Results and Discussion: Part I

DNA-ENZYMES TARGETED TO CLEAVE THE Tat/Rev OVERLAP REGION IN THE FIRST EXON OF Tat
PART I: DNA-enzymes targeted to Tat/Rev RNA

In this study, we aim to interfere specifically with the expression of Tat or Tat/Rev RNA as that would interfere with the replication of HIV. We, therefore, synthesized several mono- and one di-DNA-enzyme against the first exon of Tat where Tat and Rev ORFs overlap. The schematic representation of the sites against which the ribozymes and DNA-enzyme have been designed is shown in Fig. 1.1.

Most of the studies with DNA-enzymes including ours, has involved the use of charged lipid molecules to introduce the DNA-enzyme into mammalian cells that were subsequently challenged with the virus. Although encouraging results were obtained in tissue culture experiments but the generous use of charged lipid molecules to facilitate entry of a foreign DNA, presents a practical problem besides exerting toxic effects to mammalian cells. Clearly there is a great need to develop novel DNA-enzyme delivery systems that work in the absence of charged lipid molecules.

It is now fairly well established that macrophage tropic R5 viruses initiate the infection using the chemokine receptor—CCR5 that are present mainly on macrophages and langerhan cells (Berger et. al., 1999). Macrophages are also considered reservoirs for HIV-1 by many investigators. Given the important role of macrophages in the early establishment of HIV-1 infection and in the pathogenesis of HIV-1, it would be most appropriate to target macrophages specifically. Earlier number of studies aimed at specific interference of target gene expression exploited the affinity of the G residues at the end of the antisense constructs to the scavenger receptors (SCR) present on the macrophages (Pearson et. al., 1993; Fitzgerald et. al., 2000). Such G rich oligonucleotides are involved in the formation of G-tetrads that is sufficient to be recognized by the SCR and subsequent uptake. Prasad et al. (1999) showed that vesicular stomatitis virus replication in macrophages could be inhibited using this strategy. This property was exploited by us in this study in designing our DNA-enzyme that was targeted to cleave HIV-1 Tat/Rev RNA corresponding to the region where exon 1 of HIV-1 Tat and Rev overlapped.
Fig 1.1

SCHEMATIC REPRESENTATION OF RIBOZYME AND DNA-ENZYME CLEAVAGE SITES

<table>
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<th>T7pr</th>
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<tr>
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<td>Ribozymes</td>
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EXPERIMENTAL PROCEDURES

Plasmids used:
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. All the plasmids were purified on a Qiagen column before sequencing or for the purpose of transfection into mammalian cells.

Cell lines used:
Four cell lines (human THP-1, Cos-1, NIH-3T3-CD4-CCR5 and HeLa) were maintained in DMEM (E) in 5% fetal bovine serum (Hyclone). THP-1 cells were grown in suspension and after stimulation with Phorbol 12-myristate 13-acetate (PMA) for 24 hours (100 nM) as described before (Rando et al., 1995), were grown in 75cm² tissue culture flask (Beckton Dikinson) as a monolayer. These cells matured into macrophages and were used for virus challenge experiments. All the other cell lines were maintained as adherent cell lines.

Synthesis of mono- DNA- enzymes:
All DNA-enzyme oligonucleotides were synthesized in a DNA synthesizer (Applied Biosystems). Seven residues on either side of the AU sequence 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). Two modifications were introduced in the Dz-5970. In the first case, an extra 10 G residues were added to the 3'-end of Dz-5970 (henceforth referred to as Poly-G-Dz), the other modification being addition of a short stem loop (12 nucleotides) at 5' and 3' ends of the Dz-5970 (henceforth referred to as SL-Dz).

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 5977 respectively (Adachi et al., 1986). Predicted secondary structure of this RNA
Results and Discussion: Part I

consisted of number of stem-loop structures (Fig. 1.2) and 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 EcoR1 and Hind III and subcloned into pSGI (modified pSG5, Clonetech., CA, where a multiple cloning sites (MCS) was introduced between the EcoR1 and Bgl II site). This placed the expression of the above target gene under the influence of T7 as well as SV40 promoter (Fig. 1.3, panel A).

All DNA-enzyme oligonucleotides were synthesized in a DNA synthesizer (Applied Biosystems). DNA-enzyme-5944 (Dz-5944) was targeted against the Tat region but the DNA-enzyme-5970 (Dz-5970) was targeted in the Tat/Rev overlap region (Fig. 1.3, panel B). A Di-DNA-enzyme-70-44 was constructed by placing the above two DNA-enzymes in direct tandem with no spacer nucleotides. They both contained their 10-23 catalytic domain with flanking sequences that were complementary to the target RNA (Fig. 1.3, panel C). Seven nucleotides that are complementary to the target RNA sequence on either side of the unpaired A nucleotide in the target gene was 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) that is of the largest size (Fig. 1.3, panels D and E). Two DNA-enzymes were synthesized against this target, one is capable of hybridizing with only the loop region (Dz-6011-6-6) (panel E) and the other (Dz-6011-7-7) (panel D) with the same loop but one extra nucleotide on either side that is complementary to the target RNA. It is important to mention that 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 for designing dimer minizymes (Kuwabara et al., 1996).
Fig. 1.2

Secondary structure of HIV-1 Tat mRNA and selection of DNA-enzyme and Ribozyme target sites
Fig 1.3
DNA-ENZYMES AGAINST Tat OR Tat/Rev mRNA

A

LTR

pol

vif

Tat ex1

vpr

Rev ex1

Tat ex2

nef

pNL4-3

pol Tat ex2

pNL4-3

vpr nef

vif Tat ex1 LTR

EcoR1 HindIII

SV40Pr T7pr

pSGV-Tat

Transcription with T7 polymerase

M A G R

C F M T K A L G I S Y G R K

5'-UUGUUUCAUGACAAAGCCUUAGGCAUCUCCUAUGGAGGAAGA--3'

3' AACAAAG ACUGTTT 5'

3' TAGAGGA ACCGTCC 5'

10-23 catalytic motif

C G A

Di-Dz-70-44

Dz-5944

Dz-5970

Mono-DNAenzymes

Dz-5970MT
Fig. 1.3

DNA-ENZYMES AGAINST STEM-LOOP REGION OF Tat mRNA

D

TAT mRNA

\[
\begin{align*}
5' & \quad \text{AGUC}
\end{align*}
\]

\[
\begin{align*}
3' & \quad \text{UCAG}
\end{align*}
\]

Cleavage site (6011)

\[
\text{Dz-6011}(7+7)
\]

E

TAT mRNA

\[
\begin{align*}
5' & \quad \text{AGUC}
\end{align*}
\]

\[
\begin{align*}
3' & \quad \text{UCAG}
\end{align*}
\]

Cleavage site (6011)

\[
\text{Dz-6011}(6+6)
\]

10-23
Results and Discussion: Part I

In vitro cleavage reaction:
Equimolar concentrations (100 pmoles) of $^{32}$P labeled substrate RNA and cold DNA-enzyme were mixed in 10µl of 50mM Tris.HCl, pH 7.5 containing 10 mM MgCl₂ (standard condition). Cleavage reaction was allowed to continue at 37°C for one hour. In some experiments, the cleavage was carried out at varying concentrations of MgCl₂ as indicated. The cleavage products were analyzed as described before (Goila & Banerjea, 1998; Dash et. al., 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 50mMTris.HCl, pH 7.5, 150mM KCl, 2 mM MgCl₂) (Santoro & Joyce, 1997). Radioactive bands were visualized by autoradiography of the dried gel.

Inhibition of HIV-1 gene expression by DNA-enzymes:
Four independent experiments were carried out to determine if the DNA-enzymes could act intracellularly. The plasmid pHIV-1gpt (has a deletion in the Env gene with an SV2gpt fragment inserted at the env deletion site). On transfection produces replication defective virus particles) was used to transfect 60% confluent Cos-1 cells in a 6 well plate at a fixed concentration of 0.1µg along with varying concentration of DNA-enzymes (0.1 and 1 µg) for $1 \times 10^6$ cells. Transfected cells were incubated for 2 hours in the medium without serum followed by additional 13 hours 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 was estimated by using an ELIZA kit (Orgenon Teknika corporation, USA) and the amount of reverse transcriptase from the released particles were determined by using the nonradioactive RT-Assay kit from BMB (Germany).

NIH-3T3-CD4-CCR5 cells were cotransfected with the infectious molecular clone for HIV-1, pNL4-3, along with varying concentration of DNA-enzymes (0.5 and 1.0µg). Forty-eight hours after transfection the RNA was isolated using TRIZOL reagent. Using RT-PCR techniques, Tat and HIV-1 gag RNA were amplified using specific primers that were designed. For controls, mutant DNA-enzyme-5970 was used in equivalent amounts.
The sequence of the forward and backward primer for the HIV-1 gag (p24) gene was
(1) 5'-CCCTATAGTGAGAACCTCCA (1185 –1205 nt).
(2) 5'-CATTATGGTAGCTGGATTTGTTAC (1897 – 1920 nt).
The sequence of forward and backward primers for the amplification of Tat exon 1 were
(1) 5'- CTAGAGCCCTGGAAGCATCC (5851 – 5870 nt).
(2) 5'-GCTTGATGAGTCTGACTGTTCTG (6007 –6030 nt).

Fixed amount of the reporter gene (pNL.Luc. 0.5 μg) and DNA-enzyme at two different dozes (0.5 and 1.0 μg in 0.5 ml volume) were cotransfected into Cos-1 cells that were grown in a 6 well plate to 60% confluency. Cell lysates were prepared using a kit from the manufacturer (Promega Biotech.) and the Luciferase activity was measured 48 hours post transfection. For controls, mutant DNA-enzyme-5970 and equivalent amounts of an expression vector with no insert were used.

Labeling of oligonucleotides and cellular uptake:
All the above three DNA-enzymes (Dz-5970, Poly-G-Dz & SL-Dz) were labeled 32P γATP (Dupont / NEN, specific activity 2000ci/mmol.) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and purified on a column (Amersham). Approximately same amounts of radioactively labeled (~80000 counts per minute) DNA-enzymes in a final volume of 1 ml were added to the monolayer of cells in a 6 well plate (NUNC) in the presence or absence of lipofectin (GIBCO/BRL, MD, USA) for varying periods of time. Before harvesting, the cells were washed 5 times with phosphate buffered saline (PBS) and radioactivity was determined by standard techniques.

