Nucleic Acids Research, 1999, Vol. 27, No. 8 **1859–1865**

A conserved motif in group IC3 introns is a new class of GNRA receptor

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Received January 4, 1999; Revised and Accepted February 18, 1999

ABSTRACT

Terminal tetraloops consisting of GNRA sequences are often found in biologically active large RNAs. The loops appear to contribute towards the organization of higher order RNA structures by forming specific tertiary interactions with their receptors. Group IC3 introns which possess a GAAA loop in the L2 region often have a phylogenetically conserved motif in their P8 domains. In this report, we show that this conserved motif stands as a new class of receptor that distinguishes the sequences of GNRA loops less stringently than previously known receptors. The motif can functionally substitute an 11 nt motif receptor in the *Tetrahymena* ribozyme. Its structural and functional similarity to one class of synthetic receptors obtained from *in vitro* selection is observed.

INTRODUCTION

The long-range tertiary interactions between GNRA (N = A, G, C or U; R = A or G) tetraloops and their receptors are found frequently in large ribozymes including group I and group II introns and RNase P RNAs (1–4). Michel and co-workers identified specific combinations between the GNRA loops and their receptors by using phylogenetical and experimental approaches (1,2). They have shown that GUAA, GUGA and GAAA loops have their specific receptors that are termed CC-GG pairs, CU-AG pairs and a characteristic motif consisting of an 11 nt sequence (CCUAAG-UAUGG, termed the 11 nt motif), respectively. Kinetic analysis revealed that the affinity between a GAAA loop and an 11 nt receptor is distinctively higher than the others (2). The three-dimensional structure of an 11 nt motif receptor complexed with a GAAA loop was reported as a part of the P4–P6 domains of the *Tetrahymena* intron by X-ray crystallography (5). Free forms of an 11 nt receptor were also characterized by using NMR (6). The comparison of the two forms of the receptor suggests that dynamic structural changes occur by binding to a GAAA loop (6).

In the group I self-splicing introns, three sets of GNRA loops and their receptors have been identified; the L5b loop with a receptor in P6a (Fig. 5), the L9 loop with a receptor in P5 (Fig. 1) and the L2 loop with a receptor in P8 (Fig. 1) (2). The interaction between L5b and P6a is found only in subgroup IC, whereas the other two sets are commonly observed among subgroup IA, IB and IC introns (7,8).

More than one third of known group I introns including subgroup IA, IB and IC have a GNRA tetraloop in the P2 domain which is located at a defined distance (13 nt) from the conserved U-G base pair in the P1 domain in which U is the last nucleotide of the 5′ exon. In such introns, phylogenetical co-variations between the L2 and P8 domains were often found, implying that the P8 domain is a receptor region for the GNRA loop in L2 (8). Subgroup IC3 introns have a P2 domain with a GAAA at the L2 loop that is separated by 13 nt from a conserved U-G base pair in P1 (7,9,10). Only one intron from *Azoarcus* possessing a canonical GAAA receptor (11 nt motif) in the P8 domain is found in this subgroup (9). However, the P8 domains of the rest of IC3 introns often have a conserved motif sharing CCC-GGG base pairs with an AA bulge as shown in the boxed region in Figure 1. The conserved motif (termed the IC3 motif) seems structurally similar to the 11 nt motif. This prompted us to examine whether the conserved region functions as an unknown GNRA receptor.

MATERIALS AND METHODS

Mutant *Synechococcus* ribozyme constructs

Plasmids encoding derivatives of *Synechococcus* ribozyme having mutations in P8 domains were prepared from pTL3 (11) using PCR (12) and constructs were verified by DNA sequencing. The precursors of L-8 *Synechococcus* ribozyme lack the first 8 nt of the *Synechococcus* IC3 intron and contain the first 36 nt of the 3′ exon of the $tRNA^{Leu}$ of *Synechococcus* PCC 6301. Template DNAs for *in vitro* transcription of these precursor RNAs were generated by 20 cycles of PCR (94°C for 1 min, 55°C for 1 min, 72°C for 2 min) using *Ex Taq* DNA polymerase (Takara shuzo). For each PCR, 1 ng of a plasmid derived from pTL3 described above was used as template. Primers for preparing L2(GNRA) mutants were 5′-TAA TAC GAC TCA CTA TAG GGC CTC GAT CGC GNR AGG-3′ (where N is A, G, T or C and R is A or G) and 5′-CTC GAA CCC TCA CGA CCT TTA-3′. Primers for preparing L2(UUCG) mutants were 5′-TAA TAC GAC TCA CTA TAG GGC CTC GAT CGC UUC GGG GAT CGA GTG-3′ and 5′-CTC GAA CCC TCA CGA CCT TTA-3′. The promoter sequence for T7 RNA polymerase is underlined.

