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Inverse Regulation of the Yeast *COX5* Genes by Oxygen and Heme

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The *COX5a* and *COX5b* genes encode divergent forms of yeast cytochrome *c* oxidase subunit V. Although the polypeptide products of the two genes are functionally interchangeable, it is the Va subunit that is normally found in preparations of yeast mitochondria and cytochrome *c* oxidase. We show here that the predominance of subunit Va stems in part from the differential response of the two genes to the presence of molecular oxygen. Our results indicate that during aerobic growth, *COX5a* levels were high, while *COX5b* levels were low. Anaerobically, the pattern was reversed; *COX5a* levels dropped sevenfold, while those of *COX5b* were elevated sevenfold. Oxygen appeared to act at the level of transcription through heme, since the addition of heme restored an aerobic pattern of transcription to anaerobically grown cells and the effect of anaerobiosis on *COX5* transcription was reproduced in strains containing a mutation in the heme-biosynthetic pathway (*hem1*). In conjunction with the oxygen-heme response, we determined that the product of the *ROX1* gene, a *trans*-acting regulator of several yeast genes controlled by oxygen, is also involved in *COX5* expression. These results, as well as our observation that *COX5b* expression varied significantly in certain yeast strains, indicate that the *COX5* genes undergo a complex pattern of regulation. This regulation, especially the increase in *COX5b* levels anaerobically, may reflect an attempt to modulate the activity of a key respiratory enzyme in response to varying environmental conditions. The results presented here, as well as those from other laboratories, suggest that the induction or derepression of certain metabolic enzymes during anaerobiosis may be a common and important physiological response in yeast cells.

In *Saccharomyces cerevisiae*, subunit V of the mitochondrial inner membrane protein complex cytochrome *c* oxidase is encoded by two genes, *COX5a* and *COX5b* (2–4). Although the polypeptide products of these genes, subunits Va and Vb, respectively, are functionally interchangeable, the Va isolog is the predominant form of subunit V found in mitochondria prepared from aerobically grown yeast cells (2). This is because under these conditions, the *COX5a* gene is expressed at significantly higher levels than *COX5b* (21).

An especially interesting question regarding this small gene family concerns the biological role of *COX5b*. Gene disruptions of *COX5b* cause no readily observable phenotype; such strains appear to respire normally (2, 21). Yet *COX5b* function has been preserved throughout considerable evolutionary divergence (the genes are 67% and the proteins are 66% homologous [4]), suggesting some sort of functional selection. Moreover, all closely related *Saccharomyces* species examined thus far contain genomic copies of both genes (4). Together, these findings imply an important, but currently unknown, biological role for *COX5b*.

Since the *COX5* genes are not expressed at equivalent levels (21), a useful first approach towards elucidating the biological role of *COX5b* is to look for changes in the pattern of expression of both genes in response to different growth or physiological conditions. In particular, the effects of oxygen and heme on *COX5* expression would appear to be especially appropriate, since they are known to be involved in the expression of the yeast cytochrome *c* (*CYC1* and *CYC7*), catalase T (*CTT1*), and cytochrome oxidase subunit IV (*COX4*) genes (7–12, 23). In addition, regulatory mutations that affect oxygen or heme regulation of several yeast genes, including *CYC1*, have been identified and characterized (7, 12, 16, 17). Should these mutations also affect the expression of the *COX5* genes, they would provide a unique opportunity to examine common regulatory circuits between these genes.

In this study, we have examined the effect of oxygen, heme, and two regulatory mutations, *rox1a* and *rox1b*, on the expression of the *COX5* genes. Our results indicate that *COX5a* and *COX5b* undergo a complex pattern of transcriptional regulation in which oxygen, through heme, affects the expression of each gene in opposite ways. We have also found that the product of the *ROX1* gene is involved in *COX5* expression and that *COX5b* expression varies in certain yeast strains. The results of this as well as additional independent studies (7, 10, 12, 24, 25) suggest that many genes in *S. cerevisiae* may be controlled either positively or negatively by oxygen or heme. Particularly intriguing is the finding that a respiratory gene, *COX5b*, is controlled negatively by oxygen. While this type of regulation may appear paradoxical, it probably reflects a key cellular response to changing environmental or physiological conditions.

MATERIALS AND METHODS

Plasmids, strains, and growth media. The *Saccharomyces cerevisiae* strains used in this study are described in Table 1. Most of the plasmids used have been described previously (2, 4, 21). The β-galactosidase fusion plasmids YCp5aL and YCp5bL are similar to pCET5aL and pMC5bL (21), except that the former pair were derived from the parent plasmid pSEY102 (5). As a result, they contain a yeast centromere and are maintained at low rather than high copy number in yeast cells (20).

