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Genetics

Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation

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ABSTRACT **Genetic code differences prevent expression of nuclear genes within *Saccharomyces cerevisiae* mitochondria. To bridge this gap a synthetic gene, *ARG8*^{m}, designed to specify an arginine biosynthetic enzyme when expressed inside mitochondria, has been inserted into yeast mtDNA in place of the *COX3* structural gene. This mitochondrial *cox3::ARG8*^{m} gene fully complements a nuclear *arg8* deletion at the level of cell growth, and it is dependent for expression upon nuclear genes that encode subunits of the *COX3* mRNA-specific translational activator. Thus, *cox3::ARG8*^{m} serves as a mitochondrial reporter gene. Measurement of *cox3::ARG8*^{m} expression at the levels of steady-state protein and enzymatic activity reveals that glucose repression operates within mitochondria. The levels of this reporter vary among strains whose nuclear genotypes lead to under- and overexpression of translational activator subunits, in particular Pet494p, indicating that mRNA-specific translational activation is a rate-limiting step in this organellar system. Whereas the steady-state level of *cox3::ARG8*^{m} mRNA was also glucose repressed in an otherwise wild-type strain, absence of translational activation led to essentially repressed mRNA levels even under derepressing growth conditions. Thus, the mRNA is stabilized by translational activation, and variation in its level may be largely due to modulation of translation.**

Expression of genes encoded in mtDNA results in synthesis of a small number of proteins within the organelle that combine with nuclearly encoded polypeptides to form respiratory complexes. However, virtually nothing is known about how (or whether) organellar gene expression is coordinated with that of the nucleus. Whereas mitochondrial gene expression systems are commonly thought to resemble those of bacteria, few of the known details actually fit this generalization (1–4). For example, in *Saccharomyces cerevisiae* mitochondrial translation initiation does not employ a Shine–Dalgarno mechanism, but it does depend on mRNA-specific translational activation functions unknown in bacteria. The best-studied of these translational activators is a complex of at least three proteins, coded by the nuclear genes *PET54*, *PET122*, and *PET494*, which are bound to the inner mitochondrial membrane and are required to translate the mitochondrially coded *COX3* mRNA (5–7). This activator works by a mechanism involving *in vivo* functional interactions with both the *COX3* mRNA 5′-untranslated leader (8–10) and the small subunit of mitochondrial ribosomes (11–13), and it probably plays a role in tethering the *COX3* mRNA to the inner membrane prior to synthesis of its highly hydrophobic product.

Previous studies of mitochondrial gene regulation have been difficult and unreliable because they depended either on labeling of cells poisoned with cytoplasmic translation inhibitors or on steady-state measurements of protein levels (5). The former is clearly not a normal physiological condition, and the latter are a very poor approximation of translation rates, especially for components of multisubunit complexes whose stability depends on assembly with other proteins (14). To circumvent these problems generally, and to study the possible role of mRNA-specific translational activators in mitochondrial gene regulation, we have developed a novel reporter gene and selectable marker for yeast mitochondria, termed *ARG8*^{m}. This synthetic gene employs the yeast mitochondrial genetic code (15) to specify the same protein as the wild-type nuclear gene *ARG8*. The *ARG8* gene product is a soluble enzyme located in the mitochondrial matrix that carries out the fourth step in arginine biosynthesis and can be quantitatively assayed in yeast cell extracts (16, 17).

We have inserted *ARG8*^{m} into the yeast mitochondrial chromosome in place of the wild-type *COX3* coding sequence, where its expression complements the Arg$^-$ auxotrophy of a nuclear *arg8* deletion and is dependent upon *COX3* mRNA-specific translational activation. Quantitative measurement of reporter protein levels demonstrates that mitochondrial gene expression is subject to glucose repression. Furthermore, expression of *cox3::ARG8*^{m} is limited by expression of nuclear genes encoding the *COX3*-specific translational activator, in particular *PET494*. Because *PET494* expression is known to be modulated in response to glucose repression (18), these findings strongly indicate that mRNA-specific translational activation is used to modulate mitochondrial gene expression in yeast.

MATERIALS AND METHODS

Yeast Strains and Genetic Methods. All *S. cerevisiae* strains were congenic to the wild-type strain D273-10B (ATCC 25657) with the exception of DFS160 and DFS162, which were derived from MCC109 (8). Standard genetic methods were as described (19). The *ARG8*^{m} synthetic gene was generated from 28 overlapping oligonucleotides (Genosys, The Woodlands, TX) that fully encoded both strands (details available upon request). Four subsegments of the gene were generated independently by the PCR (20). These segments were isolated, mixed, and then joined by a second round of PCR. Full-length fragments were sequenced and an error-free gene was constructed by recombining accurate subfragments. The resulting *ARG8*^{m} gene was inserted into a plasmid carrying *COX3* flanking sequences to create pDS24. Flanking *cox3::ARG8*^{m}, pDS24 has 1.6 kb of mtDNA upstream and 2.3 kb of mtDNA downstream. pDS24 and the *LEU2* plasmid pRS315 were transformed into the *rho*^{0} strain DFS160 (*MATα ade2-101 leu2Δ ura3-52 arg8Δ::URA3 kar1-1* [ρ^0]) by microprojectile bombardment (21) using a PDS1000 apparatus (Bio-Rad). Leu$^+$ transformants that also contained pDS24 in their mito-

