Journal of General Virology (2008), **89**, 1579–1586 DOI 10.1099/vir.0.83650-0

Sequence-specific cleavage of hepatitis C virus RNA by DNAzymes: inhibition of viral RNA translation and replication

Swagata Roy,[1] Nidhi Gupta,[2] Nithya Subramanian,[1] Tanmoy Mondal,[1] Akhil Chandra Banerjea[2] and Saumitra Das[1]

Correspondence
Saumitra Das
sdas@mcbl.iisc.ernet.in

[1]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore-560012, India
[2]National Institute of Immunology, Department of Virology, New Delhi-110067, India

DNAzyme (Dz) molecules have been shown to be highly efficient inhibitors of virus replication. Hepatitis C virus RNA translation is mediated by an internal ribosome entry site (IRES) element located mostly in the 5′ untranslated region (UTR), the mechanism of which is fundamentally different from cap-dependent translation of cellular mRNAs, and thus an attractive target for designing antiviral drugs. Inhibition of HCV IRES-mediated translation has drastic consequences for the replication of viral RNA as well. We have designed several Dzs, targeting different regions of HCV IRES specific for 1b and also sequences conserved across genotypes. The RNA cleavage and translation inhibitory activities of these molecules were tested in a cell-free system and in cell culture using transient transfections. The majority of Dzs efficiently inhibited HCV IRES-mediated translation. However, these Dz molecules did not show significant inhibition of coxsackievirus B3 IRES-mediated translation or cap-dependent translation of reporter gene, showing high level of specificity towards target RNA. Also, Northern blot hybridization analysis showed significant cleavage of HCV IRES by the Dz molecules in Huh7 cells transiently transfected with the HCV–FLuc monocistronic construct. Interestingly, one of the Dzs was more effective against genotype1b, whereas the other showed significant inhibition of viral RNA replication in Huh7 cells harbouring a HCV 2a monocistronic replicon. As expected, mutant-Dz failed to cleave RNA and inhibit HCV RNA translation, showing the specificity of inhibition. Taken together, these findings suggest that the Dz molecule can be used as selective and effective inhibitor of HCV RNA replication, which can be explored further for development of a potent therapeutic agent against HCV infection.

Received 7 December 2007
Accepted 13 March 2008

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus, belonging to the family *Flaviviridae*. The viral RNA genome is approximately 9600 nt and encodes a single polyprotein of about 3000 amino acids. The long open reading frame is flanked by 5′ and 3′ untranslated regions (UTRs) that are highly conserved among the majority of HCV genotypes and contain elements that are essential for genome replication (Bartenschlager *et al.*, 2004). The translation initiation of HCV RNA is mediated by the binding of the 40S ribosomal subunit at the internal ribosome entry site (IRES) located mostly in the 5′UTR region. It has been shown that the HCV IRES can directly bind to the 40S ribosomal subunit, even in the absence of any initiation factors, in a manner similar to prokaryotic translation initiation. Subsequently, several canonical and non-canonical *trans*-acting factors facilitate the formation of a functional initiation complex during internal initiation of translation (Hellen & Sarnow, 2001). Since this mechanism is fundamentally different from the ribosome assembly at the 5′ cap-binding complex in cap-dependent translation of host cell mRNA, it serves as an attractive target for antiviral agents (Dasgupta *et al.*, 2004).

HCV causes a multitude of liver diseases in humans, including liver cirrhosis, and often leads to hepatocellular carcinoma if left untreated. Current treatment options involving interferon-α (INF-α) alone or in combination with ribavirin are not very effective. The majority of the patients do not respond well to this therapy because of the short half-life of interferon or degradation of the molecules. Failure to achieve a sustained virological response in majority of the patients has also been shown to be partly due to the varying genotypes of the infecting strain of the virus. HCV has six major genotypes with several subtypes. HCV genotype 1 has been shown to be more resistant to interferon therapy than genotype 3. Genotype 3 was found to be the most prevalent in India, followed by genotype 1 (Gupta *et al.*, 2006). Thus

developing effective antiviral therapeutics using novel approaches is the need of the hour.

Several strategies are being explored to develop antiviral agents against HCV, targeting different viral processes. Recent availability of HCV subgenomic (Lohmann *et al.*, 1999) and full-length (Blight *et al.*, 2002) replicon systems has helped immensely to assay the inhibitory effect of antiviral candidates on HCV genome replication. Earlier, several studies have shown effective inhibition of viral RNA translation when viral enzymes were targeted. Since the translation of genomic RNA is the initial obligatory step, interference with this process will have direct consequence on the viral RNA replication. HCV RNA is translated by recruitment of the ribosome at the IRES element which comprises most of the 5′UTR sequences (except the first 40 nt) and extends to a short stretch of 30–40 nt downstream of the initiator AUG. Since the IRES-mediated translation is distinct from the cap-dependent translation of host cell mRNA, this could be exploited by different approaches to achieve selective inhibition of HCV gene expression.