Mutant *Tetrahymena* ribozyme and P5abc RNA constructs

Plasmids encoding derivatives of the L-56 form of *Tetrahymena* ribozyme, the L-21 form of ΔP5abc mutant ribozyme or P5abc RNAs were prepared from pT7L-56 (13), pL-21ΔP5abc (14) or pP5abc (15) using PCR (12) and verified by sequencing. As templates for *in vitro* transcription, derivatives of pT7L-56, pL-21ΔP5abc or pP5abc were digested with *Hin*dIII, *Sca*I or *Sma*I, respectively.

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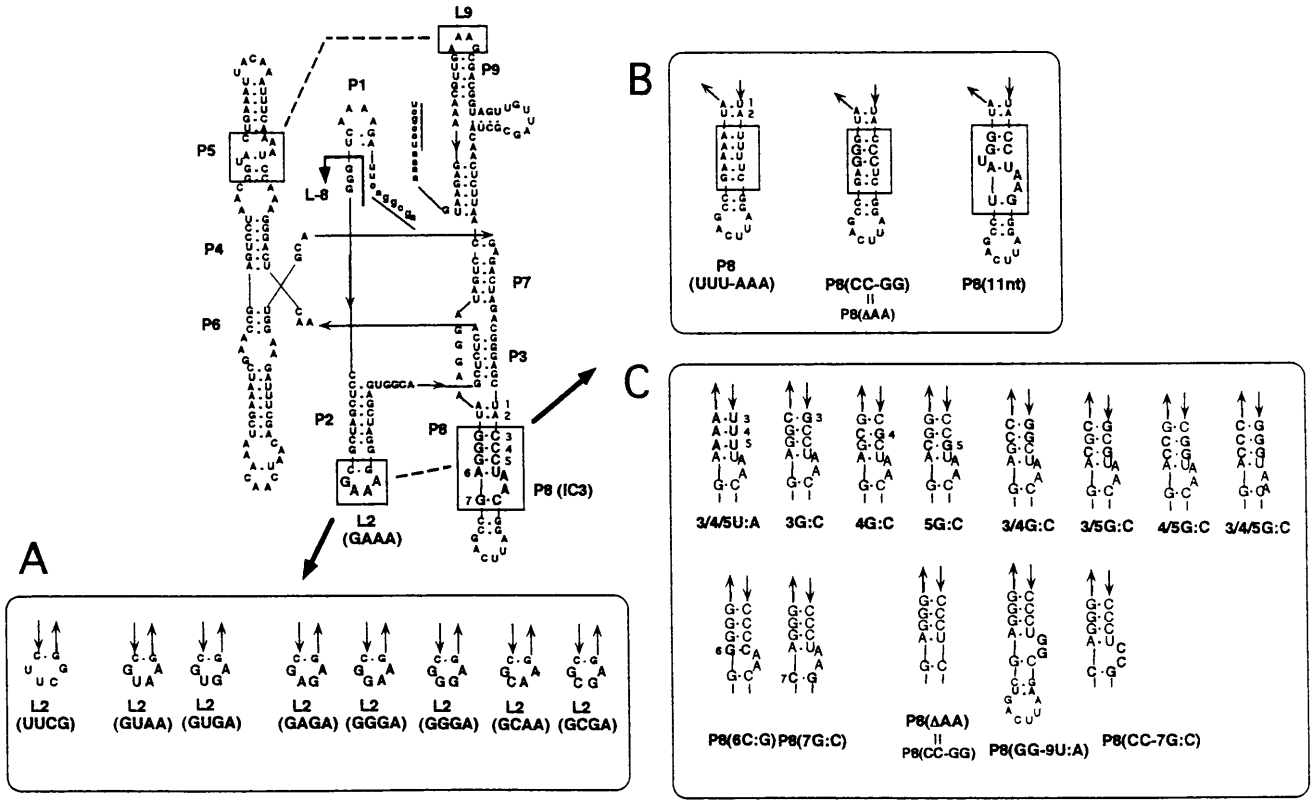

Figure 1. The secondary structure of the group IC3 intron from tRNA^{Leu} of the cyanobacterium *Synechococcus* PCC 6301. Nucleotides upstream of the lines with arrowhead, which encompass the first 8 nt of the intron as well as the 5′ exon sequence, were not included in the ribozyme constructs employed in this study. Interactions between GNRAs and the receptors (L2 × P8 interaction and L9 × P5 interaction) are indicated as dotted lines and regions participating in interactions are boxed. Sequences of L2 mutants are shown in the boxed area A. Sequences of P8 mutants in which a conserved motif of IC3 introns was replaced with other GNRA receptor motifs are shown in the boxed area B. Sequences of P8 mutants having mutations in a conserved IC3 motif are shown in the boxed area C.

Preparation of RNAs

All RNAs employed in this study were prepared by transcription *in vitro* with T7 RNA polymerase and purified by electrophoresis on 5% polyacrylamide denaturing gels as described (16). For preparation of uniformly ^{32}P-labeled or unlabeled RNAs, *in vitro* transcription was performed either in the presence or absence of [α-^{32}P]ATP, respectively.

