The Yeast GTPase Mtg2p Is Required for Mitochondrial Translation and Partially Suppresses an rRNA Methyltransferase Mutant, *mrm2*

Kaustuv Datta, Jennifer L. Fuentes, and Janine R. Maddock*

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048

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The assembly of ribosomes involves the coordinated processing and modification of rRNAs with the temporal association of ribosomal proteins. This process is regulated by assembly factors such as helicases, modifying enzymes, and GTPases. In contrast to the assembly of cytoplasmic ribosomes, there is a paucity of information concerning the role of assembly proteins in the biogenesis of mitochondrial ribosomes. In this study, we demonstrate that the *Saccharomyces cerevisiae* GTPase Mtg2p (Yhr168wp) is essential for mitochondrial ribosome function. Cells lacking *MTG2* lose their mitochondrial DNA, giving rise to petite cells. In addition, cells expressing a temperature-sensitive *mgt2-1* allele are defective in mitochondrial protein synthesis and contain lowered levels of mitochondrial ribosomal subunits. Significantly, elevated levels of Mtg2p partially suppress the thermosensitive loss of mitochondrial DNA in a 21S rRNA methyltransferase mutant, *mrm2*. We propose that Mtg2p is involved in mitochondrial ribosome biogenesis. Consistent with this role, we show that Mtg2p is peripherally localized to the mitochondrial inner membrane and associates with the 54S large ribosomal subunit in a salt-dependent manner.

INTRODUCTION

The assembly of ribosomes requires the coordinate processing and modification of rRNAs with the temporal association of ribosomal proteins. Biogenesis of ribosomes also requires a number of assembly proteins such as helicases, modifying enzymes, and GTPases. In addition to the well characterized Ras-like GTP-binding proteins, all organisms possess a number of conserved GTPases predicted to play roles in translation (Leipe *et al.*, 2002). Direct evidence for a role in ribosome function has been obtained for several of these GTPases, including the bacterial Era and Obg proteins (Tan *et al.*, 2002; Lin *et al.*, 2004; Wout *et al.*, 2004).

The Obg subfamily of GTPases has been identified in all organisms sequenced to date. Prokaryotes have one Obg protein and archaea have two paralogs, whereas eukaryotes such as the yeast *Saccharomyces cerevisiae* possess four paralogs in three distinct protein classes (Leipe *et al.*, 2002). Obg proteins possess a highly conserved GTP-binding domain, suggesting that they share a common mode of regulation. Mitochondrial and bacterial Obg proteins are likely to be orthologous because they are conserved throughout their protein lengths, in contrast to the other eukaryotic Obg proteins that possess vastly different protein sequences outside of the GTP-binding domain.

In *S. cerevisiae*, each distinct Obg protein type seems to play a specialized role in ribosome function. The nucleolar Nog1p protein copurifies with pre60S intermediates and is involved in cytosolic ribosome assembly, particularly in bio-

genesis of the pre60S particle (Fromont-Racine *et al.*, 2003; Kallstrom *et al.*, 2003; Saveanu *et al.*, 2003). In contrast, Rbg1p and Rbg2p, two cytosolic Obg proteins that are 52% similar to one another, associate with translating ribosomes (Wout and Maddock, unpublished data). Interestingly, Rbg1p also may be involved in stress response because it interacts on the translating ribosome with the stress response protein Gcn1p (Marton *et al.*, 1993) through a bridging protein, Gir2p (Wout and Maddock, unpublished data).

Here, we describe the analysis of the fourth *S. cerevisiae* mitochondrial Obg protein, encoded by YHR168W, and hereafter called Mtg2p for Mitochondrial GTPase 2. We show that deletion of *MTG2* leads to loss of mitochondrial DNA, consistent with a role in mitochondrial ribosome function. Mtg2p is found to be peripherally associated with the mitochondrial inner membrane facing the matrix compartment. Moreover, Mtg2p fractionates with the mitochondrial large ribosomal subunit in a salt-dependent manner. Yeast cells harboring a temperature-sensitive allele of *MTG2* are defective in translation and have reduced levels of mitochondrial subunits at the nonpermissive temperature. Finally, overexpression of *MTG2* partially suppresses a 21S rRNA methyltransferase mutant, $\Delta mrm2$. Together, these results suggest that Mtg2p plays a critical role in large mitochondrial ribosome function.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains used in this study are listed in Table 1. Complete media used were YEP (1% yeast extract, 2% peptone) containing 2% glucose (YPD), 6% ethanol (YPE), or 3% glycerol (YPG) as carbon source. Synthetic minimal media (0.67% yeast nitrogen base without amino acids containing 2% glucose [SD], 2% galactose [SGal], 0.1% 5-Fluoro-orotic acid, 2% glucose [5FOAD], 0.006% canavanine sulfate, 2% glucose [SCAN]) were supplemented with appropriate amino acids when required, as described previously (Guthrie and

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* Corresponding author. E-mail address: maddock@umich.edu.

