# cis-acting sequences and trans-acting factors in the localization of mRNA for mitochondrial ribosomal proteins

Annapina Russo [a], Claudia Cirulli [b], Angela Amoresano [b], Pietro Pucci [b,c], Concetta Pietropaolo [a,*], Giulia Russo [a,*]

[a] Dipartimento di Biochimica e Biotecnologie Mediche, Università Federico II, Via Sergio Pansini 5, Napoli 80131, Italy
[b] Dipartimento di Chimica Organica e Biologica, Università Federico II, Complesso Universitario Monte S. Angelo, Via Cinthia 4, Napoli 80126, Italy
[c] CEINGE Biotecnologie Avanzate S.C.a.r.l., Via Comunale Margherita 482, Napoli 80145, Italy

## ARTICLE INFO

## ABSTRACT

mRNA localization is a conserved post-transcriptional process crucial for a variety of systems. Although several mechanisms have been identified, emerging evidence suggests that most transcripts reach the protein functional site by moving along cytoskeleton elements. We demonstrated previously that mRNA for mitochondrial ribosomal proteins are asymmetrically distributed in the cytoplasm, and that localization in the proximity of mitochondria is mediated by the 3′-UTR. Here we show by biochemical analysis that these mRNA transcripts are associated with the cytoskeleton through the microtubule network. Cytoskeleton association is functional for their intracellular localization near the mitochondrion, and the 3′-UTR is involved in this cytoskeleton-dependent localization. To identify the minimal elements required for localization, we generated DNA constructs containing, downstream from the GFP gene, deletion mutants of mitochondrial ribosomal protein S12 3′-UTR, and expressed them in HeLa cells. RT-PCR analysis showed that the localization signals responsible for mRNA localization are located in the first 154 nucleotides. RNA pull-down assays, mass spectrometry, and RNP immunoprecipitation assay experiments, demonstrated that mitochondrial ribosomal protein S12 3′-UTR interacts specifically with TRAP1 (tumor necrosis factor receptor-associated protein1), hnRNPM4 (heterogeneous nuclear ribonucleoprotein M4), Hsp70 and Hsp60 (heat shock proteins 70 and 60), and α-tubulin in vitro and in vivo.

© 2008 Elsevier B.V. All rights reserved.

## 1. Introduction

Localization of mRNA to subcellular regions is a widespread mechanism of post-transcriptional control of gene expression that allows local protein synthesis to occur at a specific time and place in the cell. Maternal mRNA localization plays a crucial role in differentiation and development, in many organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus laevis*. In somatic cells, the asymmetrical distribution of mRNAs in the cytoplasm may facilitate protein import into organelles [1]. Several mechanisms for mRNA localization have been identified. However, emerging evidence suggests that most transcripts reach the protein functional site by moving along cytoskeleton elements [2,3]. In most polarized cell types, including neurons, oligodendrocytes, and astrocytes, mRNAs are transported on microtubules [4–6]. A well-studied example of actin-dependent mRNA localization is yeast where a myosin motor (Myo4p) is involved in the localization of the ASH1 mRNA [7]. Preferential use of a particular cytoskeleton element seems to be characteristic of a specific cell type. In fibroblasts, β-actin mRNA moves along a microtubule network [4], whereas in neurons the same transcript is transported on actin [8]. In higher eukaryotes, a transcript controls its sorting through a specific subset of cytoskeletal tracks [2].

A common theme shared by most localized mRNAs is that sequences within the transcript that address it to the specific cell destination lie in the 3′-UTR. The localization elements of a transcript are also known as "zip codes". They range in length from a few nucleotides to over 1 kb and represent the binding site for specific proteins that mediate the correct localization of mRNA in the cytoplasm. Although some mRNAs contain localization information in a single zip code, the correct subcellular distribution of most transcripts depends on multiple sub-elements [9]. In *Drosophila*, there are examples of transcripts in which different localization signals mediate distinct steps of the localization event. The 3′-UTR of oskar mRNA contains three different regions each of which takes part in a specific localization step [10].

Zip codes function through *trans*-acting factors that specifically bind to them. Recently, several *trans*-acting RNA-binding proteins required for mRNA localization have been identified. These include zip code-binding proteins, scaffolding proteins and motor proteins [11]. In chicken fibroblasts, ZBP1 and ZBP2 are two zip code-binding proteins that recognize and bind to β-actin zip code thereby helping to localize efficiently β-actin mRNA in the cytoplasm [12].

In yeast, several mitochondrial mRNAs, which are nuclear genome-encoded, are localized at the proximity of the organelle; their correct distribution in the cytoplasm depends on localization elements present in their 3′-UTRs [13,14] and seems to be crucial for the function of the organelle. For example, ATP2 mRNA localizes on mitochondrion-bound polysomes and its localization is essential for respiratory function [15]. This mRNA sorting process appears to be conserved from yeast to human cells [16].

We recently demonstrated that cytosolic and mitochondrial ribosomal proteins (r-proteins) are a class of localized transcripts in mammalian cells [17]. They are located near the nucleus and the mitochondrion, respectively, and their specific subcellular distribution depends on localization signals contained in the 3′-UTR region. Moreover, cytosolic r-protein mRNAs (rp-mRNAs) are transported and/or anchored to the cytoskeleton via actin microfilaments [17].

The aims of our study were to investigate the role of the cytoskeleton in the localization of mitochondrial rp-mRNAs; to identify the *cis*-acting regions of mitochondrial rp-mRNA 3′-UTR that contain the minimal localization signal essential to direct the transcripts to the mitochondrion; to isolate *trans*-acting factors that mediate rp-mRNA sorting by binding to them either directly or indirectly. Our data demonstrate that mitochondrial rp-mRNAs are associated to the cytoskeleton through the microtubule network; the localization signals in *cis* responsible for mt-rpS12 mRNA localization are located in the first 154 nucleotides of its 3′-UTR; and TRAP1 (tumor necrosis factor receptor-associated protein1), hnRNPM4, Hsp70, Hsp60 (heat shock proteins 70 and 60) and α-tubulin are four *trans*-acting factors that, in vitro and in vivo, bind to the mt-rpS12 3′-UTR and thus are probably involved in the localization process.

## 2. Materials and methods

### 2.1. Cell cultures and drug treatments

To study the association of mitochondrial rp-mRNAs with cytoskeleton, we grew HeLa cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, treated them for 20 min with compounds that disrupt the integrity of distinct components of the cytoskeleton, and then harvested the subcellular fractions. Specifically, cells were treated with 40 μM nocodazole (Calbiochem, Darmstadt, Germany) to depolymerize the microtubule network or 5 μM latrunculin A (Calbiochem) to disrupt actin filaments. To verify that the drugs resulted in the depolymerization of microfilaments or microtubules, cells were processed for western experiments using antibodies against β-actin (Sigma, St. Louis, MO) or β-tubulin (Amersham, Buckinghamshire, UK).

### 2.2. Cellular fractionation

To analyze the subcellular distribution of mitochondrial rp-mRNAs, HeLa cells were fractionated to obtain S and Ck fractions. Briefly, cells from a 35-mm plate ($2 \times 10^6$ cells) were washed with ice-cold PBS 1× and treated with 1 ml of ice-cold extraction buffer (10 mM Pipes, pH 6.8, 100 mM KCl, 2.5 mM MgCl$_2$, and 0.1% Triton X-100) at 4 °C for 1 min. The material obtained under these conditions is referred to as the "S fraction". Subsequently, the cell remnants were scraped in 1 ml of ice-cold cytoskeleton buffer (20 mM HEPES, pH 7.5, 0.5 M NaCl, 30 mM Mg-acetate, 0.5% deoxycholate, and 1% Tween-20) and left for 5 min on ice. This suspension was passed through a low-gauge needle

and centrifuged for 5 min at 4 °C at 10,000 ×g. The recovered supernatant is referred to as the "Ck fraction". Lactate dehydrogenase (LDH) activity was measured in these fractions by using a commercially available kit (Sigma-Aldrich) according to the manufacturer's instructions (Table 1a). To purify mitochondria, subcellular fractionation of cells was carried out using a standard protocol for mitochondrial isolation, slightly modified [18]. Essentially, cells from four 100-mm plates ($8 \times 10^7$ cells) were washed once with ice-cold PBS 1×; harvested in 1.3 ml of ice-cold PBS 1× and centrifuged at 250 ×g for 10 min at 4 °C. The cell pellet was resuspended by gentle pipetting in 10 volumes of ice-cold 0.133 M NaCl, 5 mM KCl, 0. 7 mM Na$_2$HPO$_4$, 25 mM Tris–HCl, pH 7.5, and centrifuged again at 250 ×g for 10 min at 4 °C. The pellet was resuspended by pipetting up and down in 500 μl of ice-cold 10 mM NaCl, 1. 5 mM CaCl$_2$, and 10 mM Tris–HCl, pH 7.5, and kept on ice for 15 min. An equal volume of ice-cold 0.7 M sucrose, 0.21 M mannitol, 2 mM EDTA, and 20 mM Tris–HCl, pH 7.5, was added, and nuclei and cell debris were removed by two sequential centrifugations at 600×g for 30 min at 4 °C to separate "raw" cytosolic (supernatant) and mitochondrial (pellet) fractions. The mitochondria were washed once with 1 ml of ice-cold 0.1 M NaCl, 50 mM Tris–HCl, pH 8.5, and 10 mM EDTA, and resuspended in a final volume of 300 μl of the same buffer immediately prior to nucleic acid isolation. The activity of LDH was measured in these fractions as described above (Table 1b).

