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Installation of orthogonality to the interface that assembles two modular domains in the *Tetrahymena* group I ribozyme

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Two modular elements (P5abc and ΔP5) in the *Tetrahymena* group I ribozyme can be separated physically to generate a two-piece ribozyme derivative consisting of a separately prepared P5abc (P5 RNA) and the rest of the intron (ΔP5 RNA). Molecular recognition in the interface assembling P5 RNA and ΔP5 RNA is strong and specific, and the catalytic ability of the two-piece ribozyme is comparable to that of the parent unimolecular ribozyme. We designed alternative P14 (L5c-L2) interacting modules participating in the assembly of P5 and ΔP5 and investigated their ability in the context of complex formation of the two-piece ribozyme and *in vivo* splicing of the unimolecular intron ribozyme. Combined use of alternative P14 and L5b-P6 interacting modules provided robust orthogonality to the P5/ΔP5 assembly interface of the bimolecular complex.

[**Key words:** Ribozyme; RNA; GNRA tetraloop; Tetraloop receptor; Kissing loop]

The formation of complex and defined three-dimensional (3D) structures by RNA molecules leads to elaborate functions involving promotion of chemical transformations (RNA enzymes) (1,2) and recognition of particular molecules (RNA aptamers) (3,4). RNA enzymes and RNA aptamers play crucial roles in the current cellular systems and also made RNA a key macromolecule in the origin of life (5–7). Moreover, nanometer-size 3D objects made of biomacromolecules are also attractive tools for nanobiotechnology (8) because they are promising platforms for multifunctional nanomedicine (9,10) and cellular nanofactory (11).

While generation of fully artificial RNA nanostructures has expanded the scope of RNA functions, naturally occurring 3D RNA structures would also be attractive as structural platforms on which to install new functions. Among a number of RNA molecules with defined 3D structures, the group I intron from *Tetrahymena thermophila* (the *Tetrahymena* group I ribozyme) is an RNA enzyme the structure–function relationship of which has been elucidated in detail (12,13). The modular architecture of the *Tetrahymena* group I ribozyme allows it to be segmented into two or more structural domains (14–17). The large modular P4–P6 domain has been employed as a structural platform from which novel RNA catalysts were generated through installation of catalytic modules by *in vitro* directed evolution (18–20).

P5abc, a subdomain of the P4–P6 domain, also has self-folding ability (21). Removal of P5abc from the full-length *Tetrahymena* ribozyme did not abolish but markedly reduced the ribozyme activity in the presence of 5 mM Mg^{2+} ions (22). The resulting mutant (ΔP5 intron), however, showed marked recovery of its *in vitro* ribozyme activity with the addition of a separately prepared P5abc domain (P5 RNA molecule) (14). This observation indicated that non-covalent assembly between P5abc and ΔP5 is of primary importance for the active 3D-structure of the intact ribozyme.

Reconstitution depends on multiple tertiary interactions between the P5abc domain (P5 RNA) and the rest of the intron (ΔP5 intron). P5 RNA and ΔP5 intron form a highly stable assembly interface (Fig. 1) (23). Modular installation of a pair of peptide recognition motifs to the P5 RNA and ΔP5 RNA allows the redesign of the P5/ΔP5 complex as an artificial RNA complex capable of facilitating the chemical ligation of a particular pair of RNA-binding peptides (24).

In this study, we designed and constructed an orthogonal interface between the P5abc and ΔP5 modules to sort and control their assembly. Such engineering would expand the utility of P5/ΔP5 bimolecular platform for RNA nanotechnology (8) and RNA synthetic biology (25,26). For example, two orthogonal interfaces would allow two P5/ΔP5 intron platforms to perform two tasks in one test tube without undesirable crosstalk. Assembly of the P5/ΔP5 interface is supported by three tertiary interactions (L5b–P6, L5c–L2, and P5a–P4) (27). The P5a–P4 interaction is structurally invariable (18) and alternative L5b–P6 interactions have been exploited (28). Therefore, we primarily focused on generating an alternative L5c–L2 interaction (also called P14 base pairs).