Table 1. Yeast strains used in this study

Strain	Relevant genotype	Source
CRY1	<i>MATa, ura3-52, trp1Δ2, leu2-3112, his3-11, ade2-1, can1-100, ρ⁺</i>	Brickner and Fuller, 1997
CRY2	<i>MATα, ura3-52, trp1Δ2, leu2-3112, his3-11, ade2-1; can1-100, ρ⁺</i>	Brickner and Fuller, 1997
SEY6210/SEY6211	<i>MATa/α, leu2-3112/leu2-3112, ura3-52/ura3-52, his3Δ200/his3Δ200, trp1-Δ901/trp1-Δ901, LYS2/lys2-801, ADE2/ade2-101, suc2-Δ9/suc2-Δ9, ρ⁺</i>	Brickner and Fuller, 1997
BY4742	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, ρ⁺</i>	ATCC
JM1837	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mtg2::kanMX4, ρ⁰</i>	EUROSCARF
JM1823	<i>MATa, ura3-52, his3-1, leu2-3112, trp1-289, mrm2::kanMX4, ρ⁰</i>	EUROSCARF
JM1931	<i>MATa, ura3-52, trp1Δ2, leu2-3112, his3-11, ade2-1, can1-100, ρ⁰</i>	This study
JM1914	<i>MATa/α, ura3Δ0/ura3-52, leu2Δ0/leu2-3112, his3Δ1/his3-11, trp1Δ2/TRP1, LYS2/lys2Δ0, ADE2/ade2-1, CAN1/can1-100, MTG2/mtg2::KanMX4, ρ⁺</i>	This study
JM1918	<i>MATα/a, ura3Δ0/ura3-52, leu2Δ0/leu2-3112, his3Δ1/his3-11, trp1Δ2/TRP1, LYS2/lys2Δ0, ADE2/ade2-1, CAN1/can1-100, MTG2/mtg2::KanMX4, pKD5 [CEN, MTG2, URA3], ρ⁺</i>	This study
JM3680	<i>MATa/α, his3/his3Δ1, leu2/leu2Δ0, lys2Δ0/lys2Δ0, ura3/ura3Δ0, ADE2/ade2-1, CAN1/can1-100, mtg2::kanMX4/mtg2::kanMX4, ρ⁰</i>	This study
JM2195	<i>MATα, lys2Δ0, ura3, leu2, his3, can1-100, mtg2::kanMX4, pKD5 [CEN, MTG2, URA3], ρ⁺</i>	This study
JM2575	<i>MATα, lys2Δ0, ura3, leu2, his3, can1-100, mtg2::kanMX4, pKD7 [CEN, MTG2, HIS3], ρ⁺</i>	This study
JM2574	<i>MATα, lys2Δ0, ura3, leu2, his3, can1-100, mtg2::kanMX4, pRS313, ρ⁺</i>	This study
JM3679	<i>MATα, lys2Δ0, ura3, leu2, his3, can1-100, mtg2::kanMX4, pKD69 [CEN, mtg2-1, HIS3], ρ⁺</i>	This study
JM3666	<i>MATa/α, ura3-52/ura3Δ0, his3-1/his3Δ1, leu2-3112/leu2Δ0, trp1-289/TRP1, LYS2/lys2Δ0, MRM2/mrm2::kanMX4, ρ⁺</i>	This study
JM3668	<i>MATα, ura3, his3, leu2, lys2Δ0, trp1-289, mrm2::kanMX4, ρ⁺</i>	This study
JM3670	<i>MATα, ura3, his3, leu2, lys2Δ0, trp1-289, mrm2::kanMX4, pKD68 [CEN, MRM2, HIS3], ρ⁺</i>	This study
JM3671	<i>MATα, ura3, his3, leu2, lys2Δ0, trp1-289, mrm2::kanMX4, pRS313, ρ⁺</i>	This study
JM3672	<i>MATα, ura3, his3, leu2, lys2Δ0, trp1-289, mrm2::kanMX4, pKD7 [CEN, MTG2, HIS3], ρ⁺</i>	This study

Fink, 1991). Semisynthetic lactate medium (0.3% yeast extract, 0.05% glucose, 0.05% CaCl_2 , 0.05% NaCl, 0.06% MgCl_2 , 0.1% KH_2PO_4 , 0.1% NH_4Cl , 2% D-lactic acid, and 0.8% NaOH, pH 5.5) was prepared as indicated previously (Glick and Pon, 1995).

Plasmid and Strain Construction

Yeast genomic DNA isolation and yeast transformation were carried out as described previously (Guthrie and Fink, 1991). The *MTG2* gene was amplified from the yeast genomic DNA (CRY1) by using polymerase chain reaction (PCR) with oligonucleotides 5' GGCACGTGCCAAATTG 3' and 5' GCAT-AGGCCCTGCAAAT 3'. The 2470-bp PCR product was cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) and the product was confirmed by sequencing in both directions. *MTG2* was subcloned into pRS316 (CEN, URA3) as a *HindIII-XhoI* fragment and into pRS313 (CEN, HIS3) as an *EcoRI* fragment to yield pKD5 and pKD7, respectively. The *MRM2* gene was PCR amplified from the yeast genomic DNA from strain CRY1 with oligonucleotides 5' GGACTAGTGCACCCGTGCATATTGAAAG 3' and 5' CCATC-GATTAGGAGATGATGCAGTCAA 3'. The 1963-bp PCR product was cloned into pCR 2.1-TOPO (Invitrogen). *MRM2* was subcloned into pRS313 (CEN, HIS3) as a *SpeI-EcoRV* fragment to yield pKD68.

JM2195, a ρ^+ strain with a chromosomal *mtg2* deletion and a plasmid-borne *MTG2* was created as follows: JM1837 (Δmtg2), a ρ^0 strain, was mated to strain CRY1 to produce a *MTG2/mtg2* ρ^+ strain (JM1914). JM1914 was transformed with pKD5 to generate JM1918. After sporulation of JM1918, haploid spores were selected on SCAN. Haploid Δmtg2 ρ^+ spores containing pKD5 were screened by patching independent spore products simultaneously on YPD plates supplemented with antibiotic G418 and SD plates lacking uracil to generate JM2195. ρ^0 derivatives of CRY1 (JM1931) were obtained by growing cultures in YPD containing 50 $\mu\text{g}/\text{ml}$ ethidium bromide.

JM3668, a ρ^+ strain with a chromosomal *mrm2* deletion, was created as follows: JM1823 (Δmrm2), a ρ^0 strain, was mated to BY4742 to produce *MRM2/mrm2* ρ^+ strain (JM3666). JM3666 was sporulated, and haploid Δmrm2 spores were selected on YPD plates supplemented with antibiotic G418 to generate JM3668.

