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Reconstitution of a group I intron self-splicing reaction with an activator RNA

(*Tetrahymena*/ribozyme/RNA enzyme/RNA–RNA interaction)

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ABSTRACT **The self-splicing rRNA intron of *Tetrahymena thermophila* belongs to a subgroup of group I introns that contain a conserved extra stem–loop structure termed P5abc. A *Tetrahymena* mutant precursor RNA lacking this P5abc is splicing-defective under standard conditions (5 mM $MgCl_2$/200 mM NH_4Cl, pH 7.5) *in vitro*. However, the mutant precursor RNA by itself is capable of performing the self-splicing reaction without P5abc under different conditions (15 mM $MgCl_2$/2 mM spermidine, pH 7.5). We have investigated the functional role of the P5abc in the mechanism of the self-splicing reaction. When an RNA consisting of the P5abc but lacking the rest of the *Tetrahymena* intron is incubated with the mutant precursor, the self-splicing reaction proceeds highly efficiently under standard conditions (5 mM $MgCl_2$/200 mM NH_4Cl, pH 7.5). Two steps of the bimolecular self-splicing reaction can be performed accurately by a shortened precursor RNA containing all essential components required in the self-splicing reaction and an activator RNA consisting of the P5abc. Gel-mobility-shift assays suggest that two molecules associate by a direct RNA·RNA interaction during the splicing reaction. The results imply that there might exist other small RNAs whose role is to activate ribozymes.**

All group I introns contain the conserved sequence elements termed P, Q, R, and S and a conserved element termed the internal guide sequence (Fig. 1). These elements have been shown to be responsible for the self-splicing activity (2, 4). Based on another conserved feature, these introns can be divided into two subgroups that are known as group IA and group IB introns (4–6) (Fig. 1 *A* and *B*). Group IA introns have an extra stem–loop or a pair of stem–loops between the base-paired segments P7 and P3. In group IB introns, P7 is immediately followed by P3, but many of them have an extension of P5 that is not found in group IA. The extension of P5, characteristic of group IB introns, contains sequence elements that are sufficiently conserved to permit alignment. It has been pointed out that this region always contains five unpaired bases forming an adenine-rich bulge separated from the P–Q pairing by a fairly constant number of nucleotides (Fig. 1 *A* and *C*) (3). The self-splicing group I intron RNA of *Tetrahymena thermophila* (7) belongs to group IB. We have shown (2) that a mutated *Tetrahymena* precursor RNA that lacks the extension of P5 (P5abc) (Fig. 1*C*) is not active under standard splicing conditions (5 mM $MgCl_2$/200 mM NH_4Cl, pH 7.5). However, this precursor RNA can be spliced accurately in the presence of 2 mM spermidine and a higher magnesium concentration (15 mM) (2). This demonstrates that P5abc does not play an essential role in the mechanism of self-splicing reaction. It is therefore conceivable that P5abc contributes to arrange the structure of the catalytic core of the intron RNA into its active form by a direct RNA·RNA interaction.

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MATERIALS AND METHODS

Construction of ΔP5abc RNA and P5abc RNA. ΔP5abc RNA was prepared as described (2, 5). P5abc RNA and P5a RNA were prepared as follows. The *Hin*cII–*Ava* II fragment containing bases from A-122 to C-204 of the *Tetrahymena* group I intron was treated with the Klenow fragment of DNA polymerase I to fill out the end. The resulting DNA was ligated into a pTZ18U plasmid (United States Biochemical) that had been linearized with *Sma* I. A clone with the correct sequence was digested with *Xba* I and transcribed *in vitro* with T7 RNA polymerase (5) for preparation of P5abc RNA. The plasmid containing the sequence of P5abc region was used as a template for the preparation of P5a RNA as described (2). Two oligonucleotides, 5′-GCT TAT TAC CAT ATA TTG GTA CTG AAC G-3′ and 5′-GGG AAT TGA CCA TGT CCG T-3′, were employed as a mutator and terminator, respectively. The transcribed RNAs were electrophoresed on 5% or 10% polyacrylamide gels containing 8 M urea, then eluted, and ethanol-precipitated. Gel filtration on Sephadex G-25 columns was performed prior to the experiments for all RNAs employed in the experiments.