Inhibition of HIV-1 gene expression by Poly G-Dz-5970 and SL-Dz-5970:
PMA stimulated adherent population (human macrophages) of THP-1 cells were employed for evaluating the bio-efficacy of the DNA-enzymes. Infectious HIV-1 clone pNL4-3 (Adachi et al., 1986) was used to transfect PMA stimulated THP-1 cells both in presence and absence of lipofectin. Total RNA was isolated using TRIZOL (GIBCO/BRL) from cells that were transfected for 6 days and divided into
two equal portions. One set was used to estimate the levels of HIV-1 Tat RNA (179 nucleotides long) and the other set for estimating the control RNA (β-actin) by RT-PCR techniques using the kit from Promega. Human β-actin specific primers (Stratagene, La Jolla, CA, USA: Catalog #302010) were used for estimating the control RNA that was 636 nucleotides long.

Supernatants that were pooled from DNA-enzyme treated and untreated cells were also used to estimate the levels of reverse transcriptase using a non-radioactive assay kit from BMB (Germany).

**HIV-1 inhibition studies using HIV-1 III B virus:**
PMA stimulated THP-1 cells that were 80% confluent and showed typical morphology of a macrophage, were infected with III B virus ~ 1PFU per cell. The virus stock was generated by transfecting HeLa-CD4 cells with the infectious molecular clone of HIV-1, pNL4-3 as described previously by one of us (Paik et. al., 1997). One μg of Poly-G-Dz was added to 1 X10⁶ cells in a volume of 0.5 ml (*no lipofectin*) and incubated for 2 hours at 37°C. Cell extracts were prepared 72 hours later and levels of HIV-1 Tat RNA were estimated by RT-PCR techniques as described earlier. PCR carried out in the absence of RT enzyme served as a control. No other DNA-enzymes could be used in this experiment as they fail to show any specific uptake.

**RESULTS**

**Cloning of target RNA sequence:**
The general scheme of cloning the target RNA and the DNA-enzyme sites is shown in Fig. 1.1 and Fig. 1.3. An *EcoR1-Hind III* fragment, spanning the region where first exons of Tat and Rev overlap was subcloned into pSGI (modified pSG5, Clonetech., CA, where a multiple cloning sites (MCS) was introduced between the *EcoR1* and *Bgl II* site), so that the gene was placed under bacteriophage T7 as well as SV40 promoter of pSG5. The colonies were screened by *EcoR1-Hind III* digestion and the putative positive clones were confirmed by sequencing (Fig. 1.4)
CLONING OF TARGET RNA SEQUENCE IN pSG I (MODIFIED pSG-V) VECTOR
In vitro cleavage of the target RNA by mono-DNAz-5944, 5970 and Di-DNAz-70-44:

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 poly linker region which also gets transcribed when linearized at the 3' end with the restriction enzyme Hind III. The expected pattern of cleavage by the action of two mono-DNA-enzymes (5944 & 5970) and Di-DNA-enzyme is shown in Fig. 1.5, 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 pmoles) of labeled substrate and unlabeled DNA-enzyme-5944 was mixed in presence of 10 mM MgCl₂ as described before (Goila & Banerjea, 1998), a faint cleaved RNA band of 200 nucleotides was observed (lane 2). In contrast, when same amounts of DNA-enzyme-5970 (that overlaps Tat and Rev) was used, almost complete disappearance of the target RNA was observed (lane 3) and specific cleaved products could be seen that were 225 and 55 nucleotides long respectively. When same amounts of Di-DNA-enzyme was 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 pmoles each) to the target RNA, specific cleavage products could be observed, as was the case with the Di-DNA-enzyme (lane 5) although with less efficiency. We conclude that both mono- and Di-DNA-enzymes cleave the target RNA in a sequence specific manner with varying efficiencies.

In vitro cleavage of the target RNA 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. 1.3, panel D & E). Fig. 1.6 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 MgCl₂ concentration (left panel, lane 4). As expected, in the absence of MgCl₂ no cleavage products could be detected (lane 1).
Fig. 1.5

CLEAVAGE OF THE 280 NUCLEOTIDE Tat/Rev TRANSCRIPT BY DNA-ENZYMES

Top

<table>
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<th>280 nt</th>
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<tbody>
<tr>
<td>200 × 80</td>
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<tr>
<td>225 × 55</td>
<td>Dz-5970</td>
</tr>
<tr>
<td>225 × 55</td>
<td>Di-Dz-7044</td>
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<tr>
<td>200 × 80</td>
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</table>

Bottom

Lane 1: Transcript
Lane 2: Dz-5944
Lane 3: Dz-5970
Lane 4: Di-Dz-70-44
Lane 5: Dz-5970+Dz-5944
DNA-ENZYMES AGAINST STEM-LOOP REGION OF Tat RNA

For both panels:
Lane 1: -MgCl₂
Lane 2: MgCl₂ (2mM)
Lane 3: MgCl₂ (10mM)
Lane 4: MgCl₂ (20mM)
The same was true in presence of 2 and 10 mM MgCl₂ (lanes 2 and 3). Essentially similar results were obtained with the DNA-enzyme-6011-6+6. The cleavage efficiency was further reduced at 20 mM MgCl₂ 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 and that too only at elevated levels of MgCl₂ (20 mM).

**In vitro cleavage of the target RNA by mono- and Di-DNA-enzyme at simulated physiological conditions:**

The relative amounts of substrate RNA and the DNA-enzymes were same as described before but the cleavage reaction was carried out at simulated physiological conditions as described in materials and methods. The cleavage results are shown in Fig. 1.7, panel A. Lane 1 shows the labeled Tat RNA of 280 nucleotides. When equimolar concentration of Dz-5944 was added (lane 2), no cleavage product was observed. Specific cleavage products could be observed with the Dz-5970 (lane 3). Under similar experimental conditions, the Di-DNA-enzyme-70-44 also failed to cleave the target RNA (lane 4). We conclude that the mono-DNA-enzyme-5970 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 under these experimental conditions.

**Cleavage of 280 nucleotide target RNA by mutant DNA-enzyme:**

In order to know if the mutant DNA-enzyme -5970 retained any sequence specific cleavage activity, equivalent amounts of it (100 pmoles) was added to the target RNA under varying concentrations of MgCl₂. No cleavage was seen in the absence of MgCl₂ (Fig. 1.7, panel B, lane 1). Lanes 2, 3 and 4 show the absence of cleavage in presence of 10, 20 and 50 mM MgCl₂ concentration respectively. Efficient cleavage was, however, obtained with equivalent amounts of Dz-5970 (lane 5) in presence of 10 mM MgCl₂ under identical experimental conditions. We conclude from this experiment that one nucleotide change in the catalytic motif had rendered the DNA-enzyme completely ineffective. This mutant -disabled DNA-enzyme was used as control for in vivo experiments.
Fig. 1.7

A  Cleavage under simulated physiological conditions

Lane 1: Transcript
Lane 2: Dz-5944
Lane 3: Dz-5970
Lane 4: Di-Dz-70-44

B  Cleavage of 280 nucleotide Tat/Rev transcript by Dz-5970MT

Lane 1: Transcript
Lane 2: Dz-5970MT (10mM MgCl₂)
Lane 3: Dz-5970MT (20mM MgCl₂)
Lane 4: Dz-5970MT (50mM MgCl₂)
Lane 5: Dz-5970 (MgCl₂; 10mM)
Effect of the concentration of MgCl₂ on cleavage efficiency of DNA-enzymes:
In presence of increasing concentrations of MgCl₂, a doze dependent increase in the cleavage products could be observed with mono-DNA-enzyme-5944, 5970 and Di-Dz-70-44 (Fig. 1.8). The most importance difference was that at 5 mM concentration of MgCl₂, only-Dz-5970 could efficiently cleave the target RNA (panel B), the rest two cleaved the target RNA very poorly under identical experimental conditions. As expected, Dz-5944 (panel A) was again the least efficient and the Di-DNA-enzyme (panel C) cleaved the substrate with intermediate level of efficiency. The same is reflected in the bottom graph (panel D) that shows about 50% cleavage of Tat RNA could be achieved with Dz-5970 at 20 mM MgCl₂ concentration and with Di-DNA-enzyme about 30% at 50mM MgCl₂ concentration. The efficiency was lowest with the Dz-5944 that was only about 5% at 50mM MgCl₂ concentration. We conclude that each DNA-enzyme may show widely varying efficiency of cleavage at different concentration of MgCl₂ and the minimum concentration of the MgCl₂ required for specific cleavage could vary significantly.

Cleavage as a function of time:
A time course experiment, using standard cleavage conditions was carried out for Dz-5970. The cleavage reaction was stopped after 0 min, 5 min, 15 min, 30 min, 1 hr, 2hrs and 4 hrs of initiation. The products were resolved by denaturing PAGE and the bands obtained were quantitated by autoradiography. A plot of % cleavage as a function of time (Fig. 1.9, panel A), shows that within five minutes 20% cleavage is observed. Cleavage efficiency increased on increasing the incubation time of the experiment and at the end of four hours about 80% of the target has been cleaved.

