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Ribozyme-Mediated Selective Induction of New Gene Activity in Hepatitis C Virus Internal Ribosome Entry Site-Expressing Cells by Targeted *Trans*-splicing

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Although hepatitis C virus (HCV) causes worldwide health problems, efficient and specific therapy is not available so far. In this study, we describe a new genetic approach to the specific HCV therapy that is based upon *trans*-splicing ribozymes that can selectively replace HCV transcripts with a new RNA that exerts anti-HCV activity. We have developed a group I intron-based ribozyme targeting the internal ribosome entry site (IRES) of HCV with high fidelity and specificity. The ribozyme was designed to *trans*-splice its 3′ tagging sequence comprising a new coding RNA, such as firefly luciferase transcript, that is linked to the 3′ part of the HCV 5′ UTR encompassing the downstream sequence of the targeted residue in the IRES. This ribozyme was then demonstrated to induce HCV IRES-dependent translation of the firefly luciferase gene selectively in HCV IRES-expressing cells with *trans*-splicing reaction. Moreover, a specific ribozyme with the coding sequence of the diphtheria toxin A chain in place of the firefly luciferase selectively triggered expression of the cytotoxin in cells expressing HCV IRES and specifically activated apoptosis of the cells. These results suggest that the *trans*-splicing ribozyme could be a potent anti-HCV agent to deliver therapeutic new gene activities specifically and selectively in HCV-infected cells.

Key Words: HCV, IRES, *trans*-splicing, ribozyme, RNA replacement, gene therapy

INTRODUCTION

Hepatitis C virus (HCV) is the main causative agent of non-A, non-B hepatitis [1,2], with approximately 170 million infected individuals worldwide [3]. The great majority of HCV infections become chronic and frequently lead to liver cirrhosis and hepatocellular carcinoma [4,5]. Current treatments for HCV infection, including interferon-α (IFN-α) in combination with rivabrin or, more recently, a polyethylene glycol-modified form of IFN-α, result in sustained viral clearance in only a limited number of patients and lead to significant toxicity [6–8]. Thus, development of more specific and effective antiviral therapies is warranted.

HCV is an ~9600-nt-long plus-strand RNA virus that belongs to the family *Flaviviridae* [9,10]. Since the viral genome is present exclusively in RNA form, several ribozyme strategies to target specifically certain HCV sequences have been proposed as anti-HCV treatments [11–14]. However, difficulty in effective cleavage and elimination of the vast majority of viral RNAs with *trans*-cleaving ribozymes in an HCV-infected cell would limit their utility in clinical circumstances. An attempt to destroy viral RNAs in an infected cell and engender production of antiviral proteins simultaneously could be more efficient to inhibit HCV replication.

The self-splicing group I intron from *Tetrahymena thermophila* has been previously demonstrated to *trans*-splice an exon attached to its 3′ end onto a separate 5′ exon RNA not only *in vitro* [15] but also in *Escherichia coli* [16] and mammalian cells [17]. Moreover, the *trans*-splicing ribozymes have been successfully employed to revise mutant transcripts associated with several human genetic diseases and human cancers [18–21]. The ribozyme was also utilized to induce selectively expression of a cytotoxin gene tagged at its 3′ end in yeast expressing a certain target RNA and hence inhibit growth of the cells [22]. Thus, *trans*-splicing ribozymes could be potentially used for the selective induction of new antiviral gene activities in HCV-infected cells while simultaneously ruining the viral RNAs.

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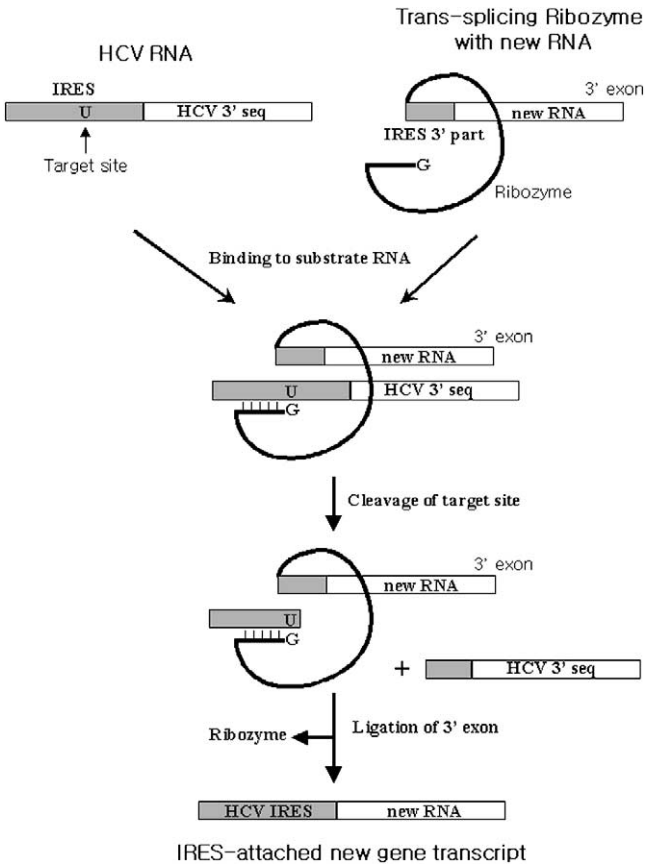

FIG. 1. Scheme of ribozyme-mediated selective induction of new RNA by targeted *trans*-splicing of HCV IRES. HCV IRES can be recognized by a ribozyme at any accessible uridine residue by base pairing to the sequence through its IGS and then cleaved. The ribozyme releases the 3′ cleavage product (HCV 3′ seq) and replaces it with a 3′ exon that encodes a new RNA sequence fused with the 3′ part of the HCV 5′ UTR sequence (IRES 3′ part).

To explore this strategy, we chose the 5′ untranslated region (5′ UTR) of HCV as a target RNA. The reason was that HCV 5′ UTR contains an internal ribosome entry site (IRES) that is highly conserved among viral genotypes [23] and mediates cap-independent viral translation important for HCV replication [24,25]. HCV IRES can be recognized by the *trans*-splicing ribozyme by base pairing to any accessible uridine residue in the RNA through the internal guide sequence (IGS) of the ribozyme. The ribozyme then cleaves the target RNA, releases the downstream HCV RNA sequence, and replaces the sequence with its 3′ exon that encodes a new gene fused with the 3′ part of the HCV 5′ UTR encompassing downstream sequence of the targeted uridine residue (Fig. 1). Because the N-terminal coding sequence of the HCV core protein is required for efficient initiation of HCV IRES-dependent translation [26], the transgene will be fused in frame with the 5′ end of the core sequence that is present immediately downstream of the HCV 5′ UTR. Therefore, correct *trans*-splicing with the target RNA would result in the restoration of an intact HCV 5′ UTR and hence induce HCV IRES-dependent translation of the new gene, yielding a hybrid protein fused with the N-terminal amino acids of the HCV core protein.

