Activity of Mitochondrially Synthesized Reporter Proteins Is Lower Than That of Imported Proteins and Is Increased by Lowering cAMP in Glucose-Grown *Saccharomyces cerevisiae* Cells

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ABSTRACT

We selected for increased phenotypic expression of a synthetic *cox2::arg8^m-G66S* reporter gene inserted into *Saccharomyces cerevisiae* mtDNA in place of *COX2*. Recessive mutations in *ras2* and *cyr1*, as well as elevated dosage of *PDE2*, allowed *cox2::arg8^m-G66S* to support Arg prototrophy. Each of these genetic alterations should decrease cellular cAMP levels. The resulting signal was transduced through redundant action of the three cAMP-dependent protein kinases, *TPK1*, *TPK2*, and *TPK3*. *ras2* had little or no effect on the level of wild-type Arg8p encoded by *cox2::ARG8^m*, but did increase Arg8p activity, as judged by growth phenotype. *ras2* also caused increased fluorescence in cells carrying the synthetic *cox3::GFP^m* reporter in mtDNA, but had little effect on the steady-state level of GFP polypeptide detected immunologically. Thus, decreased cAMP levels did not affect the synthesis of mitochondrially coded protein reporters in glucose-grown cells, but rather elevated activities in the matrix that promote efficient folding. Furthermore, we show that when Arg8p is synthesized in the cytoplasm and imported into mitochondria, it has greater activity than when it is synthesized in the matrix. Thus, mitochondrially synthesized proteins may not have the same access to matrix chaperones as cytoplasmically synthesized proteins emerging from the import apparatus.

REGULATION of the production of respiratory complexes in the mitochondrial inner membrane is an unusually complicated process (Poyton and McEwen 1996). These large, multi-subunit complexes are encoded by both nuclear and mitochondrial genes whose products are synthesized on opposite sides of the mitochondrial inner membrane (Attardi and Schatz 1988). For example, the *Saccharomyces cerevisiae* cytochrome *c* oxidase complex is composed of three mitochondrially coded subunits, Cox1p, Cox2p, and Cox3p, as well as eight smaller, nuclearly coded subunits (Capaldi 1990; Geier *et al.* 1995). The mitochondrially encoded subunits are synthesized by a genetic system composed almost entirely of nuclearly encoded proteins and mitochondrially coded RNAs (Pel and Grivell 1994; Fox 1996).

Although all three mitochondrially coded subunits of cytochrome *c* oxidase must accumulate at stoichiometric levels to produce a functioning complex, the synthesis of each is controlled by mRNA-specific translational activators that recognize the 5′-untranslated leaders of their respective mRNAs (Costanzo and Fox 1988; Mulero and Fox 1993a,b; Brown *et al.* 1994; Manthey and McEwen 1995). These activators appear to control translation rates since, at least in the case of the *COX2* and *COX3* mRNAs, the levels of the translational activators limit mitochondrial reporter gene expression (Steele *et al.* 1996; Green-Willms *et al.* 2001). In addition, the membrane-bound activator proteins (McMullin and Fox 1993; Green-Willms *et al.* 2001) apparently function to localize translation, since their targets in untranslated regions of the *COX2* and *COX3* mRNAs contain topogenic information necessary for efficient cytochrome oxidase assembly (Sanchirico *et al.* 1998). Interactions among the translational activators for the cytochrome oxidase subunits suggest that they colocalize translation of the core mitochondrially coded cytochrome oxidase subunits (Naithani *et al.* 2003).

In this study we sought to genetically identify additional functions that might serve to limit yeast mitochondrial gene expression at a translational or post-translational level by screening for overexpression of genes in mitochondrial DNA (mtDNA). However, overexpression of endogenous mitochondrial genes would not cause a predictable growth phenotype: it could increase respiratory growth if their products were limiting for respiratory function, or it could decrease respiratory growth if their overexpression caused deleterious imbalances. More likely, it would have no effect on respiratory growth. We therefore screened for chromosomal mutations and for genes on high-copy plasmids that would

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increase the level of a mitochondrially encoded reporter protein encoded by a synthetic gene inserted into mtDNA. This synthetic gene encoded a mutant form of the arginine biosynthetic enzyme Arg8p, whose stability was reduced to the point that it severely restricted growth in the absence of Arg. Selection for Arg^+ in this background yielded strains containing increased levels of the unstable reporter.

The results of our screen make it clear that lowered cAMP levels lead to increased activity of mitochondrially synthesized reporter proteins. However, analysis of this phenotype revealed that there was no increase in gene expression at the level of translation, but rather in post-translational functions affecting activity. Interestingly, our data also reveal that when the reporter proteins are synthesized in the cytoplasm and imported into mitochondria, they have greater activity than when they are synthesized in the matrix.

MATERIALS AND METHODS

Yeast strains and genetic methods: *S. cerevisiae* strains used in this study are listed in Table 1. All strains are congenic to DBY947 except SAS1B, its derivative TMD118 (both derived from D273-10B), and TF241 and its derivatives, RJS20, TMD66-68, and 73-74. Standard yeast genetic manipulations, protocols, and media were used (Sherman *et al.* 1974; Fox *et al.* 1991; Guthrie and Fink 1991). Arg^- auxotrophy was detected by printing cells to medium lacking arginine, incubating for 3 days at the indicated temperatures, and then printing a second time to medium lacking arginine, to overcome phenotypic lag. DNA was transformed into yeast using the lithium-acetate protocol (Chen *et al.* 1992) or the Frozen-EZ yeast transformation II kit from Zymo Research. *cox3::GFP^m* was introduced into strains carrying ρ^+ *cox2::arg8^m-G66S* by cytoduction with JSC10X (Cohen and Fox 2001). Successful cytoduction was confirmed by PCR with primers GFPm1 and GFPm2.

To isolate hypomorphic *cox2::arg8^m* alleles, strain TF241 (*ino1*, *pet9*, *arg8*Δ, ρ^+*cox2::ARG8^m*) was mutagenized by growth in YPD plus 2 mm $MnCl_2$ overnight at 30° (Putrament *et al.* 1973; Fox *et al.* 1991). After mutagenesis, cells were grown in YPD to early log phase and Arg^- auxotrophs were enriched for by inositol starvation (Lawrence 1991). Cells were harvested over a period of 4 days and plated on synthetic-complete (Qbiogene) glucose lacking leucine. Arginine auxotrophs were identified by replica plating to synthetic-complete glucose medium lacking leucine and arginine. On day 0, 0/797 screened colonies were Arg^-. After 1–4 days of starvation, 81/528 were Arg^-.

DNA from a *mTn::LEU2* library (Burns *et al.* 1994) was digested by *Not*I and used to transform the *cox2::arg8^m* strain TMD62 to Leu^+. Roughly 19,000 Leu^+ transformants were screened for their ability to grow on synthetic-complete glucose medium lacking leucine and arginine at 33°. To test for linkage between Leu^+ and Arg^+ in the nonrespiring transformants, these transformants were mated to TMD26C and the resulting zygotes were placed immediately on sporulation medium to prevent mitotic division before meiosis (Fox *et al.* 1991). Tetrads were dissected and only spores that were unable to respire were scored for their arginine-independent growth phenotype. Linkage between the transposon insertion and Arg^+ was indicated when almost all the respiratory-deficient spores were either Arg^+, Leu^+ or Arg^-, Leu^-.

DNA from 2μ genomic banks (Nasmyth and Tatchell 1980; Engebrecht *et al.* 1990) was transformed into TMD62. Roughly 2700 Leu^+ transformants were screened for their ability to grow on synthetic-complete glucose medium lacking leucine and arginine at 33°. Plasmids were isolated from Arg^+ transformants and reintroduced to confirm their ability to confer an Arg^+ phenotype. Genomic fragments carried by the library plasmids were identified by sequencing with the primers YEP13-1 and YEP13-3 or with YEP351-1 and YEP351-2. *PDE2* was shown to be responsible for the Arg^+ phenotype by gene disruptions in pTD15 by use of the GPS-1 kit (New England Biolabs, Beverly, MA) and by subcloning a PCR product carrying *PDE2* generated with the primers 5′*Hin*dIII-*PDE2* and 3′*Bgl*II-*PDE2* into *Hin*dIII- and *Bam*HI-cut YEp351 (Hill *et al.* 1986) to create pTD40a. The active gene on another library plasmid, pTD39, was shown to be *RTS1* by gene disruption using the GPS-1 kit (New England Biolabs) and by subcloning a 3125-bp *Bgl*II-*Cla*I fragment from pTD39 into *Bam*HI-*Nar*I-cut YEp351 (Hill *et al.* 1986) to create pTD62a. *RTS1* containing plasmids were tested in strain TMD118 because their weak phenotypic effects were more easily detectable.