YPD and SD media (supplemented as necessary) were prepared by the method of Sherman et al. (19).

Growth conditions and anaerobiosis. Unless otherwise indicated, cells were grown in liquid YPD medium (19) at 30°C. Normally, cultures were made anaerobic by bubbling with ultrapure N_2. Anaerobiosis was also induced by trans-

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TABLE 1. *Saccharomyces cerevisiae* strains used in this study

Strain[a]	Relevant genotype	Reference(s)
D273-10B	*MATα*	ATCC 24657
JM43	*MATα his4-580 trp1-289 leu2-3,112 ura3-52*	2, 3
JM43-GD5ab	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5a Δ::URA3 cox5b::LEU2*	2, 21
JM28	*MATa his4-580 trp1-289 leu2-3,112 ura3-52 cox5a-1 ade2*	2, 3
AB35-13D	*MATa his4-580 trp1-289 leu2-3,112 ura3-52 ade2*	2, 3
GM-3C-2	*MATα his4-580 trp1-289 leu2-3,112* Gal⁻ *cyc1-1 cyc7-1*	11
GM-3C-2-*rox1a*	*MATα his4-580 trp1-289 leu2-3,112* Gal⁻ *cyc1-1 cyc7-1 rox1a-1*	12
GM-3C-2-*rox1b*	*MATα his4-580 trp1-289 leu2-3,112* Gal⁻ *cyc1-1 cyc7-1 rox1b-3*	12

[a] All strains except D273-10B, JM43, and AB35-13D are respiration deficient.

ferring aerobic cultures to Brewer jars made anaerobic with GasPaks (BBL Microbiology Systems). In the latter case, anaerobiosis was indicated by the presence of an indicator strip (BBL). In general, we found that the two procedures were essentially interchangeable with respect to their effects on yeast growth and *COX5* expression. Where indicated, heme (as hemin) was added to liquid cultures at 50 μg/ml (10). When cultures were to be used for the preparation of RNA, identical aerobic cultures were first grown to early log phase, and then one culture was shifted to anaerobic conditions. Growth of both cultures was then allowed to continue for 4 h (unless otherwise indicated), at which point both cultures were immediately placed on ice, and harvested at 4°C.

RNA preparation and analysis. The preparation of RNA was carried out as described before (9). RNA fractionation was performed on 1.8% formaldehyde–agarose gels, followed by capillary transfer to nylon membranes (Biotrans). The RNA was cross-linked to the membrane with short-wave UV light (4 min at 14 cm with a hand-held lamp). When probes labeled with a random primer kit (IBI) were used, hybridization reactions were performed at 42°C in a solution containing 2.5× Denhardt solution (2), 5× SSPE (20× SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4 [pH 7.4], 20 mM EDTA), 0.2% sodium dodecyl sulfate (SDS), 50 μg of denatured salmon sperm DNA per ml, 50% formamide, and 10% dextran sulfate. Washes were done at 50°C in 1× SSC–0.2% SDS (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate); the final wash was in 0.1× SSPE–0.1% SDS. When the RNA was cross-linked and hybridized in this manner, probes could be removed and the filter rehybridized several times without detectable loss of RNA. Old probes were removed by washing the filter for 2 h at 65°C in a solution of 5 mM Tris (pH 8.0)–0.2 mM EDTA–0.5% pyrophosphate–0.1× Denhardt solution.

In certain cases, 5′-end-labeled synthetic oligonucleotides were used as hybridization probes. In order to detect the *ANB1* and *tr-1* transcripts, we used the 21-mer ANB-Pr (5′-GGCAGAACATTGCATTGGGTA-3′). The *COX5a*, *COX5b*, and actin mRNAs were detected with 5a-DSP (5′-GCAGATCCATTACAGCAGCGTTGG-3′), 5b-Pr (5′-TTTCTGTTCTAAGTTTGGCATAT-3′), and Act-Pr (5′-GCACGAGGAGCGTCGTCACCGGCA-3′), respectively. Hybridizations were carried out at 50°C in a solution of 6× SSC–10× Denhardt solution–0.5% SDS–50 mM phosphate buffer (pH 6.5)–250 μg of denatured salmon sperm DNA per ml. The final wash was at 50°C in 3× SSC–0.1% SDS. A 20-pmol amount of oligonucleotide was 5′-end labeled to a specific activity of approximately 2.5×10^6 cpm/pmol, and each hybridization reaction contained 10^6 cpm/ml.

