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HEPATOLOGY

Site-specific cleavage of HCV genomic RNA and its cloned core and NS5B genes by DNAzyme

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Key words
gene expression, HCV treatment, viral hepatitis, virology.

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Abbreviations:
CDz, Core gene specific DNAzyme; Dz, DNAzyme; DTT, Dithiothreitol; EGFP, Enhanced green fluorescent protein; FITC, Fluorescein isothiocynate (tetra-chloro-fluorescein); IRES, Internal ribosome entry site; LNA, Locked nucleic acid; MDz, Mutant DNAzyme; NS, Non-structural; NDz, NS5B gene specific DNAzyme; RDRP, RNA dependent RNA polymerase; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; UTR, Untranslated region.

Abstract

Background and Aims: The 9600 nt hepatitis C virus (HCV) genomic RNA has only one internal ribosome entry site (IRES) for translation to a single polyprotein. In search of nucleic acid-based antiviral agents, two 10-23 DNAzymes were designed to cleave the RNA in IRES and RNA dependent RNA polymerase (RDRP/NS5B) regions to prevent translation and replication of HCV RNA.

Methods: *In vitro* cleavage of HCV RNA by IRES specific DNAzyme, CDz and NS5B specific DNAzyme, NDz was carried out using HCV genomic RNA and *in vitro* synthesized runoff transcripts of core and NS5B genes. Cleavage of core and NS5B mRNAs by DNAzyme (Dz) in HepG2 cells was assessed by reverse transcription polymerase chain reaction (RT-PCR) using RNA from cells co-transfected with cloned core or NS5B gene and its respective DNAzyme. Suppression of core or NS5B protein expression due to mRNA cleavage by Dz in co-transfected cells was determined by Western blot analysis and fluorescence intensity of fluorescent-tagged expressed protein. Reduction of NS5B protein activity in NDz co-transfected cells was determined by enzymatic assays.

Results: The designed CDz and NDz cleaved HCV genomic RNA and their respective *in vitro* generated transcripts. Both mRNA and protein expressions of core or NS5B from their cloned genes reduced substantially when co-transfected with respective Dz. Reduction of RDRP expression by NDz was accompanied with its reduced enzyme activity. Increased RNA cleavage, inhibition of protein expression, and reduction of RDRP activity were observed on increasing Dz concentration.

Conclusion: Core and NS5B targeted DNAzymes can be used in controlling the replication of HCV RNA.

Introduction

The hepatitis C virus (HCV) genome is a 9600 nt uncapped single-stranded RNA with positive polarity. It contains 5′ and 3′ untranslated regions (UTRs) including regions to control translation and replication.[1] The UTRs flank an open reading frame encoding a polyprotein of 3010 or 3011 amino acids. The polyprotein is processed to structural (C, E1, E2, p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B or RDRP) proteins by host and virus encoded proteases.[2,3]

The chronic nature of HCV infection, high prevalence all over the globe, and the significant morbidity of the resulting disease make it a serious threat on public health.[4] Despite great strides in the development of an antiviral therapy, current medical treatment options are still limited. In spite of encouraging results from present day interferon (IFN)-based therapies, about 50% of the patients do not obtain sustained virological response.[5] Several small inhibitory molecules, novel immunomodulatory agents and nucleic acid-based antiviral agents that showed encouraging results are being tested, and the search for a proper combination of multiple therapeutic agents appears to be the need of the hour.

The 10–23 DNAzymes[6] are 27–33 nt synthetic deoxyribonucleotides having 15 nt conserved catalytic domain flanked by two 6–9 nt substrate binding arms. The DNAzyme cleaves the substrate RNA in between any purine-pyrimidine junction and loses its catalytic activity on mutation in the conserved catalytic domain.[7] The modification of its one or both binding arm nucleotides with the locked nucleic acids (LNA) enhances its substrate binding affinity and leads to higher catalytic activity.[8,9] Low cost of synthesis, sequence specificity, and efficient cleavage activity both *in vitro* and *in vivo* make DNAzyme an attractive tool in nucleic acid-based therapies.[10] For this study two DNAzymes, CDz

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targeting 5′UTR-Core, and NDz targeting invariable region of NS5B were designed. The NDz site is apparently free from secondary structure while CDz site has the secondary structure as indicated by computer predicted structures (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). Both these targets are important for antiviral drug development, because the 5′UTR having IRES is involved in generation of positive and negative strands, and NS5B encodes the viral replicative enzyme, RNA dependent RNA polymerase.[3,11] Here, we report the cleaving of HCV RNA at these pre-determined sites by designed Dzs and suppression of protein expressions of the targeted genes.

