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Functional Modification of a Specific RNA with Targeted *Trans*-Splicing

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Abstract

The self-splicing group I intron from *Tetrahymena thermophila* has been demonstrated to perform splicing reaction with its substrate RNA in the *trans* configuration. In this study, we explored the potential use of the *trans*-splicing group I ribozymes to replace a specific RNA with a new RNA that exerts any new function we want to introduce. We have chosen thymidine phosphorylase (TP) RNA as a target RNA that is known as a valid cancer prognostic factor. Cancer-specific expression of TP RNA was first evaluated with RT-PCR analysis of RNA from patients with gastric cancer. We determined next which regions of the TP RNA are accessible to ribozymes by employing an RNA mapping strategy, and found that the leader sequences upstream of the AUG start codon appeared to be particularly accessible. A specific ribozyme recognizing the most accessible sequence in the TP RNA with firefly luciferase transcript as a 3' exon was then developed. The specific *trans*-splicing ribozyme transferred an intended 3' exon tag sequence onto the targeted TP transcripts, resulting in a more than two fold induction of the reporter activity in the presence of TP RNA in mammalian cells, compared to the absence of the target RNA. These results suggest that the *Tetrahymena* ribozyme can be a potent anti-cancer agent to modify TP RNAs in tumors with a new RNA harboring anti-cancer activity.

Keywords: Group I intron, Ribozyme, RNA replacement, *Tetrahymena thermophila*, Thymidine phosphorylase, *Trans*-splicing

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Introduction

The self-splicing group I intron from *Tetrahymena thermophila* has been previously shown to *trans*-splice an exon attached to its 3' end onto a separate target RNA not only in test tube (Been and Cech, 1986) but also in *E. coli* (Sullenger and Cech, 1994) and mammalian cells (Jones *et al.*, 1996). Moreover, the *trans*-splicing ribozymes have been demonstrated to revise mutant transcripts associated with several human genetic and malignant diseases (Lan *et al*, 1998; Phylactou *et al.*, 1998; Watanabe and Sullenger, 2000; Shin *et al.*, 2002; Rogers *et al.*, 2002) and selectively replace hepatitis C virus (HCV) transcripts with a new RNA that exerts anti-HCV activity (Ryu *et al.*, 2003). These imply that *trans*-splicing ribozymes could be potentially used for the functional modification of any target RNA associated human diseases with a new RNA encoding therapeutic activity.

Thymidine phosphorylase (TP), which is identical to the platelet-derived endothelial cell growth factor (PD-ECGF) (Iltzsch *et al.*, 1985; Furukawa *et al.*, 1992), is expressed at much higher levels in a wide variety of tumors, compared with adjacent normal tissues (Luccioni *et al.*, 1994; Fox *et al.*, 1995; Takebayashi *et al.*, 1996). Thus, TP is a valid hallmark of cancer. Moreover, TP could be an important target for cancer therapies since TP has angiogenic activity *in vivo* (Brown *et al.*, 2000), enhances tumor progression, and confers resistance to apoptotic signal pathways (Ikeda *et al.*, 2002, 2003; Mori *et al.*, 2002).

Here, we investigated whether group I-based ribozymes could be utilized to modify TP RNA in cells to express the intended sequences tagged to their 3' end by targeted *trans*-splicing. To this effect, we first confirm the expression pattern of TP RNA in cancer patients. We next identified most accessible sites in TP RNA to the ribozymes and constructed a specific ribozyme recognizing the site. Moreover, we determined if the ribozymes could selectively induce a new reporter activity in TP-expressing cells by targeted *trans*-splicing.

Materials and Methods

Materials

Enzymes and reagents for RT-PCR, *in vitro* transcription

reaction, or cloning were purchased from Roche Applied Science or domestic suppliers, sequencing reagents from PE Applied Biosystem. Argininamide and most of other chemicals came from Sigma Chemical Co. DMEM tissue culture media and fetal bovine serum were obtained from GIBCO.

Surgical specimens and RNA analysis

Surgical samples of 11 paired normal and tumor tissues were collected from patients undergoing gastric cancer surgery at the Department of Surgery, Seoul National University Hospital. Immediately after removal, all of the tissues for molecular analysis were put in liquid nitrogen and stored at -80℃ until use.

