Mitochondria-associated Yeast mRNAs and the Biogenesis of Molecular Complexes□ⱱ

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The coherence of mitochondrial biogenesis relies on spatiotemporally coordinated associations of 800–1000 proteins mostly encoded in the nuclear genome. We report the development of new quantitative analyses to assess the role of local protein translation in the construction of molecular complexes. We used real-time PCR to determine the cellular location of 112 mRNAs involved in seven mitochondrial complexes. Five typical cases were examined by an improved FISH protocol. The proteins produced in the vicinity of mitochondria (MLR proteins) were, almost exclusively, of prokaryotic origin and are key elements of the core construction of the molecular complexes; the accessory proteins were translated on free cytoplasmic polysomes. These two classes of proteins correspond, at least as far as intermembrane space (IMS) proteins are concerned, to two different import pathways. Import of MLR proteins involves both TOM and TIM23 complexes whereas non-MLR proteins only interact with the TOM complex. Site-specific translation loci, both outside and inside mitochondria, may coordinate the construction of molecular complexes composed of both nuclearly and mitochondrially encoded subunits.

INTRODUCTION

Most mitochondrial proteins of eukaryotic cells are encoded by nuclear genes and synthesized by cytoplasmic ribosomes. However, a few proteins are encoded by the mitochondrial DNA and are translated inside mitochondria by bacterial-type ribosomes. The major mitochondrial import pathway of cytoplasmically translated proteins is now well understood (Koehler, 2004; Rehling *et al.,* 2004). Although a posttranslational mechanism for import is widely accepted, it may concern only a limited class of proteins. Indeed, it was demonstrated 30 years ago that a subclass of cytoplasmic polysomes is bound to the surface of mitochondria (Kellems *et al.,* 1975). Subsequently, experiments in yeast and human cells have suggested a cotranslational import process for some mitochondrial proteins (Fujiki and Verner, 1993; Mukhopadhyay *et al.,* 2004). The evolutionary history of mitochondria might elucidate this apparent discrepancy between post- and cotranslational import processes. Phylogenetic studies of the yeast mitochondrial proteome have demonstrated a composite origin. It is estimated that half or more of the genes that code for the modern mitochondrial proteome originated directly from the host nuclear genome, and the other half are of bacterial origin, a consequence of a massive transfer of genes from the endosymbiont to the host nuclear genome (Marcotte *et al.,* 2000; Karlberg *et al.,* 2000). This symbiotic situation required the development of protein translocation machineries (translocases) in the mitochondrial membranes (Herrmann, 2003). Possibly, the two classes of nuclear genes coding for mitochondrial products may have used the same mitochondrial import pathway. However, a recent genome-wide analysis (Marc *et al.,* 2002) suggested a strong correlation between the bacterial origin of the genes and their locus specific translation on mitochondria-linked polysomes. An index called MLR (mitochondrial localization of mRNA) was derived from microarray analyses to classify (from 0 to 100) the mRNAs according to their cellular localization. A recent analysis of the outer membrane proteome (Zahedi *et al.,* 2006) presented strong support for the MLR concept: it included both bona fide outer membrane proteins and precursors for many MLR proteins. This strongly suggests that the MLR proteins are translated next to mitochondria, although the reason for this remains obscure. In some cases, this phenomenon may favor the formation of nucleo-proteocomplexes in the vicinity of mitochondria, as for Msk1, the mitochondrial lysyl-tRNA synthetase, which guides the import of tRNA^{Lys} toward mitochondria (Entelis *et al.,* 2006).

To describe the MLR phenomenon in more detail, we developed two highly sensitive methods to: 1) quantify, in vitro, the amount of mitochondrial-associated mRNA and 2) to visualize, in situ, the localization of some mRNA species. These approaches were applied to 112 nuclear mRNAs to establish a spatial map of the sites of translation of proteins involved in the assembly of seven mitochondrial complexes. We report a close link between core proteins and mitochondrially associated translation sites. The prokaryotic origin of most of these MLR proteins emphasizes the evolutionary stability of this phenomenon.

