Inhibition of HIV-1 Integrase gene expression by 10-23 DNAzyme

NIRPENDRA SINGH[1], ATUL RANJAN[2], SOUVIK SUR[2], RAMESH CHANDRA[1] and VIBHA TANDON[2,*]

[1]*Dr BR Ambedkar Center for Biomedical Research,* [2]*Department of Chemistry, University of Delhi, New Delhi 110 007, India*

**Corresponding author (Fax, +91-11-27667794-300; Email, vibhadelhi@hotmail.com; vtandon@acbr.du.ac.in)*

HIV Integrase (IN) is an enzyme that is responsible for the integration of the proviral genome into the human genome, and this integration step is the first step of the virus hijacking the human cell machinery for its propagation and replication. 10-23 DNAzyme has the potential to suppress gene expressions through sequence-specific mRNA cleavage. We have designed three novel DNAzymes, DIN54, DIN116, and DIN152, against HIV-1 Integrase gene using Mfold software and evaluated them for target site cleavage activity on the *in vitro* transcribed mRNA. All DNAzymes were tested for its inhibition of expression of HIV Integrase protein in the transiently transfected cell lines. DIN116 and DIN152 inhibited IN-EGFP expression by 80% and 70% respectively.

[Singh N, Ranjan A, Sur S, Chandra R and Tandon V 2012 Inhibition of HIV-1 Integrase gene expression by 10-23 DNAzyme. *J. Biosci.* **37** 493–502] **DOI** 10.1007/s12038-012-9216-4

1. Introduction

Integrase is responsible for the integration of HIV-1 genome within the host DNA. Few known small molecule inhibitors of integrase such as Raltegravir (Evering and Markowitz 2008), Diketo acids derivatives and curcumin-like natural products were not found so effective owing to the high mutation rate of the viral genome and the very severe side effect of these inhibitors (Gupta and Nagappa 2003). Hence, to overcome these drawbacks, antisense therapy was put into practice to reduce the viral load and counteract the spread of infection. Since the discovery of self-cleavage and ligation activity of the group I intron for manipulating biomolecules, research interest in catalytic nucleic acids rose tremendously. DNAzymes, also known as deoxyribozymes or DNA enzymes, are single strands of DNA that have catalytic activity. While not found in nature, a wide variety of DNAzymes have been discovered by *in vitro* evolution to catalyse a broad repertoire of chemical reactions. Their potential as both therapeutic and diagnostic tools has been extensively investigated (Warashina *et al.* 1999; Wu *et al.* 1999; Hou *et al.* 2006; Saxena and Dhole 2008). It was shown earlier that certain DNAzymes molecules can also cleave RNA phosphodiester linkages (Breaker and Joyce 1994). Using a selection strategy to create a series of divalent-metal-dependent deoxyribozymes, Breaker and Joyce isolated distinct DNA motifs requiring Mg^{2+}, Pb^{2+}, Zn^{2+} or Mn^{2+} as cofactors. The 10-23 DNAzyme is able to cleave various combinations of RNA substrates wherein cleavage occurs at the 3′ side of a single unpaired purine followed by a paired pyrimidine. Kinetic studies of 10-23 DNAzyme showed that its mechanism of action is similar to that of hammerhead ribozymes (Santoro and Joyce 1997; Ota *et al.* 1998). The potential use of 10-23 DNAzymes in a wide array of gene therapeutic and industrial applications is demonstrated by a large number of studies that have inhibited gene expression (Dash and Banerjea 2004). Dash and Banerjea used 10-23 DNAzymes to interfere with the functional expression of the HIV-1 envelope (*env*) gene in a gene fusion assay. Wei Hou *et al.* (2006) have shown the inhibition of hepatitis B virus X gene expression by 10-23 DNAzyme. Banerjea and co-workers compared a hammerhead ribozyme with 10-23 DNAzymes targeting various sites in *in vitro* transcribed *CCR5* co-receptor RNA implicated in HIV-1 entry and fusion (Goila and Banerjea 1998). Zhang and co-workers targeted nine different sites in the V3 loop of *env* gene, and the most efficient molecule was tested for its ability to inhibit

Keywords. 10-23 DNAzyme; HIV IN; Integrase-EGFP expression inhibition

Published online: 25 June 2012

infection of the incoming HIV-1 virus (Zhang *et al.* 1999). A single base change in the catalytic core inactivated the DNAzyme. The sequence requirements of the catalytic centre was investigated and an unexpectedly high degree of flexibility for base substitutions in the central part of the core was found; however, the highly conserved region are likely to be directly involved in forming the catalytic site (Zaborowska *et al.* 2005). These findings were the basis of the development of a nuclease-resistant DNAzyme with improved catalytic activity. The kinetic properties of 10-23 DNAzymes compare favorably with those of RNA ribozymes (Santoro and Joyce 1997; Joyce 2001), and in a comparative study of 10-23 DNAzymes and hammerhead ribozymes directed against the same mRNA, the DNAzyme was found to be more active (Kurreck *et al.* 2002). Moreover, DNA molecules are less susceptible to degradation by nucleases in tissues and cell culture, making them considerably more stable for exogenous delivery in biological applications. To develop a potential tool for the HIV Integrase gene therapy, we used a 10-23 catalytic DNAzyme approach. The DNAzyme was originally generated as ssDNA molecules composed of 28 to 31 deoxynucleotides including 15 deoxynucleotide for the catalytic motif and eight for each of the hybridizing arm. These DNAzyme have the ability to bind and cleave the specific mRNA target in the presence of divalent metal ions between any purine and pyrimidine (R-Y) junction, more efficiently than between RU than RC. The specificity of the cleavage originates because of the complementarities between the DNAzyme and the sequences flanking the target mRNA (Roy *et al.* 2008). Although its cleavage site is ubiquitous, it is not always possible for every DNAzyme to cleave the every target with same efficiency, due to the intensive self-folding of the single-stranded RNA molecule (Chandrasekhar and Malathhi 2003) in the cellular milieu. The RNA molecule function, within a particular organic system, is principally determined by its structure. The analysis of RNA secondary structures has been an important task in molecular biology (Reeder *et al.* 2006). So, in order to get some insight into the cellular RNA structure, software-based prediction of full-length RNA structure of the target site seems to be more reliable. Mfold software performs RNA and DNA secondary structure prediction using nearest neighbour thermodynamic rules to minimize the free energy of the predicted structure with increased prediction accuracy (Zuker 2003). Mfold software (Zuker and Stiegler 1981; Mathews *et al.* 1999)-based DNA/RNA folding predictions were considered for this study to get some hint about the structure of the potential target site on the mRNA. But the actual binding and activity will differ *in vivo* from the predicted results with respect to Mfold software because of unpredictable steric and topological constraints.

