

Modulating the splicing activity of *Tetrahymena* ribozyme via RNA self-assembly

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Abstract The internal guiding sequence (IGS) is normally located at the 5' end of *trans*-splicing ribozymes that are derived from the *Tetrahymena* group I intron, and is required for the recognition of substrate RNAs and for *trans*-splicing reactions. Here, we separated the *Tetrahymena* group I intron at the L2 loop to produce two fragments: the IGS-containing substrate, and the IGS-lacking ribozyme. We show here that two fragments can complex not through the IGS interaction but under the guidance of appended interacting nucleotides, and perform *trans*-splicing. The splicing reactions took place both in vitro and in mammalian cells, and the spliced mRNA product from the self-assembled ribozyme complex can be translated into functional proteins in vivo. The splicing efficiency was dependent on the length of appending nucleotides.
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Keywords: *Tetrahymena* group I intron ribozyme; Splicing activity; β -Lactamase; L2 loop; Self-assembly

1. Introduction

Tetrahymena group I intron ribozyme can splice itself out of the RNA transcripts at the 5' splice site located in the P1–P2 domain in the presence of Mg^{2+} (~2 mM) and a guanosine nucleoside as a cofactor [1,2]. The self-splicing reaction requires a uridine at the 5' splice site, which forms a critical wobble base pair with a guanine of the 6-base internal guiding sequence (IGS) [3]. With the truncation of the L1 loop, located between the 5' splice site and the IGS, the remaining 3' fragment of *Tetrahymena* group I intron ribozyme can bind a substrate RNA bimolecularly and perform splicing reactions analogous to the reaction steps of self-splicing but in *trans*, mediated by the interaction between the substrate RNA and the IGS [4,5]. Extensive biochemical and structural studies have shed light on the mechanism of the splicing reaction [6–8], and led to the development of *trans*-splicing ribozymes

for targeting and modifying mRNA transcripts in vitro and in vivo [9–14]. There are examples of *trans*-splicing ribozymes-mediated repairing of mRNAs transcripts encoding mutant p53 [11], mutant chloride channel [12], and transcripts associated with myotonic dystrophy and sickle cell anemia in mammalian tissue culture cells [13,14]. However, biochemical engineering is needed to improve the in vivo efficiency of *trans*-splicing ribozymes for efficient RNA repairs and corrections.

We are interested in designing new variants of *Tetrahymena* group I intron ribozyme for intermolecular splicing. We began our effort by examining whether the *Tetrahymena* group I intron can be split at different sites than the L1 loop but still perform *trans*-splicing reactions. Furthermore, it would be very interesting whether a ribozyme without possessing the IGS could carry out the *trans*-splicing reaction with a substrate RNA.

We previously reported a ribozyme construct, Rz156, where the *Tetrahymena* group I intron ribozyme was inserted into the coding sequence of the cDNA of the non-secreted TEM-1 β -lactamase (Bla) [15]. Expression of Rz156 in mammalian cells resulted transcripts that can self-splice and generate enzymatically active Bla that can be detected in single mammalian cells. Based on the *cis*-splicing Rz156, we constructed a *trans*-splicing ribozyme derived from Rz156 split at the L1 loop [16]. Because this system sensitively and conveniently detects the ribozyme splicing activity both in vitro and in vivo, we used it in this study to examine whether the engineered *Tetrahymena* group I ribozyme can *trans*-splice in vitro and in vivo (Scheme 1).

