Regulators of mitochondrial translation in Saccharomyces cerevisiae

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Citation for published version (APA):
Chapter VI

General discussion
Mitochondrial translation

From genetic studies it has become clear that translation in yeast mitochondria is an extremely complicated process, requiring numerous general and specific factors. Although in recent years new biochemical approaches have added a large amount of experimental data to the information obtained by these genetic studies, the exact function of many factors involved remains obscure. In a number of aspects mitochondrial translation in yeast seems to be prokaryotic in nature (Kitakawa and Isono, 1991). The mitoribosome resembles more its prokaryotic than its eukaryotic counterpart and is sensitive to almost the same spectrum of antibiotics that interfere with translation in prokaryotes (Borst and Grivell, 1971). All general factors that have been identified, i.e. IFM1, MEF2 and TUF1, share homology with prokaryotic translation factors (Vambutas et al., 1991; Rasmussen, 1995; Nagata et al., 1983).

Most mitochondrial translation products are highly hydrophobic subunits of the respiratory chain complexes that need to be assembled in the inner membrane. These proteins have to be protected from degradation in the mitochondrial matrix and need to be targeted to the inner membrane for correct complex assembly. Specific factors for translation of individual yeast mitochondrial mRNAs play an important role in initiation of translation and localization of translation at the mitochondrial inner membrane. Further characterization of the mRNA-specific translational activators led to a generally accepted model that places translation of mitochondrial mRNAs at the inner membrane so that the synthesized proteins can be co-translationally inserted into the membrane (Fox, 1996; Michaelis et al., 1991).

So far, the best characterized yeast mitochondrial translation processes are the translation of cytochrome c oxidase subunit 3 (COX3) and apocytochrome b (COB) mRNA. For translation of COX3 mRNA three factors have been identified, Pet54p, Pet122p and Pet494p (Costanzo et al., 1986; Kloeckener-Gruissem et al., 1988). Cbs1p and Cbs2p regulate the translation of COB mRNA (Rödel, 1986). All these factors are associated with the mitochondrial inner membrane and are thought to interact with the 5'-UTR of the mRNAs. This protein-RNA interaction has been proposed to tether the mRNAs to the inner membrane. In order to translate the mRNAs at the inner membrane, the ribosome has to be targeted to the membrane-localized mRNA. Pet122p and Cbs2p are shown to interact with the mitoribosomes (Hafter et al., 1991; Michaelis et al., 1991), indicating that translation occurs at the inner membrane. The newly synthesized hydrophobic subunits of inner membrane complexes can be co-translationally inserted into the membrane thereby preventing aggregation and degradation of these proteins. Recently, studies have been described on chimeric mRNAs harbouring the COX2 or the COX3 coding region under control of the VAR1 5'-UTR (Sancherico et al., 1998). As a protein of the mitoribosomal small subunit, it is thought that the synthesis of Var1p does not depend on co-translational membrane insertion. In line with a membrane-tethering model for mitochondrial translation, both Cox2p and Cox3p were translated efficiently from the chimeric mRNAs, but their incorporation into active cytochrome c oxidase was found to be severely defective and their turnover significantly increased.

The model for translation on the surface of the mitochondrial inner membrane is based on the fact that all mitochondrial translational activators that have been studied so far are associated with the inner membrane. According to this model, the mitochondrial mRNAs need to be tethered to the inner membrane to facilitate co-translational insertion of the newly synthesized proteins. The question remains how tethering does occur. Although genetic evidence suggests that translational activators interact with the 5'-UTR of their target mRNAs, RNA-binding has not yet been demonstrated directly.
The role of NAD\(^+\)-dependent isocitrate dehydrogenase in mitochondrial translation

A search for RNA-binding proteins, possibly involved in mitochondrial translation, led to the discovery that NAD\(^+\)-dependent isocitrate dehydrogenase (Idh) is an RNA-binding protein (Elzinga et al., 1993). Idh, one of the eight enzymes of the Krebs cycle, is an octamer composed of Idh1p and Idh2p (encoded by IDH1 and IDH2, respectively). This enzyme is not only catalytically active in the TCA-cycle, it also binds specifically and with high affinity to the 5'-UTR of all mitochondrial mRNAs. Chapter II of this thesis describes the characterization of the RNA-binding activity of Idh. The value of the dissociation constant (Kd = 3 nM) that was found for the RNA-binding of Idh indicates that RNA-binding is possible even at low levels thought to exist within the mitochondria under physiological conditions and is thus likely to occur in vivo. Recently, our group showed that a strain disrupted for Idh displays a 2-3-fold increase in the synthesis of mitochondrial translation products (de Jong et al., 2000). However, the half-life of the newly produced proteins is reduced 2-3-fold. As mentioned previously, Cox2p and Cox3p showed increased turnover when their mRNAs are prevented from membrane tethering/docking. Taken together this suggests that Idh may suppress inappropriate translation of mRNAs in the mitochondrial matrix. To initiate translation, Idh should be, directly or indirectly, replaced by the membrane-bound translational activators causing the mRNAs to be tethered to the inner membrane.