MATERIALS AND METHODS

Strains and Growth Conditions

The *Saccharomyces cerevisiae* strain CW252 has an intronless mitochondrial genome and is nuclearly isogenic to W303 (Saint-Georges *et al.,* 2002). Cells were grown at 30°C in galactose medium (1% bactopeptone, 1% yeast extract,

2% galactose) for overnight precultures and FISH experiments; for purification of mitochondria, cells were grown in the same medium plus 0.1% KH$_2$PO$_4$ and 0.12% (NH4)$_2$SO$_4$.

Quantitative PCR analysis of asymmetric mRNA localization. Schematically, mitochondrion-bound polysomes were purified as in Kellems *et al.* (1975), and RNA was extracted using the hot-phenol method. Aliquots of 50 ng of either total or mitochondria-associated mRNA were used for reverse transcription (RT), and the cDNA was purified using the Macherey-Nagel PCR extract kit. Serial dilutions (1/10, 1/50, 1/100) of cDNAs were subjected to real-time quantitative PCR with oligonucleotides specific for each ORF (Supplementary Table S1) and the Sybr Green PCR kit (QuantiTech).

For a reliable evaluation of the mRNA associated with mitochondria, various correction factors were used to account for the difficulties of the purification procedure. *COX1* and *COX2* mRNAs were used to normalize the data, assuming they have a 100% mitochondrial localization. *ACT1* mRNA was used to assess the extent of contamination by nonmitochondrial fractions. The SD error by this approach varies from 0 to 5%, whereas previous microarray-based methods gave much less reproducible results (Marc *et al.,* 2002). A detailed description of this approach (PCR programs and calculations) is available in Garcia *et al.* (2006) and in supplemental document S1.

FISH Analysis and In Situ mRNA Localization

The combined hybridization of three to five antisense oligonucleotides was used to enhance the visualization of each mRNA. Typically, each oligonucleotide was designed to contain 50–55 nucleotides with five aminoallyl thymidines and a coherent Tm value (±2°C for the set of oligonucleotide). After synthesis (Eurogentec, San Diego, CA) the oligonucleotides were directly labeled with CY3 or CY5 fluorochromes as previously described (Garcia *et al.,* 2006). The probes used in this study are presented in Supplementary Table S2.

For in situ hybridization, yeast cells were grown to midlog phase and immediately fixed with a final concentration of 4% paraformaldehyde, spheroplasted with lyticase, adhered to poly-L-lysine–treated coverslips, and stored in 70% ethanol at −20°C. After overnight hybridization at 37°C, washing, and DAPI staining, coverslips were mounted with anti-fade solution and imaged on an Olympus BX61 upright microscope (Melville, NY) using a 100× 1.35 NA objective. For 3D sampling 41 images were acquired at a spacing of 200 nm in the Z-axis. ImageJ was used for image processing; several stacks can be combined using a maximum projection algorithm. We developed an extension ImageJ plug-in for calculating the distance from mRNA to mitochondrion objects (Jourdren and Garcia, unpublished results).

RESULTS

Quantitative Analysis of Asymmetric mRNA Localization

Most experimental evidence that mRNA molecules are unevenly distributed within the cell is from in situ fluorescent analyses (Darzacq *et al.,* 2003). A few studies (Diehn *et al.,* 2000; Marc *et al.,* 2002; de Jong *et al.,* 2006) used DNA microarrays to establish a list of genes whose mRNA is preferentially translated on membrane-bound polysomes. Though providing an abundance of data, these studies were limited by substantial heterogeneity of the biochemical preparations and variations in the yield due to the purification procedure. For instance, the general conclusions of the DNA microarray analyses are probably sound concerning mitochondria-bound polysomes (Marc *et al.,* 2002), but gene-specific data may be absent or inaccurate. Because it is possible to enrich mitochondrial fractions carrying associated cytoplasmic polysomes (Kellems *et al.,* 1975), we developed a protocol with better discrimination. We used real-time quantitative RT-PCR with normalization and correction of the results to take into account the purification yield and contaminating factors (see *Materials and Methods*). Mitochondrial mRNAs were analyzed to assess the exact quantity of mitochondrial products in each extract as an internal control. The technical reproducibility of the procedure was verified by conducting three independent polysomal fractionations from different yeast cultures. mRNA from 112 genes was studied, and the percentage of each mRNA associated with mitochondria-bound polysomes was between 0 and 57% (Supplementary Table S1 and Supplementary Figure S1). Three classes of gene were distinguished. The first class includes the 56 genes that have between 0 and 3% of their mRNA linked to mitochondria; these mRNAs were consid-