Currently, nucleic-acid-based antiviral approaches, which include ribozyme (Rz), DNAzyme (Dz), short hairpin RNA (shRNA) and small interfering RNA (siRNA), are being used for inhibiting the gene expression of several target RNAs (Jarczak *et al.*, 2005; Goila & Banerjea, 2004). Among these, catalytic Dzs with 10–23 catalytic motifs are increasingly being exploited over Rzs because they either match or exceed the catalytic efficiencies of the known Rzs. Deoxyribozymes or DNAzymes or DNA-enzymes (Dzs), as originally described (Santoro & Joyce, 1997), are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner (Silverman, 2005, Dash & Banerjea 2004; Joyce, 2004). Dzs are synthetic single-stranded DNA molecules which have three domains: a catalytic domain consisting of 15 nt flanked by two substrate-recognition domains which bind the target RNA through Watson–Crick base pairing. In Dzs a single nucleotide change in the 10–23 catalytic motif completely abrogates the sequence-specific cleavage activity; for example, G14C completely abolishes the catalytic cleavage (Goila & Banerjea, 2001). In some instances efficient inhibition of gene expression was achieved with 10–23 Dz (Ackermann *et al.*, 2005). Based on sequence recognition of the binding arms, Dzs can be synthesized to cleave a target gene in a sequence-specific manner similar to that of Rzs (Asahina *et al.*, 1998; Goila & Banerjea, 1998).

Compared with synthetic Rzs, Dzs are easier to prepare, less sensitive to chemical and enzymic degradation and, more importantly, easier to deliver into cells (Santoro & Joyce, 1997). Over the years, several kinds of Dzs with unique catalytic motifs have been described, but Dzs possessing the 10–23 catalytic motif have been exploited more extensively by several investigators (Banerjea *et al.*, 2004). Various studies suggest that all the target sites are not available for cleavage by a single kind of catalytic nucleic acid molecule, most probably because the secondary and tertiary structures in the target RNA prevent optimal Watson–Crick base pairing with Rzs or Dzs. More than one site is usually selected in the target RNA to get maximum cleavage by catalytic nucleic acids. 10–23 DNA-enzyme cleaves the RNA sequence at a phosphodiester bond between an unpaired purine and a paired pyrimidine residue (5′-AU-3′ most efficiently cleaved). This results in the formation of 5′ and 3′ products, which contain a 2′-3′cyclic phosphate and 5′ hydroxyl terminus, respectively (Santoro & Joyce, 1998).

METHODS

Deoxyribozyme synthesis. All the oligodeoxynucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys. The conserved 15 nt (5′-GGCTAGCTACAACGA-3′) 10–23 catalytic motif was flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. Mutant-Dz was also assembled using a 10–23 catalytic motif that possessed a single nucleotide substitution (G to C) in the 10–23 catalytic motif. This change is known to render the Dz catalytically inactive (Goila & Banerjea, 2001).

We have initially designed five DNA-enzymes, namely Dz88, Dz219, Dz305, Dz327 and Dz336, targeting different regions of HCV 5′UTR IRES (Table 1). Additionally, mutant-Dz219, possessing a point mutation as stated above, was designed, which is termed the 'mutant Dz'. These molecules were specific only for HCV genotype 1b. Later we designed another four 10–23 catalytic motif-containing Dzs, namely Dz161, Dz165, Dz285 and Dz288, that were targeted to cleave 5′UTR regions of all the currently known HCV genotypes (Table 2). The location of cleavage for each Dz is shown by arrows in the predicted 5′-UTR IRES (Fig. 1).

Cell culture and plasmid. Human hepatocellular carcinoma cells (Huh-7 and Huh-7.5 cells) monolayers (Blight *et al.*, 2002) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) at 37 °C in 5 % CO_2 atmosphere. For cells supporting the HCV full-length replicon (genotype 1b), 0.8 mg G418 sulfate ml^{-1} (Sigma-Aldrich) was added to the culture medium and for cells bearing the HCV monocistronic replicon (genotype 2a), 25 μg hygromycin B ml^{-1} was added to the culture medium. Replicon 1b carries the 1b genotype HCV 5′UTR followed by a neomycin resistance gene (*neo*), EMCV IRES and NS2–NS5 and the 3′UTR sequence. The replicon 2a carries the 2a genotype HCV 5′UTR followed by a hygromycin resistance gene (*hyg*), a ubiquitin gene (*ubi*) and NS3–NS5 and the 3′UTR. The HCV–FLuc monocistronic plasmid construct pCD (HCV–IRES–FLuc) construct carrying HCV IRES (nt 18–383) was

Table 1. Sequence of DNAzymes for subtype 1b

The mutated residue in the mutant mutDz-219 is highlighted in bold.

DNAzyme	Sequence 5′–3′
Dz88	AACGCCAGGCTAGCTACAACGAGGCTAGAC
Dz305	GCAAGCAGGCTAGCTACAACGACCTATCAG
Dz219	CCAGGCAGGCTAGCTACAACGATGAGCGG
Dz327	CTACGAGAGGCTAGCTACAACGACTCCCGG
Dz336	TGCACGGGGCTAGCTACAACGACTACGAG
mutDz219	CCAGGCAGGCTAGCTACAAC**C**ATGAGCGG

Table 2. Sequence of DNAzymes for HCV IRES based on conserved regions

DNAzyme	Sequence 5′–3′
Dz161	GTACTCAGGCTAGCTACAACGACGGTTCC
Dz165	CGGTGTAGGCTAGCTACAACGATCACCGG
Dz285	CAGTACCAGGCTAGCTACAACGAAAGGCCT
Dz288	GGCAGTAGGCTAGCTACAACGACACAAGG

cloned upstream of the gene for the firefly luciferase (Pudi *et al.*, 2003). The pCDFLuc construct contains the luciferase reporter gene and the coxsackievirus B3 (CVB3)–FLuc monocistronic plasmid construct (pCDCVB3-IRES-FLuc) contains the CVB3 5′UTR cloned upstream of the firefly luciferase reporter gene.

