Pet127 Governs a 5’ → 3’-Exonuclease Important in Maturation of Apocytochrome b mRNA in Saccharomyces cerevisiae*

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The details of mRNA maturation in Saccharomyces mitochondria are not well understood. All seven mRNAs are transcribed as part of multigenic units. The mRNAs are processed at a common 3’-dodecamer sequence, but the 5’-ends have seven different sequences. To investigate whether apocytochrome b (COB) mRNA is processed at the 5’-end from a longer precursor by an endonuclease or an exonuclease, a 64-nucleotide sequence, which is required for the protection of COB mRNA by the Cbp1 protein and is found at the 5’-end of the processed COB mRNA, was duplicated in tandem. The wild-type 64-nucleotide element functioned in either the upstream or downstream position when paired with a mutant element. In the tandem wild-type strain, the 5’-end of the mRNA was at the 5’-end of the upstream unit, demonstrating that the mRNA is processed by an exonuclease. Accumulation of precursor COB RNA in single and double element strains with a deletion of PET127 demonstrated that the encoded protein governs the 5’-exonuclease responsible for processing the precursor to the mature form.

In Saccharomyces cerevisiae, mitochondrial apocytochrome b (COB) mRNA is transcribed from the trNAglu-COB operon and is extensively processed before it reaches its mature form. Many aspects of this process have been well described; at least 15 nuclearly encoded proteins have been identified that control the metabolism of COB mRNA. As depicted in Fig. 1, the initial COB precursor is transcribed from position −1566, with the A of the AUG codon of COB defined as +1, and contains both trNAglu and COB mRNA (1, 2). Mitochondrial RNase P and a tRNA 3’-endonuclease cleave the initial precursor at positions −1170 and −1098, respectively, releasing trNAglu from the precursor mRNA (3, 4). The mature mRNA is generated by further processing of the precursor RNA at position −955 or −954 (5). This trimming step and stabilization of the mature mRNA are not completely understood; however, it is known that the nuclearly encoded protein Cbp1 specifically controls the turnover (5–9) and translation (10) of COB mRNA. In cbp1 mutant strains, the level of RNAglu is close to that of wild type, whereas precursor COB mRNA is reduced to 25% of wild-type levels and mature COB mRNA is undetectable.

The best candidate for the mitochondrial nuclease that processes the COB precursor at −954/−955 is a mitochondrial membrane-bound protein, Pet127 (11). It is a member of a large protein family that is well conserved in fungi and protists but is not present in plants or mammals (12). In pet127 strains, 5’-end processing of COB, VAR1, and ATP8/6 mRNAs, 15 S rRNA (11), and RPM1, the mitochondrial RNase P RNA (13) is blocked and unprocessed precursor RNAs accumulate to levels equivalent to the sum of processed and unprocessed RNAs in wild-type strains. These defects in processing only inhibit respiratory growth when the temperature is raised to 37 °C (11). Pet127 could itself be the RNase that trims these 5’-ends, but the protein has no RNase signature sequences or domains. Alternatively, Pet127 could modulate the activity of one or more distinct catalytic proteins that have either exonuclease or endonuclease activity.

In addition to its role in 5’-maturation of RNAs, Pet127 is implicated in the destruction of RNAs in the absence of 5’-protection. pet127 mutations were recovered as spontaneous suppressors of mutations in the 5’-UTR of COX3 (11) and COB (14) mRNA that make the mRNA unstable. COX3 precursor RNA is not trimmed at the 5’-end in a Pet127-dependent manner, so Pet127 only has a role in the turnover of COX3 mRNA, not in maturation.

