Regulation of mitochondrial translation of the ATP8/ATP6 mRNA by Smt1p

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ABSTRACT Expression of the mitochondrially encoded ATP6 and ATP8 genes is translationally regulated by F1, ATPase. We report a translational repressor (Smt1p) of the ATP6/8 mRNA that, when mutated, restores translation of the encoded Atp6p and Atp8p subunits of the ATP synthase. Heterozygous smt1 mutants fail to rescue the translation defect, indicating that the mutations are recessive. Smt1p is an intronic inner membrane protein, which, based on its sedimentation, has a native size twice that of the monomer. Affinity purification of tagged Smt1p followed by reverse transcription of the associated RNA and PCR amplification of the resultant cDNA with gene-specific primers demonstrated the presence in mitochondria of Smt1p-ATP8/ATP6 and Smt1p-COB mRNA complexes. These results indicate that Smt1p is likely to be involved in translational regulation of both mRNAs. Applying Occam’s principle, we favor a mechanistic model in which translation of the ATP8/ATP6 bicistronic mRNA is coupled to the availability of F1 for subsequent assembly of the Atp6p and Atp8p products into the ATP synthase. The mechanism of this regulatory pathway is proposed to entail a displacement of the repressor from the translationally mute Smt1p-ATP8/ATP6 complex by F1, thereby permitting the Atp22p activator to interact with and promote translation of the mRNA.

INTRODUCTION

Translation of the bicistronic ATP8/ATP6 mRNA on yeast mitochondrial ribosomes depends on F1, ATPase (Rak et al., 2009) and at least one transcript-specific translation factor encoded by ATP22 (Zeng et al., 2007a). Atp22p is one of a large number of translational activators that target mRNAs for subunits of cytochrome oxidase (Manthey and McEwen, 1995; Costanzo and Fox, 1986; Poutre and Fox, 1987), cytochrome b (Rödel and Fox, 1987), and Atp9p (Ellis et al., 1999). These factors are likely to have similar functions related to some general feature of mitochondrial translation. The requirement of F1 for translation of ATP8/ATP6 mRNA achieves two important results. First, it provides a means by which expression of the mitochondrial ATP6 and ATP8 genes is coordinated with the availability of F1, a product of the nucleocytoplasmic genetic system. Second, translational down-regulation of the ATP8/ATP6 transcript in the absence of F1 prevents accumulation of an Atp6p-Atp9p ring complex capable of equilibrating protons across the inner membrane and discharging the mitochondrial membrane potential (Godard et al., 2011).

In an earlier study (Rak et al., 2009), we reported that ATP22, when present in high copy number, was able to rescue expression in F1 mutants of the reporter gene ARG8 when the latter is substituted for ATP6 in mitochondrial DNA (mtDNA; Steele et al., 1996; Bonnefoy and Fox, 2000). Suppression was attributed to enhanced translation of ARG8*/ATP8 mRNA as a result of the high concentration of the messenger-specific translational activator Atp22p. This approach was used in the present study to screen for and characterize other suppressors. The screen enabled us to identify the product of reading frame YUL147c, here named SMT1 (Suppression of Mitochondrial Translation), as an important component of the regulatory pathway responsible for the F1-dependent translation of the ATP8/ ATP6 mRNA. On the basis of the phenotype of an smt1-null mutant...
and the properties of Smt1p, we propose that it functions to maintain the ATP8/ATP6 mRNA in a translation-mute form under F1 limiting conditions.

RESULTS
Suppression of ATP6 and ATP8 expression in F1 mutants by extragenic mutations

The translation block of the ATP8/ATP6 mRNA in strains of yeast with mutations in either the α and β subunits of F1 or the Atp11p and Atp12p factors that chaperone assembly of F1 was previously corroborated in an arg8 mutants in which either the ATP6 or ATP8 coding sequence was replaced by the ARG8m, a recoded version of the gene for acetylornithine aminotransferase (Bonnefoy et al., 2000). The arginine requirement for growth in the absence of F1 provided a simple means for isolating and identifying extragenic suppressors of the block in ATP8/ATP6 mRNA translation (Rak and Tzagoloff, 2009). As shown previously, overexpression of ATP22, coding for the translational activator of the ATP8/ATP6 mRNA, was found to partially rescue the arginine auxotrophy of MR6ΔATP12ΔATP6, an arg8 and atp12 double mutant with an ARG8m substitution for ATP6 in mitochondrial DNA (Rak et al., 2009). The double mutant was also used to screen for extragenic suppressors by selecting for arginine-independent growth on glucose as the carbon source. MR6ΔATP12ΔATP6 spread at high density (∼10⁶ cells) on minimal glucose medium containing all the growth requirements except for arginine; yielded 30–50 revertants with different growth properties after incubation of the plates for 1 wk (Figure 1A). The fastest-growing revertants grew as well as the parental strain (Figure 1B). Similar results were obtained with a double arg8, atp12 mutant harboring a mitochondrial genome in which ATP8 was replaced with ARG8m (unpublished data).