Total cellular RNA was extracted from frozen tissues by using the Tri reagent (Molecular Research Center, Inc) according to the manufacture's instruction and then treated with DNase I to remove genomic DNA. Two μg of total RNA was reverse transcribed into cDNA with MMLV reverse transcriptase and oligo $(dT)_{12-18}$. Equal amounts of cDNA was subsequently amplified by PCR in 50 μl reaction volume containing 1X PCR buffer, 200 μM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each specific primer set and 2.5 U Taq DNA polymerase. Control amplification was performed with β-actin. Amplified products were electrophoresed on 1% agarose gel.

Mapping accessible sites on TP RNA

The mapping library, called GN5 ribozyme library, was constructed by randomizing internal guide sequence (IGS) of the *Tetrahymena* group I intron so that the 5' end of the ribozyme in the library began with 5'-GNNNNN-3', where G represents guanine and N represents equimolar amounts of the four nucleotides (nt), as described (Lan *et al.*, 1998). TP RNA, target RNA, was generated by *in vitro* transcription using T7 RNA polymerase with a cDNA clone of TP (Ishikawa *et al.*, 1989, a kind gift from C.-H. Heldin, Ludwig Institute, Sweden). To map the TP RNA, 50 nM of the GN5 ribozyme library was incubated at 37℃ for 3 h under splicing condition (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$) in the presence of a guanosine (100 μM) with 100 nM of the TP RNA. The reaction products were reverse-transcribed at 37℃ for 30 min in the presence of argininamide (10 mM) with a 3' tag primer specific for the ribozyme's 3' exon *lacZ* sequence (5'-ATGTGCTGCAAGGCGATT-3') (Jones *et al.*, 1996). cDNAs were then amplified by PCR for 35 cycles using the same 3' primer and a 5' primer encompassing the 5' end of target TP RNA (5'-CCGGAATTCTAATACGACTCACTA TAGGGCAGTGGACCGCTGTG-3'). The amplified *trans*-splicing products were cloned into pUC19 vector and sequenced using the dideoxy termination method with automatic sequencer (ABI 310 Genetic Analyzer).

Construction of specific ribozymes

Specific ribozymes such as Rib73 that recognize the uridine at position 73 on the TP RNA were generated by *in vitro* transcription of DNA templates which were created from pT7L-21 by PCR with a 5' primer containing the T7 promoter and the ribozyme's IGS and with a 3' primer specific for the 3' exon *lacZ* sequence. The pT7L-21 vector encodes 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'-GGCCCA in Rib73. In addition, inactive ribozyme, R(d)73, which is devoid of the catalytic core of the enzyme (Sullenger and Cech, 1994), was constructed as negative controls. To construct enhanced ribozyme expression vector, complementary oligonucleotides containing an extended P1 plus a 7-nt-long P10 helix and 300 nt-long PCR-amplified sequence for the antisense region against TP gene were inserted upstream of IGS of Rib73. In addition, 3' exon of the modified Rib73 was replaced with a cDNA sequence encoding firefly luciferase gene (Fluc). DNA fragment consisting of Rib73 sequence with the extended IGS plus Fluc ORF was inserted between the *Eco*RI and *Xba*I sites of pSEAP that encodes alkaline phosphatase under SV40 promoter (Clontech) to generate Rib73AS-Fluc.

Analysis of *trans*-splicing reaction *in vitro*

For *in vitro trans*-splicing reaction assay of ribozymes, Rib73 (100 nM) were incubated at 37℃ for 3 h under splicing conditions with TP RNA (10 nM). The resulting RNA was reverse-transcribed at 37℃ for 30 min in the presence of argininamide (10 mM) with a 3' primer specific for the ribozyme's 3' exon *lacZ* sequence as described above. cDNAs were then amplified by PCR for 35 cycles with the same 3' primer and with a 5' primer specific for the 5' end of TP RNA. The reaction products were analyzed on a 3% agarose gel. RT-PCR products were eluted from the gel, cloned onto pUC19 vector, and then sequenced with the dideoxy termination method.

