J. Microbiol. Biotechnol. (2005), **15**(6), 1408–1413

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Re-Engineering of Carcinoembryonic Antigen RNA with the Group I Intron of *Tetrahymena thermophila* by Targeted *Trans*-Splicing

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Received: April 20, 2005
Accepted: May 18, 2005

Abstract Elevated expression of carcinoembryonic antigen (CEA) has been implicated in various biological aspects of neoplasia such as tumor cell adhesion, metastasis, blocking of cellular immune mechanisms, and antiapoptosis function. Thus, the CEA could be an important target for anticancer therapy. In this study, we developed *Tetrahymena* group 1 intron-based *trans*-splicing ribozymes that can specifically target and replace CEA RNA. To this end, we first determined which regions of the CEA RNA were accessible to ribozymes by employing an RNA mapping strategy that was based on a *trans*-splicing ribozyme library. Next, we assessed the ribozyme activities by comparing the *trans*-splicing activities of several ribozymes that targeted different regions of the CEA RNA, and then the ribozyme that could target the most accessible site was observed to be the most active with high fidelity *in vitro*. Moreover, the specific *trans*-splicing ribozyme was found to react with and altered the target CEA transcripts in mammalian cells with high fidelity. These results suggest that the *Tetrahymena* ribozyme can be utilized to replace CEA RNAs in tumors with a new RNA-harboring anticancer activity, thereby hopefully reverting the malignant phenotype.

Key words: Carcinoembryonic antigen, group I intron, RNA replacement, *Tetrahymena thermophila*, *trans*-splicing ribozyme

Carcinoembryonic antigen (CEA, CD66e, CEACAM5) is a cell surface glycoprotein that is known to be overexpressed in a majority of carcinomas and has been used for a cancer marker [6, 7, 26]. It is a member of a large family of related proteins that belong to the larger immunoglobulin super-family [8, 9]. Moreover, CEA has been shown to be involved in both homophilic (CEA to CEA) and heterophilic (CEA binding to non-CEA molecules) binding [20], suggesting that it is an intercellular adhesion molecule involved in cancer invasion and metastasis [12, 27].

In athymic nude mice, weakly metastatic colon cancer cell lines become highly metastatic when transfected with the CEA encoding cDNA [2]. In addition to its use as a blood marker for many carcinomas, elevated expression of CEA has been implicated in various biological aspects of neoplasia, especially tumor cell adhesion, metastasis, blocking of cellular immune mechanisms, and antiapoptosis functions, implying that CEA could be a specific anticancer target molecule.

Group I intron RNA is a ribozyme that can catalyze two consecutive *trans*-esterification reactions to excise itself from a precursor RNA and ligate the flanking exons. A derivative of the self-splicing intron, known as L-21 (lacking the first 21 nucleotides of the intron), was shown to *trans*-splice an exon tagged at its 3' end onto a separate RNA in mammalian cells as well as in bacteria [13, 28]. For the targeted *trans*-splicing reactions, a substrate RNA should contain the complementary sequence of internal guide sequence (IGS) of the ribozyme, and the substrate RNA and the ribozyme maintain the essential G-U base pair that defines the cleavage site.

The ribozyme has been shown to revise mutant transcripts associated with several human genetic and malignant diseases [16, 21, 22, 25, 31]. More recently, we demonstrated that a *trans*-splicing ribozyme could selectively induce any therapeutic gene activities in HCV RNA-expressing cells via specific RNA replacement of the HCV RNA [23], which implies that the strategies against a wide-range of human diseases, such as cancer, could be developed with re-engineering ribozyme through RNA replacement of the disease-associated unique RNAs.

In this study, in order to develop a therapeutic approach to CEA-associated malignant disease, we tested whether the *Tetrahymena* group I intron-based ribozyme could be generated to yield a true catalyst that cleaves and replaces the CEA RNA molecules in *trans*.