Genetic studies suggest that Cbp1 binds directly to a sequence within the AU-rich COB mRNA 5’-UTR that contains a unique CCG trinucleotide (15). The proximity of the CCG trinucleotide (−944 to −942) to the 5’-end of the mature COB mRNA prompted the hypothesis that Cbp1 binding protects the mRNA from degradation by blocking a nuclease. Single-base changes in the CCG trinucleotide eliminate COB mRNA accumulation and reduce the level of precursor RNA 5-fold, a phenotype equivalent to that of cbp1 mutants, either conditionally (ACG and CCU mutations) or at all temperatures (CAG mutation). Similar to the Δcbp1 single mutant, the Δcbp1 Δpet127 strain has no mature COB mRNA, but, unlike the single mutant strain, it accumulates substantial levels of COB precursor RNA (10). Cbp1 binding to the CCG-containing seg-

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2 The abbreviations used are: COB, apocytochrome b gene; UTR, untranslated region.

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4 G. Wiesenberger, personal communication.
Figure 1. Processing of the upstream region of the tRNA^{glu}-COB transcript. The top line represents the initial transcript of the tRNA-COB operon, which contains both COB and tRNA^{glu}. tRNA^{glu} is depicted as an open circle. The hatched box denotes the COB open reading frame; the first base pair of the COB coding sequence is numbered +1. Arrows indicate 5'-mRNA processing sites at −1170, −1098, and −954/955. The 64-nucleotide element (−961 to −898) is marked as a box. Transcription begins at the COB promoter (−1566) and proceeds through tRNA^{glu} and COB sequence. Processing at the arrows generates mature tRNA^{glu} and COB pre-mRNA with a 5'-end at −1098. The pre-mRNA is processed further to generate the mature COB mRNA with a 5'-end at −954. The goal of the present study is to determine whether this second trimming step requires an endonuclease (left side) or an exonuclease (right side). Cbp1 is known to be required for the stable accumulation and processing of COB mRNA.

EXPERIMENTAL PROCEDURES

Strains and Media—The S. cerevisiae strains used in this study are listed in Table 1. Yeast strains were grown in either YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or YEPG medium (1% yeast extract, 2% peptone, 3% glycerol). When selection was necessary, WO medium (0.17% yeast nitrogen base (without amino acids or ammonium sulfate), 0.5% ammonium sulfate, 2% glucose) was used and amino acid supplements were added to suggested final concentrations (16). Escherichia coli strains were grown in Luria Bertani medium (17). Ampicillin was added to a final concentration of 50 μg/ml when it was required. Solid media contained 2% agar.

TABLE 1

| Strain | Genotype | Reference |
|--------|----------|-----------|
| LL20/rho<sup>a</sup> | [rho<sup>a</sup>]-leu2-3 leu2-112 his3-11 | (37) |
| JC3/M9410 | [rho<sup>a</sup>M9410 mit]<b>l</b> kar1 ade2 lys2 | (5) |
| MY7/mpl232L+53R | [rho<sup>a</sup>]lys1 | (7) |
| CCG(SU6F3E) | [rho<sup>a</sup>-CCI]<b>l</b> in LL20 | (6) |
| ACG | [rho<sup>a</sup>-CCC]<b>l</b> in LL20 | (15) |
| ACG-ACG | [rho<sup>a</sup>-ACG-ACG]<b>l</b> in LL20 | This study |
| ACG-CCG | [rho<sup>a</sup>-ACG-CCG]<b>l</b> in LL20 | This study |
| ACG-CCG-ACG | [rho<sup>a</sup>-ACG-CCG-ACG]<b>l</b> in LL20 | This study |
| CCGCCG | [rho<sup>a</sup>-CCG]<b>l</b> in LL20 | This study |
| CCGCCGpet127 | [rho<sup>a</sup>-CCGpet127]<b>l</b> in LL20 | This study |
| ACGCCGpet127 | [rho<sup>a</sup>-CCGpet127]<b>l</b> in LL20 | This study |
| ACGCCGpet127 | [rho<sup>a</sup>-CCGpet127]<b>l</b> in LL20 | This study |

