CBT1* Interacts Genetically With *CBP1* and the Mitochondrially Encoded Cytochrome *b* Gene and Is Required to Stabilize the Mature Cytochrome *b* mRNA of *Saccharomyces cerevisiae

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ABSTRACT

Mutation of a CCG sequence in the 5'-untranslated region of the mitochondrially encoded cytochrome *b* mRNA in *Saccharomyces cerevisiae* results in destabilization of the message and respiratory deficiency of the mutant strain. This phenotype mimics that of a mutation in the nuclear *CBP1* gene. Here it is shown that overexpression of the nuclear *CBT1* gene, due to a transposon insertion in the 5'-untranslated region, rescues the respiratory defects resulting from mutating the CCG sequence to Δ CG. Overexpressing alleles of *CBT1* are allelic to *soc1*, a previously isolated suppressor of *cbp1^{ts}*-induced temperature sensitivity of respiratory growth. Quantitative primer extension analysis indicated that *cbt1* null strains have defects in 5'-end processing of precursor cytochrome *b* mRNA to the mature form. Cbt1p is also required for stabilizing the mature cytochrome *b* mRNA after 5' processing.

OXIDATIVE phosphorylation is carried out by enzyme complexes located in the inner mitochondrial membrane. The formation of these complexes requires the concerted participation of genes encoded in both the nucleus and the mitochondrion. The mitochondrial DNA of the yeast *Saccharomyces cerevisiae* encodes seven subunits of the various complexes directly involved in oxidative phosphorylation. The rest of the subunits are encoded in the nucleus. Nuclear gene products are also involved in many other processes of organellar biogenesis including transcription, stabilization, processing, and translation of mitochondrial RNAs. Other factors affect import of nuclear-encoded proteins into the mitochondrion and assembly of the respiratory complexes and the mitochondrial H⁺-translocating ATP synthase. The subset of nuclear genes required for respiratory functions is known as *PET* genes (COSTANZO and FOX 1990; TZAGLOFF and DIECKMANN 1990; DIECKMANN and STAPLES 1994; GRIVELL *et al.* 1999).

CBP1 is a *PET* gene required for stabilization of mitochondrial cytochrome *b* (*COB*) RNAs (DIECKMANN *et al.* 1982, 1984a,b). *COB* mRNA is transcribed as a unit with the upstream *tRNA^{gln}* (BONITZ *et al.* 1982; CHRISTIANSON *et al.* 1983). Processing of *tRNA^{gln}* from the initial transcript by RNase P and tRNA 3' endonuclease

(HOLLINGSWORTH and MARTIN 1986; CHEN and MARTIN 1988) releases the tRNA and precursor *COB* mRNA, which has a 5' end at -1098 (relative to +1 of the initiating AUG). The precursor RNAs are processed to mature mRNAs with 5' ends at -954 or -955. In *cbp1* mutant strains, *tRNA^{gln}* levels are near wild type, whereas precursor *COB* mRNA is reduced to 25% of wild type and mature *COB* mRNA is undetectable (MITTELMEIER and DIECKMANN 1993; CHEN and DIECKMANN 1994).

Previous deletion studies delineated a 64-nucleotide region of the *COB* 5'-untranslated region (UTR) from -961 to -898 that is sufficient for Cbp1p-mediated stabilization of *COB* mRNA (MITTELMEIER and DIECKMANN 1993). This sufficiency element encompasses the mapped positions of the mature 5' ends of *COB* mRNA at -955/-954. Within this otherwise AU-rich element, the CCG sequence from -944 to -942 is required for Cbp1p-mediated stabilization of the *COB* mRNA (CHEN and DIECKMANN 1997). Mutant strains with the central C changed to A are equivalent in phenotype to *cbp1* nulls, while mutation of either flanking nucleotide results in a temperature-sensitive respiratory defect. Several suppressors of the temperature-sensitive Δ CG and CCU mutations have been isolated and characterized (CHEN and DIECKMANN 1997; CHEN *et al.* 1999; ISLAS-OSUNA *et al.* 2003). Recovery of a dominant suppressor in *CBP1* that suppresses the Δ CG mutation supports the hypothesis that Cbp1 interacts with the CCG region of the *COB* 5'-UTR. Other suppressors include knockout mutations of *pet127*, which is involved in 5'-end maturation of several mitochondrial RNAs (WIESENBERGER and FOX 1997), a

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TABLE 1
Yeast strains used in this study