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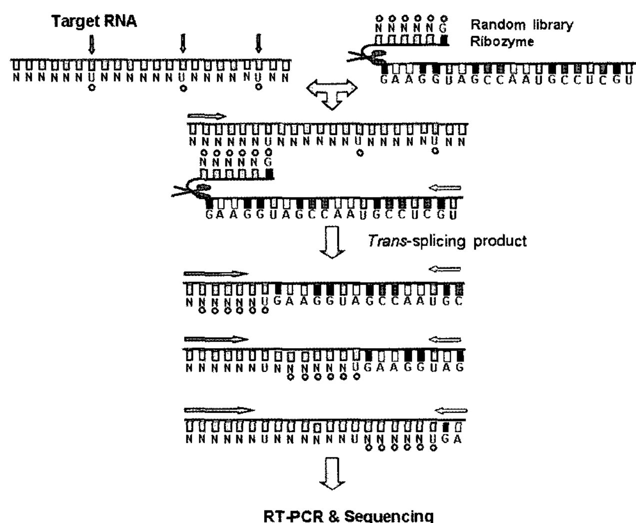

Fig. 1. Scheme for mapping accessible sites in CEA RNA *in vitro* with a *trans*-splicing ribozyme library.
The GN5 ribozyme library and target CEA transcript were incubated in the reaction buffer at 37°C for 3 h in the presence of guanosine. Reaction products were amplified with 5' primer specific for the CEA RNA and with 3' primer recognizing 3' exon tag *lacZ* sequence, and then cloned and sequenced.

To this end, we first identified the sites of CEA RNA that were most accessible to the ribozymes, and then we constructed specific ribozymes that targeted those sites and determined if the ribozymes could efficiently *trans*-splice the target RNA not only *in vitro* but also in mammalian cells.

In the splicing reaction, every uridine in the CEA RNA can be potentially targeted by the group I ribozyme of *Tetrahymena thermophila* through G-U base pairing between the IGS of the ribozymes and the target RNA. However, only a limited number of uridines on the substrate RNA could be truly accessible to the ribozyme because of the target RNA's tertiary structure and/or complex formation with proteins in cellular circumstances [17]. Thus, an RNA mapping strategy was first carried out to determine which uridines in the CEA RNA were accessible to the ribozymes. As shown in Fig. 1, the mapping method was based on a *trans*-splicing ribozyme library [16, 17, 23] and RNA tagging [13].

The ribozyme library was constructed by randomizing the IGS of the *Tetrahymena* group I intron, so that the 5' end of the ribozyme began with 5'-GNNNNN-3', where G represents guanine and N represents equimolar amount of the four nucleotides (nt), and named the GN5 ribozyme library. Thus, ribozymes in the GN5 ribozyme library would target and cleave the substrate RNA at any accessible uridine (U) residue and splice their 3' exon to the 3' and of 5' cleavage target transcript. Part of the *lacZ* gene was utilized as a 3' tagging exon in the GN5 ribozyme library that can be transferred to the target RNA's Us accessible to the ribozyme. The substrate CEA N domain RNA and B2 domain RNA were generated by *in vitro* transcription using T7 RNA polymerase with a cDNA clone of CEA (21C Frontier Human GeneBank). To map the CEA RNAs, the GN5 ribozyme library (50 nM) was reacted at 37°C for 3 h under spicing condition (50 mM HEPES, pH 7.0, 10 mM NaCl, 5 mM $MgCl_2$) in the presence of guanosine (100 μM) with the CEA RNA (1 μM). The resulting *trans*-splicing reaction products were then amplified by RT-PCR with a 3' tag primer specific for the ribozyme's 3' exon *lac*Z sequence (5'-ATGTGCTGCAAGGCGATT-3') and a 5' primer specific for the 5' end of target CEA N domain RNA (5'-CGGAATTCTAATACGACTCACTAT-AGGGAGC TCAC TATTGAATCC-3') or a 5' primer specific for the 5' end of target CEA B2 domain RNA (5'-TAATACGACTCACTATAGGGGACGACCCCACCATT TCC-3').

