CBP7 codes for a co-factor required in conjunction with a mitochondrial maturase for splicing of its cognate intervening sequence

Ivor Muroff and Alexander Tzagoloff

Department of Biological Sciences, Columbia University, Fairchild Center, New York, NY 10027, USA

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Maturases are required for the processing of their cognate intervening sequences in the mitochondrial cytochrome *b* pre-mRNA. In this paper we characterize a nuclear gene, *CBP7*, already known to be required for the translation of the cytochrome *b* transcript; we present further evidence that it is also required as a co-factor in conjunction with the maturases for the efficient processing of their intervening sequences. Results are based on Northern blot analyses which show that in *cbp7* mutants with the short cytochrome *b* gene the maturase encoding intervening sequence fails to be excised although, at the same time, at least wild-type levels of the maturase are present as indicated by antibodies specific to it in Western blot analyses.

Key words: *CBP7* gene/cytochrome *b* gene/intron processing/mRNA maturase antibodies/yeast

Introduction

The mitochondrial apocytochrome *b* gene (*cob*) in *Saccharomyces cerevisiae* is distinctive by its mosaic structure with maturase encoding introns (Lazowska *et al.*, 1980). The first intron of the short *cob* gene (three exons and two introns) (Nobrega and Tzagoloff, 1980; Bonitz *et al.*, 1982) has a long open reading frame which codes for a maturase necessary for splicing its cognate intervening sequence (Lazowska *et al.*, 1980). The second, third and fourth introns of the long *cob* gene (in which the first exon is splintered so that the gene has six exons and five introns) (Dujon 1981; Pillar *et al.*, 1983b) are the other introns endowed with maturase encoding reading frames (Lazowska *et al.*, 1980; DeLaSalle *et al.*, 1982; Holl *et al.*, 1985). The fourth intron of the long version of the *cob* gene is homologous to the first intron of the short gene.

Maturases have been postulated to facilitate processing of their cognate intervening sequences allowing for the maturation of the primary *cob* transcript to the mRNA (Lazowska *et al.*, 1980). Studies on the translation products of *cis*-dominant (*box1*, *box9* and *box2*) and *trans*-recessive (*box7*) mutants of the cytochrome *b* gene have led to a precursor model for the bI4 maturase (Weiss-Brummer *et al.*, 1982; DeLaSalle *et al.*, 1982; Lamb *et al.*, 1983). In the yeast D273-10B strain which contains the short form of the *cob* gene, the bI4 intron codes in part for a 55 kd polypeptide. This protein is translated from a reading frame that starts at the initiation codon of the first exon and ends at a stop codon in the bI4 reading frame (Weiss-Brummer *et al.*, 1982). In the case of the long version of the gene, the first three intervening sequences must first be removed

so that the first exon may be read in-frame with the bI4 reading frame. The 55 kd polypeptide is then proteolytically cleaved to generate two products of 35 kd (p35) and 27 kd (p27) (DeLaSalle *et al.*, 1982). The p27 product is derived entirely from the bI4 coding sequence and, on the basis of observations that missense mutations in the bI4 reading frame affect splicing of the cognate intervening sequence whereas mutations in the first exon do not (Weiss-Brummer *et al.*, 1982; DeLaSalle *et al.*, 1982; Lamb *et al.*, 1983), has been proposed to be the maturase responsible for promoting excision of the bI4 intervening sequence.

The expression of the apocytochrome *b* gene also requires nuclear-encoded gene products (Tzagoloff and Myers, 1986). Some of these participate either directly or indirectly in processing of the intervening sequences. Of the ~200 known nuclear complementation (*pet*) groups affecting the biogenesis of mitochondria in yeast, ~18 identity genes that affect specifically the production of cytochrome *b* (Crivellone *et al.*, 1988). Among the characterized genes are *CBP1* required for processing of the 5'-end of the primary cytochrome *b* transcript (Dieckmann *et al.*, 1984a); *CBP2* involved in splicing of the terminal intervening sequence (McGraw and Tzagoloff, 1983); *CBP6* (Dieckmann and Tzagoloff, 1985) *CBS1* and *CBS2* (Rodel, 1986) needed for the translation of the mature apocytochrome *b* RNA.

In this paper, we report on the characteristics of a set of nuclear recessive mutants that define the *pet* complementation group, G162. G162 mutants with the short cytochrome *b* gene fail to process the first intervening sequence efficiently although a low level of the mature transcript is present. The wild-type copy of the gene, designated as *CBP7*, responsible for the phenotype of these mutants, has been cloned by complementation. The sequence of the *CBP7* gene indicates that it is identical to a known gene, *CBS2*, which has previously been reported to code for a translational factor for the *cob* mRNA transcript (Michaelis *et al.*, 1988).

The role of *CBP7* (*CBS2*) was characterized more precisely by Northern and Western blot analyses. The results indicate that the effect of mutations in this nuclear gene prevent all maturase encoding intervening sequences from being excised from the cytochrome *b* pre-mRNA. Furthermore, it was determined that *cbp7* mutants fail to synthesize mature cytochrome *b* protein but can translate the cytochrome *b* pre-mRNA to produce bI4 maturase. These results imply that in addition to its probable role in translation of the cytochrome *b* mRNA, the *CBP7* product must also be involved in conjunction with the maturases in splicing of their cognate intervening sequences of the cytochrome *b* pre-mRNA.

