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P5abc of the *Tetrahymena* ribozyme consists of three functionally independent elements

YOSHIKAZU NAITO, HIDEAKI SHIRAISHI, and TAN INOUE
Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606-01, Japan

ABSTRACT

P5abc domain of *Tetrahymena* LSU intron functions as an activator that is not essential for but enhances the activity of the ribozyme either when present *in cis* or when added *in trans*. This domain contains three regions (A-rich bulge, L5b, and L5c) that have been demonstrated to interact with the rest of the intron. Although these regions are presumably important for efficient activation, the role of each element is not understood in the mechanism of activation. We employed circularly permuted introns and examined the roles of each element. The results show that each of the three elements can activate the intron independently. We also found that a correlation between the activation by P5abc and the physical affinity of P5abc to the intron exists.

Keywords: activator; complex; group I intron; tertiary interaction

INTRODUCTION

The group I introns share a highly conserved core structure that is essential for the ribozymatic activity and several less conserved peripheral domains (Cech, 1990, 1993; Jaeger et al., 1996). The *Tetrahymena* LSU intron is a group I intron ribozyme that can catalyze site-specific transesterification and hydrolysis reactions in vitro (Cech et al., 1981; Kruger et al., 1982; Zaug et al., 1984). In this intron, a peripheral domain termed P5abc has been shown to function as an activator RNA (van der Horst et al., 1991). A hardly active mutant of the intron lacking P5abc (ΔP5abc intron) (Joyce et al., 1989) can be activated by the presence of a separately prepared RNA consisting of this domain (P5abc RNA). It has been shown that the two RNAs form a stable complex via tertiary interactions (van der Horst et al., 1991). The mechanism of the activation that determines the active form of a large ribozyme is not understood.

P5abc domain of the *Tetrahymena* intron comprises three stems (P5a, P5b, and P5c) and six single-stranded regions (A-rich bulge, L5b, L5c, J5a/5b, J5b/5c, and J5c/5a) (Fig. 1A). Three single-stranded regions, A-rich bulge, L5b, and L5c, have been shown to participate in tertiary interactions within a partial or entire intron that is presumably responsible for enhancing the catalytic activity.

Reprint requests to: Tan Inoue, Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606-01, Japan; e-mail: tan@kuchem.kyoto-u.ac.jp.

A-rich bulge consisting of mostly adenosine residues is conserved among P5abc domains in IC1 and IC2 subclasses and is often observed in IB and IC3 subclasses of group I introns (Collins, 1988; Michel & Westhof, 1990). Its size and the distance from P4 stem are highly conserved. It has been shown that removal of the bulge or entire P5abc reduces the activity of the *Tetrahymena* ribozyme to the same extent (Pace & Szostak, 1991), indicating that the bulge is the most important part for the mechanism of activation. The crystal structure of a portion of the *Tetrahymena* intron termed P4–P6 RNA, consisting of P4, P5, P5abc, P6, and P6ab domains, was determined (Cate et al., 1996a). In the crystal structure, A-rich bulge interacts with P4 and the regions within P5abc.

L5b that is highly conserved in only IC1 and IC2 subclasses has a distinctive sequence (Murphy & Cech, 1994), GNRA, that has been shown to form a stable tetraloop and participate in long-range interactions (Jaeger et al., 1994; Costa & Michel, 1995; Costa et al., 1997). Footprinting experiments of P4–P6 RNA revealed that L5b interacts with P6a-J6a/6b (Murphy & Cech, 1994), suggesting that this is important for determining the structure of the RNA. By using three-component ribozyme in which the intron is assembled via tertiary interactions, it has been demonstrated that a mutation that causes J6a/6b to base pair across the loop reduces the ribozymatic activity of *Tetrahymena* intron (Cate et al., 1996b).

L5c has been demonstrated to interact with L2 in the *Tetrahymena* intron and to be important for the ribozy-