Self-Splicing Assay. Uniformly ^{32}P-labeled ΔP5abc pre-RNA {5′ exon [48 nucleotides (nt)]–intron (362 nt)–3′ exon (45 nt)} (6) was employed throughout the experiments. P5abc RNA (117 nt) consisting of a portion of the intron RNA from A-122 to C-204 (83 nt) contains an extra 21 nt (5′-GGGAAUUCGAGCUCGGUACCC-3′) at the 5′ end and 14 nt (5′-GGGGAUCCUCUAGA-3′) at the 3′ end. In P5a RNA, the nucleotides between A-140 and A-178 are replaced by 3 nt, UpApU. The precursor RNA (<10 nM) and various concentrations of the small RNAs (P5abc or P5a) were incubated with 5 mM $MgCl_2$/200 mM NH_4OAc/50 mM Tris·HCl, pH 7.5, at 30°C for 2 hr unless otherwise stated and electrophoresed on a 5% polyacrylamide/8 M urea gel. Quantitation of band radioactivity was done with a scintillation counter.

Gel-Mobility-Shift Assay. The ΔP5abc pre-RNA (<10 nM) and various concentrations of a small RNA were mixed at 4°C in the presence of 5 mM $MgCl_2$/200 mM NH_4OAc/50 mM Tris·HCl, pH 7.5, and electrophoresed on a 5% polyacrylamide gel containing 10 mM $Mg(OAc)_2$ (pH 7.5), as described by Fedor and Uhlenbeck (8).

RESULTS

To investigate whether P5abc can activate the splicing-defective precursor RNA in trans, we incubated a mutant precursor RNA (ΔP5abc pre-RNA) (Fig. 1*C*) (2) that lacks the P5abc stem–loop structure with an RNA (P5abc RNA) repre-

Abbreviation: nt, nucleotide(s).

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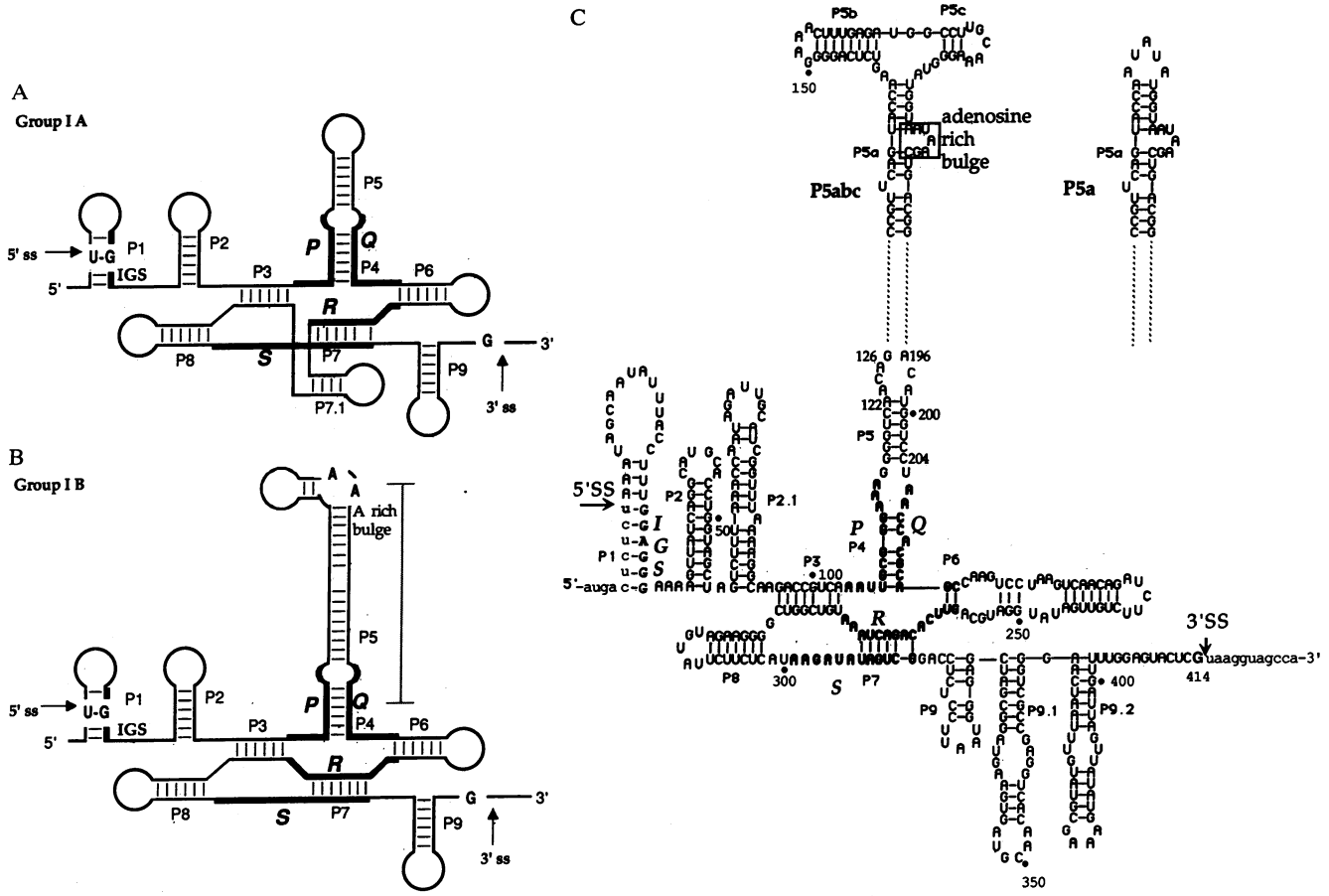

