Mam33 promotes cytochrome *c* oxidase subunit I translation in *Saccharomyces cerevisiae* mitochondria

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ABSTRACT **Three mitochondrial DNA–encoded proteins, Cox1, Cox2, and Cox3, comprise the core of the cytochrome *c* oxidase complex. Gene-specific translational activators ensure that these respiratory chain subunits are synthesized at the correct location and in stoichiometric ratios to prevent unassembled protein products from generating free oxygen radicals. In the yeast *Saccharomyces cerevisiae*, the nuclear-encoded proteins Mss51 and Pet309 specifically activate mitochondrial translation of the largest subunit, Cox1. Here we report that Mam33 is a third *COX1* translational activator in yeast mitochondria. Mam33 is required for cells to adapt efficiently from fermentation to respiration. In the absence of Mam33, Cox1 translation is impaired, and cells poorly adapt to respiratory conditions because they lack basal fermentative levels of Cox1.**

Monitoring Editor
Thomas D. Fox
Cornell University

Received: Apr 15, 2015
Revised: Jun 10, 2015
Accepted: Jun 16, 2015

INTRODUCTION

The terminal enzyme of the mitochondrial electron transport chain, cytochrome *c* oxidase, is a multimeric copper-heme enzyme that catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen. Cytochrome *c* oxidase biogenesis and function require the concerted expression of the nuclear and mitochondrial genomes. Three mitochondrially encoded proteins form the catalytic core, which is surrounded by 8–10 nuclear-encoded subunits in *Saccharomyces cerevisiae* and humans, respectively (Marechal *et al.*, 2012; Soto *et al.*, 2012a). At least 30 additional nuclear gene products are required for cytochrome *c* oxidase biogenesis, a subset of which is dedicated to translational activation of the mitochondrially encoded subunits (Fox, 2012; Soto *et al.*, 2012a).

In *S. cerevisiae*, each mitochondrial gene is controlled by one or more dedicated translational activators. Proposed mechanisms of activation include interaction with the 5′ untranslated regions (UTRs) of their target transcripts, association with the mitochondrial ribosome, and anchoring to the mitochondrial inner membrane (Herrmann *et al.*, 2013). These activities are often divided in cases in which multiple translational activators are required to promote the translation of a single mRNA transcript (Herrmann *et al.*, 2013). Accumulating data suggest that translational activators can help couple translation with membrane insertion (Bauerschmitt *et al.*, 2010; Gruschke *et al.*, 2011). Consistent with this idea, translational activators can physically interact to colocalize the synthesis of different subunits of a single respiratory complex (Naithani *et al.*, 2003). Finally, mitochondrial translational activators are part of feedback control loops that coordinate protein synthesis with assembly (Perez-Martinez *et al.*, 2003, 2009; Barrientos *et al.*, 2004; Mick *et al.*, 2007; Rak and Tzagoloff, 2009; Gruschke *et al.*, 2011). This helps prevent the accumulation of misassembled complexes, which could be deleterious to the cell.

Cox1 is a highly conserved core catalytic subunit of cytochrome *c* oxidase that bears copper and heme prosthetic groups for oxygen reduction. Cox1 synthesis is controlled by at least two translational activators, Pet309 and Mss51 (Decoster *et al.*, 1990; Manthey and McEwen, 1995; Siep *et al.*, 2000; Perez-Martinez *et al.*, 2003). Pet309 is an integral inner-membrane protein predicted to contain up to 12 pentatricopeptide repeat (PPR) motifs within its sequence that are important for RNA binding (Manthey and McEwen, 1995; Tavares-Carreon *et al.*, 2008; Zamudio-Ochoa *et al.*, 2014). Pet309 physically interacts with the *COX1* mRNA and the mitochondrial ribosome to promote *COX1* mRNA translation (Manthey and McEwen, 1995; Bauerschmitt *et al.*, 2010; Zamudio-Ochoa *et al.*, 2014). The second

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E15-04-0222) on June 24, 2015.

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Abbreviation used: qRT-PCR, quantitative reverse transcription PCR.

activator, Mss51, initiates *COX1* mRNA translation by binding its 5′ UTR (Decoster *et al.*, 1990; Siep *et al.*, 2000; Zambrano *et al.*, 2007; Perez-Martinez *et al.*, 2009). In addition, Mss51 is involved in coordinating Cox1 synthesis with cytochrome *c* oxidase assembly through its interaction with newly synthesized Cox1 protein and a number of other assembly factors (Perez-Martinez *et al.*, 2003, 2009; Barrientos *et al.*, 2004; Pierrel *et al.*, 2007; Fontanesi *et al.*, 2010, 2011; Mick *et al.*, 2010; Shingu-Vazquez *et al.*, 2010). On assembly of Cox1 with other cytochrome *c* oxidase subunits, Mss51 is released to activate *COX1* translation. Mss51 also senses heme and oxygen availability to regulate cytochrome *c* oxidase biogenesis (Soto *et al.*, 2012d).

In this study, we report the identification of a new Cox1 translational activator, Mam33, which is conserved in eukaryotic organisms. The factor is termed *m*itochondrial *a*cidic *m*atrix (Mam33) protein based on its cellular localization, charge, and molecular weight. The N-terminal 47 residues of Mam33 are removed upon import into mitochondria (Seytter *et al.*, 1998). Two previous reports were unable to assign a specific function to *MAM33* because cells lacking this gene remain viable and appear to retain most of their mitochondrial respiratory activity (Muta *et al.*, 1997; Seytter *et al.*, 1998). Here we present evidence that Mam33 promotes translation of mitochondrial *COX1*.

RESULTS

Cells require Mam33 to adapt efficiently from fermentative to respiratory carbon sources

S. cerevisiae prefers to ferment glucose, even in the presence of oxygen, which yields sufficient ATP for cell viability without mitochondrial respiration (the Crabtree effect; Gelade *et al.*, 2003; Johnston and Kim, 2005). However, in nonfermentable carbon sources (e.g., glycerol, lactate, or ethanol), yeast requires mitochondrial oxidative phosphorylation for ATP synthesis. Therefore a strain lacking a gene involved in mitochondrial respiration may grow poorly or not at all on nonfermentable carbon sources. The deletion of *MAM33* did not noticeably influence growth rate in media containing glucose or nonfermentable carbon sources (Supplemental Figure S1, A–C). However, when exponential-phase cells were transferred from glucose to a nonfermentable carbon source, *mam33*Δ cells required approximately twice the time to double their cell number as wild-type cells (Figure 1A). The reverse shift, from respiratory to fermentative growth, was unaffected by *mam33*Δ (Supplemental Figure S1D). Increased adaptation time for a cell culture population may result from some cells being unable to adapt, all cells adapting slowly, or a combination of both. After a shift to respiratory media, a comparable number of *mam33*Δ and wild-type cells were able to adapt (Figure 1B). However, the *mam33*Δ cells formed colonies more slowly (Figure 1C). In sum, these results demonstrate that *MAM33* is necessary for cells to adapt efficiently from fermentative to respiratory growth.