Luciferase assay of ribozyme activity in cells

NIH3T3 cells were plated at 3.0×10^5 cells per well in 35 mm dishes 24 h prior to transfection. TP expression vectors, TP-fl or TP-orf, were generated by cloning of the full-length cDNA sequence or ORF sequence of TP, respectively, into the downstream region of CMV promoter

of pCDNA. The cells were cotransfected with 0.1 μg TP-fl or 0.1 μg TP-orf along with 0.1 μg pRLuc encoding renillar luciferase as internal control and with 1.8 μg control vector (pSEAP) or pRib73AS-Fluc using 4 μl lipofectamine (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, NIH3T3 cells were mock-transfected or cotransfected with 0.2 μg TP-fl with or without 1.8 μg pRib73AS-Fluc or pR(d)73AS-Fluc. Total RNA was isolated from the cells 24 h after transfection with guanidine isothiocyanate (Feramisco *et al.*, 1982) supplemented with 20 mM EDTA. RNA (5 μg) was reverse transcribed with a primer specific for the 3' tagging Fluc 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'-CCGGAATTCCCGGGTACCTGGGCTCGA-3') and with a 3'-primer specific for the 3' exon sequence.

Results and Discussion

TP expression pattern in gastric cancer patients

We compared the pattern of TP gene expression between gastric tumor tissue and the adjacent non cancerous normal gastric mucosa obtained from 11 patients with gastric carcinoma using RT-PCR (Fig. 1). TP was expressed at markedly higher levels in all gastric cancer tissues than the adjacent normal tissues. Even though much larger samples should be analyzed, this indicates that TP could be a candidate for a valid marker in gastric cancers.

Trans-splicing strategy of TP RNA with group I intron

In this study, we tested if the group I-intron based ribozyme could replace and modify TP RNA with an intended RNA with new function. In the splicing reaction, the ribozyme recognizes target TP RNA by base pairing to any accessible uridine nucleotides of the RNA through IGS of the ribozyme. The ribozyme then cleaves the TP RNA, releases the downstream targeted RNA sequence, replaces the sequence with its 3' exon, and hence induce a target RNA-dependent new gene activity (Fig. 2).

TP RNA mapping for the ribozyme-accessible nucleotides

Every uridine in TP RNA can be potentially targeted by *Tetrahymena* group I intron through G-U base pairing between the IGS of the ribozyme and the target RNA. However, only a limited number of uridines on the target RNA are likely accessible to the ribozyme due to the substrate RNA's tertiary structure and complex formation with proteins in cellular milieu (Lan *et al.*, 2000). Thus, an RNA mapping strategy was carried out to determine which uridines in the TP RNA are accessible to ribozymes. The mapping method was based on a *trans*-splicing ribozyme library (Lan *et al.*, 1998, 2000; Ryu *et al.*, 2003) and RNA tagging (Jones *et al.*, 1996) as described in Materials and Methods. Sequence analyses of the splicing junction sites showed that several uridines in the leader sequence upstream of the AUG start codon (position 124 nt), appeared to be particularly accessible (Fig. 3A). Especially, the uridine at position 73 (U73) on TP RNA was the most frequently found as the splicing site. We evaluated several different ribozymes for their *trans*-splicing abilities, then Rib73 recognizing U73 on TP RNA reacted with the target RNA with the highest efficiency (data not shown). These observations strongly indicate that the relative *trans*-splicing efficiency at the selected sites corresponds with the predicted accessibility from the mapping analyses and encourage us to focus on further developing and characterizing Rib73.

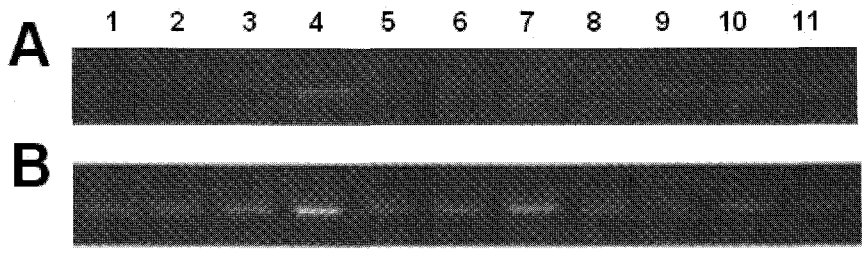

Fig. 1. RT-PCR analysis of TP gene expression of 11 gastric cancer patients. Total RNA from normal (A) and tumor (B) gastric tissue of each patient (lanes 1-11) were transcribed with reverse transcriptase and equal amount of cDNAs were amplified with TP gene specific primers.