2. Materials and methods

2.1 *Design and synthesis of 10-23 DNAzymes*

The 10-23 DNAzyme motif was the 23rd clone from round 10 during the course of 10 rounds of *in vitro* selection of a general purpose RNA-cleaving DNAzyme, accordingly termed as 10-23 (Santoro and Joyce 1997). DNAzymes were designed on the basis of three criteria. On the basis of, first, the conserved RNA sequence in the different sub-types and then, next, the presence of purine pyrimidine junction in the predicted target sequence and, at last, their open structure for the accessibility to the DNAzyme using the secondary structure prediction by M Zuker's Mfold (Zuker 2003) Web server for nucleic acid folding and hybridization prediction. So, 864 bases were submitted to Mfold server for the prediction of secondary structure, and the structure with minimum free energy was chosen and examined/gleaned for the other two characteristics. On the basis of the secondary structure prediction results of entire RNA sequence 19 putative target sites were present on Integrase for designing of 10-23 DNAzymes that have unpaired purine pyrimidine junction, specifically the adenine and uracil nucleotide. In our study we chose only those sequences with the lowest $-\Delta G$ values and which have unpaired adenine and uracil junction and scanned the structure for these attributes .We designed three 10-23 DNAzymes that had arm lengths of 14 bases (7 bases substrate recognizing arm sandwiching the 15 base 10-23 DNAzyme motif at 5′ and 3′ end). So, the total length of the antisense DNAzyme was 29 bases. These 10-23 DNAzymes were named DIN54, DIN116, and DIN152 and they are specific to HIV Integrase gene. RIN is the complementary sequence of the DIN116 target site, whereas the DIN (mut) is a mutated DNAzyme (inactive). The sequence GGCTAGCTACAACGA was a specific catalytic domain of 10-23 DNAzyme molecule, which was flanked by two substrate recognition arm of seven to nine deoxynucleotides. All the DNAzymes were synthesized by Ocimum Biosolutions, Hyderabad, India.

2.2 *Synthesis of RNA template*

The DNA template for transcription was obtained by PCR amplification and cloning of HIV Integrase gene from the plasmid pYU-2 into pGEMT Easy Vector using forward primer (5′ ATCAGGAAACATATGTTTTTAGAT 3′) and downstream primer GR (5′ CTTTTCCATCTCGAGATCCT CATC 3′) in Taq buffer (10 mM Tris-HCl, pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin) under the following temperature conditions – (94°C for 4 min)×1, (94°C for 1 min, 56°C for 30 s, 72°C for 1 min)×40, (72°C for 10 min)×1 and finally at 4°C for ∞. This DNA template

contained an Integrase gene of HIV-1 transcript, and after amplification we cloned this PCR-amplified gene into pGEMT Easy Plasmid. The integrase gene was cloned in multiple cloning site of pGEMT Easy Plasmid, which is 50 bp downstream of the T7 RNA polymerase promoter region. Then, the next round of PCR was done on the Integrase cloned pGEMT Easy Vector with the forward primer of M13 and reverse GR primer. PCR reaction was separated on 1% TAE agarose gel and purified using the QIAEX II Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. *In vitro* transcription was carried out using MAXIscript T7 transcription kit (Ambion) according to the accompanying protocol. In brief, ~1 µg of DNA template was incubated in 1×transcription buffer (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$,1 mM DTT, 10 mM NaCl and 2 mM spermidine) with 0.5 mM of each ribonucleotides, T7 RNA polymerase and RNA inhibitor. The radiolabelled transcripts were synthesized by adding 1 µL of [α-^{32}P] UTP to the transcription reaction. RNA transcripts were mixed with 2×formamide loading buffer (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide and 0.5 mM EDTA) and were separated on 4% denaturing TBE polyacrylamide gels. The RNA bands were excised by UV shadowing and were eluted overnight in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) at room temperature. After ethanol precipitation, the RNA was dissolved in RNase free water and quantified by UV-absorbance measurements or scintillation counting. Transcript stock solution was heated at 85°C for 2 min and then snap-cooled in ice; aliquots were stored at −80°C.

2.3 *In vitro RNA cleavage assays*

DNAzyme and *in vitro* synthesized RNA were heated separately for 2 min at 95°C and snap-chilled for 5 min on ice, and after that the DNAzyme and RNA were mixed together in the equimolar quantity. The concentration of RNA transcript was ~2 pmol. The RNA was first incubated at 65°C for 10 min and then at 37°C for 30 min in the reaction buffer (10 mM Na-cacodylate, pH 7.5, 120 mM NaCl, 10 mM $MgCl_2$) with varying concentration of antisense DNAzyme oligomers. After 30 min the samples were cooled on ice, mixed with denaturing loading buffer (5% Glycerol and BFB dye) and analysed on 7 M Urea PAGE containing TBE buffer (150 V at 4°C). The cleavage efficiency of DNAzyme on full-length RNA transcript was estimated with $MgCl_2$, $CoCl_2$, $NiCl_2$ and $MnCl_2$. All quantifications were performed with Image J software.

