Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process

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EUKARYOTIC CELLS are able to produce energy, in the form of ATP molecules, by two different pathways. Energy can be generated via glycolysis or by oxidation of glucose to ethanol or lactic acid. In aerobic conditions, the complete oxidation of carbohydrates, proteins, and fats to CO2 and H2O, via the or lactic acid. In aerobic conditions, the complete oxidation of ATP molecules, by two different pathways. Energy can be

...energy, resulting in a decrease of the proton gradient, which will finally lead to the stimulation of mitochondrial respiration (133, 134). However, an additional regulatory mechanism of mitochondrial respiration known as physiological control has been proposed (reviewed in Refs. 102, 124). It is based on the finding that complex IV of the respiratory chain, cytochrome c-oxidase (COX), is inhibited at a high intramitochondrial ATP-to-ADP ratio (66), through ATP binding to subunit IV of the mammalian enzyme, subunit Va of the yeast enzyme (8, 97). This mechanism of respiratory control is switched on by
cAMP-dependent phosphorylation, and it is switched off by calcium-induced dephosphorylation of COX (21). The phosphorylation event converts COX into the rate-limiting step of respiration (11, 205), thus highlighting the key role of COX in regulating the rate of respiration and ATP synthesis. This allosteric regulation of COX is short term because it results in a change of enzyme kinetics, it is immediate, and does not require protein synthesis and assembly. A long-term regulation of COX also exists and consists of controlling the number of the enzyme molecules present in the mitochondrial inner membrane and/or controlling the subunit isofrom composition that confers the different catalytic properties of enzymes. This regulation requires protein synthesis and assembly.

COX is a multimeric enzyme, with its assembly involving the interplay of two different genomes and requiring the assistance of a large number of assembly factors acting at several levels of the process. The purpose of this review is to summarize the actual knowledge about the process of COX assembly and the multiple levels of its regulation. Although some information about the bacterial and animal enzymes will also be reviewed, we will mostly focus on the S. cerevisiae system. Although still fragmentary, a large body of information about the COX assembly process and its regulation in yeast is available because of the amenability of this model organism for large-scale mutagenesis and subsequent selection of respiratory and COX mutants (130, 199) and for genomic and proteomic studies (12, 87, 158). There is no doubt that to decipher the intricacies of a biological mechanism complex fundamentally critical for cellular bioenergetics, such as the COX assembly process, the multidisciplinary approach should integrate data resulting from biochemical and genetic analyses of genome, transcriptome, proteome, and metabolome. In this light, systems biology can indeed reveal new insights and relevance on COX assembly and its regulation.

COX STRUCTURE AND ASSEMBLY

In the yeast Saccharomyces cerevisiae, COX is formed by 11 different subunits of dual genetic origin. The three largest subunits, Cox1p, Cox2p, and Cox3p, are encoded in the mitochondrial DNA (mtDNA) and form the catalytic core of the enzyme. These subunits are transmembrane proteins, embedded in the mitochondrial inner membrane with 12, 2, and 7 transmembrane α-helices, respectively, and coordinate heme A and copper cofactors, which are responsible for the electron transfer activity. Cox1p contains two redox centers, one formed by a low-spin heme A and another by a high-spin heme α3 and CuB. A third redox center of the enzyme is formed by the two copper ions present in the CuA site of Cox2p. The CuA active site accepts electrons from cytochrome c and rapidly reduces heme A. From heme A, the electrons are transferred intramolecularly to the heme α3-CuB binuclear center, where molecular oxygen binds to be reduced (11). For each electron transferred, two protons are pumped across the mitochondrial inner membrane. Cox3p does not contain prosthetic groups, and, although its function is unknown, it could be involved in the assembly and/or stability of subunits 1 and 2 or in the modulation of the access of oxygen to the binuclear center (30, 161). Interestingly, studies performed on bacterial COX, formed exclusively of the three core subunits, have shown that Cox3p could play a role in modulating proton transfer through subunits 1 and 2 (95).

In addition to the mtDNA-encoded subunits that are synthesized by the transcriptional/translational machinery present in the mitochondrial matrix, the enzyme is composed of several small nuclear-encoded subunits, synthesized in the cytoplasm and imported into mitochondria. The nuclear-encoded subunits surround the catalytic core of the enzyme and are not essential to catalyze oxygen reduction and proton vectorial transfer. The specific functions of these subunits are not yet completely understood, but it is known that they are involved in the assembly/stability and dimerization of the enzyme. They also play a significant role in protecting the catalytic core from reactive oxygen species (ROS) and in modulating the catalytic activity of the enzyme. The importance of these subunits was demonstrated by the complete loss of COX activity and, in consequence, cellular respiration of yeast strains carrying null mutations in the nuclear genes encoding for subunits Cox4p, two isoforms of Cox5p (Cox5ap and Cox5bp), Cox6p, Cox7p, and Cox9p, suggesting an essential role for these subunits in assembly/stability of the enzyme (3, 36, 62, 195, 215). Subunit Cox5ap is also involved in catalysis modulation. As mentioned above, ATP binding to the matrix domain of Cox5ap, when the intramitochondrial ATP-to-ADP ratio is high, causes a decrease in the efficiency of energy transduction or decreased H+/e− stoichiometry of proton pumping (188). Moreover, the COOH-terminal domain of Cox5p, conserved between both isoforms, interacts with subunit 2, and it was proposed to have a regulatory function preventing the backflow of water or protons from the cleft formed between Cox1p and Cox2p (32, 98, 198). COX can exist in the mitochondrial inner membrane as either a monomer or a dimer, the latter being considered the active form of the enzyme, in which two monomers are connected by a cardiolipin molecule (103, 197).

The majority of the information regarding the dimeric structure of COX was obtained by crystallographic resolution of the structure of the bovine heart enzyme (198), showing that the cytochrome c binding site (two molecules of cytochrome c bind to the dimer in a cooperative way) is located in a cleft at the interface between the two monomers (24, 57), and both the mitochondrial-encoded subunit Cox1p and some of the nuclear-encoded subunits participate to form the four subunit-subunit contacts that stabilize the dimeric structure (116). Interestingly, a strain carrying a null mutation of cox8 was found to retain a residual COX activity of 80% of the wild-type strain, resulting from the inability of the enzyme to dimerize, which reduces the efficiency of electron transfer between cytochrome c and COX (151). Finally, the deletion of COX13, coding for subunit 10, does not affect COX activity, suggesting that the enzyme can be assembled in the absence of this subunit (188).

At present, the process of COX assembly is only partially known, probably because there is a fast turnover of the hydrophobic core subunits in COX assembly-defective mutants, which is particularly exacerbated in yeast as explained below. Already in the early 1980s, studies that used rat liver mitochondria suggested that COX biogenesis occurs in a sequential process, with the different subunits being added in an ordered manner (208). Analyses of the human enzyme by Blue-Native electrophoresis have allowed a proposed assembly pathway characterized by at least three discrete assembly intermediates that probably represent rate-limiting steps in the process (143).
In accordance with the model proposed by Nijtmans et al. (143), biogenesis of Cox1p is the first step of the process and corresponds to the first assembly intermediate. The second intermediate is possibly formed by Cox1p, CoxIVp (the human homologue of the yeast subunit Cox5ap), and perhaps CoxVap (yeast subunit 6) (212). The third intermediate contains all of the subunits except subunits VIa and VIIa or VIIb (corresponding to the yeast subunits 10 and 7, respectively), the addition of which concludes the formation of the holoenzyme (143, 212). Studies performed with yeast mutants have shown that the COX assembly process is conserved between yeast and human. The fact that, in yeast COX assembly mutants, the amount of newly synthesized Cox1p is translationally downregulated (15) (explained below) probably prevents the formation of detectable amounts of assembly intermediates in most COX mutants. However, it is well known that Cox1p is necessary for the assembly of Cox2p, Cox3p, and Cox4p, and recent analyses of yeast cox2 mutants have revealed the presence of subassemblies in yeast similar to the ones described in human cells (92).