Methods

Oligonucleotides

Two pairs of nested polymerase chain reaction (PCR) primers for making cDNA clones to generate HCV 5′ UTR-Core and NS5B mRNA fragments were 5′UTR-Core: 5′-CTGTGAGG AACTACTGTCTT-3′ (Outer FP), 5′-ATGTACCCCATGAKRTC GGC-3′ (Outer RP), 5′-ACGCAGMAGCGTCTAG-3′ (Inner FP), 5′-CAYGTRAGGGTATCGATGAC-3′ (Inner RP); NS5B: 5′-TG GGGATCCCGTATGATACCCGCTGCTTTGA-3′ (Outer FP), 5′-GGCGGAAT(T/A)CCTGGTCATAGCTCCGTGAA-3′ (Outer RP), 5′- GACTCAAC(C/T)GTCACTGAACAGGACAT-3′ (Inner FP), 5′-CCACGAC(T/C)AGATC(A/G)TCTCCG-3′ (Inner RP). Real time PCR primers for core were 5′-CGGGCAGATCGTTGG TGGAG-3′ (FP) and 5′-GAGAGCCGCGAGGGGACAG-3′ and for NS5B the same inner sets of primers as written above were used. The full length NS5B was amplified using three separate sets of nested primers with overlapping sequences followed by overlapping PCR for ligation. For cloning 6X-His-tag core in pEGFP-N3 vector, forward primer 5′-ATGCATCATCACCAT CACCACATGAGCACACTT-3′ and reverse primer 5′-CGTTA GGGTATCGATGACTTTACC-3′ and for cloning 6X-His-tag NS5B in pEGFP-N3, 5′-ATGCATCATCACCATCACCACGGA ATTCCG-3′ (FP) and 5′-TCGAGCTGGCAAGAGAAAG-3′ (RP) were synthesized. For constructing the pEGFP-N3-TAG vector, the same forward primer but a modified reverse primer, 5′-CTATCGAGCTGGCAAGAGAAAG-3′ were synthesized. Different restriction sites were incorporated at the 5′ ends of the primer sets for cloning in expression vectors. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of HepG2 Cells were: 5′-AAGGCTGGGGCTCATTTGCA-3′ (FP), 5′-GTGCAGGAGGCATTGCTGATGA-3′ (RP). All the primers were synthesized from TCGA (New Delhi, India). DNAzymes, CDz: 5′-ccttggggaGGCTAGCTACAACGAaggctgccg-3′(targeting core), NDz: 5′-ctagcacggGGCTAGCTACAACGAagcggcgat-3′ (targeting NS5B) and their corresponding mutant counterparts (CMDz and NMDz) were made by Sigma (St Louis, MO,USA). Mutant DNAzymes contained TG in place of GC (underlined) in their catalytic domain. The capitalized 15 nucleotide sequence of the DNAzyme represents the catalytic domain and 9 nucleotide flanking sequences (written in small letters) indicate the substrate binding domains. All the DNAzymes and their mutants have 5′ phosphorothioate linkage and 3′ CPG-amine C7 cap. Random hexamers for the first strand cDNA synthesis were part of the cDNA synthesis kit (Clontech, Mountain View, CA, USA).

Virus strain and cell lines

Hepatitis C virus RNAs were isolated from the sera of 5 patients infected with HCV genotype 3 strains. Mammalian cell line HepG2 was obtained from the International Centre for Genetic Engineering and Biotechnology, New Delhi, India.