Here we used firefly luciferase as a reporter for investigating induction of its activity by *trans*-splicing group I ribozymes selectively in human cells with HCV IRES RNA. In addition, we set out to investigate whether the ribozymes can specifically recognize HCV IRES and induce cytotoxic activity of the diphtheria toxin A (DTA) chain, which catalytically ADP ribosylates the diphthamide group of cellular elongation factor 2, inhibits protein translation, and activates programmed cell death [27,28] selectively in human cells expressing the target RNA.

RESULTS

Mapping HCV IRES RNA for Accessible Uridines

To determine which uridines in the HCV IRES RNA are accessible, we performed an RNA mapping strategy that is based on a *trans*-splicing ribozyme library and RNA tagging [17,18]. Several uridines were revealed to be accessible to the ribozyme by sequence analyses of the splicing junctions (Fig. 2A). The uridines in the loop region of domain IIIb of the HCV 5′ UTR appear to be particularly recognized because more than half of the reaction products result from splicing at these sites. Especially, the uridine at position 199 appears to be the most accessible to the ribozyme.

To verify if the sites predicted to be accessible by mapping studies are truly the most accessible sites, we evaluated *in vitro trans*-splicing activities of four different ribozymes targeting uridines at position 195 (U195), 196 (U196), 199 (U199), or 251 (U251) in HCV IRES, which were identified via mapping analysis, or one ribozyme targeting uridine at position 329 (U329) that was not detected from our mapping study (Fig. 2B). Rib195-, Rib196-, Rib199-, Rib251-, or Rib329-3′tag ribozymes recognizing these individual sites were generated and incubated with the target HCV 5′ UTR RNA. RT-PCR of *trans*-splicing reaction mixtures with Rib195-, Rib196-, Rib199-, or Rib251-3′tag generated an amplified fragment of the expected size of 107, 108, 111, or 163 bp, respectively (Fig. 2B, lanes 2–5). Noticeably, Rib199-3′tag can *trans*-splice a 3′ exon tag onto HCV IRES with the highest efficiency. In contrast, no RT-PCR product was demonstrated from a sample with Rib329-3′tag (Fig. 2B, lane 6), indicating that U329 in HCV 5′ UTR was inaccessible to the ribozyme. These results indicate that the relative *trans*-splicing efficiency at the chosen sites corresponds with the predicted accessibility from our mapping studies. No *trans*-splicing product was detected in samples with the inactive versions of all five ribozymes that lack part of the catalytic core of the enzyme, with substrate RNA alone, or with Rib199-3′tag alone (Fig. 2B, lanes 7–13). Therefore, the

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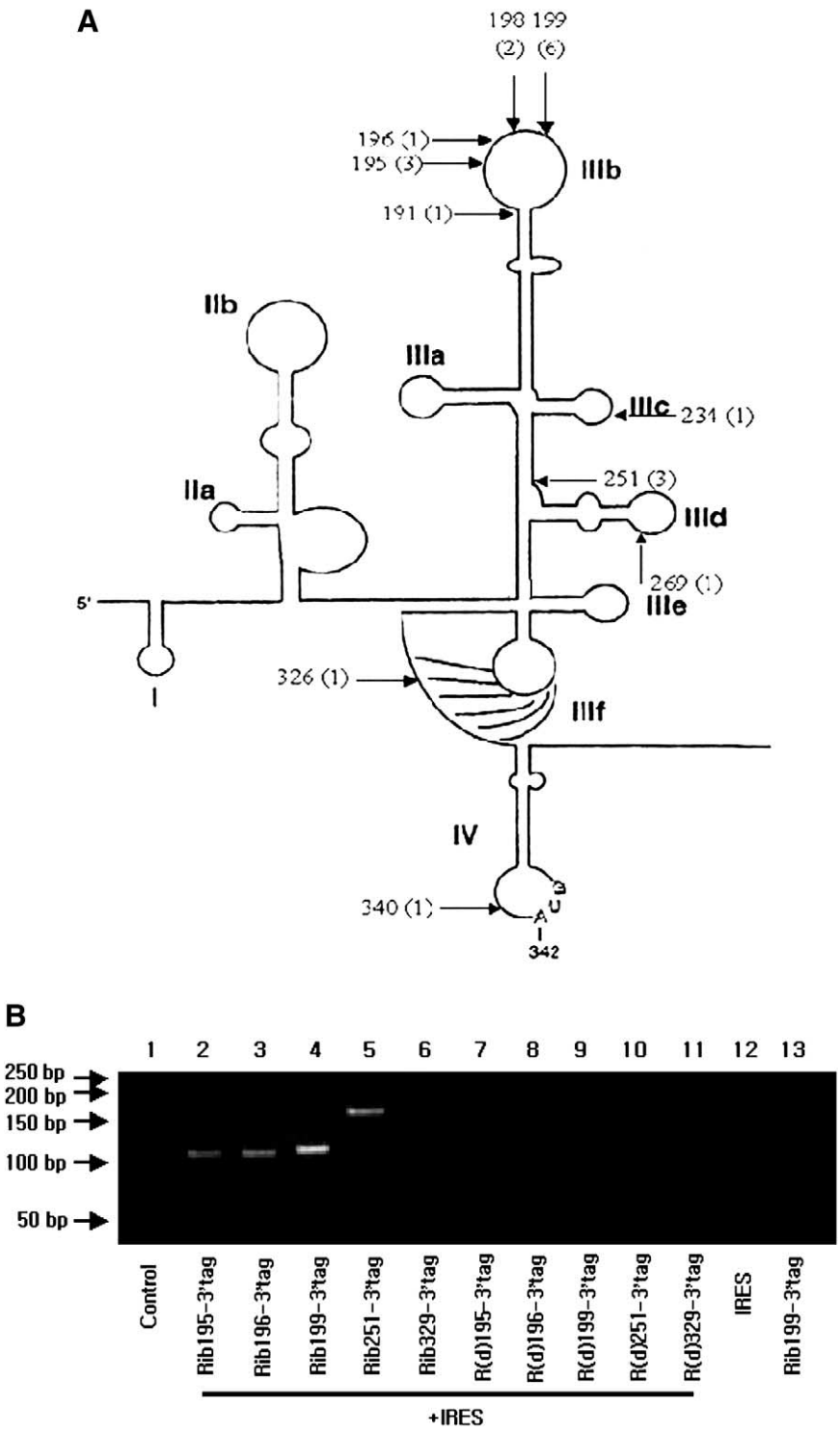