Assembling the structural subunits forming COX involves an elaborated set of pathways (Fig. 1). How the expression of subunits encoded in two physically separated genomes is coordinated, how the proteins find each other in the mitochondrial inner membrane, and at what extent COX assembly is a protein-assisted process are three examples of essential questions from which a complete answer is not yet available despite the efforts of a large number of laboratories. Several considerations allow us to understand the complexity of the COX assembly process and the complexity of its regulation. 1) Because of the dual-genetic origin of COX, the expression of COX subunits has to be coordinated and regulated to form a fully assembled enzyme in which the 1:1 stoichiometry of the structural subunits is always respected. 2) The dual-genetic origin also leads to the necessity of two different pathways of transcription, translation, maturation, and mitochondrial import. In addition to the structural subunits, the assembly of COX requires the function of a large number of protein factors necessary for all steps in the process. The analysis of assembly intermediates described above has allowed insights into the overview of the assembly process, but information about the players involved and their specific roles has been very limited. To disclose the factors involved in COX assembly, a profitable strategy has been to systematically analyze yeast mutants defective in COX assembly, with the goal of identifying the functions of the gene products responsible for this phenotype and, by this means, reconstruct the different steps of the assembly pathway. Screens of nuclear respiratory-deficient mutants have revealed the existence of >20 additional nuclear-encoded factors acting at all levels of the process (some indicated here). The process is regulated in both nuclear-mitochondrial directions in a highly unique crosstalk network. IMS, intermembrane space; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane.
these proteins during the assembly process require the activity of specific chaperones. 5) The protective role of chaperones is also emphasized by the fact that COX assembly takes place in the proximity of the sites of ROS production in the mitochondrial membrane, making both the protein subunits and the metal cofactors possible targets of oxidation. In addition, alterations in the assembly process could produce inefficient enzymes that could lead to an increase in the production of ROS. For this reason, COX biogenesis has to be precisely regulated, especially the synthesis and assembly of subunits Cox1p and Cox2p, containing active metal centers. 6) Different isoforms of nuclear-encoded subunits confer to the enzymes’ different kinetic properties, and their expression is highly regulated in response to a plethora of stimuli. In the case of the yeast *S. cerevisiae*, only one nuclear-encoded subunit exists in two isoforms, the above-mentioned Cox5a and Cox5b proteins, which are differently regulated by oxygen concentration (206). 7) Finally, multiple points of quality control of the assembly process and degradation of unassembled subunits and/or incomplete enzymes play an important role in COX biogenesis (7, 147, 160).

**REGULATION OF COX ASSEMBLY**

Regulation of COX biogenesis and activity in response to changing environmental or physiological conditions plays a central role in the physiology and metabolism of the adapting cells. The study of these regulatory processes has been approached from the perspective of multiple individual disciplines together with the methodology used by systems biology, which focuses on the iterative and integrative study of biological systems in response to perturbations, as defined by Auffray et al. (10).

Multiple levels of regulation characterize the COX assembly process, including availability of subunits and assembly factors regulated at the transcriptional and translational levels, availability of cofactors, protein import into mitochondria and membrane insertion, and coordination of sequential or simultaneous steps of the process (Fig. 1). In this section, we will summarize the present knowledge about several pathways involved in regulation of COX assembly in the yeast *S. cerevisiae*.

The yeast *S. cerevisiae* is an aerobic-anaerobic facultative organism. When grown in medium containing glucose, it produces energy and ethanol through glycolysis and fermentation, while the genes encoding proteins involved in the respiratory metabolism are repressed. When glucose is exhausted and if oxygen is present, yeast cells are able to respire by use of ethanol, previously produced as a carbon source. This requires a dramatic change of the metabolism from fermentation to respiration, called diauxic shift. During the diauxic shift, the respiratory genes are derepressed and induced by the action of several transcription factors (reviewed in Ref. 175). COX activity is modulated according to the growth conditions and cell metabolism. It is low in cells grown in the presence of glucose, and, thus with fermenting, it is induced fivefold after the diauxic shift (31, 153). COX activity is also low in cells grown in copper-depleted media (213), and it is absent in cells grown in anaerobiosis and in heme-deficient mutants (119, 164, 214). Regulation of COX biogenesis allows for the modulation of COX activity in response to substrate availability and oxygen concentration. The level of fully assembled and functional enzyme is principally controlled by the availability of the structural subunits and assembly factors, depending mostly on transcriptional regulation of nuclear and mitochondrial COX genes and by translational regulation of mitochondrial COX subunits.

**TRANSCRIPTIONAL REGULATION OF NUCLEAR COX ASSEMBLY GENES BY OXYGEN**

All of the nuclear genes encoding for COX subunits (*COX4*, *COX5a*, *COX6*, *COX7*, *COX8*, and *COX9*), with the exception of *COX5b*, are aerobic genes (31, 157). They are optimally expressed at oxygen pressure of 200 μM, corresponding to the atmospheric oxygen concentration, but they are also expressed at lower levels in anaerobic conditions (33). *COX5b*, however, is a hypoxic gene exclusively expressed at oxygen concentrations below 0.5 μM (33). The oxygen regulation of these genes was exhaustively studied in cultures grown in medium containing galactose, a nonrepressing sugar, to minimize the effects of carbon sources on the expression of COX genes (33). Analyses of the data obtained revealed that the expression of the aerobic genes is dependent on the oxygen concentration, not merely by its presence or absence. With the exception of *COX5a*, which is transcribed at stationary levels for oxygen concentrations between 200 μM and 10 μM, the other aerobic genes showed a marked decline (40–70%) of expression between 200 and 100 μM oxygen, whereas their expression remained nearly constant between 100 and 10 μM. Under 10 μM oxygen, all of the aerobic genes present a gradual decline in expression with a drastic decline at 1 μM oxygen for *COX4*, *COX6*, *COX7*, *COX8*, and *COX9* and 0.5 μM oxygen for *COX5a* (33). Given the sharp decline of expression of the dose-response curves, 1 and 0.5 μM oxygen are considered the thresholds for the expression of these aerobic genes, although they present a residual expression variable from 7% of *COX4* to 39% of *COX9*, in complete anaerobiosis (2.5% CO2 in oxygen-free nitrogen) (33). The levels of *COX5b* mRNA are undetectable for oxygen concentrations >0.5 μM, indicating that the hypoxic gene is more tightly regulated than the aerobic genes (33). Kinetic analysis of oxygen-regulated induction/repression (exemplified in Fig. 2) indicated that, although only *COX8* is fully expressed 90 min after a shift from anaerobiosis to air, the other aerobic genes reach expression between 25% (*COX9*) and 80% of their atmospheric steady-state levels after 2 h (33). Even more complex are the kinetics of gene expression after shift from air to anaerobiosis. In this case, the aerobic genes present a small increase for the first 10–20 min after the shift, followed by a rapid decrease in expression during 20 min and finally a slow continued decrease thereafter. However, the overall decrease in speed could not be totally explained by the downregulation of transcription, and it was proposed that a second mechanism involving mRNA-specific degradation exists (33, 56). The induction of the hypoxic gene *COX5b*, after a shift from aerobic to anaerobic growth conditions, is markedly slower than the induction of aerobic genes during a shift of opposite direction. The full expression of *COX5b* requires in fact 12 h (157), whereas the analysis of the repression kinetics showed that only 20 min after a shift from anaerobiosis to air *COX5b* mRNAs are undetectable (33). In summary, these data...
suggest that aerobic and hypoxic genes are regulated through different signal transduction pathways and that cells adapt faster to environmental changes in which the oxygen concentration increases rather than a switch to anaerobic conditions. It appears also surprising that, in anaerobic growth conditions, there is some expression of genes encoding for subunits of COX, an enzyme of the respiratory metabolism that requires oxygen as substrate. To try to explain this observation it is necessary to distinguish between two conditions: growth at low oxygen and growth in complete anaerobiosis. In the first case, COX expression can be explained considering the different kinetic properties that the two different isoforms of subunit 5 confer to the enzyme. The transmembrane α-helix of Cox5p interacts with Cox1p and, depending on the isoform, is able to alter the protein environment around the binuclear center in a way that does not alter the $K_m$ of the enzyme but does alter the turnover number (4). The Cox5bp isoenzyme has a higher maximal turnover number, and, although in the presence of Cox5ap the access of electrons to heme $a_1$ is limited, electron transfer from heme $a$ to heme $a_3$ is three- to four times faster in the presence of Cox5bp (4). In conditions of low-oxygen tension, the synthesis of subunit Cox5bp, instead of subunit Cox5ap, confers an advantage to the cells that can respire better because they assemble a COX enzyme catalytically more efficient for the mitochondrial production of energy (89).

