Video Article

Isolation of mRNAs Associated with Yeast Mitochondria to Study Mechanisms of Localized Translation

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Abstract

Most of mitochondrial proteins are encoded in the nucleus and need to be imported into the organelle. Import may occur while the protein is synthesized near the mitochondria. Support for this possibility is derived from recent studies, in which many mRNAs encoding mitochondrial proteins were shown to be localized to the mitochondria vicinity. Together with earlier demonstrations of ribosomes' association with the outer membrane, these results suggest a localized translation process. Such localized translation may improve import efficiency, provide unique regulation sites and minimize cases of ectopic expression. Diverse methods have been used to characterize the factors and elements that mediate localized translation. Standard among these is subcellular fractionation by differential centrifugation. This protocol has the advantage of isolation of mRNAs, ribosomes and proteins in a single procedure. These can then be characterized by various molecular and biochemical methods. Furthermore, transcriptomics and proteomics methods can be applied to the resulting material, thereby allow genome-wide insights. The utilization of yeast as a model organism for such studies has the advantages of speed, costs and simplicity. Furthermore, the advanced genetic tools and available deletion strains facilitate verification of candidate factors.

Video Link

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Introduction

Eukaryotic cells are organized in distinct compartments having specific functions. To accomplish its function, each compartment contains a unique set of proteins that are essential for its activity. A possible mechanism through which these proteins approach their compartment is by localized translation[1,2]. In this process, the protein is synthesized at its destination by ribosomes and mRNAs that are located there. Among the probable advantages of localized translation are increased efficiency of protein targeting, decreased need for protein chaperones, and enabling site-specific regulation mechanisms. Also, localized mRNAs and ribosomes can be a secluded reservoir of translation machinery in cases of cellular stress, when general translation is inhibited.

Mitochondria became in recent years a central model to study localized translation. Most mitochondria proteins are encoded in the nucleus, translated in the cytosol, and imported into the organelle. Various lines of evidence indicate that many of these proteins are produced through a local translation process. Initially, electron microscopy and biochemical fractionation studies detected ribosomes associated with mitochondria[3-5]. These studies where then corroborated *in vivo* by work on specific mRNAs, that were found to be imported only in a cotranslational manner[6,7]. Genome-wide studies of mRNAs association with mitochondria revealed that a significant fraction of mRNAs are localized to the mitochondria vicinity[8-10]. Some of these mRNAs were further characterized by *in vivo* fluorescence methods, such as FISH or mTAG[9,11]. A straight-forward interpretation of this association is that these mRNAs serve as templates for localized translation.

The mechanisms by which these mRNAs approach the mitochondria are unknown. Noncoding domains (most significantly 3' UTRs) were shown to be involved in mRNA association to the mitochondria[12]. These domains are likely to serve as a binding site to RNA-binding proteins which mediate their transport. Studies in yeast revealed that a member of the PUM family of proteins (Puf3) supports mRNA association with mitochondria[8,13]. A plausible role for Puf3, which is based on functions of other family members, is to inhibit translation while the mRNA is *en route*[14]. Thus, mRNAs may be transported in a nontranslated status, by RNA binding proteins that interact with noncoding regions. Alternatively, a large body of work suggests that transport occurs while the protein is being synthesized. In particular, translation inhibitors were shown to affect mRNAs association[8,13]. Furthermore, translated features such as the AUG, mitochondrial targeting sequence (MTS) or ORF regions were shown to assist in localization[8,11,15]. Hsp70-family protein chaperone and protein receptor on the mitochondrial outer membrane were also shown to support mRNA association, further implying that encoded-protein features are important for mRNA localization[16]. This is consistent with a model in which the ribosome-emerging protein serves as recognition element for targeting mRNA-ribosome-protein complex to the mitochondria[17].

Localized translation near the mitochondria was studied by various methods, including electron microscopy (to visualize ribosomes)[3], FISH[9], green RNA (to detect specific mRNAs)[11], and biochemical fractionation (to detect both RNA and ribosomes)[10,18]. While the former methods detect localization *in vivo* and may allow visualization of transport dynamics, the latter allows detection of many different mRNAs in a single

experiment. Furthermore, for biochemical fractionation coding or noncoding domains do not need to be altered, therefore their specific roles can be evaluated. Biochemical fractionation has been successfully used for many years, for isolation of many different cellular compartments. Its principals and limitations are well established, and one can easily modify existing protocols for different purpose. The necessary instrumentation is standard in many labs, therefore it is usually the first method of choice for studying intracellular localization. We describe a protocol that was optimized for isolation of mRNAs while ribosomes are associated with mitochondria. This protocol is therefore optimal for studying factors involved in localized translation near mitochondria.

