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The mechanism of group I self-splicing: an internal guide sequence can be provided in *trans*

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We have reconstituted a group I self-splicing reaction between two RNA molecules with different functional RNA parts: a substrate molecule containing the 5′ splice site and a functional internal guide sequence (IGS), and a ribozyme molecule with core structure elements and splice sites but a mutated IGS. The 5′ exon of the substrate molecule is ligated in *trans* to the 3′ exon of the ribozyme molecule, suggesting that the deficient IGS in the ribozyme can be replaced by an externally added IGS present on the substrate molecule. This result is different from catalysis mediated by proteins where it is not possible to dissect the specificity of an enzyme from its catalytic activity.

Key words: group I intron/mitochondrion/*Saccharomyces cerevisiae*/self-splicing

Introduction

Group I introns have the ability to promote phosphodiester bond cleavage and formation without the help of proteins. They mediate their own excision from precursor RNA and the subsequent ligation of the flanking exons in a simple salt buffer (for a review, see Cech and Bass, 1986). This self-splicing reaction is facilitated by the tertiary structure of the intron which provides the ability to recognize the splice sites of the precursor RNA and to perform the cutting and ligation reactions in a very precise manner.

The 5′ splice site is recognized by a short sequence element in the intron called the internal guide sequence (IGS) and other, strongly conserved sequence elements called P, Q, R and S are needed to 'catalyse' the cutting and ligation reactions (Been *et al.*, 1987). The 3′ splice site is also thought to be recognized by base pairing with the IGS on the basis of phylogenetic conservation of DNA sequences, but biochemical proof is still lacking (Davies *et al.*, 1982; Waring and Davies, 1984; Been and Cech, 1985; Waring *et al.*, 1986).

We are studying group I introns from mitochondrial genes of *Saccharomyces cerevisiae*. Intron aI5α from the gene coding for subunit I of the cytochrome oxidase complex is a group I intron that can perform all the reactions mentioned above (Winter *et al.*, 1988). In addition, we have found that intron aI5α is capable of promoting cutting and ligation reactions in *trans*. In this paper we show that it was possible to restore 5′ splice site selecting activity to an intron, which lacked a functional IGS, by providing a functional one in another RNA molecule along with the 5′ splice site.

Results

When the SP6 system was used to produce intron aI5α RNA, we always detected a prematurely terminated product, together with the expected full-length transcript. The shorter transcription product only contains the 5′ exon and IGS from the aI5α intron but lacks the core structure elements. Self-splicing experiments carried out with the total transcription mixture containing both transcripts showed a low level of cleavage at the 5′ splice site of the prematurely terminated product. Since this product contains no conserved group I structure elements to carry out the reaction, it appears likely that the cleavage is a *trans* reaction involving a full-length transcript and the shorter transcription product. We have investigated this reaction in more detail by using well defined molecules with special emphasis on the functional role of the IGS(s).

The aI5α intron can be roughly divided into two parts: a 5′ part of ~900 nucleotides (nt), consisting of the IGS and a large open reading frame (ORF), and a 3′ part of ~400 nt containing the 'core' structure of the intron comprising the conserved sequence elements P, Q, R and S (Figure 1). Since the ORF sequence has no function in splicing *in vitro*, the IGS can be dissected from the remainder of the active intron by separating the parts containing the IGS and the core structure in this region. By using nuclease *Bal*31 we constructed two small templates that end 128 and 160 nt downstream of the IGS (Figure 1).

Bimolecular splicing reactions were carried out by mixing unlabelled precursor RNA containing the entire intron plus flanking exons ('ribozyme') and α^{32}P-labelled substrate RNA. Before the reaction the RNAs were incubated separately in the self-splicing mixture without GTP for 10 min at 40°C to allow the molecules to fold into their respective structures. After mixing the substrate and ribozyme solutions and adding GTP and RNasin, the mixtures were incubated for 3 h at 40°C. Products were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

