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Mutations at the lariat acceptor site allow self-splicing of a group II intron without lariat formation

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The fifth intron in the gene for cytochrome *c* oxidase subunit I in yeast mitochondrial DNA is of the group II type and is capable of self-splicing *in vitro*. The reaction results in lariat formation, concomitant with exon–exon ligation and does not require a guanosine nucleotide for its initiation. It is generally assumed, but not formally proven, that the first step in splicing is a nucleophilic attack of the 2′-hydroxyl of the branchpoint nucleotide (A) on the 5′-exon–intron junction. To investigate the role of intron sequences in recognition of the 5′-splice junction and the ensuing event of cleavage and lariat formation, mutations have been introduced at and around the branchsite. Results obtained show that although branchpoint attack and subsequent lariat formation are strongly preferred events under conditions normally used for self-splicing, addition of a single T residue at intron position 856, a mutation which brings the branchpoint adenosine into a basepair, leads to a conditionally active intron, which at high ionic strength catalyses exon–exon ligation in the absence of lariat formation. Comparable behaviour is also observed with the branchpoint A deletion mutant. The implications of these findings for the mechanism of self-splicing of group II introns are discussed.

*Key words: Cox*I/branchpoint mutations/group II introns/RNA catalysis/self-splicing/yeast mitochondria

Introduction

Since the discovery of RNA splicing, various aspects of this process have been the subject of intensive study (reviewed by Cech and Bass, 1986 and Padgett *et al.*,1986) and underlying similarities in the reactions undergone by different types of precursor RNA have been brought to light. Of particular interest are the features common to the reactions displayed by the self-splicing group II introns of yeast mitochondria and nuclear pre-mRNAs in yeast as well as in mammalian cells. In each splicing pathway, the first step is cleavage at the 5′-splice site and lariat formation in which the 5′-end of the intron is attached to a branchpoint via a 2′–5′ phosphodiester bond. In the second step the 3′-splice junction is cleaved and the two exons are ligated.

In the splicing of nuclear pre-mRNA in yeast, the last adenosine of the highly conserved TACTAAC box serves as branchpoint (Domdey *et al.*, 1984). In contrast, the branchpoint sequence in mammalian pre-mRNAs and in mitochondrial group II introns is far less conserved although an adenosine remains the branchpoint nucleotide (van der Veen *et al.*, 1986; Ruskin *et al.*, 1985). The use of mutant substrates has shown that branchpoint selection in mammalian pre-mRNAs is predominantly dependent on the distance of the branchpoint from the 3′-splice junction and to a lesser extent on sequence conservation (Padgett *et al.*, 1985). Mutations in the yeast nuclear TACTAAC box, on the other hand, severely reduce splicing (Jacquier and Rosbash, 1986a).

In all group II introns so far characterized, the branchpoint adenosine is located in a single-strand bulge in a conserved stable hairpin at the 3′-end of the intron (Figure 1B). This characteristic hairpin contains a short consensus sequence that shows some resemblance to that determined for the branchpoint environment of nuclear introns (van der Veen *et al.*, 1986).

To monitor effects of mutations in the branchpoint area on self-splicing of a mitochondrial group II intron and to get a better understanding of the molecular interactions involved, we have tested the effects of two changes: a deletion of the branchpoint adenosine and introduction of a T residue at a position 25 nucleotides 5′ of the branchpoint adenosine in a sequence that is predicted to make close contact with the branchpoint sequence via hairpin folding and base pairing. Previous studies have indicated that the structure of this region is important for splicing activity *in vivo* (Schmelzer *et al.*, 1982).