Strain	Genotype		Source
	Nuclear	Mitochondrial ^a	
LL20	α <i>leu2-3,112 his3-11,15</i>	<i>rho</i> ⁺	MAYER and DIECKMANN (1989)
iLL20	α <i>leu2-3,112 his3-11,15</i>	<i>rho</i> ^{ia}	ELLIS <i>et al.</i> (2004)
Δ CG	α <i>leu2-3,112 his3-11,15</i>	<i>rho</i> ^{ACG}	CHEN and DIECKMANN (1997)
sup-a3	α <i>leu2-3,112 his3-11,15, sup-a3</i>	<i>rho</i> ^{ACG}	CHEN <i>et al.</i> (1999)
sup-a4	α <i>leu2-3,112 his3-11,15, sup-a4</i>	<i>rho</i> ^{ACG}	CHEN <i>et al.</i> (1999)
sup-a5	α <i>leu2-3,112 his3-11,15, sup-a5</i>	<i>rho</i> ^{ACG}	CHEN <i>et al.</i> (1999)
sup-u9	α <i>leu2-3,112 his3-11,15, sup-u9</i>	<i>rho</i> ^{CCU}	CHEN <i>et al.</i> (1999)
sup-u10	α <i>leu2-3,112 his3-11,15, sup-u10</i>	<i>rho</i> ^{CCU}	CHEN <i>et al.</i> (1999)
RSY1	α <i>ade1, ura3, leu2, cbp1</i> ^{ts}	<i>rho</i> ⁺	STAPLES and DIECKMANN (1993)
RSY19	α <i>ade1, ura3, leu2, cbp1</i> ^{ts} , <i>soc1</i>	<i>rho</i> ⁺	STAPLES and DIECKMANN (1994)
RSY1000	α <i>ade1, ura3, leu2</i>	<i>rho</i> ⁺	STAPLES and DIECKMANN (1994)
Tysoc1	a <i>ade1, lys1, cbp 1-20a(ts), soc1::neo</i> ^R	<i>rho</i> ⁺	R. R. STAPLES and C. L. DIECKMANN (unpublished data)
LL20 Δ <i>cbt1</i>	α <i>leu2-3,112 his3-11,15, cbt1::HIS3</i>	<i>rho</i> ⁺	This study
iLL20 Δ <i>cbt1</i>	α <i>leu2-3,112 his3-11,15, cbt1::HIS3</i>	<i>rho</i> ⁱ	This study
TG955	α <i>leu2-3,112 his3-11,15</i>	<i>rho</i> ^{TG955}	CHEN and DIECKMANN (1994)
TG955 Δ <i>cbt1</i>	α <i>leu2-3,112 his3-11,15, cbt1::HIS3</i>	<i>rho</i> ^{TG955}	This study

^aThe *i* superscript designates the *rho*⁺ mitochondrial genome that contains no introns. The TG955 superscript designates the *rho*⁺ mitochondrial genome with a deletion from –1098 to –956 of the cytochrome *b* gene (+1 is the A of the initiating ATG).

mutation linked to *DSS1*, the nuclease component of the mitochondrial 3' to 5' degradosome (DZIEMBOWSKI *et al.* 1998), and *soc1*, an unmapped suppressor of respiratory defects in a *cbp1*^{ts} strain (STAPLES and DIECKMANN 1994). Here we show that *soc1* is allelic to overexpressing alleles of the gene *CBT1*.

A previous study showed that deletion of the *CBT1* gene affects processing and stability of the *COB* mRNA (RIEGER *et al.* 1997). Here we report that overexpression of *CBT1* suppresses the temperature-sensitive phenotype of mutations in the 5'-UTR of *COB* mRNA and can also suppress a temperature-sensitive *cbp1* mutation. We show by primer extension analysis that deletion of *CBT1* leads to a decrease in 5' processing of the *COB* mRNA precursor to the mature form. Northern blot analyses of strains lacking the precursor RNA 5' extension show that Cbt1p is required for the stability of the mature mRNA after processing.

MATERIALS AND METHODS

Yeast strains and media: *S. cerevisiae* strains and their genotypes are listed in Table 1. Media used were as follows: YEPD [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose], YEPG [1% (w/v) yeast extract, 2% (w/v) peptone, 3% (v/v) glycerol], and WO [0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, and other supplements depending on the auxotrophy of the strain]. Solid media contained 2% (w/v) agar.

Disruption of *CBT1*: The *CBT1* gene was amplified from –146 to +956 (relative to +1 of the initiating ATG), using PCR with the oligonucleotides 5' CBT1 and 3' CBT1 (see Table 2), and ligated to plasmid pGEM-T Easy (Promega, Madison, WI).

The *HIS3* gene from plasmid pUC18/*HIS3* was digested with *EcoRI* and *XhoI* and ligated to the *XhoI* and *MfeI* sites at +609 and +730 of the *CBT1* reading frame. The *HIS3* reading frame is in the opposite orientation to that of *CBT1*. This *cbt1::HIS3* construct was digested with *NotI*, the enzyme was heat inactivated at 65° for 30 min, and the digested DNA was transformed into the appropriate yeast strain as described (ELBLE 1992).