### 2.3. Immunoblot analysis

Proteins were extracted from S and Ck fractions. Aliquots of samples (30 μg) were resolved by 12% SDS gel electrophoresis and transferred into nitrocellulose filters. The membranes were blocked in PBS 1×, 0.2% Tween and 5% dry milk for 2 h, and then actin (Sigma) and tubulin (Amersham) were revealed by specific antibodies and peroxidase-conjugated antibodies (Amersham). An enhanced chemiluminescence (ECL) system (Amersham) was used for detection.

### 2.4. Generation of mitochondrial rpS12 3′-UTR reporter constructs

The constructs used are shown in Fig. 4A. All mt-rpS12 constructs were generated by PCR using specific primers (Table 2). The 3′-UTR fragments obtained were subsequently inserted downstream from the

**Table 1**
LDH activity in subcellular fractions

| a | | |
| --- | --- | --- |
| | Soluble | Cytoskeletal |
| Control | 96.5 ± 0.2 | 3.5 ± 0.4 |
| Lat A | 95.8 ± 1.0 | 4.2 ± 0.4 |
| Noc | 96.8 ± 0.7 | 3.2 ± 0.7 |
| GFP | 96.5 ± 0.4 | 3.5 ± 0.6 |
| GFP/mt-rpS12 | 95.7 ± 0.3 | 2.3 ± 0.7 |
| | | |
| b | | |
| | Cytosol | Mitochondrial |
| Control | 95.6 ± 0.3 | 4.4 ± 0.5 |
| Noc | 96.2 ± 0.8 | 3.8 ± 0.2 |
| GFP | 97.3 ± 0.5 | 2.7 ± 0.3 |
| GFP/mt-rpS12 | 97.5 ± 0.5 | 2.5 ± 0.5 |
| GFP/mt-rpS12Δ1 | 95.8 ± 1.0 | 4.2 ± 0.8 |
| GFP/mt-rpS12Δ2 | 96.3 ± 0.7 | 3.7 ± 0.5 |
| GFP/mt-rpS12Δ3 | 98.2 ± 0.6 | 1.8 ± 0.5 |
| GFP/mt-rpS12Δ4 | 96.8 ± 0.4 | 3.2 ± 0.2 |

Aliquots from each fraction were assayed for LDH activity. As expected, the activity of LDH resulted within the soluble fraction (a), and the cytosolic fraction (b) preferentially. Data are expressed as a percent of the total activity. The different experiments performed in this study are indicated: HeLa cells untreated (control), cells treated with Latrunculin A or Nocodazole, cells transfected with GFP vector (GFP), chimeric constructs containing the full mitochondrial rpS12 3′UTR (GFP/mt-rpS12) or deletions of it (GFP/mt-rpS12 or GFP/mt-rpS12Δ1, GFP/mt-rpS12Δ2, GFP/mt-rpS12Δ3, GFP/mt-rpS12Δ4).

reporter gene GFP in the pEGFP-C1 expression vector (Clontech, Mountain View, California) using the HindIII and BamHI cloning sites. All fusion plasmids, prepared using QIAGEN kits (Hilden, Germany), were sequenced to verify the accuracy of the constructs.

## 2.5. Cell transfection

Transfection was carried out using LipofectAMINE™2000 (Invitrogen, Carlsbad, California). The cells were grown at 80–90% confluency in 35-mm plates or 100 mm plates and were then covered with a mixture of specific DNA and LipofectAMINE, as indicated by the manufacturer. 24 h or 48 h after transfection total RNA extracted from the cells was analyzed by semi-quantitative RT-PCR.

## 2.6. RNA extraction and semi-quantitative RT-PCR

All biochemical fractions were treated with 200 μg/ml proteinase K (Roche, Mannheim, Germany) in 0.5% SDS for 30 min at 37 °C and total RNA was purified by repeated phenol/chloroform (v/v 1:1) extraction, and ethanol precipitated. The distribution of mitochondrial rp-mRNAs among the different subcellular fractions was determined by RT-PCR. After DNAse treatment, 1 μg of total RNA was reverse-transcribed into cDNA by the random hexamers technique using 200 U of Superscript II RNAse H−Reverse Transcriptase (Invitrogen). The reaction was carried out at 42 °C for 50 min and heated to 75 °C for 15 min to terminate it. Ten microliters of the 40 μl reaction mixture were PCR-amplified in a final volume of 50 μl, using 5 μM specific primer (Table 2), 10 mM dNTPs, and 0.5 U Taq DNA polymerase (Invitrogen). Typically, 25–30 cycles of amplification were performed. In separate experiments, we ascertained that the cycle number was within the linear range of amplification. PCR products were visualized on 1% agarose gels containing the fluorescent Vistra green dye (Amersham Pharmacia Biotech) [19]. The labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the PhosphorImager STORM 840 system (Amersham).

## 2.7. RNA pull-down assay and mass spectrometry

In order to identify proteins binding specifically to mitochondrial rpS12 3′-UTR, we used RNA pull-down assay and adipic acid dehydrazide beads. Briefly, 20 μg of mt-rpS12 mRNA were placed in a 400 μl reaction mixture containing 100 mM NaOAc, pH 5.2, and 5 mM sodium *m*-periodate (Sigma), incubated for 1 h in the dark at room temperature, ethanol precipitated, and resuspended in 100 μl of 100 mM NaOAc, pH 5.2. Then 300 μl of adipic acid dehydrazide agarose beads 50% slurry (Sigma) equilibrated in 100 mM NaOAc, pH 5.2, was added to this mixture and incubated for 12 h at 4 °C on a rotator. The beads with the bound RNA were pelleted, washed twice with 1 ml of 2 M NaCl, and equilibrated in washing buffer (5 mM HEPES, pH 7.9, 1 mM MgCl₂, 0.8 mM magnesium acetate). The mt-rpS12 3′-UTR was then incubated with 7 mg of HeLa protein extract for 30 min at room temperature in 0.6 ml final volume. Heparin was also added to a final concentration of 7 μg/μl. The beads were then washed four times in 1.5 ml of washing buffer. The bound proteins were eluted in SDS sample buffer and loaded on 12.5% SDS-PAGE. Gels were stained with colloidal blue staining kit (Invitrogen Life Technologies) and protein bands were excised from the gel and destained. The samples were reduced and carboxyamidomethylated with 10 mM DTT and 55 mM iodoacetamide in 0. 1 M NH₄HCO₃ buffer, pH 7.5. Tryptic digestion of the alkylated samples was performed by adding two aliquots of enzyme for 4 hr and overnight respectively.

MALDI-MS experiments were performed on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). Typically, 1 μl of the total peptide mixture was mixed (1/1, v/v) with a 10 mg /ml solution of R-cyano-4-hydroxycinnamic acid in acetonitrile/50 mM citrate buffer (2/3, v/v).

NanoLC-MS2 experiments were carried out with a 4000 QTrap mass spectrometer (Applied Biosystems) coupled to an 1100 nanoHPLC system (Agilent Technologies, Buckinghamshire, UK). Peptides were separated on an Agilent reversed-phase column (Zorbax 300 SBC18, 150 mm), at a flow rate of 0.2 μl/min using 0.1% formic acid, 2% ACN in water as solvent A 0.1% and formic acid, 2% water in acetonitrile as solvent B. Elution was carried out with a 5–65% linear gradient of solvent B in 60 min. A micro-ionspray source was used at 2.5 kV with liquid coupling, with a declustering potential of 50 V, using an uncoated silica tip from New Objectives (Ringoes, NJ).

Peptide mass values recorded in the MALDI spectra and fragment ions recorded in ESIMSMS spectra were used for database search using the Mascot software.

