Requirements for alternative forms of the activator domain, P5abc, in the *Tetrahymena* ribozyme

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Abstract The role of P5abc domain of the *Tetrahymena* LSU self-splicing Group I intron is to enhance the activity of the intron via tertiary interactions involving A-rich bulge and terminal loops L5b and L5c. We constructed and examined alternative forms of the domain that accelerate the ribozymatic reaction. The results indicate that the characteristic structure of P5c subdomain plays an important role by forming L2×L5c interaction (P14) and that the region flanking P5c subdomain can be significantly mutable without much affecting the activity of the ribozyme.

Key words: Ribozyme; P5abc; Tertiary interaction; *Tetrahymena*

1. Introduction

Large functional RNA molecules form complex three-dimensional structures which involve many tertiary interactions. Although the nucleotides directly involved in the interactions have been identified in many RNA molecules, little is known about the structural requirements for the regions adjacent to them [1–14]. We investigated one of these regions in the *Tetrahymena* ribozyme by employing its P5abc domain.

The *Tetrahymena* LSU intron is a ribozyme that exhibits efficient self-splicing activity in the absence of proteins in vitro [15,16]. This intron consists of a conserved core structure that is responsible for the catalytic activity and several less conserved peripheral domains [17–19]. One of the peripheral domains termed P5abc has been shown to function as an activator domain that enhances the activity of the ribozyme [20]. In P5abc, three single-stranded regions, the 'A-rich bulge' and loops L5b and L5c, which participate in tertiary interactions, have been shown to be responsible for enhancing the catalytic activity [4,7,8,21–23]. The three interactions contribute independently for the activation by forming long-range interactions [24].

In the present study, we randomized P5bc domain and selected for active variants. Almost all the selected variants retained an L5c-like sequence complementary to the L2 region of the intron whereas the distance (as measured in the number of nucleotides) from the P5a segment to the L5c-like region showed a significant level of variance. Subsequent mutagenesis of these sequences and on the distance demonstrated that the complementarity is required for the activation and that in some cases the activation potential of the domain could be retained even if a full helical turn was added (or deleted), respectively.

2. Materials and methods

2.1. In vitro selection of activator domains that replace P5b and P5c domains

The upper-half RNA for the bimolecular ribozyme employed in the selection was prepared as follows. Oligonucleotides P1 (A38-C138) and P2 (complementary to C204-A113) that have 28 nt of completely randomized bases instead of P5b and P5c (41 nt) were hybridized and converted into double-stranded DNA by primer extension using Ex-Taq (Takara). The resulting DNA was purified by electrophoresis on polyacrylamide gels containing 8.3 M urea. Using this DNA (1 pmol) as a template, a PCR amplification with two oligonucleotides P3 (containing promoter for T7 RNA polymerase, 5′-exon and A2–A66) and P4 (complementary to T239-G180) was carried out. The starting pool of the upper-half RNA (0R RNA) was synthesized by transcription with T7 RNA polymerase in the presence of [α-^{32}P]ATP. The lower-half RNA was prepared as reported previously [25].

The upper-half RNAs with randomized sequence (10 pmol) and the lower-half RNA (20 pmol) were incubated in 20 μl of reaction buffer (5 mM $MgCl_2$, 50 mM Tris–Cl (pH 7.5), 200 mM NH_4OAc) at 30°C for 5 min. The ligated RNAs were purified by gel and converted to cDNA by using reverse transcriptase, Superscript II (GIBCO BRL) with oligonucleotide D8 [25] as a primer. This cDNA was used as a template for PCR reaction with oligonucleotides D6 [25] and D8 to prepare DNA template for upper-half RNA used in the next round (1R RNA) of selection.

After five rounds of selection cycle, 30 clones were isolated, cloned into plasmid pTZIVSU (p#1–p#30) and sequenced. To exclude the mutations outside of the randomized region that were presumably due to the PCR amplification, the selected clones were subcloned to remove these mutations.

2.2. Construction of mutant of selected clones

p#9CG and p#11CG that have substitutional mutation in their L5c-like sequence and p#9Δ, p#10Δ, p#11Δ1, p#11Δ2, p#11Δ3, p#11+11 bp, p#16+11 bp and p#26Δ which are altered in the regions adjacent to the L5c-like sequence were constructed from p#9–p#26 by using PCR [26].

