Regulators of mitochondrial translation in *Saccharomyces cerevisiae*

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Chapter I

Introduction
Contents of the introduction

**Saccharomyces cerevisiae: pet organism for molecular biologists**
 Mitochondrial biogenesis 11
 Mitochondrial organization 12
 Complex assembly 13

**Mitochondrial protein synthesis**

**mRNA-specific mitochondrial translational activators**

- PET111: translational activator of cytochrome c oxidase subunit 2 17
- PET54, PET122 and PET494: translational activators of cytochrome c oxidase subunit 3 18
- CBP6, CBS1 and CBS2: translational activators of apocytochrome b 19
- AEP1 and AEP2: translational activators of ATP-synthase subunit 9 19

**A model for translation initiation in yeast mitochondria**

**Translation of cytochrome c oxidase subunit 1**

- PET309 and MSS51: translational activators of cytochrome c oxidase subunit 1 21

**Aim of this thesis**

Page 11
Page 12
Page 13
Page 14
Page 17
Page 18
Page 19
Page 19
Page 21
Page 22
Eukaryotic (nucleated) cells are the basic unit of structure in plants, metazoan animals, protozoa, fungi and algae (Stanier et al., 1988). Despite the diversity that has arisen as a result of evolutionary specialization, the basic architecture of the cells in these groups has many common features. The cells are bounded by a surface membrane (the cytoplasmic membrane) and within the cytoplasm, the eukaryotic cells contain several unique membrane systems: the nuclear envelope, the endoplasmic reticulum, the Golgi apparatus, and one or two types of membrane-bound organelles that house the electron transport machinery of the cell. These membrane-enclosed organelles are the chloroplasts and mitochondria, and contain photosynthetic and respiratory electron transport systems, respectively. Both organelles share the same basic structure: an outer membrane, enclosing the topologically more complex inner membrane system in which the components of electron transport are embedded. Chloroplasts have an additional membrane system, the thylakoid, which contains the photosynthetic apparatus. Synthesis of chloroplasts and mitochondria is directed in part by the nuclear genome and in part by the genome located in the organelle itself. Organelle genomes are characteristically present in multiple copies. In the majority of organisms, chloroplast and mitochondrial genomes are covalently closed circular molecules of double stranded DNA. While chloroplast genomes are relatively constant in size and gene content, mitochondrial genomes are much more diverse.

Saccharomyces cerevisiae: pet organism for molecular biologists

The subject of the study presented in this thesis, Saccharomyces cerevisiae, is a powerful tool for molecular biologists interested in the biology of the eukaryotic cell (Botstein and Fink, 1988). The evolutionary conservation of cellular processes within higher eukaryotes and the many possibilities to manipulate the mitochondrial biogenesis make the unicellular eukaryote Saccharomyces cerevisiae an highly attractive organism. Yeast cultures are easily grown and handled under laboratory conditions, and the organism lends itself to classical genetics, transformation with foreign DNA and gene-replacement techniques. S. cerevisiae is the first eukaryote for which a complete genomic sequence was determined (Johnston, 1996). Despite advances in other systems, the facultative anaerobic yeast S. cerevisiae is still a highly attractive experimental organism for the study of mitochondrial function and assembly (Grivell et al., 1999). The fact that the mitochondrial respiratory function does not affect the viability on fermentable carbon sources as long as cells can depend on glycolysis, makes Saccharomyces cerevisiae the organism of choice to perform mutational analysis of the mitochondrial function and assembly. Additionally, the identification of novel mitochondrial proteins and the genome-wide study of changes in gene expression is greatly facilitated by availability of the full genomic sequence data. It is likely that yeast will be the first organism for which a complete inventory of mitochondrial proteins and their function can be drawn up (Grivell et al., 1999). For this reason, yeast should provide a useful testbed to verify ideas about mitochondrial dysfunction in man and other organisms.

Mitochondrial biogenesis

There are several reasons to study mitochondrial biogenesis. Most of the energy required for all cellular processes is derived from reactions taking place in the mitochondrion and key enzymes for major metabolic routes are localized in this organelle. Since mitochondria are involved in a number of central cellular processes and in metazoans play an important role in triggering events that lead to apoptosis, the organelle has gained the interest of researchers to study defects that contribute to the pathogenesis of a number of degenerative diseases in man. So far, about 50 disease-producing mutations in mtDNA
have been characterized, and an increasing number turns out to involve nuclear genes (DiMauro et al., 1998). Assembly of mitochondria depends on balanced synthesis of a handful proteins encoded by mitochondrial DNA (mtDNA) with several others encoded by nuclear genes. The nuclear and the mitochondrial genomes interact in at least two ways. First, both contribute to mitochondrial protein function and mitochondrial gene products function in multimeric complexes that contain nuclear-encoded components as well. Second, the nuclear genome encodes a large number of PET genes that function either in regulating the expression of mitochondrial genes or in the assembly of respiratory proteins (reviewed by Grivell, 1995; Poyton and McEwen, 1996).

