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Differential Effectiveness of Yeast Cytochrome *c* Oxidase Subunit V Genes Results from Differences in Expression Not Function

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In *Saccharomyces cerevisiae*, *COX5a* and *COX5b* encode two distinct forms of cytochrome *c* oxidase subunit V, V_a and V_b, respectively. To determine the relative contribution of *COX5a* and *COX5b* to cytochrome *c* oxidase function, we have disrupted each gene. Cytochrome *c* oxidase activity levels and respiration rates of strains carrying null alleles of *COX5a* or *COX5b* or both indicate that some form of subunit V is required for cytochrome *c* oxidase function and that *COX5a* is much more effective than *COX5b* in providing this function. Wild-type respiration is supported by a single copy of either *COX5a* or *COX5ab* (a constructed chimeric gene sharing 5′ sequences with *COX5a*). In contrast, multiple copies of *COX5b* or *COX5ba* (a chimeric gene with 5′ sequences from *COX5b*) are required to support wild-type respiration. These results suggest that the decreased effectiveness of *COX5b* is due to inefficiency in gene expression rather than to any deficiency in the gene product, V_b. This conclusion is supported by two observations: (i) a *COX5a-lacZ* fusion gene produces more β-galactosidase than a *COX5b-lacZ* fusion gene, and (ii) the *COX5a* transcript is significantly more abundant than the *COX5b* transcript or the *COXsba* transcript. We conclude that *COX5a* is expressed more efficiently than *COX5b* and that, although mature subunits V_a and V_b are only 67% homologous, they do not differ significantly in their ability to assemble and function as subunits of the holoenzyme.

In all eucaryotes, cytochrome *c* oxidase is a complex heterooligomer composed of subunit polypeptides encoded by both nuclear and mitochondrial genomes. In *Saccharomyces cerevisiae*, mutations in each of the three mitochondrial structural genes (*COX1*, *COX2*, and *COX3*) and in five of the six nuclear structural genes (*COX4*, *COX5a*, *COX6*, *COX8*, and *COX9*) have been shown to decrease or abolish cytochrome *c* oxidase activity (9, 14, 31, 37, 41; R. M. Wright, unpublished observations). It is generally accepted that the subunits encoded in the mitochondrion perform the catalytic functions of the holoenzyme (40), and it is assumed that the subunits encoded in the nucleus serve to modulate holoenzyme activity, either by regulating catalysis (18, 19) or affecting assembly (13). Recent studies have suggested that identical mitochondrially encoded subunits are present in cytochrome *c* oxidase from different animal tissues, but that tissue-specific isologs exist for several of the nucleus-encoded subunits (20). In addition, isologs exist for at least one of the nucleus-coded subunits of cytochrome *c* oxidase in the lower eucaryotes *S. cerevisiae* (8) and *Dictyostelium discoideum* (3). From analysis of the genes for the two subunit V isologs in yeast cells (8, 10) and from partial NH_2-terminal amino acid sequencing studies of the tissue-specific isologs in porcine and bovine tissues (21), it is clear that these isologs, like iso-1 and iso-2 cytochrome *c* in yeast cells (27), are derived from multigene families. Are the cytochrome *c* oxidase subunit isologs functionally different? Are their genes expressed differentially in response to developmental or environmental signals?

To address these questions, we have chosen to study the subunit V isologs of yeast cytochrome *c* oxidase. In the accompanying paper (10) we show that the polypeptides V_a and V_b are 67% homologous and are encoded by the single-copy genes *COX5a* and *COX5b*, respectively. As a first step in analyzing the function and expression of these polypeptides, we previously analyzed cytochrome *c* oxidase activity and respiration levels in a respiration-deficient strain, JM28, that carries a mutation in the *COX5a* gene (8, 25). This earlier study demonstrated that either *COX5a* or *COX5b* could restore cytochrome *c* oxidase activity to JM28, when expressed from high-copy-number plasmids. However, we could not assess the relative effectiveness of the two *COX5* genes by using JM28, because of the possibility that V_m, a fast-migrating mutant form of V_a that is present in JM28, interferes with the ability of V_a and V_b to assemble or function in holocytochrome *c* oxidase. In this paper, we construct mutants carrying null alleles of *COX5a* or *COX5b* or both and observe that a single copy of *COX5a* is much more effective in providing the required subunit V function than a single copy of *COX5b*. In addition, we show that the differential effectiveness of *COX5a* and *COX5b* is due to different levels of expression of the *COX5* genes and not to differences in properties of the subunit V isologs V_a and V_b.

MATERIALS AND METHODS

Plasmids. The properties of plasmids used in this study are summarized in Table 1. Plasmids YEp13-552 and YEp13-511 have been described previously (8): YEp13-552 has a 6.0-kilobase (kb) insert carrying *COX5a*, and YEp13-511 has a 4.5-kb insert carrying *COX5b*. Plasmid YCp5a was constructed by inserting the *COX5a* gene, on a 3-kb *Eco*RI fragment from YEp13-552, into the *Eco*RI site of the centromere-containing plasmid YCp19 (38). Plasmid YCp5b was constructed by inserting the *COX5b* gene, on a 3.1-kb *Bam*HI-*Bgl*II fragment from YEp13-511, into the *Bam*HI site of the centromere-containing plasmid pTC3 (from A. Brake). pTC3 has a 2-kb *CEN3* fragment inserted at the *Pvu*II site of YRp7 (39).