2.3. Preparation of RNA

The DNAs coding upper-half RNA were amplified by PCR in which the plasmids of selected clones digested with *Hin*dIII were used as templates. The templates of circularly permuted introns used for transcription were prepared through PCR as reported previously [24].

The DNAs were transcribed with T7 RNA polymerase in the presence of [α-^{32}P]ATP and the resulting RNAs were purified by electrophoresis on 5% polyacrylamide gels containing 8.3 M urea.

2.4. Assay of ribozymatic activity

Bimolecular ligation reaction was carried out as follows. The uniformly labeled upper-half RNA (2.5 pmol) and the unlabeled lower-half RNA (5 pmol) were incubated in 4 μl of water and incubated at 70°C for 10 min and 30°C for 10 min. After 1 μl of 5× reaction buffer (50 mM $MgCl_2$, 250 mM Tris–HCl (pH 7.5), 1 M NH_4OAc) was added, the mixture was incubated at 30°C for 60 min. The splicing assay was performed in 5 μl of the mixture containing uniformly

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labeled precursor RNA, 4 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 2 mM GTP at 30°C for 30 min. Hydrolysis reactions of circularly permuted introns were performed in 10 μl of the reaction buffer containing 50 mM Tris–HCl (pH 8.5) and 5 mM $MgCl_2$ at 37°C for 60 min.

The reactions were quenched by adding equal volume of stop solution (50 mM EDTA, 90% formamide and 0.25% xylene cyanol). The reaction products were loaded onto 5% polyacrylamide gels containing 8.3 M urea. The gels were quantitated by using a Bio-Image Analyzer BAS-2000 (Fuji Film).

3. Results

3.1. The activator domains obtained from in vitro selection experiments contain nucleotides that can form P5c subdomain

To investigate the mechanism of activation of the Group I intron ribozyme by a peripheral domain, we attempted to select alternative forms of P5abc of the *Tetrahymena* ribozyme by in vitro selection. For the selection, P5b and P5c domains were replaced with random sequences while the highly conserved A-rich bulge was maintained.

We employed the bimolecular ribozyme consisting of an upper-half RNA (containing 5′-exon and an intron fragment from A2 to U239) and a lower-half RNA (containing intron fragment from G234 to G414) [25] (Fig. 1). P5b and P5c (41 nt) in the upper-half RNA were replaced by completely randomized sequences (28 nt). By incubating the upper-half RNA (25 pmol) and the lower-half RNA (50 pmol) in the reaction buffer containing 5 mM $MgCl_2$, the two RNAs should be ligated to yield a longer RNA that can be isolated by gel electrophoresis if the active ribozyme was reconstituted. We performed five rounds of selection. The ligation activity of the pool of upper-half RNA (5R RNA) reached to approximately 50% of that of wild-type (Fig. 2).

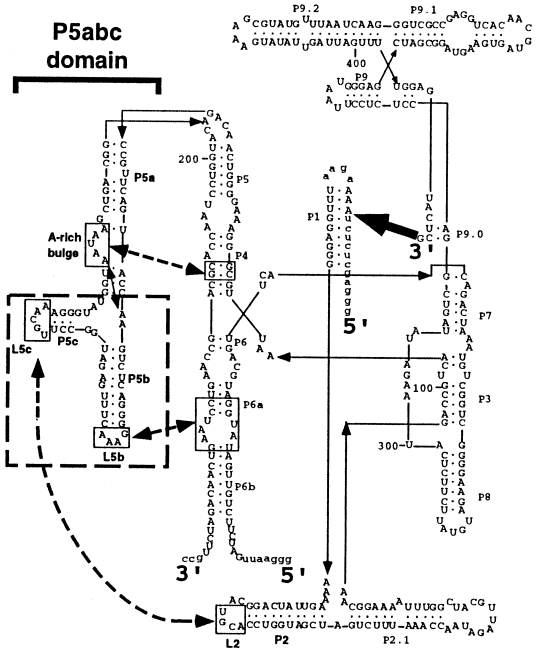

Fig. 1. The bimolecular system derived from the Group I intron of *Tetrahymena thermophila* LSU rRNA used in the selection experiment. A box with broken line indicates the P5b and P5c domains that are replaced with a completely randomized sequence consisting of 28 nucleotides in the selection. Arrows with broken lines indicate the long-range interactions of A-rich bulge×P4, L5b×J6a/6b and L5c×L2. The sequences of L1, 5′-exon, and L6 are altered as described in reference (shown in lower case) [25].