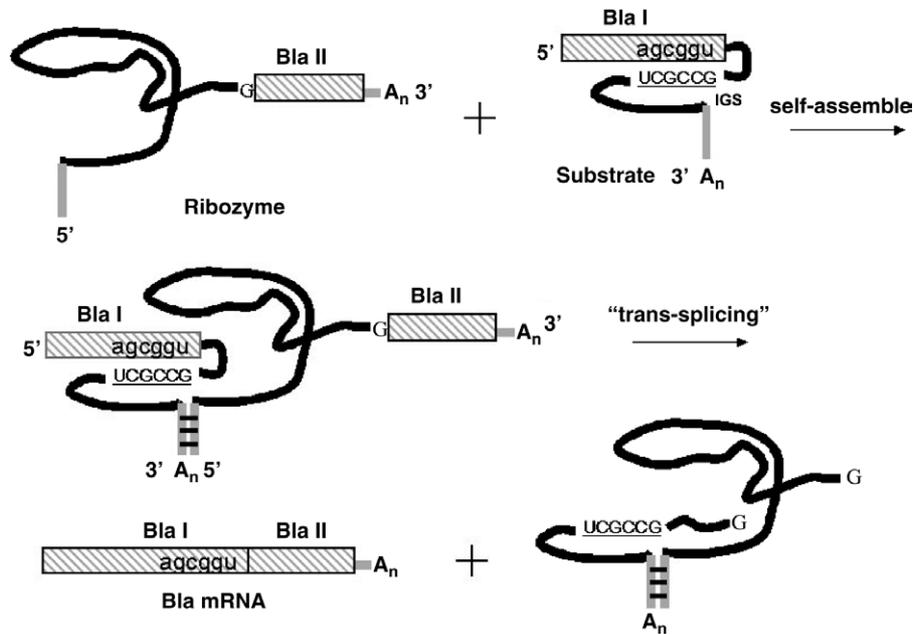
2. Materials and methods

All mammalian expression vectors were based on pCMV derived from pDsRed2-N1 (Clontech) with the DsRed cDNA removed. To construct the plasmid DNA encoding insertion mutants of the reporter ribozyme Rz156 [15], a linker was introduced into pCMV-Rz156 between positions 27 and 28, 43 and 44, 95 and 96, or 312 and 313 by polymerase chain reaction (PCR) mutagenesis. The mutant with insertion at position 43 (pCMV-Rz#111) was constructed by insertion of *KpnI* recognition sequence (GGTACC) between positions 43/44 in group I intron of the pCMV-Rz156. The 5' part of the pCMV-Rz#111 that carried *BamHI* site inside the fragment was prepared by PCR using primers containing *EcoRI* or *Sall* site and inserted in *EcoRI/Sall* site of pCMV to make pCMV-A. Appended RNA sequences were amplified by PCR and ligated into *BamHI/Sall* site in pCMV-A to construct pCMV-SA, pCMV-MA, and pCMV-LA. To prepare pCMV-ZA, pCMV-LA was self-ligated after *BamHI/KpnI* digestion. pCMV-B was constructed by self-ligation of the pCMV-Rz#111 after removal of 5' part of the pCMV-Rz#111. pCMV-B was digested by *HindIII/KpnI* and self-ligated to make pCMV-ZB. pCMV-SB, pCMV-MB, and pCMV-LB were constructed by insertion

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Abbreviations: Bla, β -lactamase; CMV, cytomegalovirus; IGS, internal guiding sequence; PCR, polymerase chain reaction; RT, reverse transcriptase



Scheme 1. Strategy for modulating the splicing activity of non-IGS-containing *Tetrahymena* ribozyme via RNA self-assembly. The IGS (GCCGCU) is located on the substrate. Bla I and Bla II are the 5' and 3' fragments of Bla mRNA, respectively. The gray bars represent the appended sense and antisense RNA sequences that are used to mediate the *trans*-splicing reaction.

of antisense sequence corresponding to the appended sequence in pCMV-SA, pCMV-MA, and pCMV-LA at *Hind*III/*Kpn*I site of pCMV-B, respectively. A point mutation (G264A) in pCMV-LBrd was created by PCR using a primer containing the mutation. pT7-LBrdel was constructed by deletion of sequence flanked by *Bgl*II/*Nhe*I site in *Tetrahymena* group I intron after replacing pCMV-LB with T7 vector. A *Bam*HI-*Kpn*I fragment containing appended sequence from pCMV-LA was ligated in *Hind*III/*Kpn*I site of pCMV-LB in blunt-cohesive ligation to prepare pCMV-LBNB. The appended sense-antisense sequences were derived from the following regions of the DsRed cDNA: nt 61–90 (for SA/SB), nt 61–149 (for MA/MB), and nt 61–259 (for LA/LB, LBrd, LBrdel, and LBNB), where A in the initiation codon is numbered as nt 1. All PCR reactions for plasmid DNA construction were done by Pfu Turbo DNA polymerase (Stratagene) to avoid undesired mutations. The Bla and ribozyme sequences in all constructs were confirmed by DNA sequencing.

