Genome-wide analysis of mRNAs targeted to yeast mitochondria

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It is agreed that nuclear-encoded mitochondrial proteins are post-translationally targeted to mitochondria, even if, in some cases, a co-translational phase can assist the import of precursor proteins. We used yeast DNA microarrays to analyse the mRNA populations associated with free and mitochondrion-bound polysomes. As expected, many mRNAs, known to encode mitochondrial proteins, are localized to free cytoplasmic polysomes, but many are localized to mitochondrion-bound polysomes. Furthermore, the 3'-UTR of six randomly chosen mitochondrion-bound mRNAs contains sufficient information to target, *in vivo*, non-translatable RNA to the vicinity of mitochondria. Interestingly, genes producing mRNAs that are targeted to mitochondria are mainly of ancient bacterial origin, whereas those producing mRNAs that are translated in the cytoplasm are mainly of eukaryotic origin. These observations, which support the recent hypotheses concerning the dual origin of the mitochondrial proteome, provide new insights into the biogenesis of mitochondria.

INTRODUCTION

The biogenesis of mitochondria involves intricate mechanisms that have been selected during evolution to ensure the correct assembly of this functionally important and structurally complex organelle. The widely accepted endosymbiont theory of the origin of mitochondria renders mitochondrial biogenesis even more intriguing, because the protein import machinery must have been refined (or re-evolved) after the transfer of most of the genes of the free-living bacterial ancestor to the host genome. The clear picture of the mitochondrial import process that has emerged in recent years should not obscure the unexplained complexity of this phenomenon. However, it is unambiguous that a basic import mechanism (Schatz and Dobberstein, 1996;

Neupert, 1997), involving a mitochondrial targeting sequence plus complexes of the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM), is responsible for the import into the mitochondrion of many proteins, although variations in this process may be required to accommodate the biochemical diversity of the 400–600 proteins transported. Several lines of evidence suggest that the mitochondrial protein import process may begin before recognition of the mitochondrial targeting signal by the proteins of the TOM machinery. In pioneering studies, Butow's group (Kellems *et al.*, 1974, 1975) demonstrated that translationally active ribosomes loaded with mRNA molecules encoding mitochondrial precursor proteins accumulate on the surface of yeast mitochondria. Although it was later suggested that such locus-specific translation may play a marginal role in the mitochondrial import (Suissa and Schatz, 1982), several recent observations support the idea that a co-translational process is involved in the mitochondrial import of some proteins (reviewed in Verner, 1993; Lithgow, 2000). The discovery, in mammalian cells, of the nascent polypeptide-associated factor NAC (Wiedmann *et al.*, 1994) and the fact that, in yeast, disruption of either the genes encoding the subunits of the NAC complex or its homologue, the ribosome-associated complex (RAC) (Gautschi *et al.*, 2001), leads to defects in protein targeting to mitochondria (George *et al.*, 1998; Funfschilling and Rospert, 1999) confirm that translation and import are associated.

The translocation of specific mRNAs to the vicinity of mitochondria appears to require *cis*-acting signals present in the RNA molecule. We have shown previously (Corral-Debrinski *et al.*, 2000) that the yeast *ATM1* mRNA contains *cis*-acting signals, in its 5' N-terminal region and 3'-UTR, which direct heterologous RNA molecules to the vicinity of mitochondria *in vivo*. mRNA

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Fig. 1. Procedure for isolating and identifying mRNAs associated with mitochondrion-bound polysomes. Yeast cells were subjected to classical lysis and the mitochondrial fraction was isolated. Mitochondrion-bound polysomes were released by incubation with detergent and purified as described previously (Ades and Butow, 1980). The post-mitochondrial supernatant fractions were used to prepare free polysomes. We synthesized cDNAs from mitochondrion-bound and free polysomes and labelled them with the fluorescent dyes Cy5 and Cy3, respectively. The labelled cDNAs were hybridized to a DNA microarray. The full protocol is available on our website (www.biologie.ens.fr/yeast-publi.html).

targeting does not require translation of the 5' sequence, suggesting that targeting occurs independently of translation.