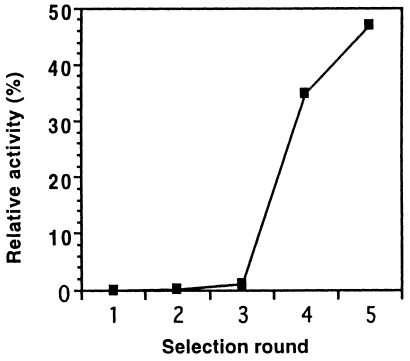

Fig. 2. The ligation activity of the pool at each round of the selection. The relative activity of the pools was quantitated by using that of the ligation system consisting of two halves of the wild-type ribozyme as the standard.

Twenty-three clones from the pool were isolated and sequenced (Table 1). The ligation activities of 15 independent clones were examined in the presence of 10 mM of $MgCl_2$ (Fig. 3). Clone #8 and clone #26 exhibited the highest activity that is comparable to that of the wild-type and the lowest activity that is 56% of that of the wild-type, respectively. Because all selected clones are more active than ΔP5bc which is a mutant lacking both P5b and P5c, the newly obtained domains of the clones can be regarded as activator domains.

The splicing activities of some clones were also measured in the presence of 4 mM of $MgCl_2$. The highest activity was observed for clone #8 (Fig. 4). The activities of the clones exceeded that of ΔP5bc, demonstrating that the new domains also function as activators in self-splicing reaction.

A sequence UGCA was identified in 16 clones out of 23 clones. Interestingly, the sequence UGCA in L5c has been reported to base-pair with a sequence UGCA in L2 in the

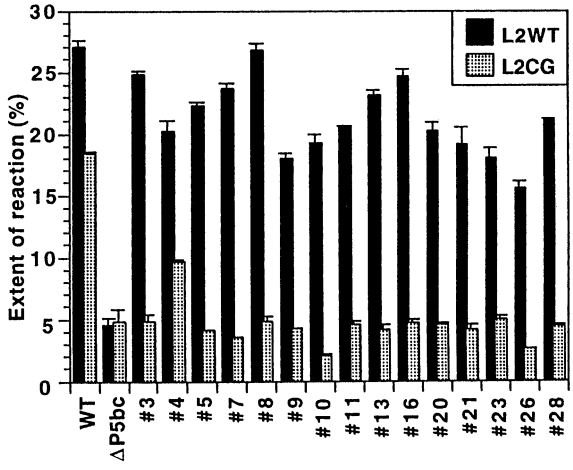

Fig. 3. Bimolecular ligation activities of several selected clones (10 mM $MgCl_2$). The activities of clones carrying a wild-type L2 sequence are shown as black bars and the activities of the clones carrying a G44C45 to C44G45 substitution in L2 are shown as shaded bars. Note: the same changes apply to legend of Fig. 5.

Table 1
Primary sequences of the randomized region in the selected clones

clone No.	Sequence
	----------1----234567----------
WT	----------GGCCU----UGCAAAGGGUAU---------
#11	---------CAACGAGGCU----UGCAAAGCGCAUGUUAAA---
#16	AGCCCACGUGGUGAACUCU----UGCAAAGAG----------
#22	--------ACACGUGUGCU----UGCAAAGACCCACUC------
#23	----------AGAAAGGUAUUAUGCAAACCUACUAAAU-----
#24	-ACCAUGCUAGUAUUCACG----UGCAAAGUGA----------
#8	----------AAUGAUGUA----UGCAAGGCAUCGAUUACAU--
#10	-----CACCCCCAGCCCGG----UGCAAGCGGGUCGU-------
#3	-----UAUGUUUUGCAGCU----UGCAAUGCUGGAA--------
#7*	--------UGUACAACACG----UGUAAGGUGCUG---------
#30	-----GGCGUUACGCGGCA----UGUAAUGCUGGA---------
#2	---AGCGUAACGUGCCCC----UGUAAUGGGUCG---------
#18	--AACUAACCACGUAGGGC----CGCACACCCAG----------
#20	---AUCCAUCUGAGGCGCA----UGCACAGCGCGU----------
#13	-UGAAGCAGACACAGGCCC----UGCACUGGCC-----------
#15	--------GGAAGAGGCCG----UGCAGGGGUCGAGC------
#9	----------ACACGGUGACGU-UGCAGGCACCUC---------
#26	----------CCAGUGCCUAGCCUGCAGAGGCCAUGC-------
#4	--AGCUACAAUGCAGCGCC----UGUGAAGUGCC----------
#6	----------AGUAGCAGC----UGCCAACUGCAAGCCCAAU--
#28	------------GGCGACUA---UGCUACGUCUCUAAGGAAGC-
#5	AAACGCCAUGCUCAGCUACUCACUAU
#19	CAUGUACGCGUGCGUAGGUCCC
#21	ACGCAGCGCAGGCGGUGCAAAGAGUAA