FIG. 1. Secondary structures of group I intron RNAs (1). Thick lines and the boldface nucleotides show the conserved elements that are required in the mechanism of the self-splicing reaction (2). 5'ss, 5' splice site; 3'ss, 3' splice site. (*A*) Simplified presentation of the secondary structure of group IA intron RNA. A conserved stem–loop structure P7.1 is emphasized. (*B*) Simplified presentation of the secondary structure of group IB intron RNA. A conserved stem–loop structure P5abc is emphasized. The bar indicates the constant distance between the P–Q pairing and the conserved adenine-rich bulge (3). (*C*) Secondary structure of the *Tetrahymena* group I intron RNA. Dotted lines show the region where P5abc RNA and ΔP5abc precursor RNA are disconnected. G-126 and A-196 are directly connected for ΔP5abc precursor RNA (2). A-122 and C-204 are the first and last nucleotide in the intron portion of P5abc RNA. The sequence of P5a RNA is illustrated.

senting the P5abc stem–loop structure under standard splicing conditions (5 mM $MgCl_2$/200 mM NH_4OAc, pH 7.5). Fig. 2*A* shows that the self-splicing reaction proceeds efficiently in the presence of P5abc RNA. The yield of the products of the self-splicing reaction increases dramatically with an increase in the concentration of P5abc RNA in the range between 0.1 μM and 5 μM (Fig. 2*A*, lanes b–h). Compared to the splicing reaction with the optimal spermidine conditions (15 mM $MgCl_2$/2 mM spermidine, pH 7.5) (Fig. 2*A*, lane k) (2), P5abc RNA is capable of activating the defective intron RNA in a much more efficient manner. When 5 μM tRNA was employed instead of P5abc RNA, no activation of ΔP5abc pre-RNA was observed (data not shown).

P5abc RNA contains the conserved adenosine-rich bulge in the P5a region, which is presumably implicated in the activation of the RNA (3, 10, 11). We prepared an RNA (P5a RNA) that lacks the P5b and P5c regions from P5abc RNA and tested its activity with ΔP5abc pre-RNA. We found that this P5a RNA is not able to induce the self-splicing of the precursor RNA under standard conditions (5 mM $MgCl_2$/200 mM NH_4OAc, pH 7.5). However, this RNA and the P5abc RNA can activate the splicing-defective precursor RNA with low efficiency if the magnesium concentration is increased to 10 mM or 25 mM (Fig. 2*B*). Under the same conditions, ΔP5abc pre-RNA by itself hardly exhibited its activity. This indicates that the activation of a splicing-defective intron RNA by the P5abc RNA depends on the stem–loop structure of P5a when it is stabilized by higher concentrations of magnesium ions. Likewise, the structure of the P5a region in the P5abc RNA could be stabilized by the P5bc stem–loop structure when it activates ΔP5abc pre-RNA under the standard conditions. However, further investigations are needed to elucidate the interaction of P5abc RNA with ΔP5abc pre-RNA under these conditions.

