***In vitro*-selected RNA cleaving DNA enzymes from a combinatorial library are potent inhibitors of HIV-1 gene expression**

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Selective inactivation of a target gene by antisense mechanisms is an important biological tool to delineate specific functions of the gene product. Approaches mediated by ribozymes and RNA-cleaving DNA enzymes (DNA enzymes) are more attractive because of their ability to catalytically cleave the target RNA. DNA enzymes have recently gained a lot of importance because they are short DNA molecules with simple structures that are expected to be stable to the nucleases present inside a mammalian cell. We have designed a strategy to identify accessible cleavage sites in HIV-1 gag RNA from a pool of random DNA enzymes, and for isolation of DNA enzymes. A pool of random sequences (all 29 nucleotides long) that contained the earlier-identified 10–23 catalytic motif were tested for their ability to cleave the

target RNA. When the pool of random DNA enzymes was targeted to cleave between any A and U nucleotides, DNA enzyme 1836 was identified. Although several DNA enzymes were identified using a pool of DNA enzymes that was completely randomized with respect to its substrate-binding properties, DNA enzyme-1810 was selected for further characterization. Both DNA enzymes showed target-specific cleavage activities in the presence of Mg²⁺ only. When introduced into a mammalian cell, they showed interference with HIV-1-specific gene expression. This strategy could be applied for the selection of desired target sites in any target RNA.

Key words: HIV-1 gag, HIV-1 replication.

INTRODUCTION

Sequence-specific cleavage activities by short DNA molecules possessing two earlier-identified catalytic motifs (10–23 and 8–17) [1] have recently been recognized as a powerful biological tool to interfere with gene expression. These were derived by *in vitro* selection from a combinatorial library of DNA sequences that were capable of cleaving 17-nucleotide-long synthetic RNAs under simulated physiological conditions. These RNA-cleaving DNA enzymes (DNA enzymes) are expected to be more stable than short catalytic RNAs (ribozymes), which are inherently more unstable. These DNA enzymes are short and have simple secondary structures. They can potentially cleave any target RNA that contains a purine and a pyrimidine nucleotide, thereby allowing far greater flexibility in choosing target sites. On the contrary, the target sites for hammerhead- and hairpin-motif-containing ribozymes are limited. After the initial discovery of DNA enzymes by Santoro and Joyce [1], a number of investigators have used them to selectively cleave and interfere with the function of target genes [2,3]. Inhibition of infection of incoming HIV-1 was reported earlier by Zhang et al. [4] by DNA enzymes that were targeted against the V3 loop of the envelope region. Most of these studies have used DNA enzymes possessing the 10–23 catalytic motif. Earlier we showed the cleavage of HIV-1 envelope RNA [5] and also the HIV-1 co-receptor CCR5 [6] in a sequence-specific manner and subsequently tested their ability to interfere with the function of the target gene when introduced into a mammalian cell. We have reported recently a functional mono-DNA enzyme against the second most important HIV-1 co-receptor, CXCR-4, and when combined with the CCR5 DNA enzyme in tandem it possessed the ability to cleave both the target RNAs (CXCR-4 and CCR5) and also interfered with the respective co-receptor functions [7]. Despite

all the success in constructing DNA enzymes, the selection and identification of DNA enzymes that might be effective in a complex cellular milieu under *in vivo* conditions has remained a problem and needs to be determined empirically. Predicted secondary structures that are thermodynamically stable may help identify loop regions, but the physiological relevance of these structures inside a mammalian cell remains uncertain.

We present a strategy to identify DNA-enzyme target sites from a random pool of DNA enzymes and demonstrate their sequence-specific cleavage activities. These DNA enzymes also interfered significantly with the expression of the HIV-1 *gag* (*p24*) gene as well as in the production of replication-competent HIV-1 in cell cultures.

EXPERIMENTAL

Cloning of the HIV-1 *gag* gene and *in vitro* transcription

The following primers were synthesized to amplify a 740 bp DNA fragment encoding the *p24* (*gag*) gene using the plasmid DNA pNL4-3 [8] and cloned into a T-tailed vector, pGEM-T-Easy (Promega Biotech, Madison, WI, U.S.A.), to yield the plasmid pGEM-gag: (i) sense, 5'-CCCTATAGTGCAGAACCCTCCA-3' (1185–1205 nt) and (ii) antisense 5'-CATTATGGTAGCTGGATTGTGTTAC-3' (1897–1920 nt).

