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Mutational evidence for competition between the P1 and the P10 helices of a mitochondrial group I intron

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ABSTRACT

A guanosine to cytosine transversion at position 2 of the fifth intron of the mitochondrial gene *COB* blocks the ligation step of splicing. This mutation prevents the formation of a base pair within the P1 helix of this group I intron—the RNA duplex formed between the 3′ end of the upstream exon and the internal guide sequence. The mutation also reduces the rate of the first step of splicing (guanosine addition at the 5′ splice junction) while stimulating hydrolysis at the 3′ intron-exon boundary. Consequently, the ligation of exons is blocked because the 3′ exon is removed prior to cleavage at the 5′ splice junction. The lesion can be suppressed by second-site mutations that preserve the potential for base-pairing at this position. Because the P1 duplex and the P10 duplex (between the guide sequence and the 3′ exon) overlap at the affected position, these results imply that the P1 and P10 pairings represent alternative structures that do not, indeed cannot, form simultaneously.

INTRODUCTION

The internal guide sequence (IGS) establishes the substrate specificity of group I ribozymes. In splicing reactions, the IGS determines the splice junctions by forming hydrogen bonds with the upstream (5′) and downstream (3′) exons, forming a pair of helices that place the splice junctions in proximity for ligation (1–4). The RNA duplex formed between the 3′ portion of the IGS and the 5′ exon is termed P1, and that between the 5′ component of the IGS and the 3′ exon is called P10 (5) (see Fig. 1).

Splicing occurs in two steps. In the first, guanosine attacks the phosphodiester bond linking the 5′ exon with the intron. In the second step, the free 3′ hydroxyl group on the 5′ exon attacks the phosphate at the 3′ splice junction, rendering ligated exons and free intron. Mutations that interfere with the base pairing between the 5′ exon and the guide sequence block the cleavage reaction (step 1) (1,2,6–11), while mutations that disrupt the hydrogen bonding between the IGS and the downstream exon may or may not inhibit the second step of splicing (ligation), depending on the strength of other determinants of the 3′ splice site (3,7,12–14).

Using site directed mutagenesis, we have determined that the P10 pairing is essential for the splicing of the fifth intron of the *COB* gene (*b*I5) of yeast (*S. cerevisiae*) mitochondria (11). The *COB* gene encodes apocytochrome b. In the course of that study, we found that replacing a C-G base pair in P10 with a G-C base pair permitted a low level of ligation and reduced the extent of the 5′ cleavage reaction that initiates splicing. The basis for the pleiotropic effect is suggested by Fig. 1: In the wild-type intron, the cytosine at position 223 of the intron has the potential to hydrogen bond with either the second nucleotide of the intron (G2) or the second nucleotide of the downstream exon (G+2). To test the hypothesis that pairing between C223 and G2 is required for splicing, we have changed the guanosine at the second position of the intron to cytosine and tested the effects of this mutation in the context of the wild-type intron and of the other mutations we generated at positions 223 (of the intron) and +2 (of exon 6). Analysis of these variants indicates that the base pair between G2 and C223 stabilizes P1 and that in its absence, ligation of exons is inhibited. This result was unexpected, because point mutations affecting P1 (and not P10) have not been known to block the second step of splicing.

MATERIALS AND METHODS

Materials

Agarose was purchased from FMC Bioproducts. BDH reagents for acrylamide gel electrophoresis came from Gallard and Schlesinger. Restriction endonucleases were purchased from American Allied Biochemicals, Bethesda Research Laboratories and New England Biolabs. T7 RNA polymerase was prepared according to the method of Grodberg and Dunn (15). Radiolabeled nucleotides were purchased from ICN and Amersham, and unlabeled nucleotides were purchased from Pharmacia. Urea was obtained from Schwarz/Mann. All other reagents came from Fisher Scientific and Sigma Chemical Company.