Assay of the 3′ splice site hydrolysis reaction

Uniformly ^{32}P-labeled precursor ribozymes (derivatives of L-8 *Synechococcus* ribozyme or L-56 *Tetrahymena* ribozyme) were dissolved in distilled water then heated at 80°C for 3 min. After cooling and incubation at the reaction temperature for 10 min, the reaction was initiated by the addition of 5× concentrated reaction buffer. The resulting mixture was incubated at the indicated temperature. Aliquots were removed at specific times and quenched on ice by the addition of an equal volume of a stop solution (150 mM EDTA, 70% formamide and 0.25% xylene cyanol). The products were electrophoresed on 5% polyacrylamide denaturing gels. For assaying the 3′ splice site hydrolysis reaction of L-8 *Synechococcus* ribozyme and its derivatives, the following two conditions were used. Condition A: 40 mM Tris–HCl pH 8.3, 2 mM $MgCl_2$, 37°C. Condition B: 40 mM Tris–HCl pH 8.3, 10 mM $MgCl_2$, 45°C. Conditions for assaying the L-56 form of the *Tetrahymena* ribozyme and its derivatives: 50 mM Tris–HCl pH 8.3, 3 mM $MgCl_2$, 6 mM NaCl, 37°C. All assays were performed at least three times. The results were reproducible so that error bars in the figures are omitted for clarity.

Gel mobility shift assay

The gel mobility shift assays using mutants of the L-21ΔP5abc intron and P5abc RNA were performed as described (14,17) on 5% polyacrylamide native gels comprising 50 mM Tris–OAc (pH 7.5) and 5, 7.5 or 10 mM $Mg(OAc)_2$ with uniformly ^{32}P-labeled P5abc RNA (<10 nM) [or its mutants (<10 nM)] and 1 μM unlabeled ΔP5abc intron (or its mutants).

RESULTS AND DISCUSSION

L2 × P8 interaction in a subgroup IC3 intron

An IC3 intron from *Synechococcus* PCC6301 pre-tRNA^{Leu} was employed for the functional and structural characterization of the P8 domain of the subgroup IC3 intron (Fig. 1; 11). The intron consisting of 240 nt has been shown to exhibit efficient self-splicing activity in the presence of a low concentration of $MgCl_2$ (11). A shortened form of this intron lacking the 5′ splice site (termed the L-8 ribozyme; Fig. 1) conducts the 3′ splice site-specific hydrolysis reaction efficiently (Fig. 2A; 18).

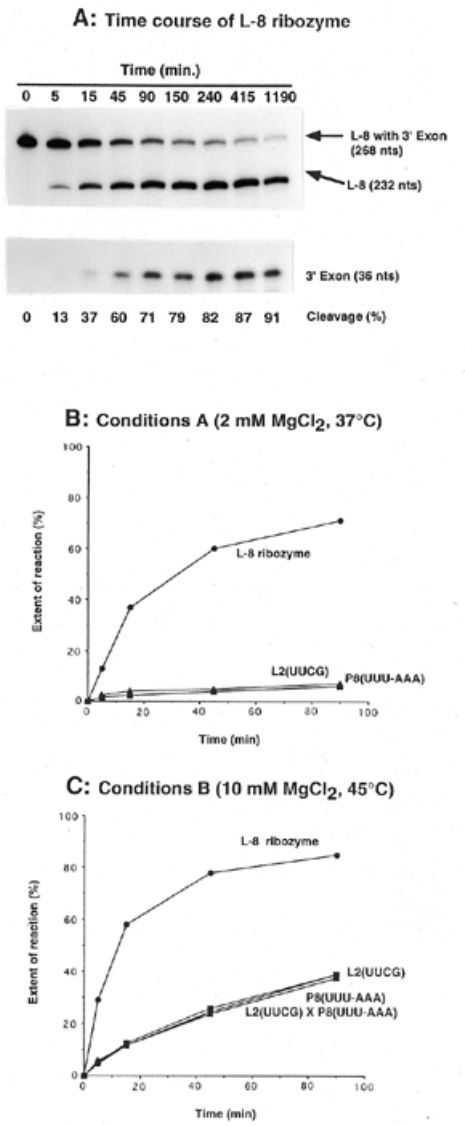

Figure 2. (**A**) Time course of the 3′ splice site specific hydrolysis reactions of L-8 ribozyme under condition A (2 mM $MgCl_2$, 37°C). After electrophoresis on a 5% denaturing gel, the gel was exposed to X-ray film with (for 3′ exon) or without (for L-8 with 3′ exon and L-8) an intensifying screen. (**B** and **C**) Effects of disrupting a hypothetical interaction between L2 and P8 regions in the *Synechococcus* ribozyme. Time courses of 3′ splice site-specific hydrolysis reactions of L-8 ribozyme (closed circle) and its mutant derivatives L2(UUCG) (closed triangle), P8(UUU-AAA) (closed square) and L2(UUCG) × P8(UUU-AAA) (open square) under condition A or B.

