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REVIEW

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Translational activator proteins required for cytochrome *b* synthesis in *Saccharomyces cerevisiae*

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Abstract In the yeast *Saccharomyces cerevisiae* translation of apocytochrome *b*, the only mitochondrially encoded subunit of ubiquinol-cytochrome *c* oxidoreductase, requires the products of at least three nuclear genes, *CBP6*, *CBS1* and *CBS2*. In this article I review available data on CBS1p and CBS2p from the initial detection of the genes up to the current investigations on interacting components and the proteins' topology.

Key words Mitochondria · Cytochrome *b* · Translational activation

RNA processing of the split gene *COB* requires translation of intron-encoded maturases

The biogenesis of the enzymes of oxidative phosphorylation (OXPHOS) is dependent on two genetic systems. While mitochondrial DNA (mtDNA) encodes about a dozen proteins, the vast majority of proteins are encoded by nuclear genes (for reviews see: Attardi and Schatz 1988; Grivell 1989; Tzagoloff and Dieckmann 1990). In the yeast *Saccharomyces cerevisiae* ubiquinol-cytochrome *c* oxidoreductase (or complex III, or co-enzyme QH_2-cytochrome *c* reductase) of the respiratory chain in the inner mitochondrial membrane (IMM) is composed of nine subunits (Ljungdahl et al. 1987). Eight of these are coded by nuclear genes; one subunit, cytochrome *b*, is encoded by the mitochondrial *COB* gene. Transcription of the *COB* gene initiates at a promoter upstream of the glutamyl-tRNA gene (Christianson et al. 1983). The resulting di-cistronic primary transcript undergoes multiple processing steps to yield mature *COB* mRNA and the tRNA. Genetic and biochemical studies revealed that in most laboratory strains the *COB* gene is mosaic, composed of six or three exons ("long" and "short" strains, respectively; Haid et al. 1979; Nobrega and Tzagoloff 1980). Three introns (bI2, bI3, and bI4 in the nomenclature used for the long *COB* gene version) are characterized by open reading frames (orfs) in-phase with the preceding exons, which encode proteins (so-called "maturases") required for the removal of the respective introns (Lazowska et al. 1980; Anziano et al. 1982; De La Salle et al. 1982; Weiss-Brummer et al. 1982; Lazowska et al. 1989). Mutations within the maturase-coding intron sequences, which interfere with the formation of functional maturases, lead to the accumulation of *COB* transcripts which still contain the respective intron as well as the orf-containing downstream introns (polar effect). Available evidence suggests that translation of the intron orfs initiates at the authentic *COB* start codon, leading to a chimeric protein composed of exon- and intron-sequences (e.g. Weiss-Brummer et al. 1982). Hence translation of splicing intermediates of *COB* pre-mRNA is an essential pre-requisite for the formation of mature *COB* mRNA and apocytochrome *b* synthesis.

Dedicated to Professor Fritz Kaudewitz, Founding Editor of *Current Genetics*, in recognition of his long service to the Journal

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Nuclear genes involved in the maturation of *COB* pre-mRNA

In an attempt to identify nuclear genes involved in the biogenesis of complex III, several laboratories including that of F. Kaudewitz screened collections of nuclear respiratory defective mutants (*pet* mutants) for strains which showed alterations in the absorption spectra of cytochrome *b* and/or cytochrome c_1, the two heme-carrying components of complex III. These screenings not only led to the isolation of all the nuclear genes encoding the subunits of complex III but, in addition, to a number of genes which are essential for some aspects of complex formation. Analysis of the "Munich collection" of *pet* mutants resulted in the isolation of the cytochrome c_1 structural gene *CYT1* (Lang and

Kaudewitz 1982; Sadler et al. 1984), the cytochrome c_1 heme lyase gene *CYT2* (Zollner et al. 1992), as well as of a number of genes involved in the maturation of *COB* pre-mRNA. Yeast strains harbouring mutations in genes of the latter category fail to produce mature *COB* mRNA at a wild-type level. Instead they either have lowered levels of *COB* transcripts due to a deficiency in 5′-end formation of *COB* mRNA (Dieckmann et al. 1984) or else accumulate *COB* pre-mRNAs which still contain one or more of the intron sequences (Pillar et al. 1983; Tzagoloff and Dieckmann 1990 for a review). While some mutants specifically fail to remove a single intron (McGraw and Tzagoloff 1983; Kreike et al. 1986), others resemble maturase-deficient *mit*⁻ strains in that they accumulate precursor forms of *COB* mRNA with the orf-containing introns. For example, mutants defective in the nuclear genes *CBS1* and *CBS2*, respectively, accumulate *COB* transcripts, which still contain introns bI2, bI3 and bI4, when combined with the long-form *COB* gene. In the context of the short *COB* gene version, the mutants fail to excise intron bI4. Two explanations can be envisaged to explain this phenotype: either CBS1p and/or CBS2p is/are directly engaged in the excision of these introns or one or both of the proteins is/are required for the formation of functional maturases and hence are indirectly involved in the processing step.