Pulled-down proteins were analyzed by western blotting with anti-TRAP1, anti-hnRNPM4, anti-Hsp70, anti-Hsp60, anti-α-tubulin (Calbiochem) and anti-SH-PTP1 (SH-protein-tyrosine phosphatase; Santa Cruz Biotechnology, Santa Cruz, California).

## 2.8. RIPA (RNP Immunoprecipitation Assay)

HeLa cells ($2 \times 10^7$) were lysed in 600 μl of RIPA buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM Na orthovanadate, 0.05 M NaF, 0.5% NP40) with protease inhibitors (Roche) for 60 min on ice and then centrifuged at 11,000 g at 4 °C for 15 min. The supernatant was precleared by incubating with 50 μl of protein A/G plus agarose for 1 h at 4 °C. The precleared cell extracts were then incubated with specific antibodies (Santa Cruz) overnight at 4 °C. Protein G-Sepharose beads (50 μl of 50% slurry) were then added for 1 h at 4 °C with gentle shaking and centrifuged. The immunoprecipitates were suspended in 100 μl TES buffer (10 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 % SDS), incubated at 65 °C for 10 min, and centrifuged for 1 min at 11,000 g. Ten microliters of the supernatant were stored as immunoprecipitated samples and subsequently fractionated by SDS-PAGE. RNA was extracted from 90 μl using Trizol procedure.

# 3. Results

## 3.1. Asymmetric distribution of mitochondrial rp-mRNAs in the soluble versus cytoskeletal cell fractions

The spatial distribution of mRNAs in the cytoplasm occurs through transport to and anchoring at specific subcellular regions where the corresponding proteins function. The cytoskeleton is widely used, in a variety of systems, to transport mRNAs from their transcription site in the nucleus to their translational and degradation sites in the cytoplasm [20].

In humans, we previously reported that mRNAs encoding cytosolic and mitochondrial r-proteins constitute a class of localized transcripts. Specifically, we demonstrated that transcripts encoding cytosolic r-proteins are transported and/or anchored to the perinuclear region and that transcripts encoding mitochondrial r-proteins accumulate near the mitochondrion. We also demonstrated that correct localization of mRNAs of cytosolic r-proteins in the cytoplasm is mediated by specific interaction of these transcripts with actin microfilaments [17]. Consequently, we asked whether the accumulation of mRNA for mitochondrial r-proteins at the proximity of the organelle was also mediated by the cytoskeleton network. To this aim, we isolated biochemically from HeLa cells two distinct fractions corresponding to a detergent-soluble fraction (S) containing cytosolic components, and a detergent-insoluble fraction (Ck), enriched in cytoskeleton elements. These two fractions were assayed for LDH activity, which is a marker of the soluble fraction. More than 90% of LDH activity occurred in the S fraction (Table 1a); thus the Ck fraction was free of S fraction contaminants. Total RNA extracted from each fraction was analyzed by semi-quantitative RT-PCR using specific oligonucleotides against mRNA for human mitochondrial r-proteins S2, S12, L3, L13 (mt-rpS2,

mt-rpS12, mt-rpL3, mt-rpL13). The mRNA for cytosolic r-protein L4, cy-rpL4, was used as a control of cytoskeleton-associated transcript and, as expected, this mRNA was recovered preferentially in the insoluble fraction Ck (Fig. 1A, B). In fact, all the tested transcripts accumulated in the Ck fraction (Fig. 1A); the quantitative analysis revealed that about 75% of these transcripts were associated with the cytoskeleton (Fig. 1B). B-tubulin and actin were associated with the Ck fraction (Fig. 1C) confirming the integrity of cytoskeleton components in the fraction preparation.

### 3.2. Mitochondrial rp-mRNAs associate with microtubules via the 3′-UTR region

Directional mRNA transport requires an intact cytoskeleton [21]. To identify the class of cytoskeleton elements involved in the asymmetric distribution of mitochondrial rp-mRNAs in the cytoplasm, we used a pharmacological approach, previously used successfully [17] to examine the effects of disruption of different cytoskeleton elements on the localization of various rp-mRNAs. Specifically, we treated HeLa cells with latrunculin A or nocodazole to induce the depolymerization of actin microfilaments or microtubules, respectively. After the treatment, cells were fractionated to obtain the S and Ck fractions. RT-PCR analysis of total RNA, extracted from each fraction, shows that only the selective disruption of the microtubule network caused by nocodazole was effective in delocalizing the transcripts (Fig. 2A). In fact, 80% of the signal was found in the S fraction following nocodazole treatment (Fig. 2B). Disruption of actin filaments did not cause changes in the subcellular distribution of mitochondrial rp-mRNAs (Fig. 2A, B). Thus, these results indicate that mRNAs of mitochondrial r-proteins cross the cell and/or anchor in the vicinity of the organelle by moving along the microtubule network. The finding that cytosolic and mitochondrial rp-mRNAs use two different classes of cytoskeleton elements, actin or microtubules, to localize in two different subcellular compartments, the perinuclear region or mitochondria surface, respectively, suggests that different protein motors are involved in mRNA transport and anchoring of these two subclasses of r-proteins.

Because the mt-rpS12 3′-UTR can localize a reporter transcript to the proximity of mitochondria [17], we asked whether this region was also responsible for the association with the cytoskeleton. To address this issue, we transfected the GFP/mt-rpS12 construct (see also Fig. 4), which contains the full 3′-UTR of mt-rpS12 transcript downstream from the GFP gene, in HeLa cells, untreated or nocodazole-treated. Cells were fractionated to separate the S and Ck fractions, and cytosolic (Cy) and mitochondrial (Mt) fractions, then the total RNA was extracted from each fraction and analyzed by semi-quantitative RT-PCR. Fig. 3A shows that the chimeric transcript was mostly (about 75%) associated to the Ck fraction in the absence of nocodazole. The addition of the drug resulted in a dramatic change in the localization of the mRNA. In fact, nocodazole induced the release of the GFP/mt-rpS12 transcript from the Ck fraction and its accumulation in the S fraction (Fig. 3A). Moreover, when mitochondria were separated from the cytosolic fraction (Fig. 3B), the GFP/mt-rpS12 mRNA was associated with mitochondria in the absence of nocodazole, whereas nocodazole caused its release into the cytosolic fraction. Thus, the data in Fig. 3 indicate that the microtubule network could be both the motor by which the transcript is transported and the anchor to keep it near mitochondria.

### 3.3. Domains necessary for the subcellular distribution of mt-rpS12 mRNA

To identify signals responsible for the localization of mitochondrial rp-mRNA, we performed a deletion analysis of the mt-rpS12 3′-UTR. Using specific oligonucleotides (Table 2) and PCR, we generated reporter constructs containing deletion mutants of mt-rpS12 3′-UTR downstream from the GFP gene (Fig. 4A). HeLa cells were transiently transfected; 48 h after transfection, the cells were biochemically fractionated to obtain the Cy and the Mt fractions. Total RNA, extracted from each fraction, was analyzed by semi-quantitative RT-PCR. The subcellular distribution of chimeric transcripts from constructs GFP/mt-rpS12Δ1 and GFP/mt-rpS12Δ2, corresponding to 756–910 nt and 911–1075 nt, respectively, was analyzed. The results of the
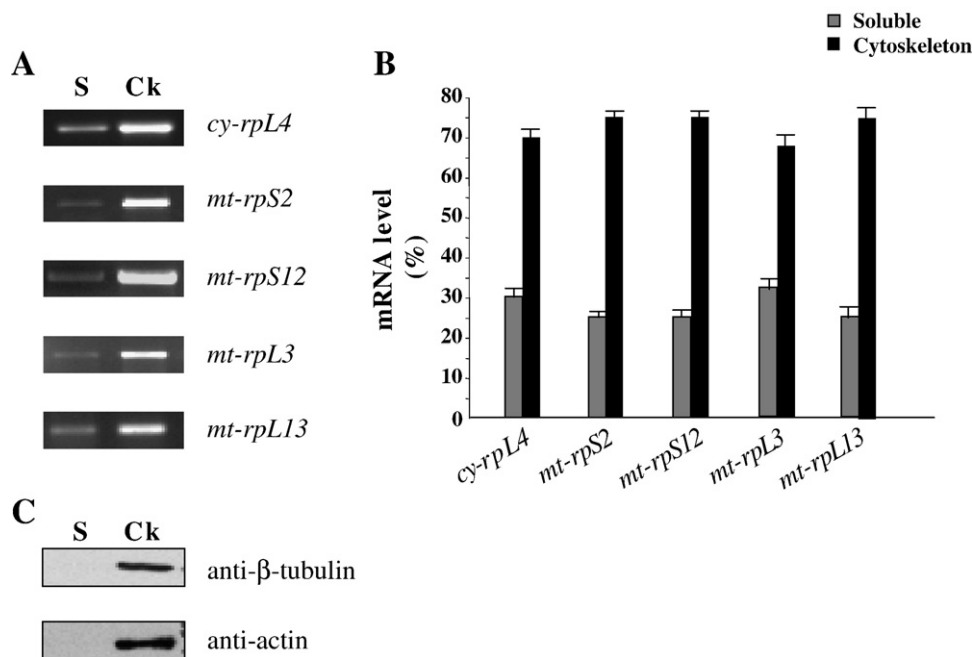


