Identification of *REO1*, a Gene Involved in Negative Regulation of *COX5b* and *ANB1* in Aerobically Grown *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae*, the *COX5a* and *COX5b* genes constitute a small gene family that encodes two forms of cytochrome *c* oxidase subunit V, Va and Vb, either of which can provide a function essential for cytochrome *c* oxidase activity and respiration. In aerobically grown wild-type yeast cells, Va is the predominant form of subunit V. The *COX5b* gene alone does not produce enough Vb to support a respiration rate sufficient to allow growth on nonfermentable carbon sources. By selecting for mutations that increase the respiratory capacity of a strain deleted for *COX5a*, we have identified a gene that is involved in negative regulation of *COX5b* expression under aerobic growth conditions. Each of four independently isolated *reo1* mutations are shown to be recessive, unlinked to *COX5b*, but dependent on *COX5b* for phenotypic expression. The mutations define a single complementation and linkage group: designated as *REO1* for regulator of expression of oxidase. *reo1* mutations increase expression of *COX5b* in aerobically grown cells, but not in anaerobically grown cells, where expression is already elevated. These mutations have no effect on *COX5a*, the other member of this small gene family which is positively regulated by heme and oxygen. The *REO1* gene does play a role in repression of *ANB1*, a gene that is normally repressed under aerobic but not anaerobic conditions. Neither *rox1* or *rox3* mutations, which have previously been shown to increase *ANB1* expression, are in the same complementation group as *reo1* mutations.

SUBUNIT V is the only cytochrome *c* oxidase subunit in *Saccharomyces cerevisiae* that is represented by alternative forms: the two subunit V isologs, Va and Vb, have 66% amino acid identity, yet either Va or Vb can provide an essential subunit V function to cytochrome *c* oxidase (CUMSKY *et al.*, 1987; TRUEBLOOD and POYTON 1987). In aerobically grown wild-type yeast, the *COX5a* gene produces enough Va to support wild-type rates of respiration and growth on nonfermentable carbon sources, whereas the *COX5b* gene produces so little Vb that yeast strains deleted for *COX5a* respire at only 10% the wild-type rate and do not grow significantly on nonfermentable carbon sources (TRUEBLOOD and POYTON 1987). Selective maintenance of two genes encoding alternative forms of cytochrome *c* oxidase subunit V could be due to differences in function between the Va and Vb polypeptides and/or due to differences in regulation of expression of *COX5a* and *COX5b*. We have observed that the *COX5* genes are oppositely regulated by heme (TRUEBLOOD, WRIGHT and POYTON 1988). In the presence of heme (that is, in aerobically grown heme prototrophs), *COX5a* is expressed at a much higher level than *COX5b*. In contrast, when heme is lacking (due to a heme auxotrophy or to anaerobic growth) (MATTOON *et al.* 1979), *COX5b* is expressed at least as well as, and probably better than, *COX5a*.

In this paper, we describe the isolation and characterization of mutations that increase *COX5b* expression in aerobically grown, heme-proficient cells. The mutations are recessive to wild type, are linked to one another and define a single complementation group; the gene identified by these mutations is designated *REO1* for regulator of expression of oxidase. The *REO* gene product is involved, directly or indirectly, in negative regulation of *COX5b* in aerobically grown, but not anaerobically grown, cells. It is also involved in negative regulation of *ANB1*, another gene that, like *COX5b*, is expressed at a higher level anaerobically than aerobically (LOWRY *et al.* 1983). In contrast, *REO1* is not involved in regulation of *COX5a*, the aerobically induced member of the *COX5* gene family.

MATERIALS AND METHODS

Strains, plasmids and growth conditions: Yeast strains (listed in Table 1) were grown in YPD, YPGE, YPL, SSG or SD. YP media contain 1% yeast extract, 2% peptone and 2% of one of the following carbon sources: D (dextrose), G (glycerol), E (ethanol) or L (lactate). SD medium contains 0.67% yeast extract without amino acids and 2% dextrose; required supplements are added at 40 μg/ml, essentially as described by SHERMAN, FINK and LAWRENCE (1979). Plasmids are listed in Table 2.

