The Cbp2 protein suppresses splice site mutations in a group I intron

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ABSTRACT

The Cbp2 protein facilitates the folding of a group I intron in the COB pre-mRNA of yeast mitochondria. Based on its ability to suppress mutations affecting the autocatalytic reaction, the protein appears to play a role in the selection of splice sites. Adding Cbp2 did not overcome the effects of mutations in P1 whose primary effect was on the first step of splicing. In contrast, most mutations affecting the ligation of exons were suppressed in vitro by Cbp2. These included mutations in P1, P9.0 and P10. In fact, a mutant transcript lacking both P9.0 and P10 ligated efficiently in the presence of Cbp2. P9.0 and P10 mutations also reduced the rate of cleavage at the splice junction, and this effect was only partially mitigated by adding Cbp2. A competitive secondary structure near the 3′ splice junction blocked Cbp2-stimulated splicing, but this mutation could be suppressed by co-transcriptional splicing in the presence of Cbp2. Our data underscore the importance of the interaction between the 5′ and 3′ splice junctions in group I introns and suggest that nucleotide–nucleotide interactions that stabilize the structure of group I introns can be superceded by protein–RNA interactions.

INTRODUCTION

In group I introns, base pairing between the 5′ splice junction and an internal guide sequence (IGS) selects the 5′ splice site, which follows a conserved U-G pair in this stem, called P1 (1–5). P1 is associated with the catalytic core of the intron through tertiary interactions including that with the bulge J4/5 (6). Such tertiary contacts are reflected by the anti-cooperative binding of the guanosine substrate and oligonucleotide analogs of the 5′ splice junction (7,8) and are described as ‘docking’ of P1 with the catalytic core (9,10). The spacing of P1 and P2 appears important in positioning the 5′ splice site with respect to the catalytic core (11,12).

Several determinants of the 3′ splice site have also been identified (Fig. 1). The first is a pairing (P10) between the 3′ exon and the internal guide sequence. That aligns the 3′ and the 5′ exons for the second step of splicing. The second, designated P9.0, associates the 3′-end of the intron with its catalytic core (13–15). P9.0 was originally described as a 2 base pair (bp) helix between the nucleotides immediately following P7 and the penultimate two nucleotides of the intron. More recently, Jaeger et al. (16) showed that P9.0 is a composite between P9.0a, which forms between a conserved guanosine that follows P7 and a cytosine near the 3′ splice site, and P9.0b, which forms between the second and third nucleotides downstream of P7 and the penultimate nucleotides of the intron. While P9.0 is observed in all group I introns, the P10 pairing is not detected in some introns (17). A G-C base pair in P7, which binds the guanosine nucleophile for the first step of splicing, has also been shown to bind the 3′ terminal guanosine for the second step (13,18,19).

The splicing of group I and II introns almost certainly employs proteins in vivo (20,21), though some group I introns splice autocatalytically in physiologic salts and temperatures (22). Intron-encoded maturases are essential for the splicing of group II and some group I introns in yeast mitochondria (23–27). In addition, other fungal introns require proteins that are synthesized on cytoplasmic ribosomes and are imported into mitochondria (28,29). It is likely that these proteins also function by stabilizing the structure of the precursor RNA leading to the appropriate phosphoryl exchange reactions at the 5′ and 3′ splice junctions (30,31). In the case of group I introns, such a structure would involve the determinants of the 5′ and 3′ splice sites like P1, P9.0 and P10.

We are studying the splicing of a group I intron from yeast mitochondria and its stimulation by a protein encoded in the nucleus. The terminal intron of the COB gene (b15) splices autocatalytically in 25 mM MgCl2 and 100 mM monovalent salt (32,33). Splicing of b15 in vivo requires the protein encoded by the nuclear gene CBP2 (34,35). The Cbp2 protein is not essential for the splicing of any other mitochondrial intron, though it stimulates the splicing of the intron in the large ribosomal RNA precursor (Shaw and Lewin, manuscript in preparation). In the test tube, addition of the Cbp2 protein permits splicing of b15 in physiologic concentrations of salt (31,35–37). Because we can compare the protein-free and the protein-facilitated splicing of b15 in vitro, we can test hypotheses about the importance of the protein in stabilizing RNA structures necessary for splicing.

