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# Designed Structural-Rearrangement of an Active Group I Ribozyme

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**The catalytic core of the *Tetrahyemena* group I ribozyme consists of two functionally different domains, P4–P6 and P3–P7, that are conjugated *via* multiple tertiary interactions. The sequence encoding the P3–P7 domain is divided into two fragments in its primary sequence although the two domains are physically separable in the three dimensional (3D-) structure of the ribozyme: The sequence encoding the P4–P6 domain is inserted into that of the P3–P7 domain. An artificial rearrangement was designed and attempted for the primary sequence of the P3–P7 domain on the basis of a 3D-structural model and the biochemical data on the ribozyme. The domain in the primary structure was relocated to form a contiguous region while retaining the 3D-structure of the ribozyme required for self-splicing. The topologically rearranged ribozyme exhibited self-splicing activity.**

**Key words: group I intron; ribozyme; self-splicing; *Tetrahymena*; topology.**

The proteins and RNAs of enzymes consist of multiple structural units termed "domains" (or "modules") that are assembled *via* multiple tertiary interactions (*1–3*). It has been reported that several enzymes can be reconstituted by the association of their separately prepared domains if the assemblage is appropriate for promoting the catalysis (*4*, *5*). This indicates that a rearrangement of the native primary amino acid or RNA sequence of an enzyme is possible while maintaining catalytic activity. In fact, two classes of such structural rearrangement are known for protein enzymes, and they provide powerful tools for characterizing or modifying the biochemical properties.

One such rearrangement is called "circular permutation", in which a circular form of a protein is generated by linking its termini, followed by a cleavage at a certain site to produce an alternative active "linear-form" (*6–15*). This method has been used widely because it can be easily performed if the termini are located in close proximity. The other method is the designed rearrangement of protein topology so that the positions of the original domains in the primary sequence are rearranged while maintaining the original 3D-structure (*16*). For example, an enzyme composed of oligomeric proteins was successfully converted to a single molecular enzyme.

The circular permutation method is also applicable to ribozymes (*17–24*). The method can improve the folding properties of P RNA, the catalytic component of RNase P (*25*). P RNA consists of catalytic (C-) and substrate binding (S-) domains that fold slowly due to kinetic folding traps. However, "circular permutation" to produce new termini in the junction region linking the two domains can dramatically improve the folding rate because escape from the kinetic traps becomes possible (*25*).

In the present study, we report a new example of a topological rearrangement of another RNA enzyme called group I self-splicing intron (Fig. 1A). The rearrangement was designed on the basis of a 3D-structural model and detailed biochemical study of the ribozyme from *Tetrahymena thermophila*. The primary sequence of the ribozyme was successfully rearranged without sacrificing its self-splicing activity.

## MATERIALS AND METHODS

*Preparation of RNAs*—Plasmids encoding the wild type *Tetrahymena* ribozyme (pTZIVSU), P3-P7 RNA (pTZΔP4-P6IVS), and P4-P6 RNA (pTZP4-P6) were described previously (*26*, *27*). The template plasmid for the P4–P6 fusion RNA (pTZP4P6fusion) was constructed from pTZIVSU by *in vitro* mutagenesis. In this plasmid, an additional sequence containing the P4–P6 domain (U107–U258) was inserted between U258 and U259 of pTZIVSU to set the P4–P6 domain sequence tandemly. Plasmids encoding tv-Tet variants (pTZtv-Tet) were prepared as follows. DNA fragments containing P4–P6 regions were prepared by PCR on pTZP4P6fusion as the template with a set of primers (5′-[A]m-CGAGCCATCT-CAAAGTTTCCCCTGA-3′ and 5′-[T]n-CGAGGTATGGT-AATAAGCTGACGGAC-3′, m = 3, 4, 5 and n = 4, 5). A DNA fragment containing the P3–P7 RNA was prepared by PCR on pTZΔP4-P6IVS with a set of primers (5′-CAC-CTGGTAGCTAGTCTTTAAACCAA-3′ and 5′-TGCCTGA-TAACTTTTCCCTCCAAAGGT-3′). The 5′ ends of the repsective P4–P6 fragment DNAs were phosphorylated and the P4–P6 and P3–P7 fragments were ligated and transformed into *Escherichia. coli* JM109. The plasmids recovered from *E. coli* were verified by dideoxy sequencing.