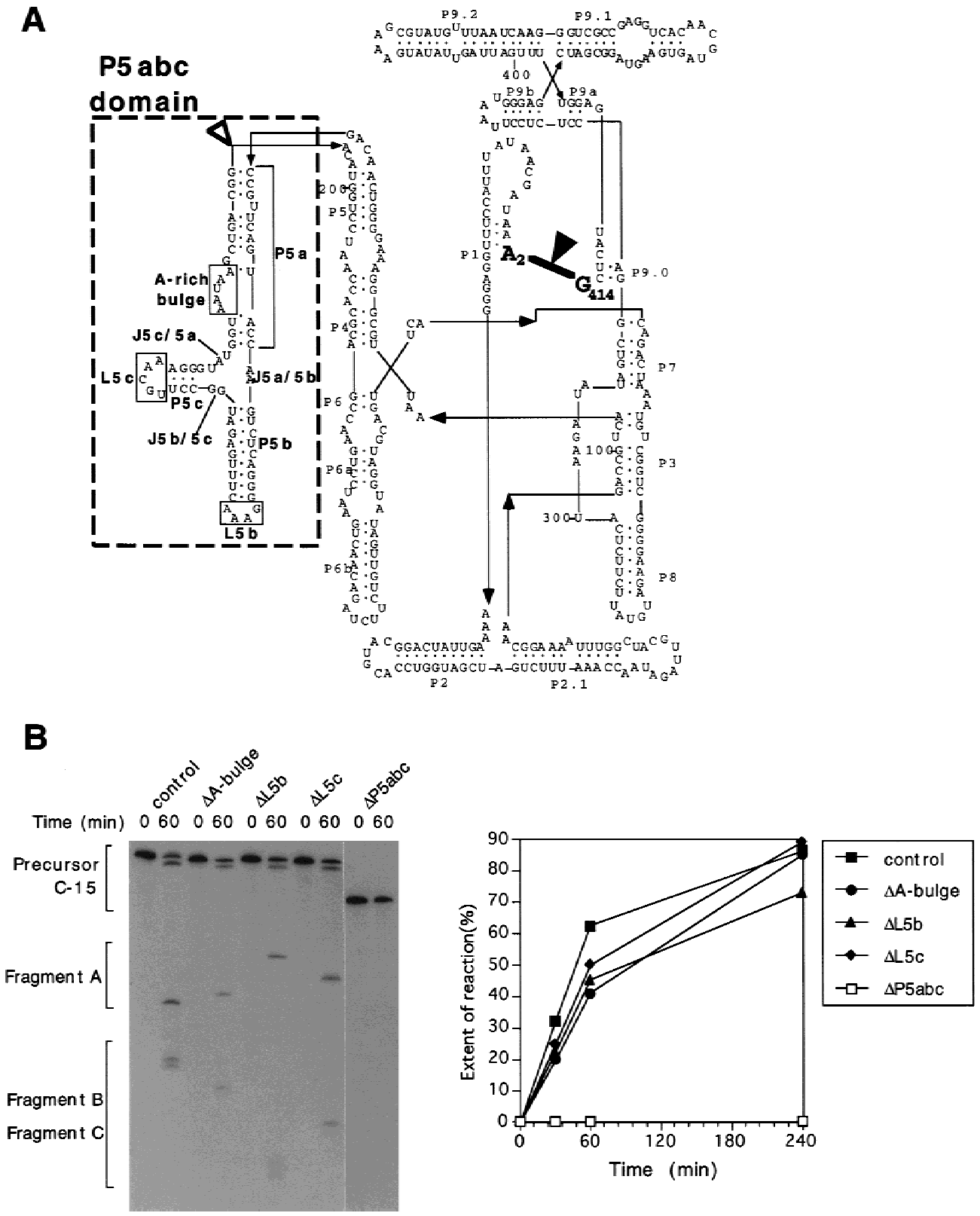


FIGURE 4. (*Legend on facing page.*)

matic activity of the intron at low magnesium ion concentration (2 mM) (Lehnert et al., 1996). By using UV crosslinking technique, we detected the link between L5c and L2 in the active form of the *Tetrahymena* intron (Y. Naito & T. Inoue, unpubl. data). L5c possesses no conserved primary sequence and the base complementarity between L5c and L2 has been found in only five IC1 introns (Lehnert et al., 1996).

The significant loss of activity by a deletion of A-rich bulge described above (Pace & Szostak, 1991) suggests a simple model for the activation by P5abc in which A-rich bulge is mainly responsible for the activation so that the contribution of L5b and L5c is only to assist the function of the bulge. This is supported by the fact that an RNA consisting of P5a alone can activate the ΔP5abc intron (van der Horst et al., 1991). However, the recent finding based on the crystal structure (Cate et al., 1996a) raises another possibility that the previous results might overestimate the role of A-rich bulge. The residues in A-rich bulge were found to interact within P5abc in P4–P6 RNA, showing that the bulge is also involved very much in determining the tertiary structure of P5abc (Cate et al., 1996a). This implies that the bulge might be setting the relative orientation of L5b and/or L5c that are contributing significantly to achieving efficient activation.

To investigate the roles of each element in the mechanism of activation, we employed circularly permuted introns in that the negative effects by deletion can be minimized. The results show that each element can independently activate the ribozyme and that the contribution of A-rich bulge dominates. In addition, we found that the physical affinity of P5abc to the intron is positively correlated with the activity of the intron by using gel-mobility shift assays and chemical modification techniques.

RESULTS

Three elements of P5abc and their role in activation

We investigated the roles of three elements in P5abc, which are A-rich bulge, L5b, and L5c, in the mechanism of the activation of the *Tetrahymena* intron. Circularly permuted mutants were employed in the experiments to avoid potential problems that are described below.

A186, which has been demonstrated as the most critical residue in A-rich bulge for the activation (Ikawa et al., 1997), interacts with J5b/5c (junction between P5b and P5c) according to recent analysis (Cate et al., 1996a), suggesting that the interaction is involved in determining the structure of P5abc domain. This raises a potential problem for analyzing the role of L5b and/or L5c in the absence of the bulge as follows. If the nucleotides at or near the ends of the bulge are simply ligated to construct a deletion mutant, it could accompany an alteration of the relative orientation of L5b and/or L5c due to the presence of a new chemical bond formed by the ligation. Circularly permuted introns were designed to avoid this potential problem.

A circular form of the *Tetrahymena* intron (Fig. 1A), in that 3′-terminal guanosine (G414) and 5′-terminal adenosine (A2) are linked, can be produced by the attack of G414 at the 5′ splice site (Inoue et al., 1986). The circular intron performs site-specific hydrolysis reaction at the circularized junction. We constructed circularly permuted *Tetrahymena* introns lacking A-rich bulge (ΔA-bulge), L5b (ΔL5c), L5c (ΔL5b), or entire P5abc domain (ΔP5abc) (Fig. 1A) and examined their activity. The mutants have no chemical bonds at the deletion sites, so that the potential problem can be avoided. A construct that has a nick between the nt G195 and A196 was employed as a control. The hydrolysis activity of the control was as efficient as that of the corresponding circular intron without the nick (data not shown).

The results of the hydrolysis reactions in the presence of 5 mM $MgCl_2$ are summarized in Figure 1B. The relative activities of ΔA-bulge, ΔL5b, or ΔL5c were 66, 73, or 80% (at the time point of 60 min) of the control, respectively, whereas ΔP5abc showed no activity under the same conditions. The mutants lacking A-rich bulge, L5b, or L5c still possess activity under the conditions where ΔP5abc is inactive. This indicates that L5b or L5c can activate the ribozyme in the absence of the bulge. Thus, it is conceivable that the three elements can function independently as an activator unit.

