The Journal of Microbiology, December 2004, p.361-364 Vol. 42, No. 4

NOTE

Comparative Analysis of Intracellular *Trans*-Splicing Ribozyme Activity Against Hepatitis C Virus Internal Ribosome Entry Site

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(Received September 15, 2004 / Accepted November 4, 2004)

Internal ribosome entry site (IRES) of the hepatitis C virus (HCV) is known to be essential for HCV replication and most conserved among HCV variants. Hence, IRES RNA is a good therapeutic target for RNA-based inhibitors, such as ribozymes. We previously proposed a new anti-HCV modulation strategy based on *trans*-splicing ribozymes, which can selectively replace HCV transcripts with a new RNA that exerts anti-HCV activity. To explore this procedure, sites which are accessible to ribozymes in HCV IRES were previously determined by employing an RNA mapping method *in vitro*. In this study, we evaluate the intracellular accessibility of the ribozymes by comparing the *trans*-splicing activities in cells of several ribozymes targeting different sites of the HCV IRES RNA. We assessed the intracellular activities of the ribozymes by monitoring their target-specific induction degree of both reporter gene activity and cytotoxin expression. The ribozyme capable of targeting the most accessible site identified by the mapping studies then harbored the most active *trans*-splicing activity in cells. These results suggest that the target sites predicted to be accessible are truly the most accessible in the cells, and thus, could be applied to the development of various RNA-based anti-HCV therapies.

Key words: gene therapy, group I intron, HCV, IRES, *Tetrahymena thermophila*, *trans*-splicing ribozyme

The hepatitis C virus (HCV) is the main causative agent of worldwide chronic liver diseases which frequently lead to liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). However, specific and effective anti-HCV therapy has not yet been developed.

HCV contains a positive single-stranded ~9,600-nt-long RNA genome. This viral genome is present exclusively in RNA form during replication. Thus, several RNA-based antiviral therapeutic strategies based on *trans*-cleavage ribozyme, antisense RNA, or siRNA have been proposed elsewhere (Hugle and Cerny, 2003). We have recently suggested a new genetic approach against HCV, which is based on *trans*-splicing ribozymes. This process could specifically replace particular HCV transcripts to selectively, as well as specifically, induce therapeutic gene activities in the HCV RNA-expressing human cells (Ryu *et al.*, 2003). The internal ribosome entry site (IRES) was targeted because the sequence is highly conserved among HCV genotypes and mediates the cap-independent translation critical for viral replication (Rosenberg, 2001). The ribozyme, which originated from self-splicing group I intron of *Tetrahymena thermophila*, has been previously shown in bacteria and mammalian cells, as well as *in vitro*, to *trans*-splice an exon tagged to its 3' end onto a separate 5' exon RNA. Those ribozymes have been utilized to repair mutant RNA associated with human genetic diseases and cancers (Lan *et al*, 1998; Phylactou *et al.*, 1998; Watanabe and Sullenger, 2000; Rogers *et al.*, 2002; Shin *et al.*, 2004). Moreover, the ribozyme reacted with and altered transcripts overexpressed in tumors such as thymidine phosphorylase (TP) RNA by transferring an intended 3' exon tag sequence onto the TP RNA (Park *et al.*, 2003). *Trans*-splicing ribozyme-based therapeutic strategies will have advantages over other methods, such that the ribozyme may selectively induce therapeutic gene activity in specific target cells, while simultaneously destroying disease-associated or related unique RNAs.

One key factor for the application of the *trans*-splicing ribozyme to efficient HCV treatment in patients will be the identification of the most accessible sites in the target RNA. To identify and confirm the most easily targeted HCV sequence in cells in this study, we evaluated intracellular *trans*-splicing activities of several group I-based ribozymes which target the different sites of HCV IRES predicted to be accessible or inaccessible from RNA map-

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ping studies in test tubes.