Construction of YEp351-*CBT1*: The *CBT1* gene was amplified from –146 to +1035, using PCR with the oligonucleotides 5' CBT1 and CBT1 + 1029BAMAS, which introduced a *Bam*HI restriction site at the 3' end of the PCR product (see Table 2). The PCR product was ligated to plasmid pGEM-T Easy (Promega) and sequenced to verify that there were no PCR-induced errors (Arizona Research Labs Sequencing Facility, University of Arizona). *CBT1* was removed from pGEM-T Easy and ligated to the 2 μ vector YEp351, using the restriction enzymes *PstI* and *Bam*HI.

Isolation of genomic DNA: Total yeast cellular DNA was isolated by a rapid glass-bead vortexing method as described (HOFFMAN and WINSTON 1987).

PCR amplifications: PCR amplification of the *CBT1* gene was carried out in 100- μ l reactions containing 1 μ l of yeast whole-cell DNA extract; 50 pmol of each of the relevant primers, dATP, dCTP, dGTP, and dTTP; and 2.5 units of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis) in 1 $\times$ PCR reaction buffer (10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl). Thirty cycles of amplification were performed as follows: denaturation, 30 sec (2.5 min in the first cycle) at 94°; annealing, 30 sec at 55°; and extension, 2 min at 72°. Amplification of the 5'-untranslated region of *CBT1* was performed with Pfu Turbo (Stratagene, La Jolla, CA) as per manufacturer's instructions with an annealing temperature of 60° and an extension time of 18 min.

Southern blot analysis of *CBT1* loci: Ten micrograms of total yeast cellular DNA was digested with the *EcoRI* restriction enzyme and separated on a 1% agarose gel. The DNA was

TABLE 2
Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')	Primer position ^a
5' CBT1	atattattcagccacaactatc	−146 to −125 (sense)
3' CBT1	cgctattctttaacatcacatgc	+933 to +956 (antisense)
ACT1 probe	ttcatggaagatggagccaagcgg	+953 to +977 (antisense)
CBT1 + 32AS	acctcactactatgacttcc	+32 to +13 (antisense)
CBT1 + 1029BAMAS	cgggatccctagcttgtgaatactgc	+1009 to +1029 (antisense)
TRNA-THR ^b	cgagtgcgatgccttacc	+17 to +34 (antisense) ^c
15S-pe	ccacatattattatcttcatca	+105 to +84 (antisense)
ATP6/8-pe	ttaaacattaataataaactga	−160 to −184 (antisense) ^d
Cob5B + 3	caattattattattattatatacataaa	−826 to −854 (antisense)
COX4242 ^e	gatactaaacctaaaataactataaa	+164 to +139 (antisense)
RPM1-pe	ttataaatattattcaatctaattatt	+156 to +130 (antisense)
VAR1-pe	gaaatatatatatatataatgcatc	−63 to −90 (antisense)

^a Primer positions are oriented where +1 is the first nucleotide of the coding region.

^b Primer anneals to *TRT2*, encoding a tRNA^{thr}.

^c The antisense strand in *TRT2* is equivalent to the sense strand in the *CBT1* 5'-untranslated region.

^d Coordinates relative to start codon of *ATP8*.

^e Primer anneals to *COX2*.

transferred to a Nytran membrane, which was probed with a random-priming-labeled fragment of *CBT1* from position −146 to position +956.

Isolation of whole-cell RNA: Total RNA was isolated from midlogarithmic cultures as described (CAPONIGRO *et al.* 1993) after growth in liquid YEPG medium at the permissive temperature (for primer extension experiments) or in YEPD medium (for Northern blot).

Isolation of poly(A)⁺ RNA: Poly(A)⁺ RNA was isolated using the batch protocol of the Oligotex mRNA minikit as described by the supplier (QIAGEN, Valencia, CA).

Northern analysis of poly(A)⁺ RNA: Ten micrograms of poly(A)⁺ RNAs was separated on a horizontal 1.25% (w/v) agarose gel in 1× TB buffer (83 mM Tris base, 89 mM boric acid). The RNAs were transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and probed in hybridization solution (6× SSC, 10× Denhardt's solution, 0.1% SDS, 50 µg/ml carrier DNA) at 50°. The *CBT1* probe was ³²P-end-labeled oligonucleotide CBT1 + 32AS (Table 2). The *ACT1* probe was ³²P-end-labeled oligonucleotide ACT1 probe (Table 2). Blots were analyzed on a Phosphorimager (Amersham, Arlington Heights, IL).

Isolation of mitochondria: Mitochondria were isolated according to the method described (FAYE *et al.* 1974), except that Zymolyase 20T (Seikagaku, Rockville, MD) was used instead of Glusulase to produce spheroplasts.