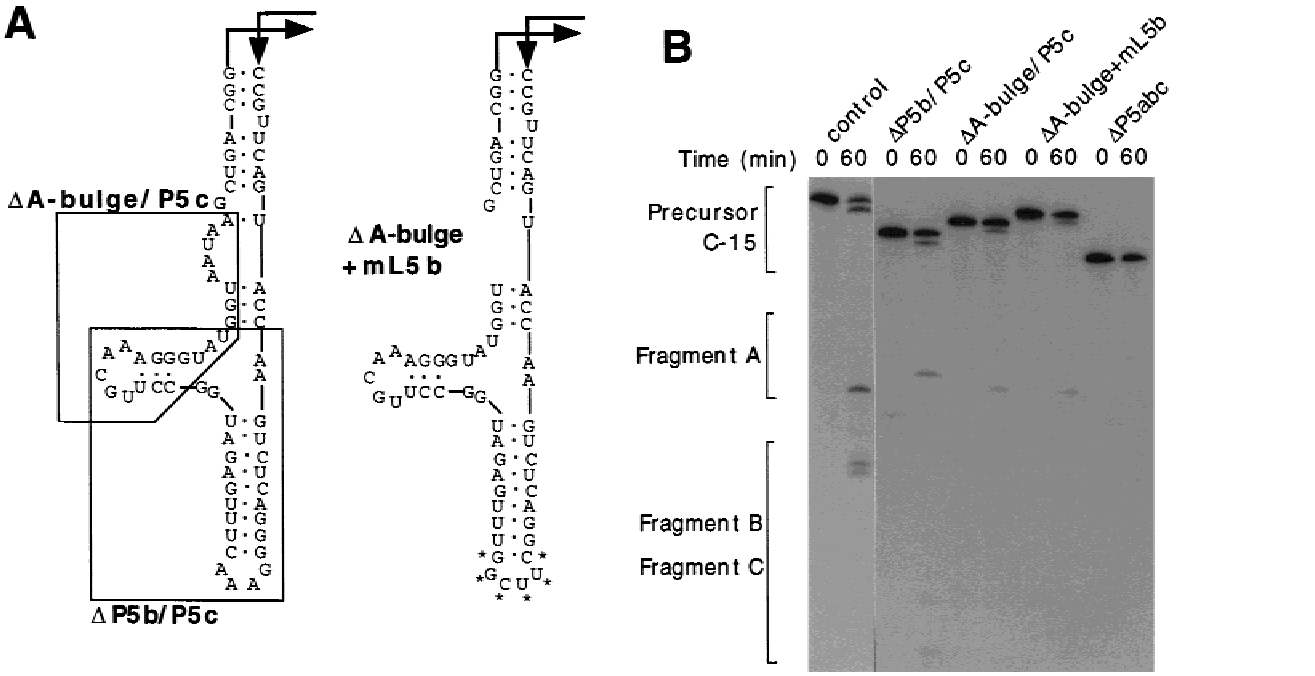
FIGURE 1. A: Secondary structure of the group I intron of *Tetrahymena thermophila* LSU rRNA. A box with broken line indicates P5abc domain. In the circularly permuted introns, G414 and A2 are connected by a phosphodiester bond (indicated by a filled arrowhead). Nucleotides deleted in circularly permuted introns (ΔA-bulge, ΔL5b, ΔL5c, and ΔP5abc) are in boxes. The control mutant has a nick between G195 and A196 (denoted by an open arrowhead). **B:** Hydrolysis reaction of circularly permuted introns. RNAs were incubated in 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$ at 30 °C for 60 min and electrophoresed on a 5% denaturing polyacrylamide gel (left). Precursor RNAs except ΔP5abc produced fragments A and B that correspond to the RNAs consisting of the nucleotides from 5′ end to G414 and from A2 to 3′ end, respectively. After the hydrolysis reaction at the junction between G414 and A2, the introns performed recyclization and reopening reactions at the phosphodiester bond between U15 and A16, resulting in C-15 introns that are 14 nt shorter than the precursors and fragments C that are 14 nt shorter than fragment B, as reported previously (Inoue et al., 1986). Time courses of the hydrolysis reaction are shown on the right.

To test the hypothesis that both L5b and L5c are independent activators, we constructed three mutants that have only one element of the three and tested their activity. They are ΔP5b/P5c (possessing only A-rich bulge), ΔA-bulge/P5c (possessing only L5b), and ΔA-bulge+mL5b (possessing L5c and an altered L5b) (Fig. 2A). Because the dual deletion of A-rich bulge and L5b is implausible, ΔA-bulge+mL5b lacking the bulge is forced to contain an altered L5b with the sequence CUUCGG, which forms a stable tetraloop closing with a C-G base pairing (Tuerk et al., 1988; Woese et al., 1990). The relative hydrolysis activities of ΔP5b/P5c, ΔA-bulge/P5c, and ΔA-bulge+mL5b were 49, 23, and 27% of the control, respectively (Fig. 2B), supporting the hypothesis that each element can activate the intron independently. Among the three elements, it was found that activation by A-rich bulge is most efficient.

A circularly permuted intron lacking A-rich bulge (ΔA-rich bulge) in this study retained the activity (66% of the control) as described. Pace and Szostak (1991) evaluated the role of A-rich bulge by analyzing a mutant intron in that A184 and G188 were directly connected so that the nucleotides between them were missing. This mutant exhibited complete loss of the activity. The difference between the results is presumably due to the alteration of the local structure that we discussed above for the design of circularly permuted introns.