Results

Properties of mutants from the G162 complementation group

Several thousand respiratory deficient mutants have been screened; of these, E67, E373, E744 and N594 were

Fig. 1. Spectrum of cytochromes in W303-1A and in *cbp7* mutants. Mitochondria were isolated from the respiratory competent haploid strain W303-1A and from three members of the G162 complementation group, E67, E373 and E744. The mitochondria were suspended at a protein concentration of 7–10 mg/ml and were extracted with deoxycholate as described previously (Tzagoloff *et al.*, 1975). Difference spectra were recorded at 23°C with a Cary Model 14 spectrophotometer after oxidizing half the extract with potassium ferricyanide and reducing the other half with sodium dithionite. The α -band of cytochrome *b* has a maximum at 562 nm and cytochromes *a* and a_3 of the cytochrome oxidase complex have maxima at 605 nm.

assigned to a single complementation group (G162) based on genetic tests. Absorption spectra of mutant mitochondria indicated an absence of cytochrome *b* at 562 nm and reduced levels of cytochromes *a* and a_3 at 603 nm. Two of the mutants, E373 and E744, had no detectable levels of cytochromes *a* and a_3 (Figure 1).

The G162 mutants revert but do so at a low rate (10^{-7} to 10^{-8}). The one exception, N594, reverts at a very high rate and for that reason has not been used in this study. About 10–25% of stationary phase cultures of all the *cbp7* mutants consist of q^- derivatives.

Northern blot analysis of cytochrome *b* transcripts

The order of splicing of the two introns of the cytochrome *b* gene in the D273-10B/A1 strain is non-obligatory. Therefore, four RNA transcripts, comprising the unspliced precursor, the two partially spliced intermediates and the fully processed mRNA are detected by Northern blot analysis of mitochondrial RNA (Bonitz *et al.*, 1982).

Northern blot analysis of all four G162 mutants reveal the same pattern; they fail to process the first intron efficiently as indicated by an overabundance of the 3.6 kb RNA species corresponding to the partially spliced product with an intact first intron. The mature RNA transcript is also present but at ~25% of the wild-type level (Figure 2).

Identity of *CBP7*

The wild-type *CBP7* gene which is defined by the G162 complementation group has been cloned by complementation and sequenced by the method of Maxam and Gilbert (1977) (data not shown). The reading frame corresponding to *CBP7* was found to be identical to *CBS2* which has been reported to code for a protein promoting the translation of the *cob* mRNA (Michaelis *et al.*, 1988). We also confirm that the phenotype of *cbp7* mutants, similarly to *cbs2* mutants, can be rescued if they contain in addition to their wild-type mitochondrial genome a q^- genome which has undergone a deletion, allowing the cytochrome *b* leader sequence to be replaced by that of the downstream *oli1* gene. Therefore, having the *oli1* leader sequence fused to the cytochrome *b* gene bypasses the need for the product of the *CBP7* (*CBS2*) gene (Rodel, 1986). This q^- genome, previously

Fig. 2. Cytochrome *b* transcripts in wild-type and *cbp7* mutants. Total mitochondrial RNA from W303-1A and the four members of the G162 complementation group, N594, E67, E744 and E373, were separated electrophoretically on a 1% agarose gel and transferred to DBM paper. The Northern blot was hybridized to a probe consisting of a 5'-end-labeled single-stranded fragment from the first exon of the cytochrome *b* gene. On the left margin are indicated the exon (B_1, B_2, B_3) and intron (I_1, I_2, I_3) compositions and on the right margin the sizes of the transcripts (Bonitz *et al.*, 1982).

designated as q^{4-35} , has also been reported to suppress the effect of mutations in *CBP1* and *CBS1* genes (Dieckmann *et al.*, 1984b; Rodel and Fox, 1987).

Properties of W303 ∇ CBP7

The *CBP7* reading frame in the respiratory competent haploid strain, W303-1A, was disrupted by a one-step gene substitution method (Rothstein, 1983) in an identical manner to that reported by Rodel *et al.* (1986). The constructed strain was designated as W303 ∇ CBP7. W303-1A has a mitochondrial genome with the long cytochrome *b* gene. Northern blot analysis of W303 ∇ CBP7 mitochondrial RNA hybridized with a cytochrome *b* exon probe shows the accumulation of a major transcript of ~6–7 kb. (Figure 3). In order to determine the intervening sequences present in this transcript, Northern blots were challenged with four probes, each derived from one of the first four introns of the long cytochrome *b* gene. As can be seen in Figure 4, the major transcript detected in W303 ∇ CBP7 lacks the first but contains the next three downstream intervening sequences. The three intervening sequences that fail to be excised in the mutant each have long open reading frames previously shown to code for maturases (Lazowska *et al.*, 1980; DeLaSalle *et al.*, 1982; Holl *et al.*, 1985).