We now present evidence that both mutant RNAs are splicing-deficient under conditions normally used to assay self-splicing activity of group II introns *in vitro*. At high ionic strength, how-

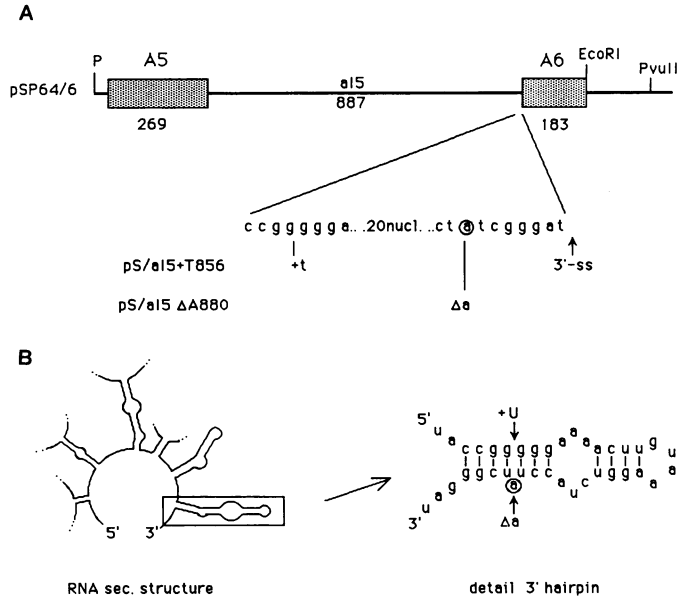

Fig. 1. Schematic representation of the $+T_{856}$ and ΔA_{880} mutant SP6 constructs. **A**: The initial pSP64/6 construct containing the aI5 intron from the gene coding for cytochrome *c* oxidase subunit I with 269 bp of exon A5 and a part of exon A6 (183 bp) behind the SP6 promoter. Filled blocks represent exons. The *Eco*RI and *Pvu*II sites used to generate different run-off precursors are also shown. The arrow indicates the 3′ splice-site. Of the two *in vitro* mutants pS/aI5+T_{856} and pS/aI5ΔA_{880} only the relevant part of the intron sequence is indicated. **B**: Schematic representation of the Michel/Dujon secondary structure with an enlargement of the 3′-terminal conserved hairpin (Michel and Dujon, 1983), in order to show the branchpoint adenosine (encircled) mutation (ΔA_{880}) and the position of the U addition in mutant $+T_{856}$.