Plasmid YRp5ab was constructed by inserting the 3.7-kb *Bam*HI-*Cla*I fragment of the chimeric gene *COX5ab* (see

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TABLE 1. Characteristics of plasmids[a]

Plasmid	Genotype	Copy no.	Parent vector
YEp13-552	*COX5a LEU2*, 2μm origin of replication	High	YEp13
YEp13-511	*COX5b LEU2*, 2μm origin of replication	High	YEp13
YCp5a	*COX5a URA3 TRP1 ARS1 CEN4*	Low	YCp19
YCp5b	*COX5b TRP1 ARS1 CEN3*	Low	pTC3
YCp5ab	*COX5ab URA3 ARS1 CEN4*	Low	YCp50
YCp5ba	*COX5ba URA3 TRP1 ARS1 CEN4*	Low	YCp19
YRp5ab	*COX5ab TRP1 ARS1*	High	YRp7
YEp5ba	*COX5ba LEU2* 2μm origin of replication	High	YEp13
pCT5aL	*COX5a-lacZ URA3* 2μm origin of replication	High	pSEY101
pMC5bL	*COX5b-lacZ URA3* 2μm origin of replication	High	pSEY101

[a] All plasmids have pBR322 sequences carrying the ColE1 origin of replication and the β-lactamase gene, which confers ampicillin resistance.

below) between the *Bam*HI and *Cla*I sites in the multiple-copy plasmid YRp7 (39). The same *Bam*HI-*Cla*I fragment of *COX5ab* was inserted between the *Bam*HI and *Cla*I sites of the centromere-containing plasmid YCp50 (30) to yield the low-copy-number plasmid YCp5ab. Plasmid YEp5ba was constructed by inserting the 3.2-kb *Bam*HI-*Hin*dIII fragment of the chimeric gene *COX5ba* (see below) between the *Bam*HI and *Hin*dIII sites of the high-copy-number plasmid YEp13 (5). The same *Bam*HI-*Hin*dIII fragment of *COX5ba* was inserted between the *Bam*HI and *Hin*dIII sites of the centromere-containing plasmid YCp19 (38) to yield the low-copy-number plasmid YCp5ba.

Plasmid pCT5aL was constructed by inserting the 1.2-kb 5′ *Xho*II fragment of *COX5a* (the same fragment used to construct *COX5ab*; see below) into the *Bam*HI site of pSEY101 (12), a plasmid that carries the yeast *URA3* gene, 2μm origin of replication, ColE1 origin, Amp^r, and the *lacZ* gene. The *COX5a-lacZ* fusion gene carried by pCT5aL has the 5′-flanking sequence and coding sequence (through the codon for amino acid 13 of mature V_a) from *COX5a* fused to the coding sequence for amino acids 8 through 1024 of β-galactosidase. Plasmid pMC5bL was constructed by inserting the 1.9-kb *Bam*HI-*Bgl*II fragment of *COX5b* into the *Bam*HI site of pSEY101. The *COX5b-lacZ* fusion gene carried by pMC5bL has the 5′-flanking sequence and coding sequence (through the codon for amino acid 13 of V_b) from *COX5b* fused to *lacZ* codons 8 through 1024.

Yeast strains. Genotypes of the *S. cerevisiae* strains are listed in Table 2. JM43 (*MATα leu2-3 leu2-112 his4-580 ura3-52 trp1-289*) was constructed as described previously (8). JM8 (*MATα ade1* [*rho*^{0}]) was constructed by J. E. McEwen (University of California, Los Angeles). GD5a, GD5b, and GD5ab are derivatives of JM43 that have chromosomal gene disruptions of *COX5a*, *COX5b*, or both *COX5a* and *COX5b* genes, respectively, as described below. Strains derived from GD5ab by transformation with low- or high-copy-number plasmids carrying *COX5a*, *COX5b*, *COX5ab*, or *COX5ba* are designated by LC (low copy number) or HC (high copy number) followed by 5a, 5b, 5ab, or 5ba, respectively, to indicate which *COX5* gene is carried on the plasmid. Strain SC5ab carries a single copy of *COX5ab* integrated at the *COX5a* chromosomal locus. This strain resulted from spontaneous integration of plasmid YRp5ab in strain HC5ab. Southern blot (hybridization) analysis indicated that integration occurred by recombination between pBR322 sequences on the YRp5ab plasmid, and pBR322 sequences flanking *URA3* in the *cox5aΔ::URA3* gene, resulting in integration of a single copy of YRp5ab near the *COX5a* locus. JM43-5aL and JM43-5bL are JM43 strains transformed with high-copy-number plasmids pCT5aL and pMC5bL, respectively.