We investigated a possible RNA–RNA interaction between ΔP5abc pre-RNA and P5abc RNA more directly by attempting gel-mobility-shift assays (Fig. 3*A*). When ^{32}P-labeled ΔP5abc pre-RNA is mixed with the P5abc RNA, a slower migrating band is observed, suggesting the formation of an RNA·RNA complex. This band was excised and material was eluted from the gel and reelectrophoresed on a 5% polyacrylamide/8 M urea gel. The isolated RNA migrated as a band corresponding to that of the ΔP5abc precursor RNA (data not shown). The P5a RNA and tRNA were found inert to ΔP5abc precursor RNA under the assay conditions.

We further compared the splicing and other related reactions of the wild-type precursor RNA and those of ΔP5abc pre-RNA with P5abc RNA at various temperatures in the range between 20°C and 42°C. Fig. 3*B* shows that the activity of the wild-type precursor increases with increasing temperature up to 37°C. In addition, it was found that the P5abc RNA has no effect on the ribozyme activity of the wild-type intron RNA. However, as indicated by the amount of unreacted pre-RNA at various temperatures, the reactions with the ΔP5abc pre-RNA exhibited an optimum between 20°C and 37°C. We attribute the low efficiency of the bimolecular

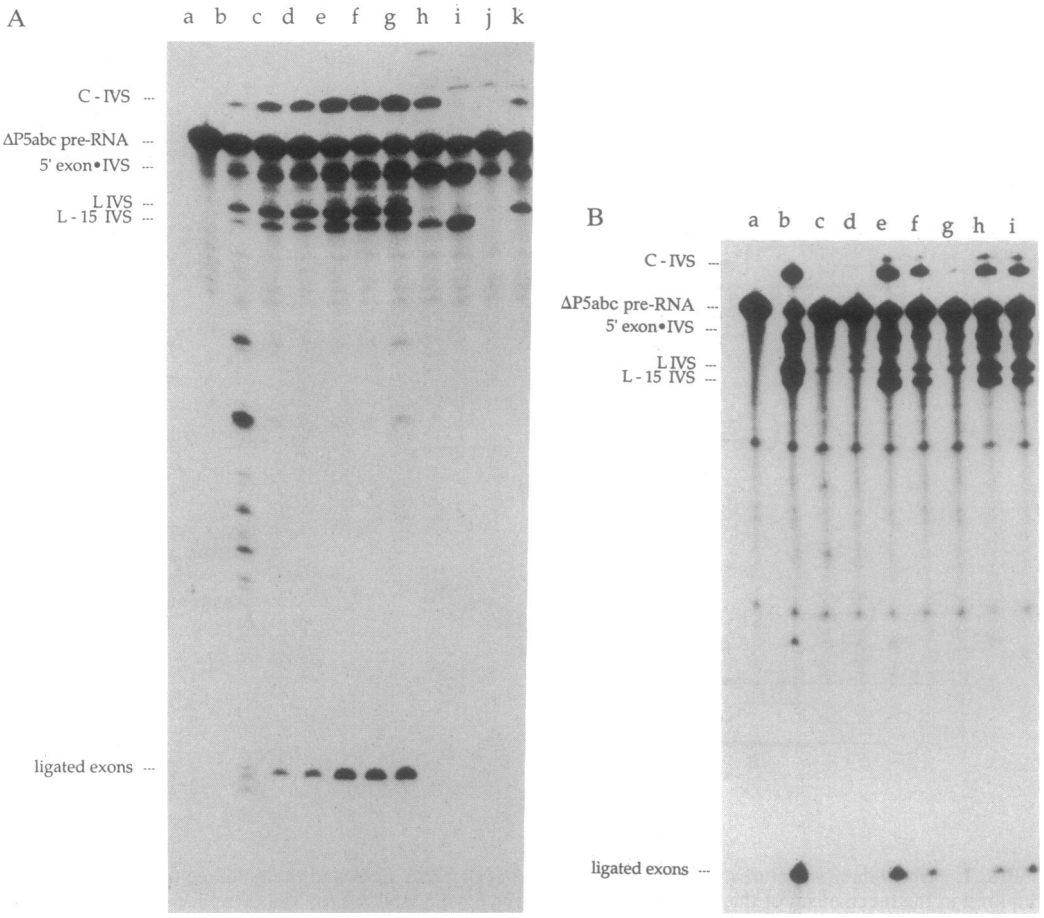