Protocol

1. Mitochondria Purification

Weigh the pellet of cells. Roughly 0.6 g are obtained from 100 ml cells.

1. Grow 100-150 ml of yeast cells to OD_{600} = 1-1.5 at 30 °C on a nonfermentable growth medium, such as galactose-based growth medium, in order to enrich for mitochondria.
2. Centrifuge cells at 3,000 x g for 5 min at room temperature and discard the supernatant.
3. Wash the pellet with double distilled water and centrifuge again. Discard the supernatant.
4. Resuspend the pellet in 1 ml DTT Buffer per 0.5 g of cells. It is important to treat the cells with a reducing agent in order to break the disulfide bonds within the cell wall, thereby improving hydrolysis.
5. Incubate the cells for 10 min at 30 °C with gentle shaking. Meanwhile, weigh 6 mg zymolyase per 1 g of cells and suspend it in Zymolyase Buffer.
6. Centrifuge cells at 3,000 x g for 5 min at room temperature and discard the supernatant.
7. Resuspend the pellet in 1 ml Zymolyase Buffer per 0.15 g of cells. Do not vortex.
8. Measure OD_{600} of 10 µl aliquot of the cells in 990 µl of water.
9. Add Zymolyase (step 1.5) to the cells to hydrolyze glucose polymers at the β-1,3-glucan linkages and generate spheroplasts. From this step, spheroplasts should be kept in an isotonic solution in order to avoid lysis.
10. Incubate cells for 15 min at 30 °C (for optimal activity of zymolyase) with gentle shaking. To verify hydrolysis of the cell wall and spheroplasts generation, mix 10 µl of the cells with 990 µl of water. Spheroplasts are expected to lyse due to osmotic difference, and the OD_{600} should be at least 10-fold less than the value determined in step 1.9. If not, continue incubation with zymolyase for another 15 min.
11. Centrifuge cells at 3,000 x g for 5 min at room temperature. Carefully discard the supernatant, as the pellet might be unstable.
12. Wash spheroplasts with Zymolyase Buffer and discard the supernatant.
13. Resuspend spheroplasts with 100 ml Recovery medium. Transfer spheroplasts to an Erlenmeyer flask and incubate for 1 hr at 30 °C with shaking. This recovery step is necessary since during the Zymolyase treatment translation is arrested and mRNA localization is disrupted (**Figure 1B**).
14. Add 0.1 mg/ml CHX and transfer spheroplasts to a precooled 50 ml conical tube. CHX addition is important to freeze ribosomes' association with mRNAs. Thus, ribosomes-dependent mRNA association with mitochondria is maintained.
15. Centrifuge spheroplasts at 3,000 x g for 5 min at 4 °C and discard the supernatant.
16. Wash twice with cold Mannitol Buffer.
17. Resuspend spheroplasts with 4 ml cold Mannitol Buffer and transfer them to a Dounce homogenizer of 15 ml capacity equipped with tight fitting pestle. Gently break spheroplasts with 15 strokes and transfer the lysate to a 13 ml tube.
18. Centrifuge the lysate at 1,500 x g for 6 min at 4 °C to pellet nuclei and unbroken cells.
19. Carefully transfer the supernatant to a new tube. Set aside 1 ml (25%) of the sample as an unfractionated sample (**"Total"** sample). Transfer 50 ml of this sample to a new tube and add 15 ml of 4X LSB for western blot analysis. Precipitate RNA from the rest of the "Total" sample (See Protocol 3, RNA precipitation).
20. Centrifuge the supernatant at 10,000 x g for 10 min at 4 °C to pellet the mitochondria.
21. Transfer the supernatant (~3 ml) to a new tube and keep it on ice. This is the **"Cytosolic"** fraction. Transfer 50 ml of this sample to a new tube and add 15 ml of 4x LSB for western blot analysis. Precipitate RNA from the rest of the "Cytosolic" sample (See Protocol 3, RNA precipitation).
22. Wash the pellet with 3 ml Mannitol Buffer and centrifuge again at 10,000 x g for 10 min at 4 °C.
23. Resuspend the pellet with 3 ml Mannitol Buffer. This sample contains the mitochondria, and referred to as **"Mitochondria"** fraction. Transfer 50 ml of this sample to a new tube and add 15 ml of 4x LSB for western blot analysis.