All *in vitro* transcription ribozyme fragments were cloned directly behind the T7 RNA polymerase promoter site in either the pTRIKAN 19 or 18 vector (Ambion). 5' pTRI primer (GTGACACTATAGAA-TACACGG) and 3' pTRI primer (CACGACGTTGTAAAACGACGG) were used to generate PCR fragments containing each ribozyme fragment with T7 RNA polymerase promoter site at the 5' end of the PCR fragment. The PCR cycle conditions used were as follows: an initial denaturing step at 94 °C for 5 min; then 35 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 90 s. A final polymerization step at 72 °C for 7 min was performed, followed by 4 °C to terminate the reaction. Each PCR product was purified from a 2% agarose gel with the GFX DNA purification kit (Amersham Biosciences). The DNA concentration was quantitated by UV absorbance. All PCR products were confirmed by DNA sequencing. Using the T7-MEGA shortscript kit (Ambion), 150–900 ng of each PCR product was used as the DNA template for *in vitro* transcription, and incubated at 37 °C for 3–4 h. 2 U of DNase I was added to each reaction and incubated at 37 °C for 15 min. The RNA transcripts were purified by electrophoresis on 8 M urea/3.5% polyacrylamide gel. The gels were stained by 0.05% toluidine blue and RNA bands were excised, crushed, and soaked in an elution buffer (0.5 M ammonium acetate, 2 mM EDTA, 0.1% SDS) at 37 °C overnight. The slurry was transferred into a Nanosep MF 0.2 µm filter (Pall Corporation) and cleared by centrifuge 14000 × *g* for 10 min. RNAs in the filtrate were ethanol-precipitated and re-suspended in nuclease-free water. Concentrations were determined by measuring the absorbance at 260 nm.

Each RNA (100 nM) transcribed *in vitro* was denatured at 95 °C for 1 min and pre-equilibrated in the reaction buffer (50 mM HEPES, pH 7.0; 150 mM NaCl; 5 mM MgCl₂) at 37 °C for 2 min. RNAs were mixed with GTP (100 µM) to start splicing reactions, which proceeded at 37 °C for 3 h. The reaction product was applied to reverse transcription by random primer (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen) in the presence of 50 mM of L-argininamide to inhibit splicing during the reverse transcription. After RNase H treatment, the resultant cDNAs were amplified by the following protocol: 94 °C for 5 min, 30 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (1 min), and final extension step at 72 °C for 7 min; primers: RT-1 forward primer (CAGAAACGCTGGTCAAAG), and RT-2 backward primer (CGTCAATACGGGATAATACC). PCR products were analyzed by electrophoresis in 2% agarose gel. The identities of all spliced products were confirmed by sequence analysis.

COS-7 cells were seeded in six-well dishes at a density of 4.0×10^5 per well for 18–24 h before transfection with 4 µg of expression vector per well by Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, total RNAs were extracted by TRIzol (Invitrogen) according to the manufacturer's instruction. Before RNA extraction, EDTA was added to TRIzol at a final concentration of 45 mM to inhibit the ribozyme activity. After treated by DNaseI (Invitrogen), extracted RNAs were reverse transcribed by oligo (dT) primer (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen) in the presence of 10 mM of L-argininamide and amplified by PCR.

To assay Bla activity, COS-7 cells were seeded in 12-well dishes at a density of 2.0×10^5 per well for 18–24 h before transfection with 1.6 µg of expression vector per well by Lipofectamine 2000. Forty-eight hours after transfection, cells were collected by centrifugation at room temperature at 2600 × *g* for 1 min. After washing the cells in 800 µl of PBS (GIBCO/BRL), cell pellets were re-suspended in 50 µl of 0.1 M phosphate buffer (pH 7.0). Cell lysates were prepared by three freezing and thawing cycles (freezing in dry ice/isopropanol for 10 min and thawing in 37 °C water bath for 10 min). The supernatant was recovered by centrifugation at 16000 × *g* for 2 min at 4 °C. Assays were conducted in 96-well black wall microtiter plates. To measure Bla activity, 45 µl of cell lysate and 5 µl of 1 mM CCI (a fluorogenic substrate for Bla synthesized in our lab [17]) were mixed in each well. Fluorescence was measured with 360 nm excitation and 465 nm emission at each time point in a SAFIRE microplate reader (TECAN, Research Triangle Park). The hydrolysis rates were converted to a unit of nM/min/µg total proteins by the calibration with the standard curve of coumarin,

the fluorophore generated in the hydrolysis of **CC1**, and the normalization against the total lysate protein contents determined by a Bradford assay (Bio-Rad).