One of the fast-growing revertants (MR6ΔATP12ΔATP6/R1) was analyzed genetically to ascertain whether the suppressor is nuclear or mitochondrial and whether it is dominant or recessive. A cross of a ρ⁰ derivative, MR6ΔATP12ΔATP6m, to the revertant produced diploid cells that failed to grow in the absence of arginine, indicative of a recessive nuclear suppressor (Figure 1C). This was confirmed by crosses of the kar1 strain with either an ATP6 or an ATP8-null mutation in mtDNA (DFSΔATP6 or DFSΔATP8, respectively) to the ρ⁰ derivative of the R1 revertant. Haploid transductants issued from the two crosses were verified to grow in the absence of arginine (Figure 1C). The suppressor of the R1 revertant was also tested directly in diploid cells harboring a mitochondrial arg8 allele in a translation-mute form under F1 limiting conditions.

FIGURE 1: Expression of the mitochondrial atp6::ARG8m allele in F1 mutants and revertants. (A) aMRSΔATP12ΔATP6 (200 µl) grown to early stationary phase in YPD was spread on minimal glucose medium supplemented with adenine, uracil, and tryptophan. The photograph of the arginine-independent revertant colonies was taken after incubation of the plate at 30°C for 7 d. (B) Serial dilutions of MRS-3A, an arg8 mutant (top), and arg8 mutants and revertants with additional mutations in ATP12 and the mitochondrial ATP6 genes. The different strains were spotted on minimal glucose medium containing adenine, uracil, tryptophan, with or without arginine. The plates were incubated at 30°C for 2 d. (C) Top, mutant and revertant strains with the indicated genotypes were grown as in B. The ρ⁰ derivative of the revertant aMRSΔATP12/R1 was crossed to the kar1 strain DFSΔATP6 containing the mitochondrial atp6::ARG8m mutation (middle) and to the kar1 DFSΔATP8 containing the atp8::ARG8m mutation (bottom). Four independent haploid transductants obtained in each transformation were checked for growth in the absence of arginine. (D) The atp2 and atp12 mutants aMRSΔATP2 and aMRSΔATP12, respectively, and aMRSΔATP12/R1, the atp12 mutant with the smt1-1 suppressor allele of R1, were labeled with [35S]methionine in vivo as described previously (Rak and Tzagoloff, 2009). Total cellular proteins were separated by SDS–PAGE on 12% (top) and 17% polyacrylamide gels (bottom), transferred to nitrocellulose, and exposed to x-ray film. (E) The Δarg8 mutant MR6 and the aMRSΔATP12/R1 revertant (Δarg8 Δatp12 smt1-1) were spotted on minimal glucose plus the indicated supplements and on rich ethanol/glycerol (YEPG) medium. The plates were incubated for 2 d at 30°C.
Regulation of ATP8/ATP6 mRNA in yeast

The plasmid DNA isolated from the R1 revertant strain was digested with SacI to get a region containing the insert of the yeast genomic DNA. Only a few survived this second screen. After replication of the transformants on minimal glucose (YPD) media, and incubation for 2 d at 30°C, the transformants harboring wild-type ATP6/R1 with a high-copy plasmid contain ectopic ATP6p, as evidenced by the accumulation of the unprocessed protein with its presequence (pre-Atp6p in Figure 2C). The identity of this band was confirmed by transformation of the R1 revertant with a high-copy plasmid containing ATP23, the gene for the protease that processes the precursor (Zeng et al., 2007b; Osman et al., 2007). The resultant transformants displayed only mature Atp6p (Figure 2C, right).