The maturase encoded by the first intron of the short cytochrome *b* gene has been shown to play a role not only in the splicing of its cognate intervening sequence but also in splicing of the fourth intervening sequence of subunit 1 of cytochrome oxidase pre-mRNA (Pajot *et al.*, 1977). It was therefore of interest to examine the effect of the disrupted *CBP7::URA3* allele on processing of the subunit 1 or *oxi3* transcript. W303 ∇ CBP7 q^0 which lacks mitochondrial DNA was crossed to three respiratory competent strains each having a different mitochondrial genome, generating the following new *cbp7* mutants: W303 ∇ CBP7/D273, a respiratory deficient mutant with the short cytochrome *b* gene and an *oxi3* gene containing the fourth intron; W303 ∇ CBP7/CB11, a *cbp7* mutant whose mitochondrial DNA had the short cytochrome *b* gene but lacked the fourth

Fig. 3. Northern blot analyses of cytochrome *b* transcripts in *cbp7* mutants with the short and long genes. Mitochondrial RNAs were prepared from the wild-type strains D273-10B/A1 (short *cob*), W303-1A (long *cob*) and from the following *cbp7* mutants: E67 (short *cob*), W303VCBP7/D273 (short *cob*), W303VCBP7 (long *cob*). The *mit*⁻ mutant M10-150 (Slonimski and Tzagoloff, 1976) with a deleted *oxi3* gene was also included in the analysis as a control. Total mitochondrial RNAs (3–5 µg) were separated on a 1% agarose gel and challenged with a 5'-end-labeled probe from the first exon of the gene following transfer to DBM paper. Lane 1: E67, lane 2; M10-150, lane 3: W303VCBP7, lane 4: W303-1A, lane 5: W303VCBP7/D273, lane 6: D273-10B/A1. The approximate sizes of the transcripts are marked in the margin. The exon/intron compositions of the 2.1, 2.9, 3.6 and 4.3 kb transcripts are described in Figure 2. The larger transcript of 6–7 kb seen in W303VCBP7 lacks the first and last intervening sequences but contains the second, third and fourth intervening sequences of the long gene (see Figure 4).

intron of *oxi3*; and W303VCBP7/KL14, a mutant with the long cytochrome *b* gene and an *oxi3* containing the fourth intron. The strains and crosses used for the construction are summarized in Table I.

Mitochondria isolated from these three mutants were analyzed for their cytochrome contents spectrally. The spectra shown in Figure 5 indicate that each mutant lacks the α -absorption band of cytochrome *b*. Only W303VCBP7/KL14 and W303VCBP7/D273 were also deficient in cytochrome oxidase as measured by cytochromes *a* and *a*₃ at 650 nm. The third strain W303VCBP7/CB11 exhibited the presence of these cytochromes. The latter mutant has a mitochondrial genome with an *oxi3* gene devoid of the fourth intron (Hensgens *et al.*, 1983a). The presence of cytochrome oxidase in this strain suggested that processing of the cytochrome oxidase subunit 1 pre-mRNA is dependent on the presence of the CBP7 protein only in the context of an *oxi3* gene with the fourth intron.

To confirm that the absence of cytochrome oxidase was caused by a block in splicing of the fourth intervening sequence of the subunit 1 pre-mRNA, Northern blot hybridizations were performed on mitochondrial RNA from wild-type yeast and from the mutants with the three different mitochondrial genomes. For these analyses probes were prepared from the first to the fifth introns of the gene. The results of the hybridizations are shown in Figures 6 and 7. As anticipated, W303VCBP7/CB11, which was found to synthesize wild-type levels of cytochrome oxidase, had a major *oxi3* transcript of ~2 kb which hybridized to an *oxi3* probe (Figure 6). This transcript was absent in W303VCBP7/D273 which has the longer gene with the fourth intron. The major transcript detected by both the exon probe and fourth intron probe (Figure 7) in the latter mutant

Fig. 4. Analysis of the high molecular weight cytochrome *b* transcript detected in *cbp7* mutants with the long gene. Mitochondrial RNA from W303-1A (WT) and from the *cbp7* mutant W303VCBP7 (M) were separated on a 1% agarose gel, transferred to DBM paper and hybridized to probes from the first to the fourth introns of the long cytochrome *b* gene. The structure of the gene and the origins of the probes used for the hybridization are shown on the drawing in the lower part of the figure. The 0.8 kb transcript detected by the first intron probe is a stable circular RNA (Halbreich *et al.*, 1980) whose excision does not depend on an intron encoded maturase (Dujon, 1981). The fifth intron was not analyzed. The excision of this intervening sequence, however, also occurs independent of any intron encoded maturase (Bonitz *et al.*, 1982).

has a size of 3.2 kb consistent with the presence in this transcript of the fourth intervening sequence. The 3.2 kb precursor was also seen in W303VCBP7/KL14 whose *oxi3* gene also contains the fourth intron. The results of the hybridizations with the other intervening sequences indicated that all the other intervening sequences were excised from the subunit 1 pre-mRNA in the three mutants (Figure 7).

Construction of a *cbp7* mutant with an intron-less *cob* gene

An intron-less version of the cytochrome *b* gene, obtained through mutagenesis, has been reported by several laboratories (Labouesse and Slonimski, 1983; Perea and Jacq, 1985; Seraphin *et al.*, 1987). Because of the absence of these introns, nuclear-encoded factors which have roles solely in the processing of intervening sequences in *cob* transcripts are not required for respiratory competence of such strains. If CBP7 is exclusively a processing factor, *cbp7* mutants carrying the intron-less *cob* gene would be expected to be respiratory competent. However, if CBP7 has any translational role, then such mutants should be respiratory deficient.