Isolation of mitochondrial RNA: Mitochondrial pellets were resuspended in 10 mM Tris pH 8.0. An equal volume of phenol was added, followed by incubation on ice for 5 min. The samples were centrifuged at 13,000 × *g* for 5 min and the supernatant was removed. The phenol extraction was repeated and the RNA-containing supernatant was dialyzed against distilled and deionized water overnight.

Primer extension analysis of mitochondrial mRNAs: Primer extension analyses were done as described (ISLAS-OSUNA *et al.* 2002) except annealing of the oligonucleotide to the RNA was performed at 45°. Oligonucleotides used for these analyses are shown in Table 2.

Determination of cell division times: Cells were grown on WO solid media, with selection for YEP351-*CBT1* where appropriate, and then inoculated into YEPG liquid media. At set

time points, absorbance measurements were taken with a Klett-Summerson colorimeter.

RESULTS

Suppressors of ACG, sup-a3, sup-a4, and sup-a5 have Tyl insertions upstream of the *CBT1* gene: In previous studies, a CCG triplet at position −944 to −942 of the *COB* 5'-untranslated region (where the A nucleotide of the initiating ATG is designated +1) was shown to be required for Cbp1p-mediated stabilization of *COB* mRNA (CHEN and DIECKMANN 1997). Suppressors of the temperature-sensitive respiratory growth phenotype of strains with the CCG triplet mutated to CCU or ACG have been linked (CHEN *et al.* 1999) to the *PET127* and *DSS1* genes, which encode products involved in mitochondrial RNA metabolism (WIESENBERGER and FOX 1997; DZIEMBOWSKI *et al.* 1998). However, the genes representing several suppressor linkage groups (III, IV, and V) were not characterized. As deletion of *CBT1* was shown to affect the biogenesis of *COB* mRNA (RIEGER *et al.* 1997), this gene appeared to be a good candidate for a locus described by one of the uncharacterized suppressor groups.

Our original hypothesis was that there would be loss-of-function mutations in the coding sequences of *CBT1*. Therefore, we planned to amplify the coding sequences of *CBT1* by PCR and determine if any base changes could be observed. Surprisingly, attempts to amplify the *CBT1* gene in the ACG suppressors sup-a3 (group III), sup-a4 (group III), and sup-a5 (group IV) by PCR using primers 5' CBT1 (−146) and 3' CBT1 (+956) proved unsuccessful, although the predicted 1101-bp product was seen in wild-type and other suppressor strains (data

FIGURE 1.—Analysis of the 5'-untranslated region of *CBT1*. (A) PCR of *tRNA^{thr}-CBT1* intergenic region in Δ CG suppressor and *soc1* strains. PCR amplification was carried out on whole-cell DNA with the oligonucleotides CBT1 + 32AS and TRNA-THR and analyzed on a 1% agarose gel. Verification of labeled products was done by extraction of the product from the gel and DNA sequencing. LL20 is the parental wild-type strain; Δ CG contains the temperature-sensitive Δ CG mutation in the 5'-UTR of the cytochrome *b* gene; sup-a3, sup-a4, and sup-a5 are independently isolated strains containing nuclear mutations that suppress the temperature-sensitive phenotype of the Δ CG mutation. sup-u9 and sup-u10 are uncharacterized nuclear-encoded suppressors of the CCU mutation in the 5'-UTR of *COB* mRNA (CHEN and DIECKMANN 1997); RSY1 is a *cbp1^{ts}* strain; RSY19 is a *cbp1^{ts} soc1* strain (STAPLES and DIECKMANN 1994); and Tysoc1 is a *cbp1^{ts} soc1 neoR* strain where neomycin resistance is linked to the *soc1* allele (R. R. STAPLES and C. L. DIECKMANN, unpublished data). (B) Southern analysis of *CBT1* loci in Δ CG suppressor and *soc1* strains. Ten micrograms of total cellular DNA was digested with *EcoRI* and separated on a 1% agarose gel. The DNA was transferred to a Nytran membrane and probed with a random-primed labeled *CBT1* –146 to +956 fragment. Markers were a mixture of λ -phage cut with *HindIII* and ϕ X174 cut with *HaeIII* and migration of appropriate fragments is shown. Strains are as described in (A).

not shown). Using primers to amplify the intergenic region between the upstream threonyl tRNA and *CBT1*, products several kilobases larger than the 415-bp product seen in wild-type LL20 and the Δ CG strain were observed in sup-a3, sup-a4, and sup-a5 (Figure 1A). The largest of these bands was sequenced in sup-a3, sup-a4, and sup-a5, and each contained a full Ty1 element. A predominant PCR product in the sup-a5 strain was 300 bp larger than the wild-type product and upon

sequencing it proved to contain only the long terminal repeat (LTR) of a Ty1 element. The vast majority of Ty1 insertions within the yeast genome occur near tRNAs or other genes transcribed by RNA polymerase III (KIM *et al.* 1998).