Cloning of SMT1

To clone the recessive suppressor, we transformed MR6ΔATP12ΔATP6/R1 with a library of yeast genomic DNA in the episomal plasmid YEp24 (Botstein and Davis, 1982). Approximately 1500 transformants were plated on 10 minimal glucose plates containing all the growth requirements of this strain plus arginine. After replication of the transformants on minimal glucose medium lacking arginine, those found to be auxotrophic for arginine were further checked for plasmid-dependent loss of growth in arginine-deficient medium. Of the several dozen putative arginine auxotrophs, only a few survived this second screen. The plasmid DNA isolated from the R1 revertant strain was determined to have a nuclear DNA insert of ~8.8 kb from chromosome X (Figure 2A). Suppression of growth in arginine-less medium was mapped to a region of the insert between the PstI and NheI sites and (pSMT1/ST9 in Figure 2A). This region contained reading frame YJL147c, reported to code for a nonessential protein associated with mitochondrial ribosomes (Kehrein et al., 2015b). A null allele of the YJL147c reading frame, henceforth referred to as SMT1 (Suppression of mitochondrial translation), was constructed by replacing the 492 nucleotides between the internal EcoRI and HindIII sites with a 1-kb fragment containing the yeast URA3 gene. This plasmid was used to replace the wild-type SMT1 of W303 with the smt1::URA3 null allele by homologous recombination (Rothstein, 1983). Uracil-dependent clones were verified by PCR amplification of the SMT1 locus to have the null allele. The smt1-null mutants grew as well as the parental wild-type strains on rich ethanol/glycerol medium (YEPL; Figure 2B, top). Deletion of SMT1 restored translation of the bicistronic ATP8/ATP6 mRNA in the F1 mutant (Figure 2, B and C). It is interesting that the smt1-null mutants displayed an increased synthesis of Atp6p, as evidenced by the accumulation of the unprocessed protein with its presequence (pre-Atp6p in Figure 2C). The identity of this band was confirmed by transformation of the smt1 mutant with a high-copy plasmid containing ATP23, the gene for the protease that processes the precursor (Zeng et al., 2007b; Osman et al., 2007). The resultant transformants displayed only mature Atp6p (Figure 2C, right).

The selection used to clone SMT1 was based on the recessiveness of the mutation in the revertant. This was confirmed by transforming the R1 revertant with SMT1 on a high-copy plasmid. Randomly picked transformants harboring wild-type SMT1 displayed the reappearance of the arginine auxotrophy (Figure 2D).

Is Smt1p the suppressor in the R1 revertant?

Because R1 suppressor and the smt1-null mutant are recessive, expression of ARG8p in a diploid mutant issued from a cross of both strains would be expected only if the two suppressors are allelic. In the complementation test, the diploid cells homozygous for the atp12 and homoathalic atp6::ARG8p and heterozygous for the smt1 deletion and R1 suppressor (MRSΔATP12,ATP6/ST9/ST1; R1) grew in the absence of arginine, indicating that the R1 suppressor is allelic with the smt1-null mutation (Figure 3A). Sequencing of PCR-amplified smt1 in the revertant showed a single base change of a G to a T, converting the glutamic acid codon 251 into a stop codon (Figure 3B). This allele will be referred to as smt1-1.
Purification and sedimentation properties of Smt1p-CH

Our failure to purify Smt1p from cells containing the protein with a single polyhistidine tag prompted us to further modify SMT1 by inserting between the end of the gene and the histidine codons a short sequence coding for the protein C epitope (Lychty et al., 2005). This new construct was integrated at the LEU2 locus of the smt1-null mutant. The SMT1-CH gene in the resultant strain aW303/SMT1-CH was verified to retain its ability to suppress translation of the ATP8/ATP6 mRNA in F1 mutants. Transformation of the Arg+ R1 revertant with integrative plasmids containing either the wild-type (pSMT1/ST19) or the modified (pSMT1/ST18) gene in both cases conferred an arginine-dependent growth phenotype on the revertant (Figure 5A).

Mitochondria of aW303/SMT1-CH were used to purify the protein from a lauryl maltoside extract by two sequential affinity steps. Most of the protein was recovered in the lauryl maltoside extract and was adsorbed on both the nickel–nitroacetic acid (Ni-NTA) and the protein C antibody–coated beads from which it was eluted under non-denaturing conditions in the presence of EDTA (Figure 5B). The elution fraction obtained by this procedure consisted predominantly of a major product that migrated as a 44-kDa protein and of a less abundant product slightly smaller in size (Figure 5, C and D). Mass spectrometric analysis of the two bands confirmed both to be Smt1p-CH. The shorter and less abundant protein may be the product of alternate processing of the N-terminal extension in the precursor during its import into mitochondria or of a proteinase-sensitive site cleaved during the purification.

The size of purified Smt1p was assessed by sedimentation of the fraction eluted from the P-C antibody beads in sucrose gradients. The protein peaked at a position of the gradient intermediate between the hemoglobin and lactate dehydrogenase size standards, with an estimated mass of 80–90 kDa corresponding an Smt1p dimer (Figure 5E, top). Similar results were obtained with a crude lauryl maltoside extract from a strain expressing HA-tagged Smt1p analyzed under similar conditions (Figure 5E, bottom).

