Inhibition of hepatitis B virus X gene expression by novel DNA enzymes

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Two mono- and a di-RNA-cleaving DNA enzymes with the 10–23 catalytic motif were synthesized that were targeted to cleave at the conserved site/sites of the X gene of the hepatitis B virus. In each case, protein-independent but Mg^{2+}-dependent cleavage of *in vitro*-synthesized full-length X RNA was obtained. Specific cleavage products were obtained with two different mono- and a di-DNA enzyme, with the latter giving rise to multiple RNA fragments that retained the cleavage specificity of the mono-DNA enzymes. A relatively less efficient cleavage was also obtained under simulated physiological conditions by the two mono-DNA enzymes but the efficiency of the di-DNA enzyme was significantly reduced. A single nucleotide change (G to C) in the 10–23 catalytic motif of the DNA enzyme 307 abolished its ability to cleave target RNA completely. Both, mono- and di-DNA enzymes, when introduced into a mammalian cell, showed specific inhibition of X-gene-mediated trans-activation of reporter-gene expression. This decrease was due to the ability of these DNA enzymes to cleave X RNA intracellularly, which was also reflected by significant reduction in the levels of X protein in a liver-specific cell line, HepG2. Ribonuclease protection assay confirmed the specific reduction of X RNA in DNA-enzyme-treated cells. Potential *in vivo* applications of mono- and di-DNA enzymes in interfering specifically with the X-gene-mediated pathology are discussed.

Key words: gene cleavage, gene silencing, gene therapy.

INTRODUCTION

Nucleic acid molecules of specific sequences possessing various catalytic motifs are able to carry out a variety of chemical reactions as efficiently as conventional enzymes that consist of proteins. Ribozymes are catalytic RNA molecules [1–3] that can cleave RNA in a sequence-specific manner [4–6]. Among the various kinds of catalytic RNAs known, the two most studied are the ones that possess the hammerhead and hairpin motifs, respectively. Both kinds of ribozyme have the potential to cleave a target RNA when provided *in trans*. Ribozymes have been synthesized against viral or cellular genes and in particular against HIV-1 [7–13]. A multi-target ribozyme designed to cleave HIV-1 Env RNA into nine regions was proposed earlier [8,13] as a means to down-regulate HIV-1 replication. Targeting nine sites at the same time would, in principle, delay the appearance of escape mutants. DNA-armed ribozymes have also been constructed with the aim of providing more stability [14]. Recently, two hairpin ribozymes that cleave conserved regions in the HIV-1 LTR (long terminal repeat) and pol gene have entered a phase-1 clinical trial [11].

Since RNA is susceptible to degradation in the cell by nucleases, the exogenous application of ribozymes has been difficult, although certain modifications in the constituent nucleotides have been found to provide stability [15]. DNA is far less susceptible to intracellular degradation by nucleases than RNA and a large number of clinical trials are being conducted using targeted antisense made up of modified nucleotides for, e.g., phosphorothioate [16]. Recently, a general-purpose RNA-cleaving DNA enzyme (henceforth referred to as the DNA enzyme) with two catalytic motifs, 8–17 and 10–23, has been identified that was shown to cleave a 13-nucleotide-long synthetic RNA substrate in a catalytic manner [17]. We recently reported the cleavage of HIV-1 envelope and HIV-1 coreceptor CCR5 RNA using these targeted DNA enzymes and also showed that, when introduced into a mammalian cell, they could also interfere with the function [18,19].

In this article, we show specific cleavage of the hepatitis X RNA by two mono-DNA enzymes and multiple specific cleavage by a di-DNA enzyme under a variety of experimental conditions, including simulated physiological conditions. We have used one of the motifs (10–23) to cleave the X gene of the hepatitis B virus (HBx) in the present study. We chose two well-conserved sites in the X gene [20] and designed two mono-DNA enzymes to cleave them individually. The same two DNA enzymes were then synthesized in tandem to cleave the target RNA at multiple sites. Furthermore, we also demonstrate that these DNA enzymes have potential to down-regulate the expression of the X gene in a liver-specific mammalian cell line that has been implicated in the formation of hepatocellular carcinoma [21]. Since the X gene is a pleiotropic transactivator [21–24] and also responsible for increasing the expression levels of other hepatitis B virus genes, any approach to specifically down-regulate the X gene product will be beneficial to patients suffering from hepatitis B and associated disorders.

MATERIALS AND METHODS

In vitro synthesis of HBx RNA

The entire X gene (465 bases) was cloned into the *Eco*RI site of the expression vector pSG5 (Stratagene, La Jolla, CA, U.S.A.) to yield plasmid pSG5-HBx. The expression of the X gene was under the control of simian virus 40 promoter [23]. This plasmid was obtained as a gift from Vijay Kumar, International Center

Abbreviations used: HBx, X gene of the hepatitis B virus; RT, reverse transcriptase; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LTR, long terminal repeat.

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for Genetic Engineering and Biotechnology, New Delhi, India. This plasmid was linearized with the *Bgl*II restriction enzyme and [^{32}P]UTP (Amersham)-labelled RNA was synthesized by T7 RNA polymerase using the transcription kit provided by Promega Biotech. (Madison, WI, U.S.A.), and as has been described before [25]. Specific activity of the labelled RNA was determined by standard procedures. These RNAs were resolved on a 7% polyacrylamide/7 M urea gel using the miniprotein gel system provided by Bio-Rad (Hercules, CA, U.S.A.). Radioactive bands were visualized on an X-ray of the dried gel. PhosphorImaging analysis was used to quantify the intensities of the radioactive bands. The X gene was also cloned into the *Eco*RI site of the expression vector pcDNA3 (Promega Biotech.) to yield plasmid pcDNA-HBx.