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U31093).
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chondria were identified by their ability to produce respiring recombinants when mated to strain GW22, which carries a deletion mutation in the COX3 5′-untranslated leader (10). A transformant, DFS162, was then mated with the *arg8*Δ rho$^+$ strain DFS142 (*MAT***a** *arg8*Δ::*URA3 leu2-3,112 his4-519 ura3*Δ [ρ^+]), and an Arg$^+$ cytoductant, DFS168, was selected that had the nucleus of DFS142 and the *cox3*::*ARG8*^{m} gene integrated by homologous double recombination into mtDNA, producing the integrant depicted in Fig. 1.

Immunological and Amino Acid Sequence Analysis of Arg8p. Antiserum was obtained from a rabbit immunized against yeast acetylornithine aminotransferase, purified as an insoluble aggregate from *Escherichia coli* expressing *ARG8* (22). The serum was treated with acetone powder (23) derived from an *arg8*Δ yeast strain and was used at a 1:1000 dilution to probe immunoblots. Immune complexes were detected with the Amersham ECL system. To obtain wild-type Arg8p, mitochondria were isolated from a strain overexpressing *ARG8* under the control of the *GAL1* promoter, and proteins were separated by SDS/gel electrophoresis. The abundant Arg8p was blotted to an Immobilon-P membrane (Millipore) and subjected to automated amino-terminal sequencing reactions at the Cornell Biotechnology Analytical and Synthesis Facility. The sequence determined, FTSILEEKAFQVTTY, begins with the 14th residue of the precursor predicted from the gene sequence (GenBank accession no. M32795).

Acetylornithine Aminotransferase Assays. Cells were washed with water and converted to spheroplasts by incubation for 30 min at 30°C in isolation buffer (1 M sorbitol/20 mM Hepes, pH 7.9/10 mM EDTA/1 mM DTT) plus Zymolyase 20T at 7.5 mg/ml. The spheroplasts were washed twice with isolation buffer and then lysed by resuspension in isolation buffer plus 10 μg/ml each of aprotinin, antipain, chymostatin, leupeptin, and pepstatin, 100 mM ammonium sulfate, and 0.1% Triton X-100. The lysates were cleared by centrifugation in a Micro-Centrifuge (Fisher) and protein concentrations were determined by "Lowry" assays (Bio-Rad DC protein assay). Acetylornithine aminotransferase activity was assayed as described (24).

RESULTS

Phenotypic Expression of a Modified Nuclear Gene Sequence Within Mitochondria. To attempt expression of a

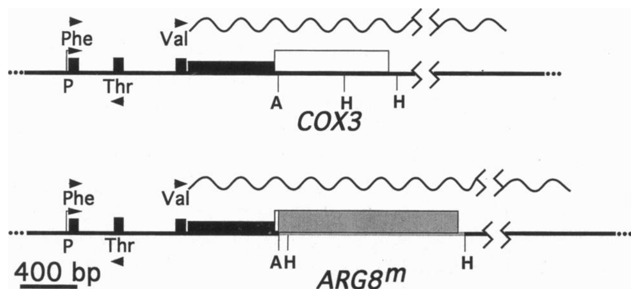


FIG. 1. Schematic representation of the substitution of *COX3* coding sequence by *ARG8*^{m} in mtDNA. The *COX3* structural gene (GenBank accession number J01478) is indicated by the open box and the mRNA 5′-untranslated leader coding sequence by the adjacent filled box. The *COX3* mRNA, processed from a transcript originating at the promoter (P), is indicated by the wavy line. The *ARG8*^{m} structural gene is indicated by the shaded box. The *cox3*::*ARG8*^{m} DNA sequence has accession number U31093. The positions of the indicated tRNA genes and the promoter are based on GenBank files L36889 and L36890 (M62622) and reviewed data (2). The upstream junction of the *cox3*::*ARG8*^{m} translational fusion was generated at the *Acc*I site in *COX3* (A) such that the first eight *COX3* codons were followed by a CAA and then an ATG corresponding to the initiation codon of the nuclear *ARG8* gene. The downstream junction was generated at the *Hin*dIII site (H) 66 bp downstream of the *COX3* termination codon (*COX3* and *ARG8*^{m} each contain addition *Hin*dIII sites as shown).