The amplified products were cloned into several pUC19 vectors. Sequence analyses of the splicing junction sites showed that several uridines, which were present in the sites downstream of the AUG start cordon, appeared to be particularly accessible, because more than 33% or 50% of the reaction products with the N domain or the B2 domain RNA resulted from splicing at these sites (Fig. 2). In particular, the most accessible site was present in the uridine at position 53 (U53) on CEA N domain RNA and 28 (U28) on CEA B2 domain RNA. Interestingly, U53 and

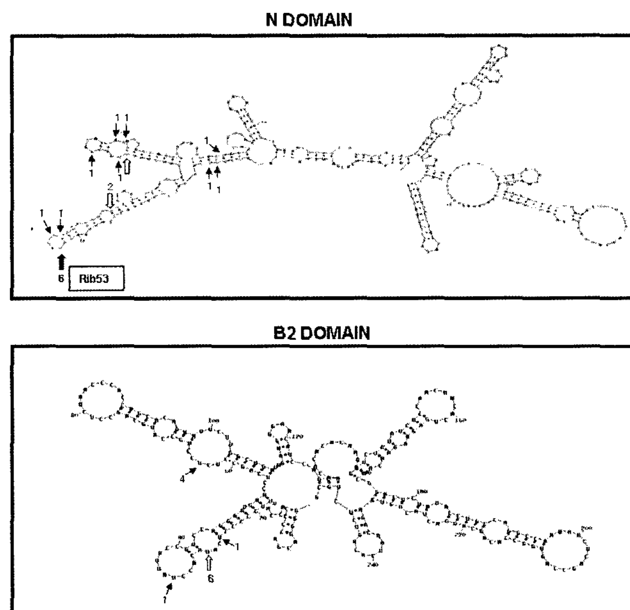

Fig. 2. Mapping of the ribozyme-accessible sites in CEA RNA.
Nucleotide positions of the accessible uridines identified from *in vitro* mapping analysis are indicated by arrows as nucleotide numbers on the secondary structure of N or B2 domain CEA RNA predicted by the computer simulating program called MFold [11]. The number of clones containing a given uridine at the splice site is presented. The thick arrow represents the most accessible site of the CEA RNA (+53 site).

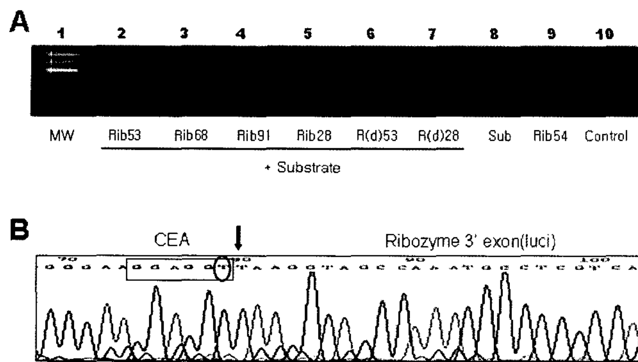

Fig. 3. Analysis of *trans*-spliced products *in vitro*. (A) RT-PCR analysis of *trans*-spliced RNA products generated *in vitro*. A series of active (lanes 2–5) or inactive (lanes 6–7) ribozymes were incubated with CEA target RNA substrate, and *trans*-spliced products were amplified. As a reaction control, CEA RNA alone (lane 8) or Rib53 alone (lane 9) was presented. Amplification products were then subjected to electrophoresis in a 2% 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 *trans*-splicing reaction between Rib53 and CEA RNA (Fig. 3A, lane 2) were isolated on a gel and cloned. The sequence of one representative clone out of 5 different clones with the same sequence is shown. The expected sequence around the splicing junction, indicated by the arrow, is shown with the ribozyme recognition sequence in CEA RNA (boxed) and the uridine at position 53 (circled).

