MRM2 encodes a novel yeast mitochondrial 21S rRNA methyltransferase

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Mitochondria of the yeast *Saccharomyces cerevisiae* assemble their ribosomes from ribosomal proteins, encoded by the nuclear genome (with one exception), and rRNAs of 15S and 21S, encoded by the mitochondrial genome. Unlike cytoplasmic rRNA, which is highly modified, mitochondrial rRNA contains only three modified nucleotides: a pseudouridine (Ψ_{2918}) and two 2'-O-methylated riboses (Gm₂₂₇₀ and Um₂₇₉₁) located at the peptidyl transferase centre of 21S rRNA. We demonstrate here that the yeast nuclear genome encodes a mitochondrial protein, named Mrm2, which is required for methylating U₂₇₉₁ of 21S rRNA, both *in vivo* and *in vitro*. Deletion of the *MRM2* gene causes thermosensitive respiration and leads to rapid loss of mitochondrial DNA. We propose that Mrm2p belongs to a new class of three eukaryotic RNA-modifying enzymes and is the orthologue of FtsJ/RrmJ, which methylates a nucleotide of the peptidyl transferase centre of *Escherichia coli* 23S rRNA that is homologous to U₂₇₉₁ of 21S rRNA. Our data suggest that this universally conserved modified nucleotide plays an important function *in vivo*, possibly by inducing conformational rearrangement of the peptidyl transferase centre.

Keywords: FtsJ/RrmJ/Pet56/rRNA modification/Spb1/Trm7

Introduction

mRNA translation occurs on large particles, ribosomes, which are made up of ~60–80 ribosomal proteins and 2–4 rRNA molecules. The ribosome is formed by the association of a small subunit (SSU) with a large subunit (LSU). The SSU contains one small molecule of rRNA (15–18S), and the LSU one large (21–28S) and 0–2 small rRNA molecules (5 and 5.8S). Peptide bond formation, the keystone of protein synthesis, occurs in the LSU and recent results suggest that it is catalysed by the large rRNA itself (Noller *et al.*, 1992; Ban *et al.*, 2000; Nissen *et al.*, 2000). The large rRNA molecule is ubiquitous and the structure of the peptidyl transferase centre is universally conserved, from Prokaryota to Eukaryota, as well as in organelle-containing DNA such as mitochondria or

plastids (Noller *et al.*, 1981). Synthesis of mature rRNA requires a complex series of post-transcriptional processing steps, including numerous nucleotide modifications (100–200), some of which take place during or immediately after transcription, while others occur once the rRNA has been assembled into pre-ribosomes (Venema and Tollervey, 1999). These modifications consist mostly of 2'-O-ribose methylation, pseudouridylation and base methylation. The latter is dependent on methyl transferases (MTases) that recognize their targets directly (Andresson and Davies, 1980; van Buul and van Knippenberg, 1985; Lafontaine *et al.*, 1994). The two other types of modification, 2'-O-ribose methylation and pseudouridylation, occur in Eukaryota through a mechanism that has been well described in the past few years. It involves a large number of small nucleolar RNAs (snoRNAs) that target the nucleotide to be methylated by forming a transient duplex with the pre-rRNA molecule (Balakin *et al.*, 1996; Bachellerie *et al.*, 2000; Kiss, 2001). However, this mechanism may not be as universal as the modifications themselves. In *Escherichia coli*, rRNA modification is achieved by several site-specific enzymes that recognize their target without a guide RNA (Ofengand and Rudd, 2000). Similarly, in mitochondria, the few rRNA modifications depend on enzymes that are not likely to be guided by small RNAs. In yeast mitochondria, the RNA component of the LSU, the 21S rRNA, contains only three modified nucleotides: one pseudouridine (Ψ_{2819}) and two 2'-O-methylated nucleotides (Gm₂₂₇₀ and Um₂₇₉₁; Klootwijk *et al.*, 1975). Ψ_{2819} is synthesized by the mitochondrial pseudouridine synthase Pus5p (Ansmant *et al.*, 2000) and Gm₂₂₇₀ is catalysed by the 2'-O-ribose MTase Pet56p (Sirum-Connolly and Mason, 1993), both enzymes being encoded by the nuclear genome. The third modified nucleotide, Um₂₇₉₁, is homologous to Um₂₅₅₂ of the peptidyl transferase centre of *E. coli* 23S rRNA. Um₂₅₅₂ is catalysed by the site-specific 2'-O-ribose MTase FtsJ/RrmJ, whose three-dimensional structure has been solved recently (Bügl *et al.*, 2000; Caldas *et al.*, 2000a,b). In yeast, three proteins exhibit sequence similarity to FtsJ/RrmJ: Spb1p (Pintard *et al.*, 2000), Trm7p (Ybr061c; L. Pintard, F. Lecoite, J. Bujnicki, C. Bonnerot, H. Grosjean and B. Lapeyre, submitted) and Ygl136c. Three-dimensional (3D) structure predictions for the three yeast proteins reveal striking similarity to the *E. coli* protein and suggest strongly that they are new 2'-O-ribose MTases. In a separate report, we have demonstrated that Trm7p is a new MTase required for modifying the tRNA anti-codon loop (L. Pintard, F. Lecoite, J. Bujnicki, C. Bonnerot, H. Grosjean and B. Lapeyre, submitted). We have shown previously that Spb1p is a nucleolar S-adenosyl-L-methionine (AdoMet)-binding protein required for 25S rRNA synthesis (Pintard *et al.*, 2000). We now hypothesize that Spb1p is required for

