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Mitochondrial presequence and open reading frame mediate asymmetric localization of messenger RNA

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Although a considerable amount of data have been gathered on mitochondrial translocases, which control the import of a large number of nuclear-encoded proteins, the preceding steps taking place in the cytosol are poorly characterized. The localization of messenger RNAs (mRNAs) on the surface of mitochondria was recently shown to involve specific classes of protein and could be an important regulatory step. By using an improved statistical fluorescent *in situ* hybridization technique, we analysed the elements of the *ATP2* open reading frame that control its mRNA asymmetric localization. The amino-terminal mitochondrial targeting peptide (MTS) and translation of two elements in the coding sequence, R1 and R2, were required for anchoring of *ATP2* mRNA to mitochondria. Unexpectedly, any MTS can replace *ATP2* MTS, whereas R1 and R2 are specifically required to maintain perimitochondrial mRNA localization. These data connect the well-known MTS–translocase interaction step with a site-specific translation step and offer a mechanistic description for a co-translational import process.

Keywords: mitochondria; membrane-bound ribosome; co-translational import

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INTRODUCTION

Recent findings have questioned the currently accepted idea that most protein localizations occur after translation. Indeed, in a previous analysis of messenger RNA (mRNA) localization in the *Drosophila* embryo, 71% of the 3,370 genes analysed were found to encode subcellularly localized mRNAs (Lecuyer *et al*, 2007). In addition, of the 780 nuclear genes coding for yeast mitochondrial proteins, 514 produce mRNAs that are translated to the vicinity of mitochondria (Saint-Georges *et al*, 2008). This supports the idea that most protein production is site-specific and suggests that mRNA localization has a prominent role in forming localized cellular machinery. Thus, mRNAs seem to contain information, not only to encode proteins, but also to regulate and guide the construction and activity of other cellular functions.

Studies on mitochondrial biogenesis are proving to be useful for addressing some of these aspects of the mRNA localization process. Site-specific translation might be particularly important in the biogenesis of mitochondria, as it could be associated with the co-translational import of mitochondrial nuclear-encoded proteins. The long standing question of whether proteins are imported into mitochondria after their translation (Neupert & Herrmann, 2007) or through a co-translational process (MacKenzie & Payne, 2007) probably differs depending on the protein considered. A recent study (Eliyahu *et al*, 2010) showed that Tom20, a peripheral component of the translocase of the mitochondrial outer membrane (TOM complex), can mediate the specific localization of mRNAs to mitochondria. More generally, the analysis of mRNA localization has shown that the 780 nuclear-encoded mitochondrial proteins identified so far are split into roughly two similarly sized groups, as a function of whether their mRNAs are attached to mitochondria or are associated with free cytoplasmic polysomes (Marc *et al*, 2002). More recent studies (Saint-Georges *et al*, 2008) found that mRNAs translated close to mitochondria can be divided into two subgroups, according to their dependence on the RNA-binding protein Puf3p—a protein that seems to be important for mitochondrial biogenesis (Gerber *et al*, 2004; Garcia-Rodriguez *et al*, 2007). Class I mRNAs have at least one Puf3p-binding site in their 3′ untranslated region sequence and their cellular localization generally depends on the presence of Puf3p, whereas the asymmetrical localization of class II mRNAs is not linked to Puf3p. Notably, the mechanistic classification of these mRNAs also reflects a separation of their functional roles; class I mRNAs code for basic elements of mitochondrial biogenesis (that is, translation machinery and assembly proteins), whereas class II mRNAs code for structural components of mitochondrial machinery (Saint-Georges *et al*, 2008). Recent analyses (Lelandais *et al*, 2009) showed that these topologically distinct classes of mRNA coincide with temporally distinct

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groups of expression when transcriptome of a continuous-grown system was studied (Tu *et al*, 2005). These observations suggest that mRNA localization linked to mitochondrial biogenesis is regulated by a group of *trans*-acting factors that recognize *cis*-acting signals of mRNA localization. The role of translation is particularly important for the biogenesis of mitochondria, in which a co-translational import process might be biologically relevant (Lithgow *et al*, 1997). The dynamics of *ATP2* mRNA in mitochondrial biogenesis can act as a model to investigate the role of translation in asymmetric mRNA localization. Yeast *ATP2* mRNA—which codes for the β-subunit of the F1 sector of ATP synthase—is a class II mRNA, the 3′ untranslated region of which seems to contain signals guiding its cellular localization. This is true for both yeast (Margeot *et al*, 2002) and mammals (Ricart *et al*, 2002), in which the control of translation seems to be linked to mRNA localization.

