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Characterization of P8 and J8/7 Elements in the Conserved Core of the *Tetrahymena* Group I Intron Ribozyme

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The universally conserved core region in the group I intron ribozymes is responsible for its catalytic activity. The structural elements in this region have been known to organize the active site of this class of ribozymes. However, it has been unclear whether all elements are requisite or some elements are dispensable for conducting the catalysis. To investigate the necessity of these elements in the catalysis, we prepared and examined a series of mutants having a nick or deletion in these elements. In this report, we show that two elements, P8 and 5′ portion of J8/7, are nonessential for activity. © 2000 Academic Press

All known group I intron ribozymes share the universally conserved core region consisting of two helical domains, P4–P6 and P8–P3–P7 (Fig. 1A, see also Ref. 1). A series of the phylogenetical and structural analyses has implicated that the catalytic site of this class of ribozymes is located within this conserved core region (2, 3, and references cited therein). Consistent with these analyses, a series of the deletion experiments which directly reveal the dispensability of a targeted region demonstrated that, in the case of an intron ribozyme from *Tetrahymena thermophila*, any structural element outside the core region can be deleted without a complete loss of the catalytic activity (4–7). However, it has been unclear whether some elements in the core are also dispensable or all the elements are requisite for conducting the catalysis.

As an initial step of our investigation on the conserved core of the *Tetrahymena* ribozyme, we attempted the deletion experiments on the elements, P8 and J8/7, located in the exterior of the P8–P3–P7 domain (Fig. 1A). For the P8 element, it was reported that the disruption of its base-pairs reduced the self-splicing activity (8), but other biochemical analyses on this element have not been reported. For the J8/7 element, its 3′ portion located in the proximity of the P7 element has been implicated to participate in the mechanism of catalysis (2). However it has been unclear whether the 5′ portion, which was shown to participate in the correct docking of 5′ splice site to the active site (9–12), is also concerned in the mechanism of catalysis.

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MATERIALS AND METHODS

Preparation of RNAs. For preparation of circularly permutated *Tetrahymena* ribozymes, a plasmid pWIVS was constructed by ligating a *Sph*I digested fragment of pTZIVSU5H (13) with pTZIVSU (8) which was linearized by *Sph*I. The resulting plasmid pWIVS bears two *Tetrahymena* introns in tandem. Template DNAs for circularly permutated ribozymes were prepared by PCR with appropriate primer sets and pWIVS. The promoter sequence for T7 RNA polymerase was included in the forward primers so that resulting PCR products can be used directly as the templates for *in vitro* transcriptions (13). In several permutated ribozymes, either A299 or A306 was replaced with G to improve the efficacy of *in vitro* transcription (see also figure legends). The A to G base-substitutions at the positions did not abolish the ribozymatic activity (1, 14, and this study). The uniformly labeled ribozymes were prepared by *in vitro* transcription with [α-^{32}P]ATP or [α-^{32}P]GTP and purified by electrophoresis on 5% polyacrylamide gels containing 8.3 M urea. For preparing J8/7-nick4, J8/7ΔA304 and J8/7Δ(G303A304) variants, the template DNAs for J8/7-nick3 and ΔJ8/7b variants were used and transcriptions were performed in the presence of excess UpG (2 mM) over GTP (0.1 mM) to incorporate UpG at the 5′ end of the transcripts.

Ribozyme activity assays. The site-specific hydrolysis reactions were performed in the mixture containing uniformly [α-^{32}P]-labeled ribozymes (approximately 3×10^4 cpm), 50 mM Tris–Cl, pH 8.3, 5 mM spermidine and indicated concentrations of $MgCl_2$ at 37°C for 3 h. The CpU dependent phosphoester transfer reactions were performed in the mixture containing uniformly [α-^{32}P]-labeled ribozymes, 50 mM Tris–Cl, pH 7.5, 5 mM spermidine, indicated concentrations of $MgCl_2$, and 1 mm CpU dinucleotide (Sigma) at 37°C for 3 h. The reactions were stopped by adding equal volume of stop solution comprising 90% formamide, 50 mM EDTA, 0.02% BPB and 0.02% XC. All reaction mixtures were electrophoresed on 5% polyacrylamide gels containing 8.3 M urea. The gels were exposed to either X-ray films or Imaging plates and quantitated by using a Bio-Image BAS-2500 analyzer (Fuji Film, Japan).