The conditions for carrying out the PCR were the same as described by us in [9]. The cloned gene was linearized at the 3' end by *Sal*I digestion (Figure 1) to obtain full-length authentic HIV-1 *gag* RNA by *in vitro* transcription using T7 RNA polymerase as described before [10]. The cloned HIV-1 *gag* gene from the pGEM-gag plasmid was excised using *Eco*RI digestion and cloned into the *Eco*RI site of pcDNA3 (Promega) to yield

Abbreviations used: Dz, DNA enzyme; RT, reverse transcriptase; hGAPDH, human glyceraldehyde phosphodehydrogenase.

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Figure 1 Strategy for isolating target sites from a pool of random DNA enzymes

HIV-1 *gag* (p24) gene was amplified from the infectious HIV-1 DNA, pNL4-3 [8] and cloned into a T-tailed vector, pGEM-T-Easy, and HIV-1 *gag* sequences were confirmed by sequencing. It was then linearized at the 3' end at the multiple cloning sites (MCS) to generate a 740-base *in vitro* transcript using T7 RNA polymerase (T7 Pr) as described earlier [10]. This RNA (0.2 μ M) was then mixed with excess amounts (20 μ M) of two different pools of DNA enzymes (Dz; see text) in the presence or absence of $MgCl_2$. The cleaved products were subjected to primer extension using labelled primers (see text). The primer-extended product was analysed alongside the sequencing gel to map the cleavage site in the target RNA. Note that both pools of DNA enzymes had a common 10–23 catalytic motif for carrying out sequence-specific cleavage of the target RNA in a catalytic manner.

the plasmid pCMV-gag. This plasmid was used for obtaining intracellular expression of HIV-1 gag. A 210-base truncated region of HIV-1 gag (1712–1922, a *Hind*III and *Eco*RI digest from plasmid pGEM-gag) was subcloned into pcDNA3. This plasmid, when linearized at the 3' end in the polylinker region, will direct the formation of a truncated transcript of 220 bases long that includes some transcribed sequences of the polylinker region also. The cloned gene was confirmed by sequencing. Labelled RNA was resolved in a 6% polyacrylamide/7 M urea gel using the Mini-PROTEAN II gel system (Bio-Rad, Hercules, CA, U.S.A.).

Synthesis of a random pool of DNA enzymes

Two kinds DNA-enzyme pool were synthesized that contained the earlier-identified 10–23 catalytic motif that had the following sequence: 5'-GGCTAGCTACAACGA-3' (Figure 1). In one case the DNA enzyme was targeted to all the possible AUGs in

the target RNA and in second case it was designed to cleave any potential DNA-enzyme target site. This was possible by either totally randomizing the seven bases on either side of the catalytic motif (see Figure 1) or having CA immediately preceding the catalytic motif (for AUG cleavage). All the DNA enzymes were 29 nucleotides long and were synthesized chemically according to standard procedures on an Applied Biosystems Oligonucleotide Synthesizer.

Cleavage reactions using the randomized pool of DNA enzymes

In vitro-synthesized labelled 740-nucleotide-long HIV-1 gag transcript that possessed the target sites was used for the cleavage reaction. This was obtained by linearizing the plasmid pGEM-gag with *Sal*II and subjecting it to *in vitro* transcription by T7 RNA polymerase. Unlabelled full-length substrate (740-bases-long HIV-1 gag RNA; 0.1 μ M) was incubated with a large excess (15 μ M) of both kinds of randomized DNA-enzyme pool in the presence and absence of 10 mM $MgCl_2$ in a buffer containing 50 mM Tris/HCl, pH 8.0, in a reaction volume of 10 μ l. After 1 h at 37 °C, cleaved RNA products were resolved by gel electrophoresis as described above.

