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Premature 3′-End Formation of *CBP1* mRNA Results in the Downregulation of Cytochrome *b* mRNA during the Induction of Respiration in *Saccharomyces cerevisiae*

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The yeast mitochondrial genome encodes only seven major components of the respiratory chain and ATP synthase; more than 200 other mitochondrial proteins are encoded by nuclear genes. Thus, assembly of functional mitochondria requires coordinate expression of nuclear and mitochondrial genes. One example of coordinate regulation is the stabilization of mitochondrial *COB* (cytochrome *b*) mRNA by Cbp1, the product of the nuclear gene *CBP1* (cytochrome *b* processing). *CBP1* produces two types of transcripts with different 3′ ends: full-length 2.2-kb transcripts and 1.2-kb transcripts truncated within the coding sequence of Cbp1. Upon induction of respiration, the steady-state level of the long transcripts decreases while that of the short transcripts increases reciprocally, an unexpected result since the product of the long transcripts is required for *COB* mRNA stability and thus for respiration. Here we have tested the hypothesis that the short transcripts, or proteins translated from the short transcripts, are also required for respiration. A protein translated from the short transcripts was not detected by Western analysis, although polysome gradient fractions were shown to contain both long and short *CBP1* transcripts. A mutant strain in which production of the short transcripts was abolished showed wild-type growth properties, indicating that the short transcripts are not required for respiration. Due to mutation of the carbon source-responsive element, the long transcript level in the mutant strain did not decrease during induction of respiration. The mutant strain had increased levels of *COB* RNA, suggestive that production of short *CBP1* transcripts is a mechanism for downregulation of the levels of long *CBP1* transcripts, Cbp1, and *COB* mRNA during the induction of respiration.

The yeast *Saccharomyces cerevisiae* is a facultative organism; it can obtain energy by either respiration or fermentation (for reviews, see references 45 and 56). Since respiration is not essential, respiratory-deficient mutant strains can be isolated and maintained on a fermentable carbon source such as glucose. Nuclear mutations describe more than 200 genes required for mitochondrial function (for a review, see reference 56). A subset of the nuclear gene products interacts with mitochondrial mRNAs to promote stability, processing, and translation (for a review, see reference 13). One example of such an interaction is that between Cbp1 and *COB* (cytochrome *b*) RNA. Cbp1 is encoded by the nuclear gene *CBP1* (cytochrome b processing) and is imported into mitochondria, where it is necessary for the production of stable *COB* mRNA (6, 12, 36, 49, 58).

The *CBP1* gene produces two types of transcripts that differ at the 3′ end (Fig. 1 and reference 32). The full-length transcripts are 2.2 kb in length; the shorter transcripts are 1.2 kb in length and have 3′ ends within the coding sequence of Cbp1. Production of the 2.2- and 1.2-kb transcripts is differentially regulated by carbon source (32, 33). When cells are switched from a fermentable carbon source (e.g., glucose) to one that requires respiration (e.g., glycerol), the steady-state level of the long transcripts decreases and that of the short transcripts increases (32). The decrease in the level of the long transcripts was unexpected since disruption of the coding sequence 3′ of the mapped ends of the short transcripts leads to respiratory deficiency (32). This respiratory deficiency is due to the truncation of full-length Cbp1, which has an apparent mature mass of 66 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gels (58). The function of short-transcript formation has remained a mystery. We hypothesized that since the level of the short transcripts increases during the induction of mitochondrial function, a product of the short transcripts or the short transcripts themselves might also be required for respiration.

Here we show that although the short *CBP1* transcripts are associated with actively translating polyribosomes, a protein translated from these transcripts could not be detected by Western blot analysis of whole-cell protein extracts. Abolition of the production of the short *CBP1* transcripts through mutagenesis of the 3′-end formation signal resulted in constitutive rather than decreased levels of long *CBP1* transcripts and a corresponding increase in the level of *COB* mRNA relative to wild-type mRNA during the induction of respiration. Therefore, production of the short transcripts appears to be a mechanism for regulating the level of long transcripts and *COB* mRNA during induction of mitochondrial function.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. Except where indicated otherwise, yeast strains were grown in the following media (all percentages for media are weight/volume): YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and WO (0.67% yeast nitrogen base without amino acids, 2% glucose) (46). The necessary amino acids, uracil, and adenine were added to WO to the suggested final concentrations (46). To repress respiration, the strains were grown to a low density in minimal salts medium (0.3% yeast extract, 0.12% NH_4SO_4, 0.07% $MgSO_4$, 0.1% KH_2PO_4, 0.05% NaCl,

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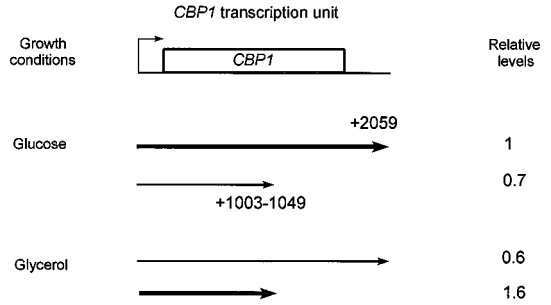

FIG. 1. Expression of the two *CBP1* transcripts is regulated by carbon source. *CBP1* produces two transcripts that share common 5′ ends but differ at the 3′ ends. The full-length transcripts are 2.2 kb long and encode a protein necessary for the accumulation of mature *COB* transcripts in mitochondria. The short transcripts are 1.2 kb long and have 3′ ends within the coding sequence of the long transcript. When wild-type yeast strains are switched from a fermentable carbon source such as glucose to a nonfermentable carbon source such as glycerol, the steady-state level of the long transcripts decreases and the steady-state level of the short transcripts increases (32).

0.04% $CaCl_2$) (40) containing 10% glucose overnight without agitation; to induce respiration, the strains were grown in minimal salts medium containing 5% glycerol with vigorous agitation. To induce transcription from the *GAL10* promoter, strains were grown in minimal salts medium containing 5% galactose. *E. coli* strains were grown in LB medium (47); ampicillin was added to a final concentration of 100 μg/ml where required. Solid media contained 2% agar.

Plasmid construction and DNA manipulation. All enzymes and restriction endonucleases were purchased from Boehringer Mannheim (Mannheim, Germany) except *Bss*HII and *Nco*I, which were purchased from New England Biolabs (Beverly, Mass.). Plasmids were constructed by using standard techniques (47). All oligonucleotides were synthesized by the Arizona Research Laboratory Biotechnology Facility (University of Arizona).