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RESULTS

Circular permutation analysis. To introduce a nick or deletion into the P8 and/or J8/7 element(s) without disturbing the structural organization of other elements in the *Tetrahymena* intron ribozyme, we employed the circular permutation analysis by using a circular form of this intron ribozyme (13). The *Tetrahymena* and several other group I introns perform a self-circularization reaction that is chemically analogous to the first step of the self-splicing (15). As schematically represented in Fig. 1B, this circular ribozyme retains the catalytic activity to perform a self-reopening reaction by a site specific hydrolysis or a dinucleotide CpU-dependent phosphoester transfer at the circularized junction. The hydrolysis or phosphoester transfer reaction is chemically analogous to the site specific hydrolysis reaction at the 3′ splice site or the exon–ligation reaction, respectively (15). When a nick or deletion was introduced in the circular ribozyme, the resulting permutated ribozyme becomes a linear form as shown in the gray inset in Fig. 1B. If the permutated mutant is still active, it performs the reopening reactions to produce two RNA fragments termed A and B (the gray inset in Fig. 1B). The fragments A and B consist of the nucleotides from A2 to the 3′ end of the permutated ribozyme and ones from the 5′ end of the permutated ribozyme to G414, respectively (note: In the phosphoester transfer reaction with a dinucleotide CpU, CpU is attached covalently at the 5′ terminus of the fragment A) (13).

Circularly permutated ribozymes with a nick in the P8 or J8/7 element. The site specific hydrolysis reaction or the CpU dependent phosphoester transfer reaction was attempted for the circularly permutated *Tetrahymena* ribozymes in which a nick was introduced in either P8 or J8/7 element (Fig. 2A). Kinetic analyses for the permutated ribozyme having a nick in P8 (termed P8-nick variant, Fig. 2A) showed that this variant efficiently performed the reopening reactions to yield two RNA fragments A and B as shown in Fig. 1B (data not shown). However, the two fragments rapidly performed a recyclization reaction at the cryptic reaction site between U15 and A16 in the L1 region (Figs. 1A and 1B, see also Refs. 13 and 15), yielding a 14 nt shorter unimolecular ribozyme (termed C-15, see Fig. 1B). C-15 ribozyme further performed a reopening reaction at the new circularized junction at G414–A16, yielding the two fragments B and A-15 (a fragment from A16 to the 3′ end of the permutated ribozyme). As a result of the reactions at the cryptic reaction site, P8-nick variant yielded the fragments A-15 and B as major reaction products after 3 h incubation (Fig. 2B).

The catalytic activities were assayed for three variants having a nick at a different position in J8/7 element (J8/7-nick1, J8/7-nick2 and J8/7-nick3, Fig. 2A). Both J8/7-nick1 and J8/7-nick2 exhibited the reopening activity in the presence of 15 mM $MgCl_2$ (Fig. 2B) whereas J8/7-nick3 which had a nick between U305 and A306 (note: the position 306 is originally A but is replaced with G in this variant. See Materials and