ered to be translated on free cytoplasmic polysomes. The second class includes 50 genes for which 9–57% of their mRNA was linked to mitochondria: the corresponding proteins are probably produced in the vicinity of mitochondria, and they were considered to be MLR proteins (Figure 1). The third class, the intermediate group (4–6%), includes six genes that cannot be unambiguously classified to either class 1 or class 2. Classes 1 and 2 contain approximately the same number of genes, 56 and 50 respectively, as previously observed in a more global analysis (Marc *et al.,* 2002). In contrast, data from the *Saccharomyces* Genome Database (Christie *et al.,* 2004) and PSI-BLAST analysis (Altschul *et al.,* 1997) indicate that 96% of the MLR genes, but only 8% of genes with nonlocalized mRNAs, are prokaryotic homologues (Figure 1 and Supplementary Table S1).

FISH Analysis of the In Situ mRNA Localization

Single-cell FISH experiments are complementary to the biochemical analyses described. New developments in probe design and in fluorophore chemistry allow detection of single molecules of mRNA in their cellular environment (Femino *et al.,* 1998, 2003). The protocol we used here is adapted from Long *et al.* (1995, 1997) and can be used to compare, simultaneously, the spatial distribution of different mRNAs relative to each other or to the mitochondrion (see *Materials and Methods*). Mitochondria were unambiguously detected using probes recognizing mitochondrial ribosomal RNAs; this allowed simultaneous detection of mRNAs and mitochondria in a single step.

Both haploid and diploid strains were examined, and because of their size, findings with diploid strains were easier to interpret. However, it was evident that in the results in the two genetic backgrounds were equivalent.

The findings of FISH experiments were entirely consistent with the results of the RT-PCR analyses. For all genes classified as MLR (>6% asymmetrical localization of the mRNA), as exemplified by *ATP1, ATP2,* and *ATP3* (Figure 2), the FISH signal localized to punctuate structures that aligned with mitochondria. Conversely *ATP16* (0% by RT-PCR) had its mRNA isotropically dispersed in the cell with no apparent association with mitochondria (Figure 2 and Supplementary Figure 2S for 3D quantification analysis). Almost all MLR genes seem to be of prokaryotic origin (Figure 1), although a few, including *ATP4* and *TIM50,* have no prokaryotic homolog (*Saccharomyces* Genome Database; Christie *et al.,* 2004); FISH analyses fully confirmed that the corresponding mRNAs of these two atypical cases were translated adjacent to mitochondria (Figure 2). This is also consistent with the detection of precursor forms of these proteins in the outer mitochondrial membrane proteome (Zahedi *et al.,* 2006).

MLR Proteins and Mitochondrial Complexes

All the mitochondrial complexes examined contained MLR proteins. We therefore examined whether these proteins have a particular role in these complexes.

The Krebs TCA cycle enzymes are believed to be highly organized in supramolecular complexes (Velot *et al.,* 1997) connected to the other supermacromolecular complex, succinate:quinone oxidoreductase (SQR, Complex II; Horsefield *et al.,* 2004). All the 16 proteins comprising these two complexes are unambiguously MLR proteins. This applies even to Tcm62, which is considered to be a putative protein chaperone required for complex II assembly.