Generation of *mtg2* Temperature-sensitive (*ts*) Alleles

ts mtg2 alleles were created using error-prone PCR as described previously (Stark, 1998). The entire coding region of *MTG2* was amplified using primers 5' AGATAGAGTTATACATACTT 3' and 5' TATATTTACTATTTACAATC

3'. Three independent PCR reactions were carried out in presence of 2 mM MgCl_2 containing a 5:1 ratio of (dTTP+dCTP):(dATP+ dGTP). The concentration of MnCl_2 was varied from 0.3 to 0.5 mM. The resulting PCR products were cloned into *BsrGI* and *SnaBI* digested pKD7 by gap repair. The PCR product and pKD7 (digested with *BsrGI* and *SnaBI*) were cotransformed into JM2195, selected on SD plates lacking histidine and uracil, and patched onto 5FOAD to counterselect the *URA3* helper plasmid bearing *MTG2*. Approximately 1800 independent cells were scored for the ability to grow at 23°C but not at 37°C on YPG. Plasmid DNA from potential *ts* alleles was amplified in *Escherichia coli* and sequenced. Plasmid DNA was also shuttled into JM2195 to create *mtg2-1*, and this was confirmed for its ability to grow at 23°C but not at 37°C.

Immunoblot Analysis

Proteins were separated on 10% SDS-PAGE and processed for immunoblot as described previously (Lin *et al.*, 2004). Antibodies to Mtg2p were generated to full-length protein expressed in *E. coli* from pET28a (Novagen, Madison, WI) (our unpublished data) and used at a dilution of 1:2000 after treatment with acetone powders generated from Δmtg2 , as described previously (Harlow and Lane, 1988). The following antibody concentrations were used: Cox2p, 1:50 (Pinkham *et al.*, 1994), F1β, 1:5000 (Emtage and Jensen, 1993), KDH, 1:2000 (Koehler *et al.*, 1998), Pgk1p, 1:300 (Molecular Probes, Eugene, OR), Tim23p, 1:10,000 (Emtage and Jensen, 1993), Mrp7p, 1:200 (Fearon and Mason, 1988), Mrp13, 1:200 (Partaledis and Mason, 1988), and Mrp49, 1:200 (Fearon and Mason, 1992).

Subcellular Localization of Mtg2p

Mitochondria were isolated from 10 l of diploid SEY6210/6211 grown in semisynthetic lactate medium at 30°C to an OD₆₀₀ of 3. Mitochondria were isolated as described previously (Glick and Pon, 1995), except that cells were incubated with Lyticase (5 mg/gm dry cell weight) in 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4, at 30°C for 2 h to produce spheroplasts.

Mitochondria were subject to extraction with sodium chloride or sodium carbonate as described previously (Sogo and Yaffe, 1994). Inner and outer membrane vesicles were obtained as described previously (Daum *et al.*, 1982). Mitoplasts were generated by diluting 100 μg of mitochondria (1 mg/ml) in buffer C (0.6 M sorbitol, 20 mM K^+ HEPES, pH 7.4) with 20 mM K^+ HEPES, pH 7.4, to a final concentration of 0.1 mg/ml followed by incubation on ice for 30 min. Protease digestion of intact mitochondria (in buffer C) or mitoplasts

(in 20 mM K⁺ HEPES, pH 7.4) were carried out on ice for 30 min at the indicated concentration of proteinase K. The reactions were inactivated by addition of trichloroacetic acid (TCA) to 15%.

Separation of Mitochondrial Ribosome

Mitochondrial ribosomes were separated by sucrose density gradient as described previously (Fearon and Mason, 1992) with slight modification. Mitochondria (15 mg) were washed three times in buffer D (10 mM Tris-Cl, pH 7.4, 10 mM magnesium acetate, 100 mM NH₄Cl, 7 mM β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) and resuspended in the same buffer. Mitochondria were incubated on ice for 10 min to allow swelling of the mitochondrial membranes. Mitochondria were recovered by centrifugation at $12,000 \times g$ for 10 min at 4°C, resuspended in buffer D + 2% NP-40, and incubated on ice 10 min to allow further swelling. Lysis was achieved by 30–50 strokes of a tight fitting pestle in a Dounce homogenizer. Mitochondrial detergent lysates were clarified by centrifugation at $40,000 \times g$ for 25 min at 4°C, and UV absorbance at 260 nm (A_{260}) was measured. Equivalent amounts of A_{260} were layered on a 10 ml of 10–30% linear sucrose gradient containing 10 mM Tris-Cl, pH 7.4, 10 mM magnesium acetate, 7 mM β -mercaptoethanol and 100, 200, or 500 mM NH₄Cl, as indicated. The gradients were centrifuged at either $135,000 \times g$ for 4 h or $250,000 \times g$ for 5 h at 4°C in a Beckman SW41Ti rotor. The gradients were fractionated into 400- μ l aliquots, and the UV absorbance at 254 nm was monitored using an ISCO continuous-flow cuvette. Protein samples were precipitated by addition of TCA to 15%, separated by SDS-PAGE, and subjected to immunoblot analysis.

Analysis of Mitochondrial Translation Products

Cell cultures (15 ml) were grown in SGal with the appropriate prototrophic requirement at 23°C to an OD₆₀₀ of 1. Cells were shifted to 37°C or maintained at 23°C, as indicated. Mitochondrial translation products were labeled with [³⁵S]methionine as described previously (Fox *et al.*, 1991) except that the cells were labeled with 1 mCi of Trans ³⁵S-label for either 1 h at 23°C or 30 min at 37°C. Mitochondria were isolated as described (Fox *et al.*, 1991). Protein concentration was estimated by the Bradford method (Bradford, 1976). Equal amounts of mitochondrial proteins were separated by SDS-PAGE on a 15% polyacrylamide gel. The gel was dried and exposed to x-ray film to visualize translation products.

Suppression of *mrm2* Deletion Strain

$\Delta mrm2 \rho^+$ cells were transformed with pKD7 (JM3672) or, as controls, either the empty vector (JM3671) or plasmid-borne *MRM2* (JM3670). Cells were grown at 37°C in SD-His for 24 h and then plated on either YPD or YPG. The fraction of total cells that was able to grow on YPG represents the percentage of nonpetite cells.