FIG. 2. *In vitro* mapping of and *trans*-splicing with HCV IRES RNA. (A) Predicted secondary structure of HCV 5′ UTR and immediately downstream ORF according to the study by Honda *et al.* [38] and mapping results of the ribozyme-accessible sites in the RNA. Nucleotide positions for the accessible uridines identified from *in vitro* mapping analysis are presented by arrows. The number of clones containing a given uridine at the splice site is indicated in parentheses. Initiator codon of the HCV core protein with the A residue at nt 342 is located in domain IV. (B) RT-PCR analysis of *trans*-spliced RNA products generated *in vitro*. Series of active (Rib-3′tag, 100 nM; lanes 2–6) or inactive (R(d)-3′tag, 100 nM; lanes 7–11) ribozymes were incubated with HCV IRES (10 nM), and *trans*-spliced products were amplified.

amplified products resulted from catalytic activity of the ribozymes. Moreover, Rib199 without 3′ exon most efficiently cleaved the majority of the target HCV IRES RNA to yield products of the expected size in the test tube (data not shown). *Trans*-splicing and *trans*-cleavage analyses, taken together with the mapping analysis, encouraged us

to focus on further characterizing and developing ribozyme Rib199.

Reducing IRES-Dependent Reporter Gene Expression by the Specific Ribozyme in Cells

To test if the ribozyme, Rib199, that recognizes specifically U199 of HCV IRES *in vitro* is also active in cells, we first assessed intracellular *trans*-cleavage activity of the ribozyme by monitoring reporter luciferase activity in cells cotransfected with Rib199 and IRES/FLuc RNA, which encodes reporter firefly luciferase (FLuc) linked to HCV IRES (Fig. 3A). The intracellular cleavage by Rib199 would result in a reduction in target RNA amount, thereby reducing the luciferase activity. To control for differences in HCV IRES-dependent FLuc expression due to transfection efficiency, toxicity, or sample recovery, we also cotransfected RNA encoding *Renilla* luciferase (RLuc). Rib199 effectively inhibited IRES-dependent expression of the reporter gene by 70% relative to a control tRNA, whereas the inactive version of the ribozyme, R(d)199, showed little inhibition (Fig. 3A). Moreover, Rib199 caused the highest reduction in luciferase activity, compared to the other ribozymes (Rib251 and Rib329), which strengthens the correlation between accessibility and function of ribozymes in cells (Fig. 3A). No sequence targeted by Rib199 (5′-UUUCUU-3′) was found in FLuc RNA. In addition, Rib199 did not inhibit the reporter activity at all in cells cotransfected with FLuc RNA (Fig. 3B). Furthermore, Rib329, which was not reactive with HCV IRES *in vitro* (as shown in Fig. 2B) could not block the expression of IRES/FLuc RNA (Fig. 3A). Thus, the inhibition of IRES/FLuc expression by Rib199 could be due mainly to the intracellular cleavage of HCV IRES RNA.

Trans-splicing Reaction by the HCV IRES-Specific Ribozyme in Cells

To determine whether Rib199 could *trans*-splice HCV IRES at the predicted U199 with fidelity in cells, we cotransfected Rib199-3′tag RNA with the HCV IRES RNA in 293T cells (Fig. 4). Total RNA was isolated from the transfected cells and analyzed by RT-PCR. A *trans*-spliced product of the expected size (151 bp) was detected in cells cotransfected with the ribozyme and the HCV IRES RNA (Fig. 4A, lane 4). By contrast, no such product was generated in cells mock transfected or transfected with the target RNA alone or the ribozyme alone (Fig. 4A, lanes 3, 6, and 7). No product was found either in cells cotransfected with the inactive ribozyme (R(d)199-3′tag) and the target RNA (Fig. 4A, lane 5). Moreover, no amplification product was detected from an RNA sample that was isolated after lysate from ribozyme-transfected cells was mixed with lysate from HCV IRES-transfected cells (Fig. 4A, lane 8). Sequence analysis of the 151-bp amplified fragment demonstrated that the ribozyme had correctly spliced its 3′ exon tag onto U199 of the HCV IRES target RNA in cells (Fig. 4B).

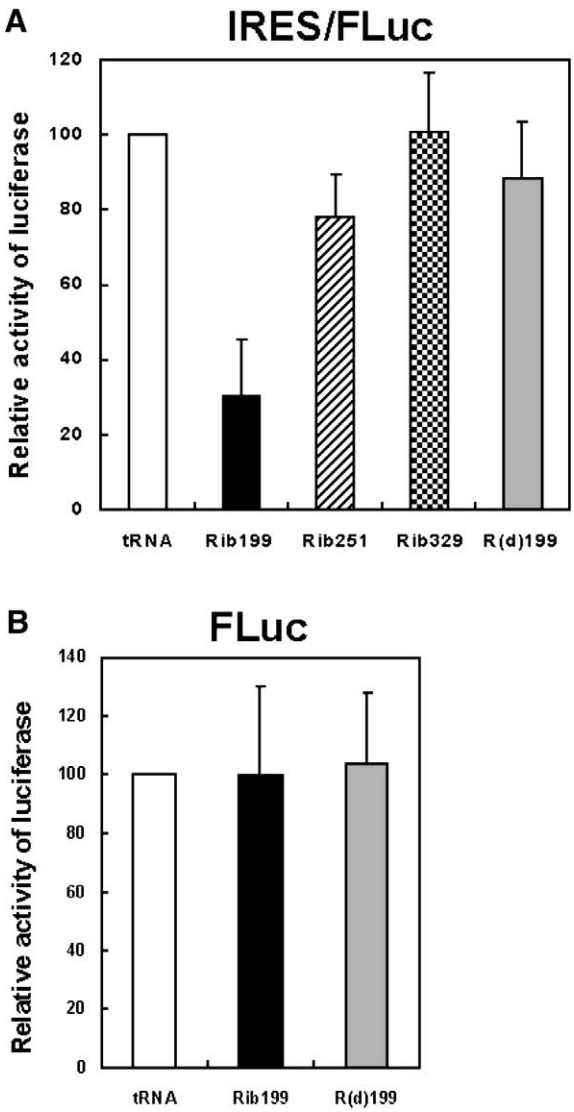

FIG. 3. Specific ribozyme cleavage activity in cells. 293T cells were cotransfected with control tRNA, active ribozyme (Rib199, Rib251, or Rib329), or inactive ribozyme (R(d)199) along with IRES/FLuc RNA (A) or FLuc RNA (B) as a reporter RNA. Relative luciferase activity was quantitated as a percentage of the sample transfected with control tRNA. Values shown represent the means ± standard deviation of three separate determinants.