W303VCBP7^{q0} was crossed to the KAR(167) strain (Seraphin *et al.*, 1987) (a gift from Gerard Faye) which contains mitochondria with an intron-less genome (including an intron-less *cob* allele) and has a wild-type nuclear background. The resulting diploids were sporulated and spores with the disrupted allele of *cbp7* were selected. These were determined to have mitochondria of the KAR(167) strain since they were able to complement strains devoid of

Table I. Genotypes and sources of *S.cerevisiae* and *Saccharomyces carlsbergensis* strains

Strain	Genotype	Source
D273-10B/A1	$\alpha, q^+, met6$	Tzagoloff <i>et al.</i> (1976)
D273-10B/A1 q^0	$\alpha, q^0, met6$	This study
E67	$\alpha, q^+, met6, cbp7-1$	This study
E373	$\alpha, q^+, met6, cbp7-2$	This study
E744	$\alpha, q^+, met6, cbp7-3$	This study
N594	$\alpha, q^+, met6, cbp7-4$	This study
W303-1A	$a, q^+, ade1-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100$	R.Rothstein ^a
W303-1B	$\alpha, q^+, ade2-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100$	R.Rothstein ^a
W303-1A q^0	$a, q^0, ade2-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100$	This study
W303-1B q^0	$\alpha, q^0, ade2-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100$	This study
KL14-4A	$a, q^+, his, trp2$	Tzagoloff <i>et al.</i> (1975)
KL14-4B	α, q^0, aux	Tzagoloff <i>et al.</i> (1975)
KL14-4B q^0	α, q^+, aux	This study
CB11	$a, q^+, ade1$	ten Berge <i>et al.</i> (1974)
CB11 q^0	$a, q^0, ade1$	This study
W303 ∇ CBP7	$a, q^+, ade2-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100, CBP7::URA3$ (W303 mitochondria)	This study
α W303 ∇ CBP7	$\alpha, q^+, ade2-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100, CBP7::URA3$ (W303 mitochondria)	W303 ∇ CBP7 $\times$ W303-1B
W303 ∇ CBP7/D273	$a, q^+, aux, CBP7::URA3$ (D273-10B/A1 mitochondria)	W303 ∇ CBP7 q^0 $\times$ D273-10B
W303 ∇ CBP7/CB11	$a, q^+, aux, CBP7::URA3$ (CB11 mitochondria)	α W303 ∇ CBP7 q^0 $\times$ CB11
W303 ∇ CBP7/KL14	$a, q^+, aux, CBP7::URA3$ (KL14-4B mitochondria)	W303 ∇ CBP7 q^0 $\times$ KL14-4B
PZ54	$a, q^+, ade, lys, box7$	P.Perlman ^b
KAR(167)	$a, intron-less q^+, kar1-1, trp5$	G.Faye ^c
MK2	$a, q^+, leu2, trp1, his, cbs1$	Pillar <i>et al.</i> (1983a)
MK2/D273	$a, q^+, leu2, trp1, his, cbs1$	This study
MK6	$a, q^+, leu2, ura3, cbs2$	Pillar <i>et al.</i> (1983a)

^aR.Rothstein, Department of Human Genetics, Columbia University, New York, USA^bP.Perlman, Department of Genetics, Ohio State University, Columbus, Ohio, USA^cG.Faye, Section de Biologie, Institut Curie, Orsay, France

mitochondria but with wild-type nuclear background to respiratory competence. However, as haploids, these spores did not have respiratory competency, suggesting that *CBP7* has a role in the translation of *cob* mRNA.

Detection of cob-related polypeptides in *cbp7* mutants

Using cytochrome *b* antiserum, mitochondrial proteins were analyzed by Western blot analysis in the following *cbp7* mutants: E67, E373, E744 and W303 ∇ CBP7/D273. As expected, the antiserum failed to detect cytochrome *b* in any of the *cbp7* mutants tested (Figure 8). All the mutants, however, revealed the presence of a 55 kd polypeptide that cross-reacted with the antiserum. The size of this protein is consistent with the bI4 maturase precursor previously

reported to be a 55 kd protein (Weiss-Brummer *et al.*, 1982). This suggests that translation of the bI4 maturases is not blocked in *cbp7* mutants.

Immunochemical detection of p27 in wild-type yeast mitochondria

The bI4 maturase protein is a minor (so far undetected) constituent of wild-type yeast mitochondria (Jacq *et al.*, 1984). The ability of the trpE/bI4 antiserum to detect this protein was therefore first tested in a *box7* mutant known to accumulate p27. This mutant, PZ54, which was kindly supplied by Dr Philip Perlman, has been shown to have a temperature sensitive mutation in the bI4 intron that allows the 55 kd precursor to be translated and proteolytically

Fig. 5. Spectra of *cbp7* mutants with the disrupted allele. Mitochondria prepared from three *cbp7* mutant strains were extracted in 50 mM Tris-HCl, pH 8, 1 M KCl, 1% deoxycholate at final protein concentrations of 9.2 mg/ml (W303 Δ CBP7/CB11), 8.1 mg/ml W303 Δ CBP7/D273) and 6 mg/ml (W303 Δ CBP7/KL14).

