MOLECULAR AND CELLULAR BIOLOGY, Feb. 1989, p. 484–491 Vol. 9, No. 2
0270-7306/89/020484-08$02.00/0
Copyright © 1989, American Society for Microbiology

Control of the *Saccharomyces cerevisiae* Regulatory Gene *PET494*: Transcriptional Repression by Glucose and Translational Induction by Oxygen

DONNA L. MARYKWAS[1] AND THOMAS D. FOX[2]*

Section of Biochemistry, Molecular and Cell Biology[1] and Section of Genetics and Development,[2] Cornell University, Ithaca, New York 14853

Received 15 July 1988/Accepted 14 October 1988

The product of the *Saccharomyces cerevisiae* nuclear gene *PET494* is required to promote the translation of the mitochondrial mRNA encoding cytochrome *c* oxidase subunit III (coxIII). The level of cytochrome *c* oxidase activity is affected by several different environmental conditions, which also influence coxIII expression. We have studied the regulation of *PET494* to test whether the level of its expression might modulate coxIII translation in response to these conditions. A *pet494*::*lacZ* fusion was constructed and used to monitor *PET494* expression. *PET494* was regulated by oxygen availability: expression in a respiration-competent diploid strain grown anaerobically was one-fifth the level of expression in aerobically grown cells. However, since *PET494* mRNA levels did not vary in response to oxygen deprivation, regulation by oxygen appears to occur at the translational level. This oxygen regulation was not mediated by heme, and *PET494* expression was independent of the heme activator protein HAP2. The regulation of *PET494* expression by carbon source was also examined. In cells grown on glucose-containing medium, *PET494* was expressed at levels four- to sixfold lower than in cells grown on ethanol and glycerol. However, addition of ethanol to glucose-containing medium induced *PET494* expression approximately twofold. *PET494* transcript levels varied over a fourfold range in response to different carbon sources. The effects on *PET494* expression of mutations in the *SNF1*, *SNF2*, *SSN6*, and *HXK2* genes were also determined and found to be minimal.

The yeast *Saccharomyces cerevisiae* can grow either by fermentation or by respiration. The production of enzymes necessary for respiration requires the simultaneous expression of genes encoded in both nuclear and mitochondrial DNA (16). For example, yeast cytochrome *c* oxidase consists of three mitochondrially encoded subunits and at least six nuclearly encoded subunits (36, 49). Thus, respiration appears to require coordination of nuclear and mitochondrial gene expression.

In *S. cerevisiae*, the levels of respiratory enzymes such as cytochrome *c* oxidase are reduced in response to environmental conditions such as anaerobiosis (63) and high glucose concentrations (47). These differences in the activity of cytochrome *c* oxidase are similar to the differences in the protein levels of its mitochondrially encoded subunits (19, 63). The mechanisms regulating mitochondrial gene expression are poorly understood. In general, the levels of mitochondrial transcripts are lower in cells grown on glucose than in galactose-grown cells (42). However, upon release from glucose repression, the level of cytochrome *c* oxidase subunit III (coxIII) was found to increase (19), while the level of its mitochondrially encoded mRNA did not (65).

Translation of at least two of the mitochondrially encoded subunits of cytochrome *c* oxidase is specifically controlled by the products of nuclear genes (4, 10, 11, 13, 14, 17, 44, 48). Several of these nuclear genes, including *PET494*, encode mitochondrial proteins (10, 58; M. C. Costanzo, E. C. Seaver, and T. D. Fox, submitted for publication). PET494, in conjunction with other nuclear gene products, activates translation of the mitochondrial mRNA encoding coxIII through a site in the 5′ untranslated leader of the coxIII mRNA (10, 11). Thus, *PET494* is a candidate for a nuclear gene involved in modulation of expression of the mitochondrial gene for coxIII at the translational level.

Since *PET494* might play a role in modulating coxIII protein levels, we have begun to examine which physiological signals regulate the expression of *PET494*, how these signals are mediated, and whether *PET494* expression is controlled by previously described regulatory systems. By monitoring the activity of a PET494–β-galactosidase fusion protein, we have found that *PET494* expression is subject to carbon catabolite repression and repressed further under anaerobic conditions. However, *PET494* expression is not controlled by several known regulators of catabolite repression in *S. cerevisiae* (5–7, 18, 34, 45). In addition, *PET494* expression is independent of the presence of heme, unlike other known genes that are regulated by oxygen levels (22, 23, 31–33, 50, 56, 64, 66). Finally, we report that the regulation of *PET494* expression by oxygen appears to occur at the translational level, whereas the control of *PET494* expression by carbon source may have both transcriptional and translational components.

MATERIALS AND METHODS

Plasmids and transformation procedures. DNA manipulations and transformation of *Escherichia coli* DH5α (Bethesda Research Laboratories, Inc.) were performed by the methods of Maniatis et al. (35). *S. cerevisiae* cells were transformed by lithium acetate treatment of intact cells (25). pSPACT was constructed by cloning a 3.25-kilobase *Bam*HI-*Eco*RI fragment from pYACTI (46) containing the yeast actin gene into pSP65 (Promega Biotec) restricted with *Bam*HI and *Eco*RI. pSP494R was constructed by cloning a 1.9-kilobase *Hin*dIII-*Sal*I fragment from pMC206 (M. C. Costanzo, unpublished results) containing the *PET494* gene into pSP64 (Promega Biotec) restricted with *Hin*dIII and