Substrate kinetics:
The radiolabeled transcripts were quantitated by TCA precipitation protocol (see materials and methods). The cleavage rate was determined by taking varying amounts of labeled substrate under enzyme saturating conditions at 37°C for 1 hour. The kinetic parameters, Kₘ and Kₖₐ₅ were calculated using the Lineweaver-Burk's plot for the Dz-5970 and are depicted in Fig. 1.9, panel B. The Kₘ value for Dz-5970 was 1.0 X 10⁻⁶ m while the Kₖₐ₅ value was 5.7 X 10⁻⁵. The Kₘ value obtained was
Fig. 1.8

EFFECT OF MgCl₂ CONCENTRATION (mM) ON CLEAVAGE EFFICIENCY

A

Dz-5944

0 2 5 10 20 50

B

Dz-5970

0 2 5 10 20

C

Di-Dz-70-44

0 2 5 10 20 50

D

Cleavage(%) vs. MgCl₂ (mM)

0 10 20 30 40 50 60

Dz-5944

Dz-5970

Di-Dz-70-44
Fig. 1.9

CLEAVAGE KINETICS OF Dz-5970

A  Time kinetics of Dz-5970

![Graph showing time kinetics of Dz-5970]

B  Substrate kinetics of Dz-5970

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<tr>
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<th>Kcat min⁻¹</th>
<th>Kcat/Km (min⁻¹ m⁻¹)</th>
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<tr>
<td>Dz-5970</td>
<td>$1.0 \times 10^{-6}$</td>
<td>$5.7 \times 10^{-5}$</td>
<td>57.97</td>
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</table>
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comparable to the values obtained by Santoro and Joyce (1997) for their DNA-enzymes.

Inhibition of HIV-1 LTR mediated luciferase gene expression by mono-and di-DNA-enzymes:
Plasmid pNL4-3 clone containing the luciferase reporter gene in the nef region (pNL.Luc) was used to transfect Cos-1 cells using lipofectin. Two concentrations of DNA enzymes were used, 0.5 μg and 1.0 μg. In both the cases the total amount of DNA was kept constant by using a non-specific DNA pUC18. A dose dependent response was observed with all the DNA-enzymes (Fig. 1.1, panel A). The inhibitory pattern in cells was as would be expected based on the in vitro cleavage efficiencies of the DNA-enzymes. Dz-5970 was the most effective followed by the di-Dz-70-44, and Dz-5944 being the least efficacious. The inhibitory effect of Dz-5970 was completely reversed by cotransfecting cells with 0.5 μg of HIV-1 Tat encoding plasmid (Fig. 1.10, panel B). The unrelated DNA in equivalent amounts showed no reduction in luciferase activity suggesting that the inhibition in activity by the DNA-enzymes was not due to DNA toxicity to the cells. The mutant-Dz-5970 showed only about 20% inhibition at 1.0 μg concentration in comparison to an 85% inhibition observed with Dz-5970 suggesting that the inhibition by Dz-5970 was not due to an antisense effect but due to its catalytic activity.

Inhibition of HIV-1 gene expression by DNA-enzymes in cells:
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 (RTPCR). Specific reduction of the HIV-1 Tat RNA was observed in the DNA-enzyme treated cells (Fig. 1.11, panel A). In the absence of Dz-5970 a prominent band for 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 the cells that had not received the HIV-1 DNA, no Tat RNA specific band could be visualized. On the contrary mutant-Dz-5970 at 1.0 μg concentration did not affect the levels of Tat
Fig. 1.10

DOWNREGULATION OF LTR MEDIATED LUCIFERASE GENE EXPRESSION BY DNA-ENZYMES

A

B
Fig. 1.11

DOWNREGULATION OF HIV-1 SPECIFIC GENES

A

Tat mRNA (179nt)

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<th>Treatment</th>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
<th>Lane 4</th>
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<tr>
<td>pNL4-3 (1.0 µg)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dz-5970 (µg)</td>
<td>-</td>
<td>(0.5)</td>
<td>(1.0)</td>
<td>-</td>
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<tr>
<td>Dz-5970MT (µg)</td>
<td>-</td>
<td>-</td>
<td>(1.0)</td>
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B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Noise 1</th>
<th>Noise 2</th>
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<tbody>
<tr>
<td>pHIVgpt (1.0 µg)</td>
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<td>+</td>
</tr>
<tr>
<td>Dz-5970</td>
<td>-</td>
<td>(0.5 µg)</td>
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C

Gag mRNA (740nt)

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<th>Lane</th>
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<tbody>
<tr>
<td>1</td>
<td>pNL 4-3 (1.0 µg)</td>
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<tr>
<td>2</td>
<td>Dz-5970 (0.5 µg)</td>
</tr>
<tr>
<td>3</td>
<td>Dz-5970 (1.0 µg)</td>
</tr>
<tr>
<td>4</td>
<td>Cell control</td>
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<tr>
<td>M</td>
<td>M</td>
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</tbody>
</table>
Results and Discussion: Part I

RNA. From this experiment we conclude that the observed inhibition in the Tat RNA is due to the catalytic activity of the DNA-enzyme.

Dz-5970 was cotransfected with pHIV-1 gpt or the infectious HIV-1 DNA-pNL4-3 (1µg for 10⁶ Cos-1 cells) in a 6 well plate. The supernatant was collected and processed for the released virus particles by estimating the reverse transcriptase and HIV-1 gag p24 antigen as described by the manufacturer (see materials & methods). Also equal amounts of cell lysates from control and DNA-enzyme treated cells were processed for HIV-1 specific RNA using specific primers for HIV-1 gag RNA by reverse transcriptase based polymerase chain reaction (RT-PCR). The results of all these experiments are shown in Fig. 1.11. Panel B: Cos-1 cells when cotransfected with HIV-1 particle encoding DNA, pHIV-gpt, and varying doze of the Dz-5970. Reduction in the levels of p24 antigen and reverse transcriptase could be seen. Note the significant inhibition of HIV-1 expression even when the concentration of Dz-5970 was 1/2 (0.5 µg) of the HIV-1 DNA. The expression was further reduced when equivalent amounts of HIV-1 encoding DNA and DNA-enzyme (1.0 µg) was used. A corresponding decrease in Gag mRNA levels was also seen. Panel C: As expected a strong HIV-1 gag RNA (entire p24 gene) signal could be observed. The intensity of this signal reduced with increasing amounts of DNA-enzymes. An 8 fold reduction was observed with 0.5 µg DNA-enzyme (lane 2) and the signal was lost when 1.0 µg DNA-enzyme-5970 was used (lane 3). No signal was detected in plain cell lysates (lane 4). In all these experiments, no toxicity of DNA-Lipofectin complex was observed in Cos-1 cells. Thus a decrease in levels of Tat protein could have contributed to an overall decrease in the levels of other HIV-1 genes.

From all the above experiments it was clear that Dz-5970 is highly efficient. With an aim to target it specifically to macrophages we modified this DNA-enzyme by tagging it with a poly-G tail at the 3' end (Fig. 1.12). We also tried to see if we could add an additional sequence that would form a stem loop structure and thus increase the stability of the DNA-enzyme.
**Fig. 1.12**

**SEQUENCE OF Poly-G-Dz-5970 and SL-Dz-5970**

3' GGGGGGGGTTAGAGG ACCGTCC 5'

A G G G c c A A A A C G

3' AACAAAG ACTGTTT
A G G G c c A A A A C G

3' TAGAGGA ACCGTCC 5'
G-C G G C-G A A A A A A C T C G

**Molecular model of a G-quartet.**

*JBC 268(5) 3546-3554*

The oxygen, nitrogen and phosphorus atoms are shown as *open, solid* and *hatched* circles respectively. Hydrogen bonds are indicated by dashed lines.
Results and Discussion: Part I

Cellular uptake of Poly-G-DNA-enzyme by PMA stimulated THP-1 cells in the absence of lipofectin:

All the three DNA-enzymes were labeled at their 5' end by polynucleotide kinase (New England Biolabs.) using $^{32}$P γATP (Amersham). Equivalent amounts of radioactively labeled DNA-enzymes were added to PMA stimulated THP-1 and HeLa cells in a 6 well plate ($1 \times 10^6$ cells) that were 80% confluent in a final volume of 0.5 ml of plain D-MEM (E) medium for the indicated periods. Cells were washed five times with plain PBS, collected by centrifugation and the radioactivity was determined. The results are shown in Fig. 1.13 for the three Dzs. Panel A1 shows the specific uptake of Poly-G-Dz (in the absence of lipofectin) only by stimulated THP-1 cells that increased with longer incubation time. No increase in the uptake was observed with HeLa cell line and most likely represents background levels of radioactivity. On the other hand Dz-5970 showed no increase in the uptake with longer incubation (panel B1) with either of the two cell lines and similar results were obtained with SL-Dz-5970 also (panel C1). All the three DNA-enzymes were, however, taken up by both the cell lines with similar efficiency in presence of lipofectin (panels A2, B2, C2). Specific uptake by Poly-G-Dz was also confirmed by adding a fluorescent tag to the Poly-G-Dz using a kit (RPN5755) from Amersham (Fig. 1.14, panel A). We conclude from the above experiments that only Poly-G-Dz is taken up by the human macrophage cell line in the absence of lipofectin.

Stability of Poly-G-Dz-5970 and SL-Dz-5970 in serum:

We next wanted to know if adding G residues at the 3'-end or adding short stem-loop structures at either end of the Dz-5970 would afford them more stability. All the three DNA-enzymes were labeled with $^{32}$P ATP (Dupont/NEN) using polynucleotide kinase (New England Biolabs.). DNA-enzymes possessing similar radioactive counts (80000 cpm) were incubated at 37°C for 48 hours in medium supplemented with of 5% fetal bovine serum and analyzed by gel electrophoresis at varying time points. Radioactivity present in the bands was calculated with the help of imaging densitometer (GS-710, Bio-rad.). Dz-5970 and Poly-G-Dz showed almost similar stability but the SL-Dz was about 20% more stable after 48 hours of incubation (Fig. 1.14, panel B).
**Fig. 1.13**

**UPTAKE OF Poly-G-Dz-5970 BY MAMMALIAN CELL LINES**

A1 Poly-G-Dz-5970 -Lipofectin

A2 Poly-G-Dz-5970 +Lipofectin

B1 Dz-5970 -Lipofectin

B2 Dz-5970 +Lipofectin

C1 SL-Dz-5970 -Lipofectin

C2 SL-Dz-5970 +Lipofectin

**Legend:**
- THP-1
- Hela
- cpm (Counts per Minute)
Fig. 1.14

A  Uptake Of fluorescent labeled Poly-G-Dz-5970 By THP-1 Cells Stimulated By PMA

B  Stability of DNA-enzymes in DMEM (10% FCS)
Sequence specific cleavage activities of Poly-G-Dz-5970 and SL-Dz-5970:
Labeled HIV-1 Tat/Rev RNA was subjected to *in vitro* cleavage by equimolar amounts (100 picomoles) of unlabeled DNA-enzyme in presence of varying concentration of MgCl₂ (Fig. 1.15, panel A). Specific cleavage products (225 and 55 nucleotides long) could be observed when the reaction was carried out with Poly-G-Dz in presence of 10mM (lane 2) and 20 mM MgCl₂ (lane 3). Under exactly similar conditions, Dz-5970 (unmodified) cleaved the target RNA more effectively in presence of 10mM MgCl₂ (lane 4). We conclude that the specificity of cleavage was maintained but the efficiency of Poly-G-Dz is about 25 – 30% lower compared to the unmodified Dz-5970.