Technological improvements now allow the distance between an mRNA molecule and the mitochondrion surface to be measured reliably by using statistical tools (Garcia *et al*, 2007a, b). We have thus taken advantage of these tools to systematically analyse the role of translation in the asymmetrical localization of *ATP2* mRNA. We observed that the asymmetric localization of *ATP2* mRNA requires translation of the open reading frame (ORF). In this ORF, the amino-terminal leader sequence and two sequence elements, R1 and R2, were required to guide the perimitochondrial localization of the *LacZ* reporter mRNA. None of these elements could achieve this alone, instead requiring cooperative action. Although the MTS was required, the MTS of this peptide sequence could be replaced with the MTS from mRNAs that are translated on free cytoplasmic ribosomes, such as *COX4* or *ATP16*, without affecting asymmetric mRNA localization. These results provide further evidence that some proteins are imported co-translationally and demonstrate a clear cooperation between the MTS–TOM machinery and the asymmetric localization process of mRNA.

RESULTS AND DISCUSSION

Methods used to study asymmetric localization of mRNA

Deciding whether an mRNA molecule is localized to the vicinity of mitochondria requires reliable experimental methods and, in the case of fluorescent *in situ* hybridization (FISH) analyses, a large number of images. One of the main difficulties arises from the experimental variability due to cellular factors such as cell heterogeneity and variations in mitochondria morphology. Thus, we analysed statistically the cell population based on FISH analyses of more than 50 cells for each experiment. An outline of our approach is shown in supplementary Fig S1 online. This approach, which used a dedicated software, was validated by the comparative analysis of two extreme situations: one negative control in which the *LacZ* mRNA was not localized and a positive control in which the fused *ATP2–LacZ* mRNA was localized asymmetrically. The two distributions of median distances between mRNA and mitochondria—256 and 88 nm, respectively—enabled us to determine the median distance threshold (140 nm) under which an mRNA is considered to be localized asymmetrically. This corresponds to a false-positive rate of 5% (supplementary Fig S1 online).

Translation of *ATP2*-coding sequence is required

To test whether the anchoring of *ATP2* mRNA at mitochondria requires the translation of the ORF, we analysed the effects of mutation of the start codon and the introduction of a −1 frameshift, which created a stop at codon 198. The asymmetric localization of the *ATP2–LacZ* mRNA reporter was abolished completely for both mutants (Fig 1A, contructs 3 and 4). Northern blot analysis and image quantification of particles show clearly that this effect was not due to mRNA degradation in absence of translation (supplementary Fig S4 online). The role of the reporter gene was assessed. For this purpose, three ORF lengths—*LacZ* = 1021 codons, $Z'' = 92$ codons and $Z' = 14$ codons—were fused to three *ATP2* constructs (supplementary Fig S3 online). We unexpectedly observed that, in the cases in which the mRNA-addressing process is weak, the fusion of a long reporter ORF might enhance the asymmetric localization (supplementary Fig S3B online). This might be connected with an increased number of translating ribosomes that mediate the mRNA–mitochondria interaction (see below).

