Molecular Biology of the Cell
Vol. 14, 3848–3856, September 2003

The Role of the 3′ Untranslated Region in mRNA Sorting to the Vicinity of Mitochondria Is Conserved from Yeast to Human Cells

J. Sylvestre,* A. Margeot,* C. Jacq,* G. Dujardin,†‡ and M. Corral-Debrinski*‡

*Laboratoire de Génétique Moléculaire, Unité Mixte Recherche Centre National de la Recherche Scientifique 8541, Ecole Normale Supérieure, Paris, France; and †Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, Gif-sur-Yvette, France

Submitted February 7, 2003; Revised May 12, 2003; Accepted May 12, 2003
Monitoring Editor: Thomas Fox

We recently demonstrated, using yeast DNA microarrays, that mRNAs of polysomes that coisolate with mitochondria code for a subset of mitochondrial proteins. The majority of these mRNAs encode proteins of prokaryotic origin. Herein, we show that a similar association occurs between polysomes and mitochondria in human cells. To determine whether mRNA transport machinery is conserved from yeast to human cells, we examined the subcellular localization of human *OXA1* mRNA in yeast. Oxa1p is a key component in the biogenesis of mitochondrial inner membrane and is conserved from bacteria to eukaryotic organelles. The expression of human *OXA1* cDNA partially restores the respiratory capacity of yeast *oxa1*$^-$ cells. In this study, we demonstrate that 1) *OXA1* mRNAs are remarkably enriched in mitochondrion-bound polysomes purified from yeast and human cells; 2) the presence of the human *OXA1* 3′ untranslated region (UTR) is required for the function of the human Oxa1p inside yeast mitochondria; and 3) the accurate sorting of the human *OXA1* mRNA to the vicinity of yeast mitochondria is due to the recognition by yeast proteins of the human 3′ UTR. Therefore, it seems that the recognition mechanism of *OXA1* 3′ UTR is conserved throughout evolution and is necessary for Oxa1p function.

INTRODUCTION

A key feature of eukaryotic cells is their organization into separate subcellular compartments, each containing distinct sets of proteins. The sorting of several proteins destined to organelles involves mRNA localization. This specific localization might be preferable to protein localization; indeed, one mRNA molecule can serve as template for multiple rounds of translation. Thus, localizing an mRNA rather than the protein to its site of action offers obvious advantages (Jansen, 2001; Tekotte and Davis, 2002). Mitochondrial biogenesis is a complex process that requires the concerted expression of both nuclear and mitochondrial genomes. More than 98% of mitochondrial proteins are encoded by the nucleus and synthesized in the cytoplasm. Mitochondrial sorting of mRNAs encoding mitochondrial proteins might likely represent a key step to ensure the functionality of the corresponding polypeptides inside the organelle. In this case, a cotranslational phase might assist the import of the precursors (Corral-Debrinski *et al*., 1999, 2000; Fünfschilling and Rospert, 1999; George *et al*., 2002). In the yeast *Saccharomyces cerevisiae*, the impairment of *ATP2* mRNA targeting to the vicinity of mitochondria leads to a respiratory deficiency due to an inefficient import of the protein (Margeot *et al*., 2002). Therefore, mRNA sorting to the vicinity of mitochondria seems to be essential for the organelle function. Yeast DNA microarrays allowed us to demonstrate that >100 mRNAs encoding mitochondrial proteins localize to mitochondrion-bound polysomes and that their 3′ untranslated regions (UTRs) are required for their localization (Marc *et al*., 2002). Herein, we present evidence indicating that in higher eukaryotes mRNAs encoding mitochondrial proteins exhibit the same distribution pattern between free and mitochondrion-bound polysomes as in *S. cerevisiae*. We then address the question of whether a common mechanism of 3′ UTR recognition exists in yeast and human cells. To do this, we used the *OXA1* gene as a model system. *OXA1* encodes a membrane protein highly conserved in both prokaryotes and

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03–02–0074. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03–02–0074.

‡ Corresponding authors. E-mail addresses: or dujardin@cgm.cnrs-gif.fr.

eukaryotes (Hermann and Neupert, 2000; Luirink *et al*., 2001; Saint-Georges *et al*., 2001). In yeast mitochondria, Oxa1p is involved in the insertion of inner membrane proteins, and a null mutation in the *OXA1* gene leads to a complete respiratory deficiency. A human Oxa1p homolog has been described that partially rescues the respiratory capacity of a yeast *oxa1* mutant, suggesting that the proteins play essentially the same role in both organisms (Bonnefoy *et al*., 1994). In this study, we first demonstrate that *OXA1* mRNA is enriched in mitochondrion-bound polysomes purified from both yeast and HeLa cells. The human cDNA described in the initial report codes for a protein devoid of the first 60 amino acids, corresponding to the mitochondrial targeting sequence (mts) (Rötig *et al*., 1997). Therefore, we decided to construct the full-length human *OXA1* cDNA and to examine the cellular distribution of the two human mRNA species in yeast cells. Both transcripts were detected almost exclusively in mitochondrion-bound polysomes, indicating that the machinery of mRNA targeting to the vicinity of mitochondria is able to recognize both human and yeast sequences. Both transcripts share the same 3′ UTR, and a human *OXA1* cDNA devoid of this sequence is unable to rescue cell respiratory capacity of yeast *oxa1* mutant, confirming the importance of this 3′ UTR to ensure the function of the human Oxa1p inside the organelle. Moreover, we show that the 3′ UTR of *OXA1* mRNA possesses mitochondrial targeting properties, because fluorescent RNAs that encompass either the yeast or the human *OXA1* 3′ UTRs are found in proximity to mitochondria in living yeast cells. Hence, these data argue for the existence of a common recognition mechanism of the *OXA1* 3′ UTR in both yeast and human cells.

MATERIALS AND METHODS

Human Cell Culture

HeLa cells were grown in American Type Culture Collection medium until confluence (Eagle's minimum essential medium with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 M nonessential amino acids, 1 mM pyruvate, and 10% fetal bovine serum). These cells have an adenocarcinoma origin and present an epithelial morphology.

Obtention of Human cDNA Clones

The truncated human *OXA1* cDNA was cloned by functional complementation of an *oxa1*− mutation, by using a human cDNA library constructed in pFL61 (Bonnefoy *et al*., 1994). The yeast expression vector pFL61 contains the replication origin of the yeast 2μ plasmid, the *URA3* gene, and the promoter and terminator of the phosphoglycerate kinase gene *PGK1* (Minet *et al*., 1992) The full-length human *OXA1* cDNA was constructed by overlap extension using polymerase chain reaction (PCR) (Ho *et al*., 1989). The first DNA fragment corresponds to the first exon of the human *OXA1* gene amplified from a genomic clone (Rötig *et al*., 1997), and the second one to the truncated human cDNA cloned by complementation. The two fragments were combined in a fusion reaction in which the overlapping ends (63 nucleotides) annealed, and the resulting fusion was amplified by PCR, cloned in pFL61 by using the unique *Not*I site, and sequenced. To obtain constructions with human *OXA1* cDNAs but devoid of their 3′ UTRs, 230 nucleotides long, we purified the complete cDNAs from the pFL61 plasmid digested with *Not*I. The inserts were then submitted to *EcoR*1 digestion, a unique site that is present 30 nucleotides downstream from the stop codon of the human *OXA1* open reading frame (ORF). The obtained fragments were subsequently cloned in pFL61 linearized by *Not*I.

