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CBP2 Protein Promotes In Vitro Excision of a Yeast Mitochondrial Group I Intron

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The terminal intron (bI2) of the yeast mitochondrial cytochrome *b* gene is a group I intron capable of self-splicing in vitro at high concentrations of Mg^{2+}. Excision of bI2 in vivo, however, requires a protein encoded by the nuclear gene *CBP2*. The CBP2 protein has been partially purified from wild-type yeast mitochondria and shown to promote splicing at physiological concentrations of Mg^{2+}. The self-splicing and protein-dependent splicing reactions utilized a guanosine nucleoside cofactor, the hallmark of group I intron self-splicing reactions. Furthermore, mutations that abolished the autocatalytic activity of bI2 also blocked protein-dependent splicing. These results indicated that protein-dependent excision of bI2 is an RNA-catalyzed process involving the same two-step transesterification mechanism responsible for self-splicing of group I introns. We propose that the CBP2 protein binds to the bI2 precursor, thereby stabilizing the catalytically active structure of the RNA. The same or a similar RNA structure is probably induced by high concentrations of Mg^{2+} in the absence of protein. Binding of the CBP2 protein to the unspliced precursor was supported by the observation that the protein-dependent reaction was saturable by the wild-type precursor. Protein-dependent splicing was competitively inhibited by excised bI2 and by a splicing-defective precursor with a mutation in the 5′ exon near the splice site but not by a splicing-defective precursor with a mutation in the core structure. Binding of the CBP2 protein to the precursor RNA had an effect on the 5′ splice site helix, as evidenced by suppression of the interaction of an exogenous dinucleotide with the internal guide sequence.

A substantial number of genes in fungal mitochondrial DNA contain group I introns (35). Although some mitochondrial group I introns are able to self-splice (splice in the absence of protein) in vitro, there is compelling evidence that in vivo splicing requires the participation of protein factors. In *Saccharomyces cerevisiae*, at least 11 nuclear gene products have been reported to be required for splicing of mitochondrial transcripts (27, 33, 40). In addition, some group I introns in yeast mitochondrial genes contain open reading frames that code for proteins (maturases) involved in the excision of the cognate intervening sequences (IVSs) from the pre-mRNAs (2, 12, 29). Protein-facilitated splicing is not confined to yeast mitochondrial transcripts. Excision of several mitochondrial group I introns of *Neurospora crassa* depends on a protein encoded by the nuclear gene *cyt18* (9). This protein has been partially purified and shown to promote splicing of the large rRNA precursor in vitro (1, 16).

The terminal intron (bI2) of the yeast mitochondrial cytochrome *b* pre-mRNA is an example of a group I intron whose in vivo excision depends on a nuclear gene product (bI2 of the short cytochrome *b* gene is identical to bI5 of the long gene). Mutations in a gene designated *CBP2* have been shown to selectively block processing of bI2 (23, 33). In vitro, however, a precursor RNA containing bI2 is capable of self-splicing (14, 37) by the two-step transesterification mechanism first described by Cech and co-workers (8) for the excision of the IVS from the precursor to the large rRNA of *Tetrahymena thermophila* and subsequently documented for a number of other group I IVSs (11, 15, 43). The protein-independent excision of bI2 from either the native cytochrome *b* pre-mRNA or from a precursor RNA containing only bI2 and flanking exon sequences occurs only at Mg^{2+} concentrations 10 times higher than physiological concentrations (10, 14).

The CBP2 protein is involved in excision of a single catalytically active IVS, providing a useful system for studying the role of proteins in splicing of pre-mRNAs with group I introns. To this end, we partially purified the CBP2 protein and studied its effect on splicing in vitro. In this report, we present evidence that at physiological concentrations of Mg^{2+}, splicing of the bI2 precursor RNA in vitro is strictly dependent on the CBP2 protein. The protein-dependent reaction utilizes a guanosine nucleoside cofactor for 5′ splice site cleavage and generates products identical to those detected under self-splicing conditions. Furthermore, a structural element in the RNA previously shown to be necessary for catalytic activity in self-splicing (5, 39, 45) is also essential for protein-dependent splicing.

MATERIALS AND METHODS

Purification of *CBP2* product. Mitochondria were prepared from bakers' yeast (Red Star; Universal Foods Corp.) by a previously described procedure (42). The purification protocol described below is based on 45 g of starting mitochondrial protein. All steps were done at 4°C.

(i) Step 1. Mitochondria suspended at a protein concentration of 20 mg/ml in 20 mM Tris chloride (pH 7.5)–1 mM EDTA–1 M NaCl were disrupted by sonic irradiation for 2 min at the maximal output of a Braun sonicator. The suspension was centrifuged at 79,000 × g_{av} for 90 min, and the soluble supernatant was collected and dialyzed against 10 volumes of 20 mM Tris chloride (pH 7.5)–1 mM EDTA (TE buffer) for 17 h with one change of buffer.

(ii) Step 2. To the dialyzed supernatant was added 0.1 volume of 10% streptomycin sulfate. The precipitate recovered after centrifugation at 17,000 × g_{av} for 10 min was washed with 900 ml of TE buffer. The washed precipitate

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was suspended in 450 ml of TE buffer containing 1 M NaCl and centrifuged at 17,000 × g_{av} for 10 min. Most of the protein in the streptomycin sulfate precipitate was extracted with the high-salt wash at this step.

(iii) Step 3. The soluble extract obtained in step 2 was fractionated with solid ammonium sulfate. The precipitate formed between 0.25 and 0.5 saturation in ammonium sulfate was dissolved in 60 ml of TE buffer. This extract (20 ml) was applied to a column (2.5 by 90 cm) of Sepharose 6B equilibrated with TE buffer containing 0.1 M NaCl. Fractions of 5 ml were collected and assayed by Western blot (immunoblot) analysis with an antibody against the trpE-CBP2 fusion protein. This step was repeated with the remaining 40 ml of extract. Peak fractions were pooled, and the protein was precipitated by the addition of solid ammonium sulfate to 0.5 saturation. The precipitate was dissolved in 21 ml of TE buffer and dialyzed against the same buffer for 5 h.

(iv) Step 4. The pool from the Sepharose 6B column was applied to a 30-ml column of DE52 (Whatman, Inc., Clifton, N.J.) equilibrated in 20 mM Tris chloride (pH 7.5). The column was washed with 30 ml of 20 mM Tris chloride (pH 7.5) followed by sequential washes with 30 ml of 20 mM Tris chloride (pH 7.5) containing (i) 0.1 M NaCl, (ii) 0.2 M NaCl, (iii) 0.4 M NaCl, and (iv) 1.0 M NaCl.