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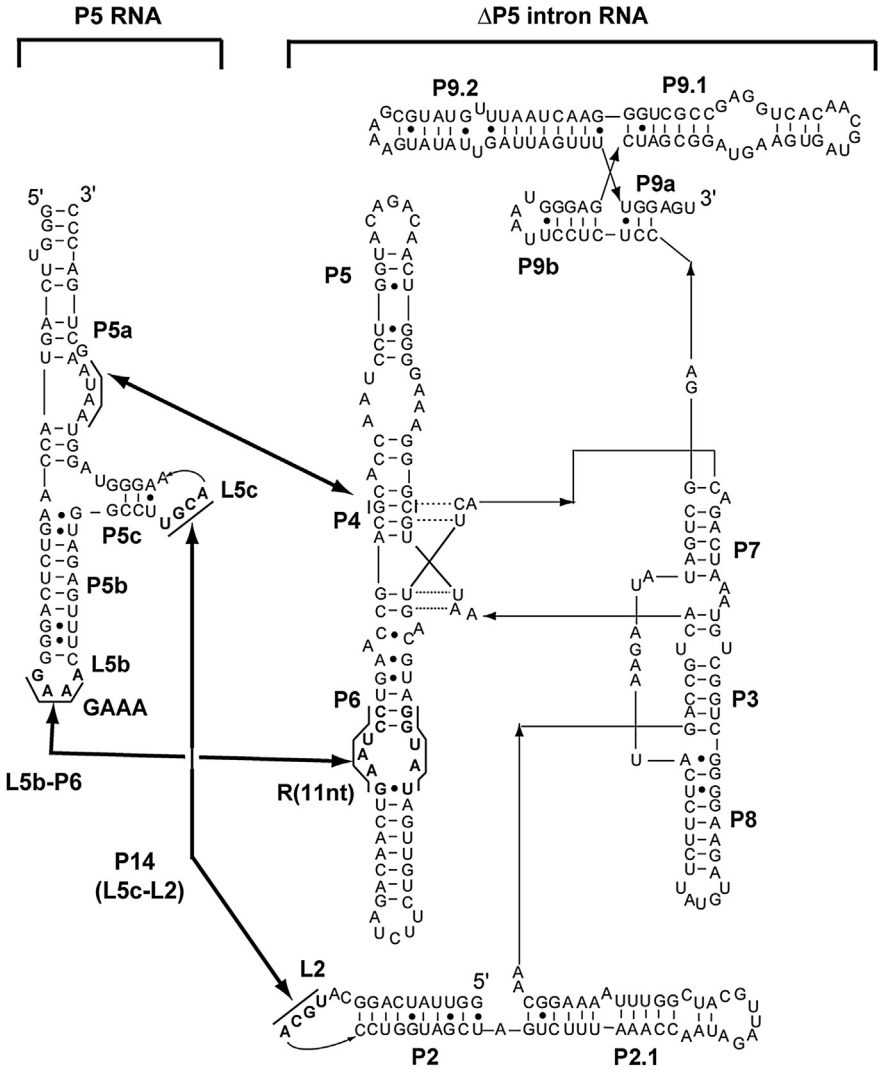

FIG. 1. A secondary structure of a two-piece derivative (P5/ΔP5 complex) of the unimolecular *Tetrahymena* group I intron RNA. The ΔP5 RNA lacks the first 30 and last 5 nucleotides of the parent *Tetrahymena* group I intron. Arrows with two arrowheads indicate the three tertiary interactions assembling the P5abc and ΔP5 elements. Closed circles indicate non-Watson–Crick base pairs.