The nuclear *arg8-G66S* mutation was introduced into TWM34 by a two-step cloning-free strategy (Storici *et al.* 2001). The CORE cassette was amplified with 5′ARG8 KANMX4 and 3′ARG8K1URA3 and integrated between bases +196 and +197 of the *ARG8* coding sequence. The CORE cassette was replaced by transformation with the primer dpAR G8G66Sa (5′-ATATATCGATTTCACCGCAGGTATTGCGGT GACCGCATTATCGCATGCAAATCCTAAAGTGGCAGAAAT TCTGCACCATC-3′)and its complement, dpARG8G66Sb. Integrants were confirmed by sequence analysis.

Genetic analysis of *TPK1*, *TPK2*, and *TPK3*: *TPK1*, *-2*, and *-3* were overexpressed by the 2μ plasmids pXP2, pXP3, and pXP4, respectively (Pan and Heitman 1999). The *tpk1::URA3* disruption from LRY520 (Robertson *et al.* 2000) was amplified with the primers TPK1A and TPK1B, the *tpk2::HIS3* knockout was amplified with the primers TPK2A and TPK2B from the strain LRY590 (Robertson *et al.* 2000), and the *tpk3::HIS3* knockout was amplified from the strain LRY636 (Robertson *et al.* 2000) with the primers TPK3A and TPK3B. The *tpk3::TRP1* disruption was released from ptpk3::TRP1+ (Toda *et al.* 1987b) by digestion with *Pvu*II. The knockout and disruption cassettes were transformed individually or pairwise into TMD62 or TMD180. pTD45 was made by subcloning the *Bam*HI fragment from pXP2 containing *TPK1* into the *Bam*HI site of pRS315 (Sikorski and Hieter 1989). pTD46 was made by subcloning the *Xba*I-*Sst*I fragment containing *TPK2* from pXP3 into *Xba*I-*Sst*I-cut pRS315 (Sikorski and Hieter 1989). pTD49 was made by subcloning the *Hin*dIII-*Sst*I fragment from pXP4 containing *TPK3* into *Hin*dIII-*Sst*I-cut pRS316 (Sikorski and Hieter 1989). pTD45 was mutagenized by QuikChange (Stratagene, La Jolla, CA) with the primers 5′TPK1KR and 3′TPK1KR to introduce a *Dra*I site and K116R into *TPK1* in plasmid pTD53. The primers 5′TPK2KR and 3′TPK2KR were used in a QuikChange reaction to disrupt a *Dra*I site and to introduce K99R into *TPK2* on pTD46 to create plasmid pTD55. Similarly in pTD61, an *Acl*I site and K117R were introduced into *TPK3* of pTD49 by the primers 5′TPK3KR and 3′TPK3KR. *tpk1-K116R*, *tpk2-K99R*, and *tpk3-K117R* were introduced into the chromosome by a two-step cloning-free strategy (Storici *et al.* 2001). The CORE cassette was amplified by 5′TPK1KANMX4 and 3′TPK1KLURA3 and replaced from −16 to +705 of *TPK1*. *TPK2* from +1 to +657 was replaced by the CORE cassette amplified with 5′TPK2KANMX4 and 3′TPK2KLURA3. The CORE cassette was amplified with 5′TPK3KANMX4 and 3′TPK3KLURA3 to replace +1 to +708 of *TPK3*. The *TPK*-flanked CORE cassettes were transformed

into TMD62. The strains containing the CORE cassettes were transformed with the *Nde*I-*Sna*BI fragment from pTD53, the *Nde*I-*Bbs*I fragment from pTD55, or the *Fsp*I-*Bam*HI fragment from pTD61. Correct integration of the catalytic missense mutations was confirmed by sequence analysis. Double mutants were made by crossing followed by tetrad dissection. The catalytic missense mutations were scored in spore progeny by PCR (Newton *et al.* 1989; http://www.ich.ucl.ac.uk/cmgs/arms98.htm). Reactions contained 200 μM of each of the dNTPs, 0.5 μM of each primer, 0.5 units/100 μl Invitrogen (San Diego) Taq polymerase, and varying amounts of $MgCl_2$. 5′TPK1116wt and TPK1D2 and 5′TPK299kr and TPK2D2 were used with 1.5 mM $MgCl_2$. 3′TPK3117kr and TPK3C2 were used with 2.5 mM $MgCl_2$, and 3′TPK3117wt and TPK3A were used with 4.5 mM $MgCl_2$ at an annealing temperature of 55°. Reactions were done for 30 cycles of 1 min at 94°, 2 min at 50°, and 3 min at 72°.

Vectorette PCR: The locations of transposon insertions were identified by Vectorette PCR followed by sequencing out of the transposon. Vectorette PCR was performed as described (http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html), with modifications (Siebert *et al.* 1995). Following digestion, DNA was purified by phenol:chloroform extraction followed by a chloroform extraction and ethanol precipitation. The ligation was followed by heat inactivation and ethanol precipitation. The pellet was resuspended in 45 μl H_2O and 2 μl were used in a 25-μl PCR reaction with primers and buffers as described previously, but with 20 cycles of 1 min at 95°, 1 min at 63°, and 1.5 min at 72°. Product from this reaction was diluted 25-fold and 8 μl was used in a 100-μl PCR reaction using UV primer and a nested primer to the transposon, mTn3-2, with 27 cycles of 1 min at 95°, 1 min at 66°, and 1.5 min at 72°. Product was gel purified and used in a sequencing reaction with mTn3-2 as the primer.

Western blot analysis: Total yeast protein was isolated from cells grown to either midlog or early saturation in synthetic-complete glucose medium lacking leucine at 33° (Yaffe 1991). Protein concentration was determined using the Bio-Rad Fast Lowry kit. Equal amounts of protein were run on 10% SDS-PAGE gels and probed. The polyclonal rabbit anti-Ssc1 and rabbit anti-Hsp60 were a kind gift from J. Herrmann and the mouse monoclonal anti-Hsp78 (SS255) was from T. Mason (Leonhardt *et al.* 1993). As a loading control, blots were also probed with rabbit anti-glucose-6-phosphate dehydrogenase (G6PDH; Sigma, St. Louis). The secondary antibodies, either goat anti-rabbit-HRP or goat anti-mouse-HRP, were detected with either the enhanced chemiluminescence (ECL) or ECL^+ kit (Amersham Pharmacia). The exposed film was photographed using an AlphaImager 950, and the signal intensity of the digitized data was determined by analysis on a Macintosh computer using the public domain National Institutes of Health (NIH) image program (developed at the NIH and available at http://rsb.info.nih.gov/nih-image/). The integrated density of a background box was subtracted from that of a box encompassing each sample. For other quantitative Western blot analysis, rabbit anti-Arg8 (Steele *et al.* 1996) was purified on a Bio-Rad DEAE column, eluted with 20 mM Na-phosphate buffer, pH 8.0, concentrated, and treated with acetone powder (Harlow and Lane 1988) from strain SAS1B. Blots were probed with this serum at 1:50 dilution, followed by AlexaFluor488 goat anti-rabbit at 1:150 (Molecular Probes, Eugene, OR). Rabbit anti-green fluorescent protein (GFP; Cohen and Fox 2001), which was purified in like manner, was used at 1:50, followed by AlexaFluor488 goat anti-rabbit at 1:100. As a loading control, blots were also probed with rabbit anti-G6PDH (Sigma) at 1:250 followed by fluorescein donkey anti-rabbit (Amersham, Buckinghamshire, UK) used at 1:65–1:150. The signals from the fluorophores were detected using a Storm PhosphorImager 840. The signals were then quantitated using ImageQuant v1.2 by subtracting the sum of pixel intensities of a background box from the sum of pixel intensities of a boxed sample. A dilution series was included with each blot for all quantitative Western blot analyses to establish the linear range of signal for each protein and to generate a standard signal curve. Because the slope of the lines and *y*-intercepts were different for each protein's standard curve, normalization could not be done by simply dividing the Arg8 signal or the GFP signal by the G6PDH signal. Rather, the formula for normalization was (*m*G6PDH/*m*Arg8p or *m*GFP)((Arg8p or GFP signal − *b*Arg8p or bGFP)/(G6PDH signal − *b*G6PDH)), where *m* is the slope of the standard curve and *b* the *y*-intercept of the standard curve (A. R. Demlow, personal communication).