Fig. 1. Sequence alignment of Mrm2p from *Saccharomyces cerevisiae* with potential homologues from different species. Ten proteins have been aligned using ClustalW (Thompson *et al.*, 1994). S.c, Mrm2p from *S.cerevisiae* (P53123); S.k, *Saccharomyces kluyveri*; K.m, *Kluyveromyces marxianus*; K.l, *Kluyveromyces lactis*; S.b *Saccharomyces bayanus*; P.a, *Pichia angusta*; P.f, *Plasmodium falciparum* (unfinished sequence from the *P.falciparum* Genome Project: PlasmoDB-<http://www.plasmodb.org>); C.a, *Candida albicans* (unfinished sequence from the Stanford Genome Technology Centre-SGTC: <http://sequence-www.stanford.edu>); S.p, *S.pombe* (P78860); E.c, FtsJ/RrmJ from *E.coli* (P28692). All the hemiascomycetous yeast sequences are unfinished sequences from Genolevures Project (<http://cbi.labri.u-bordeaux.fr/Genolevures>). Identical amino acids are on a black background and chemically equivalent groups are on a grey background. The nine motifs that form the putative AdoMet-binding domain are boxed and noted X and I–VIII. The four residues that are proposed to form the catalytic centre are indicated by a star. Segments predicted to adopt a secondary structure are indicated below the alignment: white tubes, α -helices (α 1–5); striped arrows, β -strands (β 1–7). The first insertion of 58 residues can also form an α -helix (α i) and a β -strand (β i).

2'-O-ribose methylation of U₂₉₁₈, a position of the peptidyl transferase centre equivalent to U₂₅₅₂ in *E.coli*, for which no guide snoRNA has yet been unambiguously identified in yeast (Lowe and Eddy, 1999). Here, we report that *YGL136c* is a nuclear gene encoding a mitochondrial protein required for 2'-O-ribose methylation of U₂₇₉₁ and, therefore, is the mitochondrial orthologue of FtsJ/RrmJ. As FtsJ/RrmJ, Ygl136c is active on 21S rRNA once assembled into the LSU. We propose naming this protein Mrm2p, for Mitochondrial rRNA MTase 2, and renaming Pet56p as Mrm1p.

Results

The product of the MRM2 gene exhibits striking similarities to the *E.coli* protein FtsJ/RrmJ

The yeast genome encodes three proteins presenting similarities with the *E.coli* 2'-O-ribose MTase FtsJ/RrmJ (Bügl *et al.*, 2000). Spb1p is a nucleolar AdoMet-binding

protein involved in LSU synthesis (L.Pintard, F.Lecoite, J.Bujnicki, C.Bonnerot, H.Grosjean and B.Lapeyre, 2000). Trm7p is a cytoplasmic MTase required for anti-codon loop tRNA methylation (Pintard *et al.*, submitted). Mrm2p is predicted to be a mitochondrial protein by computer analysis using PSORT (Nakai and Kanehisa, 1992), MITOPROT (Claros and Vincens, 1996) and TargetP (Emanuelsson *et al.*, 2000) programs. An alignment of Mrm2p with its putative orthologues in *E.coli* (FtsJ/RrmJ), several distantly related yeasts and a protozoan is presented in Figure 1. Nine motifs (I–VIII and X) form the putative AdoMet-binding domain (Posfai *et al.*, 1988). A group of four residues, K56, D203, K264 and E299 (K38, D124, K164 and E199, *E.coli* numbering), which are proposed to form the catalytic site (Bujnicki and Rychlewski, 2001), is conserved between different 2'-O-ribose MTases. When compared with FtsJ/RrmJ, Mrm2p exhibits two insertions of 58 (residues 136–193) and 21 (residues 207–227) amino acids. According to