Modification/protection and UV-crosslinking analyses indicate that Cbp2 binds b15 both in the catalytic core (P4) and in peripheral elements such as exon 5, the IGS, L2 and L6 (38,39). Cbp2 stabilizes helices that are otherwise susceptible to single strand-specific modifying reagents. Most importantly, the protein...
RNA ligase and restriction enzymes were purchased from Gibco-BRL or New England Biolabs. RNase A was obtained from Boehringer Mannheim. Reagents for electrophoresis and radiolabeled nucleotides were obtained from ICN. Placent RNase inhibitor was purchased from Promega. Activated nylon membranes (Hybond-N Plus) were purchased from Amersham and nitrocellulose membranes were purchased from Schleicher and Schull. Other reagents were obtained either from Sigma Chemical Company or from Fisher Scientific.

**Mutagenesis**

Several of the mutant transcripts used in this study have been described in earlier publications (42,43). Those affecting P9.0 (e.g. A677U/A678U, U736A/U737A), the extension of P1 (A3U/U4A/A5C), the 3′ exon (A+13G) and the P9.0/P10 double mutations were new and were prepared by site directed mutagenesis using the double primer method of Zoller and Smith (44). The template was a clone of hI5 and flanking exons from the HindIII site to the BglII site of COB inserted in the vector M13 mp19. All inserts were sequenced to verify that only the desired alteration had been made. Several clones of each mutation were tested to be sure that the behavior of the transcripts was consistent.

**Transcription and splicing reactions**

Transcripts were generated from wild-type and mutant templates cloned in pT7T3-18 as described by Partono and Lewin (33). Transcripts were linearized with EcoRI or Smal prior to transcription. The reaction conditions were those of Grodberg and Dunn (41) and contained 20 mM sodium phosphate buffer, pH 7.7, 8 mM MgCl2, 4 mM spermidine, 4 mM dithiothreitol, 0.8 mM ATP, CTP and GTP, 0.2 mM unlabeled UTP and 10 μCi [α-32P]UTP. Transcription reactions were conducted at 37°C for 2 h. Following transcription reactions, unincorporated nucleotides were removed by chromatography on Sephadex G-50 (Pharmacia). For co-transcriptional splicing, unlabeled nucleotides were added at 0.1 mM and reactions lasted 60 min. Polymerized RNA was recovered by precipitation with ethanol and 0.5 M NH4HCO3.

For Cbp2-assisted splicing, reactions were conducted in low salt buffer (50 mM Tris–HCl, pH 7.5, 50 mM NH4Cl, 5 mM MgCl2). For autocatalytic reactions, a high salt buffer was used (50 mM Tris–HCl, pH 7.5, 1 M KCl, 50 mM MgCl2). GTP was present at 0.2 mM in all post transcriptional splicing reactions. Cbp2-assisted reactions also contained 10 mM dithiothreitol. Reactions were usually for 1 h at 37°C and were terminated by addition of EDTA to 50 mM and precipitation with ethanol. Samples were dissolved in 90% formamide, 25 mM EDTA containing bromophenol blue and xylene cyanol and resolved on gels made of 4 or 5% polyacrylamide, 8 M urea and TBE buffer containing bromophenol blue and xylene cyanol and resolved on gels made of 4 or 5% polyacrylamide, 8 M urea and TBE buffer (89 mM Tris borate, pH 8.3, 2.5 mM EDTA). Splicing products were visualized by autoradiography.