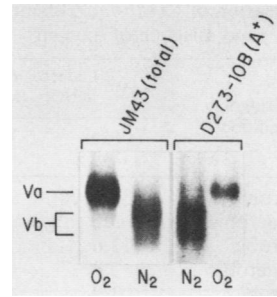

FIG. 1. Inverse regulation of *COX5* transcription by oxygen. Total or poly(A)$^+$ RNA was prepared from the indicated wild-type strains grown in YPD medium (19) either aerobically (O_2) or anaerobically (N_2). Total (30 μg) or poly(A)$^+$ (5 μg) RNA was then fractionated on 1.8% formaldehyde–agarose gels, transferred to nylon membranes, and hybridized with a chimeric *COX5a/COX5b* *Bam*HI-*Cla*I restriction fragment obtained from the plasmid YRp5ab (21). Labeling was performed by the random primer method. The positions of the *COX5a* and *COX5b* transcripts are indicated. For reasons that are not clear to us, the relative amount of each *COX5b* transcript varied; in this particular RNA preparation, the slower-migrating species predominated. Aerobic and anaerobic growth conditions and procedures for RNA preparation, blotting, and hybridization are described in Materials and Methods.

β-Galactosidase assays. All β-galactosidase assays were performed essentially as described by Guarente (6). Assays were done in triplicate on transformants grown in liquid cultures either aerobically or anaerobically at 30°C.

RESULTS

Inverse regulation of the *COX5* genes by anaerobiosis. In yeast cells, the expression of several genes has been shown to be at least partially controlled by the presence or absence of molecular oxygen (7, 11, 12). While most of these genes appear to be induced in the presence of oxygen, the *ANB1* gene (a gene lying upstream of *CYC1* whose function is presently unknown) responds in the opposite way—its transcription is repressed aerobically (11, 12). Since cytochrome *c* oxidase is a respiratory enzyme, we expected that oxygen might play a role in the expression of the genes encoding its polypeptide subunits. We therefore examined the effect of oxygen on the expression of the yeast *COX5* genes.

Total and polyadenylated [poly(A)$^+$] RNAs from two wild-type yeast strains grown in the presence or absence of oxygen were prepared (Materials and Methods) and analyzed by RNA blot (Northern) hybridization. In order to detect both *COX5a* and *COX5b* transcripts simultaneously, we used a chimeric probe containing sequences derived from both genes (21). The results of the experiment (Fig. 1) were striking. As predicted, we found that the steady-state level of the *COX5a* mRNA was high in RNA prepared from aerobically grown cells and virtually undetectable in cells grown anaerobically. Unexpectedly, however, *COX5b* transcript levels, which in this experiment were barely detectable in aerobically grown cells, dramatically increased under anaerobic conditions.

Because the probe used in the experiment in Fig. 1 contained approximately three times more sequence from

TABLE 2. Expression of *COX5-lacZ* fusions in the presence and absence of oxygen[a]

Strain	Growth conditions	β-Galactosidase activity (U)		Activity ratio, 5a/5b
		YCp5aL (5a)	YCp5bL (5b)	
JM43	Aerobic	37.0	0.36	103
	Anaerobic	5.1	2.4	2.1
JM28	Aerobic	13.0	5.8	2.2
	Anaerobic	2.8	7.8	0.36
AB35-13D	Aerobic	3.9	4.4	0.9
	Anaerobic	2.2	6.0	0.36

[a] JM43 transformants harboring either a *COX5a-* or *COX5b-lacZ* fusion (YCp5aL and YCp5bL, respectively; see Materials and Methods) were grown aerobically to stationary phase in liquid SD medium (19), then diluted 1:100 into liquid YPD medium (19). When the YPD culture reached early log phase (60 Klett units), a portion was made anaerobic with nitrogen bubbling. Growth of both cultures was allowed to continue for 2 (JM28 and AB35-13D) or 4 more hours (JM43), at which time samples of both cultures were chilled, harvested in the cold, and assayed for β-galactosidase activity as described (6). The difference in the length of the anaerobic growth periods permitted direct comparison of these results with those of the RNA blot analyses shown in Fig. 1 and 2 (for JM43) or Fig. 6 (for JM28 and AB35-13D). All assays were performed in triplicate; the results, presented as Miller units (6), represent the average of two experiments. The error was less than 20%.

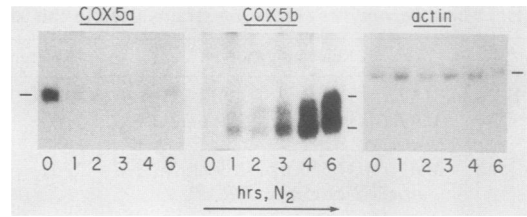

FIG. 2. Kinetics of anaerobic *COX5* transcription. The wild-type strain JM43 was grown aerobically in liquid YPD medium (19) to early log phase (60 Klett units, green filter) and then shifted to anaerobic conditions by bubbling with ultrapure N_2. At various times, samples of the culture were harvested and used for the preparation of RNA. After fractionation of the RNA (30 μg) on a 1.8% formaldehyde–agarose gel, it was analyzed by blotting and hybridization against oligonucleotide probes complementary to the indicated genes. The sequences of the various probes, hybridization and wash conditions, and the method used to remove old probes are given in Materials and Methods. The actin probe was used as a control to demonstrate that, with minor variations, equivalent amounts of RNA were loaded in each lane. Lines to the left (*COX5a*) or right (*COX5b*, actin) of each panel denote the position of each respective transcript.