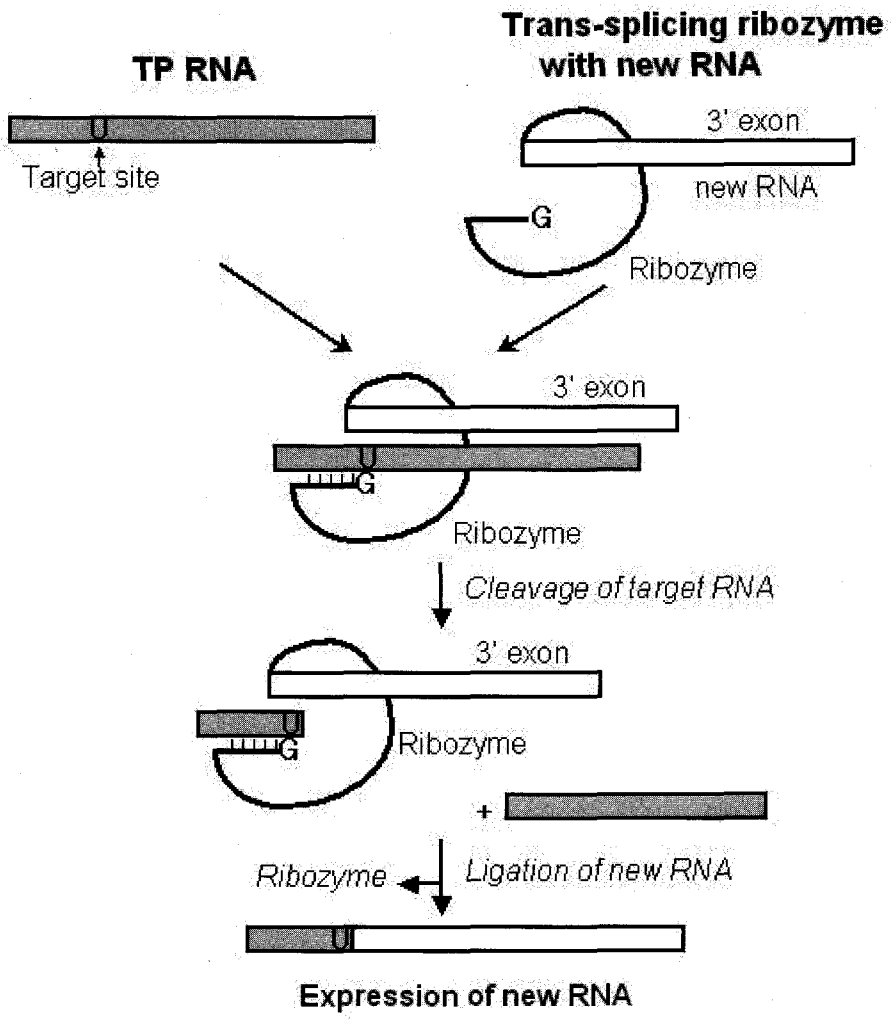

Fig. 2. Scheme to ribozyme-mediated selective expression of new RNA by targeted *trans*-splicing of TP RNA. TP RNA can be recognized by a ribozyme at any accessible uridine residue by base pairing to the sequence through its IGS, and then cleaved. The ribozyme releases the 3' cleavage product and replaces it with a 3' exon that encodes new RNA sequence.

To determine whether the specific ribozyme, Rib73, employed *trans*-splicing reaction on the target TP RNA, the active or inactive ribozymes were incubated under splicing conditions with TP RNA. RT-PCR analyses were then showed that an amplified fragment of the expected size of 102 bp was generated from reaction mixtures with TP RNA and Rib96 (Fig. 3B, lane 3). However, no such RT-PCR products were generated from either sample with TP RNA alone or Rib73 alone (Fig. 3B, lane 5 or 6). Moreover, no RT-PCR products were produced in the reaction with inactive ribozyme, R(d)73, and TP RNA (Fig. 3B, lane 4). These results suggest that the amplified RT-PCR products in the lane 3 of Fig. 3B resulted from the catalytic activity of the specific ribozyme.