Except the P4–P6 domain RNA, all template DNAs for *in vitro* transcription were prepared by PCR with appropriate primers. The sense primers consist of the T7 RNA polymerase promoter sequence, followed by the first 24 nucleotides of the respective RNA products. The anti-

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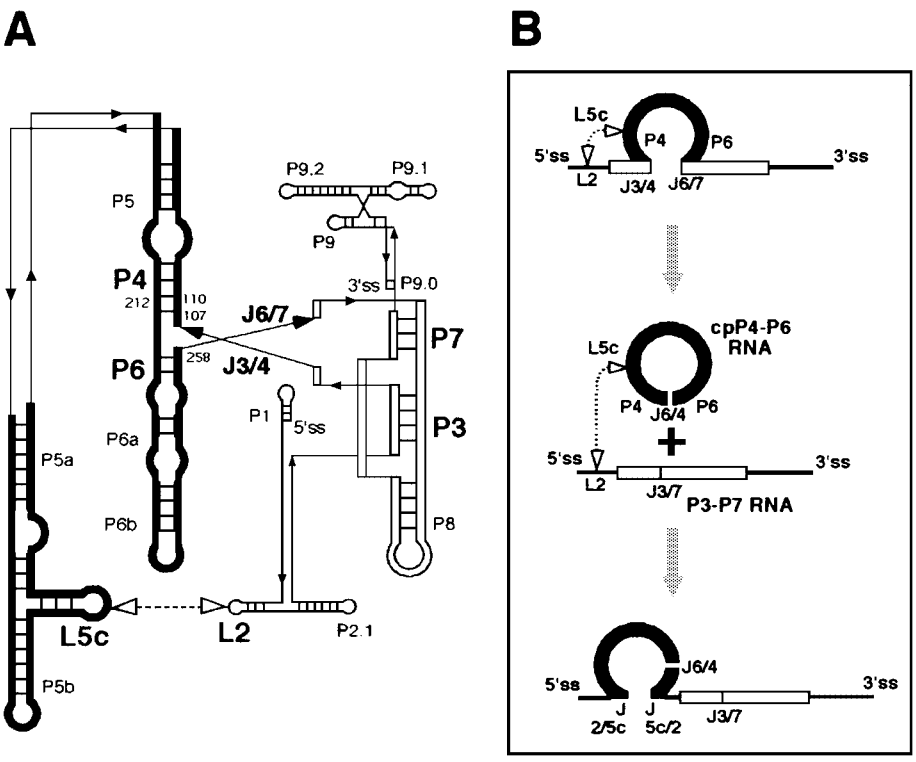


Fig. 1. **Structural features of the *Tetrahymena* group I intron ribozyme**. (A) Secondary structure of the *Tetrahymena* ribozyme (*33*). The P4-P6 and P3-P7 domains are represented by thick black and white lines, respectively. Arrowheads superimposed on the lines indicate 5′ to 3′ polarity. 5′ ss and 3′ ss indicate 5′ and 3′ splice sites, respectively. Long range base-pairings between L2 and L5c regions are represented by a broken line with white arrowheads. (B) Topological rearrangement of the *Tetrahymena* ribozyme. (top) Arrangement of the major domains of the *Tetrahymena* ribozyme in the primary structure. P4–P6 and P3–P7 domains are shown by thick black and white lines, respectively. (middle) Bimolecular ribozyme consisting of the mutant *Tetrahymena* ribozyme lacking the P4–P6 region (P3–P7 RNA) and a circularly permuted form of P4–P6 domain RNA (cpP4–P6 RNA). The linear form of RNA (P3–P7 RNA) and circularly permuted form of RNA (cpP4–P6 RNA) possess non-native junction elements J3/7 and J6/4, respectively. (bottom) Topological mutant of the ribozyme whose P4–P6 and P3–P7 domains are separated physically in the primary sequence. cpP4–P6 RNA with a nick in the L5c region was inserted into the L2 region of P3–P7 RNA via new junction elements J2/5c and J5c/2.

sense primers consist of complementary sequences of the last 24 nucleotides of the respective RNA products. They were employed as templates for *in vitro* transcription with T7 RNA polymerase, and the resulting RNAs were purified on denaturing 8% polyacrylamide gels (*28*). P4–P6 RNA was prepared by autoprocessing its precursor RNA transcribed from the template prepared by PCR as described (*27*).

Precursor RNAs for assaying the 5′ splice site cleavage reaction possess a 5′ exon (32 nucleotides) but lack a 3′ exon and the last five nulceotides of the intron. The tv-Tet [3/4] precursor ribozyme for assaying the self-circularization reaction possesses a 5′ exon (32 nucleotides) but lacks a 3′ exon. For assaying the intact self-splicing reaction the of tv-Tet [3/4] ribozyme, two forms of precursors were prepared that share the 5′ exon (32 nucleotides) but have short (38 nucleotides) and long (125 nucleotides) 3′ exons.