mitochondrially located gene encoding acetylornithine aminotransferase, we created a translational fusion of a synthetic enzyme structural gene to the *COX3* gene, at an *Acc*I site centered in the eighth codon of *COX3* (Fig. 1). Like most cytoplasmically synthesized mitochondrial proteins the precursor to acetylornithine aminotransferase is cleaved after import by proteases in the matrix (J. Rassow and N. Pfanner, personal communication). By sequencing 15 amino-terminal residues of the mature enzyme isolated from mitochondria, we found that this cleavage occurs between the 13th and 14th residues of the precursor (see *Materials and Methods*). We anticipated that a fusion protein synthesized within the matrix, containing the entire 423-amino acid residue precursor sequence, would also be processed at this site to yield an active enzyme.

Expression of nuclear genes in yeast mitochondria should be hampered by differences between the genetic codes used in the two cellular compartments (15). The yeast nuclear gene *ARG8* (17) contains 12 CUN Leu codons and 6 AUA Ile codons that would be translated in mitochondria as Thr and Met, respectively. To overcome the coding problem we synthesized *de novo* a 1.3-kb DNA fragment, *ARG8*^{m}, specifying acetylornithine aminotransferase in the yeast mitochondrial genetic code with consensus codon usage for that system (25) (Fig. 1). The two Trp residues of the enzyme were encoded by UGA, generating a strong barrier to expression of copies of *ARG8*^{m} that might escape from mitochondria to the nucleus (26).

The synthetic *ARG8*^{m} sequence was fused to the first eight codons of *COX3* in a plasmid, pDS24, containing upstream and downstream flanking mtDNA to generate the *cox3*::*ARG8*^{m} gene (GenBank accession number U31093). This plasmid was then transformed by microprojectile bombardment into the mitochondrial compartment of a karyogamy-defective (27) (*kar1*) yeast strain lacking both mtDNA (*rho*^{0}) and the nuclear *ARG8* gene. To integrate the *cox3*::*ARG8*^{m} sequence into an otherwise wild-type mitochondrial chromosome, a mitochondrial transformant containing the plasmid was mated with a strain bearing a complete mitochondrial chromosome (*rho*$^+$) and a nuclear *arg8* deletion, allowing homologous recombination between flanking mtDNA sequences. Haploid Arg$^+$ cytoductants were recovered from this cross, one of which (DFS168) is shown in Fig. 2. As expected for strains in which *COX3* had been replaced by *cox3*::*ARG8*^{m} in mtDNA, these cytoductants failed to grow on medium containing nonfermentable carbon sources. The integration of *cox3*::*ARG8*^{m} into *rho*$^+$ mtDNA was confirmed by Southern blot analysis (not shown) and the dependence of Arg$^+$ prototrophy on mitochondrial gene expression was demonstrated by eliminating mtDNA with ethidium bromide: the *arg8*Δ, [rho$^+$, *cox3*::*ARG8*^{m}] strains became Arg$^-$ upon loss of mtDNA whereas wild-type control strains did not.

If expression of *cox3*::*ARG8*^{m} reflects normal mitochondrial expression of *COX3*, then complementation of a nuclear *arg8* deletion by *cox3*::*ARG8*^{m} should be dependent upon the *COX3* mRNA-specific translational activator complex. To test this, we constructed strains lacking each of the three known subunits of this complex and determined their ability to grow in the absence of arginine. As expected, *pet54*, *pet122*, and *pet494* mutations all prevented Arg$^+$ growth of *arg8*Δ, [rho$^+$ *cox3*::*ARG8*^{m}] strains, but not *ARG8* [*rho*$^+$] strains (Fig. 2). Thus, expression of *cox3*::*ARG8*^{m} depends on the normal pathway of expression for the mitochondrial *COX3* gene.

Glucose Repression at the Level of Mitochondrial Gene Expression. To examine directly the expression of *cox3*::*ARG8*^{m} we generated an antibody against yeast acetylornithine aminotransferase and used it to probe immunoblots of total cellular protein extracts. The predicted molecular weight of the nuclearly coded acetylornithine aminotransferase precursor is 46.7 kDa. The antibody detected a single protein of approximately this size in a wild-type protein extract that was absent from *arg8*Δ strains containing either wild-type mtDNA or mtDNA