Sequence analyses of the amplified spliced products were performed to determine if the specific ribozyme could accurately employ the *trans*-splicing reaction with the target RNA. The sequence of the 102 bp RT-PCR fragment demonstrated that Rib73 had correctly reacted with the TP RNA at the predicted reaction site (5'-UGGGCU) and replaced sequences downstream of the reaction sites with the 3' exon sequences tagged at the 3' end of the ribozyme (Fig. 3C). Therefore, it was concluded that the specific ribozyme, Rib73, could replace TP RNA with a 3' exon attached to the 3' end of the ribozyme by targeted *trans*-splicing with high fidelity *in vitro*.

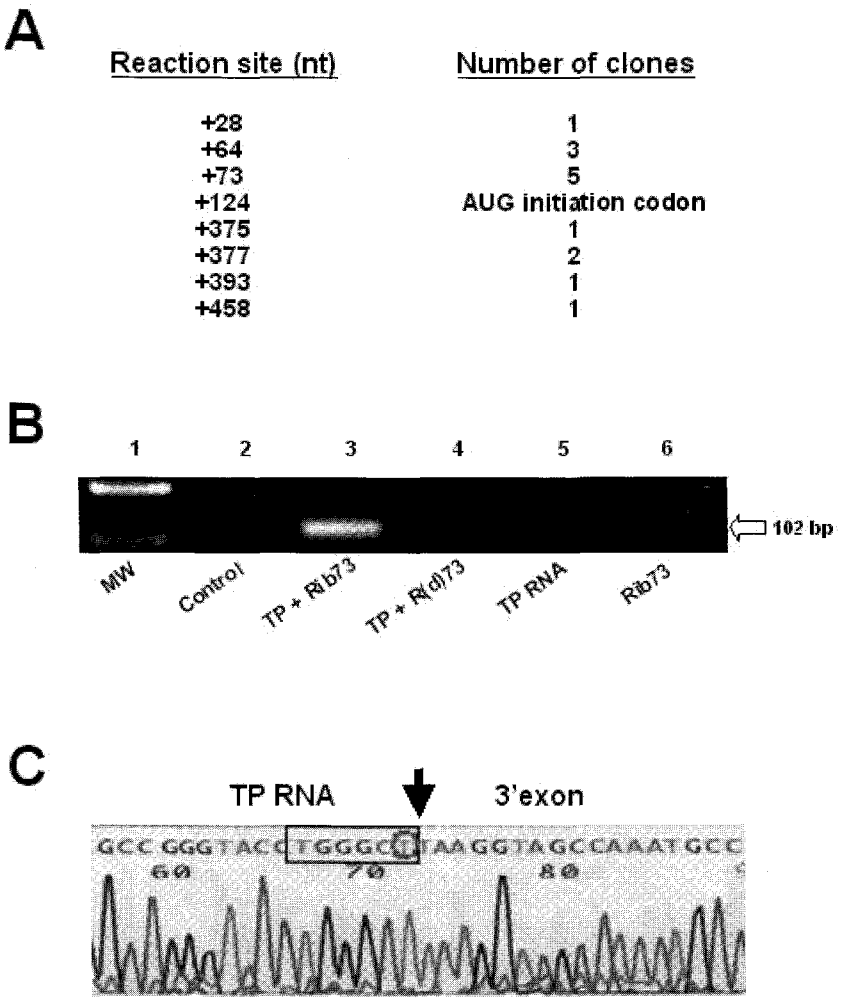

Fig. 3. *In vitro* mapping of *trans*-splicing with TP RNA. (A) Mapping results of the ribozyme-accessible sites in TP RNA. Nucleotide positions are indicated for the accessible uridines identified from *in vitro* mapping analysis. The number of individual clones containing a given uridine at the splice site is presented. (B) RT-PCR analysis of *trans*-spliced RNA products generated *in vitro*. Active (Rib73; lanes 3 and 6) or inactive (R(d)73; lane 4) ribozymes were incubated with TP target RNA substrate. As a reaction control, RT-PCR products without any RNA (lane 2), with TP RNA alone (lane 5), or Rib73 alone (lane 6) were presented. Amplification products were subjected to electrophoresis in a 3% agarose gel. The migration of 100 bp ladder is indicated as a molecular weight marker (lane 1, MW). (C) Sequence analysis of *trans*-splicing products (Fig. 3B, lane 3) produced *in vitro*. Sequence of one representative clone out of 10 different clones with same sequence is presented. The expected sequence around the splicing junction was indicated by an arrow, with the complement to the IGS boxed and the uridine at position 73 circled.