Rates of splicing were measured in duplicate time courses at 37°C in low salt buffer containing Cbp2 and various transcripts. Samples were removed after 0, 1, 2, 4, 8 and 16 min and placed in tubes containing the formamide/EDTA loading buffer described above. Samples were then separated by electrophoresis and analyzed on a PhosphorImager (Molecular Dynamics). Rates of P′ cleavage were estimated by the appearance of intron products (intron and intron–3′-exon) expressed as a fraction of the intron present in the precursor. The intron-associated precursor was corrected for spontaneous cleavage, using the small precursor as a control. Rates of splicing were calculated for each sample by comparison to the rate of cleavage of the intron in the absence of protein (39).

**Figure 1.** (A) Schematic of the secondary structure of the hI5 intron according to the representation of Cech et al. (35). P9.0a and P9.0b are not represented. Short horizontal lines reflect hydrogen bonds. The boxed region showing splice-site determinants is the area shown in detail in (B). (B) Alignment of the splice junctions are marked with plus refer to nucleotides in exon 6. Numbers refer to nucleotides preceding the intron in exon 5 and numbers marked with arrows. Nucleotide positions are indicated in small font. Negative numbers refer to nucleotides preceding the intron in exon 5 and numbers marked with a plus refer to nucleotides in exon 6.

**Materials and Methods**

**Materials**

T7 RNA polymerase was isolated as described by Grodberg and Dunn (41). The purification of Cbp2 is described elsewhere (39).
Cbp2-dependent splicing

Autocatalytic splicing

2.6

62

nd

4.7

64

2.6

62 ± 2

Nitrocellulose filter binding

A double filter method (45) was used to measure affinity of Cbp2 and intron variants. This procedure was modified by replacing the DEAE membranes with activated nylon membranes (Hybond-N Plus from Amersham). Each data point is the average of triplicate DEAE membranes with activated nylon membranes (Hybond-N). Each data point is the average of triplicate determinations from the same dot blot. Binding conditions employed low salt splicing buffer at 37°C for 15 min in the absence of GTP.

Chemical and enzymatic modification analysis

Detailed methods for modification of h5 with hydroxyl radicals and 1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-\(\rho\)-toluene-sulfonate (CMCT) and for analysis of modification sites based on reverse transcription are described in an earlier paper (39).

RESULTS

Cbp2 stimulates splicing both during and after transcription

When incubated in low salt conditions, a \(\beta\)-labeled transcript containing h5 (738 nt) and flanking exons was catalytically inert (Fig. 2, lane 1). There was no splicing during the transcription reaction or the 60 min incubation in low salt buffer. If Cbp2 was added to the transcription reaction (lane 2), co-transcriptional splicing occurred. The ligated exons were detected as a band of 454 nucleotides (nt), and intermediates included the intron–3′-exon and the free 5′ exon. Similarly, if Cbp2 was incubated with a completed transcript in low salt buffer, the same intermediates and products were formed (lane 3). These products were identical to those of the autocatalytic reaction (lane 4), with the exception that cyclization at a site 237 nt into the intron and guanosine addition within the 3′ exon were diminished. Several bi-products evident in the autocatalytic reaction were reduced, these include linear fragments of 237 and 501 nt resulting from internal cyclization and hydrolysis (33).

TABLE 1. Efficiency of Cbp2-dependent and autocatalytic splicing

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Cbp2-dependent splicing</th>
<th>Autocatalytic splicing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%5′ cleavage</td>
<td>% ligation</td>
</tr>
<tr>
<td>Wild type</td>
<td>4.7±1</td>
<td>64 ± 4b</td>
</tr>
<tr>
<td>P9.0-3′</td>
<td>3.6</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>P9.0-5′</td>
<td>2.2</td>
<td>1.4 ± 1</td>
</tr>
<tr>
<td>P9.0-5′/P9.0-3′</td>
<td>0.1</td>
<td>25 ± 10</td>
</tr>
<tr>
<td>P10</td>
<td>3.7</td>
<td>4.9 ± 2</td>
</tr>
<tr>
<td>P9.0/P10</td>
<td>1.6</td>
<td>62.5 ± 3</td>
</tr>
</tbody>
</table>