**Fig. 1.** Mitochondrial rp-mRNAs are asymmetrically distributed between cytosol and cytoskeleton. (A) HeLa cells were biochemically fractionated to separate the soluble (S) and cytoskeletal (Ck) fractions as described under "Materials and methods". Total RNA extracted from each fraction was subjected to semi-quantitative RT-PCR using probes for the indicated mitochondrial r-proteins (see Table 2). Cytosolic rpL4 mRNA was used as a typical cytoskeletal-associated transcript. (B) The signals were quantified with the PhosphorImager STORM 840 system (Amersham). For a given mRNA, the addition of both signals was considered 100% and the RNA level in each fraction was expressed as a percent of the total. The results shown represent the mean ± s.d. of a minimum of three independent experiments. (C) Western blotting analysis of actin and tubulin in the fractions.
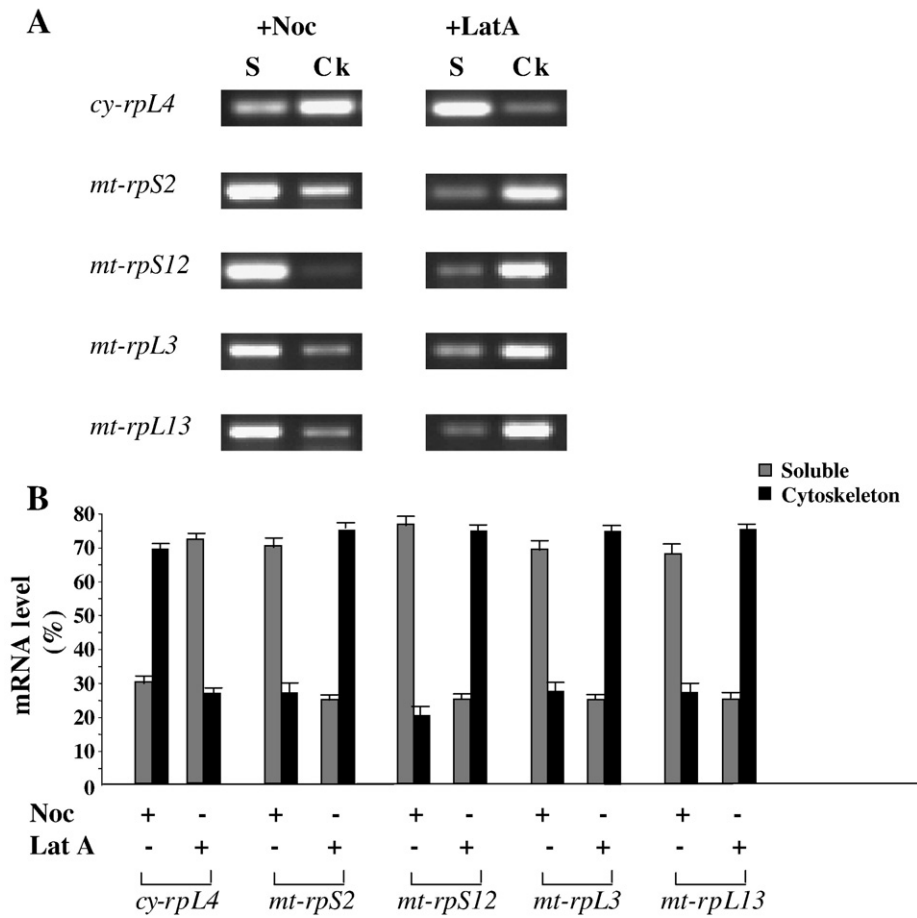
**Fig. 2.** Mitochondrial rp-mRNAs are specifically associated with cytoskeleton via microtubules. (A) HeLa cells treated with latrunculin A (Lat A) or nocodazole (Noc) were biochemically fractionated and total RNA was subjected to semi-quantitative RT-PCR as described in the legend to Fig. 1. Cytosolic rpL4 mRNA was used as a typical cytoskeletal actin-associated transcript. (B) The signals in each fraction were quantified as described in the legend to Fig. 1. The results shown represent the mean±s.d. of a minimum of three independent experiments.

experiments are shown in Fig. 4B, C. The GFP/mt-rpS12Δ1 transcript was associated to the Mt fraction (Fig. 4B). Quantification of signals indicated that this region contains the information sufficient to address the reporter gene to the vicinity of mitochondria. Instead, the GFP/mt-rpS12Δ2 transcript accumulated prevalently in the Cy fraction, suggesting that the region corresponding to 911–1075 nt does not contain any signal able to locate a reporter transcript to the proximity of the organelle (Fig. 4B). To further characterize the *cis*-regulatory element within the nucleotide positions 756–910 in the 3′-UTR, we generated two additional constructs, GFP/mt-rpS12Δ3 and GFP/mt-rpS12Δ4, containing 3′-UTR sequences corresponding to nucleotides 756–850 and 851–910, respectively. The subcellular distribution of the corresponding transcripts between Cy and Mt fractions showed that both directed about 70% of the GFP transcript to the Mt fraction, i.e., to the correct subcellular localization of the chimeric mRNA (Fig. 4B). These data led us to conclude that at least two different localization signals are present in the region defined by nucleotides 756–910 of mt-rpS12 3′-UTR, and each one is sufficient for the correct localization of the chimeric transcript.

### 3.4. Identification of proteins interacting with the mitochondrial rpS12 3′-UTR

It has been proposed that mRNAs are recognized by several proteins taking part in the different steps of the localization process [22]. We performed RNA affinity experiments in vitro to identify proteins involved, in trans, in various steps of the process leading to