Evidence that CBS1p and CBS2p are cytochrome *b*-specific translational activator proteins

A first clue as to the function of CBS1p and CBS2p came from the genetic analysis of respiratory competent revertants of *cbs1* mutants. We were able to show that a rearrangement of the *COB* gene leading to a replacement of the authentic *COB* 5′-leader sequence by the leader sequence of the mitochondrial *OLI1* gene is responsible for suppression (Rödel et al. 1985). The same rearrangement of mtDNA is also able to suppress *cbs2* mutants (Rödel 1986). The definition of the *COB* 5′-untranslated leader as the target site of both CBS1p and CBS2p favored the idea that the proteins are somehow involved in the translation of *COB* transcripts. In a subsequent study it was shown that translation of a chimeric mitochondrial gene, consisting of the intronless *COX3* coding region (which encodes subunit III of cytochrome *c* oxidase) fused to the 5′-untranslated *COB* leader requires a functional *CBS1* gene. By contrast, translation of a *COX3* mRNA derived from the *COX3* gene with its authentic leader is not dependent on CBS1p (Rödel and Fox 1987). This result proved that CBS1p is required for translation of mitochondrial transcripts with the *COB* 5′-untranslated leader sequence. In line with this interpretation Muroff and Tzagoloff (1990) reported that CBS1p and CBS2p are essential for cytochrome *b* synthesis even in case of a *COB* gene completely devoid of introns.

By analyzing the effects of deletions in the 5′-untranslated leader sequence of the *COB* gene on the translation of *COB* transcripts in vivo, Mittelmeier and Dieckmann (1995) were recently able to map the sequence elements of the *COB* leader which are necessary for translational activation by CBS1p and CBS2p. The data are consistent with the hypothesis that mitochondrial ribosomes and the translational activators bind to sites either between –170 and –104 or between –60 and the AUG at +1 of *COB* mRNA.

Further nuclear gene products required for apocytochrome *b* translation

Dieckmann and Tzagoloff (1985) reported on a mutant defective in the nuclear gene *CBP6*, which is able to form wild-type levels of mature *COB* mRNA, but shows only trace amounts of apocytochrome *b*. In contrast to *cbs1* and *cbs2* mutants, *cbp6* mutants cannot be suppressed by rearrangements of mtDNA (Tzagoloff et al. 1988). Nevertheless the authors favor the idea that *CBP6* encodes a factor which enhances apocytochrome *b* translation. In view of the dependence of *COB* mRNA maturation on translation products derived from *COB* pre-mRNA splicing intermediates, this interpretation predicts that translation of mature *COB* mRNA differs in the requirement for auxillary factors from the translation of *COB* pre-mRNA. Alternatively, the reduced concentration of cytochrome *b* may reflect enhanced proteolysis in *cbp6* mutants. There is accumulating evidence that surface hydrophobicity, which is increased in nascent polypeptide chains prior to association with chaperones or assembly with other proteins, is an important determinant of the half-lives of proteins (for a review see Bohley 1996). Perhaps different surface hydrophobicities of apocytochrome *b* and the maturase proteins can account for differential stabilities in *cbp6* mutants.

Cloning and sequence analysis of *CBS1* and *CBS2*

We isolated both the *CBS1* and the *CBS2* genes from a yeast genomic library by functional complementation of *cbs1* and *cbs2* mutants, respectively (Rödel et al. 1986). Both genes map on chromosome IV (Michaelis et al. 1988; Forsbach et al. 1989). Interestingly, the *CBS1* gene lies next to another *pet* gene (*COX9*), which encodes a subunit of cytochrome *c* oxidase (Wright et al. 1986). Sequence analysis revealed that the *CBS1* gene encodes a protein of 233 amino acids with a net positive charge (16.7% basic versus 8.6% acidic residues). The orf of the *CBS2* gene predicted a protein of 389 amino acids, again with a predominance of positively charged amino acids (15.9% basic versus 9.3% acidic residues). Both genes are characterized by low codon-bias indices (–0.02 for *CBS1* and 0.06 for *CBS2*, respectively) suggesting low expression (Bennetzen and Hall 1982). Disruption of either the *CBS1* or the *CBS2* gene in wild-type strains result in respiratory defective strains which have a phenotype identical to that of the original mutants (Rödel et al. 1986). This result indi-

cates that both gene products have no additional function beside that affected in the original mutants.