2.4 *Construction of plasmid*

The DNA fragment of HIV-1 genome, containing Integrase gene with *Xho*I and *Bam*HI restriction sites at the 5′ and 3′ end respectively, was obtained by PCR amplification using sense primer FINTEGFP (5′ ATC AGG AAA CTC CAG TTT TTA GAT 3′) having *Xho*I restriction site and antisense primer reverse GFP (5′ CTT TTC CAT GGA TCC ATC CTC ATC 3′) having *Bam*H1 restriction site. This HIV-1 IN gene was cloned in pGEM-T Easy Plasmid Vector and transformed in *E. coli* DH5α. After plasmid purification, the required DNA fragment was released by the double digestion with *Xho*I and *Bam*HI restriction enzymes. This *Xho*I–*Bam*H1–digested DNA product was ligated with *Xho*I- and *Bam*HI-digested pEGFP-N3 plasmid to generate pIN-EGFP plasmid and transformed in *E. coli* DH5α. The pIN-EGFP plasmid was purified using Endo Free Plasmid Maxi Kit (QIAGEN) according to the manufacturer guidelines and was dissolved in TE buffer (pH7 to 8) before being used for transfection of cell lines.

2.5 *Cell culture*

U-87 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) , and Jurkat was grown in RPMI 1640, and all the cell culture media were supplemented with 10% fetal bovine serum, 100 U of penicillin G/mL, 100 µg of streptomycin/mL, 0.25 µg of amphotericin-B, 0.5 mg of L-glutamine/mL, and 0.2% of sodium bicarbonate. For the growth of jurkat 50 µM β-mercaptoethanol was also added and all the cells were maintained at 37°C in 5% CO_2 at 85–90% relative humidity.

2.6 *Transient transfection of pIN-EGFP plasmid*

The transient transfection of the constructed plasmid was done by PolyFect/Fugene/PEI Transfection Reagent according to the manufacturer guidelines. Briefly, the day before transfection, 1.5×10^5 U-87, 2×10^5 Hela and 1×10^5 Jurkat cells were seeded in 35 mm plates in 2 mL complete DMEM/RPMI medium and incubated at 37°C and 5% CO_2 at 90% humidity. 2 µg of endotoxin-free pIN-EGFP plasmid and 2 µg of DNAzymes were mixed with transfection reagent for 10 min. This transfection mixture was transferred to the cells in 35 mm dish and uniformly distributed over the cells. After 24 h, the efficiency of transfection of plasmids was evaluated through the fluorescence microscopy. Cells were harvested after 48 h of transfection and lysed in 20 mM Tris and 100 mM NaCl for estimation of DNAzyme efficacy.

2.7 *Extraction of cellular protein for Western blotting*

After 48 h cells were lysed in sample lysis buffer (20 mM Tris and 100 mM NaCl pH 8.0) by fthe reeze-thawing method for 3 times in liquid nitrogen. Protein was estimated

using Bradford kit (Bangalore Genei, India). Total of 100 µg of cell lysate was loaded on 10% SDS PAGE for separation and blotting on the supported nitro cellulose membrane.

2.8 *Western blotting*

The membrane was probed overnight with polyclonal anti-Integrase antibody (1:1500). For the loading control, the housekeeping gene β-actin was probed on each blot after striping of the primary anti-Integrase antibody with strip buffer, and then this membrane was probed with mouse monoclonal anti-β-actin HRP-conjugated antibody for 45 min in the dilution of 1:22000, and the membrane was developed after three washes of 5 min each in PBS-T with Diaminobenzidine (DAB) as peroxide substrate in the presence of hydrogen peroxide.

The intensity of the bands was quantitated by Image J software (National Institute of Health, public domain software for densitometry) and densitometry data were analysed on Graph Pad-Prism software.

3. Results

3.1 *Construction of RNA-cleaving DNA enzymes and selection of target sites in the HIV Integrase gene*

The DNAzyme binds the substrate through Watson–Crick base pairing and cleaves a particular phosphodiester linkage located between an unpaired purine and paired pyrimidine residue in the presence of divalent cations. From the secondary structure prediction results of the entire RNA of the HIV Integrase gene using Mfold software, we designed three DNAzymes with the lowest ΔG values. All DNAzyme target sequences show approximately 100% similarity to all the HIV Integrase sequence known till date. Therefore, in order to make our antisense more effective, the nucleotide position at 162 (DIN54), 341 (DIN116) and 456 (DIN152) were chosen for DNAzyme target. Of the three DNAzymes, DIN54 was designed to target nucleotide position 162 at the guanine–uracil junction and DIN116 and DIN152 were targeted to cleave the adenine–uracil junction at the 341st and 456th nucleotide on the full-length Integrase mRNA transcript. The amino acid at 456th nucleotide (glutamic acid) is responsible for the tethering of the divalent metal ion. The other criterion that was considered for the designing of DNAzyme was to check the sequence for the CpG islands, because these kind of sequences have been reported to give nonspecific immune response to the body that sometimes mask the actual effect of antisense. So the DNAzyme sequences for the evaluation were made from these position, and the mutated DNAzyme was designed from the 341st nucleotide position of the integrase gene in which the two nucleotides from the catalytic domain were reshuffled with each other (position underlined) but the target recognition arm was kept intact so that it can bind the target with equal affinity as the actual catalytic DNAzyme. The complementary DNA sequence was also chosen from the 341st position of the nucleotide sequence, but the only difference with the DIN116 is that it did not contain catalytic motif (table 1).

3.2 *pIN-EGFP fusion expression plasmid construction*

Using standard recombinant techniques, HIV-1 Integrase gene with 864 bases was amplified and fused with the N-terminus of EGFP in pEGFP-N3 vector. Recombinant clones were screened for the correct size of HIV-1 Integrase insert by *Xho*I and *Bam*H1 digestion. Recombinant clones were also confirmed by sequencing (data not shown). When these recombinant plasmid were transfected into all three cell lines, Integrase-EGFP fusion protein was expressed, as shown in figure 1A. Green fluorescence was mainly located at the cytoplasm or the periphery of the nucleus in granules in jurkat cell line (figure 1 B).