***In vitro* cleavage of target RNA with Dz.** *In vitro* run off transcripts of HCV IRES RNA (387 nt) was made from linearized HCV–FLuc monocistronic construct DNA (containing nt 18–383 of HCV IRES) using the T7 RNA polymerase (Promega) in the presence of [α-^{32}P]UTP, following the manufacturer's protocol. The extra nucleotides in the labelled transcript (387 nt) came from the region between the T7 promoter and upstream of the HCV sequence (cloned in polylinker). Equimolar amounts of unlabelled Dz and labelled substrate RNA (100 pmol each) were allowed to interact in a final volume of 10 µl in a buffer containing 50 mM Tris.HCl, pH 7.5 and 10 mM $MgCl_2$ (standard conditions) as described earlier (Santoro & Joyce, 1997) for 2 h at 37 °C. The cleaved products were resolved by electrophoresis and cleavage efficiency was determined as described earlier (Goila & Banerjea, 2001).

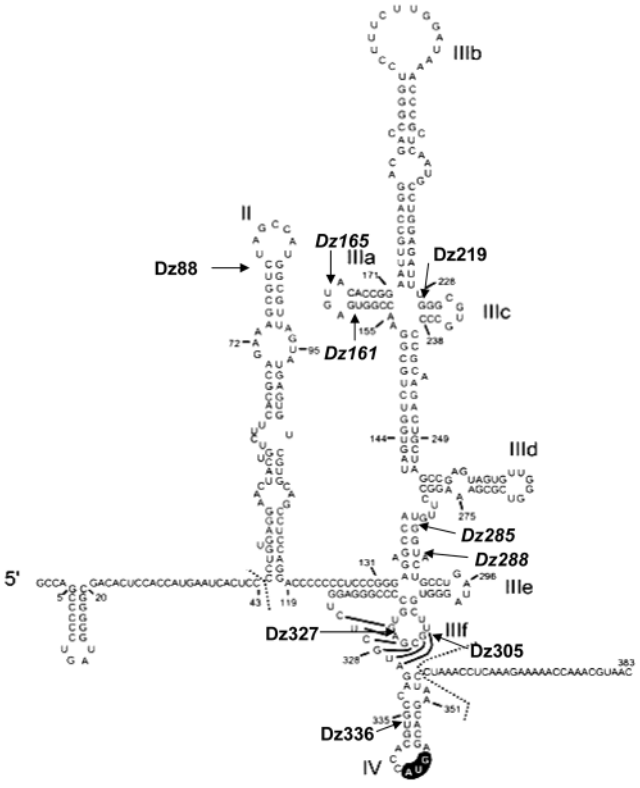

Fig. 1. Schematic diagram of HCV IRES (adopted from Brown *et al.*, 1992), showing the Dz targets. The numbers in bold are specific for genotype 1b and those in italics are conserved for all major genotypes.

Transfections and reporter assay. Monolayers (60–70 % confluent) of Huh7 cells in 35 mm dishes were co-transfected with HCV monocistronic plasmid pCDHCV-FLuc or pCDCVB3-FLuc or pCDFLuc. Dzs and pSV-β-gal plasmid were used for normalizing transfection efficiency using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection the cells were harvested using passive lysis buffer (Promega) and FLuc activity was analysed using a luciferase assay system (Promega) in a TD 20/20 luminometer (Turner Designs).

Semiquantitative RT-PCR. HCV full-length and subgenomic replicon-bearing cells were transfected with 0.4 and 0.8 µM Dzs and, 24 h post-transfection, total cellular RNAs were extracted using TRI-reagent (Sigma-Aldrich). Semiquantitative RT-PCR was performed for the HCV-IRES positive strand and actin as described earlier (Dhar *et al.*, 2007). In brief, 5 µg total RNA was reverse transcribed with the HCV 5′UTR and actin primers by annealing at 65 °C and extending at 42 °C for 50 min. After cDNA was synthesized, PCR reaction was performed using both 5′ and 3′ primers specific for HCV 5′UTR to amplify and quantify HCV RNA. The PCR products were run in 1 % agarose gel and densitometric analysis was done using MultiGauge software (Fujifilm) and the values were expressed as ratio of HCV IRES to actin.

Northern blot analysis. Total cellular RNA (20 µg) was isolated from Huh7 cells transfected with HCV–FLuc monocistronic constructs with or without Dzs and resolved on formaldehyde-agarose gel (0.8 %) under denaturing conditions. RNA were transferred and cross-linked to a nylon membrane (Sigma-Aldrich) and probed with a [α-^{32}P]-labelled firefly luciferase antisense probe, followed by autoradiography. Densitometric analysis was done and the ratio of HCV-IRES to 18S rRNA was expressed graphically.

RESULTS

Sequence-specific cleavage of HCV IRES RNA by various DNAzymes

Since the mechanism of HCV IRES-mediated translation is novel and fundamentally different from cap-dependent translation of host cell mRNA, we have designed several Dzs to target the IRES element for selective inhibition of HCV RNA translation. Furthermore, the sequence-specific cleavage in this region will consequently block viral RNA replication and therefore we designed a number of Dzs that were targeted to the predicted single-stranded loop regions within the HCV IRES element (Fig. 1, Tables 1 and 2). In order to evaluate the cleavage efficiency of the Dzs, *in vitro* cleavage reaction were performed. Out of five Dz molecules only three, Dz219, Dz305, and Dz327, have shown significant cleavage activity *in vitro* in cell-free conditions (Fig. 2a and 2b). Interestingly, the three active Dzs were found to cleave the target RNA in a sequence-specific manner with varying efficiencies (Fig. 2b, lanes 3, 4 and 5). Dz219 showed maximum cleavage activity under standard

in vitro cleavage conditions (Fig. 2b). However, Dz88 and Dz336 failed to show detectable cleavage activity.

