Long-range interaction between the P2.1 and P9.1 peripheral domains of the *Tetrahymena* ribozyme

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**ABSTRACT**

The *Tetrahymena* ribozyme possesses peripheral domains, termed P9.1 and P9.2. They are nonessential in the mechanism of the catalytic reaction but contribute to enhance the catalytic activity of the ribozyme. It has been postulated that P9.1 is capable of forming Watson–Crick base pairings with another peripheral domain, P2.1. We report here the existence of long-range base pairings between the loop regions of these two domains and show that this interaction apparently plays a role in enhancing the catalytic activity of the ribozyme.

**INTRODUCTION**

All group I self-splicing introns share a conserved secondary structure and possess peripheral elements that are characteristic of each subgroup of introns (1,2). The subgroup IC intron from *Tetrahymena thermophila* retains five peripheral elements termed P2, P2.1, P5abc, P9.1 and P9.2 (Fig. 1A). These elements have been demonstrated to be nonessential for the mechanism of the catalytic reaction of the ribozyme (3,4).

P5abc has been studied for its function in reactions mediated by the *Tetrahymena* ribozyme. We previously reported that P5abc, which is not essential in the mechanism of the catalysis, plays an important role in enhancing the activity of the ribozyme. Its enhancing effect is presumably based upon tertiary interaction with other elements of the ribozyme (5,6). Recently, the X-ray crystal structure of the P4–5–6 domains of the *Tetrahymena* ribozyme was reported, and confirmed the prediction from previous analyses: two (or three) loop regions in P5abc interact with other parts of the ribozyme via long-range tertiary interactions. These interactions presumably participate in setting the domains into an active structure (7).

In contrast to the well-characterized P5abc, the roles of the remaining peripherals, P2, P2.1, P9.1 and P9.2 (Fig. 1A) are yet unclear. However, several studies have suggested that the P9.1 and P9.2 domains may also play a role in enhancing the *Tetrahymena* ribozyme activity. Barfod and Cech demonstrated that a mutant ribozyme lacking P9.1 and 9.2 domains exhibits weak activity in reactions at both the 5′ and 3′ splice sites (3). Doudna and Cech reported that an active ribozyme can be reconstituted by assembly of the P1–P3, P4–P6 and P7–P9–P9.1–P9.2 fragments, but that this reconstitution is incomplete when P7–P9 fragment was employed in place of P7–P9–P9.1–P9.2 fragment (8). It has been shown that a mutant ribozyme lacking the P9.1 and 9.2 domains requires a high concentration of MgCl2 in order to fold itself into an active form (9,10). A study focusing on the denaturing processes revealed that a mutant ribozyme lacking P9.1 and 9.2 unfolds at lower temperatures compared to the wild type (11). Thus it is conceivable that P9.1 and/or P9.2 is involved in the formation of the active form.

It has been suggested that the loop region of P9.1 is capable of forming Watson–Crick base pairings with the loop region of P2.1 (11). Furthermore, this hypothetical interaction appears to be phylogenetically conserved among the group I introns closely related to the *Tetrahymena* intron. However, no direct evidence has been reported for the existence of such an interaction which is presumably involved in the enhancement of the ribozyme activity. To examine whether such an interaction exists, we prepared and tested mutant ribozymes in specific 3′ splice site hydrolysis reactions to determine their relative activities. Our results reveal that the P9.1 and P2.1 domains are responsible for the enhancement of ribozyme activity and that a long-range interaction between the domains does exist.

**MATERIALS AND METHODS**

**Nucleotides and enzymes**

[α-32P]ATP was obtained from ICN. *Taq* polymerase, T4 polynucleotide kinase and T4 DNA ligase were purchased from Takara shuzo. Restriction enzymes were from Takara shuzo or Toyobo. T7 RNA polymerase was prepared as described by Davanloo *et al*. (12). Synthetic oligonucleotides were purchased from Sawady technology.