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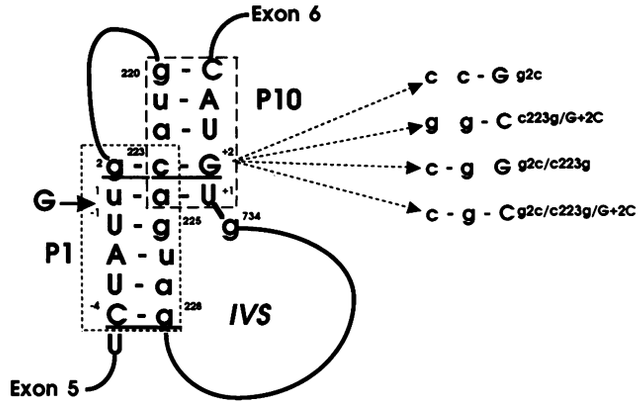

Fig. 1. Base pairing between the internal guide sequence of *COB* intron 5 and the flanking exons. The P1 and P10 helices are boxed in dotted lines. Helix P1 is comprised of 6 base pairs including four exon-intron pairs and two (above the cleavage site) between nucleotides within the intron. P10 is comprised of 5 pairs between the 3′ exon and in the IGS. The potential pairing configurations of some of the mutants studied is indicated to the right. In figures, lower case letters are used to indicate intron nucleotides, and upper case letters are used to indicate exon nucleotides.

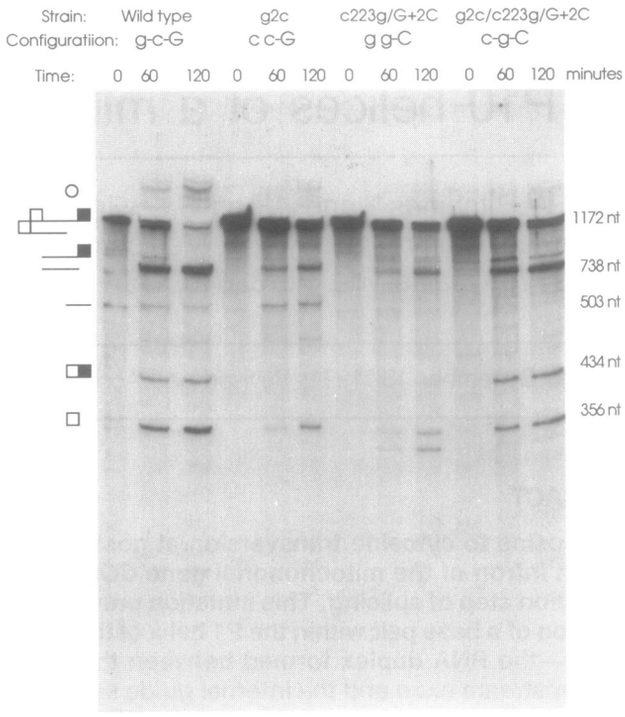

Fig. 2. Autocatalytic splicing of *b*I5 is inhibited by mutations at positions 2 and 223. The figure is an autoradiogram of a 4% polyacrylamide gel on which the ^{32}P-labeled products of autocatalytic splicing have been separated. The strains (i.e. transcript genotype) and the time of incubation under splicing conditions are indicated above the lanes: lanes 1–3 are wild-type RNA after 0, 60 and 120 minutes respectively; lane 4–6 are mutant G(2)C; lanes 7–9 are mutant C(223)G/G(+2)C; and lanes 10–12 are mutant G(2)C/C(223)G/G(+2)C. Cartoons at the margin indicate the major products of reaction: the circle represents circular intron RNA (738 nucleotides); the open box represents the 5′ exon (356 nucleotides); the closed box represents the 3′ exon (78 nucleotides) and the line represents the intron (738 nucleotides) or intron fragment (503 nucleotides) derived from the small circular form of the intron (19).