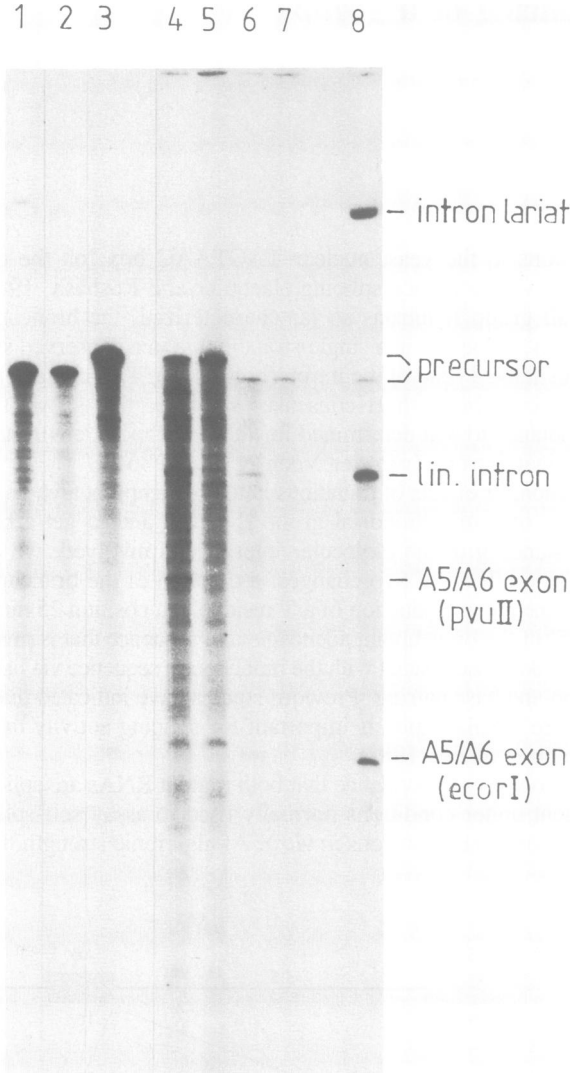

Fig. 2. Branchpoint mutants $+U_{856}$ and ΔA_{880} are disturbed in self-splicing *in vitro*. *In vitro* run-off transcripts were generated using the *Eco*RI (pSP64/6 and pS/aI5 ΔA_{880}) or *Pvu*II (pS/aI5+T_{856}) truncated template DNA and analysed on a 4% polyacrylamide-8M urea gel, either before (**lanes 1–3**) or after incubation for 1 h at 45°C in 5 mM Tris–HCl, 5 mM $MgCl_2$, 2 mM spermidine; **lane 5** (pS/aI5+T_{856} × *Pvu*II), **lane 7** (pS/aI5 ΔA_{880} × *Eco*RI) and **lane 8** (pSP64/6 × *Eco*RI) or in 5 mM Tris–HCl, 50 mM $MgCl_2$, 2 mM spermidine; **lane 4** (pS/aI5+T_{856} × *Pvu*II) and **lane 6** (pS/aI5 ΔA_{880} × *Eco*RI).

ever, correct exon–exon ligation does occur, but in the absence of lariat formation. This indicates that a change in intron structure around the branchsite can lead to a reaction in which lariat formation is unnecessary for exon–exon ligation. The role of lariat formation in the group II self-splicing mechanism will be discussed on the basis of these findings.

Results

Site directed in vitro mutagenesis of the aI5 intron branchpoint

As shown in Figure 1B, deletion of the branchpoint adenosine, as well as the insertion of an extra thymidine residue, results in a hairpin which lacks the single strand bulge present in all group II introns so far examined. If this bulge is essential for splicing, both mutants should be splicing deficient. If on the other hand only the presence of an adenosine residue is required, the T addition mutant should be splicing competent. As shown in Figure 1A, the mutations were introduced into intron aI5 of the cytochrome *c* oxidase subunit I gene at positions 856 (T-addition) and 880 (A-deletion), using oligonucleotide directed *in vitro* mutagenesis (see Materials and methods).

Branchpoint mutants are conditionally defective in self-splicing

To investigate whether mutant RNAs have retained the ability to carry out self-splicing *in vitro*, artificial precursors were generated by *in vitro* transcription of mutant template DNAs, pS/aI5 ΔA_{880} and pS/aI5+T_{856} truncated at the *Eco*RI or *Pvu*II site (Figure 1A). Incubation of these run-off RNAs under conditions normally used for splicing of group II introns (van der Veen *et al.*, 1986) failed to reveal splicing activity, as shown in Figure 2 lanes 5 and 7. However if the Mg^{2+} concentration is raised 10-fold, minor amounts of splicing products appear; the linear form of the intron as well as RNAs corresponding in length to the ligated exons (Figure 2, lanes 4 and 6). Figure 3 shows that incubation in the presence of the increased concentration of Mg^{2+}, supplemented with NH_4^+ ions (or Na^+), leads to the abundant appearance of RNA species characteristic for self-splicing, although at a slower rate than that displayed by the wild type precursor RNA. The salt effect can be mimicked by increasing the pH to 9 or higher (results not shown). As shown in Figure 4 for the ΔA_{880} mutant, self-splicing of the wild type RNA takes place within an hour (Figure 4, lanes 3–6), while appreciable amounts of precursor from the ΔA_{880} mutant still remain after two hours (Figure 4, lanes 7–10). Similar results were obtained with the $+U_{856}$ mutant (data not shown). The identity of bands predicted to correspond to the exon–exon products and the linear form of intron was confirmed by either the presence or absence of mobility shifts dependent on the run-off restriction site used (Figure 3) or directly by sequence analysis with reverse transcriptase, primed with an oligonucleotide complementary to exon A6 or to the 5′-part of the intron (van der Veen *et al.*, 1986), data not shown.

Self-splicing in the absence of lariat formation

A lariat form of the intron, which is abundantly present in incubations with wild type RNA (Figure 3A and B, lanes 5 and 6), is conspicuously absent from the products generated by both mutants. Only trace amounts of lariat RNA can be detected in the $+U_{856}$ mutant after extended exposure of the autoradiogram, as indicated in Figure 3A. We assume that the lack of lariat RNA reflects lack of formation, since the alternative, that the mutations result in the use of a cryptic branchsite resulting in highly unstable lariats seems unlikely. Alternative branchsites in lariats formed during splicing of yeast nuclear pre-mRNA do not show decreased stability (Jacquier and Rosbash, 1986a) and we have not been able to detect normal-sized lariats among the products of short incubations.