Steady-state levels of mitochondrially encoded subunits of cytochrome *c* oxidase are substantially reduced in fermenting *mam33*Δ cells

Components of the mitochondrial respiratory chain are maintained at basal levels during glucose fermentation and are up-regulated during respiratory metabolism (Ohlmeier *et al.*, 2004). For this reason, we hypothesized that *mam33*Δ cells adapt less efficiently because they do not express basal levels of respiratory chain components during fermentation. Moreover, given its mitochondrial location, we speculated that Mam33 influences respiratory components expressed from the mitochondrial genome. To test this theory,

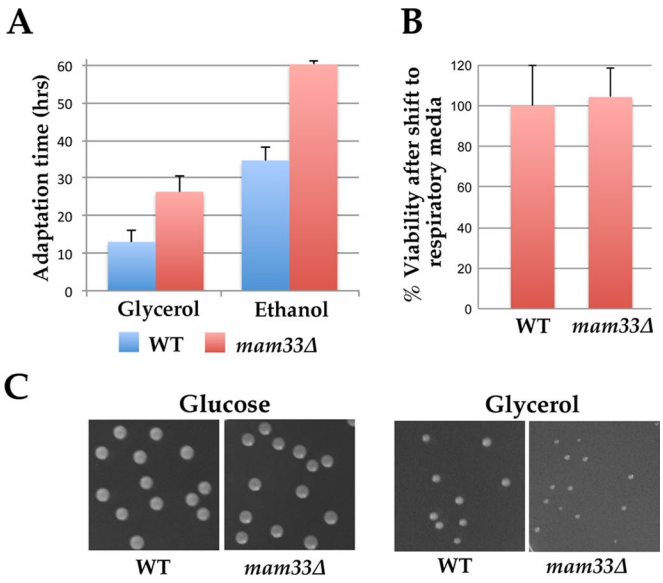

FIGURE 1: *MAM33* is required for efficient adaptation to nonfermentable carbon sources. (A) Adaptation time from a fermentable to a respiratory carbon source. Values represent the average of three independent experiments ±SEM. (B) Cell viability after transfer to a nonfermentable carbon source. Viability (percent) was calculated as number of glycerol divided by number of glucose colonies. Bars represent the average of three independent experiments ±SEM. (C) Colony formation of cells after a shift to a nonfermentable carbon source. The indicated relevant genotypes correspond to the following strains (Supplemental Table S1): wild type, BMA64-1A; *mam33*Δ, MHY1564.

we compared the levels of mitochondrial DNA (mtDNA)–encoded respiratory chain proteins in wild-type and *mam33*Δ cells. The yeast mitochondrial genome encodes seven essential subunits of three respiratory chain complexes: complex III (Cob1), complex IV (Cox1, Cox2, and Cox3) and complex V (Atp6, Atp8, and Atp9; Figure 2A; Foury *et al.*, 1998). Of interest, steady-state levels of the mtDNA-encoded subunits of complex IV (Cox1–3) were dramatically reduced in glucose-grown *mam33*Δ cells (Figure 2B). The mitochondrially encoded subunits of complex III (Cob1) and V (Atp6) were also decreased but to a lesser extent and these are likely indirect effects due to disruption of the electron transport chain. In sum, these results demonstrate that *MAM33* is required for Cox1–3 expression when cells are grown in glucose.

The abundance of nuclear-encoded complex IV subunits tested (Cox4 and Cox5) was decreased in fermenting *mam33*Δ cells but to a lesser extent than Cox1–3 (Figure 2B). The loss of the highly hydrophobic core subunits (Cox1–3) of complex IV prevents its assembly and results in the down-regulation or destabilization of the remaining subunits (Soto *et al.*, 2012a). This regulation prevents the accumulation of unassembled proteins that could form prooxidant species or aggregate and disturb membrane homeostasis. Therefore the decreases observed for the other complex subunits are likely indirect effects due to disruption of the electron transport chain.

The presence of glucose triggers a global regulatory response that represses the expression of respiratory proteins (Johnston and Kim, 2005). We predicted that if glucose repression were involved, any protein compensating for Mam33 during respiratory growth would be expressed when cells were fermenting nonrepressible sugars (e.g., galactose and raffinose). To determine whether

