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Novel mono- and di-DNA-enzymes targeted to cleave *TAT* or *TAT-REV* RNA inhibit HIV-1 gene expression

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Abstract

The regulatory proteins TAT and REV play a very important role in the transcription and replication of HIV-1. In order to se*HIV-01*lectively down regulate the expression of these genes we synthesized several mono- and one di-DNA-enzyme against the *TAT* or *TAT-REV* RNA. Several mono-DNA-enzymes possessing the 10–23 catalytic motif were assembled that were targeted to the predicted loop region of *TAT* or *TAT/REV* RNA. The cleavage efficiency of each mono-DNA-enzyme was variable and independent of the size of the predicted loop structure of the target RNA. DNA-enzyme targeted against the largest loop region cleaved the substrate RNA poorly. Mono-DNA-enzyme-5944 that targets only the *TAT* region cleaved the substrate poorly but the DNA-enzyme-5970 that overlaps *TAT* and *REV* showed potent cleavage activity. The two DNA-enzymes, when placed in tandem, cleaved the target RNA at multiple sites that were specific for the two mono-DNA-enzymes. Only Dz-5970 retained the ability to cleave the target RNA specifically at simulated physiological conditions. They were able to inhibit HIV-1 specific genes efficiently when introduced into a mammalian cell. The extent of inhibition correlated with their cleavage efficiency obtained at standard conditions of cleavage. Although DNA-enzyme-5970 showed the highest reduction ($\sim$90%), other DNA-enzymes (mono-DNA-enzyme-5944 and the di-DNA-enzyme) also showed reduction to an extent of 60 and 80% respectively. The inhibitory effect of the DNA-enzyme could be overcome by providing HIV-1 TAT to the cells.

Keywords: RNA cleaving DNA-enzyme; HIV-1 TAT; REV and ENV

1. Introduction

Nucleic acid molecules of specific sequences possessing a variety of catalytic motifs are now known to carry out a variety of chemical reactions as efficiently as enzymes that are made up of proteins. This unique property to catalytically cleave the target RNA in a sequence specific manner has been exploited by many investigators to interfere with the functional expression (Cech, 1987; Uhlenbeck, 1987; Haselhoff and Gerlach, 1988; Beck and Nassal, 1995). Ribozymes containing the hammer-head or hairpin motif has been extensively exploited to interfere with the

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replication of HIV-1 (Sarver et al., 1990; Dropulic et al., 1992; Lo et al., 1992; Ojwang et al., 1992; Welch et al., 1998). A multitarget ribozyme against HIV-1 genes has been reported (Chen et al., 1992; Paik et al., 1997) to interfere specifically with the replication of HIV-1. Despite these interesting properties the use of ribozyme as a therapeutic tool has not been very successful because the RNA is extremely susceptible to cleavage by cellular nucleases and attempts have been made to construct stable ribozymes by using modified nucleotides (Flory et al., 1996; Sioud and Sorensen, 1998). Novel chimeric ribozymes that contain the target hybridizing arms in the form of DNA have also been described (Hendry and McCall, 1995). Ribozymes are expensive and possess a short half life in plasma. Recently, Santoro and Joyce (1997) have reported sequence specific cleavage activities of a short DNA molecule with either the 10–23 or 8–17 catalytic motif. We used one of their motifs, 10–23, and showed sequence-specific cleavage activity of a DNA-enzyme that was targeted to cleave the HIV-1 coreceptor-CCR5 gene (Goila and Banerjea, 1998). Predicted secondary structures of the target RNAs have often been used to select target sites, preferably in the loop region, but it is quite possible that the target RNA may not fold in the similar manner or that the target site may not be available because of several other reasons.

HIV-1 *TAT* and *REV* are two extremely important proteins that are involved in gene expression, replication and pathogenesis of HIV-1 (Taube et al., 2000). Present efforts are underway to exploit TAT protein as a vaccine. *TAT* is translated from multiply spliced transcripts and does not depend upon REV for its transport from nucleus to cytoplasm (Jones and Peterlin, 1994). It is encoded by two exons that are present on either side of the HIV-1 *ENV* gene, the first exon consists of 72 amino acids. Another splice variant *TEV* has also been described (Benko et al., 1990).

We aimed at interfering specifically with the expression of *TAT* or *TAT*/*REV* RNA, as this would significantly interfere with the replication of HIV. We, therefore, synthesized several mono- and one di-DNA-enzyme against *TAT* and *TAT*/*REV* RNA and showed their ability to cleave the target RNA specifically. The effects of mono- and di-DNA-enzyme on the expression of HIV-1 genes were also explored.

2. Materials and methods

2.1. Cells and plasmid DNAs

Cos-1 or HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) and 10% FBS. Plasmid DNAs, pNL.Luc (luciferase gene) was inserted into the *NEF* region of pNL4-3 (Adachi et al., 1986) and pHIV-gpt (Page et al., 1990) were obtained from the NIH AIDS repository and were grown according to standard procedures. The supercoiled plasmid DNAs were purified on a Qiagen (Qiagen, GmbH, Germany) column before transfection using lipofectin reagent (GIBCO/BRL, MD). Plasmid pSV2-TAT (HIV-1 *TAT* under SV40 promoter) was a kind gift from A. Kinter, NIH, MD.