Transformation, DNA isolation and hybridization: *Escherichia coli* cells were transformed by a calcium chloride procedure (MANIATIS, FRITSCH and SAMBROOK 1982) and plasmids were selected on medium containing 50 μg/ml ampicillin. Plasmid DNA was isolated from 50–100 ml of

TABLE 1

Yeast strains

Strain[a]	Genotype
JM43	*MATα his4 trp1 leu2 ura3*
GD5a-4	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3*
GD5a-H$^+$	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3*
GD5a-101	*MATa his4 leu2 cox5a$_\Delta$::URA3*
GD5b-101	*MATa his4 leu2 ura3 cox5b::LEU2*
GD5ab-1	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 cox5b::LEU2*
CT1	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-1*
CT2	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-2*
CT3	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-3*
CT4	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-4*
CT11	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-1*
CT12	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-2*
CT13	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-3*
CT14	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-4*
CT1F*	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-1*
CT2F*	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-2*
CT3F*	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-3*
CT4F*	*MATα his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-4*
CT11F*	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-1*
CT12F*	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-2*
CT13F*	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-3*
CT14F*	*MATα trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-4*
CT39-9D	*MATa his4 leu2 cox5a$_\Delta$::URA3 reo1-3*
CT48-7D	*MATa his4 leu2 cox5a$_\Delta$::URA3 reo1-4*
DWS1-3B	*MATa his4 trp1 leu2 ura3 reo1-4*
CT40-8B*	*MATa his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-3*
CT40-3C*	*MATa trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-3*
CT43-10C	*MATa his4 leu2 cox5a$_\Delta$::URA3 reo1-1*
K399-7D	*MATa spo11 ura3 his2 leu1 lys1 met4 pet8*
K393-35C	*MATα spo11 ura3 his2 leu1 lys1 met4 pet8*
CT149-3A	*MATa his4 leu2 ura3 reo1-4*
CT149-3B	*MATα leu2 ura3*
CT39-10D	*MATa his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 cox5b::LEU2 reo1-3*
CT44-6B	*MATa his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 cox5b::LEU2*
CT48-6B	*MATa his4 trp1 leu2 ura3 cox5a$_\Delta$::URA3 reo1-4*
αLR1	*MATα his3 trp1 gal1*
αLR1-210	*MATα his3 trp1 gal1 rox1-210*
αLR1-202	*MATα his3 trp1 gal1 rox3-202*

[a] Strains K399-7D and K393-35C were obtained from R. ESPOSITO and strains αLR1, αLR1-210 and αLR1-202 were obtained from R. ZITOMER. The remaining strains are isogenic to JM43 and were constructed during the course of these studies. *The asterisk indicates the presence of two copies of *COX5b* and of *LYS1*, two genes on chromosome *IX*, as described in RESULTS.

overnight cultures of *E. coli* by a method intermediate between the small scale (1 ml) and the large scale (1500 ml) alkaline-SDS methods described by MANIATIS, FRITSCH and SAMBROOK (1982). Yeast cells were transformed using lithium acetate (ITO *et al.* 1983) and plasmids were selected on SD medium lacking uracil (for *URA3* plasmids) or tryptophan (for *TRP1* plasmids). Total genomic yeast DNA was isolated from 25 ml YPD cultures as described (MASMYTH and REED 1980). Restriction digests, agarose gel electrophoresis, transfer of DNA to nitrocellulose, and hybridization to a nick-translated ^{32}P-radiolabeled DNA fragment and autoradiography were all performed by standard methods, essentially as described by MANIATIS, FRITSCH and SAMBROOK (1982).

Growth rate determination: Growth rates on solid medium were estimated based on colony size after several days of incubation at 28°. Four phenotypes of growth on nonfermentable carbon sources are defined in Figure 1: (1) negligible growth (N), (2) slow growth (S), (3) faster-growth (F), and (4) wild-type growth (+). Growth rates in liquid medium were determined by periodic evaluation of cell density using a Klett colorimeter (Klett Manufacturing Co., New York). Klett readings were converted to dry weight by using a standard curve of Klett value *vs.* dry weight for strain JM43, grown in YPD medium. Doubling times were determined from semi-logarithmic plots of dry weight versus time.

Respiration rate and TMPD assays: Respiration rates were performed on 3 ml of cell culture using a Yellow Springs Instruments Oxygen Monitor. Rates of oxygen consumption were determined in the absence and the presence of 100 μM potassium cyanide, an inhibitor of cytochrome *c*

TABLE 2

Plasmids

Plasmid[a]	Genotype
YCp5b	*COX5b*, *TRP1*, ARS1, CEN3
YCp5ba	*COX5ba*,[b] *URA3*, *TRP1*, ARS1, CEN4
pMC5bL	*COX5b-lacZ*, *URA3*, 2 μm origin
pCT5aL	*COX5a-lacZ*, *URA3*, 2 μm origin
YCpAZ6	*ANB1-lacZ*, *TRP1*, ARS1, CEN3

[a] Plasmids YCp5b, YCp5ba, pMC5bL and CT5aL are described in TRUEBLOOD and POYTON (1987). YCpAZ-6 was a gift from RICHARD ZITOMER; it was derived from YCpCYC1 (2.4) (LOWRY and ZITOMER 1984).