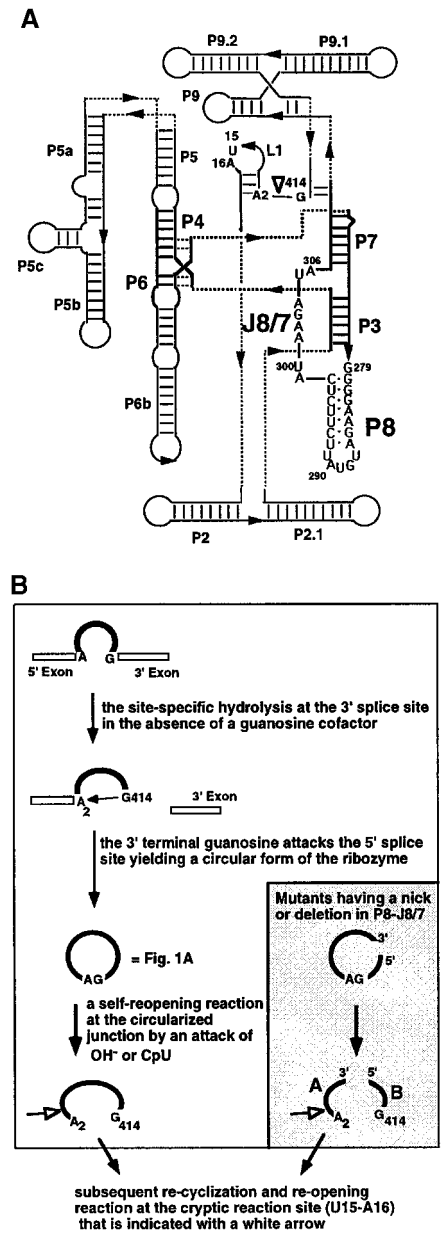

FIG. 1. The circular form and cyclization reaction of the *Tetrahymena* ribozyme. (A) Secondary structure of a circular form of the *Tetrahymena* group I intron ribozyme employed in this study. In the circular ribozyme, G414 and A2 are connected with a phosphodiester bond (indicated by white arrowhead). The nucleotides in the region investigated in this study are shown at P8 and J8/7 (G279–A306) and other structural elements are shown as lines. Thick and thin lines represent the conserved core region and non-conserved peripherals, respectively. (B) The reaction producing a circular form of the *Tetrahymena* ribozyme and the subsequent reopening reaction are shown. The reopening reaction by the circularly permutated ribozyme is shown in the gray inset.