U28 were located in the loop part on the CEA RNA secondary structure that was expected to be accessible to ribozyme.

Based on the structure prediction, U53 could be predicted as a ribozyme accessible site. However, other uridines on the loop region of CEA RNA, such as uridine at position 28 (U28), would also be expected to be possible targets for ribozymes. Thus, to verify if the sites predicted to be accessible by mapping studies were truly the most accessible sites to ribozymes, we assessed *in vitro trans*-splicing activities of two different ribozymes targeting uridines at position 28 (U28) or 53 (U53) in CEA RNA, which were identified via mapping analysis, or two ribozymes targeting uridine at position 68 (U68) or 91 (U91), which were located in the stem part on the CEA RNA secondary structure (Fig. 3A). U68 and U91 were chosen because they were present in the stem region of the CEA RNA that could be expected to be less accessible to ribozymes (Fig. 2). Rib53, Rib28, Rib68, or Rib91 ribozymes recognizing these individual sites 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 each ribozyme's IGS and also with a 3' primer specific for the 3' exon *lacZ* sequence. The pT7L-21 vector, which was kindly supplied by B.A. Sullenger at Duke University, encodes a slightly shortened version of the natural group I intron from *Tetrahymena*, called L-21 [28]. The IGS on the L-21 *trans*-splicing ribozyme (5'-GGAGGG-3') was exchanged with 5'-GCCTCC-3' in Rib53, 5'-GCAAGT-3' in Rib28, 5'-GAAAGA-3' in Rib68, or 5'-GTGAGG-3' in Rib91. In addition, inactive ribozymes, R(d)53 and R(d)28, which are devoid of the catalytic core of the enzyme [28], were constructed as negative controls. The specific ribozymes (100 nM) were incubated under splicing conditions with the target CEA RNA (10 nM). RT-PCR analyses were then performed with a 3' primer specific for the 3' exon tag sequence and a 5' primer specific for CEA RNA as described [14, 18]. Amplified fragments of expected size of 140 bp, 155 bp, 178 bp, or 115 bp were generated from the reaction mixtures with the CEA RNA and Rib53, Rib68, Rib91, or Rib28, respectively (Fig. 3A, lanes 2–5). It should be noted that Rib53 can *trans*-splice a 3' exon tag onto the CEA RNA with the highest efficiency. However, U28 is less efficiently targeted than U53, although U28 is present on the loop part of the RNA. From the RT-PCR products with both Rib68 and Rib91, U68 and U91 in the CEA RNA were less accessible to the ribozymes. Thus, these results strongly indicate that the relative *trans*-splicing efficiency at the chosen sites corresponds to the predicted accessibility from our mapping analyses. The inactive versions of ribozymes, R(d)53 and R(d)28, and substrate or ribozyme alone could not produce any *trans*-splicing products with the target RNA (Fig. 3A, lanes 6–9). These results suggest that the amplified RT-PCR products found in lanes 2–5 of Fig. 3A resulted from the catalytic activity of the ribozymes.

To determine whether the specific ribozyme could accurately perform the *trans*-splicing reaction with the target RNA, sequence analyses of the amplified spliced products found in lane 2 of Fig. 3A were carried out (Fig. 3B). The sequence of the 140 bp RT-PCR fragment demonstrated that the ribozyme, Rib53, had correctly targeted the CEA RNA at the predicted reaction site (U53) and replaced sequences downstream of the reaction sites with the 3' exon sequences attached to the 3' end of the ribozyme. Sequencing analyses of reaction products isolated from lane 3, 4, or 5 of Fig. 3A demonstrated that Rib68, Rib91, or Rib28 also correctly *trans*-spliced its 3' exon tag onto the CEA 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 replace CEA RNA with a 3' exon tagged at the 3' end of the ribozymes by targeted *trans*-splicing with high fidelity. *Trans*-splicing analyses, taken together with the mapping studies, encouraged us to focus on further studies with Rib53.