* Corresponding author.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
DAU1	*MATα ade2 ura3Δ* [*rho*$^+$]	This study
DAU2	*MAT***a** *ade2 ura3Δ* [*rho*$^+$]	This study
DAU2 [*rho*^{0}]	*MAT***a** *ade2 ura3Δ* [*rho*^{0}]	This study
DAU1-A1	*MATα ade2 ura3Δ pet494::lacZ* [*rho*$^+$]	This study
DAD1	*MAT***a***/MATα ade2/ade2 ura3Δ/ura3Δ PET494/pet494::lacZ* [*rho*$^+$]	This study
DLZ3	*MATα ura3Δ leu2-3,112 his4-519 pet494::lacZ* [*rho*$^+$]	This study
JKR102	*MAT***a** *ura3Δ leu2-3,112 his4-519* [*rho*$^+$]	This study
DLU2	*MAT***a** *lys2 ura3Δ* [*rho*$^+$]	This study
BWG1-7A	*MAT***a** *his4-519 ade1-100 leu2-3,112 ura3-52* [*rho*$^+$]	J. Pinkham and L. Guarente
LGW1	*MAT***a** *his4-519 ade1-100 leu2-3,112 ura3-52 hap2-1* [*rho*$^+$]	J. Pinkham and L. Guarente
MCY1093	*MAT***a** *his4-539 lys2-801 ura3-52 SUC2* [*rho*$^+$]	L. Neigeborn and M. Carlson
MCY1486	*MAT***a** *lys2-801 ura3-52 snf1Δ6 SUC2* [*rho*$^+$]	L. Neigeborn and M. Carlson
MCY637	*MATα his 4-539 lys2-801 ura3-52 snf2-50 SUC2* [*rho*$^+$]	L. Neigeborn and M. Carlson
MCY1372	*MATα ade2-101 ura3-52 ssn6-1 SUC2* [*rho*$^+$]	L. Neigeborn and M. Carlson

*Sal*I. pLGΔ229UP1 is a replicating plasmid (provided by J. Pinkham and L. Guarente) carrying the *URA3* gene and a *lacZ* fusion to *CYC1* upstream activation sequence 2. pRB528 (provided by H. Ma and D. Botstein) carries the *HXK2* gene disrupted by *URA3*. pYeHEM1::LEU2 (provided by R. Zitomer) carries the *HEM1* gene disrupted by *LEU2*. pT3-ANBR (also provided by R. Zitomer) carries the 5′ portion and upstream sequence of *ANB1* cloned into T3-T7-18 (Bethesda Research Laboratories). The *pet494::lacZ* fusion plasmid pNTD20 was constructed by inserting the *lacZ* gene of *E. coli* into the *PET494* gene carried on the plasmid pMKR1 (12), as follows. A deletion extending to, but not including, the *PET494* initiation codon was generated by cleavage of pMKR1 at the *Bam*HI site, followed by limited digestion with exonuclease III and treatment with S1 nuclease to generate blunt ends. Approximate sizes of the deletions were estimated by restriction digestion followed by agarose and acrylamide gel electrophoresis. A 3.0-kilobase *Bam*HI fragment carrying the *lacZ* gene was isolated from pMC1871 (8), treated with the Klenow fragment of DNA polymerase I to fill in the ends, and ligated to the deleted pMKR1 DNA.

DNA sequence analysis. The DNA sequence of the plasmid-borne *pet494::lacZ* gene fusion was determined by chemical degradation (38) of a fragment uniquely 3′ end labeled at the *Ava*I site at nucleotide −20 of the wild-type *PET494* gene (12). The DNA sequence of the *PET494-lacZ* junction of the fusion gene was determined to be 5′- ATG-GAT-CCC-GTC-GTT-3′. The ATG triplet is the initiation codon of *PET494*, and the CCC triplet is the codon for the eighth amino acid of β-galactosidase.

Strains and strain constructions. The yeast strains used in this study, their genotypes, and their sources are listed in Table 1. Standard genetic methods were used for strain constructions (53). Unless otherwise indicated, all haploid strains made for the purpose of this study were determined to be [*rho*$^+$]. All strains from this laboratory were isogenic or congenic to the wild-type strain D273-10B (ATCC 25657) except for the indicated markers. Strain DAD1 was constructed as follows. First, DAU1 was transformed with pNTD20 carrying the *URA3* gene and the *pet494::lacZ* gene fusion (see above). Integration at the *PET494* locus was confirmed by DNA hybridization analysis (data not shown). Ura$^-$ recombinants were selected on medium containing 5-fluoro-orotic acid and uracil (2). A Pet$^-$ colony (DAU1-A1) resulting from replacement of the wild-type *PET494* gene by the *pet494::lacZ* fusion was isolated and confirmed by DNA hybridization analysis (not shown). Finally, DAU1-A1 was mated to DAU2 [*rho*^{0}], and a respiring diploid colony (DAD1) was selected by its growth on ethanol and glycerol. Replacement of the wild-type *PET494* locus with the *pet494::lacZ* fusion in other strains was performed as described above for DAU1-A1. DLZ3 was a meiotic segregant from a cross of DAU1-A1 with JKR102. *hem1* derivatives of DLZ3 were constructed by disrupting the *HEM1* gene: DLZ3 was transformed with *Hin*dIII-restricted pYeHEM1::LEU2, and Leu$^+$ transformants were selected on medium that contained 20 μg of δ-aminolevulinic acid per ml to permit growth of the resulting heme-deficient cells. Individual colonies were screened for heme auxotrophy, and their construction was confirmed by DNA hybridization analysis (data not shown). *hxk2* derivatives of DAU1-A1 and DLU2 were constructed by disrupting the *HXK2* gene in each strain: cells were transformed with *Eco*RI-restricted pRB528 and slow-growing Ura$^+$ transformants were selected. Individual colonies were screened for those having high invertase activity when grown on 5% glucose-containing medium, and their construction was confirmed by DNA hybridization analysis (data not shown).

Growth conditions and media. Yeast cells were grown on complete medium (1% yeast extract, 2% peptone) or minimal medium (0.67% yeast nitrogen base without amino acids) containing the indicated carbon source(s) and appropriate supplements (53). YPD is complete medium containing 2% glucose. YPGAL is complete medium containing 2% galactose. YPEG is complete medium containing 3% ethanol and 3% glycerol. Anaerobic cultures and *hem1* mutants were grown in YPD medium supplemented with ergosterol (10 μg/ml) and Tween 80 (0.5%). Anaerobic cultures were prepared by growing with vigorous shaking under a continuous nitrogen stream in sealed flasks. Before harvesting, the anaerobic cultures were poisoned with 0.2 mg of cycloheximide per ml and chilled on ice for 15 min while still under nitrogen.