Role of Atp2p mitochondrial targeting sequence

To examine the role of the leader peptide in the asymmetry of mRNA localization, we either deleted or replaced it in the *ATP2*-coding sequence from the *ATP2–LacZ* hybrid gene (Fig 2A, construct 3). We checked that the newly created ATG start codon is functional and that the corresponding protein has the expected size and is present in comparable amounts (supplementary Figs S4,S5 online); ribosomal density and translation rate are important elements of the asymmetric localization of mRNA (Paquin *et al*, 2007). The absence of the MTS considerably reduced the asymmetric localization of the hybrid reporter mRNA for *ATP2*, *ATP3* and *ATP4* mRNAs (Fig 2E). By contrast, when Atp2p-MTS was replaced by one of the three MTSs from Cox4, Atp16 or Atp1, the corresponding fused mRNA was still localized asymmetrically (Fig 2C). This was unexpected, particularly given that *ATP16* and *COX4* mRNAs are translated on free cytoplasmic ribosomes in natural *in vivo* conditions (Garcia *et al*, 2007a; Saint-Georges *et al*, 2008). To confirm this, we examined the role of the membrane potential $\Delta\Psi$, which promotes the transport of the N-terminal matrix-targeting sequence of cleavable preproteins (Schleyer & Neupert, 1985; Martin *et al*, 1991). By using a low concentration of carbonyl cyanide *m*-chlorophenylhydrasone, we observed decreased asymmetric localization of mRNA both in cells carrying the *ATP2–LacZ* hybrid gene and *ATP4* mRNA (supplementary Fig S2 online). Interestingly, this is in agreement with the recent observation that Tom20 mediates the localization of mRNAs to mitochondria (Eliyahu *et al*, 2010). These results strongly suggest that the first steps of the mitochondrial import process—recognition of the translocase Tom20 by the MTS and $\Delta\Psi$-dependent translocation—precede the anchoring of mRNA to mitochondria. This first step corresponds to the import mechanism that is well characterized for most mitochondrial proteins (Neupert & Herrmann, 2007); it does not confer a site-specific translation process. Thus, the next question to be addressed was which elements in the ORF confer asymmetrical localization of *ATP2* mRNA.

