Cbp1 Is Required for Translation of the Mitochondrial Cytochrome b mRNA of Saccharomyces cerevisiae*

Expression of the yeast mitochondrial cytochrome b gene (COB) is controlled by at least 15 nuclear-encoded proteins. One of these proteins, Cbp1, is required for COB mRNA stability. \( \Delta \text{cbp1} \) null strains fail to accumulate mature COB mRNA and cannot respire. Since \( \Delta \text{cbp1} \) null strains lack mature COB transcripts, the hypothesis that Cbp1 also plays a role in translation of these mRNAs could not be tested previously. 5’-End trimming of precursor COB mRNA and other mitochondrial transcripts is dependent on Pet127. \( \text{pet127} \) mutants accumulate high levels of precursor COB mRNA and have no mature mRNA. \( \text{pet127} \) mutants respires well; this phenotype implies that COB precursor RNA is translated efficiently. With the expectation that a \( \Delta \text{cbp1} \Delta \text{pet127} \) strain might accumulate substantial levels of COB RNA, the double null strain was constructed and analyzed to test the hypothesis that Cbp1 is required for translation of COB RNA. The \( \Delta \text{cbp1} \Delta \text{pet127} \) strain does accumulate levels of COB precursor mRNA that are ~60% of the level of COB mRNA in the wild-type strain. However, cytochrome b protein is not synthesized, and thus the \( \Delta \text{cbp1} \Delta \text{pet127} \) strain does not respire. These results suggest that Cbp1 is required for translation of COB RNAs.

The expression of mitochondrial genes at the level of transcription, RNA processing, translation, post-translational modification, and complex assembly depends on many nuclear-encoded proteins that are synthesized in the cytoplasm and imported into mitochondria (1–3). Mutations in these nuclear genes often lead to respiratory deficiency, termed the \( \text{pet} \) phenotype because colonies are petite in size on fermentable glucose medium. To understand how mitochondrial gene expression is regulated, the function of these nuclear \( \text{PET} \) genes must first be understood.

The nuclear \( \text{PET} \) gene \( \text{CBP1} \) encodes a protein that is imported into mitochondria and is required for the stability of the mitochondrial cytochrome b (COB) mRNA (4, 5). COB mRNA is co-transcribed with the upstream tRNA\(^{60U} \). The tRNA is processed from the initial transcript by mitochondrial RNaseP and tRNA 3’-endonuclease, leaving a transcript we have called the COB precursor RNA. The precursor is further shortened at the 5’-end to produce what we have called the mature COB mRNA. In a wild-type strain, there is approximately five times more mature than precursor COB RNA. In a \( \text{cbp1} \) null strain, the mature COB mRNA is undetectable, and precursor RNA is reduced 2- to 5-fold from wild-type levels (Fig. 1). \( \text{cbp1} \) null strains are respiratory-deficient, and no apocytochrome b is synthesized, which suggests that the 5’-extension on the precursor RNA inhibits translation, the abundance of the precursor is below the threshold required for respiration (about 4% of the levels of mature mRNA in the wild-type strain), or Cbp1 is required for translation of COB RNAs.

Evidence that precursor COB RNAs can be translated has come from studies of Pet127, a nuclear-encoded protein that is localized to mitochondria, where it plays a role in RNA processing. A \( \text{pet127} \) null strain exhibits a leaky non-respiratory phenotype at 37 °C but respires well at 30 °C, the normal growth temperature for yeast. In \( \text{pet127} \) strains, 5’-end processing of COB, \( \text{VAR1} \), and \( \text{ATP8}/6 \) mRNAs and 15 S rRNA is blocked, and unprocessed COB precursor RNAs accumulate to levels equivalent to those of processed RNAs in wild-type strains (7). Since null \( \text{pet127} \) strains have no mature COB mRNA, but respire well, the 5’-unprocessed RNAs must be translated at sufficient levels to support near wild-type respiratory capability.