Microscopy: Cells were grown to early saturation in synthetic-complete glucose medium lacking leucine at 33°. Cells were washed two times with water and mounted in water on a slide. GFP was visualized with an Olympus BX60 (equipped for differential interference contrast). Images were captured using a Hamamatsu Orca 100 camera with an 8-sec exposure and analyzed via the Image Pro 4.5.0.19 software package from Media Cybernetics. Confocal microscopy was done on a Zeiss Axialvert10 microscope using a Bio-Rad MRC600 with a z-step size of 0.3 μm and 9–15 steps/sample. The signals from the confocal z-projections were quantitated using the program MetaMorph v4.5.r.4 from Universal Imaging. Cells were encircled as regions and thresholded. Fluorescing area is equal to the percentage of thresholded area and the sum of fluorescence is equal to the sum of integrated intensity of each plane for each cell. The z-series was projected with COMOS v7.1 from Bio-Rad. All images were prepared in Adobe Photoshop 6.0.

RESULTS

A screen for increased mitochondrial reporter gene expression: The reporter *cox2::ARG8^m* (Green-Willms *et al.* 2001) produces an Arg^+ phenotype under normal growth conditions. Therefore, it was difficult to screen for increased expression of this reporter by growth on minimal medium. To allow for selection of mutants with increased expression of the reporter gene, we sought a hypomorphic, or leaky, *cox2::arg8^m* missense allele. A suitable allele would not produce enough arginine to sustain growth on medium lacking arginine in an otherwise wild-type strain. However, a nuclear mutation resulting in increased expression of such a leaky *cox2::arg8^m* allele would be detectable by its Arg^+ phenotype (Figure 1).

To identify leaky *cox2::arg8^m* alleles we first subjected a strain carrying *cox2::ARG8^m* (TF241) to manganese mutagenesis (Putrament *et al.* 1973; Fox *et al.* 1991) followed by inositol starvation enrichment (Henry *et al.* 1975; Fox *et al.* 1991) for Arg^- auxotrophs (materials and methods). We next screened for mutants that would become Arg^+ when *cox2::arg8^m* expression was increased. To identify such alleles, we made use of the fact that overexpression of the *COX2* mRNA-specific translational activator *PET111* on a 2μ plasmid results in a two- to threefold increase of expression of *cox2::ARG8^m* (Green-Willms *et al.* 2001). Each *cox2::arg8^m* mutant

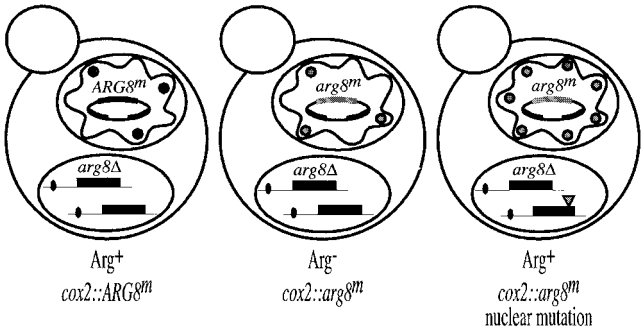

FIGURE 1.—Schematic of the screen used to detect increased *cox2::arg8^m-G66S* expression. The *COX2* coding sequence has been replaced with the recoded *ARG8^m* gene or a leaky allele, *arg8^m-G66S*. A strain with a nuclear *arg8*Δ that contains *cox2::ARG8^m* in mtDNA contains Arg8p in the matrix (solid circles) and is Arg$^+$ (left). A similar strain with *cox2::arg8^m-G66S* in mtDNA contains Arg8p-G66S (shaded circles) and is Arg$^-$ (middle). A nuclear mutation, indicated by the inverted triangle, causes increased *cox2::arg8^m-G66S* expression, resulting in an Arg$^+$ phenotype (right).

was mated to a ρ^0 strain (TMD26Cρ^0) carrying *PET111* on the 2μ plasmid pJM20 (MULERO and FOX 1993a) and the resulting diploids were scored for arginine-independent growth at various temperatures. Nine responsive *cox2::arg8^m* alleles were identified and sequenced. Three of the mutations fell within the *COX2* promoter or mRNA 5′-untranslated region. To avoid steering our screen back to RNA polymerase or mRNA-specific translational activation, these alleles were not used. The remaining mutations affected the Arg8p coding sequence (HEIMBERG *et al.* 1990; Table 1): A131T (TMD68) and a double mutation A131V, H138Y (TMD67) near the active site (BRODY *et al.* 1992); I288N (TMD66) and a double mutation A261V, H268Y (TMD73) near the B_6-binding site; G66S (RJS20) at a highly conserved position; and a frameshift at codon 96 (TMD74). The phenotypes of these alleles were examined more closely at various temperatures and in various strain backgrounds for reversion rates to Arg$^+$ and for responsiveness to *PET111* overexpression to determine the optimal screening conditions for each. Reversion rates were found to vary dramatically at different temperatures and responsiveness to *PET111* overexpression was found to be dependent on both strain background and temperature. We chose to use the *cox2::arg8^m-G66S* allele in the DBY947 strain background (TMD62) on minimal glucose medium at 33° for our selection. In a strain background known to have high mitochondrial gene expression (D273-10B) this allele was suppressed by *PET111* overexpression, whereas in the DBY947 background, which has lower expression (HE and FOX 1999), it was not.

Identification of nuclear genes affecting *cox2::arg8^m-G66S* expression: To identify possible negative regulators of mitochondrial gene expression, TMD62 was mutagenized by transformation with *Not*I fragments from two pools of a *mTn::LEU2* library (BURNS *et al.* 1994). Of ~19,000 Leu$^+$ transformants tested, 13 were Arg$^+$. Next, tetrad analysis of diploids generated by mating the mutants to TMD26C was done to test linkage of Arg$^+$ and Leu$^+$ (MATERIALS AND METHODS). In 11 cases *mTn::LEU2* was linked to Arg$^+$, and in each case the mutation was recessive. Spores derived from these tetrad analyses were then crossed to each other to test for complementation, revealing that the 13 mutants fell into two complementation groups (9 in one group and 4, including 2 whose *mTn::LEU2* was unlinked to Arg$^+$, in the second group). The location of the transposon in the 11 strains in which the transposon was responsible for the Arg$^+$ phenotype was determined by Vectorette PCR (MATERIALS AND METHODS) and DNA sequencing.

All the *mTn::LEU2* insertions in the first complementation group fell within *RAS2* at five locations (two at +113, one at +311, two at +393, one at +752, and three at +761). The insertion at +113 was used as a representative mutant for all subsequent analysis. A complete deletion of *ras2* caused the same phenotype as the *mTn::LEU2* insertions (our unpublished results). Both transposon insertions in the second complementation group fell within *CYR1* (at +1270 and +3078). Since *CYR1* is an essential gene (MATSUMOTO *et al.* 1982), these *mTn::LEU2* alleles must retain some activity. The effects of *ras2::mTn* and *cyr1::mTn* mutations on the Arg phenotype are shown in Figure 2A.

Possible positive regulators of mitochondrial reporter gene expression were identified by the introduction into TMD62 of two yeast genomic libraries in high-copy 2μ vectors (NASMYTH and TATCHELL 1980; ENGEBRECHT *et al.* 1990) and by screening for Arg$^+$ transformants (MATERIALS AND METHODS). Of the ~2700 Leu$^+$ transformants tested, 40 were Arg$^+$. After recovery of the plasmids and retesting, sequence analysis identified 10 unique plasmids that could confer some degree of Arg$^+$ growth, including 2 carrying *ARG8*. Not surprisingly, the plasmid carrying *ARG8* gave the strongest Arg$^+$ phenotype. The active gene on the plasmid that gave the next strongest phenotype was *PDE2* (MATERIALS AND METHODS; Figure 2A). *RTS1* was also identified as conferring Arg$^+$ growth, although to a much weaker degree (MATERIALS AND METHODS; Figure 2A).