Fig. 6. Analysis of *oxi3* transcripts in *cbp7* mutants with two variant forms of the gene. Mitochondrial RNA was prepared from the wild-type strain D273-10B/A1 containing an *oxi3* gene with the fourth intron (lane 1) and the following *cbp7* mutants: W303 Δ CBP7/D273 (lane 2) and W303 Δ CBP7/CB11 which lacks the first and fourth introns of the gene. The RNAs (3–5 μ g) were separated electrophoretically on a 1% agarose gel after which they were transferred to DBM paper and hybridized to a nick-translated probe from the fourth exon of the *oxi3* gene. The 2 kb transcript seen in lanes 1 and 3 has previously been shown to be the fully processed mRNA for subunit 1 of cytochrome oxidase (Hensgens *et al.*, 1983a; Bontiz *et al.*, 1980). The composition of the larger 3.2 kb transcript accumulated in W303 Δ CBP7/D273 (lane 2) has been identified as a precursor of the subunit mRNA with an intact fourth intervening sequence (see Figure 7).

processed to p27 at the permissive and non-permissive temperatures. At the non-permissive temperature, the mutation prevents splicing of the bI4 intron, and the mutant is incapable of synthesizing cytochrome *b*. Furthermore, the splicing defect leads to elevated levels of p27 (Lamb *et al.*, 1983). Western blot analyses of PZ54 mitochondria showed that the antiserum recognizes a 27 kd protein (Figure 9) and was therefore a suitable reagent for examining the presence of this protein in mitochondria of wild-type and *cbp7* mutants. The results of Figure 9 confirm the accumulation of p27 in the *box7* mutants as compared with wild-type which has substantially less of the protein.

Four different wild-type strains and their derivative ρ^0 mutants were used to test for the bI4 maturase. D273-10B, W303-1A/D273 and CB11 contain the short mitochondrial cytochrome *b* gene. W303-1A on the other hand is a respiratory competent haploid strain with the long gene.

Mitochondria were prepared from each of the above strains of yeast, as well as from their ρ^0 derivatives. Since the bI4 maturase is encoded in mitochondrial DNA, ρ^0 mutants should be deficient in the protein. The results of the Western blot, shown in Figure 10 demonstrate the presence of a 27 kd protein in all the respiratory competent strains independent of their mitochondrial DNA type but not in the isogenic strains lacking mitochondrial DNA. Two other proteins of lower molecular weights were also detected by the antibody in the wild-type but not in the ρ^0 mutants. These may be proteolytic degradation products of p27 or p35. The absence of p55 indicates that processing of the 55 kd precursor in respiratory competent yeast is an efficient process.

The ability of the antiserum used in the present study to detect the bI4 maturase in wild-type mitochondria is at variance with the experience of other laboratories. Jacq *et al.* (1984) have previously reported cloning the identical *Hae*III–*Hind*III fragment in *lacZ* expression vector. These authors indicated that although antibodies against the β -galactosidase–bI4 fusion protein detected the p27 polypeptide in mutants that overproduce the maturase, it was ineffective in doing so with wild-type yeast. This discrepancy can be accounted for either by a lower titer antiserum in their case or by a less sensitive Western blot detection system.

Detection of the bI4 maturase in *cbp7* mutants

As shown previously (Figure 8), Western blots of mitochondrial proteins from *cbp7* mutants challenged with anti-cytochrome *b* serum confirmed the presence of a protein with a molecular weight compatible with the size of bI4 maturase precursor. Since this polypeptide is currently believed to be the precursor for the bI4 maturase, it was reasonable to anticipate that *cbp7* mutants should also produce the processed 27 kd product corresponding to the active maturase. A prerequisite for this to hold true is that the *CBP7* gene product is not required for proteolytic maturation of the p55 precursor. This was verified by Western blot analysis of *cbp7* mutants. The strains examined included the *cbp7* mutants: E67, E373, E744 and W303 Δ CBP7/D273; PZ54 which overproduces the bI4 maturase and the two wild-type strains, W303-1A and W303/D273.

The results of the immunological assays indicate that the antibody to the trpE/bI4 fusion protein reacts with a 27 kd polypeptide and smaller polypeptide present in all the *cbp7* mutants (Figure 11). Additionally, the mutants exhibit the p55 precursor. This is in contrast to wild-type yeast mitochondria which contain only p27 and the smaller peptide. Even though *cbp7* mutants are capable of processing the precursor, its steady state concentration appears to be higher than in wild-type. The presence of p55 in *cbp7* mutants may be a consequence of their respiratory defect. For example, M10-150 (Slonimski and Tzagoloff, 1976) with a deletion in *oxi3* has p27 but in addition accumulates the p55 precursor (data not shown). Conceivably, proteolytic processing of the bI4 precursor may be an energy dependent step and be less efficient in strains that cannot produce ATP from oxidative phosphorylation.