Induction of transgene activity in TP-expressing cells by the specific *trans*-splicing ribozyme

To determine whether the specific ribozyme could modify the TP RNA to induce an intended transgene activity in cells, we first modified the ribozyme construct with a functional active form since group I ribozymes with only a 6-nt-long IGS have been very inactive when expressed in mammalian cells (Byun *et al.*, 2003). For use in cells, we modified Rib73 to contain an extension of P1 helix, addition of 7-nt-long P10 helix and a 300-nt-long antisense sequence against the downstream region of U73 of the TP RNA (Fig. 4A). These modifications have been demonstrated to increase specificity and activity of the group I ribozyme in cells (Kohler *et al.*, 1999). In addition, for a transgene to be readily expressed in mammalian cells, a new start codon AUG with Kozak sequences was inserted into the 5' end of the 3' exon transgene since the target sequence (U96) was present in the leader region. The expression vector

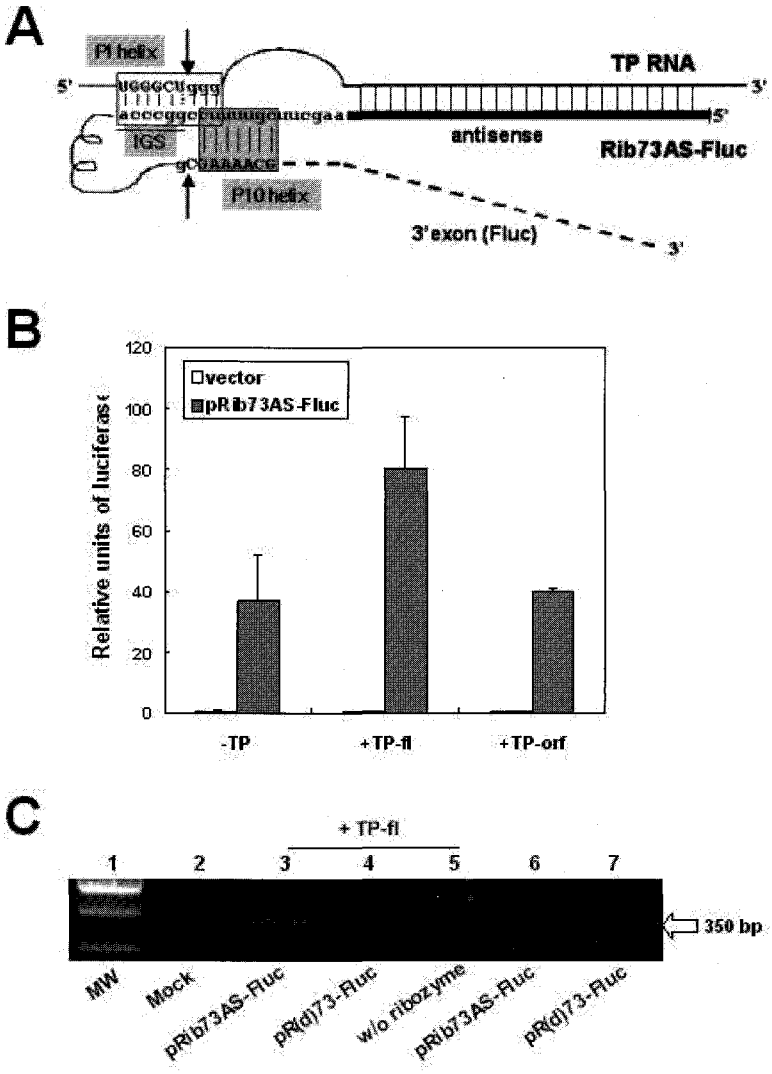

Fig. 4. Induction of luciferase activity by the specific *trans*-splicing ribozyme in TP-expressing NIH3T3 cells. (A) Modified *trans*-splicing ribozyme with extended IGS are represented with 3' exon sequences capitalized. Potential base paring between the ribozyme and the target is indicated by vertical line. Arrows indicate 5' and 3' splicing sites. (B) Cells were transfected with control vector or pRib73AS-Fluc along with or without either a plamsid encoding full-length cDNA of TP (TP-fl) or a vector encoding only open reading frame of TP (TP-orf). Luciferase activity of FLuc relative to RLuc was quantitated, and averages of three separate determinants are shown with bars indicating standard deviations. (C) RT-PCR analysis of *trans*-spliced RNA products generated in cells. NIH3T3 cells were mock-transfected (lane 2) or transfected with TP-fl vector (lanes 3-5) alone (lane 5), with the active ribozyme (pRib73AS-Fluc; lanes 3 and 6), or with the inactive ribozyme RNA (pR(d)73AS-Fluc; lane 4 and 7). *Trans*-spliced products were amplified by RT-PCR, yielding a DNA fragment of 350 bp. The migration of 100 bp ladder is shown as a molecular weight marker (lane 1, MW).