TABLE 2

| Oligo name | 5' → 3' sequence |
|------------|-----------------|
| 1717 | CGATTTCTAATAATTAAAGTTAC |
| 1718 | CGATTTCTAATAATTAATTAA |
| 5-64BsaHI | TTTATCTACGTCATAATAATAAATA |
| 3-64BsaHI | CGGGACTGCTATAGAAATAATA |
| COB68 | AATTCTTTTATATTTATATTATTTT |
| cox4242 | GACTATATCTAAATAATTATAA |
| Pet17 5'-UTR | TCATCTTTGAGTATATCACGTC |
| Pet17 3'-UTR | AAATTTATCTAATAATTATAA |

Construction of Plasmids—COB 5'-UTR sequences from −961 to −898 containing CCG or ACG sites were amplified by PCR with oligonucleotide primers 1717 and 3–64BsaHI and separately with primers 1718 and 5–64BsaHI (Table 2). The templates for CCG and ACG PCR products were mitochondrial DNA samples from the single-site CCG and ACG strains, respectively. The fragments were digested with EcoRI and BsaHI, ligated together, and then ligated into the EcoRI site of p707H at the −1096/−707 deletion junction. p707H was made from p707A (5) by changing a second EcoRI site at position +654 of COB into a HindIII site by site-directed mutagenesis. The plasmids obtained with tandem COB 5'-UTR sequences in the sense orientation were p(707-ACG-CCG), p(707-ACG-CCG), p(707-CCG-ACG), and p(707-CCG-CCG).

Generation of Yeast Mitochondrial Mutant Strains—Yeast strain LL20/rho<sup>a</sup> was transformed by high velocity microprojectile bombardment with each of the plasmids p(707-ACG-ACG), p(707-ACG-CCG), p(707-CCG-ACG), and p(707-CCG-CCG) mixed with YEp351. Selection of Leu<sup>+</sup> nuclear transformants (YEp351) and screening for mitochondrial transformants were accomplished as previously described (5). The transformants, which are termed mpACG-ACG, mpACG-CCG, mpCCG-ACG, and mpCCG-CCG, are synthetic rho strains. To recombine the mutations in the mpCOB plasmids onto the grande mitochondrial genome, the transformants were crossed to the karyogamy-deficient strain JC3/M9410, which also has a deletion from −971 to −64 in the COB gene. The recombinant cytoductants were identified by the formation of respiration-competent diplods when mated to tester strain MY7/mp232L+53R, which carries a plasmid with mitochondrial sequence from −232 to +53 of the COB gene. The presence of the tandem constructions in the mitochondrial
genome (Fig. 2) was verified by PCR amplification and sequencing.

Generation of Δpet127 Strains—The pet127::KanMX4 fragment was amplified by PCR from the BY4741 Δpet127 strain (EUROSCARF, Frankfurt, Germany) with primers Pet127 5′-UTR and Pet127 3′-UTR and ligated into the pGEM-T Easy vector (Promega). This construct was digested with NotI, and the fragment was transformed into the yeast mitochondrial mutant strains. The transformants were selected on YEPD + Geneticin plates, and the disruptions were verified by PCR.

Growth of Strains on Glycerol Plates—The strains were cultured in liquid YEPD medium at 30 °C to logarithmic phase. Cells were counted in a hemacytometer and serially diluted with distilled water to concentrations of 10⁴, 10³, 10², and 10 cells/µl. 6-µl drops of the serial dilutions were spotted onto glycerol plates and incubated at 30 °C for 4 days. The plates were photographed at days 2 and 4 of incubation.