*p#1, p#7, p#12, p#14, p#17, p#25, p#27 and p#29 share the same sequence in the randomized region. Potential base-pairs flanking L5c-like loop are underlined. The numbering in P5c subdomain same as that in Fig. 5 is shown on the top of the sequences.

wild-type intron [8]. As shown in Table 1, the nucleotides surrounding the L5c-like sequence in the 20 of the selected clones appear to share a structure called P5c subdomain [4] (Fig. 5). This subdomain consists of the five structural elements which are identified in the crystal structure of the RNA consisting of P4–P6 domain [22].

The nucleotides in the selected mutants corresponding to the five elements in the P5c subdomain can be assigned as

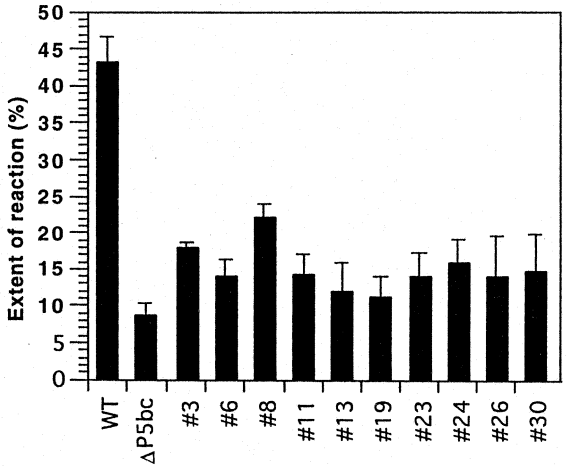

Fig. 4. Self-splicing activities of selected clones (4 mM $MgCl_2$). The relative activities of selected clones are shown.

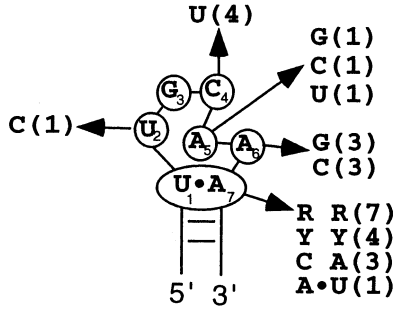

Fig. 5. The P5c subdomain in the selected clones. The nucleotides in 20 clones that form the P5c subdomain structure are indicated and newly numbered (U1 in this figure corresponds to U167 in the ordinary numbering of the *Tetrahymena* intron).

follows; the numberings of the corresponding nucleotides are in Fig. 5. For most of the selected clones: (1) a conserved flipped-out uracil, U2, which is found in the original P5c, is found; (2) G3 and C4 (U4 in four clones) are conserved that have been shown to base-pair with L2 in the original P5c [8]; (3) A5 and A6 which are highly conserved can form a side by side configuration called A-A platform of the original P5c [27]; another sequence, AC, required for forming the platform [28] is also identified; (4) U1 and A7 correspond to a non-Watson-Crick base-pair, A-U, flanking the loop in the original P5c; accordingly the nucleotides other than U1 and A7 in the selected mutants do not form Watson-Crick base-pairings; and (5) two or more of Watson-Crick base-pairs flanking U1-A7 exist as in the case of the P5c subdomain.