3.3 *Synthesis of HIV-1 Integrase RNA and in vitro cleavage reaction*

A DNA fragment of 969 bp was amplified from the pGEMT IN clone by using the M13 forward and GR primer, and this fragment was eluted from the 1% agarose gel and used for the *in vitro* transcription reaction. The schematic representation of the template amplification and *in vitro* transcription is given in figure 2. After extraction and purification of the desired DNA fragment (969 bp) from agarose gel, the purity of template was checked on the 1.2% agarose gel (figure 2B). The schematic representation of exact size and position of this DNA template is depicted in figure 2A. The HIV Integrase was synthesized by T7 RNA polymerase through *in vitro* transcription using a PCR-generated DNA template. T7 polymerase initiate the transcription from the transcription initiation site which is usually 3 to 10 bases downstream from the T7 promoter. The length of the *in vitro* synthesized RNA was 915 bases (figure 2C). The RNA transcript (915 bases) was eluted through the crush and soak method in 1×TE (pH 7.5) buffer at room temperature. The purity of extracted RNA transcript (915 bases) was found more than 99% on 4% denaturing TBE gel.

3.4 *In vitro cleavage of HIV Integrase RNA by DNAzymes DIN 116, DIN 54 and DIN 152*

The labelled transcript (915 nt long; figure 3) was subjected to *in vitro* cleavage by adding equimolar amounts

Table 1. List of DNAzymes used as antisense against HIV Integrase mRNA

DNAzyme	Sequence of DNAzyme	Position at HIV-1 RNA genome
DIN 54	5′CTGTCTA**GGCTAGCTACAACGA**TTGCCCA 3′	153–167 base
DIN116	5′TCTGTAT**GGCTAGCTACAACGA**GTATTGT 3′	334–347 base
DIN152	5′TTCATGT**GGCTAGCTACAACGA** TCTACTA 3′	451–465 base
RINTEGFP	5′ TCTGTATT----------GTATTGT 3′	334–347 base
DIN(mut)	5′TCTGTAT**GGCATGCTACAACGA**GTATTGT 3′	334–347 base

*DIN54, DIN116, and DIN152 have been designed based upon the secondary structure of respective target RNA. DIN (mut) is the sequence of DNAzyme having mutated catalytic core domain and RIN is the complementary strand of DNA without the 10-23 catalytic domain.

(100 pmol) of DNAzyme (cold) in the presence of $MgCl_2$ (10 mM). Of the three DNAzymes, only DIN116 worked efficiently at 20 mM $MgCl_2$. DIN152 and DIN54 did not work at all *in vitro* (data not shown). Almost 70% to 80% cleavage of the target RNA was observed by DIN116 in the presence of 20 mM $MgCl_2$.The appearance of 523-base-long RNA fragment and 392-base-long RNA fragment could only appear if the target RNA was cleaved at the specific site. We have also screened the cleavage efficiency of DIN116 in different divalent metal ions at varying concentration and found that DIN116 worked efficiently with $MnCl_2$ and $MgCl_2$. $ZnCl_2$ and $CoCl_2$ did not work well at lower concentration (figure 3A–D). The mutated DNAzyme did not cleave the RNA transcript. The maximum activity was found in the Mn^{++} (figure 3C) followed by Mg^{++} (figure 3A), Co^{++} (figure 3B) and Zn^{++} (figure 3D).

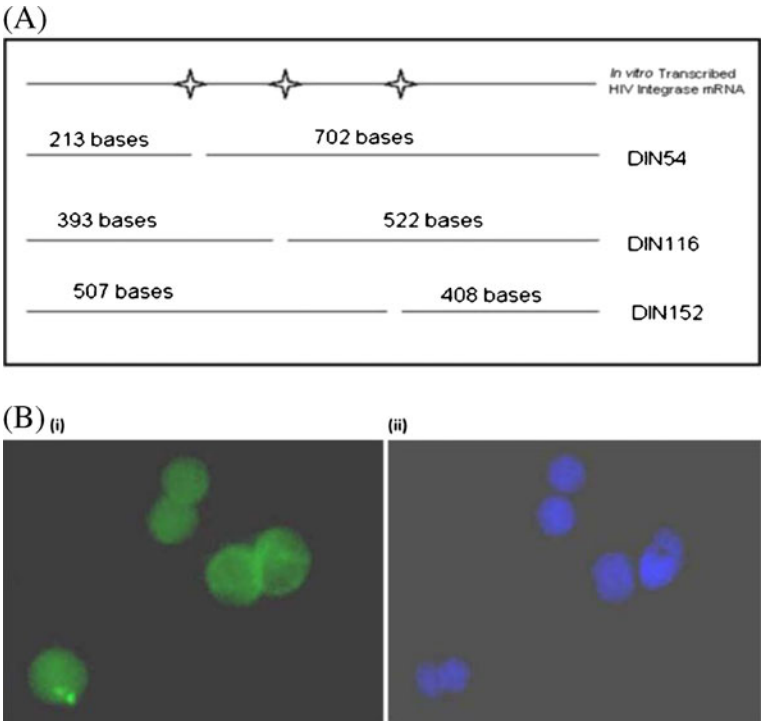

Figure 1. (**A**) Expected cleavage pattern of HIV Integrase RNA by different DNAzymes: Schematic line diagram for the different target sites of three DNAzymes used in the study and their expected product size. The full length of *in vitro* transcribed HIV Integrase mRNA is 915 bases. (**B**) HIV Integrase expression (FITC) staining in Jurkat cells: Jurkat cells were fixed after 24 h of transfection with 4% Paraformaldehyde for 10 min, and cells were permeabilized with 0.02% Triton X-100 for 5 min. These permeabilized cells were blocked with 1% mouse pre-immune sera and then probed with Anti HIV Integrase antibody (1:500 in BSA) for 2 h at 40°C. Cells were washed with PBS-T for 3 times and primary antibody was detected/captured by goat anti-mouse FITC conjugated antibody in dilution of 1:600 (**i**). Nucleus was stained with DAPI (**ii**).