4Initial rates were determined from level of total intron products (ivs and ivs-3′ exon) as a fraction of the intron present in the precursor and are presented as percent cleaved per minute. Measurements were made when <10% of the precursor had been cleaved. Duplicate determinations varied by <15% except in the case of protein dependent splicing of the P9.0 double mutant and the autocatalytic splicing of the P9.0-5′ mutant for which duplicates varied by ∼50%.

bThe percentage of exon ligated was estimated from the fraction of 5′ exon present as ligated exon as a ratio of the total cleaved 5′ exon. The range of variation for three determinations is indicated.

cnd indicates product not detected during the first 8 min of incubation at 37°C.

Cbp2 protein mitigates the effects a mutation in P10 but not in P1
Figure 3. (A) Splicing of mutant transcripts affecting P1 and P10. Each set of three lanes was from incubations in low salt buffer, low salt buffer plus Cbp2 and high salt buffer. Lanes 1–3 are P10 mutant G(+2)C; lanes 4–6 are P1 mutant G(229)C; lanes 7–9 are double mutant C(–4)G/G(229)C. Symbols are the same as in Figure 2. Several novel bands appear in the autocatalytic splicing the P1 revertant, including a ligation product between exon 5 and a shortened version of exon 6 (lane 9). The origin of these bands and of these mutant transcripts was described previously (42). The figure is an autoradiogram of a 4% polyacrylamide, 8 M urea gel. (B) Splicing of P1 mutant transcript G(2)C. The lanes were loaded in the same order as in panel B (low salt/low salt plus Cbp2/high salt). (C) Extension of the P1 helix inhibits the ligation of exons. Samples containing a three base pair extension of the P1 helix were incubated in low salt conditions with and without Cbp2 (lanes 1 and 2) and in autocatalytic conditions (lane 3). They were compared to similarly treated wild-type transcripts (lanes 4–6).

As seen in Figure 3B, Cbp2 was able to reduce the impact of this mutation on both steps of splicing (lane 2). This results implies that base-pairing at this position is not essential for Cbp2 to recognize the precursor RNA and that the riboprotein complex is active despite the weakened P1.

An extended P1 interferes with formation of P10 and prevents ligation of exons

P1 and P10 overlap with respect to the 5′ bases of the IGS (Fig. 1), and there appears to be a competition for IGS-pairing between the 5′ splice site and the 3′ splice site (43). To test the importance of this competition, we mutated positions 3–5 of the intron to the sequence U-A-C, permitting the formation of a 9 bp P1 stem that fully overlaps P10. This mutation blocked ligation of exons in both autocatalytic and Cbp2-stimulated splicing (Fig. 3C). In the presence of Cbp2 (lane 2), the true intermediates of splicing (5′ exon and intron–3′-exon) accumulated. In contrast, in high salt autocatalytic conditions (lane 3), hydrolysis at the 3′ splice site was observed, yielding free linear intron and the dead-end product consisting of the 5′-exon–intron. Hydrolysis at the 3′ splice site was twice as rapid for this mutant than for the wild type precursor (data not shown). There was also substantial cleavage at the internal cyclization site giving a fragment of 501 nt.
P1 and P10 mutations do not reduce Cbp2 binding

Since Cbp2 did not suppress the P1 mutation G(229)C, it is possible that this mutation altered the structure of the RNA so that the protein could not bind with normal affinity. This premise was tested using nitrocellulose filter binding to measure the equilibrium binding between Cbp2 and precursor RNA molecules (Fig. 4). Using the double filter method of Wong and Lohman (45), we could detect no significant differences in binding of Cbp2 to wild-type transcripts, to transcripts of P1 mutant G(229)C and to transcripts of P10 mutant C(224)G. As a control we also tested the protein or high salt protects these residues, indicating that the protein permits docking of the P1/P10 helices through tertiary interactions with the intron core (9,10).