**Mitochondrial organization**

Although the physical form of the molecule in vivo remains controversial, the sequence of the mtDNA of *S. cerevisiae* can be assembled into a circular map with a length of 85,779 bp (Foury et al., 1998). One of the most striking characteristic features of the genome is the presence of extensive intergenic spacers composed of quasi-pure A+T stretches several hundreds of base pairs in length (De Zamâroczy and Bernardi, 1986a). The genome encodes cytochrome c oxidase subunits 1, 2 and 3 (COX1, COX2 and COX3), apocytochrome b (COB), subunits 6, 8 and 9 of the mitochondrial ATP synthase (ATP6, ATP8 and ATP9) and a ribosomal protein (VAR1) (De Zamâroczy and Bernardi, 1986b). Other proteins encoded by *S. cerevisiae* mtDNA include intron-encoded proteins in COX1 and COB, that act as RNA maturases, reverse transcriptases and homing endonucleases. In addition the genome codes for 24 tRNAs that can recognize all codons, for the large and small ribosomal RNAs (21S and 15S, resp.), and for the 9S RNA component of RNase P (involved in tRNA processing). All genes are transcribed from the same strand, except tRNAthr1. Recently, the complete sequence of the mtDNA of the strain used for sequencing of the nuclear genome was published (Foury et al., 1998). The general organization of the mitochondrial genome was confirmed, while seven hypothetical ORFs were identified and hot spots of point mutations were found.

<table>
<thead>
<tr>
<th>Table I: Mitochondrial proteins in <em>S. cerevisiae</em> ¹</th>
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<tbody>
<tr>
<td>outer membrane</td>
</tr>
<tr>
<td>intermembrane space</td>
</tr>
<tr>
<td>inner membrane</td>
</tr>
<tr>
<td>matrix</td>
</tr>
</tbody>
</table>

¹ Data obtained from the Yeast Proteome Database (http://www.proteome.com), modified July 8 2001.

The majority of mitochondrial proteins are encoded by nuclear genes and imported into the mitochondria (Tzagoloff and Dieckman, 1990). Over the last year the number of genes predicted to encode mitochondrial proteins has increased from 340 (as of March 1999) to the current 470 (as of July 2001) out of a total gene complement of about 6,147 (Hodges et al., 1999). These proteins are synthesized in the cytoplasm and subsequently transported into the mitochondrion by protein complexes in the outer and inner membranes (Kubrich et al., 1995; Pfanner et al., 1994). The imported proteins account for an estimated 90% of the protein mass in mitochondria, and they are distributed in all four mitochondrial compartments: outer membrane, inter membrane space, inner membrane and matrix. The numbers of proteins of known mitochondrial localization are summarized in Table I.
Proteins involved in complex assembly and protein degradation as well as translational activators are associated with the inner membrane. The enzymes responsible for maintenance and expression of mtDNA and for function of the TCA-cycle are present in the mitochondrial matrix. A survey of the information in the Yeast Proteome Database for mitochondrial proteins grouped according to function and/or molecular environment shows that the majority of mitochondrial proteins is involved in mitochondrial gene expression. Major functional classes include proteins involved in mitochondrial protein synthesis (108, including 72 ribosomal proteins); proteins involved in translational activation (10); DNA-associated proteins (22); respiratory complex assembly factors (17). These figures emphasize the investment that is required to maintain the mitochondrial genetic system and the ability to synthesize only seven respiratory chain proteins encoded by it. Translation and respiratory complex assembly are particularly expensive processes (Grivell et al., 1999). Both are still poorly understood, with many steps occurring with the help of membrane-associated proteins.

Complex assembly

All mitochondrially encoded proteins, except the ribosomal protein Var1p and the intron-encoded proteins, are hydrophobic proteins that assemble into the respiratory complexes of the inner membrane (Grivell, 1995). NADH dehydrogenase (Complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV) together form the respiratory chain, which is indispensable for mitochondrial function (Rep, 1996). In other organisms, complete 3D structures for cytochrome c oxidase (Tsukihara et al., 1996) and ubiquinol:cytochrome c oxidoreductase (Iwata et al., 1998) have been presented. Both genetic and biochemical studies have added new factors to a growing list of nuclear-encoded factors required for the assembly of mitochondrial protein complexes (Grivell, 1995; Poyton and McEwen, 1996), however, the assembly pathways have not been elucidated.

Cytochrome c oxidase catalyzes the last electron transfer reaction of the respiratory chain: oxidation of cytochrome c and reduction of oxygen to water. Electron transfer is coupled to proton translocation across the membrane. Yeast cytochrome c oxidase consists of eleven subunits (Geier et al., 1995), three subunits are mitochondrially encoded (Cox1p, Cox2p and Cox3p: Rubin and Tzagoloff, 1973). The three mitochondrially encoded proteins are the largest subunits of the complex. It is likely that assembly of this complex occurs in the inner membrane and that it is assisted by specific assembly factors. For cytochrome c oxidase eight of these specific factors have been identified (for review: Rep, 1996; Poyton and McEwen 1996). Studies with strains carrying mutations in cytochrome c oxidase subunits suggested that assembly of the mitochondrially encoded subunits begins with insertion of Cox1p into the inner membrane, followed by association with Cox2p, Cox4p and Cox3p (Glerum and Tzagoloff, 1997; Lemaire et al., 1998). These studies indicate that Cox1p is a key subunit in the assembly of the enzyme. Loss of Cox1p has very drastic effects on the assembly of cytochrome c oxidase and the function of mitochondria. Cox1p not only forms the assembly core of cytochrome c oxidase. Together with Cox2p and Cox3p this subunit contains all redox centers (Cooper et al., 1991): two hemes (a and a3) and Cu2+ are bound by Cox1p. These three subunits, forming the functional core of cytochrome c oxidase are highly conserved across kingdoms (Kadenbach et al., 1991). Knowledge of the synthesis of subunit 1 of cytochrome c oxidase in yeast might add new insights in the assembly and (dys)function of cytochrome c oxidase in other organisms. A precise picture of the process will provide a better basis for study of mutants affecting Cox1p and cytochrome c oxidase. While 18 nuclear genes have been identified for the expression of COX1 in S. cerevisiae (reviewed by Pel et al., 1992a), little is known about
translation of the COX1 mRNA. For three mitochondrially encoded proteins (Cox2p, Cox3p, and Cobp) translation factors have been extensively studied, these specific translational activators have been suggested to localize the translation of the respiratory subunits at the inner membrane (reviewed by Fox, 1996).