Detection of p55 and p27 in *cbs1* mutants

Pillar *et al.* (1983a,b) have reported the properties of several respiratory defective *pet* strains with a phenotype similar to that of G162 mutants. One was the MK6 strain; it contains a mutant allele of *CBS2* which is the same as *CBP7*. Another

Fig. 7. Analysis of the intervening sequences present in the 3.2 kb transcript of *cbp7* mutants with an *oxl3* gene containing the fourth intron. Mitochondrial RNAs were prepared from the wild-type strain W303-1A (lane 1) and from the *cbp7* mutants W303 ∇ CBP7/KL14 (lane 2), W303 ∇ CBP7/CB11 (lane 3) and W303 ∇ CBP7/D273 (lane 4). Following electrophoresis and transfer to DBM paper, the RNA blots were hybridized to nick-translated probes from the first to the fifth introns of the gene. The structure of the *oxl3* gene in D273-10B/A1 and CB11 are shown on the lower part of the figure. The *oxl3* gene of KL14-4A is identical to that of D273-10B/A1 except for the presence of two additional introns in the A5 exon (Hensgens *et al.*, 1983b). The exons A1–A8 are shown as solid bars and the intron reading frames are open bars. The intron regions spanned by the probes are indicated above the two genes. The 2.5 kb transcripts detected by the I1 and I2 probes are stable circular excision products of the first two intervening sequences and so is the 0.8 kb transcript excised from the fifth intervening sequence (Pajot *et al.*, 1977). The transcripts detected by the I3 probe have not been characterized. The 3.2 kb transcript accumulated in the *cbp7* mutants with the D273-10B/A1 and KL14-4A mitochondrial DNAs is detected only by the I4 probe. The processed fourth intron intervening sequence is unstable and for that reason is not detected in wild-type mitochondria. The 2.5 kb circular transcript liberated from the first intervening sequence is not detected in W303 ∇ CBP7/CB11 (lane 3) because the corresponding intron is missing in the gene.

was MK2, which, like MK6, induces a deficiency in cytochrome *b* and cytochrome oxidase when the mitochondrial genome has the long *cob* gene (Pillar *et al.*, 1983b). However, with the short *cob* gene, the deficiency in the MK2 mutant is confined to cytochrome *b* and does not affect the production of cytochrome oxidase. It was of interest to examine the translational phenotype of MK2 in more detail.

The MK2 strain (a gift from Gerhard Rodel) was isolated from a respiratory competent haploid yeast with the long cytochrome *b* gene (Pillar *et al.*, 1983b). A q^0 derivative of this mutant was obtained by mutagenesis with ethidium bromide and was used to construct a new strain (MK2/D273) with the short *cob* gene by cytoduction. Mitochondria prepared from MK2 and MK2/D273 were analyzed for their protein constituents with antisera to either cytochrome *b* or the b14 maturase. The cytochrome *b* antisera revealed the presence of p55 in MK2/D273 but not in MK2 mitochondria. Similarly, p55 and p27 were detected by the trpE/b14 antiserum only in MK2/D273 mitochondria (data not shown). These results indicate that like *cbp7* (or *cbs2*) mutants, the MK2 strain can express the p55 precursor and b14 maturase only in the context of the short gene.

Discussion

Strains carrying mutations in the *CBP7* gene are respiratory deficient due to their inability to synthesize cytochrome *b* and cytochrome oxidase, as first determined by spectral assays. Further analyses of the cytochrome *b* transcripts present in point mutants and in a construct with a disrupted copy of *CBP7* indicated in each instance that the mutational block causes the accumulation of a partially spliced cytochrome *b* pre-mRNA. In the case of the short gene, the intermediate was identified as a 3.6 kb RNA species with an intact b14 intervening sequence. Mutants endowed with the long gene exhibit a larger transcript containing in addition to b14 the upstream intervening sequence corresponding to b12 and b13. Additionally, three constructed, derivative strains were analyzed. Two had the short cytochrome *b* gene but had different variants of the mitochondrial *oxl3* gene coding for subunit 1 of cytochrome oxidase. The strain W303 ∇ CBP7/D273, whose mitochondrial DNA contained the *oxl3* gene with the fourth intron, was deficient in spectral cytochrome oxidase. In contrast, the second strain (W303 ∇ CBP7/CB11) lacking the fourth intron of *oxl3* made wild-type levels of cytochrome oxidase. The third construct

Fig. 8. Western blot analysis of *cob* polypeptides in wild-type and mutant strains. Mitochondria were prepared from the *cbp7* mutant strains: E67, E373, E744 and W303 Δ CBP7/D273 and from the wild-type W303/D273. 50 μ g from each were separated in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose paper. The blot was challenged with a 1:100 dilution of the antiserum to cytochrome *b*. The 30 kD protein seen in wild-type mitochondria but not in the mutant mitochondria corresponds to apocytochrome *b*. The more weakly reacting band at 55 kD is the presumed precursor to the bI4 maturase.