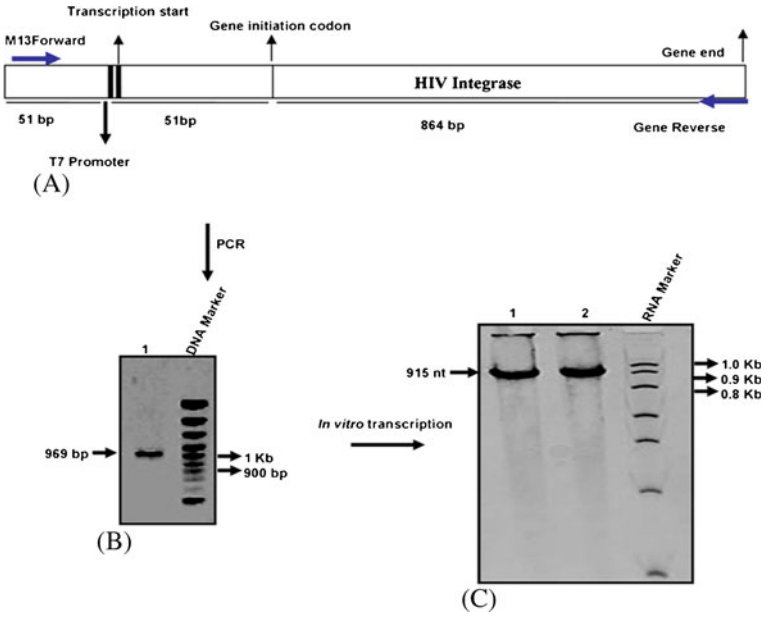

Figure 2. PCR amplification of HIV Integrase gene and synthesis of RNA transcript: PCR-amplified HIV Integrase DNA was incorporated downstream of T7 promoter in the pGEMT Easy Vector. M13 forward sequencing primer and HIV IN gene reverse primer were used to amplify the 969-base-pair-long HIV-IN DNA template. T7 polymerase was used to synthesize the RNA transcript form the transcription start site. (**A**) Schematic diagram for transcript generation strategy. (**B**) PCR amplification of HIV IN gene from the pYU-2 vector using M13 forward and gene reverse primer. Lane number 1 contains PCR-amplified DNA template for *in vitro* transcription. (**C**) The purified transcript was run on 4% Urea PAGE. Lane numbers 1 and 2 show the purified transcript, and the RNA marker is in lane number 3.

3.5 *Inhibition of IN-EGFP expression by 10-23 DNAzyme in the cell lines*

Transient transfection of U-87, HeLa and Jurkat cell with the pIN-EGFP resulted in the expression of IN-GFP fusion protein as analysed by fluorescence microscopy. As a control, expression of GFP from plasmid pEGFPN3 was verified in parallel. With both these plasmids, all the cells could be transfected with a transfection efficiency of 70% to 80%. The IN-EGFP fusion protein was probed with the polyclonal HIV Integrase antibody developed in mouse. The expression varied form one cell line to another; for example, expression of Integrase GFP fusion protein was maximum in Jurkat (figure 4A) and minimum in HeLa (figure 4B), whereas the U-87 (figure 4C) has medium level of expression in comparison with the other two cell lines. Our Western blot with these cell lines gave us two bands, one corresponds to the fusion protein which has molecular weight of about 60 kDa and the other nonspecific band at approximately at 72 kDa. DNAzymes were co-transfected along with pIN-EGFP. DIN116 showed maximum cleavage of IN-EGFP mRNA, and therefore, the expression of IN-EGFP fusion protein dropped to 75% in comparison with untreated control cells; DIN152 also gave approximately 70% inhibition of expression of IN-EGFP fusion protein in the Jurkat cells (figure 4A), whereas DIN54 showed only 20%, DIN gave 35% and RIN gave 47% inhibition. In the case of HeLa cells, DIN116 again gave 60%, DIN152 showed 50%, DIN54 10%; DIN and RIN gave 20% inhibition of IN-EGFP fusion protein in comparison with the control cells with no DNAzymes. U-87 cells also behaved in the same manner as Jurkat and gave 80% inhibition after treatment with DIN116 and DIN152, whereas in contrast to the Jurkat and HeLa cells, DIN54 showed much higher degree of inhibition and it reached approximately 52% in U-87 cells. The other two DIN (mut) and RIN showed 30% and 35% inhibition of IN-EGFP protein in comparison to the control cells at 2 µg DNA (figure 4A−C). All the experiments were repeated thrice and the densitometry values of IN-EGFP were normalized from the respective β-actin band for loading control before calculating the fold change in expression of any experiment set (figure 4 D–F).

4. Discussion

DNA has the ability to bind the substrate molecule with high affinity and specificity, which is a prerequisite of a good