(v) Step 5. The fraction eluted from DE52 with 0.4 M NaCl was dialyzed against 10 mM potassium phosphate buffer (pH 7.5) (KPO_4) and applied to a 30-ml column of hydroxylapatite (Bio-Gel HPT; Bio-Rad Laboratories, Richmond, Calif.) equilibrated in 10 mM KPO_4 (pH 7.5). The column was washed sequentially with 20 ml of (i) 10 mM KPO_4, (ii) 0.1 M KPO_4, (iii) 0.3 M KPO_4, (iv) 0.6 M KPO_4, and (v) 1.0 M KPO_4.

(vi) Step 6. The 0.6 M KPO_4 fraction from step 5 was dialyzed against TE buffer and applied to a 6-ml column of phosphocellulose equilibrated with 20 mM KPO_4 (pH 7.5). The column was washed with 8 ml of 10 mM KPO_4 followed by sequential washes of (i) 0.2 M KPO_4, (ii) 0.4 M KPO_4, (iii) 0.8 M KPO_4, and (iv) 1.0 M KPO_4.

(vii) Step 7. The 0.8 M KPO_4 eluate from step 6 was dialyzed against 20 mM Tris chloride (pH 7.5) and applied to a 1-ml column of heparin agarose (Sigma Chemical Co., St. Louis, Mo.) equilibrated in 20 mM Tris chloride (pH 7.5). The column was washed with 2 ml of 20 mM Tris chloride (pH 7.5) followed by 2 ml of 20 mM Tris chloride (pH 7.5) containing (i) 0.2 M NaCl, (ii) 0.4 M NaCl, (iii) 0.8 M NaCl, and (iv) 1 M NaCl. The 1 M NaCl eluate was dialyzed against 20 mM Tris chloride (pH 7.5) containing 7.5% glycerol and stored at −70°C.

Preparation of antibodies to trpE-CBP2 fusion protein and immunological detection of *CBP2* product. The 883-base-pair (bp) *Bam*HI-*Hin*dIII fragment encompassing the sequence coding for residues 97 to 392 of the CBP2 protein was ligated to the *Escherichia coli* expression vector pKRS101 (41) that had been linearized with *Bgl*II and *Hin*dIII. The resultant construct had an in-frame fusion of the *CBP2* coding sequence to codon 375 of the *E. coli trpE* gene. The trpE-CBP2 fusion protein was partially purified from *E. coli* transformants induced with indoleacrylic acid as described previously (13). Approximately 2 mg of the protein in complete Freund adjuvant was used to immunize rabbits. A booster injection of 1 mg in incomplete Freund adjuvant was given 4 weeks later, and blood was drawn after another week. The immunoglobulin G fraction was obtained from the antiserum by ammonium sulfate precipitation and chromatography on DEAE-cellulose.

For Western analysis, samples of protein were separated by electrophoresis on 12% polyacrylamide gels in the buffer system of Laemmli (28) and were transferred to nitrocellulose sheets. The protein blots were reacted with a 1:200 dilution of the immunoglobulin G fraction obtained from the trpE-CBP2 antiserum. Cross-reacting proteins were detected by a second reaction with ^{125}I-protein A followed by exposure to Kodak XAR X-ray film.

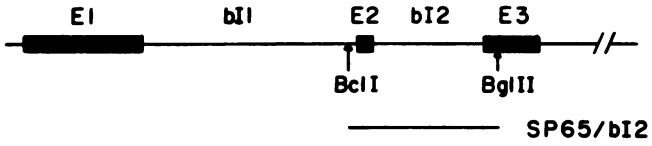

FIG. 1. Construction of SP65/bI2. The short cytochrome *b* gene from the wild-type *S. cerevisiae* D273-10B/A1 has three exons (E1, E2, and E3) and two introns (bI1 and bI2). The *BclI-BglII* fragment of mitochondrial DNA from this strain was ligated to the vector pSP65 digested with *Bam*HI. The resultant plasmid, SP65/bI2, contains 102 bp of bI1, 50 bp of E2, 733 bp of bI2, and 47 bp of E3.

Precursor RNAs. Plasmid SP65/bI2 (14) was constructed by ligating the 928-bp *BclI-BglII* fragment of the short cytochrome *b* gene (36) containing the entire terminal intron and flanking 5′ and 3′ exon sequences to the *Bam*HI site of pSP65 (Fig. 1). SP65/M6-200 and SP65/cob45 were constructed by ligating the *BclI-BglII* fragment of mitochondrial DNA from the *mit* mutants M6-200 and cob45 (M. L. Haldi, Ph.D. thesis, Ohio State University, Columbus, 1985), respectively, to the *Bam*HI site of pSP65. The SP65/E2E3 plasmid was constructed by ligating the 195-bp *BclI-BglII* fragment of mitochondrial DNA from *S. cerevisiae* E26/R29B to the *Bam*HI site of pSP65. This strain has a short cytochrome *b* gene lacking the terminal intron (23). SP65/bI2, SP65/M6-200, SP65/cob45, and SP65/E2E3 were linearized at the *Xba*I site immediately downstream of the *Bam*HI cloning site, and the linear plasmids were used as templates for runoff transcription with SP6 polymerase (34).

Splicing assays. Protein-dependent splicing assays were done at 37°C in 30 μl of a reaction mixture containing 20 mM Tris chloride (pH 7.5), 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM $CaCl_2$, 5 mM spermidine, 20 μCi of [^{32}α-P]GTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or 0.2 mM GTP, 0.25 μg of calf liver RNA per μl, 0.15 μg of unlabeled substrate RNA or 3×10^{-6} μg of ^{32}P-labeled RNA, and 0.1 to 0.3 μg of protein. Self-splicing activity was assayed under the same conditions except at 50 mM $MgCl_2$ in the absence of protein. In experiments in which RNA was analyzed by electrophoresis in 1% agarose gels, reactions were stopped by the addition of 10 μl of 20 mM EDTA (protein-dependent splicing) or 200 mM EDTA (self-splicing) and extraction with phenol-$CHCl_3$-isoamyl alcohol. Unincorporated nucleotides were removed by centrifugation of the reaction mixture through a 1-ml column of Sephadex G-50. For analysis on 4% polyacrylamide–6 M urea gels, an equal volume of acrylamide sample buffer (formamide, 20 mM EDTA, 0.02% bromophenol blue and xylene cyanol dyes) was added to RNA samples before electrophoresis. *trans* splicing activity was assayed under the same conditions except for the addition of 5′-end-labeled ApU and the omission of GTP. Reactions were stopped by the addition of 10 μl of acrylamide sample buffer and subjected to electrophoresis on 10% polyacrylamide–6 M urea gels.