When pT7 aI5α precursor RNA (ribozyme) was incubated with pT7 aI5α/128 or 160 RNA (substrate), a 5′ exon product and a product with the size of the remainder of the substrate molecules was formed (Figure 2, lanes A3 and B3). The 5′ exon fragments are characterized by the fact that they do not vary in length in the two reactions and that they comigrate with the 5′ exon product in a control reaction in which labelled ribozyme was incubated (lane C). The two other fragments of ~160 and ~190 nt were only present in the bimolecular reactions. Their length corresponds to the predicted length of the remainder of the substrate molecules when the 5′ exon is cleaved off. When substrate RNA by itself was incubated, no reaction was observed, indicating that for cleavage at the 5′ splice site the ribozyme is required (Figure 2, lanes A2 and B2). Both substrate RNAs were cleaved with similar efficiency. In the reaction with the pT7

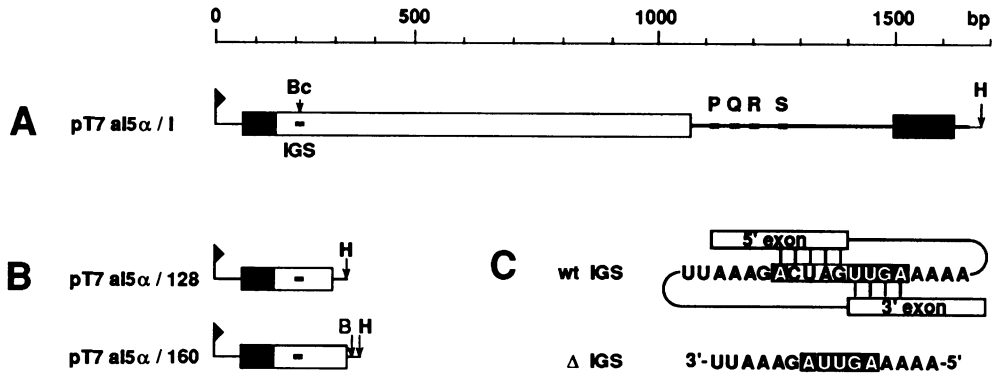

Fig. 1. Constructs of intron aI5α. (**A**) Ribozyme construct. Intron aI5α plus its flanking exons was cloned behind the T7 promoter. (**B**) Substrate constructs. The upstream parts of the ribozyme were cloned behind the T7 promoter. RNA transcripts were synthesized from *Hin*dIII or *Bam*HI linearized templates. ΔIGS constructs were made from clones pT7 aI5α/I and pT7 aI5α/128. Black boxes, exons; open boxes, internal open reading frame; IGS, internal guide sequence; P, Q, R, S, conserved sequence elements; H, *Hin*dIII; Bc, *Bcl*I; B, *Bam*HI. (**C**) The IGS and flanking sequences. The IGS is boxed. The 5′ exon binding part and the putative 3′ exon binding part are indicated. The nucleotides deleted in the ΔIGS constructs are indicated by the open box.

aI5α/128 substrate, a product was detected that comigrated with the ligated exon product in the control reaction. Since the labelled substrate molecule only has a 5′ exon and lacks a 3′ exon, this product must be the result of a *trans* splicing event in which the 5′ exon from the substrate molecule is ligated to the 3′ exon of the ribozyme molecule. In panel B this product cannot be detected because it comigrates with the substrate molecules. However, when a shorter *Bam*HI linearized pT7 aI5α/160 transcript was used this ligated exon product was also detected (results not shown).

To see whether or not the intron parts formed in the bimolecular reactions of Figure 2 were caused by GTP attack at the 5′ splice site, unlabelled substrate and ribozyme molecules were incubated in the presence of [α^{32}P]GTP (Figure 3). The intron part of the substrate molecule was labelled, as expected for the opening of the 5′ splice site (Figure 3, lane 4). The bimolecular reaction is therefore the same as the first step in the normal self-splicing reaction.