Translation of COX1 mRNA

Chapter III to V of this thesis describe the characterization of the translational activator of cytochrome c oxidase subunit 1 (COX1), Mss51p. Although we could show that Mss51p functions in an early step of Cox1p synthesis, we were unable to establish the exact function of this protein (Siep et al., 2000). Based on our extensive functional analysis two models are presented that describe the possible function of Mss51p and Pet309p in synthesis of Cox1p (Figure 1). In contrast to the situation for the COX3 mRNA-specific translational activators (Pet54p, Pet122p and Pet494p), no physical interaction between Mss51p and Pet309p could be detected by two-hybrid analysis or two-dimensional gel electrophoresis. Mutations in other translational activators are suppressible by mtDNA rearrangements that place mRNA coding sequences downstream of another 5'-UTR. The fact that no such mitochondrial suppressors could be found in mss51 mutants, is an indication that Mss51p does not interact with the 5'-UTR of COX1 mRNA. However, especially the membrane association of both Pet309p and Mss51p and requirement for these factors for translation of COX1 mRNA, place translation at the inner membrane analogous to other translation events.
Figure 1: Two hypothetical models for the role of Mss51p in the synthesis of Cox1p. This figure depicts the interactions required for membrane-associated translation of COX1 mRNA. Possible interactions that are discussed in this chapter are indicated by a dotted arrow and marked with a question mark. The assembly machinery is depicted as a 'black box'.
The membrane association of Mss51p, as depicted in both models, is based on our extensive characterization described in Chapter III of this thesis. Mss51p could only be extracted from the mitochondrial inner membrane under stringent conditions and co-fractionates with proteins known to be associated with the inner membrane, indicating a tight association with the inner membrane.

The proposed interaction with the mitoribosome is based on three observations. First, the previously identified mutant mss51-3 displays a partial deficiency in respiratory growth (DeCoster et al., 1990). The effect of the mss51-3 mutation is enhanced when this allele is combined with the pR454 mutation which confers resistance to the aminoglycoside paromomycin. This mutation is located in a conserved hairpin in the 15S mitochondrial rRNA (Li et al., 1982). Paromomycin is known to interfere with the decoding process at the ribosomal A site. The observation that this mutation enhances the translational effect of the mss51-3 mutation on Cox1p synthesis, may indicate a possible physical interaction of Mss51p with the mitochondrial ribosome, analogous to the situation for the COB mRNA translational activator Cbs2p (Michaelis et al., 1991). Second, analysis of DNA micro-array expression profiling data for yeast grown under a wide variety of conditions (Eisen et al., 1998), shows that MSS51 clusters together with a group of about 40 other genes encoding proteins involved in mitochondrial assembly or respiratory chain function, 20 of which are either proteins of the mitochondrial ribosome, aminoacyl-tRNA synthetases, or other factors closely involved in the translation process. Third, the functional analysis presented in chapter V, suggests a physical interaction between Mss51p and the mitoribosome. The results we obtained with HA-epitope tagged Mss51p suggests that Mss51p co-localizes to some extent with the mitoribosome. Mss51p could be partly detected in mitochondrial subfractions that contain ribosomes. Taken together, these observations suggest a functional interaction of Mss51p with the mitoribosome.

The functional form of Mss51p is depicted as a higher order structure. This representation is based on the migration behaviour of Mss51p in native PAGE electrophoresis. The protein is detected at a position corresponding to a molecular mass of approximately 150 kDa, while on denaturing PAGE electrophoresis, it migrates at 44 kDa (Chapter IV).

In model A an interaction of Mss51p with the growing Cox1 peptide chain is proposed. This is based on preliminary results, using 2D analysis. A comigration in the first dimension of Mss51p and (unassembled) Cox1p may suggest a direct interaction of Mss51p with Cox1p (see also below). In this model Pet309p docks the COX1 mRNA at the mitochondrial inner membrane. At the inner membrane, Mss51p interacts with the mitoribosome to target the ribosome to the right location. By interaction with the growing peptide, Mss51p directs the newly synthesized peptide to the membrane-associated assembly factors, such as Pet100p, Pet117p, Pet191p, Cox14p, Cox18p or Oxa1p. In this model the proposed function for Mss51p would then be the correct localization of translation at the inner membrane and aiding the insertion of the newly synthesized protein in the membrane similar to the function of the E. coli ‘trigger factor’. In E. coli, trigger factor acts as a chaperone that co-translationally folds newly synthesized proteins by interaction with the growing peptide chain and the ribosome (Hesterkamp et al., 1996).

