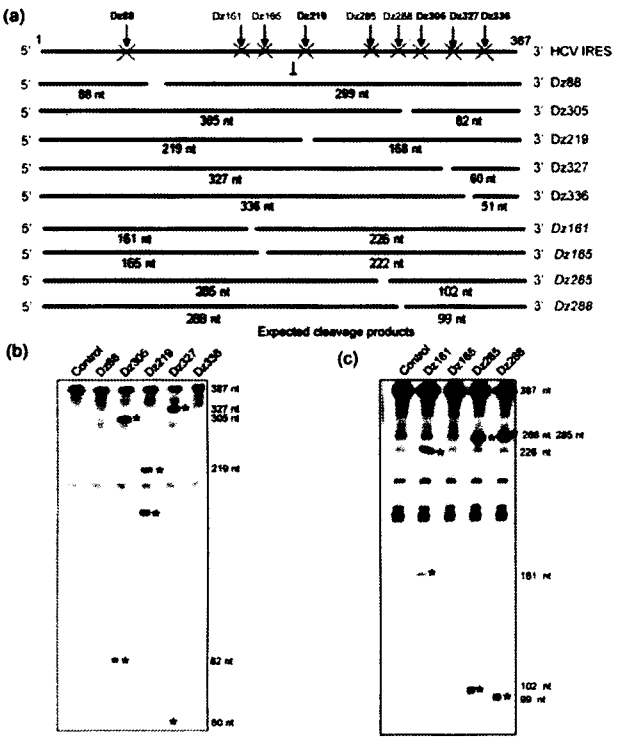

Fig. 2. *In vitro* cleavage of HCV IRES RNA by various Dz molecules. (a) Schematic representations of the Dz cleavage sites on the HCV IRES: HCV genotype 1b-specific Dzs are indicated in bold, whereas the Dzs that target the conserved sequences across the different genotypes are indicated in italics. (b) Equimolar concentrations (100 pmol) of the labelled HCV IRES RNA and respective Dz were used and the cleavage products were analysed on 8% PAGE. (c) *In vitro* cell-free cleavage products of HCV IRES by conserved Dzs were analysed on 8% PAGE. The asterisks show the cleavage products as indicated in (a) and their respective sizes are shown on the right.

Effect of Dz on HCV IRES-mediated translation *ex vivo*

In order to evaluate the intracellular cleavage efficiencies of the Dz molecules, transient co-transfection experiments were performed using plasmid HCV–FLuc monocistronic constructs and the Dz molecules in human hepatocellular carcinoma cells (Huh7). The monocistronic RNA generated *ex vivo* from the HCV–FLuc monocistronic plasmid encodes the HCV IRES element upstream of the firefly luciferase reporter gene (Pudi *et al.*, 2003). Although three Dz molecules specific for genotype 1b showed significant cleavage activity *in vitro*, only one of them, Dz219, showed impressive inhibition (81%) of HCV IRES-mediated translation. However, the mutant-Dz219 with a single substitution in the catalytic domain of Dz219 failed to inhibit HCV IRES-mediated translation, suggesting high specificity of the approach (Fig. 3a). Interestingly, two other Dzs (305 and 327) showed significant *in vitro* cleavage activity, but failed to interfere with the HCV translation (Fig. 3a). When conserved Dzs were tested for inhibition of HCV IRES function, Dz285 and Dz288 showed 38 and 35% inhibition, respectively, whereas Dz161 showed only 30% inhibition (Fig. 3b).

To investigate the cleavage of the HCV–FLuc monocistronic RNA by the Dzs in *ex vivo* conditions, Northern blot hybridization was performed. For this purpose, Huh7 cells were transiently transfected with the monocistronic DNA constructs and different Dz molecules (Fig. 4a). Total RNAs were isolated 24 h post-transfection and used for Northern assay. Dz molecules used in the assay included Dz219 and Dz285, which showed maximum activity *ex vivo*, and Dz305, Dz327 and mutant-Dz219, that did not exhibit any *ex vivo* activity. Results showed significant cleavage activity of Dz219 and Dz285; however, Dz305 and Dz327 failed to cleave HCV–FLuc RNA *ex vivo* (Fig. 4a), which is consistent with our reporter gene (luciferase) assay (Fig. 2b). The mutant-Dz219 didn't show any cleavage activity *ex vivo*, as expected. For clarity we have quantified the band intensity corresponding to the HCV–FLuc RNA and normalized it with that of the loading control band (18S rRNA). The densitometric analysis of the ratio of HCV–FLuc monocistronic RNA to the 18S rRNA (Fig. 4b), clearly demonstrated that cleavage activity of Dz219 as 48% and that of Dz285 as 25%, respectively. However, Dz305, Dz327 or the mutant-Dz219 did not exhibit any significant cleavage activity. Interestingly, the translation inhibitory activity corresponding to Dz219 and Dz285 was found to be slightly higher (Fig. 3) than the RNA cleavage activity (Northern analysis, Fig. 4), which could be due to a higher sensitivity of the luciferase assay.