Miscellaneous methods. RNA used in the competition experiment was purified from 4% polyacrylamide–6 M urea gels (18). Standard procedures were used for the isolation of

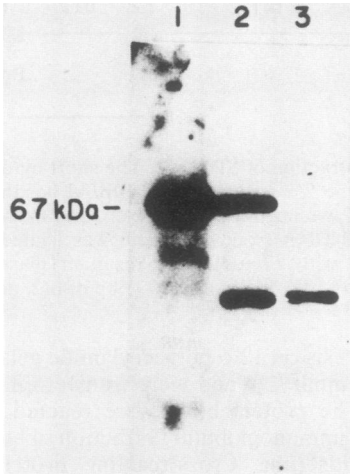

FIG. 2. Detection of CBP2 protein by Western blot analysis. Mitochondria were prepared from the parental respiratory-competent haploid strain W303-1B; from the respiratory-deficient mutant W303∇CBP2 in which the chromosomal copy of *CBP2* was replaced with a *CBP2* gene disrupted by insertion at the *Bam*HI site of a 3-kilobase fragment of DNA with the yeast *LEU* gene (14); and from W303(T-CBP2), a transformant harboring a copy of *CBP2* under the control of the *GAL10* promoter on an episomal plasmid. The two strains were grown in liquid medium containing 2% galactose, 2% peptone, and 1% yeast extract. Mitochondria were fractionated up to the 25 to 50% ammonium sulfate cut in step 3 of the purification procedure. Proteins were separated by electrophoresis on a sodium dodecyl sulfate-12% polyacrylamide gel, transferred to nitrocellulose paper, and reacted with the antibody to the trpE-CBP2 fusion protein. Lanes: 1, 2.4 μg of total mitochondrial protein of W303(T-CBP2); 2, 13.6 μg of the partially purified fraction from W303-1B; 3, 13.6 μg of the partially purified fraction from W303∇CBP2. The migration of bovine serum albumin (67 kilodaltons [kDa]) is indicated in the left-hand margin. The faster-migrating band seen in lanes 2 and 3 is an unknown protein that cross-reacts with the antibody.

plasmid DNA, digestion of DNA with restriction enzymes, separation of DNA for analytical or preparative purposes, ligation of DNA fragments, and transformation of *E. coli* (31). Protein concentrations were estimated by the method of Lowry et al. (30).

RESULTS

Purification of the *CBP2* gene product. The initial fractionation of the CBP2 protein was monitored by Western blot analysis with an antibody raised against a trpE-CBP2 fusion protein. The antibody reacts with a 68-kilodalton protein present in mitochondria of the wild-type strain of *S. cerevisiae* W303-1B (Fig. 2). This size is consistent with the molecular weight of the CBP2 protein derived from the known sequence of the gene (33). The protein is absent in mitochondria of the mutant W303∇CBP2, which harbors a disrupted copy of *CBP2*, and is present in 50- to 100-fold-elevated concentrations in mitochondria of yeast cells transformed with an autonomously replicating plasmid containing a copy of *CBP2* under the control of the *GAL10* promoter. In other experiments, the expression of the 67-kilodalton protein was found to be induced by galactose (data not shown). Since the bulk of CBP2 protein in the overexpressing strain exists as an insoluble aggregate, we used mitochondria of wild-type yeast cells for the isolation of the active protein.

TABLE 1. Recovery of protein and protein-dependent splicing activity[a]

Fraction	Protein concn (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Total units
Mitochondria	20	45,000	ND[b]	ND
Sonic supernatant	6.0	10,250	ND	ND
NaCl extract	3.0	2,150	ND	ND
25–50% Ammonium sulfate	11.2	670	ND	ND
Sepharose 6B pool	7.0	147	6.3	930
DEAE 0.4 M NaCl	0.7	21.6	4.0	86
Hydroxylapatite (0.6 M KPO_4)	0.7	9.9	8.0	79
Phosphocellulose (0.8 M KPO_4)	0.3	2.9	22	64
Heparin agarose (1.0 M NaCl)	0.083	0.50	43	22

[a] Protein-dependent splicing was assayed as described in Materials and Methods. After separation on a 1% agarose gel, reaction products were cut out from the dried gel and ^{32}P-labeled IVS was quantitated by counting in a scintillation counter. Units are defined as femtomoles of IVS formed per milligram of protein per minute.
[b] ND, Not determined.

Mitochondria were prepared on a large scale from commercial bakers' yeast. The procedure ultimately used to purify the *CBP2* product incorporated the following steps: (i) disruption of mitochondria by sonic irradiation, followed by differential centrifugation of the homogenate to remove membrane vesicles; (ii) treatment of the soluble fraction with streptomycin sulfate and extraction of the resultant precipitate with salt; (iii) fractionation of the salt extract with ammonium sulfate, (iv) separation on a sieving column, (v) ion-exchange chromatography on DEAE-cellulose, hydroxylapatite, and phosphocellulose; and (vi) adsorption chromatography on heparin agarose. Table 1 shows the recovery of protein and of splicing activity during the purification. Protein-dependent splicing was assayed in the presence of 5 mM Mg^{2+}. At this concentration of Mg^{2+}, no products were formed in the absence of protein (see below). It was not possible to measure splicing activity in the initial crude fractions due to the presence of nonspecific nucleases. The overall enrichment of the active protein throughout the purification, therefore, cannot be stated. The low recovery of splicing activity was in part due to the fact that only peak fractions were collected. Inactivation of the protein may be another factor.

The enrichment of the CBP2 protein at each step of the purification was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Western blot analysis (Fig. 3A and B). The CBP2 protein was detectable by Western analysis at all stages of the purification. The concentration of CBP2 protein was too low to be detected by Coomassie blue dye in wild-type mitochondria and in fractions obtained before the DEAE-cellulose step (Fig. 3A, lane 6). A protein with a migration indistinguishable from that of the CBP2 protein detected by Western analysis was seen in all the fractions obtained subsequent to chromatography on DEAE-cellulose.

The size of the active protein was determined by sedimentation through sucrose gradients (Fig. 4). The molecular weight of the protein was shown to be 250,000 to 300,000. Splicing activity and CBP2 protein detected by Western analysis have the same elution profile (data not shown). Since the monomer molecular weight of the *CBP2* product estimated from its migration on sodium dodecyl sulfate-polyacrylamide gels is approximately 68,000, the native protein probably exists as a complex, either in the form of a CBP2 homotetramer or in association with some other factor.