Selective Induction of Transgene Activity in an HCV IRES-Dependent Manner by the Specific *Trans*-splicing Ribozyme

To determine whether new gene activity could be selectively induced in HCV IRES-expressing cells by the *trans*-splicing ribozyme, we cotransfected a plasmid encoding HCV IRES (pR/IRES) with Rib199FL ribozyme RNA into 293T cells (Fig. 5A) or liver-derived Huh7 cells (Fig. 5B). We then monitored the induction of transgene by measuring luciferase activity of FLuc relative to RLuc used for

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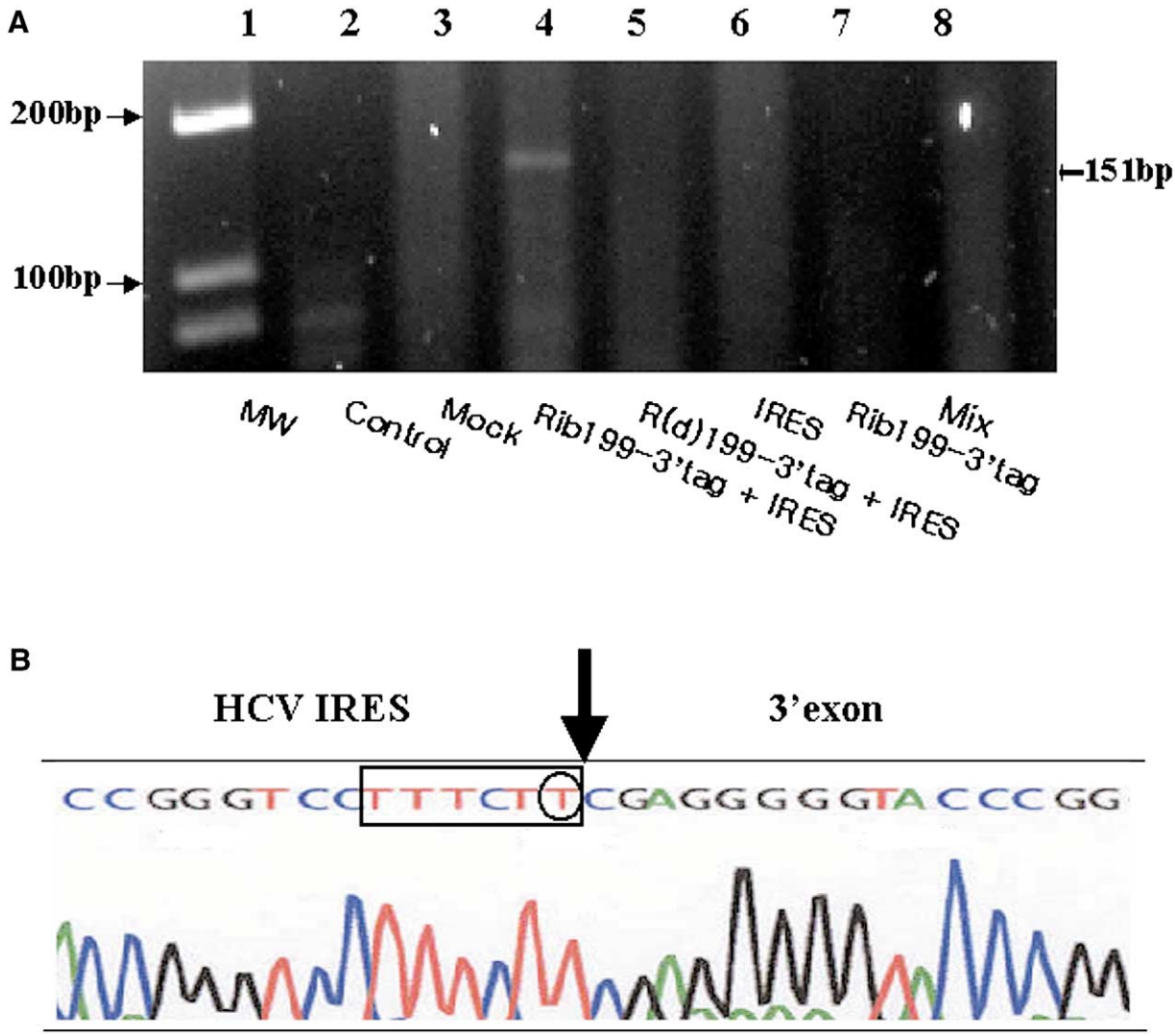

FIG. 4. *Trans*-splicing reaction with HCV IRES in cells. (A) RT-PCR analysis of *trans*-spliced RNA products generated in cells. 293T cells were mock transfected (lane 3), transfected with HCV IRES RNA (lanes 4–6 and 8) alone (lane 6), with the active ribozyme (Rib199-3′tag; lanes 4, 7, and 8), or with the inactive ribozyme RNA (lane 5). *Trans*-spliced products were amplified by RT-PCR, yielding a DNA fragment of 151 bp. (B) Sequence analysis of *trans*-spliced transcripts in cells. The amplified products in lane 4 of A were sequenced. Sequence of 1 representative clone of 10 different clones with the same sequence is shown. The correct splicing junction is indicated with an arrow along with the ribozyme recognition sequence boxed and the nucleotide at the position 199 circled.

normalization. Rib199FL was constructed by replacing the *lac*Z sequence of Rib199-3′tag with a sequence that contains FLuc RNA fused with the 3′ part of the HCV 5′ UTR (position nt 200–402) as described for Fig. 1. Transfection of Rib199FL alone into cells showed poor FLuc expression. In contrast, Rib199FL efficiently triggered luciferase activity by about 13-fold in 293T cells cotransfected with pR/IRES, compared with cells transfected with the ribozyme alone (Fig. 5A). In concordance, FLuc reporter activity was highly and selectively stimulated by Rib199FL in HCV IRES-expressing Huh-7 cells up to 6-fold (Fig. 5B). In sharp contrast, Rib199ΔFL, in which the position of nt 200–263 in the 3′ part of the HCV 5′ UTR in Rib199FL was deleted, could not stimulate the FLuc expression even in cells cotransfected with pR/IRES (Fig. 5). This could result from disruption of the stem–loop III sequence and structure in HCV IRES critical for cap-independent translation [29] in the *trans*-spliced RNA and hence suggest that selective expression of the transgene by the *trans*-splicing ribozyme in target RNA-expressing cells could be triggered by HCV IRES-dependent translation.