Synthesis of RNA-cleaving DNA enzymes

Oligonucleotides of various lengths were synthesized in a DNA synthesizer (Applied Biosystems). Altogether, three oligonucleotides were synthesized. Two of them were mono-DNA enzymes of 29 nucleotides each. They contained, in the middle, the previously identified 10–23 catalytic motif with the following sequence: 5′-GGCTAGCTACAACGA-3′. Seven residues on either side of the triplet AUG are made complementary to the target gene for Watson–Crick base pairing (the A residue is left unpaired and the cleavage is expected to occur after the A residue). The third oligonucleotide, the di-DNA enzyme, was 58 nucleotides long. Figure 1(A) shows the sites that were chosen for carrying out specific cleavage in the HBx gene and Figures 1(B)–1(D) show the complete sequences of the two mono-DNA enzymes and the di-DNA enzyme, respectively. A disabled mono-DNA enzyme 307 with the mutated base underlined is shown in Figure 1(B2). The di-DNA enzyme was linked directly (no spacer) in the order such that DNA enzyme 307 was followed by 237 (Figure 1D). It is obvious that such a chimaeric RNA alone can hybridize at both the target sites of the X RNA.

In vitro cleavage reaction

Equimolar concentrations (100 pmol each) of the [^{32}P]UTP-labelled substrate RNA and unlabelled DNA enzymes were mixed in 10 μl of 50 mM Tris/HCl, pH 7.5, containing 10 mM $MgCl_2$ and incubated at 37 °C for 2 h (standard conditions for cleavage). The cleavage products were resolved by electrophoresis on a 7% polyacrylamide/7 M urea gel in Tris/borate/EDTA buffer as described before [8]. Radioactive RNA bands were detected by autoradiography of the dried gels. The pattern of expected cleavage products obtained by the two mono-DNA enzymes and the di-DNA enzyme are depicted in Figure 2. Cleavage reactions were also carried out under simulated physiological conditions (2 mM $MgCl_2$/150 mM KCl, pH 7.5, 37 °C) [17].

Intracellular inhibition of X-gene-mediated transactivation by DNA enzymes

HepG2 or COS-1 cells (1×10^5) were grown to 80% confluency in a 24-well plate (Nunc) and transfected with plasmid DNAs (0.01–0.1 μg) encoding the X gene, the LTR-luciferase reporter plasmid (pNL4-3.Luc.R-E-DNA, abbreviated in this paper to pNL-Luc), HBx DNA enzymes or equivalent amounts of mutant DNA enzyme (which served as control) using Lipofectin (Gibco BRL, Bethesda, MD, U.S.A.) as described in [13]. Luciferase activity was determined according to the manufacturer's instructions (Promega Biotech.) and as described by us recently [26]. To ensure uniform transfection efficiency of the mammalian cells, we used reporter plasmid pSV-β-gal (Promega Biotech.) as described by the manufacturer.

Intracellular reduction of the target RNA

The intracellular decrease in HBx-specific RNA in HepG2 cells that were co-transfected with the expression vector encoding the X gene (pSG5-HBx) and indicated amounts of DNA enzymes was determined by reverse transcriptase (RT) PCR techniques. Total RNA was isolated using Trizol reagent (Gibco BRL) following the manufacturer's directions. The RNA was treated with DNase 1 (RQ1, Promega Biotech.) for 45 min at 37 °C. Equal amounts of RNA were taken and subjected to RT-PCR using the cycling conditions described by us earlier [26]. Multiple dilutions of the target RNA were carried out to determine the linear range. In order to amplify full-length X RNA (465 bases), the following primers were used: HBx primer 1, 5′-TTAGGC-AGAGGTGAAAAAGTTGCATGGTGCTGG-3′, and HBx primer 2, 5′-ATGGCTGCTAGGCTGTACTGCCAACTGGA-TCCTTCG-3′. The control RNA (509 bases), human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), was amplified using the following set of primers as described earlier [13]: forward, 5′-ACCACCATGGAGAAGGCTGG-3′, and reverse, 5′-CTCAGTGTAGCCCAGGATGC-3′.

Ribonuclease protection assay

The ribonuclease protection assay was performed using the RPA II kit (Ambion, Austin, TX, U.S.A.) by following the manufacturer's procedure. Briefly, a 550-base labelled antisense probe was synthesized by transcription of *Hin*dIII-linearized pcDNA-HBx plasmid using SP6 polymerase in the presence of [^{32}P]UTP. The probe was hybridized with the sample RNA for 15 h at 42 °C in hybridization buffer. Unhybridized probe was removed by RNase A/RNase T1 treatment for 30 min at 37 °C. Following RNase inactivation and precipitation of the protected species, the pellets were dissolved in gel loading buffer, heated for 5 min at 95 °C and electrophoresed on a 7% polyacrylamide/7 M urea gel in Tris/borate/EDTA buffer. The bands were visualized by autoradiography of the dried gel. A standard curve was prepared using known amounts of *in vitro*-synthesized sense-strand RNA that was hybridized with an excess of labelled antisense probe. The intensity of probe fragments protected by the different amounts of sample RNA was compared with the standard curve to determine the absolute amounts of the protected species in the sample RNA.

Western blotting

The human hepatoma HepG2 cells were transfected with HBx expression vector and pNL-Luc with or without the DNA enzymes. Cell extracts were prepared 48 h post-transfection and processed for Western-blot analysis using X-protein-specific monoclonal antibody B/8/2/8 [23].