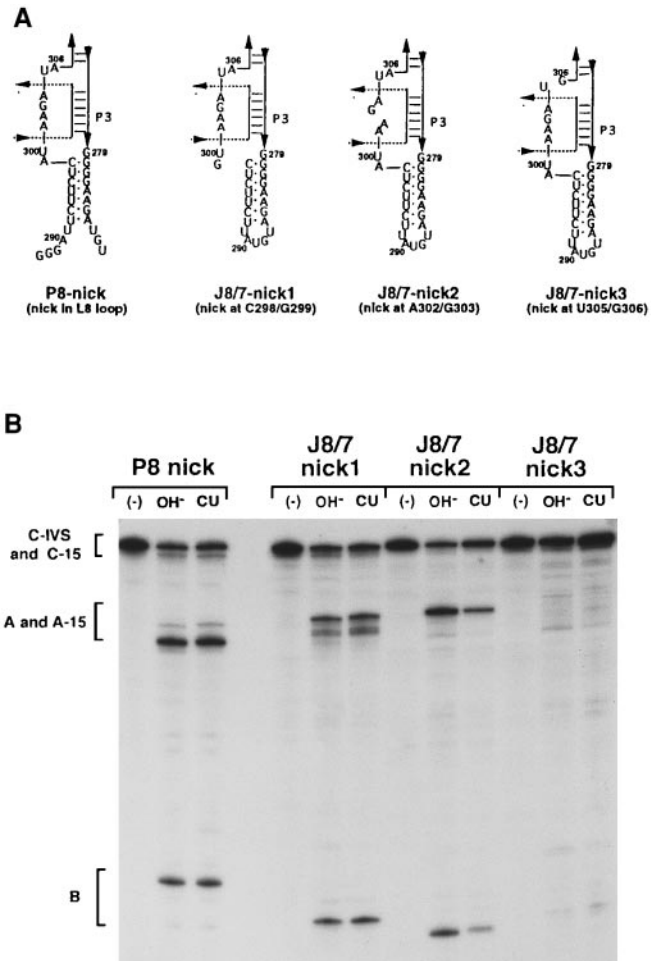

FIG. 2. The reactions of circularly permutated ribozymes with a nick in P8 or J8/7 element. (A) Circularly permutated ribozymes having a nick in L8 or J8/7. J8/7-nick1 and J8/7-nick3 have A to G base-substitutions at positions 299 and 306, respectively (see Materials and Methods). (B) The site-specific hydrolysis or the CpU dependent phosphoester transfer reaction of the circularly permutated ribozymes with nicks. The conditions for the two reactions were indicated in Materials and Methods. RNAs were incubated in the reaction buffer containing 15 mM $MgCl_2$. In the reaction of P8 nick and J8/7-nick1 variants, the two RNA fragments performed recyclization and reopening reactions at the cryptic reaction site, yielding C-15 ribozyme and fragment A-15 (Figs. 1A and 1B), as reported previously (13). In the figure, the characters, (−), OH-, and CpU, indicate no incubation, the hydrolysis reaction and the CpU dependent phosphoester transfer reaction, respectively.

Methods) was inactive even in the presence of higher concentration of $MgCl_2$ (Fig. 2B, see also Fig. 4B). The assay also showed that the catalytic activities of the three active variant ribozymes are not identical because P8-nick or J8/7 nick2 dominantly yielded the fragment A-15 or A, respectively, whereas J8/7 nick1 yielded the A and A-15 fragment comparably (Fig. 2B).

Circularly permutated ribozymes with deletion(s) in the P8 and/or J8/7. The analyses of the variants with a nick in the J8/7 element revealed that the *Tetrahymena* ribozyme is tolerant for a nick at C298–A299 or A302–G303 but not at U305–A306. To further investigate the J8/7 element, we prepared and examined two permutated ribozymes, ΔJ8/7a and ΔJ8/7b that lack the 5′ portion (positions from A299 to A302) and the 3′ portion (positions from G303 to U305) of the J8/7, respectively (Fig. 3A). ΔJ8/7a variant was active whereas ΔJ8/7b variant which lacks the covalent linkage between U305 and A306 exhibited no activity (Fig. 3B). This is consistent with the result that J8/7-nick3 variant having a nick at U305-A306 was inactive (Fig. 2B).

In addition to the variants having a deletion in the J8/7, we also prepared two variant ribozymes lacking their P8 elements (Fig. 3A). ΔP8 variant lacking the entire P8 element (positions from G279 to C298) effi-