COX5b than *COX5a*, the absolute levels of the two transcripts could not be compared directly. In order to obtain more quantitative data and to examine expression at the level of both transcription and translation, we also performed β-galactosidase assays on transformants expressing a *COX5a-* or *COX5b-lacZ* fusion. In both cases, the gene fusion was carried on a low-copy-number yeast centromeric plasmid (see Materials and Methods). The results of the assays (Table 2) indicated that after 4 h of anaerobiosis, *COX5a* expression dropped sevenfold, a figure we believe to be a minimum estimate due to the extreme stability of this fusion protein in yeast cells (M. R. Hodge, unpublished observations). In contrast, we found that *COX5b* expression increased approximately sevenfold. Overall, oxygen deprivation resulted in (minimally) a 50-fold difference in the expression ratio of *COX5a* to *COX5b* (Table 2). The general correlation between the results of the mRNA and β-galactosidase studies suggests that the effect of oxygen was largely at the level of transcription.

We next examined kinetically the effect of anaerobiosis on *COX5* expression. To accomplish this, a yeast culture was grown aerobically to early log phase (60 Klett units) in liquid YPD medium (19) and then shifted to anaerobic conditions. At various times, samples of the culture were harvested and used for the preparation of RNA. That RNA was fractionated, transferred to a membrane filter, and hybridized against, sequentially, *COX5a*, *COX5b*, and actin probes. After only 1 h of anaerobiosis, there was essentially no detectable *COX5a* mRNA; *COX5a* RNA levels remained low throughout the anaerobic growth period (Fig. 2). On the other hand, *COX5b* transcript levels increased quickly after the shift and continued to climb throughout the anaerobic growth period. They were highest at the final (6-h) time point.

It is important to note that the results of recent experiments have established that during anaerobiosis, *COX5b* expression is subject to a second level of control. In Fig. 3 we present growth profiles of yeast cells grown aerobically or anaerobically for various lengths of time. As shown in Fig. 3A (the growth curve in YPD, the medium used for the experiment in Fig. 2), the 4-h time point marked the transition from logarithmic growth to stationary phase, as after this point the growth rate of the cells declined. Our results indicate that at 4 h, during either aerobic or anaerobic growth, an additional increase in *COX5b* transcription occurred. This increase, which we estimate to be about fivefold, was specifically due to a release from glucose repression (K. Singh and G. Kim, unpublished results). Although the β-galactosidase assays reported for JM43 in Table 2 were performed at 4 h, the results reflect only the difference in *COX5* expression due exclusively to anaerobiosis. This is because the aerobic and anaerobic cultures grow at the same rate in either rich or minimal medium and derepress at precisely the same point of the growth curve (Fig. 3A and B, respectively) (M. Hodge and K. Singh, unpublished results).

The curves also demonstrate that in rich or minimal medium, our anaerobic cultures did not exhibit a requirement for ergosterol and Tween (Fig. 3). Normally, supplementation of the medium with these compounds is required to achieve yeast growth in the absence of oxygen or heme, since the biosynthesis of sterols, the desaturation of fatty acids, and heme biosynthesis itself are oxygen dependent (13, and references therein).

We believe that the growth of our cultures anaerobically without supplements can be explained by the fact that the cultures were first grown aerobically and then shifted to anaerobic conditions. We suggest that the early period of aerobic growth produces enough intracellular heme or leaves enough intracellular oxygen to ultimately synthesize enough heme to sustain growth for the remainder of the anaerobic period. We do not believe that the growth of our cultures under these conditions was due to a failure to maintain a high degree of anaerobiosis. This is because we have also grown anaerobic cultures, after an initial period of aerobic growth, in Brewer jars (Materials and Methods) that were known to be strictly anaerobic (a redox potential of less than −200 mV, indicated with a methylene blue indicator). In the latter experiments, we found the growth rate and final cell densities, in the absence of sterols and fatty acids, to be nearly identical to those shown in Fig. 3 (data not shown).