We used a whole-genome approach to characterize the mRNAs that were enriched in the vicinity of mitochondria. Our results confirmed and extended those of our previous studies: two main classes of mRNAs encoding mitochondrial proteins were identified, in which mRNA molecules were associated with mitochondrion-bound polysomes or with free cytosolic polysomes. In addition, we have shown that mRNAs located close to mitochondria had a 3'-UTR conferring mitochondrion-targeting properties. Finally, we observed that the two extreme classes of mRNA molecules encoding mitochondrial protein were related to genes of different origins: the mRNA molecules present in mitochondrion-bound polysomes were transcribed from genes with known homologues identified in bacteria, whereas the mRNA molecules present in free cytosolic polysomes were transcribed from genes of eukaryotic origin.

RESULTS

Asymmetric distribution of mRNAs encoding mitochondrial products between free and mitochondrion-bound polysomes

We purified mitochondrion-bound and free polysomes as described previously (Ades and Butow, 1980; Suissa and Schatz, 1982) (Figure 1). mRNAs from the polysome fractions were subjected to northern blotting with *ATP2* and *COX6* probes corresponding to mitochondrially and cytosolically located mRNAs, respectively, to assess the purity of fractions (see Supplementary figure 1 available at *EMBO reports* Online). cDNA molecules produced from mRNA preparations purified from free or mitochondrion-bound polysomes were labelled using the fluorescent dyes Cy3 and Cy5, respectively, and hybridized to DNA microarrays. Cy5/Cy3 ratios were analysed as described previously (Devaux *et al.*, 2001) after the elimination of non-significant data. Six independent analyses were carried out. Assuming that the Cy5/Cy3 ratio distribution of the

genes is representative of the distribution of the corresponding mRNA in both fractions, we classified all genes into centiles on the basis of their ratio. The centile standard deviation from the six experiments was very low in the extreme classes, suggesting that the corresponding mRNAs are tightly localized to the corresponding subcellular fractions. Each gene was then given a value of mitochondrial localization of mRNA (MLR) from 1 to 100, corresponding to the mean values of the centiles obtained from six independent experiments. A high MLR value, for a gene encoding a mitochondrial product, indicates a high probability of detecting the corresponding mRNA in the mitochondrial environment. The MLR values for all the genes that gave significant fluorescent signals are available on our website (www.biologie.ens.fr/yeast-publi.html). The distribution obtained is presented in Figure 2A.

MLR values and protein location

We compared the MLR values obtained with the subcellular location of the corresponding gene products, as described in the Yeast Proteome Database (YPD) (Hodges *et al.*, 1999). As expected, the low MLR classes (1–30) were enriched in mRNAs encoding cytosolic proteins (Figure 2B). About half of the 423 mRNAs identified as encoding putative mitochondrial proteins had MLR values of over 70, providing a quantitative demonstration of the importance of the mitochondrial mRNA targeting process (Figure 2C). However, mRNAs encoding mitochondrial products were also detected in free cytosolic polysomes. As observed previously (Ades and Butow, 1980; Suissa and Schatz, 1982), the protocol used for the isolation of mitochondrion-bound polysomes resulted in contamination with plasma membrane and endoplasmic reticulum fragments. Therefore, it was not surprising that, at high MLR values, we detected mRNAs encoding proteins located in these compartments. This finding is consistent with the recent study by Diehn *et al.* (2000), which showed that many mRNA species are bound to membrane-associated polysomes. These two sets of results cannot be simply compared, because our purification procedure has been designed to purify mitochondrion-bound polysomes and our cell growth conditions were different. This explains the observed

Fig. 2. Relationship between MLR and localization of proteins. A standardized MLR value was determined for each mRNA. mRNAs enriched in mitochondria-bound polysomes have the highest MLR values. (A) Distribution of mRNA according to MLR. (B and C) Percentage of mRNAs encoding cytosolic (B) or mitochondrial (C) proteins (according to the YPD) are plotted as a function of MLR value. The blue lines indicate the percentage of all localized proteins encoding either cytosolic (B) or mitochondrial (C) proteins in the YPD.

asymmetric distribution of mRNAs coding for mitochondrial proteins, a feature that was not reported in Diehn's studies (Figure 3).