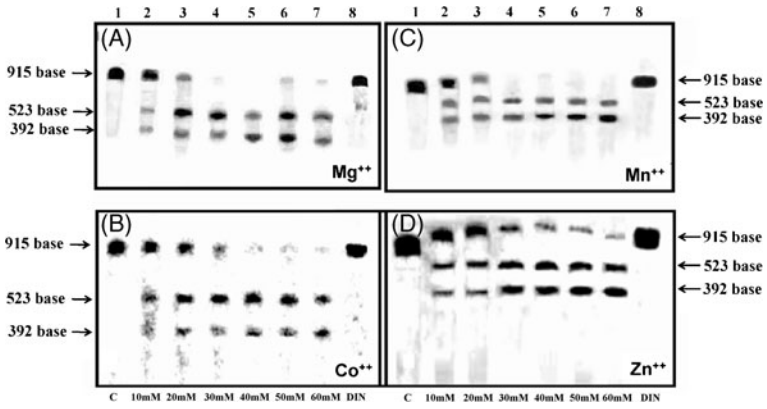

Figure 3. *In vitro* cleavage activity of DNAzyme (DIN116) in the presence of Mg^{++}, Mn^{++}, Zn^{++} and Co^{++}. Full-length α-UTP labeled RNA transcript (915 nucleotide long) was put in reaction with unlabelled DNAzyme (DIN116) in equimolar ratio to a final volume of 10 µL in 10 mM sodium cacodylate and 150 mM NaCl. Cleavage was initiated by adding $MgCl_2$, $MnCl_2$, $CoCl_2$ and $ZnCl_2$ in concentration of (10–60 mM) in lane number 2 to 7 and full-length RNA in lane number 1, whereas the mutated DNAzyme (DIN) was added in the lane number8.

enzyme. DNAzymes represent promising candidates for drug therapy in a wide range of diseases, such as cancer and cardiovascular disorders (Chan and Khachigian 2009). Breaker and co-workers (Breaker and Joyce 1994) and Santoro and Joyce (Santoro and Joyce 1997) used a unique selection strategy for identification and development of catalytic motif in the single-stranded DNA molecule. Their selection strategy yielded two different catalytic DNA sequences, 8-17 and 10-23 DNAzymes. The 10-23 DNAzymes cleave between any unpaired purine and pyrimidine of mRNA transcripts. Although they are abundant in mRNA, many of these potentially cleavable junctions are

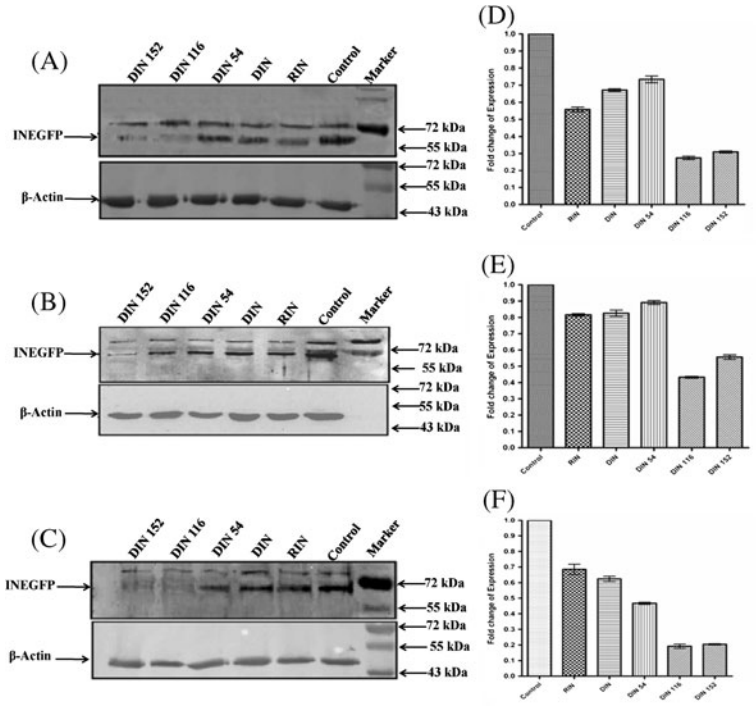

Figure 4. Effect of 10-23 DNAzymes treatment on HIV-IN expression in IN-EGFP-transfected cell lines: The figure shows the Western blot of IN-EGFP expression following DNAzymes treatment in Jurkat (**A**), HeLa (**B**) and U-87 (**C**), respectively. The densitometric analysis illustrated that DIN116 and DIN152 showed the maximum inhibition of Integrase protein expression in all three cell lines, namely, Jurkat (**D**), Hela (**E**) and U-87 (**F**).

protected from DNAzyme activity by secondary structure. The DNAzymes can be designed specifically to target the AU nucleotide of the start codon, which make them promising candidates for potential applications in functional genomics and gene therapy (Unwalla and Banerjea 2001; Achenbach *et al.* 2004; Dash and Banerjea 2004; Goodchild 2004; Hou *et al.* 2006; Joyce 2004; Lu *et al.* 2005; Mitchell *et al.* 2004; Peracchi 2004; Schubert and Kurreck 2004; Silverman 2004;Silverman 2005; Xie *et al.* 2006). DNazyme can be applicable in locations of more effective and most accessible target sites on mRNA (Cairns and Sun 2004).

The secondary structure of a nucleic acid molecule refers to the base-pairing interactions within a single molecule or set of interacting molecules. The secondary structure of biological RNAs can often be uniquely organized into stems and loops. The inaccessibility of the target sequence is a major concern in the design of therapeutic antisense strategies against highly structured viral RNA genomes. So, structural analysis of viral RNA genome can help in identification of putative target sites. For more effective DNAzymes, the complementary target sequence on mRNA must be available for hybridization. RNA nucleotides can be inaccessible when they are sequestered in secondary structure in cellular milieu. There is compelling evidence that the secondary structure determines the accessibility of mRNA for the gene regulatory mechanisms of the most antisense molecules that require complementary base-pairing for target recognition (Wang *et al.* 2011). We used Mfold software to predict HIV-1 Integrase RNA secondary structure to select the more accessible target sites. Hence, we designed and synthesized 10-23 DNAzymes according to the predicted target site with respect to Mfold analysis.