The amount of fully assembled COX decreases with decreasing oxygen concentrations, which results from the explained transcriptional repression of nuclear COX genes together with a posttranscriptional regulation of the mitochondrial genes COX1 and COX2 (158). At oxygen concentrations below 0.1 μM, COX activity is undetectable (164). However, even in complete anaerobiosis, COX genes are transcribed and, although with no direct correspondence to the mRNAs levels, both mtDNA-encoded and most nuclear-encoded COX subunits are present in protomitochondria, indicating that COX subunits are translated and imported in the complete absence of oxygen (33, 56). However, subunit 4 is detected only in very reduced amounts (0.2%), and subunit 8, involved in COX dimerization, is undetectable in protomitochondria by Western analysis (56).

Measurement of respiration by providing oxygen to anoxic cells indicated that, even in the absence of oxygen, a functional respiratory chain is maintained (58). This finding led to the hypothesis that the respiratory chain can use a final electron acceptor other than oxygen (58). This hypothesis was supported by the fact that COX is involved, probably acting as an oxygen sensor, in the induction of hypoxic genes (111) and the alternative acceptor could play a role in this induction. In oxygen-limiting growth conditions, the importance of the expression of hypoxic genes encoding for mitochondrial resident proteins can be understood considering that even during anaerobic growth the cells need to maintain many metabolic pathways that take place inside mitochondria. In addition, we can easily think that the presence of a minimum amount of fully assembled COX in the mitochondria of anoxic cells will allow for an immediate response of the cell to sudden increases in oxygen concentration, being already able to respire while increasing the extent of COX biogenesis.

The oxygen regulation of nuclear COX genes is mediated by heme that acts as an intracellular effector of the environmental signal (oxygen concentration) (165, 194, 196). Experimentally, in heme-deficient mutants, the expression of COX genes is independent of oxygen levels. In these mutants, hypoxic genes are derepressed and aerobic genes are repressed, whereas the supplementation of hemin to the medium complements the mutant phenotype (89, 140). Heme was considered the ideal effector of aerobic regulation because heme biosynthesis requires oxygen. Two enzymes of the biosynthetic pathway use oxygen as substrate (112), one of them being the product of HEM13, which catalyzes the rate-limiting step of the heme synthesis (112). Nevertheless, two interesting observations must be discussed further. First, COX genes are expressed in the complete absence of oxygen. At this respect, it is necessary to consider that Hem13p can also function in the absence of oxygen, probably by using another electron carrier as substrate (23, 111) and that a small amount of heme is also present in cells grown anaerobically (58, 90). Second, the mRNA levels of COX aerobic genes decrease with decreasing oxygen tension for concentrations between 200 and 1 μM, whereas in the same range of oxygen concentrations the intracellular level of heme remains quite constant (112). It has been suggested that heme could regulate the expression of COX genes by changes in heme concentration or by changes in heme redox states. The two models are not exclusive, and it was proposed that heme could act as the ligand of a transcription factor, modulating COX gene expression in a concentration-dependent manner for low oxygen concentrations (0–1 μM), and that heme acts as or affects the function of a transcription factor/oxygen-sensor protein depending on its redox state for higher oxygen concentrations (1–200 μM) (33). In consequence, in the...
involved in the regulation of COX activity and respiration.

Fig. 3. Transcriptional regulation of nuclear COX structural genes by oxygen concentration and by carbon source availability. In the presence of oxygen concentrations equal to or higher than 0.5 mM, COX5a is induced in a heme-dependent way by the transcriptional activator Hap2/3/4/5. Heme also induces the expression of the ROX1 gene, which is involved in the regulation of COX5b and COX6.

The inverse regulation by oxygen and heme of the genes encoding the two isoforms of subunit 5, as well as the specific repression of COX5b, is mediated by the Hap complex (34, 193, 194, 196). The inverse regulation by oxygen and heme of the genes encoding both isoforms of subunit 5 was extensively studied (Fig. 3). Although the gene encoding the aerobic isoform COX5a is induced by the Hap complex, the hypoxic gene COX5b is repressed by the heme-dependent transcriptional repressor Rox1/Reo1. In consequence, in aerobic growth conditions and in the presence of heme, Hap2/3/4/5 induces the expression of COX5a and Rox1/Reo1 represses the expression of COX5b, with the result that only the aerobic isoform of subunit 5 is synthesized in the cell. Instead, when the cells grow in anaerobic conditions or under low oxygen tension, i.e., in conditions of absence or low heme concentration, neither Hap nor Rox1/Reo1 can activate or repress the target COX genes, and the hypoxic isoform of subunit 5 is expressed (196). The oxygen-dependent action of Ord1/Ixr1, a second transcriptional repressor on COX5b gene that binds in the same target sequence as Rox1/Reo1 (113), was also reported. Ord1/Ixr1 represses specifically COX5b transcription, although it does not affect the expression of other hypoxic genes or that of aerobic genes such as COX5a. The heme-dependent repression operated by Rox1/Reo1 is not mediated through a direct effect of heme on Rox1/Reo1 protein, but it is due to a heme-dependent transcriptional activation of the ROX1 gene, operated by Hap1p (123, 225). It is interesting to note that, although the repression of COX5b is mediated indirectly by Hap1p, and therefore in a way depends exclusively on the environmental oxygen concentration, the oxygen-dependent transcriptional regulation of the COX aerobic genes is mediated by the Hap complex. Moreover, studies on the regulation of COX6 have demonstrated that, for this gene, the transcriptional regulation is independent of Hap1p (194). In consequence, the oxygen regulation is secondary to the regulation by the carbon source. Although small-scale experimental data to define the mechanism of oxygen regulation of the other COX nuclear-encoded subunits are not available, systematic analysis of yeast gene expression has indicated that the other COX genes are also regulated by the Hap complex (34, 76).

To understand whether the regulation of COX genes by oxygen was dependent on the same COX activity, mitochondrial respiration or OXPHOS, Dagsgaard et al. (56) measured the level of expression of COX aerobic genes in nuclear and mitochondrial mutants in which COX activity and consequently respiration or OXPHOS were completely abolished. The results indicated that neither COX activity, nor respiration, nor OXPHOS affects the expression of these genes. However, a marked reduction of the expression of COX genes, in both aerobic and hypoxic cells, was observed in cells devoid of mtDNA (p-strains) or containing mtDNA carrying large deletions (p-strains), indicating that the mtDNA affects the transcription of nuclear-encoded aerobic COX genes, in both normoxic and anoxic growth conditions, through a signaling pathway to the nucleus (56). In addition, the effects of oxygen...
and mtDNA on the expression of COX nuclear genes are independent of each other (56).

**TRANSCRIPTIONAL REGULATION OF NUCLEAR COX ASSEMBLY GENES BY CARBON SOURCE**

All nuclear COX structural genes are repressed by glucose and induced during the diauxic shift (61). As an example, the transcriptional regulation of COX6 gene has been extensively studied (Fig. 3). COX6 is regulated by oxygen in a heme-dependent way (194) and downregulated in the absence of mtDNA (56), as described above. Because COX6 is also repressed by glucose, cells grown in the presence of a respiratory carbon source such as lactate present four- to fivefold higher levels of COX6 transcript than shown in cells grown in glucose (216, 217). A peculiarity of COX6 expression is the length of the gene in three mRNA species with a difference in length of ~150 bp from one to the other (217). The three COX6 mRNAs show a microheterogeneity in the 5’ ends and a major diversity in the 3’ ends, accounting for the different lengths (217). More interesting is the fact that all three mRNA species are subjected to glucose repression, but with a different sensitivity. Glucose repression affects the two larger mRNAs, and derepression occurs in a very short time (~10 min). As a consequence, the relative ratio between mRNA species is different in cells grown in repressing or derepressing conditions. In glucose, the COX6 mRNAs population is enriched for the smallest mRNA class, whereas after a shift to medium containing low glucose or respiratory carbon sources, there are observed similar levels of the three different COX6 transcripts. Nevertheless, the data available do not allow discrimination between a different level of glucose-dependent transcription and different stability of the mRNA species. Transcriptional regulation of COX6 is mediated by at least four different transcriptional factors: Hap complex, Snf1p, Ssn6p, and Abf1p (Fig. 3 and Refs. 193, 194, 216). Snf1p is part of a complex essential for the release of glucose-repressible genes (46). When glucose is absent, Snf1p acts as a kinase, and it is able to phosphorylate various activators and repressors, modulating their activity (40). One possible target to Snf1p is Ssn6p, a factor involved in glucose repression (176). Because in a snf1 mutant only a slight derepression of COX6 is observed after the diauxic shift and because both a ssn6 single mutant and a snf1-ssn6 double mutant are constitutively derepressed, it is possible to conclude that both Snf1p and Ssn6p are involved in COX6 transcriptional regulation and the action of Ssn6p is epistatic to Snf1p-mediated regulation (216). Abf1p is a phosphoprotein that can have at least four different phosphorylation states, correlating with the cellular growth conditions, higher in cells grown in the presence of respiratory carbon sources. The phosphorylation state of Abf1p is also affected by Snf1p/Ssn6p because Ssn6p is essential for the dephosphorylation of Abf1p in a Snf1p-dependent manner (179). Although dephosphorylated Abf1p does not bind DNA, the level of phosphorylation determines the composition of Abf1p-containing protein-DNA complex formed on the COX6 promoters (179, 193), which modulates transcription of COX6 mRNA in a carbon source-dependent way. At present, it is not clear whether Snf1p/Ssn6p acts independently or in the same pathway as the Hap complex, which mediates the induction of COX6 after diauxic shift in a heme-dependent manner (34, 194).