Primer extension and identification of cleavage sites

Cleavage sites were mapped by primer extension of the cleavage products alongside a sequencing reaction generated using avian myeloblastosis virus reverse transcriptase (RT; 5 units/ μ l) from the RT-PCR kit from Promega Biotech (Access RT-PCR System). The transcription buffer contained 1 mM $MgSO_4$ and a 0.2 mM concentration of all the four dNTPs in a final volume of 50 μ l. The primers (antisense primer, nt 1897–1920, as described above, and one internal primer, positions 1792–1808, 5'-CCTGG-TCCCAATGCTTT-3') were labelled at the 5' ends using polynucleotide kinase (New England Biolabs) with [γ - 32 P]ATP. The terminal and internal primers generated a sequence of $\approx$ 200 nucleotides in length when analysed on a 6% PAGE/7 M urea sequencing gel. Primer-extended product and the sequencing reactions were analysed on a sequencing gel simultaneously and radioactive bands were detected by autoradiography (Figure 1).

Construction of DNA enzymes

Once the target site was identified, DNA enzymes with the 10–23 catalytic motif were synthesized. The strategy for synthesizing DNA enzymes was the same as described originally by Santoro and Joyce [1] and by us recently [5–7]. Briefly, seven nucleotides on either side of the catalytic motif were made complementary to the target gene and the A (in the case of AUG-cleaving DNA enzymes) and G (in the case of GC-cleaving DNA enzymes) were left unpaired. The cleavage was expected to take place after the A and G nucleotides, respectively (shown by arrows in Figure 4, see below). Cleavage reactions with the labelled target RNA and library-selected DNA enzyme were carried out as described by us previously [6]. A point mutation (G to C) in the catalytic motif of DNA enzyme (Dz)-1836 was also created that served as a disabled DNA-enzyme control (for details see Figure 4A below).

Inhibition of HIV-1 gag expression

COS-1 cells (1×10^6) were co-transfected with pCMV-gag (1 μ g) along with varying amounts of DNA enzymes (1 and 4 μ g) using Lipofectin (Gibco-BRL, Bethesda, MD, U.S.A.). Post-trans-

fection (48 h) cellular RNA was isolated using Trizol (Promega) reagent as described by the manufacturer. Equivalent amounts of cell lysates were subjected to RT-PCR for estimating 740-base-long HIV-1 gag RNA and 509-base-long human glyceraldehyde phosphodehydrogenase (hGAPDH) control RNA [11]. This fragment was amplified using the following primers: forward, 5'-ACCACCATGGAGAAGGCTGG-3', and reverse, 5'-CTCAGTGTAGCCCAGGATGC-3', as described earlier [12].

Inhibition of HIV-1 replication by DNA enzymes

Plasmid pNL.Luc. [8], in which the reporter gene was inserted in the Nef region, was used to transfect COS-1 cells using Lipofectin. Transfection of this infectious DNA leads to gag-pol particle production. The extent of reporter gene expression by pNL.Luc. in a mammalian cell is directly related to the amounts of gag-pol particle production. COS-1 cells were grown to 60 % confluence on a six-well plate. They were co-transfected with varying concentrations of Dz-1810 (for details see Figure 4B below) or Dz-1836 (0.5 and 2.5 μ g) and a fixed amount of pNL.Luc. DNA (0.5 μ g) for 12 h using Lipofectin in a final volume of 500 μ l. After 2 h of incubation in the presence of Lipofectin, the cells were washed with Dulbecco's modified Eagle's medium without serum and incubated further for 12 h in the same medium with 10 % fetal bovine serum. Luciferase activity was determined by the kit provided by Promega.

RESULTS AND DISCUSSION

We have used a random pool of DNA enzymes that were targeted to cleave between any A and U nucleotides, which will

Figure 2 Efficiency of cleavage *in vitro* by the two pools of DNA enzymes

HIV-1 gag RNA (740 bases) was synthesized by *in vitro* transcription as described in the text (lane 1). Excess DNA enzyme was added and the cleavage reaction was initiated in the presence (lanes 4 and 5) or in absence (lanes 2 and 3) of $MgCl_2$ (10 mM final concentration), and cleavage products were resolved by gel electrophoresis (6% polyacrylamide/7 M urea) on a Mini-PROTEAN II gel apparatus. No cleavage was observed with Dz-1810 (lane 2) or Dz-1836 (lane 3) in the absence of $MgCl_2$. Multiple cleavage was observed with Dz-1810 (lane 4) and Dz-1836 (lane 5).