Construction of *COX5a* and *COX5b* gene disruptions. Null alleles of *COX5a* and *COX5b*, *cox5aΔ::URA3* and *cox5b::LEU2*, respectively, were constructed (Fig. 1). To disrupt the chromosomal *COX5b* gene, we transformed strain JM43 with a plasmid carrying *cox5b::LEU2* that had been digested with *Bam*HI and partially digested with *Cla*I. Leu$^+$ transformants were selected. Genomic Southern blot analysis confirmed that the linear 5.4-kb *Bam*HI-*Cla*I fragment carrying *cox5b::LEU2* is integrated at the *COX5b* locus in strain GD5b (data not shown). To replace the chromosomal *COX5a* gene with *cox5aΔ::URA3*, strains JM43 and GD5b (*cox5b::LEU2*) were transformed with a plasmid carrying *cox5aΔ::URA3* that had been digested with *Xho*I. Ura$^+$ transformants were selected. Integration of the linear 5-kb *Xho*I fragment carrying *cox5aΔ::URA3* at the *COX5a* locus was confirmed by genomic Southern blot analysis of GD5a, a Ura$^+$ transformant of JM43, and GD5ab, a Ura$^+$ transformant of GD5b (data not shown).

Construction of chimeric *COX5* genes. The chimeric *COX5* genes *COX5ab* and *COX5ba* (see Fig. 3) were constructed by taking advantage of a *Xho*II (Pu/GATCPy) restriction site homology in *COX5a* and *COX5b*. The *Xho*II site occurs in both *COX5* genes at the codon for amino acid 13 of the mature subunit V isologs. In *COX5b*, the *Xho*II site is also a *Bgl*II site (A/GATCT). *COX5ab* was created by ligating a 1.2-kb *Xho*II fragment of *COX5a* (5′ to the internal *Xho*II site) into the *COX5b Bgl*II site. *COX5ab* has more than 1 kb of 5′-flanking sequence from *COX5a* and 250 base pairs (bp) of 3′-flanking sequence from *COX5b*. It codes for subunit Vab, which has the 20-amino-acid V_a leader peptide and 12 amino acids of mature subunit V_a fused to amino acids 13 through 134 of mature V_b. The reciprocal chimeric gene, *COX5ba*, was created by ligating a 1.3-kb *Xho*II fragment of *COX5a* (3′ to the internal *Xho*II site) into the *COX5b Bgl*II site. *COX5ba* has more than 1.5 kb of 5′-flanking sequence from *COX5b* and about 1 kb of 3′-flanking sequence from *COX5a*. It codes for subunit Vba, which has the 17-amino-acid V_b leader peptide and 12 amino acids of mature subunit V_b fused to amino acids 13 through 133 of mature subunit V_a.

Miscellaneous methods. Growth media (YPD, SD, YPGE, YP) for yeast strains have been described previously (10, 16). Plasmid DNA was propagated in *Escherichia coli* HB101 (4). Transformation of *E. coli*, preparation of DNA restriction endonuclease digests, and other techniques were performed by standard procedures (23, 29). Yeast transformation was performed using lithium acetate (17). Cytochrome *c* oxidase activity, cytochrome aa_3 content, and cyanide-sensitive respiration were measured as described in the footnotes to Table 3. β-Galactosidase activity was measured and calculated as described by Miller (26a), except that yeast cells were used in place of *E. coli* cells.

RNA blot analysis. Total RNA was isolated from exponential-phase yeast cultures grown aerobically in YPD (strain JM43) or SD-histidine (strain LC5ba) medium by the method of Elder et al. (14). The poly(A)$^+$ RNA was enriched by passing total RNA over an oligo(dT)-cellulose column (Collaborative Research, Inc., Waltham, Mass.) once. Poly(A)$^+$ RNA (10 or 20 μg per lane) was separated electrophoreti-

TABLE 2. *S. cerevisiae* strains used

Strain[a]	Genotype	Plasmid
JM43	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 COX5b COX5a*	
GD5b	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 COX5a*	
GD5a	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 COX5b cox5a::URA3*	
GD5ab	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	
LC5b	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YCp5b
LC5a	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YCp5a
LC5ab	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YCp5ab
LC5ba	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YCp5ba
HC5b	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YEp13-511
HC5a	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YEp13-552
HC5ab	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YRp5ab
HC5ba	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*	YRp5ba
SC5ab	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 cox5b::LEU2 cox5aΔ::URA3*, plasmid YRp5ab integrated at *cox5a::URA3* locus	
JM43-5aL	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 COX5b COX5a*	pCT5aL
JM43-5aL	*MATα his4-580 trp1-289 leu2-3,112 ura3-52 COX5b COX5a*	pMC5bL
JM8	*MATα ade1 [rho°]*	

[a] All strains except JM8 are derived from JM43.