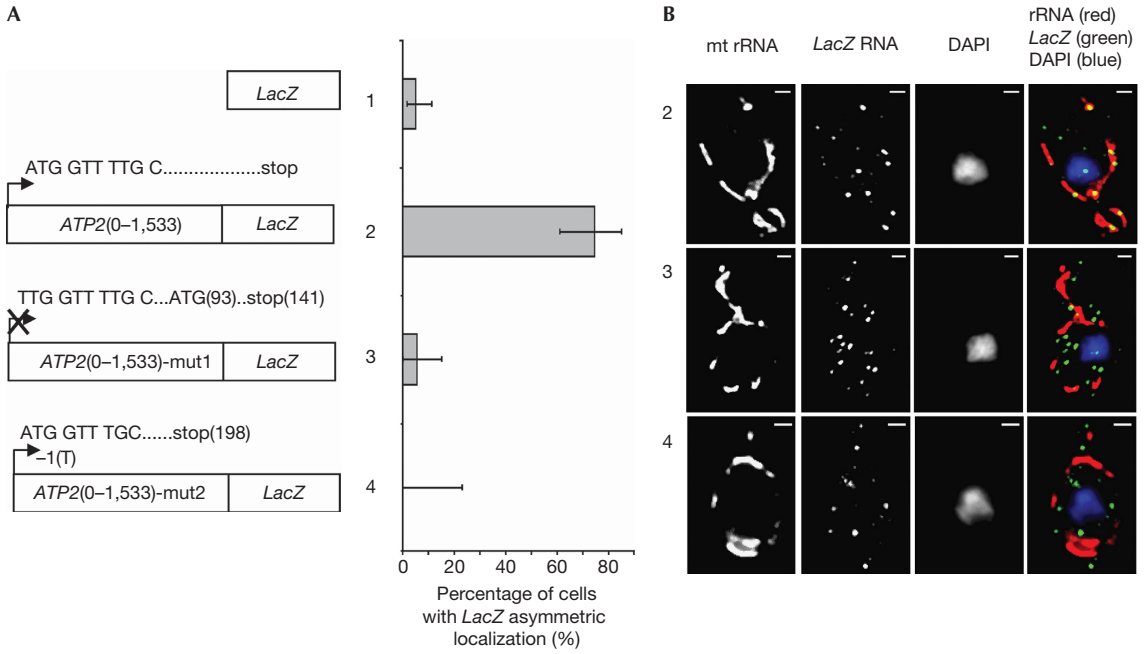

Fig 1 | Translation of the *ATP2* ORF is required for mRNA asymmetric localization. (**A**) The percentage of cells carrying an asymmetric *ATP2–LacZ* mRNA was evaluated from FISH analyses of more than 50 cells (supplementary Fig S1 online). The presence of the *ATP2* ORF (construct 2) was required to localize the hybrid *ATP2–LacZ* RNA to the vicinity of mitochondria at a level similar to that of the natural *ATP2* mRNA (Saint-Georges *et al*, 2008). Mutated forms of the *ATP2* ORF, either disrupting the ATG initiation codon (construct 3) or resulting in a −1 frameshift (construct 4), abolish the asymmetric localization of the mRNA. (**B**) FISH analysis of the hybrid constructs, each showing the major cell population. Numbers refer to the constructs in (**A**). Scale bars, 1 μm. DAPI, diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization; mRNA, messenger RNA; mt rRNA, mitochondrial ribosomal RNA; ORF, open reading frame.

Translation of two regions of the *ATP2* ORF is required

To investigate whether specific regions of the *ATP2* ORF cooperate with the N-terminal leader sequence to localize the mRNA to the vicinity of mitochondria, we used serial deletions of sequences from the ORF linked to the reporter *LacZ* mRNA. We generated two types of construct, differing in their 5′ region. One type contained the full-length *ATP2* promoter region and the entire *ATP2* MTS (Fig 3A). For the other type of construct, we used the *ZWF1* promoter as described for the above analyses, with *COX4* (MTS + 50 amino acids) fused to various regions of the *ATP2* ORF (Fig 3B). This double-approach strategy was used to assess the specificity of the leader peptide sequence and the internal ORF regions, and to determine whether a large part of *COX4* mRNA can be targeted to the mitochondrial vicinity. As we observed previously for the whole sequence, we did not find any specificity between the *ATP2*-MTS and ORF-containing signals (Fig 3). Two distinct regions, R1 (394–604 nucleotides) and R2 (756–1533 nucleotides), conferred the ability to maintain mRNA in the vicinity of mitochondria, for both *ATP2* and *COX4* constructs (Fig 3A,B). Furthermore, asymmetric localization of the mRNA was restored incrementally by the successive addition of each of the two elements R1 and R2 to the chimeric construction. Furthermore, we scanned the R2 region and identified a core R2 region (1,300–1,500 nucleotides), which can confer a significant asymmetric localization of mRNA (supplementary Fig S6 online). Interestingly, a comparison of predicted secondary structures showed that a stem–loop structure is highly conserved among five evolutionarily distant yeast species (supplementary Fig S6 online). Further analyses are required to determine whether the conserved sequence in the loop is recognized by a specific mRNA-binding protein.

Ribosomes and mRNA anchoring

Studies performed nearly half a century ago revealed that ribosomes interact with yeast mitochondria at outer–inner membrane junctions (Kellems *et al*, 1975). Here, we present a mechanistic description (Fig 4) of the main events that control this site-specific ribosomal activity. The MTS interacting with the TOM translocases is a prerequisite for mRNA asymmetric localization. This MTS, probably associated with nascent polypeptide-associated complex (George *et al*, 2002), does not carry any specific information to guide a co-translational process; rather, this information is contained in the rest of the ORF, which can stabilize the mitochondrion–ribosome interactions.