RESULTS

Mtg2p is a member of the Obg family of GTPases that shares sequence similarity with the bacterial proteins along its entire length, although with some noted exceptions. First, Mtg2p possesses 88 N-terminal amino acids not found in the bacterial proteins that are predicted to contain a mitochondrial targeting sequence (PSORT; <http://psort.nibb.ac.jp/>). Consistent with the cleavage of a mitochondrial import sequence, Mtg2p migrates as an ~50-kDa band on SDS-PAGE, approximately 5 kDa smaller than the predicted molecular mass of 54.5 kDa (our unpublished data). Second, *MTG2* includes two short insertions found in Obg proteins of only a subset of eukaryotes, including fungi and plants. Based on the structure of the *B. subtilis* Obg protein, these additional domains can be positioned between conserved structural elements within an extended domain called the OBG fold (Buglino *et al.*, 2002). Deletion of a sequence containing both regions results in a nonfunctional protein, suggesting that these insertions are essential for Mtg2p function. Surprisingly, however, relatively large protein insertions such as 3HA (Longtine *et al.*, 1998) and TAP (Rigaut *et al.*, 1999) at the end of this region (at amino acid 294) did not perturb Mtg2p function (our unpublished data). Finally, *MTG2* terminates after the conserved GTP-binding domain, whereas many bacterial Obg proteins have additional conserved C-terminal sequences. It also should be noted that the *MTG2* sequence previously deposited in GenBank contains a

frameshift at amino acid 497; the corrected sequence has been entered into GenBank (AY643812).

MTG2 Is Required for Mitochondrial Translation

To determine the role of Mtg2p in vivo, a phenotypic screen (Hampsey, 1997) was performed on a $\Delta mtg2$ strain. Cells lacking *MTG2* grew slowly on YPD and were not able to use nonfermentable carbon sources such as glycerol (YPG) and ethanol (YPE) (Figure 1A). To determine whether this phenotype was due to loss of mitochondrial DNA, a $\Delta mtg2$ haploid was mated with either wild-type or an ethidium bromide-induced ρ^0 tester strain. The $\Delta mtg2$ /*MTG2* heterozygotes grew on both glucose (YPD) and glycerol (YPG) plates, indicating that the $\Delta mtg2$ is recessive (Figure 1B). The $\Delta mtg2$ /*MTG2* ρ^0 heterozygotes, however, did not grow on YPG (Figure 1B) suggesting that deletion of *MTG2* leads to loss of mitochondrial DNA. To verify that this was the case, we examined the distribution of DNA in $\Delta mtg2$. 4,6-Diamidino-2-phenylindole (DAPI)-stained wild-type cells display a bright nuclear DNA fluorescence and punctate foci representing mitochondrial DNA. In $\Delta mtg2$ cells, however, only the bright nuclear stain was visible (Figure 1C).

Loss of mitochondrial DNA in the $\Delta mtg2$ mutant indicates that the wild-type gene product either plays a direct role in maintenance of mitochondrial DNA (mtDNA) or an essential role in a distinct mitochondrial function that when disrupted leads to eventual loss of mtDNA. To determine whether the loss of mitochondrial DNA was due to a direct or an indirect consequence of deletion of *MTG2*, spore products from a $\Delta mtg2$ /*MTG2* ρ^+ heterozygous diploid were examined for growth on YPG. All products harboring $\Delta mtg2$ failed to grow (Figure 1D). We examined whether $\Delta mtg2$ spore products possessed mtDNA by mating them with a *MTG2* ρ^0 tester strain and examined the ability of the subsequent diploid to grow on YPG. Interestingly, 70% of these diploids were able to grow on YPG (our unpublished data), thus indicating a subpopulation of the cells in the newly formed $\Delta mtg2$ spore products possessed mtDNA. These newly formed $\Delta mtg2\rho^+$ spore products lost mtDNA and converted to ρ^0 cells after subsequent growth on glucose medium (our unpublished data). To examine whether these $\Delta mtg2\rho^+$ cells had a translational defect mitochondrial translation products were labeled and compared with wild-type cells. A severe defect in mitochondrial translation was detected in $\Delta mtg2\rho^+$ cells as determined by the inability to incorporate radiolabeled methionine into newly synthesized mitochondrial protein products (Figure 1E). Thus, we conclude that Mtg2p is required for mitochondrial translation, and it is likely that the ultimate loss of mtDNA in $\Delta mtg2$ cells is a consequence of the defect in translation.

Mtg2p Is a Peripherally Associated Mitochondrial Inner Membrane Protein Facing the Matrix

The phenotype of $\Delta mtg2$ mutants and the predicted mitochondrial targeting sequence provide strong evidence that Mtg2p is a bona fide mitochondrial protein. To determine the cellular location of Mtg2p, we performed a series of biochemical fractionations. Mtg2p was enriched in the mitochondrial fraction, as was Cox2p, an integral membrane subunit of the cytochrome *c* oxidase encoded by the mitochondrial genome (Pinkham *et al.*, 1994), but not in fractions that were enriched with Pgk1p, a cytosolic marker (Figure 2A). To determine the submitochondrial localization of Mtg2p, mitochondria were fractionated into membrane pellet and soluble matrix. Mtg2p cofractionated with Cox2p (Pinkham *et al.*, 1994) but not with a soluble mitochondrial matrix protein KDH (Koehler *et al.*, 1998) (Figure 2B), indi-

Figure 1. The $\Delta mtg2$ mutant lacks mitochondrial DNA. (A) Cells lacking Mtg2p do not grow on nonfermentable carbon sources. Shown are serial spot dilutions of wild-type (*MTG2*) and $\Delta mtg2$ cells on YPD, YPG, and YPE plates. (B) The $\Delta mtg2$ mutant is ρ^0 . Serial dilutions of the indicated diploids were assayed for growth on YPD and YPG. Strains are as described in the text. (C) Representative fluorescent images of DAPI-stained DNA in wild-type and $\Delta mtg2$ haploid cells reveal a lack of mtDNA in $\Delta mtg2$ mutants. In each cell, the large focus is the cell nucleus and the smaller punctuate foci represent mitochondrial DNA. (D) Replica plate analysis of dissected tetrads from heterozygous diploid $\Delta mtg2/MTG2p^+$.

cating that Mtg2p is associated with the mitochondrial membrane. To examine the nature of association of Mtg2p with the mitochondrial membrane, mitochondria were treated with 0.1 M Na_2CO_3 , 1 M NaCl, or buffer. Sodium carbonate dislodges peripheral membrane proteins but leaves integral membrane proteins attached (Fujiki *et al.*, 1982). Like the membrane protein Cox2p and the peripheral membrane protein F1 β (Emtage and Jensen, 1993), Mtg2p remained in the membrane pellet when treated with either the buffer control or sodium chloride (Figure 2C), indicating a tight association with the membrane. In contrast, when mitochondria were treated with sodium carbonate, Cox2p was present in the pellet fraction, whereas Mtg2p and F1 β were found in the soluble fraction (Figure 2C). Therefore, Mtg2p is a peripheral membrane protein, consistent with absence of predicted transmembrane domain(s) within Mtg2p.

