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Review

Coupling of mitochondrial translation with the formation of respiratory complexes in yeast mitochondria✪

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In contrast to most other eukaryotic organisms, yeast can survive without respiration. This ability has been exploited to investigate nuclear genes required for expression of mitochondrial DNA. Availability of complete *Saccharomyces cerevisiae* genomic sequence has provided additional help in detailed molecular analysis. Seven of the eight major products encoded by mitochondrial DNA are hydrophobic subunits of respiratory complexes in the inner membrane. Localization of the translation process in the same cellular compartment ensures synthesis of mitochondrially encoded proteins near sites of their assembly into multimeric respiratory complexes. Association of mitochondrial ribosomes with the membrane is mediated by mRNA-specific translational activators, that are involved in the recognition of initation codon. The newly synthesized mitochondrial proteins are transferred to membrane by a specific export system. This review discusses the role of membrane-localized factors responsible for quality control and turnover of mitochondrially synthesized subunits as well as for assembly of respiratory complexes.

Biogenesis of the enzymes of oxidative phosphorylation in eukaryotes is dependent on two genetic systems, mitochondrial and nuclear. Mitochondria have their own genome and the machinery to express the information contained in it. The role of the mitochondrial genetic system is to supply only a few highly hydrophobic proteins of respiratory complexes embedded in the inner membrane. The majority of the subunits of energy-transducing complexes, as well as proteins involved in mitochondrial genome expression and assembly, are encoded by nuclear genes.

In the yeast *Saccharomyces cerevisiae,* all components of complex I and complex II are

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Abbreviations: MRP, mitochondrial ribosomal protein; 5′-UTL, 5′-untranslated leader sequence.

provided by nuclear genome (Fig. 1). Complex III (or ubiquinol-cytochrome *c* oxidoreductase, or bc_1 complex) is composed of nine subunits, but only one among them, cytochrome *b*, is encoded by the mitochondrial gene *COB* (Rodel, 1997). Cytochrome *c* oxidase (or complex IV) contains three mitochondrially synthesized proteins, products of the *COX1, COX2* and *COX3* genes, in addition to those provided by the nucleus (Poyton *et al.*, 1995). Three proteins encoded by mitochondrial genes *ATP6, ATP8* and *ATP9* are components of the integral membrane F_0 component of ATP synthase (or complex V or F_0F_1-ATPase). In contrast, the peripherally bound F_1 component consists only of proteins synthesized in the cytosol (Arnold *et al.*, 1999) (Fig. 1).

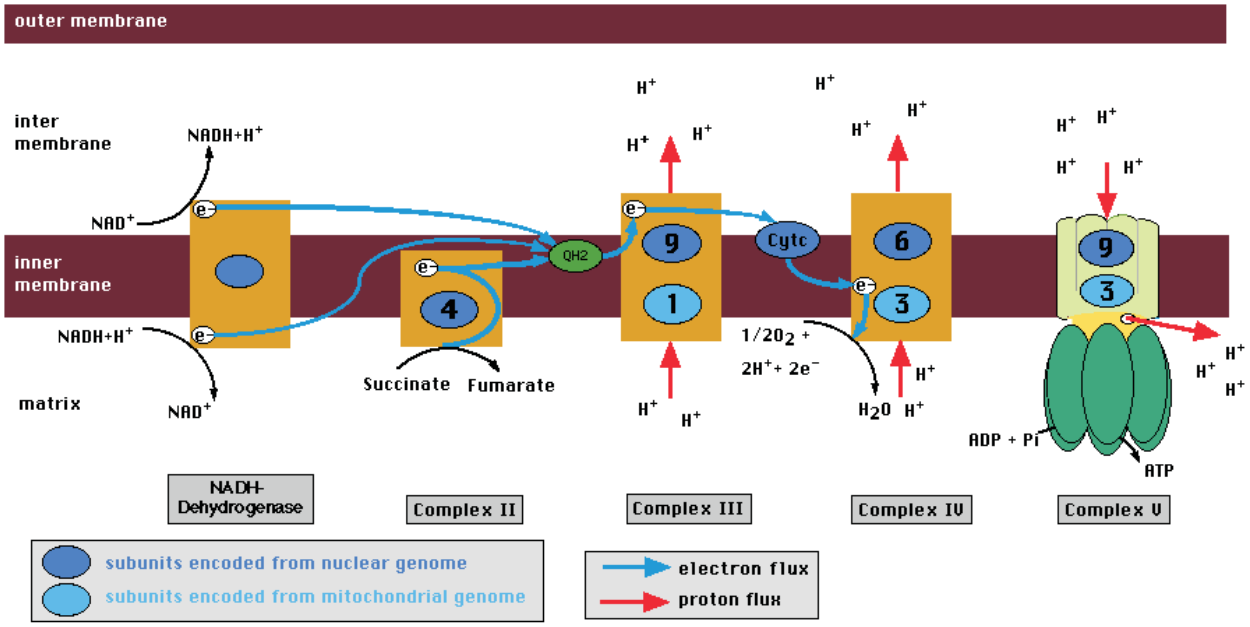

Figure. 1. Schematic representation of the respiratory chain complexes of *S. cerevisiae*.

