Generation of Temperature-Sensitive *cbp1* Strains of *Saccharomyces cerevisiae* by PCR Mutagenesis and *in Vivo* Recombination: Characteristics of the Mutant Strains Imply That CBP1 Is Involved in Stabilization and Processing of Cytochrome *b* Pre-mRNA

Robin R. Staples* and Carol L. Dieckmann*,†

*Departments of *Biochemistry and †Molecular and Cellular Biology, The University of Arizona, Tucson, Arizona 85721*

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ABSTRACT

Mitochondrial biogenesis is dependent on both nuclearly and mitochondrially encoded proteins. Study of the nuclearly encoded mitochondrial gene products and their effect on mitochondrial genome expression is essential to understanding mitochondrial function. Mutations in the nuclear gene *CBP1* of *Saccharomyces cerevisiae* result in degradation of mitochondrially encoded cytochrome *b* (*cob*) RNA; thus, the cells are unable to respire. Putative roles for the CBP1 protein include processing of precursor RNA to yield the mature 5′ end of *cob* mRNA and/or physical protection of the mRNA from degradation by nucleases. To examine the activity of CBP1, we generated temperature-sensitive *cbp1* mutant strains by polymerase chain reaction (PCR) mutagenesis and *in vivo* recombination. These temperature-sensitive *cbp1* strains lack *cob* mRNA only at the nonpermissive temperature. Quantitative primer extension analyses of RNA from these strains and from a *cbp1* deletion strain demonstrated that CBP1 is required for the stability of precursor RNAs in addition to production of the stable mature mRNA. Thus, CBP1 is not involved solely in the protection of mature *cob* mRNA from nucleases. Moreover, we found that mature mRNAs are undetectable while precursor RNAs are reduced only slightly at the nonpermissive temperature. Collectively, these data lead us to favor a hypothesis whereby CBP1 protects *cob* precursor RNAs and promotes the processing event that generates the mature 5′ end of the mRNA.

MITOCHONDRIAL biogenesis is dependent upon both nuclearly encoded and mitochondrially encoded proteins (for review see COSTANZO and FOX 1990; GRIVELL 1989; TZAGOLOFF and MYERS 1986). In the yeast *Saccharomyces cerevisiae* the expression of mitochondrial genes is a complex process involving hundreds of nuclear *PET* genes (TZAGOLOFF and DIECKMANN 1990). Some *PET* gene products participate in transcription and translation and are not specific to particular mitochondrial genes, whereas others affect the expression of individual mitochondrial genes. These gene-specific proteins may coordinate the expression of the two genomes to yield a functional mitochondrion. To understand mitochondrial-nuclear interactions, we must understand gene-specific interactions.

A number of nuclearly encoded factors are required specifically for expression of the mitochondrial gene encoding cytochrome *b* (*cob*) of *S. cerevisiae*. The nuclearly encoded mitochondrial proteins CBS1, CBS2 and CBP6 are required for translation of *cob* mRNA (DIECKMANN and TZAGOLOFF 1985; RÖDEL 1986; RÖDEL and FOX 1987). Although *cob* mRNA is present in these strains, the messages are not translated. Strains with mutations in the nuclear gene *CBP1* are respiratory deficient because *cob* transcripts are degraded (DIECKMANN, HOMISON and TZAGOLOFF 1984; DIECKMANN, KOERNER and TZAGOLOFF 1984; DIECKMANN and MITTELMEIER 1987; DIECKMANN, PAPE and TZAGOLOFF 1982; MITTELMEIER and DIECKMANN 1990). Therefore, CBP1 is required for the accumulation of stable *cob* mRNA.

Supportive of a direct role in *cob* transcript maturation or stability, CBP1 has been shown to be imported into yeast mitochondria (WEBER and DIECKMANN 1990). It has been hypothesized that CBP1 either protects *cob* mRNA from degradation, or processes precursor RNA to generate stable *cob* mRNA (DIECKMANN, HOMISON and TZAGOLOFF 1984; DIECKMANN, KOERNER and TZAGOLOFF 1984). Mature *cob* mRNA is processed from a primary transcript as shown in Figure 1 (BONITZ *et al.* 1982; CHRISTIANSON *et al.* 1983). Transcription of the *tRNA^{glu}-cob* unit and processing of tRNA^{glu} are not affected in *cbp1* strains (DIECKMANN, KOERNER and TZAGOLOFF 1984). Following removal of tRNA^{glu} (CHEN and MARTIN 1988; HOLLINGSWORTH and MARTIN 1986), the resulting transcript with a 5′ end at −1098 (relative to +1 of the initiating AUG) is processed further at −954 to generate the mature 5′ end of *cob* mRNA. Recently, sequence between −961 and −898 of the untranslated leader of *cob* has been shown to be sufficient for CBP1-

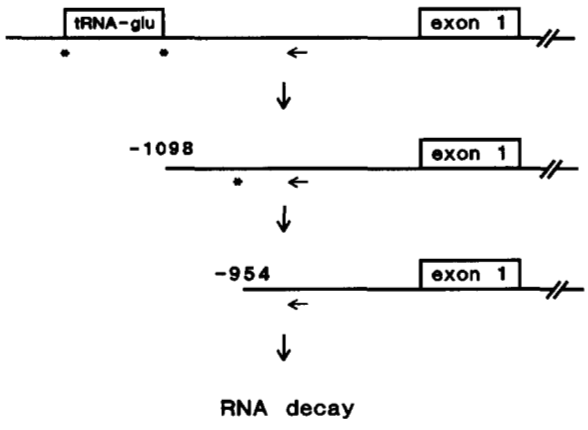

FIGURE 1.—Processing of $tRNA^{glu}$ and 5′ end formation of *cob* mRNA in yeast mitochondria. The primary transcript contains $tRNA^{glu}$ upstream of the first exon of *cob* RNA. The RNA is processed at the asterisks (*) to generate the mature tRNA and a *cob* pre-mRNA with a 5′ end at −1098 (relative to the +1 of the initiating AUG). This pre-mRNA is processed further at −954 at the asterisk (*) to generate the 5′ end of mature *cob* mRNA. ← indicates the primer binding site (at −854 to −826) for the "cob5B+3" primer used in the primer extension assays.

mediated accumulation of stable *cob* mRNA (MITTELMEIER and DIECKMANN 1993).

In the present study, we have used a genetic approach to explore the mechanism by which CBP1 acts on *cob* RNA *in vivo*. Conditional *cbp1* mutants were isolated by a method combining amplification of *CBP1* by PCR under mutagenic conditions and *in vivo* recombination as described previously (MUHLRAD, HUNTER and PARKER 1992). We have characterized the effect of such mutations on the expression of *cob*. Our data are suggestive that CBP1 protects *cob* precursor RNAs and processes or promotes processing of these RNAs to generate mature *cob* mRNA.

MATERIALS AND METHODS

Strains and media: The yeast strains used in this study are described in Table 1. Yeast strains were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol) or minimal media (WO; 0.67% yeast nitrogen base without amino acids, 2% glucose) (ROSE, WINSTON and HIETER 1990). Amino acid supplements were added to suggested final concentrations (ROSE, WINSTON and HIETER 1990). *Escherichia coli* strains were grown in LB medium (SAMBROOK, FRITSCH and MANIATIS 1989). Solid media contained 2% agar. Where ampicillin was required, a final concentration of 100 μg/ml was used.