Detection of Smt1p-ATP8/ATP6 mRNA complex

Restoration of the ATP6 and ATP8 expression in F1 mutants lacking Smt1p suggested that the latter might be a repressor that modulates translation of the ATP8/ATP6 mRNA (Figure 6A) in response to the demand of Atp8p and Atp6p for assembly with F1 and other protein constituents of the ATP synthase. The proposed function of Smt1p as a translational repressor implies its interaction with the target RNA. The existence of such a complex was tested in aW303ΔNUC1/SMT1-CH, a strain containing Smt1p double tagged with a protein C and polyhistidine and having a null mutation in NUC1, which codes for an abundant nuclease of yeast mitochondria (Vincent et al., 1988). The nuc1 mutation did not abolish but did substantially reduce the loss of mitochondrial RNA after their extraction with digitonin (Figure 6B). The procedure used to assess association of ATP8/ATP6 mRNA with Smt1p-CH (Figure 6C) entailed extraction of mitochondria with digitonin followed by affinity purification of Smt1p in
FIGURE 4: Localization of Smt1p. (A) aW303/SMT1-HA, containing Smt1p with a C-terminal HA tag, was used to isolate mitochondria (Mit) and the postmitochondrial supernatant fraction (PMS) containing soluble cytosolic proteins. Equivalent amounts of mitochondria (50 μg protein) and the soluble cytosolic protein fraction were separated by SDS–PAGE, transferred to nitrocellulose, and probed first with a monoclonal antibody against the HA and then by a secondary peroxidase-coupled anti-mouse antibody. The antigen–antibody complexes were visualized with the Super Signal Chemiluminescent Substrate Kit (Pierce, Rockford, IL). (B) Mitochondria (Mit), 0.5 ml, at a protein concentration of 10 mg/ml were disrupted by sonic oscillation and centrifuged for 20 min at 70,000 × g, into submitochondrial membrane vesicles (SMPs) and the supernatant (Sonuc sup.) consisting of soluble proteins of the intermembrane space and matrix. The submitochondrial membranes were suspended in a final volume of 0.5 ml of 0.1 M sodium carbonate and 1 mM PMSF and incubated at 4°C for 30 min. The suspension was centrifuged at 100,000 × g for 30 min. The pellets were incubated with and without protease K (Prot. K) as described previously (Zeng et al., 2007b). Samples normalized to 50 μg of starting mitochondrial protein were separated by SDS–PAGE and transferred to nitrocellulose. The Western blot was probed with a monoclonal antibody against the HA and then by a secondary peroxidase-coupled anti-mouse antibody. The antigen–antibody complexes were visualized as in A, except that the mitochondrial markers were detected with a peroxidase-coupled anti-rabbit antibody.

Additional evidence excluding the presence of the ATP8/ATP6 transcript due to nonspecific binding of RNA to the beads was excluded by lack of competition when excess yeast RNA purified from the atp6-null mutant MR10 was added to the extract (Figure 6F). Addition of this RNA increased the amount of final ATP8/ATP6 cDNA as a result of more efficient precipitation of the cDNA in the presence of the MR10 RNA.

The specificity of Smt1p for mitochondrial transcripts was also examined by testing its ability to bind the mRNA for the COB, COX3, and ATP9 for the cytochrome b subunit of the bc1 complex, subunit 3 of cytochrome oxidase, and subunit 9 of the ATP synthase, respectively. The primers internal to the COB coding sequence (Figure 7A) were verified to amplify the gene from mitochondrial DNA (Figure 7B). The COB primers also amplified a product of the right size and sequence from the RNA in the Smt1p-CH purified fraction. This product depended on the inclusion of a reverse transcriptase step before PCR amplification (Figure 7C) and was not seen in the nuc1-null mutant with the normal SMT1 gene (Figure 7C). The results obtained with PCR primers spanning the COX3 and ATP9 coding sequences could not be interpreted. Even though these primers amplified single bands of the right size with mitochondrial DNA as template, the smeared products obtained with the nuc1-negative control and with purified Smt1p-CH precluded an answer as to whether they are also associated with Smt1p.

The ATP8/ATP6 and COB mRNAs were also detected in Smt1p purified by the procedure using lauryl maltoside instead of digitonin and including purification on Ni-NTA before the protein C antibody beads (unpublished data).