To determine whether Mtg2p was associated with the outer or inner mitochondrial membrane, a series of protease sensitivity assays were performed. To examine whether Mtg2p was associated with the outer membrane, intact mitochondria were treated with increasing concentrations of proteinase K in the presence or absence of TritonX-100. Inner membrane proteins (such as Cox2p and Tim23p) were inaccessible to exogenously added protease in the absence of detergent, indicating that the outer membrane was intact in our preparation (Figure 2D). Mtg2p, also remained protease protected in intact mitochondria (Figure 2D), indicating that Mtg2p does not have domains facing the cytosol. Furthermore, when inner membrane vesicles were separated from outer membrane vesicles, Mtg2p was present predominantly with the inner membrane vesicles, as was F1 β (our unpublished data).

We next determined whether Mtg2p faces the intermembrane space or the matrix compartment. Mitochondria were subjected to hypo-osmotic shock, which disrupts the outer membrane of the mitochondria, thereby making the intermembrane space accessible to protease. The resulting mitoplasts were treated with increasing concentrations of proteinase K in presence or absence of Triton X-100. In absence of any detergent, F1 β , a peripheral inner membrane protein facing the matrix compartment remains protease protected, confirming that the inner membrane remained intact (Figure 2E). As expected Cox2p and Tim23p, inner membrane proteins with epitopes facing the intermembrane space (Emtage and Jensen, 1993; Pinkham *et al.*, 1994), were progressively degraded by increasing concentrations proteinase K (Figure 2E). Under these conditions, however, Mtg2p remained protease protected, mimicking the properties of a matrix protein (Figure 2E). Together, these studies demonstrate that Mtg2p is a mitochondrial matrix protein tightly associated with the inner membrane.

Mtg2p Fractionates with Mitochondrial Ribosomal Proteins in a Salt-dependent Manner

In mitochondria, translation takes place on ribosomes associated with the inner membrane (Spithill *et al.*, 1978; Marzuki and Hibbs, 1986). Mutations in genes involved in mitochond-

Haploid spore products (a–d) from dissected tetrads (1–10) were grown on YPD, replica plated onto YPG and YPD with G418, as indicated. (E) [^{35}S]Methionine labeling of newly synthesized mitochondrial gene products in *MTG2* or $\Delta mtg2p^+$. Mitochondria were isolated, and equivalent amounts of protein were separated by SDS-PAGE, the gel dried, and the radioactive proteins visualized on x-ray film. The position of Cox1p, Cox2p, Cytbp, Cox3p, and Atp8/9p is indicated.

Figure 2. Mtg2p is enriched on the mitochondrial inner membrane facing the matrix. Localization of Mtg2p in mitochondria. (A) Yeast cell extracts (CE) were fractionated into cytosol (C) and mitochondria (M). Proteins were separated by SDS-PAGE and subjected to immunoblot analysis. (B) Mitochondria were disrupted by sonication and further fractionated into membrane (P) and soluble matrix fractions (S) by centrifugation at $100,000 \times g$. Equivalent amounts of protein were separated by SDS-PAGE and subjected to immunoblot analysis. (C) Purified mitochondria were either treated with 0.1 M Na_2CO_3 , 1 M NaCl or buffer as indicated. Soluble (S) and membrane (P) fractions were subjected to SDS-PAGE and immunoblot analysis. (D) Intact mitochondria were treated with 0–500 μM /ml proteinase K in the presence or absence of 1% Triton X-100 (TX100), as

indicated. Samples were TCA precipitated, separated by SDS-PAGE, and subjected to immunoblot analysis. (E) Mitoplasts were generated by subjecting purified mitochondria to osmotic shock to disrupt the outer membrane. Mitoplasts were then treated with 0–200 μM /ml proteinase K at the indicated concentration in the presence or absence of 1% Triton X-100 as indicated. Samples were TCA precipitated, separated by SDS-PAGE, and subjected to immunoblot analysis. Samples were analyzed using antibodies against Mtg2p, Cox2p, Pgk1p, F1 β , KDH, and Tim23p. Cox2p and Tim23p are integral inner membrane proteins having antibody epitopes facing the intermembrane space of the mitochondria. F1 β is a peripherally associated inner membrane protein facing the mitochondrial matrix, whereas KDH is a soluble matrix protein. Pgk1p is a cytosolic marker protein.

drial ribosome function lead to either complete loss or large deletions of mitochondrial DNA (Myers *et al.*, 1985). The peripheral association of Mtg2p with the inner membrane (Figure 2), the loss of mitochondrial DNA in the Δmtg2 strain (Figure 1), and the predicted role of GTPases in translation (Leipe *et al.*, 2002) strongly suggest a role for Mtg2p in ribosome function. To examine whether Mtg2p associates with the mitochondrial ribosome, ribosomal subunits were separated on sucrose gradients. The identities of individual subunits were confirmed by subjecting fractions to immunoblot analysis by using antibodies to the small subunit (37S) protein Mrp13p (Partaledis and Mason, 1988) and the large subunit (54S) protein Mrp7p (Fearon and Mason, 1988). In buffer containing 100 mM NH_4Cl , Mtg2p cofractionated with the large subunit marker Mrp7p (Figure 3A). Increasing NH_4Cl concentration to 200 mM, however, resulted in reduced Mtg2p fractionation with the large ribosomal subunit (our unpublished data) and that association was further diminished in 500 mM NH_4Cl (Figure 3B). We conclude that Mtg2p is associated with the large ribosomal subunit but is not a core ribosomal protein.