Figure 3 Identification of DNA-enzyme target sites

In vitro-synthesized HIV-1 gag RNA (740 bases) and the random pool of DNA enzymes were mixed as described in the text and the cleaved RNA products were subjected to primer extension. Using the AU-cleaving DNA enzyme, we identified a site at position 1836 in the target RNA (A, arrow) and using the totally randomized DNA-enzyme pool with respect to target binding, a GC-cleaving site was identified at position 1810 (B, arrow). Note that the primer-extended products were observed only in the presence of Mg^{2+} in both the cases. Using the same kind of experimental protocol additional target sites were also found using primers designed to hybridize internally (see text): at nt 1725 (GU-cleaving; C) and nt 1740 (AC-cleaving; D).

target all AUG codons (initiation codons and internal methionines). We also used another pool of DNA enzymes with the potential to cleave between any purine and pyrimidine (Figure 1). This was possible by keeping the 10–23 catalytic motif constant but randomizing the flanking sequences completely or partially.

We first wanted to determine whether the pools of DNA enzymes that were synthesized chemically possessed any catalytic activity. For this purpose we carried out *in vitro* cleavage of HIV-1 gag RNA by random- and AU-cleaving DNA-enzyme pools. Figure 2 (lane 1) shows the synthesis of HIV-1 gag RNA, which is 740 bases long. Labelled substrate and excess amounts (10-fold) of both the DNA enzymes were added, and the cleavage reaction was carried out in the presence or absence of $MgCl_2$. The products were analysed by gel electrophoresis as described earlier. No cleavage was observed with either the AU-cleaving DNA enzyme (Figure 2, lane 2) or the pool of random DNA enzymes (Figure 2, lane 3) in the absence of $MgCl_2$. However, in the presence of $MgCl_2$ multiple cleavage was observed with both the random pool of DNA enzymes (Figure 2, lane 4) and the AU-cleaving DNA enzyme (Figure 2, lane 5). Multiple cleavage of the target RNA by the random pool of DNA enzymes was greater, as expected, because it can potentially hybridize to many sites in the target RNA (compare Figure 2, lanes 4 and 5).

Figure 4 Sequences of the target gene and DNA enzymes

Once the cleavage sites were identified in the target RNA (see text), the DNA enzymes were synthesized chemically. The sequences of the target RNAs for AU- and GC-cleaving DNA enzymes are shown in panels **A** and **B** respectively. They both contained the 10–23 catalytic motif [1]. Seven bases on either side of the cleavage site were synthesized that were complementary to the target sequence. In the case of the AU-cleaving DNA enzyme (**A**), the A is left unpaired, whereas in the GC-cleaving DNA enzyme (**B**), the G nucleotide is left unpaired. Both the DNA enzymes were 29 nucleotides long. A point mutation (G to C) was created in the catalytic motif of Dz-1836 to create mutant Dz-1836 (**A**, ♦).

After confirming the presence of catalytic activities in our pool of DNA enzymes, we sought to identify the target site at which the cleavage occurred. This was obtained by carrying out a primer-extension assay on the cleaved product using the same 3'

terminal primer that was used to amplify the full-length *gag* gene and one internal primer. By following the procedures described in the Experimental section we identified several target sites. As expected, the pool that was designed to identify the AU-cleaving site in the target RNA did indeed show an extended product that was targeted to cleave the AU sequence present at position 1836 (Figure 3A, arrow). This band was only present in the samples where the cleavage reaction was carried out in the presence of 10 mM $MgCl_2$. No such band was observed if $MgCl_2$ was omitted from the cleavage reaction (Figure 3A). When cleavage of the target RNA was carried out using DNA enzymes that were randomized totally with respect to their target RNA-binding sites and the 3' cleavage products extended as described earlier, only one prominent band at nucleotide position 1810 was observed (Figure 3B). Two additional cleavage sites were identified that were targeted to cleave between G and U (Figure 3C) and between A and C (Figure 3D) using the internal primer (positions 1792–1808; shown by arrows).