Further, to investigate the inhibitory effect of Dzs that would target conserved sequences in all six major genotypes of HCV, several conserved Dz molecules (including Dz161, Dz165, Dz285 and Dz288) were synthesized (Table 1 and Fig. 2a). For this purpose, HCV 5′UTR sequences of all six genotypes were aligned using the CLUSTAL W program (data not shown). The designing of Dzs was based on the secondary structures of RNA for all the strains, obtained by the mfold program (data not shown). All four conserved Dzs were first tested for RNA cleavage activity *in vitro* as described before. Among those four Dz molecules, three, Dz161, Dz285 and Dz288, were found to cleave the target sequence specifically, the last two being more efficient (Fig. 2c). Dz165 did not show significant cleavage activity.

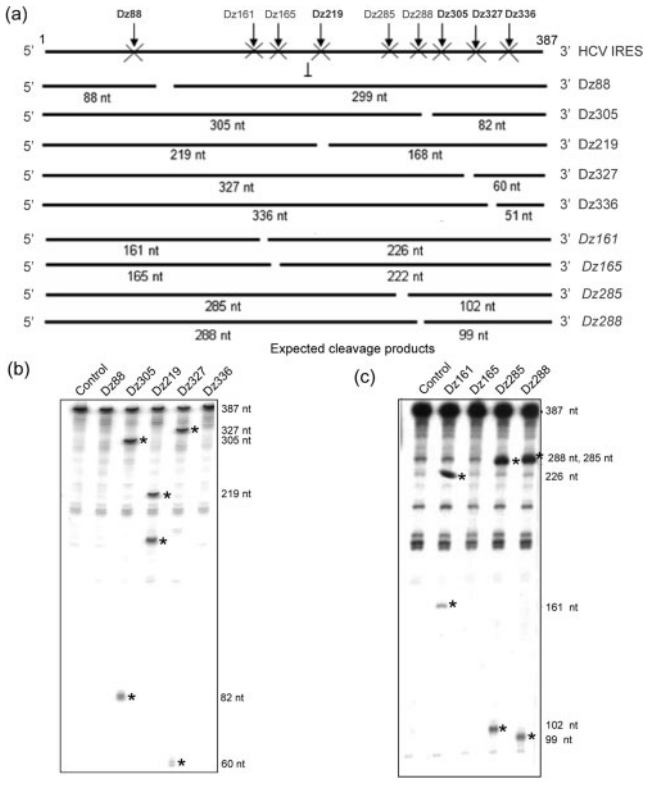

Fig. 2. *In vitro* cleavage of HCV IRES RNA by various Dz molecules. (a) Schematic representations of the Dz cleavage sites on the HCV IRES: HCV genotype 1b-specific Dzs are indicated in bold, whereas the Dzs that target the conserved sequences across the different genotypes are indicated in italics. (b) Equimolar concentrations (100 pmol) of the labelled HCV IRES RNA and respective Dz were used and the cleavage products were analysed on 8 % PAGE. (c) *In vitro* cell-free cleavage products of HCV IRES by conserved Dzs were analysed on 8 % PAGE. The asterisks show the cleavage products as indicated in (a) and their respective sizes are shown on the right.

Effect of Dz on HCV IRES-mediated translation *ex vivo*

In order to evaluate the intracellular cleavage efficiencies of the Dz molecules, transient co-transfection experiments were performed using plasmid HCV–FLuc monocistronic constructs and the Dz molecules in human hepatocellular carcinoma cells (Huh7). The monocistronic RNA generated *ex vivo* from the HCV–FLuc monocistronic plasmid encodes the HCV IRES element upstream of the firefly luciferase reporter gene (Pudi *et al.*, 2003). Although three Dz molecules specific for genotype 1b showed significant cleavage activity *in vitro*, only one of them, Dz219, showed impressive inhibition (81 %) of HCV IRES-mediated translation. However, the mutant-Dz219 with a single substitution in the catalytic domain of Dz219 failed to inhibit HCV IRES-mediated translation, suggesting high specificity of the approach (Fig. 3a). Interestingly, two other Dzs (305 and 327) showed significant *in vitro* cleavage activity, but failed to interfere with the HCV translation (Fig. 3a). When conserved Dzs were tested for inhibition of HCV IRES function, Dz285 and Dz288 showed 38 and 35 % inhibition, respectively, whereas Dz161 showed only 30 % inhibition (Fig. 3b).