Cbp2 also overcomes mutations affecting P9.0

The results presented in Figure 3 suggest that the Cbp2 protein did not eliminate the need for the P1 helix to determine the 5′ splice site. In contrast, a stable P10 helix was not required for the completion of splicing in the presence of Cbp2. P9.0b has also been implicated in the selection of the 3′ splice junction of group 1 introns (13,15,17,47). In b15, this helix would be comprised of two adenosines at positions 677 and 678 hydrogen bonded with two uridines at positions 736 and 737, the penultimate residues of the intron (Fig. 1). To determine the importance of this pairing for the second step of splicing we mutated the two A’s to U’s and the two U’s to A’s.

As seen in Figure 5A, mutating the U736A, U737A mutant (P9.0-3′) significantly reduced the ligation of exons in the autocatalytic reaction (lane 3 and Table 1) but ligation was increased in the presence of Cbp2. The A677U, A678U mutation (P9.0-5′) had a more profound effect on splicing. The first step of autocatalytic splicing (cleavage at the 5′ splice junction) was reduced and the second step (ligation of exons) was blocked completely (lane 6). Adding Cbp2 (lane 5) increased the efficiency of 5′ cleavage, but ligation was still inhibited. This inhibition may be related to the ambiguity of the 5′ cleavage site associated with this mutation. The A677U, A678U mutation led to cleavage at a site two nucleotides into the intron (i.e. following U2) in ~40% of the transcripts (lane 5). The effects of these lesions on the kinetics of splicing of b15 were quantitated by measuring the initial rate of 5′ cleavage and the extent of exon ligation (Table 1).

Ligation in the presence of P9.0 or P10 mutations

Because Cbp2 suppressed mutations in either P9.0 or P10, we monitored the effect of Cbp2 on a double mutation containing a defects in both P9.0 (A677U, A678U) and P10 (G+2C). The results are shown in Figure 5B. Lanes 1–3 show the results for the P10 mutation alone. This defect inhibited the second step of autocatalytic splicing (lane 3) but not in the presence of Cbp2 (lane 2). By contrast, the double mutant inhibited at the first step (lane 6), but was spliced efficiently in the presence of Cbp2 (lane 5). The effects of these mutations on the rate of 5′ cleavage and the efficiency of ligation are summarized in Table 1.

Structural analysis of the 3′-end of b15

In both low salt buffer and in autocatalytic conditions, the 3′ and 5′ splice junctions are resistant to modification by hydroxyl radicals, CMCT and RNase A (39). In contrast, nucleotides comprising the IGS are susceptible to modification with these reagents in low salt buffer in the absence of Cbp2. Adding the protein or high salt protects these residues, indicating that the protein permits docking of the P1/P10 helices through tertiary interactions with the intron core (9,10).

To examine the potential for base pairing and tertiary interactions within the 3′-end of b15, we analyzed this region by modification with hydroxyl radicals and CMCT. The former reagent modifies bases resulting in cleavage irrespective of secondary structure but is sensitive to solvent accessibility. CMCT modifies uridines and, to a lesser extent, guanidines and modifies unpaired residues preferentially. Figure 6 shows a portion of the results for hydroxyl radical (lanes 1–4) and for CMCT (lanes 5–8) modification with a schematic indicating the protected bases. The schematic is based on a series of experiments probing bases in a larger region than that shown in these autoradiograms.

Addition of Cbp2 to b15 in low salt buffer led to protection of P7 and P9.1. The residues surrounding the 3′ splice junction, including U736 and U737, were largely resistant to modification in non-splicing conditions. This is consistent with U736 and U737 being paired or inaccessible even in the absence of Cbp2. Similarly, A677 and A678 showed no change in susceptibility in shifting from non-splicing to splicing conditions. In contrast, G676 and C726, which are predicted to form P9.0a, both...
exhibited increased protection from hydroxyl radical upon addition of Cbp2 in low salt or upon incubation in autocatalytic conditions. C726 was also resistant to RNase A under splicing conditions (data not shown). These results suggest that P9.0a forms as a consequence of the binding of Cbp2.