Once the target sites were identified, DNA enzymes of 29 nucleotides in length were synthesized that were targeted against nucleotides 1810 and 1836 (Figure 4) only. The sequences of the target RNA and the two DNA enzymes possessing the common 10–23 catalytic motif are shown in Figure 4. We followed the same strategy to synthesize DNA enzymes with the 10–23 catalytic motif as Santoro and Joyce [1], as described in the Experimental section. Nucleotides A and G in the target site were left unpaired in Dz-1836 and Dz-1810 respectively and arrows show the expected cleavage sites in Figure 4. The mutant Dz-1836 contained a G-to-C mutation (shown by ♦, Figure 4A)

Figure 5 *In vitro* cleavage of the target RNA by AU-cleaving (A) and GC-cleaving (B) DNA enzymes in the presence of different concentrations of $MgCl_2$

Labelled target RNAs of 220 bases could be seen in lanes 1 (**A** and **B**). No cleavage was observed in the absence of Mg^{2+} (lanes 2) when equimolar amounts of target and DNA enzyme were used. Also, no cleavage was observed at 2 mM $MgCl_2$ (lanes 3). Significant cleavage, however, was observed in the presence of 10 mM $MgCl_2$ with the AU-cleaving DNA enzyme alone but not with the GC-cleaving DNA enzyme (lanes 4). The efficiency of the cleavage increased with increasing concentrations of $MgCl_2$ (lanes 4, 10 mM; lanes 5, 20 mM; lanes 6, 50 mM). Note the complete disappearance of the target RNA in lane 6 (**A**). Percentage cleavage obtained in presence of varying concentrations of $MgCl_2$ is shown in **C**. This was calculated by estimating the levels of uncleaved substrate RNA left after the reaction (substrate alone was taken as 100%).

Figure 6 *In vitro* cleavage of HIV-1 gag RNA (740 bases) by DNA enzymes

In order to determine if the same two DNA enzymes possessed the ability to cleave 740-base HIV-1 gag RNA, the labelled transcript was mixed in equimolar amounts and subjected to cleavage *in vitro* as described in the text. Lane 1 depicts the synthesis of 740 bases of HIV-1 gag RNA. Specific cleavage products (630 and 110 bases, lane 2) could be observed when mixed with equimolar amounts of Dz-1810. Dz-1836 cleaved the target RNA specifically into 656 and 84 bases (lane 3) and almost 100% cleavage was observed. Based on the amount of target RNA that remained after the cleavage reaction, we again observed that Dz-1836 was superior.

Figure 7 Inhibition of HIV-1 gag RNA expression by DNA enzymes

HIV-1 *gag*-encoding DNA (pCMV-*gag*) was co-transfected along with either equivalent amounts of unrelated DNA enzyme (Dz-CCR5 [6]) or the two DNA enzymes (Dz-1810 and Dz-1836). COS-1 cells were grown in a six-well plate, DNA transfection was carried out using Lipofectin in a final volume of 0.5 ml and lysates were prepared 48 h after transfection as described in the text. Total RNA was isolated, divided into two equal parts and subjected to RT-PCR using primers specific for the HIV-1 *gag* or *hGAPDH* genes (see text). pCMV-*gag*-transfected cells, when analysed for HIV-1 *gag*-specific RNA in the absence of RT, failed to show any *gag*-specific amplification (A, lane 6). (A) A full-length 740-base RNA could be detected when cells received only HIV-1 *gag* DNA along with 5 μ g of unrelated Dz-CCR5 (lane 1) [6]. When Dz-1810 was used in 1 μ g (lane 2) and 5 μ g (lane 3) amounts, 4- and 6-fold reductions, respectively, were observed. Reduction was $\approx$ 6-fold with a 1:1 ratio of Dz-1836 (lane 4) and $\approx$ 10-fold in the presence of 5 μ g of Dz-1836 (lane 5). This decrease was specific for the HIV-1 *gag* gene, as no reduction in the control housekeeping gene (*hGAPDH*) was observed in the corresponding lysates (B). No decrease in the levels of HIV-1 *gag* RNA was observed with 5 μ g of disabled Dz-1810 (results not shown). Note that for lanes 6 there was no corresponding lane for the control RNA.