To further investigate the specificity of the Dz activity, the Dz molecules were tested against other viral IRES as well as cap-dependent translation. For this purpose the pCDFLuc DNA construct was transiently transfected with representative Dz molecules (Dz219, 285, 288 and mutant-Dz219) and the luciferase reporter gene was assayed 24 h

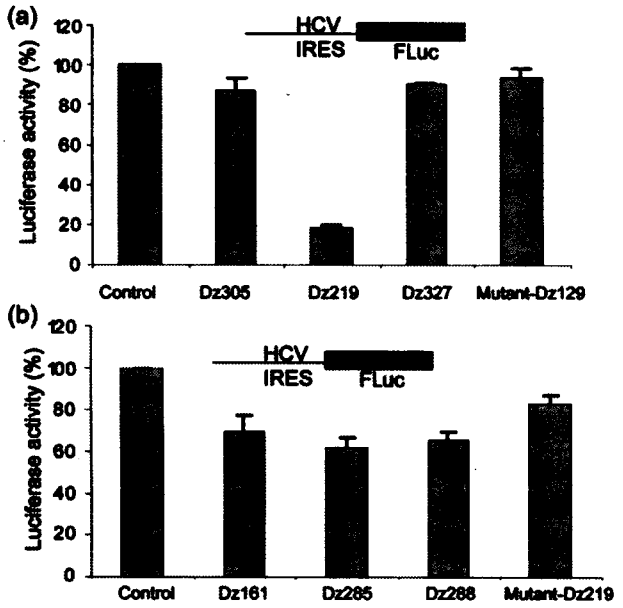

Fig. 3. Inhibition of HCV IRES-mediated translation by the Dz molecules. (a) Approximately 1 μg HCV firefly luciferase monocistronic DNA construct (HCV–FLuc) was transiently cotransfected in Huh7 cells in the absence and presence of 0.4 μM genotype 1b-specific Dz molecules. Cells were harvested and luciferase activity measured 24 h post-transfection. The transfection efficiency was normalized with β-gal activity and the normalized luciferase activity was plotted, taking the control as 100 %. (b) Similarly, 1 μg monocistronic DNA construct (HCV–FLuc) was transiently cotransfected in Huh7 cells in the absence and presence of 0.4 μM conserved Dz molecules. Luciferase activities were measured 24 h post-transfection. The results represent the average of three independent experiments done in duplicate.

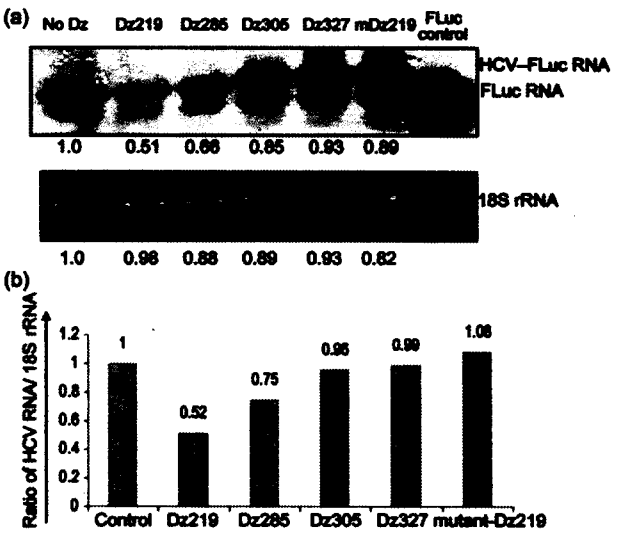

Fig. 4. Cleavage of HCV RNA *ex vivo* in Huh7 cells by the Dz molecules. (a) Northern blot hybridization of the HCV RNA isolated from Huh7 cells, co-transfected with HCV–FLuc DNA and Dzs (0.4 μM). Total RNA was isolated using TRIzol reagent 24 h post-transfection, electrophoresed on formaldehyde agarose gel (0.8 %), blotted on a nylon membrane and hybridized with α^{32}P-labelled FLuc antisense probe followed by autoradiography. The numbers below the panels represent the band intensities relative to control. (b) Graphical representation of the ratio of the densitometric values obtained from the respective band intensities corresponding to HCV-RNA and 18S rRNA.

post-transfection. Results suggest that none of the Dz molecules inhibited the cap-dependent translation of the luciferase reporter gene (Fig. 5a).