We have also considered the possibility that lariats continue to be formed during splicing and that they are opened in a rapid specific reaction to give Y-shaped molecules which are indistinguishable in migration from the linear intron (cf. Peebles *et al.*, 1986). To test this, the linear intron fraction from mutant $+U_{856}$ was recovered from gel and subjected to digestion with nuclease T2. Figure 5A shows the results of a thin layer chromatographic separation of the products obtained. The branched oligonucleotide spot, which is characteristic for nuclease-digested wild type lariat RNA (spot X, shown in Figure 5B, cf. van der Veen *et al.*, 1986) is not observed even after prolonged exposure, suggesting that this explanation does not hold.

Despite lack of normal lariat formation, minor side reactions probably occur in both branchpoint mutants. Electron microscopic examination of splicing reaction products reveals that several

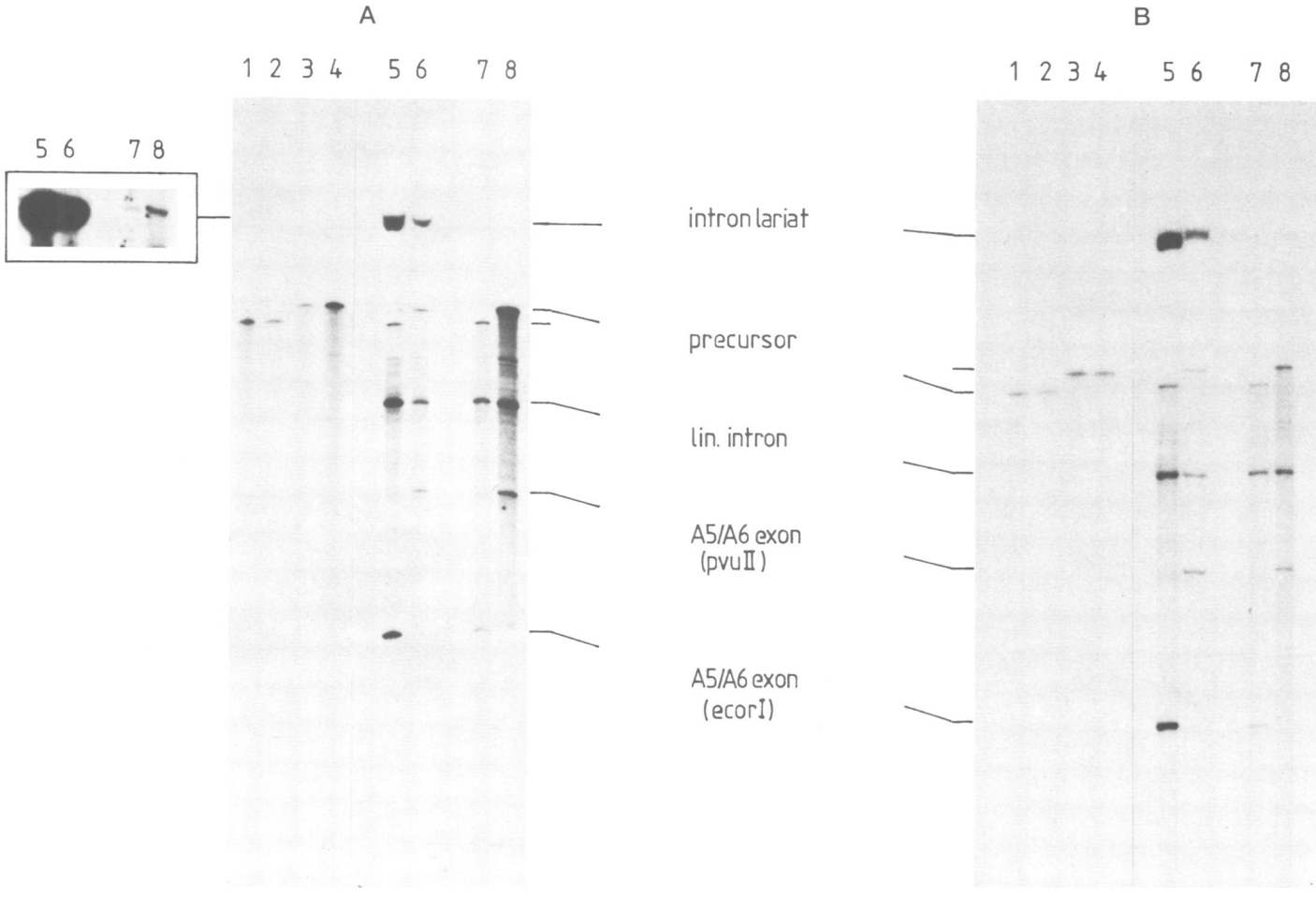

Fig. 3. Self-splicing of branchpoint mutants $+U_{856}$ and ΔA_{880} in the absence of lariat formation. **A**: Run-off transcripts of wild type intron aI5 (pSP64/6) and mutant $+U_{856}$ (pS/aI5+T_{856}) were generated by *in vitro* transcription of DNA templates truncated at the *Eco*RI or *Pvu*II site. RNA products were analysed by electrophoresis through 4% polyacrylamide-8 M urea gels, either before (**lanes 1,2**: wildtype and mutant × *Eco*RI respectively; **lanes 3,4**: wildtype and mutant × *Pvu*II respectively) or after incubation for 1 h at 45°C in 100 mM Tris−HCl, 70 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$; **lanes 5,6** wild type × *Eco*RI and *Pvu*II respectively and **lanes 7,8** mutant × *Eco*RI and *Pvu*II respectively. The lariat region of **lanes 5−8** is shown in two different exposures in order to indicate the difference in extent of lariat formation between the wild type form of the intron and the mutant form. **B**: similar to **A**, but run-off transcripts were generated of the wild type plasmid pSP64/6 (**lanes 1** and **3**) and of the mutant plasmid pS/aI5 ΔA_{880} (**lanes 2** and **4**) truncated at either the *Eco*RI or the *Pvu*II site respectively.