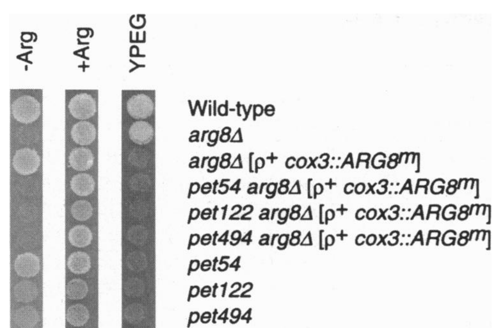


FIG. 2. Mitochondrial expression of *cox3::ARG8*^{m} depends on *COX3* mRNA-specific translational activation by *PET54*, *PET122*, and *PET494*. Yeast strains with the indicated relevant mutant genotypes were spotted and grown on complete glucose medium [yeast extract/peptone/dextrose (YPD)] (19) and then replica plated to minimal glucose medium [synthetic dextrose (SD)] (19) plus leucine, histidine, adenine, uracil, and lysine) containing arginine (+Arg), minimal glucose medium lacking arginine (−Arg), and nonfermentable medium [yeast extract/peptone/ethanol/glycerol [YPEG (8)]. The plates were incubated for 2 days at 30°C and photographed. The indicated strains are: Wild-type (JKR102, *MAT***a** *leu2-3,112 his4-519 ura3Δ* [ρ^{+}]); *arg8Δ* (DFS142, *MAT***a** *arg8Δ::URA3 leu2-3,112 his4-519 ura3Δ* [ρ^{+}]); *arg8Δ* [ρ^{+} *cox3::ARG8*^{m}] (DFS168, *MAT***a** *arg8Δ::URA3 leu2-3,112 his4-519 ura3Δ* [ρ^{+} *cox3::ARG8*^{m}]); *pet54 arg8Δ* [ρ^{+} *cox3::ARG8*^{m}] (DFS147, *MAT***a** *arg8Δ::URA3 pet54-5 ura3 leu2-3,112 his4-519* [ρ^{+} *cox3::ARG8*^{m}]); *pet122 arg8Δ* [ρ^{+} *cox3::ARG8*^{m}] (DFS174, *MAT***a** *arg8Δ::URA3 pet122::lacZ ura3 leu2-3,112 his4-519* [ρ^{+} *cox3::ARG8*^{m}]); *pet494 arg8Δ* [ρ^{+} *cox3::ARG8*^{m}] (DFS151, *MAT***a** *arg8Δ::URA3 pet494-41 ura3 leu2-3,112 his4-519* [ρ^{+} *cox3::ARG8*^{m}]); *pet54* (MCC133, *MATα pet54-5 lys2* [ρ^{+} *cox3-15*]); pet122 (BB8z, *MATα pet122::lacZ ade2 ura3Δ* [ρ^{+}]); *pet494* (MCC101, *MAT***a** *pet494-41 ade2* [ρ^{+}]).

bearing an unrecoded nuclear *ARG8* gene fused to *COX3* in a configuration otherwise identical to *cox3::ARG8*^{m} (Fig. 3). The steady-state level of the wild-type protein was lower in cells grown on the nonrepressing sugar raffinose than on glucose.

The predicted molecular weight of the primary translation product of the *cox3::ARG8*^{m} mitochondrial gene is 47.8 kDa. Cells carrying the *cox3::ARG8*^{m} mitochondrial gene (and a nuclear *arg8* deletion) contained an immunoreactive protein whose SDS gel mobility was identical to the wild type, suggesting that both the mitochondrially synthesized and wild-type proteins were processed similarly in the matrix (Fig. 3). Both proteins behaved like soluble matrix enzymes in submitochondrial fractionation experiments (not shown). However, in contrast to the wild type, the *cox3::ARG8*^{m} gene product was

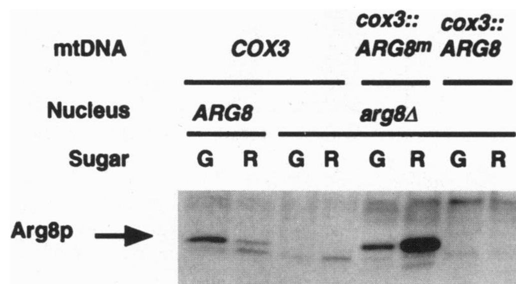


FIG. 3. Immunological detection of acetylornithine aminotransferase (Arg8p) coded either in the nucleus or mitochondria. Yeast cells with the relevant indicated mitochondrial and nuclear genotypes were grown to midlogarithmic phase in complete medium containing either 2% glucose (G) or 2% raffinose (R), and total cell protein was extracted as described (28). Eight micrograms of each protein sample were subjected to SDS/electrophoresis in an 8% polyacrylamide gel, blotted to an Immobilon-P membrane (Millipore), and probed with anti-Arg8p antiserum. The yeast strains were JKR102, DFS142, DFS168 (see legend to Fig. 2), and DFS144 (*MAT***a** *arg8Δ::URA3 leu2-3,112 his4-519 ura3Δ* [ρ^{+} cox3::*ARG8*])

present at substantially higher steady-state levels in cells grown on raffinose than on glucose.