Yeast Strains and Culture

The *S. cerevisiae* strains were the *oxa1*− mutant *NBT1* (*mat alpha*, *oxa1::LEU2*, *ade2-1*, *ura3-1*, *his3-11,-15*, *trp1-1*, *leu 2-3,-112*) and the corresponding wild-type [*WT*] *OXA1* strain *CW04* (*mat alpha*, *ade2-1*, *ura3-1*, *his3-11,-15*, *trp1-1*, *leu 2-3,-112*) (Bonnefoy *et al*., 1994). Yeast cells were transformed using a simplified lithium method (Gietz *et al*., 1992).

For the analysis of respiratory function, cells were grown in synthetic medium containing 2% glucose until OD_{600} of 2. Then, they were serially diluted (1:5) and spotted either on synthetic medium containing 2% glucose supplemented with the appropriate nutritional requirements, or on 2% glycerol medium (1% yeast extract, 2% peptone, 2% glycerol, 10% sodium phosphate buffer, pH 6.2). Plates were incubated at 28°C for either 72 or 96 h.

Biochemical Fractionation of Polysome Populations

Polysomes associated with mitochondria and free cytoplasmic polysomes of yeast cells were isolated as described previously (Margeot *et al*., 2002). For HeLa cells, we applied the following modifications: cells were collected by trypsination and spun down at 2500 rpm/min for 6 min at room temperature. Cells were resuspended in 20 ml of fresh medium, cycloheximide was added at 250 μg/ml, and the cells were incubated for 15 min at 37°C. After centrifugation, the cells were washed in mannitol-polysome buffer (0.6 M mannitol, 30 mM Tris-HCl, pH 7.4, 5 mM MgAc, 100 mM KCl, 200 μg/ml cycloheximide, 500 μg/ml heparin, 1 g/l bovine serum albumin, and 5 mM β-mercaptoethanol). The pellet was resuspended in 6 ml of mannitol-polysomes buffer, 100 μg/ml digitonin was added, and the cells were incubated on ice for 4 min. Cells were disrupted with 15 strokes by using a glass Teflon pestle. The homogenate was centrifuged at 2500 rpm for 7 min; the pellet was once again disrupted and centrifuged. Both homogenates were combined and centrifuged twice to eliminate unbroken cells and nuclei. The resulting supernatant was further centrifuged at 11,000 rpm for 30 min. The pellet of crude mitochondria associated with polysomes was washed twice in mannitol-polysome buffer before storage at −80°C. Free cytoplasmic polysomes were purified from the post-mitochondrial supernatant by sedimentation through a step gradient of 2 and 0.5 M sucrose. The gradients were centrifuged at 40,000 rpm for 20 h at 4°C by using the TST41 rotor. The pellet, containing free cytoplasmic polysomes, was used for RNA extraction.

RNA Extraction and Northern Blot Analyses

RNA extractions from mitochondrion-bound polysomes, and from free cytoplasmic polysomes, were performed using the RNEasy Protect MIDI kit (QIAGEN, Hilden, Germany). For Northern blots, 8 μg of RNA was separated by electrophoresis through denaturating formaldehyde-agarose gels and transferred to nylon membranes. The blots were stained with methylene blue before prehybridization and hybridization with specific probes (Sambrook *et al*., 1989). The PhosphorImager system and TINA software were used to compare the relative abundance of each mRNA species. Probes used were obtained by reverse transcription (RT)-PCR by using specific oligonucleotides (Table 1). For the amplifications, we used 200 ng of total RNA purified from HeLa cells and the Access RT-PCR system (Promega, Madison, WI). Labeling of DNA fragments was performed using the Nona Primer kit from Appligene (Illkirch, France).

Table 1. Human or yeast cDNA probes

ORF and function	5′ Primer (5′–3′)	3′ Primer (5′–3′)	RT-PCR product length (bp)
ACO2 Aconitase, TCA cycle	tccacgagaccaacctgaaga	ctgatggcacacgtggagct	320
AK2 Adenylate kinase, nucleotide metabolism	atgctgagggccatggtggcttct	ccccggtgatgtcatctttcatgg	363
ALDH2 Aldehyde dehydrogense 2, carbohydrate metabolism	atggcatgaccatcgccaaggagg	ccatcaatggctgagggaggaagc	374
ATP5b β subunit of F1 ATPase	atggatggtacagaaggcttggtt	catttcatggtataaatcattgcc	413
COX6c Cytochrome c oxidase subunit	atggctcccgaagttttgccaaaa	tgttaattgtttatttatcaagag	354
COX10 Heme A biosynthesis	gttgctaaataaccatttgagagc	acttaaagatagagtttcaaataa	540
OXA1 Cytochrome oxidase assembly	gtggcttctggagagactgcagatgta	gggcacaggaaggttggccatctctct	420
NDUFV1 NADH ubiquinone oxidoreductase	atcgcgccagttcctcagcctcag	ctgcagattggaggcctcattgta	579
NDUFV2 NADH ubiquinone oxidoreductase	atacaatgtataatcgaaagccag	catattttatttctctagtgacaac	438
UQCRFS1 Rieske, Ubiquinol-cytochrome c reductase	gcaggccacggtgcccgccacccc	cctctccatttgaaagccatgttc	422
SOD2 Manganese superoxide dismutase	ggtacgaccagcactagcagcatg	cagcataacgatcgtggttta	707
IRP1 Iron regulatory protein 1	tgatcgttctggctggcaaag	cgaccaagtgcacgtctccta	391
FERRITIN H chain	ttgtgtgacttcattgagacac	aagtggatgttttggtacaact	277
MtDNA (8282–13851) ATPase8, ATPase6, COXIII, ND3, ND4, ND4L, ND5	cctctagagcccactgtaaagc	ttgaggtctagggctgtta	5569
COX2	tataggctaaatcctatatat	aggtcgcctggttctaggaat	402
Yeast OXA1 Cytchrome oxidase assembly	ttccgcttctacttcggaccttatcgc	attggaatttgtagcatgggtgcggcc	420
Yeast COX3 Cytochrome oxidase subunit	atgacacatttagaaagaagtagacatcaa	tattacctgcgattaaggcatgatgactat	250
Yeast COX6 Cytochrome c oxidase subunit	tatacaacaatgttatcaagggccata	ttcatcatgtgcgtcagaatacttcct	675
Yeast ACT1 Cytoskeleton protein	gaggttgctgctttggttatt	gtggtgaacgatagatggacc	800
Yeast ATP2 β subunit of F1 ATPase	tagaagaaataaagcttaaaccaagggg	catgtccagtgggaaagcgaggcaaga	660

The 15 first ORFs correspond to human sequences, whereas the five others are from yeast. The oligonucleotides used for the RT-PCR analysis are indicated in columns 2 and 3 and the size of the PCR products in the fourth column.