cally on a 1.4% agarose gel containing formaldehyde and blotted to nitrocellulose as described previously (23). To generate a probe that hybridizes equivalently to *COX5a* and *COX5ba* mRNA, we isolated a 642-bp *Xho*II-*Bst*NI fragment of *COX5a*; the probe extends from the *Xho*II fusion junction of *COX5ba* toward the 3′ end of the *COX5a* and *COX5ba* mRNAs. The *Xho*II-*Bst*NI fragment was alkali denatured and then radiolabeled by using a random hexamer primer (Pharmacia Fine Chemicals, Piscataway, N.J.) and the Klenow fragment of *E. coli* DNA polymerase in the presence of [α-^{32}P]dATP as described previously (15). A *COX5b* probe was prepared, by the same method, from a 593-bp *Bgl*II-*Cla*I fragment of *COX5b*. The radiolabeled probes were centrifuged through a G-25 column to remove unincorporated [α-^{32}P]dATP. Hybridization (42°C, 20 h, 2 × 10^7 cpm of probe) and stringent washes (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate, 50°C) were performed as described previously (42). A yeast actin gene probe was hybridized to an identical blot to monitor the relative amounts of poly(A)$^+$ RNA loaded; we estimate that 2.3 times more JM43 RNA was loaded than LC5ba RNA. The relative specific radioactivity of the *COX5a* and *COX5b* probes was determined by hybridizing the probes to a genomic Southern blot; we estimate that specific radioactivity of the *COX5a* probe was 1.3 times that of the *COX5b* probe. The amount of *COX5a* and *COX5b* probe hybridized to specific transcripts or to

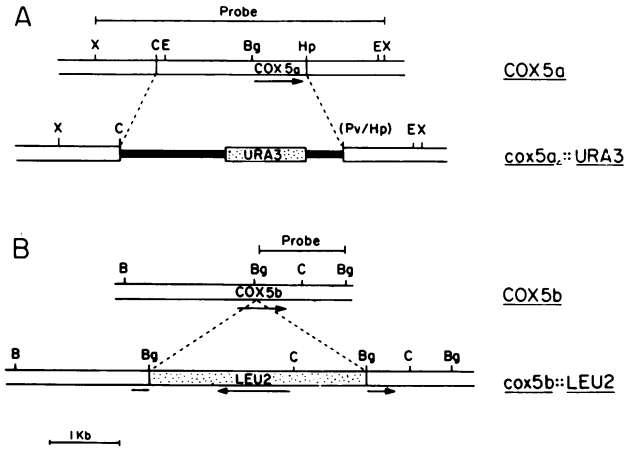

FIG. 1. Construction of *COX5a* and *COX5b* gene disruptions. (A) Replacement of *COX5a* with *URA3*. The 2.1-kb *Cla*I-*Hpa*I fragment of *COX5a* was deleted and replaced by the 3.1-kb *Cla*I-*Pvu*II fragment of YIp5 (38), which carries the *URA3* gene flanked by pBR322 sequences. The gene configuration, which completely lacks the *COX5a* coding sequence, is called *cox5aΔ::URA3*. (B) Disruption of *COX5b* with *LEU2*. As described previously (8), the 2.9-kb *Bgl*II fragment of YEp13 (5), which carries the *LEU2* gene, was cloned into the *Bgl*II site within the *COX5b* gene. The *LEU2* fragment interrupts the *COX5b* coding sequence at the codon for amino acid 13 of mature subunit V_b, hence inactivating *COX5b*. The gene configuration is designated *cox5b::LEU2*. Restriction enzyme recognition sites: X, *Xho*I; C, *Cla*I; E, *Eco*RI; Bg, *Bgl*II; Hp, *Hpa*I; Pv, *Pvu*II; B, *Bam*HI. Arrows indicate direction of transcription.

TABLE 3. A single copy of *COX5a* is more effective than a single copy of *COX5b* in supporting cytochrome *c* oxidase activity and respiration

Strain	Functional *COX5* genes	Cytochrome aa_3[a]	Cytochrome *c* oxidase sp act[b]	Respiration rate[c]
JM43	*COX5a, COX5b*	100	100	100
GD5b	*COX5a*	86	96	97
GD5a	*COX5b*	5	6	10
GD5ab	None	0	0.5	0.7

[a] The level of cytochrome aa_3 was calculated from the molar ratio of cytochrome *c* to cytochrome aa_3. Both were determined from room temperature difference spectra of whole cells (6), using extinction coefficients of 10.4 mM^{-1} cm^{-1} (Δ603 nm; reduced minus oxidized) for cytochrome aa_3 and 24.6 mM^{-1} cm^{-1} (Δ550 to 540 nm; reduced minus oxidized) for cytochrome *c*. The value corresponding to 100% for the molar ratio of *c* to aa_3 was 3.14 in JM43.

[b] Calculated as *K*, the first-order velocity constant (micromoles of cytochrome *c* oxidized per minute per milligram of mitochondrial protein) (24, 26). For JM43, K is equal to 45. Assays were performed on mitochondria isolated from cultures grown on semisynthetic galactose medium.

