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In Vitro Splicing of the Terminal Intervening Sequence of *Saccharomyces cerevisiae* Cytochrome *b* Pre-mRNA

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A region of the *Saccharomyces cerevisiae* mitochondrial cytochrome *b* gene encompassing the entire terminal intron plus flanking exonic sequences has been cloned in an SP6 vector. A runoff transcript prepared from this construct as well as the native cytochrome *b* pre-mRNA containing the terminal intervening sequence were found to act as substrates for the autocatalytic excision of the intervening sequence in vitro. This reaction proceeds under conditions previously shown by Cech and co-workers to promote protein-independent excision of the *Tetrahymena* rRNA intervening sequence. The 5′ and 3′ termini of the excised intervening sequence, determined by S1 nuclease mapping and sequence analysis, are consistent with the known sequence of the cytochrome *b* mRNA. The same region of the cytochrome *b* gene from a yeast mutant, defective in splicing due to a mutation in a critical sequence inside the terminal intron, has also been cloned in an SP6 vector. The mutant transcript fails to self-splice in the in vitro assay. These observations provide strong presumptive evidence that in vivo processing of the terminal intervening sequence of the cytochrome *b* pre-mRNA occurs by an autocatalytic mechanism analogous to that shown for other group I introns. In vivo processing of the terminal intervening sequence of the cytochrome *b* pre-mRNA, however, exhibits complete dependence on a protein factor previously shown to be encoded by the nuclear gene *CBP2*.

Cytochrome *b* is a catalytic component of the respiratory complex coenzyme QH_2-cytochrome *c* reductase (9). In *Saccharomyces cerevisiae*, cytochrome *b* is encoded by a mitochondrial gene organized into either three or six exons (11, 18). Both variants of the gene have the same terminal intron of 733 nucleotides. This intron contains sequence elements found in group I introns (17), some of which have been shown to undergo autocatalytic excision in vitro (3, 8, 23).

In previous studies, we reported that mutants with a lesion in the nuclear gene designated *CBP2* accumulate a 2.9-kilobase cytochrome *b* transcript corresponding to a partially spliced intermediate containing the terminal intervening sequence (IVS) (14). The mutant phenotype pointed to a selective requirement of the *CBP2* product for removal of the terminal IVS from the cytochrome *b* pre-mRNA. This was also supported by the observation that the respiratory defect of *cbp2* mutants could be suppressed by a precise deletion of the terminal intron from the cytochrome *b* gene (10). In an effort to better understand the role of the *CBP2*-encoded protein in splicing, we have examined whether excision of the terminal IVS is an RNA-catalyzed reaction and can occur under conditions shown by Cech et al. (3) to promote autocatalytic splicing of the *Tetrahymena* rRNA. In the present communication, we present evidence that the 2.9-kilobase cytochrome *b* pre-mRNA and an SP6-derived transcript containing the terminal IVS plus flanking exonic sequences can self-splice in vitro in the presence of GTP, Mg^{2+}, and ammonium sulfate. The precise intron/exon boundaries have been determined by characterization of the 5′ and 3′ termini of the excised IVS and sequence analysis of the ligated exons.

A mutation in the terminal intron which prevents in vivo processing also completely blocks the in vitro autocatalytic reaction. This result suggests that the reaction mechanisms in vivo and in vitro are likely to be identical.

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MATERIALS AND METHODS

Preparation of mitochondrial DNA. The strains of *S. cerevisiae* used to prepare mitochondrial DNA are described in Table 1. Both C237 (14) and its parent strain D273-10B (22) have the "short" form of the cytochrome *b* gene shown in Fig. 1. The short cytochrome *b* gene of E26/R29B (10) lacks the terminal intron. The cytoplasmic petite mutant DS400/A12 has a segment of mitochondrial DNA containing the entire short cytochrome *b* gene (18). Mitchondria were isolated by the procedure of Faye et al. (7) from stationary-phase cultures of yeasts grown in 2% glucose–2% peptone–1% yeast extract. Mitochondrial DNA was purified on CsCl gradients following lysis of mitochondria with 2% Sarkosyl (19).