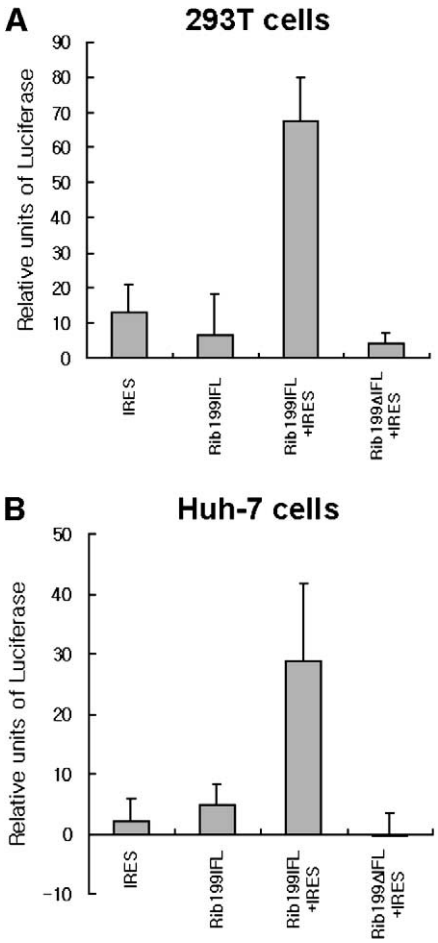

FIG. 5. Selective induction of luciferase activity by the specific *trans*-splicing ribozyme in HCV IRES-expressing 293T cells (A) or Huh-7 cells (B). Cells were transfected with HCV IRES-encoding pR/IRES plasmid (IRES) plus control tRNA, ribozyme RNA (Rib199FL) alone, IRES plus Rib199FL, or IRES plus modified ribozyme (Rib199ΔFL). Luciferase activity of FLuc relative to RLuc was quantitated, and averages of measurements performed in triplicate are shown with bars indicating standard errors.

Cell-Specific Cytotoxicity by the Specific *Trans*-splicing Ribozyme

Given the observation that the *trans*-splicing ribozyme induced transgene expression selectively in target RNA-expressing cells, we next determined if the ribozyme triggered cytotoxin activity also selectively in HCV IRES-expressing cells. We used an SV40 promoter system to express Rib199 derivatives that contain the DTA ORF fused with a sequence encompassing the 3′ part of the HCV 5′ UTR (position nt 200–402) as a 3′ exon. Because group I *trans*-splicing ribozymes with only 6-nt-long IGS were reported to be very inactive when expressed in bacteria [30], we constructed two ribozyme plasmids with extended IGS. One, called pSVR199DT, harbors an extended P1 and 7-nt-long P10 helix sequence and the other, called pSVR199DTAS, contains additional 210-nt-long antisense sequence against FLuc RNA encoded in the target RNA as well as the P1 plus the P10 helix (Fig. 6A). Cell-specific induction of cytotoxin was assessed by measuring inhibition of the expression of a cotransfected plasmid (Fig. 6B), direct cytotoxicity (Fig. 6C), and programmed cell death (Fig. 6D). For translation inhibition, Huh-7 cells were transiently cotransfected with an RLuc-expressing plasmid and the ribozyme plasmid along with or without a plasmid encoding the target HCV IRES (pIRES/F) (Fig. 6B). Approximately 70% inhibition of RLuc expression was detected in cells cotransfected with pIRES/F along with pSVR199DT, compared to those transfected pIRES/F alone. IRES-expressing cells cotransfected with pSVR199DTAS also showed efficient but less inhibition of RLuc expression than those with pSVR199DT. By contrast, only 13 or 8% inhibition of RLuc expression was observed in cells transfected with pSVR199DT alone or pSVR199DTAS alone, respectively, compared to cells without ribozyme.

To demonstrate that the selective inhibition of translation in HCV IRES-expressing cells transfected with the ribozyme plasmids corresponds to cytotoxicity, cells were cotransfected with a GFP-encoding vector and followed in culture for growth. Twenty four hours after cotransfection, the relative number of GFP-positive cells in each transfectant was compared (Fig. 6C). As shown, transient cotransfection of pSVR199DT or pSVR199DTAS along with pIRES/F resulted in a significant and selective reduction in the number of GFP-positive cells by 85.7 or 71.4%, respectively, compared to control cells transfected with pIRES/F alone. However, GFP-positive cell number was also reduced by 35.7% in cells transfected with pSVR199DT alone, compared to cells transfected without the ribozyme. In sharp contrast, the number of GFP-positive cells transfected with pSVR199DTAS alone was only 7.1% less than those transfected without the ribozyme.

To test whether selective induction of DTA by the ribozyme in the target RNA-expressing cells could effectively cause selective programmed cell death, we cotransfected cells with the above ribozyme constructs with or without pIRES/F and then employed the TUNEL assay to compare apoptotic cell fractions in each cell (Fig. 6D). The percentage of apoptotic cells transfected with the ribozyme plasmids along with pIRES/F was significantly higher than that of those transfected with the ribozymes alone or control vector. However, the percentage of apoptotic cells transfected with pSVR199DT alone was also increased by two- to threefold, compared to those with control vector. By contrast, almost no differences in the number of apoptotic cells were observed between control cells and the cells transfected with pSVR199DTAS alone, in agreement with results from translation inhibition study and cytotoxicity assay as above. Neither increase in PKR amount nor activation of PKR activity was observed