Heme restores an aerobic pattern of expression to anaerobically grown cells. As mentioned above, the growth of yeast

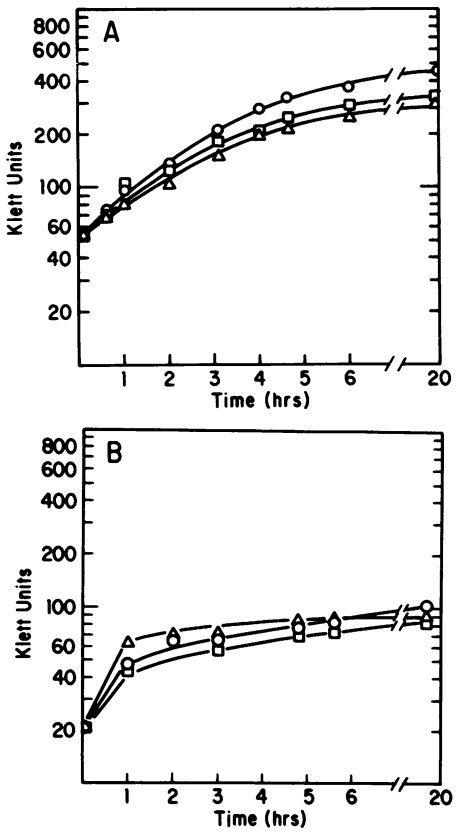

FIG. 3. Growth of yeast cells after a shift from aerobic to anaerobic conditions. (A) Aerobic cultures of the wild-type strain JM43 were grown in liquid YPD medium (19) or in YPD medium supplemented with 20 μg of ergosterol per ml and 0.2% Tween 80 (8, 13). When the cultures reached the indicated density (60 Klett units with a green filter, indicated as time zero on the graph), the ergosterol-Tween-supplemented culture (open triangles) and one of the unsupplemented YPD cultures (open squares) were shifted to anaerobic conditions as described in Materials and Methods. The third culture (open circles) was left growing aerobically. Growth was monitored for an additional 20 h. (B) Experiment identical to that in panel A except that the growth medium was synthetic dextrose (SD [19]). This medium also contained 40 μg of His, Trp, Ura, and Leu per ml in order to satisfy the auxotrophic requirements of the strain.

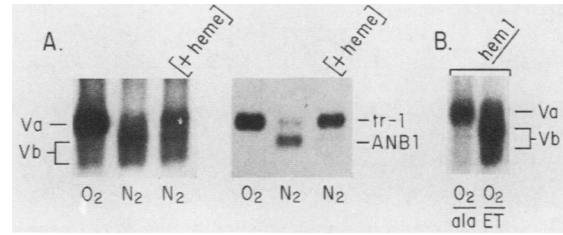

FIG. 4. Heme restores an aerobic pattern of expression to anaerobically grown cells. (A) Total RNA (30 μg) prepared from strain JM43 grown as indicated (see Materials and Methods) was fractionated, blotted, and hybridized as described in the legend to Fig. 1. In the left panel, the probe was the same chimeric *COX5a/COX5b* used in Fig. 1. The filter was then stripped of probe and rehybridized with a 5'-end-labeled oligonucleotide that hybridizes to both the *ANB1* and *tr-1* genes (right panel, 5'-GGCAGAACATTGCATTGGGTA-3'). Hybridization conditions for oligonucleotide probes are described in Materials and Methods. The positions of the various transcripts are indicated. (B) RNA (30 μg) prepared from a *hem1* strain grown to mid-log phase as indicated was analyzed with the chimeric *COX5a/COX5b* probe as described in the legend to Fig. 1. In the lane labeled ala, the culture had been grown aerobically in the presence of 50 μg of δ-aminolevulinic acid per ml, the biosynthetic intermediate whose synthesis was defective in the strain. The lane labeled ET indicates that the culture was grown in the presence of ergosterol (20 μg/ml) and Tween 80 (0.2%), which is necessary for growth of the strain (8 and 14, and references therein). The *hem1* strain used was GT38-7a (13).

cells during prolonged periods of anaerobiosis can become limited by the availability of intracellular heme, since several steps in the heme-biosynthetic pathway are oxygen dependent (13). Heme is also required for the assembly of yeast cytochrome *c* oxidase (18) and more recently has been implicated in the transcriptional activation of several yeast genes, including *CYC1*, *COX4*, and *CTT1* (7– 10, 23).