Similarly, CVB3 FLuc monocistronic DNA (pCDCVB3-FLuc) was cotransfected with the above set of Dz molecules and the luciferase assay was performed 24 h post-transfection. Results suggest no significant change in luciferase activity in presence of the above Dz molecules (Fig. 5b). Taken together, the results suggest the target specificity of the Dz molecules and the high levels of selectivity of this approach.

Effect of Dzs on the HCV RNA replication

Finally, we have analysed the inhibitory effect of the Dzs in Huh7.5 cells harbouring HCV1b replicon (Fig. 6a) (Blight *et al.*, 2002). Increasing concentration (0.4 and 0.8 μM) of Dz 219 was transiently transfected into replicon-containing cell line using Lipofectamine 2000 (Invitrogen). After 24 h, total RNA was isolated and the HCV positive-strand RNA corresponding to the 5′UTR was detected by semiquantitative RT-PCR. Results suggest approximately 70 % inhibition of the HCV1b genotype replicon RNA synthesis when 0.8 μM Dz219 was used. However, the same concentration of Dz219 failed to inhibit the HCV-RNA synthesis in Huh7 cells harbouring HCV2a genotype replicon (Lohmann *et al.*, 1999) (Fig. 6b and 6c). Upon inspection we found that the Dz219 target sequence was designed on the basis of HCV 1b sequences, which is not fully conserved in HCV2a sequence. The result also proved that bio-efficacy of Dz219 was sequence-specific. Furthermore, when the conserved Dzs (0.4 μM) were transfected into cells containing HCV replicon 1b (Fig. 6d), significant inhibition of RNA synthesis was observed with Dz285 and Dz288 (30 and 50 %, respectively). However, the inhibitory effect was relatively more pronounced (60 % for Dz285 and 70 % for Dz288) on HCV replicon 2a cell line.

DISCUSSION

A couple of studies have demonstrated previously the use of DNAzyme molecules to cleave HCV RNA *ex vivo* (Trepanier *et al.*, 2006), but this study constitutes the first report on the effect of Dzs on HCV replication in cell lines harbouring HCV subgenomic or full-length replicons.

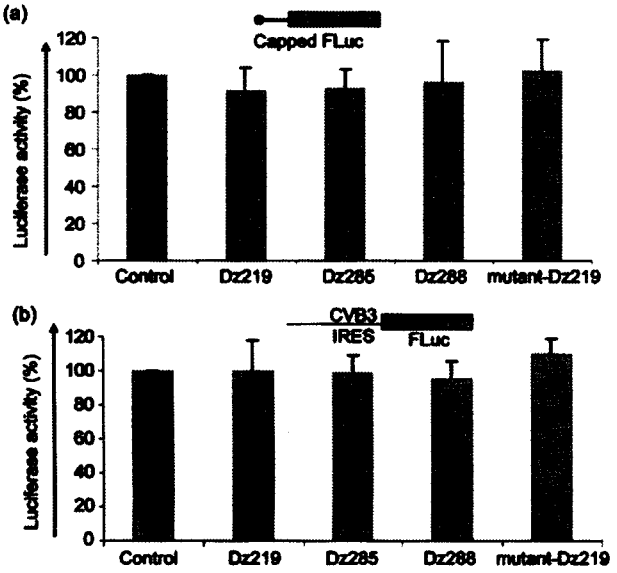

Fig. 5. Effect of Dz molecules on cap-dependent translation and CVB3 IRES-mediated translation. (a) Dz219, Dz285, Dz288 or mDz219 (0.4 μM) were co-transfected with 1 μg pCDFLuc DNA construct and β-gal DNA construct in Huh7 cells. Luciferase assay was performed 24 h post-transfection. The transfection efficiency was normalized with β-gal activity and the normalized luciferase activity was plotted taking the control as 100%. (b) Similarly, 1 μg CVB3FLuc DNA construct was cotransfected with the above Dzs and β-gal construct and the normalized luciferase values were plotted taking the control as 100%. The results shown represent the average of three independent experiments done in duplicate.