In this study, we show that constant interaction is required between ribosomes and *ATP2* mRNA to maintain the mRNA–mitochondria interaction. Indeed, stop mutants resulted in the disconnection of the ribosomes and the release of *ATP2* mRNA. This is consistent with our previous observation that puromycin releases ribosomes from mitochondria under high-salt conditions, resulting in the delocalization of more than 70% of the mRNA (Saint-Georges *et al*, 2008). How do R1 and R2 elements influence perimitochondrial mRNA localization? They can act by modulating translation rate (rare codons) or/and by interacting with RNA-

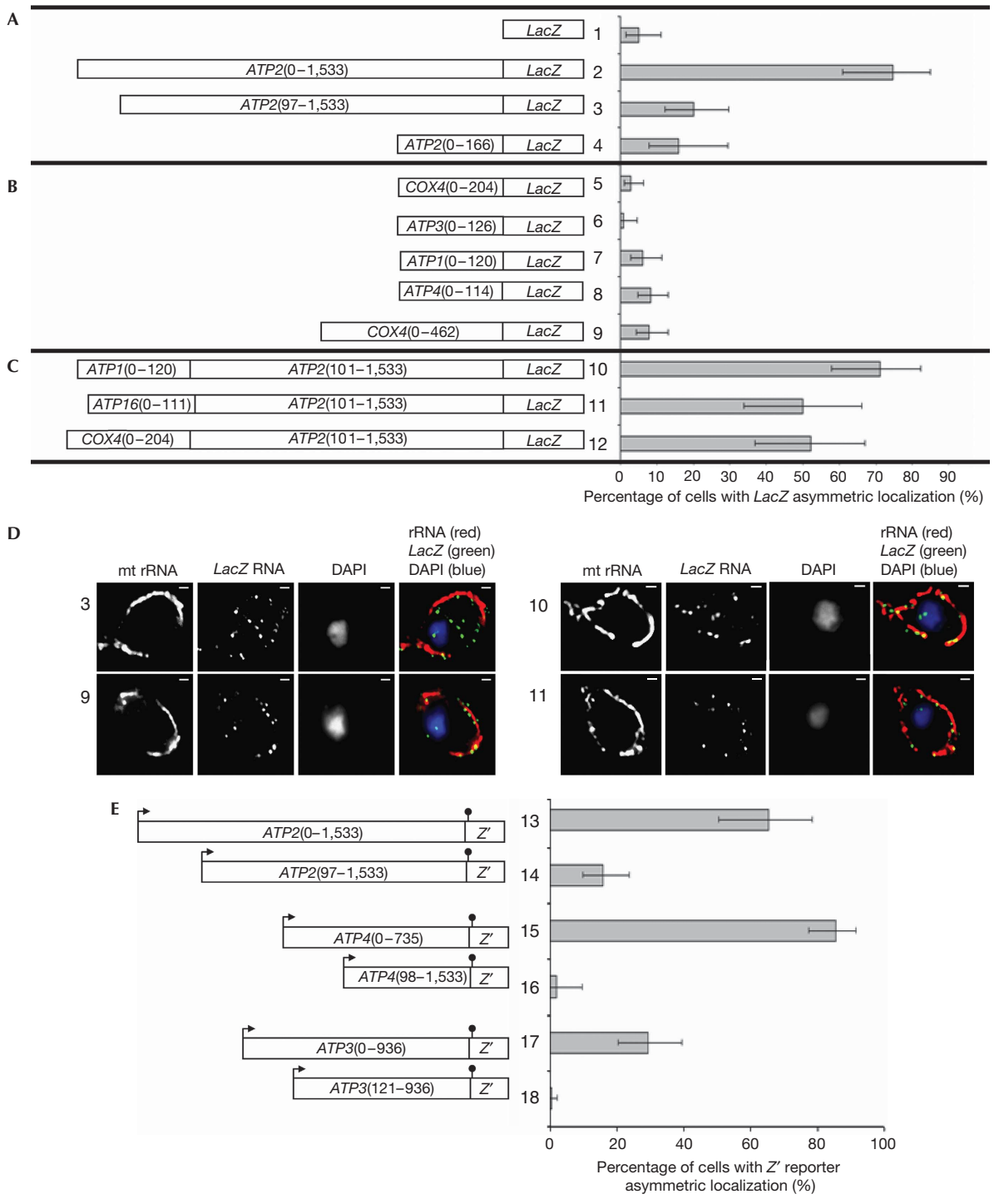

Fig 2 | The mitochondrial targeting peptide is necessary but not sufficient to guide asymmetric localization of mRNA. mRNA localization was assessed statistically by FISH analyses (more than 100 cells), as indicated in supplementary Fig S1 online. (**A**) The absence of MTS-coding sequence reduces *ATP2* mRNA asymmetric localization. (**B**) MTS-coding sequences alone are not able to guide heterologous mRNAs to the vicinity of mitochondria. The full-length *COX4*-coding sequence was also unable to promote asymmetric mRNA localization, in agreement with previous studies (Saint-Georges *et al*, 2008). (**C**) Heterologous MTS fully restore the asymmetric localization of an MTS-deleted version of *ATP2* mRNA. MTS from cytoplasmically localized *ATP16* and *COX4* was also able to confer asymmetric localization to the *ATP2* ORF sequence. (**D**) Single mRNA-resolution FISH analysis using the four hybrid mRNAs described above. The numbers 3, 9, 10 and 11 correspond to the constructs in (**B**) and (**C**). Cells were hybridized by using probes against *LacZ* (labelled with Cy3) and 16S rRNA (labelled with Cy5). Scale bars, 1 μm. (**E**) *ATP2*, *ATP3* and *ATP4* mRNA asymmetric localization is MTS-dependent. DAPI, diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization; mRNA, messenger RNA; mt rRNA, mitochondrial ribosomal RNA; MTS, mitochondrial targeting peptide.