RT-PCR Analyses

The respective amounts of human *OXA1* mRNAs in yeast and human cells were measured by RT-PCR by using the Access RT-PCR system (Promega, Madison, WI). After DNase treatment of each RNA preparation, 200 ng of RNA was used for reverse transcription. The products obtained were subjected to 25 cycles of PCR with specific oligonucleotides inside each open reading frame (Table 1). Ten percent of the amplified products were run in agarose gels, and the quantities of amplified products reflecting the amount of each mRNA in each polysomal population was measured with the TINA software.

Fluorescence Microscopy

The coat protein (CP) of bacteriophage *MS2* was fused to the green fluorescent protein (GFP) and expressed from the plasmid pCP-GFP, generously provided by D. Beach and K. Bloom (Beach *et al*., 1999). The pCP-GFP is a low copy *HIS3* selectable plasmid that produced CP-GFP under the regulation of the *MET25* promoter. Cells grown in the presence of methionine produced no detectable CP-GFP protein product. To induce CP-GFP production, cells were switched to a medium without methionine for 2 h. To obtain reporter RNA, we used the *pIIIA/MS2–2* plasmid, which contains two tandem copies of the CP-binding site. This plasmid can express a transcript tagged by the two tandem copies of the CP-binding site, under RNase P promoter control, which maintains RNA levels throughout the cell cycle. The single *Sma*I site allowed us to introduce the nucleotide sequences with potential targeting properties. The oligonucleotides used to amplify the human 3′ UTR (237 nucleotides in length) were as follows:

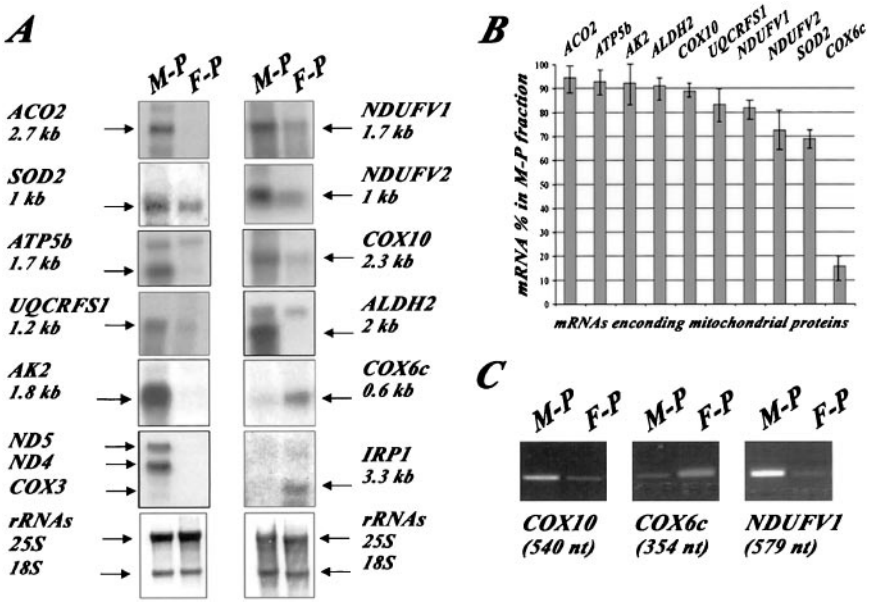

Figure 1. Subellular distribution of mRNAs encoding mitochondrial proteins in HeLa cells. (A) Northern blots were performed with RNA prepared from free polysomes (F-P) and mitochondrion-bound polysomes (M-P) by using probes for different genes encoding mitochondrial proteins (Table 1). At the bottom, methylene blue staining of the ribosomal RNAs is shown. The exposure times of the autoradiograms were ~14 h at −80°C by using Amersham intensifying screens for all the probes except the 5.6-kb mtDNA, which reveals the *ND5*, *ND4*, and *COX3* genes, the sizes of these three transcripts are 1.9, 1.45, and 1 kb, respectively, and required an exposure time of only 1 h. (B) Quantifications of the hybridization signals were made using the PhosphorImager system and TINA software. The signal obtained for an individual transcript in the M-P fraction was normalized with the *COX3* signal. Normalization for the signal obtained in the F-P fraction was performed using the *IRP1* signal. For a given mRNA, addition of both signals after normalization was considered as 100%. The approximate percentage is a mean of four independent biochemical purification experiments, and the Northern blot analyses were performed twice for each polysomal preparation. (C) RT-PCR analysis were performed with 200 ng of RNA purified from M-P and F-P by using specific oligonucleotides within *COX10*, *COX6c*, and *NDUFV1* ORFs (Table 1), the size of each amplified product is shown at the bottom. The four independent polysomal RNA purifications were subjected twice to RT-PCR analyses.

forward primer, 5′tcccccgggggacaaagtatccctggcacgacacatttg3′ and reverse primer, 5′tcccccgggggatttttgtagtacagaggtttactaact3′. To further characterize the region within the human *OXA1* 3′ UTR having targeting properties in vivo, we studied two fragments of 100 and 137 nucleotides, respectively. For the 100-nucleotide fragment close to the stop codon, we combined the precedent forward primer with the reverse primer 5′tcccccgggggactaggactggggcaaggacacagtg3′. For the 137-nucleotide fragment encompassing the polyadenylation signal, we used the forward primer 5′tcccccgggggaagtcctaggaactgtggcacacacagagat, combined with the reverse primer used to amplify the full-length 3′ UTR.

For the yeast 3′ UTR, the forward primer was 5′cccaagcttgggattaataacaaaaaatgaataaaggc 3′ and the reverse primer was 5′cccaagcttgggtccaaatgattatttcaagcaataaa 3′, which allows the amplification of the complete yeast *OXA1* 3′ UTR (158 nucleotides in length). In all cases, the binding site precedes the sequence examined. For each inserted sequence, we studied both possible orientations. *CW04* cells expressing the "green" RNAs and the CP-GFP protein were observed by fluorescence microscopy with a chilled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). For all microscopic observations, cells were harvested in early-log phase. Mitochondrial DNA and nuclear DNA were visualized using the Hoetsch reagent at 1 nM (Molecular Probes, Eugene, OR). Mitochondria were also labeled with the specific mitochondrial dye MitoTracker Red CMX Ros at 0.02 nM (Molecular Probes). We also performed the induction of the CP-GFP protein for 2 h in a medium devoid of methionine, but instead of 2% galactose, 2% glycerol was added. In this condition, several cells show mitochondria as branched tubular networks. To process the cell images, Leica 4000 software was used.