To abolish short *CBP1* transcript formation, the 180-bp 3′-end formation element (nucleotides +913 to +1092, relative to the ATG at +1) was synthesized in vitro as three pairs of complementary oligonucleotides with as many A and T residues as possible changed to C and G residues without altering the coding sequence of Cbp1. *Xho*I linkers were included on the 5′ end of the 5′ pair and the 3′ end of the 3′ pair. Each pair of oligonucleotides was ligated to appropriately restricted Bluescript KS+ (Stratagene, La Jolla, Calif.), and the inserts were sequenced with a Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio), using T3 and T7 primers. The three inserts then were removed and ligated together in Bluescript KS+ to create pMMf. Proper construction of pMMf was verified by sequence analysis. The *CBP1* insert of pMMf was excised with *Bss*HII and *Nco*I, which lie immediately adjacent to the *Xho*I sites within the mutated *CBP1* sequence. The *CBP1* fragment was ligated to the full-length *CBP1* gene in plasmid pKim3R in place of the wild-type sequence (which had a *Bss*HII site at +914 to +919 and an *Nco*I site at +1085 to +1090 added by site-directed mutagenesis). The resulting plasmid (μCH-NN/His) also contains *NUC1*, which lies immediately 3′ of *CBP1* in the genome (28), interrupted with *HIS3*$^+$ to allow for selection for integration of the *CBP1-NUC1* construct into the genome.

Transformation of yeast and *E. coli*. Yeast (16) and *E. coli* (18) strains were transformed as described previously. μCH-NN/His was integrated into the genome of S150/PL, which has a *LEU2*$^+$ insertion in *CBP1* and a wild-type copy of

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype or description	Reference or source
Strains		
Yeast		
S150-2B	*MAT***a** *ura3-52 his3*Δ *leu2-3,112 trp1-289*	
CP1L	*MAT***a** *leu2-3,112* with *LEU2* inserted at the *Pst*I site of *CBP1*	33
S150/PL	S150-2B with same disruption of *CBP1* as CP1L	This study
MMH2	S150-2B with multiple mutations between +913 and +1092 of *CBP1* (Fig. 4) and *HIS3* inserted at the *Bam*HI site of *NUC1*	This study
a70/int	*MAT***a** [*rho*$^{+(int4-35)}$][a] *ura3-52 his3-11,15 leu2-3,112*, with *HIS3* inserted at the *Bgl*II site of *CBP1*	33
yRP693	*MAT*α *ura3-52 leu2-3,112 rpb1-1*	20
E. coli RR1	F$^-$ *hsdS20* (r_B m_B) *ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* λ$^-$ *recA1 endA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 lac* [F′ *proAB lacI*^{q}ZDM15 Tn*10(tet)*]	3
Plasmids		
Yeast		
pG::−26[b]	*GAL10* promoter fused to −26 of *CBP1* (relative to ATG) in YEp52	22
pΔC3′[b]	Truncation of *CBP1* at *Cla*I in pG::−26	33
pΔGS[b]	*Bgl*II-*Sca*I deletion of *CBP1* in pG::−26	33
pΔGM[b]	*Bgl*II-*Msc*I deletion of *CBP1* in pG::−26	33
p-81CBP1	CEN plasmid containing the *GAL1* UAS directing transcription of wild-type *CBP1* starting at −81	This study
p-81MMH2	Same as p-81CBP1 except containing the MMH2 allele of *CBP1*	This study
E. coli		
Bluescript KS+	T3 and T7 RNA polymerase promoters flanking the multiple cloning site in the KS orientation, with the f1 origin in the + orientation	Stratagene
pBS+	T3 and T7 RNA polymerase promoters flank the multiple cloning site, with the f1 origin in the + orientation	Stratagene
pBS/CBP1-2	pBS+ with the 700-bp *Bam*HI-*Hin*dIII fragment of *CBP1* inserted	32
pMMf	Bluescript KS+ with a mutagenized *CBP1* insert (Fig. 4) at the *Xho*I site	This study
pKim3R	*Bam*HI-*Bgl*II fragment of the *CBP1-NUC1* region with an insertion of *HIS3*$^+$ at *Bam*HI of *NUC1*; *Bss*HII and *Nco*I sites added to *CBP1* at +914–919 and +1085–1090, respectively; fragment inserted at *Bam*HI and *Sma*I of pBS+	This study
μCH-NN/His	*Hpa*I-*Nco*I fragment of the *CBP1-NUC1* region of pKim3R containing the mutagenized *CBP1* fragment (Fig. 4), inserted between *Sma*I and *Xho*I of Bluescript KS$^+$	This study
pKS/ACT1$^+$	Bluescript KS+ containing the approximately 600-bp *Cla*I fragment of *ACT1*	49
pKS/Glu	Insertion of the *Bam*HI-*Taq*I fragment (−1350 to −1115 relative to *COB* ATG at +1) of the *COB-tRNA*^{Glu} cotranscribed region between the *Bam*HI and *Cla*I sites of Bluescript KS+	This study
pKS/COB-BT	Bluescript KS+ containing a PCR-amplified fragment of *COB* (−324 to +105) at the *Eco*RI site	This study

[a] *rho*$^{+(int4-35)}$ is a rearrangement of the mitochondrial genome such that *COB* mRNA stability is no longer dependent on Cbp1 (11).
[b] In strain a70/int. Strain and plasmid names are identical except that plasmid names begin with "p."

NUC1, by a one-step gene replacement as follows: 200 μg of plasmid was digested with *Apa*I and *Xba*I to liberate the *CBP1-NUC1* construct, phenol extracted, chloroform extracted, ethanol precipitated, and transformed directly into S150/PL. The cells were plated onto WO plates supplemented with leucine, tryptophan, and uracil to select for His^+ transformants. His^+ transformants were replica plated onto WO plates supplemented with uracil and tryptophan to ensure that they were Leu^- and were also replica plated onto YPG plates to screen for respiratory competency. MMH2 was one of many His^+ Leu^- transformants, all of which were respiratory competent.

Polysome gradients. Polyribosomes were fractionated by sucrose gradient centrifugation as described previously (1). The protein concentrations of the fractions obtained following centrifugation were determined by measurement of absorbance at 280 nm. RNA was isolated from each fraction as described previously (2).

Protein isolation and Western blot analysis. Whole-cell protein extracts were prepared by growing yeast strains to stationary phase in minimal salts medium containing 5% galactose. The cells were washed with water and vortexed for 2 min in 0.1 ml of 20% (vol/vol) trichloroacetic acid and 0.1 ml of 425- to 600-μm-diameter glass beads as described previously (31). The suspended precipitate was removed, and 100 μg of protein, as determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.), was pelleted and resuspended in 5 μl of 5× Laemmli sample buffer (0.25 M Tris Cl [pH 6.8], 40% glycerol, 20% β-mercaptoethanol, 0.024% bromphenol blue, 8% SDS)–3 μl of 1 M Tris base–10 μl of H_2O. The samples were heated for 3 min at 100°C, and half of each sample was separated by electrophoresis on duplicate SDS–10% polyacrylamide gels (27). One gel was stained with Coomassie brilliant blue, and the other was electroblotted onto nitrocellulose as described previously (58). Western blot analysis was carried out with the anti-Cbp1 polyclonal antiserum as described previously (58).