*Assay of Ribozyme Reactions*—The assays of bimolecular ribozyme reactions were performed at 100 mM $MgCl_2$ as described (*27*, *29*). The assays of self-splicing or its first step reaction in topological variant ribozymes without activator RNA were performed identically to those of bimolecular ribozyme with final concentrations of $MgCl_2$ and GTP of 100 and 5 mM for high concentration conditions or 10 and 0.5 mM for low concentration conditions, respectively. The assays of unimolecular self-circularization reactions without GTP were performed identically to those of the self-splicing reaction. Aliquots removed at specified times were treated by adding an equal volume of stop solution (150 mM EDTA, 70% formamide, 0.25% xylenecyanol), followed by electrophoresis in 5% polyacrylamide denaturing gels. The RNAs were quantified with a Bio Imaging Analyzer (BAS2500; Fuji Film, Tokyo).

*RT-PCR for the Detection of Ligated Exons from tv-Tet Ribozyme*—The tv-Tet [3/4] precursor ribozyme with a long 3′ exon (0.1 pmol) was incubated with 100 mM $MgCl_2$ and/or 5 mM GTP at 37°C for 13 h. The reaction mixtures were subjected to ethanol precipitation, and the precipitates were dissolved in 40 μl of distilled water. Ten microliters of dissolved precipitates were used for reverse transcription with primer A18 (5′-GCCAGCTGGCGAAAGGGGGA-3′) complementary to the last 20 nucleotides of the 3′ exon. The 10% or 1% volume reverse transcription mixtures were subjected to 25 cycles of PCR with primers A18 and AW1 (5′-TATAGGGAATTCGAGCTCGGGTAA-3′) complementary to the first 20 nucleotides of the 5′ exon. The resulting mixtures were electrophoresed in a 2% agarose gel using TAE buffer. The PCR product corresponding to the ligated exons was cloned into pUC18 vector, and subjected to DNA sequencing. The plasmid pTZtv-Tet[3/4] and its derivative encoding only the ligated exons were employed as templates for control PCR. PCR with primers A18 and AW1 produced DNA fragments identical to RT-PCR from the precursor RNA and ligated exons.

## RESULTS AND DISCUSSION

*Design of the Structural Rearrangement*—We attempted to convert the primary sequence of the *Tetrahymena* ribozyme to an alternative form without sacrificing its catalytic activity. The outline of the design consists of the dissection of the ribozyme into two pieces containing