We also assayed acetylornithine aminotransferase activity in detergent solubilized whole cell lysates. The specific activity in haploid wild-type cells grown on raffinose was about one-half that of glucose grown cells (0.46 unit on raffinose; 0.84 unit on glucose). In haploids carrying the *cox3::ARG8*^{m} mitochondrial gene and an *arg8* deletion in the nucleus, the specific activity in raffinose grown cells was 4.4 times that of glucose grown cells (5.7 units on raffinose, 1.3 units on glucose).

mRNA-Specific Translational Activation Is Rate-Limiting for Mitochondrial Expression of *cox3::ARG8*^{m}. Expression of *cox3::ARG8*^{m} at the level of cell growth (Fig. 2) and enzymatic activity (not shown) is completely dependent upon the nuclearly encoded *COX3* mRNA-specific translational activator complex. However, this observation sheds no light on the question of whether or not the activator complex is normally present at levels limiting for the expression of the mitochondrial gene. To approach this question, we constructed strains with altered dosage of the three nuclear genes encoding translational activator subunits, and measured expression of *cox3::ARG8*^{m}.

First, we halved the dosage of *PET54*, *PET122*, and *PET494* by constructing *arg8Δ/arg8Δ* [rho^{+} *cox3::ARG8*^{m}] diploid strains heterozygous for null mutations in the activator genes, all of which grew normally on media lacking arginine. Cells were then grown on complete medium containing either glucose or raffinose as a carbon source and acetylornithine aminotransferase specific activity in whole cell lysates was determined (Table 1). The *pet54/PET54* heterozygous diploid had essentially the same specific activity as the homozygous wild-type control. However, the reporter activity was significantly reduced in the *pet122/PET122* heterozygous diploid and essentially halved in the *pet494/PET494* strain (Table 1). Thus, the *COX3* mRNA-specific translational activator complex appears to be present in diploid cells at levels that limit expression of *cox3::ARG8*^{m} and could therefore play a role in modulating mitochondrial gene expression. Furthermore, Pet494p appears to be the translational activator subunit present at the lowest effective concentration in diploid strains.

We also studied the effects of greatly overproducing translational activator subunits by transforming a haploid *arg8Δ* [*cox3::ARG8*^{m}] strain with high copy plasmids that directed the expression of *PET54*, *PET122*, and *PET494* under the control of the strong *ADC1* promoter (28) (Table 2). Under these conditions, Pet54p and Pet122p accumulate to very high levels, whereas Pet494p is increased substantially over wild-type levels but remains undetectable by Coomassie Blue staining of electrophoretically separated mitochondrial proteins (6). Overproduction of Pet54p appeared to decrease *cox3::ARG8*^{m} expression slightly, whereas overproduction of Pet122p appeared to increase it slightly, although these effects may not be significant. However, increased levels of Pet494p caused an approximately 65% increase in *cox3::ARG8*^{m} expression in cells grown either on glucose or raffinose (Table 2), consistent with the possibility that, in wild-type cells, Pet494p levels are limiting for translation of the *COX3* mRNA and thus expression of the gene. However, some other component(s) must become limiting for synthesis of the reporter protein in the presence of excess Pet494p, because Pet494p levels are elevated many-fold under these conditions. The modest increase in reporter activity was not due to a physiological limit on accumulation of active acetylornithine aminotransferase, because extracts of a yeast strain overexpressing a plasmid-borne nuclear *ARG8* gene from the *GAL1* promoter had a specific activity 14-fold higher than the highest reported in Table 2 (not shown). The results of co-overexpression of combinations of the three translational activator subunits were also consistent with the possibility that Pet494p is limiting although, interestingly, co-overexpression of Pet54p and Pet122p in raffinose-

Table 1. Mitochondrial expression of *cox3::ARG8*^{m} in diploids containing single copies of nuclear genes for translational activator subunits.

Relevant genotype					Specific activity	
ARG8	[*cox3::ARG8*^{m}]	*PET54*	*PET122*	*PET494*	Glucose	Raffinose
+/+	−	+/+	+/+	+/+	1.21 ± 0.04	0.80 ± 0.02
−/−	−	+/+	+/+	+/+	0.04 ± 0.06	0.01 ± 0.02
−/−	+	+/+	+/+	+/+	2.07 ± 0.14	7.14 ± 0.20
−/−	+	+/−	+/+	+/+	1.88 ± 0.32	6.44 ± 0.48
−/−	+	+/+	+/−	+/+	1.26 ± 0.13	4.44 ± 0.37
−/−	+	+/+	+/+	+/−	1.02 ± 0.20	3.37 ± 0.79

Yeast cells with the indicated relevant genotypes (+ denotes functional genes; − denotes null alleles) were grown to midlogarithmic phase at 30°C in complete medium (21) containing either 2% glucose or 2% raffinose. Acetylornithine aminotransferase specific activities are expressed as the mean OD_{440}/mg of protein of a minimum of three assays, ± standard deviation. All strains used were *MAT***a**/*MAT*α *leu2-3, 112/leu2-3, 112 his4-519/his4-519 ura3*Δ/*ura3*Δ [ρ^+]. Where indicated, strains were *cox3::ARG8*^{m}, arg8Δ::*URA3*/*arg8*Δ::*URA3, pet54-5/PET54, pet122::lacZ/PET122* or *pet494-41/PET494.*

grown cells decreased reporter activity whether or not Pet494p was also overexpressed (Table 2).