The ATP synthase complex (RCC5) is composed of proteins of diverse origins, and its structure and biosynthesis are well documented (Ackerman and Tzagoloff, 2005). Three MLR proteins, *ATP1* (subunit α), *ATP2* (subunit β), and *ATP3* (subunit
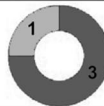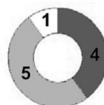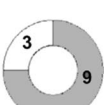
COMPLEX	ORF	GENE	RT-PCR	ORIGIN	PROTEIN DISTRIBUTION
TCA	YLR304C	ACO1	54 +/-4%	gray	
	YNR001C	CIT1	35 +/-3%	gray	
	YPR001W	CIT3	46 +/-8%	gray	
	YPL262W	FUM1	30 +/-4%	gray	
	YNL037C	IDH1	25 +/-5%	gray	
	YOR136W	IDH2	22 +/-6%	gray	12
	YIL125W	KGD1	57 +/-4%	gray	
	YDR148C	KGD2	37 +/-4%	gray	
	YFL018C	LPD1	30 +/-4%	gray	
	YOR142W	LSC1	26 +/-7%	gray	
	YGR244C	LSC2	52 +/-3%	gray	
	YKL085W	MDH1	31 +/-4%	gray	
RC2	YKL148C	SDH1	42 +/-3%	gray	1
	YLL041C	SDH2	39 +/-1%	gray	3
	YKL141W	SDH3	20 +/-3%	gray	
	YBR044C	TCM62*	14 +/-6%	gray	
RC3	YBL045C	COR1	16 +/-3%	gray	1
	YDR350C	COR2	11 +/-3%	gray	4
	YOR065W	CYT1	18 +/-1%	gray	5
	YEL024W	RIP1	21+/-4%	gray	
	YPL215W	CBP3*	11 +/-4%	gray	
RC4	YPL172C	COX10*	9 +/-5%	gray	3
	YPL132W	COX11*	13 +/-7%	gray	9
	YER141W	COX15*	32 +/-2%	gray	
	YBR037C	SCO1*	18 +/-5%	gray	
	YGR112W	SHY1*	17 +/-4%	gray	
	YER154W	OXA1*	22+/-6%	gray	
RC5	YBL099W	ATP1	39 +/-3%	gray	3 4
	YJR121W	ATP2	47 +/-7%	gray	9
	YBR039W	ATP3	38 +/-3%	gray	
	YPL078C	ATP4	22 +/-5%	white	
	YLR393W	ATP10*	29 +/-6%	gray	
TOM	YNL121C	TOM70	25 +/-2%	gray	4
	YHR117W	TOM71	30 +/-5%	gray	8
	YKL195W	MIA40	25 +/-4%	gray	
	YNL026W	SAM50	11 +/-2%	gray	
TIM	YPL063W	TIM50	29 +/-8%	white	6
	YIL022W	TIM44	24 +/-5%	gray	11
	YFL016C	MDJ1	30 +/-5%	gray	
	YJR045C	SSC1	29 +/-5%	gray	
	YJL054W	TIM54	12 +/-2%	gray	
	YOR232W	MGE1	9 +/-3%	gray	

Figure 1. MLR proteins of seven mitochondrial complexes. The association between mitochondrial-bound polysomes and 107 mRNAs coding for all the subunits of mitochondrial complexes: respiratory complexes 2, 3, 4, and 5; import complexes TOM and TIM; and TCA cycle complexes, and for assembly factors (starred) was quantified by RT-PCR analyses with several correction factors (see *Materials and Methods* and Garcia *et al.,* 2006). Only the genes that are translated in the vicinity of mitochondria (MLR proteins) are represented (all data are available in Supplementary Material). Most of these MLR genes have prokaryotic homologues (gray shaded box), whereas most of the other genes have no known prokaryotic homologue (Supplementary Material). The right column gives a schematic composition of the assembled complexes according to gene origin: mitochondrially encoded (white), nuclearly encoded either of prokaryotic (dark gray), or eukaryotic (light gray) origin.

γ) are components of the F1 subunit, and three other MLR proteins, *ATP4* (subunit b), *ATP5* (OSCP), and *ATP10* (essential chaperone for F0) are essential for F0 subunit construction. Genetic and biochemical experiments have demonstrated that the α, β, γ, b, and OSCP subunits are the basic elements of the F1 and F0 complexes (see Ackerman and Tzagoloff, 2005 for a review). The case of *ATP10* is less straightforward. There is genetic interaction between *ATP10* and *ATP6*, the mitochondrial gene for subunit a, suggesting that Atp10 may act as a subunit a-chaperone at early stages of F0 assembly (Tzagoloff *et al.,* 2004). Interestingly, the five basic subunits of the ATP synthase (α, β, γ, b, and OSCP) are all translated in the vicinity of mitochondria. Conversely, none of the 13 accessory genes required for yeast ATP synthase biogenesis (Ackerman and Tzagoloff, 2005) are MLR proteins. Thus, translation near the mitochondrion appears to be a characteristic of the early nucleation process of the core enzyme.