Site-Directed Mutagenesis

Most of the mutations used in this study were generated by the method of Sayers et al. (16) using a kit purchased from Amersham. Some have been described in an earlier publication (11). Mutation G(2)C/C(223)G was generated from a plasmid containing the single mutation C(223)G using the megaprimer method of Sarkar and Sommer (17). RNA was prepared from the amplification product directly without re-cloning. Mutagenic oligonucleotides were 20mers of the same sense as the RNA. DNA sequence analysis (18) was used to confirm that the desired nucleotide changes, and no others, had been made.

Transcription and Splicing Reactions

RNA synthesis from pT7-*b*I5 (pSPI5) and its derivatives were performed as described by Partono and Lewin (19). After transcription, RNA was purified by chromatography on Sephadex G-50, extraction with phenol/chloroform/isoamyl alcohol (50:49:1) and ethanol precipitation. Splicing reactions were performed as described (19). For time-course measurements, samples were drawn at the times indicated and reactions were terminated at by addition of Na_2EDTA to 50mM and rapid chilling in ice-water. Labeled RNA samples were separated on 4% acrylamide or 8% acrylamide, 8M urea gels. Quantitation of splicing was performed by direct scanning of dried gels using the Ambis Radioanalytic System.

RESULTS

Base Pairing Between Position 2 and Position 223 of *b*I5 is Required for Ligation

To examine the role of the G residue at the second position of the intron in the cleavage and ligation steps of splicing, we altered it to a C, and examined autocatalytic splicing in an otherwise wild-type intron and in an intron containing previously characterized mutations affecting its potential pairing partner in the IGS (C223). Some of the mutations we have analyzed are listed in Fig. 1, which also depicts the guide sequence and the P1 and P10 helices. The nucleotides of interest (G2, C223 and G+2) are underlined. ^{32}P-labeled transcripts containing various combinations of these mutations were incubated in the presence of 1M KCl, 50 mM Tris-HCl, pH7.5, 50mM $MgCl_2$, 0.2mM GTP and were analyzed by gel electrophoresis and autoradiography (Fig. 2). As shown in the first 3 lanes, the products of autocatalytic splicing of a wild-type transcript included the free linear intron, the intron plus 3′ exon, ligated exons, free exon 5, and several circular forms. The identity of these bands was established by a combination of hybridization and RNA sequence analysis (19).

When the G at position 2 was converted to C, the amount of 5′ cleavage was diminished and the ligation of exons was eliminated (Fig. 2, lanes 4–6). No ligation of exons was detected even after long exposures of autoradiograms. Since the removal of the 3′ exon from the precursor did not occur by ligation with the 5′ exon, the free intron that accumulated was a consequence of hydrolysis at the 3′ splice junction. The band appearing just below the precursor transcript in these autoradiograms is the initial hydrolysis product consisting of exon 5 linked to the intron with exon 6 removed. Most of the precursor molecule in mutant G(2)C was converted to this form within the first hour of

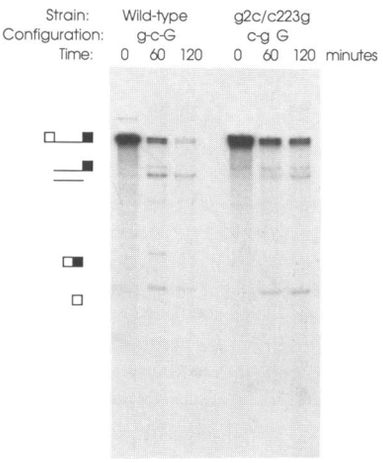

Fig. 3. P1 pairing is not sufficient for ligation of exons. Reaction of wild-type transcript (lanes 1–3) was compared with that of mutant G(2)C/C(223)G (lanes 4–6) which retains a C-G pair in P1 but disrupts a C-G pair in P10.