Enzyme assays. Yeast cell extracts were prepared by the method of Rose et al. (52). β-Galactosidase assays were performed as described by Miller (41), except that the time of assay ranged from 4 to 20 h. Under these conditions, extracts from strains that did not carry a *lacZ* fusion gene did not detectably hydrolyze *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Furthermore, β-galactosidase activity was linear with the amount of extract and with time. Units of specific activity for β-galactosidase were nanomoles of ONPG hydrolyzed per minute per milligram of protein. Each experiment was repeated several times. Standard deviations were less than 10%. Invertase assays (data not shown) were performed by the method of Goldstein and Lampen (20), and protein concentrations were determined by the method of Bradford (3).

Isolation of RNA and RNA blot analysis. Total yeast nucleic acid, primarily RNA, was prepared as described by

Sprague et al. (57) and precipitated twice with 3 M lithium chloride at 0°C for 2 h. RNA (100 μg) was denatured with glyoxal, fractionated by electrophoresis in 1.1% agarose (1.5% agarose when probing for *ANB1*) in 10 mM phosphate buffer at pH 7.0 (39), and transferred to a Biotrans nylon membrane (ICN Biomedicals Inc.). A uniformly radiolabeled RNA probe complementary to *ACT1* mRNA was prepared by SP6 RNA polymerase transcription (40) from pSPACT linearized with *Bam*HI. Similarly, an RNA probe complementary to *PET494* mRNA was prepared by using as a template pSP494R linearized with *Sal*I. An RNA probe complementary to *ANB1* mRNA was prepared by T3 RNA polymerase transcription (1) from pT3-ANBR linearized with *Eco*RI. Hybridization was done at 48°C (42°C when probing for *ANB1*) in a buffer containing 50% formamide (60) but lacking dextran sulfate. Damp blots were wrapped in plastic and autoradiographed at −70°C by using Cronex Lightning-Plus intensifying screens (Du Pont Co.). The band intensities on the autoradiograms were quantitated densitometrically by using a GS300 scanning densitometer (Hoefer Scientific Instruments).

RESULTS

Construction of a *pet494::lacZ* fusion. To assay expression of *PET494*, we have exploited the widely used method of monitoring β-galactosidase activity expressed from a fusion to the *lacZ* gene of *E. coli*. An in-frame fusion was constructed in which the *PET494* initiation codon was fused to codon 8 of *lacZ*, as described in Materials and Methods.

To ensure that the expression and regulation of the fusion gene paralleled that of the endogenous *PET494* gene, we replaced the wild-type chromosomal copy of *PET494* with the *pet494::lacZ* fusion in all strains used in this study (see Materials and Methods). The basal level of β-galactosidase expression from this fusion varied when measured in different genetic backgrounds. Therefore, each experiment was performed with isogenic or congenic strains, as indicated below.

***HAP2* does not regulate *PET494*.** The *trans*-acting gene product HAP2 has been reported to be a heme-dependent positive regulator of several genes involved in respiration (21, 27). To study the effect of a *hap2* mutation on *PET494* expression, we replaced the wild-type copy of *PET494* with the *pet494::lacZ* fusion in the *HAP2* strain BWG1-7A and the isogenic *hap2* strain LGW1 and then assayed for β-galactosidase activity. The specific activity of β-galactosidase expressed from the *pet494::lacZ* fusion was the same in both strains when grown on 2% glucose-containing minimal medium (0.20 U/mg in the *HAP2* strain versus 0.21 U/mg in the *hap2* strain), showing that *HAP2* is not required for *PET494* expression. As a control, expression of a *cyc1::lacZ* gene fusion carried on the plasmid pLGΔ229UP1 in the same two strains differed by a factor of 40 (88 U/mg in the *HAP2* strain versus 2.2 U/mg in the *hap2* strain), as expected.

***PET494* is regulated by oxygen but not heme.** The presence of oxygen is required for expression of a large number of genes involved in respiration (22, 23, 63, 66). To determine whether *PET494* expression is regulated by oxygen, the respiration-competent diploid strain DAD1, carrying one wild-type copy of *PET494* and one copy of the *pet494::lacZ* fusion, was grown aerobically and anaerobically on glucose-containing medium and assayed for β-galactosidase activity. Expression in anaerobically grown cells was one-fifth the level of expression in aerobically grown cells (Fig. 1). Since β-galactosidase is equally stable under aerobic and anaerobic conditions (22), this decrease must reflect the regulation of *PET494*.

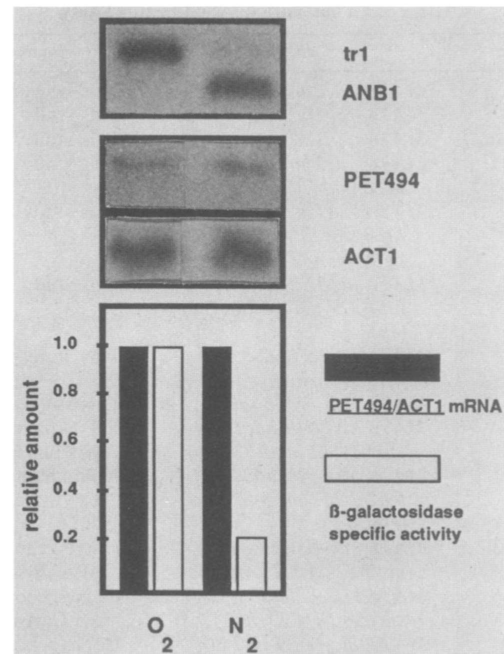

FIG. 1. Oxygen-dependent translational control of *PET494*. Expression of a *pet494::lacZ* fusion is decreased in the absence of oxygen, but the *PET494* mRNA level is not. Anaerobic cultures of strain DAD1 were grown and harvested as described in Materials and Methods. Total RNA (100 μg) prepared from cells grown aerobically or anaerobically was denatured with glyoxal, fractionated by agarose gel electrophoresis, blotted to a Biotrans nylon membrane, and probed first with a *PET494*-specific probe and subsequently with an *ACT1*-specific probe (middle panel). The transcripts were visualized by autoradiography and scanned densitometrically. The amount of *PET494* mRNA in a sample was normalized to the amount of *ACT1* mRNA (representative of the total mRNA level) in that sample. Symbols: ■, amount of *PET494* mRNA (normalized to *ACT1*) relative to the amount in the aerobically grown cells; □, amount of β-galactosidase specific activity relative to the amount expressed in the aerobically grown cells. The top panel shows an autoradiogram of a separate blot hybridized to a probe complementary to *ANB1*. The experiment was repeated three times. The blots represent one of the repeats.