Fig. 2. Deletion of *MRM2* prevents growth on glycerol at 37°C. (A) Wild-type cells (BMA64), *mrm2Δ* cells (YCB640) and *mrm2Δ* + p(*MRM2*) cells (YCB689) were spotted after serial dilutions (1/10 from left to right) on YPD plates or on YP-glycerol plates at 30 or 37°C and incubated for 4 days. (B) Individual cells were grown on YPD plates at 30°C for 4 days, then tested for their ability to grow on YP-glycerol (indicated as a plus or a minus sign). Left panel, *mrm2Δ* strain (YCB640); right panel, wild-type strain. Lower panel: western blot analysis of the tested strains grown in YPD with an anti-Cox2p antibody. Extracts were prepared from a *mrm2Δ* strain able to grow on YPGly (lane 1), from a *mrm2Δ* petite colony (lane 2) and from a wild-type strain (lane 3).

secondary structure predictions (Figure 1) and 3D modelling (L.Pintard, F.Lecoite, J.Bujnicki, C.Bonnerot, H.Grosjean and B.Lapeyre, submitted and data not shown), the larger insertion includes an α -helix and a β -strand, and could form a peripheral elaboration of the common fold, away from the AdoMet-binding and catalytic face of the protein. The shorter insertion maps to the neighbourhood of the catalytic/RNA binding site (near motif IV, which is central to catalysis in MTases) and exhibits no propensity to form regular structure elements. Interestingly, these insertions are conserved in Mrm2p homologues found in other hemiascomycetous yeasts (Souciet *et al.*, 2000), but not in *Candida albicans* or *Schizosaccharomyces pombe* (Figure 1). These insertions are likely to be present in the mature protein since the predicted molecular weight of the protein with its insertions is in good agreement with the observed electrophoretic mobility on SDS-PAGE (see below).

***mrm2Δ* is thermosensitive on glycerol-containing medium and loses mitochondrial DNA with high frequency**

A strain deleted for the *MRM2* gene was constructed using a PCR-based strategy (Baudin *et al.*, 1993). At 30°C, the *mrm2Δ* strain did not exhibit any detectable growth defect when compared with a wild-type isogenic strain, either on

glucose- or on glycerol-containing media. However, growth of the *mrm2Δ* strain was slightly reduced on glucose-containing medium at 37°C and severely reduced on glycerol-containing medium at 37°C (Figure 2A). This thermosensitive respiration phenotype was completely suppressed when transforming the mutant cells with a centromeric plasmid expressing the *MRM2* gene under the control of its own promoter (Figure 2A). After passing several generations on glucose medium, small colonies were obtained with high frequency for the *mrm2Δ* strain (Figure 2B), reminiscent of the cytoplasmic petite colonies obtained with a strain disrupted for *PET56* (Sirum-Connolly and Mason, 1993). These small colonies were unable to grow on glycerol-containing plates, suggesting that the mitochondria were no longer functional in these cells. This was confirmed by the following observations. The mitochondrial protein Cox2 was no longer detected by western blot analysis in these cells (Figure 2B) and they had lost their mitochondrial DNA, as demonstrated by fluorescence analysis after 4',6-diamidino-2-phenylindole (DAPI) staining (data not shown). Similar observations have been reported previously for mutations affecting the mitochondrial translation apparatus (Myers *et al.*, 1985; Fearon and Mason, 1992).

***Mrm2p* is induced in glycerol-containing medium and is localized to the mitochondria**

The results presented above suggested that *MRM2* was required for the mitochondrial function. Since Mrm2p was predicted to localize to mitochondria by computer analysis, we examined whether Mrm2p was induced after a shift from glucose- to glycerol-containing medium and localized to the mitochondria. To perform these experiments, a strain expressing an Mrm2-protA fusion protein under the control of its own promoter was constructed by homologous recombination (see Materials and methods). The growth rate of this strain was identical to that of an isogenic untagged strain. Western blot analysis of the tagged strain revealed a minor band (M_r 53 000) and a major band (M_r 50 000), which are likely to correspond to the precursor and the mature proteins, respectively, after cleavage of the mitochondrial signal pre-sequence. When cells were shifted from glucose- to glycerol-containing media, Mrm2-protAp was induced rapidly after the shift. Its level reached a plateau after 16 h of induction. The mitochondrial protein Cox2 was similarly induced (Poutre and Fox, 1987), while Tcm1, a protein of the cytoplasmic LSU used here as a control (Fried and Warner, 1981), was unchanged (Figure 3A).

To determine experimentally the subcellular location of Mrm2p, cell fractionation was performed with a strain expressing Mrm2-protAp. Fractions were tested by western blotting with antibodies raised against either the mitochondrial protein Cox2 or the cytoplasmic proteins Qsr1 (Tron *et al.*, 1995) and Pab1 (Adam *et al.*, 1986). Mrm2-protAp and Cox2p were detected mostly in the crude mitochondrial fraction, while the cytoplasmic proteins Qsr1 and Pab1 were recovered in the post-mitochondrial fraction only (Figure 3B). These results suggest a mitochondrial localization for Mrm2-protAp. To confirm this observation, we performed immunofluorescence experiments using a strain overexpressing Mrm2-protAp under the control of the inducible *gal1-10*