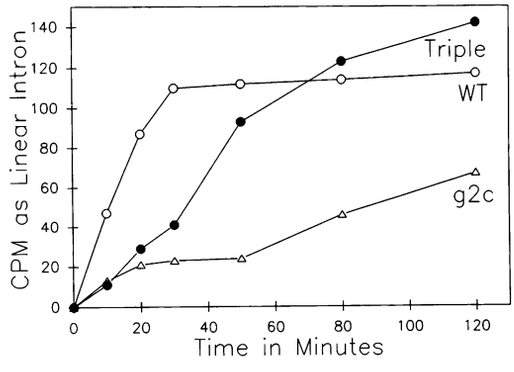

Fig. 4. The G(2)C mutation reduces the rate of processing. Autocatalytic splicing reactions were conducted using ^{32}P-labeled wild-type transcripts (open circles) and transcripts containing mutations G(2)C (triangles) and G(2)C/C(223)G/C(+2)G (closed circles). Samples were withdrawn at 0, 10, 20, 30, 50, 80, and 120 minutes and analyzed for the presence of linear intron (IVS) by gel electrophoresis. Samples were quantitated by the Ambis Radioanalytic System.

incubation. Exon 6 was only 78 nucleotides in this construction and migrated off of these gels. It was detected in denser gels (see below).

A slightly different observation was made for the mutant C(223)G/G(+2)C. This mutation prevents the formation of the 3′ G-C pair of P1, but preserves the P10 helix, substituting a G-C pairing for a C-G. This alteration reduced ligation of exons by 90%, but did not inhibit ligation completely (lanes 7–9). Incubation of this RNA under autocatalytic splicing conditions also resulted in the formation of a fragment migrating slightly faster than the 5′ exon. This fragment was most likely the result of cleavage at an aberrant site within exon 5. Several possibilities for alternative pairing with the IGS occur within the exon, and we have not established which, if any, of these is used to form this unusual product. Trace amounts of this fragment were seen in the reaction of the triple mutant [G(2)C/C(223)G/G(+2)C].

When RNA containing the triple mutant was incubated under splicing conditions, then efficient splicing was restored (lanes 10–12). Even though the extent of reaction was reduced in the triple mutant, more ligated exons accumulated than in the reaction of wild-type transcript: 46% of the cleaved 5′ exon was ligated to the 3′ exon in the mutant, compared to 34% in the reaction of wild-type transcript. This increased proportion of ligated exons was probably an indirect consequence of the reduced rate of hydrolysis at the 3′ splice junction (see below).

Each of the mutations affecting these potential pairings reduced the yield of circular intron products (Fig. 2). The reduction is attributable to the reduced rate of splicing in these constructs (see Fig. 4). After three hours of incubation, circular forms of the intron did accumulate in these reactions (*data not shown*).

Pairing Between Residues at Position 2 and at Position 223 Is Not Sufficient for Ligation

Our interpretation of the results depicted in Fig. 2 is that the IGS nucleotide at position 223 must have the capacity to pair both with the nucleotide at position 2 of the intron and that at position +2 of the downstream exon. An alternative explanation is that only the pairing in P1 is important (i.e. G2-C223). Our earlier work (11) showed that point mutations at either base 223 or at base +2 of the 3′ exon was sufficient to block ligation of exons without inhibiting the 5′ cleavage reaction. Since base changes at position +2 should not affect the potential for G2 to pair with C223, then it appears that both potential pairings are required.

To test this hypothesis further, we created mutant G(2)C/C(223)G. This transcript can form a C-G pair at the 3′ end of P1, but eliminates the potential for a standard base pair in P10 (see Fig. 1). As shown in the autoradiogram in Fig. 3, no ligation of exons was observed in this autocatalytic reaction suggesting that the P10 pairing is also required for ligation. Because the triple mutant in the C-G-C configuration spliced well, a C-G pair in P1 must be adequate as long as the P10 pairing is preserved. We noticed that reactions of transcripts containing a C-G pair at this position of P1 accumulated more of the molecule comprised of the intron and downstream exon than did wild-type transcripts (Figs. 2 and 3). In mutant G(2)C/C(223)G, almost half of the intron molecules that had been processed at the 5′ splice junction retained the 3′ exon. Because the only route for accumulation of free intron is hydrolysis at the 3′ splice site, disruption of P10 in this transcript must reduce 3′ hydrolysis.