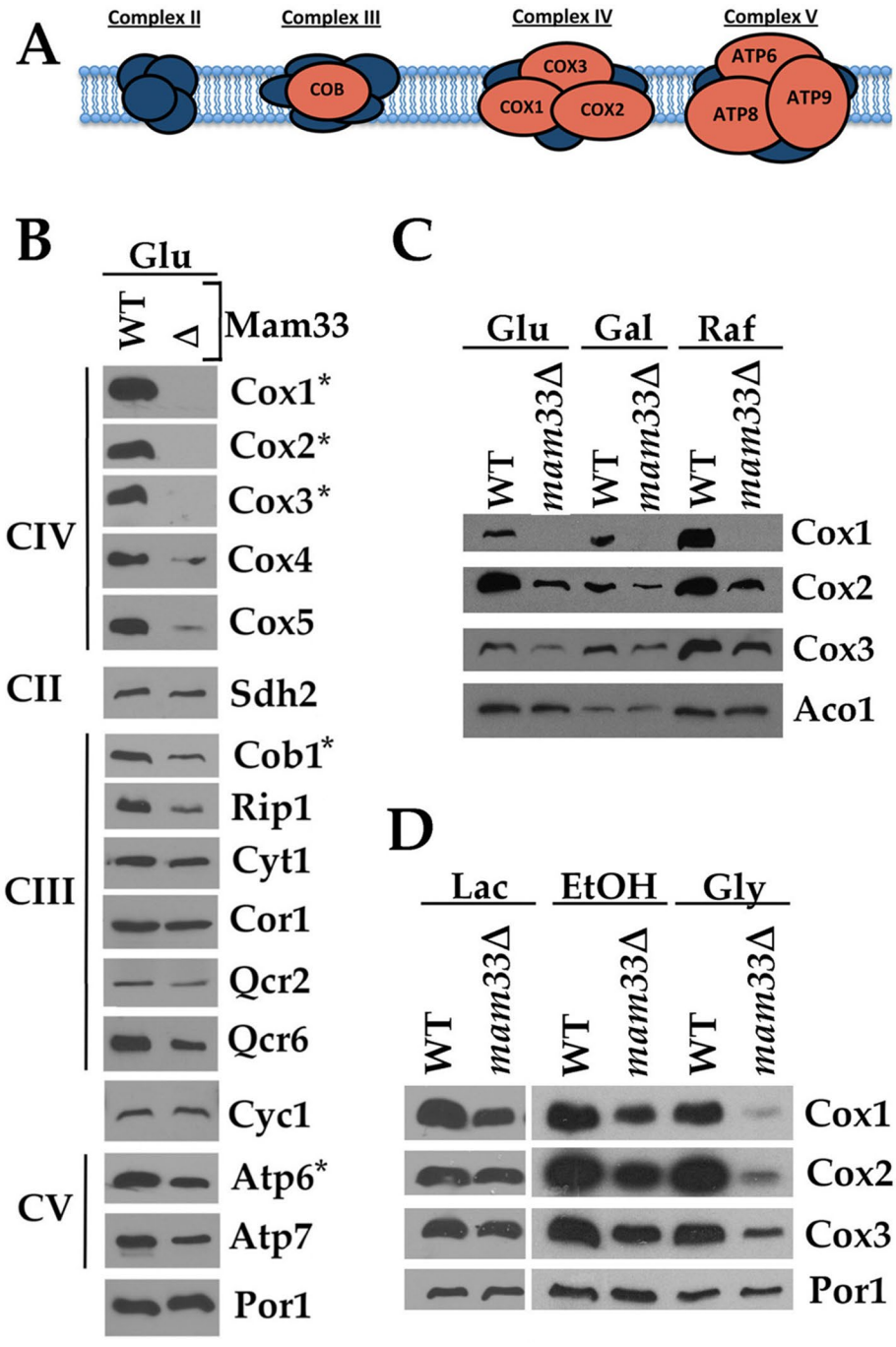

FIGURE 2: Steady-state Cox1-3 protein levels are decreased in *mam33*Δ cells. (A) Schematic representation of the *S. cerevisiae* mitochondrial respiratory chain complexes with mtDNA-encoded proteins shaded red. (B–D) Steady-state levels of mitochondrial respiratory chain proteins during fermentative and respiratory conditions. Mitochondrial proteins from wild-type (WT) and *mam33*Δ cells grown in the indicated carbon source were separated by 10% SDS–PAGE and detected by Western blotting. Asterisks indicate proteins encoded by the mitochondrial genome. CII–CV denote the respiratory chain complexes. Por1 and Aco1 were used to control for protein loading. The indicated genotypes correspond to the following strains (Supplemental Table S1): WT, BMA64-1A; *mam33*Δ, MHY1564.

Mam33-dependent Cox1–3 expression is due to glucose repression, we examined the steady-state levels of these proteins in cells grown in galactose and raffinose. All three cytochrome *c* oxidase proteins were similarly reduced in these nonrepressing sugars (Figure 2C). Thus the levels of these cytochrome *c* oxidase subunits are decreased in fermenting *mam33*Δ cells, independent of glucose repression. Although Cox1 levels are undetectable in *mam33*Δ mutants during these conditions, Cox2 and Cox3 levels appear to vary between experiments (Figure 2, B and C). The latter result suggests that the Cox2 and Cox3 decreases may be secondary effects.

Surprisingly, steady-state Cox1-3 levels were also decreased in *mam33*Δ cells grown to exponential phase in three different respiratory carbon sources (Figure 2D). The most significant decrease was observed in cells grown in glycerol. In these experiments, the reduction in Cox1 expression was the most pronounced. This result was unexpected because *mam33*Δ mutants do not have a respiratory growth defect during exponential phase (Supplemental Figure S1, A and B) and indicates that the Cox1 levels in these cells are enough to support wild-type growth. In previous reports, mutants with substantial steady-state Cox1 reductions displayed only minor cytochrome *c* oxidase activity defects (Barrientos *et al.*, 2002; Horn *et al.*, 2008).

Cells deleted for *MAM33* have modestly reduced *COX1* intron splicing

To determine whether the Cox1–3 protein reductions in *mam33*Δ cells are due to decreased transcription, RNA processing, or mRNA stability, we compared steady-state *COX1-3* mRNA levels in wild-type and *mam33*Δ cells by Northern blot analysis. Whereas all three genes are transcribed as distinct polycistronic transcripts that are cleaved into individual messages before translation, only *COX1* contains introns (Figure 3A; Lipinski *et al.*, 2010). The levels of mature *COX2* and *COX3* transcripts were equal in *mam33*Δ cells, indicating that Mam33 is not required for the transcription, polycistronic cleavage, or stability of either transcript (Figure 3B). Conversely, mature *COX1* transcript levels were decreased approximately twofold in the *mam33*Δ mutant (Figure 3B). Because *COX1* contains seven introns, this analysis poorly differentiates between transcription and overall splicing defects. Inefficient intron removal will generate a multitude of low-abundance, high–molecular weight splice variants that would appear, if visible, as a faint smear by Northern analysis.

To better quantify RNA levels and detect both mature and intermediate transcripts, we used quantitative reverse transcription PCR (qRT-PCR; Figure 3, C and D). In the first experiment, an exon region present in all intermediate and mature transcripts was amplified. The RNA levels for all three genes were unchanged in *mam33*Δ cells, indicating that Mam33 is not required

for *COX1-3* transcription or mRNA stability (Figure 3C). In the second experiment, primers spanning *COX1* exon junctions were designed to test whether *COX1* intron splicing was reduced in *mam33*Δ cells (Figure 3A). If *COX1* splicing is impaired, large introns would be present within the amplicon and prevent PCR amplification. The number of spliced transcripts decreased modestly in *mam33*Δ mutants, with those containing introns 1–4 being the most affected (Figure 3D). Although this result suggests that Mam33 is required for optimal *COX1* intron splicing, this reduction is likely indirect due to impaired translation. This interpretation is consistent with the observation that splicing of the first two *COX1* introns is most sensitive to a translation block (Decoster *et al.*, 1990).