Data regarding carbon source regulation of the other nuclear COX structural genes in *S. cerevisiae* derive essentially from systematic analyses of yeast gene expression. The results obtained from several microarray experiments have been reported over the past few years and have been analyzed for this review to extract data concerning regulation of COX subunits and COX assembly factors. DeRisi et al. (61) carried out a comprehensive investigation of the temporal program of gene expression (transcriptome) accompanying the metabolic shift from fermentation to respiration by using DNA microarrays containing virtually every gene of *S. cerevisiae* (61). Two other investigations based on the genome-wide exploration of gene expression patterns by microarray analysis were focused on the analyses of yeast gene expression in response to a large spectrum of environmental stresses ranging from osmotic and heat shock stress to oxidative stress, nutrient starvation, and acid and alkali stresses (45, 68). Gunji et al. (76) also analyzed the role of specific transcriptional factors on the reprogramming of gene expression after several environmental stress conditions. The same RNA microarrays were also used to identify genes whose expression was affected by deletion of the transcriptional activator HAP2 and HAP3 and expression patterns of genes encoding for known transcriptional factors in different growth conditions (76). By performing a similar kind of experiment, Buscien et al. (34) measured changes in the expression of the yeast transcriptome in cells grown in medium containing the fermentable, nonrepressing carbon source, galactose, induced by the deletion of HAP2 and HAP4. (34). Instead, Lascaris et al. (115) used a different approach to analyze the effect of Hap complex on the expression of yeast genes to understand their former observation that overexpression of HAP4 is able to override the signals that normally result in glucose repression of mitochondrial function. To compare the expression profile in repressing growth conditions (media supplement with glucose 3%) of wild-type cells and cells overexpressing Hap4p more than fivefold, the authors (115) performed a whole-genome expression profiling and fingerprinting of the regulatory activity network.

The analysis of expression profiles observed for COX genes in all of the experiments mentioned above have allowed us to better understand the role of COX assembly during the metabolic reprogramming that occurs during the diauxic shift and during several stress conditions. Taken together, these data trace a clear carbon source-dependent expression profile of the nuclear COX genes. Several conclusions emerge from the systematic analyses mentioned before. 1) Nuclear genes encoding COX subunits, with the exception of COX5b, are regulated by carbon sources; they are repressed by glucose and induced during diauxic shift (61). However, the glucose repression does not abolish completely their transcription, and, for example, COX6 mRNAs are still detectable in glucose-repressed cells (217). 2) The nuclear aerobic COX structural genes present a temporal pattern of expression marked by an induction coincident with the diauxic shift (glucose depletion) (61). 3) Strains carrying null mutations of *hap2* and *hap4* present a significant decrease in the expression of COX aerobic genes, ranging between 27% (COX4) and 52% (COX7) of the wild type in a *hap2* mutant and between 34% (COX4) and 62% (COX7) of the wild type in a *hap4* mutant (34). These results indicate that the Hap complex mediates the regulation of the nuclear COX genes, with the exception of COX5b (34, 76).
addition, nuclear aerobic COX structural genes are upregulated to approximately two times in wild-type cells overexpressing HAP4 (115). 4) HAP4 gene, encoding the catalytic subunit of the Hap complex, is also induced ninefold during diauxic shift, and it is one of the two transcriptional activators, among the 149 genes encoding putative transcriptional factors, to be induced more than threefold during diauxic shift (61). However, it has been demonstrated that the active Hap complex is formed by only one copy of each subunit (131), and HAP2, HAP3, and HAP5 are constitutively expressed (61, 76). 5) The Hap target sequence is present in the promoter (between −800 and −150 bp) of all the nuclear aerobic COX structural genes (34). However, binding of Hap2p/Hap3p to the Hap binding site present in the promoter of COX6 was not observed in vitro, suggesting an indirect effect of Hap2/3/4/5 on COX6 transcription (193). 6) The expression of nuclear COX structural genes changes less than twofold in response to environmental stress changes other than diauxic and oxygen concentration shift (45).

Although the analysis of the expression profiles observed for COX genes indicates clear lines of coregulation, this observation seems not to be extended to nuclear-encoded COX assembly factors. The regulation by diauxic shift of these genes is quite heterogeneous, and there are no clear traces of a common regulation pattern. For example, COX15 (involved in hydroxylation of heme o during heme A biosynthesis) and COX20 (a chaperon of Cox2p) are induced more than three times, whereas COX10 (involved in the farnesylation of protoheme during heme A biosynthesis) and COX11 (involved in the insertion of copper into the CuB center of Cox1p) are unaffected (61). The expression of some of these genes, such as COX10, COX17 (involved in the delivery of copper to mitochondria), OXA1 (necessary for the membrane insertion of the COX core subunits), and PET117 (coding for an assembly factor of unknown specific function), is practically unaffected during diauxic shift but highly induced in cells overexpressing HAP4. The promoter region of these genes does not contain the consensus Hap target sequence, suggesting an indirect effect of the Hap complex on their regulation by different environmental stimuli (115). It is possible that additional transcriptional factors play an important role in the regulation of the COX assembly factors. For example, analysis of the expression regulation of the nuclear gene PET494, coding for a translational activator of COX3 mRNA (129), showed that PET494 is repressed by glucose and induced by respiratory carbon sources such as ethanol, but its regulation is not mediated either by the Hap complex or by Snf1p, Snf2p, Snf6p, and Hxk2p, typical transcriptional factors controlling glucose repression of many nuclear genes (129).

Although there is a constant flux of information available, further analyses are necessary to understand which are the limiting factors and regulatory key points of the COX assembly process and to obtain a global view about how COX biogenesis is regulated.

TRANSLATIONAL REGULATION OF NUCLEAR COX ASSEMBLY GENES

Although the regulation of nuclear-encoded COX subunits expression is performed mostly at the transcriptional level, there are some examples in the literature of translational regulation. At this respect, it has been recently reported that the expression of COX4 is regulated posttranscriptionally by the mitochondrial content of cardiolipin, the major phospholipid component of the mitochondrial membranes, and its precursor phosphatidylglycerol in S. cerevisiae (185).

TRANSCRIPTIONAL REGULATION OF MITOCHONDRIAL COX GENES

The three subunits forming the catalytic core of eukaryotic COX are encoded in the mtDNA. As in the case of the nuclear-encoded COX subunits, the expression of the mtDNA-encoded COX subunits is affected by oxygen concentration and by carbon source.

Oxygen concentration affects expression of the three mtDNA genes in different manners. The levels of COX3 mRNA decrease two- to threefold under anaerobic growth, but the levels of COX1 mRNA are maintained constant under different oxygen concentrations. Interestingly, expression of Cox1p and Cox2p is affected by oxygen tension at the posttranscriptional level (51, 158).