The Triple Mutation Reduces the Rate of Splicing

Although more of the precursor RNA is converted to products in the reaction of the wild-type transcript, a greater fraction of the exons were ligated in the triple mutant G(2)C/C(223)G/G(+2)C (Fig. 2, lanes 11 and 12). This increase in splicing efficiency could be the consequence of a more efficient ligation step in the mutant, or it could result from a less efficient 5′ cleavage reaction, resulting in less free 5′ exon. To distinguish between these possibilities, we followed the time course of cleavage and ligation of the wild type and mutant transcripts (Fig. 4).

As expected from earlier results, splicing of wild-type *b*I5 (open circles), measured either by accumulation of intron or by decay of precursor, proceeded rapidly, and the reaction was complete by 30 minutes. In contrast, splicing of the triple mutant transcript was much slower (the initial rate was approximately one fifth that of the wild-type), but continued at the same rate for 80 minutes. The formation of the free intron in mutant G(2)C was much slower yet and appeared to be biphasic, with a second increase in free-IVS occurring between 60 and 120 minutes of

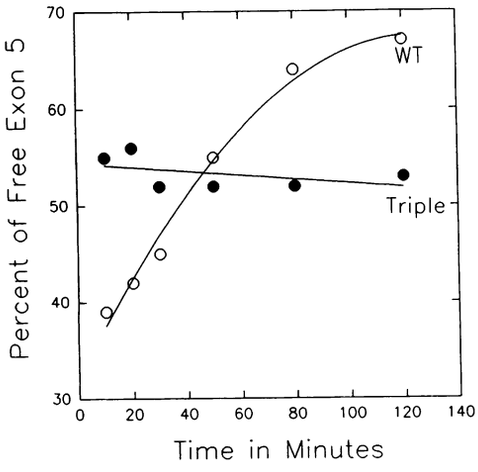

Fig. 5. The fraction of ligated exons is stable in the triple mutant. The fraction of exon 5 liberated from the intron that is ligated to exon 6 is expressed as a percentage of the total amount of exon 5 that was cleaved during the reaction. The data were taken from the same experiment that was described in Fig. 4. Open circles, wild-type transcript; filled circles, G(2)C/C(223)G/G(+2)C transcript.

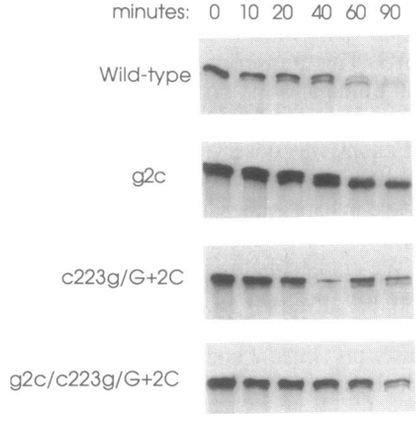

Fig. 6. Hydrolysis at the 3′ splice site is accelerated in mutant G(2)C. ^{32}P-labeled transcripts were incubated in conditions favoring hydrolysis at the 3′ splice site (11), and samples were withdrawn at the intervals indicated. The figure is taken from autoradiograms of 4% acrylamide gels. The upper band of the doublet is the full-length precursor and the lower band is intron–3′ exon. Transcript genotypes are indicated at the left.

incubation. Recovery of free intron was dependent on the presence of GTP in the reaction, and α-[^{32}P]-GTP was added to the 5′ end of the intron, so that free intron was not a product of hydrolysis at the 5′ splice site (*data not shown*).

During the course of the reaction a substantial fraction of the wild-type transcript was cleaved non-productively: At later times, the proportion of the 5′ exon not ligated to the 3′exon increased (Fig. 5). After 10 minutes of reaction, over 60% of the exon 5 cleaved from the wild-type transcript was ligated to exon 6. By 2 hours, this figure dropped to near 30%. In contrast, the fraction of ligated exons resulting from splicing of the triple mutant RNA appeared to be stable. About 45% of the processed exon 5 was linked to exon 6 in all samples. The level of free and ligated exon 5 increase at the same rate in the reaction of this transcript.