Isolation of HCV genomic RNA and reverse transcription (RT)-PCR

Genomic RNA from 5 genotype 3 strains of HCV were isolated from sera of five patients using QIA Viral RNA isolation kit, (Qiagen, Hidden, Germany) following the supplier's instructions. Regions of HCV RNA encompassing core 5′UTR-Core (–273 to +384 nt) and NS5B (8301–8593 nt) protein encoding genes of HCV strains were reverse transcribed and amplified by RT-PCR[12] using primer sets as described above. Briefly, cDNAs of core and NS5B were synthesized using the outer reverse primer of the respective region by reverse transcription reactions. The cDNAs were used as templates in the PCRs with the respective outer set of primers. The PCR product obtained (first PCR) was seeded as a template with respective inner sets of primers for amplification. The PCR product obtained after the second round of amplification (nested) was electrophorsed in 2% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator to estimate the size, and purified from agarose gel using a gel extraction kit (Qiagen, Valencia, CA, USA).

Construction of vectors

The cDNA of 657 bp (Genbank Accession no. **EF025301**) with 5′ UTR-Core (–273 to +384 nt) was cloned at *Bam*H1/*Hind*III sites of pSPT19 vector (Roche Applied Sciences, Indianapolis, IN, USA) and 293 bp of NS5B (8301–8593 nt) was also cloned at this site of the same vector after amplification of the partial sequence (732–1024 bp) from full length NS5B cDNA clone (Genbank Accession no. **DQ899730**) to generate pSPT19-core and pSPT19-NS5B vectors respectively.

The partial sequence of core gene (+1 to +381 nt) was amplified from 5′ UTR-Core clone (–273 to +384 nt) and ligated to *Eco*R1/*Kpn*1 sites of pKS(+), similarly full length cDNA of NS5B gene was also cloned in this vector at the same site and sequenced.

Both partial core (+274 to +654 nt of core clone, Genbank Accession no. **EF025301**) and full length NS5B (Genbank Accession no. **DQ899730**) cDNAs were incorporated in mammalian expression vector pEGFP-N3 (BD Biosciences Clontech, Palo Alto, CA, USA) at *Eco*RI/*Kpn*I restriction sites to generate recombinant expression vectors pEGFP-N3-Core and pEGFP-N3-NS5B to express recombinant proteins with 6X His tag at the N-terminal ends and EGFP (enhanced green fluorescence protein) at the C-terminal ends. The pEGFP-N3-NS5B-TAG vector was constructed by inserting the TAG stop codon before the EGFP coding sequence of pEGFP-N3-NS5B vector to express NS5B without EGFP at the C-terminal end. For this, the insert of the pEGFP-N3-NS5B was amplified with the same forward and reverse primers as mentioned before except that the reverse primer contained CTA overhang at the 5′ end.

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In vitro generation of short core and NS5B transcripts

DNAs of pSPT19 clones having 657 bp insert of 5′ UTR-Core (−273 to +384) and 293 bp of NS5B (+8301 to +8593 nt) having CDz and NDz recognition sites respectively were linearized with *Hind*III (NEB) and were used as templates for the generation of their runoff transcripts in *in vitro* reactions. SP6 RNA polymerase directed (α-^{32}P)-UTP-labeled runoff transcripts were synthesized and purified following the protocols described by the supplier of the *in vitro* transcription kit (Roche Applied Science).[13]

In vitro cleavage of HCV genomic RNA and short transcripts

One μg of HCV genomic RNA from each of the five patients and 0.5 μg of α-^{32}P labeled short *in vitro* core (657 nt, −273 to +384) and NS5B (293 nt, +8301 to +8593) were used in *in vitro* DNAzyme cleavage assays. For genomic RNAs 100, 300 and 500 pmoles DNAzyme and 500 pmoles mutant DNAzymes were used while for short transcripts 10, 30 and 50 pmoles DNAzymes and 50 pmoles mutant DNAzymes were used. DNAzyme reactions were carried out in a 20 μL reaction volume containing 5 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 150 mM NaCl at 37°C for 2 h.[14] HCV genomic RNA cleavage by DNAzymes was assessed by RT- PCR as described below. The *in vitro* cleavage of runoff transcripts by CDz or NDz was estimated by gel electrophoresis in a 5% urea denaturing polyacrylamide gel as described earlier.[13]

Cell culture

The HepG2 cells were propagated in RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/mL of penicillin, 100 μg/mL streptomycin and 0.25 μg amphotericin B, and 10% heat inactivated fetal calf serum, and maintained at 37°C in a humidified CO_2 incubator. Cell viability was checked by trypan blue exclusion test.