[c] Respiration was measured as O_2 consumption at 30°C by using a Yellow Springs Instruments model 53 oxygen monitor. For each measurement, the rate of O_2 consumption observed in the presence of 1 mM potassium cyanide was subtracted from the rate of O_2 consumption in the absence of potassium cyanide. For each strain, three to five measurements were performed on each of two cultures grown on semisynthetic galactose medium. The respiration rates are normalized to that of JM43; the value corresponding to 100% in JM43 was 79 pmol of O_2 consumed per min per μg of dry weight.

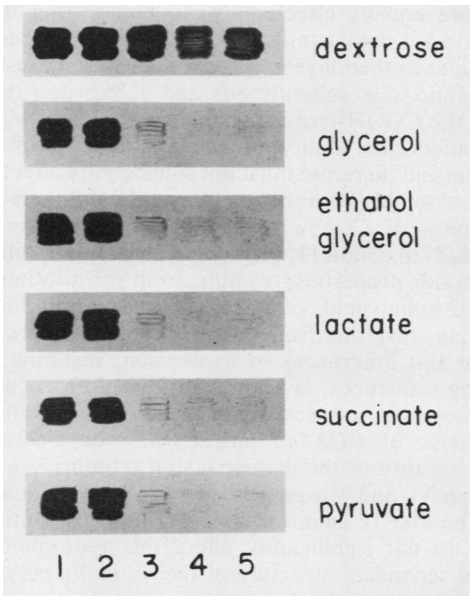

FIG. 2. Cell growth on nonfermentable carbon sources. Strains JM43, GD5b, GD5a, and GD5ab and [rho^0] strain JM8 (positions 1 through 5, respectively) were transferred from a patch of cells growing on YPD plates to solid YP medium containing the indicated carbon sources and incubated at 28°C for 3 days. All strains grow well on the fermentable carbon source dextrose. On each nonfermentable carbon source, JM43 and GD5b grow well, whereas GD5a grows only marginally. GD5ab and [rho^0] strain JM8 grow extremely poorly on nonfermentable carbon sources; the slight amount of growth detected is probably due to contamination of the media with fermentable carbon sources or to growth sustained by endogenous fermentable substrates.

DNA restriction fragment was determined by scintillation counting.

RESULTS

***COX5a* supports a higher level of cytochrome *c* oxidase activity and respiration than *COX5b*.** To study the roles of *COX5a* and *COX5b* in supporting cytochrome *c* oxidase activity, null alleles of the chromosomal copies of *COX5a* and *COX5b* were constructed in the respiration-proficient strain JM43 by the one-step gene disruption method of Rothstein (33) (Fig. 1). Strain GD5ab, with null alleles of both *COX5a* and *COX5b*, has essentially no cytochrome *c* oxidase activity or cyanide-sensitive respiration (Table 3). These results demonstrate that a form of subunit V is required for the development of cytochrome *c* oxidase activity in vivo. Spectral analysis confirms that the loss of cyanide-sensitive respiration in GD5ab is directly due to the absence of functional holocytochrome *c* oxidase molecules, since the cytochrome *c* oxidase-specific cytochrome, aa_3, is absent (Table 3). The relative contributions of *COX5a* and *COX5b* to cytochrome *c* oxidase activity can be estimated from the cytochrome *c* oxidase levels and respiration rates of strains in which only one of the two *COX5* genes has been disrupted. Strain GD5b (*COX5a*$^+$ *cox5b*::*LEU2*) exhibits wild-type cytochrome *c* oxidase activity and respiration rate, whereas strain GD5a (*cox5a*Δ::*URA3* *COX5b*$^+$) exhibits only 6% of the wild-type cytochrome *c* oxidase activity and 10% of the wild-type cyanide-sensitive respiration rate (Table 3). The levels of cytochromes aa_3 in GD5a and GD5b change in parallel with these levels of cytochrome *c* oxidase activity and respiration (Table 3), indicating that the rate of respiration in these strains is determined by their levels of functional holocytochrome *c* oxidase. Together, these results indicate that a single copy of *COX5a* is sufficient to support a wild-type level of cytochrome *c* oxidase activity and respiration, but that a single copy of *COX5b* is not.

TABLE 4. Relative effectivness of *COX5a*, *COX5b*, *COX5ab*, and *COX5ba* in supporting respiration

Strain	Functional *COX5* gene	Gene copy no.	Cyanide-sensitive respiration rate (%)[a]	
			YPGE	SD
HC5b	*COX5b*	High (2μm)	85	
HC5a	*COX5a*	High (2μm)	86	
HC5ba	*COX5ba*	High (2μm)	86	
HC5ab	*COX5ab*	High (2μm)	107	
SC5ab	*COX5ab*	Single (chromosome)		87
LC5ab	*COX5ab*	Low (CEN plasmid)	117	
LC5a	*COX5a*	Low (CEN plasmid)	89	85
LC5ba	*COX5ba*	Low (CEN plasmid)		15
LC5b	*COX5b*	Low (CEN plasmid)		11

[a] Cyanide-sensitive respiration rates (determined as described in footnote *c* of Table 3) are presented as a percentage of the cyanide-sensitive respiration rate of strain JM43 or GD5b grown in the same medium. HC5b, HC5a, HC5ba, HC5ab, LC5a, and LC5ab were grown in YPGE medium and normalized to strain JM43 (130 pmol of O_2 consumed per min per μg of dry weight) grown on YPGE. SC5ab, LC5ba, LC5a, and LC5b were grown in SD medium and normalized to strain GD5b (34 pmol of O_2 consumed per min per μg) grown on SD medium.