Since in the previous experiments both substrate and ribozyme had a functional IGS, we decided to mutate the IGS in both the ribozyme and substrate constructs. To determine which IGS is used in the bimolecular reaction, different combinations of substrate and ribozyme constructs can be incubated. The deletion that was introduced in the IGS of pT7 aI5α/I and pT7 aI5α/128 removed the 5′ exon-binding part of the IGS including the highly conserved G (Figure 1C). To test the effect of this deletion in the ribozyme molecule, the activity of this new construct was compared with the wild-type ribozyme (Figure 4, panel C). The effect of this mutation was severe and normal reactions were impaired. Opening of the 5′ splice site no longer took place, instead a cryptic 5′ splice site 9 nt upstream from the wild-type splice site was recognized and opened (Figure 4C, lanes 6 and 8), which can be explained on the basis of the alteration introduced in the IGS. Exon ligation was also abolished. Circularization was still observed, but at a very low rate. Mechanisms that could explain the action at cryptic sites and formation of these circular products will be presented in the Discussion. The observations demonstrate that the function of the 'core' elements in the ribozyme is still intact, but that the specificity of 5′ splice site selection is changed if the 5′ splice site binding part of the IGS is deleted.

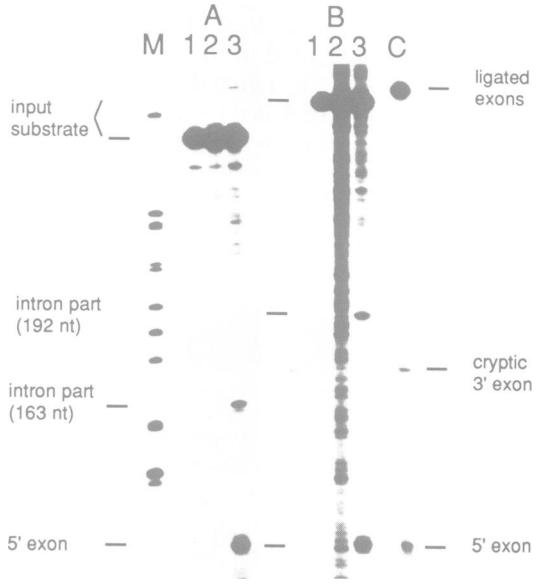

Fig. 2. Bimolecular reactions with pT7 aI5α/128 and 160. **Panel A**, reactions of pT7 aI5α/I with pT7 aI5α/128. **Panel B**, reactions of pT7 aI5α/I with pT7 aI5α/160. **Panel C**, control reaction of labelled pT7 aI5α/I. 1, input substrate; 2, substrate incubated without ribozyme; 3, reaction of the substrate with ribozyme. M, size calibration marker (see Materials and methods).

Although the mutant ribozyme is incapable of cleaving its 5′ splice site in *cis*, it is still proficient in cleavage of a substrate RNA containing a 5′ splice site along with a functional IGS that is offered in *trans* (Figure 4, panel B, lane 4). This illustrates that the function of the mutated IGS of the ribozyme can be replaced by the addition of an external IGS. The efficiency of this reaction is comparable to the one in which the functional IGS was present in the ribozyme itself and the IGS of the substrate RNA was inactivated (Figure 4, panel A, lane 3), or to the one in which the IGS in both RNAs was still intact (Figure 4, panel B, lane 3). When, as a control experiment, the mutant substrate was incubated with the mutant ribozyme, no reaction was observed (Figure 4, panel A, lane 4).

In each case when a cleavage reaction was observed we also detected a ligated exon product, showing that the *trans*

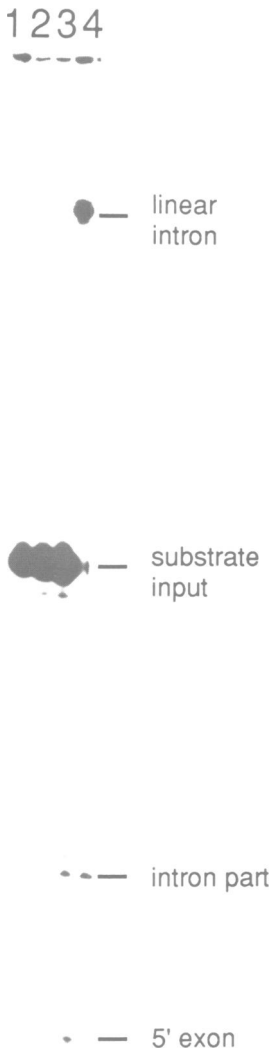

Fig. 3. Bimolecular reactions with pT7 aI5α/128. pT7 aI5α/128 was incubated with pT7 aI5α/I. 1, input substrate; 2, substrate incubated without ribozyme; 3, reaction of the substrate with pT7 aI5α/I; 4, unlabelled ribozyme and substrate were incubated with [α^{32}P]GTP.