[b] The chimeric *COX5ba* gene has the promoter, regulatory regions and coding sequence for the leader peptide and 12 amino acids of mature Vb from *COX5b* fused to the coding sequence for amino acids 13 through 133 of mature Va and 3′ flanking sequences from *COX5a*.

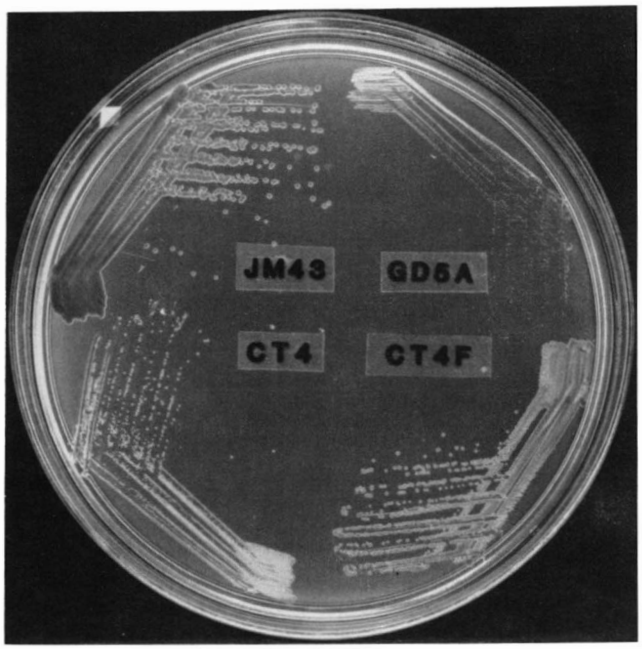

FIGURE 1.—Relative growth of respiration-impaired strains on nonfermentable medium. The indicated strains were streaked onto solid YPL medium, which contains the nonfermentable carbon source lactate, and were incubated at 28°C for four days. Four categories of growth on non-fermentable media are defined as follows: (1) *wild-type growth* (+) is defined by the colony size of the respiration-proficient strain, JM43, which is isogenic with the other strains used in these studies; (2) *negligible growth* (N) is defined by strain GD5a, which is disrupted for *COX5a* but not *COX5b*, and consequently grows slightly better than GD5ab; (3) *slow growth* (S) is used to describe the formation of small colonies as exemplified by strain CT4; (4) *faster-growth* (F) describes colonies that are smaller than wild-type but larger than CT4, as exemplified by strain CT4F. Strains CT4 and CT4F are described in RESULTS.

oxidase. The cyanide-sensitive respiration rate (pmol/min/μg) was calculated as pmol oxygen consumed per min in the absence of cyanide minus pmol oxygen consumed per min in the presence of cyanide, divided by the dry weight of cells (based on Klett readings). *N,N,N′,N′*-Tetramethyl-*p*-phenylene-diamine (TMPD) donates electrons to a cytochrome *c*/cytochrome *c* oxidase complex; in the reduced state TMPD is colorless and in the oxidized state it is blue. A filter assay for TMPD staining of yeast cells, described by MCEWEN *et al.* (1985), allows a rough determination of cytochrome *c* oxidase activity in yeast strains. The TMPD stain observed ranges from no stain (designated white or −) for strains lacking cytochrome *c* oxidase activity to a dark blue (designated wild type or ++++) for cells exhibiting wild-type cytochrome *c* oxidase activity. Intermediate levels of staining [designed weak (+), moderate (++), and strong (+++)] are observed for strains with intermediate levels of cytochrome *c* oxidase activity.

Isolation of mitochondria and immunoblot analysis: Yeast strains were grown to mid-exponential phase in 25 ml SSG medium, washed twice with H_2O, then treated with DTT and Glusulase to digest the cell wall as described by POYTON and KAVANAUGH (1976). Cell breakage was accomplished by vortexing with glass beads at high speed on a Vortex-genie for 1 min. Cell debris was removed by low speed centrifugation (1900 × *g*) and mitochondria were pelleted at 15,000 × *g* for 10 min; the latter two steps were repeated. An aliquot of 15 μg of mitochondrial protein was loaded on a 12.5% polyacrylamide gel containing 27% glycerol and 3.5 M urea; proteins were electrophoretically separated as described (CUMSKY *et al.* 1983). The immunoblot procedure of VAESSEN, KREIKE and GROOT (1981) was followed using antisera raised against cytochrome *c* oxidase subunits IV, Va and VI (isolated from commercial yeast) and ^{125}I-protein A.