RNA isolation and Northern blot analysis. Total RNA from aliquots of strains MMH2 and S150-2B frozen at each time point of the induction was isolated by the hot phenol method as described previously (25), with several modifications. The aliquots were resuspended in 25 ml of buffer A (50 mM sodium acetate, 10 mM EDTA [pH 5.0]) per liter of culture followed by the addition of 2.5 ml of 10% (wt/vol) SDS and 25 ml of buffer A-equilibrated phenol. The samples were incubated at 65°C for 4 min with agitation and occasional vortexing to keep the phases mixed. The phases were separated by a 3-min centrifugation at 3,600 × *g*, and the aqueous phases were removed to new tubes. An equal volume of phenol was added, and samples were again incubated at 65°C and centrifuged as described above. The aqueous phases were extracted with an equal volume of buffer A-equilibrated phenol-chloroform (1:1, vol/vol) followed by extraction with an equal volume of chloroform. The RNA was precipitated at −20°C for ≥1 h with 1/12.5 volume of 3 M sodium acetate and 2 volumes of 100% ethanol, washed with 70% (vol/vol) ethanol, and resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA concentration was determined by measurement of the A_{260}.

$Poly(A)^+$ RNA was enriched from total RNA by batch purification using oligo(dT)-cellulose (Pharmacia, Piscataway, N.J.). RNA samples (suspended in 1 ml of DEPC-treated H_2O) were denatured at 70°C for 5 min, placed on ice, diluted with an equal volume of 2× loading buffer (1× loading buffer is 0.02 mM Tris-Cl [pH 7.5] plus 0.5 M NaCl), added to the oligo(dT)-cellulose [prepared by washing oligo(dT)-cellulose three times with 2 ml of 1× loading buffer], and vortexed. The samples were annealed to the matrix overnight at room temperature on a Labquake Shaker (Labindustries, Inc., Berkeley, Calif.). The RNA-annealed matrices were washed three times with 5 ml of loading buffer. $Poly(A)^+$ RNA was eluted from the matrices by incubation six times on a Labquake Shaker for 0.5 to 2 h each with 2 ml of 0.01 M Tris-Cl (pH 7.5) at 42°C. The eluted fractions were pooled and precipitated with ethanol and sodium acetate at −20°C overnight. Precipitated RNA was pelleted by centrifugation at 7,100 × *g* for 30 min, washed with 70% (vol/vol) ethanol, air dried, and resuspended in DEPC-treated H_2O. RNA concentration was determined as described above.

RNA was separated by electrophoresis on vertical agarose gels (containing 10 mM methylmercuric hydroxide [Johnson Matthey Electronics, Ward Hill, Mass.] as a denaturant if indicated in the relevant figure legend). The gels were soaked in 50 mM sodium hydroxide (containing 0.1% [vol/vol] β-mercaptoethanol if the gel contained methylmercuric hydroxide), stained with ethidium bromide in TB (0.083 M Tris base, 0.089 M boric acid), and destained in TB. Northern blot analysis was performed as described previously (35) except that following hybridization with riboprobes or random-primed probes, the blots were washed for 1 h in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM trisodium citrate)–0.5% (wt/vol) SDS at 45°C and then washed for 1 h in 0.1× SSC–0.5% (wt/vol) SDS at 65°C. Blots hybridized with the scR1 oligonucleotide probe were washed in 6× SSC–0.1% SDS three times for 5 min each at room temperature followed by a 20-min wash at 45°C.

Determination of mRNA half-lives. The *rpb1-1* strain yRP693 (20) transformed with either p-81CBP1 or p-81MMH2 was used to determine the half-lives of the wild-type and mutant long *CBP1* transcripts by the transcriptional pulse-chase method as previously described (10) except that the medium used for transcription contained 2% galactose as the sole carbon source. Aliquots were harvested and frozen at 2-min intervals. Total RNA was prepared from each aliquot, and Northern analysis was performed as described above.

Preparation of probes. The *CBP1* cRNA probe pBS/CBP1-2 (32), the *ACT1* cRNA probe $pKS/ACT1^+$ (49), the *COB* cRNA probe pKS/COB-BT, and the $tRNA^{Glu}$ cRNA probe pKS/Glu were radiolabeled with [α-^{32}P]UTP (ICN, Costa Mesa, Calif.) by in vitro transcription using T3 RNA polymerase (Boehringer Mannheim) as instructed by the manufacturer. The *ATP9* sequence between positions +179 and +797 was radiolabeled with [α-^{32}P]dATP (ICN) by using a Random Primed DNA Labeling kit (Boehringer Mannheim) as instructed by the manufacturer. The scR1 RNA oligonucleotide probe (5′-GTCTAGCCGCGAG GAAGG-3′) was radiolabeled at the 5′ end with [γ-^{32}P]ATP (ICN) by using T4 polynucleotide kinase (Boehringer Mannheim) as instructed by the manufacturer. Quantitation of RNA levels was performed with a Betascope analyzer (Betagen, Waltham, Mass.) or a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The values in Tables 2 and 3 were derived by subtracting the background level from the signal for each transcript and then dividing the corrected value by the level of scR1 RNA (Table 3) or *ACT1* mRNA (Table 2) for each lane of the gel. Transcript levels at the 0-h time point in S150-2B were then set to 1.00, and the remaining values were adjusted accordingly.

RESULTS

Two types of polyadenylated RNAs are produced from the *CBP1* gene template (Fig. 1 and reference 32). The full-length, 2.2-kb RNAs encode a protein, Cbp1, that is imported into mitochondria, where it is necessary for the accumulation of mature cytochrome *b* (*COB*) mRNA (6, 12, 36, 49, 58). Truncated, 1.2-kb transcripts are also produced from the *CBP1* gene, sharing 5′ ends with the full-length transcripts but having 3′ ends that map to seven different positions within a 47-bp region in the middle of the Cbp1 coding sequence (32). Since the steady-state concentration of the 1.2-kb RNAs increases when yeast cells are induced to respire, and the steady-state concentration of the 2.2-kb RNAs decreases (Fig. 1), we hypothesized that the shorter transcripts may encode a protein that is also necessary for mitochondrial function. Therefore, we first investigated whether the 2.2- and 1.2-kb transcripts are associated with polysomes.