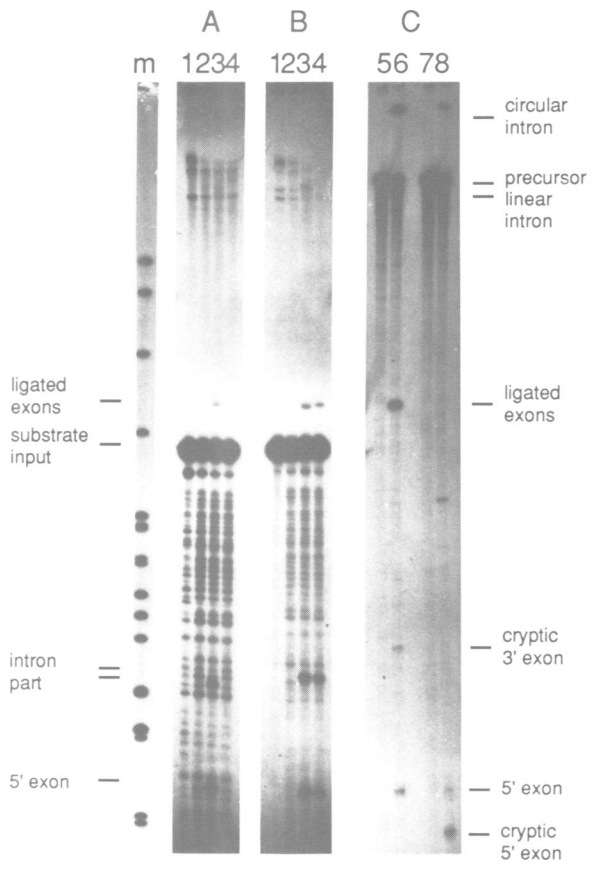

Fig. 4. The effect of deletions on the bimolecular reactions. **Panel A**, incubations with mutant substrate (pT7 aI5α/128 ΔIGS). **Panel B**, incubations with wild-type substrate (pT7 aI5α/128). **Panel C**, incubations of wild-type and mutant ribozyme alone. 1, input substrate; 2, substrate incubated without ribozyme; 3, substrate incubated with wild-type ribozyme (pT7 aI5α/I); 4, substrate incubated with mutant ribozyme (pT7 aI5α/I ΔIGS); 5, wild-type ribozyme input; 6, wild-type ribozyme incubated under self-splicing conditions; 7, mutant ribozyme input; 8, mutant ribozyme incubated under self-splicing conditions. m, size calibration marker (see Materials and methods).

splicing reaction can occur irrespective of the way in which the wild-type IGS is presented to the ribozyme. The efficiency of exon ligation in *trans* is however lower when the mutant IGS is present on the substrate RNA in contrast to the corresponding 5′ splice site cleavage itself (see above). In a reaction in which the IGS deficient ribozyme molecule was labelled and the substrate molecule unlabelled, no 5′ splice site cleavage or exon ligation was detected (results not shown). So the reconstitution of the wild-type 5′ splice site cleavage and exon ligation works in *trans* but not in *cis*.

Discussion

We have shown that a group I ribozyme with a partially deleted IGS can perform a *trans* splicing reaction with a second RNA molecule containing a 5′ splice site and functional IGS. The cleavage of the 5′ splice site and the formation of a ligated exon product in this bimolecular reaction with the wild-type substrate RNA and the mutant ribozyme can be explained by a structure in which both molecules participate in the formation of an active complex. This means that stem P1 [IGS/5′ splice site, see Burke *et al.* (1987) for details on nomenclature] of the substrate molecule must replace the corresponding structure in the ribozyme in such a way that the *trans* splicing reactions can take place. The efficiency of this reaction is low but it can nevertheless be detected. The results are schematically presented in Figure 5.