**DISCUSSION**

In this study, we used genetic and biochemical means to gain additional mechanistic details underlying the previously observed F1-dependent translation of the ATP8/ATP6 mRNA (Rak and Tzagoloff, 2009). Using a double arg8 and atp12 (F1 chaperone) mutant with a substitution of the mitochondrial ATP6 gene by recoded ARG8m for acetylornithine transaminase synthesis on mitochondrial ribosomes (Steele et al., 1996), we obtained a number of arginine-independent revertants. The R1 revertant reported here grew as well as wild type in the absence of arginine and was ascertained to have a recessive mutation in a nuclear gene. The recessive nature of the suppressor was taken advantage of to isolate SMT1, which, when mutated, confers arginine-independent growth and, like the suppressor in R1, is also recessive. Sequencing of the SMT1 in the R1 revertant indicated that the suppressor mutation creates a premature termination codon in the gene. Allelism of the R1 suppressor with the smt1-null mutation was also confirmed genetically.

Smt1p sedimented approximately midway between lactate dehydrogenase and hemoglobin, suggesting a size two times larger than the monomer estimated by SDS–PAGE. Smt1p behaves as an intrinsic inner membrane protein facing the matrix compartment.

A requisite for the proposed function of Smt1p as a translational repressor is that it interacts with the ATP8/ATP6 mRNA. The presence of the ATP8/ATP6 mRNA was confirmed by PCR amplification of a reverse transcriptase–dependent cDNA product obtained with ATP8 and ATP6 primers when the template was extracted from Smt1p-CH purified on protein C antibody beads in the presence of digitonin or lauryl maltoside. On the basis of our genetic and biochemical results, we propose that the previously observed F1-dependent regulation of At6p and At8p synthesis involves a mechanism at the core of which activation of ATP8/ATP6 mRNA translation is prevented by repressor proteins. A second important
feature of this model is the labilization of the repressor-ATP8/ATP6 mRNA complex by F₁, allowing Atp22p to exercise its function as a translational activator (Figure 8). This would imply a common or overlapping binding site on the mRNA for Atp22p and Smt1p.

Of interest, RNA binding by Smt1p is not confined to the ATP8/ATP6 mRNA. Primers internal to COB yielded a PCR product of the right sequence when RNA extracted from purified Smt1p-CH was reverse transcribed and PCR amplified. Smt1p (Mrx5p) was recently reported to be associated with ribosomes in a large complex termed Miorex (Kehrein et al., 2015a,b). Purified Smt1p is a single dimeric protein, suggesting that the purification procedure may have led to its dissociation from the Miorex complex. The presence of the COB mRNA cannot be explained by the presence of the Miorex complex with both ATP8/ATP6 and COB mRNA, as purified Smt1p-CH consists of a single protein. Instead, it suggests a possible involvement of Smt1p in translational regulation of at least one other mitochondrial gene product.

MATERIALS AND METHODS

Yeast strains and growth media

The yeast strains used in this study and their genotypes are listed in Table 1. The compositions of yeast extract/peptone/glucose (YPD),
FIGURE 6: Detection of Smt1p-ATP8/ATP6 transcript in mitochondria. (A) Map of the ATP8/ATP6 mRNA. The ATP8 and ATP6 genes are denoted by the solid bars. The locations of the RT and PCR primers are shown by the arrows. (B) Stabilization of mitochondrial RNAs in digitonin extracts of mitochondria from yeast with a Δnuc1 mutation. Mitochondria (Mito) from the Δnuc1 mutant W303ΔNUC1 were extracted with phenol and nucleic acids, precipitated with ethanol, and separated on a 1% agarose gel, without treatment and after digestion with MseI restriction endonuclease to digest DNA and with RNase I to digest RNA. Mitochondria were extracted with 2% digitonin and treated identically. The two mitochondrial ribosomal RNAs and DNA are identified in the margin. (C) Outline of protocol used to purify the Smt1p-CH-ATP8/ATP6 mRNA complex. (D) Smt1p was purified from 25 μg of starting mitochondria, separated by SDS-PAGE, and probed as in Figure 4A, except that the primary antibody was against the polyhistidine tag. The fractions shown are mitochondria (M), digitonin extract (Ex), the fraction that did not adsorb to the protein C antibody beads (FT), and eluate from the beads (EL). The samples loaded were normalized to the starting volume of mitochondria. (E) Detection of an ATP8/ATP6 cDNA after RT-PCR amplification of the fraction enriched on protein C antibody beads. Left, each lane of the gel was loaded with the PCR product obtained from the equivalent of 25 μg of starting mitochondrial protein from a W303ΔNUC1 (SMT1Δnuc1) and a W303ΔNUC1/SMT1-CH (SMT1-CH Δnuc1). The mitochondria were extracted with 2% digitonin, and the extract was purified on beads as described in Materials and Methods. One-half of the nucleic acids extracted from protein C eluate was either reverse transcribed (+RT) prior to PCR amplification or directly PCR amplified (-RT). The product shown in the extreme right lane was obtained with Atp6-29 and Atp6-24 PCR primers using purified mitochondrial DNA as the template. Right, same as the left, except that the mitochondria were a W303ΔNUC1/SMT1-CH (SMT1-CH Δnuc1) and a W303ΔNUC1/LAT1-CH (LAT1-CH Δnuc1), a strain that expresses the dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase with a CH tag. (F) Effect on cDNA synthesis of addition of mitochondrial RNA from MR10 to the protein C eluate. The conditions for purification and RT-PCR amplification were the same as in E. The indicated amounts of purified total RNA from MR10 (atp6::ARG8) were added to the nucleic acids extracted from the protein C antibody beads. The purified RNA (10 μg) was also used directly as the template for PCR amplification either without or with prior reverse transcription. A faint product was obtained in both conditions. The origin of this product is not known.
absence of F\textsubscript{ATP8/ATP6} high-copy plasmid, Atp22p is able to interact with a limited amount of ATP8/ATP6 mRNA, thereby allowing Atp22p to bind to the mRNA and activate translation. Based on its sedimentation, native Atp22p, like Smt1p, is thereby allowing Atp22p to bind to the mRNA and activate translation. F\textsubscript{ATP8/ATP6} when bound to the repressor, the mRNA is prevented from weakening the interaction of the repressor with the mRNA and partially suppress the translational block in the absence of F\textsubscript{ATP8/ATP6}.