RESULTS

Detection of mRNAs Exclusively Associated with Mitochondrion-bound Polysomes Isolated from HeLa Cells

We have previously reported that >100 mRNAs encoding mitochondrial proteins localized to the vicinity of the organelle in yeast cells (Marc *et al*., 2002). To determine whether this process is conserved throughout evolution, free cytoplasmic and mitochondrion-bound polysomal RNAs purified from HeLa cells were analyzed by Northern blot. We used a 5.6-kb mtDNA fragment (encompassing the region from nucleotide 8282 to 13851) as a mitochondrial marker and the cDNA for *IRP1*, encoding the nuclear iron regulatory protein as a cytoplasmic marker. Mitochondrial preparations seemed to be devoid of *IRP1* mRNA; furthermore, *ND5*, *ND4*, and *COX3*, mitochondrial mRNAs, were not detected in free cytoplasmic polysomes, confirming that there is little cross-contamination between the two polysomal fractions examined (Figure 1). We next investigated whether the mitochondrial fraction contained nuclear mRNAs coding for mitochondrial proteins. Ten genes covering an array of functions within the organelle were chosen for mRNA localization analysis (Table 1 and Figure 1A). Messenger RNAs encoding Aco2, Ndufv1, Ndufv2, Atp5b, Ak2, Aldh2, Sod2, Cox10, and Uqcrf1 proteins were remarkably enriched in the polysomes bound to the mitochondrial surface. Consistently, these nine transcripts were found overrepresented in polysomes bound to mitochondria in four independent experiments (Figure 1B). In contrast, the

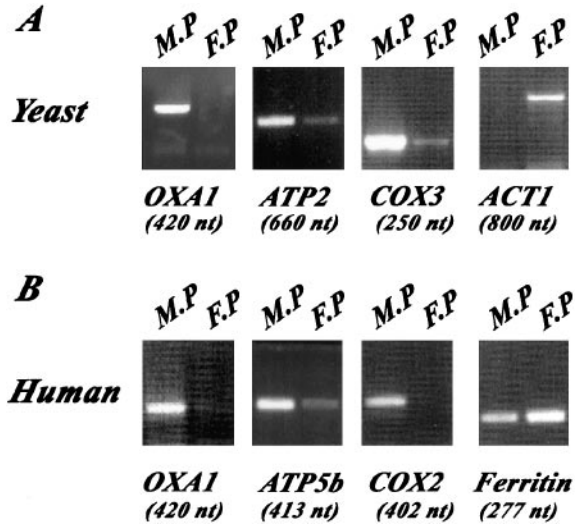

Figure 2. OXA1 mRNA localizes to the vicinity of mitochondria in both yeast and human cells. RT-PCR analyses were performed with RNAs purified from mitochondrion-bound polysomes (M-P), free cytoplasmic polysomes (F-P) in yeast and HeLa cells. Specific oligonucleotides were chosen inside each coding region for human *OXA1*, *ferritin*, and *ATP5b* genes, yeast *OXA1*, *ATP2*, *ACT1*, and *COX3* genes (Table 1) to perform RT-PCR analyses. Ten percent of the amplified products were subjected to electrophoresis; the size of each amplified product is shown at the bottom.

COX6c transcript mostly localized to free cytoplasmic polysomes, as we previously observed for the yeast homolog (Figure 1). RT-PCR analyses were performed with 200 ng of RNA purified from mitochondrion-bound polysomes or free cytoplasmic polysomes and specific oligonucleotides for *COX10*, *COX6c*, and *NDUFV1* sequences. Figure 1C shows that the cellular distribution of *COX10*, *NDUFV1*, and *COX6c* mRNAs measured by Northern blot and RT-PCR are quite similar. These results provide evidence that in HeLa cells, as in the yeast *S. cerevisiae*, several mRNAs encoding mitochondrial proteins localized to the proximity of the organelle in vivo.

Both the Yeast and Human OXA1 mRNAs Localize to the Vicinity of Mitochondria

Oxa1p is a member of the conserved *Oxa*I/Yidc/Alb protein family involved in the insertion of proteins into mitochondrial, choloroplast, and prokaryote membranes. To determine the subcellular distribution of *OXA1* mRNA, we purified mitochondrion-bound polysomes and free cytoplasmic polysomes from both yeast and HeLa cells. RNA from each polysomal fraction was subjected to RT-PCR analysis. Figure 2 shows that *OXA1* mRNA is preferentially found in mitochondrion-bound polysomes; indeed, very little signal was detected in free cytoplasmic polysomes. Additionally, it seems that mRNA localization is identical in yeast and HeLa cells. The distribution of *OXA1* mRNA was very similar to that of *COX3* and *COX2* mRNAs transcribed, respectively, from yeast and human mitochondrial genomes. Furthermore, RT-PCR products for *ACT1* and *Ferritin* confirmed that preparations of mitochondrion-bound polysomes presented little contamination with free cytoplasmic polysomes in both yeast and human cells (Figure 2). Moreover, the distribution of *OXA1* mRNA in yeast is quite similar to that of *ATP2* mRNA, a transcript almost exclusively found in the vicinity of mitochondria (Margeot *et al*., 2002). In HeLa cells, *OXA1* mRNA is particularly enriched in mitochondrion-bound polysomes, as is *ATP5b* mRNA (Figure 1). Thus, *OXA1* mRNA is mostly found at the proximity of mitochondria, and this particular subcellular distribution is conserved in yeast and human cells.

Human OXA1 mRNA Expressed in Yeast Cells Localizes to the Vicinity of Mitochondria

In *S. cerevisiae*, the *oxa1* null mutation leads to a complete respiratory deficiency. A partial restoration of cell respiration is observed when *oxa1*$^-$ cells are transformed with the human homolog, suggesting that the proteins play essentially the same role in both organisms (Bonnefoy *et al*., 1994). To address the question whether the human *OXA1* mRNA expressed in yeast cells is delivered to the proximity of mitochondria, we studied two versions of the human gene (Figure 3A). One directing the expression of the full-length protein (*HOXA1l* cDNA; see MATERIALS AND METHODS for construction) and the second allowing the expression of a truncated protein in which the first 60 amino acids coding the mitochondrial targeting sequence are absent (*HOXA1s* cDNA) (Bonnefoy *et al*., 1994). The expression of human Oxa1p was under the regulation of the yeast *PGK1* promoter in each one of the plasmids studied. We first examined the ability of each one of these sequences to complement the respiratory defect of *oxa1* yeast mutants. The *oxa1*$^-$ cells plated on glycerol medium are unable to grow at 28°C. Both the truncated and the complete forms of the human *Oxa*I protein rescue the ability to grow on glycerol. Interestingly, cells expressing the truncated version of the human protein grow better than cells expressing the full-length protein (Figure 3B). To determine the subcellular localization of the corresponding transcripts, RT-PCR analyses were performed using RNAs purified from mitochondrion-bound polysomes and free cytoplasmic polysomes (Figure 3, C and D). Several mRNAs were examined by RT-PCR to confirm that little cross-contamination between the two polysomal populations exists. As expected, *ATP2* mRNA is mainly detected in mitochondrion-bound polysomes, whereas *COX6* transcript is predominantly found in free cytoplasmic polysomes. In wild-type cells, *OXA1* mRNA is almost exclusively detected in mitochondrion-bound polysomes, as shown in Figure 2 (Figure 3, C and D, *WT*). In cells expressing the truncated version of the human protein, the corresponding transcript is strongly enriched in mitochondrion-bound polysomes (Figure 3, C and D, *HOXA1s*), very little of the transcript is detected in free cytoplamsic polysomes. The mRNA encoding the complete form of human Oxa1p is also very abundant in mitochondrion-bound polysomes, even though the mRNA level in free cytoplasmic polysomes is slightly higher than that found for the yeast mRNA and mRNA encoding the truncated form of the human protein (Figure 3, C and D, *HOXA1l*). Thus, both human *OXA1* mRNAs are recognized and delivered to the vicinity of mitochondria in yeast cells.