Three elements in the formation of the RNA–RNA complex

As described, P5abc RNA forms a stable and active complex with the mutant intron lacking P5abc domain (ΔP5abc intron) (van der Horst et al., 1991). To analyze



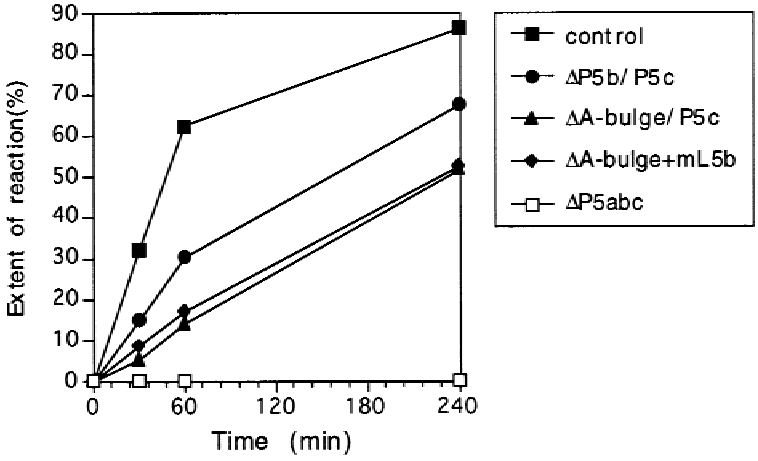


FIGURE 2. A: Circularly permuted introns possessing only one element. Nucleotides deleted for constructing ΔP5b/P5c and ΔA-bulge/P5c are boxed. ΔA-bulge+mL5b lacking A-rich bulge with the mutation in L5b (denoted by asterisks) is shown at left. **B:** Hydrolysis reaction of circularly permuted introns. RNAs were incubated in 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$ at 30 °C for 60 min and separated by electrophoresis on 5% denaturing polyacrylamide gels (above). Time courses of the hydrolysis reaction are shown (below).

the contribution of the three independent activator units in the complex formation, we performed chemical modification interference assays.

Under denaturing conditions, 5′-^{32}P end-labeled P5abc RNA was reacted with DEPC that modifies purines by N7-carboxyethylation or with hydrazine that modifies uridine, resulting in base removal. The reaction conditions were set to give one modification per one RNA molecule. After incubation with ΔP5abc intron under conditions where the intron forms a complex with P5abc, the complex consisting of the modified P5abc RNA and ΔP5abc intron was subject to gel-mobility shift assay under conditions where the complex is active (data not shown). The isolated P5abc RNA from the complex was cleaved at the modified residue with aniline and electrophoresed on a denaturing polyacrylamide gel (Fig. 3A). As a control, the modified P5abc RNA that was not included in the