An analysis of growth on the nonfermentable carbon source ethanol, glycerol, lactate, succinate, or pyruvate (Fig. 2) demonstrates that a single copy of *COX5a* alone (strain GD5b) supports a level of growth indistinguishable from that of parent strain JM43, which carries both *COX5a* and *COX5b*. In contrast, a single copy of *COX5b* (strain GD5a) supports only very marginal growth on nonfermentable carbon sources, indicating that the low level of respiration (10% of the wild-type rate) conferred by a single *COX5b* gene is not sufficient to sustain growth on nonfermentable carbon sources.

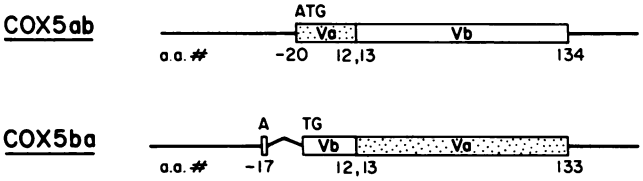

FIG. 3. Chimeric *COX5* genes. The chimeric genes *COX5ab* and *COX5ba* were constructed by joining *COX5a* and *COX5b* at a common restriction site at codon 13 of mature subunits V_b and V_a, respectively (see Materials and Methods). The chimeric gene *COX5ab* has *COX5a* sequences upstream of codon 13 and *COX5b* sequences downstream of codon 13, whereas the chimeric gene *COX5ba* has *COX5b* sequences upstream of codon 13 and *COX5a* sequences downstream of codon 13. More than 1 kb of 5′-flanking sequence from *COX5a* and *COX5b* are present upstream of *COX5ab* and *COX5ba*, respectively. The chimeric proteins V_{ab} and V_{ba}, encoded by *COX5ab* and *COX5ba*, respectively, have an fusion junction at amino acids 12 and 13 of mature subunit V. The ATG encoding the initiation codon is indicated in each case. In *COX5b*, the AUG is interrupted by an 88-bp intron (^).

We previously reported that in the *COX5a* mutant, JM28, introduction of a high-copy-number plasmid carrying *COX5b* increased respiration from 12 to 69% of the wild-type level and allowed growth on nonfermentable carbon sources (8). Respiration rates of strains HC5b and HC5a, GD5ab transformants carrying high-copy-number plasmids of *COX5b* and *COX5a*, respectively, are 85% of the wild-type respiration rate (Table 4). Both strains also grow as well as JM43 on nonfermentable carbon sources (data not shown). These observations suggest that respiration and growth defects in strains carrying only a single copy of *COX5b* are due to a deficiency in the amount of V_b assembled in cytochrome *c* oxidase, rather than to a functional inadequacy of the holoenzyme when V_b is assembled.

Which sequences are responsible for the decrease in effectiveness of *COX5b* relative to *COX5a*? In principle, the differences in the effectiveness of *COX5a* and *COX5b* in supporting cytochrome *c* oxidase activity could result from (i) differences in the properties (e.g., stability or assembly) of the polypeptides V_a and V_b and/or from (ii) differences in their level of expression. To investigate both of these possibilities, the chimeric *COX5* genes (*COX5ab* and *COX5ba*) were constructed by joining *COX5a* and *COX5b* through a common *Xho*II restriction site at codon 13 of each mature subunit V isolog (Fig. 3; see Materials and Methods). *COX5ab* has *COX5a* sequences 5′ to codon 13 and *COX5b* sequences 3′ to codon 13, whereas *COX5ba* has *COX5b* sequences 5′ to codon 13 and *COX5a* sequences 3′ to codon 13. On high-copy-number plasmids, both *COX5ab* and *COX5ba* support wild-type respiration rates (strains HC5ab and HC5ba, Table 4) indicating that both chimeric proteins, V_{ab} and V_{ba}, are capable of providing the subunit V function. The presence of the polypeptides V_{ab} and V_{ba} in these strains, as well as in strains carrying low-copy-number plasmids with the chimeric genes, has been confirmed by immunoblot analysis (data not shown).