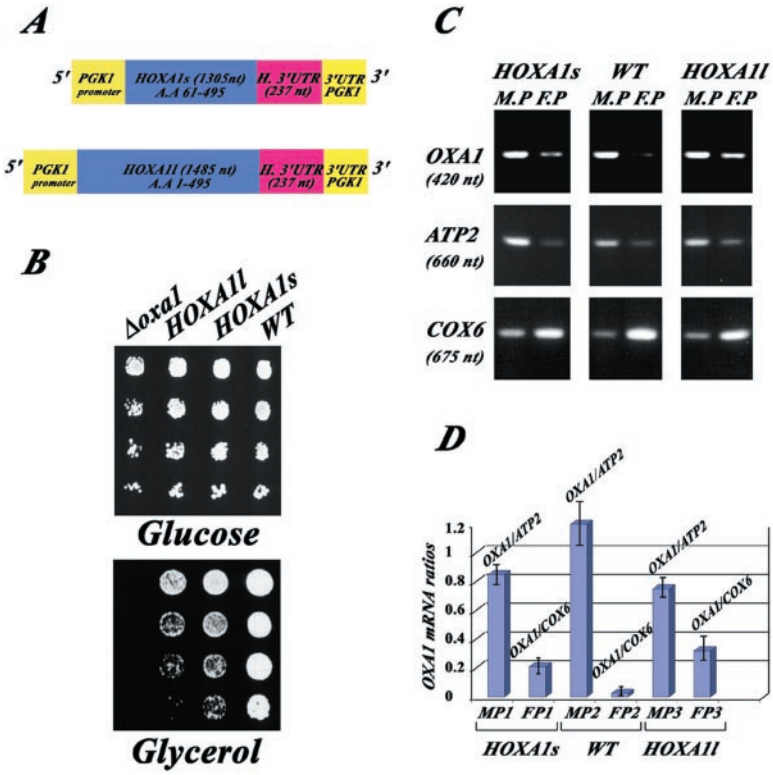

Figure 3. Human OXA1 mRNAs expressed in yeast cells are found associated with polysomes bound to the mitochondrial surface. Yeast *oxa1*$^-$ mutant cells (Δ*oxa1*, strain *NBT1*) were transformed with plasmids directing the expression of either the full-length *Oxa1* protein (*HOXA1l*) or the N-terminal truncated form (*HOXA1s*). (A) Schematic representation of the two plasmids. The expression of both human proteins is under the regulation of the yeast *PGK1* promoter. Both constructions share at their 3′ extremities 237 nucleotides of the human *OXA1* 3′ UTR sequence and the yeast *PGK1* 3′ UTR. (B) The respiratory growth of the transformants and wild-type cells (*WT*) was examined on glycerol medium at 28°C. The image represents an incubation of 3 d. (C) RT-PCR analysis were performed with RNAs from free cytoplasmic polysomes (F-P), mitochondrion-bound polysomes (M-P) purified from *WT* cells and *oxa1*$^-$ cells expressing either the *HOXA1l* or the *HOXA1s* plasmid. Specific oligonucleotides were chosen inside the coding region for human *OXA1*, yeast *OXA1*, *ATP2*, and *COX6* sequences (Table 1). Ten percent of each amplified product was subjected to electrophoresis. (D) Quantifications were made using the TINA software. For the M-P polysomes, the *OXA1* RT-PCR product signal was normalized using the *ATP2* signal. For the F-P polysomes, the *OXA1*'s RT-PCR signal was normalized using the *COX6* signal. The experiments were performed with four independent polysomal RNA purifications, and each polysomal preparation was subjected to RT-PCR analysis twice.

Presence of the Human OXA1 3′ UTR Is Essential to Allow the Human Protein to Rescue the Respiratory Function of Yeast oxa1 Mutant Cells

The two plasmids *HOXA1l* and *HOXA1s* direct the expression of human *Oxa*I proteins under the regulation of the yeast *PGK1* promoter. The 5′ UTR of the human *OXA1* gene is absent in both constructions; however, they share a 237-nucleotide stretch at their 3′ extremities that in the human gene follows the stop codon of the *OXA1* ORF (Figure 3A). To determine the role of the 237-nucleotide stretch in the ability of the human protein to rescue the respiratory capacity of *oxa1* mutants, we constructed plasmids with either the full-length or the truncated form of the proteins devoid of the entire 3′ UTR (Figure 4A). In these constructions, human *OXA1* coding regions are followed by the *PGK1* 3′ UTR. The *PGK1* gene encodes a cytoplasmic protein and its 3′ UTR did not allow a reporter RNA to localize to the proximity of mitochondria in yeast living cells (Corral-Debrinski *et al.*, 2000). Figure 4B clearly shows that the presence of the human 3′ UTR is essential for the respiratory function complementation of *oxa1*$^-$ cells; indeed, cells transformed with the plasmids, in which the human *OXA1* 3′ UTR is absent, are unable to grow on glycerol medium at 28°C. Thus, the presence of the human *OXA1* 3′ UTR is required to allow the human Oxa1p to play essentially the same role than its yeast counterparts in the inner mitochondrial membrane.