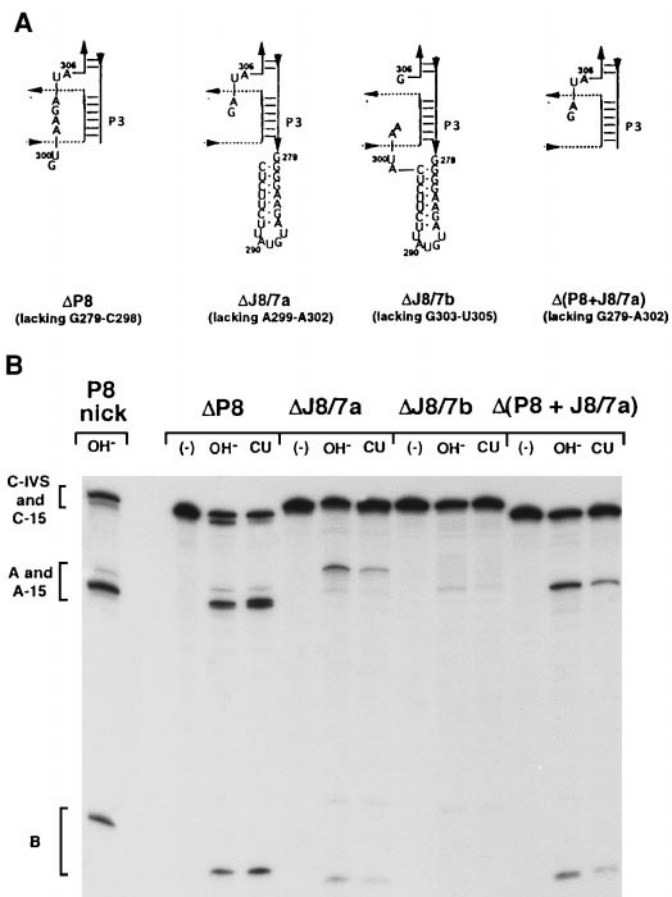

FIG. 3. The reactions of circularly permutated ribozymes with deletion(s) at P8 and/or J8/7 element. (A) Circularly permutated ribozymes with deletion(s) at P8 and/or J8/7. ΔP8 and ΔJ8/7b have A to G base-substitutions at positions 299 and 306, respectively (see Materials and Methods). (B) The site-specific hydrolysis or phosphoester transfer reaction of circularly permutated ribozymes possessing deletions. The conditions for the two reactions were indicated under Materials and Methods. RNAs are incubated in the reaction buffer containing 15 mM $MgCl_2$. P8 nick variant was also subjected to the reactions and electrophoresis as a positive control and size marker. P8 nick and ΔP8 variant performed recyclization and reopening reactions, yielding the fragments A-15 and B dominantly.