Mitochondrial protein synthesis

Looking at mitochondrial protein synthesis in *Saccharomyces cerevisiae*, the first major characteristic is the fact that the mitochondrial genetic code differs from the universal genetic code (Fox, 1987). In *S. cerevisiae*, the termination codon UGA is a sense codon and encodes Trp, while AUA encodes Met instead of the standard Ile. Yeast mitochondria use only a restricted number of 24 tRNAs that is below the minimum number of 32 considered to be necessary to translate all the codons of the genetic code according to the wobble hypothesis, but simplified decoding rules allow recognition of all codons (reviewed by Martin et al., 1990).

The mitochondrial ribosome (mitoribosome) resembles the prokaryotic ribosome. There is similarity between components of the mitochondrial translation machinery and that of bacteria, they are largely similar in size and sedimentation coefficients (Kitakawa and Isono, 1991). Especially mitochondrial elongation factors show a high similarity with bacterial elongation factors. The mitochondrial translation machinery is inhibited by almost the same spectrum of antibiotics as that of prokaryotes (Borst and Grivell, 1971). Protein synthesis in all mitochondria is inhibited by D-chloramphenicol, also antibiotics that interfere with bacterial protein synthesis inhibit the mitochondrial ribosome (tetracycline, lincomycin, erythromycin, neomycin). Mitochondrial protein synthesis is not inhibited by cycloheximide, emetine and anisomycin, which are inhibitors of the cytoplasmic protein synthesis.

The yeast mitoribosome consists of more different proteins than its cytoplasmic and prokaryotic ribosomes (Kitakawa and Isono, 1991). About half of the yeast mitoribosomal proteins (MRPs) characterized so far show a high degree of similarity to eubacterial ribosomal proteins, while they do not share any of their constituents with the cytoplasmic ribosomes. It is not clear whether the previously mentioned number of 72 yeast MRPs represents a complete set. So far, MRPs have been identified by genetic means and direct protein sequencing and both approaches are still ongoing. The availability of a complete sequence of the genome makes it possible to identify the genes encoding ribosomal proteins by computer search (Kitakawa et al., 1997). Graack and Wittman-Liebold (1998) attempted to identify the yeast MRPs directly on the basis of their association with ribosomal subunits. The yeast mitoribosome appears to contain at least 90 proteins and the actual number may exceed 100. However, proteins may have been lost from the ribosomal subunits during the isolation procedure, and due to lack of a functional assay it can not be concluded whether a protein is actually required for ribosome function. While the total molecular mass of the mitoribosomes is at least that of bacterial ribosomes, the number of MRPs is much higher than in bacteria (55 in *E. coli*) or in eukaryotic cytoplasmic ribosomes (75-76 in yeast; 70 in rat). Once all the MRPs have been identified, assignment of MRP functions within the mitoribosome is the next task. The proteins which are unrelated to ribosomal proteins from other organisms and that are essential for ribosomal function, might have been recruited during the course of evolution from other sources unrelated to ribosomes. Functions of these MRPs cannot be deduced from sequence similarity. The direct assignment of MRP function is difficult, since no *in vitro* protein synthesis system capable of accurately translating mitochondrial mRNAs is available (Dekker et al., 1993a).
Mitochondrially encoded Var1p is a mitochondrial translation product with very unusual features. The VAR1 gene is unusual in its base composition, nearly 90% A+T. Gene and gene product show a strain-dependent variation in size of up to 7 percent, but this variation does not detectably interfere with the function of Var1p (Butow et al., 1985). The tight association of Var1p with the small subunit of the mitoribosome was shown by radioactive labelling of mitochondrial translation products and comparison with ribosomal constituents (Groot et al., 1979). In a two dimensional electrophoretic separation of ribosomal proteins, Var1p is the only major labeled polypeptide species to be resolved when cells are labeled with $^{35}$SO$_4^{2-}$ in the presence of cycloheximide. Var1p is specifically associated with the 38S mitochondrial ribosomal subunit. It cannot be removed by a high salt wash, indicating that it is an integral ribosomal protein (Terpstra et al., 1979). For the assembly of the mature small ribosomal subunit, mitochondrial protein synthesis is essential. Since Var1p is the only mitochondrial translation product, this suggests that Var1p is essential for assembly or stability of the small subunit (Maheshwari and Marzuki, 1984; 1985). A marked decrease in the synthesis of Var1p in a temperature-conditional mutant resulted in loss of the small ribosomal subunit (Hibbs et al., 1987). To study the structure, function and synthesis of Var1p, a nuclear version of the VAR1 gene was engineered (Sanchirico et al., 1995). The nuclear encoded Var1p was imported into the mitochondria and was shown to be associated with the small ribosomal subunit. This relocation experiment provides a tool for molecular genetic analysis of structure function relationships in the unusual VAR1 gene, and possibly in other mitochondrially encoded genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MEF1</td>
<td>mitochondrial elongation factor</td>
<td>Vambutas et al., 1991</td>
</tr>
<tr>
<td>IFM1</td>
<td>mitochondrial translation initiation factor</td>
<td>Vambutas et al., 1991</td>
</tr>
<tr>
<td>MEF2</td>
<td>mitochondrial translation initiation factor</td>
<td>Rasmussen et al., 1995</td>
</tr>
<tr>
<td>TUF1</td>
<td>mitochondrial elongation factor</td>
<td>Nagata et al., 1983</td>
</tr>
<tr>
<td>MRF1</td>
<td>mitochondrial peptide chain release factor</td>
<td>Pel et al., 1992b</td>
</tr>
</tbody>
</table>