Mitochondrial COX genes are repressed by glucose, even in the presence of oxygen, and induced during diauxic shift (137, 201). This regulation can be assigned to the general glucose repression of mitochondrial transcription. In fact, a lower total mitochondrial RNA synthesis was observed in repressed cells than in derepressed cells both in isolated mitochondria and in vivo (39, 137, 201, 220). A general glucose repression mechanism of mitochondrial transcription is also supported by the fact that mitochondrial RNAs present the same kinetics of derepression during a shift from medium containing glucose to medium supplemented with a respiratory carbon source such as glycerol (201). After release from glucose repression, the abundance of mitochondrial transcripts increases very slowly and coincides with active cell growth (201). However, the final change in RNA abundance varies significantly between different RNA species (201). In derepressing conditions (growth in medium containing galactose), the level of COX1 mRNA is increased almost 5-fold (137); in inducible conditions (growth in medium containing glycerol), it is increased 20-fold, whereas COX2 and COX3 mRNAs are increased ~3-fold (201). The final mitochondrial RNA abundance after derepression is dependent on three additional regulatory mechanisms. First, mitochondrial mRNAs have a half-life considerably shorter than the rRNAs (132), and they do accumulate differently. Second, mitochondrial promoters of diverse transcriptional units can sustain expression with different strength, with up to 20-fold of difference between weak and strong ones (25, 207). Finally, mitochondrial transcription is also influenced by the existence of polycistronic transcription units. Probably resulting from attenuation of RNA polymerase elongation, within the polycistronic transcription units, proximal genes are more transcribed than distal genes, with a difference of up to 17-fold (136).

Two mechanisms were proposed to be involved in general derepression of mitochondrial transcription after diauxic shift: the increase of mtDNA copy number and a fivefold induction of the mitochondrial RNA polymerase encoded by the nuclear gene RPO41 (73, 210). Nevertheless, the magnitude of variation of mtDNA copy number, from zero- to threefold between repressing and derepressing conditions, is not enough to ac-
count for the full increase in RNAs level (136, 201). Moreover, 20–50 times overexpression of the genes encoding the core of mitochondrial RNA polymerase (RPO41) and its specificity factor (MTF1) in repressed cells do not result in release of mtDNA genes from glucose repression (201).

Derepression of mtDNA genes is affected indirectly by some nuclear transcription factors: Snf1p, Reg1p, and Urr1p. Mutations in SNA1 are known to prevent derepression, whereas mutations in REG1 or URR1 lead to a constitutive derepression state (201). Interestingly, overexpression of the catalytic subunit Hap4p increases 2.3-fold the COX2 mRNA level and 1.45-fold the COXI mRNA level in repressed cells (115), suggesting that the Hap complex could also indirectly affect the expression of mtDNA genes.

**TRANSLATIONAL REGULATION OF MITOCHONDRIAL COX GENES**

Mitochondria contain the translational machinery necessary for the synthesis of the mtDNA-encoded COX subunits. The genes encoding for the ribosomal RNA of the large and small ribosomal subunits and a set of tRNAs are also located in the mtDNA in yeast and higher eukaryotes. The proteins forming the mammalian mitoribosome are all encoded in the nuclear genome, whereas in the yeast *S. cerevisiae*, the Var1 protein, a component of the small ribosomal subunit, is encoded in the mtDNA. The remaining proteins necessary for expression of mtDNA genes are nuclear encoded, synthesized in cytoplasmic ribosomes, and imported into mitochondria.

Mitochondrial translation can be studied in whole cells in the presence of inhibitors of the cytoplasmic ribosomes such as emetine and cycloheximide and in isolated mitochondria (in organello) systems that we and others have used extensively (13, 15). However, a good portion of details concerning expression of mtDNA genes cannot be conveniently approached because of the lack of a true in vitro system for mitochondrial translation. Fox and colleagues (26) developed a valuable in vivo genetic system with a reporter ARGG8 gene. In this system, a mitochondrial gene is replaced by the ARGG8 gene in strains carrying a null mutation in the chromosomal arg8 gene. Arg8p is a soluble protein that is normally synthesized in the cytosol and transported into the mitochondrial matrix. A good portion of the elements that control mitochondrial translation and are described below have been found by using this methodology combined with classical in organello protein synthesis.

Most yeast genes involved in the expression of the three mtDNA-encoded proteins forming the core of the enzyme seem to be absent in high eukaryotes, particularly in humans (reviewed in Ref. 181). This can be easily explained from the qualitative differences between the two species in their mtDNA and mRNA. First, yeast COXI contains introns, whereas all human mtDNA genes are intronless. Yeast introns must be spliced before translation by specific intron-encoded maturases with the assistance of helicases and DEAD-box proteins that function as RNA chaperones to destabilize kinetic traps in RNA folding of introns in vivo (96, 128, 173, 177). Splicing of COXI introns is a fascinating process, but its description is out of the scope of this review. A second and even more significant difference resides in the mechanism of specific mitochondrial mRNA processing and translation. In yeast, translation is governed by mRNA-specific activators, which interact with the ribosomes (77) and recognize targets in the 5'-untranslated regions (5'-UTR) of the mRNAs (167), whereas in human, mitochondrial mRNAs lack 5'-UTRs (9), and the existence of specific translational activators seems to be uncertain.

Expression of the mitochondrially encoded COX subunits also requires one or more translational activator factors specific for each mRNA. Translation of COXI mRNA requires Pet509p (125) and Mss51p (60), translation of COX2 mRNA involves the action of Pet111p (156), and translational activation of COX3 mRNA requires Pet54p, Pet494p, and Pet122p (54, 107). The mRNA-specific translational activators recognize the 5'-UTR of their target mRNAs to promote translation (53). These translational activators are inner membrane proteins, either integral (Pet509p, Mss51p, Pet111p, Pet494p, and Pet122p) or peripheral (Pet54p), a localization that would facilitate a coupling of mitochondrial translation with the inner membrane, allowing for cotranslational insertion of newly synthesized hydrophobic mitochondrial gene products into the membrane (141). As explained below, most of these activators, with the exception of Mss51p, have been shown to interact among them (29, 141), suggesting some level of regulation of the expression of the different subunits forming the catalytic core of COX (65). The network of interactions among translational activator proteins is very extensive, suggesting a high level of regulation of the mitochondrial translational machinery. The physical interactions among the different translational activators are not essential for their activity because they retain it in the absence of one of the partners. However, the amount of the different activators is probably important, as suggested by the fact that overexpression of the COX2 translational activator, Pet111p, prevents translation of COXI mRNA (65).

Expression of Cox1p is regulated at the translational level depending on the availability of the other COX assembly partners (13, 15, 152). As explained above, COXI expression in yeast is under the control of two genes, MSS51 and PET309, which products are involved in the maturation and translation of COXI mRNA (60, 125). We have recently reported that mutations in MSS51 or overexpression of the wild-type gene act as suppressors of shy1-null mutants by increasing the levels of newly synthesized Cox1p (13). The function of Shy1p, related to maturation and/or assembly of Cox1p (13, 152, 180), is of high interest because mutations in its human homologue, Surf1p, have been shown to be responsible for most diagnosed cases of Leigh’s syndrome presenting with COX deficiency (191, 224). Screens of a collection of strains carrying null mutations of COX biogenesis factors showed that the amount of newly synthesized Cox1p is not only reduced in shy1 mutants but in most COX assembly mutants and restored by the mss51 suppressors of shy1 or by mutations in cox14, another COX assembly factor. The mechanism of action of Mss51p in translation of COXI mRNA differs from classical translational activators. In common with all translational activators, including Pet509p, Mss51p acts on the 5'-UTR to initiate translation (152); however, mss51 mutants, unlike pet309 mutants, cannot be suppressed by changes in the 5'-UTR of COXI mRNA (15, 152). In addition, Mss51p is required for Cox1p synthesis (i.e., translation elongation) by acting on a target coded by the protein sequence itself (152). Mss51p and Cox1p form a transient complex (15, 152) that is stabilized by Cox14p by interacting with Cox1p (15) and could function to downregulate Cox1p synthesis when COX assem-
REGULATION OF COX ASSEMBLY

The synthesis of Cox3p in yeast is governed by three specific translational activators, Pet54p, Pet122p, and Pet494p (29, 53), which interact with the 5′-UTR sequences of COX3 mRNA (52). As mentioned above, it has been shown that COX3-specific activators interact to form a COX3 mRNA-specific activator complex (141).

Although most studies on the control of mitochondrial translation concern initiation, elongation and termination are in some cases limiting steps. An interesting review on the subject can be found elsewhere (192).

REGULATION OF COFACTOR AVAILABILITY

Mitochondrial copper homeostasis, heme A biosynthesis, and insertion of both prosthetic groups into the corresponding newly synthesized subunits forming the catalytic core of the enzyme involve several pathways that must be precisely coordinated.

