Identification of the Most Accessible Sites to Ribozymes on the Hepatitis C Virus Internal Ribosome Entry Site

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Received 14 April 2003, Accepted 30 May 2003

The hepatitis C virus (HCV) is a major causative agent of chronic hepatitis and hepatocellular carcinoma. The development of alternative antiviral therapies is warranted because current treatments for the HCV infection affect only a limited number of patients and lead to significant toxicities. The HCV genome is exclusively present in the RNA form; therefore, ribozyme strategies to target certain HCV sequences have been proposed as anti-HCV treatments. In this study, we determined which regions of the internal ribosome entry site (IRES) of HCV are accessible to ribozymes by employing an RNA mapping strategy that is based on a trans-splicing ribozyme library. We then discovered that the loop regions of the domain IIIb of HCV IRES appeared to be particularly accessible. Moreover, to verify if the target sites that were predicted to be accessible are truly the most accessible, we assessed the ribozyme activities by comparing not only the trans-splicing activities in vitro but also the trans-cleavage activities in cells of several ribozymes that targeted different sites. The ribozyme that could target the most accessible site identified by mapping studies was then the most active with high fidelity in cells as well as in vitro. These results demonstrate that the RNA mapping strategy represents an effective method to determine the accessible regions of target RNAs and have important implications for the development of various antiviral therapies which are based on RNA such as ribozyme, antisense, or siRNA.

Keywords: Gene therapy, HCV, IRES, RNA mapping, Trans-splicing ribozyme

Introduction

The hepatitis C virus (HCV) infection causes chronic liver diseases that can frequently lead to hepatocellular carcinoma (Lauer and Walker, 2001). Worldwide, there are about 170 million infected individuals (World Health Organization, 1999). Presently, the only treatments for the HCV infection are α-interferon (α-IFN) or, more recently, a polyethylene glycol modified form of α-IFN in combination with rivabrin (Bisceglie et al., 2002). With these treatments, however, many chronically ill patients fail to sustain virological benefits and show significant toxicities (Manns et al., 2001; Fried et al., 2002). Therefore, more improved therapeutic modalities are needed for the HCV infection.

HCV is a member of the human flavivirus family with a positive-stranded ~9,600-nucleotide(nt) RNA genome (Miller and Purcell, 1990; Choo et al., 1991). Since the viral genome is exclusively present in the RNA form, then several ribozyme- (Lieber et al., 1996; Sakamoto et al., 1996; Welch et al., 1996; Macejak et al., 2000), antisense oligonucleotide- (Hanecak et al., 1996; Witherell, 2001), or siRNA-based strategies (Kapadia et al., 2003; Randall et al., 2003; Wilson et al., 2003) to specifically target certain HCV sequences have been proposed as HCV therapeutics. However, one key factor to successfully apply these RNA-based anti-HCV treatments to clinical settings is to find the most accessible sites in the target RNA sequences. This is because the target RNAs would form complex tertiary configurations and have proteins bound to them in cells (Lane et al., 2000).

The group I ribozyme from Tetrahymena thermophila was previously demonstrated to trans-splice an exon that is attached to its 3' end onto a separate 5' exon RNA, not only in vitro (Been and Cech, 1986) but also in E. coli (Sullenger and Cech, 1994) and mammalian cells (Jones et al., 1996). We, as well as other groups, have shown that these group I-based trans-splicing ribozymes can revise mutant transcripts that are associated with several human genetic and malignant diseases (Lane et al., 1998; Phylactou et al., 1998; Watanabe and Sullenger, 2000; Rogers et al., 2002; Shin et al., 2002).
Moreover, we demonstrated that the trans-splicing ribozymes could selectively and specifically induce therapeutic gene activities in HCV RNA-expressing cells (Ryu et al., 2003). The potential of the ribozymes to treat various human diseases is based on the ribozyme activity to specifically replace a target RNA with any RNA molecule to express. There is extremely malleable flexibility to select specific RNA molecules because virtually any U residue in a 5’ exonic RNA can be targeted for splicing by altering the nucleotide composition of the 5’ exon binding site, called internal guide sequence (IGS), on the ribozyme to make it complementary to a target sequence that is present on the substrate RNA (Sullenger, 1996). In addition, the trans-splicing ribozyme generates reaction products that are stable in cellular circumstances, and hence easily detected by RT-PCR and readily assayed, providing experimental systems to optimize ribozyme catalysis in cells (Jones and Sullenger, 1997). Therefore, the trans-splicing ribozyme library with randomized IGS can be used for mapping the most accessible reacting sites to ribozymes on any target RNA.