Figure 8 Intracellular inhibition of HIV-1 gene expression by DNA enzymes

The experimental details were same as in Figure 7, except that we used HIV-1 DNA pNL-Luc. as a reporter gene. The extent of reporter gene (luciferase) activity detected in COS-1 cells by co-transfecting 0.5 μ g of pNL-Luc. and 2.5 μ g of mutant Dz-1836 was taken as 100%. Dz-1836 in a 1:1 ratio with pNL-Luc. reduced the reporter-gene expression by 90% and increasing the dose of Dz-1836 reduced it marginally further. The same was true with Dz-1810. It is clear that, in a 1:1 ratio, Dz-1836 was slightly more effective at inhibiting reporter gene expression. An unrelated DNA enzyme (CCR5-Dz [6]) had no effect on reporter-gene expression. These are average values from two independent experiments. Mutant Dz-1810 also did not interfere with the reporter-gene expression (results not shown).

in the catalytic motif. Equimolar amounts (100 pmol) of these DNA enzymes (Dz-1836 and Dz-1810) and the target RNA of 220 bases long were mixed in either the absence or presence of increasing concentrations of $MgCl_2$ as described in [6], and the results are shown in Figure 5. Specific cleavage products that were 124 and 96 bases long with Dz-1836 (Figure 5A) and 122 and 98 bases long fragments with Dz-1810 (Figure 5B) could be observed only in the presence of 10 mM $MgCl_2$. These were the expected sizes of the fragments, which could only be formed after sequence-specific cleavage by the two DNA enzymes. Note that almost complete cleavage of the target RNA was achieved with Dz-1836 in presence of 50 mM $MgCl_2$, as compared with only 20% cleavage of the target RNA by Dz-1810 under identical conditions (Figure 5C). This difference in the efficiency of cleavage is difficult to explain in the light of results obtained from the random pool of DNA enzyme (Figure 3), which indicated that both classes of DNA enzyme worked with equal efficiency. One reason for this discrepancy could be that some molecules of DNA enzymes from the pool may be hybridizing with the target RNA close to the cleavage site in a sub-optimal manner and have potential to modulate the cleavage efficiency of the other target site. We recently described such an effect when we constructed a Di-DNA enzyme (two DNA enzymes joined in tandem) that was targeted to cleave two closely spaced target sites (HIV co-receptors CCR5 and CXCR-4) [7].

The same two DNA enzymes (Dz-1836 and Dz-1810) were tested for their ability to cleave the 740-base-long gag RNA and the results are shown in Figure 6. Lane 1 shows the synthesis of 740-base-long RNA when the pGEM-*gag* was linearized and subjected to transcription *in vitro*. When equimolar amounts of labelled gag RNA and Dz-1810 were added, specific cleavage products (630 and 110 bases) could be observed (Figure 6, lane 2). Specific cleavage products (656 and 84 bases, Figure 6, lane 3)

could be observed with Dz-1836; mutant Dz-1836, however, failed to cleave the same target completely (results not shown). It is noteworthy that Dz-1836 was only slightly more efficient, as evident by the amount of substrate RNA left after the cleavage reaction. Clearly the efficiency of the cleavage changes with the size of the target RNA, as it will have a different secondary structure.

We then wanted to address the question of whether these DNA enzymes could effectively interfere with the expression of HIV-1 genes in a cell line using the vector pCMV-gag. Lysates from each well were divided into two equal proportions and subjected to RT-PCR for the purpose of estimating the *gag* (Figure 7A) and housekeeping (*hGAPDH*; Figure 7B) genes respectively. As expected, COS-1 cells that were transfected with 1 μ g of pCMV-gag only showed prominent 740-base-long RNA (Figure 7A, lane 2). Lanes 2 and 3 in Figure 7(A) represent the amount of HIV-1 *gag* RNA that was detected after transfection of 1 and 5 μ g of Dz-1810 respectively. Lanes 4 and 5 (Figure 7A) show the amount of HIV-1 *gag* RNA when Dz-1836 was used at 1 and 5 μ g, respectively. Note that there was a significant difference in the levels of HIV-1 *gag* RNA between the two DNA enzymes when 5 μ g was used (compare Figure 7A, lanes 3 and 5). Based on these data it can be concluded that Dz-1836 was more effective at inhibiting HIV-1 *gag* gene expression at higher doses of DNA enzyme. As expected, no PCR-amplified fragment was detected in the absence of the enzyme RT (Figure 7A, lane 6). The corresponding lysates showed almost no reduction in the levels of housekeeping RNA (*hGAPDH*; Figure 7B). We conclude that DNA enzymes inhibit HIV-1 *gag* gene expression specifically.