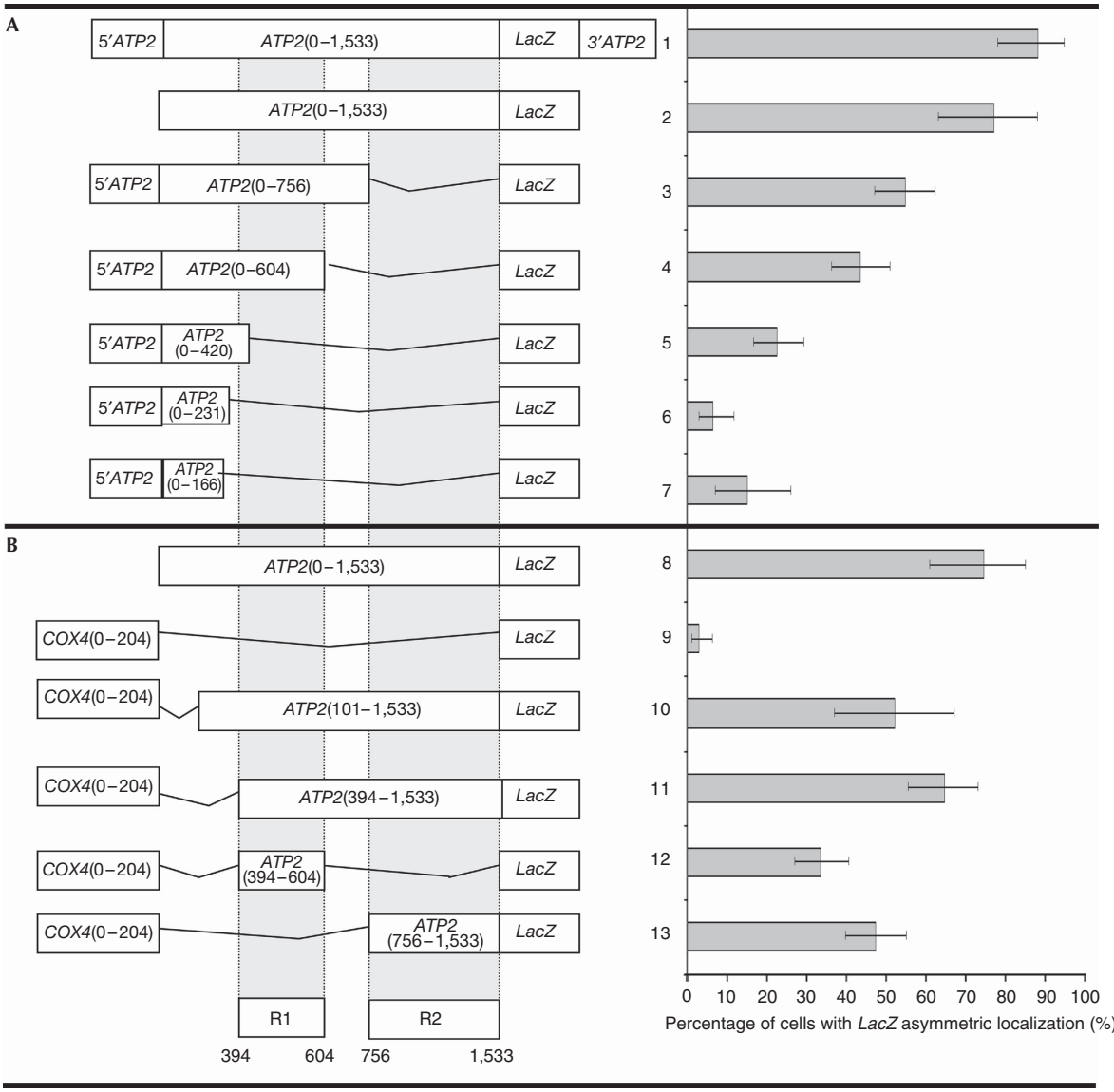

Fig 3 | Determination of the open reading frame-localized *cis*-acting elements involved in the asymmetric localization of *ATP2* mRNA. Schematic of the *ATP2* gene and various deletion constructs. These deletion constructs in the *ATP2* ORF were associated either with (**A**) the *ATP2* MTS or (**B**) the *COX4* MTS. mRNA asymmetric localization was evaluated in a statistical analysis of more than 100 cells (right). Consistent results were obtained for both types of MTS. Two localization elements, R1 (394–604 nucleotides) and R2 (765–1,533 nucleotides), in the *ATP2* mRNA ORF were each able to trigger the localization of reporter mRNA to the vicinity of mitochondria. FISH, fluorescent *in situ* hybridization; mRNA, messenger RNA; MTS, mitochondrial targeting peptide; ORF, open reading frame.

binding proteins (see the putative stem–loop structure of R2, supplementary Fig S6 online). In addition, as mRNA circularization might accelerate translation rate, the R2 element, near the ORF 3′ end, may have a special role in the regulation of the import process. It was also reported in other cellular systems that ribosomes bind specifically to mitochondria through proteins on the outer membrane in mammalian cells, through a process controlled by a GTP-dependent step (MacKenzie & Payne, 2004). In addition, the two ORF-localized *cis*-acting elements R1 and R2, which maintain the mRNA to the vicinity of mitochondria, present functional homology with the *cis*-acting elements in rat β-F1-ATPase (Ricart *et al*, 2002). The question of the interaction of ORF-localized elements with the ribosome is crucial: the recent observation that specific ribosomal proteins might be required for the translation of localized *ASH1* mRNA (Komili *et al*, 2007) gives credence to the fact that specific ribosome compositions might guide mRNA translation sites. Furthermore, the import-associated translational modulation observed in mitochondria of *Dictyostelium* (Ahmed & Fisher, 2009) underscores the importance of *ATP2* mRNA as a model to decipher the mechanism of this regulated co-translational import process.