In this study, we determined which regions of the HCV RNA sequence are most accessible to ribozymes by employing an RNA mapping strategy with the trans-splicing ribozyme library. The internal ribosome entry site (IRES), encompassing the 5’-UTR and N-terminal coding sequence of the core protein of HCV (Wang et al., 2000), was selected for targeting because the sequence is highly conserved among viral genotypes (Bukh et al., 1992) and critical for HCV replication (Rosenberg, 2001). In addition, we evaluated the mapping results by analyzing the activities of various ribozymes that target different sequences on HCV IRES in cells as well as in vitro.

Materials and Methods

Materials The restriction enzymes and reagents for RT-PCR and the in vitro transcription reaction were purchased from Roche Applied Science (Mannheim, Germany). Arginimidine and most of the other chemicals were obtained from the Sigma Chemical Co. (St. Louis, USA). The DMEM tissue culture media and fetal bovine serum came from GIBCO (Grand Island, USA).

Mapping accessible sites on HCV IRES RNA To construct the mapping library (called GN, library), IGS of the Tetrahymena group I trans-splicing ribozyme was randomized so that the 5’ end of the ribozymes in the library began with 5’-GNNNNN-3’, where G represents guanine and N represents equimolar amounts of the four nucleotides (nt) (Lan et al., 1998). Target RNA, HCV IRES (nts 18-402) of the HCV 1b genomic RNA, was generated by in vitro transcription using T7 RNA polymerase with the BamHI fragment of pH18-402CAT (Hahn et al., 1998, a kind gift from S. K. Jang, POSTECH University, Pohang, Korea). To map the HCV IRES RNA, 20 nM of the GN, library was incubated at 37°C for 3 h under splicing conditions (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM MgCl2) in the presence of a guanosine (100 μM) with 50 nM of HCV IRES RNA. The reaction products were reverse-transcribed at 37°C for 30 min in the presence of arginimidine (10 mM) with a 3’ tag primer specific for the 3’ exon lacZ sequence of the ribozyme (5’-ATGTGGTCGAAAGCGATT-3’) (Jones et al., 1996). The cDNAs were then amplified by PCR for 35 cycles using the same 3’ primer and a 5’ primer I encompassing the 5’ end of the target HCV IRES RNA (5’-GGGGGAATTCTGCGGAATTTGTTAGGTA CCG G-3’) or a 5’ primer II specific for the sequence nt 148-165 of HCV IRES (5’-GGGGGAATTCTGCGAAAGCGTTAGA-3’). The amplified trans-splicing products were cloned into a pUC19 vector and the inserted were then sequenced using the dideoxy termination method (Chung et al., 2002).