The rationale behind this work was to design effective 10-23 DNAzymes against HIV-1 Integrase, with the aim to interfere with the replication of HIV-1 in a sequence-specific manner. The major advantages of using DNAzyme over any other antisense are its simplicity of designing, target-specific cleavage, serum stability and catalytic activity. We synthesized three DNAzymes, all possessing the earlier identified 10-23 catalytic motif. For the target site selection we have scanned the full-length HIV-IN mRNA structure using Mfold software and choose DIN54, DIN116 and DIN152 to be ideal targets. These target sites have shown high degree of conservation among the different strains of HIV-1 (Unwalla *et al.* 2006) and are less compactly folded, thus allowing ample space for DNAzymes binding and activity. The DIN54 target site was present in the left-hand side of the tip of the loop where G and U nucleotides were accessible for the cleavage, whereas DIN116 and DIN152 were made to target the A and U junction on the RNA sequence, which have minimum steric hindrance for better accessibility. The target site of DIN116 and DIN152 are much more accessible to DNAzyme binding than that of DIN54, as it is evident from the Mfold structure of the HIV-IN mRNA.

The DIN116 showed the significant target-specific cleavage of full-length RNA, in cleavage experiments, whereas the other two DNAzymes did not cleave the target. Like the previously reported 10-23 DNAzyme, DIN116 also showed the maximum activity in presence of Mn^{++}. Mg^{++} showed slightly less activity than Mn^{++}, but Co^{++} and Zn^{++} had marked reduction on DIN116 cleavage activity. The efficiency of the cleavage increased significantly for DIN116 in the presence of increasing concentration of divalent metal ions (10–60 mM). As expected, a point mutation in the catalytic motif abolished the ability of the DIN116 to cleave the target RNA completely (DIN (mut)).

Another key question addressed in this work is that DNAzymes have the ability to specifically interfere with the expression of the HIV-IN when co-transfected into the mammalian cells along with the pIN-EGFP vector. Since fluorescence is most convenient marker for these kind of studies, EGFP was selected as a reporter molecule, which has already been used to study ribozyme (Passman *et al.* 2000; Beger *et al.* 2001) and RNAi (Nagy *et al.* 2003; Cao *et al.* 2004; Qin *et al.* 2004) and DNAzymes (Hou *et al.* 2006). This experiment was performed to assess the efficiency of all the three DNAzymes in physiological conditions. The inhibition was very significant in comparison with the untreated cells. Each of the DNAzymes was co-transfected in the HeLa, Jurkat and U-87 cell lines with pIN-EGFP construct. DIN116 and DIN152 reduced the level of expression of IN-EGFP expression up to 80% (0.2-fold), whereas DIN54, mutated DNAzyme (DIN mut), and a reverse complementary strand without the 10-23 motif (RIN) could inhibit the expression of INEGFP only up to 20% to 40%. In the case of U-87 cells and Jurkat cells, IN-EGFP expression dropped to 80% and 75% after treatment of the cells with DIN116 and DIN152 respectively, whereas DIN54, DIN (mut) and RIN inhibited the expression by 30%, 35% and 45% respectively in comparison withthe untreated control cells. The inhibition which we are getting in the case of DIN (mut) and RIN is due to its antisense effect rather than its catalytic activity. These two DNA sequences were used to differentiate the antisense and DNAzyme activity. Careful examination of the software-generated secondary structure revealed a difference in the target site of DIN54, DIN116 and DIN152. From the secondary structure, it seems that the target site of DIN116 and DIN152 is more accessible to DNAzymes binding but DIN54, being trapped in the secondary structure, might not be available for binding to DNAzymes, which could be the probable reason for its inefficient cleavage activity.

In addition to the activity, the specificity and inhibitory effects by these DNAzymes were also demonstrated. All the

results suggest that these 10-23 DNAzymes could inhibit the HIV Integrase gene expression *in vitro*, and could be useful strategy for gene therapy.

Acknowledgement

NS and SS are grateful to University Grants Commission (UGC), India, for the Senior Research Fellowship. AR is grateful to Council of Scientific & Industrial Research (CSIR). This work was funded by the grant obtained from UGC and Department of Science and Technology (DST), India.

References

Achenbach JC, Chiuman W, Cruz RP and Li Y 2004 DNAzymes: from creation in vitro to application in vivo. *Curr. Pharm. Biotechnol.* **5** 321–336

Beger C, Pierce LN, Kruger M, Marcusson EG, Robbins JM, Welcsh P, Welch PJ, Welte K, *et al.* 2001 Identification of Id4 as a regulator of BRCA1 expression by using a ribozyme-library-based inverse genomics approach. *Proc. Natl. Acad. Sci. USA* **98** 130–135

Breaker RR and Joyce GF 1994 ADNA enzyme that cleaves RNA. *Chem. Biol.* **1** 223–229

Cairns MJ and Sun LQ 2004 Target-site selection for the 10–23 DNAzyme. *Methods Mol. Biol.* **252** 267–277

Cao M, Ren H, Pan X, Pan W and Qi ZT 2004 Inhibition of EGFP expression by siRNA in EGFP-stably expressing Huh-7 cells. *J. Virol. Methods* **119** 189–194

Chan CW and Khachigian LM 2009 DNAzymes and their therapeutic possibilities. *Intern. Med. J.* **39** 249–251

Chandrasekhar K and Malathhi R 2003 Non-Watson Crick base pairs might stabilize RNA structural motifs in ribozymes – a comparative study of group-I intron structures. *J. Biosci.* **28** 547–555

Dash BC and Banerjea AC 2004 Sequence-specific cleavage activities of DNA enzymes targeted against HIV-1 Gag and Nef regions. *Oligonucleotides.* **14** 41–47

Evering TH and Markowitz M 2008 Raltegravir: an integrase inhibitor for HIV-1. *Expert Opin. Investig. Drugs* **17** 413–422

Goila R and Banerjea AC 1998 Sequence specific cleavage of the HIV-1 coreceptor CCR5 gene by a hammer-head ribozyme and a DNA-enzyme: inhibition of the coreceptor function by DNA-enzyme. *FEBS Lett.* **436** 233–238

Goodchild J 2004 Oligonucleotide therapeutics: 25 years agrowing. *Curr. Opin. Mol. Ther.* **6** 120–128