2.2. Target RNA and selection of DNA-enzyme cleavage sites

Two methionine codons (AUG) were selected as our target sites for cleavage by 10–23 catalytic motif-containing DNA-enzymes that were positioned at 5944 and 5970 respectively (Adachi et al., 1986). Predicted secondary structure of this RNA consisted of a number of stem–loop structures (Fig. 1); the DNA-enzyme cleavage sites present in the loop structures are shown by arrows. The DNA encoding the first exon of *TAT* and *REV* were excised from the infectious DNA-pNL4-3 using *EcoR*1 and *Hind* III and subcloned into pSGI (modified pSG5, Clonetech., CA, where multiple cloning sites (MCS) was introduced between the *EcoR*1 and *Bgl* II site). This placed the expression of the above target gene under the influence of T7 as well as SV40 promoter (Fig. 2A). DNA-enzyme-5944 was targeted against the *TAT* region but the DNA-enzyme-5970 was targeted in the *TAT*/*REV* overlap region (Fig. 2B). A di-DNA-enzyme-70-44 was constructed by placing the above two DNA-enzymes in direct tandem with no spacer nucle-

otides. They both contained their 10–23 catalytic domain with flanking sequences that were complementary to the target RNA (Fig. 2C). Seven nucleotides that are complementary to the target RNA sequence on either side of the unpaired A nucleotide in the target gene were synthesized. The cleavage is expected to take place after the A nucleotide (shown by arrows). The earlier identified catalytic motif (10–23) was placed between the two antisense flanks which possessed the following sequence: 5′-GGCTAGCTACAACGA. A single point mutation (G to C) was created in the 10–23 catalytic motif in Dz-5970 to generate Dz-5970MT.

We also targeted our DNA-enzyme to cleave between A and C nucleotide sequence (nt. position 6011) present at the tip of the largest predicted loop structure of the target RNA (shown by arrow; Fig. 1). Two DNA-enzymes were synthesized against this target, one capable of hybridizing with only the loop region (Dz-6011-6-6; Fig. 2E) and the other (Dz-6011-7-7; Fig. 2D) with the same loop but one extra nucleotide on either side that is complementary to the target RNA. In the former case the size of the DNA-enzyme was 27 nucleotides but it was 29 nucleotides long in the latter case. This computer-predicted secondary structure of *TAT* RNA was earlier used in the design of dimer minizymes (Kuwabara et al., 1996).

2.3. In vitro cleavage reaction

Equimolar concentrations (100 pmol) of ^{32}P labeled substrate RNA and cold DNA-enzyme were mixed in 10 μl of 50 mM Tris–HCl, pH 7.5 containing 10 mM $MgCl_2$ (standard condition). The cleavage reaction was allowed to continue at 37°C for 1 h. In some experiments, the cleavage was carried out at varying concentrations of $MgCl_2$ as indicated. The cleavage products were analyzed as described before (Dash et al., 1998; Goila and Banerjea, 1998) using Tris-Borate-EDTA using the mini-protein gel system from Bio-rad. Cleavage reaction was also carried out at simulated physiological conditions (10 μl of 50 mM Tris–HCl, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$; Santoro and Joyce, 1997). Radioactive bands were visualized by autoradiography of the dried gel.

2.4. In vivo inhibition of HIV-1 gene expression by DNA-enzymes

Four independent experiments were carried out to determine if the DNA-enzymes could act intra-

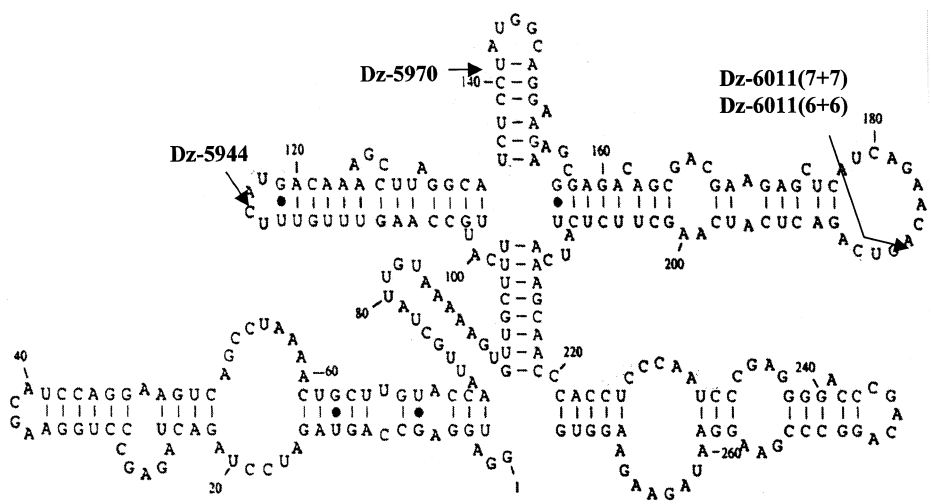

Fig. 1. Computer generated secondary structure of the *TAT* or *TAT/REV* RNA. It consists of several stem loop structures. DNA-enzymes with the earlier identified 10–23 catalytic motif, were targeted against the purine and pyrimidine nucleotides of the loop region only (shown by arrows). Two DNA-enzymes were also constructed against the largest loop region of the target RNA.

cellularly. The plasmid pHIV-1gpt was used to transfect 60% confluent Cos-1 cells in a six-well plate at a fixed concentration of 0.1 μg along with 1 μg of unrelated DNA-enzyme or varying concentration of DNA-enzymes (0.1 and 1 μg) for 1×10^6 cells. Transfected cells were incubated for 2 h in the medium without serum followed by an additional 13 h in presence of 10% fetal bovine serum. Supernatant was collected and cell lysates were prepared for RNA isolation using TRIZOL reagent (GIBCO/BRL). Levels of p24 antigen were estimated by using an ELISA kit and the