To determine whether Rib53 could also *trans*-splice CEA RNA at the predicted U53 with fidelity in cells, we designed an enhanced ribozyme construct, because group I *trans*-spicing ribozymes with only a 6-nt-long IGS were very inactive when expressed in mammalian cells [4]. We modified the ribozyme directed at U53 on CEA RNA to

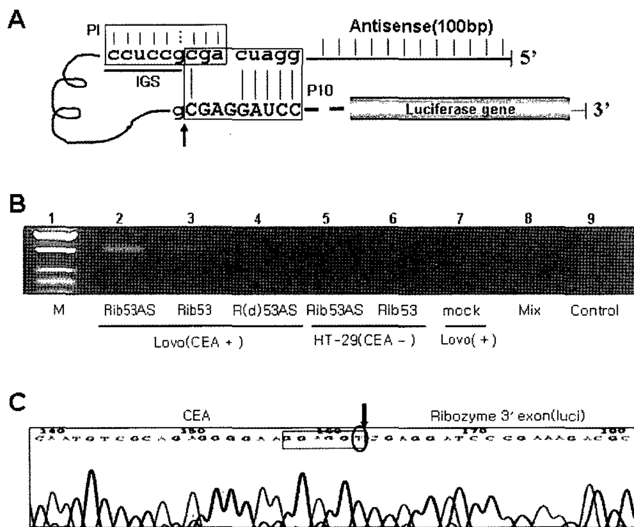

Fig. 4. *Trans*-splicing reaction with CEA RNA in cells.

(A) Schematic diagram of the *trans*-splicing ribozyme Rib53AS. The ribozyme is shown with 3' exon sequences capitalized. Potential base pairings between the ribozyme and the target CEA RNA are represented by vertical lines. Arrows depict 3' splicing sites. (B) RT-PCR analysis of the *trans*-spliced transcript generated in cells. Lovo or HT29 cells were mock transfected (lane 7), transfected with the active ribozymes (Rib53AS, lanes 2 and 5; Rib53, lanes 3 and 6), or with the inactive ribozyme (R(d)53, lane 4). *Trans*-spliced products were amplified by RT-PCR, yielding a DNA fragment of 420 bp. (C) Sequence analysis of *trans*-spliced RNA products in cells. The amplified products in lane 2 of Fig. 4B were sequenced. The sequence of one representative clone of ten 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 position 53 (circled).

contain an extension of the P1 helix, an addition of 6-nt-long P10 helix and a 100-nt-long antisense sequence complementary to the downstream region of the targeted urine of the CEA RNA and designated Rib53AS (Fig. 4A). A ribozyme without the antisense region was also constructed (Rib53). As a control, an inactive ribozyme without catalytic core sequence was generated (R(d)53AS). We constructed the CMV promoter and 3' luciferase tag expression system for their expression in cells. CEA-positive Lovo and CEA-negative HT-29 cells were either mock-transfected or transfected with 3 μg of Rib53AS or Rib53 using 9.8 μl of EXgen (Fig. 4). The total RNA was isolated from the cells 24 h after transfection as previously described [15, 19, 23, 25]. The RNA (5 μg) was reverse transcribed in the presence of 10 mM L-argininamide, together with a primer specific for the 3' tagging luciferase sequence. The resulting cDNAs were amplified for 30 cycles with a 5' primer specific for the CEA (5'-GGGAATTCCAGATGGTGCATCCCCTGGC-3') and with a 3' primer specific for the 3' exon luciferase sequence. A *trans*-spliced product of expected size (420 bp) was detected only in Lovo cells transfected with the ribozyme (Fig. 4A, lanes 2 and 3). By contrast, no such product was found in Lovo cells transfected with the inactive ribozyme, R(d)53 (Fig. 4B, lane 4). Moreover, no product was also generated in CEA-negative cells HT-29 transfected with the active ribozymes (Fig. 4B, lanes 5 and 6). Furthermore, no amplification product was detected in a "mix" RNA sample that was extracted after lysate from ribozyme-transfected HT-29 cells was mixed with lysate from mock transfected Lovo cells (Fig. 4B, lane 8). These results suggest that the observed *trans*-splicing product was generated with active ribozymes inside the target RNA-expressing Lovo cells, but not during the RNA manipulation. Moreover, ribozyme with extended IGS performed more efficiently *trans*-splicing reaction in cells. To ascertain that *trans*-splicing had occurred correctly and in frame, the RT-product of the 420 bp amplified fragment was purified and sequenced (Fig. 4C). The data showed that the specific ribozyme was able to replace the CEA target RNA in mammalian cells with a 3' exon tagged at the 3' end of the ribozyme with high fidelity.