Low cAMP signals to mitochondria through redundant activity of the cAMP-dependent protein kinase subunits: Ras2p is a GTPase required to activate adenylate cyclase, Cyr1p (THEVELEIN and DE WINDE 1999). Thus, the recessive transposon mutations result in reduced levels of cellular cAMP by eliminating or reducing Ras2p or Cyr1p activity, respectively. Pde2p is the high-affinity cAMP phosphodiesterase (SASS *et al.* 1986). When Pde2p is overexpressed, the rate of cAMP to AMP conversion is increased, resulting in lower cAMP levels. Thus, these data lead to the conclusion that low cAMP

TABLE 1

Yeast strains used in this study

Strain	Nuclear (mitochondrial) genotype	Reference
JSC10X	*MAT*α *ade2-101 ura3-52* [ρ$^-$ *pJC30X-BS(X)*]	Cohen and Fox (2001)
RJS20	*MAT***a** *ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
SAS1B	*MAT***a** *ura3-52 arg8Δ::his leu2-3,112 lys2 his3ΔHin*dIII [ρ^0]	This study
TF241	*MAT***a** *ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2* [D273-10B ρ$^+$ *cox2::ARG8^m*]	This study
TMD26C	*MAT*α *ade2-101 ura3-52 leu2Δ arg8Δ::his* [ρ$^+$]	This study
TMD26Cρ^0	*MAT*α *ade2-101 ura3-52 leu2Δ arg8Δ::his* [ρ^0]	This study
TMD62	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD66	*MAT***a** *ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2* [D273-10B ρ$^+$ *cox2::arg8^m-I288N*]	This study
TMD67	*MAT***a** ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2 [D273-10B ρ$^+$ *cox2::arg8^m-A131V H138Y*]	This study
TMD68	*MAT***a** *ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2* [D273-10B ρ$^+$ *cox2::arg8^m-A131T*]	This study
TMD73	*MAT***a** *ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2* [D273-10B ρ$^+$ *cox2::arg8^m-A261V H268Y*]	This study
TMD74	*MAT***a** *ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2* [D273-10B ρ$^+$ *cox2::arg8^m-+T frameshift (+288)*][a]	This study
TMD78	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his cyr1(3078)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD79	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(113)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD82	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his cyr1(1270)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD83	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(311)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD84	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(761)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD86	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(752)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD88	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(393)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD118	*MAT***a** *ura3-52 arg8Δ::his leu2-3,112 lys2 his3ΔHin*dIII [D273-10B ρ$^+$ *cox2::arg8^m-A261V H268Y*]	This study
TMD152b	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox2::ARG8^m*]	This study
TMD154	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(113)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::ARG8^m*]	This study
TMD162	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his ras2(113)^a::mTn::LEU2* [D273-10B ρ$^+$ *cox2::arg8^m-G66S cox3::GFP^m*]	This study
TMD180	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 trp1::hisG arg8Δ::his* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD187	*MAT***a** *ura3-52 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox2::arg8^m-G66S*]	This study
TMD189	*MAT***a** *ura3-5 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox2::arg8^m-G66S cox3::GFP^m*]	This study
TMD201	*MAT***a** *ura3-52 his3-11,15 arg8Δ::his* [DBY947 ρ$^+$]	This study
TMD215	*MAT***a** *ura3-52 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox2::ARG8^m*]	This study
TMD242	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox1::ARG8^m*]	This study
TMD243	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8Δ::his* [D273-10B ρ$^+$ *cox3::ARG8^m*]	This study
TMD343a	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 arg8-G66S* [D273-10B ρ$^+$]	This study
TWM34	*MAT***a** *ura3-52 leu2-3,112 his3-11,15* [D273-10B ρ$^+$]	This study
TWM34ρ^0	*MAT***a** *ura3-52 leu2-3,112 his3-11,15* [ρ^0]	This study

[a] The numbers in parentheses refer to the nucleotide of the coding sequence immediately upstream of insertions of the indicated nucleotide or mTn.

levels increase expression of the mitochondrial reporter *cox2::arg8^m-G66S*.

Cellular cAMP levels are known to signal downstream functions by regulating the activity of the cAMP-dependent protein kinases (PKA). In *S. cerevisiae* the catalytic subunits of PKA are Tpk1p, Tpk2p, and Tpk3p (Toda *et al.* 1987b). The activity of PKA is regulated by Bcy1p, a cAMP-binding protein (Kunisawa *et al.* 1987; Toda *et al.* 1987a). When cAMP levels are low, Bcy1p binds the catalytic subunits, repressing their activity. When cAMP levels are high, Bcy1p binds cAMP and releases the catalytic subunits, which can then phosphorylate downstream targets (Thevelein and de Winde 1999).

Overexpression of Tpk1p, Tpk2p, or Tpk3p should result in a higher level of catalytic PKA subunits preventing inhibition by normal levels of Bcy1p. Thus the pathway should respond as though cAMP levels are high, even in the presence of an upstream mutation such as *ras2::mTn* (Toda *et al.* 1987b). If the mitochondrial reporter expression were affected by cAMP through the action of PKA, then we would expect that overexpression of one of the catalytic subunits should result in a decrease of the Arg$^+$ phenotype seen in a *ras2::mTn* mutant. Indeed, the presence of *TPK1*, *TPK2*, or *TPK3* on a multicopy 2μ plasmid (Pan and Heitman 1999) reduced the Arg$^+$ growth caused by *ras2::mTn* (Figure 2B).

The three Tpk proteins are highly redundant for most cellular activities, and we found that overexpression of *TPK1* or *TPK2* or *TPK3* reduced the Arg$^+$ growth caused

by *ras2::mTn*, consistent with redundancy here. However, there is evidence for nonredundancy in Tpk regulation of pseudohyphal growth (Robertson and Fink 1998; Pan and Heitman 1999) and iron uptake, which influences respiratory capacity (Robertson *et al.* 2000). To test further for specificity in the control of *cox2::arg8^m-G66S* expression, we deleted each *TPK* gene individually and in pairwise combinations, but failed to detect Arg$^+$ growth, suggesting redundancy (deletion of all three *TPK* genes is lethal; Toda *et al.* 1987b). In addition, we chromosomally integrated (Storici *et al.* 2001) catalytically inactive missense mutations in each of the *TPK* genes, since it has been previously observed that kinase specificity may be revealed by such missense mutations, despite the artifactual observation of apparent redundancy suggested by the phenotypes of null alleles (Madhani *et al.* 1997). We changed the yeast Tpk residues equivalent to bovine protein kinase A lysine 72 to arginine (Zheng *et al.* 1993; Akamine *et al.* 2003; S. S. Taylor, personal communication), making mutations *tpk1-K116R*, *tpk2-K99R*, and *tpk3-K117R* (materials and methods). Tetrad analysis revealed that the triple mutant was inviable, confirming that these alleles are nonfunctional. However, none of the catalytically inactive *tpk* missense alleles conferred an Arg$^+$ phenotype on *cox2::arg8^m-G66S* strains, either individually or in pairwise combinations. Thus, in this system the Tpk proteins appear to be redundant.

cAMP levels post-translationally affect Arg8p-G66S and wild-type Arg8p activity: To confirm that the Arg$^+$ phenotype observed in a *ras2::mTn* mutant was due to increased accumulation of Arg8p-G66S, we carried out quantitative Western blot analysis on total cellular protein. As expected, the steady-state level of Arg8p-G66S was ~2.3-fold higher in the *ras2::mTn* mutant than in wild type (Figure 3A; Table 2). However, it was also clear that the Arg8p-G66S encoded by *cox2::arg8^m-G66S* was present at much lower steady-state levels than wild-type Arg8p encoded by *cox2::ARG8^m* (Figure 3C), suggesting that the Arg8p-G66S protein is unstable. We therefore examined the effect of *ras2::mTn* on the steady-state level of mitochondrially coded wild-type Arg8p and found very little, if any, effect of *ras2::mTn* on its level (Figure 3D; Table 2). Thus, decreased cAMP levels preferentially increased accumulation of the reporter protein encoded by *cox2::arg8^m-G66S*, but not the *cox2::ARG8^m* reporter. Since wild-type Arg8p can accumulate to high levels in yeast mitochondria (Steele *et al.* 1996; Green-Willms *et al.* 2001), this observation indicates that decreased cAMP levels caused stabilization of the unstable Arg8p-G66S, possibly by increasing the activity of protein-folding functions or by decreasing the activity of proteolytic functions in the mitochondrial matrix.