Primer Extension Analysis of mRNA 5′-Ends—Primers COB6B and cox4242 were end-labeled with T4 polynucleotide kinase and [γ-32P]ATP as recommended by the supplier of the enzyme (Fermentas), and primer extensions were done as previously described (15). Total cellular RNA was isolated from yeast strains after they grew to an optical density at 600 nm of 0.4 to 0.5 (18). 8 µg of total cellular RNA and 10 pmol 5′-end-labeled primers were mixed and brought to a final volume of 10 µl in annealing buffer (200 mM KCl, 10 mM Tris-HCl, pH 8.3, at 42 °C). The mixture was heated at 85 °C for 5 min and then annealed at 45 °C for 90 min. 15 µl of reaction mix (1 mM dGTP, 1 mM dCTP, 3 mM dATP, 3 mM dTTP, 2× reaction buffer, 7 units of avian myeloblastosis virus reverse transcriptase; the reaction buffer and enzyme were supplied by Promega) was added to the annealed primer-RNA mixture, the reaction mixture was incubated at 42 °C for 45 min, and the reaction was stopped by the addition of 12 µl of stop mix (95% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol). 8 µl of each reaction mixture was loaded on a 6% polyacrylamide-7 M urea sequencing gel.

RESULTS

Tandem Cbp1 Recognition Sites Are Functional—To determine whether the maturation of the 5′-end of COB mRNA requires endo- or exonuclease cleavage, microprojectile bombardment was used to construct a strain with CCG-containing wild-type Cbp1 recognition sites in tandem in the mitochondrial COB gene (Fig. 2). If an endonuclease is operative in the maturation of the 5′-end of COB mRNA, then at steady state the majority of the mRNA in the CCG-CCG strain will have 5′-ends that map to the downstream site. If an exonuclease is operative, we expect that only the upstream site of the CCG-CCG strain will be used for the maturation of COB mRNA (Fig. 3).

To determine whether the CCG element could function upstream or downstream of inserted sequences, strains were constructed in which the wild-type CCG element was arranged in tandem with the mutant ACG element (Fig. 2). If the CCG recognition site is operative in both the upstream and downstream position of the tandem arrangement, both ACG-CCG and CCG-ACG strains will be respiratory-competent. If either tandem arrangement disallows use of the CCG site, COB mRNA will be unstable and the strains will not respire. To compare the respiratory phenotype of the ACG-CCG and CCG-ACG strains with that of the wild-type CCG and CCG-CCG strains, the strains were grown overnight in rich glucose liquid medium (YEPD), serially diluted, and spotted on both rich glucose (YEPD) and glycerol plates (YPEG) and incubated at 30 °C (Fig. 4). Growth on medium containing non-fermentable carbon sources such as glycerol is a simple and sensitive method for measuring respiratory capability. Both the CCG-CCG and CCG-ACG strains grew very similarly to the wild-type CCG strain on YEPG, and they grew slightly better than the ACG-CCG strain. The ability of the ACG-CCG and CCG-ACG strains to grow on glycerol demonstrates that these strains have sufficient COB mRNA to support respiration. This observation
after 4 days, although spontaneous revertants arose in both samples as observed previously for the ACG strain (15).

COB Pre-mRNA 5′-UTR is Processed by an Exonuclease in a Pet127-dependent Manner—The 5′-end of COB mRNA was mapped by primer extension analysis in strains containing various combinations of 64-nucleotide elements containing either the active CCG or the mutant ACG trinucleotide. When strains were grown on the rich fermentable medium YEPD, there was no detectable precursor or mature COB mRNA in the ACG, ACG-ACG, and ACG-CCG strains (Fig. 5A). Because the ACG-CCG strain is respiratory-competent, it is unlikely that it has no COB mRNA but rather that the concentration is below the level of detection by this assay.

