Binding of the CBP2 protein to a yeast mitochondrial group I intron requires the catalytic core of the RNA

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The yeast CBP2 gene product is required for the splicing of the terminal intron (bl5) of the mitochondrial cytochrome b pre-mRNA in vivo. In vitro, bl5 RNA self-splices efficiently only at high MgCl2 concentrations (50 mM); at 5 mM MgCl2, efficient splicing requires purified CBP2 protein. To determine the sequences within bl5 recognized by the protein, we have constructed deletion and substitution mutants of the RNA. Their binding to CBP2 was assessed by their ability to inhibit protein-dependent splicing of the wild-type bl5 RNA. Several regions, including the large L1 and L8 loops, can be deleted without affecting binding. They can therefore be eliminated from consideration as critical recognition elements. In contrast, other changes prevent the RNA from binding CBP2 and also impair self-splicing. Thus, either the catalytic core contacts the protein directly, or the integrity of the core is required for proper display of other RNA sequences that bind the protein. The results are consistent with a model in which the CBP2 protein facilitates splicing by binding to and stabilizing the active structure of the RNA. However, a more specific model is proposed in which the protein specifically enhances Mg2+ binding required for catalysis.

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Many group I introns have been shown to undergo self-splicing in vitro (Cech 1990). Nevertheless, there are clear examples where splicing of these introns in vivo requires or is facilitated further by proteins. Several mitochondrial introns have open reading frames encoding maturases required for splicing of the intron containing their own mRNA (Lazowska et al. 1989; Perlman and Butow 1989). Proteins encoded in the nucleus are also required for splicing of some fungal mitochondrial introns. Two of these, the CBP2 protein of Saccharomyces cerevisiae (McGraw and Tzagoloff 1983) and the mitochondrial tyrosyl-tRNA synthetase [cyt-18 protein] of Neurospora crassa [Akins and Lambowitz 1987] must interact directly with the unspliced precursor RNA, because the purified proteins function to allow or enhance splicing in vitro (Gampel et al. 1989; Majumder et al. 1989).

Because self-splicing and CBP2 protein-dependent splicing are blocked by the same mutations within the intron and progress through the same guanosine-dependent pathway, it does not seem likely that this protein could be a splicing enzyme in the traditional sense (Gampel et al. 1989). Rather, it has been proposed that CBP2 and perhaps other group I intron splicing factors are superimposing an additional level of catalysis on an intrinsically RNA-catalyzed process. To catalyze splicing, group I intron RNA must be folded into a highly ordered structure. Of central importance to this structure is the catalytic core region comprised of highly conserved sequences that have been demonstrated, in many cases, to be critical to splicing (Cech 1990; Couture et al. 1990). In a popular model, proteins bind to and stabilize the catalytically active conformation of the RNA, promoting correct folding of the core (Gampel et al. 1989; Cech 1990; Guo et al. 1991). However, exactly what these proteins recognize in the RNA and how they facilitate splicing remain largely unknown.

The terminal intron of the S. cerevisiae cytochrome b gene [bl5] contains the sequences and structures characteristic of group IA introns [Davies et al. 1982; Michel et al. 1982]. Its splicing factor is encoded by the nuclear gene CBP2. cbp2 mutants are respiratory deficient due to a specific defect in excision of bl5 and thereby accumulate an unspliced cytochrome b pre-mRNA (McGraw and Tzagoloff 1983; Hill et al. 1985). The splicing of other mitochondrial pre-mRNAs is unaffected by the cbp2 mutation, suggesting that bl5 is the only intron that requires this particular splicing factor. Studies of the function of the CBP2 gene product have used the protein purified from wild-type S. cerevisiae mitochondria to promote splicing of bl5 in vitro. The bl5 intron is able to self-splice in vitro, although the reaction displays a
strong dependence on Mg\(^{2+}\) ion concentration with a maximum rate at 50–100 mM Mg\(^{2+}\) (Gampel and Tzagoloff 1987; Partono and Lewin 1988). Addition of purified CBP2 protein to splicing reactions at 5 mM Mg\(^{2+}\) greatly enhances the splicing rate over that in the absence of the protein (Gampel et al. 1989).

To uncover possible mechanisms of protein facilitation, we have begun to characterize the interaction between bI5 and the CBP2 protein. Attempts to directly map contact points on the RNA by protein-dependent protection of the RNA from nuclease degradation have been unsuccessful. This may be caused by a short half-life of the RNA–protein complex not allowing for sufficient trapping of the complex under the conditions of nuclease treatment. It may be necessary to perform assay binding directly with saturating concentrations of protein. This will be technically feasible only when the protein can be purified in larger quantities in a soluble form.