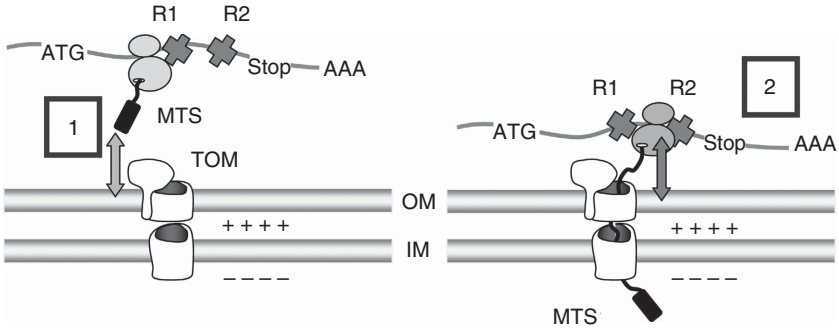

Fig 4 | The two-step model of *ATP2* mRNA addressing process. 1: translation of the MTS peptide and the inner membrane potential are necessary but not sufficient to target the mRNA–ribosome complex to the vicinity. 2: R1/2 elements, in association with the MTS and the ORF, stimulate mRNA–mitochondrion interactions. The 3′ untranslated region elements cooperate to promote asymmetric mRNA localization; they are nevertheless dispensable, whereas elements involved in either step 1 (MTS) or (R1/2 + ORF) are absolutely required. IM, inner membrane; mRNA, messenger RNA; MTS, mitochondrial targeting peptide; OM, outer membrane; ORF, open reading frame.

METHODS

Strains used and growth conditions. The *Saccharomyces cerevisiae* diploid strain Gi252 has an intronless mitochondrial genome and is isogenic to W303 (Saint-Georges *et al*, 2002). This strain was transformed by various pRS416-derived plasmids using a simplified lithium acetate method (Gietz *et al*, 1992) and selected on complete supplement mixture lacking uracil (CSM-ura; a synthetic medium supplemented with CSM) containing 2% glucose.

FISH and *in situ* mRNA localization quantitative analysis. The FISH experiments were performed as described previously (Garcia *et al*, 2007b).

Yeast cells were grown in CSM-ura containing 2% galactose for precultures and then transferred to YPGal (2% galactose, 1% bactopeptone and 1% yeast extract) for 1 night to avoid fluorescent background. Mid-log-phase cells were fixed immediately with a final concentration of 4% paraformaldehyde, spheroplasted with lyticase, adhered to poly-L-lysine-treated coverslips and stored in 70% ethanol at −20 °C. Five antisense oligonucleotide probes complementary to the *LacZ* sequence were used to detect single molecules of the reporter mRNA (supplementary Table S2 online). Mitochondrial ribosomal RNA and *ATP4* mRNA were detected by using probes as described previously (Garcia *et al*, 2007a). After overnight hybridization at 37 °C, washing and 4,6-diamidino-2-phenylindole staining, coverslips were mounted with antifade solution.

Three-dimensional (3D) imaging analysis was performed after hybridization, by acquiring 41 images 200 nm apart along the *z*-axis on an Olympus BX61 upright microscope (Rungis, France) using a ×100 numerical aperture objective. This was carried out for at least 50 cells.

To statistically analyse relative RNA localization in large cell populations, we developed software to measure the 3D distance between two fluorescent probes. Indeed, fluorescent imaging and 3D analysis by multistage acquisition enables data to be collected for the whole cell. Conclusions drawn in the absence of both 3D reconstitution and analysis of the whole cell population are highly speculative. We quantified distances between mRNA and mitochondria in an analysis involving the following steps: (1) 3D segmentation and extraction of object features (coordinate and intensity); (2) measurement of the distance between mRNA particles and mitochondrial surface; and (3) statistical analysis of generated data, with distances weighted for intensity and using the median mRNA–mitochondrion distance for each cell. Cells transformed with the pZLG plasmid expressed a non-fused *LacZ* reporter mRNA with a homogenous cytoplasmic distribution. These cells were chosen to define a reference population (100 cells) for further analyses. The maximum median mRNA–mitochondrion distance was 140 nm for more than 95% of the analysed reference cells. This median distance of 140 nm was taken as the threshold value, used to determine the potential mitochondrial localization of mRNA for each cell. The percentage of cells showing asymmetric mRNA localization could then be evaluated for the population. We calculated confidence intervals associated with this value by using a binomial test and determined whether the proportion of cells with localized mRNA was statistically different from the reference population (5%). Such analyses can be carried out routinely on more than 100 cells. Distance FISH software, called CORSEN, is an open-source software (GNU public license version 2 or later, CeCILL-A), which we want to make available to the biology community. A detailed description of CORSEN is in preparation (Jourdren *et al*, in preparation) and the application will soon be downloadable from a dedicated website. Furthermore, the source code will be available at the Google code forge.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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