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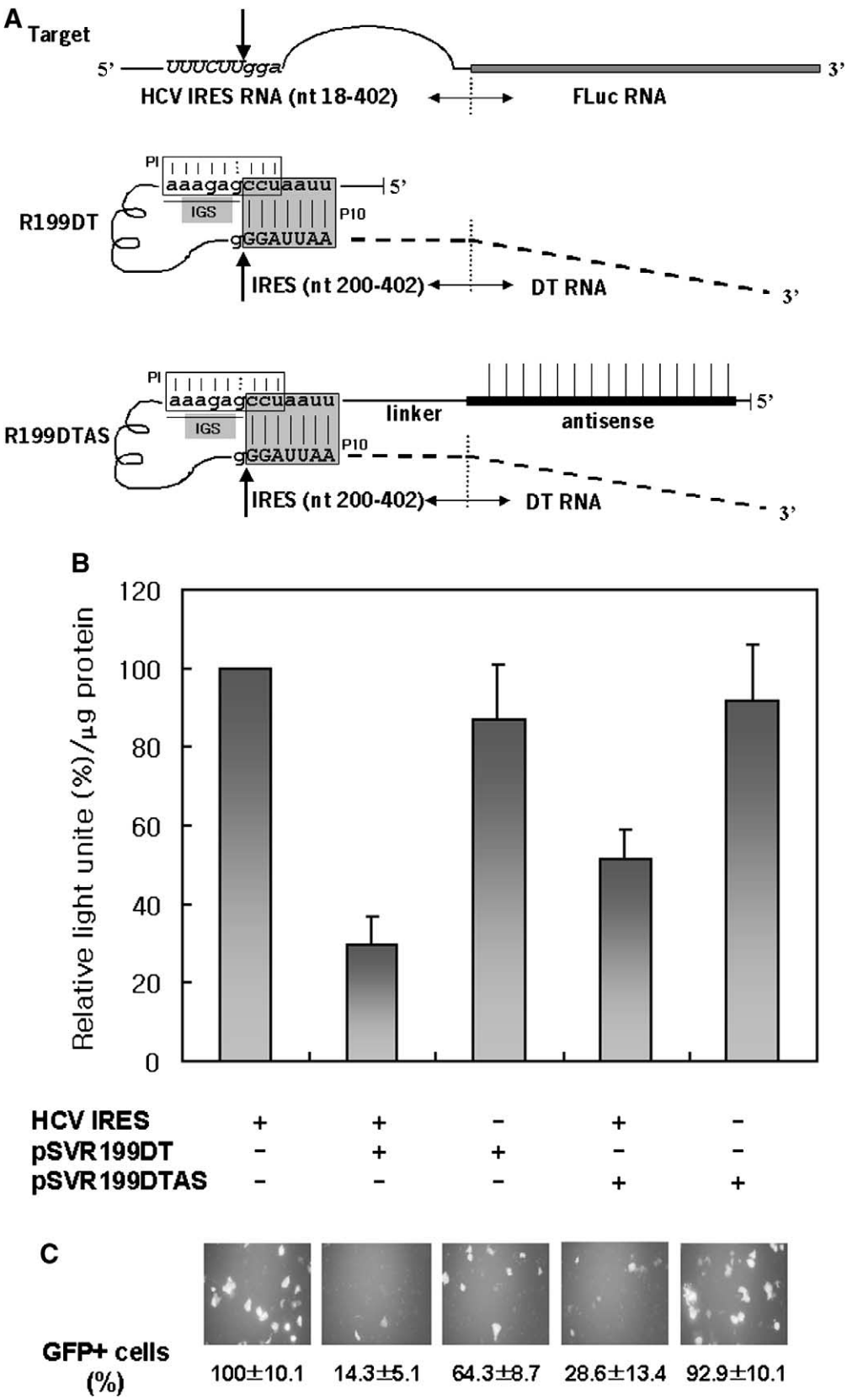

FIG. 6. Selective induction of cytotoxin by the specific ribozyme in HCV IRES-expressing Huh-7 cells. (A) Schematic diagram of the *trans*-splicing ribozymes. The target transcript is represented with sequences around the splice site shown italicized. Two *trans*-splicing ribozymes with extended IGS are shown with 3′ exon sequences capitalized. Potential base pairing between the ribozyme and the target is indicated by a vertical line. Arrows indicate 5′ and 3′ splicing sites. (B) Cells were cotransfected with pCMV/R along with pIRES/F (HCV IRES) plus control empty vector, pIRES/F plus a ribozyme plasmid encoding diphtheria toxin A chain (pSVR199DT), pSVR199DT alone, pIRES/F plus a ribozyme plasmid with 210-nt-long antisense sequence against FLuc gene (pSVR199DTAS), or pSVR199DTAS alone. Luciferase activity was expressed relative to the activity in cells cotransfected with pIRES/F alone. Error bars correspond to the SD from three independent experiments. (C) Cells were cotransfected with pEGFP-N1 along with the same set of plasmids as in B. GFP-expressing cells were observed with fluorescence microscope 24 h after transfection. The number of GFP-positive cells in each transfectant was counted and quantitated as a percentage of the number of those transfected with pIRES/F alone. Averages of triplicate measurements obtained from three separate experiments are shown ± standard deviation. (D) Cells were cotransfected with each ribozyme construct with (+IRES) or without (−IRES) pIRES/F. Apoptotic cells were examined and counted in three different fields with a fluorescence microscope 24 h after transfection. The number of the apoptotic cells was quantitated as a percentage of total nuclei. The percentage of apoptotic cells transfected with control empty vector was approximately 5%. The experiments were performed two times independently.

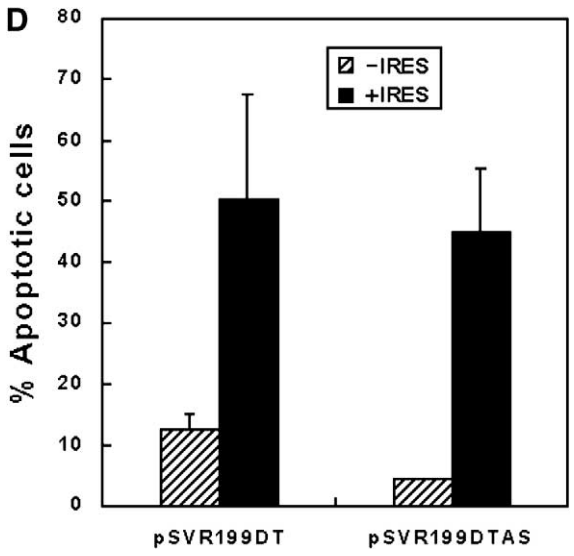

FIG. 6. (continued)

in cells coexpressing the target RNA and the ribozyme with antisense (data not shown), which strongly indicates that selective cessation of translation and induction of cell death in the cells could be due mainly to the specific DTA stimulation, but not to dsRNA-mediated nonspecific interferon response.

Discussion

In this report, a *trans*-splicing group I ribozyme has been developed to induce new gene activity selectively in human cells expressing HCV IRES RNA. In contrast to the *trans*-cleaving ribozyme, the *trans*-splicing ribozyme can perform both cleavage and ligation reactions [31] and hence could modify HCV IRES RNAs to make them encode anti-HCV agents in virally infected cells. To concur with this assumption, we observed that the ribozyme can reduce FLuc activity derived by HCV IRES in cells (Fig. 3) and induce activity of both FLuc and DTA tagged at its 3′ end selectively in HCV IRES-expressing cells (Figs. 5 and 6), which suggests that the ribozyme can simultaneously reduce the production of deleterious HCV proteins and induce the production of a functional gene product with antiviral activity in treated cells. Moreover, ribozyme-mediated intracellular *trans*-splicing reaction with high fidelity (Fig. 4) strongly indicates that the transgene expression could be induced mainly by RNA replacement in HCV-infected cells. Therefore, ribozyme-mediated selective induction of therapeutic gene activity proposed in this study could effectively render HCV-infected patients resistant to the virus by both destroying HCV RNAs and delivering an antiviral gene specifically and selectively into the virus-infected cells.