3. Results and discussion

In looking for an appropriate split site in the ribozyme structure, we chose sites located either at the junction sites of the ribozyme structural domains or in the loop region. We postulated that a split site should tolerate structural perturbations like insertions. We inserted a short sequence of nucleotides immediately after position 27, 43, 95, or 312 of the group I intron ribozyme of the Rz156, respectively. Those sites were located either at the junction sites of the domains or in the loop region, but all outside the catalytic core. These linker-inserted reporter constructs were transfected with COS-7 cells independently, and RT-PCR assay and enzymatic assay of Bla by **CC1** (a fluorogenic substrate of Bla we developed [17]) revealed that a mutant with an insertion (GGTACC) after position 43 in the L2 region of the P2 stem-loop displayed an efficient Bla production with the same activity as Rz156 (data not shown). These data indicated that an insertion at this position affects little on the ribozyme activity. This result was a little surprising in light of previous studies that nucleotides 44 and 45 of loop L2 are very sensitive to base substitutions and the L2 loop has a tertiary contact with the loop in P5 domain [18,19]. It is likely such an interaction is reserved in our insertion mutant. Therefore, this mutant was chosen for further engineering.

We split the reporter construct Rz156 between position 43 and 44 of the group I intron sequence to afford two fragments: the IGS-containing substrate, and the IGS-lacking ribozyme fragment. Each fragment was cloned into a vector with T7 RNA polymerase promoter to examine whether these RNAs were able to perform *trans*-splicing in a test tube. In vitro transcription afforded transcription units, termed ZA (5' fragment) and ZB (3' fragment). The gel-purified RNAs were mixed and reacted under splicing condition in a test tube to detect any *trans*-splicing product – Bla mRNAs. RT-PCR revealed a very faint band that matched the size of the desired *trans*-splicing product (Fig. 1). Apparently, although ZA might be able to bind ZB through the interaction of the reserved 10 base pairs to reassemble the P2 domain and thus the whole ribozyme structure, this interaction was too weak and inefficient in reassembling the ribozyme complex. Indeed, no *trans*-splicing

product was detected when we cloned ZA and ZB into a mammalian expression vector with the human cytomegalovirus (CMV) immediate early promoter and SV40 polyadenylation signal, co-transfected COS-7 cells with the vectors, and purified total cellular RNA for RT-PCR analysis (see Fig. 2).

The lack of splicing activity of the ZA and ZB mixture prompted us to investigate whether appended interacting nucleotides to each fragment at its corresponding end of the split site would help bring two fragments together and facilitate the assembly of the P2 domain and thus the final complex (see Scheme 1). Three different lengths of nucleotides (42, 101, and 211) of sense and antisense sequences were added to the 3' and 5' end of ZA and ZB, respectively. Accordingly, the 5' fragments of these constructs were named as SA, MA, LA, respectively, and the 3' fragments as SB, MB, and LB, respectively.

We conducted in vitro *trans*-splicing experiments to evaluate whether the self-assembled ribozyme complexes formed and spliced. Equal amounts (100 fmol) of in vitro transcribed corresponding RNA fragments were mixed and incubated under the splicing condition (Fig. 1). In each case, a correct *trans*-splicing product was produced and confirmed by RT-PCR analysis and sequencing. Sequence analysis revealed that the top bands with a mass of ~600 bp contained both Bla and ribozyme, which might be the strand-transferring products of reverse transcription; such RT mediated strand transfer has been observed previously with retrovirus and human immunodeficiency virus [20,21]. Therefore, the appearance of this band indicated the formation of the self-assembled complex. The middle bands with a mass of ~500 bp were consistent with the pattern of Rz156 *cis*-splicing construct with which cryptic splicing could take place [15]. These data consistently supported that appended base pairings reassembled the ribozyme complex and reconstituted its splicing activity. Indicated from the band intensities of the spliced products, of which MA/MB and LA/LB were higher than that of ZA/ZB and SA/SB, the splicing efficiency appeared to depend on the length of appended base pairs.

Control constructs, LBRdel and LBNB, were prepared to evaluate whether the observed RT-PCR products were dependent on the ribozyme activity and appended base pairings. LBRdel was a mutant of LB but with a deletion of 94 nucleotides (from nt 238 to 331, where the guanosine-binding site is located). No desired spliced products were observed in the mixture of LA and LBRdel that lacked the catalytic core of