Fig. 3. Mrm2p is located within mitochondria. (A) Induction of Mrm2p after a shift from glucose- to glycerol-containing medium. Cells expressing Mrm2-protAp (YCB642) were grown in YPD and shifted to glycerol at $t = 0$. Extracts were prepared at various times thereafter and analysed by western blotting, probed with an anti-mouse antibody to reveal Mrm2-protAp (upper blot), an anti-Cox2p antibody (middle blot) or an anti-Tcm1p antibody (lower blot). (B) Mrm2p is mostly detected in the mitochondrial fraction. Cells (YCB642) were grown in YP-glycerol until stationary phase. Differential centrifugation was performed after breaking the cells to prepare a crude mitochondrial fraction (M) and a post-mitochondrial fraction (PM). Proteins from a total cell extract (T) and from the different fractions were analysed by western blotting with anti-mouse (upper blot), anti-Cox2p (second blot), anti-Pab1p (third blot) and anti-Qsr1p antibodies (lower blot). (C–G) Microscopic examination of cells overexpressing Mrm2-protAp (YCB651) (C, D and F–G) or wild-type untagged cells (E). (C) and (F) show DAPI staining, and (D), (E) and (G) show fluorescein isothiocyanate analysis. (F) and (G) show an enlargement of one cell of (C) and (D).

promoter. DAPI, which stains both nuclear and mitochondrial DNA, was used as a marker for the mitochondria. The signal obtained for Mrm2-protAp overlaps the non-nuclear DAPI staining (Figure 3, compare F and G). Taken together, these results demonstrate that Mrm2p is a mitochondrial protein. It is noteworthy that the addition of a protA tag at the C-terminus of the protein does not prevent its transport to mitochondria.

Mrm2p co-fractionates with 21S rRNA on a sucrose gradient

Structural similarity existing between Mrm2p and FtsJ/RrmJ suggested that Mrm2p could also be an rRNA MTase. Since we have shown that Mrm2p is located within the mitochondria, we then postulated that its substrate was the mitochondrial rRNA. To investigate this possibility, we first examined whether the two molecules could be associated *in vivo*, by determining their sedimentation profile on a sucrose gradient. A native extract prepared from a strain expressing Mrm2-protAp was fractionated by centrifugation onto a sucrose gradient and the fractions analysed by western and northern blotting (Figure 4). Mrm2-protAp was detected in fractions 10 and 11 along with the majority of the 21S rRNA. This result shows that the tagged Mrm2 protein and

Fig. 4. Mrm2p co-sediments with the 21S rRNA. A cellular extract of a strain expressing Mrm2-protAp (YCB642) was fractionated on a sucrose gradient. A continuous A_{254nm} record is presented (top panel). The arrows indicate the peaks of 40S, 60S and 80S subunits. Each fraction was split into two parts: one was TCA precipitated and analysed by western blotting probed with a HRP-conjugated anti-mouse antibody (middle panel) and the other was phenol extracted and analysed by northern blotting probed with o21S1, an oligonucleotide specific for the 21S rRNA (bottom panel).

its putative rRNA substrate co-fractionate on a sucrose gradient.

Mrm2p is required for 21S rRNA methylation *in vivo*

Only two positions are 2'-O-ribose methylated in the yeast mitochondrial 21S rRNA. Gm₂₂₇₀ had already been shown to be methylated by Pet56p (Sirum-Connolly and Mason, 1993), while the enzyme responsible for the formation of Um₂₇₉₁ was still unknown. However, U₂₇₉₁ is the equivalent in mitochondria of U₂₅₅₂ in *E.coli*, a position that is methylated by FtsJ/RrmJ. Since FtsJ/RrmJ exhibits significant sequence similarity to Mrm2p, a mitochondrial protein associated with the 21S rRNA, it was logical to test the possibility that Mrm2p is responsible for the formation of Um₂₇₉₁. To map the methylated nucleotides, we took advantage of the ability of reverse transcriptase to discriminate between 2'-OH and 2'-O-methyl ribose nucleotides in the presence of low concentrations of deoxynucleotides (dNTPs) (Maden *et al.*, 1995). Primer extension was performed using two different oligonucleotides that allowed us to test the two positions known to be methylated in the 21S mitochondrial rRNA. G₂₂₇₀, which is modified in both the wild type and the *mrm2Δ* strain, serves here as a control (Figure 5, lanes 8–11). Pausing of the reverse transcriptase occurred at a position corresponding to Um₂₇₉₁ in the wild-type strain, as shown previously (Sirum-Connolly *et al.*, 1995; Figure 5, lanes 2 and 3). In contrast, no pausing could be observed in the *mrm2Δ* strain (Figure 5, lanes 4 and 5), while pausing was restored when the mutant strain was first transformed with a centromeric plasmid expressing the wild-type *MRM2* gene under the control of its own promoter (Figure 5,

Fig. 5. Mrm2p is required for methylating U_{2791} *in vivo*. AMV reverse transcriptase was used to extend 32 P-labelled primers in the presence of two different concentrations of dNTPs, either 4 μ M or 1 mM. Extension products were separated by 12% PAGE containing 7M urea and autoradiographed overnight. Extension was performed on RNA prepared from the indicated strains either with primer o21S1 specific for U_{2791} (lanes 1–7) or with primer o21S2 specific for G_{2270} (lanes 8–12). Lanes 1 and 12, unextended primers.

lanes 6 and 7). These results provide strong evidence that Mrm2p is required for the formation of Um_{2791} *in vivo*.