Cells Harboring *mtg2ts* Allele Are Defective in Mitochondrial Translation

To define the role of Mtg2p in mitochondrial ribosome function, temperature-sensitive alleles of *mtg2* were generated and expressed episomally in a Δmtg2 background. A number of temperature-sensitive alleles that grew well on YPG at 23°C but not 37°C were isolated. One such allele, *mtg2-1*, was further characterized.

At the permissive temperature, cells expressing either MTG2 or *mtg2-1* grew at equivalent rates on either YPD or YPG plates (Figure 4A). At the nonpermissive temperature, however, *mtg2-1* cells grew slowly on YPG (Figure 4A). Under these conditions, the levels of mutant protein were slightly reduced (Figure 6). Interestingly, although cells expressing *mtg2-1* were impaired for growth at the nonpermissive temperature, they remained ρ^+ , even after 48–72 h at

37°C (our unpublished data), unlike Δmtg2 that were ρ^0 (Figure 1). One likely possibility is that the *mtg2-1* expresses a partially functional protein as evidenced by the reduction, but not complete elimination, of expression of cytochrome *c*

Figure 3. Mtg2p cofractionates with the large ribosomal subunit. Mitochondrial lysates from wild-type cells were separated on 10–30% sucrose gradients containing either 100 mM NH_4Cl (A) or 500 mM NH_4Cl (B) at $135,000 \times g$ for 4 h. Gradient fractions were TCA precipitated, separated by SDS-PAGE, and subjected to immunoblot analysis. Antibodies used were against Mtg2p, Mrp7p, and Mrp13p. The top of the gradient and the migration of the small 37S and large 54S subunit peaks, based on immunoblots, are indicated.

Figure 4. The temperature-sensitive *mtg2-1* mutant displays aberrant mitochondrial translation. (A) The growth of *mtg2-1* cells on YPG is impaired at 37°C. Shown are serial dilutions of cells expressing *MTG2* or *mtg2-1* on YPD and YPG plates at 23°C and 37°C, as indicated. (B) Newly synthesized mitochondrial protein products were labeled in vivo in cells expressing *MTG2* or *mtg2-1*, with [³⁵S]methionine, at either 23°C or after shift to 37°C for either 2 or 17 h. Mitochondria were isolated, and equivalent amounts of protein were separated by SDS-PAGE, the gel dried, and the radioactive proteins visualized on x-ray film. The position of Cox1p, Cox2p, Cytbp, and Cox3p is indicated.

oxidase subunit I and III (Cox1p and Cox3p) and cytochrome *b* (Cytbp) (see below).

We next assayed whether Mtg2p was important for mitochondrial translation. Cells expressing *MTG2* or *mtg2-1* allele had equivalent amounts of mitochondrial translation products when grown at 23°C (Figure 4B). In contrast, after a shift to 37°C the synthesis of Cox1p, Cox3p, and Cytbp was reduced in *mtg2-1* cells, and synthesis of Cox2p was elevated, as determined by incorporation of radiolabeled methionine (Figure 4B). The reduction of incorporation of [³⁵S]methionine into Cox1p, Cox3p, and Cytbp in *mtg2-1* at 37°C was likely due to defects in protein synthesis rather than protein stability, because radiolabeled protein levels were lower after an extended shift to the nonpermissive temperature before addition of [³⁵S]methionine (Figure 4B). To confirm that reduced levels of translation product were a consequence of a defect in protein synthesis rather than protein stability, cells were pulse labeled at 23°C and chased for 6 h at 37°C. In this case, the level, and therefore the stability, of labeled proteins were comparable in both *MTG2* and *mtg2-1* cells (our unpublished data). Thus, although preexisting translation products are equally stable at 37°C in *mtg2-1* and *MTG2* cells, cells harboring the *mtg2-1* allele are defective in mitochondrial translation at the nonpermissive temperature.

Cells Harboring *mtg2ts* Allele Are Not Defective in Early Assembly of Ribosomal Subunits

Bacterial Obg proteins seem to play a role in the late assembly of the large ribosomal subunit (Tan *et al.*, 2002; Lin *et al.*, 2004; Wout *et al.*, 2004). Given the sequence similarity of Mtg2p and the bacterial Obg proteins, one possibility is that the translational defect observed in *mtg2-1* cells is an indirect consequence of improper ribosome assembly. To determine whether this was the case, we first examined whether there were gross alterations of mature mitochondrial ribosomal subunit ratios in *mtg2-1* cells at the nonpermissive temperature. Cells harboring either *MTG2* or *mtg2-1* at 23°C have similar ratios of 54S to 37S subunits, as well as similar total levels of ribosomal subunits (Figure 5A). Growth of either *MTG2* or *mtg2-1* at 37°C for 2 or 8 h leads to a slight accumulation of 54S relative to 37S, although there are no significant differences between these strains (Figure 5, B and C). When *mtg2-1* cells were shifted to 37°C for an extended period (17 h), however, the total level of ribosomal subunits was reduced but the relative ratio of 54S to 37S was unchanged (Figure 5D), consistent with the translational defects observed under similar conditions (Figure 4B). We conclude that Mtg2p does not play a critical role in the early assembly of either mature 37S or 54S ribosomal complexes.

To confirm that Mtg2p does not function in early ribosome assembly, we examined the steady-state levels of both early and late-assembling mitochondrial ribosomal proteins. Stable accumulation of some, but not all mitochondrial ribosomal proteins requires the presence of full-length rRNA (Fearon and Mason, 1988; Partaledis and Mason, 1988; Dang *et al.*, 1990; Fearon and Mason, 1992; Pan and Mason, 1995, 1997; Biswas and Getz, 1999). It has been proposed that the difference in stability of these ribosomal proteins is due to differences in their temporal association during mitochondrial ribosome assembly, with early assembly proteins being unstable in mutants defective for early steps of ribosomal assembly (Fearon and Mason, 1988, 1992; Biswas and Getz, 1999). For example, MRP7, a protein thought to associate at a late step in large ribosome assembly, accumulates stably in mitochondria from ρ^0 cells lacking both 21S and 15S rRNA (Fearon and Mason, 1988), whereas two early assembling proteins, MRP13 and MRP49, do not accumulate in either early ribosome assembly mutants or in strains lacking rRNA (Partaledis and Mason, 1988; Fearon and Mason, 1992; Biswas and Getz, 1999). We show here that the levels of Mrp7p, Mrp13p, and Mrp49p remained constant in mitochondria from cells harboring either a *MTG2* or *mtg2-1* allele after shift to 37°C (Figure 6), confirming that Mtg2p is not involved in a critical step in early ribosome synthesis.