FIG. 2. Self-splicing reaction with uniformly ^{32}P-labeled ΔP5abc precursor RNA and unlabeled P5abc RNA. (*A*) Splicing reaction in the presence of various concentrations of P5abc RNA. Additives were as follows. Lanes: a–g and k, 200 μM GTP; h and j, no GTP or CpU; i, 200 μM CpU (9). P5abc RNA added was as follows. Lanes: a, j, and k, none; b, 0.125 μM; c, 0.25 μM; d, 0.5 μM; e, 1.25 μM; f, 2.5 μM; g, h, and i, 5.0 μM. Conditions were as follows. Lanes: a–i, 5 mM $MgCl_2$/200 mM NH_4Cl/50 mM Tris·HCl, pH 7.5; j and k, 15 mM $MgCl_2$/2 mM spermidine/50 mM Tris·HCl, pH 7.5. The reaction mixtures were incubated at 30°C for 2 hr and electrophoresed on a 5% polyacrylamide/8 M urea gel. IVS, intervening sequence (intron). (*B*) Splicing reaction in the presence of P5a RNA. Activator RNAs were as follows. Lanes: a, d, and g, no additional RNA; b, e, and h, 5 μM P5abc RNA; c, f, and i, 5 μM P5a RNA. Conditions were as follows. Lanes: a–c, 5 mM $MgCl_2$/200 mM NH_4Cl/50 mM Tris·HCl, pH 7.5; d–f, 10 mM $MgCl_2$/50 mM NH_4Cl/50 mM Tris·HCl, pH 7.5; g–i, 25 mM $MgCl_2$/50 mM NH_4Cl/50 mM Tris·HCl, pH 7.5. The reaction mixtures were incubated in the presence of 200 μM GTP at 30°C for 2 hr and electrophoresed on a 5% polyacrylamide/8 M urea gel. IVS, intervening sequence (intron).