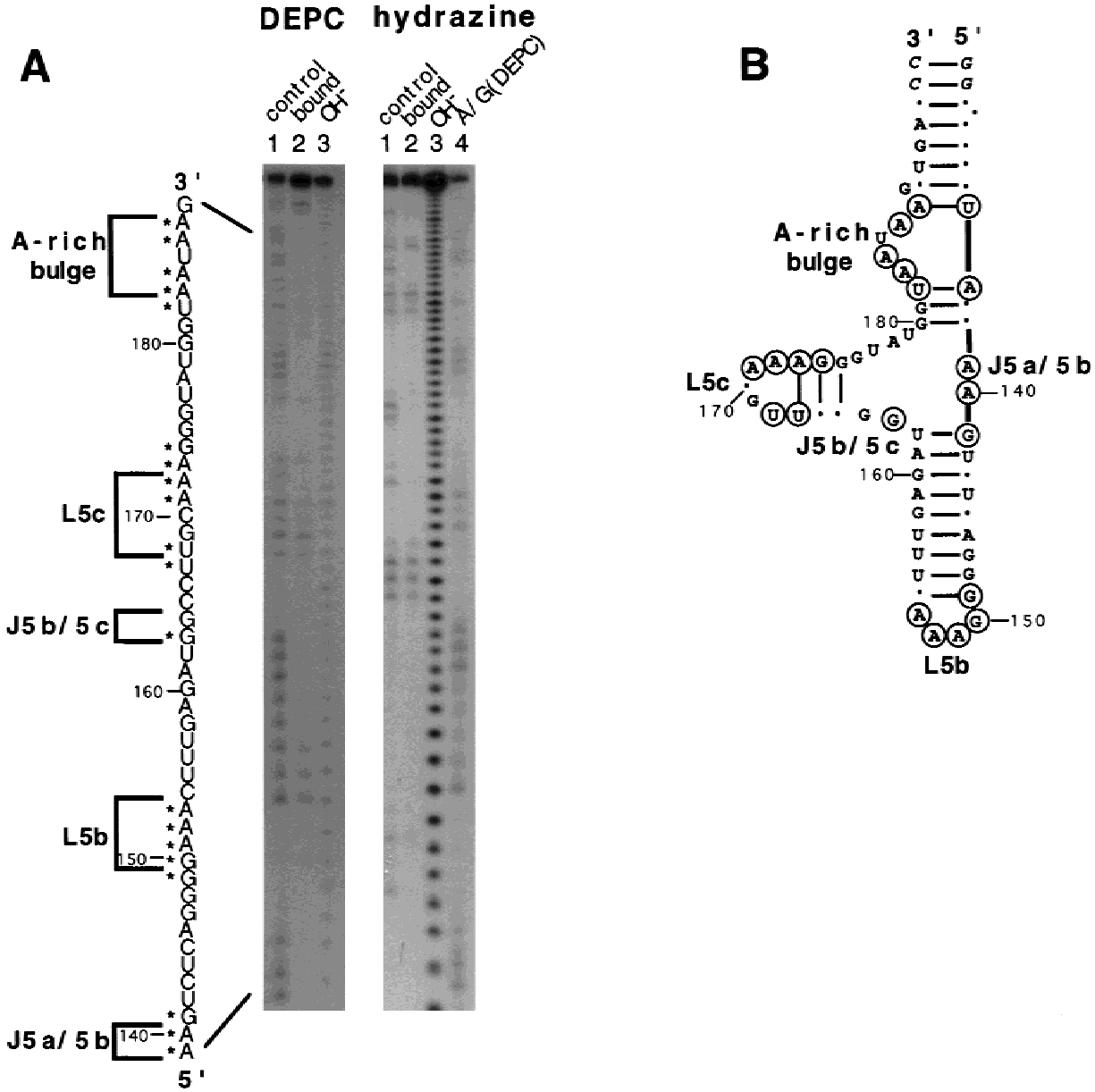


FIGURE 3. A: Modification interference experiments. Autoradiogram of aniline-induced fragments from the control P5abc (lane 1) and P5abc–ΔP5abc intron complex (lane 2) are shown. In lane 3, the fragment produced by hydrolysis of unmodified P5abc is shown, representing each residue of P5abc. Nucleotides that exhibited a significant difference between lane 1 and 2 are denoted by an asterisk on the primary sequence represented beside the gel. **B:** Summary of modification interference analysis. Nucleotides whose modification interferes with the complex formation are circled. Residues G180, G181, U142, and U162 are not circled, because the interference by the modification of these residues was not clearly observed in other experiments. Nucleotides between 5′ end and G134 as well as all cytidine residues that were not investigated in this assay are denoted by dots. For efficient transcription, the two closing base pairs of P5abc RNA are inverted from C-G to G-C (indicated in italic).

complex was also treated with aniline and electrophoresed on a denaturing polyacrylamide gel. Decreases of the intensity of the bands in the lane for P5abc from the complex compared with that in the control lane should show sites where the modifications interfere with binding. As summarized in Figure 3B, strong interferences were observed in five regions, which are, A-rich bulge, L5b, L5c, J5a/5b, and J5b/5c. Thus, it is conceivable that these regions are important for complex formation and/or forming the tertiary structure of P5abc that is required for complex formation.

The results can be evaluated by comparison with the previously reported data based on X-ray crystallography or other assay system. According to the crystal structure of P4–P6 domain of the intron (Cate et al., 1996a), A183, A184 (in A-rich bulge), A151, A152, and A153 (in L5b) have been shown to form hydrogen bonds with the residues outside the P5abc domain. A186, A187 (in A-rich bulge), A139, A140 (in J5a/5b), and G163 (in J5b/5c) have been shown to form hydrogen bonds within P5abc domain. One magnesium ion has been shown to coordinate to N7 group of G163 (in J5b/5c). G150 (in L5b) has been shown to make a hydrogen bond with A153 (in L5b) and is probably important for forming the GNRA tetraloop of L5b. A171 and A172 (in L5c) were shown to be in a stable local conformation termed A-A platform. Besides the crystal structure, a mutational analysis revealed that G169 (in L5c) has been demonstrated to base pair with C45 (in L2) (Lehnert et al., 1996), and U168 and A171 (in L5c) were proposed to base pair with A46 and U43 (in L2), respectively. It was reported that base substitutions of A184, A186, or A187 (in A-rich bulge) to pyrimidine lowered the efficiency of splicing reaction in the presence of 5 mM $MgCl_2$ (Pace & Szostak, 1991). It has been shown that both A171 and A172 (in L5c) were modified by DMS in P4–P6 RNA, but not in the intron (L-21 *Sca* I intron). A152, A153 (in L5b), and A184 (in A-rich bulge) were modified by DMS in a mutant P4–P6 RNA in that J5/J5a are base paired, but not in P4–P6 RNA. A151 (in L5b) was more strongly modified in this mutant than in P4–P6 RNA (Murphy & Cech, 1994).

The results are consistent with the notion that the interactions of A-rich bulge, L5b, and L5c with the rest of the intron are important for complex formation, indicating that the structure of P5abc in the crystal reflects the active form of P5abc. Most residues identified in this study are equal to the ones that are expected to be critical for forming the structure of the intron. Although one exception is seen for G169 (in L5c), this can be explained by assuming that the contribution of G169 on L5c-L2 interaction is smaller than the other three residues in L5c proposed to base pair with residues in L2 or that the modification by DEPC can still allow the original interaction of G169.

The relationship between the *trans*-activation and the stability of the complex consisting of P5abc RNA and ΔP5abc intron

Three elements in P5abc are involved in the mechanism of activation and involved in tertiary interactions with the rest of the intron as described above. To investigate further the relationship between the stability of the complex and the *trans*-activation, we constructed P5abc RNAs with modifications at L5b (mL5b RNA) or L5c (mL5c RNA) and examined their ability to activate ΔP5abc intron by attempting gel-mobility shift and *trans*-activation assay.

Both mL5b and mL5c RNA have a stable stem-loop structure consisting of CUUCGG instead of L5b and L5c, respectively (Fig. 4A). The new stem-loop is incapable of maintaining the original long-range interactions. The complex formation of ΔP5abc intron with the mutant P5abc RNAs was investigated by using gel-mobility shift assay. In the presence of 5 mM Mg^{2+}, P5abc RNA formed a stable complex with ΔP5abc intron, whereas mL5b RNA formed detectable amounts of complex and mL5c RNA formed no complex (Fig. 4B, above). However, in the presence of 15 mM Mg^{2+}, 85% of mL5b RNA and 12% of mL5c RNA formed the complex (Fig. 4B, below).