The case of the COX complex (RC4) deserves a special mention: none of the mRNAs of nuclear-encoded subunits were translated in the vicinity of mitochondria. However, Shy1, Sco1, Oxa1, Cox10, Cox11, and Cox15 (Figure 1), important for the assembly of this complex (Nijtmans *et al.,* 2001; Szyrach *et al.,* 2003), are undoubtedly MLR proteins.

The bc1 complex (RC3) is a complex of tripartite origin: the apocytochrome b is always encoded in the mitochondrial genome, and some of its nuclear-encoded subunits are MLR proteins, whereas others are translated on free cytoplasmic polysomes. The core proteins of the complex—Cor1, Cor2, Rip1 and Cyt1—clearly belong to the MLR protein class and they are unambiguously of a prokaryotic origin. Cbp3, like Atp10 for complex V, is a chaperone essential for complex III biosynthesis (Kronekova and Rodel, 2005), suggesting that there is in all cases at least one chaperone associated with the assembly of these core proteins. Thus, the bc1 complex shares various similarities with the ATP synthase complex.

The TOM and TIM complexes differ from the previous examples from an evolutionary point of view. This import machinery emerged as a consequence of the endosymbiosis. The
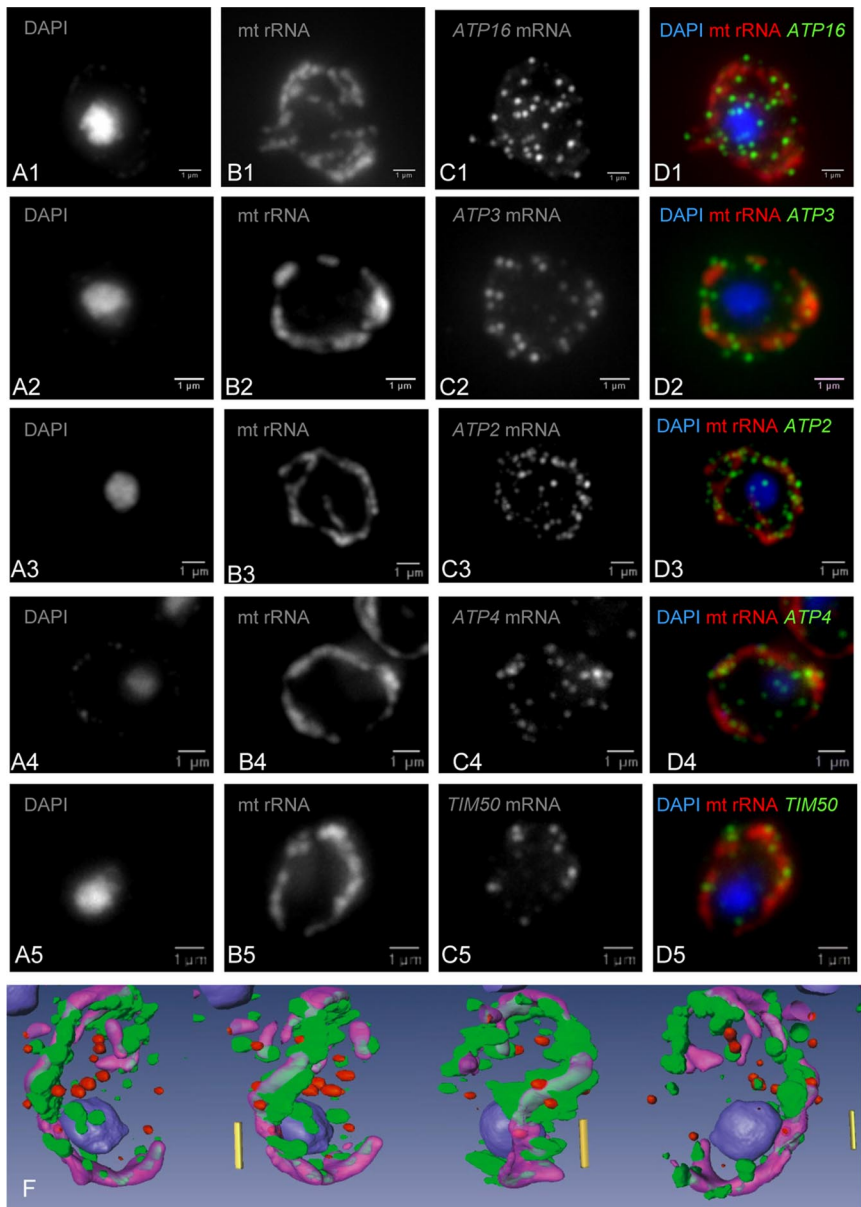
Figure 2. Live-cell imaging of nuclear-encoded mRNAs for mitochondrial proteins. Five fluorescent oligonucleotides specific for mitochondrial rRNA (lane B) and for specific mRNAs (*ATP16*-C1, *ATP3*-C2, *ATP2*-C3, *ATP4*-C4, and *TIM50*-C5) were used to probe wild-type yeast cells for hybridization. The merged figures (lane D) indicate the relative localizations of mt rRNA (red) and the various mRNAs (green). 3D quantitative analysis was used to compare *ATP16* mRNA, which is isotropically distributed, with the specifically localized *ATP3* mRNA (Supplementary Figure 2S). A 3D reconstitution of these relative localizations is represented on the bottom line for *ATP2* mRNA (green), mt rRNA (violet), and the negative control *YRA1* mRNA (red). These 3D analyses allow a quantitative assessment of the distance between a particular mRNA molecule and the edge of the mitochondrion (see Supplementary Data).