A hairpin in exon 6 specifically inhibits ligation in Cbp2-dependent splicing

As an additional test of the importance of P10, we constructed a competitor of this helix by instituting a hairpin within the downstream exon. The hairpin arose through an A to G transition at position 13 of the exon (Fig. 7) and sequestered 4 bases (GUAC) that normally pair with the IGS to form P10. Existence of this structure was supported by partial RNase A digestion of 3’-labeled precursor RNA (data not shown). Transcripts bearing the A(+13)G mutation were resistant to cleavage in the region predicted to form the stem–loop structure, as expected for this single-strand specific nuclease. This mutation did not affect the affinity of Cbp2 for bI5 (Fig. 4).

The A(+13)G mutation did not inhibit ligation during co-transcriptional splicing in the presence of Cbp2 (Fig. 8B). With increasing amounts of Cbp2 added to the transcription reaction (lanes 2–4), the efficiency of ligation equaled that of the autocatalytic reaction. The transcript did not splice during synthesis in the absence of Cbp2 (lane 1) nor did Cbp2 stimulate splicing in the transcription buffer once transcription was completed (37). Co-transcriptional splicing must tip the balance between the competing secondary structures toward that favoring ligation.

DISCUSSION

The catalytic activity of group I ribozymes depends on nucleotide–nucleotide interactions such as hydrogen bonding and base stacking. These contacts determine the secondary and tertiary structures of the ribozymes. This work demonstrates that base pairs required for autocatalytic splicing are not essential for protein-facilitated splicing. This result indicates that the binding of the protein imparts structural stability on the RNA. We have begun our analysis on the specificity of the exon binding domain of this group I intron. It is clear that the analysis can be extended to the catalytic domain as well. Mohr et al. (30) concluded that the CYT-18 protein of Neurospora can suppress mutations in the T4 Td intron by stabilizing the intron core. Gampel et al. (48) have shown that Cbp2 is not able to suppress a mutation in the guanosine nucleotide binding site of bI5. A systematic study of the bI5–Cbp2 interaction is worthwhile, since Cbp2 is the physiologic partner of this ribozyme in mitochondria. We have previously shown that Cbp2 stabilizes tertiary interactions within bI5 so that the IGS surface of P1 becomes inaccessible to solvent (39). Our present data (Fig. 6) suggest that splicing conditions also render the guanosine binding site in P7 and P9.0a less accessible to modification. We infer that Cbp2, by stabilizing P1 and leading to its docking with the intron core, also fulfills the requirements for selection of the 3’ splice site.
Figure 6. Hydroxyl radical and CMCT modification of the 3' region of bI5. Autoradiographs are presented of 10% polyacrylamide/8 M urea gels used to separate products from primer extension on hydroxyl radical (lanes 1–4) and CMCT (lanes 5–8) modified RNA. Lanes 1 and 5 are primer extension on unmodified RNA; lanes 2 and 6 are primer extension on RNA modified in low salt buffer; lanes 3 and 7 are primer extension on RNA modified in low salt in the presence of Cbp2 and lanes 4 and 8 are primer extension on RNA modified in high salt buffer. The primer employed was complementary to exon 6, and its 3' terminus annealed 21 nt downstream from the splice junction. In the schematic, squares represent nucleotides which become protected from hydroxyl radical in presence of Cbp2 or high salt and circles indicate bases protected from CMCT in splicing conditions. The diagram summarizes the results of several experiments that analyzed a large segment of the intron. The autoradiograms shown cover only a portion of the 3' region analyzed in this way. Only reductions in intensity that were observed in triplicate experiments are shown. Changes in intensity were normalized to surrounding bands as described by Shaw and Lewin (39).