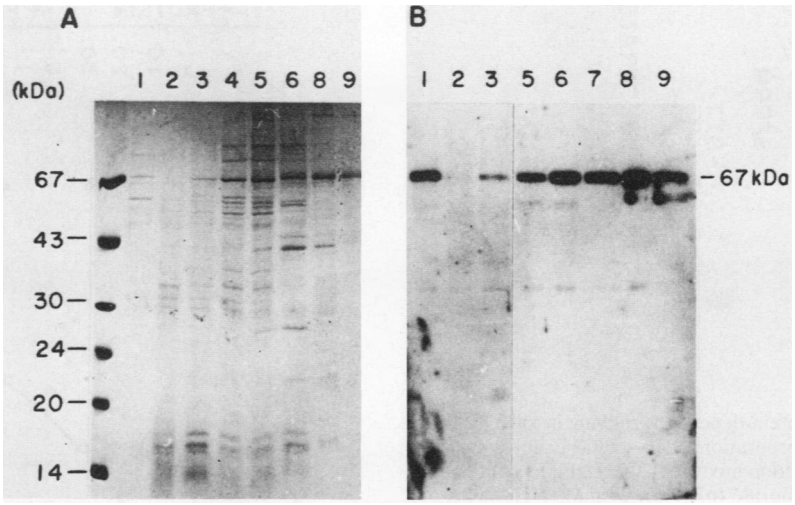

FIG. 3. Purification of CBP2 protein from wild-type yeast mitochondria. Protein fractions from each step of the purification procedure were subjected to electrophoresis on sodium dodecyl sulfate-12% polyacrylamide gels and visualized either by staining with Coomassie blue dye (A) or by Western blot analysis with the antibody against the trpE-CBP2 fusion protein (B). Lanes: 1, 1.4 μg of W303(T-CBP2) mitochondrial protein; 2, 15 μg of the sonic supernatant (step 1); 3, 15 μg of 1 M NaCl extract (step 2); 4, 15 μg of the 25 to 50% ammonium sulfate precipitate (step 3); 5, 7.5 μg of the Sepharose 6B pool (step 3); 6, 7.5 μg of the 0.4 M NaCl eluate from the DEAE-cellulose column (step 4); 7, 7.5 μg of the 0.6 M potassium phosphate eluate from the hydroxylapatite column (step 5); 8, 7.5 μg of the 0.8 M KPO_4 eluate from the phosphocellulose column (step 6); 9, 1.9 μg of the 1 M NaCl eluate from the heparin agarose column (step 7). The migrations of known molecular size standards are marked in the left-hand margin of panel A and the right-hand margin of panel B. Note that in the experiment shown, the eluate from the hydroxylapatite column was not analyzed with Coomassie blue and that the 25 to 50% ammonium sulfate fraction was not tested with the antibody. kDa, kilodaltons.

Extract enriched for CBP2 protein promotes in vitro splicing. The RNA used to assay excision of bI2 was transcribed from an SP6 recombinant plasmid containing the entire intron and flanking exon sequences (see Materials and Methods; Fig. 1). Processing of group I introns is initiated by nucleophilic attack at the 5′ splice site by the 3′ hydroxyl group of a free guanosine nucleoside, leading to covalent attachment of the guanosine nucleoside to the 5′ end of the IVS (8). Splicing can therefore by assayed by the incorporation of radioactive GTP into excised linear IVS. At 5 mM Mg^{2+} and in the presence of [^{32}P]GTP, fractions enriched in CBP2 protein promoted splicing of the bI2 precursor RNA, as determined by the formation of ^{32}P-labeled IVS (Fig. 5). Protein-dependent splicing was absolutely dependent on Mg^{2+} and GTP and was somewhat enhanced by the inclusion of Ca^{2+}. Spermidine stimulated the reaction, although the magnitude of the effect was variable in different experiments. Neither Ca^{2+} nor spermidine stimulated splicing at 5 mM Mg^{2+} in the absence of protein. Protein-dependent splicing was abolished by pretreatment of the purified fraction at 90°C.

As indicated above, Mg^{2+} is necessary for both self-splicing and protein-dependent splicing. The optimal concentration of Mg^{2+} for the protein-independent reaction was 50 mM, and virtually no linear IVS product was formed at concentrations below 10 mM (Fig. 6). In the presence of protein, however, the amount of product formed at 5 mM Mg^{2+} was 60% of that formed at 20 mM Mg^{2+}. The latter concentration was inferred to be optimal for protein-dependent splicing based on the difference in the amount of product formed in the presence and absence of protein (Fig. 6). The protein also stimulated splicing at high concentrations of Mg^{2+} as evidenced by the increase in IVS excision over and above that seen in the absence of protein. We interpret the Mg^{2+} dependence of the self-splicing reaction to reflect an inability of bI2 to assume a catalytically active

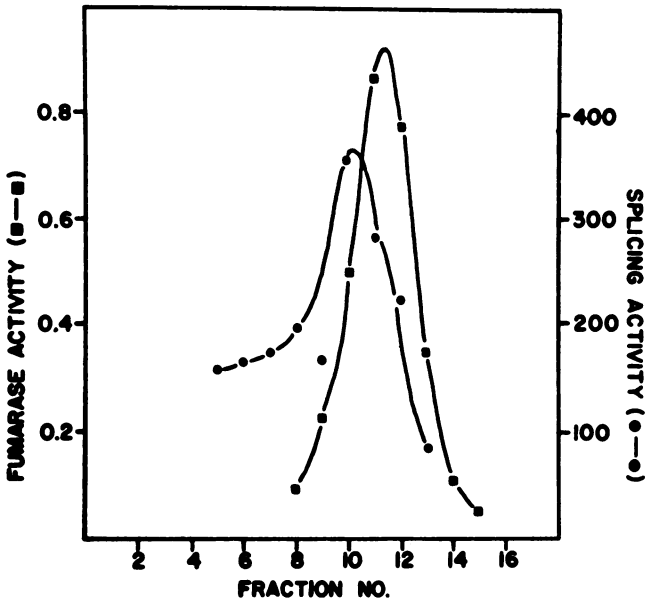

FIG. 4. Molecular weight of native CBP2 protein. A sample of the Sepharose 6B pool was mixed with purified fumarase (Sigma) and applied on a 5-ml column of a 5 to 20% linear sucrose gradient. The gradient was centrifuged at 65,000 rpm in a Beckman SW65 Ti rotor for 4 h at 4°C. Sixteen fractions were collected and assayed for fumarase by the method of Hill and Bradshaw (24) and incorporation of radioactive GTP into excised bI2 under protein-dependent splicing conditions. Activities are plotted as a function of distance traveled through the gradient. Activity units are expressed as absorption change at 240 nm per minute per 5 μl for fumarase and counts per minute incorporated into IVS per 30 min for protein-dependent splicing. The molecular weight of the native CBP2 protein was determined from the relative sedimentation of splicing activity and fumarase by the method of Martin and Ames (32). A molecular weight of 194,000 was assumed for fumarase.