From MITOP, database of mitochondria-related genes and proteins. Number of subunits encoded by nucleus and mitochondria is given.

The yeast *S. cerevisiae* as a facultative anaerobe offers distinct advantages for biochemical and genetic studies of mitochondrial gene expression and respiratory metabolism. Yeast is readily propagated on simple, well-defined media, can be grown in large quantities and is highly suitable for genetic manipulations. The respiratory function is dispensable as long as cells grow by fermentation. This means that mutations affecting expression or functioning of the respiratory chain lead to the respiratory deficient phenotype but are viable on a fermentable carbon source. The only factor complicating genetic manipulations is that the complete loss of mitochondrial translation causes, for unknown reasons, irreversible instability of mitochondrial DNA leading to deletions (rho^-) or complete lack of mitochondrial DNA (rho^0) (Myers *et al.*, 1985). Some aspects of mitochondrial gene expression cannot be studied directly due to the lack of an *in vitro* protein synthesis system derived from these organelles of any species. However, isolated mitochondria are capable of protein synthesis (McKee & Poyton, 1984).

This review focuses on the process of mitochondrial translation and its connection with subsequent membrane-specific events leading to the assembly of newly synthesized proteins into functional respiratory complexes.

MITOCHONDRIAL RIBOSOMES

Mitochondria contain their own translational machinery and use an alternative genetic code (Hudspeth *et al.*, 1982). Although the mitochondrial translation system shows unique and unusual features, it resembles more closely its prokaryotic than its eukaryotic counterpart with respect to the spectrum of antibiotics inhibiting mitochondrial translation (Borst & Grivell, 1971).

Mitochondrial ribosomes in yeast have the sedimentation coefficient of 74S and they are dissociable into 54S and 37S subunits. Like in other eukaryotes, their mass ratio of protein to RNA is higher than in Eubacteria. Most mitochondrial ribosomes contain only two RNA species; in yeast 15S and 21S rRNAs are found. Both are encoded by mitochondrial DNA and display minimal posttranscriptional modifications (Grivell, 1995). Since a large portion of mitochondrial ribosome mass is provided by proteins, it is not surprising that mitochondrial ribosomal proteins (MRPs) exceed those of bacteria in number and very often in length. Mitochondrial ribosomes do not share any constituents with the cytoplasmic ones, although all but one proteins are encoded by the nuclear genome, synthesized on cytoplasmic ribosomes and subsequently imported into the mitochondria. The only MRP encoded by the mitochondrial genome is the Var1p that has been shown to be an essential and stoichiometric component of the small subunit of the mitochondrial ribosome, involved additionally in its assembly (Mason *et al.*, 1996). MRPs were identified by different approaches including sequence analysis, genetic complementation of pleiotropic phenotypes deficient in mitochondrial protein synthesis, immunological screening of expression libraries with antibodies against MRPs or by direct biochemical methods: separation on one dimensional or two-dimensional PAGE, purification and finally amino-acid sequencing. The total number of identified MRPs is 63 (Grivell *et al.*, 1999) and it seems to be not a complete set since 50 proteins of the large and 35 proteins of the small ribosomal subunit have been detected by direct biochemical methods (Graack & Wittmann-Liebold, 1998). For the majority of MRPs genes, their inactivation leads to instability of mitochondrial DNA. Of the MRPs whose sequences are known, some are homologues of ribosomal proteins from other organisms, others have domains similar to bacterial ribosomal proteins attached to domains with no recognizable homology to known proteins, but the majority are completely unique (Graack & Wittmann-Liebold, 1998). This relatively low degree of sequence conservation is a surprising feature. It has been speculated that the conserved proteins are responsible for carrying out the central step of protein synthesis, while additional proteins or their parts have more specialized functions connected with the coupling of the translation process to the mitochondrial inner membrane, which is the final destination of 7 hydrophobic mitochondrial translation products (Fox, 1996a).

An example of a protein displaying the features described is Nam9p (Boguta *et al.*, 1992; 1997; Dmochowska *et al.*, 1995; Biswas & Getz, 1999). The N-terminal domain of Nam9p shows strong homology to a class of S4 ribosomal proteins from prokaryotes and eukaryotes. The phenotype of the *nam9-1* point mutant is a nonsense suppression of [*mit*$^-$] *ochre* mutations in different mitochondrial genes. It has been postulated that Nam9p controls the fidelity of mitochondrial translation.

GENERAL FACTORS OF MITOCHONDRIAL TRANSLATION

Mitochondrial mRNAs are uncapped and lack the poly(A) tail. They contain 5′-untranslated leader sequences (5′-UTLs) that vary in size from about 50 up to several hundred nucleotides. Little is known about the translational start site selection. Interactions