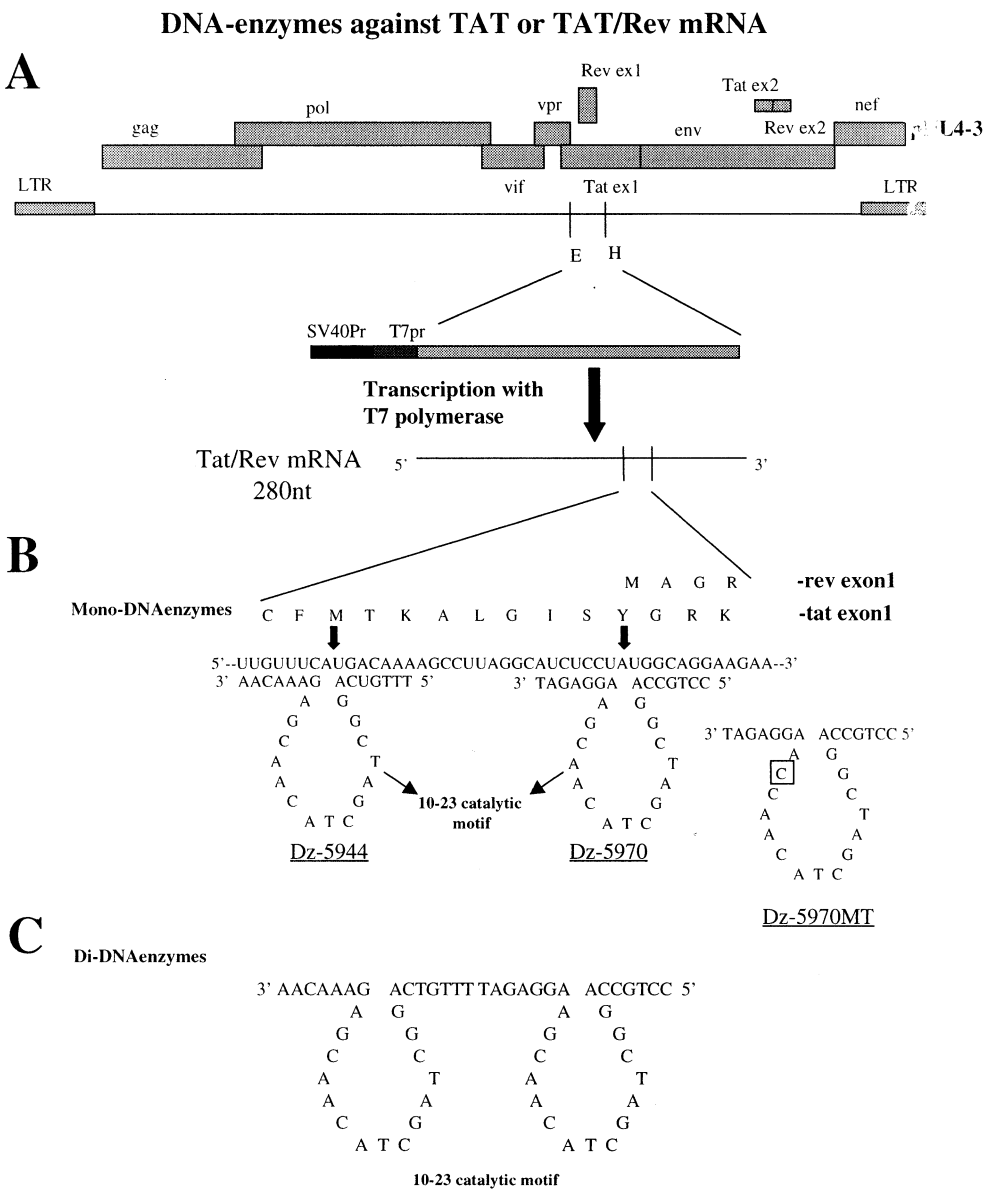

Fig. 2.

amount of reverse transcriptase from the released particles was determined by using the nonradioactive RT-Assay kit from BMB. NIH-3T3-CD4-CCR5 cells were cotransfected with the infectious molecular clone for HIV-1, pNL4-3, along with varying concentrations of DNA-enzymes. Forty-eight hours after transfection, the cell lysates were prepared as described before (Paik et al., 1997) and the RNA was isolated using TRIZOL reagent. With RT-PCR techniques, *TAT* and HIV-1 *GAG* RNA were amplified using specific primers that were designed (see Results, Section 3.6). For controls, either unrelated DNA-enzyme or mutant DNA-enzyme-5970 or equivalent amounts of the same expression vector with no insert were used. Fixed amounts of the reporter gene (pNL.Luc. 0.5 μg) and DNA-enzyme at two different doses (0.5 and 1.0 μg in 0.5 ml volume) were cotransfected into Cos-1 cells that were grown in a six-well plate to 60% confluency. Cell lysates were prepared using a kit from the Manufacturer (Promega Biotech.), and the luciferase