As a substitute for direct mapping, we have made site-directed mutations within bI5 and measured the effects of these changes on protein binding and on RNA catalysis. The binding assay uses the variant RNAs as competitive inhibitors of protein-dependent splicing of wild-type RNA. Variants that bind the protein as tightly as wild-type bI5 show that the deleted or mutated regions do not make substantial contributions to binding. Variants defective in protein binding could be of two subtypes. Where self-splicing activity of the variant RNA is also affected, it might be concluded that the altered sequences contribute directly to both the catalytically active structure and the protein-binding site. However, the diminution of protein binding could be an indirect effect of denaturation of the RNA structure. The most informative subclass with respect to protein recognition would include mutant RNAs that retain self-splicing activity but are no longer bound by the protein. In this case, the altered sequences would be good candidates for regions involved directly in the formation of the protein-binding site. If the protein binds to the catalytic core, such a phenotype may not exist.

The results of the mutagenesis experiments reported in this paper indicate that there is significant overlap in sequences contributing to the active RNA structure and to the formation of the protein-binding site. This leads to a model of protein binding that invokes recognition of a structure or structures formed only in the context of the intact group I intron core.

**Results**

**Secondary structure**

Our approach to defining the protein-binding site on bI5 RNA involved the generation of bI5 variants with deletions or substitutions. Such variants are best designed with consideration of RNA structure. The secondary structure of bI5 was predicted with reference to the model for group I introns determined on the basis of phylogenetic arguments (Davies et al. 1982; Michel et al. 1982) and largely substantiated by studies of the Tetrahymena rRNA intron (Cech 1990). For bI5, the predicted structure was verified in part by chemical modification with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMCT). This reagent modifies N3 of uridines and, to a lesser extent, N1 of guanosines with specificity for unpaired bases (Moazed et al. 1986; Ehresmann et al. 1987). Sites of modification were revealed by reverse transcription from oligonucleotide primers.

Comparison of modification under native and denaturing conditions indicates which residues are involved in base-pairing in the native state. The gel shown in Figure 1 is an example of how the secondary structure assignments were made from the CMCT modification data. The uridines of L6b (nucleotides 394, 396, 399, 402, and 405) are modified heavily under native conditions compared with their neighbors and are therefore designated as unpaired. U340 in J5/4, as well as U328 and U324 in L5, are modified heavily, indicating that they are also unpaired in the native state. The uridines of P6b[5'] (nucleotides 371–378) and P6b[3'] (nucleotides 411–424) are modified only slightly under native conditions, indicat-

![Figure 1. CMCT modification of bI5 RNA.](#)
ing that these regions are paired as predicted. Unexpectedly, the two uridines in L6a are undermodified under native conditions, suggesting that they may also be paired. The proposed tertiary interaction [P11] between L7.1 and L6a [Michel and Westhof 1990] could account for the undermodification in L6a.

A compilation of results, including studies with different oligonucleotide primers, is provided in Figure 2A. The data indicate that the predicted structure is essentially correct and the same at 5 and 50 mM Mg\(^{2+}\) [data not shown]. The conserved helical structures P2-P5 are clearly base-paired, whereas the uridines in J2/3, J3/4, J4/5, L5, J5/4, L6b, and J6/7 are unpaired and accessible to modification. Very little guanosine modification was observed under the conditions used. Consequently, it is difficult to interpret the finding that light modification is seen for the single uridine in the 3' strand of P6, as there is no information about the paired state of the rest of the helix.

The accessibility of P7 nucleotides to the CMCT reagent is consistent with earlier results of chemical modification with the Tetrahymena rRNA intron [Inoue and Cech 1985; Jaeger et al. 1990]. The importance of P7 base-pairing to catalytic activity has been demonstrated unequivocally by phylogenetic comparison [Cech 1988] and compensatory mutations [Burke et al. 1986]. Thus, the accessibility to modification can be interpreted as reflecting a dynamic equilibrium between paired and unpaired states of P7. It is even possible that the P7 residues are most often unpaired, with the helix forming only transiently at a critical step in the reaction.

The results of CMCT modification confirm the base-pairing predicted by Michel and Westhof [1990] for the P7.1-P7.1a region in group IA1 introns. This subgroup includes 16 introns, all of which potentially form very similar structures in this joining region between P3 and P7. The introns from bacteriophage T4 are included in the group IA2 subgroup and have a distinctly different predicted secondary structure in this region [Shub et al. 1988].

CBP2 binding of b15 variants

The b15 secondary structure model was used to design variant RNAs for studies to localize the protein-binding site. Each variant was tested for splicing activity and for its ability to act as an inhibitor of the protein-dependent splicing reaction. K\(_i\) values were determined under conditions where both the RNA and the protein concentrations were below the value of K\(_m\). The determinations were done by two methods, which are described in the legend to Figure 3 and in Materials and methods. Results from the two methods were in close agreement.