From the observations that cells transfected with cDNA for CEA formed aggregates and that this aggregation was completely inhibited by CEA antibody or antisense treatment of the cells [1], CEA has been suggested to function as an intercellular adhesion molecule, promoting cell aggregation by homophilic interaction. In addition, the cDNA transfectants have an ability to form liver metastases after splenic injection, and the metastases are inhibited by *in vitro* treatment with CEA antibody or antisense [3, 10]. Furthermore, CEA has been found to block cellular differentiation [5], to have tumorigenic effect in combination with other oncogene [24], and deregulated CEA expression in human colonocyte lines disrupted cell polarization, tissue architecture, and increased tumorgenicity. Thus, some anticancer drugs to block CEA production and/or expression are expected to exert apoptotic effects.

In this study, based on the *Tetrahymena* group I intron, a *trans*-splicing ribozyme was developed to specifically replace CEA RNA with the intended sequence attached to the 3' end of the ribozyme in cells as well as *in vitro*. The target specificity of the ribozyme, Rib53AS, was confirmed by the RACE RT-PCR analysis of the total *trans*-spliced products generated in the ribozyme-transfected Lovo cells. The sequence analysis of the RACE RT-PCR products revealed that all of the *trans*-splicing products (18 clones) resulted from reactions with the targeted CEA RNA only (data not shown), implying that the ribozyme exerted highly targeted RNA-specific intracellular *trans*-splicing activity. The 3' exon in the *trans*-splicing reaction can have virtually any RNA sequence changed [29], which implies that the new RNAs that exert anticancer therapeutic activity can be triggered selectively in CEA-expressing cancer cells via RNA replacement, if they are attached to the ribozyme backbone that contained the IGS identified here. Therefore, the ribozyme backbone developed in this study can be utilized for the development of modalities for specific gene

therapy against CEA-associated cancer. The ribozyme could then selectively induce anticancer gene activity such as cytotoxin or suicide gene activity in CEA RNA-expressing cancer cells, thus specifically eliminating the cancer cells. This RNA replacement would be a more attractive approach for cancer therapy, because it should inhibit or reduce the production of the CEA protein and simultaneously engender the production of therapeutic gene activity specifically in the CEA-associated cancer cells. Furthermore, other RNA-associated malignant or infectious diseases would also be treated by a *trans*-splicing ribozyme that could selectively replace the disease-associated specific RNAs with transcripts expressing therapeutic activity.

In the present study, with RNA mapping studies, we identified uridines in the CEA RNA that were most accessible to the ribozymes. Comparative *trans*-splicing analyses clearly showed that the ribozymes that recognized these isolated accessible sites were truly the most active. Recently, based on inhibitory RNA or short oligonucleotides such as *trans*-cleavage ribozyme, antisense oligonucleotides, or siRNA, many anticancer or antiviral protocols have been proposed [30]. Therefore, the RNA mapping strategy performed in this study is expected to be useful in identifying optimal accessible sites of target RNAs for various inhibitory RNAs.

Acknowledgment

The present research was conducted by the research fund of Dankook University in 2004.

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