Plasmid constructions. Standard procedures were used for the preparation of restriction fragments, their ligations to linearized plasmids, and selection and analysis of recombinant clones of *Escherichia coli* (12). SP65/bI2 and SP65/mbI2 were constructed by ligation of the 827-base pair *BclI-BglII* fragment of mitochondrial DNA from petite mutant DS400/A12 and the mit$^-$ mutant M6-200, respectively, to the *Bam*HI site of pSP65 (15). (mit$^-$ is a respiratory-deficient strain of *S. cerevisiae* with a mutation in mitochondrial DNA.) SP65/bI1 was constructed by ligation of the 1,612-base pair *Eco*RI-*Bgl*II fragment of mitochondrial DNA from

TABLE 1. Genotypes and sources of *S. cerevisiae* strains

Strain	Genotype	Source (reference)
D273-10B/A1	α ρ$^+$ *met6*	21
C237	α ρ$^+$ *met6 cbp2-1*	13
E28/R29B	α ρ$^+$ *met6 cbp2-9 cob1-20*	9
DS400/A12	α ρ$^-$ *met6*	16
M6-200/A1	α ρ$^+$ *ade cob1*	20
W303∇CBP2-1	α ρ$^+$ *ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3,112 CBP2::LEU2*	9

the deletion mutant E26/R29B into pSP65 linearized with *Eco*RI and *Bam*HI.

Assay of in vitro splicing. The SP6 constructs were linearized at the *Xba*I site downstream of the insert and were used as templates for runoff transcription by SP6 RNA polymerase (15). Unincorporated nucleotides and salt were removed from RNA samples by centrifugation through Sephadex G-50 (medium) columns (12). The self-splicing reaction mixture contained 50 mM Tris chloride (pH 8), 50 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 0.1 μg of SP6-transcribed or 10 μg of total mitochondrial RNA, and either 10 μCi of [α-^{32}P]GTP (>3,000 Ci/mmol; Amersham Corp.) or 0.2 mM unlabeled GTP, in a final volume of 20 μl. Modifications of this assay are noted in the legends to the figures. After incubation at 37°C for 30 or 60 min, the mixture was brought to 100 μl with H_2O and centrifuged through a column of Sephadex G-50. Nucleic acids in the excluded volume were precipitated by addition of 0.05 volume of 5 M NaCl and 3 volumes of ethanol. The ethanol-precipitated material was separated electrophoretically on 1% agarose gels, and products were visualized by staining with ethidium bromide. In experiments with radioactively labeled RNA, gels were dried against Whatman DE81 paper and exposed to Kodak XAR-5 film. Northern blot analyses were done by transferring RNA to diazobenzyloxymethyl paper followed by hybridization with radioactively labeled DNA or RNA probes under the conditions described by Bonitz et al. (2), except that the concentration of formamide was lowered to 30%.

Primer extension analysis. Self-splicing reaction products of SP65/bI2 RNA (1.2 μg of RNA) were mixed with 0.5 μg of 5'-end-labeled primer (13). The synthetic 24-nucleotide-long primer (5' AGCATAGAATGGTAATAAGTATCA 3') had a sequence complementary to the third exon of the cytochrome *b* mRNA. The mixture was precipitated with ethanol, dried under vacuum, and redissolved in 30 μl of 0.4 M NaCl–10 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], pH 6.4. The hybridization was done by heating at 80°C for 10 min, followed by incubation at 45°C for 3 h. The reverse transcription reaction mixture contained, in a final volume of 300 μl, 50 mM Tris (pH 8.4), 10 mM dithiothreitol, 6 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphates, 25 mM KCl, 102 U of RNasin (Promega Biotech), 66 U of reverse transcriptase (Life Sciences). After incubation at 42°C for 1 h, the reaction products were precipitated with ethanol, dried under vacuum, redissolved in 30% dimethyl sulfoxide, denatured by heating at 90°C for 10 min, and separated on a 6% polyacrylamide gel. The smaller of two prominent products was purified from the gel and sequenced by the method of Maxam and Gilbert (13).

RESULTS

Characterization of the self-splicing reaction. The exon/intron organization of the short cytochrome *b* gene of *S. cerevisiae* D273-10B is depicted in Fig. 1. To determine whether the terminal IVS is capable of autocatalytic excision, a mitochondrial DNA fragment containing 103 base pairs of the first intron (bI1), the entire second exon (E2) and second intron (bI2), and 40 base pairs of the terminal exon (E3) was cloned behind the SP6 promoter of pSP65. The resultant plasmid, SP65/bI2, was linearized at the *Xba*I site just downstream of the insert and used as a template for transcription by SP6 RNA polymerase.

Autocatalytic excision of the IVS from the SP6-derived transcript was assayed by one of several means. The results presented in Fig. 2A show that, after incubation of the precursor transcript under standard conditions for in vitro splicing, a new, smaller size RNA (0.7 to 0.8 kilobase) is formed. The new product was detected by ethidium bromide staining of the incubation mixture separated on 1% agarose (Fig. 2A, lane 2).

The mechanism of IVS excision as described by Cech et al. (3) is initiated by a nucleophilic attack of the 5' splice site by a free guanosine nucleoside, resulting in a covalent linkage between the guanosine and the 5' end of the IVS (3). Excision of the IVS by this mechanism, carried out in a reaction mixture containing [α-^{32}P]GTP, leads to the formation of a radioactively labeled IVS that can be visualized by autoradiography (Fig. 2A, lane 4).