Our knowledge of the mitoribosome contrasts with the still poorly understood characterized general factors involved in mitochondrial protein synthesis. Over the last years, only a small number of general factors has been identified by similarity, these factors are summarized in Table II. The MEF1 gene shows 20-23% identity to eukaryotic EF2, the IFM1 gene is 30-35% similar to bacterial initiation factor 2 (Vambutas et al., 1991). The MEF2 gene encodes a mitochondrial translation factor, that promotes GTP-dependent translocation of the nascent chain from the A-site to the P-site of the ribosome (Rasmussen, 1995). Using cross-hybridization with the E.coli EF-Tu gene, the gene for the mitochondrial translation elongation factor Tu, TUF1, was identified (Nagata et al., 1983). Even slight overproduction of this protein suppresses the effect of two mutations affecting tRNA maturation (Rinaldi et al., 1997). In yeast mitochondria no EF-Ts could be detected, mitochondrial EF-Tu is active in the absence of additional factors and the activity is not stimulated by the addition of E. coli EF-Ts (Rosenthal and Bodley, 1987). The gene for a mitochondrial peptide chain release factor has been cloned by a genetic approach (Pel et al., 1992b). Sequence comparison showed high similarity to E. coli release factor RF-1 and to release factors from Gram-positive and Gram-negative bacteria (Pel et al., 1992c). Mrf1p reduced nonsense suppression in yeast mitochondria and inactivation of its gene led to rho$^-$ induction (Pel et al., 1992a). The Mrf1 protein interacts with the mitoribosome (Pel et al., 1993). In addition to these general factors, each mitochondrial mRNA is likely to have specific factors for translation initiation. The specific characteristics of the 5' leaders of
mitochondrial mRNAs differ significantly from other mRNAs and this has implications for the mechanism of translation initiation in mitochondria.

The mature mRNAs are the product of processing of multigenic precursor transcripts and splicing (in the case of COX1 and COB). In contrast to the mammalian mitochondrial mRNAs that have very short or no leader sequences, yeast mitochondrial mRNAs contain long 5' and 3' untranslated regions (UTRs). These UTRs are extremely A+U rich, and vary in size from fifty up to several hundred nucleotides. Another important difference is that yeast mitochondrial mRNAs do not possess poly-A tails and are not capped at their 5'-ends. Often, many leaders contain several potential initiation sites and probably possess stable secondary structures due to G+C rich clusters. These last features seem inconsistent with a scanning model for translation initiation as proposed for eukaryotic cytoplasmic translation. In mammalian and yeast cytoplasm, stable secondary structures in the leaders of the mRNA impede the scanning by the 40S ribosomal subunit (Bairn and Sherman, 1988; Cigan et al., 1988; Kozak, 1991a). Rarely occurring upstream AUGs in cytoplasmic transcripts of both yeast and higher eukaryotes have a negative effect on translation initiation at the downstream start codon (Cigan and Donahue, 1987; Kozak, 1991b), although in some cases regulatory functions have been described (Hinnebusch and Leibman, 1991). In bacteria and picornavirus mechanisms of internal initiation have been described (Jackson et al., 1990).

In bacteria, initial binding of mRNAs to ribosomes depends on recognition and pairing between Shine-Dalgarno sequences in the mRNA 5'-UTR and a complementary sequence near the 3'-end of 16S rRNA. Similar complementary sequences between the mRNA and the small subunit rRNA can also be detected in mitochondria (Li et al., 1982). However, mRNAs lacking these elements are still active in protein synthesis (Costanzo and Fox, 1988), indicating these elements are not essential. By changing the translation initiation codon of the COX2 mRNA from AUG to AUA translation was reduced at least five-fold, without affecting the steady-state level of the mRNA. Translational initiation was still occurring at the AUA codon and not at the next AUG codon downstream (Mulero and Fox, 1994). Like in bacteria, mRNAs with non-AUG initiators were translated at reduced levels which emphasises the importance of secondary structures (Folley and Fox, 1991). All this indicates that sequences surrounding the initiation codon are not the only factors determining translation initiation in yeast mitochondria, as might also be the case in other systems.