To investigate the cleavage of the HCV–FLuc monocistronic RNA by the Dzs in *ex vivo* conditions, Northern blot hybridization was performed. For this purpose, Huh7 cells were transiently transfected with the monocistronic DNA constructs and different Dz molecules (Fig. 4a). Total RNAs were isolated 24 h post-transfection and used for Northern assay. Dz molecules used in the assay included Dz219 and Dz285, which showed maximum activity *ex vivo*, and Dz305, Dz327 and mutant-Dz219, that did not exhibit any *ex vivo* activity. Results showed significant cleavage activity of Dz219 and Dz285; however, Dz305 and Dz327 failed to cleave HCV–FLuc RNA *ex vivo* (Fig. 4a), which is consistent with our reporter gene (luciferase) assay (Fig. 2b). The mutant-Dz219 didn't show any cleavage activity *ex vivo*, as expected. For clarity we have quantified the band intensity corresponding to the HCV–FLuc RNA and normalized it with that of the loading control band (18S rRNA). The densitometric analysis of the ratio of HCV–FLuc monocistronic RNA to the 18S rRNA (Fig. 4b), clearly demonstrated that cleavage activity of Dz219 as 48 % and that of Dz285 as 25 %, respectively. However, Dz305, Dz327 or the mutant-Dz219 did not exhibit any significant cleavage activity. Interestingly, the translation inhibitory activity corresponding to Dz219 and Dz285 was found to be slightly higher (Fig. 3) than the RNA cleavage activity (Northern analysis, Fig. 4), which could be due to a higher sensitivity of the luciferase assay.

To further investigate the specificity of the Dz activity, the Dz molecules were tested against other viral IRES as well as cap-dependent translation. For this purpose the pCDFLuc DNA construct was transiently transfected with representative Dz molecules (Dz219, 285, 288 and mutant-Dz219) and the luciferase reporter gene was assayed 24 h

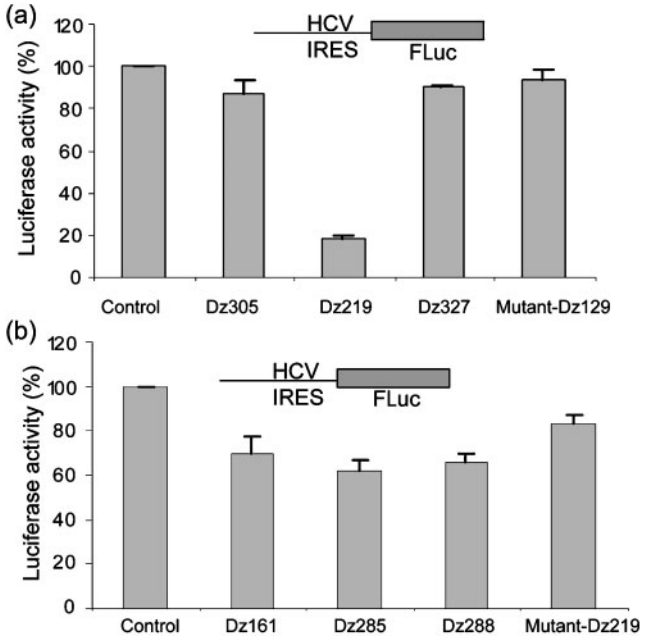

Fig. 3. Inhibition of HCV IRES-mediated translation by the Dz molecules. (a) Approximately 1 μg HCV firefly luciferase monocistronic DNA construct (HCV–FLuc) was transiently cotransfected in Huh7 cells in the absence and presence of 0.4 μM genotype 1b-specific Dz molecules. Cells were harvested and luciferase activity measured 24 h post-transfection. The transfection efficiency was normalized with β-gal activity and the normalized luciferase activity was plotted, taking the control as 100 %. (b) Similarly, 1 μg monocistronic DNA construct (HCV–FLuc) was transiently cotransfected in Huh7 cells in the absence and presence of 0.4 μM conserved Dz molecules. Luciferase activities were measured 24 h post-transfection. The results represent the average of three independent experiments done in duplicate.

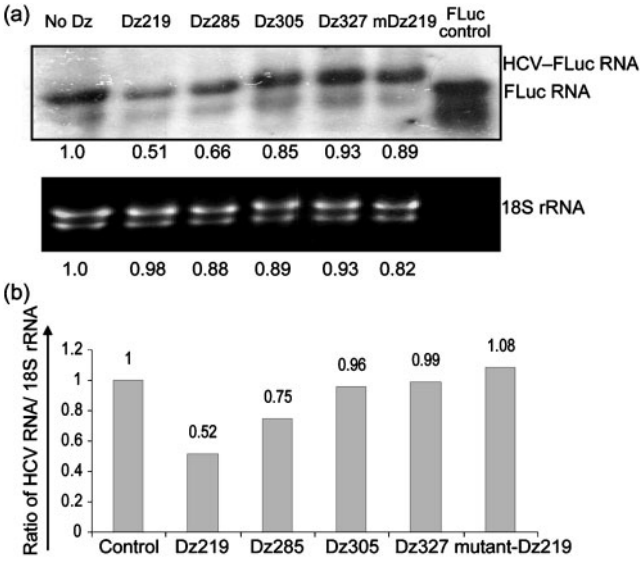

Fig. 4. Cleavage of HCV RNA *ex vivo* in Huh7 cells by the Dz molecules. (a) Northern blot hybridization of the HCV RNA isolated from Huh7 cells, co-transfected with HCV–FLuc DNA and Dzs (0.4 μM). Total RNA was isolated using TRIzol reagent 24 h post-transfection, electrophoresed on formaldehyde agarose gel (0.8 %), blotted on a nylon membrane and hybridized with α^{32}P-labelled FLuc antisense probe followed by autoradiography. The numbers below the panels represent the band intensities relative to control. (b) Graphical representation of the ratio of the densitometric values obtained from the respective band intensities corresponding to HCV-RNA and 18S rRNA.

post-transfection. Results suggest that none of the Dz molecules inhibited the cap-dependent translation of the luciferase reporter gene (Fig. 5a).