To analyze mRNA abundance and 5′-ends of the respiratory-competent strains, total RNA was isolated from the CCG, CCG-ACG, ACG-CCG, and CCG-CCG strains grown in rich non-fermentable YEPG medium. Both precursor and mature COB mRNAs were detected in all respiratory-competent strains, although total COB RNA abundance was lower in the ACG-CCG strain. The reason for reduced levels in this strain is not known, but the interpretation of 5′-end position is not affected by the reduction. The 5′-ends of both the mature and precursor COB mRNA in the CCG-CCG strain were identical to those in the CCG-ACG strain (Fig. 5, A and B), suggesting that only the upstream site defines the position of the 5′-ends of the mRNA. To verify whether the position of the 5′-end is truly defined by the most 5′-CCG element, the position of the 5′-end of COB mRNA in the ACG-CCG strain was determined. As we expected, in the ACG-CCG strain the 5′-end of the precursor COB mRNA was identical to those in the CCG-CCG and CCG-ACG strains, whereas that of the mature COB mRNA was identical to that in the CCG strain, indicating that the position of the 5′-end is indeed determined by the 5′-most CCG. These data support the hypothesis that the maturation of COB pre-mRNA is due to the action of an exonuclease that degrades the COB mRNA in the 5′- to 3′-direction until it is blocked by Cbp1.

To address the role of Pet127 in the maturation of mitochondrial COB mRNA, the nuclear gene PET127 was deleted in all six strains with single and tandem 64-nucleotide elements in the 5′-UTR of the COB gene and the 5′-ends of COB mRNAs were determined by primer extension analysis (Fig. 5C). In all Δpet127 strains, although the total amount of precursor and mature COB mRNAs did not change much, the precursor/mature ratio was inverted relative to the corresponding PET127

verifies that the CCG site is active in tandem, although the strain with the upstream CCG site grows slightly better on glycerol than the strain with the downstream site. As expected, the ACG and ACG-ACG strains did not grow on glycerol at 30 °C. The mRNA 5′-ends will have the same length as in the CCG-ACG strain, in which only the upstream site is active.

In strains containing tandem 64-nucleotide elements in both Cbp1 recognition sites are operative in strains containing tandem 64-nucleotide elements containing either the active CCG or the mutant ACG trinucleotide. When strains were grown on the rich fermentable medium YEPD, there was no detectable precursor or mature COB mRNA in the ACG, ACG-ACG, and ACG-CCG strains (Fig. 5A). Because the ACG-CCG strain is respiratory-competent, it is unlikely that it has no COB mRNA but rather that the concentration is below the level of detection by this assay.

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5′ → 3′-Exonuclease in COB mRNA Processing

Strains; there was a dramatic increase in precursor RNA and a reciprocal decrease in mature mRNA. This demonstrates that 5′-processing of COB precursor mRNA by an exonuclease largely depends on the presence of Pet127. As noted previously (13), the small amount of mature mRNA in Δpet127 strains has 5′-ends that are one or two nucleotides shorter than the equivalent PET127 strains.

DISCUSSION

The view that gene expression is controlled mainly at the level of transcription has been challenged by many findings over the last decade. Processing, modification, transport, and turnover as well as translation of mRNAs are subject to elaborate control. Among the processes that can be regulated, degradation of mRNA is of particular importance. We are beginning to gain a better understanding of mRNA degradation and its complex regulation in eukaryotic cells. Several excellent articles and reviews have been recently published describing pathways of mRNA decay and the factors and enzymes that are important in this process (19–21).

The two exoribonucleolytic mRNA decay pathways that have been described in the eukaryotic cytosol are the 5′ → 3′-Xrn1 pathway and the exosome-mediated 3′ → 5′-pathway. The relative contribution of each mechanism to overall mRNA turnover remains a subject of debate. In the 5′ → 3′-pathway, the first step is the removal of the mRNA 5′-cap structure, followed by 5′ → 3′-directed degradation of the mRNA body by the Xrn1 exoribonuclease. Xrn1 is a member of the 5PX exoribonuclease family along with another protein Rat1 (22). These two proteins are homologs and functionally interchangeable, but Rat1 is localized to the nucleus and is required for nuclear 5′ → 3′-directed decay (23, 24).