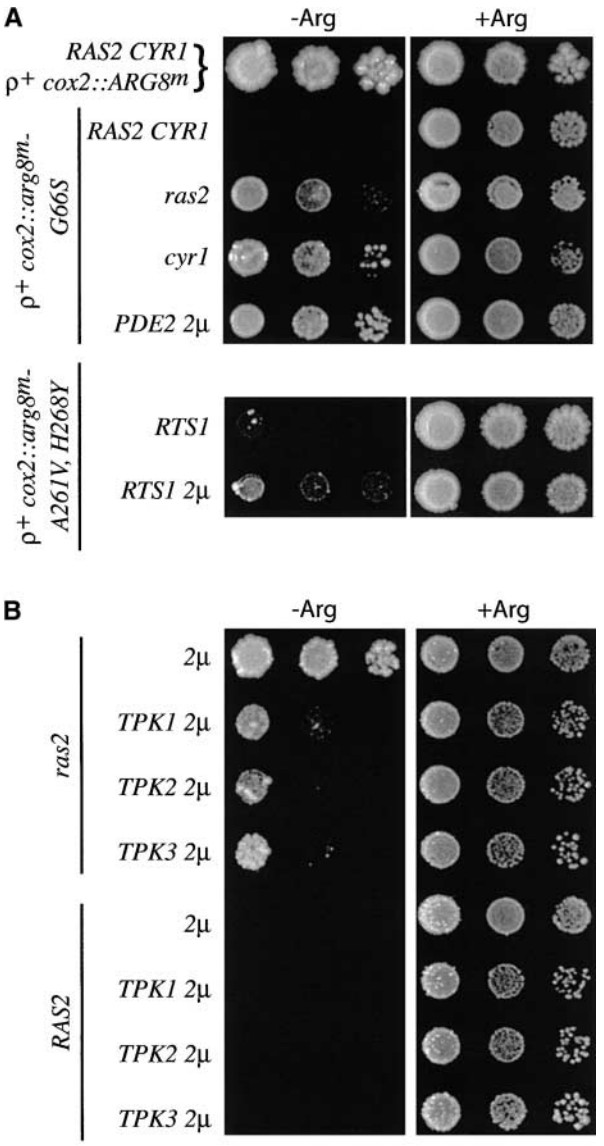

Figure 2.—Arginine-dependent growth phenotypes of a *cox2::arg8^m-G66S* strain are altered by cAMP signaling pathway mutants. (A) The *RAS2 CYR1* ρ$^+$ *cox2::arg8^m-G66S* parent strain TMD62 (Table 1) was altered by *ras2* (TMD79) or *cyr1* (TMD78) mutations. In addition, TMD62 was transformed with a *PDE2* 2μ plasmid (pTD40a). The Arg$^+$ strain TMD215 (*RAS2 CYR1* ρ$^+$ *cox2::ARG8^m*) is also shown for comparison. The *RTS1* ρ$^+$ *cox2::arg8^m-A261V H268Y* parent strain TMD118 (Table 1) was transformed with either the empty vector YEp351 (RTS1) or pTD62a (*RTS1* 2μ). Strains were grown in liquid synthetic-complete glucose medium lacking leucine, serially diluted, and then spotted on synthetic-complete glucose medium lacking leucine (+Arg) or leucine and arginine (−Arg) and incubated at 33° for 11 days (DBY947) or 9 days (D273-10B). (B) The presence of any *TPK* gene on a 2μ plasmid decreases the Arg$^+$ phenotype of a *ras2* strain. TMD79 (*ras2*) and TMD187 (*RAS2*) were transformed with empty vector (YEp352) or 2μ plasmids carrying *TPK1* (pXP2), *TPK2* (pXP3), or *TPK3* (pXP4; Pan and Heitman 1999). The strains were grown in synthetic-complete glucose lacking leucine and uracil, serially diluted, and spotted on synthetic-complete glucose lacking leucine and uracil (+Arg) or leucine, uracil, and arginine (−Arg) and grown at 33° for 15 days.

Interestingly, the Arg^+ growth of a strain containing wild-type Arg8p encoded by *cox2::ARG8^m* was improved by the *ras2::mTn* mutation (Figure 3E), even though there was very little change in Arg8p levels (Figure 3D; Table 2). Thus, the *ras2::mTn* mutation appears to increase the specific activity of the mitochondrially encoded Arg8p, suggesting that decreased cAMP levels lead to more efficient assembly of the active enzyme from the stable wild-type protein.

We also compared Arg8p-G66S levels in a *ras2::mTn* mutant to those of an isogenic strain (TMD62) containing *PET111* on a high-copy vector (pEWH107; Figure 3A; Table 2). As expected from the growth phenotypes (Figure 3B), the *ras2::mTn* mutation caused a greater increase in the Arg8p-G66S level than *PET111* overexpression did (Figure 3A). In contrast, *PET111* overexpression caused a greater increase in the steady-state level of mitochondrially coded wild-type Arg8p than the *ras2::mTn* mutation did (Figure 3D; Table 2). Thus, decreased cAMP does not appear to greatly affect the level of translation of the reporter mRNA.

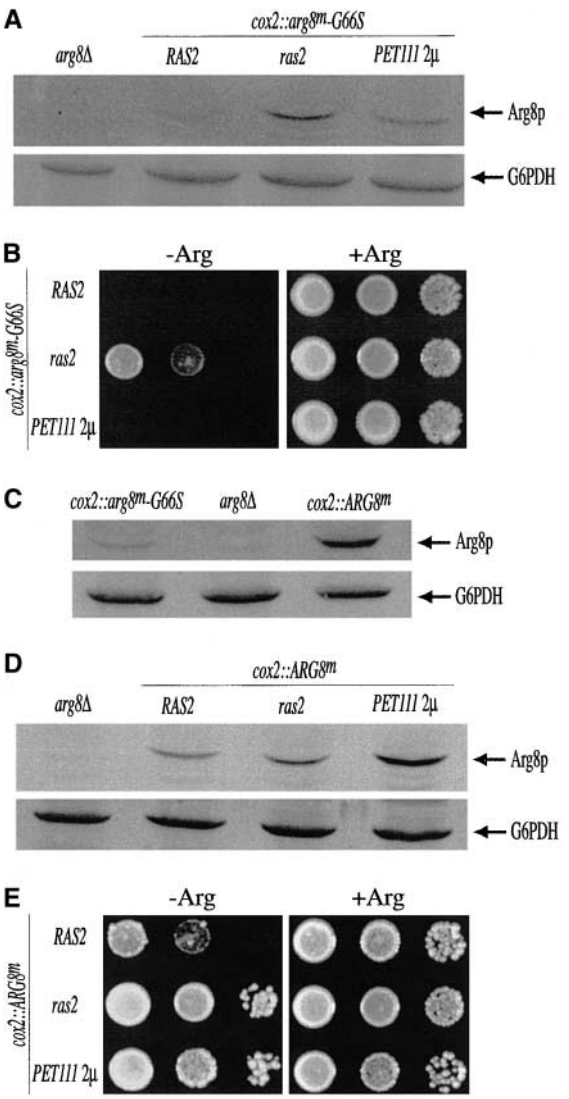

FIGURE 3.—Quantitative Western blot analysis of Arg8p and corresponding growth phenotypes. All cultures were grown to midlog phase in synthetic-complete glucose medium lacking leucine at 33°. Total yeast protein was prepared, quantitated, and analyzed by 10% SDS-PAGE electrophoresis followed by quantitative Western blot analysis (MATERIALS AND METHODS). G6PDH was used as a loading control for normalization of the Arg8p signal. Quantitative analysis of these and similar experiments is presented in Table 2. (A) Steady-state protein levels in strains carrying *cox2::arg8^m-G66S*. A total of 100 μg of TMD201 (*arg8*Δ) and 150 μg of TMD187 (*RAS2*), TMD79 (*ras2::mTn*), and TMD62 + pEHW107 (*PET111* 2μ) were loaded. (B) Arginine-independent growth of strains carrying *cox2::arg8^m-G66S*. TMD187 (*RAS2*), TMD79 (*ras2::mTn*), and TMD62 + pEHW107 (*PET111* 2μ) were grown in liquid synthetic-complete glucose medium lacking leucine, serially diluted, and then spotted on synthetic-complete glucose medium lacking leucine (+Arg) or leucine and arginine (−Arg) and incubated at 33° for 7 days. (C) Comparison of steady-state protein levels in wild-type strains expressing *cox2::arg8^m-G66S* and *cox2::ARG8^m*. A total of 100 μg of TMD187 (*cox2::arg8^m-G66S*), TMD201 (*arg8*Δ), and TMD215 (*cox2::ARG8^m*) were loaded. (D) Steady-state protein levels in strains carrying *cox2::ARG8^m*. Total protein (100 μg) from TMD201 (*arg8*Δ), TMD215 (*RAS2*), TMD154 (*ras2::mTn*), and TMD152b + pEHW107 (*PET111* 2μ) were loaded. (E) Arg^+ phenotype of strains carrying *cox2::ARG8^m*. TMD215 (*RAS2*), TMD154 (*ras2::mTn*), and TMD152b + pEHW107 (*PET111* 2μ) were grown in liquid synthetic-complete glucose medium lacking leucine, serially diluted, and then spotted on synthetic-complete glucose medium lacking leucine (+Arg) or leucine and arginine (−Arg) and incubated at 33° for 3 days.