To see whether P2 × P8 interaction exists in the *Synechococcus* ribozyme, we prepared and examined a set of mutant L-8 *Synechococcus* ribozymes in which either the L2 GAAA loop or a conserved IC3 motif in the P8 domain was replaced with a different sequence (Fig. 1): L2(UUCG) mutant having a UUCG tetraloop in L2 in place of the GAAA loop and P8 (UUU-AAA) mutant having UUU-AAA base pairs in place of a putative receptor site in P8. The 3′ splice site hydrolysis reaction was used to check for ribozymatic activity. The mutants were barely active at 37°C in the presence of 2 mM $MgCl_2$ (condition A, Fig. 2B) although their activities were partially recovered at 45°C in the presence of 10 mM $MgCl_2$ (condition B, Fig. 2C). A double mutant L2(UUCG) × P8(UUU-AAA) was as active as the corresponding single mutant L2(UUCG) or P8(UUU-AAA) (Fig. 2C). The results suggested that the reduced activity is due to the disruption of the L2 × P8 interaction.

To obtain further evidence to support the existence of the L2 × P8 interaction, we replaced a putative GAAA receptor motif conserved in the P8 domain in the *Synechococcus* intron with two well-characterized GNRA receptor motifs: the CC-GG base pair [P8(CC-GG) mutant] and 11 nt motif [P8(11nt) mutant] that are receptors for a GUAA and a GAAA loop (Fig. 1B), respectively (1,2). For each P8 mutant, we also replaced its L2 GAAA loop with GUAA or GUGA (Fig. 1A). The activities of the resulting mutants were examined. As expected, the introduction of the other GNRAs and their known receptors reconstituted the active ribozymes. The P8(11 nt) × L2(GAAA) mutant was more active than the P8(11 nt) × L2(GUAA) or P8(11 nt) × L2(GUGA) mutants (Fig. 3A). The activity of the P8(CC-GG) × L2(GUAA) mutant was higher than that of the P8(CC-GG) × L2(GAAA) or P8(CC-GG) × L2(GUGA) mutant (Fig. 3B) but lower than that of the P8(11 nt) × L2(GAAA) mutant (compare Fig. 3B to A). The interaction between the GNRA loop in L2 and GNRA receptor in P8 is thus responsible for the activity of the *Synechococcus* ribozyme.

A GNRA receptor motif conserved in the P8 domain of IC3 introns

Group IC3 introns possess GAAA loops in the L2 loop as conserved elements (7,9,10), suggesting that the IC3 motif in the P8 domain is a receptor which presumably prefers a GAAA loop. To investigate the specificity of the receptor, the hydrolysis reaction was attempted by employing the mutants possessing A or U as the second nucleotide N in the GNRA loop at L2 (Fig. 4A). Contrary to the phylogenetic prediction, a mutant with a GUAA loop is more active than a L-8 ribozyme with a GAAA loop at L2 and a mutant with a GUGA is comparably active to the L-8 ribozyme. A mutant with a GAGA loop is slightly less active than the L-8 ribozyme. These results show that the IC3 motif only weakly discriminates a GAAA from GUAA, GUGA or GAGA loop, in contrast to the 11 nt or CC-GG receptor motif that strongly prefers to bind GAAA or GUAA, respectively (Fig. 3A and B) (2). Comparison of Figure 4A with Figure 3A or B shows that a L-8 ribozyme with a GAAA loop is slightly less active than the P8(11 nt) × L2(GAAA) mutant but more active than the P8 (CC-GG) × L2(GUAA) mutant, implying that the affinity of the IC3 motif for a GAAA loop is weaker than that of the 11 nt motif and stronger than that of CC-GG base pairs for their preferred loop partner.

To further analyze the specificity of the IC3 receptor motif for GNRA loops (Fig. 1A), the activities of the mutant ribozymes with all possible GNRA loops in L2 were compared. The activities of the mutant ribozymes having a G or C as the second nucleotide N in a GNRA loop are shown in Figure 4B [note: the activity of L2(GAGA) is also shown as a standard to help compare Fig. 4A and B]. The mutants having GGRA or GCRA loops (Fig. 4B) were all less active than ones having GURA or GARA loops (Fig. 4A). The figures show that the activity was influenced by the second nucleotide N in G**N**RA (G**U**RA > G**A**RA > G**G**RA > G**C**RA) and also by the third nucleotide R in GN**R**A (GN**A**A > GN**G**A), indicating that the IC3 receptor