Translational Activation Stabilizes the *cox3::ARG8*^{m} mRNA. These findings suggest that modulated translational activation could account for glucose repression of mitochondrial genes. However, previous studies of yeast mitochondrial RNA abundance and transcription rates have revealed that both are repressed in cells grown on glucose, suggesting that transcriptional regulation could also modulate mitochondrial gene expression (31, 32). In the case of *COX3* mRNA, synthesis was approximately 3-fold higher in cells grown on the nonfermentable carbon source glycerol than on glucose, whereas the steady-state abundance of the mRNA was 4- to 6-fold higher (32).

We examined the relative steady-state abundance of *cox3::ARG8*^{m} mRNA in cells grown on glucose or raffinose, in the presence or absence of translational activation. Cells of a translationally competent *arg8*Δ [*cox3::ARG8*^{m}] strain grown on raffinose accumulated roughly 3-fold more of this artificial mitochondrial mRNA than cells grown on glucose (Fig. 4). However, in the absence of Pet54p or Pet122p the raffinose-to-glucose ratio of *cox3::ARG8*^{m} mRNA levels was reduced to roughly 1.1, and in the absence of Pet494p it was reduced to 1.75. Thus, the increase in levels of *cox3::ARG8*^{m} mRNA in cells grown on raffinose depends on translational activation of the mRNA. This suggests that translational activation stabilizes the mRNA and that the increased mRNA levels observed in raffinose grown cells may be due as much or more to increased translation as to increased transcription.

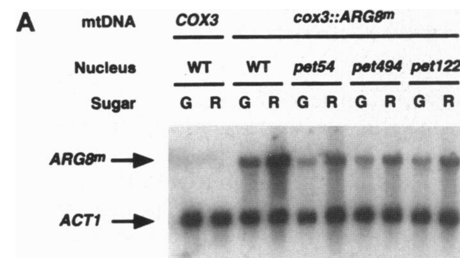
Table 2. Mitchondrial expression of *cox3::ARG8*^{m} in haploids containing high levels of translational activator subunits.

	Specific activity	
Overexpressed gene(s)	Glucose	Raffinose
None	1.37 ± 0.23	4.47 ± 0.89
PET54	1.03 ± 0.29	3.97 ± 0.58
PET122	1.68 ± 0.27	5.22 ± 1.30
PET494	2.26 ± 0.36	7.27 ± 2.08
PET54, PET122	0.99 ± 0.30	2.72 ± 0.43
PET494, PET54	2.01 ± 0.24	7.24 ± 1.94
PET494, PET122	2.21 ± 0.69	7.62 ± 1.88
PET54, PET122, PET494	1.12 ± 0.54	2.66 ± 0.61

The haploid strain DFS189 (*MAT***a** *arg8::hisG ura3-52 leu2-3,112 lys2 his3ΔHinDIII* [ρ^+ *cox3::ARG8*^{m}]) was transformed with all possible combinations of high copy (2 μ) replicating vectors to overexpress the indicated translational activator genes from the *ADC1* promoter (29): pCB1 (*PET54, HIS3*); pMC210 (30) (*PET494, LEU2*); pEAD122 (6) (*PET122, URA3*). All strains studied were transformed to His$^+$, Leu$^+$, Ura$^+$ prototrophy using empty replicating vectors where appropriate. The presence of plasmid-borne genes in the overproducing strains was confirmed by PCR analysis of total cellular DNA, using primers (5′-GGTGAATTCCGGGTGTACAAT and 5′-GGTGGATCCTCTGAGGACATA) specific for the *ADC1* promoter and terminator sequences surrounding the plasmid-borne genes (not shown). Cells were grown in minimal medium (Difco yeast nitrogen base) containing lysine and either 2% glucose or 2% raffinose. Acetylornithine aminotransferase specific activity was determined as described in the legend to Table 1.