Mam33 is required for efficient *COX1* mRNA translation

To ascertain whether Mam33 influences the translation of *COX1-3* mRNAs, we compared mitochondrial protein synthesis in wild-type and *mam33*Δ cells. For this experiment, mitochondrial translation products were labeled in vivo with [^{35}S]methionine and cysteine while blocking cytosolic translation with cycloheximide. Cox1 synthesis was strongly diminished in *mam33*Δ mutants during fermentation (Figure 4A). The translation of Cox2 and Cox3 was also reduced but to a lesser extent, again suggesting an indirect effect. In contrast, Cox1 synthesis was unaffected in respiring cells.

Mitochondrial gene expression is especially susceptible to indirect effects because transcription and translation are tightly coupled (Dieckmann and Staples, 1994; Wallis *et al.*, 1994; Rouillard *et al.*, 1996; Rodeheffer *et al.*, 2001; Rodeheffer and Shadel, 2003; Bryan *et al.*, 2002; Williams *et al.*, 2007; Markov *et al.*, 2009). Because Cox1 splicing and translation are both decreased in *mam33*Δ cells, it is possible that a defect in just one process indirectly disrupts the other process. For example, an increase of partially processed *COX1* transcripts could delay translation of the mature mRNA. To test this possibility, we compared mitochondrial translation profiles of wild-type and *mam33*Δ strains carrying an intronless mitochondrial genome. The level of *COX1* mRNA translation remained low in *mam33*Δ cells, at a level comparable to that of the intron-containing *mam33*Δ strain (Figure 4B). Taken together, these results suggest that Mam33 acts at the level of *COX1* translation and the observed splicing defect is indirect.

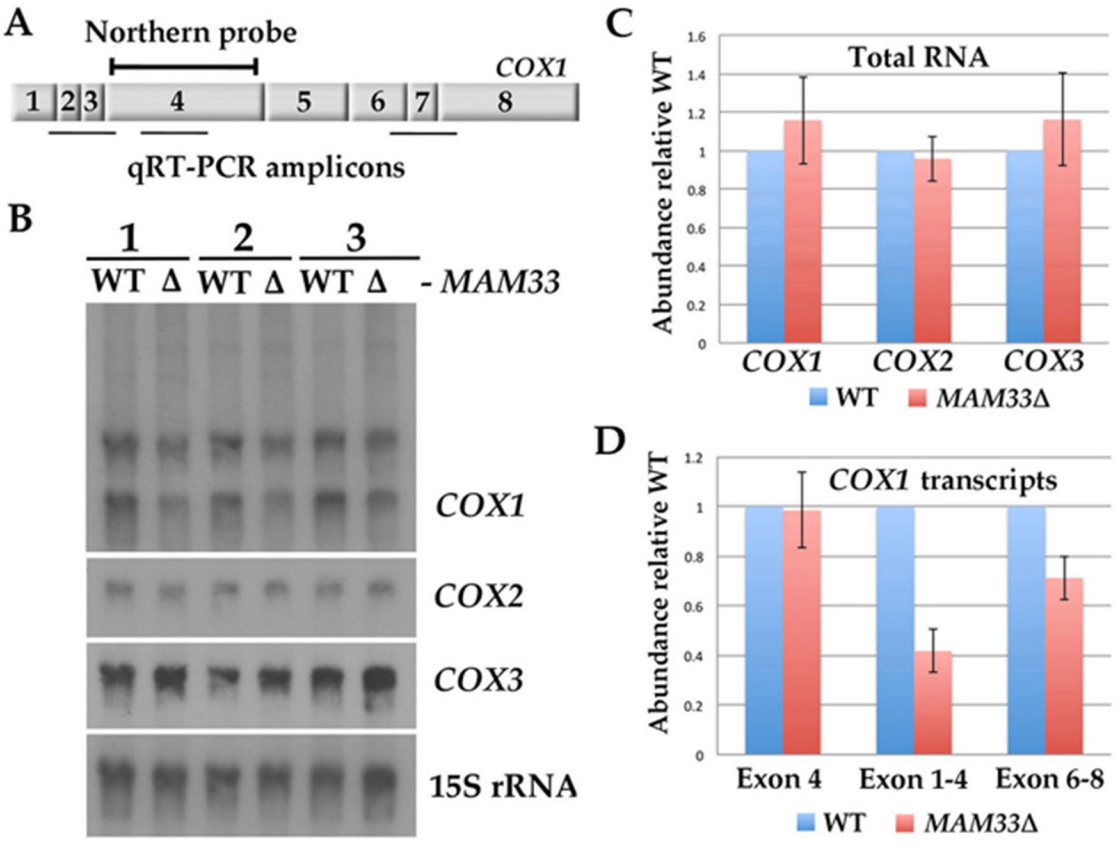

FIGURE 3: Cox1 splicing is impaired in *mam33*Δ mutants. (A) Diagram of the *COX1* Northern probe and qRT-PCR amplicons used in B–D. Because *COX2* and *COX3* do not contain introns, the Northern probes and qRT-PCR amplicons were simply designed within the coding sequence of each gene. (B) Northern blot analysis of *COX1-3* mRNA in WT and *mam33*Δ cells grown in glucose. The numbers above the blot indicate three independent biological replicates and correspond to the three replicates used in C. The gel area above the mature *COX1* transcript (bottom band) is presented to show the absence of specific splicing intermediates. 15S rRNA served as a mitochondrial loading control. (C) qRT-PCR analysis of total *COX1-3* transcript levels. The *COX1* amplicon spanned a region within exon 4 as shown in A. The values were normalized to *ACT1*, and WT was set equal to 1. Bars represent the average fold change of three biological replicates, and error bars represent SEM. (D) qRT-PCR analysis of specific *COX1* splicing intermediates. Each *COX1* amplicon is indicated directly below the chart. Data were normalized and statistically analyzed as described for C. The indicated genotypes correspond to the following strains (Supplemental Table S1): WT, BMA64-1A; *mam33*Δ, MHY1564.