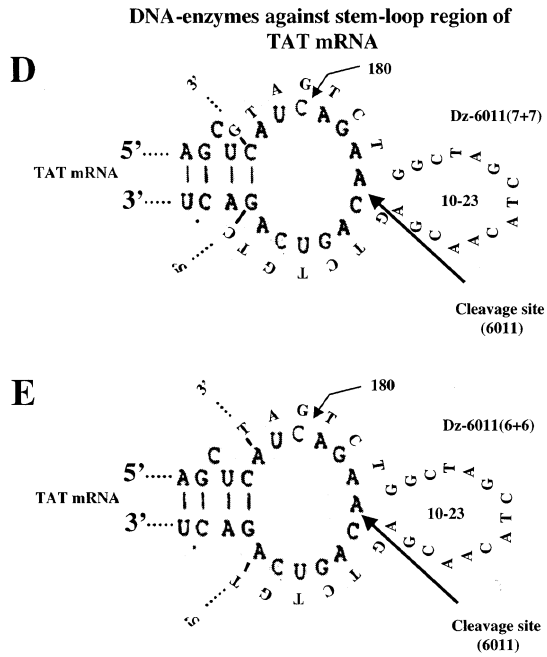

Fig. 2. (contd.) (A) Infectious clone of HIV-1, pNL4-3, was used to isolate the *TAT*/*REV* exons by double digestion with *EcoR*1 and *Hind* III. The fragment was cloned into a modified pSG5 vector (see text; Clonetech., CA) in a manner that it was under T7 as well as SV40 promoter. This plasmid was linearized with *Hind* III before subjecting it to in vitro transcription using T7 RNA polymerase (Promega). A 280 nucleotide long transcript is expected. (B) The sequence of the target RNA and the corresponding amino acids are shown. It is clear that both the DNA-enzymes were targeted to cleave between the A and U nucleotides. Dz-5944 was targeted against the *TAT* and Dz-5970 against the overlap regions of *TAT* and *REV*. Three DNA-enzymes were chemically synthesized that were 29 nucleotides long. All of them possessed the 10–23 catalytic motif earlier described (Santoro and Joyce, 1997). A single point mutation (G to C) was introduced in the catalytic motif of the Dz-5970. Seven bases long antisense flanks that were complementary to the target RNA was synthesized in each case. (C) A di-DNA-enzyme 70-44 was synthesized by combining the two mono-DNA-enzymes in a specific order such that Dz-5944 was placed down stream of the Dz-5970. Such a DNA-enzyme was exactly 58 nucleotides long. Note that they are in direct tandem with each other with no spacer nucleotides in between. (D) and (E) Two kinds of DNA-enzymes were synthesized against the largest predicted loop structure of the *TAT* RNA using the same 10–23 catalytic motif. In one case the Dz-6011 (7 + 7) could hybridize with the entire loop region and an additional pair of nucleotides with the target RNA (D). Such a DNA-enzyme possessed the antisense flanks that were seven bases on each side of the target site. The second Dz-6011 (6 + 6) was also targeted to cleave at the same site in the target RNA but was designed to hybridize with only the single stranded loop region (E).

activity was measured 48 h post transfection. In order to know if the observed inhibition was a reversible process, pSV2-TAT was used in 0.5 μg quantity along with the indicated amounts of DNA-enzymes. The cell viability was checked for each experiment by trypan blue exclusion method that was uniformly above 90%.

3. Results

3.1. In vitro cleavage of the target RNA by DNA-enzymes

In vitro cleavage efficiency of the two mono-DNA-enzymes were tested against a 280 nucleotides long labeled target RNA possessing the target sites that includes sequences of the polylinker region also. The expected pattern of cleavage by the action of two mono-DNA-enzymes (5944 and 5970) and di-DNA-enzyme is shown in Fig. 3A, top panel. The bottom panel shows the cleavage products obtained that were analyzed by gel electrophoresis. Lane 1 shows the synthesis of labeled HIV-1 *TAT* RNA that is 280 nucleotides long. When equivalent amounts (100 pmol) of labeled substrate and unlabeled DNA-enzyme-5944 was mixed in presence of 10 mM $MgCl_2$ as described before (Goila and Banerjea, 1998), a faint cleaved RNA band of 200 nucleotides was observed (lane 2). In contrast, when the same amount of DNA-enzyme-5970 (that overlaps *TAT* and *REV*) was used, almost complete disappearance of the target RNA was observed (lane 3), and specific cleavage products could be seen that were 225 and 55 nucleotides long, respectively. When the same amounts of di-DNA-enzyme were used, the efficiency of the cleavage was less but mono-DNA-enzyme specific cleavage products could be observed (lane 4). When the two mono-DNA-enzymes were added simultaneously (100 pmol each) to the target RNA, specific cleavage products could be observed as was the case with the di-DNA-enzyme (lane 5). We conclude that both mono- and di-DNA-enzymes cleave the target RNA in a sequence-specific manner with varying efficiencies.

3.2. In vitro cleavage by DNA-enzyme directed against the largest loop region

Two DNA-enzymes, Dz-6011-7 + 7 and DNA-enzyme-6011-6 + 6, were synthesized against the predicted loop region of the TAT RNA (Fig. 2, panel D and E). Fig. 3B shows the in vitro cleavage efficiency of the two DNA-enzymes. DNA-enzyme that can potentially hybridize with the entire loop region and one more nucleotide, Dz-6011-7 + 7, showed sequence-specific cleavage (265 and 15 bases long RNA fragments) only in presence of 20 mM $MgCl_2$ concentration (left panel; lane 4). As expected, in the absence of $MgCl_2$ no cleavage products could be detected. The same was true in presence of 2 and 10 mM $MgCl_2$. Essentially similar results were obtained with the DNA-enzyme-6011-6 + 6. The cleavage efficiency was further reduced at 20 mM $MgCl_2$ concentration (compare left with right panel; lane 4). We conclude that the DNA-enzyme that was exclusively designed against the largest predicted loop structure cleaved the target RNA very poorly.

3.3. In vitro cleavage by DNA-enzymes at simulated physiological conditions

The relative amounts of substrate RNA and the DNA-enzymes were the same as described before but the cleavage reaction was carried out at simulated physiological conditions as described in Materials and Methods. When an equimolar amount of Dz-5944 was added, no cleavage product was observed. Specific cleavage products could be observed with the Dz-5970 only. Under similar experimental conditions, the di-DNA-enzyme-70-44, also failed to cleave the target RNA (data not shown). We conclude that the mono-DNA-enzyme-5970 alone could cleave the target RNA specifically and that mono-DNA-enzyme-5944 and the di-DNA-enzyme-70-44, completely failed to cleave the target RNA.

3.4. Mutant-DNA enzyme-5970 failed to cleave the target RNA

In order to know if the mutant Dz-5970 retained any sequence-specific cleavage activity,