RESULTS

Isolation of mutants: In order to identify *trans*-acting genes and *cis* elements involved in regulation of *COX5b*, we selected for the ability of strain GD5a-4, which has a gene deletion of *COX5a* (see Table 1), to grow on YPlactate (YPL) and YPglycerol + ethanol (YPGE) media. GD5a-4 grows extremely poorly on all nonfermentable carbon sources because, as described previously (TRUEBLOOD and POYTON, 1987), it lacks sufficient cytochrome *c* oxidase subunit V and hence respires at a low level. Four independent spontaneous mutants of GD5a-4 that are able to grow slowly on nonfermentable carbon sources were isolated and characterized.

Mutant strains carry recessive mutations that are unlinked to *COX5b*: To determine whether the mutations are dominant or recessive, each of the four mutants, CT1, CT2, CT3 and CT4, was crossed to GD5a-101 (*cox5a*$_\Delta$::*URA3*, *COX5b*$^+$). In each case, the resulting diploid strain grows extremely poorly on nonfermentable carbon sources, indicating that the mutant alleles are recessive to wild type. Each mutant was crossed to GD5b-101 (*COX5a*, *cox5b*::*LEU2*) and subjected to tetrad analysis (Table 3) in order to determine whether the mutations in strains CT1, CT2, CT3 and CT4 are linked to *COX5b* and whether the *COX5b* gene is required for growth of CT1, CT2, CT3 and CT4 on nonfermentable carbon sources. Spores carrying *COX5a* cannot be scored for the presence or absence of the mutations since *COX5a* alone is sufficient to support wild-type respiration rates; therefore, such spores are excluded from the analysis presented in Table 3. One-half of the Ura$^+$, Leu$^-$

TABLE 3

Phenotype of slow growth on YPGE requires a functional *COX5b* gene *and* a mutation at a locus unlinked to *COX5b*

Cross[a]	*COX5b* genotype of *cox5aΔ*::URA3 spores	YPGE growth[b]/TMPD stain[c] phenotype		
		YPGE^S/ TMPD^M	YPGE^N/ TMPD^W	YPGE$^-$/ TMPD$^-$
CT1 × GD5b-101	*COX5b*	4	5	0
	cox5b::LEU2	0	0	7
CT2 × GD5b-101	*COX5b*	3	2	0
	cox5b::LEU2	0	0	5
CT3 × GD5b-101	*COX5b*	3	3	0
	cox5b::LEU2	0	0	12
CT4 × GD5b-101	*COX5b*	5	4	0
	cox5b::LEU2	0	0	9

[a] Diploids were formed by crossing the indicated parent strains. See Table 1 for genotypes of parental haploid strains.

[b] YPGE growth determined based on colony size relative to respiration-proficient strain JM43 after three to four days growth on YP glycerol + ethanol solid medium. Slow growth (YPGE^S) is typified by the growth phenotype of strains CT1, CT2, CT3 and CT4. Negligible growth (YPGE^N) is typified by strain GD5a-4 (*cox5a*$_\Delta$*::URA3, COX5b*). No growth (*YPGE*$^-$) describes the phenotype of GD5ab-1 (*cox5a*$_\Delta$*::URA3, cox5b::LEU2*). See Figure 1 for a more detailed description of growth phenotypes.

[c] Staining of yeast colonies is indicative of the level of cytochrome *c* oxidase activity. Our respiration proficient strain, JM43, exhibits dark blue staining with TMPD in less than 2 min, whereas GD5ab-1, a strain lacking cytochrome *c* oxidase activity due to disruption of *COX5a* and *COX5b*, is colorless even after extended incubation (designated TMPD$^-$). Intermediate levels of staining are observed for strains with intermediate levels of cytochrome *c* oxidase activity. GD5a-4 (*cox5a*$^-$, *COX5b*$^+$), which has cytochrome *c* oxidase activity between 5% and 10% of the wild-type rate (TRUEBLOOD and POYTON 1987), exhibits weak staining with TMPD (designated TMPD^W), turning light blue within about 4 min. Strains CT1, CT2, CT3 and CT4 exhibit moderate staining (designated moderate or TMPD^M).

(*cox5a*$_\Delta$*::URA3, COX5b*$^+$) spores grow slowly on nonfermentable carbon sources and stain moderately with TMPD as do the mutants, whereas the other half exhibit the extremely poor growth and weak TMPD staining of GD5a-4. These results indicate that in each strain (CT1, CT2, CT3 and CT4) the ability to grow on nonfermentable carbon sources is due to a single mutation or to multiple linked mutations and that the mutations in each of these strains are not tightly linked to *COX5b*. None of the spores that are Ura$^+$ and Leu$^+$ (*cox5a*$_\Delta$*::URA3, cox5b::LEU2*) grow on YPGE, nor do they stain with TMPD; thus, the mutations do not bypass the subunit V requirement but rather depend on the presence of *COX5b* to be expressed phenotypically.