As a control to show that anaerobic conditions were achieved, we monitored the transcription of *ANB1*, a gene normally transcribed only under anaerobic conditions (32, 33). *ANB1* is homologous to and cross-hybridizes with another gene, *tr-1*, which is transcribed in much greater quantities under aerobic conditions (32, 33). As expected, *ANB1* transcript was present only in the anaerobically grown cells, whereas the *tr-1* transcript level was dramatically reduced (Fig. 1). The anaerobically grown cells also required Tween 80 and ergosterol for growth, confirming that conditions were anaerobic.

The regulation of *PET494* expression by oxygen could be direct or indirect. Since oxygen is required for the biosynthesis of heme in *S. cerevisiae* (37), cells grown anaerobically lack heme. Therefore, the signal regulating *PET494* could be oxygen or heme or both. Although *PET494* was not controlled by *HAP2*, the possibility remained that *PET494* is regulated by heme independently of *HAP2*.

To test the expression of *PET494* in cells grown aerobically in the absence of heme, we used mutants deficient in

TABLE 2. Elevation of expression of a *pet494::lacZ* fusion in cells grown on non-glucose-containing medium[a]

Carbon source	β-Galactosidase specific activity[b]	Activity relative to 2% glucose
Glucose (2%)	0.076	1.0
Glucose (15%)	0.061	0.8
Galactose (2%)	0.15	2.0
Ethanol (3%) plus glycerol (3%)	0.32	4.2
Ethanol (3%)	0.45	5.9
Glucose (2%) plus galactose (2%)	0.054	0.7
Glucose (2%) plus ethanol (3%)	0.15	2.0

[a] Cells were grown in minimal medium containing the indicated carbon source(s) and appropriate supplements. The respiration-competent diploid strain DAD1 was used for these experiments.
[b] β-Galactosidase specific activity is expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein.

heme biosynthesis. As described in Materials and Methods, we disrupted the *HEM1* gene in a haploid strain, DLZ3, which contained the *pet494::lacZ* fusion. The level of β-galactosidase activity was unaffected in the *hem1* gene disruptants grown aerobically on glucose-containing medium (0.059 U/mg in the *HEM1* strain versus 0.076 U/mg in the *hem1* strain) and was decreased under anaerobic conditions (data not shown). These results indicate that *PET494* expression is regulated by oxygen independently of heme.

Glucose repression of *PET494*. Cytochrome *c* oxidase activity is glucose repressed (47), as is the synthesis of coxIII protein (19). In the respiration-competent strain DAD1, the *pet494::lacZ* fusion was repressed by glucose (Table 2). Expression relative to cells grown on glucose was twofold higher on galactose and four- to sixfold higher on ethanol and glycerol. Furthermore, the addition of glucose to galactose- or to ethanol-containing medium caused a reduction in β-galactosidase activity. Interestingly, the addition of glucose to ethanol-containing medium did not lower the level of β-galactosidase activity to the fully repressed level of cells grown on 2% glucose. However, the addition of glucose to galactose-containing medium did fully repress the expression of β-galactosidase. These results indicate that *PET494* expression is regulated by glucose or carbon catabolite repression. In addition, *PET494* expression appears to be induced slightly by the presence of ethanol.

***SNF1*, *SNF2*, *SSN6* and *HXK2* do not control *PET494*.** Since *PET494* expression was repressed by glucose, we wanted to determine whether *PET494* was regulated by other genes known to be involved in carbon catabolite repression. *SNF1* and *SNF2* encode positive activators of glucose-repressible genes (6, 45), whereas *SSN6* apparently functions to decrease expression of genes subject to glucose repression (7). To study the effect of mutations in *SNF1*, *SNF2*, and *SSN6* on *PET494* expression, we replaced the wild-type copy of *PET494* with the *pet494::lacZ* fusion in a series of four congenic haploid strains, one wild-type and three mutant strains for each of these three loci. We then measured β-galactosidase activity in cells grown under repressed and derepressed conditions. Mutations in all three genes caused a slight increase in *PET494* expression (Table 3). Mutations in *SNF1* and *SNF2* did not block *PET494* expression, as they did for secreted invertase (6, 45) and as would have been expected if they regulated *PET494* positively. Furthermore, the mutation in *SSN6* did not cause high constitutive expression of *PET494*, as it did for secreted invertase (7) and as would have been expected if it regulated *PET494* negatively. The regulation of *PET494* in response to different amounts of glucose was not dramatically altered by mutations in any of these genes. As in the wild-type strain, the level of expression of the *pet494::lacZ* fusion in each mutant strain when shifted to 0.5% glucose was about twofold higher than the level of expression when grown on 15% glucose. Clearly, the mutations in *SNF1*, *SNF2*, and *SSN6* did not change the twofold derepression of *PET494* expression in response to glucose limitation. Invertase activity in the transformant strains was measured as a control and behaved as expected (data not shown).

TABLE 3. Effect of mutations in *SNF1*, *SNF2*, and *SSN6* on expression of a *pet494::lacZ* fusion[a]

Relevant genotype[b]	β-Galactosidase specific activity[c] in medium with following amt of glucose:			Expression in cells grown on 0.5% glucose relative to expression in cells grown on 15% glucose
	0.5%	2%	15%	
WT	0.046	0.025	0.023	2.0
snf1	0.059	0.055	0.024	2.5
snf2	0.087	0.076	0.044	2.0
ssn6	0.067	0.047	0.032	2.1

[a] Repressed cells were grown to exponential phase in complete medium containing either 2% or 15% glucose. Derepressed cells were prepared by shifting repressed cells from 2% to 0.5% glucose for 2 h.
[b] The parental wild-type (WT) strain used in this study was MCY1093. The parental *snf1* strain was MCY1486. The parental *snf2* strain was MCY637. The parental *ssn6* strain was MCY1372. These were congenic strains. The *pet494::lacZ* fusion replaced the wild-type chromosomal copy of *PET494* in each strain.
[c] β-Galactosidase specific activity is expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein.