In vivo localization of mRNAs of high MLR value

We used an RNA-labelling system to analyse *in vivo* the results obtained by microarrays (Bertrand *et al.*, 1998; Beach *et al.*, 1999). We showed previously, when studying the *ATM1* mRNA (Corral-Debrinski *et al.*, 2000), that the 3'-UTR contains sufficient information to target RNA to the vicinity of mitochondria. Therefore, we investigated the *in vivo* RNA targeting properties of eight 3'-UTRs from mRNAs that were found to be in close vicinity to mitochondria (MLR from 84 to 98). These mRNAs were selected from those for which the location of the product protein was not known. We tagged the various 3'-UTRs with two coat protein (CP)-binding sites and expressed a chimeric gene encoding the MS2 coat protein fused to green fluorescent protein (CP-GFP). We then analysed the eight constructs and the corresponding negative control. Six of the eight RNAs gave images in which discrete fluorescent spots that closely

Fig. 3. mRNAs encoding mitochondrial proteins found in the two extreme cytosolic and mitochondrion-bound fractions. Top 25 highest (A) and lowest (B) MLR values measured for mRNAs encoding mitochondrial proteins. MLR values are shown in the first column, and the ratios found in Diehn's experiments, from cells grown in glucose, are shown in the second column. Colour scales are provided for MLR and for ratios, ranging from red (mitochondrial or membrane localization) to green (cytosolic localization). The clear asymmetric mRNA localization observed in cells grown with galactose (the present work) contrast with the results observed when the carbon source is glucose (Diehn *et al.*, 2000).

matched the distribution of mitochondria were observed, as determined by Hoechst staining (Figure 4). These results imply that the 3'-UTRs contain sufficient information to address, *in vivo*, the corresponding mRNAs to mitochondria vicinity as it was observed in the microarray experiments. This strongly suggests that the corresponding translation products do encode mitochondrially localised products. This point has been recently confirmed for COQ4 (Belogrudov *et al.*, 2001) and MRF1' (Torkko *et al.*, 2001).

MLR values and the origin of mitochondrial proteins

With a stringent BLAST cut-off value ($E < 1 \times 10^{-10}$), it was observed recently (Karlberg *et al.*, 2000) that 50.6% of the 423 yeast mitochondrial proteins have homologues in prokaryotes, whereas 49.2% have neither eubacterial nor archaeal homologues. We investigated the relationships between the subcellular location of mRNA species and the two phylogenetic classes of mitochondrial proteins described by Karlberg *et al.* (2000). We observed a clear correlation between the MLR value and the origin of the corresponding gene (Figure 5). The mRNAs of bacterial origin were mostly located in the vicinity of mitochondria, whereas mRNAs of eukaryotic origin were translated on free cytosolic ribosomes. We also found that the divergent ancestry of these two classes of proteins is reflected in their functional contributions. This was clearly the case for the enzymes of the TCA cycle, where all the mRNA molecules encoding these enzymes had a high MLR value cycle (see Supplementary figure 2).

Fig. 4. 3'-UTR of mRNAs with highest MLR can target a chimeric RNA to the vicinity of mitochondria. The 3'-UTR of six ORFs with high MLR values (84–98) and unknown function were analysed by the 'green RNA method' (Bertrand *et al.*, 1998; Bloom *et al.*, 1999). As expected, the gRNA-induced fluorescent speckles are co-localised with mitochondria when 3'-UTR is cloned in the 5'3' orientation. *ATM1*/3'-UTR is the positive control. *BGL2* is a cell wall protein, its 3'-UTR constitute a negative control. Each 3'-UTR was cloned in both the 3'5' and 5'3' orientation (control shown only for *COQ4*). For each construct, gRNA (g), Hoechst staining of the DNA (H) and Nomarski (N) view are provided.