Ribozyme construction Specific ribozymes (such as Rib86-, Rib195-, Rib199-, Rib251-, Rib329-, or Rib380-3’tag that recognize the uridine at position 86, 195, 199, 251, 329, or 380, respectively, on the HCV IRES RNA) were generated by in vitro transcription of the DNA templates which were created from pT7L-21 by PCR with a 5’ primer that contained the T7 promoter and IGS of each ribozyme, as well as with a 3’ primer specific for the 3’ exon lacZ sequence. The pT7L-21 vector encoded a slightly shortened version of the natural group I intron from Tetrahymena, called L-21 (Sullenger and Cech, 1994). The IGSs on the L-21 trans-splicing ribozyme (5’-GGAGGG-3’) was exchanged with 5’-GUGGCU-3’ in Rib86-3’tag, 5’-GAGGCAC in Rib195-3’tag, 5’-GAGAAA-3’ in Rib199-3’tag, 5’-GGCAGU-3’ in Rib251-3’tag, 5’-GGCCAGA-3’ in Rib329-3’tag, or 5’-GCGUUU-3’ in Rib380-3’tag. In addition, inactive ribozymes [R(d)86-, R(d)195-, R(d)199-, R(d)251-, R(d)329-, or R(d)380-3’tag, which lack part of the catalytic core of the enzyme (Sullenger and Cech, 1994)], were constructed as negative controls.

In vitro assay of ribozyme activity For the in vitro trans-splicing reaction assay of the ribozymes, the individual ribozymes with 3’tag (100 nM) were incubated at 37°C for 3 h under splicing conditions with HCV IRES RNA (10 nM). The resulting RNA was reverse-transcribed at 37°C for 30 min in the presence of arginimidine (10 mM) with a 3’ primer specific for the 3’ exon lacZ sequence of the ribozyme, as previously described. The cDNAs were then amplified by PCR for 35 cycles with the same 3’ primer and with either a 5’ primer I (specific for the 5’ end of HCV IRES RNA) for the reaction with Rib86 or R(d)86, or a 5’ primer II (specific for the sequence nt 148-165 of HCV IRES) for the reaction with the other ribozymes. The reaction products were analyzed on a 3% agarose gel. The RT-PCR products were eluted from the gel, cloned onto a pUC19 vector, and then sequenced with the dideoxy termination method.

For the in vitro trans-cleavage reaction assay, each ribozyme without a 3’tag was produced by in vitro transcription of the Scal-digested DNA fragments of the templates that were used for the generation of ribozymes with a 3’tag. The ribozymes (50 nM) were incubated at 37°C for 4 h under splicing conditions with 5’ end radio-labeled HCV IRES RNA (500 pM). The resulting products of the cleavage reactions were resolved on 5% acrylamide/7 M urea gel and analyzed by autoradiography.

Trans-cleavage reaction in cells The substrate RNA, IRES/FLuc RNA, which encodes HCV IRES followed by firefly luciferase (FLuc) RNA, was created by the in vitro transcription of the DNA
templates that were amplified by PCR from the pR/HCV/F plasmid that is a dicistronic vector expressing renilla luciferase (RLuc) under the CMV promoter and firefly luciferase (FLuc) under the control of HCV IRES. This was kindly donated by S. K. Jang. RLuc RNA was generated by the \( in \text{ vitro } \) transcription of the Salt-digested pR/HCV/F plasmid. The RNAs that were used for the transfection into cells were modified at its 3’ end by the addition of poly(A) using poly(A) polymerase (Amersham Biosciences, Piscataway, USA) at 37°C for 20 min. For the \( trans \)-cleavage reaction in the cells, the 293T cells were plated at 3.0 \( \times \) 10⁵ cells per well in 35 mm dishes 24 h prior to transfection. The cells were cotransfected with 0.5 \( \mu \)g IRES/RLuc RNA along with 0.5 \( \mu \)g RLuc RNA and 4 \( \mu \)g tRNA, active or inactive ribozymes using 3 \( \mu \)l DMRIE-C (Invitrogen, Carlsbad, USA). The cell lysates were harvested 24 h after transfection, and the reporter gene activities were assessed by measuring relative light units using a luminometer TD-20/20 (Turner Designs Instrument) and dual-luciferase reporter assay system (Promega, Madison, USA) (Kim and Park, 2002).