We next investigated the *trans*-activation by the mutant P5abc RNAs. In the presence of 5 mM $MgCl_2$, mL5b RNA (10 μM) weakly activated ΔP5abc intron (14% of that by P5abc RNA) and mL5c RNA hardly activated the intron, suggesting the importance of L5b and L5c in the activation (Fig. 4C, above). However, in the presence of a higher concentration of $MgCl_2$ (15 mM), both mL5b and mL5c RNA activated the intron more efficiently than P5abc RNA (Fig. 4C, below). Thus, under the conditions where the complex of ΔP5abc intron with the mutant P5abc RNA can be formed, the intron is efficiently activated by the derivatives. In the presence of 15 mM $MgCl_2$, the activation by the derivatives was less efficient than that by the original P5abc RNA if their concentration is lowered to 0.1 μM (data not shown). The results suggest that the affinity between P5abc and ΔP5abc intron directly determines the activity of the intron.

DISCUSSION

Our analysis revealed that the elements of P5abc function independently in activating the intron. On the basis of previous studies, it is reported that A-rich bulge, L5b, and L5c interact with P4 (Cate et al., 1996a), P6a-J6a/6b (Murphy & Cech, 1994), and L2 (Lehnert et al., 1996), respectively, in the active form of the intron. In addition, the clear relationship is seen between the activity of the ΔP5abc intron–P5abc RNA complex and the stability of the complex that is accomplished by interactions of the three elements. By combining these,

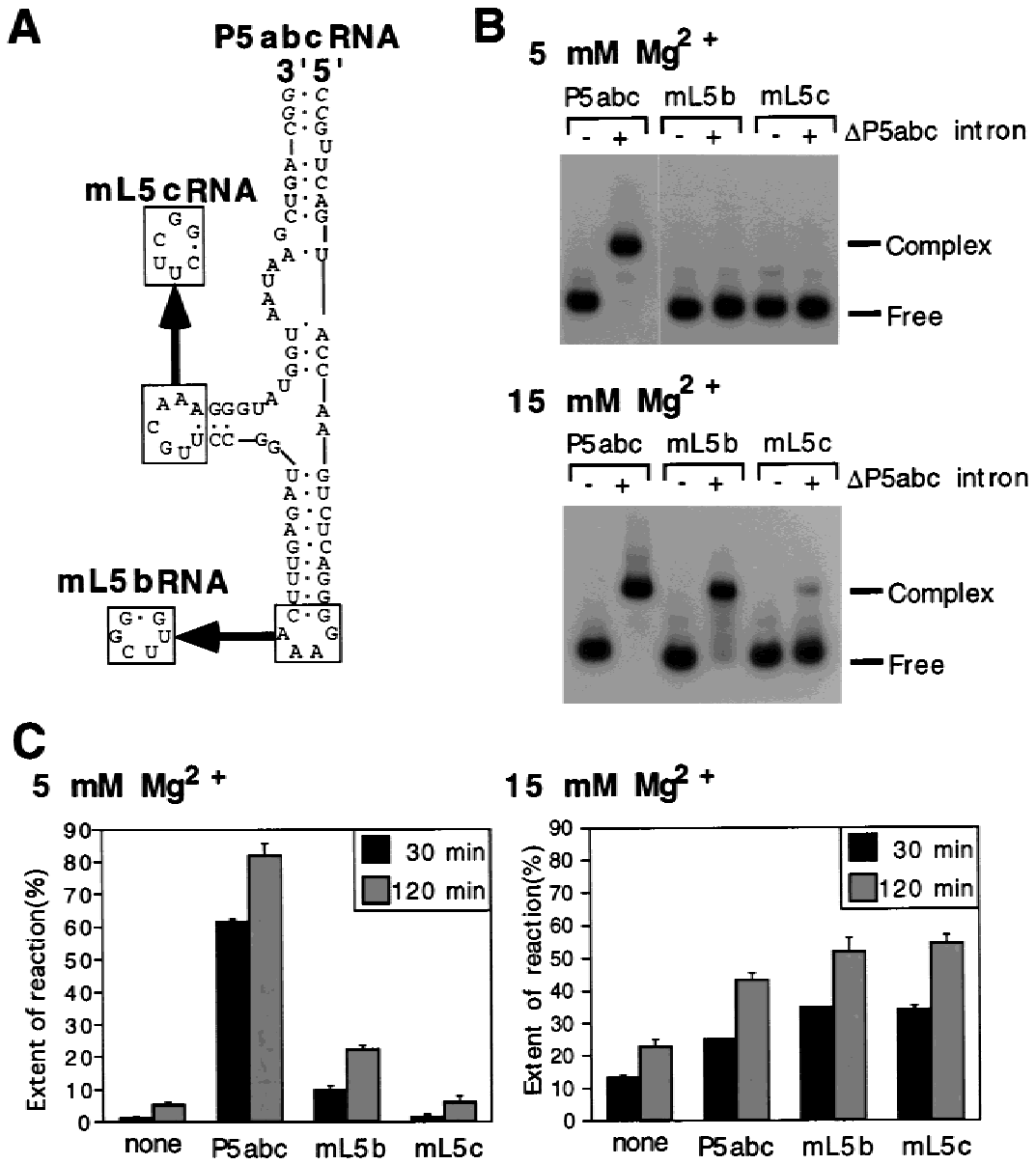


FIGURE 4. A: Secondary structure of P5abc, mL5b, and mL5c RNA. Altered sequences are boxed. **B:** Gel-mobility shift assay of P5abc, mL5b, and mL5c RNA performed in the presence of 5 mM Mg^{2+} (above) or 15 mM Mg^{2+} (below). **C:** *Trans*-activation by P5abc, mL5b, or mL5c RNA in the presence of 5 mM $MgCl_2$ (left) or 15 mM $MgCl_2$ (right). 5′ Exon·ΔP5abc *Sca* I intron were incubated in the mixture containing 10 μM of P5abc, mL5b, mL5c RNA, or no additional RNA. The fraction corresponding to the product of the first step of self-splicing reaction was quantitated.

we conclude that the efficient activation by P5abc is attributed to the integration of the three independent long-range interactions.