synthesis of mitochondrial proteins in the cytosol required the development of protein translocation systems to ensure appropriate localization. Consequently, few of these translocase components have obvious bacterial homologues and most are of eukaryotic origin. Systematic analysis of the MLR subunits composing these complexes provided an interesting observation. At least one of the components of each subcomplex of the translocase machinery (Koehler, 2004) is unambiguously an MLR protein (SAM = *SAM50*, TOM = *TOM70*, TIM22 = *TIM54*, Export = *OXA1*). This is consistent with the suggestion that the ability of mitochondria to bind mRNAs for the genes coding for targeted proteins would alleviate the problem of targeting the prokaryotic proteins (Herrmann, 2003). As in the respiratory complexes, most of the MLR proteins are critical to the biogenesis of the complex. *SAM50*, for instance, is required for viability and probably forms the core of the SAM complex (Paschen *et al.*, 2003; Kozjak *et al.*, 2003; Pfanner *et al.*, 2004). The TOM translocase is composed of seven proteins and the MLR proteins Tom70 and Tom40 are crucial to the properties of the

complex. Tim50, an essential protein of the complex TIM23, has no prokaryotic homolog but its MLR localization and the FISH analyses identify it as an MLR protein. This might be an example of a protein becoming MLR through acquisition of specific targeting signals. Finally, Mia40, a recently discovered protein (Chacinska *et al.*, 2004), is a central component of the protein import and assembly machinery. Mia40 is required for the assembly of small proteins of the intramembrane space (Rissler *et al.*, 2005). The identification of Mia40 as an MLR protein (of prokaryotic origin) is entirely consistent with its importance in the construction of this mitochondrial domain.
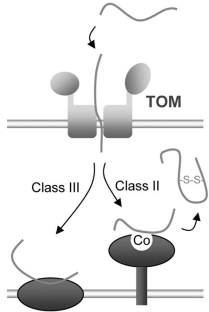
DISCUSSION

The Functional Implications of Site-specific Translation of MLR Genes

Forty-eight of the 50 MLR proteins we identified are of prokaryotic origin, according to the phylogenic data avail-

A) Class I IMS proteins
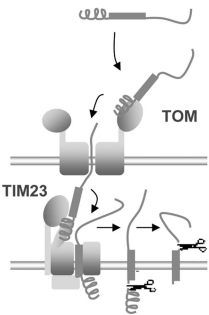
B) Class II and III IMS proteins