To test the effectiveness of the *COX5ab* gene, we introduced it into GD5ab (*cox5aΔ::URA3 cox5b::LEU2*) in two ways: (i) on CEN plasmid YCp5ab and (ii) by integration into the chromosome in single copy (see Materials and Methods). The resulting strains, LC5ab and SC5ab, both grow well on nonfermentable carbon sources (data not shown) and respire at wild-type rates (Table 3). These respiration and growth phenotypes are similar to those exhibited by strains carrying low copy numbers or a single copy of *COX5a* (LC5a and GD5b) and are much higher than the 11 and 10% respiratory rates exhibited by strains carrying low copy numbers or a single copy of *COX5b* (LC5b and GD5a) (Tables 3 and 4). Thus, *COX5ab* is as effective as *COX5a*, the gene with which it shares 5′ sequences, and is much more effective than *COX5b*, the gene with which it shares 3′ sequences. Just as *COX5a* and *COX5ab* share 5′ sequences and are equally effective, *COX5b* and *COX5ba* share 5′ sequences and are equally ineffective in supporting respiration. On centromere plasmids, *COX5ba* and *COX5b* support respiration rates of approximately 15 and 11%, respectively, of the wild-type rate, and on high-copy-number plasmids both genes support 85% of the wild-type respiration rate (Table 4). In contrast, *COX5a*, the gene with which *COX5ba* shares 3′ sequences, supports 85% of the wild-type respiration rate on either a centromere plasmid or a high-copy-number plasmid.

By a comparison of the effectiveness of *COX5a*, *COX5b*, *COX5ab*, and *COX5ba*, it is clear that the ability of these genes to support respiration is determined by sequences 5′ to codon 13 of *COX5a* and *COX5b* and not by sequences 3′ to codon 13. Since sequences 3′ to codon 13 of *COX5a* and *COX5b* are equally effective, we conclude that these sequences do not significantly affect either the properties of the polypeptides or their level of production. It is interesting that the 38 amino acid substitutions and 1 insertion that exist between the COOH-terminal 120 amino acids of V_a and V_b do not affect the ability of each subunit V to support respiration and therefore must not significantly affect subunit stability, assembly, or function. Since the differences in the effectiveness of *COX5a* and *COX5b* are determined by sequences 5′ to codon 13, they could be due to (i) differences in polypeptide properties, resulting from substitutions within the first 12 amino acids of mature V_a and V_b; (ii) differences in targeting, resulting from leader peptide sequence divergence; or (iii) differences in expression, resulting from 5′ noncoding sequences. It is unlikely that differences in stability, assembly, or function are responsible for differential effectiveness of *COX5ab* and *COX5b* or *COX5ba* and *COX5a*, because (i) the mature fusion proteins V_{ab} and V_{ba} differ from V_b and V_a, respectively, by only 6 amino acids (within the first 12 amino acids), and (ii) these amino acids changes do not significantly affect the hydrophobicity or predicted secondary structure of the V_a or V_b polypeptides (10). Moreover, if V_b were less competent than V_a to function as a subunit of the holoenzyme, an increase in the copy number of its gene would not be expected to restore the rate of respiration to a wild-type level, as it does in strain HC5b (Table 4). Differences that result from targeting are also unlikely because a chimeric gene, in which the V_a leader peptide coding sequence alone has been replaced by the V_b leader peptide coding sequence, supports a wild-type respiration rate when expressed from a low-copy-number (CEN) plasmid (S. M. Glaser et al., J. Cell. Biochem., in press). Thus, the leader peptide from *COX5b* does not, by itself, diminish the effectiveness of *COX5a*. From the results of this chimeric *COX5* gene experiment, we deduce that the differential effectiveness of *COX5a* and *COX5b* does not result from differences in targeting, stability, assembly, or function of the V_a and V_b polypeptides.

Differential expression can account for differences in effectiveness of *COX5a* and *COX5b*. The chimeric *COX5* gene experiment strongly suggests that the differential effectiveness of the two *COX5* genes is due to different levels of expression that result from differences in the 5′ noncoding regions. To more directly investigate expression levels and to specifically determine whether transcript abundance differs from the *COX5* genes, we performed RNA blots that allowed a comparison of the levels of *COX5a*, *COX5ba*, and *COX5b* transcripts (Fig. 4). The *COX5a* transcript in strain JM43 (lane 1) is clearly more abundant than the *COX5ba* transcript in strain LC5ba (lane 2); after correcting for the twofold difference in amounts of poly(A)$^+$ RNA loaded, we estimate a difference in transcript abundance of roughly sevenfold. In JM43, the *COX5b* transcripts (lane 3) are substantially less abundant than the *COX5a* transcripts (lane 1); after correcting for specific activity differences in the two radiolabeled probes, we estimate a sixfold difference in transcript abundance. From these results, we conclude that the low abundance of the *COX5b* and *COX5ba* transcripts can account, to a large degree, for the decreased effectiveness of these genes. Additional evidence for differential expression of *COX5a* and *COX5b* comes from measurements of β-galactosidase activity in yeast strains JM43-5aL and JM43-5bL, which carry *COX5a-lacZ* and *COX5b-lacZ* fusion genes, respectively. The fusion genes contain precisely the same *COX5a* and *COX5b* sequences that were used to construct *COX5ab* and *COX5ba* (see Materials and