Because of the link between heme biosynthesis and growth, and between heme and gene expression, we wondered whether oxygen indirectly affected *COX5* expression by altering intracellular heme levels. In order to test this possibility, we performed an experiment similar to that done by Lowry and Leiber for the yeast *ANB1* gene (10). Yeast cells were grown aerobically to early log phase and then shifted to anaerobic conditions in either the presence or absence of heme. RNA from cells grown in either condition was then prepared and analyzed by blotting and hybridization (Fig. 4A). The results indicated that the presence of heme caused an increase in the levels of the *COX5a* transcript and a decrease in that of *COX5b*. As a control, we stripped the blot of the *COX5* probe and reprobed it with one derived from the yeast *ANB1* gene. The result (Fig. 4A) was consistent with that of Lowry and Leiber (10). We found that heme addition caused an increase in the levels of the *tr-1* transcript (an aerobically expressed gene) and a concomitant decrease in the level of the *ANB1* mRNA.

The effect of heme on *COX5* expression was also tested a second way. We reasoned that if heme had a direct regulatory role, biosynthetic defects which resulted in heme deficiency should mimic the effect of anaerobiosis on *COX5* transcription. We therefore made RNA from a yeast strain containing a lesion in *HEM1*, the structural gene for δ-aminolevulinate (δ-ALA) synthetase and the first step in the heme biosynthetic pathway (8, 13). That RNA was analyzed by blotting and hybridization against the chimeric *COX5* probe. The results (Fig. 4B) demonstrated that an effect identical to that observed anaerobically was reproduced in a *hem1* strain grown aerobically. Specifically, we found that the steady-state level of the *COX5a* mRNA was reduced, while that of *COX5b* mRNA was elevated. Importantly, in independent studies, Trueblood et al. demonstrated an identical effect of the *hem1* mutation (22), while Myers et al. reported that a different heme-biosynthetic mutation (*hem2*) also caused a decrease in the level of the *COX5a* mRNA (14). Together, these results suggest that the effect of oxygen on *COX5* transcription is indirect and that heme, presumably as a coeffector, is involved in the inverse regulation of the *COX5a* and *COX5b* genes.

***COX5* expression is affected by the *ROX1* locus.** The product of the *ROX1* gene, in conjunction with heme, is involved in the transcription of yeast genes expressed in either the presence or absence of oxygen (so-called "aerobic" and "anaerobic" genes, respectively [10, 12]). Strains carrying the *rox1a* allele have the phenotype of expressing aerobic genes, like *CYC1*, *tr-1*, and *SOD*, anaerobically, as well as expressing an anaerobic gene, *ANB1*, aerobically (12). Strains harboring the *rox1b* allele have the phenotype of expressing *ANB1* aerobically (12).

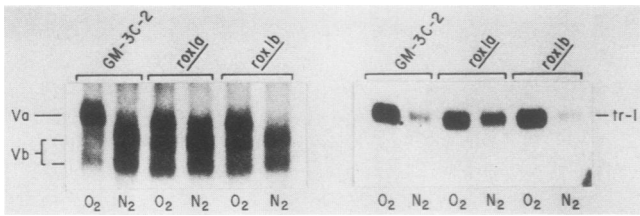

FIG. 5. *COX5* transcription is affected by the *ROX1* gene product. Total RNA (30 μg) prepared from the indicated strains grown in YPD medium either aerobically (O_2) or anaerobically (N_2) was analyzed as described in the legend to Fig. 1, except that growth was for 2 h after the shift to anaerobiosis (see Materials and Methods). The probes for the left and right panels were the same as those used in Fig. 4A. GM-3C-2 is the parent from which the *rox1a* and *rox1b* mutants were derived (see Table 1). It was included in this experiment to demonstrate that the expression of the *COX5* genes is not appreciably affected by this genetic background. GM-3C-2 carries a chromosomal deletion of the entire *CYC1* region, and thus the *ANB1* transcript is not seen in this blot (11).

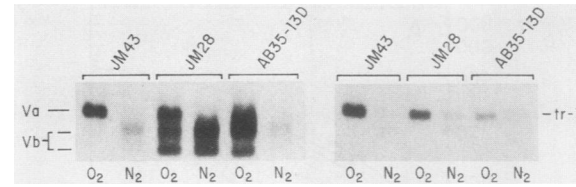

FIG. 6. *COX5* transcript levels vary in different strains. The indicated yeast strains were grown, and RNA was prepared and analyzed as described in the legend to Fig. 1, except that growth was for 2 h after the shift to anaerobiosis. Total RNA (30 μg) was loaded per lane. The probes used for the left and right panels were the same as those for Fig. 4A. In order to accentuate the difference in transcript levels between the strains, this blot was exposed for a shorter time (9 h with an intensifying screen) than those shown in previous figures. The less abundant transcript in the right panel (below *tr-1*) corresponds to *ANB1*. The genotypes of JM28 and AB35-13D are given in Table 1.