Our observation that each element functions independently helps to understand the mechanism of activation in detail. The structural studies strongly indicate that A-rich bulge and L5b are involved in the long-range interactions within P4–P6 domain and are important for the folding of the domain (Murphy & Cech, 1994). The interactions should bring two possible effects for the P4–P6 domain. (1) The bulge and/or L5b alter the local structure that modifies the activity. (2) The interactions help to stabilize the tertiary structure of the catalytic core or parts of it. The mechanism of the activation by A-rich bulge is likely to be the former case because P5a RNA can activate the ΔP5abc intron *in trans* (van der Horst et al., 1991). For L5b, we cannot deny either one of the two possibilities at present. In contrast to A-rich bulge and L5b, L5c activates the ribozyme via long-range interactions with the rest of the intron, as it has been demonstrated that L5c interacts with L2 located outside the P4–P6 domain (Lehnert et al., 1996). It remains to be determined whether this interaction stabilizes the core region or assists the proper docking of P1 to the active site, as in the case of P2–P8 interaction in GIA introns (Peyman, 1994).

Our results show that A-rich bulge, L5b, or L5c can be deleted without significant loss of the activity in the presence of 5 mM of $MgCl_2$, demonstrating that the *Tetrahymena* intron can form an active structure in the absence of one of these elements. The result is consistent with the previous report of the structural analysis in that the mutations in A-rich bulge or L5b have no effect on the stability of the structure of the ribozyme (L-21 *Sca* I intron) in the presence of 2 mM $MgCl_2$ (Laggerbauer et al., 1994). However, it was reported that nucleotide substitutions in A-rich bulge or L5b result in a complete alteration of the original structure of P4–P6 RNA even in the presence of 13 mM of $MgCl_2$ (Murphy & Cech, 1994). Although P4–P6 RNA itself is sensitive to the loss of the interaction of either the bulge or L5b, it seems that the intron is assured to form the active form in its absence under the conditions we employed. This implies that the long-range interactions between P4–P6 domain and the rest of the intron contribute to the further stabilization of the domain or that the structure of the isolated P4–P6 RNA is not exclusively identical to the corresponding local structure in the intron.

The P5abc RNA derivatives with substitution in either L5b or L5c can hardly activate the ΔP5abc intron *in trans* in the presence of 5 mM $MgCl_2$. In contrast, the circularly permuted introns with deletion in the corresponding regions still retain significant levels of the activity under similar reaction conditions. The difference of the activity can be explained by assuming that the intramolecularly integrated P5abc in the circularly permuted introns is equivalent to the condition where the extremely high concentration of P5abc exists for the *trans*-activation. If so, the observed tolerance for the circularly permuted mutants can be accomplished because the affinity is much less influential in the *cis*-activation.

L5b and L5c can function in the absence of the other two elements. A-rich bulge is the most highly conserved element in P5abc of IB and IC subclasses (Collins, 1988; Michel & Westhof, 1990) of group I intron. In contrast, L5b is conserved only in IC1 and IC2 (Murphy & Cech, 1994) and the base complementarity between L5c and L2 can be found in only five IC1 introns (Lehnert et al., 1996). In this context, one can imagine that the *Tetrahymena* LSU intron acquired A-rich bulge first in its evolution and, at the later stage, L5b and L5c were added to complete the architecture of P5abc. It is interesting to note that L5b and L5c exist as physically independent activator domains from A-rich bulge.

MATERIALS AND METHODS

Plasmids

For preparation of circularly permuted introns, a plasmid termed pTZIVSU5H was constructed from pTZIVSU (Williamson et al., 1989) by using in vitro mutagenesis technique. In this plasmid, an additional sequence containing the upper half of the *Tetrahymena* intron (from A2 to U239) was tandemly inserted after G414 of *Tetrahymena* intron in pTZIVSU. pmL5b and pmL5c that have substitutional mutation at L5b or L5c, respectively, with CUUCGG were constructed from pP5abc (Williams et al., 1992) by using the PCR method described previously (Imai et al., 1991). pL-21ΔP5abc that has deletion of 5′ exon and the sequence from A2 to T21 of the *Tetrahymena* intron was constructed from pTZIVSU by using the PCR method (Imai et al., 1991).

Preparation of nucleic acids

Templates of circularly permuted introns used for transcription were prepared through PCR amplification by using pTZIVSU5H that was digested with *Pvu* II as a template. The oligonucleotides used were as follows:

P1, TAATACGACTCACTATAGACATGGTCCTAACCAC GCAGC;
P2, TAATACGACTCACTATAGCTGACGGACATGGTCCTAA;
P3, TAATACGACTCACTATAGCTTTGAGATGGCCTTGCAA;
P4, TAATACGACTCACTATAGAGGGTATGGTAATAAG CTGAC;
P5, TAATACGACTCACTATAGGTAATAAGCTGACGGA CATGGT;
P6, CCGTCAGCTTATTACCATA;
P7, ACCATACCCTTTGCAAGGCCA;
P8, CCCTGAGACTTGGTACTGAACG;
P9, AGGCCATCTCAAAGTTTCCC;
P10, GGTACTGAACGGCTGTTGAC;
P11, CCATCTCAAAGTTTCCCC;
P12, ACCATACCCTTTGCAAGGCCATCTCAAACCGAAG CCTGAGACTTGGTACTGAA;
P13, CTGTTGACCCCTTTCCCGCAA

(T7 promoters are underlined). The template DNAs for control, ΔA-bulge, ΔL5b, ΔL5c, ΔP5b/P5c, ΔA-bulge/P5c, ΔA-bulge+mL5b, and ΔP5abc RNA were amplified through PCR by employing the primers (P1+P6), (P2+P7), (P3+P8), (P4+P9), (P5+P10), (P2+P11), (P2+P12), and (P1+P13), respectively.