The observation of selective expression of DTA by the ribozyme (Fig. 6) suggests that ribozyme transfer into HCV-infected cells could selectively cause the death of the cells and thereby specifically impede HCV replication and spread to healthy cells. Regarding a report that a very small amount of DTA introduced to a mouse cell was sufficient to kill the cell [32], only a fraction of viral transcripts will probably need to be reacted and changed to ones encoding DTA for effective elimination of the virally infected cells. Moreover, the *trans*-splicing ribozyme generates reaction products that would be stable in cells, easily detected by RT-PCR (Fig. 4), and readily assayed (Fig. 5), providing experimental systems to optimize ribozyme catalysis in cells [33]. For these reasons, targeted cytotoxin delivery with the *trans*-splicing ribozyme might be one method to treat HCV infection genetically.

To apply the strategy of ribozyme-mediated toxin delivery successfully to clinical settings, specificity should be an important aspect. Cytotoxicity could result from nonspecific toxin expression via *cis*-splicing of the ribozyme, *trans*-splicing with cellular RNAs, direct translation of exon sequences, or other indirect effects. To minimize this unintended toxicity, the 3′ part of the HCV 5′ UTR encompassing the downstream sequence of the targeted U199 of IRES (nt 200–402) was inserted at the front of a transgene in the ribozyme's 3′ exon. Selectivity and specificity of the ribozyme that can highly stimulate the transgene activity in cells expressing the target RNA are probably due to the presence of two stop codons in-frame upstream of the HCV core initiation codon (at nt 294 and 297), which would cause premature termination of direct translation initiated within the ribozyme construct and/or inhibition of translation of RNA products generated by *trans*-splicing with unintended cellular RNAs. In addition, no activity of a modified ribozyme truncated at nt 200–263 of the 3′ part of the HCV 5′ UTR (Fig. 5) indicates that an intact HCV IRES should be restored for expression of the transgene by the ribozyme and highlights the speculation that nonspecific transgene induction could be minimized with our ribozyme construct. Although expression of a ribozyme with an extended P1 and P10 helix was shown to induce DTA activity specifically from measurement of cotransfected plasmid expression (Fig. 6B), the number of survival cells transfected with the ribozyme alone, which would more sensitively indicate toxin activity of both translation inhibition and cytotoxicity, was greatly reduced, compared to cells without the ribozyme (Fig. 6C). Moreover, apoptosis of the cells transfected with the ribozyme alone was more activated than in those transfected with vector control (Fig. 6D). This nonspecificity would probably result from illegitimate *trans*-splicing with very upstream unintended cellular RNAs. This is consistent with the report of nonspecific *trans*-splicing activity in cells expressing a ribozyme that consists of only 6-nt-long IGS [17]. To cir-

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cumvent this limitation, we further extended the IGS of the ribozyme such that it would contain an antisense sequence against the target RNA (Fig. 6A) as described [21,22,30]. In contrast to the ribozyme without the antisense domain, a ribozyme harboring a more extended IGS with the antisense region clearly reduced nonspecific toxicity (Figs. 6C and 6D), indicating enhancement in the specificity of transgene induction by increasing the length of base pairing between the ribozyme and the target RNA. Studies on the molecular analysis of the ribozyme's efficacy and specificity in cells are currently in progress.

From a practical point of view, however, killing HCV-infected cells by selective delivery of cytotoxin into the cells with *trans*-splicing ribozyme may be inappropriate for gene therapy to treat patients due to the possibility of massive destruction of liver cells. Instead, selective induction of a dominant negative mutant of viral proteins or immune modulating proteins could be more proper to control specifically the cytopathic effects of HCV. Studies here with cytotoxin though could supply a highly sensitive experimental model to assess the specificity of *trans*-splicing ribozymes in target cells and hence have important implications for developing more effective ribozymes. In addition, the 3′ exon in the *trans*-splicing reaction can be composed of virtually any RNA sequence [34], which implies that gene activity of any therapeutically relevant transcripts could be engendered selectively in HCV-infected cells if they are attached to the ribozyme backbone developed in this study.

We have here provided proof of principle that the *trans*-splicing group I ribozyme can be applied for a new delivery system of antiviral activity selectively and specifically into HCV-infected human cells via RNA replacement. The *trans*-splicing ribozyme could be exploited to deliver specifically therapeutic gene activity also in other virus-infected or tumor cells that express unique RNAs. For HCV therapy, it is noteworthy that the target sequence for the ribozyme identified here is present in all of the major HCV genotypes. This implies that the ribozyme could be used in the majority of infected patients. With further improvement of ribozyme activities and development of an efficient gene transfer system, RNA replacement would represent a useful approach to the modulation of HCV infection. In addition, therapeutic validity of the *trans*-splicing ribozyme will have to be determined in future comparative studies with other potentially useful anti-HCV agents, such as antisense oligonucleotides, *trans*-cleavage ribozyme, RNA aptamer, or siRNA [35].

MATERIALS AND METHODS

Mapping IRES RNA of HCV. The mapping library (GN_5) was generated as previously described [18]. HCV 5′ UTR RNA encompassing HCV IRES (nt 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 [36] (a kind gift from S. K. Jang, POSTECH University, Korea). The GN_5 ribozyme library (50 nM) and HCV 5′ UTR RNA (50 nM) were each denatured at 95°C for 1 min and then equilibrated in reaction buffer containing 50 mM Hepes (pH 7.0), 150 mM NaCl, and 5 mM $MgCl_2$ at 37°C for 5 min. The substrate RNA was then added to the ribozyme library along with guanosine (100 μM) to start the *trans*-splicing reaction, which proceeded at 37°C for 3 h. The reaction products were amplified by RT-PCR, cloned, and sequenced as described [17].