of "Shine-Dalgarno" type between the 3′-end of 15S rRNA and the messenger are unlikely to play a role in this process due to variable distance of the Shine-Dalgarno box from the initiation codon. Indeed, a chimeric mRNA lacking the putative Shine-Dalgarno box was translated normally *in vivo* (Costanzo & Fox, 1988). Simple scanning of mRNA by ribosome can be ruled out since 5′-UTLs contain additional upstream AUG codons, short open reading frames and a stable secondary structure (Pel & Grivell, 1994). Other mechanisms, such as entry at an internal landing site may be rather used to guide the ribosome to the initiation AUG codon, as shown in studies carried out with *COX2* and *COX3* mRNAs in which the initiator AUG had been mutated to AUA, the alternative methionine codon in yeast mitochondrial DNA. Translation from such mutated mRNAs was strongly impaired leading to a leaky nonrespiratory phenotype (Folley & Fox, 1994; Mulero & Fox, 1994). Interestingly, the residual translation in such mutants was initiated at the altered initiation codon, not at the next downstream AUG (Mulero & Fox, 1994; Bonnefoy & Fox, 2000). This finding led to the important conclusion that, in addition to AUG, some sequence or structural information must be used for selection of the proper initiation site. Thus, when the initiator AUG is mutated to AUA, the surrounding nucleotides provide sufficient information to select the proper start site, albeit at a low efficiency.

The mechanism of start site selection remains obscure, but it is known that initiation of translation requires mRNA-specific activator proteins whose targets lie in the 5′-UTLs (reviewed by Fox, 1996a). The messenger-specific translational activators may be involved in positioning of the ribosome over the correct AUG initiation codon. The role and features of the mitochondrial translational activation system, which is one of the most unusual mechanisms involved in gene expression, will be discussed in detail in the following section.

Besides specific activators some general components of the protein synthesis machinery are also involved in the initiation of translation. Certain mutations in 5′-UTLs of *COX2* and *COX3* mRNA were suppressed by mutations in genes encoding proteins of the small ribosomal subunit, *MRP21* and *MRP51*. The suppression by ribosomal proteins did not by-pass the mRNA-specific translational activation system and was not due to a general increase in translational efficiency since the *mrp21* and *mrp51* mutants failed to suppress the leaky non-respiratory phenotype of the *COX2* and *COX3* initiation codon mutants (Green-Willms *et al.*, 1998). These data indicate that the ribosome plays an active role in the recognition of translation start signals. As the suppression was not gene specific, the mitochondrial ribosome might recognize a common element in 5′-UTLs. A candidate for such an element is the octanucleotide sequence UAUAAAUA that has been identified in *COX2* mRNA and found in other 5′-UTLs (Dunstan *et al.*, 1997). Ribosomal proteins Mrp21 and Mrp51 could be involved in the association of the ribosome with mRNA which additionally requires specific activator proteins, resulting in selection of the proper site for the initiation of translation.

The nuclear gene *IFM1* encodes the yeast mitochondrial homologue of prokaryotic initiation factor IF-2. The protein probably functions in mitochondrial translation initiation since mutants display features commonly found in strains defective in mitochondrial protein synthesis (Vambutas *et al.*, 1991), although the connection of Ifm1p with other elements specifying proper translation initiation has not been demonstrated so far.

The elongation step of mitochondrial translation requires products of two nuclear genes *MEF2* and *MEF1* that are homologous to prokaryotic elongation factors EF-Tu and EF-G, respectively (Nagata *et al.*, 1983; Vambutas *et al.*, 1991). Both these yeast mitochondrial elongation factors display activity

in the presence of *E. coli* ribosomes (Rosenthal & Bodley, 1987).

Frame-shift mutations at a specific run of U residues in the coding sequence of the *COX2* gene that disturb elongation lead to a leaky phenotype (Fox & Weiss-Brummer, 1980). Interestingly, ribosomal frame-shifting is reduced in the genetic background of the paramomycin resistance mutation in the mitochondrial gene encoding 15S rRNA (Weiss-Brummer & Huttenhofer, 1989).

Up to date the only protein involved in the termination of mitochondrial translation is Mrf1p. It is homologous to prokaryotic RF1 specific for UAA and UAG and should be sufficient for the recognition of UAA and UAG in mitochondria; UGA is not the stop codon in mitochondrial translation (Pel *et al.*, 1992b). When overproduced, Mrf1p reduced the nonsense suppression caused by two well characterized ribosomal ambiguity mutations: *nam9-1* in a gene encoding the mitochondrial ribosomal protein responsible for translation fidelity (Boguta *et al.*, 1992; Dmochowska *et al.*, 1995; Chacinska *et al.*, 2000) and *MSU1,* in a gene specifying 15S rRNA (Pel *et al.*, 1992b; 1993; Fox, 1996a). These data provide strong evidence that Mrf1p is a mitochondrial translation release factor. This antisuppression phenotype is in good agreement with the observation found previously for RF1 in *E. coli* that an increased level of the peptide release factor changes the balance between termination and elongation, reducing readthrough of stop codons (Weiss *et al.*, 1984).

As expected for the proteins generally required for translation, deletion of *MRF1* leads to the destabilization of mtDNA. Two known *mrf1* missense mutations appear to define the ribosome binding domain in the Mrf1 protein and lead to the non-respiratory phenotype although mtDNA remains intact. Interestingly, both mutations cause defects in Cox2p synthesis and in the splicing and/or translation of *COX1* mRNA, which is not in agreement with expectations based on studies of other mutant general translational factors, *ifm1* and *mef1*, displaying an overall reduction in mitochondrial protein synthesis (Vambutas *et al.*, 1991; Pel *et al.*, 1992a; 1992b; 1993). The non-respiratory phenotype of *mrf1* mutants on the background of intron-less mitochondrial DNA allowed the authors to hypothesize that the splicing impairment is a secondary effect of the lack of intact maturases due to the disturbances in mitochondrial protein synthesis. The molecular basis of this specific translational phenomenon in *mrf1* mutants remains unknown.