2. RNA Extraction

1. Add to each sample one volume of 8 M guanidinium-HCl and two volumes of 100% ethanol. Vortex and incubate for at least 2 hr at -20 °C.
2. Centrifuge samples at 10,000 x g for 20 min at 4 °C. Discard supernatant. Be careful as the pellet might be unstable.
3. Wash the pellet with 70% EtOH.
4. Resuspend the pellet with 400 ml of RNase-free water and transfer the sample to a new Eppendorf tube.
5. Precipitate the RNA again by adding 0.1 volume of 3 M sodium acetate pH 5.2 and two volumes of 100% EtOH. Vortex and incubate for at least 2 hr at -20 °C.
6. Centrifuge samples at 20,000 x g for 20 min at 4 °C. Discard the supernatant and wash with 70% EtOH.
7. Air dry the pellet and resuspend with RNase-free water. Store RNA samples at -80 °C. Samples can be used for northern analysis[19] or microarray analysis[18] (See Protocol 3).

3. Preparing the RNA for Microarray Analysis

1. Resuspend the mRNA from step 2.2 with 650 ml of RNase-free water.

2. To remove any residual DNA or proteins, add equal volume (650 ml) of acidic phenol:chloroform (5:1, pH 4.7) and vortex vigorously. Centrifuge at maximum speed for 2 min.
3. Transfer 500 ml of the upper phase (the aqueous phase) to a new tube. Avoid taking the interphase, as it contains DNA. Also be careful of taking phenol, which can inhibit the reverse transcription reaction.
4. The RNA sample contains a significant amount of heparin, which is a potent inhibitor of reverse transcriptase. Thus, for RT-PCR or microarray labeling it needs to be removed. Remove heparin by LiCl precipitation: add LiCl to a final concentration of 2 M and incubate samples over night at -20 °C.
5. Thaw the samples at 4 °C and centrifuge at 20,000 x g for 20 min at 4 °C.
6. Carefully discard the supernatant and wash the pellet with 80% ethanol. Centrifuge again as described in step 3.5.
7. Discard the supernatant, air dry and resuspend the pellet in 150 ml of RNase-free water.
8. To remove any residual LiCl, precipitate again with 0.1 volume of 3 M sodium acetate pH 5.3 and 3 volumes of 100% ethanol. Incubate at -20 °C for at least 2 hr.
9. Centrifuge at top speed for 20 min at 4 °C. Wash carefully the transparent pellet with 80% ethanol and air dry.
10. Resuspend the pellet with 25 ml RNase-free water and keep the samples at -80 °C.
11. Follow the steps for RNA labeling and hybridization described in[18,20].

Representative Results

This protocol allows separation of a mitochondria-containing fraction from cytosolic components. The best way to test its success is to perform northern analysis and western analysis (**Figure 1**) to samples from the different isolation steps. Three parameters for the isolation quality are derived from these analyses. First, whether the RNA or proteins in the samples are intact – these will be detected as distinct bands in the analyses. Degradation events will induce the appearance of additional, shorter bands or smears. Second, comparing the signals from each step with the signal from the input (*i.e.* Total) provides important information regarding losses during preparation. Severe losses will be observed as a significant difference between the Total and the relative amount of signal. Lastly, and most important, the quality of separation between mitochondrial and cytosolic components can be determined. Isolation of intact mitochondria will result in signal for mitochondrially-transcribed transcript only in the mitochondria fraction and not in the cytosolic fraction (**Figure 1A**). Furthermore, proteins that are mitochondria-associated will appear by western blot only at the mitochondria fraction, and cytosolic proteins in the cytosolic fraction (**Figure 1B**).