Plasmid constructions: All enzymes and restriction endonucleases were purchased from Boehringer Mannheim except as noted otherwise. pRS415/$CBP1^+$ was constructed from pRS415 (*LEU2*$^+$ *CEN6*) (SIKORSKI and HIETER 1989) and Yep13/T31 (DIECKMANN, HOMISON and TZAGOLOFF 1984). Yep13/T31 was digested with *Nhe*I and *Sal*I, and a 2.8-kb fragment containing $CBP1^+$ was recovered after electrophoresis through an agarose gel. The ends of the fragment were treated to generate blunt ends using dNTPs (Boehringer Mannheim) and Klenow enzyme. This fragment was ligated into the *Apa*I and *Sac*I sites (also treated to generate blunt ends using Klenow enzyme) of pRS415.

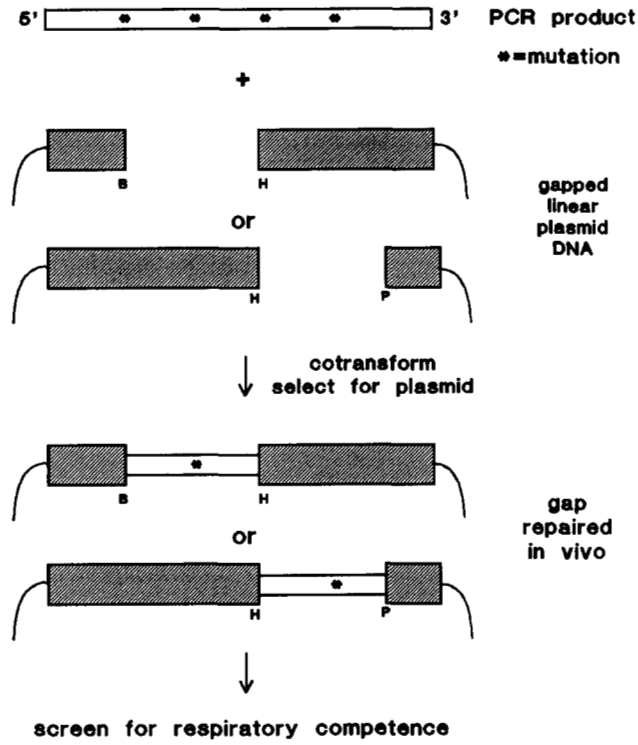

FIGURE 2.—Strategy for cloning PCR products *in vivo* in yeast by gap repair. The entire coding sequence of *CBP1* was amplified under mutagenic conditions as described in the text and is depicted in the open box containing asterisks (*). This sequence is homologous to that in the shaded boxes of pRS415/$CBP1^+$. Plasmid pRS415/$CBP1^+$ (*LEU2*$^+$, *CEN6*) was linearized and gapped in *CBP1* coding and flanking sequences (shaded boxes) by digestion of the plasmid with *Bam*HI (B) and *Hind*III (H) or *Hind*III (H) and *Pst*I (P). The linearized plasmid DNA and the PCR products were mixed together and used to transform a strain that was deleted for *CBP1*, and thus respiratory deficient. LEU^+ transformants were selected on WO plates, forcing the gap to be repaired *in vivo* by homologous recombination. These transformants were then tested for respiratory capability at all temperatures. Although mutations are depicted here as being recoverable only within the gap, recombination can occur anywhere between the ends of the gap and the PCR products. Thus, mutations may be recovered downstream or upstream of the target site in addition to the target site.

pKS/$ACT1^+$ was constructed from pGEM/ACT1 (MAYER and DIECKMANN 1989), which contains a 600-bp *Cla*I fragment of $ACT1^+$ (spanning part of the intron and part of the second exon) and Bluescript vector (Stratagene, La Jolla, California). The *Eco*RI-*Pst*I fragment of pGEM/ACT1 was ligated into the *Eco*RI and *Pst*I sites of Bluescript vector.

Construction of temperature-sensitive *cbp1* strains using polymerase chain reaction (PCR) mutagenesis and *in vivo* recombination in yeast: The entire coding sequence of *CBP1* in pRS415/$CBP1^+$ was amplified by PCR under mutagenic conditions after linearization of the plasmid with *Eco*RI. T7 and T3 promoter-specific primers (The Arizona Research Laboratory Biotechnology Facility, Tucson, Arizona) were used for the PCR. PCR reactions were done according to manufacturer's instructions (GeneAmp, Perkin-Elmer-Cetus, Norwalk, Connecticut), except that for mutagenic PCR, Mn^{++} was added to a final concentration of 0.25 mM or 0.5 mM, and Mg^{++} to a final concentration of 4.25 mM or 6.0 mM. The PCR products were transformed directly into yeast as described previously (MUHLRAD,

TABLE 1

Strains and genotypes

Strains	Genotype	Source or reference
S. cerevisiae		
DS10LL	*MAT***a** *his3-11,15 leu2-3,112 lys2 Δtrp1 ura3-52* [*rho*$^+$]	Gift from M. WERNER-WASHBURN
20HΔB3	*MATα leu2-3,112 his3-11,15 Δcbp1::HIS3* [*rho*$^+$]	LIU and DIECKMANN (1989)
DCL/LΔKG	*MATα ade1 leu2-3,112 Δcbp1::LEU2* [*rho*$^+$]	DIECKMANN and TZAGOLOFF (1985)
MY7	*MAT***a** *lys1* [*rho*$^+$]	MITTELMEIER and DIECKMANN (1993)
JC3/M9410	*MAT***a** *ade2 lys2 kar* [*rho*$^+$ *Δcob*]	MITTELMEIER and DIECKMANN (1993)
M10-152	*MATα* [*rho*$^+$ *cob*]	SLONIMSKI and TZAGOLOFF (1976)
YS2	*MATα leu2-3,112 his3-11,15 Δtrp1 ura3-52 Δcbp1::HIS3* [*rho*$^+$]	Spore of DS10 × LL20HΔB3, this study
YS1	*MAT***a** *ade1 leu2-3,112 his3-11,15 Δcbp1::HIS3* [*rho*$^+$]	Spore of LL20HΔB3 × CB11, this study
RS1	*MAT***a**/*MATα ade1/ADE1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 Δcbp1::HIS3/Δcbp1::HIS3* [*rho*$^+$]	Diploid of YS1 × LL20HΔB3, this study
RSY2000	*MATα ade1 leu2-3,112 ura3-52 Δcbp1::LEU2* [*rho*$^+$]	Spore of DCL/LΔKG × DS10, this study
RSY1000	*MATα ade1 leu2-3,112 ura3-52* [*rho*$^+$]	This study
RSY1000a	*MAT***a** *ade1 leu2-3,112 ura3-52* [*rho*$^+$]	R. STAPLES (unpublished data)
RSY1	*MATα ade1 leu2-3,112 ura3-52 cbp1-26(ts)* [*rho*$^+$]	This study
RSY5	*MATα ade1 leu2-3,112 ura3-52 cbp1-27(ts)* [*rho*$^+$]	This study
RSY7	*MATα ade1 leu2-3,112 ura3-52 cbp1-28(ts)* [*rho*$^+$]	This study
RSY8	*MATα ade1 leu2-3,112 ura3-52 cbp1-29(ts)* [*rho*$^+$]	This study
RSY20	*MATα ade1 leu2-3,112 ura3-52 cbp1-30(ts)* [*rho*$^+$]	This study
RSY33	*MATα ade1 leu2-3,112 ura3-52 cbp1-31(ts)* [*rho*$^+$]	This study
S. carlsbergensis		
CB11	*MAT***a** *ade1* [*rho*^{0}]	TEN BERGE, ZOUTEWELLE and NEEDLEMAN (1974)