The group I ribozyme of *Tetrahymena thermophila* can recognize accessible uridine residues in target RNA through G-U base pairing between the internal guide sequence (IGS) of the ribozyme and the target RNA. To determine which HCV IRES RNA uridines are accessible to ribozymes in our recent study, an RNA mapping strategy was employed *in vitro* (Ryu and Lee, 2003). The mapping method was based on the sequence analyses of *trans*-splicing junction sites using a ribozyme library with randomized IGS of the *Tetrahymena* group I intron (Lan *et al.*, 1998; Ryu *et al.*, 2003) and RNA tagging (Jones *et al.*, 1996). Then, the residues between nt 191 and 199, which are present at loop IIIb domain of HCV IRES (Honda *et al.*, 1999), appeared to be particularly accessible with the uridine at position 199 (Ryu and Lee, 2003).

To verify if the sites predicted to be accessible by mapping studies in the test tube are truly the most accessible sites to ribozymes in cells, we first assessed intracellular *trans*-splicing activity of three different ribozymes targeting uridines at position 196 (U196), 199 (U199), or 251 (U251) in HCV IRES RNA, which were identified from mapping study, or two ribozymes targeting uridine at position 86 (U86) or 329 (U329), which were not detected from the mapping analysis (Fig. 1). The IGS of each *trans*-splicing ribozyme is as follows: Rib86, 5'-GAAGGA-3'; Rib195, 5'-GAGGAC; Rib199, 5'-GAGAAA-3'; Rib251, 5'-GGCAGU-3'; Rib329, 5'-GCGAGA-3'. Intracellular *trans*-splicing activity of the ribozymes was observed by monitoring the induction rate of expression of the reporter gene, firefly luciferase (FLuc) in this study, which was tagged at the 3' end of each ribozyme. We cotransfected each ribozyme (5 μg) consisting of the specific IGS along with or without HCV IRES RNA (5 μg) into liver-derived Huh7 cells. We then assessed the induction of the transgene by measuring FLuc activity relative to *Renillar* luciferase (RLuc) cotransfected for normalization 24 h after transfection using a luminometer TD-20/20 (Turner Designs Instrument, USA) and a dual luciferase reporter assay kit (Promega, USA). The 3' exon of each ribozyme contains FLuc RNA fused with the 3' part of the HCV IRES which encompasses downstream sequence of the targeted residue (Fig. 1A). Thus, correct *trans*-splicing can induce FLuc gene expression in an HCV IRES-dependent manner. Transfection of ribozyme alone into cells poorly induced FLuc expression. With Rib86FL and Rib329FL targeting U86 and U329 of the HCV IRES, respectively, little stimulation of FLuc was shown even in the presence of HCV IRES RNA. In contrast, Rib196FL, Rib199FL, and Rib251FL efficiently triggered FLuc activity in the presence of the target RNA (Fig. 1B). Notably, FLuc activity was the most highly induced by cotransfection of the HCV IRES RNA with the Rib199FL ribozyme up to 6-fold, compared with cells transfected with ribozyme alone. The ribozyme was predicted to target the most accessible site of the HCV IRES RNA from mapping analyses. Therefore, relative ribozyme accessibility of the target sites in cells corresponded with the predicted accessibility from mapping studies.

In our previous study, the *trans*-splicing ribozyme tagged with diphtheria toxin (DTA) RNA at its 3' end was able to selectively trigger cytotoxin activity in HCV IRES-expressing cells, thus inducing apoptosis in the cells (Ryu *et al.*, 2003). This cell-specific expression of DTA by the ribozyme could discriminately cause the deaths of HCV-infected cells, thereby suppressing HCV replication and spreading to normal and adjacent cells. To confirm the relative accessibility of the ribozyme to the substrate in cells, we next determined and compared the cytotoxin induction rate of ribozymes targeting different U residues of the HCV IRES RNA. The SV40 promoter system was used to express ribozyme derivatives harboring DTA ORF