**Preparation of the mutant RNAs**

All mutant ribozymes employed in this study were prepared from the pT7L-21 plasmid using the polymerase chain reaction as described (13), and the constructs verified by DNA sequencing. Plasmids were linearized with either *Hind*III for the hydrolysis assay or *Sca*I for DMS modification. RNAs were transcribed *in vitro* with T7 RNA polymerase and purified through electrophoresis on 5% polyacrylamide denaturing gels.

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**Figure 1.** (A) The secondary structure of the group I intron from *T.thermophila* LSU rRNA. Nucleotides upstream of the dashed lines, which encompass the first 21 nucleotides of the intron as well as the 5′ exon sequence, were not included in the ribozyme constructs employed in this study. Boxed areas demarcate the regions deleted in the ΔP2.1T, ΔP9.1T, ΔP9.2T and ΔP9.1/9.2 mutants as indicated. (B) Schematic representation of the Tetrahymena LSU rRNA precursor and its derived L-21 ribozyme.

**Mutant ribozyme constructs**

ΔP2.1T and ΔP9.1/9.2 mutants lack nucleotides 64A–86U or 332C–402U, respectively. The ΔP2.1T/9.1/9.2 mutant lacks 64A–86U and 332C–402U. In the ΔP9.1T and ΔP9.2T mutants, nucleotides 338G–361C in P9.1 or 375U–402U in P9.2, respectively, are replaced with a hexanucleotide 5′-CUUCGG-3′ which forms a stable stem–loop structure. The L-56 and L-95 mutants lack the first 56 or 95 nucleotides and have base substitutions A57G and A97G, respectively. P2.1m1, P2.1m2, P9.1m1, P2.1/9.1m1, P9.1m2 and P2.1/9.1m2 constructs are described in the text.

**Assay of the 3′ splice site hydrolysis reaction**

Uniformly 32P-labeled precursor ribozymes were dissolved in distilled water and heated at 80°C for 3 min. After cooling and incubation at 37°C for 10 min, the hydrolysis reactions were initiated by addition of 5× concentrated reaction buffer to the reaction (50 mM Tris–HCl pH 8.3, 5 mM MgCl2, 200 mM NaCl) and incubation at 37°C was continued. Aliquots were removed at specific time points and quenched on ice with the addition of an equal volume of stop solution (50 mM EDTA, 80% formamide and 0.25% xylene cyanol). The reaction products were electrophoresed on 5% polyacrylamide denaturing gels and quantitated with a Fuji BAS100 imaging analyzer. All assays were performed at least three times and the results were reproducible (see Fig.5). Error bars in Figure 2 were omitted for the clarity of the figure.

**Chemical modification by DMS**

Chemical modifications of RNAs by DMS were performed as described with a modification buffer containing 50 mM Tris–HCl pH 7.5, and 12 mM MgCl2. A primer complementary to A390–U409 was used for the reverse transcriptions as described (6).

**RESULTS**

**Deletion of P9.1, P9.2 and P2.1 domains**

We first attempted to determine whether P9.1 or P9.2 are important for the enhancement of the ribozyme activity since it had been pointed out that deletion of these domains in the Tetrahymena ribozyme decreases the catalytic activity (3). Three mutants, one lacking both P9.1 and P9.2 domains (ΔP9.1/9.2), another lacking the terminal region of P9.1 (ΔP9.1T) and the third lacking the terminal region of P9.2 (ΔP9.2T) were prepared based upon the secondary structural model by Michel and Westhof (Fig. 1A) (1). Their activities were compared to examine the effect of the deletions on the ribozyme.

To avoid superfluous bias from other long-range interactions, the activity of the mutants was assayed by means of the 3′ splice site specific hydrolysis reaction in the absence of the P1 and P10 elements (Fig. 1B). These elements have been shown to be involved in long-range interactions that are important for self-splicing but not required for the hydrolysis reaction (14–19).