Interestingly, a mutation of the COB translational activator Cbs2p was found to be suppressible by overexpression of ABC1 (Bousquet et al., 1991). ABC1p is an assembly factor of apocytochrome b, essential for the correct conformation and function of the cytochrome bc1 complex (Brasseur et al., 1997). Mss51p and Cbs2p might have a similar function in mitoribosome interaction. Suppression of a mutation in Cbs2p by an assembly factor indicates that (Cbs1p and) Cbs2p is not the only factor that promotes correct
synthesis of apocytochrome b. Since such suppressors could not be found for \textit{mss51} mutants, Mss51p might combine functions in translation and in assembly in itself. Through an interaction with the growing peptide chain Mss51p may stabilize the peptide prior to association with chaperones or assembly with other proteins.

In model B, as in model A, Pet309p docks the COX1 mRNA at the inner membrane. In contrast to model A, an interaction of Mss51p with the COX1 mRNA is proposed. Although it has been concluded that it is unlikely that Mss51p binds RNA, this possibility is formally not excluded. In favour of such a RNA-interaction is the finding that part of the MSS51 sequence has a weak similarity with the pre-mRNA splicing factor PRP19 of \textit{Arabidopsis thaliana}, over a region of 200 amino acids. Part of this homologous region is also shared by \textit{S. cerevisiae} PRP19, a known splicing factor. Although Prp19p does not contain any recognizable RNA-binding motifs, it is likely to bind RNA. Since Mss51p is involved in translation it might also bind RNA. Based on the effect of the paromomycin mutation, it could be hypothesized that Mss51p might play a role in the elongation phase of translation. Paromomycin interferes with the decoding process at the ribosomal A site, which indicates interference with the elongation process. A physical interaction of Mss51p with the mitoribosome suggests that Mss51p might be part of the mitoribosome. To promote correct elongation of translation of the COX1 mRNA by the mitoribosome, Mss51p might interact with the mRNA downstream of the AUG codon to keep the mitoribosome at the membrane-localized COX1 mRNA. In this model, the proposed function for Mss51p would be to ensure continuation of synthesis of the growing peptide chain on the inner membrane. This membrane-localized synthesis of Cox1p promotes co-translational insertion of the newly synthesized protein into the inner membrane.

Mss51p is depicted as a multimeric structure. From 2D experiments, it was concluded that Mss51p is in a higher order complex (Chapter IV). This complex might consist either of a multimer of Mss51p, or of hetero-multimeric structures with different proteins. A homo-multimeric (3-4 Mss51p subunits) structure does not seem unlikely since increased expression of Mss51p is directly reflected in the amount of the 150 kDa complex. However, association of Mss51p with other proteins can not be ruled out. The most likely candidates for this proposed interaction would be either Pet309p or Cox1p. Since migration of the Mss51p-containing complex is unaffected by disruption of PET309, Pet309p is unlikely to be a component of this complex. As mentioned before, comigration of unassembled Cox1p with the 150 kDa complex can be interpreted in favour of a direct interaction of Mss51p and Cox1p. This interpretation seems to be in conflict with the before mentioned data obtained with disruption of PET309, since in \textit{pet309-null} strains Cox1p synthesis is supposed to be completely abolished. If this would be true, Cox1p could not be associated with Mss51p. However, we repeatedly have observed accumulation of small amounts of fully assembled cytochrome c oxidase complex in strains lacking Mss51p or Pet309p (and also in the absence of other translational activators such as Pet111p and Pet494p; M. Artal, M. Farhoud, personal communication). This would mean that synthesis (of strongly reduced amounts) of Cox1p could occur, even in the absence of Pet309, and this would formally leave open the possibility of interaction with Mss51p.

Beside Pet309p and Cox1p, other candidate proteins may be involved. Manthey \textit{et al.} (1998) suggested that Nam1p acts as a carrier, bringing COX1 mRNA to the membrane. In that case an interaction between Mss51p and Nam1p might exist. On basis of model A one might argue that the protein that inserts Cox1p into the inner membrane might be the complex partner of Mss51p. Recently it was shown that Cox1p, Cox3p and apocytochrome b interact with Oxa1p (Hell \textit{et al.}, 2001). Newly synthesized Cox1p displays strong dependence on Oxa1p for its membrane insertion and interacts physically with Oxa1p during its insertion process. The interaction of Oxa1p with the peptide chain is enhanced...
by the presence of the associated ribosome. However, an interaction of Oxa1p with the ribosome has not yet been shown (Hell et al., 2001).