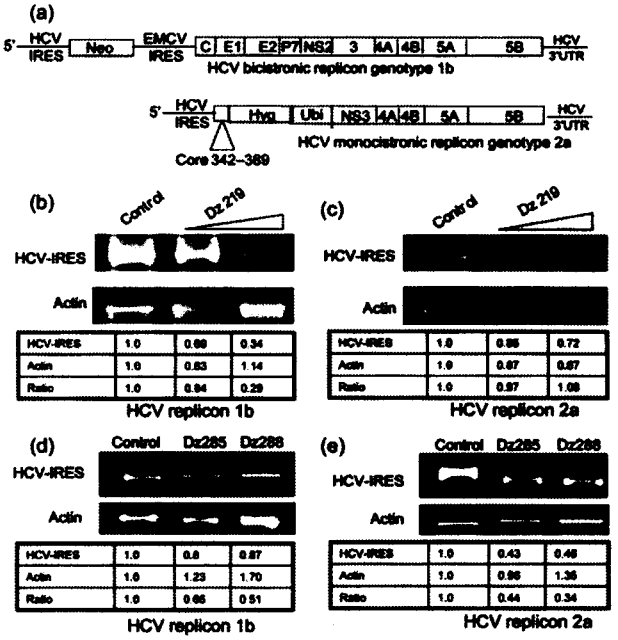

Fig. 6. Effect of Dz molecules *ex vivo* in replicon cell line. (a) Schematic representation of the HCV replicon genotype 1a and 1b. (b and c) Increasing concentrations (0.4 and 0.8 μM) of Dz219 were transfected either in Huh7.5 cells harbouring replicon 1b genotype (b) or Huh7 cells harbouring replicon 2a genotype (c). RNA was isolated using TRIzol reagent 24 h post-transfection, and semiquantitative RT-PCR was performed using HCV-5'UTR-specific primers or the actin primers. The products were analysed on 1% agarose gel. (d and e) Similarly, the effects of Dz285 and Dz288 (0.4 μM) were analysed with HCV replicon 1b genotype (d) and HCV replicon 2a genotype (e).

Although we have designed several Dzs targeting different regions of HCV IRES and tested their activities *in vitro* as well as *ex vivo* in cell lines harbouring HCV replicon, only a couple of them were found to be more effective in the *in vitro* and *ex vivo* assays. Interestingly, when all the Dzs used in the study were mapped to the target sequences/structures within HCV IRES (Fig. 1), it appears that the Dz285 and Dz288, targeting HCV SLIIId loop, and Dz219, targeting SL IIIb, achieved maximum inhibition, perhaps due to the importance of the target site in ribosome assembly during internal initiation of translation. This could be also due to the fact that all target sites are not available for efficient cleavage by a single kind of catalytic nucleic acid molecule, most probably because the secondary and tertiary structures in the target RNA prevent optimal base pairing. Base pairing and cleavage activity also depend on the arm length of the RNA-binding site of the Dzs. Enzymes with longer arms sometimes showed higher cleavage activity compared with enzymes with shorter arms (Oketani *et al.*, 1999). Modifications in the 5′ and 3′ termini of these molecules help in preventing nuclease degradation without affecting its catalytic activity (Oketani *et al.*, 1999). Interestingly, it has been demonstrated earlier that the efficiency of some Dz molecules can be enhanced by using them in combination with some oligodeoxynucleotides (ODNs) which would hybridize the target RNA near the Dz cleavage site to facilitate the cleavage reaction (Sood *et al.*, 2007). Thus, it would be interesting to explore whether the apparently inactive/inefficient Dz molecules in our study could also be used in combination with ODNs to potentiate catalytic efficiency for the RNA cleavage.

It appears that, if required, Dz molecules can be used at higher concentration to achieve maximum inhibition of viral protein synthesis with minimum effect on host cell RNA translation (data not shown). It is also possible that the effective concentration required to achieve 50% inhibition of viral RNAs could be lowered to a large extent by using a cocktail of Dz molecules in the line of combination therapy.

Dz molecules have been shown to be a more stable antiviral agent compared with Rz or siRNA (Santoro & Joyce, 1997). Unlike siRNA or shRNAs, Dz molecules are not expected to activate double-stranded RNA-activated protein kinase

(PKR) and result in attenuation of host cell RNA translation due to phosphorylation of eIF2 by PKR (Gil & Esteban, 2000). It is also possible to make more stable derivative of the Dzs molecules such as morpholino- or phosphorothio- derivatives etc. Remarkable stability was also achieved by modifying (inverting) the first and last nucleotide residues, especially at the 3′-end of the Dz, which will have serum stability enhanced tenfold (Sun *et al.*, 1999). In this connection, it has been shown also that efficient uptake of macrophage tropic-anti-HIV-1 Dz by human macrophages in the complete absence of charged lipid molecules can be enhanced by attaching ten G residues at the 3′-end of a 10–23 catalytic motif-containing Dz. G residues form G quartet-like structures that are recognized by the scavenger receptor present on macrophages (Unwalla & Banerjea, 2001).