Previously, we have shown that a nuclear encoded protein binds specifically and with high affinity to the 5'-UTR of all mitochondrial mRNAs (Papadopulou et al., 1990; Dekker et al., 1992). This protein was identified as the TCA-cycle enzyme (NAD⁺-)dependent isocitrate dehydrogenase (Idh) (Elzinga et al., 1993). A stem-loop structure in the 5'-UTR was shown to be recognized by Idh (Dekker et al., 1992). The enzyme consists of a heterodimer formed between related subunits encoded by the nuclear IDH1 and IDH2 genes (Cupp and McAllister-Henn, 1991; 1992). Both catalytic and RNA-binding activities depend on a native heterodimeric enzyme. This double role of Idh makes the enzyme one of a growing list of dual-function RNA-binding proteins. Many other enzymes have been found to be RNA-binding proteins, e.g. aconitase (Roualt et al., 1988;1990), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Singh and Green, 1993), thymidylate synthase (TS; Chu et al., 1993), enoyl-CoA hydratase (Nakagawa et al., 1995), MnSOD (Fester and Schuster, 1995), catalase (Clerch et al., 1996), glutamate dehydrogenase (GDH; Preiss et al., 1997; Bringaud et al., 1997). It has been suggested that the RNA-binding domain present in these enzymes evolved from a dinucleotide binding site, or vice versa (Hentze, 1994). We have hypothesized that the combination of dehydrogenase and RNA-binding activities in a single protein may form a regulatory link between the need for mitochondrial function and
rate of biogenesis (Elzinga et al., 1993). In vitro translation experiments showed increased translation of mitochondrial translation products in mitochondria of an idh-null strain (De Jong et al., 2000). Binding of Idh to mitochondrial mRNAs might modulate or repress the level of translation of the hydrophobic respiratory subunits by keeping the mRNAs in the mitochondrial matrix. At the moment of need for more respiratory capacity the mRNAs are tethered to the inner membrane by the specific translational activators to synthesize the proteins of the respiratory subunits.

mRNA-specific mitochondrial translational activators

The translation of mitochondrially encoded transcripts depends on mRNA-specific translational activators encoded in the nucleus (reviewed by Fox, 1996). For four of the eight major mRNAs one or more nuclear-encoded gene-specific translational activators have been identified (Table III). These activator proteins are associated with the inner membrane, recognize sites in the 5'-UTR and interact with the mitoribosomal small subunit. Through these interactions the insertion of translation products into the appropriate respiratory complexes might be facilitated.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Activator</th>
<th>Localization</th>
<th>Interaction</th>
<th>References</th>
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<tbody>
<tr>
<td>COB</td>
<td>CBS1</td>
<td>inner membrane</td>
<td>5'-UTR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBS2</td>
<td>associated with inner membrane</td>
<td>5'-UTR, small subunit of mitoribosome</td>
<td>Rödel, 1986a</td>
</tr>
<tr>
<td></td>
<td>CBS2</td>
<td>inner membrane</td>
<td>under investigation</td>
<td>Rödel, 1986b</td>
</tr>
<tr>
<td>COX1</td>
<td>MSS51</td>
<td>associated with inner membrane</td>
<td>5'-UTR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PET309</td>
<td>inner membrane</td>
<td>5'-UTR</td>
<td></td>
</tr>
<tr>
<td>COX2</td>
<td>PET111</td>
<td>inner membrane</td>
<td>5'-UTR</td>
<td></td>
</tr>
<tr>
<td>COX3</td>
<td>PET54</td>
<td>associated with inner membrane</td>
<td>5'-UTR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PET122</td>
<td>inner membrane</td>
<td>5'-UTR, small subunit of mitoribosome</td>
<td></td>
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<td></td>
<td>PET494</td>
<td>inner membrane</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PET111</td>
<td>inner membrane</td>
<td>5'-UTR</td>
<td></td>
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</table>

PET111: translational activator of cytochrome c oxidase subunit 2

The nuclear gene PET111 was shown to be required for translation of the mitochondrial mRNA encoding cytochrome c oxidase subunit 2 (Poutre and Fox, 1987). Studies with chimeric mRNAs showed that Pet111p acts specifically at a site in the 54 nucleotides 5'-UTR of COX2 mRNA (Mulero and Fox, 1993a). Mutations generated in the 5'-UTR of COX2 mRNA, could be suppressed by a missense mutation in PET111 (Mulero and Fox, 1993b). Levels of Pet111p are rate-limiting for Cox2p expression and overproduction of PET111 partially suppresses the respiration-deficient phenotype of a mutant in which the start codon of COX2 is changed from AUG to AUA (Mulero and Fox, 1994). Translation of heterologous mitochondrial mRNAs containing bases -16 to -47 of the COX2 5'-UTR can be activated by Pet111p (Dunstan et al., 1997). This stretch contains four regions that are crucial to translation: a possible stem-loop structure at -20 to -35, the -16 to -19 region, the C-residue at -46 and the -36 to -46 region. Interestingly, the possible stem loop at -20 to -35 contains a conserved UCUAA sequence which is part of the COX2 mRNA sequence that is bound by Idh (Dekker et al., 1992). Binding of Pet111p and Idh could be mutually exclusive, translation might be activated when Idh is displaced by Pet111p.
PET54, PET122 and PET494: translational activators of cytochrome c oxidase subunit 3