HUNTER and PARKER 1992) and illustrated in Figure 2 with the following specific modifications. To target the mutagenesis to different regions, the plasmid pRS415/*CBP1*$^+$ was linearized and gapped by digestion with either *Bam*HI and *Hind*III or *Hind*III and *Pst*I. The large 7.8- and 7.9-kb fragments were purified twice by agarose gel electrophoresis. The entire 2.8-kb PCR product and approximately 1 μg of gapped linear plasmid DNA were mixed and used to transform *S. cerevisiae* strain RS1 (Table 1). LEU$^+$ transformants were selected at 30° on WO plates and screened for respiratory competence at 15°, 23°, 30° and 36° on YPG plates.

Transformation of yeast and *E. coli* strains: *S. cerevisiae* strains were transformed as described previously (GEITZ and SCHIESTL 1991). *E. coli* strains were transformed as described previously (COHEN, CHANG and HSU 1972).

RNA preparations and Northern blot analysis: Total cellular RNA was isolated as described (CAPONIGRO, MUHLRAD and PARKER 1993) from mid-logarithmic cultures grown in YPD or YPG. Approximately 8 μg of RNA was subjected to electrophoresis on 1.0% agarose gels containing 6.7% formaldehyde. Mitochondrial RNA was prepared as described (MITTELMEIER and DIECKMANN 1990) from overnight cultures grown at 30° in YPD and subjected to electrophoresis on nondenaturing 1% agarose gels. Following electrophoresis, Northern analysis was performed as described previously (MAYER and DIECKMANN 1989; MITTELMEIER and DIECKMANN 1990).

DNA probes: An *ACT1*$^+$ DNA probe was generated by digesting pKS/ACT1$^+$ with *Bam*HI and *Hind*III. A fragment of approximately 600 bp was purified by agarose gel electrophoresis. The *cob* DNA probe, which spans both the untranslated leader sequence and *cob* coding sequence (−1350 to +319 relative to the initiating AUG of *cob* coding sequence), was generated by digesting pKS-Mb2-1A (T. MITTELMEIER, unpublished data) with *Bam*HI and *Eco*RI. A fragment of approximately 1.7 kb was purified by agarose gel electrophoresis. A *cox3* specific probe was generated by digestion of pLSF600 (FOLLEY and FOX 1991) with *Xba*I; the 1.9-kb fragment spanning the entire coding region of *cox3* was purified by agarose gel electrophoresis. Each probe was radiolabeled with [α^{32}P]dATP (ICN, Costa Mesa, California) using the Random Primed Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Primer extension analysis of *cob* transcripts: For quantitative analysis by primer extension, 12 μg of total cellular RNA was hybridized to 15 pmoles of the "cob5B+3" primer (5′-CAA TTA TTA TTA TTA TTA TTA TAC ATA AA-3′; MITTELMEIER and DIECKMANN 1993). This primer:RNA ratio was shown to be saturating for *cob* mRNA (data not shown). The primer is antisense to the *cob* untranslated leader sequence from −854 to −826 relative to the initiating AUG of the *cob* coding sequence. Primer extensions were carried out as described previously (MITTELMEIER and DIECKMANN 1993), except that the hybridization reaction was incubated at 58° for 45 min. The primer extension products were subjected to electrophoresis on a 6% polyacrylamide 9 M urea sequencing gel, dried and autoradiographed. Radioactive markers were generated by end-labeling either φX174 *Hae*III markers (New England Biolabs, Beverly, Massachusetts) or pBR322 *Msp*I markers (New England Biolabs) with [γ^{32}P]ATP (ICN) in a kinase exchange reaction using polynucleotide kinase (Pharmacia LKB Biotechnology, Piscataway, New Jersey) according to the manufacturer's instructions.

Quantitation of Northern blots and primer extension products: *cob*, *cox3* and *ACT1* mRNAs from Northern blots and cDNAs from primer extension gels were quantitated using a Betascope Analyzer (Betagen, Waltham, Massachusetts). For Northern blot analyses of total cellular RNA, *cob* mRNA was normalized to *ACT1* mRNA. For Northern blot analyses of mitochondrial RNA, *cob* mRNA and splicing intermediates were normalized to *cox3* mRNA. Primer extension products were normalized to a cDNA that was

independent of *CBP1* expression and present in all reactions including those in which RNA from JC3/M9410 (Table 1) was used. JC3/M9410 contains a deletion of *cob* untranslated leader sequence from −975 to −64, and thus the primer binding site is deleted.

RESULTS

Isolation of conditional *cbp1* strains: It was shown previously that *cob* transcripts were degraded in *cbp1* strains (DIECKMANN, HOMISON and TZAGOLOFF 1984; DIECKMANN, KOERNER and TZAGOLOFF 1984; DIECKMANN and MITTELMEIER 1987; DIECKMANN, PAPE and TZAGOLOFF 1982; MITTELMEIER and DIECKMANN 1990). There are two nonexclusive mechanisms by which CBP1 might act. The first is physical protection of the mRNA from nucleolytic degradation and the second is processing of precursor RNA to yield stable mature *cob* mRNA. We proposed that conditional mutations of *CBP1* might be used to distinguish these models. To this end, we sought to isolate and characterize temperature-sensitive *cbp1* strains.

The entire coding sequence of *CBP1* was amplified by PCR under mutagenic conditions as described in MATERIALS AND METHODS. As outlined in Figure 2, the PCR products were cloned by gap-repair (MUHLRAD, HUNTER and PARKER 1992) of pRS415/*CBP1*$^+$. Two different gaps were used in separate transformations to target mutations to the 5′ or 3′ half of *CBP1*. Of 2300 transformants, 559 were respiratory deficient at all temperatures; 72 showed conditional respiratory deficiency at either 15°, 36° or both, but respired at 24° and 30°; the remainder were respiratory competent at all temperatures. All of the conditional transformants grew on glucose at all temperatures and therefore exhibited conditional growth only under respiratory conditions.