Taken together, these results provide proof of the concept that the HCV IRES could be an effective and selective target using conserved DNA-enzyme molecules to develop novel antiviral therapeutics against hepatitis C virus infection. It would be interesting to couple this with organ-specific delivery approaches. Liver-specific delivery of Dz molecules using Sendai virus virosome- (Ramani *et al.*, 1997) or lentivirus- (Kusunoki *et al.*, 2003) based vectors would be ideal for developing Dz-based antiviral therapeutics against hepatitis C virus infection.

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X Protein of hepatitis B virus potently activates HIV-1 subtype C long terminal repeat promoter: implications for faster spread of HIV-1 subtype C

Several genetic subtypes of HIV have been identified throughout the world, but it is predominantly subtype C that is responsible for causing the epidemic in India, some regions of south Asia and Africa. Besides HIV-1 infection, individuals are also co-infected with other pathogens such as hepatitis B virus (HBV), and other bacterial and yeast pathogens. HBV encodes the X gene (*HBx*) whose product is known to activate several heterologous promoters, including the HIV-1 long terminal repeat (LTR) promoter [1–4]. HIV-1 gene expression is controlled by the LTR promoter, which is rich in various transcription factor binding sites [5]. The HIV-1 LTR sequence of genetic subtype B is significantly different than subtype C, and it is therefore reasonable to assume that *HBx* may have different effects on promoter activity [5].

In order to study the possible impact of HBx protein on HIV-1 subtype-specific LTR-mediated activation, we co-transfected HIV-1 LTR-B and C reporter constructs (pBlue-3′-LTR-B and C-Luc) along with *HBx* encoding DNA, pSG5.HBx (465 nucleotide long X gene is placed under SV40 promoter that allows intracellular expression) [6] into human 293 cells using lipofectin (Invitrogen, Carlsbad, California, USA) for 48 h. Cell lysates were prepared and the amounts of luciferase were determined according to the manufacturer's instructions (Promega Biotech, Madison, Wisconsin, USA); the results are shown in Fig. 1a (mean of three independent experiments). To ensure uniform transfection efficiency we included an internal reporter control plasmid (pSV-β-gal; Promega). Control cells or cells transfected with 100 ng pSG5.HBx alone showed background luciferase activity. LTR-B and LTR-C reporter plasmids showed basal promoter activity, and the latter showed approximately twofold more basal activity. When co-transfected with 100 ng of either LTR-B or LTR-C plasmids along with 100 ng pSG5.HBx construct, the latter combination (LTR-C plus HBx) showed more than sevenfold more activation when compared with basal activity, whereas it was less than twofold with LTR-B plus *HBx* combination. When 100 ng of each LTR and 100 ng of pSG5.HBx DNA were used for transfection, the pattern and the extent of activation remained unchanged. When co-transfected with HBx plus B-Tat (cytomegalovirus promoter driven Tat derived from pNL4-3DNA) plus reporter construct (LTR-B-luciferase), a strong synergistic effect was observed. It is noteworthy that the level of LTR-B promoter activation by the HBx protein is