The first gene to be identified as necessary for translation of COX3 mRNA was the nuclear PET494 gene (Müller et al., 1984). Mitochondrial rearrangements that replace the normal 5'-UTR with the leaders of other mitochondrial mRNAs suppressed pet494 mutations. These same rearrangements suppressed pet54pet494 double mutants, suggesting that PET54 acts together with PET494 in COX3 translation (Costanzo et al., 1986). Suppression by rearrangements in the mRNA indicates an interaction with the 5'-UTR of the COX3 mRNA. Localization studies showed that Pet494 protein is an integral inner membrane protein, while half of the Pet54 protein is associated with the inner membrane and the other half is in soluble form (McMullin and Fox, 1993). As a third nuclear encoded protein necessary for expression of COX3 mRNA, PET122 has been identified (Kloekener-Gruissem et al., 1988; Ohmen et al., 1988). Pet122p is integral inner membrane protein (McMullin and Fox, 1993). Pet54p is also involved in splicing intron a15β of COX1 pre-mRNA (Valencik et al., 1989). Insertion of 4 amino acids within the Pet54 polypeptide identified different functional domains (Valencik and McEwen, 1991). Levels of Pet54p are not rate-limiting for COX3 expression, while levels of Pet122p and Pet494p are rate-limiting (Folley and Fox, 1991; Steele et al., 1996). Respiratory growth of a mutant with reduced COX3 translation was sensitive to decreased dosage of genes PET122 and PET494. Levels of a mitochondrial reporter gene vary between strains under- and overexpressing translational activator subunits, in particular Pet494p (Marykwas and Fox, 1989). Regulation of Pet494p appears to occur at the translational level. Glucose grown cells express four to six fold lower levels of Pet494p than cells grown on ethanol and glycerol.

Mutations in one of these three genes abolished translation of a chimeric mRNA bearing the COX3 5'-UTR and the coding sequence of COB, showing that all three products act within the COX3 5'-UTR (Costanzo and Fox, 1988). Interaction of Pet54p, Pet122p and Pet494p with the 5'-UTR occurs at least 172 nucleotides upstream of the initiation codon. Mutation of the translation initiation codon AUG to AUA had no effect on the levels of Cox3p, showing that the site of interaction is specified by other sequence or structural features (Folley and Fox, 1991). Deletion of the entire 5'-UTR completely prevented translation. A region of 151 bases, between -480 and -330, containing sequences that are sufficient for translational activation could be defined (Wiesenberger et al., 1995). The respiratory defect of a pet54 missense mutation was enhanced by a T insertion between bases -428 and -427 (Costanzo and Fox, 1995). This suggests that the -400 region is important for Pet54p-COX3 mRNA interaction. Overproduction of Pet494p weakly suppresses several COX3 5'-untranslated leader mutations affecting translation, co-overproduction of Pet122p more strongly suppresses such mutations (Costanzo and Fox, 1993; Wiesenberger and Fox, 1995). A dominant mutation in PET122 and overproduction of Pet122p suppress the respiratory defect of a pet54 missense mutant (Brown et al., 1994). Taken together, these data indicate that the three translational activators interact with the COX3 mRNA.

Analysis of C-terminal truncations of Pet122p identified PET123, MRP1 and MRP17 as suppressors of these mutations (Hafter et al., 1990; McMullin et al., 1990; Hafter et al., 1991; Hafter and Fox, 1992). All three proteins are mitoribosomal proteins of the small subunit. Pet123p and Mrp1p interact functionally with each other (Hafter et al., 1991). These findings indicate that Pet122p promotes translation initiation of the COX3 mRNA through an interaction with the small subunit of the mitoribosome. Two-hybrid analysis showed interactions between Pet54p, Pet122p and Pet494p (Brown et al., 1994). It was suggested that the activators form a complex that mediates the interaction of the COX3 mRNA with the mitoribosome at the surface of the inner membrane.
Translation of apocytochrome b (COB) mRNA requires the products of three nuclear genes: CBP6, CBS1 and CBS2 (reviewed by Rödel, 1997). In a mutant expressing wild type levels of COB mRNA the absence of the Cob protein was the result of missense mutations in CBP6 (Dieckmann and Tzagoloff, 1985). Mutants in CBP6 cannot be suppressed by mitochondrial gene rearrangements that place heterologous 5'-UTRs on the COB mRNA. Cbp6p has been suggested to stabilize the nascent polypeptide chain prior to association with chaperones or assembly with other proteins (Rödel, 1997).

CBS1 and CBS2 were isolated from a yeast genomic library by functional complementation of cbs1 and cbs2 mutants, respectively (Rödel, 1986a). Mutations in both genes can be suppressed by fusion of the COB gene with the 5'-UTR of the ATP9 gene (Rödel, 1986b), indicating that unlike Cbp6p these two proteins interact with the 5'-UTR of COB mRNA. Analysis of deletions in the 5'-UTR mapped the site of action of the translational activators either between -170 and -104 or between -60 and the AUG at +1 of the COB mRNA (Mittelmeier and Dieckmann, 1995). Cbs1p behaves as an integral inner membrane protein, while Cbs2p behaves as a soluble protein with some characteristics of a membrane-associated protein (Michaelis et al., 1991). There is some evidence that Cbs2p may be in contact with the small ribosomal subunit (Michaelis et al., 1991). The C-terminus of Cbs2p is important for its function (Tzchoppe et al., 1999). At the transcriptional level, the expression of both genes is lower in cells cultured under anaerobic growth, expression of CBS1 is subject to regulation by glucose (Rödel, 1997).