The b15 variant \(\Delta L1-B\) has entirely wild-type sequences but is missing the 5' exon and the 223 nucleotides between the 5'-splice site and the 5' terminus of the 5'-exon-binding site [Fig. 2B]. The K\(_i\) for \(\Delta L1-B\) was determined by method A to be 14 nm [Fig. 3] and by method B to be 2-8 nm [Table 1]. These numbers are equal within experimental error to each other and to the K\(_i\) value determined for the full-length b15 RNA. We therefore interpret \(\Delta L1-B\) binding as representative of wild-type b15 binding.

The large loops, L1 (221 nucleotides) and L8 (152 nucleotides), are unusual features of the b15 intron because of their size and richness in adenosines and uridines [94% for L1; 79% for L8]. Deletion of either of these loops [\(\Delta L1-10\) and \(\Delta L8\) variants] does not affect binding [Table 1]. In contrast, deletion of the P6a, P6b, and L6b region [\(\Delta L6\), P7.1-P7.1a [\(\Delta P7.1\)], or sequences 3' to P8[5'] [\(\Delta L8/BgIII\)], results in drastic disruption of binding to CBP2 (>200 nm reflecting no detectable inhibition). All of these deletion variants are defective in self-splicing as well as protein-dependent splicing. CMCT modification of these variant RNAs gave patterns of modification indistinguishable from the wild-type pattern outside the secondary structure elements directly affected by the given deletion [data not shown]. Thus, the secondary structure is still formed in the context of the deletion mutants.

The specificity of recognition was tested by adding other group I introns to the protein-dependent splicing reaction. The group IA introns from bacteriophage T4 [nrdB and td; Shub et al. 1988] and the group IB intron from the Tetrahymena rRNA [L-21 Scal ribozyme; Zaug et al. 1988] did not inhibit the reaction detectably at concentrations up to 200 nm.

In the mutant designated RP5td, the J4/5, P5, L5, and J5/4 regions of b15 were replaced with the same regions from the td intron [Fig. 4A]. This mutant RNA self-splices, although it has an increased requirement for Mg\(^{2+}\). Splicing rates for RP5td approach that for the wild-type RNA as the concentration of Mg\(^{2+}\) is increased to 200 mM [Fig. 4B]. In the presence of the protein at 5 mM Mg\(^{2+}\), splicing of this RNA is barely detectable [<10% wild type; data not shown]. However, the RP5td

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**Figure 2.** Secondary structure of b15 RNA. The structure is drawn according to the convention for group I introns with conserved stem-loop structures P1-P9 and L1-L9 [Burke et al. 1987]. (A) Compilation of CMCT modification results with primers in addition to the one shown in Fig. 1. Circles indicate residues preferentially modified under native conditions [heavy lines indicate 2- to 5-fold higher modification than average, e.g., nucleotide 396-408 [heavy] vs. nucleotide 394 [light]]; open boxes indicate undermodified uridines [e.g., 386 and 388], shaded boxes indicate residues modified under denaturing conditions and undermodified under native conditions [e.g., nucleotides 367-380]. Modification was not determined for P1[5'], L1, L8, P8, P9, P9.1, and the 3' end of the intron. (B) Sequences deleted or altered by mutagenesis. \(\Delta L1-B\) is missing all sequences preceding the 5' exon-binding site. In \(\Delta L6\), the indicated region is deleted and replaced with a small site, 5'-CCCGGG-3'. \(\Delta P7.1\) is a complete deletion of all sequences between P7 and P3. In \(\Delta L8\), the 152-nucleotide wild-type L8 is replaced with a BgIII site, 5'-AGAUCU-3'. The \(\Delta L8/BgIII\) variant is a runoff transcript from the \(\Delta L8\) template linearized at the BgIII site inserted downstream of P8 [5'-AGATCT-3' in the DNA template]. The G-site mutants are described in Fig. 5.
Protein facilitation of RNA self-splicing

Figure 2. (See facing page for legend.)
Figure 3. Kinetic analysis. (A) Gels showing the kinetics of splicing product formation in the protein-dependent reaction as a function of precursor and inhibitor RNA concentrations. Because the unspliced precursor and excised IVS RNAs are not resolved on these 10% polyacrylamide gels, the upper portion of the gel containing these bands is not shown. Reactions were carried out at the indicated concentrations of uniformly labeled wild-type b15 RNA in the presence of unlabeled AL1-B RNA as inhibitor (I) at the indicated concentrations. Aliquots were taken at 2, 5, 10, and 20 min of reaction. Products are the ligated exons and the 5' exon. Concentrations of RNA (as indicated) and protein (15 nM) were very small relative to the approximate Km value of 200 nM estimated from determinations of reaction rate as a function of RNA concentration >10 nM (A. Gampel, unpubl.). (B) Graphic analysis of the initial rate of 5'-splice site cleavage as a function of RNA concentration. Rates were determined from quantitative analysis of autoradiographs in A (for calculations, see Materials and methods). The slope of the line gives a value of $V_{\text{max}}/K_m$ in the absence of inhibitor and $V_{\text{max}}/K_m(\text{app})$ in the presence of inhibitor. For competitive inhibition, $K_m(\text{app}) = K_m (1 + [I]/K_i)$ and the value of $K_i$ can be calculated. From the data at $I = 10$ nM, the calculated $K_i$ is 14 nM and from $I = 50$ nM, the calculated $K_i$ is 13 nM. The low extent of reaction at $I = 200$ nM did not allow for the calculation of rates or $K_i$ from these data.