Since sup-a5 has been previously linked to *soc1*, a suppressor of a *cbp1^{ts}* allele, the intergenic region of two *soc1* mutant strains was also amplified. RSY19 harbors the original spontaneous *soc1-1* suppressor mutation (STAPLES and DIECKMANN 1994). Tysoc1 was isolated in an attempt to map the *soc1* locus with a Ty1-neomycin resistance insertion element (R. R. STAPLES and C. L. DIECKMANN, unpublished observation). As shown in Figure 1A, both *soc1* strains had large PCR products that when sequenced proved to include full Ty1 elements as well as the smaller band containing only an LTR insertion, whereas the *cbp1^{ts}* strain RSY1 had a wild-type-sized PCR product.

To further investigate the arrangement of the DNA encompassing the Ty1 insertions, genomic DNA in the threonyl tRNA-*CBT1* intergenic interval was analyzed by Southern blotting (Figure 1B). Total DNA isolated from wild-type strain LL20 yielded two *EcoRI* fragments when probed with a random-primed *CBT1* fragment (–146 to +956), one of 1730 bp (5' end of *CBT1* ORF and 5'-untranslated region), and one of 2329 bp (3' end of ORF and untranslated region). The wild-type strain LL20 and the *cbp1^{ts}* strain RSY1, as well as sup-u9 and sup-u10 (suppressors of CCU), have fragments of these sizes. The sup-a3, sup-a4, and sup-a5 strains, as well as the two *soc1* strains RSY19 and Tysoc1, have the 3' ORF 2329-bp fragment. However, instead of the 5' ORF 1730-bp fragment, two larger fragments of ~4000 and 6500 bp were observed. No smaller band was observed in sup-a5, RSY1, or Tysoc1 as might have been expected from the smaller PCR product (Figure 1A). The PCR products containing only the LTR of Ty1 are likely amplified from a minor deletion product carried in the sup-a5, RSY1, and Tysoc1 strains.

Sequencing of the PCR-amplified 5' region of *CBT1* in the Δ CG-suppressor strains and the *soc1* strains demonstrated that there were two distinct Ty1 insertion sites in these strains. Sup-a3 and sup-a4 have a Ty1 element inserted into position –124 (relative to the *CBT1* initiating ATG) and have a duplicated 5-bp sequence, CTATC. Sup-a5 and the *soc1* strains RSY19 and Tysoc1 contain Ty1 insertions at position –151 and have a duplicated GAACA sequence. In the original sorting of the suppressor strains into linkage groups (CHEN *et al.* 1999), sup-a3 and sup-a4 were in group III, while sup-a5 was in group IV and was linked to *soc1*. Subsequently, a cross of sup-a3 to *soc1* showed linkage in 39 tetrads analyzed (M. A. ISLAS-OSUNA, unpublished data). Taken together, the analysis of the *CBT1* upstream region and crosses to *soc1* suggests that sup-a3, sup-a4, and sup-a5 are all in the *soc1* linkage group.

Ty1 insertion in the 5'-UTR of *CBT1* results in over-expression: To determine whether the insertion of the

FIGURE 2.—Northern analysis of *CBT1* mRNA levels in Δ CG suppressor strains. Ten micrograms of poly(A)⁺ mRNA was separated on a 1.25% agarose gel. The RNA was transferred to a Nytran membrane and probed with end-labeled oligonucleotides homologous to *ACT1* (*ACT1* probe) and *CBT1* (*CBT1* + 32AS). After normalization with the *ACT1* control, the levels of *CBT1* mRNA in each strain were quantified relative to LL20 as follows: LL20, 100%; sup-a3, 2500%; sup-a4, 2000%; sup-a5, 1900%; and Δ CG, 130%.

Ty1 elements into the 5'-UTR had an effect on *CBT1* expression, poly(A)⁺ RNA was analyzed by Northern blot (Figure 2). *CBT1* mRNA levels in sup-a3, sup-a4, and sup-a5 were 19- to 25-fold higher than those in the wild-type LL20, as standardized to the amount of *ACT1* mRNA in each strain.

Plasmid-borne overexpression of *CBT1* suppresses the temperature-sensitive phenotype of the Δ CG strain and a *cbp1*^{ts} strain: The Northern blot data suggested that overexpression of *CBT1* is responsible for the rescue of the temperature-sensitive phenotype of the Δ CG strain. To test this idea, the Δ CG strain was transformed with the multicopy 2 μ plasmid YEp351-*CBT1*. YEp351-*CBT1* partially rescued the temperature-sensitive phenotype of the Δ CG strain (Table 3), while the control YEp351 plasmid did not. Likewise YEp351-*CBT1* suppressed the temperature-sensitive defect of a *cbp1*^{ts} strain (Table 3). Thus overexpression of *CBT1* can rescue conditional mutations in either the *COB* mRNA or *CBP1*.