Decreased cAMP increases the fluorescence of GFP coded by *cox3::GFP^m*, but not the steady-state level of GFP: To ask whether decreased cAMP could affect expression of other mitochondrial reporter genes, we looked at the effect of a *ras2::mTn* mutation on expression of GFP specified by a synthetic coding sequence, *GFP^m*, inserted at the *COX3* locus in mtDNA (COHEN and FOX 2001). Cells bearing the *cox3::GFP^m* reporter were grown to early saturation in minimal glucose medium at 33° and were examined by fluorescence microscopy (Figure 4, A–C). The cells carrying the *ras2::mTn* mutation were strikingly brighter than the wild-type control. It also appeared that the *ras2::mTn* mutant cells were larger and contained greater mitochondrial volume than wild-type cells. These observations were confirmed by analysis of optical z-sections obtained by confocal microscopy of the cells (Figure 4, D–F; Table 3). *ras2::mTn* mutant cells appear to contain 1.4-fold more fluorescent mitochondrial volume and are ~2.4-fold brighter than wild-type cells (Table 3). Interestingly, however, the steady-state levels of mitochondrially coded GFP, detected by quantitative Western blot analysis of total cell extracts prepared from the same cultures used for microscopy, were only 1.2-fold higher in the *ras2::mTn* mutant (Figure 4G; Table 3). Thus, while decreased cAMP caused increased expression of *cox3::GFP^m* at the level of fluorescence, this does not appear

TABLE 2

Quantitation of mitochondrially synthesized Arg8p-G66S and Arg8p accumulation

	Ratio	
	Arg8p-G66S	Arg8p
ras2 vs. RAS2	2.3 ± 0.5	1.0 ± 0.2
2μ-*PET111 vs. PET111*	1.7 ± 0.2	2.3 ± 0.4

These numbers represent the average of three quantitative Western blot experiments (MATERIALS AND METHODS) as shown in Figure 3. Ratios are given with the standard deviation.

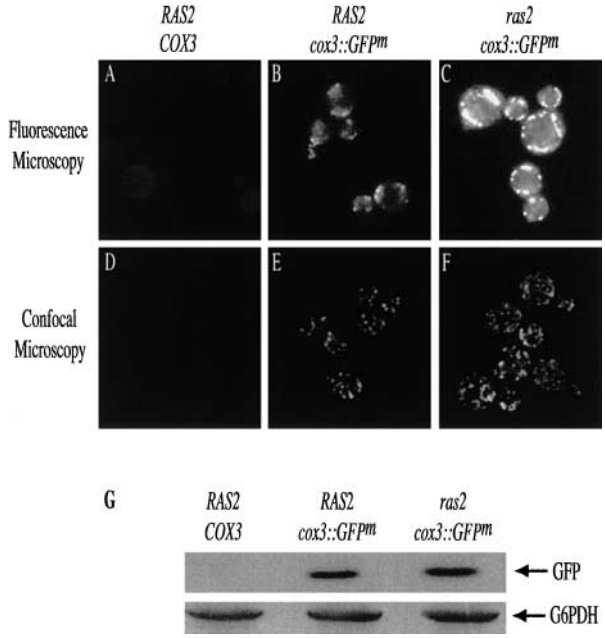

FIGURE 4.—Examination of fluorescence and steady-state levels of GFP expressed from *cox3::GFP*^{m} in mtDNA. Quantitative data from this and similar experiments are presented in Table 3. (A–C) Standard fluorescence microscopy on TMD187 (*RAS2 COX3*), TMD189 (*RAS2 cox3::GFP*^{m}), and TMD162 (*ras2 cox3::GFP*^{m}). (D–F) Projected z-series of confocal fluorescence microscopy on TMD187 (*RAS2 COX3*), TMD189 (*RAS2 cox3::GFP*^{m}), and TMD162 (*ras2 cox3::GFP*^{m}; MATERIALS AND METHODS). (G) Steady-state protein levels in cultures used for microscopy. Cultures were grown to early saturation in synthetic-complete glucose medium lacking leucine at 33°. Total protein was prepared and 75 μg was analyzed by 10% SDS-PAGE electrophoresis followed by quantitative Western blot analysis (MATERIALS AND METHODS). G6PDH was used as a loading control for normalization of the GFP signal.

to be due to increased translation of the *cox3::GFP*^{m} mRNA.

Mitochondrially synthesized Arg8p has less activity than cytoplasmically synthesized and imported Arg8p: We have observed varying degrees of arginine-independent growth with the mitochondrial reporter *ARG8*^{m} at different mitochondrial loci. In the DBY947 strain background, strains carrying *cox1::ARG8*^{m} and *cox3::ARG8*^{m} grow similarly to a ρ^0 strain with nuclearly encoded *ARG8*, whereas a strain with *cox2::ARG8*^{m} grows more weakly (Figure 5A). [Strain background affects these differences since in another strain background, D273-10B, *cox3::ARG8*^{m} grows better in the absence of arginine than *cox2::ARG8*^{m} does, which grows better than *cox1::ARG8*^{m} (our unpublished results).] We examined steady-state levels of Arg8p in the DBY947 background and found that when Arg8p is synthesized in the mitochondria it accumulates to higher steady-state levels than when it is synthesized in the cytoplasm and imported into the mitochondrial matrix (Figure 5B; Table 4). Although there is almost twice as much Arg8p in the *cox2::ARG8*^{m} strain as in the nuclear *ARG8* strain, the *cox2::ARG8*^{m} strain grows much less well in the absence of arginine, indicating that the imported Arg8p has a much higher specific activity. Thus, although the mitochondrially synthesized Arg8p is stable, it appears that imported Arg8p is folded into active enzyme more efficiently than mitochondrially synthesized Arg8p.

Mitochondrially synthesized Arg8p-G66S is much less stable than mitochondrially synthesized wild-type Arg8p, resulting in Arg$^-$ growth (Figure 3). To ask whether import of this hypomorphic mutant protein would improve its stability and activity, we chromosomally integrated a nuclear *arg8-G66S* allele by a two-step cloning-free strategy (STORICI *et al.* 2001) and compared its growth phenotype to wild-type nuclear *ARG8* in respiring ρ^+ strains (Figure 6A). The arginine-independent growth of these two strains was indistinguishable at 33° (Figure 6A) and at 16° (our unpublished results). Addi-

TABLE 3

Quantitation of cells, fluorescence, and relative GFP levels

	z-series steps	Fluorescing area	Sum of fluorescence	Steady-state GFP
ras2 vs. RAS2[a]	1.2	1.4	2.4	1.2
Range of ratios	1.1–1.5[a]	1.1–1.9[a]	1.7–3.7[a]	1.0–1.5

Ratios represent the average of ratios of four experiments as shown in Figure 4.

[a] These ratios were derived by dividing the average value of *ras2* (TMD162) cells by the average value of *RAS2* (TMD189) cells for each experiment. Cells examined in four experiments were *RAS2*, 169 (34, 49, 52, 34); *ras2*, 190 (47, 55, 56, 32).

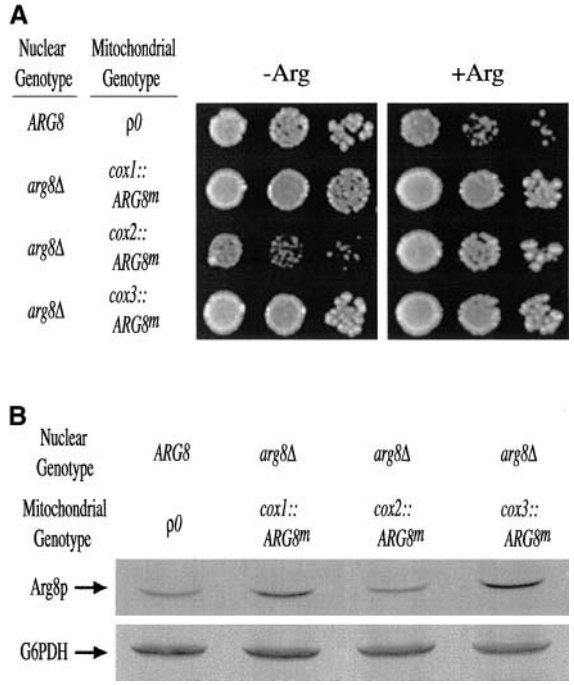

FIGURE 5.—Examination of arginine-independent growth and Arg8p accumulation when *ARG8* is expressed from different genetic loci. (A) Arginine-independent growth of TWM-34ρ^0 (*ARG8*), TMD242 (*arg8Δ cox1::ARG8^m*), TMD152b (*arg8Δ cox2::ARG8^m*), and TMD243 (*arg8Δ cox3::ARG8^m*). The strains were grown in liquid synthetic-complete glucose medium lacking leucine, serially diluted, and then spotted on synthetic-complete glucose medium lacking leucine (+Arg) or leucine and arginine (−Arg) and incubated at 33° for 4 days. (B) Comparison of steady-state Arg8p levels. The same strains as in A were grown to midlog phase in synthetic-complete glucose medium lacking leucine at 33°. Total yeast protein was prepared and quantitated and 100 μg were analyzed by 10% SDS-PAGE electrophoresis followed by quantitative Western blot analysis (MATERIALS AND METHODS). G6PDH was used as a loading control for normalization of the Arg8p signal. Table 4 contains the quantitative analysis of data from this and similar experiments.

tionally, quantitative Western blot analysis revealed that the nuclearly synthesized Arg8p-G66S accumulated to similar levels as nuclearly or mitochondrially synthesized wild-type Arg8p (Figure 6B; Table 5). However, it was able to support Arg$^+$ growth better than the mitochondrially synthesized wild-type Arg8p (Figure 6A). Furthermore, the cytoplasmically synthesized Arg8p-G66S is apparently far more stable than the mitochondrially synthesized Arg8p-G66S. Thus, we conclude that the import process is important for helping Arg8p to attain a fully active conformation.