The short *CBP1* transcripts are present on polysomes. Polysomes were prepared from G::−26, a strain that overexpresses both types of *CBP1* transcripts several hundredfold under the direction of the galactose-inducible *GAL10* upstream activation sequence (UAS) (for a review, see reference 23). The ratio of 2.2-kb to 1.2-kb *CBP1* transcripts in this strain is 1.6, as determined by Northern blot analysis (33). As seen in Fig. 2A, the A_{280} profile of a sucrose gradient separation of polysomes and ribosomes shows that polysomes were present in fractions 2 to 11.

Total RNA was extracted from each of the sucrose gradient fractions and subjected to Northern blot analysis using a probe that detects both long and short *CBP1* transcripts. Both types of *CBP1* transcripts were found in fractions 2 to 14, which include both polyribosomes and monoribosomes (Fig. 2B). As a control, the blot was subsequently hybridized with a probe for the actin (*ACT1*) mRNA without prior stripping of the *CBP1* probe (Fig. 2C). *ACT1* transcripts of 1.4 kb were apparent in all fractions displaying the 2.2- and 1.2-kb *CBP1* mRNAs. These data are suggestive that both types of *CBP1* transcripts are translation-competent mRNAs.

A protein product of the short transcript is not apparent in whole-cell extracts. Development of antisera to the amino-terminal portion of Cbp1 (58) allowed us to investigate whether both of the *CBP1* mRNAs produce stable proteins. The wild-type long and short *CBP1* transcripts should produce proteins that are 66 and 31.5 kDa, respectively, in the overexpression strain G::−26 (Fig. 3A) (58). Overexpression of wild-type *CBP1* resulted in the detection of a very abundant band at 66 kDa, which corresponds to the product of the long transcripts, whereas no band was apparent at 31.5 kDa, the predicted size of the product of the short transcripts (Fig. 3B). We estimate the limit of detection to be 1% of the signal of the 66-kDa band; thus, if a protein is made from the short transcripts, it must be a very unstable relative to the 66-kDa pro-

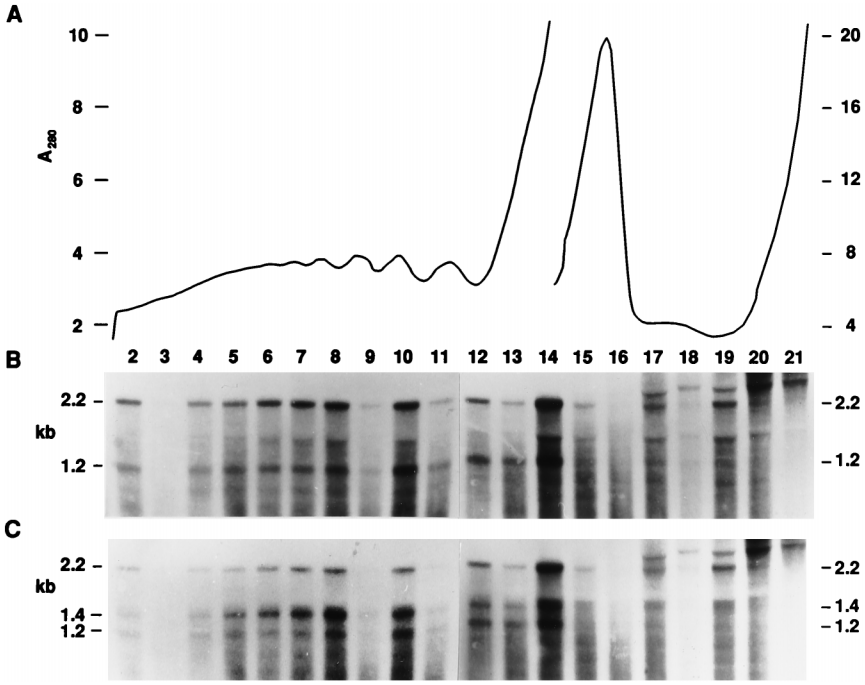

FIG. 2. Both *CBP1* transcripts are associated with actively translating polysomes. (A) Fractionation of polysomes from a strain overexpressing *CBP1* transcripts. *CBP1* transcripts were induced by growing strain G::−26 on galactose medium. Polysomes were fractionated by centrifugation on a sucrose gradient. Fractions were collected from the bottom of the tube so that the top of the gradient is at the right, and absorbance at 280 nm was measured by an in-line spectrophotometer. The break in the curve in fraction 13 represents the point where the pen went off the page, and the curve was resumed at the point in fraction 14 where the pen came back down onto the page. Units are corrected for a twofold change in scale to the right of the discontinuity. (B) Northern blot analysis of *CBP1* transcripts in polysome fractions. Total RNA was extracted from each fraction collected from the polysome gradients. Half of each sample was separated by electrophoresis on a denaturing gel, transferred to Nytran (Schleicher & Schuell, Keene, N.H.), and hybridized to a *CBP1* cRNA probe. The extra band between the two *CBP1* bands is the 18S rRNA, which hybridizes to the *CBP1* probe. (C) Without stripping the *CBP1* probe used in panel B, we subsequently probed the blot with a cRNA probe complementary to the 1.4-kb actin (*ACT1*) transcript. The specific activity of the actin probe was approximately 2% that of the *CBP1* probe.

tein. Truncation of the *CBP1* reading frame at the *Cla*I site in strain ΔC3′ disrupted the length and sequence of only the 2.2-kb transcript and resulted in the production of a 54-kDa protein with an abundance similar to that of the 66-kDa protein in strain G::−26 (Fig. 3B). The short transcripts were not interrupted by the truncation and, as in the wild-type strain, also did not produce a detectable amount of stable 31.5-kDa protein.

The addition of a stop codon in the middle of the long transcript allows short protein production. The short *CBP1* transcripts have seven different polyadenylation sites, as mapped by cDNA sequencing (33). Only one 3′ end creates an in-frame UAA stop codon by the addition of the poly(A) tail; the other six transcripts lack stop codons. To determine whether a 2.2-kb *CBP1* transcript with a stop codon located in the middle of the transcript and a poly(A) tail farther downstream would produce a stable short polypeptide of length similar to that which could be generated by the short transcripts, we examined whether short proteins were produced from two strains with frameshift mutations that introduce premature stop codons. To avoid confusion with possible translation products from the short transcripts, the 3′-end formation signal of the 1.2-kb transcript was partially deleted (ΔGS) or completely destroyed (ΔGM) when the frameshifts were created (33) (Fig. 3A). As shown in Fig. 3B, we observed a protein that was in the range expected (30.4 kDa) for the product of the interrupted long transcript in strain ΔGS, which has a stop codon four codons downstream of the *Bgl*II site at position +976 (relative to the ATG at position +1). Less of the 30.4-kDa protein was present in the ΔGS strain than either the 66-kDa protein in the wild-type G::−26 strain or the 54-kDa protein in the ΔC3′ strain. In the ΔGS strain, a very low level of short transcripts was made (5% of the total *CBP1* transcripts, compared with 39% in the wild-type strain G::−26 [33]) because the short-transcript 3′-end formation element was only partially deleted. Therefore, it is possible that the low level of short protein observed was produced from the altered short transcripts.