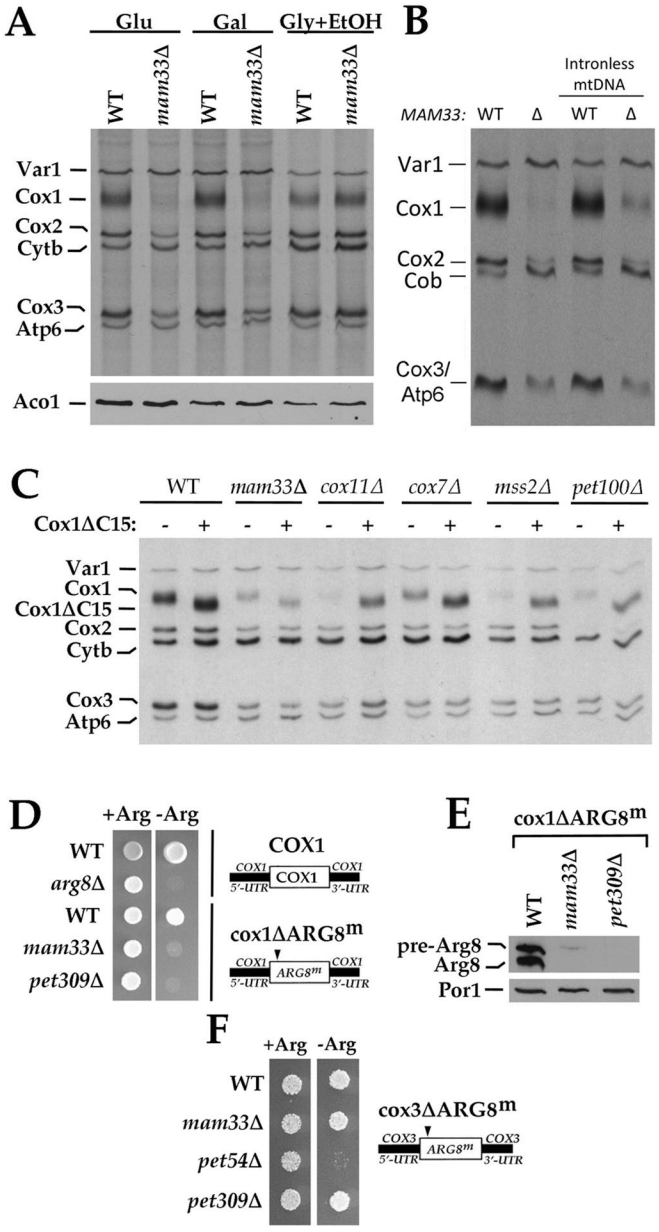

FIGURE 4: Mam33 is required for efficient *COX1* translation during fermentation. (A) Mitochondrial translation products of wild-type (BMA64-1A) and *mam33*Δ (MHY1564) cells grown in glucose, galactose, or a combination of glycerol and ethanol. Cells were labeled in vivo with [^{35}S]methionine and cysteine for 10 min in the presence of cycloheximide at 30°C (as described in *Materials and Methods*). Proteins were resolved by 19% SDS–PAGE and analyzed by autoradiography. The top of the gel was transferred to a nitrocellulose membrane and probed with the anti-Aco1 antibody to normalize the signals for protein loading. Each mitochondrially encoded translation product is denoted in the left margin. (B) Mitochondrial translation products in *mam33*Δ cells containing an intronless mitochondrial genome. Cells were grown in galactose and processed as described in A. The denoted genotypes (from left to right lanes) correspond to the following strains (Supplemental Table S1): BMA64-1A, MHY1564, MHY1954, and MHY1985. (C) Cox1 (–) or Cox1ΔC15 (+) cells with a deletion in the indicated genes were grown in galactose, and mitochondrial translation products were radiolabeled as described for A. The denoted relevant genotypes (from left to right lanes) correspond to the following strains (Supplemental Table S1): NB40-36a, XPM295a, MHY2021, MHY2036, MHY2037, MHY2038, MHY2043, MHY2044, MHY2045, MHY2046, MHY2047, and MHY2048. (D) Growth phenotypes of strains carrying an Arg8^{m} reporter under control of the *COX1* 5′ and 3′ UTRs. Relevant nuclear and mitochondrial genotypes are indicated on the left and right, respectively. Yeast cells were grown to exponential phase in yeast extract/peptone/dextrose and then spotted to glucose minimal medium containing (+Arg) or lacking (–Arg) arginine. Plates were incubated for 2 d at 30°C. The cleavage site for the pre-Arg8^{m} matrix-targeting signal is denoted with a black triangle. The indicated relevant genotypes correspond (from top to bottom) to the following strains (Supplemental Table S1): BMA64-1A, NB40-36a, EHW463, MHY2053, and MHY2072. (E) Steady-state accumulation of the Arg8^{m} reporter in cells grown in glucose. Total mitochondrial proteins were separated by 10% SDS–PAGE and analyzed by Western blotting. When expressed inside the mitochondrial matrix (i.e., not imported), the Arg8^{m} mitochondrial targeting signal is inefficiently cleaved, and Arg8^{m} migrates as two bands. Por1 was used as a loading control. The indicated relevant genotypes correspond in order of lanes to the following strains (Supplemental Table S1): EHW463, MHY2053, and MHY2072. (F) Growth phenotypes of strains carrying an Arg8^{m} reporter under control of the *COX3* 5′ and 3′ UTRs. Cells were processed as described in D. The indicated relevant genotypes correspond (from top to bottom) to the following strains (Supplemental Table S1): EHW465, MHY2093, MHY2096, and MHY2095.

A negative feedback mechanism coordinates Cox1 translation with complex assembly. Mss51 activates *COX1* translation, binds newly synthesized unassembled Cox1 nascent polypeptide, and remains bound during complex assembly (Perez-Martinez *et al.*, 2003, 2009; Barrientos *et al.*, 2004; Pierrel *et al.*, 2007; Fontanesi *et al.*, 2010, 2011; Mick *et al.*, 2010). At a point late in complex assembly, Mss51 is released to initiate a new round of translation. If assembly is compromised, Mss51 remains sequestered with the unassembled complex and is unable to reactivate *COX1* translation. Thus Cox1 synthesis could be diminished in *mam33*Δ mutants because of cytochrome *c* oxidase assembly defects. To test this possibility, we eliminated this feedback control mechanism using a truncated version of Cox1 (Cox1ΔC15). Mss51 is unable to bind newly synthesized Cox1ΔC15 and is constitutively free to activate its translation (Shingu-Vazquez *et al.*, 2010). This appears to be the only Cox1 assembly feedback mechanism, because the Cox1ΔC15 bypass significantly rescued all of the numerous assembly mutants tested (Shingu-Vazquez *et al.*, 2010). In *mam33*Δ mutants, the translational defect was not rescued by the *COX1ΔC15* allele, whereas translation in the assembly mutants (*cox11*Δ, *cox7*Δ, *mss2*Δ, and *pet100*Δ) was significantly restored (Figure 4C). These results demonstrate that *mam33*Δ mutants exhibit a Cox1 translation defect without complex assembly feedback. Of interest, the translation profiles of the assembly mutants were strikingly similar to those of the *mam33*Δ mutant, in that Cox2 and Cox3 labeling was weakly reduced. Because this assembly feedback mechanism specifically targets Cox1 translation, it is likely that Mam33 also targets Cox1 and that the Cox2 and Cox3 reductions are indirect.