Another protein involved in mitochondrial translation, the product of the *PET112* gene, probably performs a general function, since the null mutation destabilizes mitochondrial DNA. Interestingly, a point mutation in this gene specifically blocks the synthesis of Cox2p, which resembles the effect of *mrf1* mutations (Mulero *et al.*, 1994).

Two other mitochondrial proteins, Mss1p and Mto1p, that form a complex seem to control translation. Both genes were cloned by complementation of mutations that result in a non-respiratory phenotype only in the presence of a point mutation in mitochondrial 15S rRNA conferring resistance to paramomycin (par^{R454}) (Decoster *et al.*, 1993; Colby *et al.*, 1998). Both proteins were dispensable for respiratory growth in the genetic background of wild type mtDNA. In the paramomycin-resistant background the *mss1* and *mto1* mutations partially blocked the splicing of *COX1* mRNA and caused a complete absence of Cox1p, although synthesis of other mitochondrial translation products was increased. The widespread occurrence of homologous proteins in prokaryotes argues for the general role of the Mto1p/Mss1p complex in the process of translation. Probably the Mto1p/Mss1p complex has a proof-reading function slowing down the rate of translation, and perhaps interacts transiently, since no stable association could be detected, with the small subunit of the ribosome. In the presence of the paramomycin-resistance mutation this interaction is weakened or prevented, resulting in a high rate of

amino-acid substitutions in mitochondrially translated proteins. Therefore a large part of translation products cannot be functional, possibly including intron-encoded maturases involved in the processing of *COX1* mRNA. The complete lack of the Cox1 subunit could be explained by its more rapid turn-over. Thus, in addition to the increase in translation rate of each product, the rate of degradation may account for the pattern of mitochondrially synthesized proteins in the mutant Mto1p/Mss1p (Decoster *et al.*, 1993; Colby *et al.*, 1998).

TRANSLATIONAL ACTIVATION

The requirement for gene-specific activator proteins for translation of most, if not all, mRNAs is the unusual feature of the mitochondrial expression system. To date the translation of five of the seven major mitochondrially encoded membrane proteins has been shown to depend on specific nuclear genes. Mutants defective in either of these genes showed the respiratory defective phenotype due to the absence of one particular translation product, although the corresponding mRNA was present (Costanzo & Fox, 1990). The specificity of translational activation is determined by 5′-UTL of mRNAs, as shown by *in vivo* translation experiments of chimeric transcripts with 5′-UTLs of *COX2*, *COX3* and *COB* fused to each other's coding sequences. Translation clearly depended on the translational activator proteins specified by the 5′-UTL (Rodel & Fox, 1987; Costanzo & Fox, 1988; Mulero & Fox, 1993a).

Respiratory deficiencies due to alterations in Cbs1p or Cbs2p, two proteins required for translation of *COB* mRNA, were overcome by mitochondrial mutations replacing the original leader by the 5′-UTL of *ATP9* (Rodel, 1986; 1997). Mutation in the third gene *CBP6*, whose product is apparently involved in translation of *COB* mRNA, was not by-passed by attaching the 5′-UTL of *ATP9*, indicating that not all translational activators interact with 5′-UTLs (Dieckmann & Tzagaloff, 1985; Dieckmann & Staples, 1994). A similar situation was reported for *COX1*-specific activators, Mss51p and Pet309p (Decoster *et al.*, 1990; Manthey & McEwen, 1995). The phenotype of *pet309* null mutation was suppressed by mtDNA rearrangements that placed a new 5′-UTL in *COX1* mRNA, but no such bypass of the *mss51* mutation was observed. This may suggest an additional function or a different role of Mss51 and Cbp6 proteins in the translational activator complexes.

There has been limited success in determining the target sequences within 5′-UTLs of *COX3, COB* and *COX2* mRNA, the three best studied genes (Costanzo & Fox, 1993; Wiesenberger *et al.*, 1995; Mittelmeier & Dieckman, 1995; Dunstan *et al.*, 1997).

Genetic data strongly support the idea of a direct interaction between translational activators and 5′-UTLs. The mutations localized in *COX3* 5′-UTL were suppressed by alterations of Pet122 or by overproduction of Pet494 activator proteins. Similarly, a mutation affecting 5′-UTL of *COX2* mRNA was overcome by a single substitution or by an increased dosage of wild-type Pet111 activator protein (Mulero & Fox, 1993b; Costanzo & Fox, 1993; Wiesenberger *et al.*, 1995).

For one activator protein, Pet122p, a direct interaction with the mitochondrial ribosome was suggested on the basis of genetic studies. The truncation of Pet122p led to the respiratory incompetence, but was suppressed by mutations in the genes encoding proteins of the ribosomal small subunit, Mrp1p, Mrp17p and Pet123p, as well as the Pet127 protein affecting mitochondrial RNA stability (Haffter & Fox, 1992; McMullin *et al.*, 1990; Haffter *et al.*, 1991; 1990). It is interesting to note that all ribosomal proteins that were picked up in this screen do not show any homology with known proteins and seem to fulfil functions unique to the mitochondrial system. The membrane-localized Pet127p is involved in mitochondrial mRNA surveillance system

(Wiesenberger & Fox, 1997; Węgierski *et al.*, 1998).