types of lariats arise in very limited amounts. Among these are lariats with variable tail and loop size and in the T-addition mutant, these are additional to normal-sized lariats. The low levels of these unusual molecules have so far precluded their further characterization.

Discussion

Unlike group I introns, self-splicing of group II introns does not depend on a guanosine nucleotide for its initiation and results normally in lariat formation concomitant with exon−exon ligation (van der Veen *et al.*, 1986; Peebles *et al.*, 1986; Schmelzer and Schweyen, 1986).

As for splicing of nuclear pre-mRNAs, it is assumed that the first step in group II intron splicing is a nucleophilic attack of the 2′-OH of the branchpoint nucleotide on the 5′-exon−intron junction resulting in cleavage at the 5′ splice-site and lariat formation. The sequences conferring specificity on this event are largely unknown, although results obtained with 5′-exon deletion mutants suggest important roles for sequences located within the exon and at the 5′-end of the intron (van der Veen *et al.*, 1987).

To investigate the role of intron sequences in recognition of the 5′-splice junction and the events of cleavage and lariat formation, we have introduced mutations at and around the branch-site. In mammalian pre-mRNAs, use of mutant substrates has shown that the branchpoint mutations lead not to splicing deficiency, but to splicing at a reduced rate. Deletion of the entire branchpoint sequence causes the activation of nearby cryptic branchpoint adenosines (Ruskin *et al.*, 1985; Padgett *et al.*, 1985). Replacement of the branchpoint A to a G or T residue results in the use of the adjacent A residue in branch formation, while the A to C transversion is tolerated to a certain extent (Hornig *et al.*, 1986). In yeast nuclear pre-mRNA splicing most mutations at the TACTAAC box affect *in vitro* splicing dramatically and no cryptic branchpoint sites are used (Newman *et al.*, 1985; Jacquier *et al.*, 1985; Jacquier and Rosbash, 1986a).

Mechanistically, lariat formation in self-splicing group II introns is likely to be different from either the nuclear yeast or the mammalian pre-mRNA splicing, because during self-splicing phosphodiester bond formation occurs in the absence of an added energy source. This is energetically impossible, unless it can be coupled via transesterification to the cleavage of a second bond.