Figure 1 demonstrates two additional outcomes that are standard for this protocol: first, association of nuclear-encoded mRNAs with mitochondria varies between genes. One can see that ACO1 mRNA, which encodes a mitochondrial protein, appears mostly in the mitochondria fraction, while the mRNA encoding the cytosolic actin protein (ACT1) is mostly in the cytosolic fraction (**Figure 1C**). Genome-wide analyses revealed variation among many genes[8,10,21,22] and the basis for this diversity is under extensive research. Second, significant amounts of ER-related material are coisolated in the mitochondrial fraction. The mRNA encoding SEC61, which is ER-associated, is detected by northern blot (**Figure 1A**) and the ER-membrane protein Cue1 is detected by western blot (**Figure 1B**). This may be an outcome of the known physical contacts between ER and mitochondria. While further purification of mitochondria is possible, it may introduce some limitations; these are discussed in the Discussion.
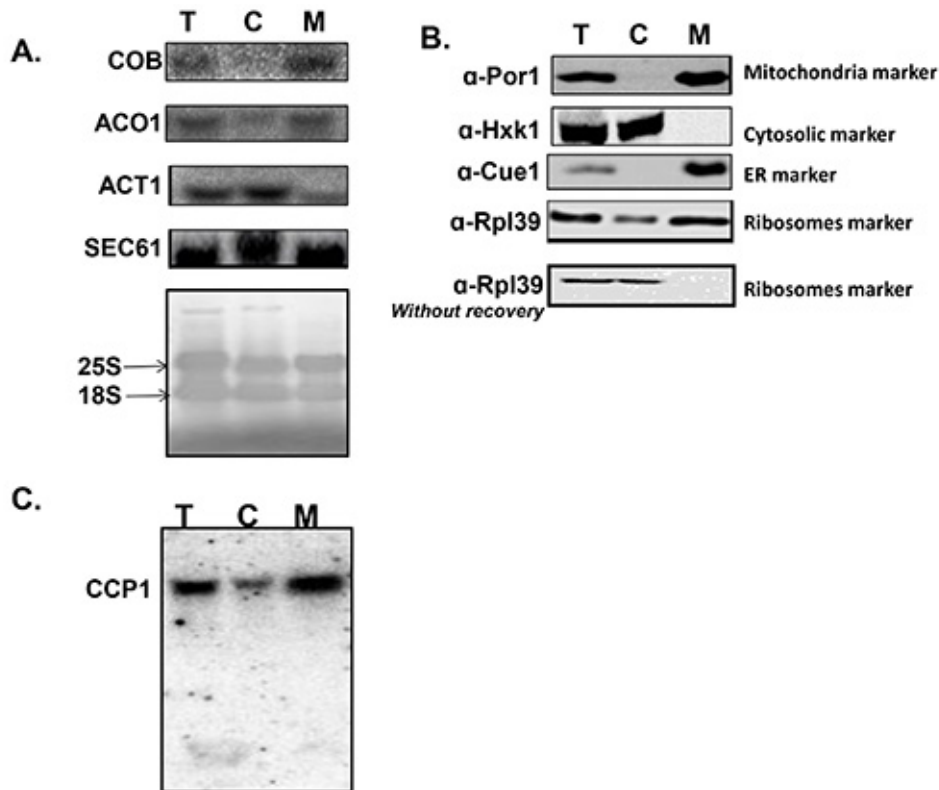
Figure 1. Quality verification of isolation. Quality is tested for samples taken in three representative steps of the procedure: Before fractionation (Total [step 1.20], T; cytosolic fraction [step 1.22], C; and mitochondrial fraction [step 1.24], M). The samples are subjected to northern analysis (**A, C**) or western analysis (**B**). **A)** Northern analysis for the following mRNAs: COB is an RNA that is transcribed inside the mitochondria therefore its signal should be exclusively in the mitochondria. Its appearance in the C fraction will indicate of mitochondria lysis during preparation. ACT1 is an mRNA that encodes a cytosolic protein, and therefore translated by cytosolic ribosomes and appears mostly at the C fraction. ACO1 is an mRNA that encodes a protein that is imported to the mitochondria, and appears mostly at the mitochondria fraction. SEC61 is an mRNA that encodes ER resident protein; its presence in the M fraction indicates the presence of ER components in this fraction. The bottom panel presents a methylene blue staining of the northern membrane, is which two rRNAs (18S and 25S) are detected. This panel demonstrates that significant amount of ribosomes appear in the M fraction. **B)** Western analysis for the following proteins: Por1 is a mitochondria outer membrane protein therefore its signal is expected only in the M fraction. Signal in the C fraction will suggest dissociation of mitochondrial components during preparation. Hxk1 is a cytosolic protein and Cue1 is an ER protein. Both report on the copurification of these fractions with the mitochondria fraction. Rpl39 is a ribosomal protein, again demonstrating the presence of ribosomes in both fractions. The panel "Rpl39 without recovery", presents the fractionation of ribosomes when the protocol is excluded of the recovery step (step 1.14). **C)** Northern analysis for CCP1, an mRNA that encodes a protein that is imported to the mitochondria. The entire gel is presented to demonstrate the lack of shorter, degradation-originated bands. Please click here to view a larger version of this figure.