the association of mRNAs of mitochondrial r-proteins to mitochondria, namely, recognition of the localization sequences, transport along cytoskeleton elements, and anchoring at the protein functional site. The mt-rpS12 3′-UTR was used as bait to evaluate its ability to bind specific proteins. The beads coated with mt-rpS12 3′-UTR were incubated with whole protein extract from HeLa cells. After stringent washing, RNA-associated proteins were eluted and analyzed by SDS-PAGE. Several specific bands (ranging from 42 kDa to 75 kDa) were associated with the mt-rpS12 3′-UTR beads but not with control beads (Fig. 5A, panel Coomassie, bands 1–5). The bands were excised from the gel, reduced, alkylated and digested in situ with trypsin. The resulting peptide mixtures were directly analyzed by MALDI/MS according to the peptide mass fingerprinting procedure. Peaks detected in the MALDI spectra were used to search for a non-redundant sequence database using the in-house MASCOT software, and exploiting the specificity of trypsin and the taxonomic category of the samples. The set of experimental mass values was compared to the theoretically predicted peptides from the proteins in the explored database. The number of measured masses that matched within the given mass accuracy of 200 ppm was recorded and the proteins that had the highest number of peptide matches were examined. The probability score of the second best candidate identified by the program was several orders of magnitude lower. Additional data were obtained in nanoLC/MS/MS experiments. Several spots could not reliably be identified by peptide mass fingerprinting. Thus, the peptide mixtures were fractionated by nanoHPLC and sequence information was obtained by tandem mass spectrometry on a 4000 QTrap linear
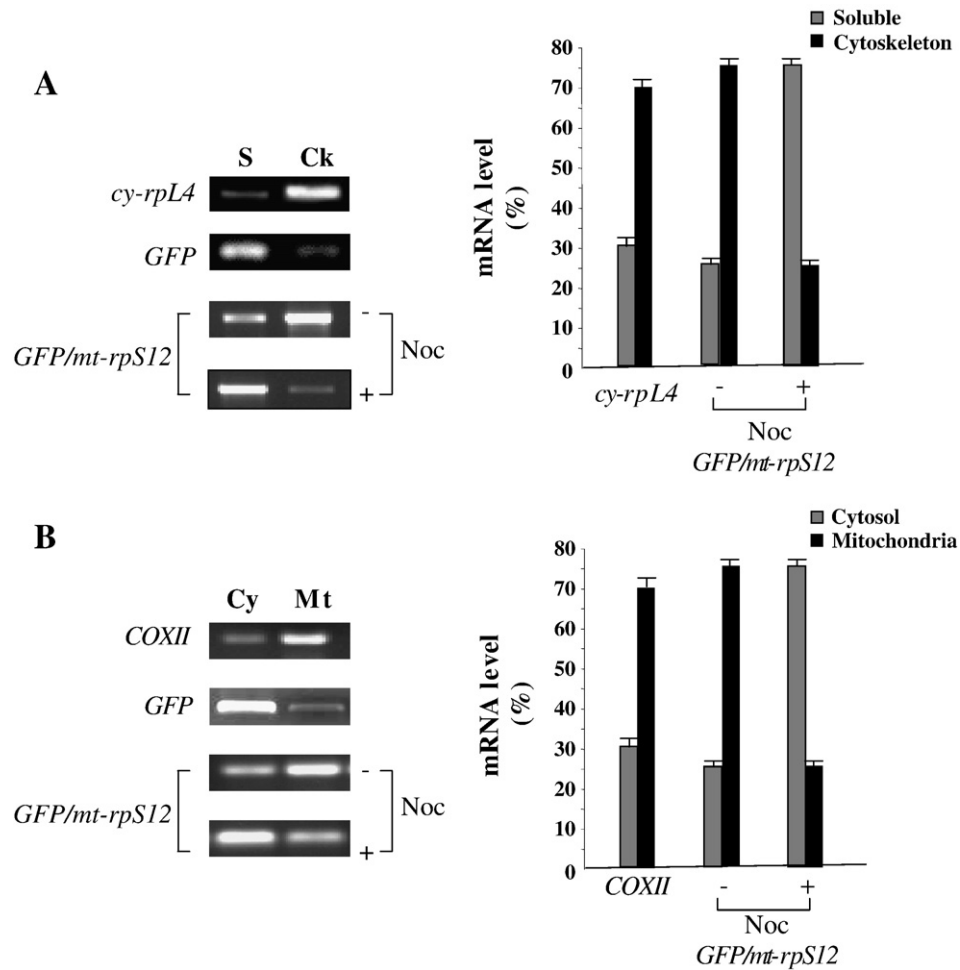
**Fig. 3.** The 3′-UTR of mt-rpS12 is required for the association to the cytoskeleton. HeLa cells, untreated or Noc treated, were transfected with GFP/mt-rpS12 DNA (see also Fig. 4), biochemically fractionated and total RNA was subjected to semi-quantitative RT-PCR analysis as described in the legend to Fig. 1. (A) left panel, Cytosolic rpL4 mRNA and GFP mRNA were used as cytoskeletal (Ck) and soluble (S, Cy) markers, respectively. (B) left panel, Mitochondrial-encoded COXII was used as control of mitochondrial fraction (Mt). (A) and (B) right panel, The signals in each fraction were quantified as described in the legend to Fig. 1. The results shown represent the mean±s.d. of a minimum of three independent transfection experiments.

ion trap instrument so leading to the unambiguous identification of the candidate protein. Among the putative interactors identified with this integrated approach, here we report the identification of TRAP1, hnRNPM4, Hsp70 and Hsp60, and α-tubulin (Fig. 5A).

To verify mass spectral identifications, the SDS gel was probed with the antibodies against the identified *trans*-acting factors (Fig. 5A, Western panel). We carried out RIPA experiments to verify, in vivo, the existence of a specific interaction between mt-rpS12 mRNA and the identified proteins. We immunoprecipitated TRAP1, hnRNPM4, Hsp70,

Hsp60, α-tubulin, or SH-PTP1 from HeLa cell extract (Fig. 5B) and looked for the mt-rpS12 mRNA in the RNA–protein immunoprecipitate complexes using RT-PCR (Fig. 5C) and specific oligonucleotides against the 3′-UTR region (Table 2). Amplification of the signal corresponding to this mRNA in the samples indicated that mt-rpS12 mRNA is able to bind TRAP1, hnRNPM4, HSP70, HSP60, and α-tubulin in vivo and that these interactions are mediated by the 3′-UTR region. Moreover, the absence of signal in the immunoprecipitate with anti-SH-PTP1, a control antibody, confirmed the validity of this assay (Fig. 5C). These data are consistent with a model in which mt-rpS12 mRNA is transported on microtubules to reach the mitochondrion. However, although the binding appears specific, a detailed analysis of the binding site is required to establish whether there is a direct interaction between the mt-rpS12 mRNA and the identified putative protein partner or whether one or more adapter proteins contribute to the binding.

## 4. Discussion

The regulation of eukaryotic gene expression is a complex process that occurs at different levels, from pre-mRNA transcription and processing in the nucleus, export from the nucleus to a subcellular localization, mRNA translation and decay. At post-transcriptional level, a central role is increasingly attributed to mRNA localization since it enables local synthesis of proteins at a subcellular region close

**Table 2**
Oligonucleotides sequences

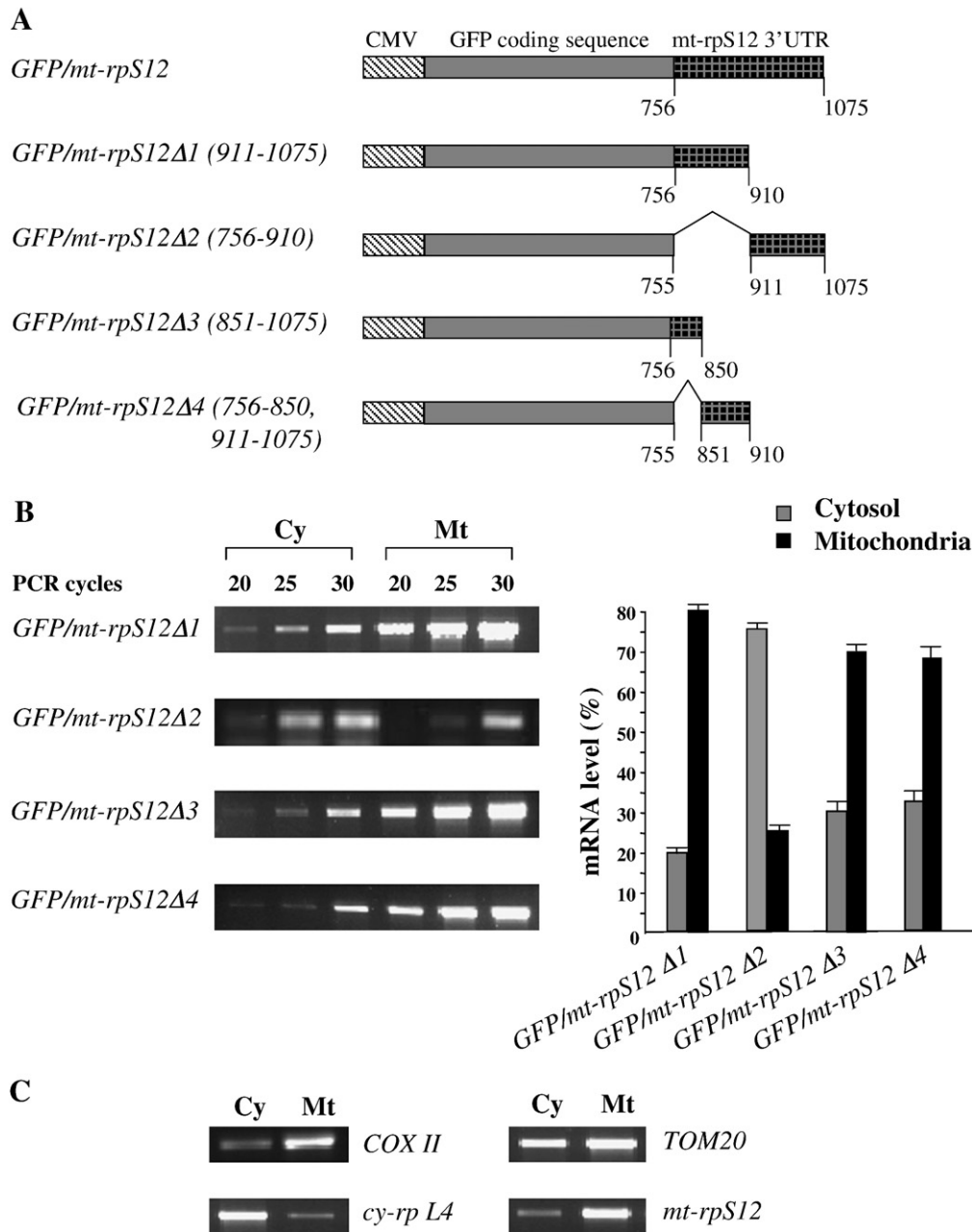|  | 5′-Primer (5′–3′) | 3′-Primer (3′–5′) |
| --- | --- | --- |
| β-actin | ggcaccaccttctaca | caggaggacaatgat |
| cy-rpL4 | atacgccatctgttctgc | tgtttggcttgtagtgc |
| mt-rpS12 3′UTR | ctcagaagaagtgacggt | cacagaagaagtgacggctg |
| mt-rpS2 | gcaggacgcttggaagcg | gagagcctcaacctgctg |
| mt-rpL3 | taacatctttggaggtggcaga | actgttccacaaccactatttc |
| mt-rpL13 | tcctgctagtagaggaat | tagccgataatcttcagg |
| GFP | taccggtcgccaccatgg | cttgtacagctcgtccat |
| COX II | ttattcctagaaccaggc | ggctctagaggggtaga |
| TOM20 | tggacagccacagcagttac | cccagagctgctcaactacc |
| GFP/mt-rpS12Δ1 | catcagaagaagtgacggctg | tagtggtcctgatggaa |
| GFP/mt-rpS12Δ2 | ttaagccataggagtcctg | acatcacaagcgccaggg |
| GFP/mt-rpS12Δ3 | catcagaagaagtgacggctg | ggcgcaaaggccagcatc |
| GFP/mt-rpS12Δ4 | tctagaggcagccactcatg | tagtggtcctgatggaa |