RNA binds well ($K_i = 30$ nM) to the CBP2 protein at 5 mM Mg$^{2+}$ (Table 1). A possible unified explanation for all of these effects is that the RPStd replacement has perturbed the RNA structure required for catalysis, leading to an increased Mg$^{2+}$ requirement for both self-splicing and protein-facilitated splicing, but that the contribution of CBP2 to splicing is largely the same.

G-site mutants

To further assess the importance of the core of the group I intron structure in CBP2 protein recognition, point mutants within P7 were made. The single-nucleotide change, C263A (Tetrahymena numbering), replaces the P7 bulged C residue conserved among group IA introns to an A as is found in group IB introns (Fig. 5A). This replacement could affect the intron structure either by changing the identity of the bulged nucleotide or by producing six contiguous base pairs in P7 coupled to an extension of J6/7. The splicing rate for this mutant RNA is less than that for the wild type (5- to 10-fold decreased at 0.2 mM GTP), but both protein-dependent and self-splicing reactions occur with Mg$^{2+}$ dependence similar to that of the wild-type RNA (data not shown). Results of competition experiments show the $K_i$ for the C263A RNA to be approximately equal to wild-type RNA (Table 1).

The mutation G264A alters the G of the G : C pair, a critical component of the guanosine-binding site (Michel et al. 1989). Unlike the change in the bulged nucleotide, the G264A mutation drastically affects protein binding. G264A mutant RNA does not inhibit the protein-dependent reaction even at concentrations up to 900 nM. This mutant is also completely defective in guanosine-dependent splicing at high Mg$^{2+}$ levels or in the presence of the protein.

The results with the G264A mutant suggested that formation of the guanosine-binding site might be critical for CBP2 protein recognition. Further experiments were done to distinguish between a direct contact of the protein at the G site, dependent on the identity of the G : C pair, versus an indirect dependence of protein binding on the structural integrity of the G site. Two additional G-site mutations were made, changing C311 of the G : C pair to a U in the wild-type background (generating a G : U wobble pair at the G site) and in the G264A mutant background (generating an A : U base pair at the G site).

The set of variants with G : C (wild type), A : C, G : U, or A : U at the G site were tested for splicing with guanosine (G), 2-aminopurine ribonucleoside (2-AP), or
2-amino-6-chloropurine ribonucleoside (6-Cl-2-AP) as the cofactor for 5'-splice site cleavage. For the *Tetrahymena* ribozyme, the cofactor preference is determined by the identity of the base pair at the G site. The G : C or G : U pair prefers G as a cofactor, whereas the A : C or A : U pair prefers 2-AP (Michel et al. 1989) or 6-Cl-2-AP, the latter having a twofold lower *Km* (M. Hanna, pers. comm.). For b15, the A : U variant does not splice with G, but 5'-splice site cleavage proceeds efficiently with either of the other cofactors [Fig. 5C]. No exon ligation occurs with the A : U variant. This is expected, because the A : U pair does not bind exogenous G, it is not expected to bind the 3'-terminal G as would be necessary for exon ligation (Michel et al. 1989). Cleavage was not detected for the A : C variant with 2-AP or for the G : U variant with G under the conditions tested (data not shown). These results differ from those obtained with the *Tetrahymena* intron where the analogous variants retain splicing activity with appropriate cofactor preference.

In addition to self-splicing, the A : U variant is able to undergo protein-dependent splicing with 2-AP or 6-Cl-2-AP [Fig. 5C]. This renewal of protein recognition as compared with the A : C mutant occurs as a result of the single-base change, C311U, which restores a base pair at the G site. This leads to the interpretation that the identity of the base pair at the G site is not important to CBP2 protein recognition, because b15 RNAs with either G : C or A : U at the G site are able to undergo protein-dependent splicing. However, the integrity of the cofactor-binding site must be critical because RNAs with a mismatch at the G site do not bind the CBP2 protein.