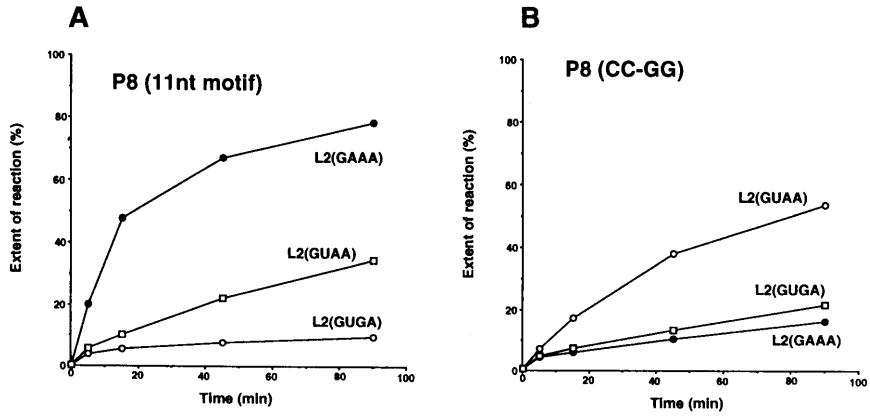

Figure 3. Effects of replacing a conserved motif in the P8 domain with previously known GNRA receptors. Time courses of mutant ribozymes under condition A. (**A**) Mutant ribozymes whose P8 domains have an 11 nt GAAA receptor motif. (**B**) Mutant ribozymes whose P8 domains have CC-GG base pairs that are the receptor motif specific for a GUAA loop.

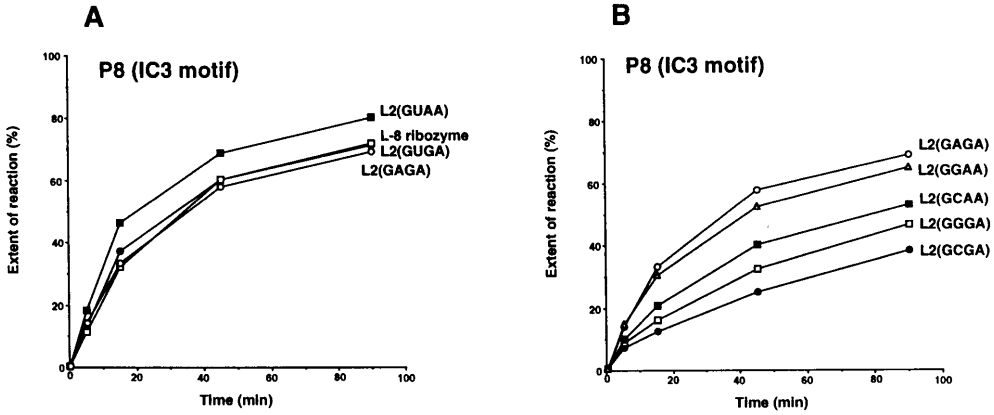

Figure 4. Effects of replacing the GAAA loop in the L2 region with other GNRA loops. Time courses of mutant ribozymes under condition A. (**A**) L-8 ribozyme (L2 loop is GAAA, closed circle) and its mutants whose L2 loops are GUAA (closed square), GUGA (open square) or GAGA (open circle). (**B**) L-8 ribozyme mutants whose L2 loops are GAGA (open circle), GGAA (open triangle), GCAA (closed square), GGGA (open square) or GCGA (closed circle).

discriminates the second and third nucleotides (N and R) in the GNRA loop. The discrimination of these nucleotides by the IC3 motif is less stringent than that by the 11 nt motif or CC-GG pairs (Figs 3 and 4).

An IC3 motif can substitute the 11 nt motif receptor in P6 in the *Tetrahymena* ribozyme

The *Tetrahymena* ribozyme has a large P5 extension consisting of P5a, P5b and P5c regions (19). The long-range interaction between the P5b region (L5b loop) and P6 domain utilizes a GAAA loop and 11 nt receptor motif (Fig. 5A; 2,5,20). We substituted the 11 nt motif in the P6a region of the *Tetrahymena* ribozyme with the IC3 motif or other sequences and tested the activity of the resulting mutants. By using a shortened form of the *Tetrahymena* ribozyme (termed the L-56 ribozyme; Fig. 5A), the 3′ splice site-specific hydrolysis reaction was attempted for the mutant L-56 ribozyme with an IC3 motif [L-56P6(IC3) mutant], CC-GG base pairs [L-56P6(CC-GG) mutant] or UU-AA base pairs [L-56P6(UU-AA) mutant] (Fig. 5B). Under the conditions we employed (40 mM Tris–HCl pH 8.3, 3 mM $MgCl_2$, 6 mM NaCl at 37°C), the IC3 mutant [L-56P6(IC3)] was more active than the CC-GG mutant but less active than the wild-type 11 nt motif (Fig. 6). The results demonstrate that the IC3 motif can function as a GAAA receptor in the *Tetrahymena* ribozyme. Consistent with the results from the mutant *Synechococcus* ribozymes, the affinity of the IC3 motif for a GAAA loop is weaker than that of the 11 nt motif but is stronger than that of CC-GG base pairs.