Comparison of the DNA sequences with those contained in current data bases shows no homology to other gene sequences except for an orf (YHR063c) of unknown function identified on chromosomeVIII of *S. cerevisiae.* This orf has a significant degree of similarity to the *CBS2* orf (Ouzounis et al. 1995). Minor sequence similarities between CBS1p and the yeast mitochondrial translational activator proteins CBP6p (Dieckmann and Tzagoloff 1985), PET111p (Strick and Fox 1987), and PET54p (Costanzo et al. 1989) were noted (Grivell 1989), but the significance of this observation is unclear. The failure to identify homologous genes in other organisms may hint at the possibility that translational activator genes like *CBS1* and *CBS2* are unique in yeast. In this respect it may be worth noting that the unusally long AT-rich leader sequence of the *COB* gene (the target of CBS1p and CBS2p) seems also to be a unique feature of yeast mtDNA.

Subcellular localization of CBS1p and CBS2p

The intracellular localization of both proteins was examined with the aid of specific antibodies. CBS1p was identified as a 23.5-kDa protein within mitochondria, suggesting cleavage of a 3.5-kDa pre-sequence during mitochondrial import. This interpretation is in line with the observation that in vitro translation of *CBS1* mRNA results in the formation of a 27-kDa protein, which is processed to the mature 23.5-kDa form during in vitro import into isolated mitochondria (Körte et al. 1989). Determination of the amino-terminal amino acids of the mature protein allowed the exact determination of the proteolytic processing site between Tyr^{29} and Ile^{30} (Körte et al. 1991). The pre-sequence of the CBS1p precursor protein has all the characteristics of a mitochondrial targeting sequence, i.e. several positively charged amino-acid residues, no acidic residues, and the potential to form an amphiphilic helix (for a review see Hartl et al. 1989).

CBS2p was also identified as a mitochondrial protein. In contrast to CBS1p, there is no evidence for the presence of an amino-terminal pre-sequence. Import of the in vitro synthesized 45-kDa protein into isolated mitochondria is not accompanied by a detectable proteolytic processing protein (Michaelis and Rödel 1990).

A detailed study on the solubilization characteristics showed that both proteins are associated with the IMM: while CBS1p behaves like an integral membrane protein, CBS2p seems to be loosely associated with the membrane by electrostatic forces (Michaelis et al. 1991). There is some evidence that CBS2p may be in contact with the small mito-ribosomal subunit. Based on all these available data we proposed a model, according to which the intra-mitochondrial localization of CBS1p and CBS2p, as well as their postulated affinity to the *COB* RNA leader, ensure that translation of the *COB* transcripts initiates only in the context of the membrane (Michaelis et al. 1991). In this way co-translational insertion into the membrane can occur and aggregation of the hydrophobic apocytochrome *b* in the soluble compartment of the mitochondrial matrix can be avoided.

As a consequence of our model, not only apocytochrome *b* but also the maturase proteins would be synthesized at the membrane, suggesting that the IMM is the preferential site of *COB* RNA processing.

A link between translation and assembly of cytochrome *b*

During assembly of the cytochrome bc_1 complex, apocytochrome *b* is incorporated in a sub-complex, which is composed of six of the nine subunits (Crivellone et al. 1988; Schmitt and Trumpower 1991). Covalent attachment of heme seems to occur at a later stage of assembly of the enzyme (Tzagoloff et al. 1988). As in the case of cytochrome *c* oxidase, nuclear genes have been identified which are specifically required for the assembly of complex III (Wu and Tzagoloff 1989; Bousquet et al. 1991). Of special interest is the observation that one of these genes, *ABC1*, can suppress the respiratory deficiency of a *cbs2* mutant, when present in multicopy (Bousquet et al. 1991). The *ABC1* gene product cannot substitute for the CBS2p, because the suppressor activity is specific for the *cbs2-223* allele. It was proposed that ABC1p might represent a chaperonin, which is involved in the correct folding and/or assembly of apocytchrome *b*. As *ABC1* in multicopy can restore the respiratory capacity in a mutant defective in a cytochrome *b* translational activator gene, folding and assembly of apocytochrome *b* seem to occur during the translation process.