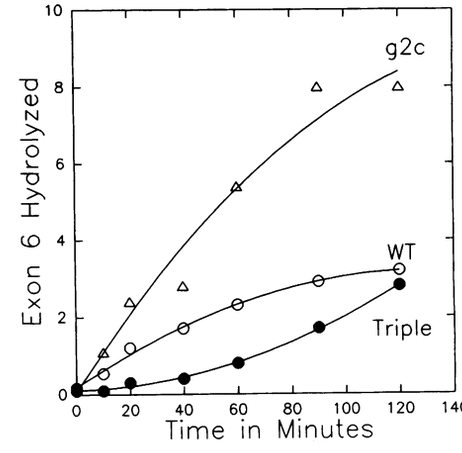

Fig. 7. Rate of accumulation of exon 6. Samples from splicing reactions were taken at the intervals shown and were fractionated on 8% polyacrylamide gels. Bands corresponding to exon 6 were quantitated by direct scanning of the gel. They are expressed as a fraction of the input precursor RNA band present at the beginning of the reaction and quantitated from the same gel.

Hydrolysis at the 3′ Splice Junction is Rapid in Mutant G(2)C

A significant fraction of wild-type *bI5* precursor RNA is lost with respect to the ligation step because the 3′ splice junction is susceptible to hydrolysis under autocatalytic conditions (11,20). To determine whether the G to C mutation at position 2 affected this susceptibility, we assayed the rate of hydrolysis in the wild-type transcript, the G(2)C mutation and in its revertant [G(2)C/C(223)G/G(+2)C]. Representative autoradiograms are shown in Fig. 6: The top band corresponds to the precursor RNA and the lower band to the 5′ exon—intron. The rate of hydrolysis of the G(2)C mutant transcript appeared to be more rapid than that of the wild-type RNA: by 60 minutes, most of the mutant transcript was hydrolyzed at the 3′ splice junction while the approximately half of the remaining wild-type precursor was intact.

To quantitate the rate of 3′ hydrolysis, samples were fractionated on an 8% polyacrylamide gel, and the rate of accretion of the free 3′ exon (exon 6) was measured. Exon 6 is cleaved by guanosine addition at an internal site resembling the 5′ splice junction (11), so that the sum of the exon-related products was used to estimate the level of hydrolysis (Fig. 7). Hydrolysis at the 3′ splice junction occurred at twice the rate in the P1 mutant G(2)C than in the naturally-occurring transcript. The rate of hydrolysis in the triple mutant [G(2)C/C(223)G/G(+2)C] was reduced to approximately wild-type levels but exhibited a lag for the first 20 minutes.

These results are consistent with those reported earlier that mutations in P1 enhance the 3′ hydrolysis reaction in this intron (11) and in the pre-ribosomal RNA of *Tetrahymena thermophila* (10). Because the 5′ cleavage reaction in mutant G(2)C was much slower than in the normal transcript (Fig. 4) and the hydrolysis reaction was faster, this mutation probably blocked ligation by allowing cleavage at the 3′ splice junction to outpace that at the 5′ junction. The triple mutant remedied the ligation defect by reducing the rate of the 3′ hydrolysis and increasing the rate of G addition at the 5′ splice site. In the mutant C(223)G/G(+2)C, which exhibits a low level of both cleavage and ligation (Fig. 2), the rate of hydrolysis at the 3′ splice site is somewhat slower

than that observed using wild-type transcripts. The lower rates of 3′ cleavage in this double mutant and in the triple mutant imply that the wild-type C-G base pair stabilizes P10 more than does the G-C configuration possible in these RNA molecules.