Comparison of GNRA–receptor interactions by RNA–RNA gel mobility shift assay

A separately prepared P5abc domain RNA of the *Tetrahymena* intron is able to form a stable RNA–RNA complex with a mutant intron lacking the domain (ΔP5abc intron) and this complex functions as a ribozyme consisting of two RNA molecules (14,21). This bimolecular ribozyme was employed for comparing the physical affinity between GNRA loops and their receptors (Fig. 5A). The gel mobility shift assays were performed by using mutants of the L-21 *Sca*I form of the ΔP5abc intron and P5abc RNA. An 11 nt motif in the ΔP5abc intron and a GAAA loop in

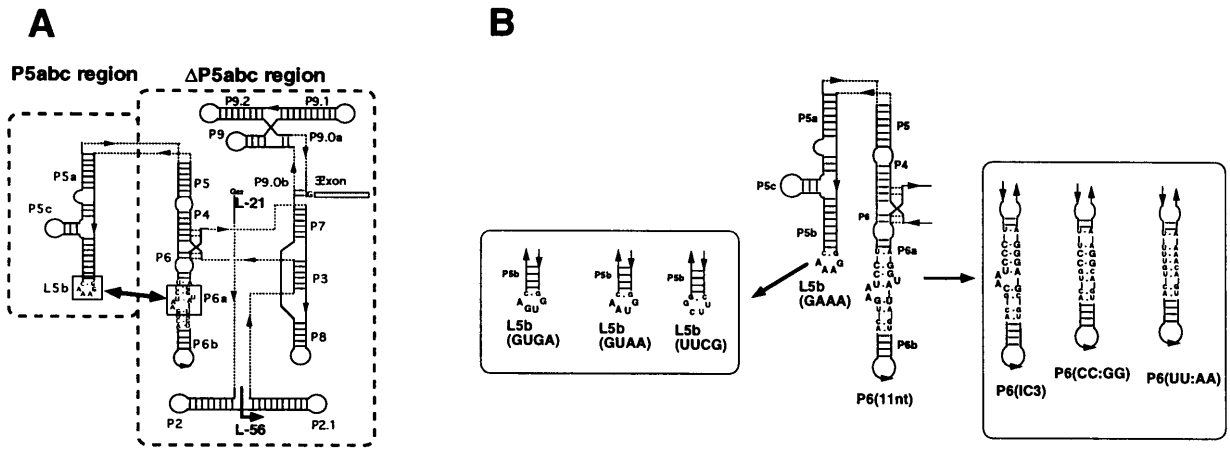

Figure 5. (**A**) The secondary structure of the L-21 ribozyme derived from the group I intron from the *Tetrahymena* LSU. A bold line with two black arrowheads indicates the interaction between L5b and P6a that utilizes GAAA and its 11 nt receptor. L-56 ribozyme that lacks the P1 and P2 regions is schematically shown in the figure. (**B**) Mutant *Tetrahymena* ribozymes whose L5b or P6 region was replaced with other tetraloops or their receptors.

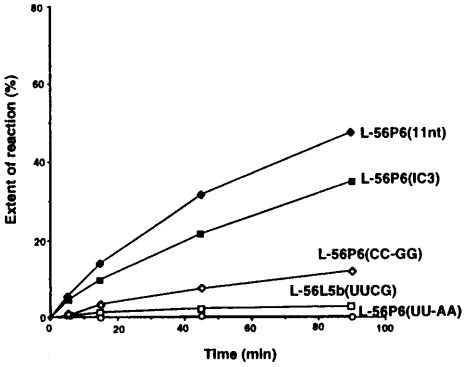

Figure 6. Effects of replacing the 11 nt GAAA receptor motif in P6 of the *Tetrahymena* ribozyme with other GNRA receptors. Time courses of 3′ splice site-specific hydrolysis reactions of the L-56 form of the *Tetrahymena* ribozyme having the 11 nt motif in P6a (closed diamond) and its three mutants whose GNRA receptors in P6a are IC3 motif (closed square), CC-GG pairs (open diamond) and UU-AA pairs (open circle), respectively. L-56L5b(UUCG) is a control variant in which a GAAA tetraloop in the L5b region is replaced with a UUCG tetraloop to disrupt the L5b × P6a interaction.

P5abc RNA were replaced with other GNRA receptor motifs and other tetraloops, respectively (Fig. 5B). In the presence of 5 mM Mg^{2+}, no complex formation was observed except for the combination of GAAA [P5abc-L5b(GAAA)] and the 11 nt motif [ΔP5abc-P6(11nt)] (data not shown). In the presence of 7.5 mM Mg^{2+}, a mutant ΔP5abc intron having an IC3 motif [ΔP5abc-P6(IC3)] formed a stable complex with P5abc RNA having either a GAAA or GUAA loop (Fig. 7A). Under the same conditions, the ΔP5abc intron having an 11 nt motif or CC-GG pairs was unable to form a stable complex with P5abc RNAs except for the combinations of P5abc-L5b(GAAA) and ΔP5abc-P6(11nt). In the presence of 10 mM Mg^{2+}, ΔP5abc-P6(IC3) formed a stable complex with the mutant P5abc having a GUGA loop (Fig. 7B). The result is consistent with the conclusion described in the case of the *Synechococcus* ribozyme in that the IC3 motif discriminates a GAAA from a GUAA or GUGA less stringently than the 11 nt motif or CC-GG pairs. Gel mobility shift assays using P5abc RNA indicate that differences in the affinity between a GAAA loop in L5b and various receptors in P6a (11 nt motif > IC3 motif > CC-GG pairs) is proportionally related to differences in the activity of corresponding versions of the L-56 form of the *Tetrahymena* ribozyme.