Fig. 9. Detection of the bI4 maturase in an overproducing mutant. PZ54 is a temperature sensitive mutant which overproduces the bI4 maturase (p27) due to a single amino acid change which suppresses splicing of the bI4 intervening sequence (Lamb *et al.*, 1983). W303-1A is a respiratory competent strain with the long *cob* gene. Mitochondria (50 μ g protein) from wild-type and from the *mit*⁻ mutant were analyzed as described in the legend to Figure 8, except that antibody to the trpE/bI4 fusion protein was used. The antibody reacted with a protein of 27 kD which was present at higher concentrations in the mutant. A second more weakly reacting band of slightly lower molecular weight is also seen. The relationship of this protein to p27 has not been investigated.

W303 Δ CBP7/KL14 with the long cytochrome *b* gene and the fourth *oxi3* intron also failed to express cytochrome oxidase. These results are consistent with previous studies

Fig. 10. Detection of the bI4 maturase in wild-type strains. Mitochondrial protein (100 μ g) from the wild-type strains containing the short *cob* gene (D273-10B, W303/D273 and CB11) and from another strain with the long gene (W303-1A) were analyzed for the presence of the bI4 maturase. In the same experiment mitochondria were also prepared from the isogenic strains lacking mitochondrial DNA (ρ^0). The trpE/bI4 antiserum was used at a 1:100 dilution. The antibody detected a protein which migrated like p27 and several smaller proteins in the wild-type mitochondria but not in the ρ^0 . The identities of the smaller polypeptides are not known.

Fig. 11. Detection of the bI4 maturase in *cbp7* mutants. Mitochondria were prepared from the wild-type strains W303-1A (long *cob* gene), W303/D273 (short *cob* gene), from the *cbp7* mutants: W303 Δ CBP7/D273, E67, E373 and E744 and from the bI4 maturase overproducing mutant, PZ54. For each, 100 μ g of sample were separated electrophoretically on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose paper and reacted with the trpE/bI4 antibody at a 1:100 dilution. The positions of the 55 kD precursor (p55) and the processed maturase (p27) are marked in the margin.

indicating that processing of the fourth intron from the *oxi3* pre-mRNA requires a functional maturase encoded in the first intron of the short cytochrome *b* gene or the equivalent fourth intron of the long gene (Pajot *et al.*, 1977).

Additional experiments were undertaken to clarify the possible requirements of the *CBP7* protein in translation and/or splicing of the cytochrome *b* pre-mRNA. If the primary function is in translation, mutations in *CBP7* should cause a deficiency of the bI4 maturase. By the same reasoning, a direct role in splicing should not affect translation of the bI4 maturase. Two different antibody probes were obtained to test for the presence of either 27 kd bI4 maturase or the 55 kd precursor in wild-type and *cbp7* mutants. An antibody was prepared against native cytochrome *b*. This probe should react with the primary 55 kd translation product which contains the amino terminal half of cytochrome *b* (DeLaSalle *et al.*, 1982). The second antibody against a *trpE*/bI4 fusion protein was a more selective probe for the bI4 maturase. The results of the immunological assays indicate the presence of the 27 kd bI4 maturase in all of the *cbp7* mutants when their mitochondrial DNA contains the short cytochrome *b* gene. Since translation of the 55 kd precursor polypeptide is initiated from the ATG of the 5'-exon (DeLaSalle *et al.*, 1982), and continues through the first three exons of the long gene before reaching the termination codon of the bI4 intron, the resultant protein encompasses both exon and intron sequences and therefore should be detected by antibodies to cytochrome *b* and to the bI4 maturase. This expectation was borne out by the Western blot results. Mutants with the short gene contain in addition to the bI4 maturase a 55 kd protein that cross-reacts with antisera to cytochrome *b* and to the *trpE*/bI4 fusion protein.

One of the hallmarks of *cbp7* mutants is their accumulation of the 3.6 kb transcript corresponding to the mRNA for the maturase. A second prediction is that *cbp7* mutants with the short gene should have elevated amounts of the bI4 maturase. This was confirmed by the stronger signal of the 27 kd protein in the mutants compared with the wild-type. Mutant mitochondria also have higher concentrations of the 55 kd precursor suggesting that its conversion to the 27 kd bI4 maturase may be a rate limiting step in respiratory deficient strains.

Mitochondria carrying intron-less alleles of the *cob* gene do not alleviate the effect of a *cbp7* mutation, strongly supporting the hypothesis that *CBP7* is a translation factor. Additional evidence for this hypothesis comes from the fact that the ρ^{4-35} genome is able to suppress *cbp7* mutants. This is also implied by the apparent inability of these mutants to process the second and third intervening sequence of the long *cob* gene. Such strains accumulate a high molecular weight transcript with the second, third and fourth intervening sequences and show no evidence of the *cob* mRNA as opposed to a 25% level of *cob* mRNA seen in isogenic strains with the short *cob* gene. However, at least wild-type levels of bI4 maturase are seen in *cbp7* mutants; thus, *CBP7* also appears to have some processing role since in *cbp7* mutants maturases are unable to process their cognate intervening sequences efficiently, again evidenced by the fact that only a 25% level of mRNA is seen in strains with the short *cob* mRNA. Perhaps the translational role of *CBP7* is specific to the second round of translation that takes place in the synthesis of cytochrome *b*, that is from the mature mRNA transcript.