To see whether L2, a possible partner of the L5c-like sequence, is involved in the activation in the selected clones, a substitution from GC to CG was introduced at L2 of 15 clones and the activities of the resulting mutants were examined (Fig. 3). The mutation decreased the activity of the wild-type ribozyme but that of ΔP5bc was uninfluenced. In the mutant clones except clone #4, the mutation decreased the activities to the level comparable to or lower than that of ΔP5bc, suggesting that the L5c-like sequence of the clones interacts with L2.

To obtain more direct evidence that the L5c-like sequence interacts with L2, compensatory mutation experiments analogous to that performed by Lehnert et al. in the wild-type intron were attempted by employing clone #9. Either substi-

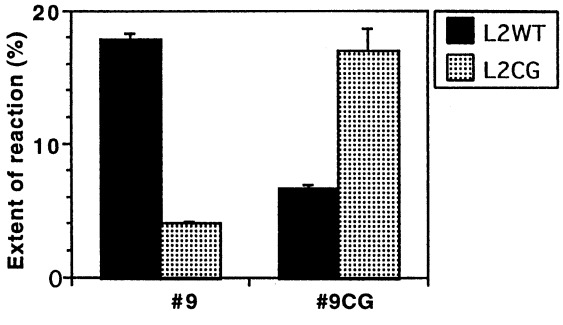

Fig. 6. Restoration of base-pairings between L5c-like sequence and L2 in #9 restores the activity (10 mM $MgCl_2$). #9CG has base substitution (GC to CG) in L5c-like sequence. The activities of mutants that have original sequence in L2 are shown in black bars and that have substitutional mutation (G44C45 to CG) in L2 are shown in shaded bars.

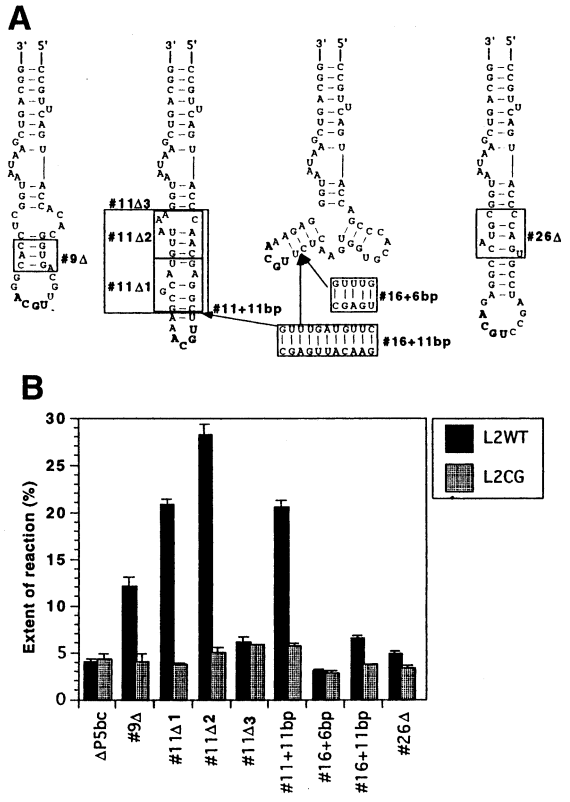

Fig. 7. A: Predicted secondary structure of #9Δ, #11Δ1, #11Δ2, #11Δ3, #26Δ, #16+6 bp and #16+11 bp. The L5c-like sequences are shown in bold type. The deleted or inserted sequences are boxed. B: The bimolecular ligation activities of mutants of selected clones (10 mM $MgCl_2$).

tution of GC to CG at either L2 (#9/L2CG) or the L5c-like loop (#9CG) decreased the activity of clone #9 to a level comparable to that of ΔP5bc (Fig. 6). However, when two mutations were introduced simultaneously (#9CG/L2CG), the activity of the mutant was recovered, demonstrating that the L5c-like sequence in #9 interacts with L2 as in the case of the wild-type.

3.2. Alteration of the region between P5c subdomain and P5a of the selected mutants

The L5c-like sequence is conceivably responsible for activating the selected clones by interacting with L2. However, both the primary sequences and the predicted secondary structures of the region between P5c subdomain and P5a are highly variable in the corresponding clones (data not shown). These results suggest that this region can be altered structurally without much affecting the activity of the ribozyme. This possibility was tested by examining activities of mutants derived from several selected clones in which the region between P5c subdomain and P5a is altered.