Transfections

The confluent HepG2 cells were transfected with pEGFP-N3 vector DNA or co-transfected with recombinant expression vector DNA of pEGFP-N3-Core or pEGFP-N3-NS5B / pEGFP-N3-NS5B-TAG (1 μg/mL) and respective Dz or mDz (1, 3 or 5 μg; 1 μg ~ 150 picomoles) using lipofectamine reagent (GIBCO BRL, Gaithersburg, MD, USA) following the supplier's instructions in a 24 well plate. Transfection or co-transfections were carried out in Opti-MEM I serum free medium (GIBCO BRL) for 5 h followed by incubation in 10% serum containing complete medium for 48 h.

Quantitation of EGFP fused core and NS5B mRNAs in transfected cells

Core and NS5B mRNA expressions in Dz or MDz co-transfected with vector DNAs in HepG2 cells were quantitated by real time PCR (RT-PCR) using a light cycler system (Rotor Gene PCR-3000, Corbett Research, Wasserburg, Germany) with SYBR Green I fluorescent dye as described by Ruelle *et al*., 2004.[15] Gene specific primers that were used for the two mRNAs are as given before. Normalization of RT-PCR was done taking cellular GAPDH mRNA as the internal control. The cDNA of Dz untreated control cells was normalized to the level of GAPDH mRNA and ascribed as 100%.

Quantitation of core and NS5B proteins

DNAzyme transfected and mock transfected cell extracts of HepG2 cells expressing His-tagged core and NS5B were prepared by the method of Luo *et al*., 2000.[16] Briefly, a washed cell pellet was lysed in 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10 mM dithiotheritol, 50% glycerol, 1 mM phenylmethylsulphonyl fluoride, 2%Triton X-100, 200 mM NaCl, $MgCl_2$ 10 mM, DNase 15 μg/mL, sonicated, and then centrifuged. Aliquots of supernatant were boiled for 5 min in the sample buffer containing 62.5 mM Tris-HCl, pH 6.7, 20% glycerol, 2% SDS, and 10 mM 2-mercaptoethanol. Protein estimation of the supernatant was done by Bradford reagent (Bio-Rad, Richmond, CA, USA) using bovine serum albumin as standard. Aliquots of supernatant containing 25 μg protein were fractionated in 10% denaturing SDS-PAGE[17] and blotted to nitrocellulose membrane. The blot was probed with 1:1000 diluted mouse monoclonal anti-His-tag antibody (Sigma) and developed with secondary antibody (peroxidase conjugated goat anti-mouse IgG, Sigma) as described by Sambrook *et al*.[18] The housekeeping gene GAPDH was probed with the human polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The core and NS5B protein expressions in mock and DNAzyme transfected cells were initially ascertained by Western blot analysis using 15, 30 and 40 μg of total proteins (results not shown). Core and NS5B protein expressions in Dz or mDz transfected cells were also quantified by the fluorescence intensity of EGFP fused core and NS5B proteins.

Purification of RNA dependent RNA polymerase (RDRP or NS5B) and assays

For purification of RDRP to assay, HepG2 cells were co-transfected with pEGFP-N3-NS5B-TAG vector and NDz or mutant NDz. Cells were lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10 mM dithiothreitol, 50% glycerol, 1 mM phenylmethylsulphonyl fluoride, 2% Triton-X-100, 200 mM NaCl, 10 mM $MgCl_2$, 15 μg/mL DNase and sonicated after 48 h of incubation in serum containing medium following 5 h of co-transfection. Cell lysates were centrifuged at 13 000 rpm in a microfuge at 4°C and supernatants were collected. Supernatants were then incubated with TALON (BD Biosciences) affinity resin to purify the 6 X His tagged NS5B protein as per supplier's protocol. The resin bound protein in the column was washed thoroughly with 1x wash buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) and then eluted with 1x elution buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7.0) and the eluted fractions were dialysed in the same buffer without imidazole. Peak fractions were pooled and concentrated by a Centricon (Millipore) concentrator. Equal amounts of proteins (5 mg) from mock, Dz or MDz co-transfected cell lysates were used for purification of expressed RDRP protein.

