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

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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

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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 (Lan *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 (Lan *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' exon 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). Argininamide 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$_5$ 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 *Bam*HI fragment of pH(18-402)CAT (Hahm *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$_5$ library was incubated at 37°C for 3 h under splicing conditions (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM MgCl$_2$) 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 argininamide (10 mM) with a 3' tag primer specific for the 3' exon *lacZ* sequence of the ribozyme (5'-ATGTGCTGCAAGGCGATT-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'-GGGGAATTCGGGCGAATTGGGTA CCG G-3') or a 5' primer II specific for the sequence nt 148-165 of HCV IRES (5'-GGGGAATTCCTGCGGAACCGGTGAGTA-3'). The amplified *trans*-splicing products were cloned into a pUC19 vector and the inserts 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 IGS on the L-21 *trans*-splicing ribozyme (5'-GGAGGG-3') was exchanged with 5'-GUGGCU-3' in Rib86-3'tag, 5'-GAGGAC in Rib195-3'tag, 5'-GAGAAA-3' in Rib199-3'tag, 5'-GGCAGU-3' in Rib251-3'tag, 5'-GCGAGA-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 argininamide (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 *Sca*I-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 vitro* transcription of the *Sal*I-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 3.0×10^5 cells per well in 35 mm dishes 24 h prior to transfection. The cells were co-transfected with 0.5 µg IRES/FLuc RNA along with 0.5 µg RLuc RNA and 4 µg tRNA, active or inactive ribozymes using 3 µl 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$_5$ library (constructed based on the *Tetrahymena* group I intron), contains a randomized IGS. Thus, ribozymes in the GN$_5$ 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$_5$ 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$_5$ library was incubated under splicing conditions with the substrate HCV IRES RNA that is generated by *in 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.

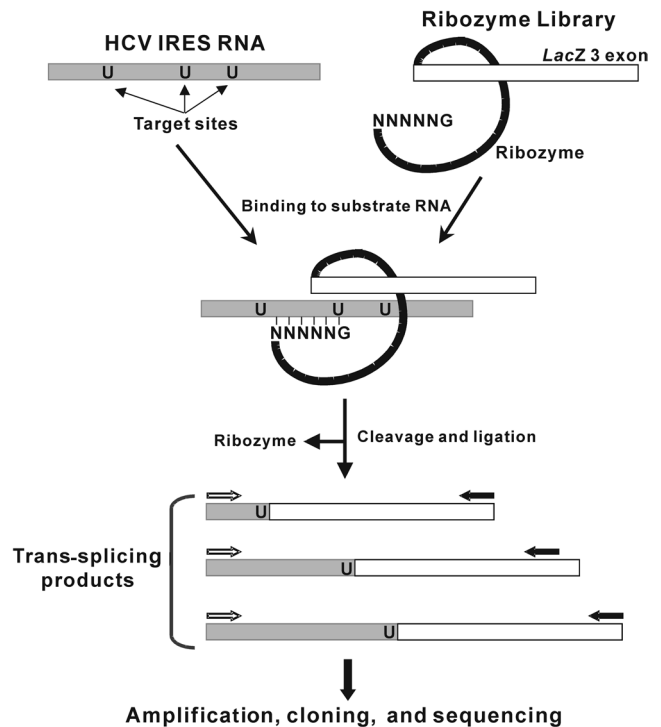Sequence analyses of the splicing junction sites showed that

Fig. 1. Scheme for mapping the accessible sites in HCV IRES RNA *in vitro* with a *trans*-splicing ribozyme library. The GN5 ribozyme library (20 nM) and target HCV IRES transcript (50 nM) were incubated in the reaction buffer at 37°C for 3 h in the presence of guanosine (100 µM). The reaction products were amplified with a 5' primer, which was specific for HCV IRES RNA, and with a 3' primer that recognized the 3' exon tag *lacZ* sequence, cloned, and sequenced.

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 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
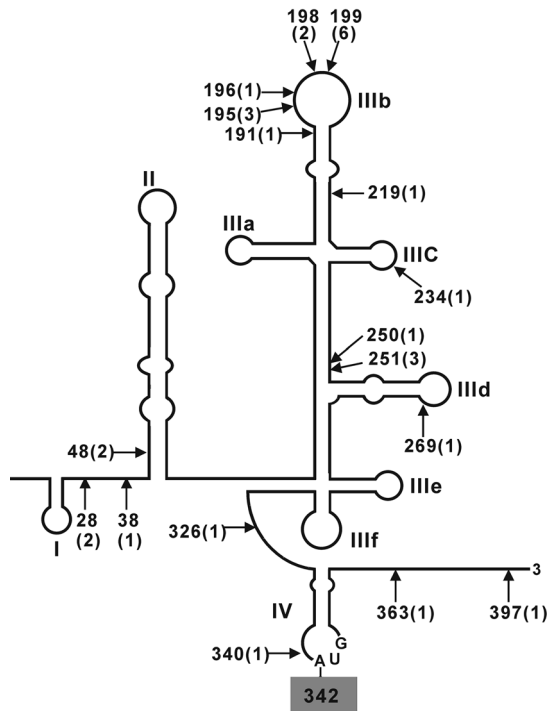
Fig. 2. Mapping results of the ribozyme-accessible sites in HCV IRES RNA. Nucleotide positions of the accessible uridines that were identified from the *in vitro* mapping analysis are indicated as nucleotide numbers by arrows on the predicted secondary structure of HCV 5'UTR and immediately downstream 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.