### Table 1. CBP2 binding and splicing activity of b15 variants

| Inhibitor               | *Km* [μM] | Self-splicing activity |
|-------------------------|-----------|-----------------------|
|                         | method A  | method B  | Binding*              |
| Wild-type b15           | n.t.      | 2-8       | +                     |
| ΔL1-10                  | n.t.      | 2-8       | +                     |
| ΔL1-8                   | 14        | 2-8       | +                     |
| ΔL8                     | 10        | 2-8       | +                     |
| ΔL6                     | n.t.      | >200      | -                     |
| ΔP7.1                   | n.t.      | >200      | -                     |
| ΔL8/BglII               | n.t.      | >200      | -                     |
| RPStd                   | 30        | n.t.      | (+)                   |
| C263A                   | 9-13      | 2-8       | (+)                   |
| G264A                   | >900      | n.t.      | -                     |
| T4 nrdB                 | n.t.      | >200      | -                     |
| T4 td                   | n.t.      | >200      | -                     |
| *Tetrahymena*           | n.t.      | >200      | -                     |

[n.t.] Not tested, [n.a.] not applicable.

*Results of *Km* determinations by methods A [see Fig. 3] and B [see Materials and methods] are summarized.

*Self-splicing activity was determined as described in Materials and methods and is indicated as + (at least 80% of wild-type activity) or − (<10% of wild-type activity) except for RPStd [see Fig. 4] and C263A (10–20% activity).

### Discussion

We have investigated the binding specificity of the CBP2 protein for b15 by mutational analysis of the RNA. Because the intron self-splices, it has been possible to characterize mutant RNAs with respect to the effect of the mutation on catalytic activity as well as protein binding. To a first approximation, the mutants fall into two categories. Some changes affect neither catalytic activity nor binding, whereas others drastically reduce both. The variants that retain wild-type binding and activity allow us to eliminate several sequences as direct contact points for the protein. Both of the large loops, L1 and L8, can be deleted from b15 without disrupting protein binding, indicating that the combined 368 nucleotides comprising these loops do not contribute to formation of the binding site. Also, we were able to replace L4/5, P5, and L5 with the analogous regions from the td intron and generate a hybrid intron capable of binding the protein. The td sequences are very dissimilar to the b15 sequences, we conclude that this region, above the P4 stem, is unlikely to contact the protein in a sequence-specific manner. Although a protein contact involving recognition of secondary structure with minimal sequence requirement cannot be ruled out, the sizes of the L4/5 internal loop and P5 stem are changed considerably by the td replacement. Finally, it appears that the identity of the bulged nucleotide in P7 and the identity of the base pair at the G-binding site are not important for protein recognition, as the C263A and G264A : C311U variants undergo protein-dependent splicing.

Many of the variants fell into the class of mutants that did not bind the protein and were also unable to self-splice. This group included RNAs with deletions or mutations in P6, P7, and P7.1. One interpretation for these results is that these variants can be classified as denaturation mutants. Because the secondary structures of these RNAs are not altered from the wild type, as indicated by reactivity of uridines with CMCT, denaturation implies a loss of tertiary structure or unfolding of the RNA. The loss of the catalytic core structure would account for the self-splicing defect. By extension, the defect in binding would indicate that formation of the protein-binding site occurs only in the context of the correctly folded intron. A similar situation exists for the interaction between yeast tRNA*Phε* and phenylalanyl tRNA synthetase. Although the synthetase does not specifically contact any of the nucleotides involved in tertiary interactions, the integrity of those interactions is required for protein recognition as well as for tertiary structure formation (Sampson et al. 1990).

The activities of the G-site variants A : C and A : U lend further support to the argument that the core structure of the RNA is required for formation of the protein-binding site. The A : C variant is completely deficient in self- and protein-dependent splicing and does not bind the protein. The compensatory mutation that restores base-pairing, concomitant with a change in base-pair identity to A : U, permits both self- and protein-dependent splicing provided that the cofactor is 2-AP or 6-Cl-
2-AP. Thus, the altered identity of the nucleotide (G264A) in the A:C variant cannot account for the defect in protein binding. Instead, it appears that CBP2 protein binding is sensitive to the base-paired state at the G site.

Having a base pair at the G site might be required for protein binding in one of two ways. First, the G site might be a nucleating element for the active structure so that the entire core is unable to form without prior formation of the G site. This leaves open a wide range of possibilities for the exact location of the protein-binding site, excluding only L1, L8, the P7 bulge, and the region above P4. A second possibility is that the base pair at the G site only affects the local structure of the inner core. In effect, then, the overall tertiary structure forms, but the local geometry of P7 and the G site is altered with respect to other core internal sequences and structures. This leads to a model whereby the protein binds to the active core, perhaps even entering the catalytic pocket. The possible sequences contributing directly to the protein-binding site according to this model would be limited to those in close contact with and structurally dependent on the G site. Further experiments will be required to distinguish between these two interpretations.