Similarly, CVB3 FLuc monocistronic DNA (pCDCVB3-FLuc) was cotransfected with the above set of Dz molecules and the luciferase assay was performed 24 h post-transfection. Results suggest no significant change in luciferase activity in presence of the above Dz molecules (Fig. 5b). Taken together, the results suggest the target specificity of the Dz molecules and the high levels of selectivity of this approach.

Effect of Dzs on the HCV RNA replication

Finally, we have analysed the inhibitory effect of the Dzs in Huh7.5 cells harbouring HCV1b replicon (Fig. 6a) (Blight *et al.*, 2002). Increasing concentration (0.4 and 0.8 μM) of Dz 219 was transiently transfected into replicon-containing cell line using Lipofectamine 2000 (Invitrogen). After 24 h, total RNA was isolated and the HCV positive-strand RNA corresponding to the 5′UTR was detected by semiquantitative RT-PCR. Results suggest approximately 70 % inhibition of the HCV1b genotype replicon RNA synthesis when 0.8 μM Dz219 was used. However, the same concentration of Dz219 failed to inhibit the HCV-RNA synthesis in Huh7 cells harbouring HCV2a genotype replicon (Lohmann *et al.*, 1999) (Fig. 6b and 6c). Upon inspection we found that the Dz219 target sequence was designed on the basis of HCV 1b sequences, which is not fully conserved in HCV2a sequence. The result also proved that bio-efficacy of Dz219 was sequence-specific. Furthermore, when the conserved Dzs (0.4 μM) were transfected into cells containing HCV replicon 1b (Fig. 6d), significant inhibition of RNA synthesis was observed with Dz285 and Dz288 (30 and 50 %, respectively). However, the inhibitory effect was relatively more pronounced (60 % for Dz285 and 70 % for Dz288) on HCV replicon 2a cell line.

DISCUSSION

A couple of studies have demonstrated previously the use of DNAzyme molecules to cleave HCV RNA *ex vivo* (Trepanier *et al.*, 2006), but this study constitutes the first report on the effect of Dzs on HCV replication in cell lines harbouring HCV subgenomic or full-length replicons.

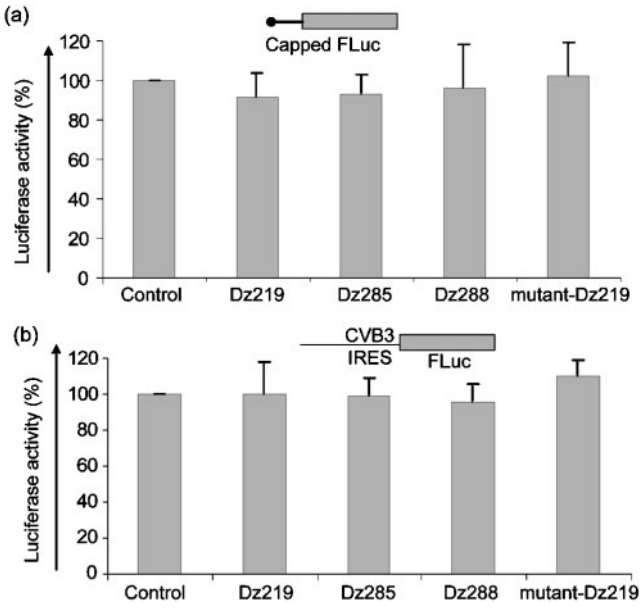

Fig. 5. Effect of Dz molecules on cap-dependent translation and CVB3 IRES-mediated translation. (a) Dz219, Dz285, Dz288 or mDz219 (0.4 μM) were co-transfected with 1 μg pCDFLuc DNA construct and β-gal DNA construct in Huh7 cells. Luciferase assay was performed 24 h post-transfection. The transfection efficiency was normalized with β-gal activity and the normalized luciferase activity was plotted taking the control as 100 %. (b) Similarly, 1 μg CVB3FLuc DNA construct was cotransfected with the above Dzs and β-gal construct and the normalized luciferase values were plotted taking the control as 100 %. The results shown represent the average of three independent experiments done in duplicate.