The results with the ΔGS strain were confirmed by those with the ΔGM strain, in which all of the short-transcript 3′-end formation signal was removed (33). The stop codon created by this frameshifting deletion is 29 codons beyond the *Bgl*II site. The product of this mRNA was quite visible at 31 kDa, within the range expected (32.9 kDa) (Fig. 3B). Therefore, contrary to the result with wild-type (short-transcript-containing) strains in which the short protein is not detectable, strains with a stop codon in the middle of the long transcripts produce stable polypeptides, albeit at lower abundances than those of full-length Cbp1 proteins.

Formation of the 1.2-kb *CBP1* transcript is abolished by mutagenesis. Though no stable protein was apparently produced from the wild-type 1.2-kb transcripts, we sought to determine whether undetectable levels of translation products from the short transcripts or the short transcripts themselves are necessary for respiration by eliminating the mid-gene 3′-end formation signal. A collection of random single point mutations within the previously described 180-bp regulatory element for short-transcript 3′-end formation (33) failed to eradicate short-transcript production (48). Therefore, a more intensive mutagenesis strategy was designed to abolish produc-

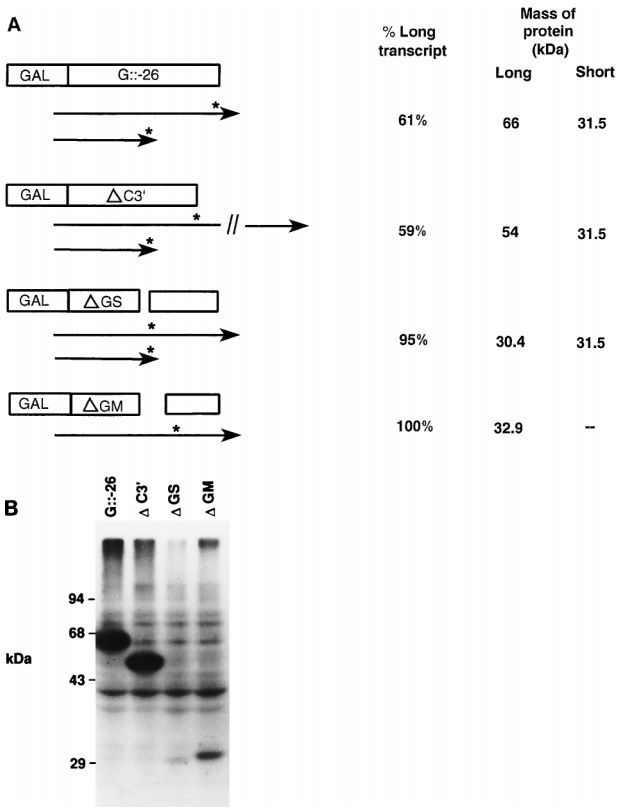

FIG. 3. The short *CBP1* transcripts do not produce detectable proteins. (A) Expected sizes of proteins translated by strains carrying plasmids with different *CBP1* overexpression constructs (33). The asterisks represent stop codons. G::−26 contains the entire *CBP1* gene under the control of the *GAL10* promoter in YEp52. ΔC3′ has a deletion of the 3′ end of *CBP1* including the 3′-end formation signal of the long transcript. A new 3′-end formation signal was provided in the plasmid (mRNA of approximately 6 kb). A stop codon follows shortly after the deletion. ΔGS has a deletion of a *Bgl*II-*Sca*I fragment which shifts the reading frame and introduces a stop codon four codons beyond the *Bgl*II site. ΔGM has a deletion of a *Bgl*II-*Msc*I fragment. A stop codon is introduced 29 codons beyond the *Bgl*II site. "% Long transcript" refers to the percentage of total transcripts that are long. The expected sizes are based on measurements made from in vitro-translated products of *CBP1* transcripts, adjusted for approximately 2 kDa processed from the amino terminus during transport into the organelle (58). (B) Western blot analysis of whole-cell extracts of strains overexpressing *CBP1* transcripts. *CBP1* transcription was induced by growth of the strains on galactose. Fifty micrograms of protein was separated by electrophoresis on an SDS–10% polyacrylamide gel and blotted to nitrocellulose. Cbp1 was detected with a polyclonal antiserum previously described (58).

tion of the short transcripts by mutating the 3′-end formation signal without altering the coding sequence of the long transcripts. Unlike pre-mRNAs from animal cells which require the conserved hexanucleotide AAUAAA and a GU/U-rich sequence for cleavage and polyadenylation (for a review, see reference 24), yeast pre-mRNAs lack a single consensus sequence for 3′-end formation; 3′-end formation signals in yeast appear to be degenerate and redundant (for a review, see reference 17). While general A+T richness is not sufficient to direct 3′-end formation, most proposed 3′-end formation signals are A+T rich (17). Therefore, 50 A and T residues within the 180-bp regulatory element were changed to C and G residues without altering the coding sequence of the long transcripts (Fig. 4), and the altered sequence was introduced into wild-type strain S150-2B to create the mutant strain MMH2. Transformants were selected in such a way that there was no selection either for or against respiratory competency (see Materials and Methods). Replacement of the wild-type sequence with the mutant regulatory element in the genomic copy of *CBP1* was confirmed by Southern blot analysis (data not shown). The mutant strain MMH2 had a growth rate similar to that of the wild-type strain S150-2B in liquid medium containing glycerol (data not shown), which requires mitochondrial function for use as a carbon source.

It was shown previously that the level of short *CBP1* transcripts increases upon the induction of respiration of wild-type strains (32). To determine whether MMH2 produces short *CBP1* transcripts, strains MMH2 and S150-2B were induced to respire during an 8-h time course on glycerol medium after a switch from medium containing repressing levels of glucose. Poly(A)$^+$ RNA isolated from aliquots of each strain 0, 4, and 8 h after the initiation of induction was analyzed by Northern blotting with a *CBP1* cRNA probe, which can detect both 2.2- and 1.2-kb transcripts (Fig. 5). During induction of the wild-type strain S150-2B, the abundance of the long transcripts decreased while the abundance of the short transcripts increased, as had been seen previously (32). The mutant strain MMH2 produced no short transcripts under either repressing (0 h) or inducing (4 and 8 h) conditions. Moreover, the abundance of the long transcripts in strain MMH2 did not decrease upon induction of respiration (Table 2); the level of long transcripts in MMH2 remained at approximately 65 to 70% of the wild-type repressed level throughout the induction. Thus, in the absence of a short-transcript 3′-end formation signal, carbon source has no effect on long-transcript levels.