**Construction of the smt1-null allele**

The 3-kb B\textsubscript{cl}l fragment of pSMT1/T1 containing the entire SMT1 coding sequence plus 5' and 3' flanking sequences was cloned into the BamH\textsubscript{I} site of YEp352B, a shuttle plasmid identical to YEp352 (Hill et al., 1986) except for the presence of a single BamH\textsubscript{I} site instead of the multiple cloning sequence. The resultant plasmid pSMT1/ST2 was digested with EcoR\textsubscript{I} plus HindIII to remove 500 base pairs from the middle of the gene. The gapped plasmid was ligated to a 1-kb EcoR\textsubscript{I}-HindIII fragment containing the yeast URA3 gene to yield pSMT1/ST3. The smt1::URA3 allele plus flanking sequences was removed from pSMT1/ST3 as a BsrU\textsubscript{I}-Nhel fragment and was used to transform W303-1A. Several uracil-independent and respiratory-competent clones (aw303\textsubscript{ΔSMT1}) obtained from the transformation were verified by PCR amplification of the SMT1 locus to carry the smt1-null allele. The alpha mating type smt1-null mutant was obtained from a cross of aw303\textsubscript{ΔSMT1} to W303-1B.

**Construction of yeast strains that synthesize Smt1p with a C-terminal hemagglutinin and a double protein-C plus polyhistidine tag**

Primers Smt1-1 and Smt1-2 (Table 2) were used to amplify SMT1 fused at the 3' end with a sequence coding for the HA tag. The PCR product consisting of ~500 nucleotides of the 5'-untranslated region (5'-UTR), the entire SMT1 coding sequence fused to the sequence of the tag, was digested with a combination of ScaI and PstI and ligated to Ylp351 (Hill et al., 1986) linearized with the same enzymes, yielding plasmid pSMT1/ST5. Similarly, primers Smt1-1 and Smt1-3 were used to amplify the 5'-UTR and coding sequence of SMT1 without the termination codon. The PCR product was digested with a combination of ScaI and PstI and ligated to Ylp351-CH cut with the same enzymes, yielding plasmid pSMT1/ST6. The PCR product containing the entire SMT1 coding sequence fused to the polyhistidine tag, was digested with a combination of BamHI and EcoRI and ligated to YEp352 (Hill et al., 1986) linearized with the same enzymes, yielding plasmid pSMT1/ST7. Similarly, primers Smt1-1 and Smt1-4 were used to amplify the entire SMT1 coding sequence fused to the polyhistidine tag and ligated to Ylp351-CH cut with the same enzymes, yielding plasmid pSMT1/ST8. The PCR product containing the entire SMT1 coding sequence fused to the polyhistidine tag, was digested with a combination of BamHI and EcoRI and ligated to YEp352 (Hill et al., 1986) linearized with the same enzymes, yielding plasmid pSMT1/ST9. The PCR product containing the entire SMT1 coding sequence fused to the polyhistidine tag, was digested with a combination of BamHI and EcoRI and ligated to Ylp351-CH cut with the same enzymes, yielding plasmid pSMT1/ST10.