Copper is a metal prosthetic group essential for COX assembly and function (19). The enzyme contains three copper atoms. Two are bound to Cox2p, constituting the CuA site, and the third, referred to as CuB, is associated with the heme A group of heme d1 in Cox1p. In living cells, copper ions are mainly bound to small soluble proteins, called copper chaperones, which are responsible for intracellular copper trafficking and compartmentalization. In yeast, mitochondrial copper acquisition and insertion into the apoenzyme have been shown to depend on at least five evolutionary conserved proteins. Three of these are copper chaperones necessary for the delivery of copper to mitochondria: Cox17p, Cox19p, and Cox23p. Yeast Cox17p is a small hydrophilic protein containing a CxC metal-binding motif (70) that binds copper (20, 81, 150). Involvement of COX17 in copper homeostasis was originally proposed because the respiratory-deficient phenotype of cox17Δ null mutant strains can be rescued by supplementation of copper to the medium (70). The protein localizes in both the cytoplasm and the mitochondrial intermembrane space (70), a localization that is consistent with a function as a soluble copper chaperone, probably shuttling copper from the cytoplasm into the mitochondrial intermembrane space (20, 70). This hypothesis has been challenged by the observation that, although apo-Cox17p is predominantly a monomer with a simple hairpin structure, the protein-copper complex exists in a dimer-tetramer equilibrium (82), making unlikely its free passage through the outer membrane protein channel. In addition, it has been recently shown that tethering of Cox17p to the inner mitochondrial membrane does not affect COX assembly (50).

Two homologues of Cox17p, the small soluble proteins Cox19p and Cox23p, exhibit a cellular distribution similar to Cox17p and contain a twin Cx9C metal-binding motif (70). Although Cox23p is able to assemble COX, but their respiratory-deficient phenotype is complemented by exogenous copper supplementation, albeit only with concomitant overexpression of COX17 (17). Although Cox23p assembly and translation of mitochondrial COX1 mRNA in *S. cerevisiae*. COX1 mRNA translation requires the action of 2 proteins, Pet309p and Mss51p. Both act on the 5′-untranslated region (UTR) to initiate translation (145). In addition, Mss51p is required for Cox1p synthesis (i.e., translation elongation) by acting on a target codon by the protein sequence itself (145). Mss51p and Cox1p form a transient complex (13, 145) that is stabilized by Cox14p (13) and could function to downregulate Cox1p synthesis when COX assembly is impaired (13). In this model, the release of Mss51p from the complex to make it available for Cox1p synthesis occurs at a downstream step in the assembly pathway, most likely catalyzed by Shy1p (15).

The expression of Cox2p in yeast is one of the better characterized steps of COX metabolism. Translation of yeast COX2 mRNA is initiated by Pet111p (138, 156), which is present in the mitochondrial membranes at a low concentration, limiting the synthesis of Cox2p (74). The yeast protein is synthesized as a precursor (pCox2p), containing an NH2-terminal sequence that plays a role in targeting Cox2p into the inner membrane and is subsequently proteolytically removed (explained below). The first 14 codons in NH2-terminal leader peptide of Cox2p are also involved in the regulation of downstream translation of COX2 mRNA. Interestingly, the secondary structure of the mRNA rather than the amino acid sequence is more important for the function of the positive element (209). Three additional sequences located in the Cox2p NH2-terminal region of the mature protein have been described to play an inhibitory role in the absence of the positive element (211). Details about the maturation of the Cox2p precursor are described below.

The synthesis of COX1 mRNA in *S. cerevisiae* is one of the better characterized steps of COX metabolism. Translation of yeast COX1 mRNA translation requires the action of 2 proteins, Pet309p and Mss51p. In addition, Mss51p is required for Cox1p synthesis (i.e., translation elongation) by acting on a target codon by the protein sequence itself (145). Mss51p and Cox1p form a transient complex (13, 145) that is stabilized by Cox14p (13) and could function to downregulate Cox1p synthesis when COX assembly is impaired (13). In this model, the release of Mss51p from the complex to make it available for Cox1p synthesis occurs at a downstream step in the assembly pathway, most likely catalyzed by Shy1p (13).

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Two homologues of Cox17p, the small soluble proteins Cox19p and Cox23p, exhibit a cellular distribution similar to Cox17p and contain a twin Cx9C metal-binding motif (17, 145). The recombinant form of Cox19p was reported to bind copper (50), but the COX assembly defect of cox19Δ null mutant could not be rescued by supplementation of copper to the medium (145). Mutants of cox23Δ also fail to assemble COX, but their respiratory-deficient phenotype is complemented by exogenous copper supplementation, albeit only with concomitant overexpression of COX17 (17). Although Cox23p
does not physically interact with Cox17p in a stable complex, recent data indicates that it is also required for mitochondrial copper homeostasis, functioning in a common pathway with Cox17p acting downstream of Cox23p. (81). In the same line, Cox19p would participate in a different portion of the copper distribution pathway (17). Two additional chaperones facilitate copper insertion into the COX CuA and CuB active sites, respectively, Sco1p and Cox11p, which directly receive copper from Cox17p (93). In vitro experiments have shown that a soluble truncated form of both Cox11p and Sco1p are able to bind copper (41, 144); however, it is still not clear how the transfer of copper occurs between these proteins because physical interactions among them have not been detected (93). However, both Cox11p and Sco1p are anchored to the mitochondrial inner membrane through a transmembrane α-helix and expose the copper binding side in the intermembrane space (20, 42) where they could receive copper directly from the copper chaperones. The metallochaperone for the formation of the CuB site of Cox1p is the product of COX11 (41, 88), which, at least in the prokaryote Rhodobacter sphaeroides, also participates in the formation of the Mg/Mn centers present at the interface of subunits 1 and 2 (88). The metallochaperone for the formation of the CuA center of Cox2p is the product of SCO1 (71), which transfers copper from Cox17p to Cox2p. Sco1p has a metal binding CxxxC motif analogous to the copper binding motif of Cox2p, and this motif is essential for its function, as demonstrated by site-directed mutagenesis (159). SCO1 was originally identified as a multicopy suppressor of a cox17-null mutant (70) and has been shown to directly interact with Cox2p (122). In addition, because the CuA center is formed by a Cu(I) ion and a Cu(II) ion, it remains to be elucidated whether Sco1p mediates the transfer of both different valent ions or, alternatively, whether two Cu(I) are inserted in Cox2p by Sco1p and the active site is successively oxidized. It was demonstrated that Sco1p is able to bind both Cu(I) and Cu(II), although it is not clear whether Sco1p receives both cations or only the monovalent copper Cu(I) from Cox17p (94). Although the experimental data could support a role for Sco1p in copper insertion, considering its structural similarity with the protein family of disulfide reductases, it has been considered that it is more likely to be involved in the reduction of cysteines in the Cox2p copper binding site. This reduction is necessary for the cofactor incorporation (1, 219).

Heme $a$ is a unique heme compound present exclusively in COX. It differs from protoporphyrin (heme $b$) because it has a farnesyl instead of a vinyl group at carbon C2 and a formyl instead of a methyl group at carbon C8 (44). The first step of the heme $A$ biosynthetic pathway is the conversion of heme $b$ in heme $O$ catalyzed by the farnesyl-transferase Cox10p (200). The subsequent oxidation of heme $O$ to heme $A$ occurs in two discrete monoxygenase steps. The first consists on a monoxygenase-catalyzed hydroxylation of the methyl group at carbon position 8, resulting in an alcohol that would then be further oxidized to the aldehyde by a dehydrogenase. The first step is catalyzed by Cox15p, in concert with ferredoxin (Yah1p) and the putative ferredoxin reductase Arh1p, which are probably necessary for the electron supply to the oxygenase Cox15p (16, 18, 28). The identity of the putative gene product involved in the oxidation of the alcohol resulting from the Cox15p action to the corresponding aldehyde to yield heme $A$ remains unknown at present. The biosynthesis of heme $A$ is regulated by downstream events in the COX assembly process. Barros and Tzagoloff (19) measured the amount of heme $A$ and heme $O$ in different yeast COX mutants and observed that all of the mutants analyzed showed a drastic reduction of steady-state levels of heme $A$, with the exception of shy1-, cox20-, and cox5a-null mutant, in which heme $A$ was still detectable at 10–25%, probably associated with residual assembled COX. The overexpression of COX15 significantly increased the amount of heme $A$ in COX mutants, including mutants in which Cox1p was not synthesized, suggesting that the absence of heme $A$ in the mutants is not due to a rapid turnover of the cofactor in the absence of COX subunit one but due to a feedback regulation of the heme $A$ synthesis when the COX assembly process is blocked. The COX mutants analyzed, with the obvious exception of cox10, showed also an accumulation of heme $O$, indicating that this compound is stable. In addition, the cox15-null mutant presented a very low amount of heme $O$, a phenotype that was not rescued by the overexpression of COX10. This observation suggested that the first step of the heme $A$ biosynthesis is also positively regulated in a Cox15p-dependent manner (19).