Because *COX5a* and *COX5b* can clearly be classified as an aerobic and an anaerobic gene, respectively, and because heme is involved in both *COX5* expression and *ROX1* function, it was of interest to determine the effect of the *ROX1* locus on *COX5* expression. To address this, we prepared RNA from yeast strains carrying the *rox1a* and *rox1b* mutations as well as from an isogenic strain that was wild type at that locus (these strains were kindly supplied by R. Zitomer). The RNA was then analyzed by blotting and hybridization with the chimeric probe. The results (Fig. 5) showed that the *COX5* genes responded to the presence of the *rox* mutations in a manner similar to *CYC1* and *ANB1*. That is, in a *rox1a* background, the *COX5a* transcript was present under anaerobic conditions, while the *COX5b* RNA was expressed at higher levels aerobically. Furthermore, the presence of the *rox1b* mutation resulted only in higher levels of *COX5b* transcription aerobically; *COX5a* transcription was unaffected.

We again stripped the blot of the *COX5* probe and rehybridized it with the *ANB1* probe used previously. That experiment (Fig. 5) indicated that the *rox* mutants behaved in the predicted way with respect to the *tr-1* transcript (this strain contained a deletion of the endogenous *CYC1* and *ANB1* genes and thus the probe recognized only *tr-1* [12]). It is apparent, therefore, that the product of the *ROX1* gene is involved in the expression of the *COX5a* and *COX5b* genes.

***COX5* expression varies with strain.** Experiments performed on different wild-type yeast strains from our laboratory indicated that *COX5b* expression varied with the genetic background of the strain. We therefore decided to analyze the endogenous level of *COX5* expression in several of our strains. The results of that study (not shown) indicated that *COX5a* expression did not usually vary from strain to strain. However, a severalfold variation in *COX5b* levels, especially under aerobic conditions, was common. In particular, a pair of related strains JM28 and AB35-13D (2), exhibited a particularly dramatic response. AB35-13D (originally obtained from A. Brake and J. Thorner) is a respiration-proficient strain previously used to construct several appropriately marked transformation recipients (2) (Table 1). JM28 is a haploid strain derived from a cross between AB35-13D and E4-238, a cytochrome *c* oxidase-deficient strain carrying the *cox5-1* allele (2) (Table 1).

In Fig. 6 we present the results of a hybridization experiment on RNA prepared from AB35-13D and JM28 grown under aerobic and anaerobic conditions. As apparent from the figure and β-galactosidase assays (Table 2), *COX5b* expression was elevated under both conditions. Comparing the results for JM28 with those for the wild-type strain, JM43, the increase was approximately 16-fold aerobically and 3-fold anaerobically. For AB35-13D, we observed a 12-fold aerobic and 2.5-fold anaerobic increase in *COX5b* expression. In general, the results for the two strains and between the two assays were in good agreement, although anaerobically we found a higher level of β-galactosidase activity than suggested by the hybridization study. As noted earlier, the high degree of stability of these fusion proteins in yeast cells may, at least in part, explain the apparent difference.

In contrast to the majority of strains tested previously, we found that *COX5a* expression was also affected in JM28 and AB35-13D. Although somewhat difficult to see in Fig. 6, but supported by β-galactosidase data (Table 2), the expression of *COX5a* was down about two- to threefold in JM28 and ninefold in AB35-13D. Stripping the blot from Fig. 6 and reprobing it with the *ANB1* probe demonstrated that the transcription of *tr-1* was similarly affected (right portion of Fig. 6). Interestingly however, the pattern of *ANB1* transcription did not follow that of *COX5b*. Therefore, the element(s) responsible for the dramatic increase in the expression of *COX5b* in JM28 and AB35-13D is not necessarily common to the expression of all anaerobic genes.

At present, it is not clear why *COX5* expression is altered in these two strains. An obvious possibility is that *COX5a* and *COX5b* are structurally different in AB35-13D and JM28. We do not favor this interpretation, however, because first, our β-galactosidase results suggest that the regulatory effect observed is *trans* rather than *cis* acting. Second, we have been able to isolate regulatory mutations which result in the overexpression of *COX5b* (M. G. Cumsky, G. Kim, and M. R. Hodge, manuscript in preparation). Most of these mutations are recessive, and many do not affect *COX5a*. We find it more likely, therefore, that AB35-13D and JM28 carry a cryptic regulatory mutation(s) that affects the expression of the *COX5* genes. Currently, we are in the process of performing complementation analyses on these mutants as well as on JM28 and AB35-13D in order to determine whether the phenotypes observed are the result of the same or different mutations. We are also disrupting one or both of the *COX5* genes in AB35-13D and JM28. These studies should facilitate

our understanding of both the expression and function of the yeast *COX5* genes.