**Fig. 4.** Targeting domains within the mt-rpS12 3′-UTR. (A) Schematic representation of gene constructs producing deletion mutants of the mt-rpS12 3′-UTR. The coding region of GFP was linked to the entire mt-rpS12 3′-UTR or to its deletion mutant DNA. Relevant nucleotide positions are shown. (B) Left panel, example of Vistra Green-stained agarose gels of semi-quantitative RT-PCR of the deletion mutants obtained from cytosol (Cy) and mitochondrial (Mt) fractions. Right panel, mRNA levels were quantified with the PhosphorImager STORM 840 system (Amersham). (C) Cross-contamination of the fractions was checked by revealing the cytosolic rpL4 as an example of cytosolic protein, mitochondrial-encoded COXII and endogenous mt-rpS12 mRNAs as mitochondrial markers. The TOM20 transcript is distributed similarly in the fractions. The results shown represent the mean±s.d. of a minimum of three independent transfection experiments.

to or at the functional site of the protein. mRNA localization is a multistep process that needs distinct transport and anchoring components. Based on the data available, the first step occurs in the nucleus where specific *trans*-acting factors recognize localization elements of a transcript and give rise to ribonucleoprotein (RNP) particles. These RNP particles are storage complexes that contain multiple mRNA molecules under translational arrest and components of the translational machinery [23]. After export to the cytoplasm, the complexes undergo a highly dynamic maturation process during which some proteins shuttle back to the nucleus, whereas others stay associated with the mRNA. Then, in several cell types, most mature RNP particles are translocated along cytoskeleton filaments via interactions of *cis*-acting elements and motor proteins and/or

accessory *trans*-acting factors. Finally, the RNP particles are released from the motor protein and the transcript is anchored to the functional site of the protein, or in its proximity, in the cytoplasm where it can be actively translated [24]. In eukaryotic cells, microtubules are cytoskeleton components that play a crucial role in events such as cell motility and division, organelle transport and cell morphology and organization. The transport of mRNAs along microtubules has been described in such systems as *Drosophila* oocytes, yeast, neurons and fibroblasts [25–27]. Recent data indicate that mRNA distribution occurs in a microtubule-dependent manner also in cardiocytes [28].

The microtubule network serves as a directional track to guide mitochondria to discrete areas of the cell in response to a specific cell
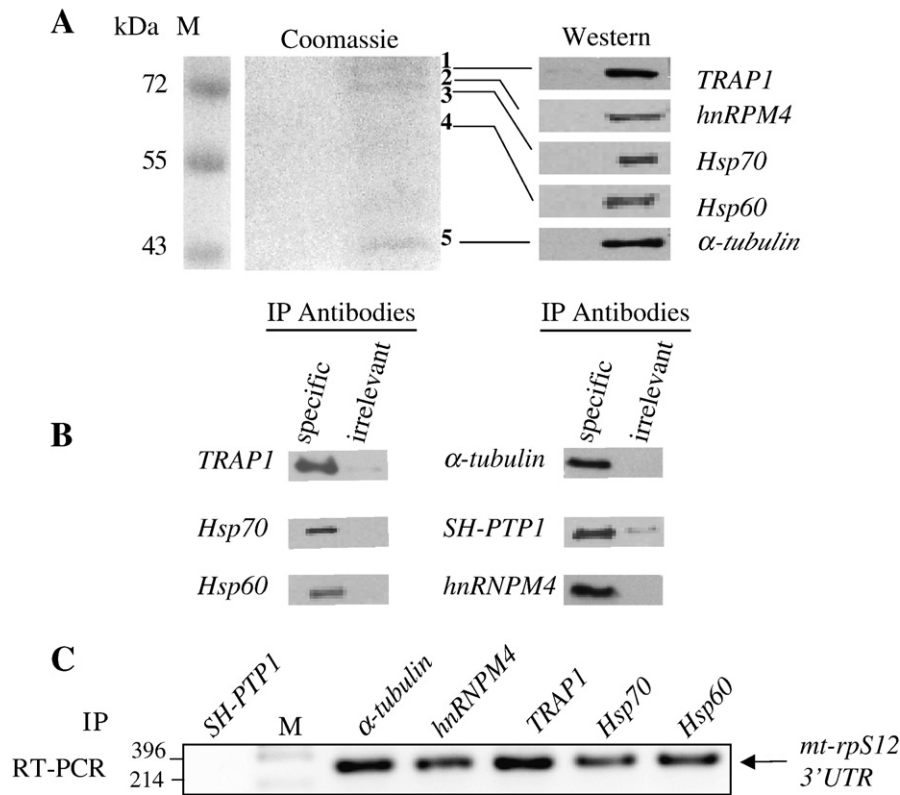
Fig. 5. The mt-rpS12 3′-UTR interacts in vitro and in vivo with TRAP1 (band 1), hnRNPM4 (band 2), Hsp70 (band 3), Hsp60 (band 4) and α-tubulin (band 5). (A, panel on the left) Coomassie Blue staining of RNA pull-down assay using adipic acid dehydrazide agarose beads coated with mt-rpS12 3′-UTR after incubation with HeLa protein extract (7 mg). (A, panel on the right) The nature of the bands, predicted by mass spectrometry, was confirmed by western blotting of the same RNA pull-down experiment by using specific antibodies. The mobility of protein size markers is shown (M). (B) Western blotting of anti-TRAP1, anti-hnRNPM4, anti HSP70, anti-HSP60, anti-α-tubulin or anti-SH-PTP1 immunoprecipitate from HeLa cells. The control of nonspecific binding was performed with immunoprecipitation reaction using normal IgG. (C) RT-PCR of total RNA extracted from the indicated immunoprecipitate using primers against mt-rpS12 3′-UTR (Table 2). Note the absence of signal in RNA–SH-PTP1 immunoprecipitate complex.

request. Microtubule remodeling affects mitochondrial distribution patterns and energy production in the cell. Most nuclear mRNAs that code for mitochondrial proteins seem to be translated on polysomes that are associated with the mitochondrial outer membrane. Studies in yeast showed that >100 nucleus-encoded transcripts of mitochondrial proteins are associated to polysomes on the mitochondrion surface [16]. Since we previously found that mRNAs encoding mitochondrial r-proteins localize to the proximity of mitochondria and that the cytoskeleton is involved in the perinuclear localization of the mRNA of cytosolic r-proteins [17], we asked whether the cytoskeleton could be involved in the transport of nucleus-encoded transcripts whose proteins are necessary for mitochondria activity. Our data demonstrate that, in HeLa cells, mRNAs for mitochondrial rpS2, rpS12, rpL3 and rpL13 are asymmetrically distributed (Fig. 1A), and 70% of each of these transcripts is associated with the Ck fraction (Fig. 1B). Next, we investigated whether a specific class of cytoskeleton elements was responsible for the long-range transport of transcripts, and a different class of cytoskeleton elements was responsible for local delivery of mt-rp-mRNAs; or, since mitochondria seem to use both microtubules and actin to travel within the cell [29], whether actin and the microtubule cytoskeleton interact to localize mitochondrial rp-mRNAs. We found that pharmacological disruption of the microtubule network substantially altered the distribution of these transcripts. In fact, after microtubule disruption, all tested mRNAs were redistributed from the Ck to the S fraction (Fig. 2A, B). The selective depolymerization of actin did not affect mitochondrial rp-mRNA localization. These results indicate that the two subclasses of ribosomal proteins mRNAs, cytosolic rp-mRNAs [17] and mitochondrial rp-mRNAs, use a common railway, the cytoskeleton, but two different tracks, namely, actin microfilaments to reach the perinuclear

region, and the microtubule network to localize near the mitochondrion. Moreover, the shift of signal from the Mt to the Cy fraction upon nocodazole treatment indicates that microtubules are involved not only in the transport step but also in the anchoring of the mitochondrial r-protein mRNAs to the mitochondrion surface (Fig. 3A, B).