**Purification of Smt1p**

The procedure of Herrmann et al. (1994) was used to prepare mitochondria from aw303/SMT1-CH grown to early stationary phase in YPGal. In a typical purification, 20 ml of the mitochondrial suspension at a protein concentration of 10 mg/ml was adjusted to 2 mM phenylmethylsulfonyl fluoride (PMSF) with a 0.2 M ethanolic solution and 1% lauryl maltoside with a 10% solution. After centrifugation at 70,000 × g\textsubscript{av} for 10 min, the supernatant was adjusted to pH 8.2 with 1.5 M Tris-Cl, pH 8.8, and added to 1 ml of packed Ni-NTA beads that had been prewashed with Ni-NTA wash buffer (10 mM Tris-Cl, pH 7.5, 10 mM imidazole, pH 8, 100 mM NaCl). The mixture was rotated at 4°C for 2 h, and nonadsorbed proteins were removed by centrifugation at 1000 rpm for 10 s. The beads were washed three times with 5 ml of Ni-NTA wash buffer containing 0.1% lauryl maltoside and eluted with 2 ml YP plus ethanol/glycerol, YP plus galactose, and minimal glucose media have been described (Myers et al., 1985).
TABLE 1: Genotypes and sources of Saccharomyces cerevisiae strains.

| Strain            | Genotype                  | mtDNA | Source                                |
|-------------------|----------------------------|-------|---------------------------------------|
| W303-1A           | MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ | R. Rothstein (Columbia University, New York, NY) |
| W303-1B           | MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ | R. Rothstein |
| DFSΔATP6          | MATα kar1 ade2 leu2 ura3 arg8::URA3 | ρ⁺ atp6::ARG8n | This study |
| DFKΔATP8          | MATα kar1 ade2 leu2 lys2 ura3 arg8::URA3 | ρ⁺ atp8::ARG8n | This study |
| MR6ΔATP6          | MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 | ρ⁺ atp6::ARG8n | Rak and Tzagoloff (2009) |
| MR6ΔATP8          | MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 | ρ⁺ atp8::ARG8n | Rak and Tzagoloff (2009) |
| aW303ΔSMT1        | MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 | ρ⁺ | This study |
| W303ΔSMT1         | MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 | ρ⁺ | This study |
| aW303/SMT1-HA     | MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 leu2::pSMT1/ST5 | ρ⁺ | This study |
| aW303/SMT1-CH     | MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 leu2::pSMT1/ST13 | ρ⁺ | This study |
| aW303/SMT1-Chp⁰   | MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 leu2::pSMT1/ST13 | ρ⁺ | This study |
| aW303ΔNUC1        | MATα ade2-1 his3-1,15 leu2-3,112 nuc1::TRP1 trp1-1 ura3-1 | ρ⁺ | This study |
| aW303ΔNUC1ΔSMT1   | MATα ade2-1 his3-1,15 leu2-3,112 nuc1::TRP1 smt1::URA3 trp1-1 ura3-1 | ρ⁺ | This study |
| aW303ΔNUC1/SMT1-CH| MATα ade2-1 his3-1,15 leu2-3,112 nuc1::TRP1 smt1::URA3 trp1-1 ura3-1 leu2::pSMT1/ST13 | ρ⁺ | This study |
| aW303ΔNUC1/LAT1-CH| MATα ade2-1 his3-1,15 lat1::HIS3 leu2-3,112 nuc1::TRP1 trp1-1 ura3-1 leu2::pLAT1/ST6 | ρ⁺ | This study |
| MR6               | MATα ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ | Rak et al. (2007) |
| MR10              | MATα ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ atp6::ARG8n | Rak et al. (2007) |
| MRSI⁰             | MATα ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺, intronless | Rak and Tzagoloff (2009) |
| aW303ΔATP1/SMT1-CH| MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 leu2::pSMT1/ST13 | ρ⁺ | This study |
| aW303ΔCOX2/SMT1-CH| MATα ade2-1 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 leu2::pSMT1/ST13 | ρ⁺ cov2::ARG8n | This study |
| MRS-3A            | MATα ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ | Rak and Tzagoloff (2009) |
| aMRSΔATP2ΔATP6    | MATα ade2-1 arg8::HIS3 atp1::LEU2 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ atp6::ARG8n | Rak and Tzagoloff (2009) |
| aMRSΔATP2ΔATP6/R1 | MATα ade2-1 arg8::HIS3 atp1::LEU2 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 | ρ⁺ atp6::ARG8n | This study |
| aMRSΔATP12        | MATα ade2-1 arg8::HIS3 atp1::LEU2 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ | Rak and Tzagoloff (2009) |
| aMRSΔATP12/R1     | MATα ade2-1 arg8::HIS3 atp1::LEU2 his3-1,15 leu2-3,112 smt1::URA3 trp1-1 ura3-1 | ρ⁺ | This study |
| aMRSΔATP12ΔATP6   | MATα ade2-1 arg8::HIS3 atp1::LEU2 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ⁺ atp6::ARG8n | Rak and Tzagoloff (2009) |
Primer sequence  