DISCUSSION

Our data on organellar expression of the synthetic mitochondrial reporter gene *cox3::ARG8*^{m} strongly suggest that it is controlled at the level of translation by the activity of the nuclearly encoded *COX3* mRNA-specific translational activator complex. As judged by the effects of under- and overpro-



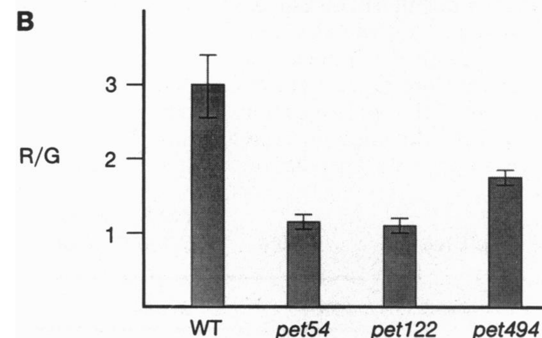


FIG. 4. Elevation of *cox3::ARG8*^{m} mRNA levels in raffinose grown cells is dependent on translational activation. (*A*) Yeast strains carrying a nuclear *arg8* deletion and the indicated relevant genotypes were grown to midlogarithmic phase in complete medium containing either 2% glucose (G) or 2% raffinose (R) and total RNA was prepared by hot phenol extraction (33). Four micrograms of each sample was electrophoresed (10), blotted to nitrocellulose, hybridized to ^{32}P-labeled DNA produced by random priming (Boehringer Mannheim) of an *ARG8*^{m} template and an *ACT1* (34) template, and autoradiographed. The strains, described in the legend to Fig. 2, were DFS142, DFS168, DFS147, DFS151, and DFS174. (B) Three such blots were quantitatively analyzed with a Betascope 603 blot analyzer (Betagen, Waltham, MA) and the *ARG8*^{m} hybridization signals were normalized to *ACT1*. The bar graph indicates the average ratio and standard deviation (R/G) of raffinose-grown to glucose-grown *ARG8*^{m} hybridization for the four strains carrying the *cox3::ARG8*^{m} mitochondrial gene and the indicated nuclear genotypes.

duction of individual subunits of the complex, Pet494p appears to be the most limiting factor. This suggests that regulation of expression of the nuclear gene *PET494* might in turn modulate *COX3* mRNA translation. Consistent with this idea, *PET494* is expressed at a very low level in yeast cells and is subject to glucose repression (18). Whereas Pet54p levels had little effect on expression of *cox3::ARG8*^{m}, the *PET54* gene is known to be expressed approximately 10-fold more strongly than *PET494*, such that its levels should not be limiting (35). Alterations in Pet122p levels affected *cox3::ARG8*^{m} expression, but more modestly than changes in Pet494p. The regulation of *PET122* expression has not been studied carefully, but appears to occur at a low level comparable to *PET494* (B.A. Barlow and T.D.F., unpublished). Thus the *COX3* mRNA-specific translational activator may be a dynamic complex whose level is modulated in response to growth conditions primarily by regulation of the nuclear gene encoding one of its subunits, Pet494p.

While transcription rates and steady-state mRNA levels are modulated in yeast mitochondria (32), it is not clear that these effects regulate gene expression *per se*. For example, artificial overproduction of *COX2* mRNA does not cause an apparent increase in synthesis of the protein product (36). Indeed, translational activation can affect mRNA levels posttranscriptionally. *COX2* mRNA stability is apparently dependent upon translational activation, because its steady-state level is substantially reduced in the absence of its mRNA-specific activator, Pet111p (37). The levels of wild-type *COX3* mRNA are not greatly affected by the presence or absence of translational activators (38): apparently the structure of the *COX3* mRNA makes it intrinsically stable. However, the artificial *cox3::ARG8*^{m} mRNA, like the native *COX2* mRNA, is stabilized by translational activation. Decreased stability of some untranslated mRNAs has also been observed in chloroplasts of *Chlamydomonas reinhardtii* (39). These observations contrast with many in eukaryotic cytoplasms where interference with translation generally stabilizes mRNAs (40).

Our studies strongly suggest that yeast mitochondrial mRNA-specific translational activators are likely to play a dual role, both tethering translated mRNAs to the inner membrane and controlling the synthesis levels of mitochondrial gene products. However, the reasons for mRNA-specificity are not clear, particularly where the subunits of a single enzyme, e.g. cytochrome *c* oxidase, are regulated separately: perhaps independent control of organellar mRNA translation allows topological distinctions that assist in the assembly of membrane complexes. In any event, there is now abundant evidence that chloroplasts, like yeast mitochondria, also employ nuclearly coded functions to activate translation of specific organellar mRNAs, through sites in their 5′-untranslated leaders (41, 42). Translational modulation occurs in chloroplasts (43, 44), and seems likely to involve these activators. Thus, mRNA-specific translational activation appears to be a general mechanism controlling organellar gene expression.