Additional data suggestive of a requirement for Cbp1 in COB RNA translation have come from analyses of point mutations in the COB 5’-untranslated leader. Previously, we have defined a CCG trinucleotide in the otherwise AU-rich COB RNA leader that is especially important for Cbp1-dependent accumulation of COB RNAs. This CCG is located just downstream of the 5’-end of mature COB mRNA (Fig. 1). We have hypothesized that Cbp1 interacts with COB RNAs in the region containing the CCG trinucleotide. \( \text{ACG} \) and \( \text{CCU} \) mutant strains are temperature-sensitive for respiration and have very low levels of COB mRNA whereas the \( \text{CAG} \) mutant strain is respiratory-incompetent at all temperatures and has undetectable levels of mRNA (8). \( \text{pet127} \) null mutations arose as spontaneous suppressors of the conditionally respiratory-deficient \( \text{ACG} \) and \( \text{CCU} \) mutations but were unable to suppress the \( \text{CAG} \) mutation. However, all three of the \( \text{pet127} \) mutant strains (\( \text{ACG} \), \( \text{CCU} \), \( \text{CAG} \)) accumulated similar increased levels of COB precursor RNA;\(^2 \) Thus, the accumulated COB precursor RNAs must be translated in the \( \text{pet127} \) \( \text{ACG} \) and \( \text{CCU} \) strains but not in the \( \text{CAG} \) strain. We have interpreted the respiratory-deficient phenotype of \( \text{CAG} \) as a loss of Cbp1 function in translation of COB RNAs.

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‡ The abbreviations used are: \( \text{pet} \), \( \text{pet127} \); \( \text{COB} \), cytochrome b; \( \text{COX}1 \), cytochrome c oxidase subunit I; \( \text{COX}2 \), cytochrome c oxidase subunit II; \( \text{COX}3 \), cytochrome c oxidase subunit III.

\(^{2}\) M. A. Islas-Osuna and C. L. Dieckmann, unpublished results.
Cbp1 Is Required for Translation of COB mRNA

RNA was isolated as described previously from mid-logarithmic cultures grown in YPD (11). For quantitative analysis by primer extension, 8 μg of total RNA was hybridized to 10 pmol of 32P-diradiolabeled COB “Cob68” primer (12) and COX2 “Cox4242” primer (13). The extension reactions were carried out as described previously (12) using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) except that the hybridization reaction was incubated at 47 °C for 90 min. 9 μl of each of the reaction mixtures were loaded on a 7 M urea, 6% polyacrylamide wedge sequencing gel. The signals obtained from precursor and mature COB mRNAs were quantitated using a PhosphorImager (Amersham Biosciences) and normalized to the signal from cytochrome c oxidase subunit II (COX2) transcripts in the same strain. In Δpet127 strains, COX2 levels are reduced modestly. Northern analysis done in triplicate showed that the COX2 mRNA level is 70% of wild-type in the Δpet127 strain (data not shown). COB levels in the primer extension analyses of pet127 strains were corrected for this reduction in COX2 levels.

[14]S/Methionine Labeling of Mitochondrial Gene Products—In vivo pulse-labeling of mitochondrial proteins was performed as described previously (14). The cultures were labeled with 12.5 μCi/ml L-[14]S-methionine for 2 h (Amersham Biosciences) in the presence of cycloheximide, which inhibits cytosolic protein synthesis. Translation products were fractionated by 10% SDS-PAGE, dried, and visualized by exposing the gel to film for 14 days.

RESULTS

The Double Deletion Strain Δcbp1Δpet127 Does Not Respond—Growth on media containing non-fermentable carbon sources such as glycerol is a simple and very sensitive method for measuring respiratory capability. Metabolism of glycerol requires a functional respiratory chain and ATP synthase in the mitochondrial compartment. To compare the respiration phenotype of the Δcbp1Δpet127 strain with that of the single mutant and wild-type controls, the strains were grown overnight on rich glucose liquid medium (YPD) and then serially diluted and spotted on rich glycerol plates (YPEG) and incubated at 25, 30, or 33 °C (Fig. 2). The wild-type and the Δpet127 strains grew very similarly at 30 °C on YEPG. The wild-type strain grew slightly better than the Δpet127 strain at both 25 and 33 °C. The Δcbp1 and Δcbp1Δpet127 strains did not grow on glycerol at any of the three temperatures.

The double Δcbp1Δpet127 deletion could lead to respiratory incompetence by affecting the stability, processing, and/or translation of COB transcripts. Either COB mRNA is destroyed (epistasis of the cbp1 COB RNA instability phenotype) or COB RNA accumulates in the Δcbp1Δpet127 strain in an unprocessed form (epistasis of the pet127 unprocessed COB RNA phenotype) but is not translated.