3′ UTR of the Human OXA1 mRNA Is Able to Localize a Reporter RNA to the Vicinity of Mitochondria in Living Yeast Cells

Because we demonstrated that human *OXA1* transcripts localized to mitochondrion-bound polysomes and that the presence of the human *OXA1* 3′ UTR is necessary for the function of the human protein in yeast mitochondria, we next determined whether this sequence possesses mitochondrial targeting properties, by visualizing fluorescent RNAs in living yeast cells (Beach *et al.*, 1999). The 237-nucleotide human sequence, as well as a stretch of 158 nucleotides downstream the stop codon of the yeast *OXA1* ORF were fused to two CP (coat protein from the *MS2* bacteriophage) binding sites in the *pIIIA/MS2-2* plasmid, which directs the synthesis of reporter RNAs. These RNAs can be visualized by fluorescence microscopy when cells express the CP-GFP fusion protein. Cells expressing RNAs in which the 3′ UTRs is inserted in the opposite orientation, such that the noncoding sequence is transcribed, showed a low amount of fluorescence distributed throughout the cytoplasm (our unpublished data). Figure 5A clearly shows that the reporter RNA containing the yeast *OXA1* 3′ UTR, in the coding orientation, produced a punctuate distribution of fluorescent speckles or reticulum structures representing mitochondria as confirmed by staining with the MitoTracker probe. The overlay of gRNA and MitoTracker labeling (Figure 5A, merge) indicates that the hybrid RNA mostly colocalized with the mitochondria. The human *OXA1* 3′ UTR is also able to target a reporter RNA to close contact to mitochondria (Figure 5B). When cells were grown on galactose, mitochondria look like small, discrete fluorescent spots within the cytoplasm. A 2-h incubation in 2% glycerol allows visualization of mitochondria as more elaborated networks (Figure 5, A and B, merge). Reporter RNAs containing either the yeast or the human sequence were often visualized in proximity to mitochondria under both conditions examined (Figure 5A and

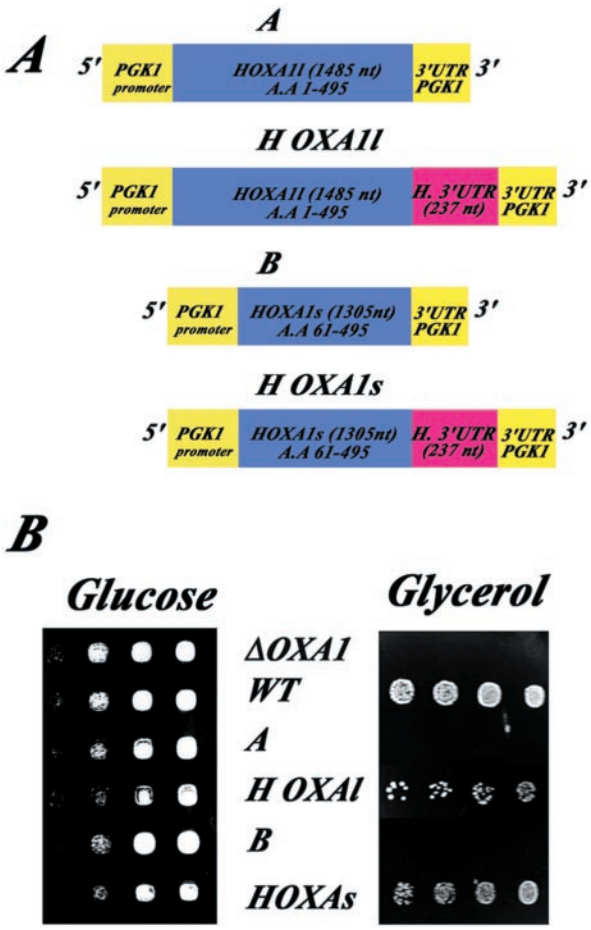

Figure 4. The human OXA1 3′ UTR is essential to rescue the respiratory function of yeast oxa1 mutant cells. (A) Schematic representation of the four constructions. The complete human *OXA1* 3′UTR was deleted from the original constructions directing the expression of either the full-length human *Oxa1* protein (*HOXA1l*) or the N-terminal truncated version (*HOXA1s*) to give *HOXA1/A* et *HOXA1s/B*. (B) Yeast *oxa1*-mutant cells (Δ*oxa1*, strain NBT1) were transformed with either one of the four plasmids (A) and the respiratory capacity of the transformed cells was tested by serially diluting cells at OD_{600} of 2 and plating in both glucose medium or glycerol medium. Images represent an incubation of 4 d at 28°C.

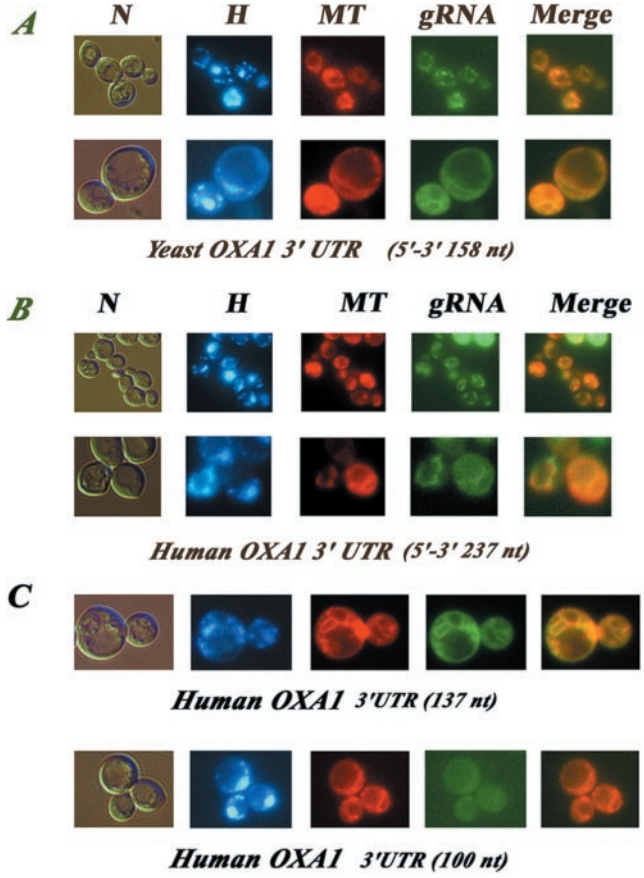

Figure 5. Imaging fluorescent RNAs in living yeast cells. Coexpression of CP-GFP plasmid and reporter RNAs, leads a GFP-labeled RNA, which was visualized using fluorescence microscopy techniques. Cells were grown in 2% galactose medium or incubated in a 2% glycerol medium to detect cells in which mitochondria consist of a branched tubular network and visualized at early log phase. gRNA indicates the green RNA labeling, H the Hoechst staining, N the cells photographed with Nomarski optics, and MT cells labeled with the MitoTracker dye. The merge at the right of the figure represents the superposition of both green RNA fluorescence and mitochondrial labeling with MitoTracker. (A) Reporter RNA with the yeast *OXA1* 3′ UTR of 158 nucleotides in length cloned in the coding orientation. Top line, CP-GFP expression was induced for 2 h in 2% galactose medium devoid of methionine Bottom line, CP-GFP expression was induced for 2 h in 2% glycerol medium devoid of methionine. (B) Reporter RNA with the human 3′ UTR of 237 nucleotides in length cloned in the coding orientation. Top line, CP-GFP expression was induced for 2 h in 2% galactose medium devoid of methionine. Bottom line, CP-GFP expression was induced for 2 h in 2% glycerol medium devoid of methionine. (C) Reporter RNAs encompassing two subfragments of 100 and 137 nucleotides of the human *OXA1* 3′ UTR amplified with specific oligonucleotides, and cloned in the unique *Sma*I site of the *pIIIA/MS2–2* plasmid (see MATERIALS AND METHODS). The insert orientations inside the plasmid were checked by PCR by using an internal *pIIIA/MS2-2* oligonucleotide. The CP-GFP expression was induced for 2 h in 2% glycerol medium devoid of methionine.