The *HXK2* gene product, hexokinase II, is also required for carbon catabolite repression of several genes in *S. cerevisiae* (18, 34). To study the effect of a null *hxk2* mutant on the expression of *PET494*, we disrupted the *HXK2* gene in a haploid strain, DAU1-A1, containing the *pet494::lacZ* fusion and measured β-galactosidase activity compared with that in the undisrupted parental strain. Although the *hxk2* lesion caused constitutive high invertase activity (data not shown), the level and pattern of expression of β-galactosidase expressed from the *pet494::lacZ* fusion were not affected by this mutation (Table 4). Similar results were obtained with a respiration-competent diploid strain homozygous for the *hxk2* mutation (Table 4). Thus, the *HXK2* gene product does not control *PET494* expression.

Effect of growth conditions on *PET494* mRNA levels. To better understand the mechanisms by which *PET494* is regulated, we have examined the effects of oxygen and carbon source on *PET494* mRNA levels in the respiration-competent diploid strain DAD1. With this strain we could compare the steady-state level of bona fide *PET494* mRNA with the level of *PET494* gene expression as determined by the level of β-galactosidase expressed from the *pet494::lacZ* fusion gene.

To examine the effects of oxygen on *PET494* transcription, total RNA was prepared from cells grown aerobically or anaerobically on glucose, subjected to agarose gel electrophoresis, and blotted to a nylon membrane. The membrane was then hybridized to both a *PET494*-specific probe and, as an internal control, an actin-specific probe (Fig. 1). Surprisingly, the *PET494* transcript level did not vary in response to oxygen deprivation, whereas β-galactosidase activity decreased fivefold, as reported above. This result indicates that oxygen regulation of *PET494* occurs at the translational level.

To examine the effects of carbon source on *PET494*

TABLE 4. Effect of a *hxk2* mutation on expression of a *pet494::lacZ* fusion

Relevant genotype[a]	Carbon source[b]	β-Galactosidase specific activity relative to WT on 2% glucose
HXK2 pet494::lacZ (WT)	Glucose (15%)	0.57[c]
	Glucose (2%)	1.0[c]
	Glucose (0.5%)	1.4[c]
hxk2 pet494::lacZ	Glucose (15%)	0.61[c]
	Glucose (2%)	0.92[c]
	Glucose (0.5%)	1.2[c]
HXK2/HXK2 PET494/ pet494::lacZ (WT)	Glucose (2%)	1.0[d]
	Galactose (2%)	2.1[d]
	Ethanol (3%) + glycerol (3%)	8.1[d]
hxk2/hxk2 PET494/ pet494::lacZ	Glucose (2%)	1.2[d]
	Galactose (2%)	1.5[d]
	Ethanol (3%) + glycerol (3%)	7.1[d]

[a] The parental haploid HXK2 strain was DAU1-A1. The *pet494::lacZ* fusion replaced the wild-type (WT) chromosomal copy of *PET494* in this strain. The *hxk2* haploid strain was derived from DAU1-A1 by disrupting the *HXK2* gene. The diploid *HXK2/HXK2* strain was derived by crossing DAU1-A1 with DLU2, which contains a wild-type copy of *PET494*. The *hxk2/hxk2* diploid was derived by disrupting the *HXK2* gene in strain DLU2 and then crossing this with the *hxk2 pet494::lacZ* haploid strain.

[b] Repressed cells were grown to exponential phase in complete medium containing either 2% or 15% glucose. Derepressed cells were either grown directly in galactose- or ethanol-and-glycerol-containing complete medium or prepared by shifting repressed cells from 2% to 0.5% glucose for 2 h.

[c] Expression relative to that in the *HXK2 pet494::lacZ* haploid strain grown on 2% glucose.

[d] Expression relative to that in the *HXK2/HXK2 PET494/pet494::lacZ* diploid strain grown on 2% glucose.

transcription, we performed a similar hybridization analysis on total RNA prepared from cells grown on either glucose-, galactose-, or ethanol- and glycerol- containing medium. In general, the level of *PET494* transcript increased under conditions where *pet494::lacZ* expression increased (Fig. 2), suggesting that the carbon source-dependent regulation of *PET494* involves control at the level of transcription.

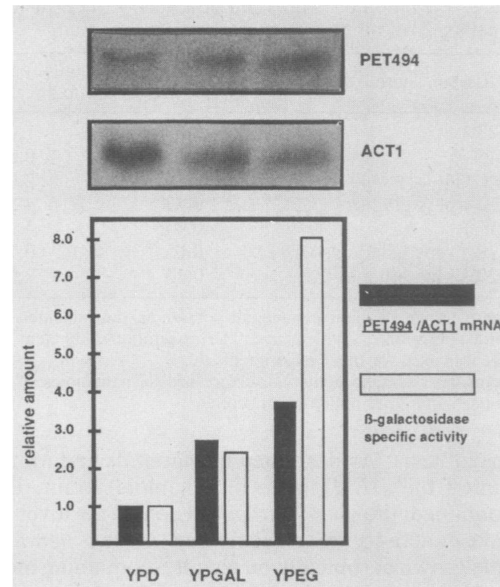

FIG. 2. Effects of carbon source on *PET494* transcript levels. Total RNA was prepared from yeast cells of the strain DAD1 grown in complete medium containing either glucose, galactose, or ethanol plus glycerol. A 100-μg portion of each sample was denatured with glyoxal, fractionated by agarose gel electrophoresis, transferred to a Biotrans nylon membrane, and probed first with a *PET494*-specific probe and subsequently with an *ACT1*-specific probe. The transcripts were visualized by autoradiography and scanned densitometrically. Symbols: ▬, amount of *PET494* mRNA (normalized to *ACT1*) relative to the amount in cells grown on glucose; ▭, amount of β-galactosidase specific activity relative to the amount expressed in glucose-grown cells. The RNA determination was repeated three times. The blots represent one of the repeats.