Δcbp1Δpet127 Accumulates High Levels of COB Precursor RNA—To determine whether the respiratory deficiency of the double deletion strain Δcbp1Δpet127 was a result of the instability of COB transcripts, the steady-state levels of COB precursor and mature RNAs were determined by quantitative primer extension analysis (Fig. 3). As observed previously, the respiratory-deficient Δcbp1 strain had no detectable mature COB mRNA and reduced levels of the COB precursor RNA (6). The respiratory-competent Δpet127 strain had only COB precursor RNA at a level equal to the sum of precursor and mature mRNA in the wild-type strain (128% of wild-type levels of mature COB mRNA). Like the Δpet127 single mutant, the Δcbp1Δpet127 strain had no mature COB mRNA but had substantial levels of COB precursor RNA (58% of wild-type levels of mature COB message). Slow respiratory growth on glycerol plates has been observed for strains that have as little as 4% of wild-type levels of mature COB mRNA (12). The inability of the Δcbp1Δpet127 strain to respire, despite relatively high levels of COB precursor transcripts, supports the hypothesis that Cbp1 is required for translation of COB RNAs as well as their stability.

Cytochrome b Protein Does Not Accumulate in the
Cbp1 Is Required for Translation of COB mRNA

TABLE I
Names and genotypes of yeast strains

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![Fig. 2. Growth on medium requiring respiration.](image)

The strains wild type (WT), Δcbp1, Δpet127, and Δcbp1Δpet127 were cultured in YPD media to logarithmic phase. Cells were washed, diluted in water, counted, and serially diluted. 10 μl of the dilutions were spotted on YEPG plates to yield dots with 10⁵, 10⁴, 10³, 10², and 10 cells. The plates were incubated for 5 days at 25, 30, and 33 °C.

![Fig. 3. Primer extension analysis of 5’-ends of COB transcripts.](image)

Total RNA was isolated from the strains, annealed to end-labeled COB (cob6B) and COX2 (cox422) primers simultaneously, and extended by reverse transcriptase. The corresponding positions of the 5’-ends of the COB pre-mRNA (Pre-COB), mature COB mRNA (Mat-COB), and COX2 are marked on the left. As a control for the amount of RNA loaded, COX2 (the mitochondrial gene coding for cytochrome c oxidase subunit II) was extended together with COB RNAs. The COB levels were normalized with respect to COX2 levels with the appropriate correction for differences of COX2 levels in the Δpet127 strains (see “Experimental Procedures”). The level of mature COB mRNA in the wild-type (WT) strain was set to 100%, and the level of mature and COB pre-mRNA in the mutant strains was compared with it. Numbers were obtained from the averages and standard deviations of three gels. COB precursor RNA levels are: wild type, 18.6 ± 11.6; Δcbp1, 9.1 ± 6.2; Δpet127, 128.0 ± 44.3; and Δcbp1Δpet127, 58.0 ± 6.2.

![Fig. 4. [35S] methionine-labeled mitochondrial gene products.](image)

Mitochondrial gene products in the mutant strains were labeled with [35S] methionine in the presence of cycloheximide, an inhibitor of cytoplasmic translation. Mitochondria isolated from each strain were suspended in Laemmli buffer, and the labeled proteins were separated by 10% SDS-PAGE. The positions of major mitochondrial gene products are indicated on the left, whereas the positions and sizes of protein molecular size standards are indicated on the right. WT, wild type.

Δcbp1Δpet127 Strain—To determine whether the cytochrome b gene product was present in the Δcbp1Δpet127 strain, mitochondrial gene products in the double and single mutant strains were labeled in vivo with [35S] methionine and analyzed by SDS-PAGE (Fig. 4). As expected, apocytochrome b protein was detected at robust levels in both the wild-type and Δpet127 strains (lanes 2 and 3), confirming efficient translation of COB precursor RNA in the Δpet127 strain. In contrast, no apocytochrome b was detected in the Δcbp1Δpet127 strain (Fig. 4, lane 4), suggesting that Cbp1 is required for translation of COB RNAs.

The absence of cytochrome c oxidase subunit I (Cox1) in the Δcbp1Δpet127 strain is another indicator that Cbp1 is required for translation of COB RNAs, as opposed to assembly/stability...
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of cytochrome b protein. Translation of an intron-excision maturase, encoded in the fourth intron of COB (bi4), is required for splicing of the bi4 intron and also for splicing of the fourth intron of COX1. Therefore, if bi4 maturase is not translated from the COB precursor RNA containing introns, fully spliced COX1 mRNA is not produced, and Cox1 protein cannot be translated (15).