It is possible that short transcripts are made in MMH2 but are very unstable and fail to accumulate. To test this possibility, a 10-min pulse of transcription from overexpression constructs of the wild-type (p-81CBP1) and MMH2 (p-81MMH2) alleles of *CBP1* was performed. No short transcripts were produced by the MMH2 allele (Fig. 6B, lanes U and I), while the wild-

```
+913    TCT GCA CAT GAT TCC AGA ATC ATT TAT AAA    S150
        AGC GCG CAC GAC AGC AGG ATC ATC TAC AAG    MMH2

+943    GTC TTC AAG ATT TGT ACC CAT AAT AGA GCC    S150
        GTC TTC AAG ATC TGC ACC CAC AAC CGG GCC    MMH2

+973    TAT AGA TCT ATA TGT AGC GAA ATG TTT TGG    S150
        TAC CGA TCG ATC TGC AGC GAG ATG TTC TGG    MMH2

+1003   CAA TTA ACT CCA GAG TAC TAT TGT AAT AAC    S150
        CAG CTG ACC CCC GAG TAC TAC TGC AAC AAC    MMH2

+1033   CCT TTG ATA TTA CCG GCA ATT ATT GAC TTC    S150
        CCA CTG ATC CTG CCG GCC ATC ATC GAC TTC    MMH2

+1063   ATT ACA AAG CAA GAC TCT TTA ACA ATG GCC    S150
        ATC ACC AAG CAG GAC AGC CTC ACC ATG GCC    MMH2
```

FIG. 4. Sequences of the wild-type and mutagenized regulatory elements for *CBP1* short-transcript formation. *CBP1* sequence between +913 and +1092, which encompasses the 3′ ends of the short transcripts, was previously shown to be necessary and sufficient for carbon source-dependent production of the short transcripts (33). The wild-type sequence (upper rows, plain type) contains a perfect match to the bipartite signal TAG. . .TATGTA proposed to be an efficiency element of a 3′-end formation signal and another sequence with one mismatch to another efficiency element consensus sequence, TATATA (overlined) (17). Five possible positioning elements (17) (double underlined) all contain at least one mismatch to the proposed consensus sequences (TT)AAGAA(C) or AATAAA. The short transcript has seven different polyadenylation sites (boldface) (33), five of which are of the consensus type Py(A)$_n$ (where Py represents a pyrimidine) (17). The mutagenized form (lower rows, italicized) contains 50 silent changes from the wild-type sequence.

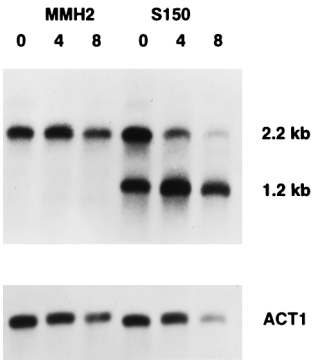

FIG. 5. Northern blot analysis reveals that the mutant strain MMH2 does not produce detectable levels of the short *CBP1* transcripts. MMH2 and S150-2B were grown overnight in medium containing 10% glucose to repress respiration. Half of each culture was frozen at −80°C (0-h time point), while the other half was resuspended in medium containing 5% glycerol and incubated with vigorous agitation to induce respiration. Aliquots were harvested and frozen at 4 and 8 h following induction. Poly(A)$^+$ RNA was prepared from each aliquot. Approximately 15 μg of poly(A)$^+$ RNA was separated by nondenaturing electrophoresis, transferred to Nytran, and hybridized to a uniformly labeled *CBP1* cRNA probe. The blot was exposed to X-ray film and then hybridized to a uniformly labeled *ACT1* cRNA probe. *CBP1* mRNA levels were normalized to those of *ACT1* mRNA (Table 2). The sizes of the bands were estimated by comparison to RNA size standards run on the same gel (not shown).

type allele made short transcripts (Fig. 6A, lanes U and I). Since MMH2 does not make short transcripts but grows at a wild-type rate on glycerol, we conclude that short-transcript production is not necessary for respiration.

We observed that long-transcript levels in MMH2 remained

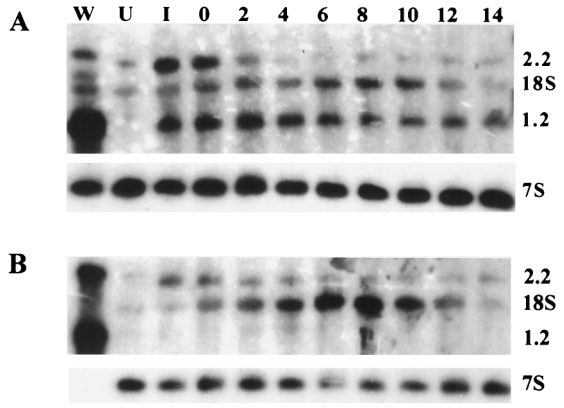

FIG. 6. A transcriptional pulse-chase assay reveals that the long transcripts produced by the wild-type and MMH2 alleles of *CBP1* are degraded at the same rate. Plasmids containing the wild-type and MMH2 alleles of *CBP1* driven by the *GAL1* UAS were transformed into a yeast strain (20) with a temperature-sensitive mutation in the largest subunit of RNA polymerase II (42). Cells were grown overnight in the neutral carbon source raffinose at the permissive temperature (uninduced sample; lanes U), and a pulse of transcription was induced by incubating the cells in galactose at the permissive temperature for 10 min (induced samples; lanes I). Transcription was repressed by the addition of glucose and a rapid shift to the restrictive temperature. Aliquots were quick-frozen at 2-min intervals after repression of transcription (numbered lanes). Approximately 5 μg of total RNA was separated on a nondenaturing gel, transferred to Nytran, and hybridized with a uniformly labeled *CBP1* cRNA probe. *CBP1* mRNA levels were quantitated on a PhosphorImager and normalized to scR1 RNA levels (the 7S RNA component of the signal recognition particle [19], labeled "7S") to account for loading differences. (A) Wild type; (B) MMH2. Lanes W contain RNA from strains transformed with p-81CBP1 (total RNA; A) or pG::−26 [poly(A)$^+$ RNA; B] grown overnight in galactose to be used as size markers for *CBP1* transcripts. This figure was made with Adobe Photoshop version 4.0.