TABLE 3

Generation times of Δ CG and *cbp1*^{ts} strains rescued by plasmid-borne overexpression of *CBT1*

Strain	Plasmid	Growth temperature	Generation time (hr)
LL20 ^a	None	30°	3.2
Δ CG	None	30°	>48 ^c
Δ CG	YEp351	30°	>48 ^c
Δ CG	YEp351- <i>CBT1</i>	30°	7.2
RSY1000 ^b	None	34.5°	4
RSY1 (<i>cbp1</i> ^{ts})	None	34.5°	>48 ^c
RSY1 (<i>cbp1</i> ^{ts})	YEp351	34.5°	>48 ^c
RSY1 (<i>cbp1</i> ^{ts})	YEp351- <i>CBT1</i>	34.5°	4.1

^a The parental wild-type strain of Δ CG.

^b The parental wild-type strain of RSY1.

^c No growth over the course of the experiment.

FIGURE 3.—Primer extension analysis of *COB* mRNAs in Δ cbt1 strains. Quantitative primer extension analyses were done on total RNA essentially as described (ISLAS-OSUNA *et al.* 2002) except that primer Cob5B + 3 was used in place of Cob6B. Pre-*COB*, precursor cytochrome *b* mRNA; Mat-*COB*, mature cytochrome *b* mRNA. *COX2* was used as a loading control. Quantitation of RNAs in strains with mtDNA containing introns (LL20 and LL20 Δ cbt1) relative to the mature *COB* mRNA in LL20 is as follows: mature *COB*, LL20 100%, LL20 Δ cbt1 29%; precursor *COB*, LL20 16%, LL20 Δ cbt1 39%. Quantitation of RNAs in strains containing intronless mtDNA (iLL20 and iLL20 Δ cbt1) relative to the mature *COB* mRNA in iLL20 is as follows: mature *COB*, iLL20 100%, iLL20 Δ cbt1 34%; precursor *COB*, iLL20 8%, iLL20 Δ cbt1 29%.

Deletion of *CBT1* affects 5' processing of *COB* mRNA: The product of the *CBP1* gene interacts with the 5' end of *COB* mRNA (CHEN and DIECKMANN 1997). The finding that overexpression of *CBT1* rescues mutations in either the 5' end of *COB* mRNA or *CBP1* infers that Cbt1p also acts on the 5' end of the *COB* mRNA. To test this hypothesis, *cbt1* was disrupted in LL20 and iLL20, which is isogenic to LL20 in the nucleus but contains an intronless mitochondrial genome, and the 5' end of *COB* mRNA was analyzed by quantitative primer extension analysis. In wild type, the level of precursor *COB* mRNA is ~10% of the level of mature *COB* mRNA (CHEN *et al.* 1999) (Figure 3). Similar amounts of precursor and mature RNAs are in the *cbt1* null strains and the total abundance of *COB* mRNA is lower (Figure 3). The processing defect is slightly greater in an intron-containing strain (Figure 3). Therefore, Cbt1p is involved in the 5' processing of *COB* mRNA to the mature form. A strain containing the overexpression allele of *CBT1* and wild-type mtDNA (sup-a3 ρ ⁺) showed no

effect on the *COB* mRNA levels, the ratio of mature to precursor RNAs, or growth on nonfermentable substrates (data not shown and CHEN *et al.* 1999), suggesting that overexpression of *CBT1* has a phenotype only when the Cbp1p-*COB* mRNA interaction is perturbed and that *CBT1* is not a rate-limiting step in *COB* mRNA processing or stabilization.

Deletion of *CBT1* results in accumulation of 15S and *RPM1* RNAs with unprocessed 5' ends: The defect in 5' processing of *COB* in the *cbt1* deletion strains is similar to that previously shown for *pet127* mutants (WIESENBERGER and FOX 1997), although it is not as pronounced. No detectable mature *COB* mRNA is in *pet127* strains, whereas some *COB* mRNA with mature 5' ends is in the *cbt1* deletion strains. *pet127* mutants also have defects in the processing of other mitochondrial RNAs that require 5' processing, including *VARI*, the bicistronic *ATP8/6* mRNA, 15S rRNA (WIESENBERGER and FOX 1997), and *RPM1*, encoding the RNA component of RNase P (G. WIESENBERGER, personal communication). To test whether deletions of *CBT1* also caused processing defects in these RNAs, primer extension analysis was performed with primers specific for these RNAs (Figure 4). Primer extension of the LL20 Δ *pet127* strain was included to show the positions of bands resulting from extension of precursor mRNAs (WIESENBERGER and FOX 1997). Bands larger than the mature band are unprocessed, partially processed, or artifacts resulting from premature halting of the reverse transcriptase. However, as they are all larger than the mature RNA, the relative amounts of these bands demonstrate the presence or absence of a processing defect. There was no discernable difference between *ATP8/6* mRNAs in a *cbt1* null strain and a wild-type strain. There was a significant increase in the levels of *VARI* message in the *cbt1* null strain compared to LL20 but no defect in 5'-end processing (Figure 4, Table 4). This increase in *VARI* mRNA levels is possibly the result of a compensatory mechanism that attempts to rescue respiration by increasing levels of Var1p, a mitochondrial ribosomal protein, and thus mitochondrial translation rates.