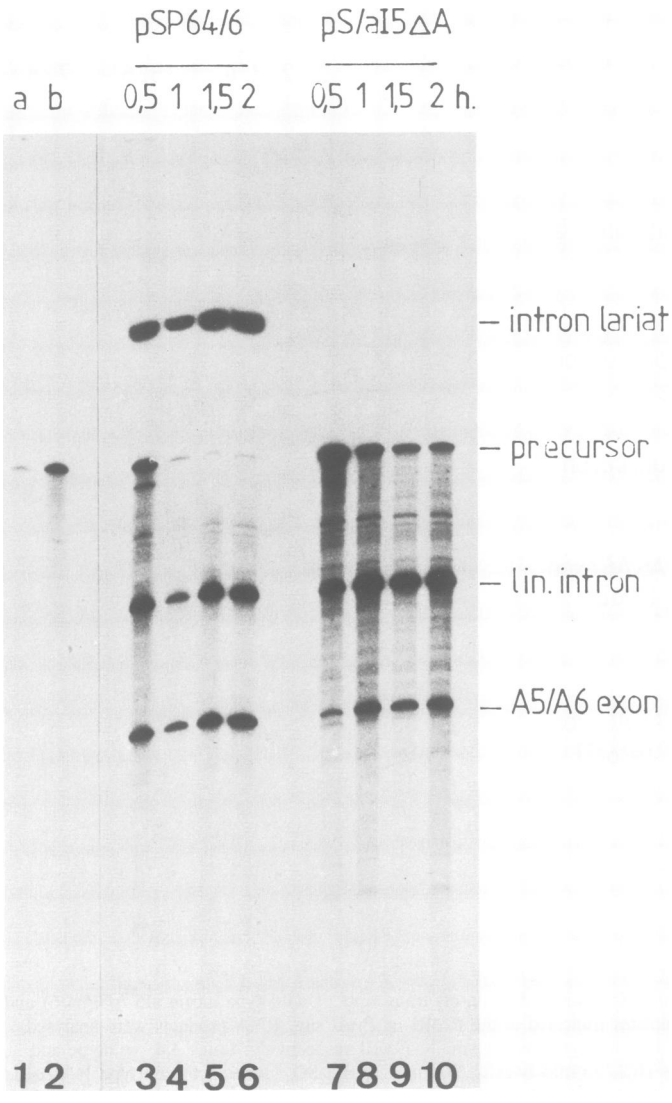

Fig. 4. Time dependence of the *in vitro* splicing reaction. Comparison of self-splicing rate of mutant ΔA_{880} to wild type. Run-off transcripts were generated on pSP64/6 × *Pvu*II and pS/aI5 ΔA_{880} × *Pvu*II DNA respectively and analysed on a 4% polyacrylamide-8 M urea gel, either before (**lanes a** and **b**) or after incubation for up to 2 h (**lanes 3–10**) in 100 mM Tris–HCl, 70 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$.