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RNA templates for RNA dependent RNA polymerase assays were transcribed from linearized murine inducible nitric oxide synthase (iNOS) clone having 400 nt insert in an *in vitro* transcription reaction using SP6 RNA polymerase, and purified (Roche Applied Science) as described earlier.[13] The 3′-OH group of the purified template RNA was blocked by cordycepin triphosphate and assays were carried out following the method of Luo *et al*.[16] in a 20 μL reaction volume containing 20 mM HEPES, pH 8.0, 1.5 mM $MnCl_2$, 100 mM ammonium acetate, 1 mM DTT, 500 μM each of GTP, CTP, ATP, 10 μM UTP, 0.5 μCi (α-^{32}P) UTP, RNA template, iNOS reverse primer, 10U RNasin and equal amount of partially purified NS5B protein preparation were incubated at 30°C for 2 h. The reaction was stopped by digestion with 50 μg/mL of proteinase K (Boehringer Mannheim, Mannheim, Germany) in proteinase K buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5% SDS) for 30 min. The labeled RNA product was recovered by spin column chromatography (Roche Applied Science) and analyzed by 6% SDS-PAGE urea denaturing gel and autoradiographed.

Results

Cleavage of *in vitro* generated runoff transcript

In vitro synthesized runoff transcripts of HCV 5′-UTR-core (−273 to +384 nt) and NS5B (+8301 to +8593 nt) RNA cleaved into 466 and 191 nt, and 162 and 131 nt fragments respectively by their respective DNAzyme but neither mDzs showed the RNA cleavage activity. The increased RNA cleavage activities of both CDz and NDz were observed on increasing their concentration as shown in the Fig. 1b. The nucleotide sequences of designed DNAzymes with their target sites in HCV core and NS5B RNA genes are shown in Fig. 1a.

In vitro cleavage of HCV genomic RNA by core and NS5B site specific DNAzymes

Quantitation of uncleaved HCV RNA of genotype 3 by real time PCR after *in vitro* cleavage reactions with 100, 300, and 500 picomoles of CDz and NDz indicated ~35%, 55% and 80% cleavage of core and ~30%, 60% and 85% cleavage of RDRP respectively. RNA cleavage by either mutant DNAzymes was observed to be less than 5% even with 500 picomoles of the enzyme (Fig. 1c).

Intracellular cleavage of expressed core and NS5B mRNAs by DNAzyme

Intracellular cleavage of expressed EGFP-fused core and NS5B mRNAs in HepG2 cells by their respective DNAzyme was determined by measuring uncleaved mRNAs using real time PCR. Quantitation of these mRNAs in DNAzyme co-transfected cells indicated their decreased intracellular levels or increased RNA cleavage activity. Like in *in vitro* reactions, increased intracellular RNA cleavage activities of both DNAzymes were observed on increasing their concentration. The highest level of DNAzyme cleavage activity was observed when 5 μg of CDz or NDz were used. However, none of the mutant DNAzymes showed appreciable mRNA cleavage activity (Fig. 1d).