Steady-state levels of at least one mitochondrial chaperone increase in a *ras2::mTn* mutant: Since cAMP levels appear to affect the folding of mitochondrial reporter proteins, we asked whether several known chaperones could be responsible by overexpressing and/or deleting their genes where possible. We tested *SSC1*, *ECM10* (*SSC3*), *HSP78*, and *TCM62* for effects on the phenotype

TABLE 4

Quantitation of accumulation of mitochondrially synthesized Arg8p relative to cytoplasmically synthesized Arg8p

ARG8 allele	Ratio
cox1::ARG8^m vs. nuclear *ARG8*	3.7 ± 1.7
cox2::ARG8^m vs. nuclear *ARG8*	2.4 ± 1.3
cox3::ARG8^m vs. nuclear *ARG8*	5.0 ± 1.8

These numbers represent the average of three quantitative Western blot experiments (MATERIALS AND METHODS) as shown in Figure 5B. Ratios are given with the standard deviation.

of *cox2::arg8^m-G66S* or *cox2::ARG8^m* in *RAS2* and *ras2* strains, but in no case did we detect any differences (our unpublished results). Thus, we cannot attribute the observed increased folding to the activity of any of these individual mitochondrial chaperones.

In addition, we examined the steady-state levels of three mitochondrial chaperones in *RAS2* and *ras2::mTn* strains (Figure 7). While there was no clearly significant difference in the levels of Hsp60p and Ssc1p, the level of Hsp78p was increased in the *ras2::mTn* mutant. Thus, cAMP does appear to affect the steady-state levels of at least one, but not all, mitochondrial chaperones.

DISCUSSION

We have developed a screen for nuclear mutations that increase the activity of a mitochondrially coded reporter protein in nonrespiring cells growing on glucose. This reporter protein is an unstable form of the arginine biosynthetic enzyme Arg8p, which when normally expressed from the *COX2* locus in mtDNA cannot support Arg$^+$ growth. The Arg$^+$ prototrophs selected from this parent strain after mutagenesis with a transposon library were due to *ras2::mTn* and *cyr1::mTn* mutations. Cyr1p is the adenylate cyclase, whose full activation requires Ras2p (MATSUMOTO *et al.* 1982; UNO *et al.* 1987). Thus, both mutations should cause decreased intracellular cAMP levels. A parallel screen for genes causing Arg$^+$ prototrophy when present on a high-copy vector yielded *PDE2*, the high-affinity cAMP phosphodiesterase. Elevated levels of Pde2p should also decrease cAMP levels. cAMP levels are known to modulate many downstream functions through the activity of PKA (THEVELEIN and DE WINDE 1999). We confirmed that decreased PKA activity is likely necessary for increased expression of our mitochondrial reporter, since overexpression of any of the three genes coding catalytic subunits of the enzyme, *TPK1*, *TPK2*, or *TPK3*, reversed the Arg$^+$ growth phenotype caused by the *ras2::mTn* mutation. We were unable to detect any specificity of *TPK1*, *TPK2*, or *TPK3* in this connection, either by deletion of the genes or by introduction of catalytic missense mutations. Taken together, our results clearly establish

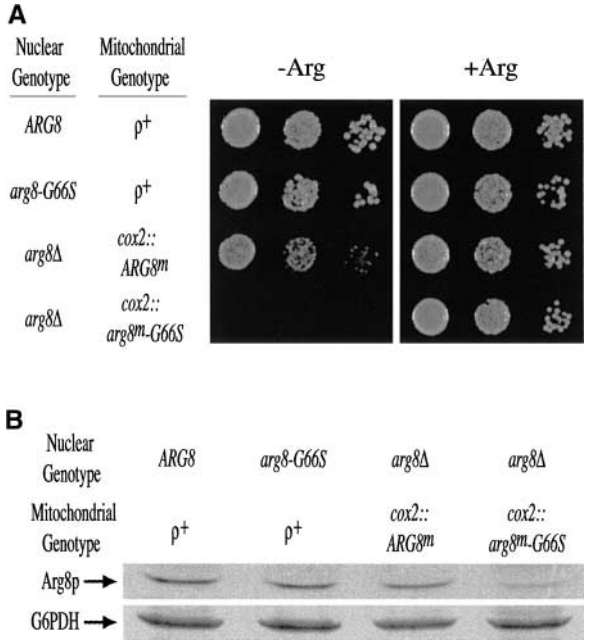

FIGURE 6.—Comparison of Arg8p and Arg8p-G66S expressed from the nuclear and mitochondrial genomes. (A) Arginine-independent growth of TWM34 (*ARG8*), TMD343a (*arg8-G66S*), TMD152b (*cox2::ARG8*^{m}), and TMD62 (*cox2::arg8*^{m}*-G66S*). These strains were grown in liquid synthetic-complete glucose medium, serially diluted, and spotted to synthetic-complete glucose medium (+Arg) or synthetic-complete glucose medium lacking arginine (−Arg) and grown for 2 days at 33°. (B) Quantitative Western blot analysis on 150 μg total protein prepared from the strains shown in A grown to midlog phase in synthetic-complete glucose medium at 33°.

TABLE 5

Quantitation of accumulation of Arg8p and Arg8p-G66S synthesized in the cytoplasm or mitochondrial matrix

ARG8 allele	Ratio
Nuclear *arg8-G66S vs.* nuclear *ARG8*	0.98 ± 0.12
cox2::ARG8^m *vs.* nuclear *ARG8*	0.98 ± 0.04
cox2::arg8^m*-G66S vs.* nuclear *ARG8*	0.34 ± 0.03

These numbers represent the average of three quantitative Western blot experiments (MATERIALS AND METHODS) as shown in Figure 6B. Ratios are given with the standard deviation.

that decreased cAMP can increase the activity of this mitochondrially coded reporter protein by decreasing the activity of PKA.

The increased mitochondrial reporter activity we observed does not appear to be due to increased levels of translation for several reasons. First, the level of wild-type Arg8p, which is substantially more stable than the mutant Arg8p-G66S, was not significantly altered by lowered cAMP levels, although it was increased by overexpression of the *COX2* mRNA-specific translational activator protein, Pet111p, as previously reported (GREEN-WILLMS *et al.* 2001). Nevertheless, lowered cAMP levels increased the ability of a strain synthesizing wild-type Arg8p in the matrix to grow in the absence of arginine, indicating increased specific activity of the enzyme. Furthermore, although lowered cAMP increased GFP fluorescence, it did not significantly increase the steady-state level of mitochondrially coded GFP polypeptide as detected by Western analysis. Thus, our evidence indicates that cAMP levels do not affect mitochondrial gene expression at the level of reporter protein synthesis.

Our observations of increased mitochondrial reporter activity in cells containing decreased cAMP levels are most easily understood in terms of the hypothesis that decreased cAMP causes increased activity of matrix-localized protein chaperones, which are then better able to interact with the reporter proteins. According to this view, more efficient folding of the unstable variant Arg8p-G66S would lead to the higher steady-state levels of protein observed and increased activity. Wild-type Arg8p made in the mitochondria is not further stabilized by decreased cellular cAMP, but the improved Arg$^+$ growth indicates that the enzyme is more active, which could also be a result of improved folding efficiency. We made similar observations with mitochondrially synthesized GFP. The GFP chromophore forms relatively slowly during protein folding, and a substantial fraction of GFP within a cell can be in a nonfluorescing state (CORMACK *et al.* 1996; REID and FLYNN 1997). Decreased cAMP could therefore increase the rate at which mitochondrially coded GFP forms its active chromophore. Thus, the behaviors of mitochondrially coded Arg8p-G66S, wild-type Arg8p, and GFP in *ras2::mTn* cells are consistent with the hypothesis that decreased cAMP levels increase chaperone functions in the mitochondrial matrix.