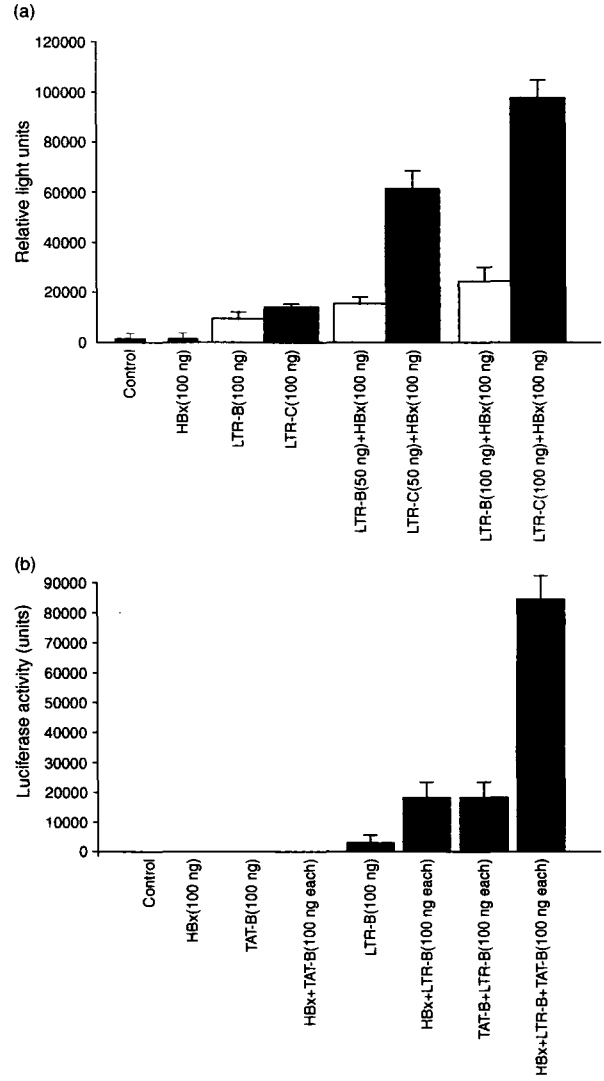

Fig. 1. Amounts of luciferase determined from prepared cell lysates according to the manufacturer's instructions. (a) Human 293 cells were grown in Dulbecco's modified essential medium plus 10% fetal bovine serum, grown to 80% confluence in a 12-well plate. They were co-transfected with the indicated amounts of pSG5.HBx [6] and HIV-1 subtype B and C specific long terminal repeat (LTR)-luciferase constructs using lipofectin as described by the manufacturer. Twenty-four hours after transfection, cell lysates were prepared and the amounts of luciferase from equivalent fractions were determined as described by us earlier [3]. (b) Various combinations of plasmid DNA as indicated (100 ng each) were co-transfected and luciferase activity was determined as described in (a).

equivalent to what we observed with LTR plus B-TAT interaction (mean ± SD from three experiments, Fig. 1b).

This is the first study that suggests that the HBx protein might play an important role in upregulating HIV-1 subtype-C LTR-mediated gene expression compared with subtype-LTR-B-driven expression. These observations have strong implications for the increased replication of subtype C virus.

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The first reported case and management of multicentric Castleman's disease associated with Kaposi's sarcoma in an HIV-2-infected patient

The multicentric variant of plasma-cell Castleman's disease (MCD) often coexists with Kaposi's sarcoma (KS) in HIV-1-infected patients, and this association could be explained by the possible involvement of human herpes virus 8 (HHV-8) in their pathogenesis. MCD has never been described in an HIV-2-infected patient. We report the first case of MCD associated with HIV type 2 infection and its successful management with rituximab.

A 46-year-old man was referred for asthenia and anorexia with important loss of weight. This homosexual patient originating from Cape Verde had no past medical history. A few days before his admission, seropositivity for HIV-2 was discovered. Physical examination revealed enlargement of all lymph nodes, mild hepatomegaly and palpable spleen. The initial laboratory evaluation included the following: moderate normocytic aregenerative anaemia and positive direct Coombs tests without haemolysis, hypergammaglobulinemia, elevated C-reactive protein (45 mg/l), and a high ferritin level with low serum iron level. HIV-2 was confirmed, with a high viral load (93 100 copies/ml) and low CD4 lymphocyte count (133 cells/μl). The HHV-8 viral load was detectable (3231 copies/150 000 cells).

Histological examination of the biopsy specimen from axillary lymph nodes confirmed the diagnosis of Castleman's disease, and staining for HHV-8 (Fig. 1) underlined HHV-8-positive cells in the mantle zone, large HHV-8-positive cells dispersed in interfollicular areas and foci of KS with HHV-8 and latent nuclear antigen type 1 stain being present in fusiform-shaped cells. The patient was treated with two cycles of vinblastine, with a dramatic improvement, but symptoms relapsed within 2 weeks. At this time, bone marrow analysis showed evidence of toxic aplasia and a large amount of cytomegalovirus (290 000 copies/ml) in spite of cytomegalovirus viraemia negativity. HAART combining tenofovir, emtricitabine, lopinavir and ritonavir was initiated one week after the beginning of vinblastine; it was interrupted a few weeks later because of an episode of massive bloody diarrhoea with important loss of weight. Histological analysis of a colic biopsy sample showed non-specific ulcerated colitis with marked nuclear dystrophy, evocating a viral disease, but imunohistochemical methods failed to isolate any