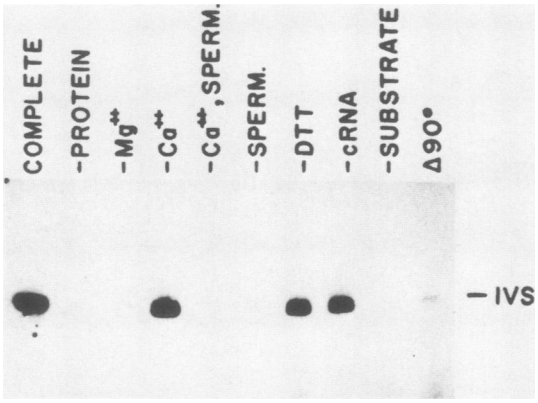

FIG. 5. Conditions for protein-dependent splicing in vitro. Splicing was assayed by the incorporation of [α-^{32}P]GTP into excised linear IVS. The complete reaction mixture (COMPLETE) contained 5 mM $MgCl_2$, 20 mM Tris chloride (pH 7.5), 20 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol (DTT), 5 mM spermidine (SPERM.), 5 mM $CaCl_2$, 20 μCi of [α-^{32}P]GTP, 0.25 μg of calf liver RNA (cRNA) per μl, 0.15 μg of SP65/bI2 precursor RNA (substrate), and 0.3 μg of protein in a final volume of 30 μl. The protein used was the 1 M NaCl eluate from the heparin-agarose column. The omissions from the complete assay mixture are indicated. The heat sensitivity of the protein extract was tested by treatment of the protein at 90°C for 5 min before addition to the reaction mixture (Δ90°). RNA was separated on a 1% agarose gel. The migration of the linear IVS is marked in the right-hand margin. A radioactive product corresponding to the IVS with a 5′ G and a ligated 3′ exon (IVS-3′ exon in Fig. 8) comigrates with the free IVS in this gel system. This is also true in Fig. 6, 7, and 10.

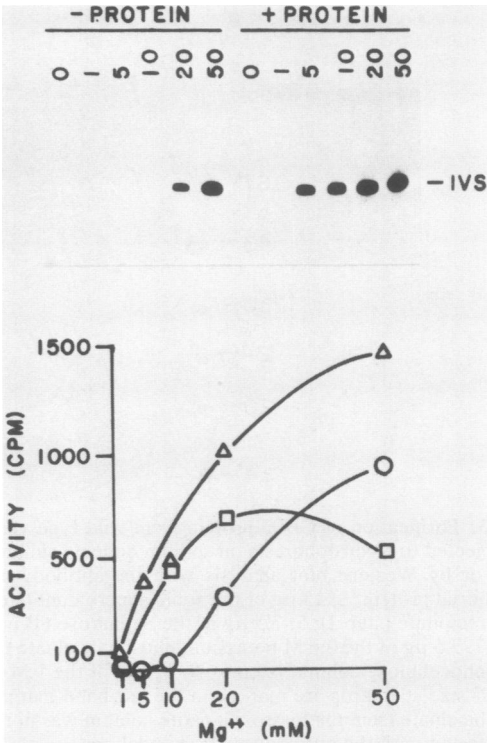

FIG. 6. Optimal concentration of Mg^{2+} for self-splicing and protein-dependent splicing. Splicing was assayed as described in the legend to Fig. 5 (see COMPLETE) except that the concentration of Mg^{2+} was varied as indicated at the top of the gel and protein was omitted in the self-splicing assay (− PROTEIN). The bands corresponding to IVS were excised from dried gels, and the radioactivity was measured by scintillation counting. The splicing activities measured in the presence (△) and absence (○) of protein and as a difference of the two (□) are expressed in counts per minute (cpm) recovered in the linear IVS.

conformation under physiological conditions (see Discussion). In this case, the CBP2 protein obviates the high Mg^{2+} requirement by stabilizing the active RNA structure.

The purest preparation of the CBP2 protein is still contaminated by other proteins. It could not be excluded, therefore, that the observed protein-dependent splicing resulted from a component other than the CBP2 protein. Several lines of evidence argue against this possibility. First, splicing activity copurified with CBP2 protein, as determined by Western analysis, at all stages of the purification procedure (data not shown). Second, splicing activity was not detected in a partially purified fraction isolated from the mutant W303∇CBP2 (Fig. 7) which lacked immunologically detectable CBP2 protein (Fig. 2). Mitochondria were prepared from the wild-type respiratory-competent strain W303-1B and the isogenic respiratory-deficient strain W303∇CBP2. The soluble extracts obtained after sonic irradiation of mitochondria were carried through step 2 of the purification procedure and were tested for their ability to promote splicing in the presence of 5 mM Mg^{2+}. The fraction obtained from the mutant strain was completely deficient in splicing activity. These results constitute strong evidence that the CBP2 protein is required for in vitro splicing of the bI2 precursor RNA under protein-dependent conditions.

Protein-dependent splicing is an RNA-catalyzed reaction. The products of self-splicing and of protein-dependent splicing could be visualized when the reactions were carried out in the presence of a uniformly labeled precursor RNA (Fig. 8). In addition to the linear IVS and the ligated exons, two partial products were detected which probably resulted from a single cleavage of the precursor at the 5′ splice site. Both RNA species have previously been reported to be products of the self-splicing reaction (37). One of the products migrated faster than the ligated exons and presumably corre-

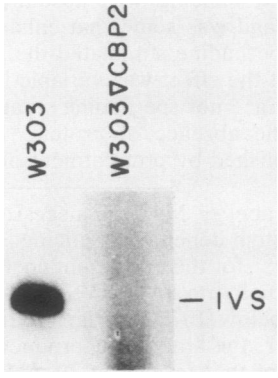

FIG. 7. Absence of splicing activity in W303∇CBP2 mitochondria. Mitochondria were prepared from haploid strain W303-1B and from W303∇CBP2. The two mitochondrial preparations were subjected to the first two steps of the purification procedure followed by ammonium sulfate fractionation. The protein fraction precipitated at 25 to 50% saturation in ammonium sulfate had sufficiently reduced levels of nonspecific nucleases to permit assays of splicing activity. Reactions were carried out under protein-dependent splicing conditions as described in the legend to Fig. 5.

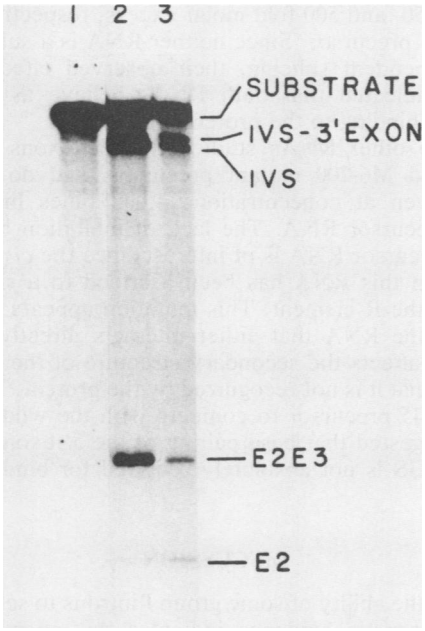

FIG. 8. Products of self-splicing and protein-dependent splicing. Uniformly labeled wild-type precursor RNA was prepared as described in the Materials and Methods section. The radioactive precursor (SUBSTRATE) was incubated under protein-dependent splicing conditions except that 0.2 mM unlabeled GTP was substituted for radioactive GTP. Splicing was assayed in the absence of protein at 5 mM Mg^{2+} (lane 1) and at 50 mM Mg^{2+} (lane 2) and in the presence of CBP2 protein at 5 mM Mg^{2+} (lane 3). Reaction products were separated on a 4% polyacrylamide gel containing 6 M urea. The products detected are the 733-nucleotide IVS (IVS), the 227-nucleotide ligated exons (E2E3), the 780-nucleotide IVS ligated to the 3′ exon (IVS-3′EXON), and the 47-nucleotide 5′ exon (E2).

sponds to free 5′ exon (E2). The second product was slightly larger than the linear IVS and is likely to be the IVS with the attached 3′ exon (IVS-3′EXON). The same products were detected under self-splicing and protein-dependent splicing conditions and only when GTP was included in the reactions, suggesting that the same reaction mechanism is used in the presence or absence of protein.