Our Western blot analysis and in vivo radiolabeling experiments do not exclude the possibility that Cox1 is translated normally but then rapidly degraded in *mam33*Δ cells. To test Cox1 stability, we monitored expression of an *ARG8^{m}* reporter under the control of the *COX1* 5′ and 3′ UTRs (*cox1Δ::ARG8^{m}*). In cells with functional translational activators, the mitochondrial *cox1Δ::ARG8^{m}* gene supported growth on minimal media lacking arginine (Figure 4D). In contrast, when the *COX1* translational activator *PET309* was

deleted in this strain, the cells became Arg⁻. The *mam33*Δ, *cox1*Δ::*ARG8*^{m} strain was also Arg⁻, indicating that the decrease in Cox1 synthesis in fermenting *mam33*Δ cells is due to a defect in translation and not stability. Consistent with the Arg⁻ growth phenotype, Arg8^m steady-state protein levels were severely decreased in the *mam33*Δ mutant (Figure 4E).

In some in vivo translation assays, decreased Cox1 translation is accompanied by reduced Cox2 and Cox3 labeling (Pierrel *et al.*, 2007; Zambrano *et al.*, 2007; Soto *et al.*, 2012d). To test whether the affects on the other cytochrome *c* oxidase subunits are indirect, we chose to assay *COX3* translation in *mam33*Δ mutants by monitoring expression of the *ARG8*^{m} reporter under the control of *COX3* 5′ and 3′ UTRs (*cox3*Δ::*ARG8*^{m}; Williams *et al.*, 2005). Deletion of the *COX1* translational activator *PET309* or *MAM33* did not affect cell growth on minimal medium lacking arginine (Figure 4F). As expected, deletion of a *COX3* translational activator, *PET54,* generated Arg⁻ cells (Figure 4F). Therefore the decrease in *COX3* translation observed in *mam33*Δ cells (Figure 4A) is indirect and due to protein instability.

DISCUSSION

Here we show that Mam33 activates translation of mitochondrially encoded Cox1, a core subunit of the cytochrome *c* oxidase complex. Whereas cells deleted for *MAM33* do not exhibit any noticeable growth defects during exponential phase, they have difficulty adapting from fermentative to respiratory growth. How do we explain this distinctive phenotype? In wild-type cells, components of the mitochondrial respiratory chain are maintained at basal levels during fermentation and are increased when cells shift to respiratory metabolism (Ohlmeier *et al.*, 2004). When cells are rapidly shifted from a fermentable to a respiratory carbon source, this basal level of respiratory chain activity provides enough energy, as the cells increase their respiratory capacity. Respiratory adaptation is more difficult for *mam33*Δ cells because Cox1 is virtually absent during fermentation. Because the electron transport chain is dispensable during fermentative growth, these mutants do not exhibit a discernible growth defect when fermenting. Once acclimated, respiring *mam33*Δ cells are not appreciably affected because an ~50% reduction of cytochrome *c* oxidase activity is enough to support wild-type growth in nonfermentable media (Horn *et al.* 2008).

The translation of an Arg8 reporter flanked by the *COX1* UTRs is Mam33 dependent (Figure 4, D and E). This result indicates that the UTRs are sufficient for Mam33 activation and strongly suggests that Mam33 has a specific target within the UTRs of *COX1* mRNAs. Future work will determine which UTR is necessary and whether Mam33 acts directly on this mRNA.

Since the early 1970s, genetic screens for petite *S. cerevisiae* mutants have identified numerous factors critical for mitochondrial gene expression (Ebner *et al.*, 1973; Tzagoloff *et al.*, 1975). The analysis of respiration-deficient mutants exhibiting gene-specific translation defects led to the concept of translational activators (Muller *et al.*, 1984; Tzagoloff and Myers, 1986; Tzagoloff and Dieckmann, 1990; Korte *et al.*, 1989; Costanzo and Fox, 1990; Fox 1996; Haffter *et al.*, 1990; McMullin *et al.*, 1990; Poyton and McEwen, 1996). For the past 20 years, Mss51 and Pet309 have been the only recognized Cox1 translational activators (Decoster *et al.*, 1990; Manthey and McEwen, 1995). However, mitochondrial translational activators with overlapping functions, or those only conditionally required, would have been missed in genetic screens for petite mutants. *MAM33* is an example of such a gene.

Given that Mam33 activates translation of mitochondrially encoded Cox1, how do *mam33*Δ cells retain respiratory competence? A likely explanation is that a second factor, which is down-regulated during fermentative growth, can compensate for Mam33 during respiration. Although the Cox1 translational activators Mss51 and Pet309 are reasonable candidate factors, both are required for Cox1 expression in fermenting cells (Decoster *et al.*, 1990; Manthey and McEwen, 1995; Siep *et al.*, 2000; Perez-Martinez *et al.*, 2003, 2009; Zamudio-Ochoa *et al.*, 2014). Another candidate is *YGR021W*, a protein highly homologous to human TACO1. TACO1 is a *COX1* mRNA–specific translational activator whose absence causes a cytochrome *c* oxidase deficiency and late-onset Leigh syndrome (Weraarpachai *et al.*, 2009; Seeger *et al.*, 2010). However, *mam33*Δ *ygr021w*Δ double mutants retain respiratory competence (unpublished data). Further studies in this lab aim to identify this factor.

Although steady-state Cox1 levels were decreased in respiring *mam33*Δ cells, translation was unaffected. This result suggests that Mam33 may also directly or indirectly affect complex stability during respiration. The complex may also be unstable in fermenting cells, but this phenotype would be masked because translation is impaired.

Much remains unknown regarding the molecular functions of translational activators in mitochondria. Translational activators are believed to organize the translation machinery by 1) recognizing specific mitochondrial mRNAs and altering secondary structure, 2) interacting with ribosomal proteins, and 3) tethering translation to the inner membrane (Herrmann *et al.*, 2013). Given that Mam33 is weakly associated with the inner membrane (Seytter *et al.*, 1998), activation of Cox1 translation likely occurs by either of the first two or a novel mechanism. Future studies in this lab will examine whether Mam33 physically interacts with the Cox1 transcript or the mitochondrial ribosome. In support of a model in which Mam33 physically interacts with mitochondrial ribosomes, a search of the STRING protein interaction database identified five mitochondrial ribosomal proteins among the 10 highest-scoring Mam33 partners. This database predicts interactions based on several methods, including gene coexpression and high-throughput interaction experiments (Jensen *et al.*, 2009).