Discussion

Technological advancements in imaging had yielded high resolution tools to study mRNA localization. Today, one can measure the movement of even a single mRNA molecule at the msec time scale[23-26]. Yet, traditional biochemical approaches, as the one described above, are also informative and are preferable in some cases. Biochemical isolation allows purification of a large repertoire of mRNAs and proteins, and is therefore preferable for genome-wide studies. In a single isolation, one can obtain sufficient mRNA levels to allow characterization of thousands of genes, either by DNA microarrays or RNA-seq[18,27]. In parallel, one can isolate proteins and perform a proteomic analysis to characterize the proteome on mitochondria[28]. Biochemical fractionations can be adapted to separate mitochondria from other cellular organelles, in particular the ER[29]. This may be advantageous in some types of cells were the high spatial overlap between ER and mitochondria obstructs microscopic localization analyses. An important advantage of biochemical fractionations is their low cost, which allows analysis of many different samples. A related advantage is their simplicity – biochemical fractionation, as the one described above, usually do not require laborious fluorescent tagging of mRNAs, as is the case in *in vivo* imaging studies. Finally, many pitfalls and artifacts are already diagnosed for these traditional protocols, therefore the necessary controls are well-established.

The presented protocol entails a recovery step (step 1.14) that follows the zymolyase treatment. This step is critical because the stress induced during the zymolyase treatment leads to almost immediate translational arrest and ribosomes' dissociation from mRNAs. The actual trigger for ribosomes dissociation can any of the steps that included in this treatment, namely removal of the carbon source, exposure to high g during centrifugation, treatment with reducing agents (DTT), cell-wall removal by zymolyase or introduction of high osmotic conditions; each of these is

known to induce a translational arrest. This translational arrest probably affects the association of ribosomes with mitochondria. Accordingly, we found that mRNA association with mitochondria is low after zymolyase treatment, and increases during recovery (not shown).

The mitochondrial fraction that is isolated by this protocol contains a significant amount of ER, as evident from the presence of various ER markers (*e.g.* **Figure 1**). An obvious desire is to further remove ER components and to obtain pure mitochondria, using one of several standard protocols that utilize gradients[29-33]. However, these protocols necessitate stripping of ribosomes of the mitochondria, either due to the stressful zymolyase treatment (**Figure 1B**), or the inclusion of EDTA in the buffers. Ribosomes' stripping has significant impact on mRNA association and therefore may introduce severe biases. It is therefore unwanted here. Intriguingly, we found that these protocols are not effective in separating ER from mitochondria when ribosomes are associated with these organelles. For example, when we applied gradient separation after the recovery from the zymolyase treatment (*i.e.* when ribosomes reassociate with the mitochondria), both ER and mitochondria markers sedimented to the same fraction. This is probably because the associated ribosomes obscure the differences in their densities (data not shown). Thus, for ribosome association studies, alternative methods for mitochondria isolation will need to be developed.

For many studies, however, the presence of ER components is not necessarily problematic. Association of mRNAs that encode well-established mitochondrial proteins (*e.g.* Aconitase, ATP2, Oxa1) is likely to be with the mitochondria and not the ER component in this crude preparation. It is therefore recommended to use one of these typical mitochondrial mRNAs as readouts for impact on mitochondria. Proteins that were not verified as mitochondrial should not be used as reporters; this is important in particular when genome-wide mRNA studies are performed (*e.g.* microarray analysis), as mis-assigned mRNAs may introduce an error.

The major technical challenge of this protocol is minimizing mRNA degradation. While using RNAse-free reagents is always advised, one should remember that upon lysis of cells enormous amounts of RNAses are unleashed into the solution. Heparin is a very effective RNAse inhibitor and is added at significant amounts. When large extract volumes are prepared, its relatively low cost provides additional advantage compared to other RNAse inhibitors. Heparin however, also inhibits reverse transcriptase, and therefore should be removed for studies that involve such enzymatic activity (*e.g.* RT-PCR or DNA microarray labeling). Herein we present a removal procedure that is based on LiCl precipitation (steps 3.4-3.10). This precipitation effectively removes heparin, but apparently also significant amounts of mRNAs are lost. Alternatively, there are various spin-columns that are supposed to remove heparin from RNA samples, yet we had a limited success with these.

Disclosures

The authors declare no competing financial interests.

Acknowledgements

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