AEP1 and AEP2: translational activators of ATP-synthase subunit 9
Two nuclear genes are required for the expression of subunit 9 of the ATP-synthase subunit 9 (Atp9p). At the non-permissive temperature temperature-conditional mutants showed deficiencies in subunits of the ATP-synthase complex, primarily Atp9p (Payne et al., 1991; Ziaja et al., 1993). The mutants indicated that two nuclear genes, AEP1 and AEP2, are required for synthesis of Atp9p (Finnegan et al., 1991, Payne et al., 1993,). Mutations in the AEP2 gene suggested that this factor is most likely to affect either translation or processing of the transcript (Ackerman et al., 1991). A mutation in the 5'-UTR of the ATP9 gene could suppress a temperature-sensitive mutation in the AEP2 gene, indicating that Aep2p may interact with the 5'-UTR of the ATP9 mRNA (Ellis et al., 1999).

A model for translation initiation in yeast mitochondria
The fact that there are distinct translational activators for the mitochondrially encoded mRNAs led to the speculation that translation of these mRNAs occurs at a discrete number of sites on the inner membrane. It has been suggested that there are only a very limited number of sites where translation of COX2 and COX3 mRNA can initiate, in this way, the expression of respiratory chain subunits might be modulated in response to the expression level of the translational activators (Fox, 1996). Michaelis et al. (1991) proposed a model for the organization of these translation initiation sites based on the available data for the COB translational activators Cbs1p and Cbs2p. The membrane association and the affinity for the COB 5'-UTR of both activators and the ribosome interaction of Cbs2p, ensure that translation of the COB mRNA initiates at the inner membrane (Figure 1). Newly synthesized apocytochrome b can be co-translationally inserted into the membrane and aggregation of this hydrophobic protein is prevented. This model is directly applicable to the COX3 situation, the available data for Pet54p, Pet122p and Pet494p indicate the same level of organization for translation of this mRNA (Figure 1). Translation of the other mitochondrial mRNAs, encoding respiratory chain subunits, is very likely to occur through the same mechanism. Var1p is the only mitochondrial gene product that is not inserted into
the membrane. Although this hydrophilic protein is also synthesized on membrane-associated mitoribosomes (Marzuki and Hibbs, 1986), nothing is known about what, if any, specific activators are required. The ultimate destination of Var1p indicates that synthesis does not depend on the membrane insertion mechanism and that the 5'-UTR does not contain information to localize translation at the inner membrane. Recently, studies with chimeric mRNAs with the VAR1 5'-UTR and the coding sequence of COX2 or COX3 confirmed the hypothesis on membrane-associated translation of respiratory chain subunits (Sanchirico et al., 1998). Strains carrying these chimeric mRNAs synthesize Cox2p and Cox3p, but are deficient in cytochrome c oxidase activity and accumulation of Cox2p and Cox3p. The mislocalization of Cox2p and Cox3p leads to unassembled subunits which are degraded rapidly. This indicates that the 5'-UTRs of COX2 and COX3 mRNA contain membrane targeting information and confirms the importance of membrane targeting during synthesis of respiratory chain subunits for the correct insertion in the inner membrane.

Figure 1: Model for the function of mitochondrial mRNA-specific translational activators. The activator proteins are associated with the inner membrane, recognize sites in the 5'-UTR and interact functionally with the mitoribosomal small subunit. Through these interactions the insertion of translation products into the appropriate respiratory complexes might be facilitated (based on Michaelis et al., 1991 and Fox, 1996).
Translation of cytochrome c oxidase subunit 1

The mitochondrially encoded subunit 1 of cytochrome c oxidase (COX1) is the largest subunit of the cytochrome complex and forms an integral part of the catalytic core of the enzyme (Capaldi, 1990). Synthesis of the subunit is strictly regulated at different levels. The COX1 gene in Saccharomyces cerevisiae is transcribed as a polycistronic precursor RNA containing cistrons COX1, AAP1, OL12 and ENS2, encoding cytochrome c oxidase subunit 1, ATPase subunit 8, ATPase subunit 6 and an endonuclease, respectively. The COX1-containing transcripts are released from the AAP1-OL12-ENS2 cistrons by processing between the COX1 and AAP1 cistrons. The COX1 coding region contains introns, which must be removed before translation. To synthesize mature COX1 mRNA numerous RNA-processing steps are involved (Dieckmann and Staples, 1994).