B, merge). Hence, both yeast and human 3′ UTR sequences are recognized by yeast proteins that allow the targeting of reporter RNAs to the proximity of mitochondria in vivo.

To map more precisely *cis*-acting elements within the 237 nucleotides of human *OXA1* 3′ UTR, the subcellular distribution of two deletion RNAs was analyzed. The first one contains the 100 nucleotides immediately downstream of the stop codon, and the other contains the 137 nucleotides close to the putative polyadenylation signal at the end of the human cDNA sequence. When either one of these sequences was placed in the opposite orientation, such that the noncoding sequence was transcribed, both reporter RNAs were diffuse in their distribution throughout the cytoplasm (our unpublished data). The hybrid RNA containing the 100 nucleotides downstream the stop codon did not localize to the vicinity of mitochondria; indeed, a diffuse and weak fluorescent staining in the cytoplasm was consistently observed (Figure 5C). In contrast, the hybrid RNA containing the last 137 nucleotides of the 3′ UTR

close to the polyadenylation signal was as efficient as the complete 3′ UTR (Figure 5B). Indeed, we were able to visualize the reporter RNA in branched and elaborated cytoplasmic networks also stained by MitoTracker, which represented mitochondrial structures (Figure 5C, MT, gRNA, and merge). These results indicate that the mechanism by which the human Oxa1p rescues respiratory function of yeast *oxa1*$^-$ cells implies that the corresponding mRNA localizes to the proximity of the organelle in vivo. To achieve this specific localization the human 3′ UTR is required and is probably recognized by yeast proteins allowing its accurate targeting.

DISCUSSION

Subcellular RNA localization is used by eukaryotic cells to achieve high local concentrations of protein products. More than 90 localized mRNAs are known so far. Mounting evidence shows that mRNA localization is not restricted to cell fate determination; it is also required for the efficient import of proteins destined to the nucleus and organelles in somatic cells (Jansen, 2001). A classic example has been found in oligodendrocytes, which are involved in myelination of neurons. In these cells, the mRNA encoding myelin basic protein is localized to the peripheral processes as granules containing multiple mRNA molecules and some components of the translation machinery (Bassell *et al*., 1999; Tekotte and Davis, 2002). Another example well studied in yeast is the regulation of mating-type switching, which requires the concentration of Ash1p within the daughter nucleus. This asymmetric distribution is achieved by the localization of *ASH1* mRNA to the bud tip (Chartrand *et al*., 1999; Jansen, 2001).

We recently found that yeast mRNAs transcribed in the nucleus and coding for mitochondrial proteins are asymmetrically distributed in the cytoplasm (Corral-Debrinski *et al*., 2000; Marc *et al*., 2002). To determine whether this mRNA sorting process is conserved in human cells, we purified polysomes bound to mitochondria from HeLa cells. We clearly demonstrated that, as in yeast, several mRNAs encoding mitochondrial proteins are sorted to the vicinity of mitochondria. Transcripts encoding Aco2, Aldh2, Ak2, Atp5b, Cox10, Uqcrf1, Ndufv1, and Ndufv2 are almost exclusively found in polysomes specifically associated with the mitochondrial surface. In contrast, *COX6c* mRNA was mostly found in the free cytoplasmic polysomes. Interestingly, the Aldh2 and Ak2 precursors have been shown to use a cotranslational pathway of import (Nobumoto *et al*., 1998; Ni *et al*., 1999). Both mRNAs seemed remarkably enriched in polysomes bound to the mitochondrial surface in HeLa cells, confirming the link between mRNA delivery to mitochondrial surface and cotranslational import. Furthermore, 8 of 10 transcripts examined possess yeast counterparts, and their sorting in yeast is similar to that observed in human cells, showing the conservation of this process throughout evolution. In yeast, no simple explanation can be found to account of the specific mitochondrial targeting of mRNAs, except that they code for proteins of procaryotic origin (Marc *et al*., 2002). Although, we have only analyzed a limited set of human mRNAs, this correlation could also hold true in human cells because Cox6c, a complex IV subunit is not conserved in bacteria and all the other proteins have prokaryotic homologs.

The YidC/Oxa1p/Alb3 protein family is required for the proper biogenesis of a subset of bacterial, mitochondrial, and thylakoid membrane proteins (Luirink *et al*., 2001). In this study, we showed that two forms of the human *OXA1* mRNA, one encoding a truncated version of the protein, devoid of the first 60 amino acids, and the other the full-length protein are mainly targeted to the proximity of yeast mitochondria. Surprisingly, cells expressing the truncated mRNA grow even more rapidly on respiratory medium than those expressing the full-length RNA and the relative amount of RNA bound to mitochondria also seems higher. The two forms of human *OXA1* mRNA have no human 5′ UTR sequence but share a 237-nucleotide sequence at their 3′ extremities. We demonstrated in this study, that the deletion of this 3′ UTR sequence abolishes the respiratory growth of the *oxa1* mutant cells. Thus this 3′ UTR human sequence seems essential for the mitochondrial function of the human *Oxa*I proteins, whereas the 5′ UTR and the beginning of the ORF are not. Furthermore, the 237 nucleotides and even a subfragment corresponding to the last 137 nucleotides of this 3′ UTR are able to deliver a reporter RNA to the vicinity of mitochondria in living yeast cells, showing that yeast proteins involved in mRNA sorting to the vicinity of mitochondria are able to recognize the human *OXA1* 3′ UTR. Therefore, as in the case of the *ATP2* mRNA (Margeot *et al*., 2002), there is a direct correlation between human protein function inside the organelle and human *OXA1* mRNA localization to the vicinity of yeast mitochondria.

Interestingly, yeast proteins involved in mRNA sorting to the vicinity of mitochondria are able to recognize the human *OXA1* 3′ UTR. It has already been shown that stem-loop structures are required for the localization of *ASH1* mRNA in yeast (Chartrand *et al*., 1999; Gonzalez *et al*., 1999). In an attempt to define a conserved structural motif in the 3′ UTR involved in the specific targeting of *OXA1* mRNA to the vicinity of mitochondria, we folded stretches of 100 nucleotides within the putative 3′ UTRs (Zuker, 1989) of five *OXA1* orthologs, presenting similar functions: *Homo sapiens*, *S. cerevisiae*, the two genes of *Schizosaccharomyces pombe* (Bonnefoy *et al*., 2000), *Neurospora crassa* (Nargang *et al*., 2002), and *Arabidopsis thaliana* (Hamel *et al*., 1997). Even though a perfectly conserved structure cannot be defined, motifs encompassing stem-loop structures formed by stems and asymmetrical bulges were observed for all the sequences folded (our unpublished data). This conserved stem loop structure might be recognized by transacting factors involved in mRNA transport to the vicinity of mitochondria.