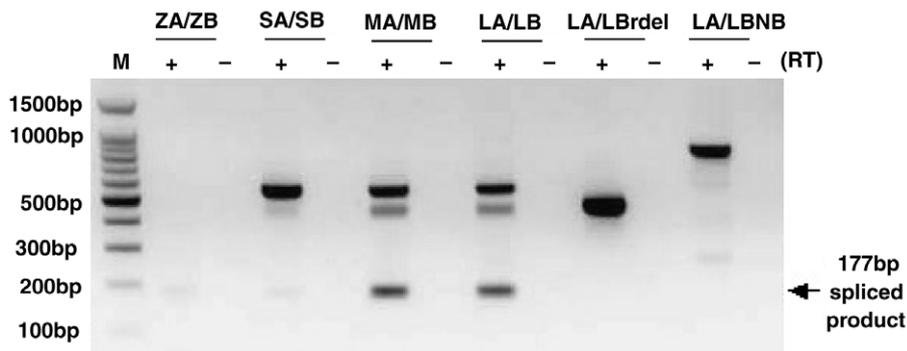


Fig. 1. RT-PCR analyses of in vitro *trans*-splicing reactions of the assembled ribozyme complexes. Arrow indicates 177 bp DNA of the expected RT-PCR products from *trans*-splicing reactions. RT, reverse transcriptase; M, DNA marker.

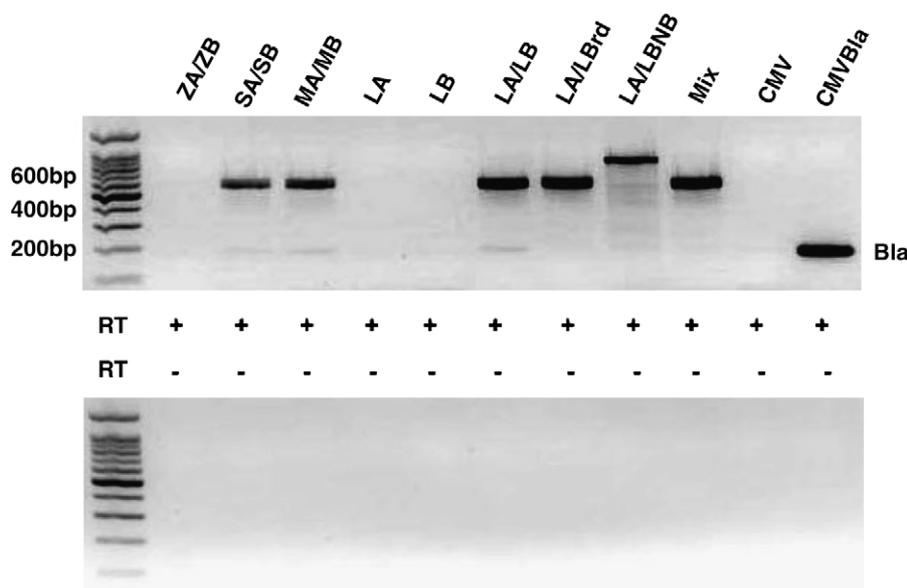


Fig. 2. RT-PCR assay of COS-7 cells transiently transfected with indicated constructs. Reverse transcriptase (RT) was present in upper samples but not in lower. The expected RT-PCR product from *trans*-splicing reaction (Bla) was as indicated.

the ribozyme, confirming that the reconstituted ribozyme catalytic activity was responsible for the spliced products (Fig. 1). The other control construct, LBNB, carried the same 211 nucleotides of LA and thus should not form a correctly assembled ribozyme complex with LA. Indeed, when it was incubated with LA, no correct splicing occurred, which confirmed that appropriate RNA/RNA interaction and alignment were required for the correct splicing (Fig. 1). The top band had a mass of ~ 800 bp containing Bla, ribozyme, and the 211 bp appended nucleotides, likely the strand-transferring reverse transcription product of an incorrectly assembled complex.

Having demonstrated this assisted assembling of ribozyme activity *in vitro*, we asked whether it could take place *in vivo*. To do this, we generated mammalian expression vectors for each construct and tested them in COS-7 cells. The RT-PCR assays of extracted RNAs from transfected cells showed that co-expression of LA and LB produced the *trans*-spliced products at the expected size (see Fig. 2). Sequence analysis confirmed the accurate *trans*-splicing of Bla mRNA. The correctly *trans*-spliced product (Bla mRNA) was also detectable in the co-expression of SA/SB or MA/MB, the constructs with shorter base-pairing interaction than LA/LB, but with lower intensity. No spliced product was detected when LA was co-transfected with a ribozyme dead mutant LBrd which carried a single point mutation (G264A) in its guanosine-binding site of the ribozyme, suggesting that the spliced production of Bla mRNA was due to the reassembled ribozyme's catalytic function. Co-expression of LA and LBNB did not produce the spliced mRNA of Bla either. Moreover, no *trans*-splicing product was detected in a "mix" control, where cells separately transfected with LA and LB, respectively, were mixed shortly after lyses, indicating that *trans*-splicing did not take place during the RNA extraction and the RT reaction. These results, together with the results in ZA and ZB, support that the engineered split ribozymes with appended base pairings are capable of reassembling and *trans*-splicing in

mammalian cells and that the efficiency may depend on the length of sense–antisense interaction.