HBx RNA stability

To rule out the possibility of involvement of any cellular RNase in the degradation of X RNA, labelled target RNAs were allowed to hybridize individually with the DNA enzymes (equimolar amounts) and added to HepG2 cell lysate as described earlier [27]. After incubation at 30 °C for 15 min, the RNA was extracted and analysed by gel electrophoresis and visualized by autoradiography.

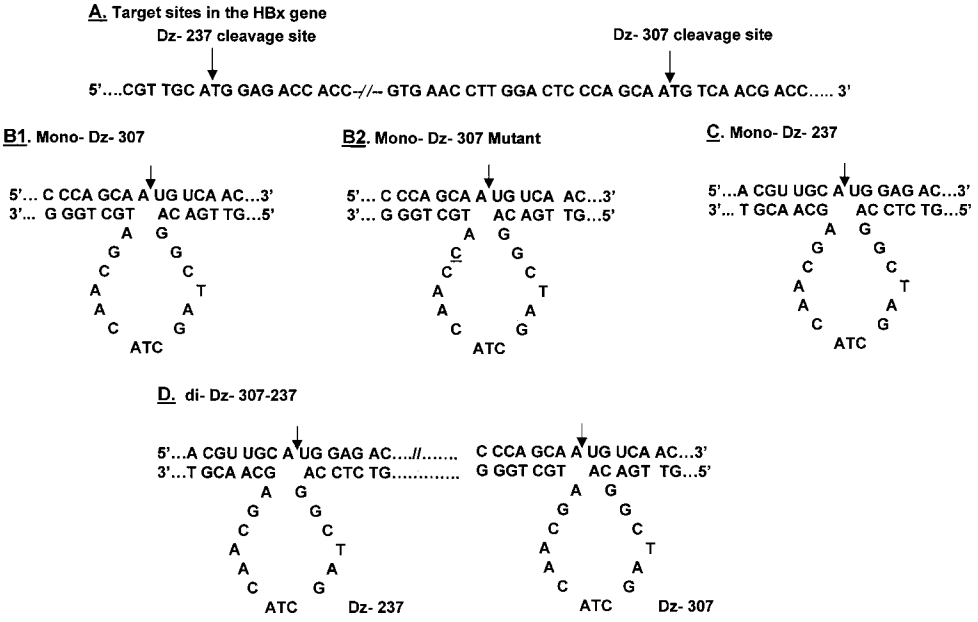

Figure 1 Target-site selection and construction of HBx DNA enzymes

(**A**) Selection of target sites within the X gene. The two target sites selected for making two mono- and a di-DNA enzyme are shown with arrows. Two internal initiation codons (AUG, methionine) were selected for constructing DNA enzymes which were from the most conserved region of the gene [20]. The cleavage is predicted to take place after the A residues at the two sites, as shown by arrows. (**B1**) Construction of HBx DNA enzyme (Dz) 307; seven bases on either side of the A residue (left unpaired) of the AUG is provided along with the 10–23 catalytic motif of 10–23 type. (**B2**) A disabled mutant mono-DNA enzyme was constructed by changing the G residue in the catalytic motif to C (underlined). (**C**) Construction of HBx DNA enzyme 237. The strategy for constructing this DNA enzyme was essentially the same as described for DNA enzyme 307, which also uses the 10–23 catalytic motif and seven bases of flanking sequences to hybridize specifically with the chosen target site. (**D**) Construction of the di-DNA enzyme (di-Dz-307-237). This construct contains the above two DNA enzymes, 307 and 237 (in the 5′–3′ direction), connected directly in tandem with no spacer nucleotides. They both contain their individual 10–23 catalytic motifs. Such a di-DNA enzyme is expected to give rise to multiple cleavage products.

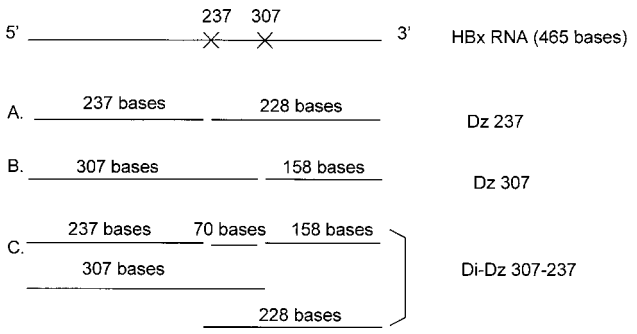

Figure 2 HBx RNA cleavage pattern obtained by the mono- and di-DNA enzymes

The expected cleavage pattern with mono-DNA enzymes (Dz) 237 and 307, and the di-DNA enzyme, using X RNA as substrate. Mono-DNA enzyme 237 is expected to generate cleaved RNA fragments of 237 and 228 nt long (**A**), whereas, mono-DNA enzyme 307 would generate RNA fragments of 307 and 158 nt long (**B**). The di-DNA enzyme is expected to generate all the above fragments. An additional 70-base fragment would be seen if the two target sites in the X RNA are cleaved either simultaneously or sequentially (**C**).

Kinetic analysis

Kinetic parameters for cleavage by various DNA enzymes were determined using various concentrations of the HBx RNA (465 bases) in the presence of excess amounts of DNA enzymes under standard conditions of cleavage as reported earlier [17–19]. The cleaved RNA fragments were quantified with the help of a PhosphorImager and kinetic parameters were calculated from a Lineweaver–Burk plot according to standard procedures.