	GENES	Processing	RT-PCR (this work)	Prokaryote homologues	MLR protein
Class I	CCP1	Yta10 and Pcp1	29+/-11%	YES	
	MCR1	Mpp and Imp1	32+/-7%	YES	
	CYB2	Mpp and Imp1	26+/-8%	YES	
	MGM1	Mpp and Imp1	16+/-6%	NO	
Class II	CYC1	NO	0+/-0%	YES	
	COX17	NO	0+/-0%	NO	
	COX19	NO	1+/-1%	NO	
	COX23	NO	5+/-5%	NO	
	ERV1	NO	0+/-0%	NO	
	SOD1	NO	0+/-0%	YES	
	TIM8	NO	0+/-0%	NO	
	TIM9	NO	1+/-2%	NO	
	TIM10	NO	0+/-0%	NO	
	TIM12	NO	0+/-0%	NO	
	TIM13	NO	0+/-0%	NO	
Class III	CYT2	NO	0+/-0%	NO	
	COX12	NO	0+/-1%	NO	
	QCR8	NO	0+/-0%	NO	

Figure 3. Class I intermembrane space proteins are MLR proteins. Protein-translocation pathways into the intermembrane space (IMS) of mitochondria have been classified into three classes (Herrmann and Hell, 2005). Class I proteins have typical mitochondrial presequences followed by hydrophobic sorting domains and are directed to the TIM23 complex. Class II and III proteins are imported independently of the inner-membrane TIM23 complex. Polysome localization of mRNA coding for the IMS proteins described was examined by RT-PCR analysis. Class I proteins were clearly produced from mRNA translated in the vicinity of mitochondria (MLR proteins-RT-PCR value above 6%, indicated in dark gray in the right column), whereas none of the mRNAs for the class II or III proteins were significantly linked to mitochondria (non-MLR proteins, white; RT-PCR value <6%).

able from SGD (Christie *et al.*, 2004), whereas 53 of the 62 non-MLR proteins translated on free cytoplasmic polysomes are of eukaryotic origin (no homology found in the available prokaryotic sequences). This suggests that a strong selective pressure has maintained a mitochondria-linked translation mechanism for MLR proteins. This apparently ancestral process raises the following various interesting considerations.

MLR Proteins and Protein-Translocation Pathways

A recent survey (Herrmann and Hell, 2005) of the mechanisms by which proteins of the mitochondrial intermembrane space (IMS) are transported from the cytosol has revealed three main classes. These classes differ in the energy sources used to drive the translocation reactions. We examined the MLR values of most of these proteins (Figure 3) and found that class I proteins

are exclusively MLR proteins, whereas the class II and III proteins are non-MLR proteins. The well-characterized class I proteins have bipartite presequences composed of matrix-targeting N-terminal sequences followed by hydrophobic sorting domains. Unlike class II or III proteins, class I protein importation into IMS is TIM23 complex dependent, and consequently class I (MLR) protein importation relies on a physical chain of interactions between TOM and TIM23 complexes. Thus, class I protein import may require a tight connection between cytoplasmically localized translation and sites of outer–inner membrane association.

MLR Proteins and Mitochondrial Complexes

We describe several examples of essential core elements of the early steps of the complex biogenesis being MLR pro-
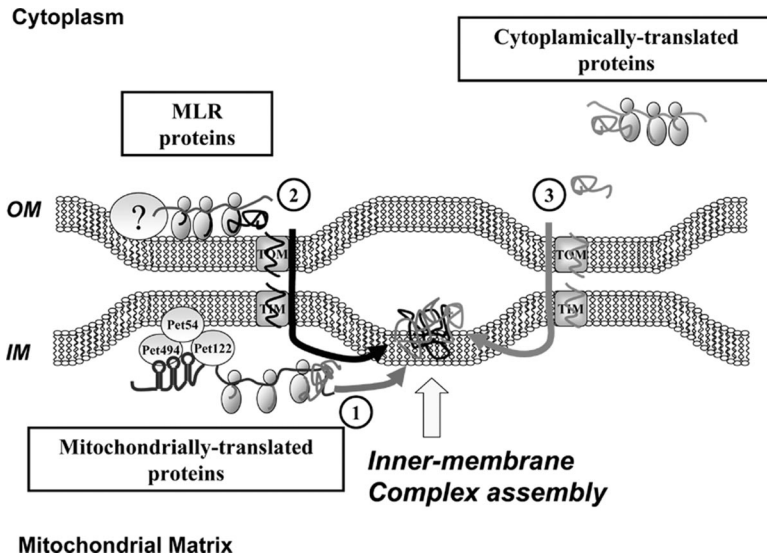
Figure 4. Proposed model of inner-membrane mitochondrial complex biogenesis. Three types of proteins compose the inner-membrane complexes: (1) Mitochondrial-encoded proteins translated on membrane-bound mRNA, depending on specific activator proteins. (2) MLR proteins, identified in this work, are nuclear-encoded and translated in the vicinity of the outer mitochondrial membrane. (3) Nuclear-encoded proteins translated on free cytoplasmic polysomes and that are posttranslationally imported. The model assumes that pathways 1 and 2 are spatially connected, thus facilitating the formation of the core protein complex with which accessory proteins (pathway 3) will combine.