In addition to localizing the protein-binding site, we would like to understand the mechanism by which the protein facilitates splicing of a specific group I intron. The commonly invoked working hypothesis for this and other systems is that the protein binds to the RNA and stabilizes a catalytically active conformation. Implicit in this hypothesis is the assumption that the predominant structure present under physiological conditions is inactive for splicing. The AU-rich nature of bI5, combined with several large and potentially unstructured nonconserved sequences, might have been expected to make the active conformation less favored at low Mg$^{2+}$. However, we could not distinguish the secondary structure formed under conditions where the intron does and does not self-splice. Thus, unless there is some subtle secondary structure defect at low Mg$^{2+}$ levels that is corrected or...
Protein facilitation of RNA self-splicing

This will be tested in the future with probes more revealing of tertiary structure, such as free radicals generated by Fe(II)-EDTA.

Here, we propose a new model whereby the defect in bI5 relative to other self-splicing introns resides at a specific Mg2+ binding site. The Mg2+ profile of the self-splicing and protein-dependent splicing reactions (Gampel et al. 1989) is consistent with Mg2+ having a saturable binding site. According to this model, the high Mg2+ requirement in self-splicing reflects a low affinity for that ion at a specific site that must be filled for splicing to occur, perhaps because the metal ion participates directly in active site chemistry. The function of the protein would be to strengthen, directly or indirectly, the Mg2+ site. One possible type of direct interaction is the donation of a ligand for Mg2+ by an amino acid side chain of CBP2. That is, the correctly folded bI5 RNA might lack a ligand required for tight binding of Mg2+, which is provided by RNA functional groups in the Tetrahymena intron and others that self-splice at low Mg2+. Possible candidates for Mg2+ ligands provided by proteins include aspartate and glutamate side chains, which have been shown to function in Mg2+ coordination at the 3′→5′ exonuclease active site of the DNA polymerase I Klenow fragment (Beese and Steitz 1991). An indirect effect could be structural alteration upon CBP2 protein binding that puts an RNA functional group into the correct geometry to act as a Mg2+ ligand. This model, invoking either a direct or an indirect effect of CBP2 protein on Mg2+ binding, and the standard model involving Mg2+ or protein in structural stabilization are not mutually exclusive. It is possible that the protein acts both to stabilize the correct folded structure of the RNA and to strengthen a specific Mg2+ site at or near the active site.

Materials and methods

Plasmid construction

The plasmid pBS+/bI5 contains the entire insert from the previously described SP65/bI2 plasmid (Gampel and Tzagoloff 1987) ligated into the polylinker of the vector pBS+ (Stratagene). The terminal intron of the yeast mitochondrial cytochrome b gene is referred to in the literature as either bI2 or bI5 depending on the number of cytochrome b introns in the strain used in the study (Gampel et al. 1989). Thus, bI2 and bI5 describe the same intron. pBS+ is linearized with XbaI to generate a template for T7 transcription. Wild-type bI5 RNA contains, in order from 5′ to 3′, 26-nucleotide pBS+ polylinker, 104-nucleotide bI4, 50-nucleotide exon 5, 738-nucleotide bI5, 42-nucleotide exon 6, and 6-nucleotide pBS+ polylinker. Earlier studies describe the bI5 intervening sequence (IVS) as 733 nucleotides in length (Nobrega and Tzagoloff 1980). Incorporation of sequence revisions (Bonjardim and Nobrega 1984) in the L8 region reveals the correct length of the bI5 IVS to be 738 nucleotides. pTD1 was constructed by ligation of the 1.6-kb HindIII–EcoRV fragment of pUC9–Td (Chu et al. 1984) into the pT7-2 vector (U.S. Biochemical). The 9-nucleotide polylinker, 392-nucleotide 5′ exon, 1016-nucleotide bI5, 202-nucleotide 3′ exon, and 28-nucleotide polylinker. pNi contains the 879-bp BglII–HpaI frag-
Intron, 200-nucleotide exon 2, and 17-nucleotide poly(A) tract. The phagemid pTZ18U/bi5 used for site-directed mutagenesis contains the mitochondrial DNA insert of pBS +/bi5 subcloned into the phagemid vector pTZ18U [Meade et al. 1986].

**Preparation of RNA**

Transcription reactions for preparation of unlabeled RNA contained 40 mM Tris (pH 7.5), 6 mM MgCl₂, 5 mM DTT, 1 mM spermidine, 0.5 mM each ribonucleotide, 0.2 μg/ml of linear template, and 2000 units of T7 RNA polymerase per microgram of template. T7 polymerase was isolated from Escherichia coli strain BL21 containing plasmid pAR1219 [Davanloo et al. 1984]. Preparation of uniformly labeled RNA was the same except ATP was at 10 μm and [α-³²P]ATP was included at a final specific activity of 0.2 Ci/μmole. RNA was isolated from 4% polyacrylamide/8 M urea gels by soaking gel slices in 10 mM Tris (pH 7.5), 250 mM NaCl, and 1 mM EDTA, followed by ethanol precipitation.