RESULTS

Construction of mono- and di-RNA-cleaving DNA enzymes and selection of target sites in the HBx gene

All the RNA-cleaving DNA enzymes contained the 10–23 type of catalytic motif described earlier [17]. The target sites chosen for cleavage were two internal initiation codons (AUG) from the central conserved region of the gene representing the 79th and 103rd amino acids (Figure 1A) [20,23]. Seven nucleotides at either end of the catalytic motif were synthesized which were complementary to the target RNA for the two mono-DNA enzymes (Figures 1B and 1C). A di-DNA enzyme was synthesized in tandem; DNA enzyme 307 followed by DNA enzyme 237 (Figure 1D). A point mutation in the catalytic motif of DNA enzyme 307 was created which served as a control for *in vitro* and *in vivo* studies (Figure 1B2). The expected cleavage pattern of the X RNA by mono-DNA enzymes and the di-DNA enzyme is shown in Figure 2.

In vitro cleavage of HBx RNA by DNA enzymes 237 and 307, and the di-DNA enzyme

As expected, a 465-base-long labelled X transcript was synthesized (Figure 3A, lane 1). Equimolar concentrations of labelled substrate and unlabelled DNA enzyme 307 were added

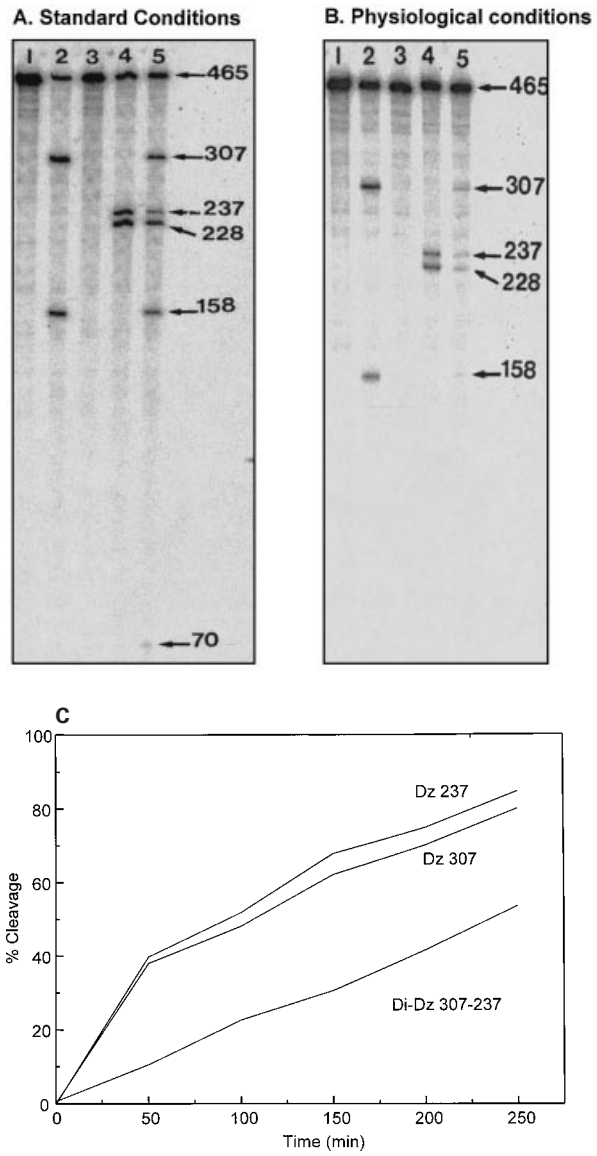

Figure 3 ***In vitro*** **cleavage of HBx RNA by DNA enzymes**

Lane numbers in panels (**A**) and (**B**) correspond to each other. Standard cleavage conditions (see text) were used in (**A**) whereas (**B**) shows simulated physiological conditions. RNA fragments were analysed on a sequencing gel (Gibco BRL). Lanes 1 show the synthesis of 465-base labelled X RNA by *in vitro* transcription. Equimolar amounts of mono-DNA enzyme 307 (unlabelled), when mixed with the labelled X RNA, yielded specific products of 307 and 158 bases long (lanes 2). Mutant mono-DNA enzyme 307 failed to cleave the target RNA (lanes 3). Mono-DNA enzyme 237 also yielded specific cleavage products (237 and 228 bases; lanes 4). When equivalent amounts of di-DNA enzyme were used, specific cleavage products were observed that were obtained with both mono-DNA enzymes. Note that, in general, the efficiency was slightly lower under physiological conditions (compare lanes 2 and 4) with the two mono-DNA enzymes but the difference was significantly greater when the di-DNA enzyme was used. (**C**) The efficiency of the cleavage was compared by carrying out the cleavage reactions for 4 h under standard conditions for cleavage under similar experimental conditions. Samples collected at multiple time points were analysed as described earlier by us [18,19]. Mono-DNA enzymes 237 and 307 possessed similar kinetics for cleavage but the di-DNA enzyme was less efficient.

to a final 10 μl volume of buffer as described before [18,19]. Cleavage was initiated by adding $MgCl_2$ (final concentration 10 mM; standard conditions) at 37 °C for 2 h and the cleaved RNA products were analysed on a sequencing gel. DNA enzyme

Table 1 Kinetic analysis of HBx DNA enzymes

Kinetic measurements were carried out for all three DNA enzymes by taking various amounts (0.1–0.01 μM) of the substrate RNA (labelled 465-base HBx RNA) in 50 mM Tris/HCl, pH 7.5, and 10 mM $MgCl_2$ under DNA enzyme saturating conditions at 37 °C for 1 h. The substrate and the cleaved products were separated on 7% polyacrylamide/7 M urea gels and detected by autoradiography. The extent of cleavage was determined by quantification of radioactivity present in the bands of substrate and products with the help of a PhosphorImager. The kinetic parameters were calculated from a Lineweaver–Burk plot. Shown are mean values from two experiments.