We next wanted to know if DNA enzymes could interfere with the production of replication-competent HIV-1. For this purpose we used infectious HIV-1 DNA, pNL4-3 [8]. COS-1 cells were co-transfected with pNL.Luc. (along with mutant Dz-1836) and the two DNA enzymes in a ratio of either 1:1 (0.5 μ g each) or 5 times more DNA enzymes, 1:5 (results not shown). Unrelated DNA enzyme (Dz-CCR5) [6] in a 1:1 ratio was used as a control. Mutant Dz-1810 did not interfere with the expression of the reporter gene (results not shown). After transfection (48 h) cell lysates were checked for the reporter-gene expression, and the results are shown in Figure 8. Even in a 1:1 ratio of HIV-1 DNA to Dz-1836, more than 90% inhibition could be seen (where luciferase activity with pNL4-3 and 2.5 μ g of mutant Dz-1836 was set at 100%), which was reduced further to $\approx$ 95% when the amount of Dz-1836 was increased 5-fold, indicating that saturation level was already reached with the lower dose of DNA enzyme. Inhibition with Dz-1810 was 75% at a 1:1 ratio of pNL.Luc. In contrast, an unrelated Dz-CCR5 [6] at 0.5 or 2.5 μ g (results not shown) did not show any reduction in reporter-gene activity. Similar results were obtained with 14-nucleotide antisense constructs of both the DNA enzymes that lacked the 10–23 catalytic motif (results not shown). We conclude from these experiments that both the DNA enzymes possess potent anti-HIV-1 properties that are mainly due to the catalytic nature of such molecules. Zhang et al. [4] reported the inhibition of incoming HIV-1 by V3-loop-targeted DNA enzymes that contained the same catalytic motif. Out of nine potential AU cleavage sites in the loop region, only four showed activity and some failed to show any cleavage activity under conditions *in vitro*. This illustrates further the point that not all potential cleavage sites based solely on the sequence of RNA in the target RNA are accessible for cleavage by DNA enzymes. DNA enzymes selected from a random pool by *in vitro* cleavage activity may overcome this problem. Since the HIV-1 *gag* region is the most conserved among all the genetic subtypes, we chose it

region to test the antiviral activities of our DNA enzymes. Moreover, these two DNA enzymes (Dz-1810 and Dz-1836) could be combined to target two sites simultaneously. Mutations at two sites simultaneously in the target gene may be a rare event and this approach may delay significantly the appearance of viral mutations that allow escape from the antiviral effects of DNA enzymes.

In conclusion, we have developed an *in vitro* screening assay for isolating DNA enzymes that are likely to be more effective in their ability to cleave a target RNA. By randomizing the target-binding sites or introducing specific nucleotides in the design of the pool of DNA enzymes, it may be possible to select DNA enzymes that are targeted against a particular pair of purine and pyrimidine nucleotides. Using such an approach, several DNA-enzyme cleavage sites were identified and corresponding DNA enzymes were synthesized. All of them possessed sequence-specific cleavage activities (results not shown). Two such DNA enzymes (one from each pool) were tested for their anti-HIV-1 activities. The two cleavage sites (positions 1810 and 1836) identified are very closely positioned in the target RNA, which suggests strongly that this region is accessible for targeting purposes using other approaches (ribozymes, antisense expression, etc.) too. Usually one has to use excess amounts of ribozyme or antisense molecules to observe anti-viral effects in a mammalian cell line. These DNA enzymes (Dz-1836 and Dz-1810), even in a 1:1 ratio, could interfere very significantly with HIV-1 gene expression and therefore be potentially useful for therapeutic purposes.

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