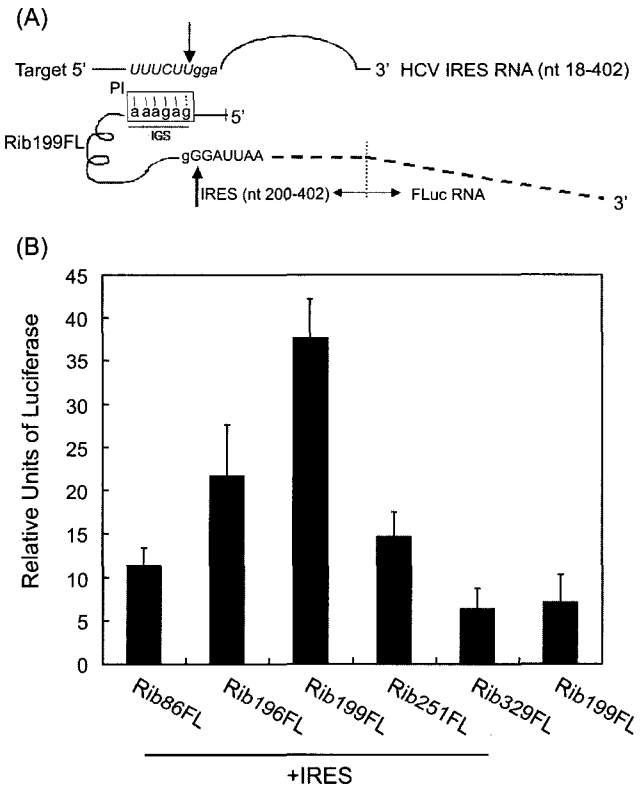

Fig. 1. Comparison of luciferase activity induction by specific *trans*-splicing ribozymes in HCV IRES-expressing Huh7 cells. (A) Schematic diagram of the *trans*-splicing ribozyme. The target transcript; HCV IRES RNA, is represented with sequences around the splice site shown italicized. Rib199FLuc targeting U199 of the target RNA is shown with 3' exon sequences capitalized. Potential base paring between the ribozyme and the target is indicated by vertical line. Arrows indicate 5' and 3' splicing sites. (B) Cells were transfected with ribozyme alone or with IRES RNA plus various ribozyme RNAs. Luciferase activity of FLuc relative to RLuc was quantitated, and averages of measurements, performed in triplicate, are shown with bars to indicate standard errors.

fused with the 3' part of the HCV IRES which encompassed the downstream sequence of the targeted residue. To express functional *trans*-splicing ribozyme, we extended the IGS of each ribozyme by adding 210-nt-long antisense sequences against FLuc RNA encoded in the target RNA, as well as three more nucleotides of the P1 helix and the 7-nt-long P10 helix sequence (Fig. 2A). It was reasoned that intracellular expression of a ribozyme with only a 6-nt-long IGS was highly inactive (Byun *et al.*, 2003). Moreover, such extension of IGS increased specificity and efficiency of the *trans*-splicing ribozyme in cells by several hundred fold (Kohler *et al.*, 1999). For assessment of cytotoxin induction, Huh7 cells were transfected with 300 ng of GFP-encoding plasmid, pEGFN-1 (Clontech, USA), plus 1.4 μg of different SV40 promoter-derived ribozyme expression vectors with or without 300 ng pIRES/F which harbors the CMV promoter and HCV IRES followed by the FLuc gene. Along with pSEAP, the control SV40 promoter-containing empty vector, pEGFN-1 was cotransfected with pIRES/F as a control. Cytotoxicity due to cytotoxin induction by each ribozyme was compared by observation of relative GFP-positive cells 24 h after transfection (Fig. 2B). For each sample, 20 random fields of view were analyzed and the number of GFP-expressing cells was counted using fluorescence microscope. In the control cells without ribozymes, approximately 1.5×10^3 cells were counted as GFP-positive. As shown, transfection of ribozyme expression plasmid alone decreased the number of GFP-positive cells by only 5%, compared with cells transfected with control vector. This suggests the specificity of the ribozyme in cells. Cotransfection of expression vector encoding Rib329, pSVR329-DTAS, with pIRES/F showed no influence on cell cytotoxicity. However, the number of GFP-positive cells was reduced by cotransfection of target RNA-encoding plasmid, along with expression vector encoding Rib196, Rib199, or Rib251, which is predicted to efficiently target the HCV IRES from mapping analyses. The targeting of the most accessible site of target HCV IRES RNA, U199, *in vitro* by pSVR199DTAS, which encodes Rib199, showed the most effective cell cytotoxicity in the presence of the target RNA, which causes a significant and selective reduction in the number of GFP-positive cells by up to 72%. These results, together with the reporter gene stimulation analysis, strongly indicated that the *trans*-splicing ribozyme activities in cells correlated very well with the ribozyme accessibility predicted from mapping studies.