The third method (Fig. 2C) makes use of radioactively labeled precursor RNA synthesized by transcription of the SP65/bI2 template in the presence of [α-^{32}P]GTP. In this experiment, three different size RNAs were detected by autoradiography: the full-size precursor, the excized IVS, and a smaller transcript which was determined by primer extension analysis (see below) to be the ligated E2-E3 exons.

In vitro excision of the IVS is time dependent (Fig. 2C) and shows a strict requirement for GTP. Trace amounts of the processed IVS occasionally detected in the absence of added GTP probably arise during the synthesis of the substrate since GTP is used in the transcription reaction. Figure 2B shows the effect of GTP concentration on IVS excision. The optimum GTP concentration for the self-splicing reaction is approximately 0.2 mM, a value similar to that reported for self-splicing of other group I intervening sequences (3, 8, 23). At the highest concentration of GTP (5 mM), a second, slightly larger excision product was observed. This RNA has not been characterized; it may result from an attack of GTP at a site in exon E2. Similar studies indicated that IVS excision occurs within a narrow range of Mg^{2+} concentrations, exhibiting a maximum activity at 50

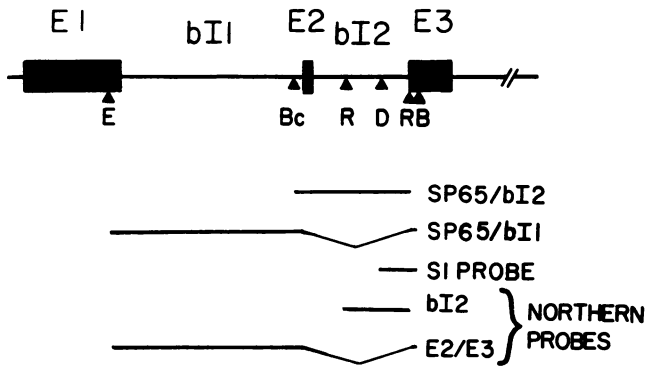

FIG. 1. Organization of the cytochrome *b* gene in *S. cerevisiae* D273-10B. The short cytochrome *b* gene is composed of three exons (E1, E2, and E3) and two introns (bI1 and bI2) depicted by the solid bars and lines, respectively. SP65/bI2 was constructed by ligating the *Bcl*I-*Bgl*II fragment of wild-type mitochondrial DNA to the *Bam*HI site of pSP65. SP65/bI1 contains the *Eco*RI-*Bgl*II fragment of mitochondrial DNA from the deletion mutant E26/R29B ligated to the *Eco*RI-*Bam*HI sites of pSP65. The *Dra*I-*Xba*I fragment of SP65/bI2 was used as a probe for S1 nuclease analysis. The *Xba*I site is part of the multiple cloning region of pSP65, downstream of the mitochondrial DNA insert of SP65/bI2. The *Rsa*I fragment of SP65/bI2 containing only bI2 sequences was used as an intron-specific probe in Northern analyses. A transcript from SP64/bI1, which directs transcription from the SP6 promoter in the opposite orientation of SP65/bI1, was used as an exon-specific (E2-E3) probe. The locations of restriction sites for *Bcl*I (Bc), *Eco*RI (E), *Rsa*I (R), *Dra*I (D), and *Bgl*II (B) are indicated.