Previous reports have shown that the self-splicing products of group I intron are not translated efficiently in mammalian cells [22]. To investigate whether any functional Bla protein was translated from the *trans*-spliced product, we assayed the enzymatic activity of Bla in cell lysates by CC1 (Fig. 3). The Bla activity in the lysates of COS-7 cells co-transfected with SA/SB, MA/MB or LA/LB was readily detectable; the activity of LA/LB was about 46-fold of that of the controls (0.93 ± 0.34 vs. 0.02 ± 0.01 nM/min/ μ g proteins). The Bla activity of LA/LB was twofold of that from MA/MB, and 19-fold of that from SA/SB, indicating its dependence on the length of the introduced nucleotide sequence. Consistent with the RT-PCR results, neither LBrd nor LBNB produced any Bla activity when co-expressed with LA. These data strongly suggested that the *trans*-spliced Bla mRNA from the reconsti-

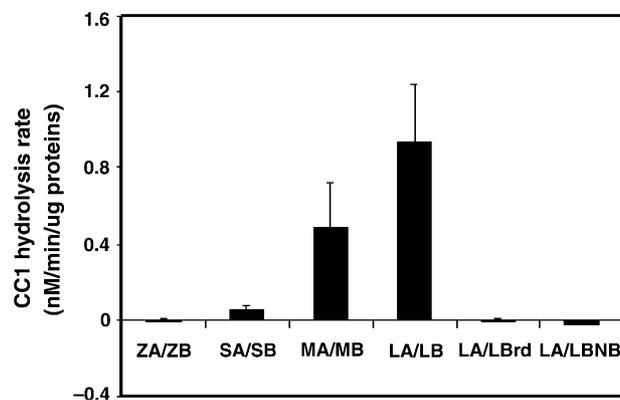


Fig. 3. The enzymatic activity assay of COS-7 cells transiently transfected with indicated constructs. The hydrolysis rates of CC1 were determined from six independent transfections and normalized against total lysate protein contents in nM/min per μ g protein.

tuted MA/MB or LA/LB be translatable and the protein be enzymatically active.

We have demonstrated here that two separated *Tetrahymena* group I intron ribozyme fragments – the substrate fragment including the IGS, and the ribozyme fragment – can be assembled into a functional complex both in vitro and in mammalian cells under the guidance of appended interacting nucleotides. The assembled complex possessed the splicing activity both in vitro and in vivo. This self-assembled *trans*-splicing reaction differs from previously known *trans*-splicing *Tetrahymena* group I intron ribozymes in that the association is not dependent on the IGS [9,13,23].

The observed dependence of the splicing efficiency of the assembled complex on the length of the appended nucleotides has also been reported for *trans*-splicing ribozymes [16,24–26]. Although the binding energy of appended 42 bp in SA/SB would be strong enough for stable complexation, its splicing efficiency was much less than that of MA/MB and LA/LB, suggesting that appended nucleotides may involve more than just aiding the complex association. Further experiments especially structural study would help to fully understand the roles of appended nucleotides during the splicing reaction.

The split site we chose here is located in the middle of the L2 loop, which was reported to be important to the tertiary structure of the active ribozyme [19]. If no additional nucleotides are appended to each fragment at the split site, the splicing activity cannot be reconstituted, and the appending of a certain length of nucleotides led to the restoration of the splicing activity. Therefore, the *Tetrahymena* group I intron ribozyme may be similarly split at other loop sites and reassembled under the guidance of appended interacting nucleotides.

We appended nucleotides to assemble the ribozyme complex in our study, and other interacting groups such as proteins, or small molecules may also be exploited to control the complex formation. Separated domains of proteins can complement together with partially or completely restored activity [27,28]. Based on this phenomenon, several enzyme complementation assays have been developed to monitor protein folding and protein–protein interactions [27–29]. This system may thus be applied to design a similar ribozyme-based assay to detect these RNA-involved interactions in vitro and in cells.

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