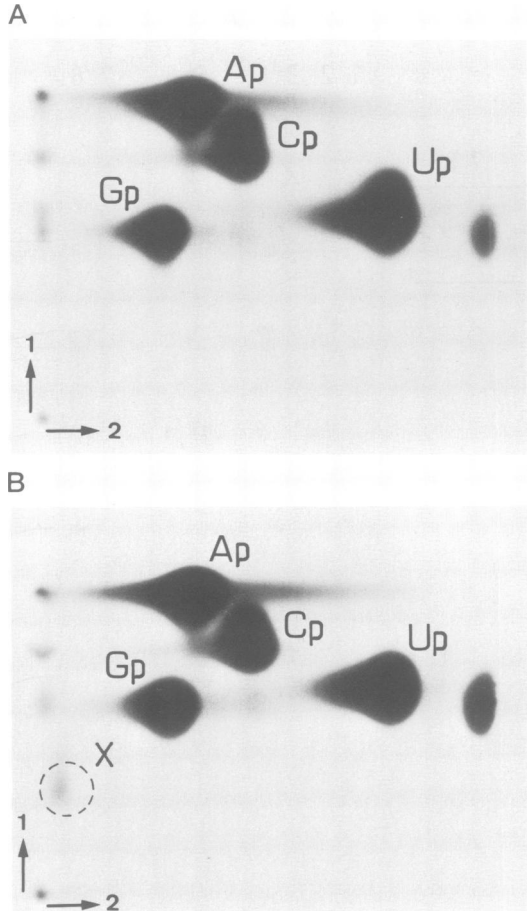

Fig. 5. No branch site is present in the linear intron RNA of mutant $+U_{856}$. Nuclease T2 digests were spotted onto cellulose thin layer plates and chromatography was carried out in the following solvents: first dimension 0.5 N NH_4OH:isobutyric acid (3:5, v:v), second dimension isopropanol:conc. HCl:water (70:15:15, v:v:v). **A**: T2 digest of linear intron RNA fraction of branchpoint mutant $+U_{856}$. **B**: T2 digest of lariat intron RNA from the pSP64/6 wild type. The nuclease-resistant component is indicated by X.

Such cleavage may not be easy to achieve, since as pointed out by Cech and Bass (1986), internal 2′-OH groups in RNA are relatively poor nucleophiles. The ability of the branchpoint adenosine to promote efficient lariat formation *in vitro* must therefore be due to a combination of factors, including straining of the 5′exon–intron junction by intron folding, activation of the 2′-OH by a neighbouring proton-abstracting group and exclusion of water or OH^- from the active site. The distances and geometry required to generate such interactions are obviously critical since the results presented above show that trapping of the branchpoint adenosine in a basepair (mutant $+U_{856}$) reduces lariat formation to an extremely low level. In line with this, model studies with a synthetic RNA hairpin derived from *Escherichia coli* 16S rRNA show that the presence of a single-strand bulge contributes significantly to the ability of the hairpin to adopt different conformations (White and Draper, 1987).

Until now only one comparable mutation in the group II bI1 intron has been described (Schmelzer and Schweyen, 1986). In this, the branchpoint bulge was enlarged by deletion of a G opposite the branchpoint adenosine. In this mutant (M4873) splicing is abolished *in vivo* and *in vitro*. No lariat formation is detectable.

No detectable lariat formation occurs in the ΔA_{880} mutant, whereas at high ionic strength both the $+U_{856}$ and the ΔA_{880} mutant have normal amounts of correctly ligated exons. We conclude that unlike in mammalian- and nuclear yeast pre-mRNAs, deletion or alteration of branchpoint sequences does not activate cryptic branchsites and moreover that lariat formation is not strictly necessary for *in vitro* group II self-splicing. The conditional nature of the mutants is of great interest, since it implies that *in vitro*, the conformation of their introns is dependent on ionic strength.

In these branchpoint mutants release of the 5′-exon is presumably the result of site-specific hydrolysis catalysed by free OH^- instead of the 2′–OH group of the branchpoint adenosine (Cech and Bass, 1986). OH^--mediated cleavage has also been observed in *trans*-splicing reactions catalysed by intron aI5 (Jacquier and Rosbash, 1986b). These authors speculate that binding of an exon analogue *in trans* may distort intron folding sufficiently to permit access of OH^- ions to the catalytic centre of the intron. Mutant $+U_{856}$ shows, however, that OH^- catalysed 5′-exon cleavage can occur in what appears to be a normal *cis*-splicing reaction.

In both *cis*- and *trans*-catalysed reactions, it is remarkable that the second step in splicing, cleavage of the 3'-splice-site and ligation of the exons, continues to occur efficiently. The 5'-exon binding site (van der Veen *et al.*, 1987; Jacquier and Michel, 1987) is apparently unaffected by the mutations we have introduced, so that the exon can be positioned for attack on the 3' intron–exon splice-site and is still used in preference to external OH^- ions.

The fact that efficient exon–exon ligation can be induced to occur in virtual absence of lariat formation raises the question why group II introns should have maintained this step as part of their splicing mechanism.

One possibility is that the same active region of the intron is involved in both 5'-exon cleavage and the subsequent attack of the released exon on the 3'-exon. Exclusion of OH^- ions is possibly essential for the success of this second step, because attack by OH^- will prevent exon ligation. Folding of the active centre may thus create an environment in which not only water is excluded, but also raises the nucleophilicity of the branchpoint adenosine above a critical level necessary for attack on the 5'-splice junction and subsequent lariat formation.