Contrary to our results (Supplemental Figure S1, A and B), two previous studies reported reduced *mam33*Δ growth on a single nonfermentable carbon source. One reported an effect on glycerol at low temperatures (Muta *et al.*, 1997), whereas the other observed a mild defect on lactate but not glycerol at 30°C (Seytter *et al.*, 1998). Both studies measured growth rate as the time required for cells to form colonies after plating fermenting cultures onto respiratory plates. This method does not distinguish between adaptation and growth rate.

The crystal structures of yeast Mam33 and two homologues, human p32/gC1qR/ C1QBP /HABP1 and *Trypanosoma brucei* p22, have been solved (Jiang *et al.*, 1999; Sprehe *et al.*, 2010; Pu *et al.*, 2011). Each forms a doughnut-shaped homotrimeric structure that displays an unusual asymmetric charge distribution such that one surface of the doughnut is covered with negatively charged residues. The most prominent feature is a central hydrophilic channel that may function as a pore. For molecules to traverse this channel, it was proposed that protein-binding partners might induce the two α-helices covering this central region to undergo a conformational change (Jiang *et al.*, 1999). It is intriguing to envision a mechanism by which proper ribosomal alignment of *COX1* mRNA would require passage through the Mam33 channel. Despite the weak sequence homology between yeast Mam33 and human p32 (*E* value = 0.04), the human protein complements the adaptation defect of *mam33*Δ cells grown in glycerol medium (Muta *et al.*, 1997).

Although human p32 is by far the most-studied Mam33 homologue, its cellular localization and proposed activities are conflicting.

The p32 protein is primarily in the mitochondria (Dietmeier *et al.*, 1993; Dedio and Muller-Esterl, 1996; Muta *et al.*, 1997; Matthews and Russell, 1998; Jiang *et al.*, 1999; Itahana and Zhang, 2008; Li *et al.*, 2011; Hu *et al.*, 2013) but has also been reported to be present in the nucleus (Matthews and Russell, 1998; van Leeuwen and O'Hare, 2001), cytoplasm (Krainer *et al.*, 1991; Matthews and Russell, 1998), and endoplasmic reticulum (Dedio and Muller-Esterl, 1996) and on the cell surface (Gupta *et al.*, 1991; Ghebrehiwet *et al.*, 1994; Manthey and McEwen, 1995; Kittlesen *et al.*, 2000; Mahdi *et al.*, 2001). Some of these differences may be attributed to the different tissue types or cancer cell lines examined. Furthermore, the proposed activities of p32 are ubiquitous and sometimes incompatible with its localization. These activities range from influencing cellular and viral transcription/mRNA splicing in the nucleus (Krainer *et al.*, 1991; Luo *et al.*, 1994; Yu *et al.*, 1995; Tange *et al.*, 1996; Wang *et al.*, 1997; Petersen-Mahrt *et al.*, 1999; Van Scoy *et al.*, 2000; Hall *et al.*, 2002; Zheng *et al.*, 2003; Chattopadhyay *et al.*, 2004; Liang *et al.*, 2004; Berro *et al.*, 2006; Heyd *et al.*, 2008; Huang *et al.*, 2008), gene expression (Fogal *et al.*, 2010), and apoptosis in the mitochondria (Itahana and Zhang, 2008; Rizvi *et al.*, 2011; Eckart *et al.*, 2014; Xiao *et al.*, 2014) and binding small molecules and peptides on the cell surface (Ghebrehiwet *et al.*, 1994; Fogal *et al.*, 2008). Of interest, p32 has also been shown to be a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation, but its physiological role remains unknown (Fogal *et al.*, 2010; Amamoto *et al.*, 2011). Given that p32 complements Mam33 function in yeast, a detailed study of *MAM33* in yeast cells will permit a better understanding of its underlying biological function, which is conserved in eukaryotes (Muta *et al.*, 1997).

MATERIALS AND METHODS

Strains, media, and genetic methods

S. cerevisiae strains used in this study are listed in Supplemental Table S1. Yeast cells were grown at 30°C in rich medium (YP) containing 1% yeast extract and 2% peptone or synthetic complete (SC) medium containing 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and the appropriate amino acids. Media contained 2% glucose, 2% galactose, 2% raffinose, 2% lactate, 3% glycerol, or 3% ethanol as indicated for each experiment. Chromosomal deletions of *MAM33*, *COX11*, *COX7*, *MSS2*, *PET100*, and *PET54* were performed by transforming cells with a *KANMX4*, *TRP1*, or *URA3* cassette PCR amplified with homologous flanking sequences for recombination (Baudin *et al.*, 1993; Wach *et al.*, 1994; Gietz *et al.*, 1995; Manthey and McEwen, 1995).

Adaptation experiments

To determine the time required for cells to adapt from a fermentative to respiratory carbon source, log-phase (OD_{600} = 0.3) cells grown in 50 ml of SC plus glucose at 30°C were washed twice with distilled H_2O (dH_2O) and resuspended in the same medium containing either glycerol or ethanol. The cells were diluted in half (OD_{600} = 0.15) in the same medium, incubation with shaking was continued, and the time required for the cell density to double was determined by measuring OD_{600}. To determine the time required for cells to shift from respiratory to a fermentative growth, the same experiment was performed, except that cells were first grown in SC medium containing either glycerol or ethanol and then switched to SC medium containing glucose.

To assay cell viability and colony formation after transfer to a nonfermentable carbon source, log-phase cells in SC glucose medium (OD_{600} = 0.2) were washed twice with dH_2O and diluted. Approximately 200 cells were spread onto SC glucose or SC glycerol medium and incubated at 30°C for 2 or 3 d, respectively. Viability (percent) was calculated as the number of glycerol colonies divided by glucose colonies. Wild type was set equal to 100%.

Northern blotting and qRT-PCR

For RNA extraction, 60-ml cultures were grown to exponential phase (OD_{600} = 0.4) in YP medium containing glucose. RNA was isolated using TRIzol reagent (Life Technologies, Waltham, MA) according to the manufacturer's instructions.