The cis-acting signal responsible for mitochondrial rp-mRNA localization lies in the 3′-UTR region [17], which is the case of most localized mRNAs identified so far. One or more restricted segments of the 3′-UTR are required for localization, not the entire region [9]. The results of selective disruption of microtubules indicate that the mt-rpS12 3′-UTR can mediate the association of the reporter GFP mRNA to the cytoskeleton (Fig. 3A), and, furthermore, that the same region is responsible for the interaction between the chimeric transcript and the microtubules in the proximity of the mitochondrion (Fig. 3B). These findings suggest that microtubules play also an anchoring role. A specific population of microtubules surrounding the mitochondrion might be responsible for maintaining the specific localization of mitochondrial rp-mRNAs in the proximity of the organelle. In fact, different populations of microtubules, in Drosophila and in the wasp Nasonia, are responsible for the localization of specific mRNAs to different oocyte regions [30,31].

Analysis of deletion mutants produced to dissect the 3′-UTR in the reporter GFP/mt-rpS12 DNA (Fig. 4) showed that deletion of nucleotides 756–910 (GFP/mt-rpS12Δ2) resulted in a complete loss of the mRNA distribution pattern and the chimeric transcript accumulated in the Cy fraction, whereas deletion of the remaining 3′-UTR portion of GFP/mt-rpS12 mRNA did not affect its subcellular distribution (GFP/mt-rpS12Δ1 in Fig. 4). A more detailed analysis of the 154 nucleotides spanning a region potentially containing localization

signals revealed that the removal of either the sequence containing nucleotides 756–850 (GFP/mt-rpS12Δ3) or the sequence containing nucleotides 851–910 (GFP/mt-rpS12Δ4) did not cause loss of localization (Fig. 4). Thus, each mutant contained the information sufficient to target the GFP transcript to the organelle and the efficiency appeared only slightly lower than the efficiency of the whole region.

Since these regions do not contain any conserved sequences, our data are consistent with the hypothesis that the secondary structure may be relevant for the generation of a localization signal. A stem-and-loop structure in the 3′-UTR of ATP2 mRNA has been shown to be responsible for its localization in the vicinity of mitochondria [15]. Recent in-silico studies have predicted a structure that is common to most transcripts that have a high mitochondrial localization ratio (MLR) in yeast. This predicted consensus structure is short, has a high MLR and a high AU content, which is typical for untranslated regions of mRNA [32]. Furthermore, a 10 nt (CYTGTAAATA) sequence located in the 3′-UTR of 256 nuclear genes, whose products are targeted to mitochondria, has been shown to interact with the RNA-binding protein Puf3p, which is involved in the localization of the cognate mRNAs [33]. This sequence is not present in the mt-rpS12 3′-UTR.

The localization message in the 3′-UTR region of an mRNA is deciphered by specific RNA-binding proteins that associate with and/or are released from the transcript in a very dynamic manner during each step of the localization process, from the site of transcription in the nucleus to its final destination in the cytoplasm. A central issue in RNA localization is to identify the proteins involved in the process. We have isolated five proteins by an in vitro RNA affinity-based method in which mt-rpS12 3′-UTR was used as bait. Bound proteins were identified by mass spectrometry as human TRAP1, hnRNP M4, Hsp70, Hsp60 and α-tubulin. The specific interaction of these proteins with mt-rpS12 mRNA was confirmed in vivo by using RIPA experiments (see Fig. 5).

Mitochondria contain several chaperones that play an important role in mitochondrion function, namely, chaperones Hsp10, Hsp60, a form of Hsp70 (mitochondrial Hsp70/Ssq1), chaperones of the Clp/Hsp100 family [34], and TRAP1, also known as Hsp75 [35]. Hsp10, Hsp60 and Hsp100 function as general chaperones for protein folding [36], and Hsp70 plays an important role in protein import [37]. TRAP1 is a poorly characterized mitochondrial protein that shows strong sequence homology to the Hsp90 family. Despite the essential function in facilitating protein folding and import, knowledge about mitochondrial molecular chaperones is limited. There is evidence that these proteins can regulate a number of cellular processes, including cell survival and apoptosis [38,39]. TRAP1 has recently been implicated in protecting mitochondria against damage caused by reactive oxygen species [40,41]. PINK1, a protein kinase seems to phosphorylate TRAP1 to protect cells against apoptosis and promote cell survival [42]. Our study reveals a new function for Hsp70, Hsp60 and TRAP1. Indeed, their interaction with mt-rpS12 mRNA suggests that they are involved in mRNA localization. Data from *D. melanogaster* embryos showing that the molecular chaperone Hsp90 is required for mRNA localization support this hypothesis. It has been demonstrated that Hsp90 functions as a factor in trans in the correct spatial position of nanos and pgc mRNAs [43]. However, the mechanism by which the molecular chaperones act in this context remains to be elucidated.

HnRNP M4 belongs to the hnRNPM protein family. It appears as a cluster of four proteins (M1–M4) by two-dimensional electrophoresis and seems to play an important role in transcript-specific packaging, alternative splicing of pre-mRNAs and heat shock response [44,45]. Some shuttling hnRNPs are implicated in the localization of specific mRNAs in the cytoplasm. A case in point is hnRNPA2 that binds to the localization element in the 3′-UTR of MBP mRNA thereby determining its correct subcellular distribution [46]. These findings suggest that hnRNPM4 could play a role in the early steps of the localization process, e.g., mRNP export from the nucleus to the cytoplasm.

Another interesting putative *trans*-acting factor is α-tubulin. In fact, a proposed model hypothesizes that mitochondrial rp-mRNAs are transported along microtubules and anchored to the organelle via microtubules network. Compelling evidence indicates that tubulin can associate to the mitochondrial membrane and that it is associated with VDAC, the main outer membrane component of the permeability transition pore [47,48]. In the light of these observations, we propose that cellular tubulin might be used for the transport of the mt-rp-transcript from the nucleus to the vicinity of the mitochondrion, whereas mitochondrial tubulin might be used for anchoring by providing a solid structure with which to bring the mRNA in contact with regulatory factors as TRAP1.

Further studies are required to investigate the relevance of the secondary structure of the first 154 nucleotides of 3′-UTR mt-rpS12 in its subcellular localization; and the functional significance of the interaction between mt-rpS12 mRNA and TRAP1, hnRNPM4, Hsp70, Hsp60 or α-tubulin in the transport of mitochondrial rp-mRNAs from the nucleus to their correct localization near mitochondria.