A number of the factors involved in these processes have been identified and characterized. MSS116 encodes a putative RNA helicase required for splicing of group II introns of COX1 and COB (Séraphin et al., 1989). The homology of the MSS116 gene with translation factor eIF-4A outside the helicase boxes suggests that Mss116p may have an additional function to its role in splicing. MRS1, MSS18 and PET54 are involved in excision of intron a15β from the COX1 transcript (Herbert et al., 1992; Johnson and McEwen, 1997; Valencik et al., 1989). NAM1 is involved in splicing of the COX1 mRNA (Asher et al., 1989; Groudinsky et al., 1993), and might be involved in directing mRNAs to the membrane (Wallis et al., 1994). The SUV3 gene is needed for stability and processing of COX1 and COB mRNA (Conrad-Webb et al., 1990; Golik et al., 1995), a null-mutant displays severe inhibition of mitochondrial translation (Dziembowski et al., 1998). Expression of PET127 in this null-mutant can suppress this effect (Wegierski et al., 1998). PET127 is a mitochondrial membrane-associated protein involved in stability and processing of mitochondrial mRNAs (Wiesenberger and Fox, 1997). This gene has also been identified as a suppressor of carboxy-terminal truncations of Pet122p (Hafer and Fox, 1992). MSS1 encodes a mitochondrial GTPase which is involved in translation of COX1 mRNA (Decoster et al., 1993). The product of the MT01 gene also specifically affects COX1 expression (Colby et al., 1998), Mto1p and Mss1p form a heterodimeric complex which may play a general role in mitochondrial translation. In total more than 18 nuclear-encoded proteins control the expression of COX1 (reviewed by Pel, 1992a).

PET309 and MSS51: translational activators of cytochrome c oxidase subunit 1

Two of these 18 proteins are specifically needed for translation of COX1 mRNA, analogous to the situation for COX2, COX3, COB and ATP9. The PET309 gene encodes a protein that is required for two steps in the expression of COX1 (Manthey and McEwen, 1995). Pet309p is required for production or stability of precursor and mature transcripts from intron-bearing alleles of COX1 and, through interaction with the 5'-UTR of COX1 mRNA, Pet309p functions in initiation of translation. Mitochondrial revertants were found, containing a rearranged COX1 gene, such that most of the 5'-UTR of COX1 was replaced by the 5'-UTR of COB. Pet309p was localized as an integral membrane protein, spanning the inner membrane with part exposed to the intermembrane space and part exposed to the mitochondrial matrix (Manthey et al., 1998). The N. crassa Cya-5 protein shares a region of homology with Pet309p, CYA-5 is also specifically required for a post-transcriptional step in Cox1p expression (Coffin et al., 1997). This region of homology contains several tandem copies of a PPR (pentatricopeptide) motif consisting of a pair of anti-parallel β-helices that form a superhelix that might be able to bind RNA (Small and Peeters, 2000). Loss of Cox1p in a pet309-null strain does not affect Cox4p and Cox6p accumulation, but both proteins are soluble since they are not assembled into cytochrome oxidase, and Cox2p and Cox5p are unstable, because assembly of cytochrome oxidase is prevented (Glerum and Tzagoloff, 1997).
The MSS51 gene was isolated by complementation of a nuclear pet mutant without cytochrome oxidase activity in an intron-containing strain (Faye and Simon, 1983). The Mss51 protein turned out to be necessary for the correct maturation of the mitochondrial pre-mRNA of COX1. Originally, Mss51p was thought to be a component of the mitochondrial splicing system, specifically involved in splicing of introns of COX1 pre-mRNA (Simon and Faye, 1984). Studies on strains without mitochondrial introns showed that Mss51p is in fact necessary for the translation of the COX1 transcript (Séraphin et al., 1987). The apparent splicing defect in the absence of a functional Mss51 protein, is in this case thus merely a secondary due to the fact that the maturases of COX1 are not translated (Decoster et al., 1990). As described for other translational activators, suppressors of some nuclear pet mutations are associated with rearrangements of mitochondrial DNA. However, revertants of this type are not found for mss51 mutations. This may indicate that the Mss51 protein interacts very near the AUG start codon, so that non-homologous recombination cannot readily occur (Decoster et al., 1990). Mss51p might also interact with the 3'-UTR or elements within the coding region. In a strain resistant to paromomycin, the translation defect of COX1 mRNA by the mss51-3 mutant is enhanced (Decoster et al., 1990). Paromomycin is an antibiotic that interferes with the decoding process at the ribosomal A site. The paromomycin-resistant mutation, a C to G transposition, occurs at a position corresponding to the first base pair of the imperfect helix in the secondary structure model of the 15S rRNA (Li et al., 1982). This indicates that the translation machinery may interfere, directly or indirectly, with the action of Mss51p (Decoster et al., 1990).

**Aim of this thesis**

To further elucidate the process of translation initiation in yeast mitochondria, two proteins involved in different aspects of this process were chosen as the subject of study. Chapter II of this thesis describes the characterization of the RNA-binding properties of Idh and discusses the implications for the possible function of the RNA-binding activity in regulating mitochondrial gene expression. To look at activation of mitochondrial translation, MSS51 was chosen as subject of study. To characterize the MSS51 gene product, different biochemical approaches were applied. The localization and the possible function of Mss51p are discussed in chapter III. Using epitope-tagging it is shown that Mss51p is associated with the mitochondrial inner membrane and in *in vitro* translation experiments it is shown that Mss51p functions in an early step of Cox1p synthesis. This chapter also describes the characterization of the mutant allele mss51-3. Another factor that is involved in synthesis of Cox1p is Pet309p, in chapter IV it is shown that Mss51p and Pet309p have distinct roles in synthesis of Cox1p. Chapter V describes the attempts to show a possible interaction of Mss51p with the mitoribosome and a functional analysis of MSS51 using a mutagenesis approach. In Chapter VI, a hypothetical model for the function of Mss51p is presented, based on the experimental data discussed in Chapters III-V.