Deletion of *CBT1* had a slight effect on the ratio of unprocessed to mature 15S rRNA and a more pronounced effect on processing of the *RPM1* RNA (Figure 4, Table 4). Neither effect of Δ *cbt1* was as great as that observed in the *pet127* strain. Interestingly, both the mature 15S and *RPM1* RNAs in the *pet127* null strain were shorter than that observed in either LL20 or LL20 Δ *cbt1*. These data suggest that processing to the shorter RNA in the *pet127* mutant strain occurs via some other, normally minor, nuclease activity. Overexpression of *CBT1* in a strain with wild-type mtDNA displayed no effect on the processing of any of the RNAs tested when compared to LL20 (data not shown).

Cbt1p is required to stabilize mature cytochrome *b* mRNA: The TG955 strain contains a mitochondrial genome in which the sequence between the 3'-processing site of *tRNA^{glu}* and the 5' end of mature *COB* mRNA

FIGURE 4.—Primer extension of 15S rRNA, *RPM1* RNA, *VARI* mRNA, and *ATP8/6* mRNA in Δ *cbt1* strains. Quantitative primer extension analyses were done essentially as described (ISLAS-OSUNA *et al.* 2002) except that mitochondrial RNA was used as the template and annealing was performed at 45°. Mat, mature form of RNA. Arrows represent RNAs that are not processed to the mature form. *COX2* was used as a loading control. Quantitation of RNAs is shown in Table 4.

is deleted (CHEN and DIECKMANN 1994). Removal of *tRNA^{glu}* from the multigenic precursor by the 3' tRNA endonuclease results in the formation of a *COB* mRNA with a mature 5' end at position -955 that requires no further processing. Deletion of *CBP1* in this strain resulted in destabilization of the *COB* mRNA, demonstrating that Cbp1p is required for stabilizing the mature *COB* mRNA after processing (CHEN and DIECKMANN 1994). To determine whether Cbt1p is also required after processing for stabilization of the mature *COB* mRNA, *CBT1* was deleted in the TG955 strain. Northern analysis showed that the double TG955 Δ *cbt1* mutant had lowered

TABLE 4
Quantitation of mitochondrial RNAs in a $\Delta cbt1$ strain

RNA	% of wild-type mature RNA	
	LL20	LL20 $\Delta cbt1$
<i>ATP8/6</i>		
Mature	100	140
Precursor	5	7
<i>VAR1</i>		
Mature	100	400
Precursor 1	7	29
Precursor 2	17	34
Precursor 3	1	6
Precursor 4	1	2
Precursor 5	2	9
<i>RPM1</i>		
Mature	100	126
Precursor 1	16	104
Precursor 2	12	47
15S rRNA		
Mature	100	117
Precursor	2	11

Quantitation of bands is shown from quantitative primer extension shown in Figure 4.

levels of *COB* mRNA similar to the $\Delta cbt1$ strain (Figure 5). We hypothesize that Cbt1p, like Cbp1p, is required to stabilize the mature *COB* mRNA.

DISCUSSION

CBT1 is the third nuclear gene known to affect the 5' processing and stabilization of mitochondrial *COB*

FIGURE 5.—Northern analysis of *COB* mRNA in a $\Delta cbt1$ strain with no precursor form of *COB*. Approximately 5 μ g of mitochondrial RNA was loaded onto a 1.25% agarose gel, electrophoresed, blotted onto Nytran, and probed separately with the various probes. Blots were analyzed on a Phosphorimager. The *ATP9* signal was used as a loading control for quantitation purposes. TG955 strains have cytochrome *b* mRNA precursor sequence deleted from -1098 to -956 (where the A of the initiating ATG is $+1$). Levels of *COB* mRNA were quantified relative to LL20 as follows: LL20, 100%; LL20 $\Delta cbt1$, 16.4%; TG955, 116%; and TG955 $\Delta cbt1$, 2%.