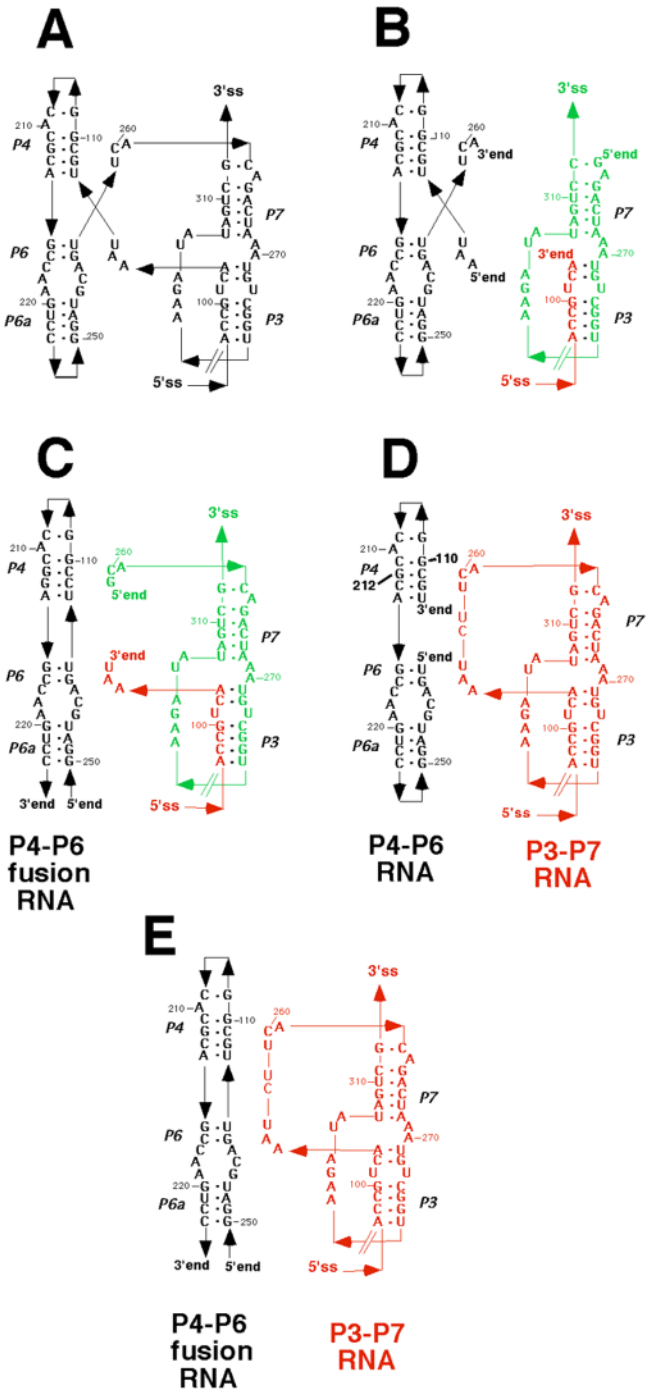


Fig. 2. **Structural features of the region joining the P4–P6 and P3–P7 domains of the *Tetrahymena* ribozyme and its derivatives.** In B–F, green and red sequences do not belong to the same molecule. (A) The secondary structure of the P4–P6 and P3–P7 domains (B), (C) Three piece ribozymes by Doudna and Cech (*29*). (D) A two-piece ribozyme by Ikawa *et al*. (*27*). (E) A newly designed bimolecular ribozyme without a nick at the core region where the P4–P6 and P3–P7 domains are assembled.

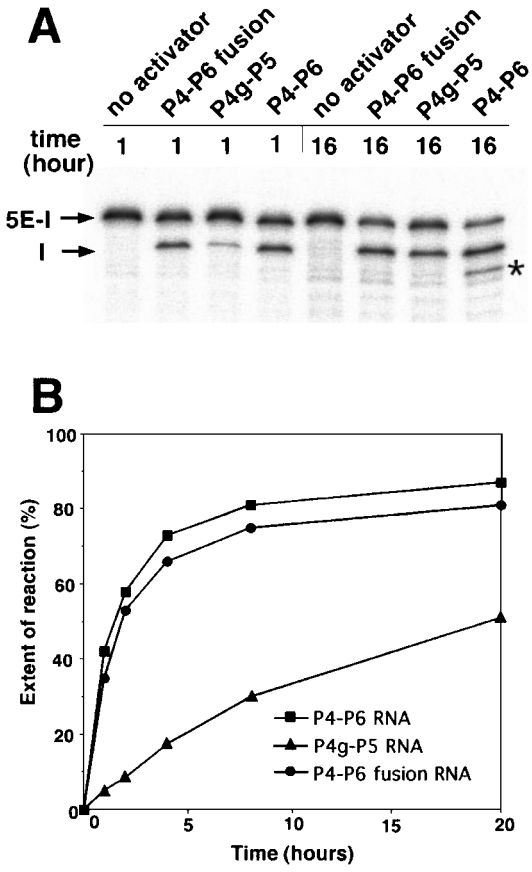


Fig. 3. **The 5′ splice site cleavage reaction of P3–P7 RNA with activator RNAs.** (A) and (B) are an autoradiogram and time courses of the reaction, respectively. P4–P6 and P4g–P5 activator RNAs consist of U107–U258 and G110–G212 of the *Tetrahymena* ribozyme, respectively. The P4–P6 fusion activator RNA is a circularly permuted form of P4–P6 RNA in which U107 and U258 are joined covalently by a 3′-5′ phosphodiester bond and that has a break at L6b region. 5E and I represent the 5′ exon and intron, respectively. The asterisk corresponds to a product presumably resulting from cleavage at the internal processing site (*29*).

either P4–P6 or P3–P7 and the connection of the resulting pieces at a newly designed site as presented schematically in Fig. 1B; The P4–P6 and P3–P7 regions are the universally conserved elements composing the catalytic core of group I ribozymes (Fig. 1A). The two regions are separated in the crystal structure [(*30*), see also Fig. 1A: the secondary structure reflecting the corresponding 3D-form] although they are connected via single stranded regions with a complex topology (Fig. 2A); P4–P6 is inserted in the P3–P7 region in the primary sequence (Fig. 1B-top). We first attempted to see whether the ribozyme could tolerate dissection into the two regions.