Evidence that protein-dependent splicing is catalyzed by bI2 RNA was obtained from studies with mutant precursor RNAs. The mutant precursors were transcribed from templates consisting of the region of mitochondrial DNA with bI2 and flanking exons of two *mit* mutants, M6-200 and cob45 (Haldi, Ph.D. thesis). The mutation responsible for the in vivo splicing deficiency of M6-200 has been shown to be a G-to-A transition (nucleotide +2668 in reference 36) in the conserved sequence element R of bI2. The mutation in cob45 is a base change (nucleotide +2222 in reference 36) in the exon upstream of bI2 within a sequence predicted to base pair with the internal guide sequence (IGS). Both the R-S and 5′ splice site-IGS base-pairing interactions have been shown to be critical for self-splicing (5, 39, 44, 45). Neither mutant RNA was processed under self-splicing or protein-dependent splicing conditions (data not shown).

The CBP2 protein does not promote *trans* splicing. The two transesterifications responsible for the autocatalytic excision of group I introns are not obligatorily coupled to one another (26). Cleavage at the 3′ splice site is uncoupled from cleavage at the 5′ site in reactions carried out in the absence of GTP and in the presence of a dinucleotide with the 3′-terminal sequence of the 5′ exon. In this assay, the 3′

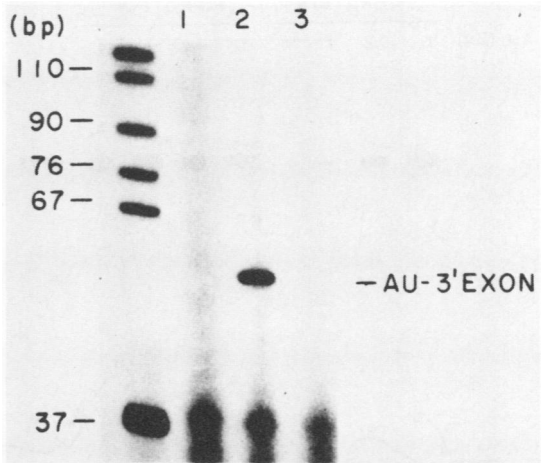

FIG. 9. *trans* splicing with the dinucleotide ApU. Splicing reactions were done in 30 μl of a reaction mixture containing 20 mM Tris chloride (pH 7.5), 20 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol, 5 mM spermidine, 5 mM $CaCl_2$, 0.15 μg of unlabeled bI2 precursor RNA, and 10 μCi of [^{32}P]pApU (approximately 5,000 Ci/mol) in the absence of protein at 5 mM Mg^{2+} (lane 1) and at 50 mM Mg^{2+} (lane 2) and in the presence of 0.3 μg of protein at 5 mM Mg^{2+} (lane 3). The migration of the resultant ^{32}P-labeled product (AU-3′EXON) is indicated in the right-hand margin. pBR322 DNA digested with *Hpa*II was 5′ end labeled and used as molecular weight markers.

hydroxyl of the dinucleotide initiates a nucleophilic attack at the 3′ splice site, resulting in the formation of two RNA products; the 5′ exon attached to the IVS and the dinucleotide ligated to the 3′ exon.

A synthetic dinucleotide containing the last two nucleotides (AU) of the 5′ exon (E2) was used to study *trans* splicing of the precursor RNA. A radioactive product was detected when unlabeled precursor was incubated under *trans* splicing conditions in the presence of 50 mM Mg^{2+} and 5′-end-labeled pApU (Fig. 9, lane 2). The labeled product was confirmed by sequence analysis to be the dinucleotide ligated to the 3′ exon (data not shown). The cleavage site in the *trans* reaction is identical to the 3′ splice site used in *cis* splicing (14). Both *trans* splicing and *cis* splicing exhibit the same Mg^{2+} dependence with an optimum at 50 mM (data not shown). At 5 mM Mg^{2+}, no *trans* splicing product was formed in the absence or presence of CBP2 protein (Fig. 9, lanes 1 and 3).

The failure of CBP2 protein to promote *trans* splicing was examined further by testing the effect of ApU on the protein-dependent *cis* splicing reaction. In *trans* splicing, the dinucleotide base pairs with the IGS, also referred to as the 5′ exon binding site, thereby displacing the natural 5′ exon (3, 17). Since GTP addition depends on the interaction of 5′ exon sequences with the IGS (3, 44), the dinucleotide should inhibit GTP addition by virtue of its ability to compete with 5′ exon for binding to the IGS (Fig. 10). The ability of ApU to occupy the 5′ exon binding site under self-splicing and protein-dependent splicing conditions was assayed by measuring the incorporation of radioactive GTP into linear IVS in the presence of excess unlabeled dinucleotide (Fig. 10). As expected, ApU diminished product formation in the self-splicing assay. In contrast, the dinucleotide had no effect on the protein-dependent reaction. This suggested that the 5′ exon binding site formed in the presence of 50 mM Mg^{2+} recognizes and binds ApU, whereas the binding site formed in the presence of protein at 5 mM Mg^{2+} interacts only with the natural 5′ exon.