Functional dissection of the IC3 GNRA receptor

To identify the nucleotides in the IC3 motif that participate in recognizing a GNRA loop, we investigated a series of mutant *Synechococcus* ribozymes having mutations in the IC3 motif. First, a mutant in which conserved CCC-GGG base pairs at positions 3, 4 and 5 (note: base pairs in the P8 domain were numbered as shown in Fig. 1) were replaced with UUU-AAA pairs was prepared (Fig. 1C) and its activity was tested by attempting the hydrolysis reaction at the 3′ splice site (Table 1). The mutant was hardly active, indicating that the CCC-GGG segment participates in the interaction.

Table 1. Effects of base substitutions of conserved CCC-GGG base pairs in the IC3 motif

	% Cleavage in 90 min (or in 15 min)		
	L2(GAAA)	L2(GUAA)	L2(GUGA)
P8(IC3)	71 (37)	79 (46)	71 (32)
3/4/5U:A	**14 (5.4)**	**8.6 (4.1)**	**8.8 (4.0)**
3G:C	61 (27)	69 (37)	**41 (15)**
4G:C	**39 (12)**	63 (27)	64 (26)
5G:C	69 (37)	74 (44)	62 (22)
3/4G:C	**39 (13)**	64 (27)	59 (21)
3/5G:C	67 (30)	75 (36)	**42 (14)**
4/5G:C	**32 (9.2)**	**45 (13)**	**34 (8.4)**
3/4/5G:C	**27 (8.0)**	**51 (15)**	**25 (7.8)**

Reactions were carried out under condition A and mutants whose activity was considerably weaker than the P8(IC3) series are indicated in bold.

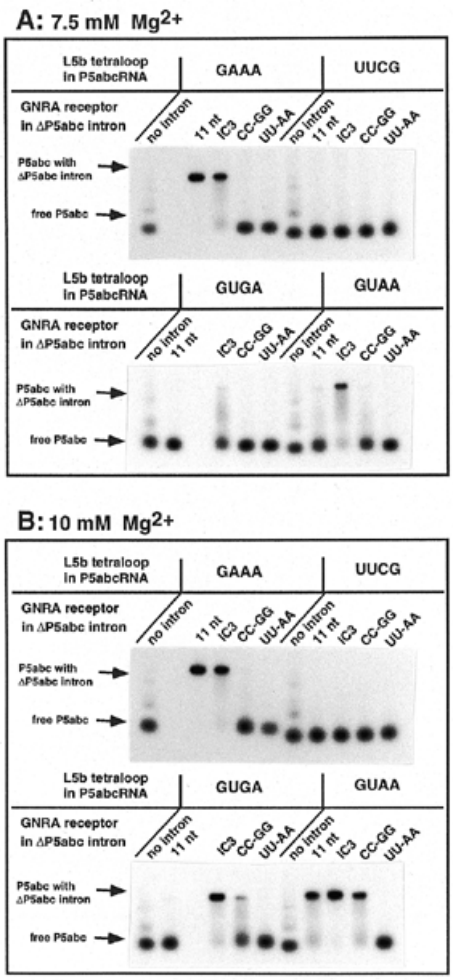

Figure 7. Gel mobility shift assays of P5abc RNAs and the L-21 form of ΔP5abc introns. Sequences of the wild-type and mutant P5abc RNAs are shown in Figure 5A and B. Sequences of the wild-type and mutant ΔP5abc introns are also shown in Figure 5A and B. (**A**) Gel mobility shift assay in the presence of 7.5 mM magnesium ions. (**B**) Gel mobility shift assay in the presence of 10 mM magnesium ions.

On the basis of these observations, we systematically replaced one, two or all three C-G base pair(s) at position 3–5 with a G-C base pair(s) (Fig. 1C) and tested its effect on the 3′ splice site hydrolysis reaction by employing GAAA, GUAA and GUGA loops (Table 1). The substitution at position 3 reduced the activity of the L2(GUGA) mutant moderately and those of the rest slightly. In a similar manner, substitution at position 4 reduced the activity of the L2(GAAA) mutant moderately and that of the rest slightly. The substitution at position 5 resulted in a slight decrease in activity for all three loops.

Mutants with substitutions of 2 or 3 bp at positions 3, 4 and/or 5 were prepared and their activities were tested. The mutant with double substitutions at positions 3 and 4 exhibited an activity similar to that of the one having a substitution at position 4. The mutant with double substitutions at positions 3 and 5 exhibited an activity similar to the one having the substitution at position 3. However, the mutant with double substitutions at positions 4 and 5 exhibited an effect different from that of either the one with single substitution at position 4 or position 5. The substitutions considerably decreased the activity of both the L2(GAAA) and L2(GUGA) mutants whereas it influenced the activity of the L2(GUAA) mutant to a lesser extent. The substitutions of all three C-G base pairs to G-C pairs lowered the activity of the resulting mutant to an extent similar to that of the mutant with double substitutions at positions 4 and 5. However, the mutant 3/4/5G-C was still distinctively more active than the 3/4/5U-A mutant (Table 1). In summary, it is conceivable that the substitutions at position 3 or 4 or double substitutions at positions 4 and 5 resulted in a considerable decrease in the affinity for GUGA and/or GAAA, but the affinity for the GUAA loop was influenced less severely. The results show that the IC3 receptor motif is tolerant of substitution(s) from C-G(s) to G-C(s) for receiving a GNRA loop.