**Results and Discussion**

Mapping of ribozyme-accessible uridines in HCV IRES RNA  
HCV IRES RNA can be recognized by the \( trans \)-splicing ribozyme by base pairing to any accessible uridine residue in the RNA through IGS of the ribozyme. However, only a limited number of uridines on the target RNA can be actually accessible to the ribozyme, due to the substrate RNAs complex but stable tertiary structure (Lan et al., 2000). To determine which uridines in the HCV IRES RNA are accessible to ribozymes, an RNA mapping strategy was conducted. The mapping method was based on a \( trans \)-splicing ribozyme library (Lan et al., 1998, 2000) and RNA tagging (Jones et al., 1996) (Fig. 1).

The ribozyme library, called the GN, library (constructed based on the *Tetrahymena* group I intron), contains a randomized IGS. Thus, ribozymes in the GN library will react with and cleave to the substrate RNA at any accessible uridine (U) residue and transfer a 3’ exon to the 3’ end of a 5’ cleavage target product. Part of the lacZ gene was employed as a 3’ exon and molecular tag in the GN library that can be spliced onto the target RNA’s Us that are accessible to the ribozyme. To map the HCV IRES RNA, the GN library was incubated under splicing conditions with the substrate HCV IRES RNA that is generated by \( in \text{ vitro } \) transcription (nt 18-402). The \( trans \)-splicing reaction products were amplified by RT-PCR. Two different amplification reactions were performed with a 3’ primer (specific for the ribozyme’s 3’ exon lacZ sequence) and two different 5’ primers, 5’ primer I (encompassing the 5’ end of target HCV IRES RNA) or a 5’ primer II (specific for the sequence nt 148-165 of HCV IRES), in order to exclude possibilities of missing any long amplified products from ribozyme reactions with the 3’ part of the target RNA. The amplified products were then cloned and sequenced.

Sequence analyses of the splicing junction sites showed that several uridines that are present in the loop regions of domain IIIb appeared to be particularly accessible, because almost 50% of the reaction products resulted from splicing at these sites (Fig. 2). Particularly, the most accessible site was present in the uridines at position 199 on the HCV IRES RNA.

**Evaluation of RNA mapping studies**  
To verify if the sites that were predicted to be accessible by the mapping studies were truly the most accessible sites to ribozymes, we assessed the \( trans \)-splicing activities of three different ribozymes that target uridines at positions 195 (U195), 199 (U199), or 251 (U251) in HCV IRES that were detected via a mapping analysis, or one ribozyme that targeted uridine at position 380 (U380) that was not identified from our mapping study (Fig. 3A). The Rib195-, Rib199-, Rib251-, or Rib380-3’tag ribozymes that recognized these individual sites were generated by \( in \text{ vitro } \) transcription and incubated under splicing condition with the target HCV IRES RNA. RT-PCR analyses were then performed with a 3’ primer that was specific for the 3’ exon tag sequence and a 5’ primer that was specific for HCV IRES, as previously described. An amplified fragment of the expected size of 87 bp, 91 bp, or 143 bp was generated from the reaction mixtures with the HCV IRES.
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RNA and Rib195-, Rib199-, or Rib251-3’tag, respectively (Fig. 3A, lanes 3-5). It should be noted that the Rib199-3’tag can trans-splice a 3’ exon tag onto HCV IRES with the highest efficiency. However, it was difficult to detect this RT-PCR product from a sample with Rib380-3’tag (Fig. 3A, lane 6), indicating that U380 in HCV IRES was barely accessible to the ribozyme. These results, therefore, indicate that the relative trans-splicing efficiency at the chosen sites is the same as the predicted accessibility from our mapping analyses.

Inactive versions of all four ribozymes that lack part of the catalytic core of the enzyme produced no RT-PCR products in the reaction with the target RNA (Fig. 3A, lanes 7-10). Moreover, no trans-splicing product was generated from either sample with the RNA substrate alone or the Rib199-3’tag alone (Fig. 3A, lanes 11, 12). These results suggest that the amplified cDNA products that are found in lanes 3-5 in Fig. 3A are the result of the catalytic activity of the ribozymes.