Figure 7. A potential stem–loop structure in exon 6 near the 3' splice junction resulting from the A to G transition at position +13. The arrow indicates the position of the 3' splice junction and the numbers indicate the nucleotide distance from the junction.

results suggest that the formation of P9.0a correlates with the tertiary contact required for 3' splice site selection. Our results are consistent with those of Jaeger et al. (16) who suggested that P9.0a contributes to the stability of the sunY ribozyme. The potential for forming a G·C pair between analogous positions is conserved among group I introns (17).

There is evidence from the Tetrahymena ribozyme that the guanosine binding site in P7 binds the 3’ terminal guanosine of the intron following the first step of splicing (13,18,49). The rate of the ligation reaction is significantly reduced by mutating the conserved guanosine at the 3'-end of the intron (18,19,49). Tetrahymena introns disrupted in both P9.0 and P10 could catalyze exon ligation at rates between 1 and 30% that of the wild-type intron (13). We found that Cbp2 suppressed mutations in both P9.0 and P10 in bI5 (Fig. 5B) that block ligation under autocatalytic conditions. The effect of the double mutation was surprising for two reasons. First, Cbp2 permitted the second step of splicing in a transcript lacking two determinants of the 3' splice site. Second, Cbp2 stimulated ligation of the double mutant was more efficient than that of either of the single mutants. This result suggests that the interference imposed by the P9.0-5' mutation (A677U, A678U) was reduced by the P10 mutation. The P9.0-5' mutation leads to a misalignment of P1 with the intron core [as suggested by its effect on the 5' splice site (Fig. 5A, lane 5)]. The
P10 mutation G+2C affects a position at which P1 and P10 are in competition for binding the IGS (Fig. 1) (43). Reducing the P10 mutation G+2C affects a position at which P1 and P10 are in′ affinity of the 3′ component of the catalytic core of Tetrahymena. In addition, Cbp2, by constraining the flexibility of the 3′ end of the A677U, A678U mutant to the guanosine binding site in P7 (Fig. 1), it is not surprising that mutations directed at the 3′ splice site should influence the 5′ splice site and, conversely, that a mutation in P1, such as G2C, should reduce the second step of splicing (43). Some, but not all, mutations affecting P1 were suppressible by Cbp2. In particular, the base pair between C(−4) in the 5′-G and G(228) in the IGS was essential. While Cbp2 appears to make contacts with the IGS and the 5′ exon (38,39), these positions do not form UV-crosslinks with the protein.

The capacity of intron 5 to splice during the transcription reaction (Fig. 2) and the ability of the exon 6 mutation A(+13)G to ligate co-translationally (Fig. 3B) implicate the protein in the sequential folding of the intron. We have demonstrated that co-transcriptional addition of Cbp2 alters the folding of L1 during transcription (37) and that Cbp2 stabilizes the secondary structure of L1 and P1 (39). The A(+13)G mutation inhibited the ligation of exons in the Cbp2-stimulated reaction, but did not affect the first step of splicing (Fig. 8A), and had no impact on either step of autocatalytic splicing (lane 3). A(+13)G does not change a known binding site of the protein on this RNA (38,39). The difference in the protein-stimulated and the autocatalytic reactions probably reflects the increased flexibility of the RNA in the high-salt conditions relative to that in the protein complex. Emerick and Woodson (52) noted that RNA precursors containing the Tetrahymena IVS sometimes misfolded during transcription and must be re-folded for splicing to occur. It is apparent that the stem–loop in exon 6 causes a similar phenomenon for h5. Adding Cbp2 during transcription permits the correct folding of the determinants of the 3′ splice-site. In low salt conditions, the alternative secondary structure otherwise predominates. Burke and colleagues, using a model system based on a group I intron from Azotobacter, demonstrated that the substrate for the ligation reaction is a P1–P10 complex (53,54). Because of the overlap between P1 and P10 in h5, we view this complex as a dynamic rather than a static structure. The role of Cbp2 in determining the 3′ splice site seems related to its ability to secure P1 and to promote tertiary interactions between P1 and the catalytic center of the intron (38,39).

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