TABLE 2. Levels of the long and short transcript of *CBP1* in wild-type and mutant strains during the induction of respiration

Strain	Transcript	Transcript level[a]		
		0 h[b]	4 h	8 h
S150-2B	Long	1.00	0.50	0.56
	Short	0.70	1.17	1.59
MMH2	Long	0.63	0.70	0.65
	Short	≤0.06[c]	≤0.06[c]	≤0.06[c]

[a] Adjusted for background and normalized to *ACT1* mRNA levels. The long transcript level at the 0-h time point in S150-2B was set to 1.00, and the remaining transcript levels were adjusted accordingly. Units are arbitrary.
[b] Time after induction of respiration.
[c] The level of detection is estimated to be 1/10 of the value of the long transcript in S150-2B at the 8-h time point, or 0.06.

at approximately 70% of the wild-type repressed level (Table 2). We assumed that the 30% decrease in long-transcript abundance in MMH2 may be due to a slight decrease in transcript half-life. To determine whether the mutations that abolished short-transcript formation altered the stability of the long transcripts in MMH2, the half-lives of the transcripts produced by the overexpression constructs p-81MMH2 and p-81CBP1 were measured during a chase of the 10-min transcriptional pulse (10) (Fig. 6, numbered lanes). The long transcripts produced by both strains have half-lives of 1 to 3 min (assayed on two blots). Thus, the mutations in MMH2 did not drastically change the stability of the mRNA, although differences in very short half-lives are difficult to measure. The decrease in long-transcript levels and the concomitant increase in short-transcript levels in the wild type could be due to a reciprocal change in the stability of the two transcripts during the switch to glycerol growth, something that we were not able to measure due to the galactose-driven pulse/glucose-shutoff chase assay. The constitutive level of long transcripts in MMH2 could be due then to mutation of a glycerol-induced instability element in the long-transcript template. We favor the simpler hypothesis that the change in abundance of the two transcripts in the wild type is due primarily to the increased use of the short-transcript 3′-end formation element during the switch to glycerol.

Abolition of the short *CBP1* transcripts allows accumulation of increased levels of *COB* RNA. It had been shown previously that Cbp1 is necessary for the accumulation of mature mitochondrial *COB* mRNA (6, 12, 36, 49). *COB* is cotranscribed from a common promoter with the upstream *tRNA*^{Glu} (7). The tRNA is released from the primary transcript by mitochondrial RNase P and tRNA 3′-endonuclease to generate *COB* pre-mRNA (5, 21, 30), which is then processed at the 5′ end to produce the stable, mature *COB* mRNA in a Cbp1-dependent manner. In strains with temperature-sensitive alleles of *CBP1*, reduced levels of *CBP1* mRNA led to a decrease in *COB* mRNA abundance (50). These data are indicative that Cbp1 is rate limiting for *COB* mRNA stability. We hypothesized that in strain MMH2, the constant level of long *CBP1* transcripts during the induction of respiration might result in an increase in the levels of Cbp1 and *COB* RNA relative to the wild type without altering the rate of *COB* transcription. To test this hypothesis, total RNA isolated from aliquots of MMH2 and S150-2B at 4-h intervals during the induction of respiration was analyzed by Northern blotting using *COB* and *tRNA*^{Glu} cRNA probes (Fig. 7). The steady-state level of *COB* RNA in MMH2 relative to that in S150-2B increased during the induction of respiration (Table 3). However, the levels of tRNA^{Glu} in the two strains remained virtually identical, indicative that the rate

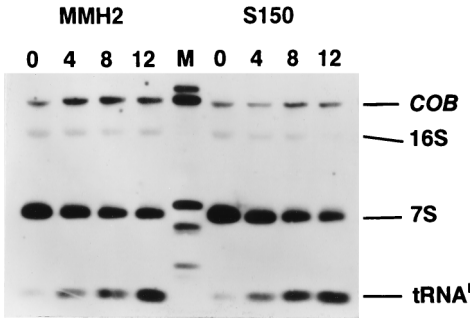

FIG. 7. Northern blot analysis reveals that *COB* RNA levels are higher in MMH2 than in S150-2B whereas $tRNA^{Glu}$ ($tRNA^{E}$) levels are similar in the two strains. Total RNA was prepared at 4-h intervals after the induction of respiration and blotted as described in the legend to Fig. 5. The blot was hybridized to uniformly labeled *COB* and $tRNA^{Glu}$ cRNA probes and exposed to X-ray film. The *COB* and $tRNA^{Glu}$ levels were quantitated on a Betascope analyzer and normalized to those of the scR1 RNA (labeled "7S"; see Table 3). The sizes of the bands were estimated by comparison to RNA size standards run in lane M.

of transcription of the $tRNA^{Glu}$-*COB* unit and the release of the tRNA from the primary transcript were unaffected by the loss of the short *CBP1* transcripts. The loss of the short *CBP1* transcripts also had no effect on the level of another mitochondrially encoded transcript, *ATP9*, which encodes subunit 9 of the mitochondrial ATPase. Thus, the loss of short *CBP1* transcript formation affects accumulation of only *COB* mRNA, which is a primary function of Cbp1 (6, 12, 36, 49). The constant level of long *CBP1* transcripts during the induction of respiration leads specifically to an increase in *COB* mRNA, indicative that the role of short-transcript formation is to decrease the level of long *CBP1* transcripts, Cbp1, and *COB* mRNA during the induction of respiration.

DISCUSSION

Identification of 3′-end formation signals in yeast has been slow because these signals appear to be redundant and highly degenerate (for a review, see reference 17). The signal consists of three parts: an efficiency element (putative consensus sequence TAG. . .TATGTA or TATATA), a positioning element [putative consensus sequence (TT)AAGAA(C), where parentheses indicate less conserved parts of the consensus sequence, or AATAAA], and the polyadenylation site itself [consensus sequence $Py(A)_n$] (17). The 180-bp segment of *CBP1* that is required for short-transcript 3′-end formation contains a perfect match to one of the putative consensus sequences for the efficiency element (TAG. . .TATGTA) and another sequence that contains one mismatch to the other efficiency sequence (TATATA) (Fig. 4). However, five possible positioning elements within the segment all contain at least one mismatch to the putative consensus sequence; four of the possible positioning elements are upstream of the perfect-match efficiency element rather than downstream, and the fifth one is downstream of five of the short-transcript polyadenylation sites. Perhaps the weakness of the possible positioning elements explains why there are seven different polyadenylation sites for the short transcripts. The aberrant location of the perfect-match putative efficiency element with respect to the putative positioning elements or the mismatch in the other putative efficiency element could result in the observed inefficient use of the short-transcript 3′-end formation element, thus allowing production of the long transcripts as well. Regardless of the exact identity of the 3′-end formation signal, this study describes an efficient way to destroy 3′-end formation simply by reducing the A+T richness of the sequence surrounding the mapped 3′ ends of transcripts.