DNA enzyme	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m
HBx DNA enzyme 237	200	2.15	0.011
HBx DNA enzyme 307	100	3.7	0.037
HBx di-DNA enzyme	310	1.6	0.005

307 yielded the expected sizes cleaved RNA fragments, 307 and 158 bases (Figure 3A, lane 2). The same DNA enzyme with a single base mutation at the catalytic motif rendered the DNA enzyme completely inactive, as no cleavage was observed (Figure 3A, lane 3). Also, no cleavage was observed in the presence of 50 mM $MgCl_2$ with the mutant DNA enzyme (results not shown). The other mono-DNA enzyme 237 yielded RNA fragments that were 228 and 237 bases long, as expected (Figure 3A, lane 4). When a similar molar ratio of di-DNA enzyme was added, specificity of the cleaved RNA fragments was the same as that of the mono-DNA enzymes individually (Figure 3A, lane 5). Also, an RNA fragment of 70 bases could be seen by the di-DNA enzyme alone (Figure 3A, lane 5, arrow). Figure 3(B) is similar to Figure 3(A), the only difference being the experimental conditions of cleavage. In the case of Figure 3(B), all the cleavage experiments were carried out at simulated physiological conditions. Cleavage efficiency by individual mono-DNA enzymes was only affected slightly (compare lanes 2 and 4 of both Figures 3A and 3B). This difference was much more pronounced with the di-DNA enzyme, where significantly less cleavage was obtained under physiological conditions (compare lanes 5 of Figures 3A and 3B). A comparison of the kinetics of cleavage activities of all the three DNA enzymes under standard conditions is shown in Figure 3(C). DNA enzymes 307 and 237 were equally efficient in cleavage but the di-DNA enzyme showed somewhat delayed kinetics of cleavage (Figure 3C). With prolonged incubation (4 h), nearly complete cleavage of the X RNA was obtained with the mono-DNA enzymes but only 65–70 % cleavage was obtained with the di-DNA enzyme under standard conditions (results not shown).

Kinetics analysis of HBx DNA enzymes

The kinetic parameters K_m and k_{cat} for the two mono-DNA enzymes and the di-DNA enzyme are presented in Table 1. The K_m values for DNA enzymes 237 and 307 were 200 and 100 nM, respectively, whereas the K_m value for the di-DNA enzyme was 310 nM (Table 1). Standard cleavage conditions were employed as described earlier.

HBx gene product transactivates HIV-1 LTR promoter-directed expression

In HepG2 cells (1×10^5/well) co-transfected with fixed amount of reporter gene (pNL-Luc, 0.1 μg) but increasing amounts of X-

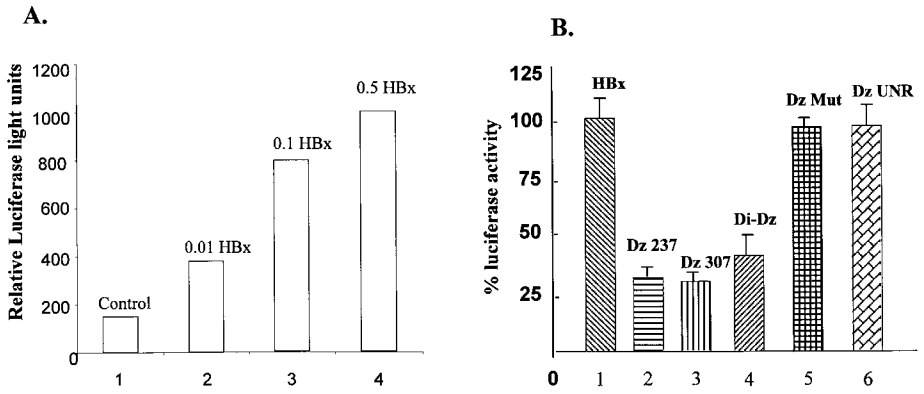

Figure 4 HBx-dependent luciferase gene expression and its inhibition by DNA enzymes

(**A**) HepG2 cells (1×10^5) were grown to 80% confluency in a 24-well plate, and increasing amounts (in μg) of X-gene-encoding DNA and a fixed amount (0.1 μg) of reporter gene (pNL-Luc) was transfected using Lipofectin. After 15 h of transfection, the samples were processed according to the method described earlier [18,26] for determining the luciferase activity. Column 1 shows the background activity when 0.5 μg of unrelated plasmid DNA was transfected. Note that the luciferase activity increased with the increasing concentrations of the X-gene-encoding plasmid DNA. Unrelated DNA had no effect on the levels of luciferase activity (results not shown). (**B**) Inhibition of X-gene-mediated transactivation of the reporter gene placed under the HIV-1 LTR promoter. HepG2 cells were transfected with various combinations of DNA enzymes or unrelated DNA along with a constant amount of reporter plasmid DNA (0.1 μg of pNL-Luc). Both the mono-DNA enzymes (Dz 237 and Dz 307) and the di-DNA enzyme (Di-Dz) interfered very efficiently with reporter gene expression. The disabled DNA enzyme (Dz Mut) at equivalent concentrations had no effect on the levels of luciferase activity obtained. Unrelated DNA enzyme (Dz UNR, 29-mer against HIV-1 coreceptor CCR5) at an equivalent concentration failed to interfere. Shown are means $\pm$ S.D. from three separate experiments.