In order to know what effect the addition of short stem-loop structures had on the cleavage efficiency, cleavage reactions were carried out with equimolar amounts of substrate and enzyme in presence of varying amounts of MgCl₂ and the results are shown in Fig. 1.15, panel B. Here lane 1 depicts the synthesis of substrate RNA (280 bases long). Lanes 2 and 3 show the extent of cleavage obtained in presence of 5 and 10mM MgCl₂ respectively. Lane 4 is same as lane 3 except that the substrate and the SL-Dz were subjected to brief denaturation (90°C for 2 min) before subjecting them for cleavage. As expected, excellent cleavage was obtained with Dz-5970 (lane 5) in presence of 10 mM MgCl₂. Addition of stem loop structures (12bases) affected the cleavage potential of the Dz-5970 very significantly. Additional denaturation before cleavage reaction increased the cleavage only marginally (~5%).

Inhibition of LTR mediated reporter gene expression by Poly-G-Dz-5970 and SL-Dz-5970:
We wanted to compare the efficacy of all the three DNA-enzymes in HeLa cells that required the use of lipofectin for the introduction of DNA-enzymes into mammalian cells. Equivalent amounts (1.0 µg each) of the pNL.Luc and DNA-enzymes were used in presence of 10µl of lipofectin for 1X 10⁶ cells and the results are shown in Fig. 1.16. The values (mean +/- standard deviation) represent average from three independent experiments. The amount of luciferase detected after transfecting pNL.Luc. was taken as 100% (control) (column 1). Both the DNA-enzymes, Dz-5970 (column 2) as well as Poly-G-Dz (column 3), interfered very significantly with
Fig. 1.15

CLEAVAGE OF 280 nt Tat/Rev TRANSCRIPT
BY Poly-G-Dz-5970 AND SL-Dz-5970

A  Poly-G-Dz-5970

Lane 1 : Transcript (280nt)
Lane 2 : Poly-G-Dz-5970 (10mM MgCl₂)
Lane 3 : Poly-G-Dz-5970 (20mM MgCl₂)
Lane 4 : Dz-5970 (10mM MgCl₂)

B  SL-Dz-5970

Lane 1 : Transcript (280nt)
Lane 2 : SL-Dz-5970 (10mM MgCl₂)
Lane 3 : SL-Dz-5970 (20mM MgCl₂)
Lane 4 : SL-Dz-5970 +Δ90°C (20mM MgCl₂)
Lane 5 : Dz-5970 (10mM MgCl₂)
the reporter gene expression (>80% reduction). In comparison, the Dz-SL (column 4) as well as Dz-5970 MT. (column 5), under identical experimental conditions failed to interfere with the reporter gene expression. We conclude that Poly-G-Dz was just as effective as Dz-5970 in inhibiting specifically the HIV-1 LTR mediated reporter gene expression in HeLa cells as well wherein the uptake of the all the DNA-enzymes depend upon the presence of lipofectin. Very poor interference by SL-Dz and mutant DNA-enzyme correlated with the poor in vitro cleavage data.

Reduction of HIV-1 TAT RNA by Poly-G-Dz in human macrophage cell line in the absence of lipofectin:

Infectious clone of HIV-1 DNA, pNL4-3, was cotransfected with same amounts of DNA-enzymes in presence or absence (only with Poly-G-Dz) of lipofectin. The total RNA was isolated and divided into two equal parts. One part was used to estimate the HIV-1 Tat RNA and the other for the control RNA (β-actin, 636 nt) by RT-PCR technique as described by the manufacturer (Promega). Lane 1 of Fig. 1.17A shows a Tat specific amplified product (179 bases) when stimulated THP-1 cells were transfected with pNL4-3 DNA. Dz-5970 in presence of lipofectin, interfered with the expression of Tat gene very efficiently (> 10 fold) (lane 2). Poly-G-Dz (1.0 μg) treated cells in presence (lane 3) or in absence of lipofectin (lane 4), reduced the target gene expression by 10 and 8 fold respectively (compare lane 1 with lanes 3 and 4). Using similar conditions, both the SL-Dz (lane 5) as well as the mutant-Dz-5970 (lane 6) failed to interfere with the gene expression significantly (less than 2-fold reduction). On the contrary, the corresponding lanes that show the levels of a house keeping gene from equivalent amounts of RNA, β-actin, remained essentially unchanged (bottom panel), suggesting specific antiviral effects of our DNA-enzymes. Tat gene was not amplified from THP-1 cells that were transfected with pNL4-3 in the absence of RT (lane 7). Lane 8 shows the size of the standard DNA markers (PCR markers, Promega Biotech.).
Fig. 1.16

DOWNREGULATION OF LTR MEDIATED LUCIFERASE GENE EXPRESSION BY DNA-ENZYMES

<table>
<thead>
<tr>
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<tr>
<td>pNL Luc(1.0μg)</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Dz-5970(1.0μg)</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poly-G-Dz-5970(1.0μg)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SL-Dz-5970(1.0μg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dz-5970 MT(1.0μg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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**Fig. 1.17A**

DOWNREGULATION OF HIV-1 SPECIFIC GENES IN THP-1 CELLS TRANSFECTED WITH INFECTIOUS HIV-1 CLONE pNL4-3

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<tbody>
<tr>
<td>pNL4-3 (1.0 µg)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Dz-5970 (1.0 µg)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly-G-Dz-5970 (1.0 µg)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>Dz-5970SL (1.0 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Dz-5970MT (1.0 µg)</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
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</tr>
<tr>
<td>Lipofectin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
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</tr>
</tbody>
</table>

- **Tat RNA** (179nt)
- **β-actin RNA** (636nt)

**Marker**

1000  
750  
500  
300  
150  
50
Results and Discussion: Part I

Down regulation of HIV-1 proteins by Poly-G-Dz-5970:
Infectious DNA, pNL4-3, when transfected into THP-1 cells is able to generate infectious virus as has been reported for many other mammalian cells (Adachi et. al., 1986). The experimental protocol is same as followed for earlier experiment with the exception being the length of incubation period after transfection of stimulated THP-1 cells that was for 6 days. HIV-1 gag-pol particles were recovered from the pooled supernatants as described earlier (Paik et. al., 1997) and amount of the reverse transcriptase was determined using an ELISA kit (non-radioactive, BMB). The values were compared with pNL4-3 transfected cells (Fig. 1.17B, column 1) that was taken as 100% (control). Both, Dz-5970 (column 2) and Poly-G-Dz (column 3) interfered significantly (>80%) in the presence of lipofectin. In the complete absence of lipofectin, the Poly-G-Dz interfered to the extent of 60% (column 4). On the other hand, Dz-SL (column 5) as well as the mutant Dz-5970 (column 6) failed to shown any significant reduction in the RT activity. Both these DNA-enzymes were, as expected, completely inactive in the absence of lipofectin (data not shown). Thus the difference in the RT activity with Poly-G-Dz in presence and absence of lipofectin is about 20%. It must be mentioned that in this macrophage cell line, replication competent virus is generated that can initiate multiple rounds of infection. We usually found peak RT production 4 or 5 days after transfection as described by us earlier (Paik et. al., 1997). These are average from three separate experiments.

HIV-1 TAT RNA is reduced in THP-1 cells infected with HIV-1 III B strain:
HIV-1 III B strain was grown in HeLa-CD4 cells and the dose of the virus for 1X10^6 THP-1 cells was standardized according to the method described by Paik et al. (1997). The infection was carried out using DEAE-dextran as earlier described by Kimpton and Emerman (1992). Cells were harvested 3 days after infection and total RNA was isolated for the purpose of simultaneous detection of HIV-1 Tat as well as control (β-actin RNA) as determined by RT-PCR techniques. As expected, the cells that were infected with IIIB strain of virus, showed a prominent band specific for Tat RNA (Fig. 1.18, lane 1). This band was not detected if the RT was omitted from the PCR (lane 3). Poly-G-Dz treated THP-1 cells interfered very significantly with the expression of HIV-1 Tat RNA (lane 2) and more than 10 fold reduction was observed
Fig. 1.17B

DOWNREGULATION OF HIV-1 SPECIFIC GENES IN THP-1 CELLS TRANSFECTED WITH INFECTIOUS HIV-1 CLONE pNL4-3

**Table:**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (%)</th>
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<tbody>
<tr>
<td>pNL 4-3 (1.0 µg)</td>
<td>++ ++ ++ ++ ++</td>
</tr>
<tr>
<td>Dz-5970 (1.0 µg)</td>
<td>- + - - - -</td>
</tr>
<tr>
<td>Poly-G-Dz-5970 (1.0 µg)</td>
<td>- - + + - -</td>
</tr>
<tr>
<td>SL-Dz-5970 (1.0 µg)</td>
<td>- - - - + -</td>
</tr>
<tr>
<td>Dz-5970MT (1.0 µg)</td>
<td>- - - - - +</td>
</tr>
<tr>
<td>Lipofectin</td>
<td>++ ++ - + +</td>
</tr>
</tbody>
</table>
Fig. 1.18

DOWNREGULATION OF HIV-1 Tat mRNA BY Poly-G-Dz-5970 IN THP-1 CELLS INFECTED WITH III B STRAIN OF HIV-1

Lane 1: HIV-1 IIIB
Lane 2: Poly-G-Dz-5970
Lane 3: Cell control
Results and Discussion: Part 1

(compare lane 2 with lane 1). The plain cells showed no HIV-1 Tat specific amplification. No reduction in control RNA (β-actin) was observed from equivalent cell lysates.