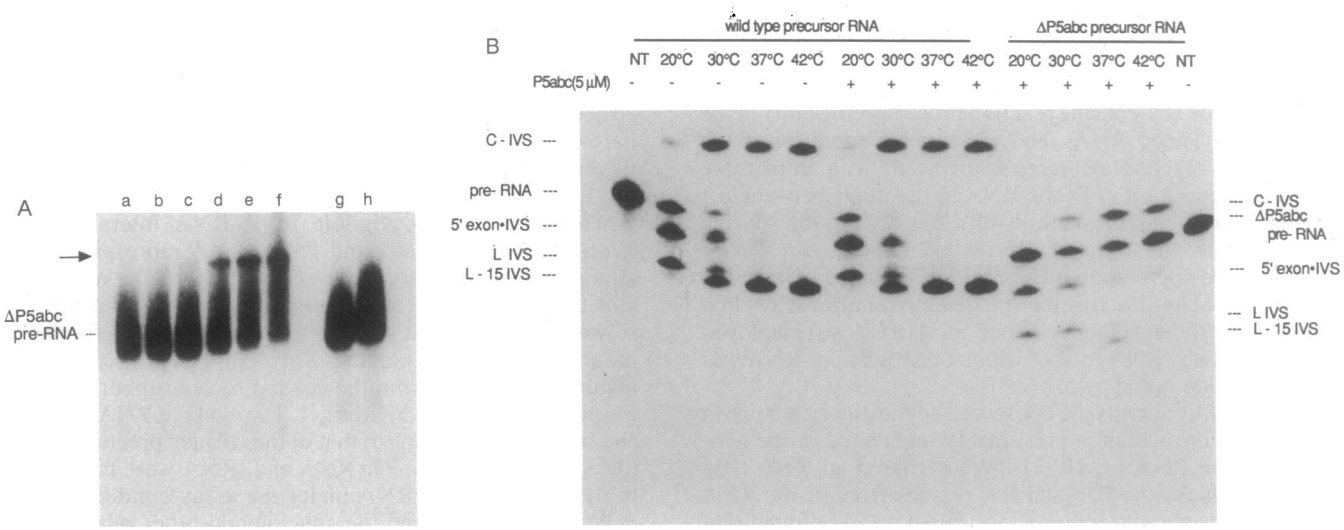

FIG. 3. Interaction of P5abc RNA with ΔP5abc precursor RNA. (*A*) Nondenaturing gel electrophoresis of uniformly ^{32}P-labeled ΔP5abc precursor RNA in the presence of various concentrations of unlabeled P5abc RNA. Lanes: a, no P5abc RNA; b, 0.25 μM P5abc RNA; c, 1.25 μM P5abc RNA; d, 2.5 μM P5abc RNA; e, 5 μM P5abc RNA; f, 10 μM P5abc RNA; g, 5 μM P5a RNA; h, 5 μM tRNA. An autoradiogram of the gel is shown. A slow migrating band (arrow) appeared related to the increase of the concentration of P5abc RNA, indicating the formation of the RNA·RNA complex. (*B*) Temperature dependence. Uniformly ^{32}P-labeled wild-type precursor RNA [5′ exon (48 nt)–intron (413 nt)–3′ exon (45 nt)] (2) or ΔP5abc precursor RNA was incubated in the presence or absence of unlabeled P5abc RNA at various temperatures for 2 hr under standard conditions (5 mM $MgCl_2$/200 mM NH_4Cl/50 mM Tris·HCl, pH 7.5). The resulting mixtures were electrophoresed on a 5% denaturing polyacrylamide gel. C-IVS, a circular form intron RNA produced by the cyclization reaction of the excised linear intron RNA (12); 5′ exon·IVS, an RNA consisting of the 5′ exon and the intron produced by the group I intron-mediated specific hydrolysis reaction (13) at the 3′ splice site; L IVS, the excised linear intron RNA by splicing; L-15 IVS, a linear form RNA produced by the hydrolysis of the circular form intron RNA (C-IVS) (12); NT, not incubated.

reactions at 42°C to the dissociation of the two RNAs. This indicates that a direct RNA·RNA interaction suggested by gel-mobility-shift assays exists when the bimolecular self-splicing reaction proceeds.

The kinetics of the splicing reaction of the wild-type precursor RNA and that of the ΔP5abc pre-RNA with P5abc RNA are compared in Fig. 4. The rate of self-splicing of the wild-type RNA was approximately twice as high as that of the ΔP5abc pre-RNA. A similar difference was found for the specific hydrolysis reaction (13) at the 3′ splice site that is known as a major side reaction mediated by the intron RNA. The ligation of the exons proceeds to the same extent (≈30%) for both reaction systems after 2 hr of incubation. These results demonstrate that the efficiency of the bimolecular self-splicing reaction is comparable to that of the original group I self-splicing system.

DISCUSSION

We have shown that, in an efficient manner, the P5abc RNA can activate a shortened form of a group I intron RNA that contains all essential components required in the mechanism of the self-splicing. This demonstrates that a group IB intron RNA is physically separable into two components: the core unit and the activator unit.

Group IA introns possess extra P7.1 and/or P7.2 stem–loop(s) (P7.1/P7.2) instead of the P5abc of group IB introns as shown in Fig. 1*A*. Like the *Tetrahymena* group IB intron,