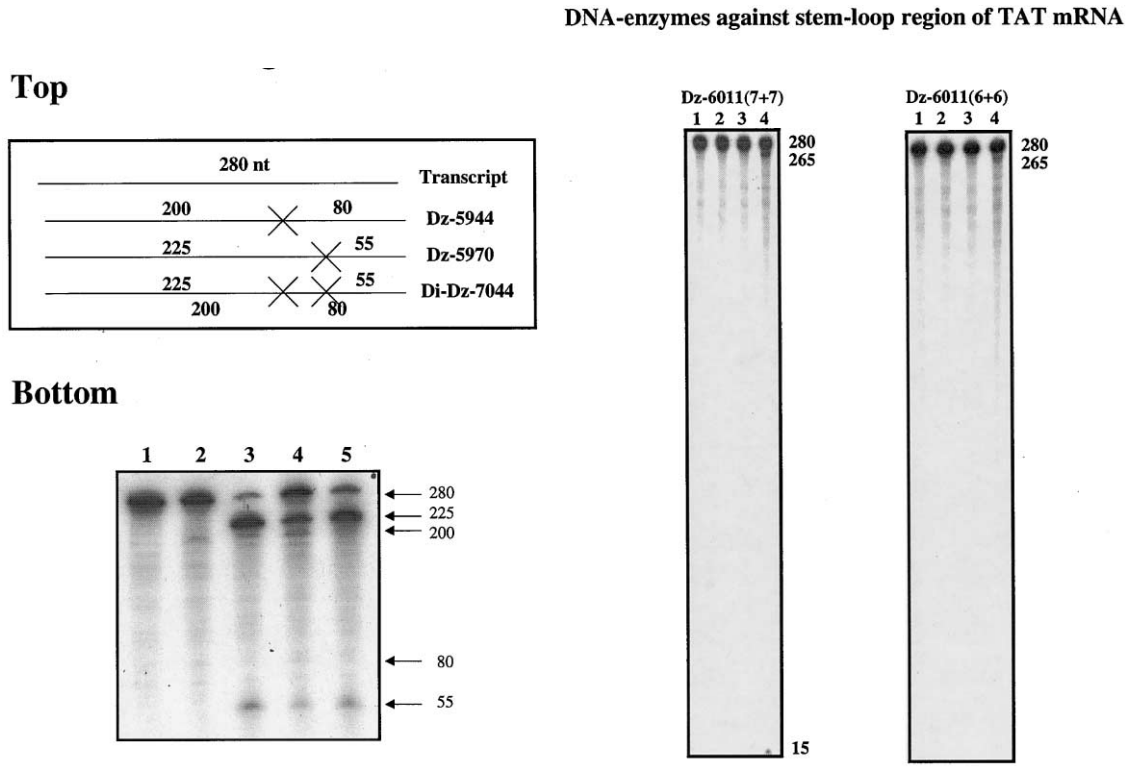

Fig. 3. (A) Top panel: the cleavage pattern that is expected after cleavage of the 280 nt long *TAT* RNA with the two mono-DNA-enzymes and the di-DNA-enzyme is shown. Cleavage by Dz-5944 is expected to generate a 5′ product of 200 bases and 80 bases long 3′ product. DNA-enzyme-5970 is expected to cleave the target into 225 and 55 bases long fragments. The di-DNA-enzyme is expected to generate mono-DNA-enzyme (5944 and 5970)-specific RNA fragments. Bottom panel: the results of the cleavage of the target RNA by mono- and di-DNA-enzyme is shown. Lane 1 shows the synthesis of 280 nucleotides long labeled transcript that possess the target sites for cleavage. Cleavage was initiated in presence of 10 mM $MgCl_2$ using equimolar amounts (100 pmol) of labeled substrate and cold DNA-enzyme. Lane 2 shows the cleavage obtained with the Dz-5944. A faint RNA band of 200 bases could be observed. Under exactly similar conditions of cleavage, Dz-5970 was very efficient. Almost complete cleavage of the substrate RNA could be seen that yielded specific products (RNA fragments of 225 and 55 bases). Lane 4 shows the extent of cleavage by di-DNA-enzyme-70-44 obtained under similar experimental conditions. Mono-DNA-enzyme-specific cleaved RNA fragments could be seen. Lane 5 shows the extent of cleavage obtained when the two mono-DNA-enzymes (5944 and 5970) were used simultaneously (100 pmol each) to cleave the target RNA. (B) A comparison of cleavage efficiency of two DNA-enzymes directed against the largest predicted loop structure of the *TAT* RNA. DNA-enzyme that could potentially hybridize with the entire loop region and an additional pair of nucleotides in the target RNA (Dz-6011-7 + 7) could specifically cleave the target RNA into 265 and 15 bases long fragments, albeit inefficiently and only in presence of 20 mM $MgCl_2$ (lane 4). No cleavage was observed in the absence of $MgCl_2$ (lane 1) or in presence of 2 (lane 2) and 10 (lane 3) mM $MgCl_2$ (left panel). The second Dz-6011-6 + 6 was designed in a manner that it could hybridize only with the single stranded region of the loop. Under exactly similar experimental conditions, the efficiency of cleavage decreased further as specific cleavage could be observed only in presence of 20 mM $MgCl_2$ (lane 4, right panel). In presence of lower concentrations of $MgCl_2$ (lanes 2 and 3) or in the absence of $MgCl_2$ (lane 1), no cleavage was detected.

equivalent amounts (100 pmol) were added to the target RNA under varying concentrations of $MgCl_2$. No cleavage was seen in the absence of $MgCl_2$ (Fig. 4, lane 1). Lanes 2, 3 and 4 show the absence of cleavage in presence of 10, 20 and 50 mM $MgCl_2$ concentration, respectively. Efficient

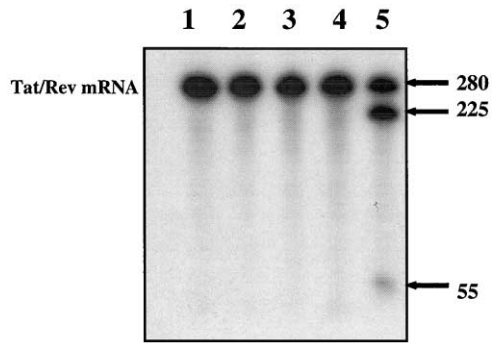

Fig. 4. A point mutation was created in the catalytic motif of the Dz-5970 and tested for its ability to cleave the target RNA as was done previously. As expected, no cleavage was observed in the absence of $MgCl_2$ (lane 1) or in presence of 10 (lane 2), 20 (lane 3) and 50 mM $MgCl_2$ (lane 4). Efficient cleavage was, however, obtained in presence of 10 mM $MgCl_2$ with the Dz-5970 (the non-mutated version) (lane 5) under identical experimental conditions.

cleavage was, however, obtained with equivalent amounts of Dz-5970 in the presence of 10 mM $MgCl_2$ under identical experimental conditions. We conclude from this experiment that one nucleotide change in the catalytic motif had rendered the Dz-5970 completely ineffective. This mutant-disabled DNA-enzyme, was used as control for in vivo experiments.