Taken together the final conclusion on the function of Mss51p is that this protein is different from the other translational activators. Most important argument for this conclusion is the absence of mitochondrial revertants. No mitochondrial 5'-UTR rearrangements have been found to suppress mutations in MSS51, unlike other translational activators. This may mean that Mss51p does not act via direct interaction with the 5'-UTR. Unlike most translational activators, Mss51p may be present in appreciable excess. Only Pet494p, another peripheral membrane associated translational activator, shows comparable expression levels. This might indicate distinct roles for the peripheral membrane translational activators and the integral membrane translational activators. Our data and the recent data on Oxa1p leave the opportunity to speculate that Mss51p interacts with the mitoribosome and Oxa1p to couple translation and membrane insertion of Cox1p. Further experiments need to be performed to test this hypothesis. These experiments might include confirmation of the interaction of Mss51p and Cox1p and/or the ribosome, using the HA- or GFP-tagged Mss51p in immune precipitations and 2D gel electrophoresis. In combination with an antibody against Oxa1p, the interaction of Mss51p and Oxa1p needs to be investigated. For analyzing the putative Mss51p-COX1 RNA interaction, a combination of genetic (three hybrid system) and biochemical (band-shift assays, cross-linking) approaches can be employed.

Translational activators in mitochondria

For the translational activators of COX1 mRNA possible homologues could be found through database searches. We found a homologue for MSS51 in Schizosaccharomyces pombe and based on the sequence conservation this protein most probably represents the S. pombe orthologue of Mss51p. For the other translational activator protein for COX1 mRNA, Pet309p, functional homologues have been found in C. albicans and S. pombe, while proteins with significant sequence similarity have been found in maize and N. crassa (Coffin et al., 1997; Fisk et al., 1999; Costanzo et al., 2000). Although the function of this S. pombe orthologue of MSS51 has to be determined, it is striking that we find these homologues in distantly related yeast and other fungi since for the translational activators of other mitochondrial mRNAs possible homologues could only be found by screening for complementation of mutations in conserved genes adjacent to the PET genes (Costanzo et al., 2000). The authors showed that in the closely related yeasts Saccharomyces kluveri and Kluyveromyces lactis the mRNA-specific translational activators PET111 and PET122 are highly diverged in sequence, while their functions are orthologous. Not only the translational activator genes, but also the 5'-UTRs of the COX2 and COX3 mRNAs in S. kluveri and K. lactis have little similarity to each other or to those of S. cerevisiae. This implies that through co-evolution of the yeast mitochondrial mRNA translational activators and the yeast mitochondrial mRNAs, membrane-bound translation has been retained, while the sequence of the factors involved have diverged. The fact that for MSS51 and PET309 homologues with significant similarity could be found in distantly related fungi, while for the COX2 and COX3 translational activators no significant matches could be found, indicates that proteins involved in synthesis of Cox1p may be subject to more stringent evolutionary constraints. A reason for this might be that Cox1p is the key subunit in the assembly of cytochrome c oxidase. Complex assembly is assumed to begin with insertion of Cox1p into the inner membrane, followed by association with Cox2p, Cox4p and Cox3p (Glerum and Tzagoloff, 1997; Lemaire et al., 1998). In addition to this it can be argued that since Cox1p is the largest and most hydrophobic mitochondrially translated subunit, extra control mechanisms have to be operative. The hydrophobic nature of Cox1p
would require such special control mechanisms to prevent excess of potentially hazardous unassembled Cox1p in the mitochondrial inner membrane.

Taken together, it looks like the mitochondrial specific translational activators lack evolutionary conservation, mRNA-specific mitochondrial activators appear to be among the most rapidly diverging proteins in budding yeasts, despite their conserved functions (Costanzo et al., 2000). This conservation of function is the result of co-evolution of the target RNAs. The question remains why they are so variable, even in different yeast strains. And why don't we find counterparts in other genetic systems? As mentioned before, at least between different yeasts the system is there, only the players have diverged. Only in other fungi homologues of mRNA-specific activators could be found, indicating that mRNA-specific translational activation may be a feature of fungal mitochondrial gene expression. Orthologous proteins might be present in more distantly related species, however, the divergence between closely related species indicates that detection by sequence similarity alone will be difficult. In bacteria docking seems to occur at the protein level. While no mRNA-specific translational activators have been found, proteins have been found that dock newly synthesized proteins at their site of assembly. In the mammalian system little is known on how translation initiation in mitochondria occurs. Mammalian mitochondrial mRNAs lack 5’UTRs (Attardi and Schatz, 1988). While the loss of similarity of 5’-UTRs between different yeast strains already led to co-evolution of the translational activators, one could imagine that in the mammals the whole system of mitochondrial translation might have changed. Two possible changes might have occurred during evolution. First, although mammals might have a similar system using specific translational activators for translation of mitochondrial mRNAs, these factors might have diverged so drastically that they are unrecognizable. Second, the system itself changed to a totally different mechanism with other factors that localize translation at the inner membrane, regulate translation, and protect the newly synthesized proteins from degradation.