Several yeast nuclear genes produce multiple transcripts that differ at the 3′ end. These include *GAL1* (52), *URA3* (4), *ALG7* (26), *MCM1* (14, 44), *RNA14* (34), *SIR1* (53), *AEP2*/*ATP13* (15), and *CBP1* (32). Of these, *RNA14*, *SIR1*, *AEP2*/*ATP13*, and *CBP1* produce truncated short transcripts that have 3′ ends within the coding region of the long transcripts. The best-characterized example is *CBP1*. The abundance of the two *CBP1* transcripts is regulated reciprocally by carbon source (Table 2, Fig. 5, and reference 32). When respiration is induced, the steady-state level of the long transcripts decreases while that of the short transcripts increases. Like *CBP1*, *AEP2*/*ATP13* is necessary for respiration (15), and the level of the short *AEP2*/*ATP13* transcripts increases during the induction of respiration (48). This result is suggestive that carbon source-regulated alternative 3′-end formation might be a more general phenomenon involved in the modulation of respiratory function. It has been shown that alternative 3′-end formation in the adenovirus major late transcription unit is caused by a decrease in the activity of the ubiquitous mammalian 3′-end formation factor CstF during late adenovirus infection in combination with two poly(A) sites of different strengths (29). Perhaps a similar situation exists in yeast; the activity of a component of the general 3′-end formation machinery could be regulated by carbon source.

Since the protein translated from the long *CBP1* transcripts is necessary for respiration (32), the decrease in the level of long transcripts upon switching cells to a medium that requires respiration was unexpected. The increase in abundance of short transcripts upon switching cells to glycerol medium implied that the short transcripts might also have a function that is necessary for respiration. As a first step toward elucidating the function of the short *CBP1* transcripts, we investigated whether a protein was translated from the short transcripts. Although both long and short *CBP1* transcripts were associated with polysomes, a protein translated from the short transcripts was not detected. One possible explanation for the lack of short-protein production is that six of the seven short transcripts lack a stop codon, and the UAA stop codon of the seventh transcript is formed by the addition of the poly(A) tail. It has been shown that stalled ribosomes are unable to release a nascent polypeptide in the absence of a stop codon and release factors (8, 43, 55), and the nascent polypeptide may be degraded on the ribosome. The proximity of the stop codon to the poly(A) tail in the seventh type of short transcript may also interfere with the release of the nascent protein from the ribosome and target the protein for degradation. Alternatively,

TABLE 3. Ratios of the levels of several mitochondrial RNAs in mutant strain MMH2 to those in the wild-type strain S150-2B

RNA	Level[a]			
	0 h[b]	4 h	8 h	12 h
COB	0.78	1.6	1.4	1.7
$tRNA^{Glu}$	1.0	1.2	1.1	1.1
COB/$tRNA^{Glu}$	0.73	1.2	1.4	1.7
ATP9	0.97	0.90	1.3	ND

[a] Ratio of the level of the indicated RNA in MMH2 to the level in S150-2B, adjusted for background and normalized to scR1 RNA levels. *COB*, $tRNA^{Glu}$, and *ATP9* levels at the 0-h time point in S150-2B were set to 1.00 to adjust for different probe specific activities, and the remaining values were adjusted accordingly. Values are the means of five blots (0, 4, and 8 h) or four blots (12 h), except for the *ATP9* values, which are from one blot.

[b] Time after induction of respiration. ND, not done.

a carboxyl-terminal polylysine tract, which would be translated from the poly(A) tail in the absence of a stop codon, may prevent proper folding of the protein and/or act as a signal for degradation. A third possibility is that six of the seven types of short transcripts lack 3′ untranslated regions (UTRs) since the poly(A) tail would be translated, and the 3′ UTR of the seventh type consists only of the poly(A) tail itself. The lack of a 3′ UTR or the presence of an unusual 3′ UTR may prevent efficient translation of the messages (54). However, initiation is not affected in this case since the short transcripts are associated with polysomes; also, the ratio of long to short transcripts in each of the polysome fractions is similar to that seen in total RNA. That short Cbp1 proteins are detectable in strains ΔGS and ΔGM that have stop codons in the middle of long transcripts is suggestive that restoration of a stop codon and 3′ UTR to the short transcripts would render them able to produce detectable amounts of protein. However, the short proteins of strains ΔGS and ΔGM are less abundant than the long proteins produced by strains G::−26 and ΔC3′. This result is suggestive that either the carboxyl half of Cbp1 is important for the stability of the proteins or the presence of an unusual and very long 3′ UTR affects translation adversely.

Since wild-type cells lack a detectable protein translated from the short *CBP1* transcripts, there are three possibilities for the function of the short transcripts. First, the protein may be active at levels below the limit of detection. Second, the short transcripts may have a function at the RNA level. Third, the short transcripts may have no function. Therefore, as a second step toward elucidating the function of the short transcripts, we abolished the production of the short transcripts without altering the coding sequence of the long transcripts. We have shown here that the short transcripts themselves have no function; however, abolition of short-transcript formation results in constitutive long-*CBP1* transcript production during the induction of respiration, which results in higher levels of the Cbp1 target, mitochondrial *COB* mRNA. In other words, production of the short *CBP1* transcripts is important for downregulating the levels of long *CBP1* transcripts, Cbp1, and *COB* mRNA during the induction of respiration. This result is supportive of our hypothesis that *CBP1* mRNA and Cbp1 are limiting for *COB* mRNA abundance, though Cbp1 levels cannot be accurately measured due to low abundance (58). We have not measured cytochrome *b* protein levels or ubiquinol-cytochrome *c* reductase (complex III) activity; however, neither must be rate limiting for growth of yeast on glycerol medium since the growth rate of the mutant during the induction of respiration is the same as that of the wild type. Cytochrome *b* protein that is not assembled into complex III is degraded (9). Similarly, CoxII subunits that are not assembled into cytochrome *c* oxidase complexes also are degraded (41).

Why would the cell modulate the level of *COB* mRNA downward during the period when the mass and volume of the organelle increase (45)? It is clear that transcription rates of mitochondrial operons increase during the induction of mitochondrial function, resulting in an increase in abundance of all mitochondrial RNAs (38, 39, 57). However, mitochondrial $tRNA^{Glu}$ abundance increases by as much as 16-fold whereas *COB* mRNA increases only 3- to 5-fold in the same period. As shown by our results, more than half of the difference between the $tRNA^{Glu}$ and *COB* mRNA levels can be attributed to the downregulation of *COB* mRNA stability by premature 3′-end formation of *CBP1* mRNA. This lesser increase in *COB* mRNA relative to that of $tRNA^{Glu}$ is tolerated because, as shown by our previous studies, wild-type *COB* mRNA levels on glycerol are not rate limiting for growth; in fact, up to a sixfold reduction in *COB* mRNA levels (in mutants with unstable *COB* mRNA) can be tolerated before the rate of growth on glycerol is slowed (36, 37, 49, 51). In general, perhaps increasing the ratio of rRNAs and tRNAs to mRNAs via mechanisms similar to the limitation of *COB* mRNA stability by Cbp1 could result in optimal rates of protein synthesis, and the increase in turnover of the mRNAs could provide nucleotides to the pool for the increase in transcriptional activity.

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