Several lines of evidence demonstrated that the *COX3*-specific activators, Pet54p, Pet122p and Pet494p, form a complex. First, the missense *pet54* mutation was suppressed in an allele-specific manner by a missense mutation affecting Pet122p (Brown *et al.*, 1994). Second, pairwise interactions between Pet54p and Pet122p, as well as between Pet54p and Pet 494p, were detected in the yeast two-hybrid system (Brown *et al.*, 1994). Although Pet122p and Pet494p failed to interact with each other in the two-hybrid approach, the Pet122 as well as Pet54 proteins were found to be bound to Pet494p in co-immunoprecipitation experiments with specific Pet494p-directed serum, which argues strongly for the presence in the inner membrane of a trimeric complex (Fox, 1996a). So far there is no data supporting the existence of activator complexes specific for other mitochondrially translated proteins.

It is very likely that translational activation is the rate-limiting step in the whole mitochondrial gene expression process. The level of expression of some activator proteins is very low. A calculation based on a study of the *PET494* promoter fused to a reporter sequence encoding beta-galactosidase gave the number of molecules between 2 and 60 per cell, depending on growth conditions. This result can be even overestimated since beta-galactosidase is very stable while Pet494p appears to be rather unstable (Fox, 1996b). Based on a comparison of expression levels the abundance of Pet111p is as low as that of Pet494p, which opens the possibility of subtle modulation of synthesis of the individual translation products in response to environmental changes. This, in fact, takes place in the case of some activator proteins regulated by the availability of oxygen and/or carbon source (reviewed by Grivell, 1995; Fox, 1996a).

Another very important feature of mitochondrial translational activators is their relatively common association with mitochondrial inner membrane. Two *COX3*-specific translational activators, Pet122p and Pet494p, are integral membrane proteins, substantially resistant to alkaline carbonate extraction. The third, Pet54p, is bound to the membrane fraction and can be extracted with alkaline carbonate which indicates its peripheral association with the membrane (McMullin & Fox, 1993). A similar tendency has been observed for two activator proteins that are required for Cobp synthesis. Both Cbs1p and Cbs2p are membrane bound: Cbs2p is peripherally associated whereas Cbs1p is an integral membrane protein (Michaelis & Rodel, 1990; Michaelis *et al.*, 1991). Pet111p and Pet309p behave like integral mitochondrial membrane proteins when overexpressed to allow their detection (Fox, 1996a; Manthey *et al.*, 1998).

It appears that mitochondrial ribosomes cannot simply initiate translation by interacting, with unassisted mitochondrial mRNAs in the matrix. The initiation of translation requires specific activators bound to the 5′-UTL which probably support the positioning of ribosomes over the correct initiation codon. Since the activators are associated with the membrane, the translation should occur on the surface of the mitochondrial inner membrane. Indeed, such coupling of the mitochondrial protein synthesis machinery with inner membrane has been considered for decades as an explanation for the firm binding of ribosomes to membranes (Bunn *et al.*, 1970; Spithill *et al.*, 1978; Marzuki & Hibs, 1986). Additionally, there is evidence indicating co-translational insertion into membrane of at least some mitochondrial translation products (reviewed by Poyton *et al.*, 1992). This would allow the nascent polypeptide chains to by synthesized near their site of assembly into multimeric respiratory complexes.

The data supporting such a scenario came from two sets of experiments. First, a mutation affecting *CBS2* was partially suppressed by overproduction of the protein encoded by the *ABC1* gene, implicated in the correct fold-

ing or assembly of cytochrome *b* (Bousquet *et al.*, 1991).

Second, the productive tethering of the ribosome and translated mRNAs to the inner membrane facilitating the co-translational insertion of newly synthesized polypeptides was considered as a possible interpretation of experiments described recently by Sanchirico *et al.* (1998). This study was performed on chimeric *COX2* and *COX3* mRNAs with the 5′ untranslated region derived from the mRNA encoding the soluble and hydrophilic ribosomal protein Var1. DNA sequences specifying these chimeric mRNAs were inserted into mtDNA at the *VAR1* locus and expressed in the strains containing a plasmid that supplied a functional form of the Var1 protein, imported from the cytosol. Although both Cox2p and Cox3p were actively synthesized, such strains remained respiratory deficient and defective in the accumulation of Cox2 and Cox3 subunits. This can be explained by assuming that both *COX2* and *COX3* mRNAs are translated at a location, probably in the matrix, that does not allow the productive assembly of subunits into the cytochrome oxidase complex leading to their rapid degradation. Interestingly, Cox2p synthesized as a precursor was correctly processed to the mature form which suggests that translocation, at least of the N-terminal part, through the inner membrane was not prevented, but could be either aberrant or in a wrong place.