For Northern blot analysis, 20 µg of total RNA was separated on a denaturing 1% formaldehyde-agarose gel, transferred to a Hybond-N+ nylon membrane, ultraviolet cross-linked, hybridized sequentially with gene-specific ^{32}P-labeled probes, and analyzed by autoradiography (Brown, 2004). Gene-specific probes were labeled from PCR products (Supplemental Table S2) by random oligomer labeling according to the manufacturer's instructions (Ready-To-Go Labeling Beads dCTP; Amersham Pharmacia Biotech, Piscataway, NJ). Unincorporated nucleotides were subsequently removed using MicroSpin G-25 columns (Amersham Pharmacia Biotech).

cDNA synthesis and subsequent qRT-PCR were performed with 30 ng of total RNA using an Applied Biosystems StepOne Real-Time PCR system according to manufacturer's instructions (iTaq Universal SYBR Green One-Step Kit; Bio-Rad, Hercules, CA). After a reverse transcription cycle at 50°C for 10 min, DNA was amplified by 1 cycle at 95°C for 1 min and then 40 cycles consisting of 95°C for 15 s followed by 60°C for 1 min. A melt-curve analysis was performed to confirm primer specificity. Reactions were run in triplicate in three independent experiments. The primer sequences are listed in Supplemental Table S2.

Isolation of mitochondria

Yeast cells were grown to exponential phase (OD_{600} = 0.6) in YP medium containing the indicated carbon source, harvested by centrifugation (3000 × *g* for 5 min), resuspended (2 ml/g of cells) in reducing buffer (100 mM Tris-SO_4, pH 9.4, and 10 mM dithiothreitol [DTT]), and then incubated with shaking for 40 min at 30°C. Cells were then washed once with 1.2 M sorbitol, resuspended (6.7 ml/g of cells) in Zymolyase buffer (1.2 M sorbitol, 20 mM KPi, pH 7.4, and Zymolyase 100T [1 mg/g cells; Amsbio, Abingdon, UK]), and incubated with gentle shaking for 40 min at 30°C. All of the subsequent steps and centrifugations were performed on ice or at 4°C, respectively. Spheroplasts were harvested by centrifugation (2500 × *g* for 5 min), resuspended in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 0.2% fatty acid–free bovine serum albumin [Sigma-Aldrich, St. Louis, MO], and 1 mM phenylmethanesulfonyl fluoride [Sigma-Aldrich]), and homogenized by 20 strokes in a tight-fitting glass douncer. The homogenate was centrifuged (3000 × *g* for 5 min), and the supernatant was transferred to a new tube. The pellet was homogenized a second time, and the supernatants, containing mitochondria, were combined. The combined supernatants were centrifuged (3000 × *g* for 5 min) repeatedly until no pellet was observed, and then mitochondria were harvested by centrifugation (12,000 × *g* for 10 min). Protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976).

Analysis of mitochondrial proteins

Isolated mitochondria (20 µg of protein) were resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride [Sigma-Aldrich]), and proteins were resolved on a 10% SDS–PAGE gel (Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane (Amersham Hybond ECL) and analyzed by

Western blotting (Towbin *et al.*, 1979). The antibodies were gifts from the following people and used at the indicated dilutions: anti-Arg8 rabbit polyclonal antibody (1:1000) from Tom Fox at Cornell University (Steele *et al.*, 1996), anti-Cob1 rabbit polyclonal antibody (1:10,000) from Brian Robinson at the Hospital for Sick Children Research Institute (Lee *et al.*, 2001), anti-Cor1 (1:10,000), anti-Cyt1 (1:2000), anti-Qcr6 (1:5000), and anti-Qcr2 (1:10,000) rabbit polyclonal antibodies from Rosemary Stuart at Marquette University (Cruciat *et al.*, 2000), anti-Cox5a (1:2000), anti-Rip1 (1:2000), anti-Cyc1 (1:1000), and anti-Sdh2 (1:5000) rabbit polyclonal antibodies from Antoni Barrientos at the University of Miami (Fontanesi *et al.*, 2011), anti-Atp6 (1:10,000) and anti-Atp7 (1:50,000) rabbit polyclonal antibodies from Jean Velours at Universite de Bordeaux, and anti-Aco1 (1:100,000) rabbit polyclonal antibody from Ophry Pines at Hebrew University of Jerusalem (Regev-Rudzki *et al.*, 2005). The anti-Cox1 (11D8B7; 1:1000), anti-Cox2 (4B12A5; 1:2000), anti-Cox3 (DA5BC4; 1:1000), and anti-Cox4 (1A12A12; 1:1000) mouse monoclonal antibodies were obtained from Abcam. The anti-Por1 mouse monoclonal antibody (16G9; 1:20,000) was obtained from Invitrogen. Blots were incubated with horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies (1:5000), and signals were detected with ECL Western blotting reagents (Amersham).

Labeling of mitochondrial translation products in vivo

Cells were grown in minimal medium lacking methionine and cysteine to exponential phase (OD_{600} = 0.6), and 1 ml of cells was collected by centrifugation (10,000 × *g* for 15 s). Cells were resuspended in 500 µl of the same medium supplemented with cycloheximide (0.2 mg/ml) and incubated at 30°C for 2 min with gentle agitation. Mitochondrial proteins were then labeled for 10 min at 30°C by adding 33 µCi of [^{35}S]methionine and cysteine (EXPRES^{35}S^{35}S protein labeling mix; PerkinElmer, Waltham, MA). Labeled cells were collected by centrifugation (10,000 × *g* for 15 s), resuspended in ice-cold solubilization buffer (1.8 M NaOH, 1 M β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride), and mixed by vortexing for 10 s, and the suspension was subsequently diluted with 845 µl of ice-cold water. Proteins were precipitated by the addition of 10% trichloroacetic acid solution and 1 h of incubation on ice with occasional vortexing. Precipitated proteins were collected by centrifugation (18,000 × *g* for 20 min at 4°C), and pellets were rinsed once with ice-cold 0.5 M Tris-base and then once with ice-cold water. The pellets were resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and proteins were resolved on a 19% acrylamide/1% bisacrylamide gel and analyzed by autoradiography or Western blotting.

ACKNOWLEDGMENTS

We are grateful to Thomas D. Fox, Xochitl Perez-Martinez, Alexander Tzagoloff, Rosemary Stuart, Antoni Barrientos, Brian Robinson, Jean Velours, and Ophry Pines for strains and antisera. This work was supported by a grant from the New Jersey Health Foundation (PC130-13) to M.F.H.

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