The mutant and wild type ribozymes (L-21) were incubated under hydrolysis conditions (30 mM Tris–HCl pH 8.3, 5 mM MgCl2, 200 mM NaCl) at 37°C (Fig. 2A) and assayed. The ΔP9.1/9.2 and ΔP9.1T mutants were similar in their significantly lower levels of activity compared to that of the wild type L-21 intron. In contrast, the ΔP9.2T mutant exhibited a level of activity comparable to wild type. These results indicate that the weak activity of the ΔP9.1/9.2 mutant may be due to the lack of the P9.1 terminal region.
Figure 2. Comparison of L-21 and mutant ribozymes in 3′ splice site hydrolysis reactions. (A) Time course of the relative activities of L-21 (closed square), ΔP9.1T (closed circle), ΔP9.2T (open square) and ΔP9.1/9.2 (open circle) are shown. (B) Time course of the relative activities of L-21 (closed square), ΔP9.1/9.2 (open circle), ΔP2.1T (open square) and ΔP2.1T/9.1/9.2 (closed circle).

Based upon this observation that P9.1 plays an important role in enhancing the ribozyme activity, we next investigated the existence of a proposed interaction between P9.1 and P2.1. Phylogenetical studies have suggested that P9.1 may interact with P2.1 through long-range base pairings, although no direct evidence has been reported (11). Given that the proposed interaction may be solely responsible for activation of the *Tetrahymena* ribozyme, mutants lacking P2.1, the putative counterpart for this interaction, should exhibit a decrease in activity resembling that of the ΔP9.1T mutant. To test this hypothesis, two mutants were prepared which lack the terminal region of P2.1 (ΔP2.1T) or both P9.1/9.2 and the terminal region of P2.1 (ΔP2.1T/9.1/9.2) and analyzed (Figs 1A and 2B). The activity of the ΔP2.1T mutant was significantly decreased compared to that of the wild type and comparable to that of the ΔP9.1T mutant (compare Fig. 2B to 2A). This hypothesis would also predict that a mutant lacking both P2.1 and P9.1/9.2 should exhibit a level of activity similar to that of the ΔP9.1/9.2 mutant. As anticipated, the activity of the mutant ΔP2.1T/9.1/9.2 was virtually indistinguishable from that of the ΔP9.1/9.2 mutant, thus supporting our hypothesis. Based on the secondary structure analysis of the *Tetrahymena* intron (21), one can conceive that the respective terminal-loop regions of P2.1 and P9.1 are capable of forming four Watson–Crick base pairings, though the comparative studies suggest the existence of up to seven base pairings (1,11) (Fig. 3).

**Evidence for base pairing between P2.1 and P9.1**

To obtain evidence for the existence of the postulated long-range interaction between domains P9.1 and P2.1, we attempted a chemical modification experiment, employing L-21 and three mutant ribozymes that lack various 5′ peripheral elements (Fig. 4). Dimethyl sulfate (DMS) which preferably modifies N-1 of the adenosine residue was employed under conditions where the mutants are catalytically active (6,20). Notable differences were observed at nucleotides A351 and A352, which are positioned within the loop region of P9.1 and presumably capable of forming base pairings with the two U residues in the loop region of P2.1 (Fig. 3). The adenosines of L-95 or the ΔP2.1T mutant lacking the loop region of P2.1 were modified unambiguously, whereas the corresponding ones in L-21 or the L-56 mutant containing the loop region of P2.1 were protected. These results are consistent with the hypothesis that the terminal region of P9.1 interacts with that of P2.1.
To determine whether the two adenosines (A351 and A352) contribute towards activation of the ribozyme, a compensatory mutation experiment was attempted. Three mutants were prepared, P9.1m1 in which A351 and A352 were substituted by U351 and U352, P2.1m1 in which the putative complementary pair U75 and U76 were replaced by two adenosines, and P2.1/9.1m1 which was designed to restore the putative base pairings by mutations at all four nucleotides (U351, U352, A75 and A76). The mutant introns were examined by employing the hydrolysis reaction (Fig. 5A), and as anticipated, both the P2.1m1 and P9.1m1 mutants were distinctively less active than L-21, whereas the P2.1/9.1m1 mutant exhibited an activity comparable to the wild type, suggesting that this interaction may be responsible not for the activation of hydrolysis at the 3′ splice site but also for reactions at the 5′ splice site.