Three deletion mutants were prepared from clone #11 (#11Δ1, #11Δ2 and #11Δ3) (Fig. 7A). The mutants of clones #9, #10 and #26 were also constructed by deleting the corresponding stem regions (#9Δ, #10Δ and #26Δ). Two more mutants of clones #11 and #16 were constructed by inserting 11 bp stem derived from rRNA into their stems (#11+11 bp and #16+11 bp).

The mutants #11Δ1, #11Δ2 and #11+11 bp showed activities comparable to that of clone #11. The mutant #9Δ exhibited 50% of the activity of the wild-type, which is much higher than that of ΔP5bc (20% of wild-type) (Fig. 7B). The activities of the three mutants decreased by the substitution of GC to CG in L2, indicating the existence of the interaction

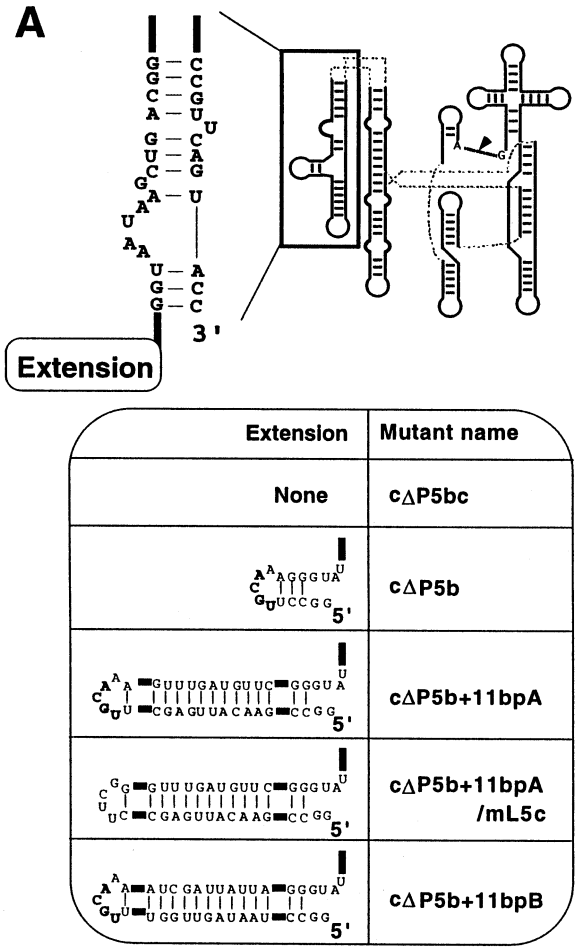

Fig. 8. A: The secondary structures corresponding to P5b and P5c regions of mutants of circularly permuted intron are presented. The corresponding sequences for the mutants in a box at 5′-end of ΔP5bc are shown in the table. The inserted base-pairs are indicated for the mutants cΔP5b+11 bp A, cΔP5b+11 bp B and cΔP5b+11 bp A/mL5c. B: Hydrolysis activities of circularly permuted introns (5 mM $MgCl_2$).

between L5c-like sequence and L2. Thus, it appears that the region between P5c subdomain and P5a can be altered without causing much effect on the activity of the ribozyme in these examples.

The activity of #10Δ, #11Δ3, #26 or #16+11 bp was comparable to that of ΔP5bc (Fig. 7B). For these mutants, the mutations might have unexpectedly altered the original structure of the region or the local conformation required for the interaction with L2 because of the difficulty of predicting the tertiary structure of an RNA molecule.

3.3. Alteration of P5c region in the wild-type ribozyme

On the basis of the results described above, we prepared and tested mutants with a modification in the P5abc domain of wild-type intron by using circularly permutation. In these mutants, the 3′-end of G414 and 5′-end of A2 are ligated by a phosphodiester bond which can be specifically hydrolyzed if the ribozyme is active [24]. The mutants lacking either P5b (cΔP5b) or P5bc (cΔP5bc) were also constructed for the comparison (Fig. 8A). The activity of cΔP5bc for the hydrolysis reaction was 43% of that of cΔP5b (Fig. 8B).