Bimolecular reactions have also been reported for the *Tetrahymena* intron but they differ from our results in several ways. Most of these experiments deal with oligoribonucleotides which can be cleaved and ligated by the *Tetrahymena* ribozyme (Inoue *et al.*, 1985; Kay *et al.*, 1987). In that case no large conformational changes are needed. Other bimolecular reactions involve the oligomerization of the *Tetrahymena* intron (Zaug and Cech, 1985) and the cleavage of an RNA substrate by a core ribozyme of the *Tetrahymena* intron (Szostak, 1986; Doudna and Szostak, 1989). In the first case oligomerization is only detected if the introns are denatured before the experiment is carried out. The explanation proposed for this is that this procedure allows the RNA molecules to fold in dimeric and even higher order structures, which can then effectively react with each

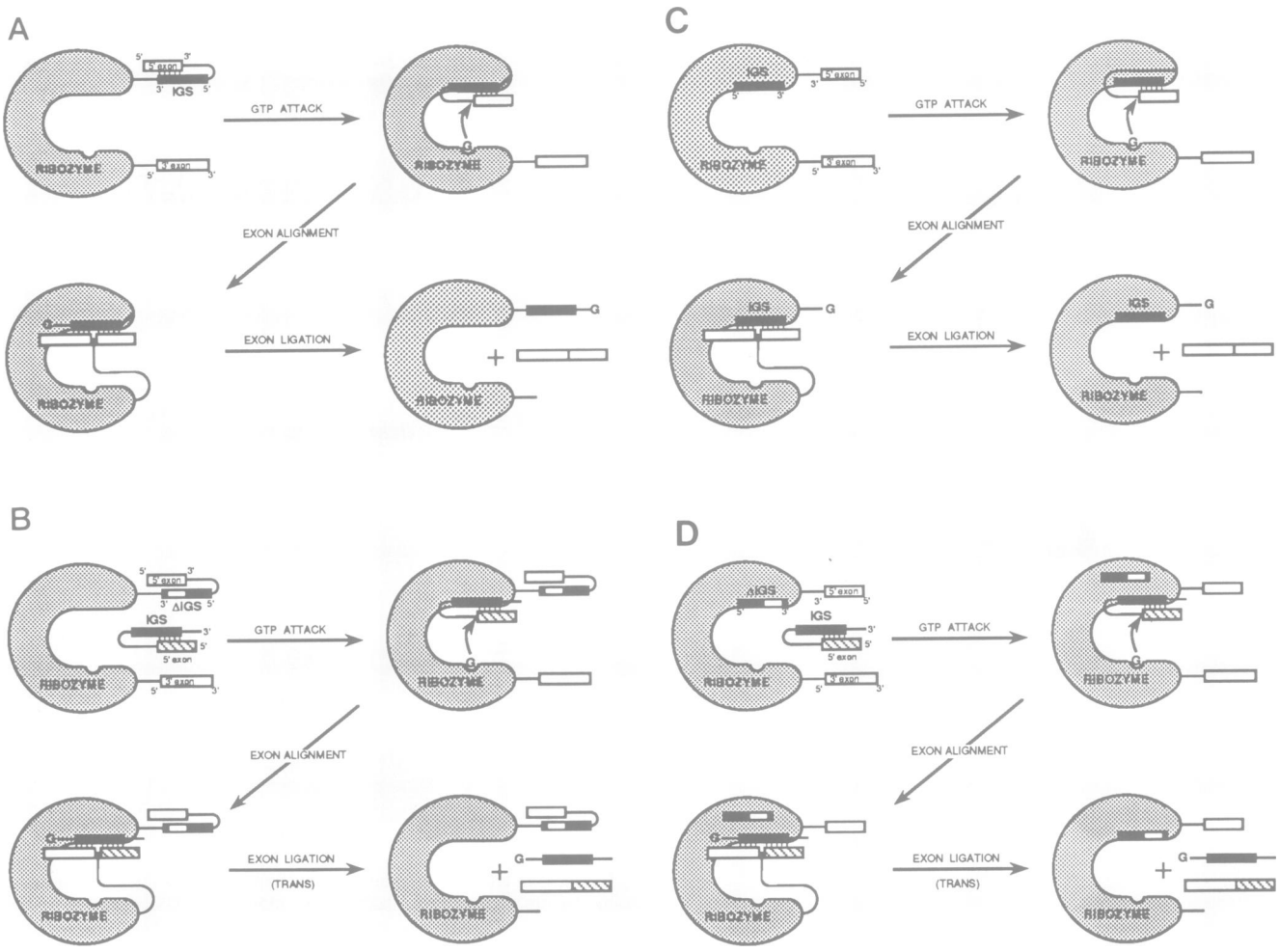

Fig. 5. Schematic representation of two models for the normal and the reconstitution reactions. (**A**) and (**C**) Two different models for the normal splicing reaction of wild-type pT7 aI5α/I transcripts. (**B**) and (**D**) Two different models for the *trans* splicing reaction of mutant pT7 aI5α/I transcripts with wild-type substrate RNAs. The exons of the ribozyme are represented by open boxes, the 5′ exon of the substrate by a striped box. The remainder of the structure (grey areas) represents the intron. The black boxes represent the wild-type IGS. The deletion in the IGS is indicated by an interruption in the black box.

other. In another case a truncated form of the *Tetrahymena* intron with most of the conserved structure but without the IGS is used to cleave a substrate molecule containing the 5′ splice site and the IGS. Because the two RNA molecules do not overlap, an active bimolecular complex can be formed without major distortions in the RNA structure.