encoding the modified *trans*-splicing ribozyme with Fluc sequence as its 3'exon was denoted pRib73AS-Fluc3.

For the functional analysis of the specific ribozyme in cells, we cotransfected a plasmid encoding TP (TP-fl or TP-orf) with pRib73AS-Fluc into NIH3T3 cells (Fig. 4B). We then assessed transgene induction by monitoring luciferase activity of Fluc relative to Rluc used for normalization. TP-fl is an expression plasmid encoding full-length cDNA of TP, thus containing the target residue (U73). However, TP-orf does not harbor the target sequence since it encodes only open reading frame sequence of TP. Transfection of pRib73AS-Fluc alone (-TP) showed a slight expression of Fluc. This nonspecific expression could be due to direct translation of exon sequences and/or translation of self-splicing products. Further studies will be needed to elucidate the molecular

mechanism of this little nonspecific translation. In contrast, pRib73AS-Fluc efficiently stimulated luciferase activity by about more than 2-fold in cells cotransfected with TP-fl, compared to the cells transfected with the ribozyme alone (Fig. 4B). In sharp contrast, pRib73AS-Fluc could not trigger the FLuc expression in cells cotransfected with TP-orf (Fig. 4C). This could result from the absence of U73 in the target RNA expressed from TP-orf. Therefore, these results strongly indicate that the expression of the transgene by the *trans*-splicing ribozyme in cells could be highly triggered with target RNA-specific manner.

To determine whether the transgene induction in cells with the specific ribozyme would be due to the *trans*-splicing reaction of Rib73AS-Fluc with TP RNA with fidelity in cells, we cotransfected pRib73AS-Fluc with TP-fl in NIH3T3 cells (Fig. 4C). Total RNA was isolated from the transfected cells and analyzed by RT-PCR. A *trans*-spliced product of expected size (350 bp) was detected only in cells cotransfected with the ribozyme vector and TP-fl (Fig. 4C, lane 3). By contrast, no such product was generated in cells mock-transfected or transfected with the target plasmid alone, the ribozyme vector alone, or the inactive ribozyme alone (Fig. 4C, lanes 2 and 5-7). Moreover, no product was also detected in cells cotransfected with the inactive ribozyme plasmid (pR(d)73-Fluc) and TP-fl (Fig. 4C, lane 4). Sequence analysis of the amplified fragment showed exact *trans*-splicing of the ribozyme with U73 residue of the TP RNA in cells (data not shown). Thus, these results suggested that the specific group I ribozyme was able to modify the target TP RNA to highly induce transgene activity in mammalian cells with *trans*-splicing reaction.

In this study, we showed that TP RNA could be a valid gastric cancer marker. In addition, we developed a specific group I intron ribozyme to modify such a specific target RNA associated with human disease to selectively induce transgene activity in cells. The 3' exon can be exchanged with any RNA sequence in the *trans*-splicing reaction (Sullenger and Cech, 1995). Thus, any new RNAs with anti-cancer therapeutic activity, for example, cytotoxin RNA, can be highly and selectively expressed in TP-expressing cancer cells, if they are tagged at the ribozyme backbone containing IGS identified here. The ribozyme could then remove target TP RNA and simultaneously deliver cytotoxin activity selectively in cancer cells expressing the RNA. Therefore, this RNA replacement may be an attractive approach for cancer therapy because it should inhibit or reduce the production of the TP protein and simultaneously engender the production of therapeutic gene activity much higher and selectively in the TP-associated cancer cells. Specific messages associated with a wide range of human diseases are now being intensively identified through the functional genomics and bioinformatic studies. Together with these advances, *trans*-splicing ribozymes that can modify the disease-associated unique transcripts would be general tools for the treatment of diverse human diseases such as cancer.

Acknowledgments

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