The proposed role of the protein in both translation and splicing creates an obvious dilemma in view of the presence of the bI4 maturase in *cbp7* mutants with the short *cob* gene. This apparent discrepancy can be rationalized by the following argument. In the case of the short gene, the bI4 maturase is synthesized following the first round of translation of the 3.6 kb pre-mRNA. If translation of this transcript is not completely blocked in the *cbp7* background and some bI4 maturase is translated, the subsequent defect in splicing of the bI4 intervening sequence by virtue of maintaining a higher concentration of the 3.6 kb transcript (mRNA for maturase) could account for the somewhat higher expression of the maturase in *cbp7* mutants. The absence of cytochrome *b* in *cbp7* mutants with the short gene follows from the lower steady state level of the processed mRNA. Finally, the absence of any *cob* mRNA in *cbp7* mutants with the long gene and the inability of such strains to express bI4 maturase may be a function of the presence of two additional upstream introns with encoded maturases. Thus the production of bI4 maturase in these mutants requires two additional rounds of translation to form the 3.6 kb transcript. Lowered rates of translation of these maturases might result in a significant decrease of the bI4 maturase and in turn of the *cob* mRNA.

Materials and methods

Strains and media

The *S. cerevisiae* strains used in this study are listed in Table I. *pet* mutants were derived from the wild-type strain, D273-10B/A1, by ethylmethane sulfonate or nitrosoguanidine mutagenesis (Dieckmann *et al.*, 1984a). The media used had the following compositions: YPD (1% yeast extract, 2% peptone, 2% glucose) YPGal (1% yeast extract, 2% peptone, 2% galactose) YPEG (1% yeast extract, 2% peptone, 3% glycerol) and WO [0.67% nitrogen base without amino acids (DIFCO), 2% glucose]. For solid media, 2% agar was added.

Northern blot hybridization

The extraction of mitochondrial RNA has been described previously (Bonitz *et al.*, 1980). Total mitochondrial RNA separated by electrophoresis in 1% agarose gels was transferred to diazobenzoyloxymethyl paper by the method of Alwine *et al.* (1977). Single-stranded DNA used as probes were 5'-end-labeled.

Western blotting analyses

Wild-type and mutant strains of yeast were grown aerobically at 30°C in liquid YPGal to early stationary phase. Mitochondria were purified by the method of Faye *et al.* (1974). Mitochondrial proteins were separated on 12% SDS-PAGE gels by the method of Laemmli (1970) and transferred to nitrocellulose. The blots were reacted with antisera and the bound antibodies visualized by a secondary reaction with [¹²⁵I]protein A (Schmidt *et al.*, 1983).

Preparation of polyclonal antibodies to yeast cytochrome *b*

Cytochrome *b* was purified from commercial baker's yeast (Red Star) following extraction of mitochondria with deoxycholate and fractionation of the extract on hydroxylapatite and DEAE cellulose by an unpublished procedure (F. Foury and A. Tzagoloff). Antibodies were raised in rabbits against this purified cytochrome *b*.

Preparation of polyclonal antibodies against the bI4 maturase

Antibodies against the bI4 maturase protein were obtained as follows. A 651 bp *Hae*III–*Hind*III fragment containing part of the bI4 sequence was cloned in the *Escherichia coli* expression vector pATH11 (Dieckmann and Tzagoloff, 1985). The resultant construct, pI4/HH, has the bI4 coding region of the *Hae*III–*Hind*III fragment fused in-frame to the upstream *trpE* gene. The reading frame in the fragment has two TGA codons which specify tryptophan in the yeast mitochondrial genetic code (Nobrega and Tzagoloff, 1980) but are recognized as translational stops in *E. coli*. The first TGA, however, occurs 104 codons past the *Hae*III site. The pATH construct should

therefore express a fusion protein of ~49 kd of which the amino terminal 336 residues are derived from the *TrpE* gene and the carboxyl terminal 104 residues from the *bl4* coding sequence. This fusion protein was designated *trpE/bl4*.

MC1061E *E. coli* transformed with the *pl4/HH* plasmid were grown in 300 ml of culture and induced with indolacrylic acid to overexpress the fusion protein by a previously reported method (Dieckmann and Tzagoloff, 1985). Samples of the insoluble protein fractions were obtained and fractionated by SDS-PAGE. A major protein band (estimated to represent 50% of the insoluble protein fraction) migrating with an estimated size of 49 kd was present in the material recovered from *E. coli* cells transformed with *pl4/HH*. The *trpE/bl4* fusion protein was further purified to ~70–80% homogeneity by chromatography on a Bio-Gel A-0.5 column. Approximately 12 mg of fusion protein was recovered. The precipitated protein was solubilized in 1.2 ml of 1% SDS and used for inoculation of rabbits. Two rabbits were each injected with ~2.0 mg of protein, followed by two booster injections of 2.0 mg every 2 weeks. Only one of the two rabbits produced antiserum that reacted with the *trpE/bl4* antigen.

Miscellaneous methods

Standard methods were used for digesting DNA with restriction endonucleases, purification of DNA from agarose gels, ligation of DNA restriction fragments, transformation of *E. coli* with plasmid DNA and preparation of plasmid DNA (Maniatis *et al.*, 1982). The method of Maxam and Gilbert (1977) was used to sequence single-stranded DNA with labeled 5'-ends.

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