The stability of mitochondrially synthesized proteins is also affected by alteration in mitochondrial ribosome. It was recently documented for some yeast strains that single amino acid substitution in the ribosomal protein Nam9 correlates with a decrease in the steady state level of some mitochondrially translated proteins and a lack of Cox2p, although *de novo* mitochondrial protein synthesis is not changed. This finding suggests that Nam9p, like the translational activator proteins and the 5′-untranslated region of the mRNA, is involved in productively attaching the ribosome to the mitochondrial inner membrane. Nam9-1p might be defective in this process, leading to problems in the biogenesis of Cox2p and possibly other respiratory chain components (Chacinska *et al.*, 2000).

However, it is important to emphasize that 5′-UTLs in mRNAs coding for hydrophobic proteins are interchangeable because Cox3 translated from chimeric mRNA with *COB* 5′-UTL finds its way into the active cytochrome *c* oxidase, allowing cells to respire (Fox, 1996a). On the other hand, translation of a mitochondrial mRNA under the control of membrane-bound activators is not sufficient to insert a soluble reporter protein into the inner membrane (Steele *et al.*, 1996).

The facts presented above lead to speculations about the advantages of such a complicated and specialized system of distinct translational activators for assembly of respiratory enzymes. The higher order organization of the mitochondrial protein synthesis machinery, including translational activators, and proteins required for post-translational events, may exist at discrete sites in the inner membrane allowing synthesis and membrane insertion of the polypeptides in a defined topological orientation.

EXPORT OF PROTEINS FROM THE MITOCHONDRIAL MATRIX

Among the proteins synthesized on mitochondrial ribosomes, the subunit 2 of cytochrome *c* oxidase (Cox2) is particularly interesting because it is synthesized as a precursor with an N-terminal leader peptide consisting of 15 amino-acid residues, that is removed upon translocation through the inner membrane (Sevarino & Poyton, 1980; Pratje & Guiard, 1986). The leader peptide is essential for the accumulation of functional Cox2p and most likely initiates its insertion into the membrane (Torello *et al.*, 1997; He & Fox, 1997). In the *cox2* mutants lacking the presequence the defect is bypassed by mtDNA re-

arrangement that results in a fusion of the sequence coding for the amino-terminal part of the Cob protein to the Cox2 coding sequence (Torello *et al.*, 1997).

The products of five nuclear genes, *IMP1*, *IMP2*, *SOM1*, *OXA1* and *COX20* were found to be involved directly or indirectly in the removal of the signal sequence since mutations affecting these genes prevented the processing of the Cox2 precursor.

IMP1 and *IMP2* code for subunits of the mitochondrial inner membrane protease 1 that is responsible not only for the maturation of the Cox2 subunit but also for the second proteolytic step of processing of some intermembrane space proteins synthesized and imported from the cytosol (Behrens *et al.*, 1991; Schneider *et al.*, 1991). Two subunits of this enzyme are localized on the intermembrane face of the inner membrane and display non-overlapping specificity. Imp1p recognizes Cox2p, cytochrome b_2 and NADH cytochrome b_5 reductase (Mcr1p) as the substrates whereas Imp2p is required for the processing of cytochrome c_1 and cytochrome *c* peroxidase as well as for the stability of Imp1p (Pratje & Guiard, 1986; Hahne *et al.*, 1994; Nunnari *et al.*, 1993).

Another protein most likely required for the activity of the mitochondrial inner membrane protease is Som1p. *SOM1* was identified as a multicopy suppressor of a mutation in the *IMP1* gene. Deletion of the *SOM1* gene led to the non-respiratory phenotype, strong reduction of cytochrome b_2 and accumulation of the Cox2 precursor. Thus, the small 8.4 kDa inner membrane Som1 protein appears to be a factor essential for correct functioning of the Imp1 protease (Esser *et al.*, 1996; Bauerfeind *et al.*, 1998).

Since the cleavage of the Cox2 precursor occurs on the outside surface of the inner membrane and other products of mitochondrial translation contain at least one hydrophilic tail exposed into the intermembrane space (Poyton *et al.*, 1992), a mechanism responsible for their translocation out of the matrix should be present in mitochondria. In opposition to extensive studies on the mechanisms of protein import from the cytoplasm into the mitochondria, very little is known about proteins responsible for the export of mitochondrially synthesized polypeptides (reviewed by Tokatlidis & Schatz, 1999). The only known component involved in this process is Oxa1p, a conserved integral inner membrane protein (Bonnefoy *et al.*, 1994a; 1994b; Kermorgant *et al.*, 1997). Oxa1p is required for the export of N- and C-terminal tails of Cox2p, being responsible for an important step of cytochrome *c* oxidase biogenesis, and also plays a role in ATP-synthase formation (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994b; Altamura *et al.*, 1996; He & Fox, 1997; Hell *et al.*, 1997; Kermorgant *et al.*, 1997; Meyer *et al.*, 1997). Oxa1p has been reported to interact physically with nascent chains synthesized in mitochondria, including Cox2p and Cox3p. In addition to its function in the biogenesis of mitochondrially encoded proteins, Oxa1p appears to play an even more general role in translocation of some imported nuclear encoded proteins, including Oxa1p itself. The export from the matrix, like other pathways involved in the translocation across the inner mitochondrial membrane, requires a membrane potential and resembles the sec-independent sorting of membrane proteins in prokaryotes (Hell *et al.*, 1998; Herrmann *et al.*, 1997; Rojo *et al.*, 1999). Thus, Oxa1p represents a central component of the general machinery that translocates hydrophobic proteins from the matrix into the inner mitochondrial membrane. It is worth to note in this context that, surprisingly, the conserved function of Oxa1p was by-passed by specific mutations affecting cytochrome c_1 that decreased its hydrophobicity and membrane association but not electron transfer (Hamel *et al.*, 1998).