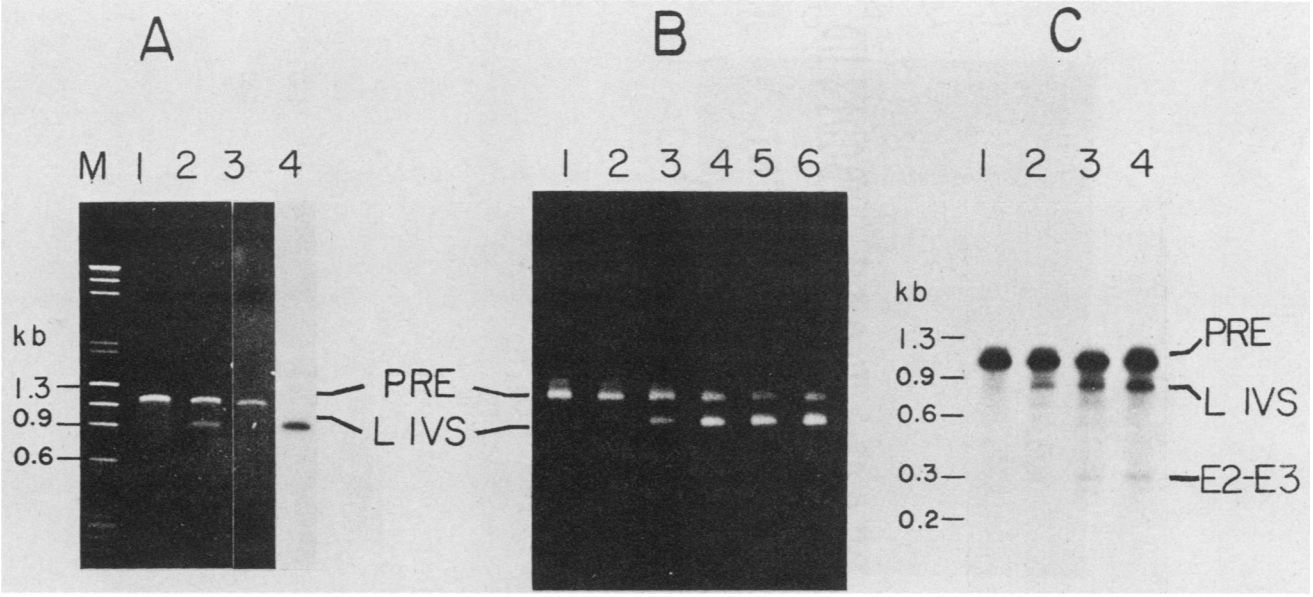

FIG. 2. In vitro splicing of the SP65/bI2 transcript. The construct, SP65/bI2, was linearized with *Xba*I and used as a template for runoff transcription of the precursor RNA substrate (PRE) for in vitro splicing. In (A), the precursor transcript was incubated in the presence or absence of GTP and the products of the reaction were separated by electrophoresis on 1% agarose. Lane M, Size standards consisting of λ DNA digested with *Hin*dIII and φX phage DNA digested with *Hae*III; lane 1, ethidium bromide-stained products formed in a reaction mixture containing all components of the standard in vitro splicing assay as described in Materials and Methods except for the omission of GTP; lane 2, same as lane 1 except for the addition of GTP; lane 3, same as lane 2 except that [α-^{32}P]GTP was substituted for the unlabeled GTP; lane 4, same as lane 3 except that the gel was exposed to X-ray film. In (B), the precursor RNA was incubated under standard conditions with various concentrations of GTP. Lane 1, No GTP; lane 2, 10 μM GTP; lane 3, 50 μM GTP; lane 4, 0.2 mM GTP; lane 5, 1 mM GTP; lane 6, 5 mM GTP. In (C), radioactive precursor RNA was synthesized by inclusion of [α-^{32}P]GTP in the transcription mixture. The radioactive precursor was incubated in the presence or absence of 0.2 mM GTP for various times. The products were separated on 1% agarose and the gel was exposed to X-ray film. Lane 1, No GTP; lane 2, 15 min in the presence of 0.2 mM GTP; lane 3, 30 min in the presence of 0.2 mM GTP; lane 4, 60 min in the presence of 0.2 mM GTP. The autoradiography shows the precursor (PRE), the excised linear IVS (L IVS), and the ligated exons (E2-E3). kb, Kilobases.

mM (data not shown). Whereas the Mg^{2+} dependence of autoexcision of the *Tetrahymena* rRNA IVS is similar in that the reaction occurs efficiently within a narrow concentration range, the optimal Mg^{2+} concentration for excision of the rRNA IVS is 10 mM (3). Self-splicing of the cytochrome *b* IVS is stimulated by ammonium sulfate concentrations of up to 100 mM, similar to the salt dependence of the *Tetrahymena* rRNA IVS reaction (3).

A mutation in the IVS prevents splicing. The mit$^-$ mutant M6-200 has been shown to have a mutation in the terminal intron of the cytochrome *b* gene that blocks in vivo processing of the IVS from the pre-mRNA (2). The mutation responsible for the processing defect has been sequenced and shown to be a G-to-A transition at nucleotide 2668 (M. L. Haldi, Ph.D. thesis, Ohio State University, Columbus, 1985) in the conserved sequence element R proposed to participate in base pairing interactions in the core structure of group I IVSs (6, 16, 17, 24). Alteration of this sequence element by site-specific mutagenesis blocks the self-splicing reaction of the *Tetrahymena* rRNA IVS (25). To test whether the single base substitution in M6-200 affects in vitro excision of the terminal IVS of cytochrome *b* pre-mRNA, the *Bcl*I-*Bgl*II fragment from M6-200 mitochondrial DNA was ligated to the *Bam*HI site of pSP65. This construct was used as a template for synthesis of a mutant precursor by runoff transcription. Self-splicing of the precursor was examined either by ethidium bromide staining or by the more sensitive [α-^{32}P]GTP incorporation assay. The results of these experiments (Fig. 3) indicate that the intron mutation blocks the autocatalytic excision reaction.