3.5. Effect of the concentration of $MgCl_2$ on cleavage efficiency of DNA-enzymes

In the presence of increasing concentrations of $MgCl_2$, a dose-dependent increase in the cleavage products could be observed with all the DNA-enzymes. The most important difference was observed at a 5 mM concentration of $MgCl_2$, only Dz-5970 could cleave the target RNA, the other two failed to do so under identical experimental conditions. As, expected, the Dz-5944 was again the least efficient and the di-DNA-enzyme cleaved the substrate with intermediate level of efficiency (data not shown). We conclude that each DNA-enzyme may show widely varying efficiency of cleavage at different concentrations of $MgCl_2$, and the minimum concentration of the $MgCl_2$ required for specific cleavage could vary significantly.

Fig. 5. (A) Fixed amount (0.5 μg) of luciferase reporter gene containing HIV-1 DNA, pNL.Luc., was used in a cotransfection assay using Cos-1 cells. All the three DNA-enzymes (Dz-5944, 5970 and 70-44) in two different dozes (0.5 and 1.0 μg) were used keeping the total concentration of DNA same for all the wells (see text). They were tested for their ability to interfere with the HIV-1 gene expression that is directly proportional to the amount of luciferase detected. At 48 h after transfection, the cells were harvested and the amount of luciferase was determined using a kit from Promega. When compared with the values obtained with the reporter gene alone with equivalent amounts of unrelated DNA (pGEM-3z, Promega), a significant decrease in the reporter gene activity was observed with all the three DNA-enzymes that was dose-dependent (compare left side with right). The most efficient inhibition was observed with the Dz-5970, followed by di-Dz-70-44 and the Dz-5944 was least effective. Mutant-Dz-5970 failed to interfere with the expression of the reporter gene. (B) The nature of the experiment is exactly same as described in Fig. 5A. Dz-5970 was selected for causing inhibition of the reporter gene expression. This observed inhibition was completely reversible if the cells were cotransfected with 1 μg of HIV-1 *TAT* encoding plasmid DNA (pSV2-TAT). (C) Cos-1 cells were grown to 60% confluency in a six-well plate and cotransfected with pHIV-1gpt and indicated amounts of DNA-enzyme using lipofectin (GIBCO/BRL) in a volume of 0.5 ml. Two hours later, the cells were washed twice with plain medium and replenished with DMEM (E) with 10% fetal bovine serum. After 48 h of transfection, the supernatant was collected and assayed for p24 antigen using the instructions of the Manufacturer. The amount of reverse transcriptase released was assayed using a kit from BMB. DNA-enzyme-5970 at 0.5 and 1 μg amounts, interfered significantly with the expression of both *TAT* and *REV*. The reduction was specific as the same amount of mutated Dz-5970 failed to interfere with the expression of HIV-1 genes (data not shown). The cell control that received no HIV-1 DNA, did not show any evidence of the production of HIV proteins. (D) Cos-1 cells were grown in a six-well plate as described earlier and cotransfected with 1 μg of infectious pNL4-3 DNA along with unrelated or Dz-5970 at the indicated amounts using lipofectin. Cell lysates were prepared for carrying out RT-PCR for estimating the levels of *TAT* RNA. In all the wells the concentration of pNL4-3 was kept constant at 1 μg. Cells that had been exposed to the pNL4-3, showed a prominent *TAT* RNA specific band. When cotransfected with 0.5 or 1 μg DNA-enzyme (210 nM) in a 0.5 ml volume, a dose-dependent decrease in the signal could be observed. The mutant disabled DNA-enzyme, under similar conditions, showed no reduction in the levels of HIV-1 *TAT* RNA.

3.6. Inhibition of HIV-1 gene expression by mono- and di-DNA-enzyme

Plasmid pNL4-3 clone containing the luciferase reporter gene in the NEF region (henceforth referred to as pNL.Luc) was used to transfect Cos-1 cells using lipofectin as described earlier. The DNA-enzyme was used either in equivalent amounts as that of reporter plasmid (0.5 μg each, left panel, Fig. 5A) or twice the amount (1.0 μg, right panel). In both the cases the total amount of DNA was kept constant by using a non-specific DNA pUC18. When cells were transfected with 0.5 μg of pNL.Luc DNA, the final concentration of DNA was 1 μg. When 1 μg of pNL.Luc DNA was used, the final concentration of DNA was 2 μg per well. Furthermore, to ensure uniform transfection efficiency we employed pSV-β-gal (Promega) that indicated that the variation was less than 10%. As expected, in both cases, DNA-enzyme-5970 was most effective, as measured by the luciferase activity. This was followed by di-Dz-70-44, and Dz-5944 was least efficacious. The mutant-Dz-5970 or unrelated DNA in equivalent