Because copper and heme $A$ are COX cofactors and together form the metal active centers of the enzyme, it is possible to hypothesize that copper is involved in the regulation of heme $A$ biosynthesis. In fact, some circumstantial evidence has pointed to a possible specific link between copper and heme $A$. To address this question, an interesting study recently showed that the presence of copper does not affect expression, stability, and activity of Cox10p and Cox15p (135), concluding that the biosynthesis of heme $O$ and heme $A$ are not regulated by copper.

COORDINATION OF THE DIFFERENT STEPS IN THE COX ASSEMBLY PROCESS OCCURRING INSIDE MITOCHONDRIA

In the previous sections, we have exposed that COX biogenesis involves the intrinsic coordination of a large number of pathways, including mitochondrial transcription, translation, import, and metal metabolism, which are precisely localized in a limited number of foci in a defined mitochondrial area, the matrix face of the inner membrane. Yeast two-hybrid analyses and coimmunoprecipitation experiments have allowed for the description of a network of multiple protein-protein interactions necessary for the coordination of the events in COX assembly. First of all, the efficient expression of the mtDNA-encoded genes COX1, COX2 and COX3 requires a tight coordination of transcription and translation, which is achieved through the localization of active transcription complexes to the inner membrane, where translational factors are present (Fig. 5). The coordination between transcription and translation results from protein-protein interactions among the RNA polymerase Rpo41p, the matrix protein Nam1p, involved in RNA processing and translation, and the mitochondrial membrane protein Sls1p, a component of the nucleoids, structures that facilitate the interaction of mtDNA with the inner face of the inner membrane (162, 163). Yeast two-hybrid analyses have shown additional interactions between Nam1p and the COX translational factors Pet309p, Pet111p, and Pet494p, which further interact with Pet54p and Pet122p. The interaction between Nam1p and Pet309p was confirmed by coimmuno-
The product of the conserved protein family that mediates the export of NH$_2$-terminal domains of membrane proteins, such as Cox2p, across the inner membrane to the intermembrane space of mitochondria. Yeast strains carrying null mutations in mba1 exhibit a respiratory-deficient phenotype, similar to oxa1-null mutants, due to the severe impairment on the membrane insertion of mtDNA-encoded proteins (85, 149). However, both ribosomal subunits remain bound to the inner membrane in the absence of Oxa1p and Mba1p, indicating that other factors collaborate to the tethering of ribosomes to the membrane (149). One of these additional factors was recently identified as Cox11p, necessary for the formation of the Cu$_2$ center in Cox1p (105). This observation is particularly interesting, taking into account that the two metal active sites of Cox1p, a protein containing 12 transmembrane helices, are buried in a hydrophobic pocket deeply embedded below the membrane surface (98, 198). Considering the localization of the heme a$_3$ and copper cofactors in Cox1p, it is reasonable to think that they are cotranslationally inserted (reviewed in Refs. 43, 50). This model is supported by the observation of a weak interaction between Cox11p and the ribosome that would facilitate the insertion of copper concomitantly with the translation of Cox1p (105). The two metal active sites of Cox1p require the stabilizing presence of the other two subunits (99, 148).

The three mtDNA-encoded COX subunits are highly hydrophobic. To avoid their unproductive aggregation in the matrix hydrophilic environment, the synthesis of these proteins is strictly coupled to membrane insertion (Fig. 5). The COOH-terminal domain of Oxa1p interacts with the large subunit of the mitoribosome, promoting the contact between the translational apparatus and the membrane insertion machinery to facilitate the insertion of the nascent hydrophobic COX subunits into the mitochondrial inner membrane right after their synthesis (100, 187). Oxa1p cooperates with the mitochondrial membrane protein Mba1p to recruit mitochondrial ribosomes to the inner membrane. Yeast strains carrying null mutations in mba1 exhibit a respiratory-deficient phenotype, similar to oxa1-null mutants, due to the severe impairment on the membrane insertion of mtDNA-encoded proteins (85, 149). Mba1p functions as a ribosome receptor and helps Oxa1p in the orientation of the ribosome exit site toward the inner membrane insertion machinery. Mba1p was shown to interact with the large ribosomal subunit, interaction that does not require Oxa1p or the neosynthesized protein (149). However, both ribosomal subunits remain bound to the inner membrane in the absence of Oxa1p and Mba1p, indicating that other factors collaborate to the tethering of ribosomes to the membrane (149). One of these additional factors was recently identified as Cox11p, necessary for the formation of the Cu$_2$ center in Cox1p (105). This observation is particularly interesting, taking into account that the two metal active sites of Cox1p, a protein containing 12 transmembrane helices, are buried in a hydrophobic pocket deeply embedded below the membrane surface (98, 198). Considering the localization of the heme a$_3$ and copper cofactors in Cox1p, it is reasonable to think that they are cotranslationally inserted (reviewed in Refs. 43, 50). This model is supported by the observation of a weak interaction between Cox11p and the ribosome that would facilitate the insertion of copper concomitantly with the translation of Cox1p. Because heme a$_3$ forms the binuclear active site of Cox1p with Cu$_2$, a cotranslational mechanism of heme insertion could also occur. In any case, there is no doubt that the
insertion of heme $a_3$ into Cox1p takes place before the addition of subunit 2, because the farnesyl group of heme $a_3$ is packed between subunits 1 and 2 (43). The factors involved in the insertion of heme $a_3$ into Cox1p have not yet been characterized in eukaryotes. Heme A has been shown to auto-insert in a four helix bundle protein mimicking the hydrophobic pocket of Cox1p (69). However, recent studies performed in R. sphaeroides have shown that the homologue of yeast Shy1p is necessary for the insertion of heme $a_3$ and stability of the binuclear center (180).

The CuA active site is located above the inner membrane surface, in a domain of Cox2p folded in a $\beta$-barrel and protruding into the mitochondrial intermembrane space. The Sco1p-mediated insertion of copper into Cox2p probably takes place after the extrusion of the Cox2p copper binding domain in the intermembrane space and before the addition of this subunit to a COX assembly intermediate (50). However, the cleavage of the precursor form of Cox2p by Imp1/2p is not affected by copper insertion (86).

Another interesting question is how the nuclear-encoded structural subunits and assembly factors find their way to the intramitochondrial site of COX assembly. The localization of the nuclear-encoded COX subunits and COX assembly factors, such as Cox10p, Cox11p, Cox15p, Cox19p, and Cox23p, is facilitated by the fact that they are synthesized by cytoplasmic ribosomes tightly associated to the mitochondrial outer membrane (126, 127). Because COX assembly takes place at discrete foci on the matrix face of the mitochondrial inner membrane, it is possible to imagine that these sites are located close to the nucleoids that anchor the mtDNA to the membrane and close to the TOM/TIM import translocation contacts to allow for the coordination between import of the cytosolic subunits and biogenesis of the core subunits.

Finally, unassembled and misfolded membrane protein subunits, which could have deleterious effects if accumulated in the mitochondrial inner membrane, are degraded by energy-dependent proteases, which include the conserved AAA ATP-dependent proteases (7, 49, 114). Their function has been extensively studied in S. cerevisiae, where pleiotropic phenotypes have been observed in strains carrying inactive variants of these enzymes (7, 49, 114). In general, the AAA proteases combine proteolytic and chaperone-like activities, forming a membrane-integrated quality-control system. Cells lacking an AAA protease loose COX activity and respiratory competence (186, 203). Two AAA proteases are present in the inner membrane of mitochondria, the i-AAA and m-AAA proteases (147). They both are membrane bound and formed by homologous subunits with ATP-dependent metalloprotease activity but are exposed to opposite membrane surfaces. The i-AAA protease is composed of Yme1 subunits and is active on the intermembrane side (117). The m-AAA proteases constitute hetero-oligomeric complexes that are composed of Yta10 (Afg3) and Yta12 (Rca1) subunits in yeast and expose their catalytic sites to the matrix (6). Strains carrying mutations in the i- and m-AAA protease subunits are COX and respiratory deficient (5, 38, 190). Proteolysis by AAA proteases is modulated by another membrane-protein complex that is composed of prohibitins in eukaryotic cells (142, 183). Recently, a second important role for the mAAA protease has been described. The mitochondrial ribosomal protein MrpL32 is processed by the m-AAA protease, allowing its association with preassembled ribosomal particles and completion of ribosome assembly in close proximity to the inner membrane (147). The dual role of the m-AAA protease in protein quality control and ribosome assembly in mitochondria suggest the possibility that mitochondrial translation is regulated by a negative-feedback loop (147). Accumulating nonassembled or altered proteins, the substrates of the m-AAA protease may compete with MrpL32 for binding to the m-AAA protease and thereby impair MrpL32 processing and mitochondrial protein synthesis (147). In this way, mitochondrial protein synthesis, quality-control, and ultimately MRC assembly, including COX biogenesis, are tightly coordinated.