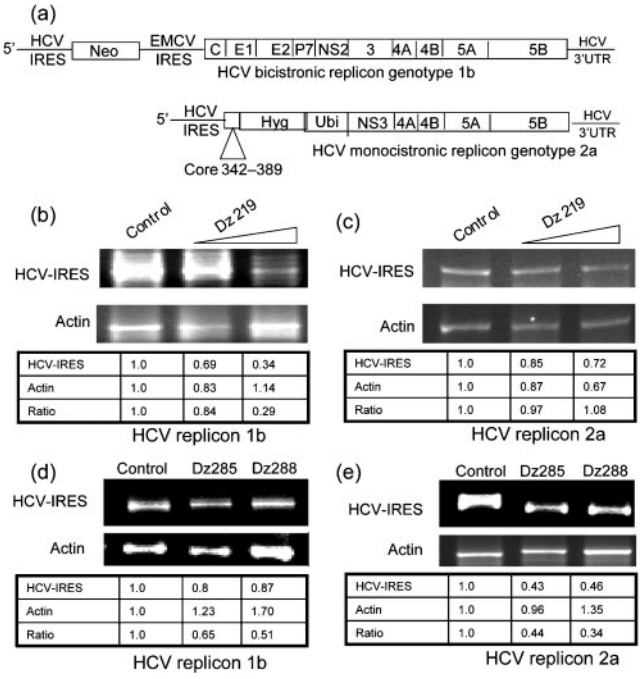

Fig. 6. Effect of Dz molecules *ex vivo* in replicon cell line. (a) Schematic representation of the HCV replicon genotype 1a and 1b. (b and c) Increasing concentrations (0.4 and 0.8 μM) of Dz219 were transfected either in Huh7.5 cells harbouring replicon 1b genotype (b) or Huh7 cells harbouring replicon 2a genotype (c). RNA was isolated using TRIzol reagent 24 h post-transfection, and semiquantitative RT-PCR was performed using HCV-5′UTR-specific primers or the actin primers. The products were analysed on 1 % agarose gel. (d and e) Similarly, the effects of Dz285 and Dz288 (0.4 μM) were analysed with HCV replicon 1b genotype (d) and HCV replicon 2a genotype (e).

Although we have designed several Dzs targeting different regions of HCV IRES and tested their activities *in vitro* as well as *ex vivo* in cell lines harbouring HCV replicon, only a couple of them were found to be more effective in the *in vitro* and *ex vivo* assays. Interestingly, when all the Dzs used in the study were mapped to the target sequences/structures within HCV IRES (Fig. 1), it appears that the Dz285 and Dz288, targeting HCV SLIIId loop, and Dz219, targeting SL IIIb, achieved maximum inhibition, perhaps due to the importance of the target site in ribosome assembly during internal initiation of translation. This could be also due to the fact that all target sites are not available for efficient cleavage by a single kind of catalytic nucleic acid molecule, most probably because the secondary and tertiary structures in the target RNA prevent optimal base pairing. Base pairing and cleavage activity also depend on the arm length of the RNA-binding site of the Dzs. Enzymes with longer arms sometimes showed higher cleavage activity compared with enzymes with shorter arms (Oketani *et al.*, 1999). Modifications in the 5′ and 3′ termini of these molecules help in preventing nuclease degradation without affecting its catalytic activity (Oketani *et al.*, 1999). Interestingly, it has been demonstrated earlier that the efficiency of some Dz molecules can be enhanced by using them in combination with some oligodeoxynucleotides (ODNs) which would hybridize the target RNA near the Dz cleavage site to facilitate the cleavage reaction (Sood *et al.*, 2007). Thus, it would be interesting to explore whether the apparently inactive/inefficient Dz molecules in our study could also be used in combination with ODNs to potentiate catalytic efficiency for the RNA cleavage.

It appears that, if required, Dz molecules can be used at higher concentration to achieve maximum inhibition of viral protein synthesis with minimum effect on host cell RNA translation (data not shown). It is also possible that the effective concentration required to achieve 50 % inhibition of viral RNAs could be lowered to a large extent by using a cocktail of Dz molecules in the line of combination therapy.

Dz molecules have been shown to be a more stable antiviral agent compared with Rz or siRNA (Santoro & Joyce, 1997). Unlike siRNA or shRNAs, Dz molecules are not expected to activate double-stranded RNA-activated protein kinase

(PKR) and result in attenuation of host cell RNA translation due to phosphorylation of eIF2 by PKR (Gil & Esteban, 2000). It is also possible to make more stable derivative of the Dzs molecules such as morpholino- or phosphorothio- derivatives etc. Remarkable stability was also achieved by modifying (inverting) the first and last nucleotide residues, especially at the 3′-end of the Dz, which will have serum stability enhanced tenfold (Sun *et al.*, 1999). In this connection, it has been shown also that efficient uptake of macrophage tropic-anti-HIV-1 Dz by human macrophages in the complete absence of charged lipid molecules can be enhanced by attaching ten G residues at the 3′-end of a 10–23 catalytic motif-containing Dz. G residues form G quartet-like structures that are recognized by the scavenger receptor present on macrophages (Unwalla & Banerjea, 2001).

Taken together, these results provide proof of the concept that the HCV IRES could be an effective and selective target using conserved DNA-enzyme molecules to develop novel antiviral therapeutics against hepatitis C virus infection. It would be interesting to couple this with organ-specific delivery approaches. Liver-specific delivery of Dz molecules using Sendai virus virosome- (Ramani *et al.*, 1997) or lentivirus- (Kusunoki *et al.*, 2003) based vectors would be ideal for developing Dz-based antiviral therapeutics against hepatitis C virus infection.

ACKNOWLEDGEMENTS

We thank Dr Akio Nomoto for HCV plasmid constructs. We also thank Dr Ralf Bartenschlager and Dr Volker for providing the Huh7 cells harbouring the HCV2a replicon and Dr Charles M. Rice for providing the Huh7.5 cells harbouring the HCV1b replicon. We gratefully acknowledge our lab members for their help and discussion. This work was supported by grant from the Department of Biotechnology and Life Science Research Board, India, to S. D.

REFERENCES

Ackermann, J. M., Kanugula, S. & Pegg, A. E. (2005). DNAzyme-mediated silencing of ornithine decarboxylase. *Biochemistry* **44**, 2143–2152.

Asahina, Y., Ito, Y., Wu, C. H. & Wu, G. Y. (1998). DNA ribonucleases that are active against intracellular hepatitis B viral RNA targets. *Hepatology* **28**, 547–554.