Two mutants in which an 11 bp stem derived from either a stable stem of rRNA (cΔP5b+11 bp A) or an unstable stem from a Group II intron (cΔP5b+11 bp B) is inserted at P5c of the wild-type ribozyme were prepared and tested. cΔP5b+11 bp A exhibited the activity which is 88% of that of the wild-type (Fig. 8B) whereas cΔP5b+11 bp B exhibited the activity comparable to that of cΔP5bc, indicating that the stability of the stem is important for the activation in this case. When a CG mutation was introduced at L2 of cΔP5b+11 bp A, the hydrolysis activity decreased to the level of cΔP5bc (Fig. 8B). A mutant of cΔP5b+11 bp A (cΔP5b+11 bp A/mL5c) was also constructed in which an altered L5c exists with the sequence CUUCGG; this sequence forms a stable tetraloop closed by a C-G base-pair. The activity of this mutant was comparable to that of cΔP5bc. Taken together, the results indicate that the activation depends on an L2×L5c interaction but not to the size of the stem.

4. Discussion

Various forms of the activator domain that substitutes P5bc of the *Tetrahymena* ribozyme were obtained from in vitro selection experiment. Most of the selected activator domains contain the L5c-like sequence that can interact with L2 region and appear to form the characteristic P5c subdomain structure whereas the region connecting the P5c subdomain and P5a is highly variable. This suggests that the region connected to P5c subdomain can be altered without much affecting the activity of the ribozyme. This hypothesis was tested by investigating the various forms of the selected mutants or the wild-type ribozyme. It was concluded that it is possible to alter the region between P5c subdomain and P5a into various forms without causing much effect on the activity of the *Tetrahymena* ribozyme. Surprisingly, in one case, even the insertion of an 11 bp influenced the activity of the ribozyme moderately. This may suggest that, in some cases, an active form of the ribozyme is uninfluenced by the insertion of relatively long extra stem structure, if the originally existing long-range interactions are maintained.

If the interaction is not transient, our results imply that the structural alteration in P5c does not or only weakly affect the rest of the long-range interactions. In the P4–P6 crystal structure, the single strand connecting P5c to P5a makes a loose turn towards the solvent at the surface of the molecule, suggesting that it should be possible to add or remove several nucleotides from it without disturbing the original location of L5c. The significant level of variance of the region between P5c subdomain and P5a in our selected clones could be attributed to this characteristic feature of the junction.

Alternatively, the L2×L5c interaction might be a transient one. If the interaction is transient, the drastic structural alteration in P5c might have little effect on the active form of the ribozyme. It is noted that the original L2×L5c interaction is relatively weak [29,30]. Downs and Cech suggested a possibility that a local rearrangement of domain 2 precedes the cyclization of the *Tetrahymena* ribozyme [31].

Twenty of our selected clones are able to adopt the structure of P5c subdomain in the crystal structure of P4–P6 RNA from the *Tetrahymena* ribozyme [22,27]. The sequence UGCA in L5c is complementary to UGCA in L2 in these mutants. For the corresponding UGCA of P5c subdomain in the crystal, the U is flipped out of the loop, the GC in L5c makes base-pairs with GC in J6/6a of another molecule and the A is in the configuration called A-A platform. In the wild-type intron, the first nucleotide in A-A platform in L5c (A5) is susceptible to methylation in the absence of L2 but is protected in its presence, suggesting that the A-A platform exists only in the presence of L2×L5c interaction [32]. We have identified the long-range interactions between the GC (G3 and C4) in L5c-like region and the GC in L2 in the selected mutants that is responsible for activating the ribozyme. By combining these evidences together, we would like to propose that the characteristic structure of P5c subdomain found in the crystal of P4–P6 RNA is equivalent to that in the wild-type intron.

Because the site of the modification is located in the peripheral regions, the drastically modified region around the site could not provide serious distortion onto the catalytic core region of the ribozyme in some cases. This explains why Group I introns have obtained a variety of peripheral regions during the course of its evolution. In five naturally occurring Group IC1 introns, it is found that the sequences of L5c and L2 are complementary [8]. In these introns, the lengths of P5c (3–5 bp) and P2 (8–10 bp) are conserved. In an intron of ND4L gene in *Podospora anserina* [33], six nucleotides complementary to L5c are started at the fourth base of P2 although the complementary sequence in L5c starts at fifth base of P5c. This might be a naturally occurring example which suggests that the structure adjacent to L2 can be variable in order to keep the L2×L5c interaction.

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