We also examined whether the first IVS of the short cytochrome *b* pre-mRNA, another group I IVS, can self-splice in vitro (16). Plasmid SP65/bI1 used to prepare the substrate RNA contains the first intron (bI1) bracketed by 100 nucleotides of the upstream first exon (E1), the entire second exon (E2), and part of the third exon (E3) (Fig. 1). The runoff transcript synthesized from this plasmid did not self-splice when incubated under standard conditions (Fig. 3). Varying the concentration of ammonium sulfate (0 to 0.5M) or $MgCl_2$ (0 to 0.2M) did not promote autocatalytic excision of the first IVS from the precursor (data not shown).

Splice junctions at the E2/E3 boundaries. Even though the nucleotide sequence of the *S. cerevisiae* cytochrome *b* mRNA is known (2), the existence of three alternative pairs of splice sites, each capable of generating the identical mRNA sequence, precludes the identification of the exon/intron boundaries in the gene (2). To determine these boundaries, the 5′ end of the excised linear IVS was sequenced. The 5′-end-labeled IVS, prepared by incubation of SP65/bI2 RNA under self-splicing conditions with [α-^{32}P]GTP, was subjected to partial cleavage by base-specific RNases (20). The products were separated on a sequencing gel next to a ladder generated by partial alkaline hydrolysis of the labeled IVS (Fig. 4, right panel). The partial cleavage products obtained by T_1 RNase revealed the first nucleotide to be the nonencoded G which is liberated as guanosine tetraphosphate (23). The second nucleotide in the sequence is a U corresponding to the first encoded nucleotide at the 5′ end of the IVS. The 3′ end of the excised IVS was determined by S1 nuclease mapping. A probe spanning the bI2/E3

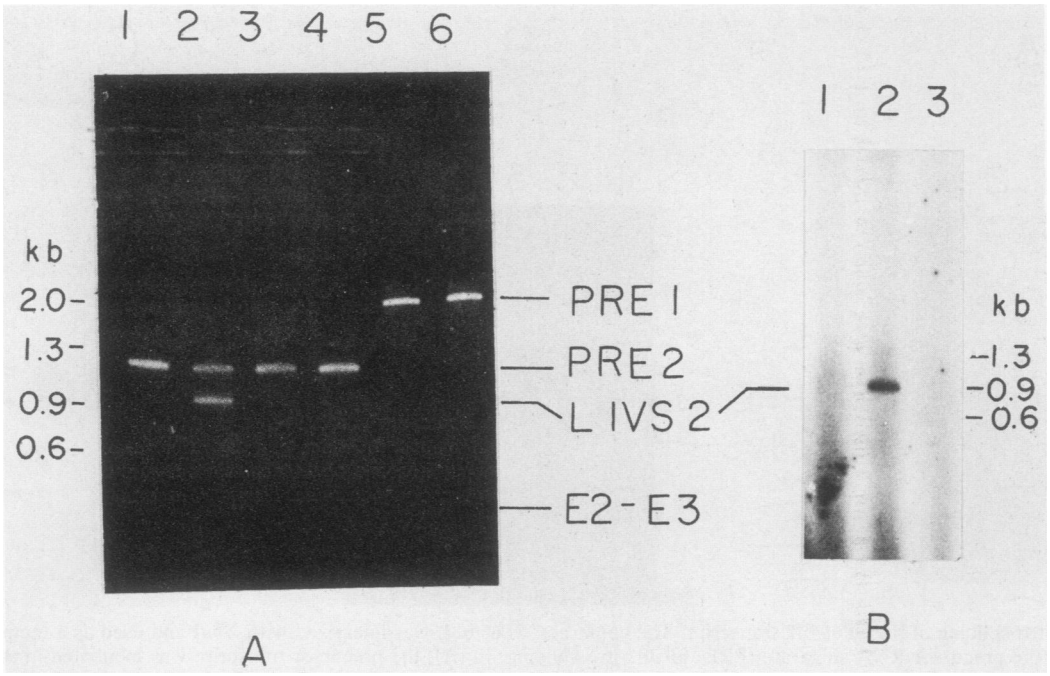

FIG. 3. Effect of a mutation in the terminal cytochrome *b* IVS on self-splicing. Precursor RNAs were transcribed from SP65/bI1 (PRE 1), SP65/bI2 (PRE 2), or SP65/mbI2 (PRE 2). In (A), the three different RNAs were assayed under standard self-splicing conditions in the presence or absence of 0.2 mM GTP. The reaction products were separated by electrophoresis on 1% agarose and stained with ethidium bromide. Lane 1, PRE 2 transcribed from the wild-type template incubated in the absence of GTP; lane 2, same as lane 1 except for the presence of 0.2 mM GTP; lane 3, PRE 2 transcribed from the mutant template incubated in the absence of GTP; lane 4, same as lane 3 except for the presence of 0.2 mM GTP; lane 5, PRE 1 incubated in the absence of GTP; lane 6, same as lane 5 except for the presence of 0.2 mM GTP. In (B), the in vitro splicing reactions were carried out in the presence of [α-^{32}P]GTP and the products were visualized by autoradiography. Lane 1, PRE 1; lane 2, PRE 2 transcribed from the wild-type template; lane 3, PRE 2 transcribed from the mutant template. The migrations of the substrates (PRE 1, PRE 2), of the linear intron from PRE 2 (L IVS 2), and of the ligated exons from PRE 2 (E2-E3) are marked in the margin. kb, Kilobases.