gene-encoding plasmid DNA, a dose-dependent increase in luciferase activity was observed (Figure 4A, columns 2–4). We failed to detect any luciferase activity when unrelated plasmid DNA was transfected (results not shown). The plasmid pNL-Luc contains the luciferase gene that is inserted in the Nef region [28]. The concentration of this plasmid was kept constant at 0.1 μg but the HBx-encoding plasmid DNA was used in three different concentrations (0.01, 0.1 and 0.5 μg in Figure 4A, columns 2, 3 and 4, respectively). Column 1 shows the extent of luciferase activity when cells were transfected with the reporter plasmid DNA along with equivalent amounts of an unrelated DNA, pGEM3Z (Promega Biotech.; control). We did not try a DNA concentration higher than 0.5 μg/well because of the toxicity. Both cell lines tested (HepG2 and COS-1) gave essentially similar results (results not shown). We conclude that the X gene product increases the HIV-1 LTR-mediated reporter gene expression in a dose-dependent manner.

Intracellular inhibition of X-gene-mediated transcription by DNA enzymes

Equivalent amounts of HBx plasmid and HBx DNA enzyme (0.01 μg each) were used to transfect HepG2 cells in the presence of the reporter gene-encoding plasmid DNA (pNL-Luc). As expected, both the mono-DNA enzymes were very efficient at inhibiting LTR-mediated transcription (Figure 4B, compare column 1 with columns 2 and 3). The di-DNA enzyme also showed good interference, although it was less efficient than either of the two mono-DNA enzymes (Figure 4B, column 4). The same amount of either the point-mutated DNA enzyme (Dz Mut, Figure 4B, column 5) or an unrelated DNA enzyme (29 nt long) against an unrelated gene, CCR5 [18], failed to show any inhibition of LTR-mediated luciferase gene expression (Figure 4B, column 6). Both the mono-DNA enzymes were more effective in causing the inhibition of reporter gene expression than the di-DNA enzyme. Shown are means $\pm$ S.D. from three separate experiments.

Intracellular cleavage of the X RNA

Whether or not DNA enzymes were able to reduce intracellular levels of HBx RNA upon co-transfection of the target gene and DNA enzymes in HepG2 cells was examined by quantifying the levels of X RNA by RT-PCR from equal amounts of cell lysates using different sets of primer combinations (Figure 5A). Lane 1 from Figure 5(B) shows the level of full-length X RNA when pSG5-HBx plasmid was transfected along with equal amounts of unrelated DNA. Figure 5(B) lanes 2–4 represent levels of X RNA observed with the di-DNA enzyme, and mono-DNA enzymes 307 and 237 respectively. Figure 5(B) lane 5 shows the amplification reaction carried out in the absence of RT enzyme. As expected, there was no amplification observed in this lane. Figure 5(B) lane 6 shows the level of X RNA when transfected with the mutant (disabled) DNA enzyme 307 that served as a control. All these results strongly support our conclusion that the reduction in the levels of full-length X RNA is most likely due to specific intracellular cleavage. The hGAPDH was also amplified from equivalent amounts of cell lysates that served as an internal control. Since the levels of hGAPDH RNA observed after RT-PCR remained the same in all corresponding lanes (Figure 5C), we conclude that the reduction observed in the levels of X RNA by DNA-enzyme-treated cells was specific.

Ribonuclease protection assay

A ribonuclease protection assay was performed in order to quantify the amount of X RNA present in total cellular RNA preparations that were obtained from control cells or cells transfected with DNA enzymes. An antisense probe of X RNA (550 nt long) was prepared by linearizing the pcDNA-HBx plasmid with *Hin*dIII and subjecting it to *in vitro* transcription using SP6 RNA polymerase in the presence of [^{32}P]UTP (Figure 6A). The probe was hybridized to the sample RNA as described by the manufacturer (Promega Biotech.). After nuclease digestion the protected fragments were analysed by PAGE and autoradiography. Figure 6(B) lane 1 shows the 550 nt probe, which gets completely degraded after nuclease treatment when mixed with

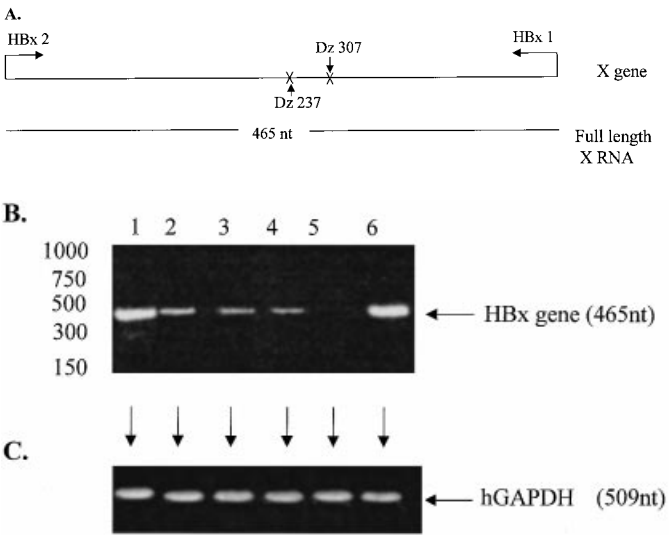

Figure 5 Decrease in HBx-speciifc RNA by DNA enzymes

(**A**) To address the question of intracellular cleavage of the X RNA by the DNA enzymes, two primers were designed to amplify the full-length X gene (465 bases). Simultaneous RT-PCR of treated cells would enable one to address the question of intracellular cleavage. (**B**) Total cellular RNA was extracted from HepG2 transfected cells using Trizol (Gibco BRL) and a RT-PCR was set up using HBx primers using the earlier-described cycling conditions for PCR [26]. Lane 1, the amount of X-RNA detected from cells transfected with pSG5-HBx plasmid (0.1 μg) in the absence of DNA enzymes. When co-transfected with pSG5-HBx and DNA enzymes (0.5 μg each; lane 2, di-DNA enzyme; lane 3, DNA enzyme 307; lane 4, DNA enzyme 237), reduced expression of X RNA was observed. Lane 5, RT-PCR carried out in the absence of RT. When equivalent amounts of disabled DNA enzyme were used, no reduction in the levels of full-length X RNA was observed (compare lane 6 with lane 1). (**C**) The same amounts of lysates were used as in (**B**) and levels of the control RNA were determined for the hGAPDH gene using the primers described earlier. A 509-base DNA fragment of equal intensity was detected in all the corresponding lanes.