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
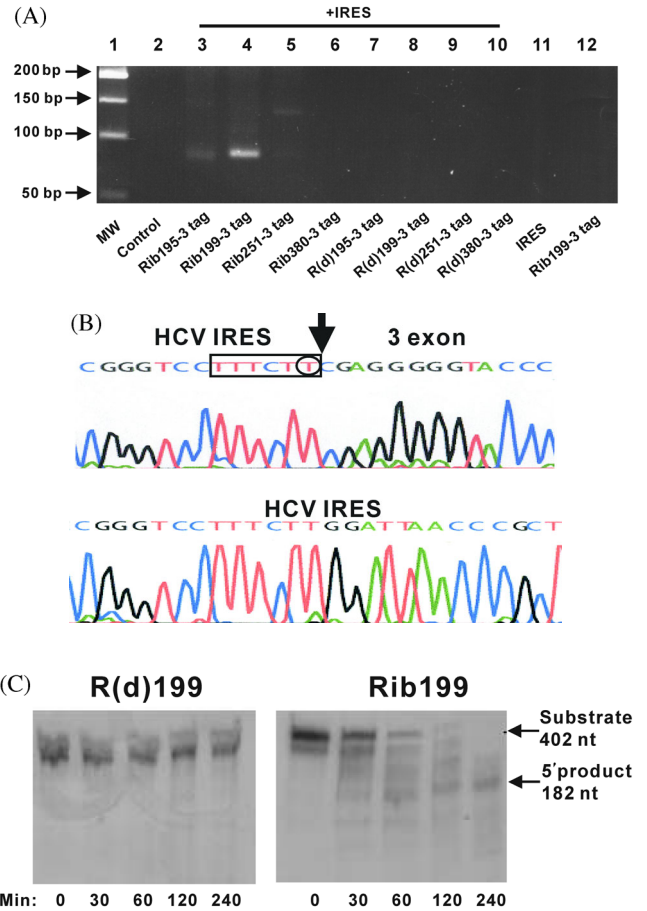

Fig. 3. *In vitro trans*-splicing and *trans*-cleavage activities. (A) RT-PCR analysis of trans-spliced RNA products generated *in vitro*. A series of active (100 nM; lanes 3-6) or inactive ribozymes (100 nM; lanes 7-10) were incubated with the HCV IRES target RNA substrate (10 nM), and the *trans*-spliced 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*-cleavage 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
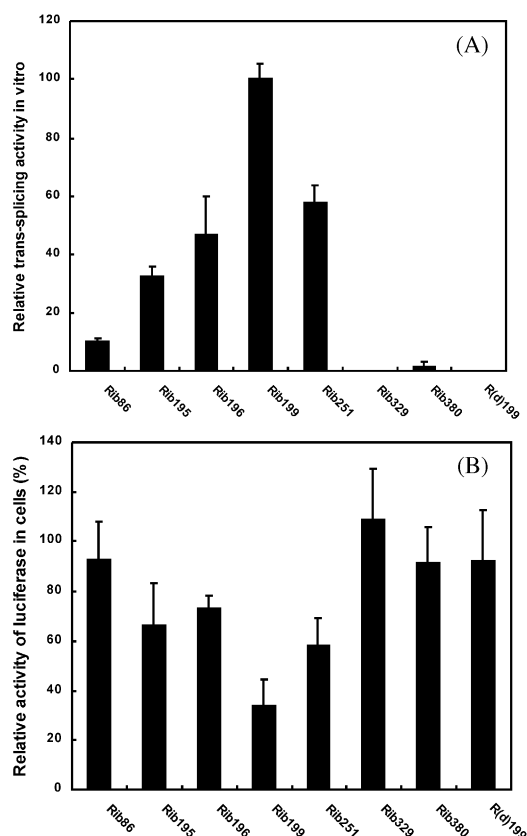

Fig. 4. Ribozyme activity *in vitro* and in cells. (A) Ribozyme *trans*-splicing activity *in vitro*. Specific ribozymes that recognize individual sites on HCV IRES or the inactive version of the ribozymes were incubated with HCV IRES. The *trans*-splicing reaction products were amplified and analyzed, described in Fig. 3A. The PCR products were quantified using Alphaimager™ 2200 (Alpha Innotech). The relative *trans*-splicing activity of each ribozyme was represented as the *trans*-splicing products that were normalized with the amount of PCR products of the reaction with Rib199. (B) Ribozyme cleavage activity in cells. The 293T cells were co-transfected with the control tRNA, specific ribozymes, or inactive ribozymes along with IRES/FLuc RNA as a reporter RNA. The relative luciferase activity was quantitated as a percentage of the sample that was transfected with the control tRNA. The values represent the means plus and minus standard deviation of three separate measurements.

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

encodes renilla luciferase (RLuc). Rib195, Rib196, Rib199, and Rib251 reduced the HCV IRES-dependent expression of the reporter gene; whereas, the inactive version of the ribozyme, R(d)199, showed little inhibition. Rib199 in particular blocked the IRES-dependent expression of the reporter gene the most effectively and reduced the expression by 70% relative to the control tRNA. Furthermore, Rib86, Rib329, and Rib380, which were poorly reactive with HCV IRES *in vitro* (as shown in Fig. 4A), also barely inhibited the expression of IRES/FLuc RNA. Thus, the reduction of the IRES/FLuc expression by Rib195, Rib196, Rib199, and Rib251 could be mainly due to the intracellular cleavage of HCV IRES RNA. These results also strongly indicated that the relative ribozyme-accessibility of the target sites in the cells corresponded with the accessibility which were predicted by 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 inhibitory 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.

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