Affinity-purified Mrm2p methylates U_{2791} of the 21S rRNA, only when assembled into the LSU

To investigate further the precise function of Mrm2p, we tested the activity of affinity-purified Mrm2–protAp on various substrates. To optimize the chance of detecting an MTase activity, the substrates were prepared from a *mrm2Δ* strain and thus were not methylated on position 2791. It has been shown previously that FtsJ/RrmJ is not active on deproteinized 23S rRNA, but requires the LSU to methylate the rRNA (Bügl *et al.*, 2000; Caldas *et al.*, 2000b). Therefore, we compared the activity of Mrm2–protAp on deproteinized 21S rRNA and on mitochondrial LSU purified on a sucrose gradient (equivalent to fraction 11 of Figure 4). Affinity-purified Mrm2–protAp was incubated with the two different substrates, then total RNA was extracted and used as a template in a primer extension experiment to analyse the methylation state of U_{2791} . Affinity-purified Mrm2–protAp was able to methylate the 21S rRNA assembled within the LSU *in vitro* (Figure 6, lanes 3 and 5). No methylation was observed with deproteinized rRNA (lanes 8 and 9) that gave a signal comparable to a control experiment in which no affinity-purified enzyme has been added (lane 7). Thus, this result demonstrates that Mrm2p methylates the 21S rRNA at position U_{2791} *in vitro*, when it is assembled with proteins into the LSU or pre-LSU, as previously reported for FtsJ/RrmJ.

Discussion

We report here the characterization of Mrm2p, a new yeast mitochondrial MTase that is required for the formation of

Fig. 6. Mrm2p methylates U_{2791} *in vitro*. Various RNA substrates have been prepared from a *mrm2Δ* strain (YCB640) and treated (lanes 3–6 and 8–9) or not (lanes 2 and 7) with the affinity-purified Mrm2–protA protein. To test for the MTase activity of Mrm2p, the substrate was either the whole 54S ribosomal subunit (lanes 2–6) or the deproteinized 21S rRNA (lanes 7–11). Then methylation status was determined by primer extension mapping using o21S1 as described in Figure 5. Lane 1, primer alone; lanes 3, 4 and 8 were treated with Mrm2p for 30 min, and lanes 5, 6 and 9 for 60 min; lanes 10 and 11, untreated control RNA from a wild-type strain. To detect the methylated nucleotides, primer extension was performed with 4 μ M dNTPs, except lanes 4, 6 and 11, which were incubated with 1 mM dNTPs.

Um_{2791} on the 21S rRNA. Sequence analysis predicted a mitochondrial location for Mrm2p and it had been reported that a *mrm2Δ* strain had a growth defect on glycerol-containing medium at 37°C, as expected for a protein having a mitochondrial function (Escribano and Mazon, 2000). We have now demonstrated experimentally that Mrm2p is a mitochondrial protein, both by cell fractionation analysis and immunolocalization.

A noteworthy feature of Mrm2p is its striking 3D structure similarity to other known 2'-O-MTases, particularly the FtsJ/RrmJ family (Bügl *et al.*, 2000; Caldas *et al.*, 2000b; L.Pintard, F.Lecointe, J.Bujnicki, C.Bonnerot, H.Grosjean and B.Lapeyre, submitted). FtsJ/RrmJ methylates U_{2552} of the A-loop of domain V of the universally conserved peptidyl transferase centre of *E.coli* 23S rRNA, a position equivalent to U_{2918} in yeast cytoplasmic 25S rRNA and U_{2791} in mitochondrial 21S rRNA. Amongst the three yeast proteins exhibiting similarity to FtsJ/RrmJ, Mrm2p is the most closely related to the *E.coli* protein. It has been proposed that mitochondria originated from bacteria that lived endosymbiotically within primitive cells (Yang *et al.*, 1985), supporting the view that some mitochondrial proteins are more similar to bacterial proteins than to their eukaryotic counterparts. Therefore, we decided to test the hypothesis that Mrm2p is the mitochondrial orthologue of FtsJ/RrmJ and methylates the homologous position of the peptidyl transferase centre.