DISCUSSION

The product of the yeast nuclear gene *PET494* has been shown to specifically activate translation of the mitochondrially encoded mRNA for coxIII (44). We are interested in determining whether the *PET494* gene product plays a role in modulating coxIII translation in response to environmental stimuli such as oxygen and carbon source. To do this, it is necessary to determine how *PET494* expression is regulated. Experiments reported in this paper show that *PET494* expression is subject to oxygen-dependent, heme-independent control. In addition, *PET494* is subject to carbon source-dependent regulation which appears to involve both repression by glucose and induction by ethanol. Since the responses of *PET494* to environmental stimuli are similar to those of cytochrome *c* oxidase, these findings are consistent with the idea that *PET494* acts to modulate translation of the mitochondrially encoded protein coxIII.

Cells grown under anaerobic conditions expressed β-galactosidase from a *pet494::lacZ* fusion at a much lower level than did cells grown in the presence of oxygen. Since β-galactosidase appears to be equally stable in vivo under aerobic and anaerobic conditions (22), this decrease reflects the regulation of *PET494*. Surprisingly, *PET494* mRNA levels did not respond to oxygen starvation, suggesting that oxygen regulates *PET494* at the level of translation. The conclusion that *PET494* is subject to oxygen-dependent translational control would be strengthened if we could demonstrate that the level of the *pet494::lacZ* mRNA did not respond to anaerobiosis. However, we have been unable to detect this very large low-abundance mRNA by probing RNA blots with high-specific-activity probes.

In several well-studied cases of yeast genes whose expression is controlled by oxygen levels, heme mediates the regulatory response. (Oxygen is needed for heme biosynthesis in *S. cerevisiae* [37].) Heme is required to activate transcription of several aerobically induced genes such as *CTA1*, *CTT1*, *CYC1*, and *CYC7* (22, 23, 50, 56, 66), which encode catalase A (9), catalase T (55), iso-1-cytochrome *c* (54), and iso-2-cytochrome *c* (15), respectively. Heme is also required to repress transcription (during aerobic growth) of the anaerobically induced gene *ANB1* (31–33) and the gene encoding coproporphyrinogen oxidase (64).

In striking contrast to these oxygen-regulated genes, *PET494* expression was independent of heme. Strains lacking heme as a result of a *hem1* mutation had normal aerobic levels of *PET494* expression and reduced levels of *PET494* expression under anaerobic conditions. Consistent with these findings, *PET494* expression was independent of *HAP2*, which regulates the expression of other respiratory genes in response to heme (21, 27). Furthermore, although all other known instances of oxygen regulation involve transcriptional control, our data indicate that oxygen regulation of *PET494* occurs at the translational level.

PET494 expression was also regulated by carbon source. The expression of the *pet494*::*lacZ* fusion was greatest in cells grown on nonfermentable carbon sources. Moreover, expression decreased with increasing amounts of glucose in the medium. Interestingly, the addition of glucose to galactose-containing medium fully repressed PET494–β-galactosidase levels, whereas addition of glucose to ethanol-containing medium did not. These data indicate that *PET494* expression is repressed by glucose but appears also to be partially induced by ethanol. Despite being repressed by glucose, *PET494* was not controlled by the products of the *SNF1*, *SNF2*, *SSN6*, or *HXK2* genes, which control the glucose repression of many other genes in *S. cerevisiae* (5–7, 18, 34, 45).

Although the extent of the carbon source-dependent regulation of *PET494* was not large (two- to sixfold), similar small effects have been observed for other glucose-repressible genes in *S. cerevisiae*. For example, the steady-state levels of the mRNAs encoding the α and β subunits of the mitochondrial ATPase were repressed 2.5- to 7-fold by glucose (59). The steady-state level of cytochrome *c* oxidase activity was shown to be repressed two- to sixfold by glucose when cells grown exponentially under repressing and derepressing conditions were compared (47). The effects we observed on the expression of *PET494* were similar in magnitude to the reported effects of carbon source on cytochrome *c* oxidase activity and thus were consistent with its possible role as a regulator of coxIII synthesis.

The effects of carbon source on *PET494* transcription are difficult to interpret. Relative to glucose-grown cells, galactose-grown cells showed a 2.5-fold increase in both *PET494* mRNA level and *pet494*::*lacZ* expression. However, in the experiment shown in Fig. 2, cells grown on nonfermentable carbon sources exhibited only a 3.7-fold increase in the *PET494* transcript level relative to glucose-grown cells, despite their relative 8-fold increase in β-galactosidase expression. Although these changes were small, they suggest that regulation of *PET494* by carbon source may involve both transcriptional and posttranscriptional controls.

Translationally controlled eucaryotic genes generally have unusually long 5′ untranslated mRNA leaders (24, 26, 29, 30, 43, 51, 61, 62). In some cases, such as in the mRNAs transcribed from the yeast nuclear genes *GCN4* and *CPA1*, short open reading frames within the 5′ leaders play a role in translational regulation (43, 61, 62). In contrast, the *PET494* mRNA has a short 5′ leader that does not contain open reading frames (12). Nevertheless, our data suggest that *PET494* expression may be translationally controlled by a mechanism that operates through this short 5′ leader.

In addition to *PET494*, two other nuclear genes, *PET54* and *PET122*, are required to specifically activate translation of the mitochondrially encoded coxIII mRNA (13, 14, 28). Recent experiments have shown that *PET54* expression is repressed by glucose to the same extent as *PET494* expression and is similarly reduced by oxygen starvation (S. Zonghou and T. D. Fox, unpublished data). It will be interesting to see whether *PET494*, *PET54*, and *PET122* are coordinately regulated in response to the same environmental signals and thereby modulate coxIII translation.

ACKNOWLEDGMENTS

We thank D. Botstein, M. Carlson, L. Guarente, H. Ma, L. Neigeborn, J. Pinkham, and R. Zitomer for their gifts of plasmids and strains.

This work was supported by National Research Service Award training grant GM07273, Public Health Service grant GM29362, and Research Career Development Award HD00515 from the National Institutes of Health.