To ensure that the deletions in the plasmid had been repaired correctly, plasmids were isolated from 11 temperature-sensitive transformants and transformed into *E. coli* strain RR1 (BOLIVAR *et al.* 1977). Restriction digests of the plasmids confirmed that these transformants had a wild-type restriction map, indicating that the gaps were repaired correctly (data not shown). To ensure that the temperature-sensitive mutations were plasmid-encoded, plasmids were transformed into YS1 (*MAT***a**) and YS2 (*MAT*α) (Table 1), each of which have deletions of *CBP1* and flanking sequences. LEU$^+$ transformants were selected and screened for temperature-sensitive growth on glycerol plates. All 11 plasmids caused YS1 and YS2 to exhibit temperature-sensitive growth on glycerol medium. We concluded that the temperature-sensitive mutations were plasmid-encoded.

To determine whether the temperature-sensitive mutations encoded by the plasmids were identical or different, each strain was crossed to itself (of the opposite mating type) and to each of the other mutant strains. The resulting diploids were temperature-sensitive for growth on glycerol, indicating that the mutations could not complement each other. However, papillae arose at 36° from the patches of diploids on YPG plates in which the different mutant strains had been crossed to each other, but no papillae arose from diploids in which the strains had been crossed to themselves. Presumably, these papillae are the result of crossover or gene conversion events, and thus it is likely that no two of the 11 temperature-sensitive mutations are identical. We conclude that each of the mutations is a new allele of *CBP1*.

For ease in strain manipulation, as well as to verify whether the temperature-sensitive phenotype was expressed when the mutant alleles were in single copy, six of the temperature-sensitive mutations were recombined onto the yeast genome by gene replacement (ROTHSTEIN 1991). Specifically, plasmids encoding each of the temperature-sensitive *cbp1* mutations and a wild-type *CBP1*$^+$ allele were digested with *Pvu*II, and the 3.2-kb fragment containing *CBP1* sequence was purified by agarose electrophoresis. This fragment was used to transform RSY2000, which contains a *LEU2*$^+$ gene disruption of *CBP1* (Table 1); respiratory-competent transformants were selected on YPG supplemented with 0.05% glucose. These transformants were screened for loss of the *LEU2*$^+$ marker and for temperature-sensitive growth on glycerol medium. The following strains were obtained (shown in Figure 3): RSY20, in which the mutagenesis had been targeted to the 5′ half of *CBP1*; and RSY1, RSY5, RSY7, RSY8 and RSY33, in which the mutagenesis had been targeted to the 3′ half of *CBP1*. An isogenic wild-type strain, RSY1000, was constructed by transformation with a wild-type *CBP1*$^+$ gene from pRS415/*CBP1*$^+$.

To confirm integration of the temperature-sensitive *cbp1* mutations, the transformants were crossed to an isogenic wild-type strain of opposite mating type (RSY1000a) and sporulated. The temperature-sensitive respiratory-deficient phenotype segregated 2:2 (2 temperature sensitive for respiration: 2 wild type for respiration, an average of 6 tetrads analyzed), indicating that integration into the genome was successful. In addition, Southern analyses of DNA from the transformants confirmed integration at the correct locus (data not shown).

Temperature-sensitive *cbp1* strains lack *cob* mRNA at 36° and have decreased levels of *cob* mRNA at 24°: Since strains with *cbp1* null alleles are respiratory deficient because *cob* transcripts are degraded (DIECKMANN, HOMINSON and TZAGOLOFF 1984; DIECKMANN, KOERNER and TZAGOLOFF 1984; DIECKMANN and MITTELMEIER 1987; DIECKMANN, PAPE and TZAGOLOFF 1982; MITTELMEIER and DIECKMANN 1990), we tested whether *cob* mRNA was lacking at the restrictive temperature in the temperature-sensitive *cbp1* strains. Total RNA was isolated from

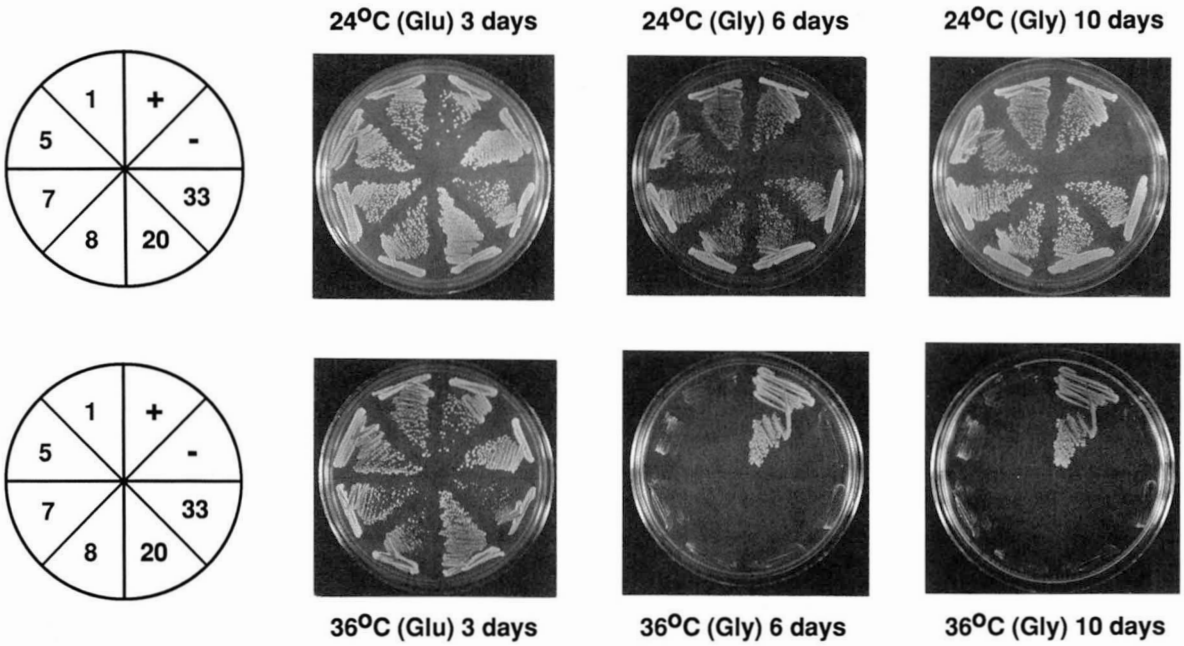

FIGURE 3.—Temperature-sensitive *cbp1* strains on fermentable and nonfermentable carbon sources at permissive and restrictive temperatures. The plate diagrams to the left of the photos indicate the orientation of the plates and the strains present. +, RSY1000 (isogenic wild type); −, RSY2000 (isogenic *cbp1* deletion strain); 1, RSY1; 5; RSY5; 7,RSY7; 8, RSY8; 20, RSY20; 33, RSY33. Glu, YPD medium (fermentable); Gly, YPG medium (nonfermentable).

the temperature-sensitive strains, RSY1000 (isogenic wild type) and RSY2000 (isogenic Δ*cbp1*) grown in YPD at 24° and 36° and in YPG at 24°. Northern analysis of RNA showed that there was no detectable *cob* mRNA present in the temperature-sensitive strains when grown at 36° in YPD (Figure 4C) except for a faint band present in RSY7. These results indicate that, when grown at 36°, most of the temperature-sensitive *cbp1* strains lack *cob* mRNA, a phenotype similar to that of *cbp1* deletion strains. Northern analysis showed that *cob* mRNA was present in temperature-sensitive strains when grown at 24° in either glycerol or glucose (Figure 4, A and B), although at lower levels than in wild type. As assayed by Northern blot analyses, the levels of $tRNA^{glu}$, *oli1*, *cox1*, *cox2* and *cox3* were not affected by the temperature-sensitive *cbp1* mutations at 36° (data not shown). Thus, the mutations affect only *cob* transcripts.