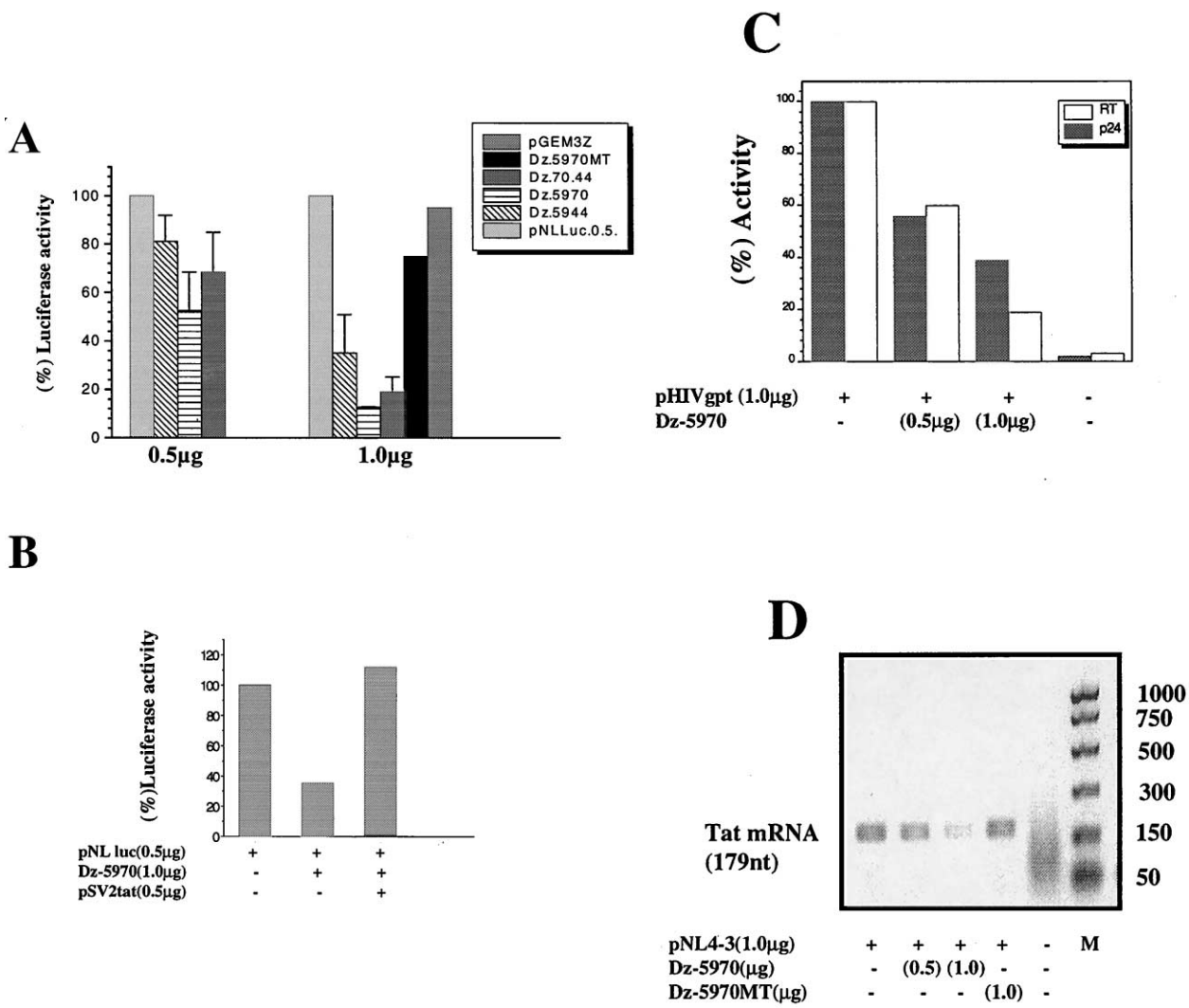

Fig. 5.

amounts showed no reduction in the luciferase activity when the cells were challenged with either 1 μg of pNL.Luc or when 0.5 μg was used (data not shown). We conclude that the two mono- as well as di-DNA-enzymes were able to specifically interfere with the expression of HIV-1 genes in a dose-dependent manner, although, they differed significantly in their efficacy. The inhibitory effect of the Dz-5970 was completely reversed by co-transfecting the cells with 0.5 μg of HIV-1 *TAT* encoding plasmid DNA (Fig. 5B). We then wanted to know if the reduction in the reporter gene expression correlated with the reduction of structural proteins of HIV-1. This was carried out using the most efficient Dz-5970 and pHIV-1 gpt that releases HIV-1 gag-pol particles when transfected into mammalian cells. It is clear from Fig. 5C that both reverse transcriptase as well as the p24 gag antigen levels were reduced in a dose-dependent manner.

3.7. In vivo inhibition of HIV-1 gene expression by DNA-enzymes

Cell lysates from control and DNA-enzyme-treated cells were processed for HIV-1 specific RNA using specific primers for HIV-1 *GAG* and *TAT* RNA by reverse transcriptase based polymerase chain reaction (RT-PCR). The sequence of the forward and backward primer for the HIV-1 *GAG* (p24) gene was: (1) 5′-CCCTATAGTGCAGAACCTCCA (1185–1205 nt). (2) 5′-CATTATGGTAGCTGGATTTGTTAC (1897–1920 nt). The sequence of the primer pairs for the amplification of *TAT* exon 1 were: 5′-CTAGAGCCCTGGAAGCATCC (5851–5870 nt)-forward 5′-GCTTGATGAGTCTGACTGTTCTG (6007–6030 nt)-reverse. Specific reduction of the HIV-1 *TAT* RNA was observed in the DNA-enzyme treated cells (Fig. 5D). In the absence of Dz-5970, a prominent band specific for *TAT* RNA could be seen. A slight reduction of the *TAT* RNA signal with 0.5 μg, but a 10-fold reduction, could be seen when 1.0 μg DNA-enzyme was cotransfected. In cells that had not received the HIV-1 DNA, no *TAT* RNA specific band could be visualized. On the contrary, mutant-Dz-5970 at 1 μg concentration did not affect the levels of *TAT* RNA. Essentially similar levels of reduction were observed with HIV-1 *GAG* RNA (data not shown). From these experiments we conclude that the observed inhibition in the levels of HIV-1 *TAT* or *GAG* RNA is specific, dose-dependent and due to the catalytic activity of the DNA-enzyme.