LITERATURE CITED

1. **Bailey, J. N., and W. T. McAllister.** 1980. Mapping of promoter sites utilized by T3 RNA polymerase on T3 DNA. Nucleic Acids Res. **8**:5071–5088.
2. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**:345–346.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72**:248–254.
4. **Cabral, F., and G. Schatz.** 1978. Identification of cytochrome *c* oxidase subunits in nuclear yeast mutants lacking the functional enzyme. J. Biol. Chem. **253**:4396–4401.
5. **Carlson, M.** 1987. Regulation of sugar utilization in *Saccharomyces* species. J. Bacteriol. **169**:4873–4877.
6. **Carlson, M., B. C. Osmond, and D. Botstein.** 1981. Mutants of yeast defective in sucrose utilization. Genetics **98**:25–40.
7. **Carlson, M., B. C. Osmond, L. Neigeborn, and D. Botstein.** 1984. A suppressor of *SNF1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107**:19–32.
8. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100**:293–308.
9. **Cohen, G., F. Fessl, A. Traczyk, J. Rytka, and H. Ruis.** 1985. Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the *cta1* mutation. Mol. Gen. Genet. **200**: 74–79.
10. **Costanzo, M. C., and T. D. Fox.** 1986. Product of *Saccharomyces cerevisiae* nuclear gene *PET494* activates translation of a specific mitochondrial mRNA. Mol. Cell. Biol. **6**:3694–3703.
11. **Costanzo, M. C., and T. D. Fox.** 1988. Specific translational activation by nuclear gene products occurs in the 5′ untranslated leader of a yeast mitochondrial mRNA. Proc. Natl. Acad. Sci. USA **85**:2677–2681.
12. **Costanzo, M. C., P. P. Mueller, C. A. Strick, and T. D. Fox.** 1986. Primary structure of wild-type and mutant alleles of the *PET494* gene of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **202**:294–301.
13. **Costanzo, M. C., E. C. Seaver, and T. D. Fox.** 1986. At least two nuclear gene products are specifically required for translation of a single yeast mitochondrial mRNA. EMBO J. **5**:3637–3641.
14. **Costanzo, M. C., E. C. Seaver, D. L. Marykwas, and T. D. Fox.** 1988. Multiple nuclear gene products are specifically required to activate translation of a single yeast mitochondrial mRNA, p. 373–382. *In* M. F. Tuite, M. Picard, and M. Bolotin-Fukuhara (ed.), Genetics of translation. Springer-Verlag KG, Berlin.
15. **Downie, J. A., J. W. Stewart, N. Brockman, A. M. Schweingruber, and F. Sherman.** 1977. Structural gene for yeast iso-2-cytochrome *c*. J. Mol. Biol. **113**:369–384.
16. **Dujon, B.** 1981. Mitochondrial genetics and functions, p. 505–635. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Ebner, E., T. L. Mason, and G. Schatz.** 1973. Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. II. Effect of nuclear and extrachromosomal mutations on the formation of cytochrome *c* oxidase. J. Biol. Chem. **248**:5369–5378.
18. **Entian, K.-D.** 1980. Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression in yeast. Mol. Gen. Genet. **178**:633–637.
19. **Falcone, C., M. Agostinelli, and L. Frontali.** 1983. Mitochondrial translation products during release from glucose repression in *Saccharomyces cerevisiae*. J. Bacteriol. **153**:1125–1132.
20. **Goldstein, A., and J. O. Lampen.** 1975. β-D-Fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42C**:504–511.
21. **Guarente, L., B. LaLonde, P. Gifford, and E. Alani.** 1984.

Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene of *S. cerevisiae*. Cell **36**:503–511.