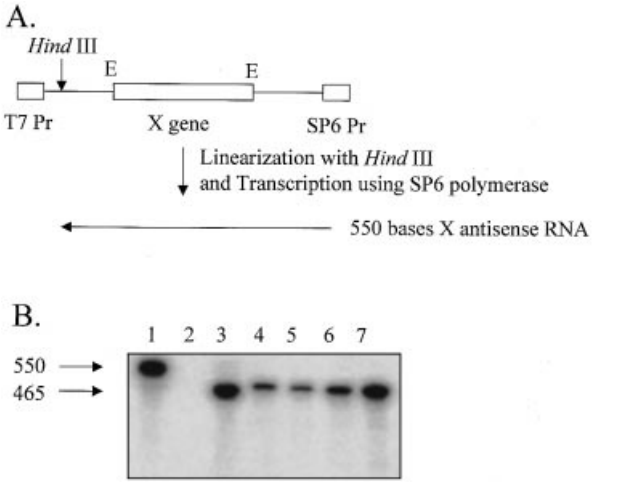

Figure 6 Intracellular reduction of HBx RNA as measured by ribonuclease protection assay

Plasmid pcDNA-HBx was linearized at the restriction site *Hin*dIII and subjected to *in vitro* transcription in the presence of labelled UTP using SP6 RNA polymerase. Labelled transcript of 550 bases was obtained that served as the antisense probe for carrying out ribonuclease protection assays (**A**). The probe was hybridized to total cellular RNA obtained from HepG2 cells. After nuclease digestion the protected fragments were analysed on 7% polyacrylamide/7 M urea gels. (**B**) Lane 1, full-length 550 nt probe. Lane 2, the probe hybridized to yeast RNA (control) and subsequently digested with RNase. No signal is observed in this lane due to complete degradation of the probe by RNase. HepG2 cells that received the pSG5-HBx plasmid DNA (0.1 μg) alone showed the 465-base protected RNA fragment (lane 3). Cells that were treated with the 0.5 μg each of DNA enzyme 237 (lane 4), DNA enzyme 307 (lane 5) and di-DNA enzyme (lane 6) showed reduction in the levels of target RNA. Note that the mono-DNA enzymes were more effective in reducing the levels of X RNA (compare lanes 4 and 5 with lane 6). On the contrary, equivalent amounts of mutant DNA enzyme 307 (lane 7) showed the same amounts of target RNA as observed in lane 3.

yeast RNA (Figure 6B, lane 2). RNA from HepG2 cells transfected with pSG5-HBx plasmid DNA (0.1 μg) protected the probe to give a 465 nt long fragment (Figure 6B, lane 3). HepG2 cells that were treated with DNA enzyme 237 (Figure 6B, lane 4), DNA enzyme 307 (Figure 6B, lane 5) and di-DNA enzyme (Figure 6B, lane 6; 0.5 μg each) showed reduced amounts of target RNA. Mutant DNA enzyme 307-treated cells showed no reduction in the levels of target RNA (Figure 6B, compare lane 7 with lane 3). The relative amount of X RNA present in a mixture of total cellular RNA was calculated and found to be 10 ng (Figure 6B, lane 3). The DNA enzyme treatment significantly reduced the levels of X-specific RNA. X RNA levels were found to be 2 ng in DNA enzyme 237-treated cells (Figure 6B, lane 4), 1.8 ng in DNA enzyme 307-treated cells (Figure 6B, lane 5) and 4.5 ng in cells treated with the di-DNA enzyme (Figure 6B, lane 6). The mutant DNA enzyme 307 failed to reduce the intracellular levels of X RNA, which were approx. 9.98 ng (Figure 6B, lane 7). These values are means from two separate experiments. We conclude that an approx. 5-fold reduction in the levels of X RNA was obtained with the two mono-DNA enzymes but that only an approx. 2-fold reduction with the di-DNA enzyme was seen under similar experimental conditions.

DNA enzyme-treated HepG2 cells show reduced levels of X protein

Equal amounts of lysates from the cells that were co-transfected with the plasmid pSG5-HBx and DNA enzymes were processed

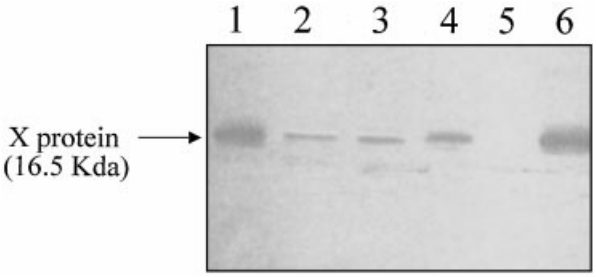

Figure 7 Reduction in intracellular HBx protein levels by DNA enzymes

Total cell extracts from transfected cells were taken and Western-blot analysis was carried out using HBx-specific monoclonal antibody B/8/2/8 [23]. Lane 1, the presence of immunologically reactive X protein as an 18 kDa band. Lanes 2, 3 and 4, the reduced levels of X protein when the cells were transfected with mono-DNA enzymes 307 and 237, and the di-DNA enzyme, respectively. No protein band was observed in the lysates derived from HepG2 cells alone (lane 5). Under identical experimental conditions, the disabled DNA enzyme did not show any reduction in the levels of the X protein (lane 6).