Previously, three ways of dissecting the core region designed to cause a minimal structural perturbation to the 3D-structure were attempted (Figs. 2, B–D)(*27*, *29*). Unfortunately, these dissections are inapplicable for the present study because the primary sequences of the P4–P6 and P3–P7 regions must not be fragmented in the core region to produce the alternative form of the ribozyme (Fig. 1B, bottom). To overcome this problem, a new method of dissection (Fig. 2E) was planned on the basis of the previously reported type C and D dissections. The newly designed P3–P7 and P4–P6 subunit RNAs were examined to see whether they exhibit the catalytic capacity of a bimolecular ribozyme *in vitro*.

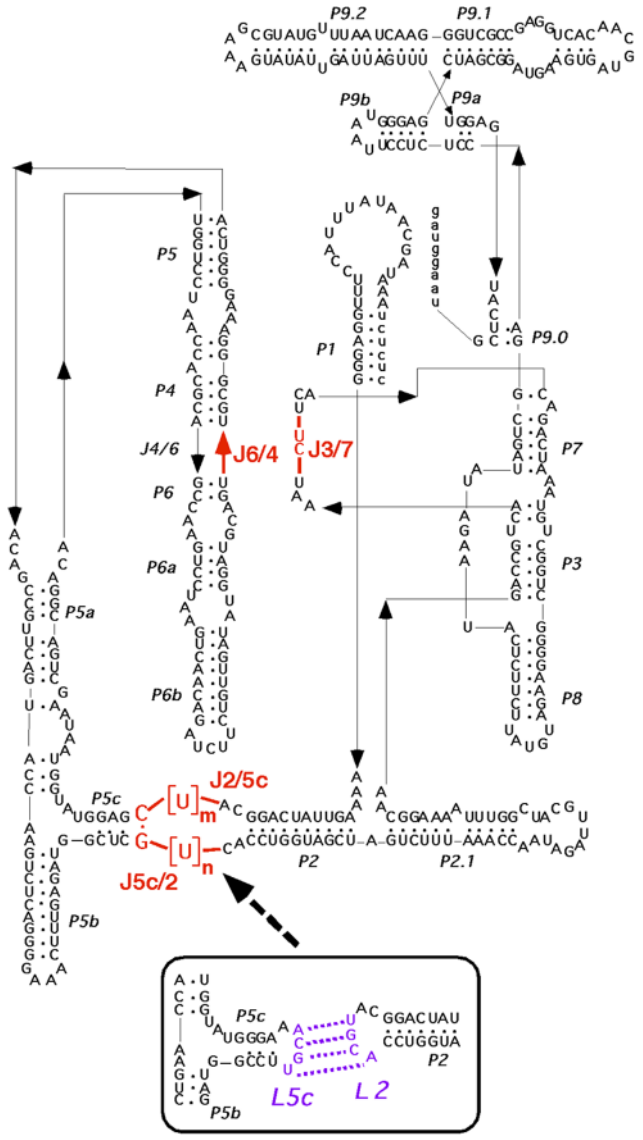


Fig. 4. **Secondary structure of the topological variant of the *Tetrahymena* ribozyme.** $[U]_m$ and $[U]_n$ indicate numbers of uridines consisting of newly generated junction elements J2/5c and J5c/2, respectively. Original base-pairs between L2 and L5c regions are shown in the inset.