boundary was 3′-end labeled and hybridized to the linear IVS. The protected 5′ end of the DNA probe was estimated to be at nucleotide 2956 of the DNA sequence (data not shown). The 5′ and 3′ splice sites predict a sequence in agreement with the previously determined sequence of the cytochrome *b* mRNA (2). This was confirmed by sequence analysis of the E2-E3 ligation product formed in the self-splicing reaction. A synthetic oligonucleotide encompassing the sequence from nucleotides 2967 to 2990 in the terminal exon of the gene was hybridized to the total products formed from the SP65/bI2 runoff transcript. After reverse transcription, the smaller of the two resultant cDNAs was purified and sequenced. The E2-E3 product has a sequence at the splice junction consistent with the 5′ and 3′ ends of the excised IVS and of the reported sequence of the cytochrome *b* mRNA (2) (Fig. 4).

Self-splicing of the native cytochrome *b* pre-mRNA. The *cbp2* mutant C237 was previously found to accumulate a 2.9-kilobase cytochrome *b* precursor RNA containing the entire terminal IVS (14). This mutant provided a source of native precursor that made it feasible to examine whether this RNA is a suitable substrate for autocatalytic excision in vitro. Total mitochondrial RNA obtained from C237 was incubated under self-splicing conditions, and IVS excision was monitored by either incorporation of [α-^{32}P]GTP or Northern hybridization analysis. A minor radioactive species which comigrated with the linear IVS liberated from the SP6 transcript was detected in the GTP incorporation assay (data not shown). The identity of this product as the excised terminal IVS was confirmed by carrying out the reaction in the presence of 0.2 mM unlabeled GTP and analyzing the products by hybridization to an intron-specific probe. The probe hybridizes to a novel RNA whose appearance is GTP dependent (Fig. 5), suggesting that it is formed by the same mechanism responsible for the autocatalytic removal of the IVS from the SP6-derived precursor.

Although the native precursor contains the necessary sequence information to direct self-splicing in vitro, we have been unable to detect the presence of mature cytochrome *b* message in mitochondria from a strain with a disrupted copy of the *CBP2* gene (*CBP2*::*LEU2*). A radioactive RNA probe containing bI2, E2, and E3 sequences failed to detect cytochrome *b* mRNA in total mitochondrial RNA of the disrupted strain W303∇CBP2-1 even on long exposures of Northern blots (Fig. 6).

DISCUSSION

As detailed in the present study, the terminal intron of the mitochondrial cytochrome *b* gene of *S. cerevisiae* undergoes autocatalytic excision in vitro by the mechanism first described for the excision of the intervening sequence from the *Tetrahymena* rRNA precursor (3) and several other group I introns of mitochondrial genes (8, 23). It was previously noted that the sequence of the yeast cytochrome *b* mRNA at the E2-E3 boundary can be created by three different combinations of 5′ and 3′ splice sites (2). Sequence analysis of the excised linear IVS formed in the self-splicing reaction shows that only one of the three alternative pairs of splice sites is used. The experimentally determined exon/intron

boundaries conform to the secondary structure model of group I introns proposed by Waring, Davies, and co-workers (24, 26) in which the internal guide sequence aligns the exon/intron junctions such that the target of the nucleophilic attack by GTP is the phosphate at the 3′ position of a U that