In conclusion, in human cells, mRNAs encoding a subset of mitochondrial proteins are sorted to the vicinity of mitochondria, as we first demonstrated for the yeast *S. cerevisiae*. Because motifs within the 3′ UTR of the human *OXA1* RNA are functional in yeast cells, we can conclude that the recognition mechanism is also conserved in both cells. The conservation of this process throughout evolution suggests that it is essential for the organelle biogenesis in higher eukaryotes. Therefore, mutations affecting this machinery are likely responsible for some mitochondrial disorders.

ACKNOWLEDGMENTS

We thank Michèle Kermorgant for technical assistance. We thank Drs. Elvira Carvajal, Thierry Delaveau and Olga Groudinsky for helpful discussions and critical reading of the manuscript and Dr. Yu-Chun Lone for kindly providing HeLa cells. This study was supported by funds from the Centre National de la Recherche

Scientifique, the Ecole Normale Supérieure, and the Association Française contre les Myopathies (grants MNM 2000 to M.C.-D. and G.D.). A.M. is the recipient of a Centre National de la Recherche Scientifique/Bourse de l'Ingénieur fellowship.

REFERENCES

Bassell, G.J., Oleynikov, Y., and Singer, R.H. (1999). The travels of mRNAs through all cells large and small. FASEB J. *13*, 447–454.

Beach, D.L., Salmon, E.D., and Bloom, K. (1999). Localization and anchoring of mRNA in budding yeast. Curr. Biol. *9*, 569–578.

Bonnefoy, N., Kermorgant, M., Groudinsky, O., and Dujardin, G. (2000). The respiratory gene *OXA1* has two fission yeast orthologs which together encode a function essential for cellular viability. Mol. Microbiol. *35*, 1135–1145.

Bonnefoy, N., Kermorgant, M., Groudinsky, O., Minet, M., Slonimski, P.P., and Dujardin, G. (1994). Cloning of a human gene involved in cytochrome oxidase assembly by functional complementation of an oxa1$^-$ mutation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *91*, 11978–11982.

Chartrand, P., Meng, X.-H., Singer, R.H., and Long, R.M. (1999). Structural elements required for the localization of Ash1 mRNA and of a green fluorescent protein reporter particle *in vivo*. Curr. Biol. *9*, 333–336.

Corral-Debrinski, M., Belgareh, N., Blugeon, C., Claros, M.G., Doye, V., and Jacq, C. (1999). Overexpression of yeast karyopherin Pse1p/Kap121p stimulates the mitochondrial import of hydrophobic proteins *in vivo*. Mol. Microbiol. *31*, 1499–1511.

Corral-Debrinski, M., Blugeon, C., and Jacq, C. (2000). In yeast, the 3′ Untranslated region or the presequence of *ATM1* is required for the exclusive localization of its mRNA to the vicinity of mitochondria. Mol. Cell. Biol. *20*, 7881–7892.

Fünfschilling, U., and Rospert, S. (1999). Nascent polypeptide-associated complex stimulates protein import into yeast mitochondria. Mol. Biol. Cell *10*, 3289–3299.

George, R., Walsh, P., Beddoe, T., and Lithgow, T. (2002). The nascent polypeptide-associated complex (NAC) promotes interaction of ribosome with the mitochondrial surface in vivo. FEBS Lett. *516*, 213–216.

Gietz, D., St. Jean, A., Woods, R.A., and Schiestl, R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. *20*, 1425

Gonzalez, I., Buonomo, S.B.C., Nasmyth, K., and Ahsen, U.V. (1999) *ASH1* mRNA. localization in yeasts involves multiple secondary elements and Ash1 protein translation. Curr. Biol.*9*, 337–340.

Hamel, P., Sakamoto, W., Wintz, H., and Dujardin, G. (1997). Functional complementation of an oxa1$^-$ yeast mutation identifies an *Arabidopsis thaliana* cDNA involved in the assembly of respiratory complexes. Plant J. *12*, 1319–1327.

Hermann, J.M., and Neupert, W. (2000). Protein transport into mitochondria. Curr. Opin. Microbiol. *3*, 210–214.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pearse, L.R. (1989). Site-directed mutageneses by overlap extension using the polymerase chain reaction. Gene *77*, 51–59.

Jansen, R.-P. (2001). mRNA localization: message on the move. Nature reviews. Mol. Cell. Biol. *2*, 247–256.

Luirink, J., Samuelsson, T., and Gier, J.-W.D. (2001) YidC/Oxa1p/Alb3: evolutionarily conserved mediators of membrane protein assembly. FEBS Lett. *501*, 1–5.

Marc, P., Margeot, A., Devaux, F., Blugeon, C., Corral-Debrinski, M., and Jacq, C. (2002). Genome-wide analysis of mRNAs targeted to yeast mitochondria. EMBO Rep. *3*, 159–164.

Margeot, A., Blugeon, C., Sylvestre, J., Jacq, C., and Corral-Debrinski, M. (2002). In *Saccharomyces cerevisiae*, *ATP2* mRNA sorting to the vicinity of mitochondria is essential for respiratory function. EMBO J. *21*, 6893–6904.

Minet, M., Dufour, M.-E., and Lacroute, F. (1992). Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. *2*, 417–422.

Nargang, F.E., Preuss, M., Neupert, W., and Herrmann, J.M. (2002). The Oxa1 protein forms a homooligomeric complex and is an essential part of the mitochondrial export translocase in *Neurospora crassa*. J. Biol. Chem. *277*, 12846–12853.

Ni, L., Heard, T.S., and Weiner, H. (1999). In vitro mitochondrial import: a comparison of leader sequence charge and structural relationships with the in vitro model resulting in evidence for co-translational import. J. Biol. Chem. *274*, 12685–12691.

Nobumoto, M., Yamada, M., Song, S., Inouye, S., and Nakasawa, A. (1998). Mechanism of mitochondrial import of adenylate kinase enzymes. J. Biochem. *123*, 128–135.

Rötig, A., Parfait, B., Heidet, L., Dujardin, G., Rustin, P., and Munnich, A. (1997). Sequence and structure of the human *OXA1L* and its upstream elements. Biochem. Biophys. Acta *1361*, 6–10.

Saint-Georges, Y., Hamel, P., Lemaire, C., and Dujardin, G. (2001). Role of positively charged transmembrane segments in the insertion and assembly of mitochondrial inner-membrane proteins. Proc. Natl. Acad. Sci. USA *98*, 13814–13819.

Sambrook, K.J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Tekotte, H., and Davis, I. (2002). Intracellular mRNA localization: motors move messages. Trends Genet. *18*, 636–642.

Zuker, M. (1989). On finding all suboptimal foldings of an RNA molecule. Science *244*, 48–52.