In our reactions, denaturation is not needed prior to incubation. The RNA molecules are allowed to fold in their respective structures before they are mixed. Moreover, the sequences of the RNA substrate molecules are also present in the ribozyme molecule itself, the only difference being a small deletion of 4 nt within the IGS. To investigate whether an active complex is formed by intermolecular base pairing between the nucleotides immediately downstream of the substrate IGS with nucleotides within the ribozyme, we tested two other constructs. In the first one the nucleotides downstream of the substrate IGS were removed to exclude the base pairing possibility between substrate and ribozyme. This construct reacted in the same way as pT7 aI5α/128 (results not shown). In the second one the IGS was also deleted. This construct reacted in the same way as pT7 aI5α/128 ΔIGS (results also not shown). These results show that the interaction between ribozyme and substrate is not a trivial one in which base pairing of P2 is composed of a substrate and a ribozyme part. The real nature of this interaction is still unknown, but it could involve tertiary or non-canonical base pairing to facilitate the reaction. So it is impossible to form an active complex without major distortions in the ribozyme since the sequences in the substrate are also present in the ribozyme itself. The observation that stem P1 in the substrate molecule can replace the corresponding structure in the ribozyme indicates that the structure of the ribozyme probably consists of modules with different functions. P1 (and flanking sequences) would comprise a module containing the information to recognize a 5′ splice site, the core of the ribozyme would be a module with the 'catalytic' activity. It is possible that the 3′ splice site is located in another module.

Two possible artist's views illustrating our findings are given in Figure 5. In the first model (Figure 5A and B), the module comprising the 5′ exon and IGS is shown separately from the 'catalytic core' indicated by the grey shape. In this representation it is quite conceivable that instead of the non-functional module of the ribozyme itself, a module presented in *trans* can have access to the catalytic centre of the ribozyme (Figure 5B). Arguments favouring