**Chemical modification with CMCT**

Reactions under native conditions were done with 0.1 μM RNA in 50 mM potassium borate (pH 8), 5 mM MgCl₂, 50 mM KCl, and 5 mM of CMCT (Sigma) at 37°C for 1–30 min. Denaturing conditions were with 0.1 μM RNA in 50 mM potassium borate (pH 8), 1 mM EDTA, 50 mM KCl, and 0.4 mg/ml of CMCT at 95°C for 0.5–10 min. Reactions were stopped by the addition of 25 μl of 1.5 M NaOAc, 5 μl of yeast tRNA (0.6 mg/ml), and 375 μl of ethanol on ice. Samples were transferred to dry ice for 3 min, centrifuged for 5 min, rinsed with 70% ethanol, dried, and dissolved in 10 mM Tris (pH 8.0) and 0.1 mM EDTA. Sites of modification were revealed by reverse transcription from the synthetic oligonucleotide primers IP257-20, IP337-21, IP386-24, IP437-20, IP499-32, and IP697-21. The 3' nucleotide of IP257-20 is complementary to nucleotide 257 of bI5 (bI5 numbering system with the first nucleotide of the IVS as it exists in the precursor as number 1), and the primer contains a total of 20 nucleotides. All primers are named according to the same rationale. Each 5'-³²P-labeled primer (1 pmole) was annealed to 0.5 pmole of modified RNA in 25 mM Tris (pH 8.3), 30 mM NaCl, and 5 mM DTT by heating at 95°C for 3 min followed by quick cooling on ice. Primer extension was done at 37°C in annealing buffer plus 15 mM MgOAc, 0.25 mM each dNTP, and 0.2 U/μl of AMV reverse transcriptase (Life Sciences). Reaction products were separated on 8% polyacrylamide/8 M urea gels.

**bi5 variants**

Site-directed mutagenesis of pTZ18U/bi5 was carried out by the method of Kunkel (1985). For ΔL1 and ΔL1-10, nucleotides 3–223 and 5–221, respectively, were deleted and replaced with a BamHI restriction site. ΔL1-10 contains the BamHI-EcoRI fragment of ΔL1 ligated to the pBS+ vector. In ΔL8, L8 is replaced by a BglII restriction site. In ΔL6, nucleotides 355–436 are removed and replaced with a Smal restriction site. ΔL8/BglII is the transcript from the ΔL8 variant plasmid linearized at the BglII site. ΔP7.1 is described in Figure 2B, and the replacement variant RPStd is shown in Figure 4. The G : U mutant (C311U) was made in the wild-type pTZ18U/bi5 background. The A : C mutant was cloned from the mitochondrial DNA of the in vivo-isolated mutant M6-200 [P. Perlman, unpubl.] and in addition to the G264A change at the G site, this variant has a second base change in the core, G427U. The A : U compensatory mutant (G264A: C311U) was constructed in the M6-200 background and therefore also contains the G427U change. Mutant sequences were verified by sequencing with reverse transcriptase from the primers described above.