DISCUSSION

One characteristic feature of HIV-1 is its genetic variability, caused by low fidelity of its reverse transcriptase. This variability constitutes a major obstacle both for vaccine and drug development. During the past decade, a variety of anti-HIV gene therapy strategies have been developed. These include antisense RNA (Cagnon, et al., 1995), ribozymes (Yu, et al., 1995; Zhou et al., 1994), RNA decoys (Bahner, et al., 1996), transdominant mutant proteins (Bonyhadi, et al., 1997), bacterial toxin (Banda, et al., 1998) and intracellular antibodies (Inouye, et al., 1997; Kitamura, et al., 1999) and DNA-enzymes.

The purpose of this work was to design effective mono- or multi-target DNA-enzymes against the two most important genes, Tat and Rev, of HIV-1 with the hope 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 with relatively small amounts of it. 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. 1.2) where A and U nucleotides were accessible for the cleavage. The same secondary structure was earlier used 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 (Fig. 1.5). 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 (Fig. 1.7, panel A). 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) (Fig. 1.3, panel D and E). 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 (Fig. 1.6). 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 MgCl₂ (20mM) that is at least 5 to 10 fold more than the physiological levels for humans. All these observations point out clearly 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 presence of 2 -5 mM MgCl₂ 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 MgCl₂. The efficiency of the cleavage increased significantly for all the three DNA-enzymes in presence of increasing amounts of MgCl₂ (Fig. 1.8). It is noteworthy that Dz-5970 alone could exhibit significant cleavage activity at 5 mM MgCl₂ 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 (Fig. 1.7, panel B). 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 (Fig. 1.11). The signal for target RNA disappeared completely when HIV-1 DNA and the DNA-enzyme-5970 was used in equivalent amounts. No toxic effect was observed at these concentrations of plasmid DNA. All the earlier studies required excess amounts of antisense DNA or the ribozyme expressing DNA (10 to 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 was used. 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 by Santoro and Joyce (1997) for their DNA-enzymes and earlier by us (Dash et al., 1998). 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 dependant decrease in reporter gene activity was observed (Fig. 1.10). The most efficient inhibition was observed with Dz-5970, followed by the Di-Dz-70-44 and the Dz-5944 was lease effective. This pattern of efficiency matches closely with 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.

We report for the first time the feasibility of using Poly-G-Dz for the purpose of specifically down regulating the most important HIV-1 regulatory proteins, namely Tat and Rev, in human macrophages in the absence of any charged lipid molecules. We have clearly shown that addition of 10 G residues at the 3'-end of the DNA-enzyme that contained the earlier described 10-23 catalytic domain, still retained significant cleavage activity besides possessing the unique ability of being taken up specifically by human macrophages (Fig. 1.13). A slight reduction in the cleavage potential with Dz-5970 containing 10 G residues is expected as it has now potential to form additional secondary structures that can affect its binding with the
Results and Discussion: Part I

target RNA (Fig. 1.15). We have not explored the minimum number of G residues in this study that would be required for it to maintain its ability to be taken up by macrophages. It depends upon the ability of formation of quadruplex structures with the G nucleotides. It may be possible to still retain the specificity to macrophages by reducing the G residues to 5, as has been reported earlier (Pearson et al., 1993). This not only reduces the cost of the synthesis of oligonucleotides but might also increase the efficiency of cleavage as less number of secondary structures could be generated in principle. DNA-enzymes lacking the G residues at their end showed no evidence of cellular uptake in the absence of lipofectin (Fig. 1.13). Furthermore, adding stem loop structures at both the ends of the DNA-enzyme, not only affected adversely its \textit{in vitro} cleavage potential but also failed to interfere with the intracellular expression of HIV-1 genes (Figs 1.16 and 1.17). The amount of reduction in HIV-1 Tat RNA both in presence or absence of lipofectin in THP-1 cells, was very similar. The reduction was due to the catalytic nature of the DNA-enzyme because the mutant-DNA-enzyme failed to interfere with the expression of HIV-1 Tat RNA. This reduction was specific because the levels of control RNA (\(\beta\)-actin) remained unchanged. Interestingly, in presence of lipofectin in HeLa cells, both the DNA-enzymes (with & without Poly-G tracks) showed very efficient interference with the levels of reporter gene expression, with Poly-G-Dz being only slightly less efficient. When compared with the production of structural proteins, reverse transcriptase (RT), (Fig. 1.17B) with that of reporter gene activity (Fig. 1.16), it seems that the level of interference is not very efficient in the former case. In this connection it is important to mention that the reporter gene activity was studied in HeLa cells that were transfected for only 48 hours with pNL.Luc., whereas the structural protein RT was estimated in a macrophage cell line from the pooled supernatant collected over a period of 6 days that might allow for multiple rounds of replication as the input DNA-enzyme may have been degraded by then. This was necessary so as to allow the detection of structural proteins by ELISA. Here also, the mutant-Dz as well as one that contained the stem loop structures, failed to interfere with the expression of HIV-1 specific genes.

Whether PMA treated cells, that were treated with Poly-G-Dz in the absence of lipofectin could be protected against HIV-1 challenge, was studied by infecting III
Results and Discussion: Part I

B strain of HIV-1. Poly-G-Dz treated cells showed remarkable protection 3 days after the infection with HIV-1 as evident by estimating the levels of HIV-1 Tat RNA by RT-PCR (Fig. 1.18). This reduction was specific because the amounts of control RNA (β-actin) remained unchanged among all the corresponding lysates. It is possible that this protection is transient in nature and may require continuous presence of this DNA-enzyme.

Thus our study strongly suggests that targeting the two most important regulatory proteins 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. We show that by simply adding Poly (G) at the 3'-end of the Dz-5970, it could be taken up specifically by human macrophages without significantly altering its catalytic property to cleave a target RNA in a sequence specific manner. We also show by number of experiments that such a novel DNA-enzyme possesses potent anti-HIV-1 activity and show significant protection when challenged with HIV.
Results and Discussion: Part II

RIBOZYMES AND DNA-ENZYMES TARGETED TO CLEAVE Tat/Rev/Env OVERLAP REGION IN THE SECOND EXON OF Tat
PART II: Ribozymes and DNA-enzymes targeted to Tat/Rev/Envelope RNA

We designed two non-GUC hammerhead ribozymes and a mono-DNA enzyme to cleave in the second exon of Tat where the Tat, Rev and Envelope ORFs overlap. The schematic representation of the sites against which the ribozymes and DNA-enzyme have been designed is shown in Fig. 2.1. We have also designed a di-ribozyme having both the mono-ribozymes placed in tandem and tried to compare their in vitro cleavage efficiencies and their potential to inhibit HIV-1 gene expression in cells.

EXPERIMENTAL PROCEDURES:

Plasmids used:
Plasmid DNAs, pNL 4-3 and pNL.Luc (Luciferase gene was inserted into the Nef region of pNL4-3; Adachi et al., 1986) were obtained from the NIH AIDS repository.

Cell lines used:
Human THP-1, Cos-1 and HeLa were maintained in DMEM (E) in 5% fetal bovine serum (HyClone). THP-1 cells were grown in suspension and after stimulation with Phorbol 12-myristate 13-acetate (PMA) for 24 hours (100 nM) as described before (Rando et al., 1995), were grown in 75cm² tissue culture flask (Beckton Dikinson) as a monolayer. These cells matured into macrophages and were used for virus challenge experiments. All the other cell lines were maintained as adherent cell lines.

Target site selection and construction of mono-ribozyme expression vectors:
Two non-GUC hammerhead ribozymes, Rz-8366 and Rz-8377 against nucleotide positions 8366 and 8377 in the region where the second exons of Tat, Rev and the Envelope ORFs overlap, were designed using the following strategy. Rz-8366 was targeted against UUC nucleotide sequence, Rz-8377 was targeted against a CUC
Fig 2.1
RIBOZYMES AND DNA-ENZYMES AGAINST Tat/Rev /Env mRNA

PCR amplification and cloning in pTarge T vector

Transcription with T7 polymerase

mRNA 107 nt.

-DPPPNPPEG
-PLSFQTHLPIPR
-PTSQSRG

5'-CCATTATTTCAGACCCACCTCCCATCCTCCGAGGGG--3'
Rz-8366        Rz-8377  Dz-8381
Results and Discussion: Part II

nucleotide sequence (Fig. 2.1). The oligonucleotide encoding the ribozyme containing the hammerhead catalytic motif flanked on either side by 8 nucleotides complementary to the target RNA was synthesized chemically with its corresponding forward and reverse primers. A di-ribozyme was also synthesized having both the above mono-ribozymes placed in tandem. The ribozyme constructs made are shown in Fig. 2.2. PCR amplified ribozyme encoding DNA fragments were initially cloned into a T-tailed vector (pGEM-T, Promega Biotech., WI, USA) and Rz-8377 was also cloned in the T-tailed vector pTarge-T (Promega Biotech., WI, USA) under T7 and CMV promoters for cell culture studies. The Ribozyme oligonucleotide was amplified using two terminal primers. The following primers were synthesized.

a. **Rz-8366 (ribozyme nucleotide)**

  (1) 5'-GTGGGTCT **CTGATGAGTCCGTGAGGACGAA** AAACGATA- 3'

  The sequence of the catalytic motif of hammerhead ribozyme is shown in bold letters. The sequence of 8 bases on either side of this motif is also shown which provides specificity for the target RNA.

  (2) Forward primer: 5'- GTGGGTCTCTGATG -3'
  (3) Reverse primer: 5'- TATCGTTTTTCGTCCTC -3'

b. **Rz-8377 (ribozyme nucleotide)**

  (1) 5'-GGGATTGG **CTGATGAGTCCGTGAGGACGAA** AGGTGGGT -3'

  The sequence of the catalytic motif of hammerhead ribozyme is shown in bold letters. The sequence of 8 bases on either side of this motif is also shown which provides specificity for the target RNA.