MATERIALS AND METHODS

Plasmid construction for gel mobility shift assay and *in vivo* splicing assay Plasmids encoding the full-length *Tetrahymena* group I intron (pTZ-IVSU) (29), its mutant lacking the P5abc domain (pTZ-ΔP5) (30), and P5 RNA (pP5abc) (30) were used as PCR templates to prepare DNA fragments of the L30 intron RNA, ΔP5 intron RNA, and P5 RNA, respectively. Plasmids encoding mutants of full-length intron, ΔP5 RNA, and P5 RNA were generated by PCR-based mutagenesis of pTZ-IVSU, pTZ-ΔP5, and pP5abc, respectively. The sequences of the plasmids thus prepared were confirmed using a 4300 DNA analyzer (Li-COR, Lincoln, NE, USA).

Preparation of RNAs Template DNAs for *in vitro* transcription reactions of L30 intron (Fig. S1A), ΔP5 intron, P5 RNA, and their variants were prepared by PCR using appropriate plasmid DNAs as templates. The transcription products were purified by electrophoresis on 8% (P5 RNA) or 6% (L30 intron and ΔP5 intron) polyacrylamide gels (29:1 acrylamide:bisacrylamide) containing 7 M urea. The concentrations of RNAs were determined from the absorption at 260 nm (A_{260}).

3′ end labeling with BODIPY fluorophore The 3′ ends of the parent and variant P5 RNAs and L30 intron were labeled with BODIPY fluorophore (31). After transcription and purification of RNAs, solutions containing RNA, 100 mM NaOAc (pH 5.2), and 3 mM $NaIO_4$ were incubated at room temperature for 1 h in the dark. After recovery of RNAs by ethanol precipitation, solutions containing RNA, 20 mM imidazole, 0.1 mM BODIPY in DMSO, and 10 mM $NaBH_3CN$ were incubated at 37°C for 1 h. RNAs were recovered by ethanol precipitation.

Gel mobility shift assay Aqueous solutions of the BODIPY-labeled P5 RNA (5 pmol, final concentration 0.5 μM) and nonlabeled ΔP5 intron (10 pmol, final concentration 1.0 μM) were heated separately at 80°C for 2.5 min. The two RNA solutions were mixed. To this solution was added a 10× concentrated folding buffer to adjust the mixture to 50 mM Tris–OAc (pH 7.5) and appropriate concentrations of $Mg(OAc)_2$. The resulting mixture was incubated at 37°C for 30 min. After adding 6× concentrated loading buffer consisting of glycerol and xylene cyanol (XC) (0.1%), the samples were loaded onto a 5% nondenaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide) containing 50 mM Tris–OAc (pH 7.5) and appropriate concentrations of $Mg(OAc)_2$. Electrophoresis was carried out at 4°C, 200 V for the initial 5 min and then 75 V for 5 h. The resulting gels were analyzed with a FluoroImager Pharos FX (BioRad, Hercules, CA, USA).

β-Galactosidase assay Plasmid bearing the wild-type intron (pTZ-IVSU) or its mutant was transformed into *Escherichia coli* JM109. Individual colonies were allowed to grow overnight at 37°C with shaking in Terrific Broth (TB) containing ampicillin (50 μg/mL). Cultures were diluted to an optical density at 600 nm (OD_{600}) of ~0.1 and allowed to grow to OD_{600} of ~0.6 before performing β-galactosidase assays. Cell cultures (20 μL) were mixed with 80 μL of permeabilization solution (0.8 mg/mL hexadecyltrimethylammonium bromide, 0.4 mg/mL sodium deoxycholate, 200 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, and 5.4 μL/mL β-mercaptoethanol). The permeabilization mixture was kept for 30 min at 30°C; then 1.9 mL of 30°C prewarmed substrate mixture (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 20 mg/mL hexadecyltrimethylammonium bromide, 10 mg/mL sodium deoxycholate, 1 mg/mL *o*-nitrophenyl-β-D-galactoside, and 2.7 μL/mL β-mercaptoethanol) was added to initiate the reaction. After 2 h at 30°C, reactions were terminated by addition of 1 mL of 1 M Na_2CO_3. Enzyme activities were expressed as the rate change in the absorption at 420 nm (A_{420}) per the absorption at 600 nm (A_{600}) unit of the assay mixture.