In E. coli, degradation of mRNA can follow several pathways, but these are very different from those in eukaryotic cells and they are mediated by the combined action of endo- and exoribonucleases. In prokaryotes, degradation of mRNA often exhibits a net 5′ → 3′-directionality. Most bacterial transcripts are degraded in a pathway initiated by RNase E. RNase E is a 5′-end-dependent endonuclease (25), the major component of the degradosome. In addition to RNase E, the bacterial degradosome includes the degradative PNPase and the RNA unwinding DEAD box RNA helicase B, RhhB. The complex also contains enolase as an integral component, which has an unknown function in the degradation process (26, 27). RNase E-dependent degradation begins with an endonucleolytic cleavage, often near the 5′-end of the mRNA, followed by successive endonucleolytic cleavages along the body of the mRNA. Each fragment is then degraded by the action of PNPase and other exonucleases in the 3′ → 5′-direction (28). In addition to the RNase E-dependent pathway, a 5′ → 3′-exonuclease was identified recently (29).

How is mRNA turnover regulated in mitochondria? Despite the monophyletic, α-proteobacterial origin of mitochondria and the conservation of most of their biological roles, recent data have revealed that mitochondrial genome organization and gene expression are extraordinarily divergent among eukaryotes (30). Similarly, the regulation of mitochondrial mRNA turnover in diverse species is surprisingly different (20). One enzymatic activity that affects turnover in all systems is 3′ → 5′-exoribonuclease activity.

The mitochondrial degradosome (mtEXO) is the main 3′ → 5′-exoribonuclease in yeast mitochondria. The degradosome is composed of two large subunits: an RNase and an RNA helicase encoded by nuclear genes DSSI and SUV3, respectively (31). Lack of Suv3 or Dss1 causes an increase in excised intron stability, a resulting inhibition of splicing, strong inhibition of mitochondrial translation, respiratory incompetence, and finally, loss of mitochondrial genomes (32). In addition to the effect on turnover, suv3 or dss1 mutations affect 5′- and 3′-processing of mitochondrial RNAs (33). This suggests that turnover and processing of mitochondrial transcripts may be linked.

To date, there is only one protein that is known to be involved in a 5′-end-dependent degradation pathway in mitochondria, Pet127. Nuclear mutations in PET127 suppress mutations and deletions in the 5′-UTR of COX3 mRNA (11) as well as in COB mRNA (14) that make the mRNA unstable. Pet127 is necessary for the efficient 5′-end processing of several precursor transcripts in a 5′-end-dependent manner, including COB mRNA (11, 13). The 5′-ends trimmed in a Pet127-dependent manner have no similar common sequence or structure. Our model for the turnover of COB pre-mRNA is that the 5′-UTR of COB mRNA is trimmed in a Pet127-dependent manner until it is obstructed by Cbp1, a protein that exclusively stabilizes COB mRNA. When the interaction between the mRNA and Cbp1 is attenuated (e.g. by mutation in the mRNA or in the protein), the mRNA continues to be degraded in the 5′ → 3′-direction. Our results clearly support the hypothesis that the maturation and decay of COB pre-mRNA are due to the action of a 5′ → 3′-exonuclease. Although this type of mechanism is similar to the mechanism of mRNA decay observed in the cytosol and nucleus of eukaryotic cells, it is the first 5′ → 3′-exoribonucleolytic activity observed to date in mitochondria.

Knocking out components of either the 3′ → 5′ or the 5′ → 3′-cytosolic degradation pathway in yeast has minimal effect on...
the transcriptome, which implies redundancy (34, 35). Interestingly, the deletion of either Suv3 or Dss1 can be suppressed by overexpression of Pet127 (36), suggesting that mRNA degradation in the mitochondrial compartment of yeast also proceeds by redundant pathways.

Although proteins homologous to Pet127 are very well conserved within lower eukaryotes, no orthologous proteins are detectable in higher eukaryotes. This raises the possibility that the mechanisms responsible for mitochondrial mRNA turnover in yeast differ from those of higher eukaryotes.

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