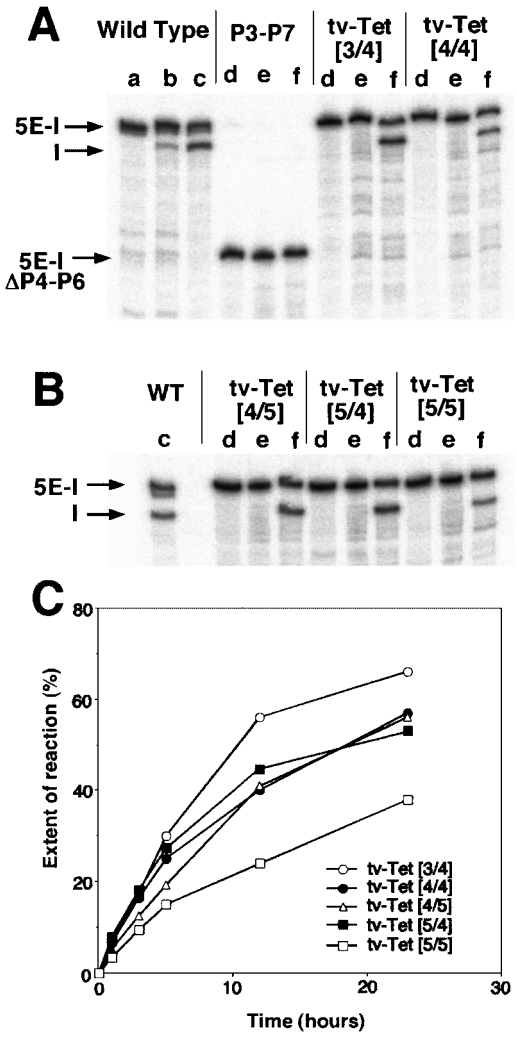


Fig. 5. **Assay of the 5′ splice site cleavage activity of the topological variants of the *Tetrahymena* ribozyme.** (A) and (B) are autoradiograms of the 5′ splice site cleavage reactions of the wild type, P3-P7 RNA and the topological variants. 5E and I represent the 5′ exon and intron, respectively. The reactions were performed at 37°C (a) without $MgCl_2$ and GTP for 1 h, (b) with 10 mM $MgCl_2$, 0.5 mM GTP for 1 h, (c) with 100 mM $MgCl_2$, 5 mM GTP for 1 h, (d) without $MgCl_2$ and GTP for 13 h, (e) with 10 mM $MgCl_2$, 0.5 mM GTP for 13 h and (f) with 100 mM $MgCl_2$, 5 mM GTP for 13 h. (C) Time courses of the reactions of the variants under the conditions in (f).

The P3–P7 subunit was inactive by itself but an active ribozyme was formed in the presence of the previously designed P4–P6 RNA (Fig. 2D) or its P4g–P5 fragment [positions G110–G212, see Figs. 1 and 2D (*27*)]. The new bimolecular system consisting of the P3–P7 RNA and the P4-6 fusion RNA (Fig. 2E) was subjected to a guanosine-dependent 5′ splice-site cleavage reaction by employing the P3–P7 subunit together with either the P4–P6 RNA or its P4g–P5 fragment. The cleavage reactions corresponding to the first step of the self-splicing reactions were performed using a shortened form of precursor P3–P7 RNA possessing a 5′ exon but lacking the 3′ splice site (Fig. 3A). The new complex was distinctly more active than that comprising P3–P7 and P4g–P5, but slightly less active than that comprising P3–P7 and P4–P6 (Fig. 3B).

*Construction of an Alternative form of Ribozyme by Alternating the Sequence Topology*—The two RNAs were connected covalently to produce an alternative linear form while maintaining the catalytic activity (Fig. 1B, bottom). As connection sites, L2 of the P3–P7 RNA and L5c of the P4–P6 fusion RNA were chosen because the two loops are located very close to each other in the 3D-structural model of the wild type ribozyme (*31*). They form four Watson-Crick base-pairs (small inset in Fig. 4) that contribute to the activation of the wild type ribozyme (*31*), and are essential for constituting a bimolecular ribozyme consisting of the P3–P7 and P4g–P5 RNA (*27*).

To produce the topological variant of the *Tetrahymena* ribozyme (tv-Tet ribozyme), we designed breaks in the