Multiple Copies of *MTG2* Partially Suppress a Methyltransferase Mutant, Δ rmr2

In *E. coli*, overexpression of the Obg protein CgtA_E suppresses both the growth and ribosome assembly defects of Δ rmrJ mutant, providing evidence that CgtA_E is involved in the late assembly of the large ribosomal subunit (Tan *et al.*, 2002). RmrJ is an rRNA methyltransferase responsible for methylation of U₂₅₅₂ within the peptidyl transferase center of the 23S rRNA (Bugl *et al.*, 2000; Caldas *et al.*, 2000). Nucleotide modification of the large rRNA molecule within the peptidyl transferase center is a universally conserved feature in prokaryotic, eukaryotic, as well as mitochondrial translation system. The 21S mitochondrial rRNA contains three modified nucleotides: one pseudouridine (ψ_{2819}) and two 2'-O-methylated nucleotides (Gm₂₂₇₀ and Um₂₇₉₁) (Sirum-Connolly and Mason, 1993; Ansmant *et al.*, 2000; Pin-

Figure 5. The levels of both 37S and 54S ribosomal particles are reduced in *mtg2-1* mutants at the nonpermissive temperature. Mitochondrial ribosome profiles from cells expressing either *MTG2* or *mtg2-1* grown (A) continuously at 23°C or shifted to 37°C for (B) 2h, (C) 8h or (D) 17h. Mitochondrial ribosomal subunits were fractionated on 10–30% linear sucrose gradients containing 200 mM NH_4Cl at $250,000 \times g$ for 5 h and monitored by UV absorbance at 254 nm. The positions of the 37S and 54S peaks are labeled. The relative ratios of the 54S/37S peak areas are listed below each profile. Asterisks indicate contaminating peaks that do not correlate with mitochondrial ribosomal proteins (data not shown).

tard *et al.*, 2002). In *S. cerevisiae*, the RrmJ ortholog Mrm2p is responsible for methylation of U_{2791} in 21S rRNA (analogous to position U_{2552} in *E. coli*) both in vivo and in vitro (Pintard *et al.*, 2002). *S. cerevisiae* cells lacking *MRM2* rapidly lose mitochondrial DNA, becoming ρ^0 at 37°C (Pintard *et al.*, 2002), a phenotype consistent with a role in late 54S assembly.

Figure 6. The stability of early assembling large ribosomal proteins is not affected in *mtg2-1*. Mitochondrial protein extracts obtained from cells expressing *MTG2* or *mtg2-1* allele grown at either 23°C or 37°C (for 2 or 17 h), as indicated, were separated by SDS-PAGE and subjected to immunoblot analysis. The relative levels of Mtg2p, the early assembling proteins Mrp49p and Mrp13p, and the late assembling protein Mrp7p were detected using protein specific antibodies. Loading controls include Cox2p and Tim23p, two proteins that should remain unaffected in this assay.

To provide evidence that Mtg2p functions late in 54S assembly, we tested whether multiple copies of *MTG2* would suppress the $\Delta mrm2$ mutant. To do this, we monitored the rapid conversion from ρ^+ to ρ^0 in $\Delta mrm2$ cells incubated at 37°C in cells harboring either complementing *MRM2* or *MTG2*, on plasmids. The addition of *MTG2* on a CEN plasmid resulted in an approximately twofold increase in Mtg2p levels (Figure 7A). As shown previously (Pintard *et al.*, 2002), $\Delta mrm2$ cells harboring a control plasmid rapidly lost mitochondrial DNA at 37°C and became ρ^0 at a high frequency (monitored by growth on YPG), whereas $\Delta mrm2$ mutants complemented with *MRM2* remained ρ^+ (Figure 7, B and C). Interestingly, the introduction of *MTG2* partially suppressed the loss of mitochondrial DNA in $\Delta mrm2$ mutants (Figure 7, B and C). The expression of *MTG2* from a 2μ plasmid did not result in further suppression (our unpublished data). Thus, the introduction of extra copies of *MTG2* partially suppresses the thermosensitive loss of mitochondrial DNA of $\Delta mrm2$ and is consistent with a role of Mtg2p in late ribosome assembly.

DISCUSSION

Ribosome biogenesis, in general, is a complex process requiring numerous factors that, although they interact with precursor ribosome intermediates and aid in their maturation into functional subunits, often are not a part of the mature ribosome. For example, in *S. cerevisiae* the assembly of cytoplasmic ribosomes requires the coordinated action of ~170 assembly proteins (Fromont-Racine *et al.*, 2003). Many auxiliary proteins are required for temporal processing and modification of rRNA, coordinated loading of ribosomal proteins onto rRNA to form functional subunits and ribo-

Figure 7. Partial suppression of $\Delta mrm2$ by multiple copies of *MTG2*. (A) Mitochondrial protein extracts from $\Delta mrm2$ cells harboring either *MRM2* or *MTG2* on a CEN plasmid were grown at 30°C, separated by SDS-PAGE, and subjected to immunoblot analysis by using antibodies against Mtm2p and Tim23p (as a loading control). (B) *MTG2* partially suppresses the formation of p^0 cells in $\Delta mrm2$ mutants at 37°C. Serial dilution of $\Delta mrm2$ cells harboring an empty vector, *MRM2*, or *MTG2* were assayed for growth on YPD and YPG plates at 37°C as indicated. (C) Quantitative analysis reveals the partial suppression of $\Delta mrm2$ by *MTG2*. Multiple cultures of $\Delta mrm2$ cells harboring an empty vector, *MRM2* or *MTG2* were grown in glucose media at 37°C for 24 h and plated on YPD and YPG plates. The number of nonpetite cells, as assayed by growth on both YPG and YPD, was counted. Shown are the average $\pm$ SD of the results corresponding to six independent starting cultures.