The templates for 5′ exon·ΔP5abc *Sca* I intron and L-21ΔP5abc *Sca* I intron were prepared by digesting pΔP5abc (Joyce et al., 1989) and pL-21ΔP5abc with *Sca* I. The templates for P5abc RNA, mL5b RNA, and mL5c RNA were prepared by digesting pP5abc, pmL5b, and pmL5c with *Sma* I.

The uniformly labeled RNAs were prepared by in vitro transcription with T7 RNA polymerase and [α-^{32}P] ATP and purified by electrophoresis on polyacrylamide gels containing 8.3 M urea. Unlabeled L-21 ΔP5abc *Sca* I intron, P5abc, mL5b, and mL5c RNA were transcribed in the absence of [α-^{32}P] ATP. For modification interference assay, P5abc RNA having unphosphorylated 5′ end was transcribed in the buffer containing excess CpG over GTP to introduce CpG at the first nucleotide of the transcript (Axelrod & Kramer, 1985).

Ribozyme activity assays

Hydrolysis reactions were performed in the mixture containing uniformly labeled circularly permuted introns (approxi-

mately 5×10^3 cpm), 50 mM Tris-HCl, pH 8.5, and 5 mM $MgCl_2$ at 37 °C for 30, 60, or 240 min. *Trans*-activation assays were performed in the mixture containing uniformly labeled 5′ exon·ΔP5abc *Sca* I intron (approximately 5×10^3 cpm, less than 50 nM), 50 mM Tris-HCl, pH 7.5, 5 or 15 mM $MgCl_2$, and 200 μM GTP in the presence of various concentrations of unlabeled P5abc, mL5b, or mL5c RNA at 30 °C for 30 or 120 min. The reactions were terminated by addition of two volumes of stop solution (90% formamide, 50 mM EDTA, 0.02% BPB, and 0.02% XC). All reaction mixtures were loaded onto 5% polyacrylamide gels containing 8.3 M urea. The gels were exposed on X-ray film and quantitated by using a Bio-Image Analyzer BAS-100 (Fuji Film).

Gel-mobility shift assay

Uniformly labeled P5abc, mL5b, or mL5c RNA (approximately 5×10^3 cpm, 100 nM) was incubated with unlabeled ΔP5abc intron (10 μM) in the buffer containing 50 mM Tris-HCl, pH 7.5, and 5 or 15 mM $MgCl_2$ at 30 °C for 60 min. In this assay, the ΔP5abc intron that lacks the first 21 nt and last 5 nt (L-21ΔP5abc *Sca* I) was used. The mixtures were electrophoresed on 5% polyacrylamide gels containing 50 mM Tris-HOAc, pH 7.5, and 5 or 15 mM $Mg(OAc)_2$. The gels were exposed to X-ray film and quantitated by using a Bio-Image Analyzer BAS-100.

Modification interference analysis

Unlabeled P5abc RNA was 5′ end-labeled by using [γ-^{32}P] ATP and T4 polynucleotide kinase. DEPC and hydrazine modification were performed as described by Peattie (1979). For purine modification, 1 μL of DEPC was added to 150 μL of the solution containing the 5′ end-labeled P5abc RNA (0.4 pmol, approximately 10^5 cpm), 10 μg of yeast tRNA, 50 mM sodium acetate, pH 4.0, and incubated at 90 °C for 5 min. For the uracil modification, 5 μL of hydrazine was added to 5 μL of water containing the 5′ end-labeled P5abc RNA (0.4 pmol, approximately 10^5 cpm), 10 μg of yeast tRNA, and incubated on ice for 4 min. The modified RNAs were recovered by ethanol precipitation.

The modified P5abc RNAs (0.2 pmol, approximately 5×10^4 cpm) were incubated in 5 μL of the solution containing 50 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$ in the presence of 0.1 μM of L-21ΔP5abc *Sca* I intron at 30 °C for 60 min. The mixtures were electrophoresed on 4% polyacrylamide gels containing 50 mM Tris-HOAc, pH 7.5, and 5 mM $Mg(OAc)_2$. Bands containing P5abc–intron complex were recovered from the gel. As a control, the modified P5abc RNA was incubated in the absence of L-21ΔP5abc *Sca* I intron, electrophoresed on a 4% polyacrylamide gel, and recovered from the gel as described above.

To cleave the RNAs at modified residues, the modified RNAs were dissolved in 20 μL of 1 M aniline acetate, pH 4.5, and incubated at 60 °C for 20 min. For preparation of partially hydrolyzed P5abc that represents every nucleotide within P5abc in the sequencing gel, the 5′ end-labeled P5abc RNA was partially hydrolyzed by incubation in the buffer containing 50 mM sodium bicarbonate, pH 9.0, and 1 mM EDTA at 90 °C for 10 min. The cleaved RNAs were precipitated by ethanol twice, dissolved in loading buffer (50% formamide, 0.025% BPB, and 0.025% XC) and loaded onto 10% polyacrylamide sequencing gels (0.35-mm thick) containing 8.3 M urea. The gels were exposed to X-ray film and relative intensities of each band were quantitated by using a Bio-Image Analyzer BAS-100.

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