**RETROGRADE REGULATION: COMMUNICATION FROM MITOCOCHONDRIA TO NUCLEUS**

COX is the final product to the coordinate expression of the nuclear and mitochondrial genomes. To achieve this coordination a bidirectional communication is necessary between the two genomes: an anterograde signaling from nucleus to mitochondria and a communication in the opposite direction, called retrograde regulation. The retrograde regulation acts as a sensor of mitochondrial functionality. In fact, it acts on the transfer of information from mitochondria to the nucleus in response to changes in the functional state of the organelle, which allows the cells to appropriately adjust their metabolism (35). For example, the drastic reduction of the expression of nuclear COX genes in mutants lacking fragments or all of mtDNA strongly argues in favor of a retrograde regulation of these genes (56). The description of the current knowledge regarding retrograde regulation is beyond the purpose of this paper but extensive reviews on the subject can be found elsewhere (35). Briefly, several factors that mediate retrograde regulation have been characterized in yeast, including three positive regulators, the RTG1, RTG2 and RTG3 genes (101, 118), and four negative regulators (120, 121, 189). The RTG1 and RTG3 products are transcription factors that bind to an upstream activation sequence called R box of target genes while the role of the RTG2 product is unknown although it is necessary for the function of Rgt1p/Rgt3p (reviewed in Ref. 35). Because not all the genes encoding for mitochondrial proteins are regulated by the RTG genes it has been suggested that other factors are probably involved (63).

**COX AND THE ORGANIZATION OF THE MRC**

The assembly of monomeric COX is just the first step toward the building of a maximally efficient functional enzyme. It is known that COX acts as a dimer to maximize its efficiency (158). In addition, its arrangement with respect to other complex and mobile electron carriers forming the respiratory chain (MRC) at a higher organizational level probably also influences the efficiency at which electrons transfer from one to the other finally reaching the pocket in the COX complex where oxygen is bound and sequentially reduced to water.

In general, two alternative models for the arrangement of the MRC complexes in the membrane have been proposed. Chance and Williams (47) proposed in 1955 their “solid-state” model in which the MRC was acting as a single unit and the substrate is channeled directly from one enzyme to the next in the MRC. This concept changed gradually because the enzymes of the
MRC were found individually active after isolation (79) and a new “random collision” model emerged. According to the random collision model, all components of the MRC diffuse individually in the mitochondrial inner membrane and the transfer of electrons depends on random, transient encounter of the individual protein complexes and the smaller electron carriers coenzyme Q and cytochrome c (48). In this model, the rates of diffusion and appropriate concentrations of redox components limit the maximum rates of electron transport in the inner membrane. The random collision model is mostly favored, although the concept of a solid-state organization has been recently supported by the observation of preferential associations between specific complexes. Isolation of multi-complex units or supercomplexes, consisting of the association of COX (complex IV) with the bc1 complex (complex III) and complex I in various stoichiometries, suggests a solid-state model based on direct electron channeling between complexes. The two models for the organization of the MRC are kinetically distinguishable; the random collision model implies that the components exhibit “pool behavior” of the electron carriers, whereas the solid-state model implies the opposite (37). Kinetic evidence by inhibitor-titration studies and the use of metabolic flux control analysis in yeast (27) and bovine heart mitochondria showed consistency with both models and suggested that both coexist (22).

MRC supercomplexes of different compositions have been described in bacteria [e.g., Paracoccus denitrificans (184)] and in mitochondria from S. cerevisiae (55, 172), other fungi (109), higher plants (64, 108), and mammals (171, 172) by means of blue-native-PAGE, gel filtration and coimmunoprecipitation (55), and by comparing the detergent dependence of cytochrome c-reductase, COX, and coupled ubiquinol oxidase activities of mitochondria (172). In S. cerevisiae mitochondria, almost all COX is found bound to the bc1 complex independently of the growth conditions, although changes in the molar complex III-to-IV ratio were observed. This ratio seems to be adjusted by varying the ratio of two supercomplexes containing dimeric complex III and one and two copies of complex IV, respectively (55, 172). In bovine mitochondria, supercomplexes containing monomeric complex I associated with dimeric complex III and zero to four copies of complex IV, respectively, have been identified (170). Recently, electron microscopy has allowed for the characterization of the two major MRC supercomplexes I1III2 and I1III2IV1 in bovine heart mitochondria, which are also two major supercomplexes detected in human mitochondria (169). In addition to the functional implications of a supercomplex organization of the MRC, it has been proposed that these MRC supercomplexes may play a role in the stabilization of the individual complexes, mostly complex I (2, 109, 171, 184). However, instability of individual complexes does not affect COX, and, in the yeast S. cerevisiae, in which complex I is absent, the formation of supercomplexes is not required for the stability of individual bc1 complex and COX.

What is holding COX together to the bc1 complex? Studies performed in yeast showed that deletion of genes encoding for bc1 complex subunits QCR6, QCR9, QCR10, and RPI1 or COX subunits COX8, COX12, and COX13, all nuclear encoded, did not affect the assembly of supercomplexes. Although cytochrome c is essential for COX assembly in a structural capacity (14), it is not the glue between bc1 and COX complexes because, even when experimentally removed, it does not affect the stability of supercomplexes (172). Supercomplexes have been identified in P. denitrificans (184), in which only homologues of the mitochondrial-encoded subunits exist, suggesting that these subunits could play a key role in the assembly of supercomplexes (170). On the other hand, studies performed in yeast cardiolipin mutants have shown that this mitochondrial membrane phospholipid plays an essential role in the assembly of bc1 and COX complexes (222, 223). Studies performed in yeast have shown that cardiolipin does not seem to be essential for the formation of MRC supercomplexes, but it is important for the stability of supercomplexes containing functional COX as well as the individual complex (154). In addition, cardiolipin is required to prevent formation of the resting state of COX in the membrane (154). Finally, it has been recently shown that cardiolipin biosynthesis and mitochondrial MRC function are interdependent. Cardiolipin biosynthesis is regulated at the level of cardiolipin synthase activity by the pH component change of the proton-motive force generated by the functional electron transport chain (72). Supercomplex assembly and its regulation are other examples of the large number of players in the network of pathways that leads to the biogenesis of functionally efficient mitochondrial COX.

In conclusion, most of the ATP used for the different processes performed by eukaryotic cells are derived from the metabolic pathways housed in mitochondria. The function of COX, the terminal enzyme of the MRC, is essential for the regulation of ATP production within mitochondria. Alterations in COX assembly have a great impact in cell performance and survival and, although not reviewed here, also in human health. A better understanding of COX biogenesis is essential for elucidating the molecular basis of aerobic energy production in normal and disease conditions. Although there is a large body of information concerning the biogenesis of COX, our knowledge about how the specific pathways involved and the process as a whole are regulated is still fragmentary. The list of genes involved in COX assembly in yeast is probably close to completion, but their products are still mostly uncharacterized. Many laboratories are making a continuous effort to understand the specific function of the many factors involved and to discover the remaining ones. This effort must combine classical biochemical and molecular biology approaches with genome-wide analyses to uncover the effect of altering specific pathways on the whole process of COX assembly. In this regard, a systems biology approach can be foreseen as the key to obtain such knowledge.

ACKNOWLEDGMENTS

We thank Karine Gouget for critically reading the manuscript.

GRANTS

This study was supported by National Institute of General Medical Sciences Grant GM-071775A and a research grant from the Muscular Dystrophy Association (both to A. Barrientos).

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