**DISCUSSION**

Nuclear-encoded factors that promote translation of specific mitochondrial messenger RNAs have been described: for COB mRNAs (Cbs1 and Cbs2), for ATP9 mRNAs (Aep1 and Aep2), for COX1 mRNAs (Pet309), for COX2 mRNAs (Pet111), for COX3 mRNAs (Pet54, Pet494, and Pet122), and for ATP8/6 mRNAs (Nca2/Nca3) (reviewed in Ref. 3). In many cases, the deletion of the nuclear gene results in a decrease in the abundance of the mitochondrial mRNA as well as a block to initiation of translation. Here we have shown that Cbp1 falls into this class of “translation activator” proteins as it affects both stability and translation of COB RNAs. In respiratory-competent Δpet127 strains, COB precursor RNAs are translated to produce wild-type levels of cytochrome b protein. However, in Δcbp1Δpet127 strains, precursor RNAs accumulate but are not translated to produce either the COB-encoded maturases or cytochrome b.

The translation of wild-type levels of cytochrome b in the Δpet127 strain indicates that precursor RNAs with extended 5′-ends are translated as efficiently as 5′-trimmed mRNAs. All evidence to date has pointed toward an internal entry mode of translation initiation for mitochondrial mRNAs (3). For example, mitochondrial mRNAs often have quite long 5′-untranslated regions with multiple AUG sequences upstream of the bona fide start codon. The messages do not have 5′-cap structures that would be required for a cap-dependent initiation scheme similar to that for eukaryotic cytoplasmic translation (16). Especially for COX2 and COB, sites near the start codon have been delineated through mutagenesis as being required for translation initiation (17, 18). Thus, it should not be surprising that mRNAs that are extended in length at the 5′-end, many nucleotides away from the ribosome entry site, can be translated. It has been known for some years that unspliced precursor mRNAs are translated, translation of the maturases in the introns of COB and COX1 is required for subsequent excision of the introns (15).

So why do COB, ATP8/6, and VAR1 mRNAs have 5′-extensions that are shortened in a Pet127-dependent manner, whereas COX1, COX2, COX3, and ATP9 do not? COX1, COX2, COX3, and ATP9 may not be susceptible to shortening by Pet127 because they have 5′-triphosphate ends (the RNA is not processed after transcription). For example, Escherichia coli RNase E is much more active on RNAs with monophosphate 5′-ends than triphosphate ends (19). COX3 transcripts are processed by cleavage of the upstream tRNA55. This 5′-processed mRNA may not be susceptible because the 5′-end is protected by RNA secondary structure or by proteins, such as the translational activators Pet494, Pet54, and Pet122 (20). COB, ATP8/6, and VAR1 mRNAs are susceptible up to the point where protection is provided. It may be that the sequences between the long and short mRNA 5′-ends of ATP8/6 and VAR1 are dispensable as has been shown for COB (13), but these sequences have not yet disappeared over evolutionary time, or there may be some subtle necessity for these sequences that has yet to be discovered.

**COB** precursor RNAs were 2-fold higher in the Δpet127 than in the Δpet127Δcbp1 strain. This implies that Cbp1 protects against one or more degradation pathways that are not governed by Pet127, and/or the rate of transcription of COB is decreased in cbp1 mutant strains. Lower rates of COB transcription could be a specific effect of the loss of Cbp1, or they could be a general effect of the respiratory deficiency of this strain.

Does Cbp1 work together with the COB-specific translational activators Cbs1 and Cbs2 to promote translation of the RNAs? Cbp1 acts through a sequence that maps to the 5′-end of the mature COB mRNA (~961 to ~988), whereas Cbs1 and Cbs2 act through a site that maps to positions between ~232 to ~60 and ~33 to ~4 relative to the start codon at +1 (18). COB mRNA is stable in cbs1 and cbs2 mutants, but the mRNA is not translated (21). Cbp1 is found in the soluble fraction when mitochondria are sonicated in buffer lacking salt, whereas Cbs1 is firmly embedded in the membrane and Cbs2 is peripherally attached (22). The three proteins may interact at the surface of the inner membrane in a complex that includes COB mRNA. Cbp1 could become associated with the RNA during transcription, and through its affinity for the other two proteins, it could deliver the RNA to the membrane complex of Cbs1 and Cbs2, which promotes association with mitochondrial ribosomes. Testing of this model requires further genetic and biochemical experimentation.

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