Expression of *CBS1* and *CBS2*

As outlined above the low codon-bias indices of *CBS1* and *CBS2* indicate low expression. Northern analyses confirmed this prediction (Michaelis et al. 1988; Forsbach et al. 1989). Expression of *CBS1* is subject to regulation by glucose and by oxygen. There is evidence that regulation of *CBS1* expression is mediated at least in part by the HAP2/3/4 system, which plays an important role in the communication between the nucleus and the mitochondria (see Forsburg and Guarente 1989 for a review; Schlapp 1992). As for *CBS1* RNA, the concentration of *CBS2* RNA is lower in cells cultured under anaerobic growth conditions compared to aerobically grown cells, although the reduction (2-fold) is less pronounced than for *CBS1* (9-fold). The promoter region of *CBS2* is characterized by an oligo(dA-dT) tract of 23 bp, a sequence motif which is suggested to interfere with nucleosome formation (Kunkel and Mortinson 1981) and to act as an upstream promoter element for constitutive transcription in yeast (Struhl 1985). There is experimental evidence that the oligo(dA-dT) se-

quence of the *CBS2* promoter defines a DNase-hypersensitive site, presumably reflecting a nucleosome-free region in the chromatin (W. Hörz, personal communication). A promoter fragment covering the (dA-dT) tract is bound by DAT1p, a DNA-binding protein which recognizes such sequences (Winter and Varshavsky 1989; Schlapp 1992). It is currently unknown whether DAT1p also binds to the *CBS2* promoter in vivo. An interesting aspect of *CBS2* expression comes from the observation that a transcript of the divergently transcribed 5′-flanking gene is complementary to the *CBS2* transcript over a distance of 77 bases (Schlapp and Rödel 1990). It is not known whether this natural antisense RNA has any influence on *CBS2* expression.

We have currently no indication for post-transcriptional regulation of *CBS1* and *CBS2* expression as observed in the case of *PET494*, which encodes one of the activator proteins of *COX3* mRNA translation (Costanzo and Fox 1986). Regulation of *PET494* expression by oxygen appears to occur at the translational level (Marykwas and Fox 1989). Translational regulation of *CBS1* expression via a small orf in the 5′-leader of the *CBS1* transcript, which initiates with an AUG initiation codon, could be excluded by site-directed mutagenesis (Krummeck et al. 1991). There was no indication that this orf affects the scanning of ribosomes.

It is presently unknown whether the translational activator proteins CBS1p and CBS2p – in addition to their postulated function as determinants of the site of apocytochrome *b* translation – are involved in the regulation of cytochrome *b* expression. This would be the case if the concentration of one or both of the proteins is lower than that of *COB* transcripts and thus becomes rate-limiting for the synthesis of apocytochrome *b* and the maturases, respectively.

Current studies

Current studies primarily aim at the identification of mitochondrial proteins which physically interact with CBS1p and/or CBS2p by genetical means as well as by use of the two-hybrid system. In the case of the *COX3* mRNA-specific translational activator proteins the latter assay was successful in establishing interactions between PET54p and PET122p as well as between PET54p and PET494p (Brown et al. 1994). In the two-hybrid system proteins are fused to either the *GAL4* DNA-binding (DB) domain or the *GAL4* transcriptional activating domain (AD) and tested for physical association as measured by the expression of a reporter gene dependent on a reconstituted GAL4p (Fields and Song 1989). Interestingly, high-level expression of a GAL4-DB-CBS1p fusion protein, devoid of the mitochondrial targeting sequence, can complement the respiratory deficient phenotype of *cbs1* mutants (Krause et al., unpublished results). This observation confirms reports on the entry into mitochondria of proteins lacking amino-terminal targeting signals upon over-expression (Körte et al. 1989; Dircks and Poyton 1990; Brown et al. 1994) and suggests that the CBS1p moiety can fold into its normal conformation. Biological activity is lost when the 18 carboxy-terminal amino acids of CBS1p are removed (Krause et al., unpublished results). Studies are underway to test whether this loss of activity results from an inability of the protein to associate with the mitochondrial membrane.

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