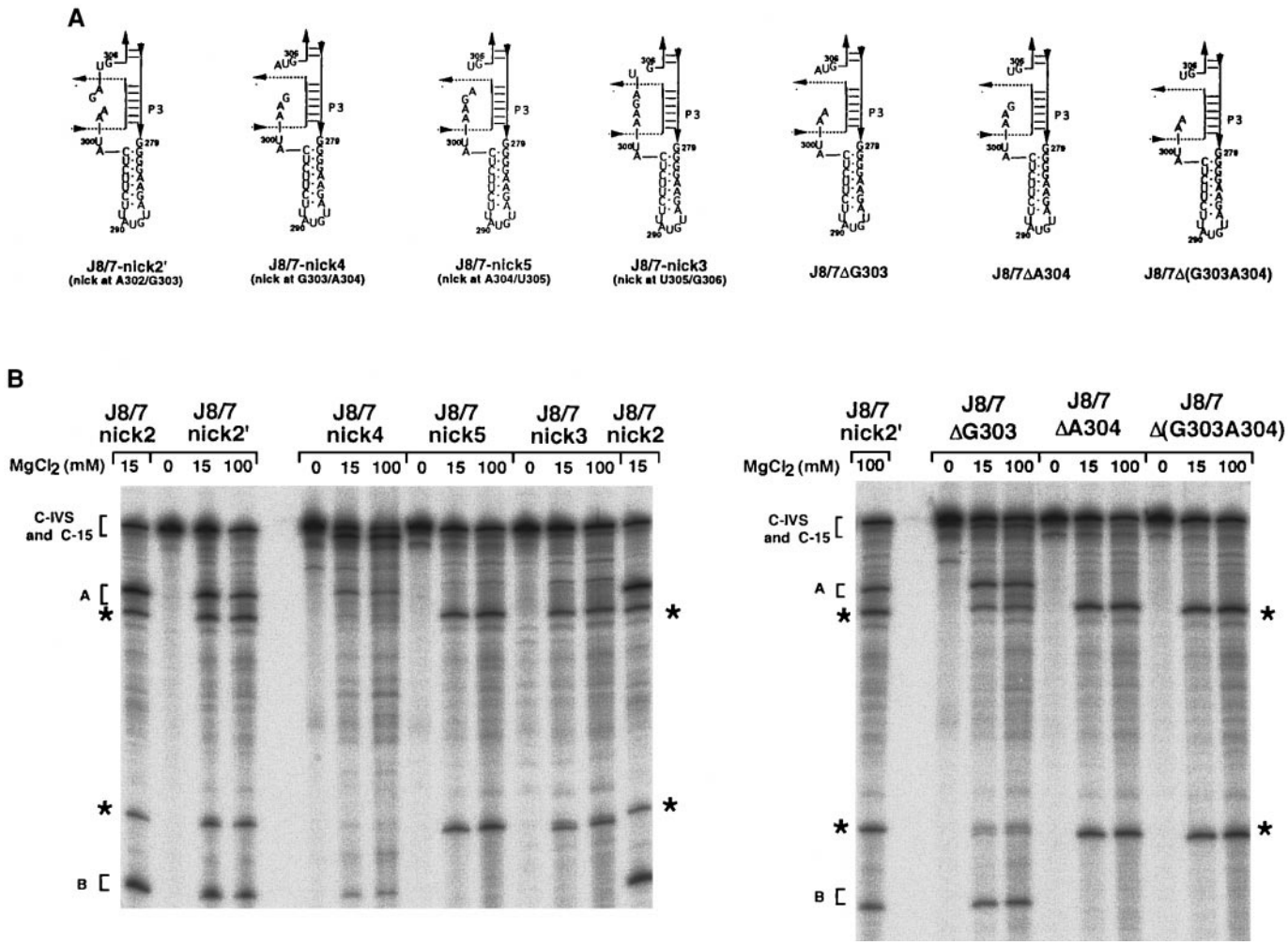

FIG. 4. The hydrolysis reactions of circularly permutated ribozymes with a nick or deletion in the J8/7 region. (A) Circularly permutated ribozymes possessing a nick or deletion in the 3′ portion of J8/7. In this series of permutated ribozymes, A306 is replaced with a guanine (see Materials and Methods). (B) The site specific hydrolysis reaction of circularly permutated ribozymes with a nick or deletion in the 3′ portion of J8/7. The conditions for the reaction were indicated under Materials and Methods. RNAs were incubated in the reaction buffer containing either 15 or 100 mM $MgCl_2$. Asterisks indicate the products that were not produced by the ribozymatic activity of the group I intron. The cleavage occurs nonreproducibly (compare J8/7 nick3 variant in Fig. 2B with that in Fig. 4B) at a site more internal than the cryptic reaction site at U15-A16. A similar cleavage of the *Tetrahymena* ribozyme was observed previously (24).

ciently performed the reopening reaction, yielding the fragment A-15 dominantly (Fig. 3B). Moreover, Δ(P8+J8/7a) lacking the nucleotides between G278 and C303 was also active although it lacks the entire P8 element as well as the 5′ portion of the J8/7 element (Fig. 3B).

Identification of the critical elements in the 3′ portion of J8/7. To evaluate the role of the 3′ portion of J8/7 in the mechanism of catalysis, we prepared and tested the permutated ribozymes having a nick or deletion within this region (Fig. 4A). The assay using the site-specific hydrolysis reaction revealed that J8/7-nick4 variant having a nick between G303 and A304 is active in the presence of 15 mM $MgCl_2$ (Fig. 4B). However, J8/7-nick5 variant having a nick between A304 and U305 was inactive even in the presence of 100 mM $MgCl_2$ (Fig. 4B). The results indicate that the covalent linkage between A304 and U305 is critical for the hydrolysis reaction. This observation is consistent with other results that J8/7ΔG303 variant having the covalent linkage at A304–U305 was active whereas two variants J8/7ΔA304 and J8/7Δ(G303A304), which lack the covalent linkage at A304–U305, were inactive (Fig. 4B).

J8/7-nick5, J8/7ΔA304, and J8/7Δ(G303A304) which are inert to the hydrolysis reaction were subjected to the CpU dependent phosphoester transfer reaction (Fig. 5). Interestingly, J8/7-nick5 and J8/7ΔA304 exhibited the activity whereas J8/7Δ(G303A304) was inactive (Fig. 5). The GTP dependent phosphoester