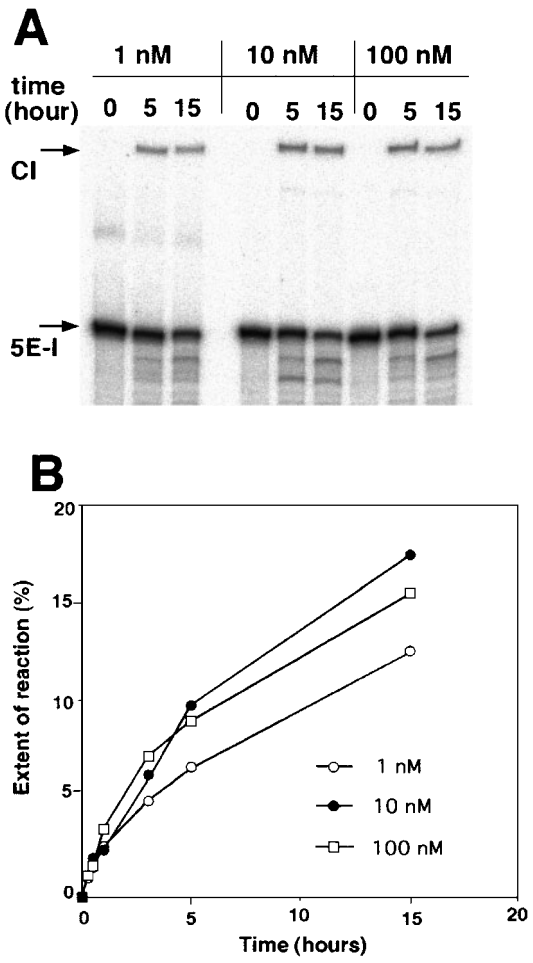


Fig. 6. **Self-circularization reaction of the tv-Tet[3/4] variant at different RNA concentrations.** An autoradiogram (A) and time courses (B) of the reactions at 1, 10 and 100 nM of tv-Tet[3/4] with 100 mM $MgCl_2$ at 37°C. 5E and I, and C-I correspond to 5′ exon, intron and circular form of intron, respectively.

two loop regions of the RNAs and joined them to form new regions termed J2/5c and J5c/2 as shown in Figs. 1B and 4. The lengths of J2/5c and J5c/2 were optimized in the newly created RNA, termed the tv-Tet ribozyme because the crystal structure of the native L2 X L5c base-pairs has not been determined. Five tv-Tet derivatives different J2/5c and/or J5c/2 sequence(s) were constructed and their activities were investigated (Fig. 4).

Shortened forms of the tv-Tet ribozymes lacking the 3′ splice site were prepared and subjected to the guanosine-dependent 5′ splice-site cleavage reaction. The tv-Tet ribozymes were inactive under low $Mg^{2+}$ ion conditions but active under high $Mg^{2+}$ ion conditions, although the degree of activity of each ribozyme varied (Fig. 5). Among the five tv-Tet ribozymes, the most active [3/4] variant, which possesses three or four uridines, J2/5c and J5c/2, respectively (Fig. 4), was employed for subsequent analysis.

The ribozyme reaction was performed by varying the RNA concentration to see whether catalysis proceeded in a unimolecular or bi- (or multi-) molecular manner (Fig. 6). To avoid unnecessary effects due to the concentration of the exogenously added guanosine cofactor, a self-circularization reaction was employed. For circularization, the guanosine-dependent 5′ splice-site cleavage reaction is performed by employing the endogenous guanosine at the 3′ end of the intron (*32*). Under three different RNA concentrations, the tv-Tet [3/4] ribozyme performed self-circularization at three different RNA concentrations (Fig. 6A) with efficacies at middle (10 nM) and high RNA (100 nM) concentrations slightly higher than that at low RNA concentration (1 nM). The improvement in the efficacy (1.4–1.5-fold) was small compared with the increase in the RNA concentration (100-fold) (Fig. 6B). The tv-Tet [3/4] ribozyme exhibited considerable activity at 1 nM, a concentration distinctively lower than the previously reported dissociation constant between P3–P7 RNA and P4–P6 activator RNA (88 nM) (*27*). Thus it can be concluded that at least the majority of tv-Tet [3/4] ribozyme RNAs catalyze the reaction unimolecularly.

*Topological Mutant Can Perform Intact Self-Splicing Reaction*—Two precursor tv-Tet [3/4] RNAs possessing a short and long 3′ exon were prepared and characterized to see whether the tv-Tet [3/4] ribozyme can perform the intact self-splicing reaction. The data indicated that the tv-Tet [3/4] can yield an excised intron (Fig. 7A). Bands presumed to correspond to two forms of the ligated exons were also observed (bands with dots and asterisks in Fig. 7B).

To confirm the ligated exons, we performed RT-PCR using primers for the amplifications of the precursor RNA and the ligated-exons (Fig. 7C). The RT-PCR fragment corresponding to the ligated exons was amplified as well as the fragment corresponding to the precursor RNA when the reaction mixture was employed directly as a source of the templates. PCR products corresponding to the ligated exons were not observed if control mixtures without GTP or $Mg^{2+}$ were used, indicating that the production of the ligated exons depends on GTP and $Mg^{2+}$ ions. The putative ligated exons were isolated and their sequences were determined by RT-PCR followed by dideoxy sequencing (data not shown). Thus it is concluded that the topological variant *Tetrahymena* intron, tv-Tet [3/4], can perform the intact self-splicing reaction.

## CONCLUSION

A topological mutant ribozyme was designed and constructed without sacrificing its self-splicing activity on the basis of the 3D-structure of the *Tetrahymena* ribozyme and the its biochemical parameters. The construction of a bimolecular ribozyme consisting of two designed modular domains was performed, followed by the assemblage of the two domains via two new single-stranded RNAs to produce an alternative unimolecular ribozyme. This class of topological variants should serve as a useful tool for fully elucidating the highly complicated structure-function relationships of large ribozymes.