The amount of *cob* mRNA present in each strain shown in Figure 4 was quantitated and normalized to *ACT1* mRNA and is expressed as a percentage of the level of *cob* mRNA in wild-type RSY1000 (Table 2). The levels of *cob* mRNA in the temperature-sensitive *cbp1* strains ranged from 2–15% as compared with the isogenic wild-type strain when grown at 24° in glucose, whereas the levels ranged from 9–29% of that in the wild-type strain when grown at 24° in glycerol. This decrease in *cob* mRNA levels compared with the isogenic wild-type strain appeared to have no effect on the growth rate of the strains in glycerol at 24° as the doubling time was similar in all strains (data not shown). These data suggest that the abundance of

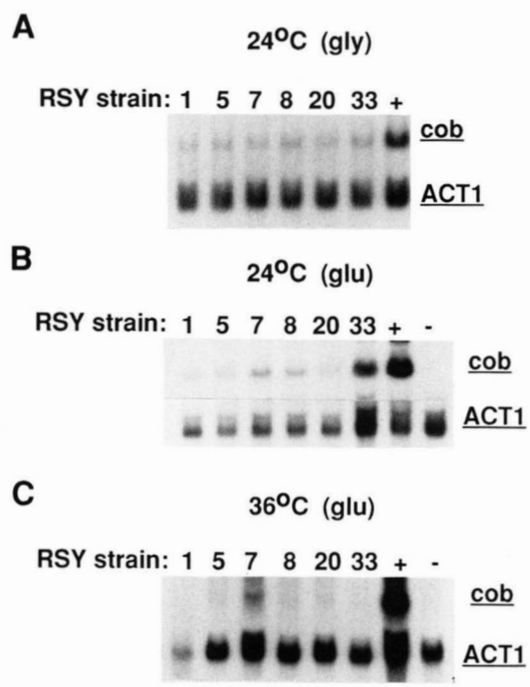

FIGURE 4.—Northern analyses of *cob* and *ACT1* mRNAs in temperature-sensitive *cbp1* strains grown in fermentable and nonfermentable carbon sources at permissive and restrictive temperatures. (A) RSY strains grown at 24° in YPG medium (Gly); (B) RSY strains grown at 24° in YPD medium (Glu); (C) RSY strains grown at 36° in YPD (Glu). Lane designations are as described in the legend to Figure 3. *cob*, mature cytochrome *b* mRNA; *ACT1*, actin mRNA.

cytochrome *b* mRNA in wild-type yeast cells is not rate-limiting for growth under respiratory conditions.

The levels of *cob* precursor RNAs are affected by temperature-sensitive *cbp1* mutations: To begin the

TABLE 2

Levels of *cob* mRNA[a] at permissive and restrictive temperatures in temperature-sensitive *cbp1* strains

Strain	24° (Gly)[b]	24° (Glu)[c]	36° (Glu)
RSY1000	100	100	100
RSY1	9.8	2.4	[d]
RSY5	19.2	7.2	[d]
RSY7	15.7	10.2	[d]
RSY8	27	9.1	[d]
RSY20	18.5	4.3	[d]
RSY33	28.9	15	[d]

[a] Percent of wild type, determined from Northern analyses of total cellular RNA.
[b] Glycerol.
[c] Glucose.
[d] Not determined.

dissection of the role of CBP1 in 5′ end processing and protection of *cob* mRNA, we decided to assay processing of *cob* pre-mRNA in the temperature-sensitive *cbp1* strains. As Northern blot analysis of total cellular RNA did not reveal the presence of 5′ unprocessed precursors to mature *cob* mRNA in either the *cbp1* deletion, temperature-sensitive or wild-type strains, we turned to the more sensitive method of quantitative primer extension analyses of total RNA from these strains. Using an antisense primer that hybridizes between −854 and −826 as shown in Figure 1, cDNAs were detected that correspond to mRNAs with mature 5′ ends at −954 (128 nucleotides [nt]) and to the RNAs with 5′ ends generated by the 3′ endonucleolytic cleavage of $tRNA^{glu}$ at −1098 (272 nt) (lanes labeled "+" in Figure 5). cDNAs corresponding to primary transcripts with 5′ ends at −1566 (740 nt) and the 5′ ends of the RNAs generated by the 5′ endonucleolytic cleavage of $tRNA^{glu}$ at −1170 (344 nt) were not detected (not shown in figure). We conclude that the only measurable precursors to mature *cob* mRNA are the RNAs generated by the 3′ endonucleolytic cleavage of $tRNA^{glu}$ which have 5′ ends at −1098.

In wild type, these precursor RNAs had a steady-state level that was approximately 50% (precursor/mRNA = 0.5) the level of mature mRNA (Table 3). The abundance of these transcripts is surprising because, at this level, they should be detectable by Northern blot analysis. Perhaps the cDNAs with 5′ ends at −1098 represent a collection of unspliced RNAs. As each of the unspliced RNA species is not as abundant as the mRNA (BONITZ *et al.* 1982), they are undetectable in these Northern blot analyses of total cellular RNA.

If the sole function of CBP1 is to process the −1098 precursors to the −954 mature molecules, and the rate of decay of precursors is not dependent on precursor concentration, then the mutant strains should exhibit increased steady-state levels of the −1098 precursors at the restrictive temperature. If processing at −954 is blocked and the precursor decay rate is dependent on precursor concentration, the steady-state level of precursor in mutant strains should be approximately the same as in wild type. However, if CBP1, in binding to the precursor protects it from degradation, or nucleates a complex that has a protective role, the steady-state level of precursor RNAs would be expected to decrease in the mutant strains at the restrictive temperature. As shown in Figure 5 and quantitated in Table 3, the abundance of the −1098 precursors in the *cbp1* deletion strain, as well as in each of the temperature-sensitive *cbp1* strains, was reduced when cells were grown at 24° in glycerol (Figure 5A), at 24° in YPD (Figure 5B), and at 36° in YPD (Figure 5C) (see Table 3). We conclude that CBP1 is required to stabilize directly or indirectly the precursor RNAs that have 5′ ends at −1098.