Recently, the *COX20* gene was identified by complementation in a mutant defective in Cox2 processing. The *cox20* mutations resulted in accumulation of Cox2 precursor although they did not affect export or process-

ing directly. Cox20p was found to be bound to the precursor and mature form of Cox2p which suggested its chaperone-like function necessary for the cleavage and subsequent interaction of mature Cox2 subunit with other polypeptides of cytochrome *c* oxidase in the assembly step (Hell *et al.*, 2000).

In addition to the pathway of cytochrome *c* oxidase formation, intriguing data came from study of the export of artificial reporter protein, Arg8p, fused to Cox2-presequence. The translocation of this mitochondrially encoded fusion protein across the inner membrane was blocked by mutation in the *PNT1* gene. The product of this gene, originally identified as conferring resistance of the cells to the antibiotic pentamidine, was shown to be an integral inner membrane protein. Although the null mutant did not affect either the respiratory growth capacity or the export of wild-type Cox2 subunit in *S. cerevisiae*, the deletion of its homologue in *Kluyveromyces lactis* resulted in a clearly non-respiratory phenotype and a defect in the assembly of KlCox2 subunit that appeared to be due to a block in translocation of the C-terminal part. This demonstrates that the Pnt1 protein in *K. lactis* plays an important physiological role and suggests that an overlapping function may exist in *S. cerevisiae*. Since the *pnt1* deletion caused increased sensitivity to H_2O_2 and accumulation significantly more [*rho*$^-$] mutations under respiratory growth conditions, one may hypothesize that Pnt1p plays a role in the mitochondrial "detoxification" system promoting the transport of components damaged by oxidative stress out of the matrix (He & Fox, 1999).

TURN-OVER AND QUALITY CONTROL OF MITOCHONDRIAL MEMBRANE PROTEINS

It has been known for a long time that mitochondria contain their own protein degradation system and now it is clear that all known mitochondrial proteases are homologous to prokaryotic ATP-dependent proteases. Mitochondrial soluble and membrane-bound enzymes differ in specificity.

The inner mitochondrial membrane contains proteins related to bacterial FtsH ATP-dependent metalloproteases, which are members of the AAA family of ATPases associated with diverse cellular functions. These proteins are responsible for the degradation of incomplete and unassembled newly synthesized mitochondrial translation products (reviewed by Suzuki *et al.*, 1997). The *YME1* gene was identified by complementation of the mitochondrial DNA escape phenotype (Thorsness *et al.*, 1993) and independently by complementation in a mutant defective in the degradation of unassembled Cox2 subunit of cytochrome *c* oxidase (Nakai *et al.*, 1995). Yme1p forms an inner membrane complex of approximately 850 kDa with the active sites on the intermembrane space surface, that can degrade a reporter protein anchored in the mitochondrial inner membrane (Leonhard *et al.*, 1996). Two genes, *AFG3* and *RCA1,* encode components of the second complex present in the inner membrane that exposes its catalytic sites to the mitochondrial matrix. A coordinated proteolytic and chaperone-like function has been reported for the Afg3/Rca1 complex (Pajic *et al.*, 1994; Guzelin *et al.*, 1996; Arlt *et al.*, 1996; 1998). Both *rca1* and *afg3* mutants synthesize subunits of respiratory complexes at normal levels and import the cytoplasmically synthesized subunits as efficiently as the wild type, but the assembly of respiratory complexes is affected (Paul & Tzagoloff, 1995). Additionally, the proteolytically inactive variants of Afg3p and Rca1p were able to complement the respiratory deficiency caused by the lack of Afg3p and Rca1p, suggesting that the protease function is not essential for the maintenance of respiratory competence (Guzelin *et al.*, 1996; Arlt *et al.*, 1996). Indeed, the chaperone-like activity of the Afg3/Rca1 complex was required for the assembly of mitochondrial ATP synthase (Arlt *et al.*, 1996).

The observation that the *OXA1* gene acts as a multicopy suppressor of respiratory deficiency of *afg3*- and *rca1*-null mutants suggests an intriguing connection between the efficiency of translocation and the Afg3p/Rca1p-dependent events, supporting the assembly function of the protease complex (Rep *et al.*, 1996). Such a bypass of *afg3*- and *rca1*-null mutations was also possible when the *MBA1* gene encoding an inner membrane-associated protein was overexpressed, indicating its related role in the biogenesis of the respiratory chain (Rep & Grivell, 1996).

Assembly and degradation of proteins appear to be opposite processes, but ATP-dependent proteases may be involved at the step of decision between assembly or degradation events, carrying out the quality control of polypeptides and regulating the subunit stoichiometry of protein complexes. This could be achieved by holding of newly synthesized proteins until other partner subunits become available (Suzuki *et al.*, 1997; Grivell *et al.*, 1999).