DISCUSSION

In this paper we have presented evidence that oxygen, through heme, exerts an inverse effect on the expression of the yeast *COX5* genes. In addition, we have shown that the *ROX1* gene product may also be involved in this inverse response. The pattern of expression exhibited by *COX5a* was similar to that seen for *CYC1* (7, 12). It also corresponds to what might be predicted for a gene encoding a respiratory protein; that is, efficient expression in the presence of oxygen, and little or no expression in its absence.

On the other hand, *COX5b* expression seems, at first glance, paradoxical. It is not immediately clear why a gene whose product is involved in respiration would be expressed maximally when the cells cannot respire and when there appears to be no functional cytochrome *c* oxidase (18). In our view, the effect of anaerobiosis and heme limitation on *COX5b* expression is an important one. By understanding the functional significance of this key regulatory response, we hope to gain important insights into the biological role of *COX5b* and into the overall biology of the cytochrome *c* oxidase complex. We believe that the induction (or derepression) of *COX5b* has probably evolved to occur under conditions of reduced or low oxygen tension and not strict anaerobiosis. The corresponding synthesis of subunit Vb might then serve to change the polypeptide composition of the holoenzyme. A cytochrome *c* oxidase complex containing subunit Vb rather than subunit Va might then be expected to be enzymatically better suited to conditions of low oxygen tension, for example, by having a lower k_m for oxygen.

The identification of *COX5b* as a gene whose expression increases anaerobically is also important in another regard. While *ANB1*, a gene of unknown function, was previously the sole known anaerobic gene, it is now becoming clear that anaerobic genes represent an interesting and clearly important family of yeast genes. In addition to *COX5b*, it was recently reported that the expression of the yeast *HEM13* gene also increased anaerobically (24, 25). This finding has direct bearing on our results, since the *HEM13* product, coproporphyrinogen oxidase (the enzyme that catalyzes the sixth step in the heme-biosynthetic pathway), also requires oxygen for enzymatic activity (13, 24, 25). Furthermore, we have recently learned that there is yet another example of a physiologically important anaerobic gene. *HMG2*, one of two yeast genes encoding 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme catalyzing the rate-limiting step in sterol biosynthesis, is also induced severalfold anaerobically (1, 13; J. Rine, personal communication).

We suggest that the anaerobic expression of *HEM13* and *HMG2*, as we have suggested for *COX5b*, represents an important cellular response to changing environmental conditions, specifically, conditions of reduced oxygen tension. It is intriguing that the *COX5b* and *HEM13* products are directly involved in oxygen-dependent biochemical reactions and that the biosynthesis of sterols in yeast cells is also oxygen dependent (13). It is tempting to speculate that the link between the regulation of these genes and the oxygen dependence of their respective functions is not coincidental. Certainly more than speculation is the distinct possibility that in yeast cells heme is used as an internal barometer of oxygen tension and, through its interaction with certain regulatory proteins like *ROX1*, as a means to regulate gene expression.

We are actively involved in identifying and studying the regulatory proteins that control the expression of the *COX5* genes. We are particularly interested in determining whether the same or a different protein(s) brings about the inverse regulation of the *COX5* genes and how many different *trans*-acting proteins are involved in *COX5* expression. Although all the factors that control the expression of the *COX5* genes have not yet been identified, the available data suggest that there are specific as well as shared elements involved in expressing both genes. From the data presented in this study, it appears that *ROX1*, possibly in conjunction with other proteins and the presence or absence of heme, can inversely affect both *COX5a* and *COX5b*. However, certain of our regulatory mutations appear to affect only the expression of *COX5b*. It should be mentioned that the product of another yeast gene, *HAP1*, has also been implicated in the heme-mediated control of *CYC1* (7, 16, 17). At present, workers in our laboratory and that of R. O. Poyton of the University of Colorado (personal communication) are studying the effect of *HAP1* on *COX5* expression.

Finally, it should be mentioned that preliminary results from our laboratory for a set of mutants that overexpress *COX5b* and for a *cis*-acting regulatory element upstream of the *COX5b* gene suggest that the control of *COX5b* transcription is largely negative (M. G. Cumsky, G. Kim, and M. R. Hodge, in preparation; M. R. Hodge and M. G. Cumsky, in preparation). That is, the expression of this gene is repressed in the presence of oxygen and derepressed in its absence. Whether activation elements are present upstream of *COX5b* is, at present, uncertain. Because *COX5b* is normally expressed at relatively low levels endogenously, it is not inconceivable that expression is driven from only a weak basal-type element. Future work will hopefully define more clearly the *cis*- as well as *trans*-acting elements that control transcription of *COX5b*. Our ultimate goal is to understand in detail how both of these genes are regulated and, in turn, how subunits Va and Vb individually affect the properties of holocytochrome *c* oxidase.

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