Our experiments comparing the growth of strains containing mitochondrially synthesized Arg8p or Arg8p-G66S with those containing cytoplasmically synthesized Arg8p or Arg8p-G66S that is imported into the matrix indicate that the imported protein has greater specific activity. It is clear from a large body of work that imported proteins emerging from the translocation machinery on the matrix side of the inner membrane are engaged by mitochondrial chaperones, which participate in the import process and in folding of the imported proteins (KOEHLER 2000; LIU *et al.* 2001; PFANNER and GEISSLER 2001; NEUPERT and BRUNNER 2002). There is also evidence for participation of the mitochondrial Hsp70, Ssc1p, in the folding of the mitochondrially synthesized ribosomal protein Var1p (HERRMANN *et al.* 1994; WESTERMANN *et al.* 1996; KLANNER *et al.* 2000). However, the fact that mitochondrially synthesized Arg8p has lower specific activity than imported Arg8p strongly suggests that this single protein has less access to folding functions when it emerges from mitochondrial ribosomes than when it emerges from the import channel. Thus,

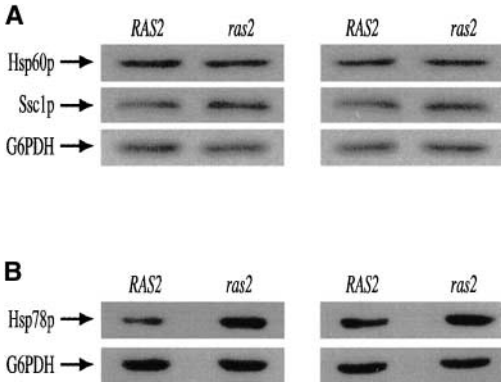

FIGURE 7.—Effect of *ras2::mTn* on the steady-state levels of three mitochondrial chaperones. Total protein was prepared from cultures grown to midlog phase in synthetic-complete glucose medium lacking leucine at 33°, quantitated, and analyzed by 10% SDS-PAGE electrophoresis followed by Western blot analysis (MATERIALS AND METHODS). Duplicate cultures are shown. (A) A total of 12 μg TMD187 (*RAS2*) and TMD79 (*ras2::mTn*) were analyzed with anti-Ssc1p and anti-Hsp60p (J. Herrmann) and anti-G6PDH as a loading control. (B) A total of 50 μg TMD187 (*RAS2*) and TMD79 (*ras2::mTn*) were analyzed with anti-Hsp78p and anti-G6PDH as a loading control. Quantitative analysis of digitized signal strength (MATERIALS AND METHODS) indicates a ratio of 1.6 ± 0.3 for the level of Hsp78p in the *ras2* mutant relative to *RAS2*.

it appears that mitochondrial translation of the *cox2::ARG8*^{m} mRNA, which appears to be localized by topogenic signals in its untranslated regions (SANCHIRICO *et al.* 1998), limits chaperone accessibility in the matrix. This is the first evidence we are aware of that a protein synthesized in different cellular compartments is folded with different efficiencies in the mitochondrial matrix.

We were unable to affect reporter activity by individual manipulation of the genes *SSC1*, *ECM10* (*SSC3*), *HSP78*, and *TCM62*, which encode known mitochondrial chaperones. The effects we observed in response to decreased cAMP could be due to alterations in the levels of any combination of these or other mitochondrial chaperones. We examined the steady-state levels of three mitochondrial chaperones and found that the level of Hsp78p was increased in a *ras2* mutant. However, we have not established a cause-and-effect relationship between increased Hsp78p and increased reporter protein activity when cAMP is decreased. Hsp78 has been implicated in cooperating with Ssc1p in several mitochondrial chaperone functions (MOCZKO *et al.* 1995; KRZEWSKA *et al.* 2001). It has also been shown to be able to interact with imported proteins at the early stage of import and to prevent aggregation of misfolded proteins when Ssc1p activity is limiting (SCHMITT *et al.* 1995). Ssc1p, the mitochondrial form of Hsp70, has previously been shown to assist in the folding of a soluble protein (Var1p) coded and translated in the mitochondrial matrix (HERRMANN *et al.* 1994), along with Mdj1p and Tcm62p (WESTERMANN *et al.* 1996; KLANNER *et al.* 2000). In addition, it is known to participate in the import of proteins into the mitochondrial matrix as well as to assist in their folding (KANG *et al.* 1990; FOLSCH *et al.* 1998). Whether it plays a role in increasing reporter protein activity here is not clear.

Another chaperone of potential interest is Hsp60p, an abundant and essential mitochondrial matrix protein (HALLBERG *et al.* 1993). We were unable to detect a change in Hsp60p steady-state levels in a *ras2::mTn* mutant. Interestingly, however, we obtained the gene *RTS1* in our screen for genes that would increase reporter expression when present in high copy, although its effect was very weak. Elevated dosage of *RTS1* has been shown to suppress the temperature-sensitive phenotypes of some *hsp60* missense mutations, although it does not appear to increase *HSP60* expression (SHU and HALLBERG 1995). Presumably, elevated Rts1p affects mitochondrial chaperones through its action as the B′ regulatory subunit of protein phosphatase A (PP2A), as discussed below (ZHAO *et al.* 1997; ZABROCKI *et al.* 2002).

The involvement of cAMP and PKA in controlling yeast mitochondrial functions is not well understood. In general, PKA activity is high in cells growing on glucose and lower in cells growing on respiratory carbon sources requiring active respiration (THEVELEIN and DE WINDE 1999). An analysis of gene expression in *tpk* deletion strains suggested that Tpk2p preferentially regulates respiratory growth and carbon source utilization in a negative fashion (ROBERTSON *et al.* 2000). Activation of yeast adenylate cyclase requires Ras1p or Ras2p, although carbon source modulation of adenylate cyclase is effected by a G-protein-coupled receptor system that responds to the addition of glucose to the environment (THEVELEIN and DE WINDE 1999). [*ras2* mutants fail to grow well on nonfermentable carbon sources, but this is apparently due to the fact that *RAS1* is strongly downregulated in the absence of glucose causing lethality due to the absence of ras activity rather than to a respiratory defect *per se* (BREVIARIO *et al.* 1986).] cAMP levels normally decrease transiently during the diauxic shift from glucose (fermentative conditions) to ethanol (respiratory conditions) during the growth of yeast cultures, and this presumably helps to control the induction of respiratory functions (BOY-MARCOTTE *et al.* 1996, 1998).

In our experiments, the cells lacked respiratory ability due to the deletion of mitochondrial genes encoding cytochrome *c* oxidase subunits and their replacement with reporter genes. The cells were grown on glucose and studied during logarithmic growth, conditions under which PKA activity would normally be high. Thus, it appears that the reduction of steady-state cAMP caused by the genetic alterations we generated created conditions that at least partially mimic those in cells adapting to growth on nonfermentable carbon sources.

Previous studies on the effects of cAMP on mitochondrial functions are not easily compared to ours. Transcription of mitochondrial rRNA genes has been re-

ported to be positively controlled by cellular cAMP (McEntee *et al.* 1993), although this is not true for mRNAs (Dejean *et al.* 2002b). In any case, expression of the *COX2* and *COX3* mitochondrial genes appears to be limited at the level of translation in yeast mitochondria, not at the level of transcription (Pinkham *et al.* 1994; Steele *et al.* 1996; Green-Willms *et al.* 2001). More recently, elevated dosage of *RAS2* has been shown to suppress the nuclear *atp1-2* missense allele, affecting the α-subunit of the ATP synthase complex (Mabuchi *et al.* 2000). However, increased ATP synthase activity was observed only in cells growing by respiration on nonfermentable medium, not in cells grown on glucose, and may have been due to increased synthesis of the mutant protein (Mabuchi *et al.* 2000). Consistent with this conclusion, cells grown on the nonfermentable carbon source lactate exhibited increased mitochondrial enzyme content and respiratory capacity when cAMP concentrations were elevated (Dejean *et al.* 2002a,b).

It appears, therefore, that the cAMP-PKA pathway can influence mitochondrial respiratory functions in different ways under different conditions. As we have found in the present study, decreased cAMP levels and decreased PKA activity in nonrespiring cells growing on glucose results in elevated activity of matrix-localized functions that can stabilize and fold mitochondrial translation products. This response is likely to play a role in the adaptation of yeast cells to the stress of the diauxic transition from glucose to ethanol in the environment. On the other hand, in cells already growing under respiratory conditions in the absence of fermentable carbon sources, elevated cAMP apparently globally increases the levels of respiratory complexes.

Recent evidence has suggested a role for PP2A in PKA-mediated nutrient-induced signaling (Sugajska *et al.* 2001; Zabrocki *et al.* 2002). The fact that we isolated *RTS1*, which encodes one of several regulatory subunits of PP2A, as a weak dosage-dependent activator of *cox2::arg8*^{m} expression is consistent with this idea. Elevated levels of the catalytic PP2A subunit Pph22p mimic the phenotype caused by high PKA activity (Sugajska *et al.* 2001; Zabrocki *et al.* 2002), while we observe that elevated *RTS1* dosage mimics a low PKA activity phenotype. Perhaps increased expression of the Rts1p regulatory subunit lowers the activity of another form of PP2A necessary to activate PKA-dependent genes.

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