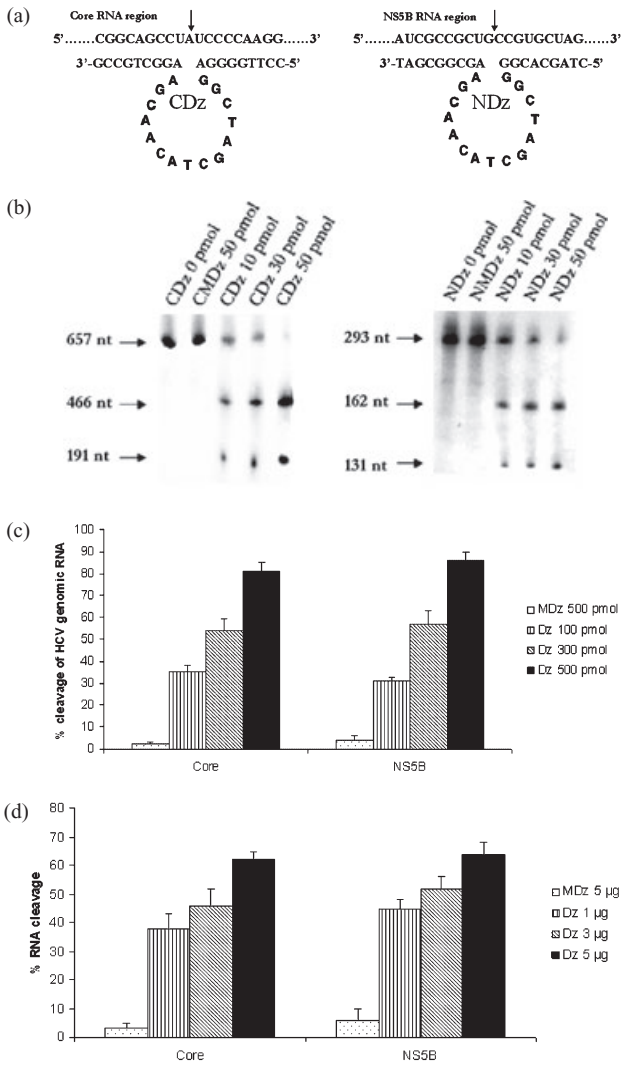

Figure 1 (a) Cleavage sites of CDz and NDz on hepatitis C virus (HCV) Core and NS5B RNA regions. (b) *In vitro* cleavage analysis of (α^{32}P)-UTP labeled short transcripts of HCV Core (657 nt) and NS5B (short 293 nt) by DNAzymes CDz and NDz respectively and their corresponding mutant DNAzyme (MDz) in 5% urea-polyacrylamide gel. This is a representative figure of the activities of these DNAzymes on short transcripts of one of the five strains of genotype 3. (c) *In vitro* cleavage analysis of five strains of HCV genotype3 genomic RNA by core specific CDz or NS5B specific NDz as measured by real time polymerase chain reaction (PCR). P values of the HCV RNA cleavage plotted above with genomic RNAs of five strains of genotype 3 were < 0.05 when repeated five times with each RNA. (d) Intracellular cleavage of HCV Core and NS5B RNAs on co-transfection of designed DNAzyme in HepG2 cells having recombinants pEGFP-N3-core or pEGFP-N3-NS5B expression vectors as described under 'Methods'. RNA cleavage was measured by real time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. P values of the RNA levels plotted above with five independent sets of experiments were < 0.05.

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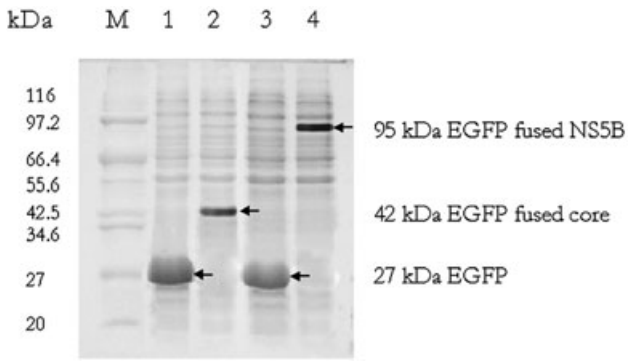

Figure 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of HepG2 cell lysates transfected with pEGFP-N3, pEGFP-N3-core or pEGFP-N3-NS5B DNAs. Expressions of enhanced green fluorescence protein (EGFP) is seen in lanes 1 and 3 in cells transfected with pEGFP-N3 DNA while expressed EGFP fused core (lane 2) and EGFP fused NS5B (lane 4) protein bands are visible in pEGFP-N3-core and pEGFP-N3-NS5B transfected cells respectively.