  (2) Forward primer: 5'- GGGATTGGCTGATG -3'
  (3) Reverse primer: 5'- GATCGGATCCCTGCCGG -3'

85
Fig 2.2
RIBOZYMES AND DNA-ENZYMES AGAINST Tat/Rev/Env mRNA

Rz-8366

Rz-8377

Rz-77-66

Dz-8381
c. Di-ribozyme 77-66 (ribozyme nucleotide)

(1) 5' -GGGATTGG CTGATGAGTCCGTGAGGACGAA AGGTGGGTCT CTG ATGAGTCCGTGAGGACGAA AAACGATA- 3'
The di-ribozyme was synthesized as both the above ribozyme sequences placed in tandem.

(2) Forward primer: 5'- GGGATTGGCTGATG- 3'
(3) Reverse primer: 5'- TATCGTTTTTCGTCCTC -3'

PCR conditions were same as described in materials and methods. The recombinant plasmids were checked for the insert by digestion with SalI and NcoI. The positive clones were confirmed by sequencing. Both the mono-ribozymes, Rz-8366 and Rz-8377 were placed under SP6 promoter and were named as pGem-Rz-8366 and pGem-Rz-8377 respectively (Fig. 2.3, A and B). The di-ribozyme was also found to have been cloned under SP6 promoter and named pGEM-Rz-77-66 (Fig. 2.3C) Rz-8377 was also cloned in the T-tailed vector pTarge-T under T7 and CMV promoters and was named pTarge-Rz-8377. (Fig. 2.3 D).

Cloning of target RNA sequence:
The general scheme of cloning target RNA for in vitro cleavage analysis has been shown in Fig 2.1. A 63 base pair region spanning from nucleotide positions 8352 to 8414 was PCR amplified with its respective forward and reverse primers and cloned in the T-tailed vector pTarge-T vector under T7 promoter and the clones were confirmed by sequencing (Fig 2.4).

(2) Forward primer: 5'- CACCATTATCGTTTCAG -3'
(3) Reverse primer: 5'- CTATTCCTTCGGGCCTG -3'
The plasmids were purified on a Qiagen column (Qiagen, USA) and used for in vitro or cell culture experiments. The T7 promoter was used to obtain in vitro generated transcripts using T7 RNA polymerase after linearization with NotI.
Fig 2.3A
SEQUENCE OF Rz-8366 CLONED IN pGEM-T VECTOR UNDER SP6 PROMOTER

SEQUENCE UNDER SP6 Pr
5'--- ATTGTGGGTCTCTGATGAGTCCGTGA GGACGAAAAACGATAAACTC---3'
Fig 2.3B

SEQUENCE OF Rz-8377 CLONED IN pGEM-T VECTOR UNDER SP6 PROMOTER

SEQUENCE UNDER SP6

5' ---GGGATTTGGCTGATGAGTCCGTGAGGACGAAAGGTGGGT --- 3'

TGAGGACGAAAGGTGGGT --- 3'

T7 Pr

3' C C T A A C C C G G U U U C C U C C C A A

SEQUENCE UNDER SP6

5' ---GGGATTTGGCTGATGAGTCCGTGAGGACGAAAGGTGGGT --- 3'
Fig 2.3C

SEQUENCE OF Rz-77-66 CLONED IN pGEM T VECTOR UNDER SP6 PROMOTER

SEQUENCE UNDER SP6 Pr

5'----GGGATTTGGCTGATGAGTCCCGTGAG
GACGAAAGG TGGGTCTCTGATGAG
TCCGTGAGGACGAAAAACGATA----3'

---GGGATTGGCTGATGAGTCCGTGAG
GACGAAAGGTGGATCTCTGATGAG
TCCGTGAGGACGAAAAACGATA---3'
Fig 2.3D
SEQUENCE OF Rz-8377 CLONED IN pTarge-T VECTOR UNDER T7 and CMV PROMOTER

Sequence under T7 and CMV Promoter

5' ---GGGATTGGCTGATGAGTCCG
TGAGGACGAAAGGTGGGT --- 3'
SEQUNCE OF 63 bp SYNTHETIC TARGET CLONED IN pTARGET-T VECTOR UNDER T7 PROMOTER

Sequence under T7 Promoter

5'...GATTCAACCATTATCGTTTCAGACCCACCTCCCAAT
CCCGAGGGGACCCGACAGGCCCGAAGAATAGAAT..3'
In vitro transcription of the ribozyme constructs:

All the ribozyme plasmids were Qiagen purified and linearized at the 3' end with Ncol prior to in vitro transcription. Transcription of the linearized plasmid DNA was carried out as described in materials and methods.

Ribozyme cleavage reaction:

Ribozyme cleavage reaction was carried out by adding equimolar concentration (100 pmoles each) of the labeled target RNA and cold ribozyme in a reaction buffer containing 50mM Tris.HCl, pH 7.5, in a volume of 10μl. The reaction mixture was briefly heated at 94°C and the cleavage reaction was initiated by adding MgCl₂ (10mM, final concentration) at 37°C for 2hrs, referred to as standard conditions. The reaction was stopped by adding the stop buffer (95% Formamide, 0.05% Xylene Cyanol FF, 0.05% Bromophenol Blue, 20mM EDTA). The samples were denatured at 55°C for 10 min before loading on to the gel. The cleaved products were analyzed by 7M Urea-6% Polyacrylamide gel electrophoresis using either the Mini-PROTEAN II system (BIO-RAD, U.S.A.) or sequencing gel apparatus (model S2, GIBCO/BRL). The vacuum dried gel was exposed to an X-Ray film (BIOMAX™ MS, Kodak, U.S.A.) and the bands were analyzed qualitatively by autoradiography or quantitatively by densitometry (GS-710 Calibrated Imaging Densitometer, BIO-RAD, U.S.A.) of the autoradiograms. Cleavage was also studied under simulated physiological condition: 150mM KCl, pH 7.5, 2mM MgCl₂, 37°C for 2 hrs.

Inhibition of HIV-1 gene expression by ribozymes and Dz-8381:

PMA stimulated adherent population (human macrophages) of THP-1 cells were employed for evaluating the bio-efficacy of the ribozymes and Dz-8381. Infectious HIV-1 clone pNL4-3 (Adachi et al., 1986) was used to transfect PMA stimulated THP-1 cells both in presence and absence of lipofectin. Total RNA was isolated using TRIZOL (GIBCO/BRL) from cells that were transfected for 6 days and divided into two equal portions. One set was used to estimate the levels of HIV-1 Tat RNA (179 nucleotides long) and the other set for estimating the control RNA (β-actin) by RT-PCR techniques using the kit from Promega. Human β-actin specific primers
Results and Discussion: Part II

(Stratagene, La Jolla, CA, USA: Catalog #302010) were used for estimating the control RNA that was 636 nucleotides long.
The sequence of forward and backward primers for the amplification of Tat exon 1 were
(1) 5'-CTAGAGCCCTGGAAGCATCC (5851–5870 nt).
(2) 5'-GCTTGATGAGTCTGACTGTTCTG (6007–6030 nt).

Supernatants that were pooled from ribozyme or DNA-enzyme treated and untreated cells were also used to estimate the levels of reverse transcriptase and p24 gag antigen. Levels of p24 antigen were estimated by using an ELIZA kit (Orgenon Teknika Corporation, USA) and the amount of reverse transcriptase from the released particles were determined by using the nonradioactive RT-Assay kit from BMB (Germany).

RESULTS

1. Ribozyme design and cloning:
Two hammerhead motif containing mono-ribozymes, Rz-8366 and Rz-8377, were designed to cleave at nucleotide positions 8366 (UUC) and 8377 (CUC). Both are non-GUC hammerhead ribozymes and the cleavage was designed to take place after C nucleotide (Fig. 2.2). The ribozymes were cloned as has been described in experimental procedures. The sequence of the cloned ribozymes was found to exactly correspond to the sequence of the ribozyme oligonucleotide that was used for PCR (Fig. 2.3A, B, C and D). A mutant DNA-enzyme, targeted to Tat exon I (Dz-5970MT) was used as an antisense control. The mutant DNA-enzyme was shown to lack all cleavage activity in vitro (discussed in 'Results and Discussion Part I').

2. In vitro cleavage of 107 nucleotide target RNA by ribozymes:
The catalytic activity of the ribozyme was tested in an in vitro cleavage assay. Plasmid DNA encoding the Tat/Rev/Env gene was linearized by Not I, which after subjecting to an in vitro transcription with T7 RNA polymerase in the presence of α-32P UTP is expected to generate a 107 nucleotide long transcript. The ribozyme
specific RNAs were obtained by linearizing the plasmids encoding it (by using the restriction enzymes as described previously) and transcribing using SP6 RNA polymerase. A schematic representation of the cleavage reaction by one of the ribozymes is shown in Fig. 2.5. The expected cleavage pattern obtained by the ribozymes is shown in Fig. 2.6, panel A. Equimolar concentration of labeled target RNA was mixed with the cold ribozyme RNA individually in a reaction buffer containing 50mM Tris.HCl, pH 7.5 and the cleavage reaction was initiated by adding MgCl$_2$ (10mM) at 45°C for 2 hrs. Fig. 2.6, panel B, lane 1 shows the synthesis of 107 nucleotide long RNA synthesized (target substrate). Specific 5’ and 3’ cleavage products were obtained as expected with the two mono-ribozymes (lane 2: Rz-8366; and lane 3: Rz-8377) and the di-ribozyme showed multiple but specific cleavage products that retained the specificity of the two individual mono-ribozymes (lane 4). The DNA-enzyme Dz-8381 also cleaved to generate its specific 5’ and 3’ cleavage products (lane 5). A comparative cleavage analysis showed that Rz-77-66 and Dz-8381 were the most efficient followed by Rz-8377 and Rz-8366. All ribozyme reactions were carried out at 45°C while DNA enzyme cleavage was carried out at 37°C.