Fig. 5. The dual origin of the mitochondrial proteome matches the dual location of mRNAs. mRNAs encoding mitochondrial proteins were separated according to MLR values in six classes containing an equivalent number of ORFs. For each class, the proportions of genes of eukaryotic origin, including yeast genes, are displayed as black bars, and those of prokaryotic origin are displayed as blue bars (Karlberg *et al.*, 2000). The mRNAs associated with mitochondrion-bound polysomes (high MLR values) correspond mainly to genes of ancient prokaryotic origin, whereas cytosolically translated mRNAs correspond to genes of eukaryotic origin.

DISCUSSION

We demonstrated here, by means of a genome-wide approach, the extent to which mitochondrion-bound polysomes are important in mitochondrial biogenesis. Almost half the studied mRNAs from nuclear genes encoding mitochondrial products were found to be translated in the vicinity of mitochondria. This confirms and considerably extends previous observations based on a limited number of genes (Ades and Butow, 1980; Suissa and Schatz, 1982; Corral-Debrinski *et al.*, 2000). It has already been shown that such genomic-wide approaches can produce crucial information concerning transcripts known to encode secreted or membrane proteins present in large amounts in membrane-bound fractions (Diehn *et al.*, 2000). The approach

used in this study was specifically adapted to the study of mitochondrial biogenesis. A list of ORFs encoding mRNA molecules with MLR values of over 80 is available on our website (www.biologie.ens.fr/yeast-publi.html). These ORFs are very likely to encode mitochondrial products, because we demonstrated that six of these genes encoded mRNA species that targeted a heterologous RNA to the vicinity of mitochondria *in vivo*. This *in vivo* targeting property of most of the relevant 3'-UTRs: (i) is fully consistent with the DNA microarray analyses presented in this work; (ii) is in agreement with previous studies on *ATM1*mRNA and *ASH1*mRNA (Long *et al.*, 1997; Takizawa *et al.*, 1997) demonstrating the importance of this 3'-UTR; and (iii) strongly suggests that all mitochondrion-targeted mRNAs with high MLR values are likely to possess a similar 3'-UTR. We looked for the common 'zip code' present in these 3'-UTRs. As observed previously in other organisms (Jansen, 2001), it was difficult to identify convincing common primary sequences in the different 3'-UTRs. Several zip codes present in secondary or tertiary structures may make up the signal recognized by the protein machinery involved in mRNA transport. More generally, given the specific and important role of this 3'-UTR sequence, studies of the *in vivo* mitochondrial import of these proteins should take into account the corresponding 3'-UTR, and the results of studies of chimeric genes should be interpreted with caution.

The reasons for the inequalities in targeting of mRNAs encoding mitochondrial proteins were puzzling. Two studies (Karlberg *et al.*, 2000; Marcotte *et al.*, 2000), using two different approaches, have recently presented convincing evidence supporting a dual prokaryotic and eukaryotic origin for the mitochondrial proteome. Our observation (Figure 5) that mRNAs encoding proteins of putative prokaryotic origin are mainly translated on mitochondrion-bound polysomes, whereas mRNAs encoding proteins of putative eukaryotic origin are rather translated on free cytosolic polysomes, is novel and gives new insights into old paradigms.

First, co-translational import is thought to facilitate the import of highly hydrophobic proteins, which have a strong tendency to aggregate (Fujiki and Verner, 1993). However, this attractive

hypothesis is not consistent with the observation that the membrane proteins are mainly of eukaryotic origin (Marcotte *et al.*, 2000) and do not always correspond to high MLR values, whereas the proteins of prokaryotic origin are rather soluble and correspond to high MLR values.