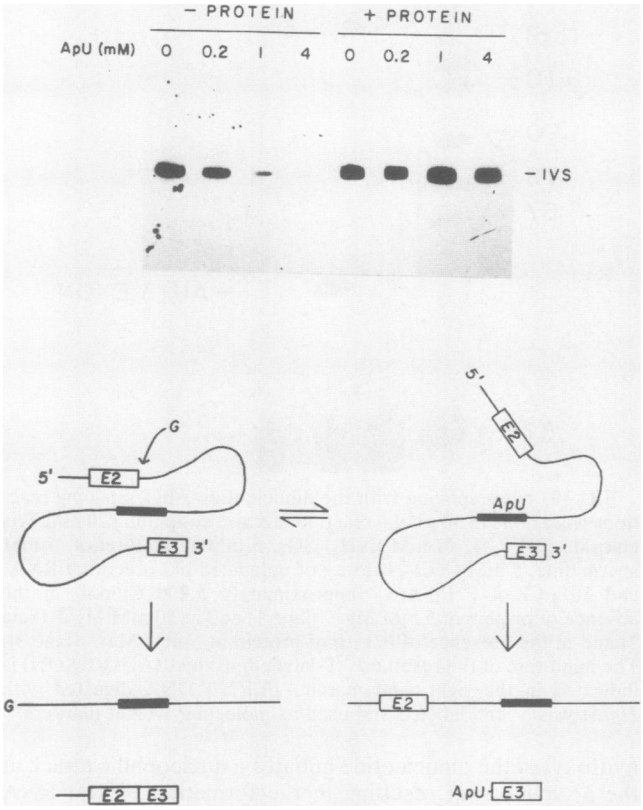

FIG. 10. Effect of ApU on *cis* splicing in the presence of GTP. Splicing reactions were done under standard conditions in the presence of [α-^{32}P]GTP with various amounts of unlabeled ApU as indicated. Reaction mixtures contained either 50 mM Mg^{2+} (- PROTEIN) or 5 mM Mg^{2+} and protein (+ PROTEIN). The position of the ^{32}P-labeled IVS product is indicated. The predicted alignments required for *cis* and *trans* splicing are shown in the diagram at the bottom of the figure. The internal guide sequence (filled box) is shown to base pair with the 5′ exon in *cis* splicing, allowing guanosine attack at the 5′ splice site. In *trans* splicing, ApU displaces the 5′ exon, allowing attack by the 3′ hydroxyl of ApU at the 3′ splice site. Under these experimental conditions, the dinucleotide and the 5′ exon presumably compete for binding to the IGS.

Binding of CBP2 protein to bI2 precursor RNA. Attempts to demonstrate a physical complex of CBP2 protein and the precursor RNA by mobility shift or filter binding assays were not successful. As an alternative, binding of the protein to the precursor was studied indirectly by measuring the effect of excess unlabeled RNA on splicing of a radioactively labeled bI2 precursor RNA. The amount of radioactive IVS and ligated exons formed in the protein-dependent splicing reaction diminished with increasing concentration of unlabeled wild-type bI2 precursor (Fig. 11, left panel). At an approximate molar ratio of 250 of unlabeled-to-labeled RNA, product formation was decreased by 50%. Unlike protein-facilitated splicing, self-splicing was unaffected by the addition of unlabeled precursor (see last two lanes of each panel in Fig. 11). The lack of dependence of self-splicing on RNA concentration was expected since the catalytic activity of the RNA at 50 mM Mg^{2+} does not depend on a saturable factor. To gain some information about the regions of the RNA that interact with the protein, we measured splicing of the labeled precursor RNA in the presence of unlabeled mutant precursor or product RNAs. The linear IVS and the cob45 mutant substrate elicited a 50% reduction in product formed when added in 250- and 500-fold molar excess, respectively, over the labeled precursor. Since neither RNA is a substrate for protein-dependent splicing, their observed effects on the reaction indicated that both RNAs behave as inhibitors capable of binding to the protein.

The two other RNAs studied, ligated exons (data not shown) and M6-200 mutant precursor, had no effect on splicing even at concentrations 4,000 times higher than labeled precursor RNA. The lack of inhibition by M6-200 mutant precursor RNA is of interest since the *cis* dominant mutation in this RNA has been ascribed to a single base change in the R element. This mutation appears to be in a region of the RNA that either interacts directly with the protein or affects the secondary structure of the RNA in a way such that it is not recognized by the protein. The ability of the cob45 precursor to compete with the wild-type precursor suggested that base pairing of the 5′ exon sequence with the IGS is not absolutely required for binding to the protein.

DISCUSSION

Despite the ability of some group I introns to self-splice in vitro (4), genetic evidence indicates that in many cases proteins are required for splicing in vivo (9, 12, 27, 29). One such documented case is exemplified by the terminal intron of the yeast mitochondrial cytochrome *b* pre-mRNA which is capable of self-splicing in vitro (14, 37) but requires a protein encoded by the yeast nuclear gene *CBP2* for in vivo splicing (23, 33). The sequence of the CBP2 protein, inferred from the sequence of the gene, is not homologous to any known aminoacyl-tRNA synthetase, nor does it exhibit significant sequence similarity to any protein in the most current translated version of GenBank. Furthermore, *cbp2* mutants retain the ability to translate all the products of the mitochondrial genome except cytochrome *b*. In this respect, the CBP2 protein differs from splicing factors such as the *N. crassa* tyrosyl-tRNA synthetase (1) and the leucyl-tRNA synthetase (22), both of which have recently been reported to facilitate splicing of group I introns.

The requirement for CBP2 protein in processing of the cytochrome *b* pre-mRNA was implicit from the phenotype of *cbp2* mutants, but biochemical evidence supporting a direct role of the protein in splicing was lacking. The present studies were undertaken to answer the question of whether *CBP2* codes for a splicing factor involved in excision of bI2 and, if so, to clarify the role of the protein. Our results indicated that at physiological concentrations of Mg^{2+}, splicing of the bI2 precursor is absolutely dependent on a protein factor. The factor is present in mitochondrial extracts of wild-type yeast, is absent in extracts of a *cbp2* mutant, and copurifies with CBP2 protein. These results provide strong corroborating evidence for a direct participation of CBP2 protein in excision of bI2. Two lines of evidence argue in favor of the notion that CBP2 protein does not affect the basic mechanism of the self-splicing reaction but rather has an auxiliary function most likely concerned with stabilizing or conferring a catalytically active secondary or tertiary structure or both in the RNA. First, CBP2 protein-dependent splicing requires GTP and generates products identical to those observed in the self-splicing reaction. The use of a guanosine nucleoside as the nucleophile for 5′ splice site cleavage is the distinguishing characteristic of the two-step transesterification mechanism catalyzed by group I introns (7). Second, a mutant precursor (M6-200) with a base change in a region of the RNA shown to be essential for the catalytic