Deletion of the *MRM2* gene is viable, although it leads to instability of the mitochondrial genome and the formation of cytoplasmic petite colonies. However, it is possible to grow the deleted strain on glycerol-containing medium, in order to select for the presence of functional mitochondria. Primer extension analysis, performed with limiting concentrations of nucleotides, revealed that methylation at U₂₇₉₁ is completely abolished in the *mrm2Δ* strain. This defect in 21S methylation, as well as the thermosensitive respiration of the *mrm2Δ* strain, was suppressed when the cells were transformed by a centromeric plasmid expressing the wild-type *MRM2* gene. Moreover, affinity-purified Mrm2p was able to restore methylation of U₂₇₉₁ *in vitro*. Methylation occurred when the 21S rRNA was assembled within the LSU, as reported previously for FtsJ/RrmJ (Bügl *et al.*, 2000; Caldas *et al.*, 2000b). Alternatively, methylation could occur on pre-LSU while subunits are being assembled, as is the case for some other rRNA modifications (Wrzesinski *et al.*, 1995). Interestingly, the position equivalent to U₂₇₉₁ on the cytoplasmic rRNA, U₂₉₁₈, is methylated once the rRNA is assembled into pre-ribosomal particles (Brand *et al.*, 1977).

While cytoplasmic rRNAs contain a large number of modified nucleotides (Maden, 1990), yeast mitochondrial 15S rRNA probably contains none and 21S rRNA contains only two methylated riboses and one Ψ. These nucleotides are all located within the universally conserved peptidyl transferase centre, at the site of interaction with the 3'-terminal end of the tRNA (the CCA region). The fact that there are very few modifications, located at the heart of the ribosome and conserved from Prokaryota to Eukaryota and cellular organelles, has led to speculation that they must fulfil some essential function(s). In *E.coli*, the SSU and LSU have been assembled successfully from their natural components (Traub and Nomura, 1968). However, when using synthetic transcripts, only active SSU were assembled *in vitro* (Krzyzosiak *et al.*, 1987). Large complexes presenting some physical resemblance to the LSU were obtained, which were inactive. By constructing chimeric molecules containing synthetic transcripts and naturally modified rRNA, the minimal region necessary for activity was narrowed down to a short segment of domain V (2445–2523, *E.coli* numbering) that contains only six modified nucleotides, none of which is universally conserved (Green and Noller, 1996). According to these results, one can speculate that the three modified nucleotides that are conserved in the mitochondrial 21S rRNA are dispensable for the ribosome activity. Along this line, deletion of *PUS5*, which encodes the pseudouridine synthase responsible for the formation of Ψ₂₈₁₉, has no detectable phenotype (Ansmant *et al.*, 2000). In contrast, *PET56*, which encodes the 2'-O-ribose MTase responsible for the formation of Gm₂₂₇₀, is essential for the synthesis of the mitochondrial LSU and for mitochondrial function (Sirum-Connolly and Mason, 1993; Sirum-Connolly *et al.*, 1995). We report here that deletion of *MRM2* abolishes the formation of the third 21S rRNA modification, Um₂₇₉₁, and leads to instability of the mitochondrial genome. Therefore, *MRM2* must fulfil an important function *in vivo*. It has been reported that the so-called 2550-loop, which contains Um₂₅₅₂ and G₂₅₅₃, plays an essential role in translation by base pairing with C75 of the tRNA 3'-terminal region. Mutation of G₂₅₅₃, not U₂₅₅₂, abolishes

the interaction with tRNA and thus the peptidyl transferase activity (Kim and Green, 1999). Since mutation at position 2552 has little effect, it is conceivable that it is not the nature of the nucleotide that is important, but rather the presence of a methyl group at that position. Recently, solution structure of the A-loop of 23S rRNA has demonstrated that methylation at U₂₅₅₂ modifies the configuration of C₂₅₅₆ and U₂₅₅₅, which, in turn, affects the tertiary interactions of the A-loop within ribosome structure (Blanchard and Puglisi, 2001).

Cytoplasmic ribosomes, which contain highly modified rRNAs, synthesize several thousands of proteins. In contrast, yeast mitochondrial ribosomes, whose 21S rRNA contains three modifications, synthesize only eight different polypeptides. Nissen *et al.* (2000) have shown recently that nascent polypeptides exit from the ribosome through a tunnel whose average diameter is ~15 Å, implying that protein folding cannot occur within this tunnel. Interestingly, in the ribosome of *Haloarcula marismortui*, 80% of the methylated nucleotides are located on the inner face of this tunnel. These modifications could play a role in the export of the nascent polypeptide chain from the ribosome. Hydrophobic coating of the inner face of the tunnel would prevent sticking of the unfolded amino acid chain and would allow it to slip outside the ribosome. The lower number of modifications of the mitochondrial rRNA would reflect the small variety of proteins synthesized by the organellar machinery, which faces only a limited number of combinations of charged residues on the emerging polypeptides.