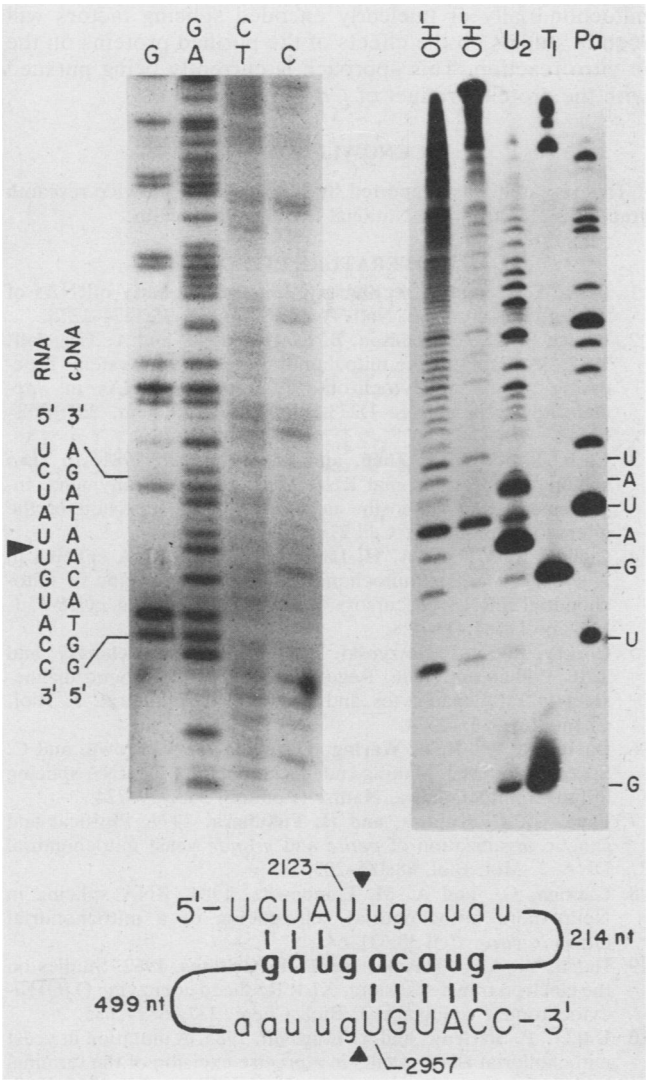

FIG. 4. Determination of precise splice junctions of bI2. The terminal IVS of the cytochrome *b* gene was labeled by incubation of the SP65/bI2 precursor RNA with [α-^{32}P]GTP under self-splicing conditions. The reaction products were passed sequentially through four Sephadex columns to remove unincorporated [α-^{32}P]GTP. The radioactive IVS was treated with alkali (OH^-), RNase U_2 (U_2), RNase T_1 (T_1), or pancreatic RNase (Pa), and the cleavage products were separated on a 20% sequencing gel as shown in the right panel. Primer extension analysis of the small RNA product of the self-splicing reaction is shown in the left panel. 5′-End-labeled primer corresponding to the noncoding strand sequence between nucleotides 2967 and 2990 (the numbering convention used is that of reference 18) was hybridized to a mixture of RNA formed under self-splicing conditions. Reverse transcription of the resultant hybrids yielded two products: a large cDNA presumed to be a full-length copy of the precursor RNA, and a smaller cDNA corresponding to the ligated "exons." A partial sequence of the smaller cDNA is shown in the left panel, where the arrowhead indicates the junction between exons 2 and 3 of the cytochrome *b* gene.

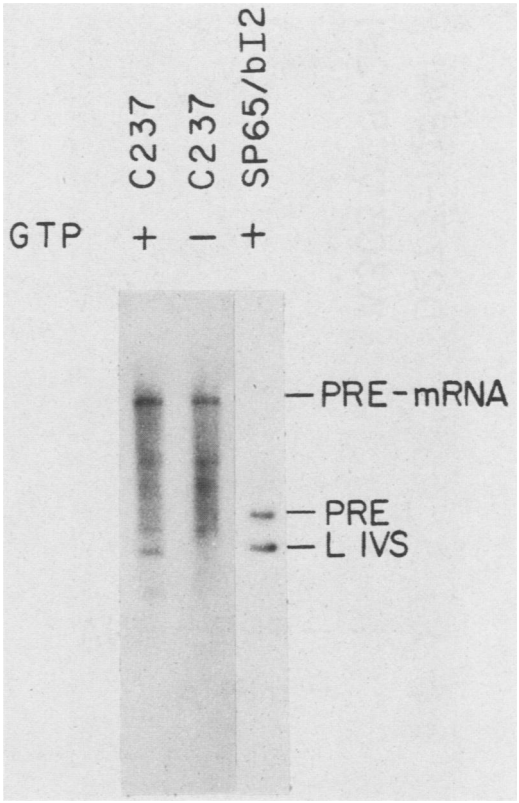

FIG. 5. In vitro splicing of native cytochrome *b* pre-mRNA. Total mitochondrial RNA from the *cbp2* mutant C237 was incubated under self-splicing conditions in the absence (−) or presence (+) of 0.2 mM GTP. Precursor RNA prepared by runoff transcription of SP65/bI2 was incubated under the same conditions. After electrophoretic separation on 1% agarose, the RNAs were transferred to diazobenzyloxymethyl paper and hybridized to a radioactive probe from the terminal intron of the cytochrome *b* gene. The results of the Northern hybridization show a GTP-dependent product in C237 mitochondrial RNA whose migration is identical to that of the IVS formed from the artificial substrate.

lies in apposition to a G in the guide sequence (Fig. 4). A second invariant feature of the self-splicing group I introns, also found in the terminal IVS of the cytochrome *b* pre-mRNA, is cleavage at the 3′ splice site after a G, resulting in a linear IVS with a 3′-terminal G (3, 8, 23).