4. Discussion

The purpose of this work was to design effective mono- or multi-target DNA-enzymes against two important genes, *TAT* and *REV*, of HIV-1 with the aim that using this strategy it would be possible to interfere with the replication of HIV-1 in a sequence-specific manner. The DNA-enzyme being catalytic in nature, may be able to do so at relatively small concentrations. We synthesized several DNA-enzymes, all possessing the earlier identified 10–23 catalytic motif. While selecting the target sites, we reasoned that the loop region of the highly structured *TAT* or *TAT*/*REV* RNA, would be an ideal target. We chose Dz-5944 and 5970 that were present in the tip of the small loop (see Fig. 1) where A and U nucleotides were accessible for the cleavage. The same secondary structure was used earlier by others to design dimer minizymes (Kuwabara et al., 1996). A di-DNA-enzyme was constructed by placing the two DNA-enzymes in tandem. Both the mono-DNA-enzymes and the di-DNA-enzyme were able to cleave the target RNA into specific products. The Dz-5970 was most efficient in cleavage, followed by the di-DNA-enzyme; and the Dz-5944 showed very poor cleavage activity under exactly similar experimental conditions. Quite remarkably, the di-DNA-enzyme retained the cleavage specificity of the two mono-DNA-enzymes. Difference in the secondary structures at the target sites in the *TAT* RNA could account for the varying effects of each DNA-enzyme. When the same three DNA-enzymes were tested for their ability to cleave the same target RNA under simulated physiological conditions, only Dz-5970 could cleave the target. The failure of the di-DNA-enzyme could be explained on the basis of the large size (58 nucleotides) that might assume additional secondary structures not favorable for Watson–Crick base

pairing between DNA-enzyme and the target RNA.

Careful examination of this computer-predicted secondary structure of *TAT* RNA revealed a relatively big loop structure that contained 13 nucleotides. We, therefore, designed two DNA-enzymes, one that was targeted to hybridize with the single stranded regions of the loop (Dz-6011-6-6) and other with an extra nucleotide (6011-7 + 7). It was surprising to observe that the longer DNA-enzyme (29 nucleotides) was able to cleave the target RNA more efficiently than the shorter one (27 nucleotides) that was exclusively designed to hybridize with the single stranded region of the loop. One of the factors contributing to this variation could be shorter antisense flanks with the latter DNA-enzyme that may have created less stable hybrid with the target RNA. It is noteworthy that these DNA-enzymes cleaved the target RNA very inefficiently, and evidence for specific cleavage could be seen only in presence of high concentration of $MgCl_2$ (20 mM), that is at least five- to tenfold more than the physiological levels for humans. All these observations clearly point out that computer-predicted secondary structure-based approaches are not likely to be very useful. The ability of the DNA-enzymes to cleave the target RNA in the presence of 2–5 mM $MgCl_2$ is considered an important feature as it is close to our physiological levels. We, therefore, carried out cleavage reaction with both the mono- and the di-DNA-enzyme in presence of varying amounts of $MgCl_2$. The efficiency of the cleavage increased significantly for all the three DNA-enzymes in presence of increasing amounts of $MgCl_2$. It is noteworthy that Dz-5970 alone could exhibit significant cleavage activity at 5 mM $MgCl_2$ and also at simulated physiological conditions where the concentration was only 2 mM. As expected, a point mutation (G to C) in the catalytic motif abolished the ability of Dz-5970 to cleave the target RNA completely. We then addressed the question whether these DNA-enzymes have the ability to specifically interfere with the expression of the HIV-1 genes when introduced into mammalian cells. We chose Dz-5970 for most of our experiments as that was most efficient under standard conditions of cleavage. A dose-dependent decrease in HIV-1 *TAT* and *GAG* RNA was observed. This inhibition was very impressive and significant because even at 1:1 ratio of DNA-enzyme and HIV-encoding DNA, an almost eight- to tenfold reduction in the target RNA was observed. No toxic effect was observed at these concentrations of plasmid DNAs. All the earlier studies required excess amounts of antisense DNA or the ribozyme-expressing DNA (10–100-fold more) to interfere with the gene expression (Chen et al., 1992). This remarkable efficiency of the DNA-enzyme-5970 to exert its anti-HIV-1 effects at such low concentrations of DNA-enzyme, could only be explained by its potent catalytic nature. This was not the case when equivalent amounts of mutant DNA-enzyme-5970 were used. We then tested the presence of HIV-1 *TAT* RNA in Cos-1 cells that were cotransfected with infectious HIV-1 DNA, pNL4-3. A dose-dependent decrease was again observed. The mutant DNA-enzyme at the same concentration as pNL4-3 (both 1 μg each) failed to show any reduction in the levels of *TAT* RNA, strongly suggesting its catalytic role inside the mammalian cell. This inhibition of *TAT* RNA is significant because it was able to reduce the intracellular levels of *TAT* RNA in cells that were actively supporting the formation of replication-competent wild-type HIV-1.

The kinetic properties of the Dz-5970 was very similar to the ones reported earlier (Dash et al., 1998; data not shown). When the two mono-DNA-enzymes and the di-DNA-enzyme were compared for their ability to interfere with the HIV-1 gene expression, DNA-enzyme dose-dependent decrease in reporter gene activity was observed. The most efficient inhibition was observed with Dz-5970, followed by the di-Dz-70-44 and the Dz-5944 was least effective. This pattern of efficiency closely matches the in vitro cleavage conditions and not what was observed at the simulated physiological conditions. Thus, it is possible to achieve good inhibition of gene expression by DNA-enzymes in a mammalian cell that failed completely to cleave the target RNA under simulated physiological conditions.

In summary, we have demonstrated efficient cleavage of the *TAT* or *TAT*/*REV* RNA by two

mono- and a di-DNA-enzyme. The di-DNA-enzyme retained the cleavage specificity of the mono-DNA-enzymes. DNA-enzyme based on the largest predicted loop structure cleaved the target RNA very poorly. Both the mono- and di-DNA-enzyme inhibited the expression of HIV-1 genes very effectively at much reduced concentrations compared to the earlier studies where large amounts of antisense DNA was used. Targeting the important regulatory genes of HIV-1, *TAT* and *REV*, could be the most promising strategy that could be combined with other known anti-retroviral approaches to reduce the viral burden in humans infected with HIV.

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