TABLE 2: Genotypes and sources of Saccharomyces cerevisiae strains. Continued

| Strain            | Genotype | mtDNA   | Source       |
|-------------------|----------|---------|--------------|
| MRSΔATP12ΔATP6    | MATα ade-2-1 arg8::HIS3 atp12::LEU2 his3-1,15 leu2-3,112 trp1-1 ura3-1 | ρ- atp6::ARG8α | This study   |
| aMRSΔATP12ΔATP6/R1 | MAT a ade-2-1 arg8::HIS3 atp12::LEU2 his3-1,15 leu2-3,112 smt1-1 trp1-1 ura3-1 | ρ+ atp6::ARG8α | This study   |
| aMRSΔATP12ΔATP6/R1p0 | MAT a ade-2-1 arg8::HIS3 atp12::LEU2 his3-1,15 leu2-3,112 smt1-1 trp1-1 ura3-1 | ρ0 | This study   |
| aMRSΔATP12ΔATP6/R2p0 | MAT a ade-2-1 arg8::HIS3 atp12::LEU2 his3-1,15 leu2-3,112 trp1-1 ura3-1 R2sup | ρ0 atp6::ARG8α | This study   |
| aMRSΔATP12,ATP6//R1/SMT1-1.CH | MAT a ade-2-1 his3-1,15 leu2-3,112 smt1-1 trp1-1 ura3-1 arg8::HIS3 atp12::LEU2 trp1::pSMT1/ST18 | ρ+ atp6::ARG8α | This study   |
| aMRSΔATP12,ATP6//R1/SMT1 | MAT a ade-2-1 arg8::HIS3 atp12::LEU2 his3-1,15 leu2-3,112 smt1-1 trp1-1 ura3-1 | ρ+ atp6::ARG8α | This study   |
| a/αMRSΔATP12,ATP6,ΔATP6 | MAT a ade-2-1/ade-2-1 arg8::HIS3/arg8::HIS3 atp12::LEU2/atp12::LEU2 his3-1,15/15 his3-1,15 leu2-3,112/leu2-3,112 smt1-1/smt1::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 | ρ+ atp6::ARG8α | This study   |

Pull down of Smt1p and RT-PCR amplification of ATP8/ATP6 cDNA

A mitochondrial suspension representing 250 μg of protein was centrifuged at 10,000 × gav for 10 min and used to purify Smt1p as described, with the following modifications. The pellet was extracted in 50 μl of 2% digitonin in PC wash buffer. The digitonin extract was added to 30 ml of packed protein C antibody beads and rotated for 30 min. After four washes of the beads with PC buffer containing 0.5% digitonin, proteins were eluted with 60 μl of PC elution buffer containing 0.5% digitonin.

The eluate was extracted with an equal volume of water-saturated phenol, and nucleic acids were precipitated by addition of 5 M NaCl to a final concentration of 0.25 M and three volumes of ethanol. The pelleted and dried nucleic acid was dissolved in 10 μl of water and reverse transcribed in a final volume of 20 μl with the Atp6-24 primer using the Script cDNA synthesis kit (BioLab). The resultant cDNA was precipitated by addition of salt and ethanol and PCR amplified with primers Atp6-29 and Atp6-24 in a final volume of 50 μl. The cDNA was separated on a 1% agarose gel and visualized by ethidium bromide staining.

Miscellaneous procedures

Yeast was transformed by the LiAc procedure of Schiestl and Gietz (1989). Standard protocols were used for DNA cloning, purification of plasmids from Escherichia coli, and colony hybridization (Sambrook et al., 1989). The conditions for in vivo labeling of yeast mitochondrial gene products with [35S]methionine have been described (Rak et al., 2009). Proteins were separated by SDS–PAGE in the buffer system of Laemmli (1970). Protein concentration was determined by the procedure of Lowry et al. (1951).

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