ASSEMBLY OF RESPIRATORY COMPLEXES

In contrast to the availability of a broad spectrum of structural data on ATP synthase, cytochrome *c* oxidase and bc_1 complexes, very little is known about the processes involved in their assembly.

The list of nuclear genes that have been identified as affecting late steps of the respiratory chain biogenesis is still growing. The Cox10 and Cox11 proteins that were originally reported to be essential for a posttranslational step of cytochrome *c* oxidase biogenesis are in fact required for the biosynthesis of heme A (Nobrega *et al.*, 1990; Tzagaloff *et al.*, 1993; Glerum & Tzagaloff, 1994; Hiser *et al.*, 2000). Cox17p and Sco1p function as carrier proteins in the delivery of copper from the cytosol to the mitochondrial Cox1 and Cox2 subunits of cytochrome *c* oxidase (Glerum *et al.*, 1996a; 1996b; Rentzsch *et al.*, 1999). The point mutants in *COX17* and *SCO1* genes can be rescued by alterations in the Sco2 protein that shows homology to Sco1p and has a potential copper-binding domain (Glerum *et al.*, 1996a). The above mentioned Cox20p acts probably as a membrane-bound chaperone (Hell *et al.*, 2000).

In the case of several other proteins including Pet100, Pet117, Pet191, Cox14 and Cox15 it is known only that they play an undefined role at some stage after the synthesis of the mitochondrially encoded subunits of cytochrome *c* oxidase. Strains bearing mutations in the genes encoding those proteins lack, or have a decreased level, of the studied subunits or of the whole complex, and display respiratory incompetence, although the *de novo* synthesis of proteins is not impaired (Church *et al.*, 1996; McEwen *et al.*, 1993; Glerum *et al.*, 1995; 1997).

It has been demonstrated recently that prohibitins, ubiquitous, abundant and strongly conserved proteins, play a role in the stabilization of mitochondrially synthesized polypeptides (Steglich *et al.*, 1999; Nijtmans *et al.*, 2000). The yeast homologues of mammalian prohibitin and prohibitin-related protein, Phb1p and Phb2p, respectively, form a complex in the inner mitochondrial membrane, which probably contains 12–16 copies each of Phb1p and Phb2p and does not contain any other proteins (Snyers *et al.*, 1998; Nijtmans *et al.*, 2000). Pulse-chase experiments with mitochondria showed that the Phb1/2 complex is able to stabilize newly synthesized Cox2p and Cox3p that remain associated with Phb1/2 until being assembled into the oxidase complex. This suggests the possibility that the Phb1/2 complex protects newly synthesized mitochondrial proteins from proteolysis and functions as a membrane bound chaperone (Nijtmans *et al.*, 2000). Moreover, expression of the Phb1/2 complex is increased in a yeast strain with altered Mss51p, the translational activator of *COX1*. Transient induction of the complex due to imbalance in the mitochon-

drially encoded subunits in the *mss51* mutant, might suggest a holding function, preventing the synthesized peptides from misfolding (Nijtmans *et al.*, 2000).

The assembly of F_0F_1-ATPase (complex V) requires the Atp10, Atp11 and Atp12 proteins. Atp11p and Atp12p are localized in the matrix and are responsible for the biogenesis of F_1, whereas the membrane localized Atp10p functions in the assembly of the F_0 component of mitochondrial ATP synthase (Ackerman & Tzagaloff, 1990; Ackerman *et al.*, 1992; Bowman *et al.*, 1991; Wang & Ackerman, 1998; 2000).

The bc1 complex is dependent on the action of the *ABC1* gene product that was originally identified as a multicopy suppressor of a *COB* mRNA translational defect. Additionally, three proteins, Cpb3, Cpb4 and Bcs1, seem to be involved in the process of assembly. In the mutants affecting the Abc1, Cpb3 and Cpb4 proteins the absence of either NADH-succinate dehydrogenase activity or spectrally detectable cytochrome *b* were observed, whereas alteration in Bcs1p, a member of the AAA family, led to a decrease in the FeS protein content indicating its role specifically in biogenesis of the Rieske iron-sulfur protein (Wu & Tzagaloff, 1989; Bousquet *et al.*, 1991; Nobrega *et al.*, 1992; Crivellone, 1994; Brasseur *et al.*, 1997).

A remarkable feature of the whole set of factors discussed above is that the majority of them display no similarity to known chaperone proteins. The only exception known so far are prohibitins that show weak homology to members of hsp60 family (Nijtmans *et al.*, 2000). Thus, it seems reasonable to suppose that, in contrast to the matrix environment where the "classical" chaperones function, other mechanisms, specific for mitochondrial inner membrane, are involved in the formation of the respiratory chain complexes.

CONCLUDING REMARKS

Correct localization and assembly of newly synthesized product into higher order structures is one of the fundamental processes of the cell. The importance for the cellular economy of coordination between translation and those post-translational events has driven the evolution of unique and sometimes unexpected regulatory systems. Coupling of mitochondrial translation with export, quality control and assembly might be advantageous for formation of respiratory complexes in yeast mitochondria. A still growing list of genes involved in this process suggests that so far we really have little idea of what is going on at the late stage of proteins biogenesis in the inner mitochondrial membrane.

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