somal export from the nucleus. These assembly factors include nucleases, ATP-dependent RNA helicases, and modifying enzymes, as well as a number of recently identified GTPases (Kressler *et al.*, 1999; Venema and Tollervey, 1999; Fatica and Tollervey, 2002; Fromont-Racine *et al.*, 2003). In *E. coli*, although ribosomal subunits can be reconstituted in vitro by using purified components, this process is slow and requires nonphysiological conditions (Herold and Nierhaus, 1987; Culver and Noller, 2000). In vivo, however, additional nonribosomal proteins (rRNA-modifying enzymes, chaperones, and GTPases) aid in efficient assembly of bacterial ribosomal subunits (Bugl *et al.*, 2000; Tan *et al.*, 2002; Inoue *et al.*, 2003).

The assembly of mitochondrial ribosomes requires the coordinated synthesis of mitochondrially encoded rRNAs and nuclear encoded proteins as well as the import of these cytosolic proteins into the mitochondria and association into functional subunits. Interestingly, only few mitochondrial

ribosome assembly factors have been identified (Sirum-Connolly and Mason, 1993, 1995; Sirum-Connolly *et al.*, 1995; Pintard *et al.*, 2002; Barrientos *et al.*, 2003). These include the 21S rRNA-modifying enzymes that are responsible for two 2'-O-methylated nucleotides (Gm₂₂₇₀ and Um₂₇₉₁) and one pseudouridine (ψ_{2819}) that form a part of the conserved peptidyl transferase center (Sirum-Connolly and Mason, 1993; Ansmant *et al.*, 2000; Pintard *et al.*, 2002). More significantly, two novel GTPases, Mss1p and Mgt1p, have been identified that functionally interact with 15S and 21S rRNA, respectively (Decoster *et al.*, 1993; Barrientos *et al.*, 2003). In addition, Mgt1p, is involved in biogenesis of the large ribosomal subunit (Barrientos *et al.*, 2003). Mgt1p is a member of the YawG/YlqF subfamily of GTPases (Leipe *et al.*, 2002) that includes the 60S biogenesis GTPases Nug1p and Nog2p.

In this study, we show that another putative mitochondrial GTPase, Mgt2p, also seems to play a role in the assembly of the large ribosomal subunit. We demonstrated that, as expected for a ribosome assembly factor, Mgt2p is localized to the mitochondria and peripherally associated with the inner membrane facing the mitochondrial matrix (Figure 2). Furthermore, deletion of *mtg2* led to a decrease in mitochondrial translation and subsequent loss of mitochondrial DNA (Figure 1), a phenotype commonly found in cells defective in translation (Myers *et al.*, 1985; Fearon and Mason, 1992).

Although the precise function of Mgt2p is unknown, it is likely that Mgt2p is either involved directly in the biogenesis of the mitochondrial large ribosomal subunit or plays an as yet undefined role in translation. We favor the former hypothesis for several reasons. First, Mgt2p is required for mitochondrial translation, as shown indirectly by the loss of mtDNA in a $\Delta mtg2$ mutant (Figure 1) and directly in the analysis of mitochondrial translation products in the temperature-sensitive *mtg2-1* mutant (Figure 4). Second, Mgt2p associates specifically with the 54S subunit but is not a core ribosomal protein, as demonstrated by its dissociation in 500 mM NH_4Cl . Third, Mgt2p is not necessary for early ribosome assembly, because yeast cells harboring the *mtg2-1* allele did not display a drop in the stability of large ribosomal proteins that assemble early (Figure 6). Fourth, *mtg2-1* mutants do not exhibit an altered ribosomal subunit ratio, but it did show reduced levels of both 34S and 54S ribosomal subunits at the nonpermissive temperature (Figure 5). It has been proposed that the relative levels of mitochondrial ribosomal subunits are coordinately controlled (Biswas and Getz, 1999). Thus, a reduction in the assembly of the 54S subunit may coordinately lower 37S subunit to maintain the optimal subunit ratio. Finally, extra copies of Mgt2p partially suppress the thermosensitive formation of petite cells in a $\Delta mrm2$ mutant (Figure 7). Mrm2p is responsible for 2'-O-ribose methylation of U₂₇₉₁ on the 21S rRNA which is, in turn, required for optimal mitochondrial ribosome function (Pintard *et al.*, 2002). In vitro, Mmr2p methylates 21S rRNA that has already been assembled into the large 54S ribosomal subunit but not free rRNA, indicating that it is required for a late step in ribosome assembly (Pintard *et al.*, 2002). Thus, suppression of growth defects of $\Delta mrm2$ by overexpression of Mgt2p is consistent with a role of Mgt2p in a late step of ribosome biogenesis.

A role for Mgt2p in mitochondrial ribosome function is also consistent with the proposed function of Obg proteins in general, as well as the association of bacterial Obg proteins with the 50S subunit (Lin *et al.*, 2004; Wout *et al.*, 2004) and the implied role of these proteins in a late step of 50S subunit biogenesis (Tan *et al.*, 2002; Lin *et al.*, 2004; Wout *et al.*, 2004), specifically. Interestingly, in addition to a role in ribosome maturation, the bacterial Obg proteins also interact

with proteins involved in stress response (Scott and Haldenwang, 1999; Scott *et al.*, 2000; Wout *et al.*, 2004), a function that has not been reported for and is unlikely in mitochondria. One possibility is that the cytosolic Obg proteins Rbg1p and Rbg2p are involved in stress response (Wout and Maddock, unpublished data) whereas the nucleolar (Fromont-Racine *et al.*, 2003; Kallstrom *et al.*, 2003; Saveanu *et al.*, 2003) and mitochondrial Obg proteins (Nog1p and Mtg2p, respectively) may be exclusively involved in ribosome assembly. We are currently pursuing studies to determine the specific molecular role of Mtg2p in ribosomal synthesis.

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