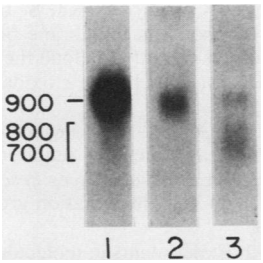

FIG. 4. *COX5a* transcripts are much more abundant than *COX5ba* or *COX5b* transcripts. Poly(A)$^+$ RNA, isolated from aerobically growing exponential cultures, was separated by electrophoresis on a 1.4% agarose gel containing formaldehyde. Lanes: 1 and 3, 20 μg of RNA from strain JM43; 2, 10 μg of RNA from strain LC5ba. After transfer to nitrocellulose, lanes 1 and 2 were hybridized to a radiolabeled *COX5a* probe that hybridizes equally well to *COX5a* and *COX5ba* RNA. Lane 3 was hybridized to a radiolabeled *COX5b* probe. After normalization for the amount of RNA loaded per lane (as determined by hybridization to an actin gene probe), we estimate that the *COX5a* mRNA is roughly 7 times more abundant than the *COX5b* mRNA. In lane 3, the disperse mRNAs in the 700- to 800-bp region are the *COX5b* transcripts. After correcting for the specific activity difference in the probes (1.3 times greater for *COX5a* than for *COX5b*), we estimate that the *COX5a* transcript is roughly 6 times more abundant than the *COX5b* transcripts in the JM43 poly(A)$^+$ RNA preparation (cf. lanes 1 and 3). The band at 900 bp is an unidentified transcript that is detected with this probe but not with the *COX5b* coding sequence-specific oligonucleotide used in the accompanying paper (10). Since this transcript is observed in strain GD5ab (data not shown), it is not a *COX5a* or *COX5b* transcript; it appears to originate downstream of the *COX5b* gene.

Methods). When grown aerobically to the midexponential phase in SD medium, JM43-5aL and JM43-5bL exhibit approximately 120 and 4 U of β-galactosidase activity, respectively. This substantial difference in expression has been observed with several independently derived pools of transformants.

DISCUSSION

In the accompanying paper (10), we found that the two *COX5* genes in *S. cerevisiae* encode proteins that are substantially different in primary sequence and have nonhomologous flanking sequences. In experiments described in this paper, we have analyzed the function and expression of these genes. Studies with strains carrying null mutations, strains carrying multiple copies of each gene, and strains carrying chimeric genes clearly demonstrate that both *COX5a* and *COX5b* encode proteins that can function as subunit V of cytochrome *c* oxidase. However, it is also clear that single copies of *COX5a* and *COX5b* differ markedly in their ability to support cytochrome *c* oxidase activity and yeast cell growth on nonfermentable carbon sources. These differences can be attributed to differences in the level of expression of *COX5a* and *COX5b* and not to differences in stability, targeting efficiency, assembly, or function of subunits V_a and V_b. Transcriptional efficiency or transcript stability is responsible, at least in part, for the differential expression of *COX5a* and *COX5b*. It is not yet clear whether transcript abundance is the only level at which expression differs. Sequence features in the 5′ end of the *COX5b* mRNA (10), which are also present in the *COX5ba* and the *COX5b-lacZ* mRNAs, suggest that differences in transcript processing and translation may also contribute to differential expression. The *COX5b* mRNA contains an 88-bp intron (between the A and U of the V_b initiation codon) that could be inefficiently removed due to an unusual 5′ splice junction: GCAUGU rather than the canonical GUAUGU. However, since the *COX5ba* transcript appears to be efficiently spliced (i.e., it migrates with the same mobility as the *COX5a* transcript; Fig. 4), we find it unlikely that inefficient splicing significantly affects *COX5b* expression. The 5′ end of the *COX5b* mRNA also contains a short open reading frame (AUG, GAA, UAA; 30 nucleotides upstream of the V_b initiation codon) that could interfere with or possibly regulate V_b translation, as has been found for *GCN4* (28). The role of the upstream AUG is under investigation.

Why are there two *COX5* genes? The *COX5* genes share similarities with the small multigene families in yeast that encode the cytochrome *c* isologs (22, 27), *ras* proteins (11, 32), α-tubulins (34, 35), alkaline phosphatases (2), α-factors (36), and the RP51 ribosomal proteins (1). In each case, two different genes encode proteins that differ in primary sequence, have similar or identical functions, and are expressed at different levels. It is not clear why cells carry two genes for these proteins. Selective advantages could include subtle functional differences in the protein products or increased capacity for regulation of gene expression. From the results described here, it is clear that either subunit V isolog, V_a or V_b, can provide an essential subunit V function in vivo. Yet, because the mature subunit V_a and V_b polypeptides share only 67% homology in primary sequence and predicted secondary structure (10), it is reasonable to suspect that they may confer different structural or catalytic properties on holoenzyme molecules into which they are assembled. It is quite possible that the in vivo assays for function described in this paper are inadequate to reveal subtle but important functional differences (e.g., in K_m or V_{max}) that may become manifest only when cells are grown under certain conditions. Clearly, biochemical and biophysical studies with holoenzyme molecules carrying each subunit V isolog are needed to determine whether sequence divergence between subunits V_a and V_b is functionally important. Studies on regulation of expression of *COX5a* and *COX5b*, particularly an investigation of environmental conditions and mutations that increase *COX5b* expression, may also help address the question of why yeast has two functional *COX5* genes.

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