Ribozyme construction. Rib195-, Rib196-, Rib199-, Rib251-, and Rib329-3′tag ribozymes were transcribed using templates generated from pT7L-21 [16] by PCR with a 5′ primer containing the T7 promoter and each ribozyme's IGS and with a 3′ primer specific for the 3′ exon sequence as described [16,17]. A form deleted in the catalytically critical region of each ribozyme was constructed for an inactive control as described [16]. Rib199 ribozyme without 3′ exon was generated by *in vitro* transcription with the *Sca*I fragment of template used for Rib199-3′tag. Rib199FL was transcribed with the *Bam*HI fragment of pRib199FL that was generated by exchanging wild-type group I intron IGS 5′-GGAGGG-3′ with 5′-GAGAAA-3′ and inserting a 3′ exon sequence encompassing the 3′ part of the HCV 5′ UTR (nt 200–402) plus the FLuc gene, which was generated by PCR with pR/HCV/F (a dicistronic vector expressing RLuc under the CMV promoter and FLuc under the control of HCV IRES and kindly donated by S. K. Jang), into the multicloning sites of pT7L-21. To generate Rib199ΔFL, the 3′ exon of Rib199FL ribozyme was replaced by a fusion sequence consisting of a truncated 3′ part of the HCV 5′ UTR RNA (nt 264–402) and FLuc RNA. To construct pSVR199DT, complementary oligonucleotides containing an extended P1 plus a 7-nt-long P10 helix were synthesized and inserted upstream of the IGS of pRib199FL. FLuc ORF present in this modified pRib199FL was then replaced with DTA ORF, and a DNA fragment consisting of Rib199 sequence with the extended IGS plus the 3′ part of the HCV 5′ UTR followed by DTA ORF was inserted between the *Eco*RI and the *Xba*I sites of pSEAP, which encodes alkaline phosphatase under SV40 promoter (Clontech). pSVR199DTAS was created by insertion of a 210-nt-long PCR-amplified sequence for the antisense region against the FLuc gene between the *Hin*dIII and the *Eco*RI sites of pSVR199DT and adding PCR-amplified 200-nt-long linker DNA from the CAT gene into the *Eco*RI site of the plasmid.

Substrate construction. IRES/FLuc RNA composed of HCV IRES linked to FLuc RNA was generated by *in vitro* transcription with the *Not*I-digested template of pR/HCV/F. FLuc RNA was created by *in vitro* transcription of DNA template that was constructed by deletion of the RLuc gene and the HCV IRES from pR/HCV/F. RLuc RNA was generated by *in vitro* transcription of *Sal*I-digested pR/HCV/F plasmid. pR/IRES plasmid, which encodes a CMV promoter-derived RLuc gene followed by the HCV IRES, was generated by digesting pR/HCV/F with *Xba*I and *Not*I, filling in the 5′ overhangs with Klenow fragment of *E. coli* DNA polymerase I, and self-ligating the blunt ends. The pIRES/F vector, which harbors the CMV promoter plus HCV IRES followed by the FLuc gene, was constructed by removal of the RLuc gene with *Pst*I digestion of pR/HCV/F and self-ligation. pCMV/R encoding the RLuc gene under the control of the CMV promoter was created by *Sal*I and *Not*I digestion of pR/HCV/F, Klenow filling, and blunt-end self-ligation.

Trans-cleavage reaction and trans-splicing reaction. RNAs used for transfection into cells were modified at the 3′ end by addition of poly(A) using poly(A) polymerase (USB) at 37°C for 20 min. For *trans*-cleavage reaction in cells, 293T cells were plated at 3.0×10^5 cells per well in 35-mm dishes 18–24 h prior to transfection. The cells were cotransfected with 0.5 μg IRES/FLuc RNA or 0.5 μg FLuc RNA along with 0.5 μg RLuc RNA and 4 μg tRNA, Rib199, or R(d)199 (inactive version of Rib199) using 3 μl DMRIE-C (GIBCO BRL). Cell lysates were harvested 24 h after transfection, and 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). For *trans*-splicing reaction in cells, 293T cells were mock transfected or cotransfected with 1 μg HCV IRES RNA with or without 4 μg Rib199-3′tag or R(d)199-3′tag. In the "mix" sample, two cultures separately transfected with HCV IRES alone or

Rib199-3′tag alone were lysed and mixed. Total RNA was isolated from the cells 24 h after transfection with guanidine isothiocyanate [37] supplemented with 20 mM. RNA (5 μg) was reverse transcribed with a primer specific for the 3′-tagging *lac*Z sequence in the presence of 10 mM L-argininamide. The resulting cDNAs were amplified for 40 cycles with a 5′ primer specific for the *trans*-splicing junction (5′-GGGGAATTCAC-CGGGTCCTTTCTTCGAG-3′) and with a 3′ primer specific for the 3′ exon *lac*Z sequence, cloned, and sequenced.

Ribozyme assays in cells. 293T cells or Huh7 cells were seeded in 35-mm plates at a density of 3.0×10^5 cells per plate 24 h before transfection. The cells were cotransfected with 1 μg pR/IRES with 5 μg control tRNA, Rib199FL, or Rib199ΔFL. For the assessment of ribozyme activity in the absence of substrate RNA, cells were cotransfected with 1 μg pTKR (containing the RLuc expression cassette under TK promoter) with 5 μg tRNA or Rib199FL. Cell lysates were harvested 24 h after transfection, and reporter gene activities were assessed as above. Inactive ribozyme was not appropriate for control because transfection of the ribozyme alone into cells resulted in a slight increase in luciferase activity (data not shown) as described [21], probably due to direct translation initiation of the FLuc gene in this construct. For toxin assay, Huh-7 cells were cotransfected with 0.2 μg pCMV/R and 0.15 μg pIRES/F along with or without 0.75 μg pSVR199DT or pSVR199DTAS. Cells were transfected with pCMV/R plus pCMV-βgal (Clontech) along with only 0.75 μg each ribozyme plasmid for the analysis of toxin activity without substrate HCV IRES. RLuc activities were analyzed as above. Alternatively, cells were cotransfected with 300 ng pEGFR-N1 (Clontech) plus 1.4 μg pSVR199DT or pSVR199ASDT along with or without 300 ng pIRES/F, and GFP-positive cells were observed 24 h after transfection. As a control, pEGFP-N1 was cotransfected with pIRES/F along with 1.4 μg control empty vector (pSEAP). For analysis of apoptosis, cells were cotransfected with 1.4 μg of each ribozyme construct with or without 300 ng pIRES/F. Then, the TUNEL staining protocol was performed to visualize apoptotic nuclei as specified by the manufacturer (Apoptosis Detection System, Fluorescein; Promega). In parallel, the same cells were counterstained with propidium iodide (1 μg/ml in phosphate-buffered saline) and viewed with a fluorescence microscope (Olympus Optical). The number of apoptotic cells was expressed as a percentage of total nuclei.

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