RNAs. *PET127* is required for processing of the 5' end of the precursor RNA to the mature form. In *pet127* strains, precursor RNA accumulates to levels equivalent to the combined level of precursor and mature RNAs in the wild-type strains, and there is no processing to the shorter mRNA. *pet127* strains are also defective in 5' processing of *ATP8/6*, *VAR1* mRNAs, 15S rRNA (WIESENBERGER and Fox 1997), and the mitochondrially encoded component of RNase P, *RPM1* RNA (G. WIESENBERGER, personal communication, and Figure 4). *pet127* strains can respire except at high temperature (37°). *CBP1* is required for the stabilization of *COB* RNAs (DIECKMANN *et al.* 1982, 1984a,b; MITTELMEIER and DIECKMANN 1993; CHEN and DIECKMANN 1997). In *cbp1* strains, the precursor *COB* mRNA, in which the 5' end extends to the 3' end of *tRNA^{gln}* at position -1098 relative to the ATG at $+1$, is reduced to $\sim 10\%$ of wild-type levels. The mature mRNA, in which the 5'-UTR extends to nucleotide -954 or -955 , is undetectable. *cbp1* strains cannot respire. Cbp1p likely interacts with the 5'-UTR of *COB* RNAs to protect them from degradation (CHEN and DIECKMANN 1997).

Deletion of *CBT1* results in a decrease in the mature *COB* mRNA/precursor *COB* mRNA ratio (Figure 3). These data suggest that Cbt1p is required for efficient 5' processing of *COB* precursor RNA. In the original report describing the characterization of *CBT1* (RIEGER *et al.* 1997), Northern analysis showed that the major *COB* mRNA species in *cbt1* null strains is ~ 200 nucleotides longer than the mature *COB* mRNA in wild-type strains. The authors attributed the increase in length to a 3'-end extension on the *COB* mRNA. The primer extension analysis presented here (Figure 3) shows that most of the *COB* mRNA in *cbt1* null strains is unprocessed at the 5' end and is 143 or 144 nucleotides longer than the mature form (BONITZ *et al.* 1982). These data suggest that the size difference observed in the Northern blots is due at least partially to a 5'-end processing defect, but do not exclude the possibility that *cbt1* null strains have a defect in 3'-end processing as well. Mitochondrial 5'- and 3'-end processing factors have been shown to interact genetically. Overexpression of *PET127*, a 5'-processing factor (WIESENBERGER and Fox 1997), can suppress deletions in *SUV3* or *DSS1*, which encode the helicase and exonuclease components of the 3' to 5' mitochondrial degradosome (WEGIERSKI *et al.* 1998). Additionally, *suu3* or *dss1* deletions result in accumulation of mRNAs with extensions at both the 3' and 5' ends (DZIEMBOWSKI *et al.* 2003).

Here we show that *CBT1* overexpression from Ty1-stimulated transcription or from 2 μ plasmid transcripts suppresses a temperature-sensitive defect in the 5'-UTR of *COB* mRNA or a temperature-sensitive mutation in *CBP1*. The overexpressing suppressor cannot rescue a null CAG mutation in the 5'-UTR of *COB* (CHEN *et al.* 1999), nor can it suppress a deletion of *CBP1* (T. P. ELLIS, unpublished observation). We also show that

deletion of *CBT1* lowers *COB* mRNA levels in strains carrying either a wild-type mitochondrial genome or a mitochondrial genome in which the region between the 3' end of the *tRNA^{glu}* and the mapped 5' ends of the mature *COB* mRNAs is deleted (Figure 5). Together, these data suggest that, like Cbp1p, Cbt1p stabilizes *COB* mRNA. When overexpressed in *cbp1^{ts}* or Δ CG strains, Cbt1p could compensate for the partial loss of affinity between Cbp1p and *COB* mRNA. Such a parallel factor could be specific to *COB* mRNA or it could be a more general mitochondrial mRNA-stabilizing factor. In combination with a *cbp1^{ts}* allele, Ty1-induced overexpression of *CBT1* (*soc1*) resulted in a 1.5- to 3-fold increase in several of the different mitochondrial mRNAs (STAPLES and DIECKMANN 1994). That study supports the idea that overexpression of Cbt1p has a more general stabilizing effect on several mitochondrial mRNAs.

Alternatively, Cbt1p may be part of a processing complex with Pet127p. Loss of Cbt1p affects processing of several RNAs, similar to Pet127p. However, unlike deletions of *CBT1*, Δ *pet127* suppresses the same *COB* or *cbp1^{ts}* mutations that overexpression of *CBT1* does. This may suggest that, if both proteins are part of the same complex, Pet127p is a destructive nuclease and Cbt1p confers specificity to the complex. When Pet127p is absent there is no processing of *COB* mRNAs and when Cbt1p is absent Pet127p is less active as a specific 5'-precursor-processing enzyme but is more destructive of RNA in general. When Cbt1p is overexpressed, Pet127p degradation activity is suppressed. How both the degradation activity of Pet127p and the stabilizing function of Cbt1p promote RNA processing will require additional experimentation.

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