Once it was ascertained the specific ribozymes performed the trans-splicing reaction to transfer their 3’ exon onto the target HCV IRES RNA, we then attempted to determine if the

Fig. 2. Mapping results of the ribozyme-accessible sites in HCV IRES RNA. Nucleotide positions of the accessible uridines that were identified from the in vitro mapping analysis are indicated as nucleotide numbers by arrows on the predicted secondary structure of HCV 5’UTR and immediately downstream ORF (Honda et al., 1999). The number of clones that contain a given uridine at the splice site is presented in parentheses. The AUG residue that is located at nt 342-344 in domain IV denotes the initiator codon of the HCV core protein.

Fig. 3. In vitro trans-splicing and trans-cleavage activities. (A) RT-PCR analysis of trans-spliced RNA products generated in vitro. A series of active (100 nM; lanes 3-6) or inactive ribozymes (100 nM; lanes 7-10) were incubated with the HCV IRES target RNA substrate (10 nM), and the trans-spliced products were amplified. As a reaction control, the RT-PCR products without RNA (lane 2), with HCV IRES alone (lane 11), or Rib199 alone (lane 12) were presented. Amplification products were then subjected to electrophoresis in a 3% agarose gel. The migration of 50 bp ladder is indicated as a molecular mass marker (lane 1, MW). (B) Sequence analysis of trans-splicing products produced in vitro. The amplified products from the trans-splicing reaction between Rib199 and HCV IRES (Fig. 3A, lane 4) were isolated on a gel and cloned. Sequence of one representative clone (from 10 different clones with the same sequence) is shown. The expected sequence around the splicing junction, indicated by an arrow, is shown with the ribozyme recognition sequence in HCV IRES RNA boxed and the uridine at position 199 circled. The intact HCV IRES sequence is also represented in parallel. (C) In vitro cleavage of HCV IRES RNA. Active ribozyme, Rib199, or inactive ribozyme, R(d)199, (50 nM each) were incubated with the 5’ end radiolabeled substrate HCV IRES RNA (0.5 nM) and the aliquots were removed at 0, 30, 60, 120, and 240 min, as indicated. The cleavage reactions then were resolved on a 5% acrylamide/7 M urea gel. The uncleaved substrate and 5’ cleavage products are indicated.
ribozyme reaction could occur with fidelity. To this effect, sequence analyses of the spliced products (detected at lane 4 in Fig. 3A) were carried out. A sequence analysis of the 91-bp amplified fragment demonstrated that the ribozyme, Rib199-3'tag, had correctly reacted with HCV IRES at the predicted reaction site (U199) and replaced sequences downstream of the reaction sites with the 3' exon sequences tagged at the 3' end of the ribozyme (Fig. 3B). Sequencing of the reaction products that were isolated from lanes 3 or 5 of Fig. 3A demonstrated that the Rib195- or Rib251-3'tag also correctly spliced its 3' exon tag onto the HCV IRES target RNA at the predicted reaction site (data not shown). From these results, it was concluded that specific ribozymes that target the predicted accessible sites could react with high fidelity with the target RNA.

To confirm the activities of the ribozymes that target the sites that are predicted to be most accessible by mapping studies, the trans-splicing activities of the ribozymes were next tested. To this effect, we constructed ribozymes without 3' exon (described in Materials and Methods) and incubated them with 5' end radio-labeled HCV IRES RNA. Rib199 without 3' exon were revealed to efficiently cleave a majority of the target HCV IRES RNA and yield products of the expected size in vitro (Fig. 3C). In contrast, no cleavage products of the target RNA were found with the inactive ribozyme (R(d)199), indicating that the cleavage products that were detected in the sample with Rib199 resulted from the catalytic activity of the ribozyme. Moreover, trans-cleavage activity Rib199 was the most efficient among the ribozymes that targeted different accessible sites of HCV IRES (data not shown).