Several modification enzymes play a dual role in ribosome maturation. For instance, Dim1p is a base dimethylase required for modification and processing of yeast 18S rRNA. Conditional mutants in which the two functions are uncoupled have demonstrated the bifunctionality of the protein (Lafontaine *et al.*, 1998). A similar hypothesis has been formulated for Pet56p (Mason, 1998) and it is noteworthy that other modification enzymes from *E.coli* have a dual function, such as for instance the tRNA methylase TrmA (Persson *et al.*, 1992) and the pseudouridine synthases TruB and RluD (Gutgsell *et al.*, 2000, 2001). Unlike *PET56*, deletion of *MRM2* does not significantly affect the production of the LSU, since the ratio of SSU versus LSU in the *mrm2Δ* strain appears normal (data not shown). However, cells become unable to respire at 37°C and rapidly lose the mitochondrial DNA when grown in YPD at 30°C, indicating that Um₂₅₅₂ must fulfil an important function *in vivo*. Altering the mitochondrial translation ability, even slightly, could have a dramatic effect and lead to the loss of mitochondrial DNA after a few generations, as shown previously for other mitochondrial components (Myers *et al.*, 1985; Fearon and Mason, 1992). Further work will address two questions: (i) is the loss of rRNA modification at U₂₇₉₁ in the *mrm2Δ* strain sufficient to explain the observed mitochondrial defect or does Mrm2p play other functions; and (ii) which determinants render Mrm2p, as well as FtsJ/RrmJ, active only on the LSU, not on deproteinized rRNA? It is possible that some factors present in the ribosome cooperate with Mrm2p. Alternatively, it is conceivable that proper folding of the rRNA structure is necessary for Mrm2p to recognize its target.

Table I. Strains used in this study^a

Strain	Relevant genotype	Origin
BMA64	<i>MATa</i> α <i>trp1</i> Δ	F.Lacroute
YCB640	<i>MATa</i> <i>mrm2</i> Δ :: <i>TRP1</i>	this work
YCB642	<i>MATa</i> <i>MRM2-protA</i> :: <i>TRP1</i> <i>pep4</i> :: <i>URA3</i>	this work
YCB651	<i>MATa</i> <i>HIS3</i> :: <i>GAL1-10</i> :: <i>MRM2-protA</i> :: <i>TRP1</i> <i>pep4</i> :: <i>URA3</i>	this work
YCB689	<i>MATa</i> <i>mrm2</i> Δ :: <i>TRP1</i> p(<i>CEN,LEU2,MRM2</i>)	this work

^aAll strains are *ade2-1 his3-11 leu2-3 112 trp1* Δ *ura3-1 can1-100*.

Materials and methods

Microbiological methods

The strains used in this study were handled by standard techniques (Guthrie and Fink, 1991). They were constructed for this study (Table I) from a derivative of W303 (Baudin *et al.*, 1993). Yeast transformation was performed using the lithium acetate method (Gietz *et al.*, 1992). PCR was performed with *Taq* DNA polymerase (Gibco-BRL); the products were purified on Sepharose CL6B spin columns (Pharmacia) and precipitated prior to use. Disruption of *YGL136c* was performed by using a PCR-based strategy with the diploid strain BMA64 (Baudin *et al.*, 1993) and oligonucleotides U-YGL136 (TATCCGAGGTTTGAAGTTT-TCTCCT) and L-YGL136 (TAGGTGTGAAAATGAACGTGACT-TGAAACTTTGCGCGCAAACAAATACGGGTGTTGGCGGGTGTGTC) to amplify a *TRP1* cassette. Sporulation of the *Trp*⁺ diploid cells led to four viable spores for each tetrad analysed, with a similar growth rate. Two successive backcrosses were performed before analysis of the strain YCB640. Tagging with two IgG-binding domains of *Staphylococcus aureus* protein A (*MRM2-protA*) at the chromosomal *MRM2* locus was performed by a PCR-based strategy with oligonucleotides YGL136-A-U (GATTAAGAAGAAAAGAAATGTAGATAAACTGGACGTTAATT-TCAAAGCTGGAGCTCAAAAC) and YGL136-A-L (GTGTGAAAAT-GAACGTGACTTGAAACTTTGCGCGCAAACAAATCATATTATAC-GACTCACTATAGGG), and plasmid pBS1173 as a template (Puig *et al.*, 1998). A *pep4*::*URA3* mutation was introduced by genetic crossing to reduce the degradation of proteins in native extracts. Two successive backcrosses were performed before analysis of the strain YCB642. For immunofluorescence studies, a strain overexpressing the tagged Mrm2 protein was constructed. The *GAL1* promoter was introduced by a PCR-based strategy within the tagged strain, with oligonucleotides Gal1-Y-G-L136-U (AAAATGTCAGCCTCTGATATAGATGGAAGTCGTAGC-CA-TCAAGATGGGCTGAATTCGAGCTCGTTTAAAC) and Gal1-Y-G-L136-L (CGACCTAAAGAAGACGATATTATACTCCTGATACGAT-TGTACACTAAAATCATTTTGAGATCCGGGTTTT) and pFA6a-His-3MX6-PGAL1 as a template (Longtine *et al.*, 1998). Two successive backcrosses were performed before analysis of the strain YCB651.