Protein expressions of cloned core and NS5B genes in HepG2 cells

HCV core and NS5B genes were observed to express efficiently under human cytomegalovirus (CMV) promoter of pEGFP-N3 vector in HepG2 cell line. Both partial Core and full length NS5B genes expressed as 42 kDa and 95 kDa proteins respectively with fused N-terminal 6Xhis and C-terminal 27 kDa EGFP tags as seen in the Coomassie Brilliant Blue stained 10% SDS-PAGE (Fig. 2). Expression of 68 kDa full length RDRP without EGFP tag is not shown.

Inhibition of core and NS5B protein expressions

Both fluorescent intensity and Western blot analyses of core and NS5B protein expressions in respective DNAzyme co-transfected HepG2 cells indicated the suppression of their protein expressions (Fig. 3a,b) while their mutant counterpart had no effect. Suppression of NS5B protein expression was also evident from its decreased enzymatic activity as shown in the Fig. 3c. Reduction of core and NS5B protein expressions and, NS5B activity increased almost linearly with the increase of their respective Dz concentration. These results are in agreement with the observed decreased mRNA levels in DNAzyme co-transfected cells harboring the expression plasmid carrying core or NS5B gene (Fig. 1d).

Suppression of NS5B activity in NDz co-transfected cells

RNA dependent RNA polymerase (NS5B) assays of affinity purified protein expressed in HepG2 cells without EGFP fusion tag showed the enzymatic activity of the expressed protein. Enzymatic activity of NS5B was found to be decreased substantially on co-transfection with NDz. With 1, 3 and 5 μg NDz 31%, 53%, and 69% decrease in NS5B activity were respectively observed. However, no appreciable reduction in NS5B activity was observed with mNDz co-transfected or in mock transfected cells (Fig. 3c,d).

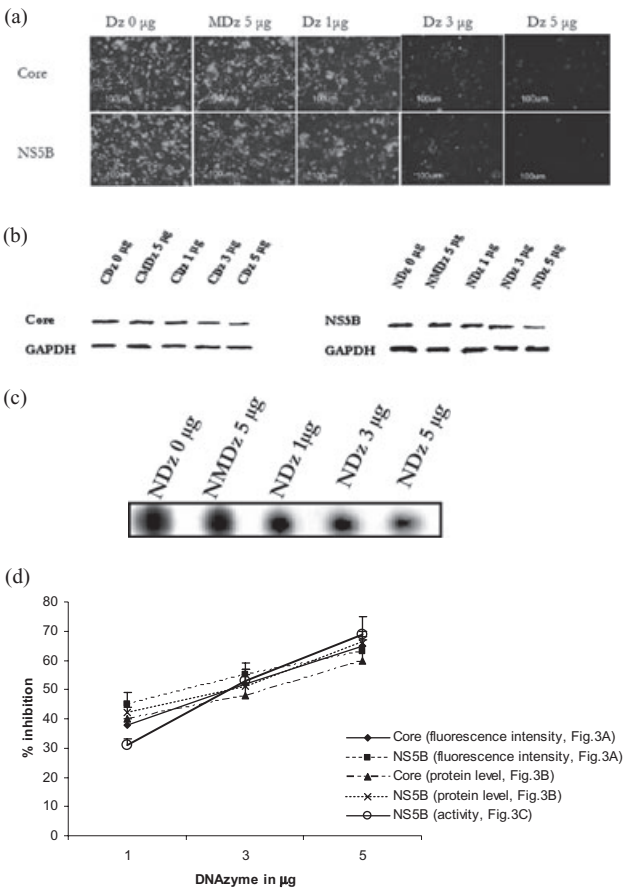

Figure 3 (a) Expressions of enhanced green fluorescence protein (EGFP) fusion proteins of core or NS5B in Dz/MDz co-transfected or mock transfected HepG2 cells. (b) Western blot analysis of hepatitis C virus (HCV) core and NS5B protein expressions in DNAzyme transfected and mock transfected HepG2 cells as described under 'Methods'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (c) RNA dependent RNA polymerase (NS5B) activities in cytosol of HepG2 cells on co-transmission with pEGFP-N3-NS5B-TAG and NDz or NMDz as described under 'Methods'. (d) Percent inhibitions of protein expression and NS5B activity as measured by integrated density values (IDV) of the Western blots, fluorescent intensities, and bands of labeled RNA transcripts produced by purified RDRP as observed in the autoradiograph. P values of the percentage inhibitions plotted above with five independent sets of experiments were < 0.05.