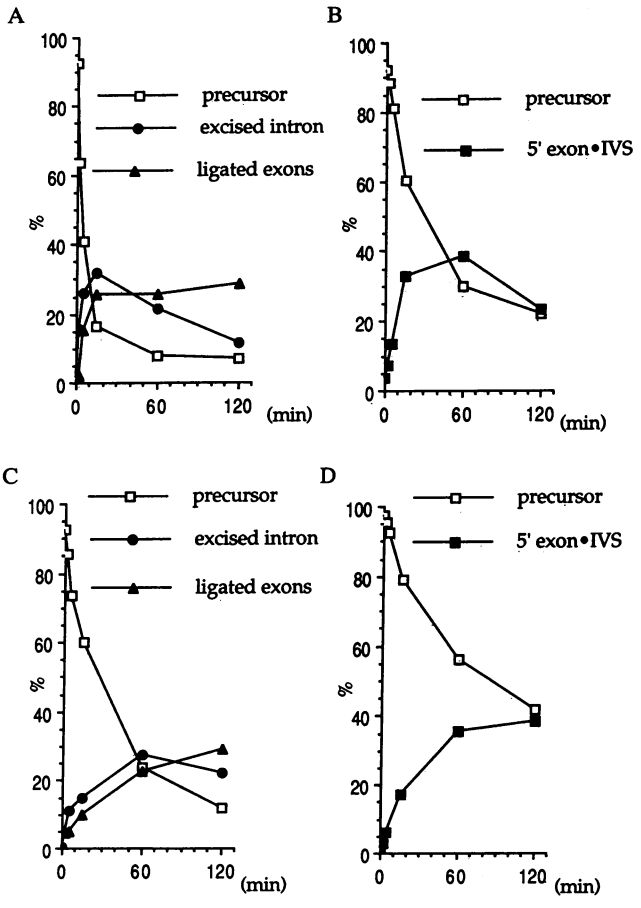

FIG. 4. Time courses of the self-splicing reactions and the specific hydrolysis reactions at the 3′ splice sites of the precursor RNAs. Wild-type precursor RNA was incubated in the presence of 200 μM GTP (*A*) or in the absence of GTP (*B*). ΔP5abc precursor RNA with 5 μM P5abc RNA was incubated in the presence of 200 μM GTP (*C*) or in the absence of GTP (*D*) under standard conditions (5 mM $MgCl_2$/200 mM NH_4Cl/50 mM Tris·HCl, pH 7.5).

a group IA intron RNA in *sunY* gene of bacteriophage T4 can be self-spliced in the presence of guanosine and magnesium ions *in vitro* (14). When the P7.1/P7.2 of this intron is replaced by a short string of nucleotides, it has been shown that the activity of the shortened intron RNA becomes significantly lower than that of the wild type (15). A group IA intron RNA lacking P7.1/P7.2 is identical to a group IB intron RNA lacking P5abc as shown in Fig. 1*A*. These shortened intron RNAs can still function as ribozymes but are much less active when compared with the corresponding self-splicing RNAs containing the two regions. P5abc RNA is an activator RNA for a group IB intron. This suggests that the P7.1/P7.2 might also function as an activator for the group IA intron RNA in trans. The sequence and structure of the P5abc are very different from those of the P7.1/P7.2. The P5abc and the P7.1/P7.2 are located at distinctly different sites in the introns. If the role of the P7.1/P7.2 is equivalent to that of the P5abc in self-splicing reaction, it will be interesting to see how they activate the core of the intron RNA.

It has been demonstrated that a small stem–loop structure of a self-splicing group II intron RNA from yeast mitochondria can stimulate a hydrolysis reaction at the 5′ splice site of an inactive mutant precursor RNA in trans (16). In this case, the mutant precursor RNA consisting of the 5′ exon and the 5′ half of the self-splicing group II intron RNA lacks the stem–loop structure in the 3′ half of the intron. The sequence of the small portion of the RNA is well conserved among group II introns. The function of this RNA has been concluded to be essential for the reaction. Our results support the hypothesis that this RNA may have a role as an activator RNA that is comparable to the P5abc of group IB intron. If one conserved portion of group I or group II intron RNA is an activator unit, it leads to a speculative hypothesis that activator RNA components also exist in other ribozymes.

RNase P in *Escherichia coli* is an enzyme consisting of an RNA component termed P RNA, which by itself can function as a catalytic RNA, and a protein component termed C5 (17, 18). The role of the C5 protein has been shown as an activator molecule that enhances the activity of the P RNA. In the self-splicing reaction, the function of the P5abc RNA is comparable to that of this protein. Activator proteins for group I intron were identified. In the splicing reactions *in vivo*, tyrosyl-tRNA synthetase and CBP2 protein were found to activate group I intron RNAs in *Neurospora* and yeast, respectively (19, 20). For RNase P and the *Neurospora* group I introns, the nature of the interaction between the RNAs and the proteins is not fully understood at present. In the splicing reactions of many group II introns, the intron RNAs require protein components to activate their catalytic cores (21). The splicing pathway and the conserved sequences of the introns of precursor mRNAs in the nucleus of eukaryotes show similarity to those of self-splicing group II intron RNAs. A spliceosome, which consists of multiple RNAs and proteins, splices precursor mRNAs (3) in higher eukaryotes. On the basis of these resemblances between a group II intron and a spliceosome, it has been speculated that the RNA components in the core of the spliceosome are responsible for the splicing reaction and that the protein components function as activator molecules for the RNA (22–24). Our discovery of the activator RNA supports the hypothesis that some RNA component(s) of the spliceosome might also function as activator RNA(s) whose role is to assist the other RNAs (23–25) to perform the splicing reaction. In addition to the RNA components of large RNA–protein complexes such as the spliceosome, there are many reports on other stable small RNAs whose exact functions are unknown at molecular level (16, 26). Since both RNA and protein can function as activator molecules for ribozymes, the interaction between the activators and ribozymes should be investigated in conjunction with the mechanism of highly complicated splicing

reactions by the spliceosome and other systems involving small RNAs.

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