**Kinetic analysis**

For the purposes of a kinetic analysis of binding, the CBP2 protein was treated as the enzyme, and the RNA was treated as the substrate of the reaction. The analysis was done under subsaturating conditions for both enzyme and substrate, where the initial rate of protein-dependent splicing is a linear function of either protein or RNA concentration. This was demonstrated for the ranges 0.1–10 nM RNA and 4–30 nM protein. The Kₘ value was estimated to be >200 nM because plots of splicing rate versus RNA concentration deviated from linearity above 200 nM RNA. The exact value has not been determined because of small quantities of purified protein. The inhibition constant Kᵢ was determined by two methods. For method A, the concentration of bi5 RNA was varied at fixed inhibitor RNA concentration [I], and the splicing rates [v] were plotted as a function of [bi5 RNA] [see Fig. 3B]. For method B, [bi5 RNA] was fixed while I was varied, and v was plotted as a function of I. For both methods, precursor RNA was incubated in 20 mM Tris (pH 7.5), 20 mM [NH₄]SO₄, 5 mM MgCl₂, 5 mM CaCl₂, 2 mM spermidine, 5 mM DTT, 60 μg/ml of yeast tRNA, 0.5 mM GTP, and 15 mM CBP2 protein at 37°C. For method A, splicing assays contained 0.1–10 nM uniformly labeled bi5 RNA and 10, 50, 200, or 900 nM (G264A only) inhibitor RNA. Each determination of Vₘₐₓ/Kₘ included data from a minimum of four bi5 concentrations. Aliquots were removed into urea/dye mix at 1- to 10-min time points, and reaction products were separated on 10% polyacrylamide/8 M urea gels. Counts in each of the two product bands, ligated exons [LE] and 5' exon [5'E], and in the upper band containing precursor, IVS-3' exon, and IVS were quantitated by radioanalytical imaging (Molecular Dynamics Phosphorimager) of dried gels. Counts in the upper band, LE, and 5'E were converted to molar amounts according to the number of adenine nucleotides [because the RNA was A-labeled] in each: Counts in the upper band were divided by 409 moles of A/mole of RNA [the value characteristic of the precursor RNA]; counts in LE were divided by 78 moles of A/mole of RNA; and counts in 5'E were divided by 65 moles of A/mole of RNA. These corrected counts were then used to calculate percent product = [LE + 5'E] x 100/[upper band + LE + 5'E]. The approximation that the upper band contained only precursor RNA does not lead to significant error, because the reaction proceeded to <5% completion. Splicing rates were determined from direct plots of percent product versus time. Vₘₐₓ/Kₘ and Vₘₐₓ/Kₘ(app) were determined from plots of splicing rate versus RNA concentration. This method of Kᵢ calculation does not require determinations of Vₘₐₓ or Kₘ, but, rather, relies on the ratio. For method B, reactions contained 0.5 nM uniformly labeled bi5 and unlabeled inhibitor at 0.6–200 nM in fivefold increments. Products were separated on 4% polyacrylamide/8 M urea gels. At low concentrations of S relative to Kₘ, v/S is equal to Vₘₐₓ/Kₘ. In the presence of inhibitor [I], v/S is equal to Vₘₐₓ/Kₘ(1 + I/Kᵢ). Thus, the reaction rate in the presence of inhibitor, vᵢ, is equal to v₀ [half the rate in the absence of inhibitor] when the factor [1 + I/Kᵢ] is equal to 2. This is true when the concentration of inhibitor is equal to Kᵢ. The concentration of inhibitor that re-
duced splicing by 50% was estimated from autoradiograms to generate values for \( K_i \) by method B.

**Splicing assays for G-site variants**

Reactions were done under the same conditions as for the \( K_i \) determinations, with 0.5 nM uniformly labeled b5S except for the variations in Mg\(^{2+}\) concentration, presence or absence of protein, and added cofactor as indicated. Cofactor concentrations were 2.5 mM G, 2-A'P (Sigma), or 6-Cl-2-A'P (Aldrich; Robbins and Uzunanski 1981). Quantiﬁcation for the experiment with RPS3d was done by AMBIS radioanalytic scanning of dried polyacrylamide gels. The identity of the reaction products has been described previously (Gampel and Tzagoloff 1987; Partono and Lewin 1988).

**Self-splicing assays**

Self-splicing activities reported in Table 1 were determined under conditions identical to those for protein-dependent splicing except at 50 mM Mg\(^{2+}\). Assays were done with measurements at one or two time points between 10 and 60 min.

**Purification of CBP2 protein**

The puriﬁcation procedure is modiﬁed from that described previously [Gampel et al. 1989]. Mitochondria were prepared from α-W303-1B cells [R. Rothstein] grown in YPG (2% galactose, 2% Bacto-peptone, 1% yeast extract) to late log phase. Mitochondria were harvested from 9 liters of cells [average yield of 140 grams wet weight], resuspended in 15 ml of 20 mM Tris [pH 7.5], and disrupted by sonication [Heat Systems-Ultrasonics sonicator W-225] at the maximum output with a microtip, three rounds of 10 sec each. All procedures were carried out on ice or at 4°C. Disrupted mitochondria were centrifuged in a Beckman TL-100 centrifuge, TL-100 rotor, at 80,000 rpm for 30 min. The supernatant (0.1 volume of 10%) and incubated on ice for 10 min. Precipitate was collected by centrifugation in 15-ml Corex tubes at 12,000 rpm for 30 min in JS13.1 rotor and washed once in 12 ml of 50 mM Tris [pH 7.5] and 1 mM EDTA. Protein was extracted from the pellet by homogenizing gently in 6 ml of 50 mM Tris (pH 7.5), 1 mM EDTA, and 60% glycerol. One-milliliter fractions were collected and dialyzed overnight into 10 mM Tris (pH 7.5), 0.1 mM EDTA, and 60% glycerol. β-Mercaptoethanol and phenylmethylsulfonyl fluoride were added to 250-μl dialyzed fractions to final concentrations of 10 mM and 0.1 mM, respectively. The last 2 ml from the 0.6 M NaCl eluate and the first 1 ml from the 2.0 M NaCl eluate contained most of the activity (>90% with the remainder in the flowthrough fraction) in equal amounts. These three fractions were pooled and used in the protein-dependent splicing assays. A protein concentration of 15 nM was estimated by density on a silver-stained gel compared with a BSA standard, with the assumption that silver staining of the CBP2 protein and that of the standard are comparable. Because all of the protein might not be active, 15 nM represents an upper limit for the concentration of active protein.

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