teins. This is especially clear for the ATP synthase complex for which the assembly of the subunits during the biogenesis of the complex is well described (Ackerman and Tzagoloff, 2005). Subunits α (*ATP1*), β (*ATP2*), and γ (*ATP3*) of the F_1 subunit and subunit b (*ATP4*), OSCP (*ATP5*), and the essential chaperone Atp10 (*ATP10*) of the F_0 subunit are all essential for the early steps of ATP synthase biogenesis, and are all MLR proteins. MLR proteins appear to be critical early during biogenesis of most of the complexes described above. This strongly suggests that the MLR-specific translation sites (the punctate structures in Figure 2) might also be the sites of initiation of the early steps of complex construction. Systematic relative localization analysis of these translation sites may further elucidate the space and time-controlled biogenesis of mitochondrial complexes.

MLR Protein Translation and Membrane Contact Sites

We can suggest a model (Figure 4) in which MLR proteins are translated at specific sites near the outer mitochondrial membrane. This is reminiscent of the mitochondrial mRNA translation process: for some mRNAs, including those of the *COX1*, *COX2*, and *CYTb* genes, specific translation sites at the inner mitochondrial membrane have been demonstrated. This site-specific translation depends on the mRNA associating with identified protein complexes (Naithani *et al.*, 2003; Krause *et al.*, 2004). The protein Oxa1 establishes, in some cases, a link with ribosomes to ensure cotranslational insertion of the nascent mitochondrial-encoded polypeptides into the membrane (Szyrach *et al.*, 2003). Possibly, mitochondrial genes translocated to the nucleus after the symbiotic event have continued to use the same translation process. This membrane-controlled production might have facilitated the early steps of symbiosis when primitive mitochondrial genes became nuclear at a time when the mitochondrial import machineries did not exist.

There is no evidence for a mechanism coordinating the output of the mitochondrial and nuclear gene products in yeast. We propose that MLR protein translation is a key event in the early step of the biogenesis of these mitochondrial complexes. The corresponding prediction is that each mitochondrial complex is constructed at particular sites where cotranslationally imported nuclear-encoded proteins congregate with proteins of the same complex translated within the mitochondria. This implies that outer and inner mitochondrial membranes contact at sites allowing a process of cotranslation on both sides of the membranes. Cytoplasmic ribosomes have been observed bound to regions of the outer membrane that are in intimate association or contact with the inner membrane (Kellems *et al.*, 1975). Also the fact that, in the intermembrane space, MLR proteins are imported via a chain of physical contacts between the two membranes gives further credence to this idea (see above and Figure 3). The improved FISH methodology presented in this work should allow us to address this issue experimentally.

MLR Proteins and Cellular Compartments

There may be damaging effects of site-specific translation of MLR proteins. The location of many mRNAs close to mitochondria may be deleterious if reactive oxygen species (ROS) production alters these molecules. For example, in Alzheimer's disease, it was observed that ROS production in the mitochondrial environment may cause excessive mRNA oxidation (Shan *et al.*, 2003). Peri-mitochondrial translation might thus promote exposure of the mRNAs concerned to free radicals, with subsequent aggregation of the translation products (Swerdlow and Khan, 2004). However, a healthy

wild-type cell is likely to minimize this possibility by a time-dependent compartmentalization process that disconnects mitochondria biogenesis and ROS production (Tu *et al.*, 2005). It would be interesting to study how MLR protein production is regulated temporally.

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