DISCUSSION

Most analyses of mutations in the P1 stem of group 1 introns considered their effects on the 5′ cleavage step. Doudna et al. (21) used a *trans*-cleavage assay and determined that the 5′ cleavage reaction follows a U-G base pair occurring within the central portion of P1 of the *Tetrahymena* ribozyme. They also found that the base pair following the U-G pair affects the efficiency of cleavage: Replacing an A-U pair at this position with a C-G pair reduced cleavage by 80%. Barfod and Cech (13), studying the same intron, reported that the conserved U-G pair was required for the first step of splicing but that this pair was superfluous for the ligation step. They found that base-pairing between the last nucleotide of the 5′ exon and the IGS was not required for ligation and that 5′ exons ending in any nucleotide were competent for ligation. In contrast, Price *et al.* (10) reported that limiting the potential for base-pairing between the 5′ exon and the IGS of the *Tetrahymena* ribozyme reduced the joining of exons and increased hydrolysis at the 3′ splice site and guanosine addition at cryptic sites within the exon.

In these experiments we did not study the pairing between the 5′ exon and its binding site in the intron. Rather, we examined a potential G-C base pair occurring entirely within the intron—two positions past the 5′ cleavage site in stem P1. Our rationale was that this is one of only 2 G-C pairs in a 6-membered stem and that the C at position 223 (in the IGS) had the potential to pair with a G at position +2 of the 3′ exon in addition to the G at position 2 of the intron. Indeed, our previous work suggests that the C(223)-G(+2) pair is required for exons to ligate (11). The results described above indicate that preventing the G-C pair at the 3′ end of P1 reduces the rate of the 5′ cleavage reaction but does not inhibit this step completely. However, in the absence of this pairing, no ligation of exons was observed. There are several possible explanations for this inhibition of step 2, including the possibility of a tertiary base-pairing interaction among these nucleotides. We favor the hypothesis that ligation cannot occur because hydrolysis of the 3′ exon removes it as a substrate. This conclusion is supported by the observation that hydrolysis is stimulated in RNA substrates which lack the potential to form stable P1 duplexes.

Intron 5 of *COB* has the potential to form a 5 base pair P10 helix that overlaps with P1 in two positions. Overlap between P1 and P10 is characteristic of the *Tetrahymena* ribozyme and, in fact, may be a conserved feature of all group I introns (1,4,22). This potential for alternative pairing suggests that P1 and P10 form sequentially during the splicing pathway and that the 5′ and the 3′ splice sites are in *competition* for the binding of the internal guide sequence (IGS). P1 is synthesized before P10. In most group 1 introns, the loop region L1 is quite short, stabilizing P1 in relation to P10. Kinetic modeling of the folding of *COB* intron 4 has suggested that a competition between P1 and P10 does exist for that group I intron (23). Our results support this hypothesis by demonstrating a mutation that retards the guanosine-dependent 5′ cleavage reaction (requiring P1) and hastens hydrolysis at the 3′ splice site. By reducing the stability of P1, P10 may form prior to cleavage at the 5′ splice site and intron-catalyzed hydrolysis at the 3′ splice site may ensue. Conversely, a mutation that disrupts P10 while retaining fully duplexed P1 [G(2)C/C(223)G], appears to accumulate molecules processed at the 5′ splice site but not at the 3′ splice site.

In the normal splicing pathway, guanosine addition at the 5′ end of the intron cleaves P1 and introduces an additional non-pairing nucleotide. This event would destabilize P1 and permit a stable interaction between the IGS and the 3′ splice junction. It should be noted that other interactions, such as pairing between a dinucleotide in J7/9 and the dinucleotide preceding the terminal G of the intron, also situate the 3′ splice junction near the catalytic site of the intron (12,24,25). Our data suggest that the internal guide sequence plays a dynamic rather than a static role in the selection of splice sites: a portion of the P1 helix must be displaced by the 3′ exon before ligation can result. This concept is consistent with models for catalysis by group I introns that require the terminal guanosine of the intron to displace the guanosine added to the 5′ end of the intron in the G-binding site of the intron for ligation or cyclization to occur (26).

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