The results obtained with the RNA precursor from the mutant M6-200 show that a G-to-A transition in the conserved sequence element R, as defined by Waring and Davies (24), within the terminal intron of the gene prevents in vitro splicing. This mutant was found earlier to be blocked in processing of the intervening sequence in vivo (2). These observations constitute strong evidence that in vivo processing of the cytochrome *b* pre-mRNA involves the same two-step transesterification mechanism described for in vitro self-splicing of the *Tetrahymena* rRNA precursor. In vivo, however, this process requires a protein factor previously shown to be encoded by the nuclear gene *CBP2*. A similar situation has been described for the mitochondrial cytochrome *b* gene of *Neurospora crassa* which contains a group I intron capable of self-splicing in vitro (8). Maturation of the precursor in vivo is blocked by mutations in a nuclear gene presumed to code for an auxiliary splicing factor (4, 8).

Although the precise role of such proteins in splicing is not known at present, several explanations seem reasonable.

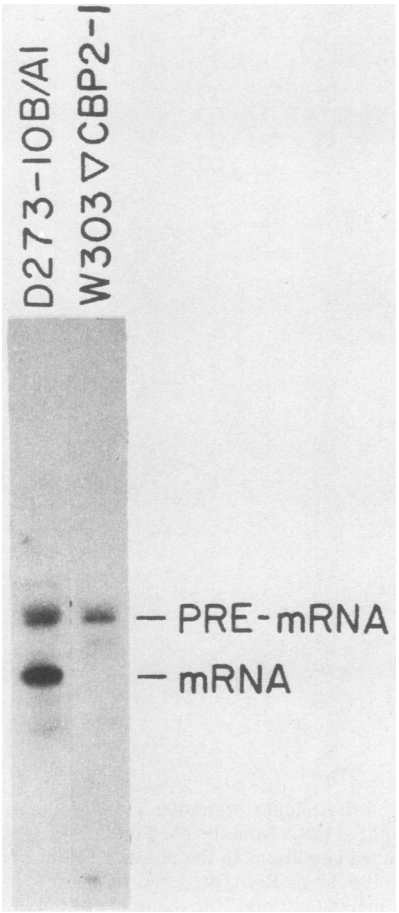

FIG. 6. Self-splicing of native cytochrome *b* pre-mRNA in vivo. Mitochondrial RNA from the disrupted strain W303∇CBP2-1 and wild-type strain D273-10B were separated on 1% agarose, transferred to diazobenzyloxymethyl paper, and hybridized to a probe containing sequences from the flanking exons (E2/E3 probe in Fig. 1). The RNA species corresponding to the native precursor with unexcised bI2 (PRE-mRNA) and the fully-spliced message (mRNA) are marked in the margin.

Under optimal conditions for in vitro splicing, which include concentrations of Mg^{2+} two orders of magnitude greater than exist in vivo (5), the efficiency of the excision reaction for the terminal IVS of cytochrome *b* is comparable to that of the *Tetrahymena* IVS. The high concentration of Mg^{2+} in the in vitro reaction may act to stabilize the RNA secondary structure necessary for the autocatalytic reaction, whereas the *CBP2* product and other splicing factors may stabilize this splicing-competent structure in the RNA in vivo. The difference in requirements for Mg^{2+} ions in the self-splicing reaction of the terminal IVS of cytochrome *b* pre-mRNA (50 mM), and some other group I IVSs (>20 mM) (8, 23), as compared with that of the *Tetrahymena* rRNA IVS (10 mM), may reflect a different capacity for protein-independent splicing in vivo.

Splicing proteins (maturases) have also been proposed to prevent interaction of ribosomes with the pre-mRNA that could disrupt the secondary structure of the RNA (6). According to this interpretation, the splicing factor binds to the IVS, preventing movement of the ribosome into this critical region of the RNA without affecting the secondary structure required for splicing. Excision of the IVS would result in a concomitant removal of the splicing protein and permit continuation of translation through the newly spliced exons. The attractiveness of this hypothesis has been lessened by the recent finding that the IVS of the *Tetrahymena* rRNA can self-splice in vivo in *E. coli* even when it is introduced into the coding region of the β-galactosidase gene (25). A more definitive judgment of the precise function of mitochondrially or nuclearly encoded splicing factors will require studies on the effects of the purified proteins on the in vitro reaction. This approach is currently being pursued with the protein product of *CBP2*.

ACKNOWLEDGMENT

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