The levels of *cob* splicing intermediates are affected by the temperature-sensitive *cbp1* mutations: To assess the effects of the temperature-sensitive mutations on splicing intermediates, we isolated mitochondrial RNA from RSY1000 (wild type), M10-152 (a nonisogenic strain in which splicing of *cob* introns is slowed), RSY20 (temperature-sensitive *cbp1*) and RSY2000 (*cbp1* deletion strain) grown at 30° in YPD to stationary phase. Northern analyses of *cob* and *cox3* RNAs are shown in Figure 6A. In the wild-type strain, RSY1000 ("+"), and in M10-152 ("mit⁻"), *cob* splicing intermediates containing both intron 4 and intron 5 (bI4,bI5) (4.3 kb), containing only bI4 (3.6 kb), and containing only bI5 (2.9 kb) are present. However, in wild type the intermediate that contains the bI5 intron is clearly a doublet, of which the lower band may represent a precursor with a processed (−954) 5′ end. Figure 6 also shows that when compared to the wild-type strain, RSY1000 ("+"), all splicing intermediates of RSY20 ("20") and RSY2000 ("−") were decreased in abundance. mRNA and splicing intermediates were quantitated and normalized to *cox3* as described in MATERIALS AND METHODS and are shown in Table 4 (expressed as percentages of the mature wild-type RNA). RNAs from RSY20 that contained only bI4 and the intermediates that contained bI4 and bI5 were each 1% of wild-type levels. However, the splicing intermediates that contained only bI5 were undetectable in RSY20 (estimated level of detection: <0.5%). The amount of total precursors were reduced to 2% of mature wild-type levels in RSY20. We conclude that CBP1 is required for increasing the stability of splicing intermediates and RNAs that lack a processed 5′ end. The simplest hypothesis is that CBP1 protects the precursors by binding to them, or nucleates a complex that protects the RNAs.

***cob* mRNA levels are affected dramatically by the temperature-sensitive mutations:** The levels of precursor and mature RNAs were 8- and 10-fold lower on average in the temperature-sensitive strains than in wild type when the strains were grown at the permissive temperature in YPD (Table 3). Raising the

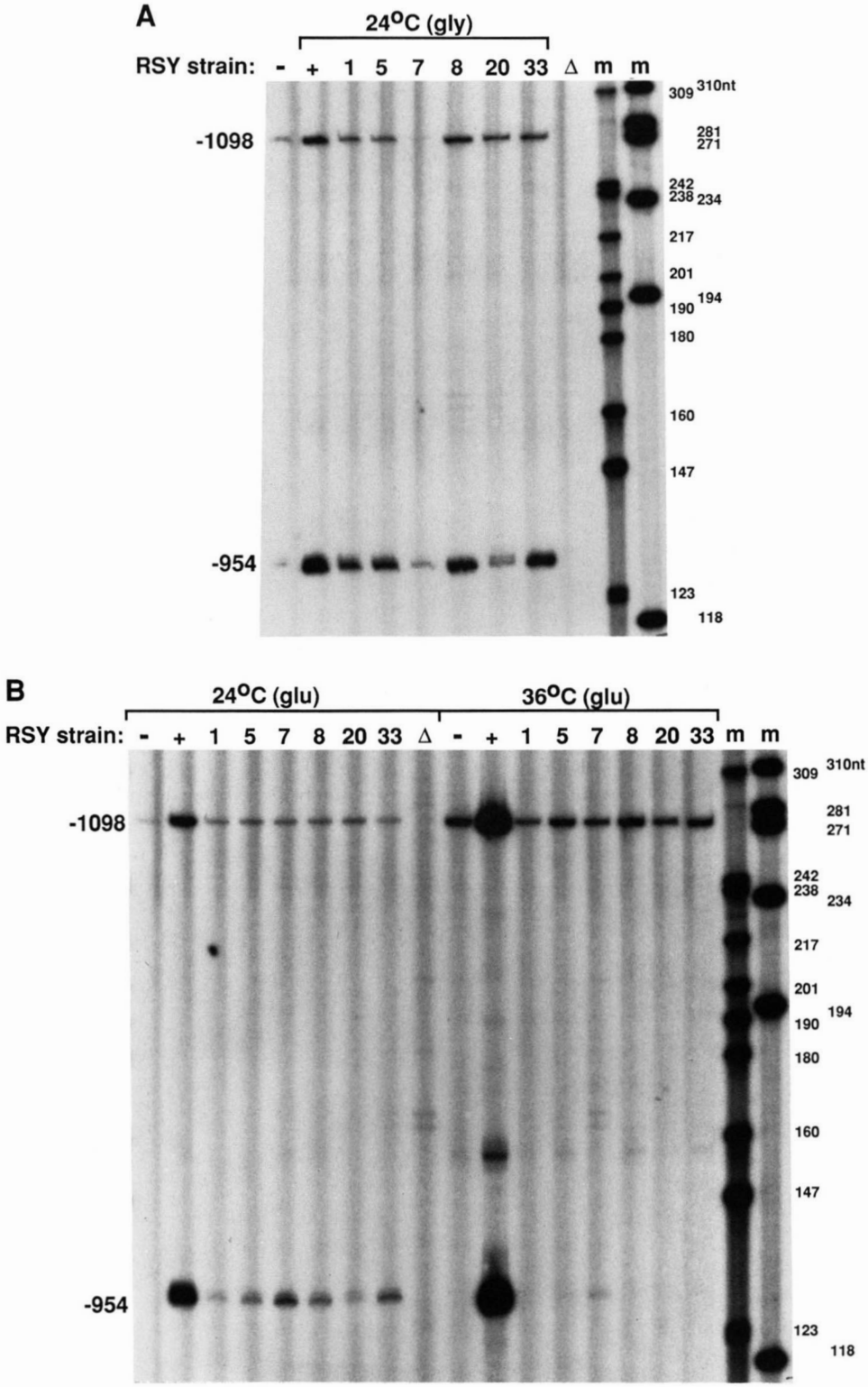

FIGURE 5.—Primer extension analyses of *cob* RNAs from temperature-sensitive *cbp1* strains at permissive and restrictive temperatures. Primer extension analyses were done as described in the MATERIALS AND METHODS. Lane designations are as described in the legend to Figure 3; Δ, RNA from JC3/M9410; m, markers from pBR322 digested with *Msp*I and ϕX174 digested with *Hae*III. −1098, primer extension product of 272 nucleotides (nt), the 5′ end of which corresponds to *cob* pre-mRNA generated by the 3′ endonucleolytic cleavage of $tRNA^{glu}$; −954, primer extension product of 128 nt, the 5′ end of which corresponds to that of mature *cob* mRNA. (A) RSY strains grown in YPG (Gly) at 24°; (B) RSY strains grown in YPD (Glu) at 24° and 36° as indicated.

growth temperature of the temperature-sensitive strains from 24° to 36° had an additional threefold effect on precursor levels (average decrease was from 14% to 5% of wild-type levels), whereas the effect on mature mRNA levels was a much more dramatic 25-fold (average decrease was from 13% to 0.5% or less). These data suggest that CBP1 affects the mature and precursor mRNA levels by different mechanisms. The simplest model is that CBP1 affects the level of mature mRNA by being required for processing of precursor RNAs to generate the 5′ end of mature mRNA. The data do not rule out the alternative hypotheses that the affinity of CBP1 for −1098 precursors and −954 RNAs is different and/or that the inherent stabilities of the two RNAs are different.

