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# Evidence of a tertiary interaction functional in group I 3′-splicing

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It has been recently shown that the schedule of 3′-splicing events for yeast mitochondrial group I introns requires that conserved helix P10 materializes only after 5′-cleavage has taken place. A scenario compatible with experimental findings has been proposed [(1992) FEBS Lett. 297, 201–204; (1990) Proc. Natl. Acad. Sci. USA 87, 8192–8196] where the formation of P10 is postponed by competition for standard basepairing with an extended P1 interaction engaging the same portion of the internal guide sequence (IGS) which is subsequently involved in P10. Although the formation of P10 in the fifth intron of yeast apocytochrome *b* gene (YCOB5) has been confirmed by site-directed mutagenesis, we cannot be confident that this interaction is merely stabilized by Watson–Crick base-pairing, involving the 3′-exon and the IGS, especially given that its formation requires closure of an intron loop of 159 unpaired bases. Thus, our aim is to establish the participation of the 5′-extremity of the intron in the formation of P10. By deoxyribose substitution at positions 1 and 2 of the 5′-extremity of the intron, we are able to confirm the existence of tertiary interactions stabilizing the 3′-splicing site. We show that selective deoxyribose substitution renders the intron inefficient for 3′-splicing when compared with wild type levels.

Tertiary interaction; Mitochondrial group I intron; Sequential folding; Structure competition

## 1. INTRODUCTION

One distinctive structural feature of yeast mitochondrial introns appears to be the formation of conserved helix P10 [1,2]. This interaction has been inferred by phylogenetic analysis [3] and has only been confirmed experimentally for the fifth intron of the apocytochrome *b* gene (YCOB5) by means of site-directed mutagenesis and compensatory basepairing [2]. The interaction involves the 5′-extremity of the 3′-exon and a portion of the internal guide sequence (IGS) (Fig. 1). Moreover, computer simulations on the closely-related species YCOB4 [1] reveal that whenever the helix P10 is required for 3′-splicing, its formation should be *postponed* until 5′-cleavage has occurred, otherwise, a prolonged strain applied on the 3′-splicing phosphodiester linkage due to adjacent interactions P9.0 and P10 causes 3′-hydrolysis to prevail over exon–exon ligation. The computer simulations which helped establishing this picture mimick the trans-assisted folding of the intron in a sequential manner. This is probably a reasonable assumption since all attempts carried out so far to find in vitro catalytic activity by recombination of the (already folded) RNA species and the *trans*-acting factor have failed [1].

The chronology of events leading to the postponement of P10 formation is actually fulfilled and could be explained invoking a scenario revealed by simulations and probed by site-directed mutagenesis: the 3′-exon competes for base pairing to a portion of the IGS with nucleotides 1 and 2 at the 5′-extremity of the intron (Fig. 1). Once 5′-cleavage has taken place and concurrently, the 5′-extremity of the intron is no longer engaged in basepairing, P10 may form thereby inducing exon–exon ligation. This is compatible with the fact that a perturbed intron forced away from this splicing pathway becomes prone to 3′-hydrolysis [3]. Further experimental probes have been designed [1] by showing that P10 may be allowed to form prematurely (that is, prior to habilitation of the 3′-extremity of the 5′-exon as a nucleophilic agent) by selectively destabilizing competing interaction P1. Moreover, the scenario of P1–P10 competition has been confirmed recently by site-directed mutagenesis in a rather conclusive manner [4].

Although, in the light of the evidence mentioned above, the formation of P10 appears to be confirmed for this class of mitochondrial introns, its actual stabilization by mere Watson–Crick basepairing as suggested by Fig. 1 seems debatable. This is so since the loss of conformational entropy which results from the required loop closure is substantial. Rough estimates [5,6] give $T\Delta S^\circ \approx -9.80$ kcal/mol at a typical in vitro splicing temperature $T$=42°C [7], making P10 only marginally stable by $\Delta G^\circ \approx -1.08$ kcal/mol. Such estimates are probably not very reliable given the size of the loop involved (cf. [6]).

Our aim is to provide experimental evidence for further stabilization of P10 by tertiary interactions. The nucleotides to be engaged in such additional interaction

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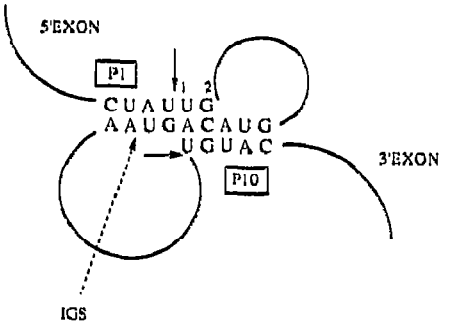


Fig. 1. Intramolecular splicing substrate for YOCB5. The arrows indicate the splicing sites.

may be singled out by inspection of the competitive scenario proposed earlier. Residues 1 and 2 have been displaced from base pairing with the IGS precisely to enable formation of P10 [1] and therefore, it is likely that they participate in shaping the intramolecular 3'-splicing substrate. In view of this, *it seems natural to probe tertiary interactions by deoxyribose substitution at positions 1 and 2 of the 5'-extremity of YCOB5*. On the other hand, it seems improbable that the ribose of a residue engaged in the P10 helix itself might use its 2'-OH for tertiary interaction with the intron 5'-extremity since this group is engaged itself in hydrogen bonding with the O4' of the next ribose in the 3'-direction, stabilizing the C3'-endo conformation of the A-RNA [8].