Secondly, despite the known versatility of the mitochondrial import machinery (Pfanner and Geissler, 2001), no specific routes for the import of proteins originating from prokaryotes have been identified. However, fumarase (Fum1p), a typical protein of prokaryotic origin, clearly requires the coupling of translation and translocation for its import into mitochondria (Stein *et al.*, 1994; Knox *et al.*, 1998). This is consistent with the high MLR value of 81 obtained here for *FUM1*mRNA. Another protein of putative prokaryotic origin, malate dehydrogenase (Mdh1p), requires the presence of the ribosome-associated NAC for full import (Funfschilling and Rospert, 1999); this is also consistent with the high MLR value of 95 obtained in this study for *MDH1*mRNA.

Thirdly, these results also open the way to a new investigation area. If co-translational import into mitochondria is the general rule for mitochondrial proteins of prokaryotic origin, what is the relationship between this process and the ancestral origin of these proteins? The transfer of genes from α -proteobacteria to the nuclear genome necessitated a switch from prokaryotic to eukaryotic ribosomes for translation of the message. The unexpected discovery of mitochondrion-type ribosomes outside mitochondria in the germplasm of *Drosophila* embryos (Amikura *et al.*, 2001) suggests that specific cellular loci may control atypical translation processes. Is the translation of mRNAs associated with mitochondrion-bound ribosomes derived from such an ancestral process?

Finally, analyses of the mRNA populations of mitochondrion-bound polysomes from diverse origins should allow us to attempt a rigorous reconstruction of the evolution of the relationships between the nuclear and mitochondrial genomes. The study of most bacterium-like organisms, such as *Reclinomonas americana* (Gray *et al.*, 2001), might help us to trace the evolution of this important phenomenon. Our recent observation that a similar asymmetric distribution of mRNAs encoding mitochondrial products is also observed in human cells is consistent with the generalization of this phenomenon to all eukaryotes.

METHODS

Yeast strain and growth conditions. The *CW04* strain of *Saccharomyces cerevisiae* was used in this study. This strain is isonuclear with the strain *W303-1B* (*MATa*, *leu2-3*; *ura 3-1*; *trp1-1*, *ade1-2*, *his3-11*, *can1-100*). *CW04* cells were grown at 30°C in rich medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose and 30 µg/ml adenine), galactose-rich medium [1% Bacto-yeast extract, 1% Bacto-peptone, 0.1% KH_2PO_4 , 0.12% $(\text{NH}_4)_2\text{SO}_4$ and 2% galactose] or synthetic medium containing 2% galactose supplemented with the appropriate nutritional requirements.

Living cells expressing the green RNAs were observed by fluorescence microscopy with a chilled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) as described previously (Corral-Debrinski *et al.*, 2000). For all microscopic observations, cells were harvested in early log phase.

Plasmids and DNA manipulations. The 3'-UTR of selected genes were obtained by 16-cycle PCR amplification using as template yeast genomic DNA (100 ng) and the rTth DNA polymerase XL (Perkin-Elmer Cetus) and 20 nucleotide-long oligonucleotides. The amplified regions spanned between the last 10 bp of the considered ORF and the first 10 bp of the next one. PCR fragments were then inserted into the pIII/MS2-1 plasmids using the *Sma*I restriction site.

Array production and hybridization. Array production and hybridization were conducted as described previously (Devaux *et al.*, 2001) on full-length ORF microarrays.

Visualization of 'green-RNA' in live cells. The 'green-RNA' technique was used as described by Corral-Debrinski *et al.* (2000). Reporter RNA was produced from the pIII/MS2-1 plasmid (Beach *et al.*, 1999), which contains two tandem copies of the CP-binding site. The single *Sma*I site was used to introduce the complete 3'-UTR of YAR027w, YBR026c/*MRF1'*, YGR141w, YBR159w, YDL204w, YDR204w/*COQ4*, YDR275w, YEL052w/*AFG1* and YGR282c/*BGL2*. In all constructs, the binding site preceded the sequence examined. We studied both possible orientations (5'3' and 3'5') for each inserted sequence. The cells expressing the 3'5' constructs were used as negative controls.

Online supplementary information. Full and detailed protocols for free and mitochondrion-bound polysomes purification are available at www.biologie.ens.fr/yeast-publi.html, which also contains supplementary information and a database to search the full dataset.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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