DISCUSSION

Some mitochondrial transcripts of *S. cerevisiae* that encode structural proteins are processed from polycis-

TABLE 3

Levels of precursor and mature *cob* mRNA[a] and the precursor/mRNA ratios in temperature-sensitive *cbp1* strains and a *cbp1* deletion strain

Strain	24° (Gly)[b]			24° (Glu)[c]			36° (Glu)		
	P[d]	M[e]	P/M[f]	P	M	P/M	P	M	P/M
RSY2000	[g]	[g]	[g]	5.5	[h]	[g]	4.3	[h]	[g]
RSY1000	32	100	0.32	50	100	0.50	85	100	0.85
RSY1	14	44	0.32	6.5	9	0.72	1.7	[h]	[g]
RSY5	16	64	0.25	6.0	11	0.55	5.1	[h]	[g]
RSY7	4.8	19	0.25	7.5	24	0.31	3.4	0.5	6.8
RSY8	12	33	0.36	8.0	15	0.53	6.0	[h]	[g]
RSY20	18	27	0.67	9.5	5	1.9	2.6	[h]	[g]
RSY33	7.4	31	0.24	4.0	15	0.27	4.3	[h]	[g]

[a] Percent of mature wild-type *cob* mRNA, determined by primer extension analyses of total cellular RNA.
[b] Glycerol.
[c] Glucose.
[d] Precursor RNA.
[e] Mature RNA.
[f] Precursor/mature ratio.
[g] Not done.
[h] Not detectable.

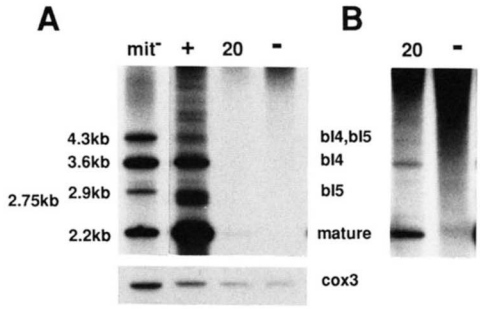

FIGURE 6.—Northern analyses of *cob* and *cox3* RNAs from mitochondrial RNA preparations. Mitochondrial RNA was electrophoresed on a 1% nondenaturing agarose gel as described in MATERIALS AND METHODS. bI4,bI5 refers to the splicing intermediate that contains both intron 4 (bI4) and intron 5 (bI5); bI4 refers to the intermediate that contains only intron 4; bI5 refers to the intermediate that contains only intron 5. (A) mit⁻, strain M10-152 in which splicing is slowed; +, RSY1000 (wild type); 20, RSY20; and −, RSY2000. (B) Overexposure of A, showing *cob* transcripts of RSY20 (20) and RSY2000 (−).

tronic precursor RNAs as *cob* mRNA is from the *tRNA^{glu}-cob* transcript (see GRIVELL 1989 for review of transcription units). These primary transcripts are processed to yield monocistronic messages and tRNAs. Processing of primary transcripts in yeast mitochondria includes endonucleolytic cleavages involved in tRNA maturation, intron processing, 3′ end processing of mRNAs at conserved dodecamer sequences, and 5′ end maturation (COSTANZO and FOX 1990). In addition to *cob* mRNA, the *cox3*, *var1* and *aap1-oli2* mRNAs have been proposed to have processed 5′ ends (THALENFELD, HILL and TZAGOLOFF 1983; ZASSENHAUS, MARTIN and BUTOW 1984; SIMON and FAYE 1984). However, very little is known about 5′ end maturation of mitochondrial mRNAs. We have used PCR mutagenesis along with *in vivo* recombination (MUHLRAD, HUNTER and PARKER 1992) to isolate

TABLE 4

Levels of *cob* splicing intermediates[a] and precursor/mRNA ratios in a temperature-sensitive *cbp1* and a *cbp1* deletion strain

Strain	M[b]	4.3 kb[c]	3.6 kb[d]	2.9 kb[e]	P$_t$[f]
RSY2000	[g]	[g]	[g]	[g]	[g]
RSY1000	100	8	14	13[h]	35
RSY20	3	1	1	[g]	2

[a] Percent of mature wild-type *cob* mRNA, determined by Northern analyses of mitochondrial RNA.
[b] Mature *cob* mRNA.
[c] Splicing intermediate that contains both bI4 and bI5.
[d] Splicing intermediate that contains only bI4.
[e] Splicing intermediates that contains only bI5.
[f] P$_t$ is total percentage of splicing intermediates.
[g] Not detectable.
[h] Includes both the 2.9-kb and 2.75-kb splicing intermediates.

conditional alleles of *CBP1*. This technique has proven to be a quick and useful method of obtaining these types of alleles. We envision that in combination with a plasmid shuffle (SIKORSKI and BOEKE 1991), conditional alleles of any gene, including essential ones, could be obtained. In the present study, using strains generated by this method, we have implicated the nuclear gene product CBP1 in the cleavage that forms the mature 5′ end of cytochrome *b* mRNA and shown that CBP1 is necessary for the stability of precursor RNAs.

That a protein can increase the stability of an RNA is not unique to CBP1 and *cob* transcripts. The stability of nonmitochondrial mRNAs of both yeast and mammalian cells has been observed to be affected by mutations in particular genes (BERNSTEIN *et al.* 1992; KLAUSNER, ROUAULT and HARFORD 1993; MINVIELLE-SEBASTIA *et al.* 1991). Also, nuclear genes have been identified by mutations in *Chlamydomonas* that affect the stability of specific chloroplast mRNAs

(DRAPIER, GIRARD-BASCOU and WOLLMAN 1992; KUCHKA *et al.* 1989; SIEBURTH, BERRY-LOWE and SCHMIDT 1991).

Composition of the −1098 cDNAs detected by primer extension analysis: Primer extension analysis of the 5′ ends of mature and pre-mRNAs in *CBP1*$^+$ strains revealed that the major detectable precursors to mature *cob* mRNA are molecules generated by 3′ endonucleolytic cleavage of tRNA^{glu}. These RNAs have 5′ ends at −1098, 144 nucleotides upstream of the mature 5′ end of *cob* mRNA. Other precursors, such as the primary transcript and the RNA generated by cleavage 5′ of tRNA^{glu}, were not observed. Thus, tRNA^{glu} is processed extremely rapidly, perhaps even cotranscriptionally. In contrast to undetectable levels of primary transcripts containing the tRNA, the −1098 precursors are present at 50% of the level of transcripts processed at −954.

Limitations of primer extension analysis include the possibility that the reverse transcriptase is unable to melt the higher order structure of the tRNA, and thus the −1098 cDNA could represent not only the −1098 RNA, but the −1170 and the −1566 RNAs as well. If a large percentage of the −1098 cDNA were composed of cDNA from the −1170 and −1566 precursor RNAs, then a proportional decrease in the levels of tRNA^{glu} in the temperature-sensitive strains should be detectable by Northern blot analysis. Little difference in the levels of tRNA^{glu} between the temperature-sensitive and wild-type strains was detected (data not shown).