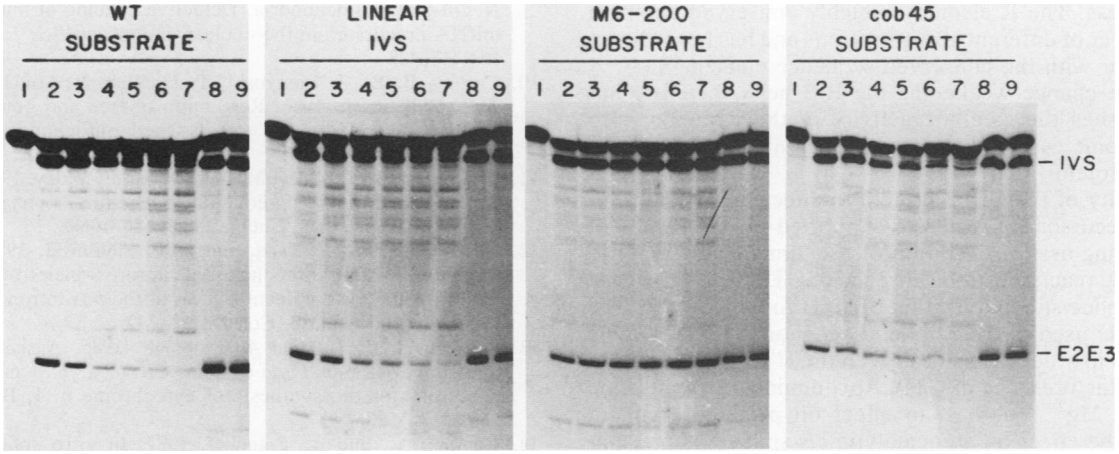

FIG. 11. Splicing of uniformly labeled precursor RNA in the presence of unlabeled precursor and product RNAs. Protein-dependent and self-splicing reactions were carried out under standard conditions with uniformly labeled wild-type precursor RNA (see the legend to Fig. 8) except for the addition of excess unlabeled RNA as indicated at the top of each panel. The following competitor RNAs were used: wild-type bI2 precursor RNA (WT SUBSTRATE), excised linear IVS (LINEAR IVS), and precursor RNAs transcribed from the mutant templates SP65/M6-200 (M6-200 SUBSTRATE) and SP65/cob45 (cob45 SUBSTRATE). Lanes: 1, 5 mM Mg^{2+}, no protein, no competitor RNA; 2, 5 mM Mg^{2+}, plus protein, no competitor RNA; 3, same as lane 2 plus 250-fold excess unlabeled RNA; 4, same as lane 2 plus 500-fold excess unlabeled RNA; 5, same as lane 2 plus 1,000-fold excess unlabeled RNA; 6, same as lane 2 plus 2,000-fold excess unlabeled RNA; 7, same as lane 2 plus 4,000-fold excess unlabeled RNA; 8, 50 mM Mg^{2+}, no protein, no competitor RNA; 9, same as lane 8 plus 4,000-fold excess unlabeled RNA. Reaction products were separated by electrophoresis on 4% polyacrylamide–6 M urea gels. Bands corresponding to unprocessed precursor RNA, IVS, and ligated exons (E2E3) were excised from the gels, and their radioactivity was measured by scintillation counting.

activity of group I introns (4) is not spliced in the presence of CBP2 protein. Splicing under both conditions thus appears to depend on folding of the RNA into a catalytically competent structure.

Self-splicing and protein-dependent splicing of the bI2 precursor RNA both require Mg^{2+}, although the concentration needed for optimal activity is lower in the presence of CBP2 protein. The difference in Mg^{2+} concentration optima most likely reflects two different functions of the divalent metal in self-splicing, catalytic and structural. It has been proposed that Mg^{2+} is required at the catalytic center of the *Tetrahymena* rRNA precursor (6). In protein-dependent splicing, the requirement at the catalytic center is probably satisfied at 5 mM Mg^{2+}. At the higher concentrations needed for self-splicing, Mg^{2+} probably has the additional function of stabilizing a catalytically active form of the RNA. Mg^{2+} and other divalent metals are known to affect the structure of RNA in solution. There is also evidence that specific base-pairing interactions relevant to the catalytic activity of RNA are affected by Mg^{2+} (25). The concentration at which Mg^{2+} stabilizes a specific higher-order structure is likely to depend on a number of factors including the sequence and base composition of the RNA. Precursor RNAs containing mitochondrial group I introns generally require higher concentrations of Mg^{2+} for optimal self-splicing activity than the *Tetrahymena* pre-rRNA (15, 43). This may simply be a reflection of nonproductive RNA structures being favored at 5 mM Mg^{2+}. According to this interpretation, the function of CBP2 protein is to promote formation of the catalytically active structure of bI2 at the relatively low concentrations of Mg^{2+} which prevail under physiological conditions. A dual requirement of Mg^{2+} for in vitro catalysis by RNA is not without precedent. The ribonucleoprotein complex RNase P processes the 5′ termini of tRNA precursors (38). This 5′ endonucleolytic cleavage reaction is also catalyzed by the naked RNA moiety of RNase P, but only in the presence of high concentrations of Mg^{2+} or other divalent metals (20). These observations have been interpreted in terms of two functionally different metal-binding sites in the RNA component of RNase P (21). One of the binding sites is at the active center and is satisfied at low concentrations of Mg^{2+}. At higher concentrations, divalent metals are thought to bind to other sites, thereby affecting the structure of the RNA component of RNase P (19). The catalytically active structure of the RNA can also be formed as a result of the specific protein-nucleic acid interactions in the RNase P ribonucleoprotein complex.

The postulated role of the CBP2 protein in stabilizing a splicing-competent structure of the RNA implies the formation of a specific protein-RNA complex during catalysis. The existence of such a complex is consistent with evidence that protein-dependent splicing is saturable with the bI2 precursor. Although the regions or structural features of the RNA recognized by the CBP2 protein have not been identified, they are probably defined by sequences in the intron rather than at the splice sites. This is suggested by the observation that the excised linear IVS and a precursor with a 5′ exon mutation (cob45) both acted as inhibitors in protein-dependent splicing. Recognition by the CBP2 protein of some general structure common to group I introns is unlikely for two reasons. First, splicing of all introns other than bI2 is normal in *cbp2* mutants (23). Second, the CBP2 protein does not promote in vitro excision of the group I intron bI1 (bI4 in the long cytochrome *b* gene) nor does a transcript containing bI1 act as an inhibitor of the bI2 excision reaction (data not shown).

Although structural features of group I introns in themselves are not sufficient for recognition by the CBP2 protein, one of the conserved sequence elements appears to be absolutely necessary. The M6-200 mutant substrate, which has a base change in the R element, is not an inhibitor in protein-dependent splicing, even when present in very high

molar excess. The R element is highly conserved among a large number of different group I introns and has been shown to base pair with the conserved sequence element S (4). A single base change weakening the R-S helix therefore not only abolishes the catalytic activity of the RNA but also perturbs some essential structure necessary for interaction with the protein.

The ability of the CBP2 protein to affect the structure of the bI2 precursor RNA is also supported by the results of *trans* splicing assays. Addition of the dinucleotide ApU to self-splicing reactions in the absence of GTP incurs cleavage at the 3′ splice site and ligation of ApU to the 3′ exon. The dinucleotide used in *trans* splicing reactions must resemble the 5′ exon in order to base pair with the 5′-exon-binding site (3, 17). In the presence of GTP, ApU diminishes *cis* splicing at 50 mM Mg^{2+} but has no effect on protein-dependent splicing. The effect on autocatalytic *cis* splicing is presumably due to competition of the dinucleotide and the natural 5′ exon for binding to the IGS. Several explanations can be invoked to account for the observed inability of ApU to compete with the endogenous 5′ exon at 5 mM Mg^{2+} in the presence of protein. Protein-dependent conditions may be more stringent than self-splicing conditions. In this case, the dinucleotide and the IGS would only be capable of forming a 5′ splice site helix at high Mg^{2+} concentrations. This interpretation is suggested by the relative weakness predicted for the two A · U base pairs (dinucleotide-IGS pairing) as compared with the four base pairs in the natural 5′ exon. Alternatively, the protein may physically shield the 5′-exon-binding site, allowing entry of the natural 5′ exon but not of the dinucleotide.

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