## 2. MATERIALS AND METHODS

Initially, two deoxyoligonucleotides, d(CUAUUG) and d(CUAU), complementary to the IGS of YCOB5 were synthesized corresponding respectively to the fragments (−4...+2) and (−4...−1) of the 5'-splicing substrate. The fragments are denoted according to the standard 5'→3' direction, assigning positive contour values to positions within the intron sequence. Thereafter, the deoxyoligonucleotides were ligated with the aid of T4 ligase to the RNA species I-2 and I, respectively. The latter RNA species coincides with the intron–3'-exon molecule and the former results from I by deletion of the first two nucleotides at the 5'-extremity. Construction of plasmids pT7-(I-2), pT7-(I-1) and pT7-I as well as transcription into the RNA species by T7 polymerase were performed following standard procedures [2]. The deoxyoligonucleotides d(CUAUUG), d(CUAUU) and d(CUAU) were synthesized on an Applied Biosystems 380 B DNA kit, following an existing protocol [9]. In order to test the functionality of the hybrids resulting from ligation of a DNA and an RNA species, the deoxyoligonucleotides were labelled with $^{32}P$ at their 5'-extremity. The incubation of all hybrid or chimaeric molecules was performed at Mg(II) concentration of 20 mM, that is, considerably higher than the standard splicing concentration [7]. These conditions seem adequate considering that deoxyribose substitution has a deleterious effect on 5'-cleavage when contrasted with the pure RNA species. The mobilities of chimaeric species and deoxyoligomers replacing the 5'-exon fragments were found to be identical, within experimental error, to those of the RNA counterparts. In order to estimate the fraction of molecules which were able to splice at the 5'-site, the chimaeric species d+I, d+(I−1) and d+(I−2) (Fig. 2) were incubated and after 22 min the reactions were stopped by adding a large excess of a solution mixture 90% (w/v) dimethylformamide/10 mM EDTA–10% xylene cyanol/Bromophenol blue.

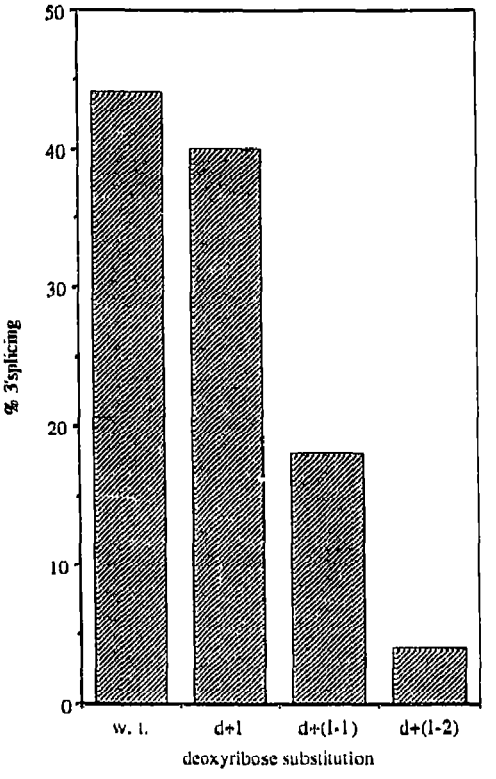


Fig. 2. Effect of deoxyribose substitution at the 5'-extremity of the intron on the ability to ligate autocatalytically the flanking regions. A shorthand notation has been adopted where different deoxyribose fragments ligated to RNA species are denoted 'd'. The effect of +1 and +1, +2 substitution is assessed by determining the percentage of molecules which, having been able to initiate splicing, are capable of 3'-splicing. Thus, the effect is *normalized* to account for exon–exon ligation. The results reveal a dramatic decrease of exon–exon ligation activity, depending on the degree of deoxyribose substitution at the 5'-extremity.

## 3. RESULTS

Incubation at special splicing conditions (42°C; 20 mM Mg(II)) reveals that scarcely 14% of the molecules of the chimaeric species d(CUAU)-I initiated splicing or revealed 5'-splicing activity and 40% of that population was able to ligate both regions flanking the intron (Fig. 2). This level of 3'-catalytic activity, normalized relative to the population active in 5'-splicing, is similar to that of the wild type [2]. Incubation of chimaeric species d(CUAUUG)-(I−2) revealed approximately the same percentage of splicing initiation or 5'-cleavage but, in this case, a dramatic decrease (to 4%) in 3'-splicing yields was measured for the population previously proven active in 5'-splicing (Fig. 2). These results support the involvement of ribose 2'-OHs in tertiary interactions which are functional for 3'-splicing.

An additional test was performed to rule out the possibility that a single nucleotide at the 5'-extremity of the

intron might be responsible for stabilizing the 3′-splicing site. This time, only the first residue at the 5′-extremity was subject to deoxyribose substitution, ligating the deoxyoligonucleotide d(CUAUU) to species I−1, that is, to the intron–3′-exon molecule with the 5′-end residue deleted. In this case, 18% of those hybrids which have proven capable of 5′-splicing were actually capable of exon–exon ligation. Such results indicate a pronounced reduction in autocatalytic activity when compared with chimaeric species d(CUAU)-I, yet not as pronounced as that found for the chimaeric species d(CUAUUG)-(I−2).

The extra stabilization provided by two tertiary hydrogen bonds would be in itself not particularly significant ($\Delta\Delta H^\circ \approx -2.2$ kcal/mol). However, the entropic contribution due to stacking of tertiary interactions must provide some degree of cooperativity not explored hitherto (cf. [10]). The significance of this extra stabilization is suggested by two facts: (i) the substantial decrease in splicing activity which seems to hold for the chimaeric species; and (ii) the experimental confirmation of the P1–P10 competitive scenario [4].

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