Banerjea, A. C., Chakraborti, S., Unwalla, H., Goila, R., Shrabani, B., Dash, B. C. Sriram, B., Parasivam, M. & Viswanathan, S. (2004). Potential therapeutic application of DNA enzymes and siRNAs against viral and cellular genes. In *Synthetic Nucleic Acids as Inhibitors of Gene Expression: Mechanisms, Applications, and Therapeutic Implications*, pp. 115–134. Edited by L. M. Khachigian. Boca Raton, FL: CRC Press.

Bartenschlager, R., Frese, M. & Pietschmann, T. (2004). Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res* **63**, 71–180.

Blight, K. J., McKeating, J. A. & Rice, C. M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**, 13001–13014.

Brown, E. A., Zhang, H., Ping, L. H. & Lemon, S. M. (1992). Secondary structure of the 5′ nontranslated regions of hepatitis C virus and pestivirus. *Nucleic Acids Res* **20**, 5041–5045.

Dasgupta, A., Das, S., Izumi, R., Venkatesan, A. & Barat, B. (2004). Targeting internal ribosome entry site (IRES)-mediated translation to block hepatitis C and other RNA viruses. *FEMS Microbiol Lett* **15**, 189–199.

Dash, B. C. & Banerjea, A. C. (2004). Sequence-specific cleavage activities of DNA enzymes targeted against HIV-1 Gag and Nef regions. *Oligonucleotides* **14**, 41–47.

Dhar, D., Roy, S. & Das, S. (2007). Translational control of the interferon regulatory factor 2 mRNA by IRES element. *Nucleic Acids Res* **35**, 5409–5421.

Gil, J. & Esteban, M. (2000). Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* **5**, 107–114.

Goila, R. & Banerjea, A. C. (1998). Sequence specific cleavage of the HIV-1 coreceptor CCR5 gene by a hammer-head ribozyme and a DNA-enzyme: Inhibitions of the coreceptor function by DNA-enzyme. *FEBS Lett* **436**, 233–238.

Goila, R. & Banerjea, A. C. (2001). Inhibition of hepatitis B virus X gene expression by novel DNA enzymes. *Biochem J* **353**, 701–708.

Goila, R. & Banerjea, A. C. (2004). Sequence-specific cleavage of hepatitis X RNA in *cis* and *trans* by novel monotarget and multitarget hammerhead motif-containing ribozymes. *Oligonucleotides* **14**, 249–262.

Gupta, R., Subramani, M., Khaja, M. N., Madhavi, C., Roy, S., Habibullah, M. C. & Das, S. (2006). Analysis of mutations within the 5′ untranslated region, interferon sensitivity region, and PePHD region as a function of response to interferon therapy in hepatitis C virus-infected patients in India. *J Clin Microbiol* **44**, 709–715.

Hellen, C. U. T. & Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev* **15**, 1593–1612.

Jarczak, D., Kofr, M., Beger, C., Manns, M. P. & Kruger, M. (2005). Hairpin ribozymes in combination with siRNAs against highly conserved hepatitis C virus sequence inhibit RNA replication and protein translation from hepatitis C virus subgenomic replicons. *FEBS J* **272**, 5910–5922.

Joyce, G. F. (2004). Directed evolution of nucleic acid enzymes. *Annu Rev Biochem* **73**, 791–836.

Kusunoki, A., Miyano-Kurosaki, N. & Takaku, H. (2003). A novel single-stranded DNA enzyme expression system using HIV-1 reverse transcriptase. *Biochem Biophys Res Commun* **301**, 535–539.

Lohmann, V., Körner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Oketani, M., Asahina, Y., Wu, C. H. & Wu, G. Y. (1999). Inhibition of hepatitis C virus-directed gene expression by a DNA ribonuclease. *J Hepatol* **31**, 628–634.

Pudi, R., Abhiman, S., Srinivasan, N. & Das, S. (2003). Hepatitis C virus internal ribosome entry site-mediated translation is stimulated by specific interaction of independent regions of human La autoantigen. *J Biol Chem* **278**, 12231–12240.

Ramani, K., Bora, R. S., Kumar, M., Tyagi, S. K. & Sarkar, D. P. (1997). Novel gene delivery to liver cells using engineered virosomes. *FEBS Lett* **404**, 164–168.

Santoro, S. W. & Joyce, G. F. (1997). A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci U S A* **94**, 4262–4266.

Santoro, S. W. & Joyce, G. F. (1998). Mechanism and utility of an RNA-cleaving DNA enzyme. *Biochemistry* **37**, 13330–13342.

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

Sood, V., Unwalla, H., Gupta, N., Chakraborti, S. & Banerjea, A. C. (2007). Potent knock down of HIV-1 replication by targeting HIV-1 Tat/Rev RNA sequences synergistically with catalytic RNA and DNA. *AIDS* **21**, 31–40.

Sun, L. Q., Cairns, M. J., Gerlach, W. L., Witherington, C., Wang, L. & King, A. (1999). Suppression of smooth muscle cell proliferation by a c-*myc* RNA-cleaving deoxyribozyme. *J Biol Chem* **274**, 17236–17241.

Trepanier, J., Tanner, J. E., Momparler, R. L., Le, O. N. L., Alvarez, F. & Alfieri, C. (2006). Cleavage of intracellular hepatitis C RNA i the virus core protein coding region by deoxyribozymes. *J Viral Hepat* **13**, 131–138.

Unwalla, H. & Banerjea, A. C. (2001). Inhibition of HIV-1 gene expression by novel macrophage-tropic DNA enzymes targeted to cleave HIV-1 TAT/Rev RNA. *Biochem J* **357**, 147–155.