3. Cleavage of 107 nucleotide RNA at simulated physiological condition:
Efficiency to cleave the target RNA was determined at simulated physiological conditions (150 mM KCl, pH 7.5, 2mM MgCl$_2$, 37°C for 2 hrs) by all the ribozymes and Dz-8381. Fig. 2.7 shows the cleavage of the 107 nucleotide long transcript by the ribozymes and Dz-8381 under simulated physiological conditions. Lane 1 in all panels shows the synthesis of 107 nucleotide long RNA synthesized (target substrate) Lane 2 shows the cleavage carried out in complete absence of MgCl$_2$ and Lane 3 shows cleavage reaction carried out under simulated physiological conditions. Specific cleavage products could be observed with the Dz-8381 (panel D). Under similar experimental conditions, Rz-8377 (panel B) and Rz-7766 (panel C) could also cleave the target RNA although the di-ribozyme showed slightly poor cleavage as compared to Rz-8377. Rz-8366 did not show any detectable cleavage products (panel A). We conclude that both the DNA-enzyme as well as the Rz-8377 could cleave the target RNA to a similar extent under these experimental conditions and
Fig. 2.5

SCHEMATIC REPRESENTATION OF IN VITRO CLEAVAGE REACTION

63 bp target RNA cloned in pTarge-T vector

Not I

CMV T7 Tat/Rev/Env (63 nt)

Digestion with Not I and in vitro transcription

Target RNA 107 nt

+ Mg^{2+}

Cleavage

Cleavage products

Ribozyme oligonucleotide cloned in pGEM-T vector

Nco I

T7 Rz SP6

Digestion with Nco I and in vitro transcription

Rz RNA
Fig 2.6

COMPARATIVE CLEAVAGE BY MONO- AND DI-RIBOZYMES AND Dz-8381

A

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Transcript (107nt)
Rz-8366
Rz-8377
Dz-8381

B

Lane 1: Transcript (107 nt.)
Lane 2: Rz-8366
Lane 3: Rz-8377
Lane 4: di-Rz-77-66
Lane 5: Dz-8381
Fig 2.7

CLEAVAGE OF 107 nt Tat/Rev/Env TRANSCRIPT UNDER SIMULATED PHYSIOLOGICAL CONDITIONS

Lane 1: Transcript (107nt)
Lane 2: Ribozyme/DNA-enzyme (No MgCl₂)
Lane 3: Ribozyme/DNA-enzyme (simulated physiological conditions)
these were used for subsequent cell culture experiments. The overall efficiency of all the ribozymes and Dz-8381 were, in general, ~20-30% less under physiological conditions than what was achieved under standard conditions of cleavage (compare Fig. 2.6 with 2.7). This difference in efficiency in cleavage was not due to different amounts of ribozymes being used in cleavage reaction. All the ribozymes and Dz-8381 failed to cleave in complete absence of MgCl₂.

4. *In vitro* cleavage kinetics:

a) Effect of Magnesium ion concentration:

The cleavage efficiency of all ribozymes was tested under varying Mg²⁺ concentration (Fig. 2.8). The reaction conditions were similar for all the ribozymes so the individual lanes in the three panels can be compared. The cleavage reaction was done using increasing Mg²⁺ concentration from 0 mM, 1 mM, 2 mM, 5 mM, 10 mM and 20 mM. Lane 1 in panels A, B, C and D shows cleavage in absence of MgCl₂. As expected no cleavage was observed in complete absence of Mg²⁺ ions. Efficiency of cleavage increased on increasing the Mg²⁺ ion concentration in the reaction buffer for all ribozymes and Dz-8381 although the dose dependent response was different in each case. The bands were quantitated for percentage cleavage after developing them on an autoradiogram. A plot of % cleavage as a function of MgCl₂ concentration (Fig. 2.8, panel E) showed that the di-ribozyme and the Dz-8381 showed an excellent dose dependent response with almost 90% cleavage at 20 mM MgCl₂ concentration, followed by Rz-8377 with almost 80% cleavage and lastly by Rz-8366 with approximately 60% cleavage. It was clear that Rz-77-66 is most efficient followed by Rz-8377 and then Rz-8366. Also Dz-8381 which has a cleavage site only 4 bases downstream of Rz-8377 was found to be more active than the ribozyme.

b) Cleavage as a function of time:

A time course experiment, using standard cleavage conditions was carried out for all the ribozymes. The cleavage reaction was stopped after 0 min, 5 min, 15 min, 30 min, 1 hr, 2 hrs and 4 hrs of initiation. Fig. 2.9 shows the time kinetics of Rz-8366, Rz-8377, di-Rz-77-66 and Dz-8381 (panels A, B, C and D respectively). The bands
Fig 2.8

EFFECT OF MgCl$_2$ CONCENTRATION (mM) ON CLEAVAGE EFFICIENCY

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Rz-8366

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Rz-8377

C

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Rz-77-66

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Dz-8381
**Fig 2.8**

**EFFECT OF MgCl$_2$ CONCENTRATION (mM) ON CLEAVAGE EFFICIENCY**

![Graph showing the effect of MgCl$_2$ concentration on cleavage efficiency.](image-url)
Fig 2.9

CLEAVAGE AS A FUNCTION OF TIME (RIBOZYMES AND Dz-8381)

A

B

C

D

Rz-8366

Rz-8377

Rz-77-66

Dz-8381
Fig 2.9

CLEAVAGE AS A FUNCTION OF TIME (RIBOZYMES AND Dz-8381)
were quantitated for percentage cleavage after developing them on an autoradiogram. A plot of % cleavage as a function of MgCl₂ concentration (Fig. 2.9, panel E) showed that di-ribozyme, Rz-77-66 was able to reach a plateau within 30mins showing almost 90% cleavage. Rz-8377 and Dz-8381 showed 80% cleavage after 4hrs while Rz-8366 was less efficient in comparison with 70% of the substrate cleaved. The experiments revealed that the di-ribozyme is able to start the cleavage reaction as early as 5min (panel C, lane 2), while the mono-ribozymes and Dz-8381 start showing discernible products at the 15 minute time point.

c) Substrate kinetics:
The radiolabeled transcripts were quantitated by TCA precipitation protocol (see materials and methods). The cleavage rate was determined by taking varying amounts of labeled substrate under enzyme saturating conditions at 45°C for 1 hour. The reaction for Dz-8381 was carried out at 37°C. The kinetic parameters were calculated using the Lineweaver-Burk's plot for the Rz-8366, Rz-8377 and Di-Rz-77-66. The kinetic parameters, Kₘ and Kₖₐₜ for the two mono ribozymes are depicted in Table 2.1. The Kₘ values for Rz-8366 was 5.0 X 10⁻⁴ m and 1.4 X 10⁻⁵ m for Rz-8377. The di-ribozyme, Rz-77-66 had a Kₘ value of 5.0 X 10⁻⁴ m. Comparison of the kinetic parameters revealed that the mono-Rz-8377 had a more favorable Kₘ value as compared to mono-Rz-8366 and di-Rz-77-66. Dz-8381 however, had the most favorable Kₘ value of 4.2 X 10⁻⁶ m.

5. Combined cleavage of target RNA by Rz-8377 and Dz-8381:
Since Rz-8377 and Dz-8381 were found to be more efficient under simulated physiological conditions subsequent cell culture experiments were carried out with these two. To determine if both the DNA-enzyme and the ribozyme cleave in a synergistic manner or are mutually exclusive, we carried out a combined cleavage experiment where both the ribozyme and the DNA-enzyme were added. Fig. 2.10, lane 1 shows the 107 nucleotide transcript. lanes 2 and 3 show cleavage with Dz-8381 and Rz-8377 respectively. Lane 4 shows the cleavage of the target RNA when both the DNA-enzyme and ribozyme were added. As can be seen from lane 4 the
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Fig. 2.10

CLEAVAGE OF 107 nt. TRANSCRIPT BY Rz-8377 AND Dz-8381

Lane 1: Transcript (107 nt.)
Lane 2: Dz-8381
Lane 3: Rz-8377
Lane 4: Rz-8377 + Dz-8381
addition of both the DNA-enzyme and the ribozyme did not bring about a significant increase in cleavage when compared to cleavage in presence of only ribozyme or DNA-enzyme. Thus from this we conclude that both the ribozyme and DNA-enzyme cleave in a mutually exclusive manner and did not exhibit a synergistic effect. This can be expected as the cleavage sites of Rz-8377 and Dz-8381 are separated by just four bases and cleavage by one will reduce the efficiency with which the other could hybridize as some of the bases to which the hybridizing arms bind would have been cleaved off.

6. **Comparative inhibition of HIV-1 gene expression by a ribozyme and a DNA-enzyme:**

With an aim to compare the ability of a ribozyme and a DNA-enzyme to inhibit HIV-1 gene expression we carried out cell culture experiments with Rz-8377 and Dz-8381. The cleavage sites of these two nucleic acid anti-virals is just separated by four nucleotides and also lie in the same loop region of the predicted mRNA secondary structure for Tat (Fig. 1.2). The use of both DNA-enzymes and ribozymes has its pros and cons in that while DNA enzymes are easy to synthesize and deliver and are more resistant to nuclease action, ribozymes could be continuously transcribed and hence maintain a steady level of ribozyme RNA in the cells even though RNA is highly susceptible to nuclease degradation. The inhibitory activity of Rz-8377 and Dz-8381 was measured as a function of their ability to inhibit LTR mediated reporter gene expression and their ability to bring about a decrease in viral titers in supernatant of cells transfected with the wild type infectious clone of HIV-1, pNL4-3. Finally the levels of Tat RNA were measured by carrying out a semi quantitative RT-PCR using Tat specific primers. Total RNA was isolated from transfected THP-1 cells using TRIZOL reagent (GIBCO/BRL) following the procedure described by the manufacturer. The RNA was treated with DNase I (RQ1; Promega) for 45 min at 37°C. 1μg of total RNA was taken for RT-PCR analysis of Tat mRNA and the same amount was used to estimate the levels of β-actin mRNA.