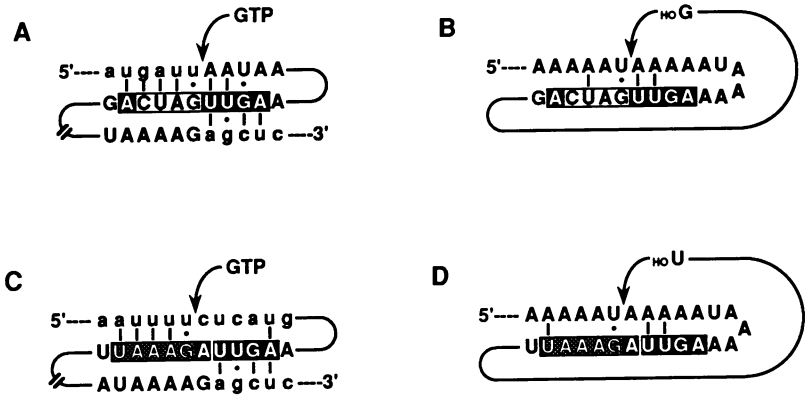

Fig. 6. IGS alignments in intron aI5α. (**A**) IGS alignments of the 5′ and 3′ splice sites in wild-type pT7 aI5α/I transcripts. (**B**) IGS alignments of the wild-type circularization sites in pT7 aI5α/I transcripts. (**C**) IGS alignments of the cryptic 5′ splice site and wild-type 3′ splice site in the pT7 aI5α/I ΔIGS transcripts. (**D**) Possible alignments of the circularization sites in pT7 aI5α/I ΔIGS transcripts. Lower case letters represent exon sequences, capitals represent intron sequences. The IGS sequences are boxed. The open box represents the four nucleotides of the wild-type IGS that are deleted in the ΔIGS. The shaded box represents upstream sequences that can replace the deleted nucleotides.

such a model can be found in the putative three-dimensional reconstruction of the *Tetrahymena* intron consisting of interacting RNA helices (Kim and Cech, 1987) and the experiments reported by Doudna and Szostak (1989) in which an IGS with base paired short RNA oligonucleotides is presented in *trans* to a core containing *Tetrahymena* ribozyme. In the second model, shown in Figure 5C and D, the IGS is shown as an integral part of the core structure. We postulate that the non-functional IGS with flanking sequences is pushed aside by the external IGS/5′ splice site structure, which is then properly positioned in the catalytic core (Figure 5D). Such a view, of an IGS with a position more intimately associated with the catalytic centre, is supported by the fact that the *Tetrahymena* intron can accept oligonucleotides as short as tri- or dinucleotides as a substrate (Inoue *et al.*, 1985; Kay and Inoue, 1987) and by recent studies based on probing the intron RNA with Fe(II)-EDTA, suggesting the existence of defined internal and external regions in the RNA structure (Latham and Cech, 1989).

The two models have in common, however, that the tertiary structure of the group I introns must be very flexible and that the postulated RNA modules involved in the splicing reaction can interact in a dynamic way. Such a view is supported also by the ease with which alternative splice sites can be used, resulting for instance in the formation of interlocked RNA circles consisting of intron parts (Tabak *et al.*, 1987a). It is furthermore supported by our observations in this paper that the deletion of four nucleotides in the IGS had effects on the self-splicing characteristics of intron aI5α itself: first, removal of the nucleotides involved in base pairing with the 5′ exon resulted in loss of wild-type 5′ splice site recognition (Figure 6A and C). This is due to the loss of the base pairing possibility with the 5′ exon and loss of the highly conserved G residue in the IGS which defines the exact position of the 5′ splice site. The newly created sequence, however (Figure 6C, shaded area), contains a new G residue and this results in a new IGS which is able to recognize and support cleavage at a site 9 nt upstream from the wild-type 5′ splice site, albeit at low efficiency. Second, the circularization is altered: in the wild-type ribozyme circular products are formed when the 3′ guanosine of the intron attacks the circularization sites in the intron (Winter *et al.*, 1988). These circularization sites are also defined by the IGS (Figure 6B). It is unlikely that in the case of the mutant ribozyme a terminal guanosine is the attacking group in the circularization reaction since no products with such a 3′ residue were detected. However, it has been reported for truncated forms of the *Tetrahymena* intron that circular products are formed by attack at a circularization site by 3′ terminal residues other than guanosine (Joyce and Inoue, 1987). The terminal nucleotide of the ribozyme run-off transcript is a uridine and we propose that this uridine residue is responsible for the attack at an internal circularization site (Figure 6D). Earlier work done in our laboratory on intron aI3 from the same gene as intron aI5α also suggests that a terminal uridine can be responsible for circularization (Tabak *et al.*, 1987b). Third, deletion of the highly conserved guanosine residue involved in base pairing with the 3′ U residue of the 5′ exon results in a shift of the IGS with respect to its putative 3′ exon binding part since another G residue is now proposed to define the splice sites. In the proposed alignment of the 3′ exon to the IGS by Davies *et al.* (1982), the 3′ terminal G residue of the intron is aligned with the highly conserved G residue in the IGS. In the case of the 4 nt deletion, the 3′ exon is no longer properly aligned to the IGS and we propose that this results in an inability to recognize the wild-type 3′ exon. Therefore, no ligated exon product is found in which the cryptic 5′ exon is ligated to the wild-type 3′ exon. It is also unlikely that a cryptic 3′ splice site can be recognized, since only one site 122 nt downstream of the 3′ exon meets the sequence specificity to base pair with the mutant IGS in case it is not involved in some sort of secondary or tertiary structure. The conclusions of the experiment are that the 5′ splice site is no longer recognized by the intron, but the core of the ribozyme and its capability of promoting phosphodiester bond cleavage and formation remained intact. This is even more clearly demonstrated by our finding that this core ribozyme could catalyse a reaction in *trans* when a functional IGS was offered in another RNA molecule together with a 5′ splice site.