Gupta SP and Nagappa AN 2003 Design and development of integrase inhibitors as anti-HIV agents. *Curr. Med. Chem.* **10** 1779–1794

Hou W, Ni Q, Wo J, Li M, Liu K, Chen L, Hu Z, Liu R and Hu M 2006 Inhibition of hepatitis B virus X gene expression by 10–23 DNAzymes. *Antiviral Res.* **72** 190–196

Joyce GF 2001 RNA cleavage by the 10–23 DNA enzyme. *Methods Enzymol.* **341** 503–517

Joyce GF 2004 Directed evolution of nucleic acid enzymes. *Annu. Rev. Biochem.* **73** 791–836

Kurreck J, Wyszko E, Gillen C and Erdmann VA 2002 Design of antisense oligonucleotides stabilized by locked nucleic acids. *Nucleic Acids Res.* **30** 1911–1918

Lu ZX, Ye M, Yan GR, Li Q, Tang M, Lee LM, Sun LQ and Cao Y 2005 Effect of EBVLMP1 targeted DNAzymes on cell proliferation and apoptosis. *Cancer Gene Ther.* **12** 647–654

Mathews DH, Sabina J, Zuker M and Turner DH 1999 Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* **288** 911–940

Mitchell A, Dass CR, Sun LQ and Khachigian LM 2004 Inhibition of human breast carcinoma proliferation, migration, chemoinvasion and solid tumour growth by DNAzymes targeting the zinc finger transcription factor EGR-1. *Nucleic Acids Res.* **32** 3065–3069

Nagy P, Arndt-Jovin DJ and Jovin TM 2003 Small interfering RNAs suppress the expression of endogenous and GFP-fused epidermal growth factor receptor (erbB1) and induce apoptosis in erbB1-overexpressing cells. *Exp. Cell Res.* **285** 39–49

Ota N, Warashina M, Hirano K, Hatanaka K and Taira K 1998 Effects of helical structures formed by the binding arms of DNAzymes and their substrates on catalytic activity. *Nucleic Acids Res.* **26** 3385–3391

Passman M, Weinberg M, Kew M and Arbuthnot P 2000 In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis Bx sequence in cultured cells. *Biochem. Biophys. Res. Commun.* **268** 728–733

Peracchi A 2004 Prospects for antiviral ribozymes and deoxyribozymes. *Rev. Med. Virol.* **14** 47–64

Qin ZL, Zhao P, Zhang XL, Yu JG, Cao MM, Zhao LJ, Luan J and Qi ZT 2004 Silencing of SARS-CoV spike gene by small interfering RNA in HEK 293 T cells. *Biochem. Biophys. Res. Commun.* **324** 1186–1193

Reeder J, Hochsmann M, Rehmsmeier M, Voss B and Giegerich R 2006 Beyond Mfold: recent advances in RNA bioinformatics. *J. Biotechnol.* **124** 41–55

Roy S, Gupta N, Subramanian N, Mondal T, Banerjea AC and Das S 2008 Sequence-specific cleavage of hepatitis C virus RNA by DNAzymes: inhibition of viral RNA translation and replication. *J. Gen. Virol.* **89** 1579–1586

Santoro SW and Joyce GF 1997 A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. USA* **94** 4262–4266

Saxena V and Dhole TN 2008 Preventive strategies for frequent outbreaks of Japanese encephalitis in Northern India. *J. Biosci.* **33** 505–514

Schubert S and Kurreck J 2004 Ribozyme- and deoxyribozyme-strategies for medical applications. *Curr. Drug Targets* **5** 667–681

Silverman SK 2004 Breaking up is easy to do (if you're a DNA enzyme that cleaves RNA). *Chem. Biol.* **11** 7–8

Silverman SK 2005 In vitro selection, characterization, and application of deoxyribozymes that cleave RNA. *Nucleic Acids Res.* **33** 6151–6163

Unwalla H and Banerjea AC 2001 Novel mono- and di-DNA-enzymes targeted to cleave TAT or TAT-REVRNA inhibit HIV-1 gene expression. *Antiviral Res.* **51** 127–139

Unwalla H, Chakraborti S, Sood V, Gupta N and Banerjea AC 2006 Potent inhibition of HIV-1 gene expression and TAT-mediated apoptosis in human T cells by novel mono- and multitarget anti-TAT/Rev/Env ribozymes and a general purpose RNA-cleaving DNA-enzyme. *Antiviral Res.* **72** 134–144

Wang Y, Shen J, Shang X, Wang J, Li J, Yin J and Zou C 2011 Ezrin mRNA target site selection for DNAzymes using secondary structure and hybridization thermodynamics. *Tumour Biol.* **32** 809–817

Warashina M, Kuwabara T, Nakamatsu Y and Taira K 1999 Extremely high and specific activity of DNA enzymes in cells with a Philadelphia chromosome. *Chem. Biol.* **6** 237–250

Wu Y, Yu L, McMahon R, Rossi JJ, Forman SJ and Snyder DS 1999 Inhibition of bcr-abl oncogene expression by novel deoxyribozymes (DNAzymes). *Hum. Gene Ther.* **10** 2847–2857

Xie YY, Zhao XD, Jiang LP, Liu HL, Wang LJ, Fang P, Shen KL, Xie ZD, Wu YP and Yang XQ 2006 Inhibition of respiratory syncytial virus in cultured cells by nucleocapsid gene targeted deoxyribozyme (DNAzyme). *Antiviral Res.* **71** 31–41

Zaborowska Z, Schubert S, Kurreck J and Erdmann VA 2005 Deletion analysis in the catalytic region of the 10–23 DNA enzyme. *FEBS Lett.* **579** 554–558

Zhang X, Xu Y, Ling H and Hattori T 1999 Inhibition of infection of incoming HIV-1 virus by RNA-cleaving DNA enzyme. *FEBS Lett.* **458** 151–156

Zuker M 2003 Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31** 3406–3415

Zuker M and Stiegler P 1981 Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* **9** 133–148