Discussion

Nucleic acid based gene silencing has received special attention and revolutionized the field of gene therapy owing to their extraordinary substrate-specificity and less chances of off-target effects as substrate-based pharmacological inhibitors cannot differentiate various isoforms of their targets. A designed DNAzyme has stringent RNA substrate specificity to cleave at pre-determined

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site by virtue of the obligatory requirement of its hybridization with the target site. Post-transcriptional silencing of several genes including HIV-1 gag RNA, HIV-1 TAT/Rev RNA, hepatitis B virus X gene, murine inducible nitric oxide synthase (iNOS) have been shown in recent years.[10,13,19–22] The regulatory role of one gene on the other can also be studied by post-transcriptional silencing of the gene by DNAzyme. Recently we have shown the down regulation of hyperactive polyhedrin gene of *Autographa californica* nucleopolyhedrovirus by down regulating the viral serine/threonine (pK1) gene by pK1 specific DNAzymes.[23]

The access of Dz to the target site of RNA may be obstructed by the secondary or higher order structure around it. In the case of murine iNOS RNA we have shown that in spite of secondary structure in the translation initiation codon region, the designed Dz cleaved the phosphodiester bond between A and U of AUG codon efficiently. This is believed to be due to the loss of secondary structure on binding with the ribosomes.[13,14] The efficient cleavage of HCV core RNA having secondary structure around IRES by CDz structure may also be due to the loss of secondary structure on ribosome binding.

The increased enzymatic activity of CDz and NDz on increasing their concentration in addition to the inactivity of their mutant counterparts in cell free system and intracellularly indicated the reliability of the DNAzyme activity. As CDz and NDz were found to cleave genomic RNAs of all five strains of genotype 3 HCV and the short runoff transcripts of core and NS5B genes with equal efficiency in *in vitro* reactions, core and NS5B genes of one of these strains was used for their intracellular mRNA cleavage and protein expression studies. Both fluorecein isothiocyanate (FITC) labeled CDz and NDz were found to be stable in HepG2 cells for more than 72 h (results not shown). Both the cloned genes efficiently express in HepG2 cells under human cytomegalovirus promoter (Fig. 2). Expressed viral replicative enzyme, RNA dependent RNA polymerase (NS5B protein) is enzymatically actve (Fig. 3c). Like *in vitro* cleavage of HCV genomic RNA by CDz or NDz, mRNA transcripts of core and NS5B expressed in HepG2 cells are also cleaved on co- transfection with their respective Dz. Intracellular cleavage of expressed mRNA by their respective DNAzyme are also evident from reduced protein expressions of the targeted genes (Fig. 3). Suppression of NS5B gene activity in NDz co-transfected cells further confirmed the selective action of this DZ on NS5B gene. Like protein expressions of (Fig. 1d) core and NS5B genes, and activity of NS5B protein reduced almost linearly with the increase of NDz concentration (Fig. 3d).

Around 170 million people across the world are suffering from HCV infection and the majority of cases become chronic due to the asymptomatic nature of the acute infection, making early diagnosis difficult. Only a fraction of the total infected people respond to interferon based therapies, which is also coupled with adverse side effects. The treatment of cancer, genetic diseases and viral infections including HIV, Hepatitis B, influenza and asthma are currently under development using DNAzymes.[24–27] The therapeutic potential of DNAzymes is also reported for studies on cardiovascular disease.[28] Frequent occurrence of mutant strains of the virus due to error-prone replication have made it almost impossible until now to produce any effective vaccine.

The high fidelity of DNAzymes in cleaving RNAs at predetermined site and subsequent inhibition of protein expression as observed in this study, strongly suggest that these two DNAzymes could be useful in suppressing the replication and translation of the hepatitis C virus.

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