RNA-catalysed cleavage reactions have attracted general attention as potential agents to selectively destroy unwanted mRNAs *in vivo*. Due to their extreme simplicity viroids have already been explored for such purposes (Haseloff and Gerlach, 1988). Although the in *trans* reaction we have

documented here is not (yet) very efficient, cleavage mediated by group I introns may merit further investigation because the reaction depending on recognition of '5′ splice site and IGS' can be made extremely specific.

In conclusion, our experiments show that it is possible to dissect the splice site selection from the 'cleaving activity' of group I intron aI5α. This is a result that is quite different from catalysis by proteins for which it is not possible to dissect the substrate binding capacity from catalytic activity.

Materials and methods

Restriction enzymes, ligase and Klenow enzyme were from BRL. S1 nuclease was from Sigma. [α^{32}P]GTP (400 Ci/mmol) and [α^{32}P]UTP (3000 Ci/mmol) were from Amersham. RNasin nuclease inhibitor was from Promega. T7 RNA polymerase was home-made, using an overproducing *Escherichia coli* strain kindly provided by F.W.Studier, according to the protocol from Davanloo *et al.* (1984). Size calibration in gel electrophoresis experiments was obtained by using end-labelled pBR322 DNA cut with *Msp*I: (622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160 (2×), 147 (2×), 122, 110, 90, 76, 67, 34, 26, 15 and 9 bp).

Constructs of intron aI5α

Ribozyme constructs were made by cloning intron aI5α plus its flanking exons behind the T7 promoter in vector pT713 (Figure 1). Substrate constructs were made by cloning the 5′ parts of the ribozyme behind the T7 promoter in pT713. Ribozymes were transcribed from *Hin*dIII linearized templates. Substrate RNAs were synthesized from *Hin*dIII or *Bam*HI linearized templates. Constructs with deleted IGS (ΔIGS) were made by digesting pT7 aI5α/I and pT7 aI5α/128 with *Bcl*I, removing the 5′ protruding ends with S1 nuclease and rejoining the blunt ends with ligase. The sequence of both mutants was verified.

Transcription and self-splicing reactions

RNA was synthesized using T7 RNA polymerase in the presence of one unit of RNasin. Self-splicing was carried out at 40°C in 20 μl of a mixture containing 0.2 mM GTP, 100 mM $(NH_4)_2SO_4$, 50 mM $MgCl_2$ and 50 mM Tris−HCl (pH 7.5) and one unit of RNasin. GTP end-labelled molecules were formed by incubating unlabelled precursor RNAs with 30−40 μCi [α^{32}P]GTP under the same conditions. The reaction was stopped by adding EDTA to a final concentration of 75 mM in a total volume of 100 μl. The products of self-splicing were recovered by passing this mixture through a Sephadex G-50 column and precipitating the RNA with ethanol. Gel electrophoretic analysis of RNA was carried out as described by Tabak *et al.* (1987a).

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