Human β-actin specific primers (Stratagene, La Jolla, CA, USA: Catalog #302010) were used for estimating the control RNA that was 636 nucleotides long.
a) Inhibition of LTR-mediated luciferase gene expression:
Tat is a transcriptional activator of HIV-1. Thus if Tat were to be downregulated we should see a corresponding downregulation of transcription from HIV-1 LTR. For our LTR-mediated reporter gene downregulation experiments, we used pNL Luc plasmid where, the reporter gene Luciferase that was inserted into the Nef region of pNL4-3, was used to transfect HeLa-Tat cells (1 µg for 1 X 10^6 cells) as described in materials and methods. Two concentrations of Dz-8381 and the Rz-8377 expressing plasmid, pTarge-Rz-8377, were used, 0.5 µg and 1.0 µg. As expected both the ribozyme and Dz-8381 inhibited LTR-mediated luciferase gene expression to a comparable extent (Fig. 2.11). A dose dependent response was observed with 0.5 µg showing 60% inhibition while 1 µg of DNA-enzyme or ribozyme showed almost 80% inhibition of luciferase activity. Dz-5970MT on the other hand which was used as an antisense control showed only 20% inhibition of luciferase activity at 1 µg concentration suggesting that inhibition with Rz-8377 and Dz-8381 was by virtue of their catalytic activity and not by an antisense effect. The control DNA or unrelated DNA, used in equivalent amounts showed no reduction in the luciferase activity suggesting that the inhibition was not due to DNA toxicity to the cells. We conclude that both, Rz-8377 as well as Dz-8381 were able to specifically interfere with the expression of HIV-1 in a dose dependent manner, and the inhibition was by virtue of their catalytic activity and not via an antisense effect. It is important to mention that no cellular toxicity was observed at these concentrations of DNA. The data obtained was the mean of three experiments.

b) In vivo inhibition of HIV-1 gene expression by DNA-enzymes:
Dz-8381 and pTarge-Rz-8377 were cotransfected with the infectious HIV-1 DNA-pNL 4-3 (1 µg for 10^6 THP-1 cells) in a 6 well plate. The supernatant was collected and processed for the released virus particles by estimating the HIV-1 Reverse transcriptase and HIV-1 gag p24 antigen as described by the manufacturer (see materials & methods). Mutant Dz-5970 was used as a control. Also equal amounts of cell lysates from control and DNA-enzyme treated cells were processed for HIV-1
INHIBITION OF HIV-1 LTR-MEDIATED LUCIFERASE GENE EXPRESSION BY Rz-8377 AND Dz-8381

Fig 2.11

% Luciferase activity

0.5 µgs  1.0 µg

pGEM3Z  Dz.5970MT  Dz.8381  Rz.8377  pNLLuc
specific RNA using specific primers for HIV-1 Tat RNA by reverse transcriptase based polymerase chain reaction (RT-PCR).

The results of all these experiments are shown in Fig. 2.12. Panel A and panel B show HIV-1 RT and p24 levels which is an index of the viral load present in the supernatant. The results are a mean of three experiments. HIV-1 reverse transcriptase levels are expressed as percentage of positive control while p24 levels are expressed as picograms. As can be seen from the graph there is a dose dependent response to the concentration of ribozyme expressing plasmid, pTarge-Rz-8377 and Dz-8381. 0.5 μg of pTarge-Rz-8377 or Dz-8381 showed almost 60% inhibition while at 1μg a significant inhibition of almost 80% was observed. Where the mutant DNA-enzyme was used there was very little inhibition of p24 antigen levels which is not significant when compared to inhibition by equivalent amounts of Rz-8377 and Dz-8381, suggesting that the inhibition is by virtue of their catalytic property and not via an antisense effect. Fig. 2.13, shows the Tat RNA levels as seen when 1μg of total RNA was used for RT-PCR using Tat exon I specific primers. The Tat message shows almost a 40% inhibition with 0.5μg of pTarge-Rz-8377 or Dz-8381 and almost a complete disappearance of the signal when cotransfected with 1μg of the same. These results in fact mimic the levels of p24 or HIV-1 RT assays. All the corresponding lanes (1 to 7) showed almost similar levels of the housekeeping b-actin mRNA (control RNA). We conclude from this experiment that both Rz-8377 and Dz-8381 showed a dose dependent inhibition of viral gene expression and the inhibition was comparable. Both the ribozyme and the DNA-enzyme were able to reduce the level of Tat mRNA in a dose dependent manner and again to a comparable extent. The inhibition of Tat and Rev could be bringing about a subsequent decrease in the levels of all other viral proteins since these proteins are important for transcription and viral RNA export. And hence an overall decrease in viral titres is observed as shown by HIV-1 p24 and reverse transcriptase levels.
DOWNREGULATION OF HIV-1 SPECIFIC GENES IN THP-1 CELLS TRANSFECTED WITH INFECTIOUS HIV-1 CLONE pNL4-3

A  HIV-1 Reverse Transcriptase assay

B  p24 ELISA
Fig 2.13

DOWNREGULATION OF HIV-1 Tat RNA IN THP-1 CELLS TRANSFECTED WITH INFECTIOUS HIV-1 CLONE pNL4-3

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<tr>
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<tr>
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P-actin RNA
DISCUSSION

The use of catalytic RNA and DNA provides a unique approach towards achieving selective destruction by cleavage of specific target RNAs. Many catalytic motifs have been defined and studied, but the hammerhead catalytic motif has been used extensively for inhibiting the viral and cellular gene expression. The purpose of this work was to design effective mono- or multi-target ribozymes and DNA-enzyme, against the three most important genes, Tat, Rev, and Envelope of HIV-1 with the hope that using this strategy it would be possible to interfere with the replication of HIV-1 in a sequence specific manner. We also wanted to compare the efficacies of ribozyme versus the DNA-enzyme in inhibiting HIV-1 gene expression in cells and delineate the pros and cons of either of them. Both, the ribozyme and the DNA-enzyme being catalytic in nature, may be able to do so with relatively small amounts of it. Also by targeting them to a region where all three open reading frames overlap we aimed to synthesize a multi-target mono-ribozyme or DNA-enzyme which would have its target sequence on all the species of HIV-1 RNA. Moreover such regions are less prone to sequence variation, as any mutation in these regions would affect all the three open reading frames. We synthesized two mono-ribozymes, a di-ribozyme and a DNA-enzyme (possessing the earlier identified 10-23 catalytic motif). Although all the ribozymes including the di-ribozyme and the DNA-enzyme were able to cleave the target RNA into specific products, they did so with varying efficiencies (Fig. 2.6) with Rz-77-66 and Dz-8381 being highly efficient followed by Rz-8377. The efficiency of the cleavage increased significantly for all the three ribozymes and Dz-8381 in presence of increasing amounts of MgCl$_2$ (Fig. 2.8). All our in vitro cleavage data suggested that Rz-8366 was not as efficient as our other ribozymes in cleaving the target RNA as has been reflected in the kinetic studies of the ribozymes using varying parameters of time and MgCl$_2$ concentration (Figs. 2.6, 2.8 and 2.9). Difference in the secondary structures at the target sites in the Tat RNA could account for the varying effects of the ribozyme and DNA-enzyme. Quite remarkably, the di-ribozyme although showing good response to MgCl$_2$ concentration, showed an unfavourable Km and Kcat/Km value (Table 2.1). Also it was found to cleave the
Results and Discussion: Part II

target RNA about 20% less as compared to Rz-8377 and Dz-8381 under simulated physiological conditions (Fig. 2.7). Both, Rz-8377 and Dz-8381 cleaved the target RNA in a mutually exclusive manner since they shared the hybridizing region on the target RNA. Thus it can be assumed that the difference in cleavage efficiency by virtue of the target RNA being accessible to one but not the other could be ruled out and must be attributed to the efficacy of the DNA-enzyme or ribozyme per se. When the mono-ribozyme and the DNA-enzyme were compared for their ability to interfere with LTR mediated reporter gene expression, a doze dependant decrease in reporter gene activity was observed for both ribozyme and DNA-enzyme and the inhibition was almost similar for both. We then addressed the question whether they have the ability to specifically interfere with the expression of the HIV-1 genes when introduced into mammalian cells. A doze dependent decrease in HIV-1 Tat RNA was observed (Fig. 2.13). This inhibition was very impressive and 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. This inhibition was reflective of similar decrease in HIV-1 RT and p24 antigen levels (Fig. 2.12 panel A and B) which is an index of the viral load in the cell supernatant. Thus it could be construed that inhibition of Tat and Rev could in turn be inhibiting the formation of other viral proteins which resulted in decreased viral levels. Both the ribozyme as well as the DNA-enzyme were found to be equally efficient at inhibiting HIV-1 specific gene expression when used in a 1:1 ratio with infectious HIV-1 DNA. All the earlier studies required excess amounts of antisense DNA or the ribozyme expressing DNA (10 to 100 fold more) to interfere with the gene expression (Chen et al., 1992). This was not the case when equivalent amounts of mutant DNA-enzyme-5970 was used, thus suggesting that inhibition was due to the catalytic activity of the ribozyme and DNA-enzyme. The ability of the ribozyme and DNA-enzyme to inhibit HIV-1 gene expression to a similar extent is very interesting since the ribozyme is delivered to the cells as a ribozyme expressing plasmid under a strong eukaryotic promoter which ensures a continuous transcription and availability of ribozyme RNA within the cell. The DNA-enzyme on the other hand is delivered as an oligonucleotide, and is not synthesized de novo in the cell. This can be explained on the basis of the inherent susceptibility of RNA to nuclease degradation. Thus
while the ribozyme mediated inhibition would require a continuous synthesis of ribozyme RNA within the cell to replace the molecules degraded, the DNA-enzyme does not and a one time administration at a particular dose can suffice to show a comparable effect. Moreover in case of HIV-1 one can selectively target the DNA-enzymes to macrophages by attaching a poly G tract at the 3'end (Unwalla and Banerjea, 2001). Thus while a ribozyme would require complex delivery mechanisms in the form of viral vectors, a DNA-enzyme can be administered parenterally just like any other drug.

In summary, we have showed efficient cleavage of the Tat/Rev/Envelope RNA by two mono- and a di-ribozyme and a DNA-enzyme. The Di-ribozyme retained the cleavage specificity of the mono-DNA-enzymes. Both the mono-ribozyme and 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 to inhibit the expression of the target gene. Targeting regions in HIV where ORFs of different exons overlap with ribozymes and DNA-enzymes 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.