## References

[1] S.M. Du T.G., R.P. Jansen, Why cells move messages: the biological functions of mRNA localization, Semin. Cell Dev. Biol. 18 (2007) 171–177.
[2] S.L. Bullock, Translocation of mRNAs by molecular motors: think complex? Semin. Cell Dev. Biol. 18 (2007) 194–201.
[3] I.M. Palacios, How does an mRNA find its way? Intracellular localisation of transcripts, Semin. Cell Dev. Biol. 18 (2007) 163–170.
[4] G.J. Bassell, Y. Oleynikov, R.H. Singer, The travels of mRNAs through all cells large and small, FASEB J. 13 (1999) 447–454.
[5] J.H. Carson, H. Cui, W. Krueger, B. Schlepchenko, C. Brumwell, E. Barbarese, RNA trafficking in oligodendrocytes, Results Probl. Cell Differ. 34 (2001) 69–81.
[6] S. Medrano, O. Steward, Differential mRNA localization in astroglial cells in culture, J. Comp. Neurol. 430 (2001) 56–71.
[7] H.A. Müller M, D. Niessing, Directional mRNA transport in eukaryotes: lessons from yeast, Cell Mol. Life Sci. 64 (2007) 171–180.
[8] G.J. Bassell, H.L. Zhang, A.L. Byrd, A.M. Femino, R.H. Singer, K.L. Taneja, L.M. Lifshitz, I.M. Herman, K.S. Kosik, Sorting of beta-actin mRNA and protein to neurites and growth cones in culture, J. Neurosci. 18 (1998) 251–265.
[9] D.J. Jambhekar A, *cis*-acting determinants of asymmetric, cytoplasmic RNA transport. RNA 13 (2007) 625–642.
[10] W.P. Kim-Ha J, J.L. Smith, P.M. Macdonald, Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. Development 119 (1993) 169–178.
[11] I.M. Palacios, D. St Johnston, Getting the message across: the intracellular localization of mRNAs in higher eukaryotes, Annu. Rev. Cell Dev. Biol. 17 (2001) 569–614.
[12] H.S. Pan F, R.H. Singer, W. Gu, ZBP2 facilitates binding of ZBP1 to beta-actin mRNA during transcription, Mol. Cell Biol. 27 (2007) 8340–8351.
[13] P. Marc, A. Margeot, F. Devaux, C. Blugeon, M. Corral-Debrinski, C. Jacq, Genome-wide analysis of mRNAs targeted to yeast mitochondria, EMBO Rep. 3 (2002) 159–164.
[14] M. Corral-Debrinski, mRNA specific subcellular localization represents a crucial step for fine-tuning of gene expression in mammalian cells, Biochim. Biophys. Acta 1773 (2007) 473–475.
[15] A. Margeot, C. Blugeon, J. Sylvestre, S. Vialette, C. Jacq, M. Corral-Debrinski, In Saccharomyces cerevisiae, ATP2 mRNA sorting to the vicinity of mitochondria is essential for respiratory function, EMBO J. 21 (2002) 6893–6904.
[16] J. Sylvestre, A. Margeot, C. Jacq, G. Dujardin, M. Corral-Debrinski, The role of the 3′ untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells, Mol. Biol. Cell 14 (2003) 3848–3856.
[17] A. Russo, G. Russo, M. Cuccurese, C. Garbi, C. Pietropaolo, The 3′-untranslated region directs ribosomal protein-encoding mRNAs to specific cytoplasmic regions, Biochim. Biophys. Acta 1763 (2006) 833–843.
[18] D.P. Tapper, R.A. Van Etten, D.A. Clayton, Isolation of mammalian mitochondrial DNA and RNA and cloning of the mitochondrial genome, Methods Enzymol. 97 (1983) 426–434.
[19] A.O. Gramolini, G. Belanger, B.J. Jasmin, Distinct regions in the 3′ untranslated region are responsible for targeting and stabilizing utrophin transcripts in skeletal muscle cells, J. Cell Biol. 154 (2001) 1173–1183.
[20] K. Czaplinski, R.H. Singer, Pathways for mRNA localization in the cytoplasm, Trends Biochem. Sci. 31 (2006) 687–693.

[21] M. Lopez de Heredia, R.P. Jansen, mRNA localization and the cytoskeleton, Curr. Opin. Cell Biol. 16 (2004) 80–85.

[22] R.P. Jansen, mRNA localization: message on the move, Nat. Rev., Mol. Cell Biol. 2 (2001) 247–256.

[23] A.M. Krichevsky, K.S. Kosik, Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation, Neuron 32 (2001) 683–696.

[24] J.E. Wilhelm, R.D. Vale, RNA on the move: the mRNA localization pathway, J. Cell Biol. 123 (1993) 269–274.

[25] R. Smith, Moving molecules: mRNA trafficking in mammalian oligodendrocytes and neurons, Neuroscientist 10 (2004) 495–500.

[26] W.M. Saxton, Microtubules, motors, and mRNA localization mechanisms: watching fluorescent messages move, Cell 107 (2001) 707–710.

[27] K.L. Farina, S. Huttelmaier, K. Musunuru, R. Darnell, R.H. Singer, Two ZBP1 KH domains facilitate beta-actin mRNA localization, granule formation and cytoskeletal attachment, J. Cell Biol. 160 (2003) 77–87.

[28] D. Scholz, P. McDermott, M. Garnovskaya, T.N. Gallien, S. Huettelmaier, C. DeRienzo, G.t. Cooper, Microtubule-associated protein-4 (MAP-4) inhibits microtubule-dependent distribution of mRNA in isolated neonatal cardiocytes, Cardiovasc. Res. 71 (2006) 506–516.

[29] R.L. Morris, P.J. Hollenbeck, Axonal transport of mitochondria along microtubules and F-actin in living vertebrate neurons, J. Cell Biol. 131 (1995) 1315–1326.

[30] D. St Johnston, Moving messages: the intracellular localization of mRNAs, Nat. Rev., Mol. Cell Biol. 6 (2005) 363–375.

[31] E.C. Olesnicky, C. Desplan, Distinct mechanisms for mRNA localization during embryonic axis specification in the wasp *Nasonia*, Dev. Biol. 306 (2007) 134–142.

[32] R.K. Ejsmont, P. Golik, P.P. Stepien, Prediction of the structure of the common perimitochondrial localization signal of nuclear transcripts in yeast, Acta Biochim. Pol. 54 (2007) 55–61.

[33] Y. Saint-Georges, M. Garcia, T. Delaveau, L. Jourdren, S. Le crom, S. Lemoine, V. Tanty, F. Devaux, C. Jacq, Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf3p in mRNA localization, PloS ONE 3 (2008) e2293.

[34] W. Voos, K. Rottgers, Molecular chaperones as essential mediators of mitochondrial biogenesis, Biochim. Biophys. Acta 1592 (2002) 51–62.

[35] J.D. Cechetto, R.S. Gupta, Immunoelectron microscopy provides evidence that tumor necrosis factor receptor-associated protein 1 (TRAP-1) is a mitochondrial protein which also localizes at specific extramitochondrial sites, Exp. Cell Res. 260 (2000) 30–39.

[36] B. Bukau, A.L. Horwich, The Hsp70 and Hsp60 chaperone machines, Cell 92 (1998) 351–366.

[37] F. Moro, C. Sirrenberg, H.C. Schneider, W. Neupert, M. Brunner, The TIM17.23 preprotein translocase of mitochondria: composition and function in protein transport into the matrix. EMBO J. 18 (1999) 3667–3675.

[38] H.M. Beere, Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways, J. Clin. Invest. 115 (2005) 2633–2639.

[39] P.J. Muchowski, J.L. Wacker, Modulation of neurodegeneration by molecular chaperones, Nat. Rev. Neurosci. 6 (2005) 11–22.

[40] C.N. Im, J.S. Lee, Y. Zheng, J.S. Seo, Iron chelation study in a normal human hepatocyte cell line suggests that tumor necrosis factor receptor-associated protein 1 (TRAP1) regulates production of reactive oxygen species, J. Cell. Biochem. 100 (2007) 474–486.

[41] G. Hua, Q. Zhang, Z. Fan, Heat shock protein 75 (TRAP1) antagonizes reactive oxygen species generation and protects cells from granzyme M-mediated apoptosis, J. Biol. Chem. 282 (2007) 20553–20560.

[42] J.W. Pridgeon, J.A. Olzmann, L.S. Chin, L. Li, PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1, PLoS Biol. 5 (2007) e172.

[43] Y. Song, L. Fee, T.H. Lee, R.P. Wharton, The molecular chaperone Hsp90 is required for mRNA localization in *Drosophila melanogaster* embryos, Genetics 176 (2007) 2213–2222.

[44] R. Gattoni, D. Mahe, P. Mahl, N. Fischer, M.G. Mattei, J. Stevenin, J.P. Fuchs, The human hnRNP-M proteins: structure and relation with early heat shock-induced splicing arrest and chromosome mapping, Nucleic Acids Res. 24 (1996) 2535–2542.

[45] K.V. Datar, G. Dreyfuss, M.S. Swanson, The human hnRNP M proteins: identification of a methionine/arginine-rich repeat motif in ribonucleoproteins, Nucleic Acids Res. 21 (1993) 439–446.

[46] T.P. Munro, R.J. Magee, G.J. Kidd, J.H. Carson, E. Barbarese, L.M. Smith, R. Smith, Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking, J. Biol. Chem. 274 (1999) 34389–34395.

[47] F. Bernier-Valentin, B. Rousset, Interaction of tubulin with rat liver mitochondria, J. Biol. Chem. 257 (1982) 7092–7099.

[48] M. Carre, N. Andre, G. Carles, H. Borghi, L. Brichese, C. Briand, D. Braguer, Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel, J. Biol. Chem. 277 (2002) 33664–33669.