22. **Guarente, L., and T. Mason.** 1983. Heme regulates transcription of the *CYC1* gene of *S. cerevisiae* via an upstream activation site. Cell **32**:1279–1286.
23. **Hörtner, H., G. Ammerer, E. Hartter, B. Hamilton, J. Rytka, T. Bilinski, and H. Ruis.** 1982. Regulation of synthesis of catalases and iso-1-cytochrome *c* in *Saccharomyces cerevisiae* by glucose, oxygen and heme. Eur. J. Biochem. **128**:179–184.
24. **Hultmark, D., R. Klemenz, and W. J. Gehring.** 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene *hsp22*. Cell **44**:429–438.
25. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
26. **Katz, R. A., B. R. Cullen, R. Malavarca, and A. M. Skalka.** 1986. Role of the avian retrovirus mRNA leader in expression: evidence for novel translational control. Mol. Cell. Biol. **6**: 372–379.
27. **Keng, T., and L. Guarente.** 1987. Constitutive expression of the yeast *HEM1* gene is actually a composite of activation and repression. Proc. Natl. Acad. Sci. USA **84**:9113–9117.
28. **Kloeckener-Gruissem, B., J. E. McEwen, and R. O. Poyton.** 1988. Identification of a third nuclear protein-coding gene required specifically for posttranscriptional expression of the mitochondrial *COX3* gene in *Saccharomyces cerevisiae*. J. Bacteriol. **170**:1399–1402.
29. **Lindquist, S., and T. J. McGarry.** 1986. Translational control of heat shock proteins in *Drosophila*, p. 86–90. *In* M. B. Mathews (ed.), Translational control. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA **81**:3655–3659.
31. **Lowry, C. V., and R. H. Lieber.** 1986. Negative regulation of the *Saccharomyces cerevisiae ANB1* gene by heme, as mediated by the *ROX1* gene product. Mol. Cell. Biol. **6**:4145–4148.
32. **Lowry, C. V., and R. S. Zitomer.** 1984. Oxygen regulation of anaerobic and aerobic genes mediated by a common factor in yeast. Proc. Natl. Acad. Sci. USA **81**:6129–6133.
33. **Lowry, C. V., J. L. Weiss, D. A. Walthall, and R. S. Zitomer.** 1983. Modulator sequences mediate oxygen regulation of *CYC1* and a neighboring gene in yeast. Proc. Natl. Acad. Sci. USA **80**:151–155.
34. **Ma, H., and D. Botstein.** 1986. Effects of null mutations in the hexokinase genes of *Saccharomyces cerevisiae* on catabolite repression. Mol. Cell. Biol. **6**:4046–4052.
35. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Mason, T. L., and G. Schatz.** 1973. Cytochrome *c* oxidase from bakers' yeast II. Site of translation of the protein components. J. Biol. Chem. **248**:1355–1360.
37. **Mattoon, J. R., W. E. Lancashire, H. K. Sanders, E. Carvajal, D. R. Malamud, G. R. C. Braz, and A. D. Panek.** 1979. Oxygen and catabolite regulation of hemoprotein biosynthesis in yeast, p. 421–435. *In* W. S. Caughey (ed.), Biochemical and clinical aspects of oxygen. Academic Press, Inc., New York.
38. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
39. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4838.
40. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
41. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Mueller, D. M., and G. S. Getz.** 1986. Steady state analysis of mitochondrial RNA after growth of yeast *Saccharomyces cerevisiae* under catabolite repression and derepression. J. Biol. Chem. **261**:11816–11822.
43. **Mueller, P. P., S. Harashima, and A. G. Hinnebusch.** 1987. A segment of *GCN4* mRNA containing the upstream AUG codons confers translational control upon a heterologous yeast transcript. Proc. Natl. Acad. Sci. USA **84**:2863–2867.
44. **Müller, P. P., M. K. Reif, S. Zonghou, C. Sengstag, T. L. Mason, and T. D. Fox.** 1984. A nuclear mutation that posttranscriptionally blocks accumulation of a yeast mitochondrial gene product can be suppressed by a mitochondrial gene rearrangement. J. Mol. Biol. **175**:431–452.
45. **Neigeborn, L., and M. Carlson.** 1984. Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. Genetics **108**:845–858.
46. **Ng, R., and J. Abelson.** 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77**:3912–3916.
47. **Polakis, E. S., W. Bartley, and G. A. Meek.** 1965. Changes in the activities of respiratory enzymes during the aerobic growth of yeast on different carbon sources. Biochem. J. **97**:298–302.
48. **Poutre, C. G., and T. D. Fox.** 1987. *PET111*, a *Saccharomyces cerevisiae* nuclear gene required for translation of the mitochondrial mRNA encoding cytochrome *c* oxidase subunit II. Genetics **115**:637–647.
49. **Power, S. D., M. A. Lochrie, K. A. Sevarino, T. E. Patterson, and R. O. Poyton.** 1984. The nuclear-coded subunits of yeast cytochrome *c* oxidase. I. Fractionation of the holoenzyme into chemically pure polypeptides and the identification of two new subunits using solvent extraction and reversed phase high performance liquid chromatography. J. Biol. Chem. **259**:6564–6570.
50. **Prezant, T., K. Pfeifer, and L. Guarente.** 1987. Organization of the regulatory region of the yeast *CYC7* gene: multiple factors are involved in regulation. Mol. Cell. Biol. **7**:3252–3259.
51. **Rao, C. D., M. Pech, K. C. Robbins, and S. A. Aaronson.** 1988. The 5′ untranslated sequence of the c-*sis*/platelet-derived growth factor 2 transcript is a potent translational inhibitor. Mol. Cell. Biol. **8**:284–292.
52. **Rose, M., M. J. Casadaban, and D. Botstein.** 1981. Yeast genes fused to β-galactosidase in *Escherichia coli* can be expressed normally in yeast. Proc. Natl. Acad. Sci. USA **78**:2460–2464.
53. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. **Sherman, F., J. W. Stewart, E. Margoliash, J. Parker, and W. Campbell.** 1966. The structural gene for yeast cytochrome *c*. Proc. Natl. Acad. Sci. USA **55**:1498–1504.
55. **Spevak, W., F. Fessl, J. Rytka, A. Traczyk, M. Skoneczny, and H. Ruis.** 1983. Isolation of the catalase T structural gene of *Saccharomyces cerevisiae* by functional complementation. Mol. Cell. Biol. **3**:1545–1551.
56. **Spevak, W., A. Hartig, P. Meindl, and H. Ruis.** 1986. Heme control region of the catalase T gene of the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **203**:73–78.
57. **Sprague, G. F., Jr., R. Jensen, and I. Herskowitz.** 1983. Control of yeast cell type by the mating type locus: positive regulation of the alpha-specific *STE3* gene by the *MATalpha1* product. Cell **32**:409–415.
58. **Strick, C. A., and T. D. Fox.** 1987. *Saccharomyces cerevisiae* positive regulatory gene *PET111* encodes a mitochondrial protein that is translated from an mRNA with a long 5′ leader. Mol. Cell. Biol. **7**:2728–2734.
59. **Szekely, E., and D. L. Montgomery.** 1984. Glucose represses transcription of *Saccharomyces cerevisiae* nuclear genes that encode mitochondrial components. Mol. Cell. Biol. **4**:939–946.
60. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
61. **Tzamarias, D., D. Alexandraki, and G. Thireos.** 1986. Multiple *cis*-acting elements modulate the translational efficiency of *GCN4* mRNA in yeast. Proc. Natl. Acad. Sci. USA **83**:4849–

4853.

62. **Werner, M., A. Feller, F. Messenguy, and A. Piérard.** 1987. The leader peptide of yeast gene *CPA1* is essential for the translational repression of its expression. Cell **49**:805–813.
63. **Woodrow, G., and G. Schatz.** 1979. The role of oxygen in the biosynthesis of cytochrome *c* oxidase of yeast mitochondria. J. Biol. Chem. **254**:6088–6093.
64. **Zagorec, M., and R. Labbe-Bois.** 1986. Negative control of yeast coproporphyrinogen oxidase synthesis by heme and oxygen. J. Biol. Chem. **261**:2506–2509.
65. **Zennaro, E., L. Grimaldi, G. Baldacci, and L. Frontali.** 1985. Mitochondrial transcription and processing of transcripts during release from glucose repression in "resting cells" of *Saccharomyces cerevisiae*. Eur. J. Biochem. **147**:191–196.
66. **Zitomer, R. S., J. W. Sellers, D. W. McCarter, G. A. Hastings, P. Wick, and C. V. Lowry.** 1987. Elements involved in oxygen regulation of the *Saccharomyces cerevisiae CYC7* gene. Mol. Cell. Biol. **7**:2212–2220.