Comparison of the loop region sequence of P2.1 and P9.1, predicts that Watson–Crick base pairings may also be formed between G77–C78 and C350–G349 residues (Fig. 3). To examine whether such base pairings may also contribute to the activation of the ribozyme, we prepared mutants with substitutions designed to either disrupt or restore the putative base pairings at these residues in a manner similar to that described above (termed P2.1m2, P9.1m2 and P2.1/9.1m2, respectively). The results of the hydrolysis reaction indicate that nucleotides G77 and C78 in P2.1 and nucleotides G349 and C350 in P9.1 do contribute to the enhancement of the activity of the intron by long-range interaction (Fig. 5B).

To further confirm our observations, we examined the activity of mutant ribozymes in which all four base pairings between the P2.1 and P9.1 domains of the Tetrahymena intron can form seven base-pairings according to the comparative studies (11). However, this is inconsistent with the secondary structure analysis of P2.1 and P9.1 (21). The structure of P2.1 that includes a characteristic A–C pair is found in several other IC1 introns and its existence has been confirmed experimentally (21–23). On P9.1 domain, the chemical modification at A347 that should be involved in seven P2.1–P9.1 base pairings is uninfluenced by the existence of the terminal region of P2.1 (Fig. 4). Although the possibility that the seven P2.1–P9.1 base pairings may form transiently is not excluded, our and previous observations are consistent with the idea that P2.1–P9.1 interaction consists of four base pairings.

It is still unclear whether reactions at the 5′ splice site are also affected by this interaction. We attempted guanosine dependent cleavage reactions at the 5′ splice site that are equivalent to the first splicing step by employing mutant introns lacking the terminal region of P2.1 or P9.1. Our preliminary results show that the activities of these mutants are lower than the corresponding wild type, suggesting that this interaction may be responsible not only for the activation of hydrolysis at the 3′ splice site but also for reactions at the 5′ splice site. It should be noted, however, that further analyses are necessary to elucidate whether this interaction is involved in the enhancement of all of the Tetrahymena ribozyme catalytic reactions.

DISCUSSION

We examined the importance of the P9.1, P9.2 and P2.1 peripheral elements for Tetrahymena ribozyme activity by employing mutants lacking P1 and P10 in 3′ splice site specific hydrolysis reactions. The tertiary interactions in that the P1 and P10 elements are involved have been shown to affect the efficiency of the reaction at the 5′ splice site and trans exon-ligation reaction at the 3′ splice site. To eliminate superfluous bias that might conceal the effects of putative P2.1–P9.1 interaction, we employed 3′ splice site specific hydrolysis reactions that is unaffected by deleting P1 and P10 elements (Fig. 1B) (19). We demonstrated that the loop region of P9.1 interacts with that of P2.1 through Watson–Crick base pairings and that this interaction enhances the hydrolysis activity of the ribozyme (Fig. 3).

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In group I introns, the existence of long-range interactions other than the ones involving P2.1, P9.1 and P5abc have been indicated. As described, P10 which consists of base pairings with the 5′ exon is important in conjugating the first and second step of the splicing (17). It has been shown, however, that if one of these interactions is disrupted, the corresponding mutant intron is still capable of performing the reactions albeit with lower activity. This evidence
indicates that the activity of the wild type ribozyme is highly dependent upon yet unidentified long-range interactions that consort with the basal activity of the catalytic core. We propose that peripherals other than P5abc, P2.1 and P9.1 are involved in the activation mechanism of the *Tetrahymena* ribozyme.

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**NOTE ADDED IN PROOF**

After we completed this work, an article describing the P2.1–P9.1 interaction was reported by Lehnert et al. (24).

**REFERENCES**