The effect of the conserved base pairs at positions 6 (U:A) and 7 (C:G) was examined accordingly (Fig. 1C and Table 2). Substitutions at position 6 (U:A→G:C) or position 7 (C:G→G:C) resulted in a slight decrease in the activities for all three GNRA loops, suggesting that they are involved weakly or indirectly in the interaction (Table 2).

Table 2. Effects of introducing mutations into conserved nucleotides within the IC3 motif

	% Cleavage in 90 min (or in 15 min)		
	L2(GAAA)	L2(GUAA)	L2(GUGA)
P8(IC3)	71 (37)	79 (46)	71 (32)
6C:G	63 (28)	72 (39)	62 (25)
7G:C	66 (31)	66 (32)	55 (19)
ΔAA	**15 (5.4)**	53 (17)	**21 (6.8)**
GG	**43 (11)**	**47 (12)**	**32 (8.3)**
CC+7G:C	63 (29)	56 (23)	**38 (12)**

Reactions were carried out under condition A and mutants whose activity was considerably weaker than the P8(IC3) series are indicated in bold.

The effect of the conserved AA bulge (Fig. 1) was examined accordingly. As shown in Table 2, deletion of the AA bulge reduced the activity severely for the mutant with a GAAA or GUGA loop but less severely for the mutant with a GUAA loop. The mutants whose AA bulge was replaced with GG or CC (Fig. 1C) also exhibited a reduction in the activity except for the mutant with a GUAA loop, although the effect was rather weak when compared with that of the deletion mutant [Table 2; note: to prevent possible structural alterations predicted by the Zucker MFOLD program (23), base substitutions were introduced additionally at the stem region of the P8 domain of the mutants]. The results indicate that the AA bulge is likely to contribute to the mechanism of GNRA recognition but its role is unclear. Unfortunately, results obtained from the substitution experiments were insufficient to depict a three-dimensional model for the interaction between a GNRA loop and the IC3 receptor motif. However, it seems possible to determine the three-dimensional structure of an IC3 receptor complexed with a GNRA loop if the analysis by X-ray crystallography is attempted using a mutant P4–P6 domain RNA in which the 11 nt receptor motif is replaced with an IC3 receptor (5).

A perfect copy of the IC3 motif has not been observed in naturally existing RNAs other than group IC3 introns at present. However, the motif resembles a new class of synthetic GAAA

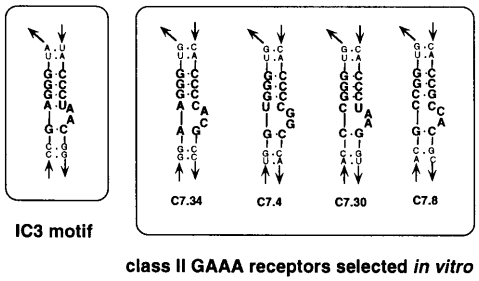

Figure 8. Comparison of the IC3 GNRA receptor and GAAA class II receptors obtained from *in vitro* selection (22).

receptors termed class II obtained from an *in vitro* selection experiment (22). The class II receptors share a consensus sequence CCC-GGG at the position corresponding to positions 3–5 of the IC3 receptor followed by an asymmetric internal loop (Fig. 8) (22). The CCC-GGG base pairs are conserved in the IC3 motif but another conserved AA bulge element is not conserved in the class II receptors. The specificity of one clone of class II receptor (C7.34) for GNRA loops seems similar to that of the IC3 motif. For example, C7.34 discriminates GUAA, GAGA or GGAA from GAAA less stringently than the 11 nt motif (22). The IC3 motif also discriminates GUAA, GUGA or GAGA from GAAA less stringently than the 11 nt motif. However, the specificities for GNRA loops are not precisely identical. C7.34 prefers GNRA loops in the order GGAA > GAAA > GUAA (22) whereas the IC3 motif prefers the loops in the order GUAA > GAAA > GGAA. Although further investigation is needed, it is tempting to propose that the class II and IC3 motifs share some parts of the mechanism for receiving a GNRA loop. (It has been noted by Costa and Michel that very few natural molecules with a structure similar to that of C7.34 exist; 22.)

ACKNOWLEDGEMENTS

We thank Dr M. Sugiura for the gift of plasmid pTL3 and Y. Naito, T. Shimizu and Dr Ruth Yu for critical reading of the manuscript. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas and Encouragement of Young Scientists from the Ministry of Education, Science, Sports and Culture, Japan.

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