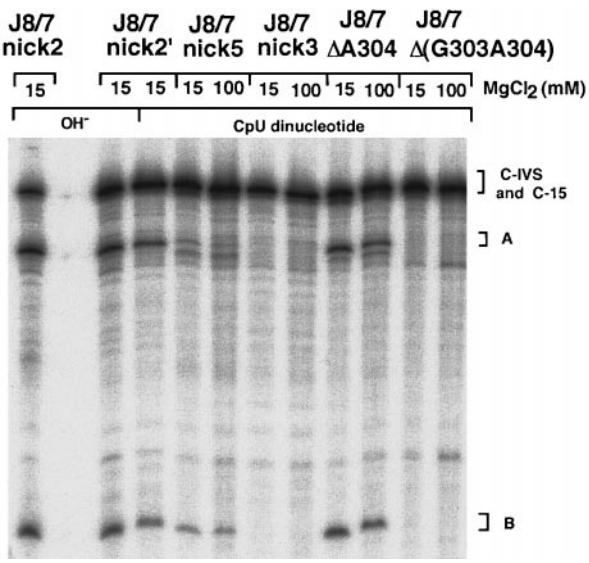

FIG. 5. The CpU dependent phosphoester transfer reactions of circularly permutated ribozymes that are inert to the site specific hydrolysis reaction. The conditions for the reaction with CpU were indicated under Materials and Methods. RNAs were incubated in the reaction buffer containing either 15 or 100 mM $MgCl_2$.

transfer reaction that is chemically analogous to the first step of the self-splicing also attempted, but no activity was detected for the three variant (data not shown). These results show that the phosphodiester bond at A304–U305 is dispensable for the phosphoester transfer reaction with CpU whereas the bond at U305–A306 is essential for both the hydrolysis and phosphoester transfer reaction of the permutated ribozymes.

DISCUSSION

Our present results show that the catalytic activity of the *Tetrahymena* ribozyme is retained despite a complete deletion of the P8 element (ΔP8 variant in Fig. 3B). According to the crystal structure of the active site of the *Tetrahymena* ribozyme, P8–P3–P7 domain forms a bent helix that wraps around the coaxially stacked P4–P6 domain (2). Our present result suggests that the two continuous helical domain consisting of P3 and P7 is sufficient for organizing the active site with P4–P6 domain by forming the bent helical structure.

The 5′ portion of J8/7 element was showed to participate in the correct docking of the 5′ splice site to the active site. A302 was identified to interact directly with U(-3) in the P1 helix which contains the 5′ splice site (9). More recently, Strobel and co-workers identified an interaction between A301 and G25 in the P1 helix via their 2′ OH groups (10). They also suggested that U300 and G303 interact with G26 and C(-2) in the P1 helix, respectively (11, 12). Our analyses demonstrate that the *Tetrahymena* ribozyme is catalytically active in the absence of either U300-A301-A302 (ΔJ8/7a variant in Fig. 3B) or G303 (J8/7ΔG303 variant in Fig. 4B), demonstrating that they are not critical for the catalysis and supporting the previous notion that their primary role is the correct docking of the P1 helix.

Our analyses demonstrate that the phosphodiester bond at U305–A306 is essential for the *Tetrahymena* ribozyme to perform the hydrolysis or phosphoester transfer reaction (Figs. 2B, 4B, and 5). Previous biochemical analyses have shown that a phosphate at U305–A306 coordinates a magnesium ion that is critically important for the activity of this ribozyme (19–23). The phosphate at U305–A306 was also demonstrated to be positioned in close proximity to the guanosine binding site in the crystal structure of the *Tetrahymena* ribozyme (2). Taken these observations together, it is conceivable that the phosphate at U305–A306 might coordinate a magnesium ion which directly participates in the catalysis.

Our analyses also demonstrate that the phosphodiester bond at A304–U305 is critical for the hydrolysis reaction and the phosphoester transfer reaction with GTP but is dispensable in the phosphoester transfer reaction with CpU (Figs. 4B and 5). Mutational analyses of U305 demonstrated that this nucleotide formed a base-triple with P4 element (20). The analyses also showed that disruption of this base-triple slowed the first step of the self-splicing but accelerated the second step, implying that the base-triple regulates the sequential splicing reaction (20). If the base-triple involving U305 is weakened or disrupted by a nick at A304-U305, it is conceivable that the resulting permutated ribozyme shares some catalytic properties with a variant ribozyme having a mutation at U305. The phosphoester transfer reaction of the circular group I ribozyme with GTP or CpU is chemically analogous to the first or the second step of the self-splicing reaction, respectively (15). This implies a possible reason why the reaction with CpU was allowed to proceed whereas the reaction with GTP was inhibited in the absence of the covalent linkage between A304 and U305.

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