CBP1 affects the stability of the −1098 *cob* precursor RNAs and unspliced precursor RNAs: Temperature-sensitive *cbp1* strains of *S. cerevisiae* were isolated that affect *cob* mRNA expression at high temperatures. As determined by quantitative primer extension analysis, we found that the stability of the −1098 precursor RNAs was CBP1-dependent in these strains. In the temperature-sensitive *cbp1* strains, the level of precursor was approximately 5% (36°) to 14% (24°) of that expressed in *CBP1*$^+$ wild-type strains, a level similar to that in a *cbp1* deletion strain (Table 3). The values for the deletion strain are similar to those observed previously in a *cbp1* insertion null mutant strain (MITTELMEIER and DIECKMANN 1993). Northern blot analyses of mitochondrial RNA revealed that splicing intermediates were reduced in the temperature-sensitive strain RSY20 when compared to the wild-type strain, RSY1000. The 4.3-kb (bI4,bI5) and the 3.6-kb (bI4) intermediates were less than 15% (Table 4) of the corresponding splicing intermediates in the wild-type strain. Furthermore, the 2.9-kb (bI5) intermediate was undetectable. Similar to previously characterized null point mutants (DIECKMANN, KOERNER and TZAGOLOFF 1984; R. R. STAPLES and C. L. DIECKMANN, unpublished data), the *cbp1* deletion strain RSY2000 contained no measurable splicing intermediates. Thus, the effect of temperature-sensitive *cbp1* mutations on the level of *cob* splicing intermediates is less severe than that observed in *cbp1* null strains. As illustrated in Figure 7, we propose that CBP1 protects *cob* transcripts that contain introns and/or that have unprocessed 5′ ends by binding to the RNAs.

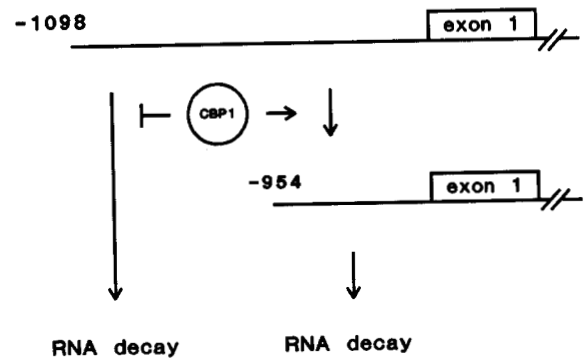

FIGURE 7.—Model of CBP1 activity. We propose that CBP1 interacts either directly or indirectly with the −1098 *cob* precursor RNAs. This interaction inhibits RNA decay of the precursors (⊢) and promotes processing of the RNA (→) to generate a mature *cob* mRNA with a 5′ end at −954.

CBP1 is necessary for production of *cob* mRNA: *cob* precursor levels are reduced 2- to 7-fold when cells are grown at the restrictive temperature rather than at the permissive temperature, but the level of mature mRNA decreases at least 10- to 50-fold. The simplest hypothesis to explain these data is that CBP1 interacts with precursor RNAs to promote the processing event that generates the mature 5′ end of *cob* mRNA (Figure 7). By this model, at 24°, mutant CBP1 can interact with the −1098 precursor RNAs but with a low affinity, and mature *cob* RNAs are produced at low levels. At 36°, mutant CBP1 may have no affinity for the precursors, and, as a result, mature *cob* mRNA is never produced or produced at levels below the limit of detection.

Evidence in support of a role in processing of *cob* precursor RNA by CBP1 comes from the precursor/mRNA ratios in the temperature-sensitive *cbp1* strains. A prediction of this model is that the ratio of precursor RNAs/mRNA (P/M ratio) in the temperature-sensitive *cbp1* strains should change. The P/M ratio has been shown previously (PIKIELNY and ROSBASH 1985; RYMOND *et al.* 1990) to be a sensitive assay for measuring the rate of processing in mutations that affect splicing. The wild-type P/M ratio was 0.5 at 24° on YPD (Table 3). For five of the temperature-sensitive strains, the P/M ratio ranged from 0.3–0.7 at 24° when the strains were grown on glucose. The exception was RSY20, which had a P/M ratio of 1.9. At 36°, mature mRNA was measurable only in RSY1000 (wild type) and RSY7 where the P/M ratio was 0.85 and 6.8, respectively. In all other temperature-sensitive strains, the level of −954 RNA was below the level of detection (<0.5%). Using 0.5% as the upper

limit, the P/M ratios of the five other mutants ranged from 3.4–12. That the ratio increased in RSY20 at 24° and in all strains at 36° supports the hypothesis that CBP1 is involved in the cleavage at −954.

The model as shown in Figure 7 assumes that the steady-state level of precursor RNA is affected by three rates: (1) the rate of synthesis, (2) the rate of processing at −954, and (3) the rate of decay. In the absence of CBP1 we assume that the rate of synthesis is unchanged, the rate of processing is nil, and the rate of decay is faster than in the presence of CBP1. The change in rates of processing and decay in the mutant strains leads to the observed reduced level of *cob* precursor RNA and complete absence of mature *cob* mRNA.

We cannot rule out that in addition to its role in precursor protection and cleavage, CBP1 may be required for the stability of mature *cob* mRNA following cleavage. Since *cob* mRNA is not present at 36°, we have no method of assessing whether CBP1 remains associated with the RNA following cleavage. One might hypothesize that cleavage at −954 allows a stable secondary structure to form whereby *cob* mRNA is now resistant to nucleases. Similar to this latter hypothesis, the stability of some *E. coli* RNAs is dependent on secondary structures at the 5′ end of the RNA (BELASCO and HIGGENS 1988; BOUVET and BELASCO 1992; EMORY, BOUVET and BELASCO 1992).

Our data do not rule out the alternative hypothesis that CBP1 stabilizes both the precursor RNA and the mature *cob* mRNA, and has no role in processing of *cob* pre-mRNA. For the data to be explained by this model, in wild-type strains the affinity of CBP1 for the mature mRNA must be stronger than the affinity for precursor RNA because the temperature-sensitive mutations nearly destroy CBP1-precursor interaction at the permissive temperature. However, the mutations completely destroy the CBP1-mRNA interaction only at the restrictive temperature. Not only must we invoke different binding affinities for CBP1 to the two RNAs, but we must also invoke different inherent stabilities of precursor RNAs and mature RNA, since in the absence of CBP1, precursor RNAs are much more abundant than mature mRNA.

***cob* mRNA is not rate-limiting for the expression of cytochrome *b* in the temperature-sensitive strains:** Even though *cob* mRNA levels in the temperature-sensitive strains were reduced to 10–30% of that expressed in wild-type strains, the strains grew in glycerol at 24° at rates similar to wild-type. Thus, a 3- to 10-fold decrease in the the level of *cob* mRNA is not rate limiting for growth at this temperature. Either translation or stability of cytochrome *b* protein may be up-regulated in the temperature-sensitive mutants to compensate for the mRNA decrease; alternatively, lower cytochrome *b* protein levels may not be rate limiting for growth. Future experiments can address this issue by quantitating cytochrome *b* levels relative to total cellular protein in the mutant strains.

In summary, we have characterized new temperature-sensitive strains of *cbp1* in which the stability of 5′ unprocessed *cob* transcripts is decreased at all temperatures. At the restrictive temperature, there are no 5′ processed *cob* transcripts. These data are suggestive that CBP1 promotes the stability and 5′ processing of *cob* precursor RNAs.

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