Many anti-HCV protocols based on inhibitory RNAs, such as antisense, ribozyme, or siRNA, were recently proposed. For this approach to be successful when applied to clinical settings, accessibility of the target HCV RNA to the inhibitory RNAs in cells will be one essential factor which must be overcome. In this study, we clearly showed that the *trans*-splicing ribozymes targeting most accessible sites identified by *in vitro* mapping analyses were most active in cells in the induction of the activity of the transgene tagged at the 3' end of the ribozymes. Therefore, mapping analyses could be a useful way to identify recognizable sites of the HCV RNA and, potentially, other unique disease-related target RNAs for the RNA-based inhibitors, thus harnessing the therapeutic development of various infectious, genetic, or acquired human diseases. Comparative studies with other inhibitory RNAs targeting various HCV IRES sites, identified here as accessible in cells, will have to be studied to determine whether the mapping analysis could generally be utilized for the foundation of accessible sites to the RNA inhibitors. In addition, the cytotoxin induction study performed here could be a highly sensitive experimental method for the

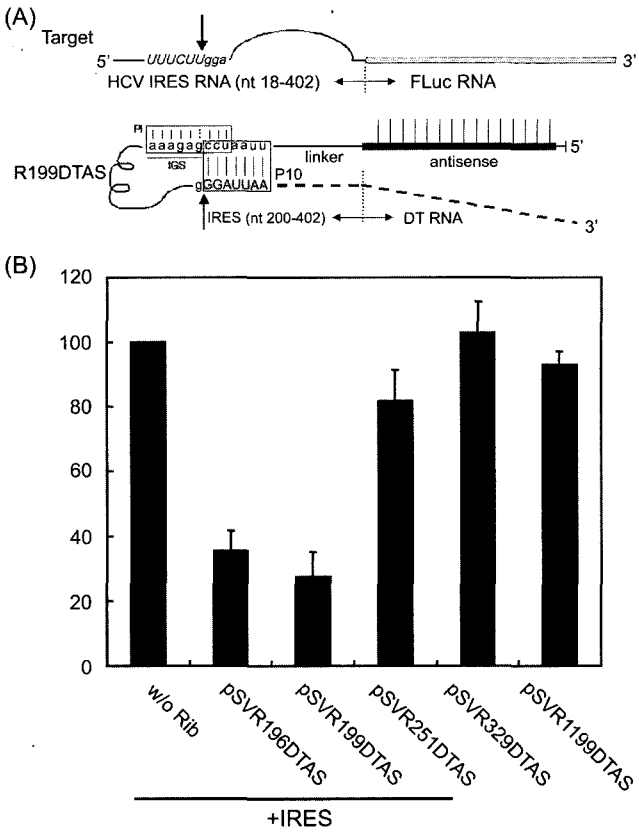

Fig. 2. Comparison of cell cytotoxicity induction by specific ribozymes in HCV IRES-expressing Huh7 cells. (A) Schematic diagram of the *trans*-splicing ribozyme. The target transcript, HCV IRES plus FLuc RNA, is represented with sequences around the splice site shown italicized. *Trans*-splicing ribozymes with extended IGS are shown with 3' exon sequences capitalized. Potential base paring between the ribozyme and the target is indicated by vertical line. 5' and 3' splicing sites were indicated by arrows. (B) Cells were cotransfected with pEGFP-N1 along with pIRES/F (IRES) plus control empty vector, pIRES/F plus various ribozyme plasmids encoding diphtheria toxin A chain (pSVR-DTAS), or pSVR199DTAS alone. The number of GFP-expressing cells in each transfectant was counted 24 h after transfection and quantitated as a percentage of the number of those transfected with pIRES/F alone. Error bars correspond to the SD from three independent experiments.

assessment of specificity and efficacy of *trans*-splicing ribozymes in target cells, applying confirmation of ribozyme accessibility in cells for ease and, moreover, development of more effective ribozymes.

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