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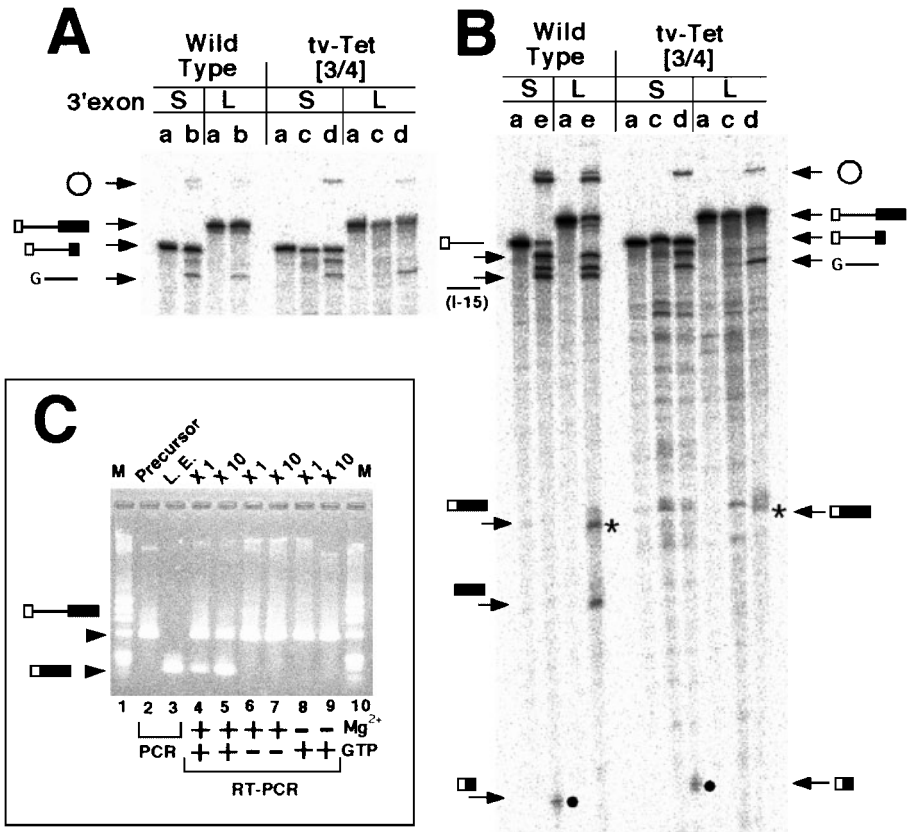


Fig. 7. **Self-splicing reaction by the tv-Tet[3/4] variant.** (A) An autoradiogram of the reactions of the precursor form of the wild type ribozyme and tv-Tet[3/4] with 30 minutes exposure. (B) An autoradiogram of the reactions of the precursor form of the wild type ribozyme and tv-Tet[3/4] with 6 h exposure. S and L represent short (38 nucleotides) and long (125 nucleotides) 3′ exons of the precursor, respectively. Thick white or black lines indicate the 5′ and 3′ exons, respectively. The thin line indicates the linear intron. G indicates a cofactor GTP attached at the 5′ end of the linear intron. The circle indicates the circular intron. Reaction conditions; a, with no $MgCl_2$, 5 mM GTP at 37°C for 12 h; b, with 100 mM $MgCl_2$, 5 mM GTP at 50°C for 15 min; c, with 100 mM $MgCl_2$, no GTP at 37°C for 12 h; d, with 100 mM $MgCl_2$, 5 mM GTP at 37°C for 12 h; and e, with 100 mM $MgCl_2$, 5 mM GTP at 37°C for 45 min. (C) The ligated exons of the self-splicing reaction by tv-Tet[3/4]. Lanes 1 and 10, size markers (λ phage DNA digested with *Hin*dIII); lane 2, PCR from the plasmid encoding tv-Tet[3/4] precursor; lane 3, PCR of the plasmid encoding the ligated exons; lanes 4 and 5, RT-PCR of the reaction products with GTP and $MgCl_2$, lanes 6 and 7, RT-PCR of the reaction products without GTP; lanes 8 and 9, RT-PCR of the reaction products without $MgCl_2$. In lanes 4, 6, and 8, the RT products used for PCR were one-tenth of those in lanes 5, 7, and 9, respectively.

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