For complementation analysis, the YGL136c ORF was amplified by PCR from the genomic DNA using oligonucleotides oLP4 (AACTGC-AGCTCTTCTTGGTTTCGTTGGA) and oLP3 (TCCCCGGGAG-GGGTTGGTCACTTTAG). The amplified PCR product was cleaved with *Pst*I and *Sma*I, and subcloned in a pFL36 centromeric plasmid (Bonneaud *et al.*, 1991) cleaved by the same restriction enzymes. The resulting plasmid was introduced into the *mrm2* Δ strain to generate YCB689.

Protein analysis, western blotting and immunoprecipitation

Protein extraction and analysis were as previously described (Bonnerot *et al.*, 2000). Antibodies were added at the following dilutions: mouse anti-Cox2 (Molecular Probes), 1/250; mouse anti-Tcm1 (Vilardell and Warner, 1997), 1/5000; mouse anti-PAB1 (Adam *et al.*, 1986), 1/10 000; rabbit anti-Qsr1 (Tron *et al.*, 1995) 1/1000. Anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase (HRP) were obtained from Sigma and used at 1/10 000 and 1/7 000 dilutions, respectively. Immunoprecipitation was performed with a native extract from strain YCB651 with 20 μ l of immunoglobulin G-Sepharose beads (Pharmacia) as previously described except that two final washes were performed with polysome buffer (Bonnerot *et al.*, 2000).

Preparation of mitochondria-enriched fractions

Cells were cultivated in 2% glycerol-containing medium until saturation. After two washes in water, cells were washed and resuspended in

breaking buffer [0.44 M sorbitol, 1 mM EDTA, 0.3% bovine serum albumin (BSA) in 10 mM PIPES pH 6.7]. Zirconium beads were added and cells were lysed by vortexing at 4°C for 6 min on a multimixer. Debris was removed by centrifugation at 4000 g for 5 min. Centrifugation at 20 000 g for 30 min gave a pellet that was resuspended in breaking buffer, clarified at 4000 g for 5 min, then centrifuged at 20 000 g for 30 min. The resulting pellet corresponds to the mitochondria-enriched fraction.

Polysome analysis and preparation of the LSU

Polysome analysis was performed as described previously (Bonnerot *et al.*, 2000). LSUs were prepared from the disrupted YCB640 strain. After centrifugation on a sucrose gradient, two fractions corresponding to the LSU peak were pooled and pelleted by centrifugation at 226 000 g for 40 min in a TL100.3 (Beckman). LSUs were resuspended in polysome buffer and stored at -80°C until use.

Immunofluorescence microscopy

Cells were prepared as described previously (Pintard *et al.*, 2000). Immunodetection was achieved as follows. Alexa 488 rabbit anti-mouse IgG (Molecular Probes) was added as a primary antibody at 1/250 dilution. After three washes in phosphate-buffered saline (PBS)-Tween 0.1%, alexa 488 goat anti-rabbit IgG (Molecular Probes) was used as the secondary antibody at 1/250 dilution. After three more washes in PBS-0.1% Tween, the slide was mounted in 80% glycerol in PBS containing DAPI (Sigma). The slide was viewed on a Leitz microscope and images were acquired with a high-resolution camera.

RNA extraction and primer extension analysis

RNA extraction was performed as described previously except that dichloromethane was used instead of chloroform. Primer extension was carried out on 5 μ g of total yeast RNA with 100 000 c.p.m. purified (QIAquick Nucleotide Removal Kit; Qiagen) end-labelled oligonucleotides o21S1 (CGTATTTAACCAACTCACGTAACA) or o21S2 (ATGGACTTATTCAGATACTTTTGTC) in buffer for AMV reverse transcriptase supplied by the manufacturer (Promega). After 5 min at 85°C, the mix was allowed to reach 55°C, then deoxyribonucleotides (1 mM or 4 μ M) and the enzyme were added. The reaction was allowed to proceed at 42°C for 1 h before ethanol precipitation. Products of the primer extension were resolved on a 12% acrylamide-urea gel and quantified with a phosphorimager.

Methylation assay

Affinity-purified Mrm2-protAp was incubated in polysome buffer containing 33 μ M AdoMet with either RNA or LSUs prepared from the disrupted strain (YCB640) for 30–60 min at 37°C. RNA was then extracted by phenol-dichloromethane and analysed by primer extension as described above.

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