In vivo, exclusion of water may additionally be achieved by electrostatic shielding by proteins, since it may be impossible to achieve the configuration required for splicing without lariat formation under the ionic conditions prevailing in mitochondria. It may be significant in this respect that in many other RNAs (like *E.coli* ribosomal 16S RNA and 5S RNA), adenosine residues in unpaired bulges are often involved in protein binding (Peattie *et al.*, 1981).

An alternative explanation for the maintenance of lariat formation in evolution has a basis in the probable differences in catalytic rate *in vitro* and *in vivo*, lariat formation may stabilize an intron configuration that promotes attack of the 5'-exon on its 3'-counterpart and differences in efficiency between linear and branched forms may not be evident at the lower rates of catalysis *in vitro*.

Although the data presented in this report imply direct interactions between the branchpoint area and the intron splice-sites, changeable by introduction of mutations, these interactions remain to be proven by compensatory intron alterations. Hopefully, this will eventually lead to the identification of sequences involved in the formation of an active catalytic site.

Materials and methods

Restriction enzymes were from New England Biolabs or Boehringer Mannheim. Polynucleotide kinase and SP6 polymerase were purchased from New England Nuclear; reverse transcriptase from Life Sciences Inc. DNA polymerase, Klenow fragment was obtained from Boehringer Mannheim, while T4 DNA ligase was from New England Biolabs.

Recombinant plasmids

Plasmid pSP64/6 (van der Veen *et al.*, 1986) contains the 1340 bp *BclI–EcoRI* fragment derived from yeast strain D273-10B. This fragment consists of part of exon A5 (269 bp), all of intron aI5 (887 bp) and part of exon A6 (183 bp) from the *coxI* gene. The orientation of the *coxI* fragment behind the SP6 promoter is such that the coding strand is transcribed.

Plasmid M13mp19 aI5 contains the *HindIII–EcoRI* insert of pSP64/6. Plasmids pS/aI5+T_{856} and pS/aI5 ΔA_{880} are *in vitro* mutated versions of pSP64/6; respectively insertion of a T residue at intron position 856 and deletion of the branchpoint adenosine at intron position 880. Both mutations have been confirmed by double-strand sequence analysis (van der Veen *et al.*, 1986).

Plasmid DNA was isolated as described by Birnboim and Doly (1979). Single stranded template DNA, for use in the dideoxy sequence method of Sanger *et al.* (1977), was prepared from recombinant phage M13 plaques (Sanger *et al.*, 1980).

Oligonucleotide directed mutagenesis

To create mutations, single-stranded DNA of M13mp19 aI5 served as template DNA. After second strand synthesis in the presence of Klenow polymerase, T4 DNA ligase, deoxynucleoside triphosphates, ATP, the universal M13 sequence primer and the oligonucleotide carrying the desired nucleotide substitution, the resulting double stranded DNA was digested with *HindIII* and *EcoRI* and recloned in M13mp19 (Sims *et al.*, 1985). Mutant clones were selected by sequence analysis. Of both mutants the complete 1340 nucleotide yeast insert was sequenced before recloning in the *HindIII* and *EcoRI* digested SP64 vector to ensure that no additional mutations had occurred.

In vitro transcription and splicing of precursor RNA

DNA of the mutants and plasmid pSP64/6 were linearized by digestion with *EcoRI* or *PvuII*. The DNA was extracted with phenol, precipitated and redissolved in water. The *in vitro* transcription reaction with SP6 polymerase was carried out as described by van der Horst and Tabak (1985). The resulting [α^{32}P]UTP-labelled precursor RNA was incubated under group II self-splicing conditions as described by van der Veen *et al.* (1986) or as stated in the text.

Primer extension analysis

RNA species isolated from gel slices were co-precipitated with an appropriate 5'-end labelled synthetic DNA fragment (van der Veen *et al.*, 1986). The sequence reactions were carried out as described by Tabak *et al.* (1984).

Nuclease T2 digestion

Nuclease T2 digestions were carried out as described previously by van der Veen *et al.* (1986).

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