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1. Attardi, G. & Schatz, G. (1988) *Annu. Rev. Cell Biol.* **4,** 289–333.
2. Dieckmann, C. L. & Staples, R. R. (1994) *Int. Rev. Cytol.* **152,** 145–181.
3. Grivell, L. A. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30,** 121–164.
4. Fox, T. D. (1996) in *Translational Control*, eds. Hershey, J. W. B., Matthews, M. B. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 733–758.
5. Costanzo, M. C. & Fox, T. D. (1990) *Annu. Rev. Genet.* **24,** 91–113.
6. McMullin, T. W. & Fox, T. D. (1993) *J. Biol. Chem.* **268,** 11737–11741.
7. Brown, N. G., Costanzo, M. C. & Fox, T. D. (1994) *Mol. Cell. Biol.* **14,** 1045–1053.
8. Costanzo, M. C. & Fox, T. D. (1993) *Mol. Cell. Biol.* **13,** 4806–4813.
9. Costanzo, M. C. & Fox, T. D. (1995) *Curr. Genet.* **28,** 60–66.
10. Wiesenberger, G., Costanzo, M. C. & Fox, T. D. (1995) *Mol. Cell. Biol.* **15,** 3291–3300.
11. McMullin, T. W., Haffter, P. & Fox, T. D. (1990) *Mol. Cell. Biol.* **10,** 4590–4595.
12. Haffter, P., McMullin, T. W. & Fox, T. D. (1991) *Genetics* **127,** 319–326.
13. Haffter, P. & Fox, T. D. (1992) *Mol. Gen. Genet.* **235,** 64–73.
14. Dowhan, W., Bibus, C. R. & Schatz, G. (1985) *EMBO J.* **4,** 179–184.
15. Fox, T. D. (1987) *Annu. Rev. Genet.* **21,** 67–91.
16. Jauniaux, J.-C., Urrestarazu, L. A. & Wiame, J.-M. (1978) *J. Bacteriol.* **133,** 1096–1107.
17. Heimberg, H., Boyen, A., Crabeel, M. & Glansdorff, N. (1990) *Gene* **90,** 69–78.
18. Marykwas, D. L. & Fox, T. D. (1989) *Mol. Cell. Biol.* **9,** 484–491.
19. Rose, M. D., Winston, F. & Hieter, P. (1988) *Methods in Yeast Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
20. Jayaraman, K. & Puccini, C. J. (1992) *BioTechniques* **12,** 392–398.
21. Fox, T. D., Folley, L. S., Mulero, J. J., McMullin, T. W., Thorsness, P. E., Hedin, L. O. & Costanzo, M. C. (1991) *Methods Enzymol.* **194,** 149–165.
22. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89.
23. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
24. Vogel, H. J. & Jones, E. E. (1970) *Methods Enzymol.* **17A,** 260–264.
25. Dujon, B. (1981) in *The Molecular Biology of the Yeast Saccharomyces, Life Cycle and Inheritance*, eds. Strathern, J. N., Jones, E. W. & Broach, J. R. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 505–635.
26. Thorsness, P. E. & Fox, T. D. (1990) *Nature (London)* **346,** 376–379.
27. Conde, J. & Fink, G. R. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3651–3655.
28. Yaffe, M. P. (1991) *Methods Enzymol.* **194,** 627–643.
29. Ammerer, G. (1983) *Methods Enzymol.* **101,** 192–201.
30. Costanzo, M. C. & Fox, T. D. (1986) *Mol. Cell. Biol.* **6,** 3694–3703.
31. Mueller, D. M. & Getz, G. S. (1986) *J. Biol. Chem.* **261,** 11816–11822.
32. Ulery, T. L., Jang, S. H. & Jaehning, J. A. (1994) *Mol. Cell. Biol.* **14,** 1160–1170.
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993) *Current Protocols in Molecular Biology* (Current Protocols, Boston).
34. Gallwitz, D. & Seidel, R. (1980) *Nucleic Acids Res.* **8,** 1043–1059.
35. Fox, T. D. & Shen, Z. (1993) in *Protein Synthesis and Targeting in Yeast*, eds. McCarthy, J. E. G. & Tuite, M. F. (Springer, Berlin), pp. 157–166.
36. Pinkham, J. L., Dudley, A. M. & Mason, T. L. (1994) *Mol. Cell. Biol.* **14,** 4643–4652.
37. Poutre, C. G. & Fox, T. D. (1987) *Genetics* **115,** 637–647.
38. Costanzo, M. C., Seaver, E. C. & Fox, T. D. (1986) *EMBO J.* **5,** 3637–3641.
39. Sakamoto, W., Chen, X., Kindle, K. L. & Stern, D. B. (1994) *Plant J.* **6,** 503–512.
40. Sachs, A. B. (1993) *Cell* **74,** 413–421.
41. Staub, J. M. & Maliga, P. (1994) *Plant J.* **6,** 547–553.
42. Zerges, W. & Rochaix, J.-D. (1994) *Mol. Cell. Biol.* **14,** 5268–5277.
43. Berry, J. O., Breiding, D. E. & Klessig, D. F. (1990) *Plant Cell* **2,** 795–803.
44. Gillham, N. W., Boynton, J. E. & Hauser, C. R. (1994) *Annu. Rev. Genet.* **28,** 71–93.