for Western blot analyses using the procedure described in [29]. Lane 1 of Figure 7 shows the level of X protein from cells transfected with pSG5-HBx along with equal amounts of unrelated DNA. Figure 7 lanes 2–4 show the levels of protein from cell lysates that were transfected with DNA enzymes 237 and 307, and the di-DNA enzyme (0.5 μg each) respectively. Figure 7, lane 5, represents HepG2 cell lysates alone, and, as expected, shows no X-protein-specific band. Figure 7, lane 6, shows the

levels of X protein detected when equivalent amounts of the mutant DNA enzyme 307 (disabled) was used. Mutant DNA enzyme failed to reduce the expression of the X protein (Figure 7, compare lane 1 with lane 6). An approx. 4–6-fold reduction in the levels of X protein was observed with the two mono-DNA enzymes (Figure 7, compare lane 1 with lanes 2 and 3). Di-DNA-enzyme-treated cells showed an approx. 2-fold reduction (Figure 7, compare lane 1 with lane 4). We conclude that mono-DNA enzymes were more effective at specifically reducing the levels of X protein than the di-DNA enzyme.

HBx RNA stability

To rule out the possibility of any RNase activity in the cells that may have degraded the X RNA upon hybridization with the target RNA, all the DNA enzymes were hybridized individually with a ^{32}P-labelled X RNA and added to HepG2 cell lysate. After a 15 min incubation at 37 °C, the amount of radioactivity was quantified, as described earlier. No non-specific degradation of the target RNA was obtained with any of the three DNA enzymes, disabled DNA enzyme or unrelated DNA enzyme (results not shown).

DISCUSSION

We report specific cleavage of a biologically relevant full-length X RNA by two mono-DNA enzymes and multiple cleavages by a di-DNA enzyme, which possessed the 10–23 catalytic motif, described earlier [17]. We showed the cleavage specificity under two different sets of experimental conditions: (i) under standard conditions where the concentration of $MgCl_2$ was 10 mM, and (ii) under simulated physiological conditions with 2 mM $MgCl_2$ (see the Materials and methods section). The efficiency of cleavage under simulated physiological conditions was only slightly lower for the two mono-DNA enzymes but the efficiency of the di-DNA enzyme was significantly lower. Thus use of the two mono-DNA enzymes simultaneously may be a better choice for the inactivation of the target RNA. It is noteworthy that, under physiological conditions, the specificity of cleavage by all three DNA enzymes was strictly retained. Lower cleavage efficiency by the di-DNA enzyme was expected because it is capable of generating more secondary structures (because of its longer size) that could potentially interfere with the hybridization with the target RNA under simulated physiological conditions. The fact that cleavage occurred under simulated physiological conditions suggests that these DNA enzymes could be attractive candidates for therapeutic application in humans. The presence of a 70-base-long RNA fragment after cleavage by the di-DNA enzyme could be due to either simultaneous cleavage at two target sites or sequential cleavage.

These DNA enzymes were very efficient at interfering specifically with HIV-1 LTR-mediated transcription, as no inhibition was observed when equivalent amounts of an unrelated DNA enzyme of similar length (29 nt) or the mutant (disabled) DNA enzyme was used. The di-DNA enzyme was also found to be only slightly less effective than the two mono-DNA enzymes. The inhibition observed was largely because of the catalytic nature of cleavage of the target RNA by these DNA enzymes, as the mutant DNA enzyme 307 failed to interfere with the levels of X RNA or protein. The same was true when a completely unrelated DNA enzyme was used. We have also ruled out the possibility of a role of any double-stranded-RNA-dependent ribonuclease in the intracellular degradation of X RNA that may account for the reduced expression of X RNA or protein. Evidence was obtained for specific intracellular cleavage of the target RNA by the DNA enzymes. A significant reduction in the X RNA levels was observed by carrying out a RT-PCR reaction or by ribonuclease protection assay. The amounts of intracellular X RNA in mono-DNA-enzyme-treated cells were about 4–5-fold lower than in the control cells or the mutant-DNA-enzyme-treated cells. Similar observations were made when the DNA-enzyme-treated cells were analysed for the amounts of X protein by Western-blot analyses. It is noteworthy that, in all three intracellular assays, mono-DNA enzymes were more efficient than the di-DNA enzyme, which correlated with the *in vitro* cleavage efficiency of DNA enzymes under simulated physiological conditions. The efficacies of DNA enzymes were tested initially in COS-1 cells and later on in a liver-specific cell line, HepG2, where the expression of the X gene has been linked with hepatocellular carcinoma [21]. Almost identical results were observed with both cell lines.

Multi-target DNA enzymes could potentially be used to cleave the same 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 proof-reading mechanisms and this kind of approach could potentially delay the appearance of resistant populations. We have shown clearly that a di-DNA enzyme efficiently cleaved the HBx RNA at specific multiple sites. Thus the di-DNA enzyme retained the specificity of the two mono-DNA enzymes. These multi-target DNA enzymes could also be exploited to down-regulate cellular RNAs as well by multiple specific cleavages and provide new opportunities to study the functional importance of essential cellular or viral genes.

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