The trans-splicing and trans-cleavage analyses indicated, therefore, that the relative ribozyme accessibility of the target sites corresponded with the predicted accessibility from the mapping analyses.

Comparison of ribozyme activities in vitro and in cells To compare the Rib199 ribozyme activity with other ribozymes, we incubated various ribozymes that targeted different sites in HCV IRES with the substrate RNA in vitro, amplified trans-splicing products, and analyzed the relative ribozyme activity by assessing the relative amounts of the reaction products (Fig. 4A). Rib195, Rib196, Rib199, and Rib251, which targeted uridines in HCV IRES that were detected via the mapping analysis, efficiently employed the trans-splicing reaction with the target RNA. In contrast, Rib86, Rib329, and Rib380, which targeted uridines that were not identified from our mapping study, barely performed a trans-splicing reaction with the HCV IRES RNA. Noticeably, Rib199 that targeted the site that was predicted to be the most accessible by the mapping study harbored the highest ribozyme activity in vitro among all the ribozymes that were tested in this study.

Once it was determined that the ribozymes that specifically recognized the most accessible sites of the substrate efficiently reacted with the target RNA in vitro, we then determined if the ribozymes could also be active in cells. To this effect, we assessed the intracellular trans-cleavage activity of the ribozymes by measuring and comparing the reporter firefly luciferase activity in the cells that were co-transfected with individual ribozyme and IRES/FLuc RNA that encoded the reporter firefly luciferase (FLuc) linked to HCV IRES (Fig. 4B). If the ribozymes were truly active in cells, then the intracellular cleavage by the ribozymes would result in a reduction in the target RNA amount, thereby reducing the luciferase activity. To control and normalize for differences in the HCV IRES-dependent FLuc expression that is due to transfection efficiency, we also co-transfected RNA that

![Graph](image-url)
based viral suppression is the accessibility of the substrate. One key factor that influences the efficiency of this RNA-siRNA) have been proposed (Sullenger and Gilboa, 2002). trans-splicing ribozyme, antisense oligonucleotides, or short oligonucleotides (such as trans-cleavage ribozyme, many anti-viral protocols that are based on inhibitory RNA or HCV IRES RNA for the inhibitory RNA binding. Therefore, mapping and in vitro analyses.

In this study, by the use of mapping studies, we identified the loop IIIb regions, especially uridine at position 199, in HCV IRES as the most accessible to ribozymes. Moreover, the trans-splicing and trans-cleavage analyses in vitro, and the trans-cleavage reaction in cells clearly showed that the ribozymes that recognize these identified accessible sites are truly most active. Other groups also demonstrated that hammerhead ribozymes that targeted the loop IIIb of HCV IRES were more effective than others (Macejak et al., 2000). However, they chose ribozymes randomly, not by rational mapping studies, and also did not analyze the activity of ribozyme that recognized uridine at position 199. Recently, many anti-viral protocols that are based on inhibitor RNA or short oligonucleotides (such as trans-cleavage ribozyme, trans-splicing ribozyme, antisense oligonucleotides, or siRNA) have been proposed (Sullenger and Gilboa, 2002). One key factor that influences the efficiency of this RNA-based viral suppression is the accessibility of the substrate RNA for the inhibitory RNA binding. Therefore, mapping studies, as performed here, could be generally used in a wide range of studies for optimizing the intracellular anti-HCV activity with various inhibitory RNAs. Furthermore, the mapping analyses that were developed in this study could be potentially exploited to isolate the most accessible sites, also in other viral RNAs or tumor-associated unique RNAs, for the therapeutic development of other infectious or malignant diseases.

Acknowledgments We are deeply thankful to S. K. Jang (POSTECH University) for his generous gift of the pH(18-402)CAT vector and pR/HCV/F, and B. Sullenger (Duke University) for p7TL-21. The present research was conducted by the research fund of Dankook University in 2001.

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