Complementation and linkage analysis: Complementation tests were performed by mating *MAT***a** derivatives of CT3 or CT4 (CT39-9D or CT48-7D, respectively) to spontaneous His$^+$ revertants of CT1, CT2, CT3 and CT4 (CT11, CT12, CT13 and CT14). All eight diploids grow slowly on YPGE medium (Table 4), indicating that complementation has *not*

TABLE 4

Four mutations are recessive to wild type and fall into a single complementation group, designated *REO1*

Cross[a]	Growth on YPGE[b]	Alleles in diploid[c]
CT11 × CT39-9D	Slow	*reo1-1/reo1-3*
CT12 × CT39-9D	Slow	*reo1-2/reo1-3*
CT13 × CT39-9D	Slow	*reo1-3/reo1-3*
CT14 × CT39-9D	Slow	*reo1-4/reo1-3*
GD5a-H$^+$ × CT39-9D	Negligible	*REO1/reo1-3*
CT11 × CT48-7D	Slow	*reo1-1/reo1-4*
CT12 × CT48-7D	Slow	*reo1-2/reo1-4*
CT13 × CT48-7D	Slow	*reo1-3/reo1-4*
CT14 × CT48-7D	Slow	*reo1-4/reo1-4*
GD5a-H$^+$ × CT48-7D	Negligible	*REO1/reo1-4*

[a] A spontaneous His4$^+$ revertant of GD5a-4(GD5a-H$^+$) and of each mutant CT1, CT2, CT3 and CT4 (yielding CT11, CT12, CT13 and CT14, respectively) was crossed to CT39-9D (a spore from diploid strain CT39, which was formed by crossing CT13 and GD5b-101), and CT48-7D (a spore from diploid strain CT48, which was formed by crossing CT14 and GD5b-101). See Table 1 for genotypes of haploid strains. Diploids were selected on SD + leucine.

[b] "Negligible" growth colonies were as tiny as those of haploid strain GD5a-4; "slow" growth colonies were at least 4 times smaller than those of wild-type strain JM43 and were equivalent in size to colonies of the haploid *reo1*$^-$ mutants, CT1, CT2, CT3 and CT4.

[c] Designations *reo1-1*, *reo1-2*, *reo1-3* and *reo1-4* indicate mutant alleles originally isolated in strains CT1, CT2, CT3 and CT4 respectively. *REO1* is the wild-type allele. *REO* stands for regulator of expression of oxidase.

occurred and that the four mutations constitute a single complementation group. The mutations in strains CT1, CT2,CT3 and CT4 are designated *reo1-1*, *reo1-2*, *reo1-3* and *reo1-4*, where *reo* stands for regulator of expression of oxidase.

To exclude the possibility that these diploid strains exhibit intergenic noncomplementation (*i.e.*, that nonallelic mutations are dominant enhancers of one another), linkage analysis was performed. Since the diploids used for complementation analysis lack the *COX5a* gene and have a low level of respiration, they sporulate extremely poorly. Consequently, it was necessary to create diploid strains that carried the *COX5a* gene and pairwise combinations of the *reo1* alleles; such diploids were formed by mating a *COX5a*$^+$ *reo1*$^-$ strain derived from CT4 (DWS1-3B) to each *reo1*$^-$ mutant (CT1, CT2, CT3 and CT4). The *COX5a* gene, contributed by DWS1-3B, supports a wild-type respiration rate so that sporulation can proceed efficiently. As expected, analysis of 15 to 20 tetrads from each of these diploids yielded two *COX5a*$^+$ spores that grow at a wild-type rate on YPGE and two spores that grow slowly on YPGE, indicating the presence of a *reo1* allele (Table 5). Six tetrads from the control cross, in which DWS1-3B was mated to the *REO1*$^+$ strain GD5a-4, produced two *COX5a*$^+$ spores with wild-type growth on YPGE, one *reo1*$^-$, *cox5a*$^-$ spore that grows slowly on YPGE and one *REO1*$^+$, *cox5a*$^-$ spore that fails to grow on YPGE. From this analysis it is clear

TABLE 5

reo1-1*, *reo1-2* and *reo1-3* are each linked to *reo1-4

Cross[a]	Relevant genotype	YPGE growth phenotypes of tetrads[b] 2+:2S	2+:1S:1N	2+:2N
CT1 × DWS1-3B	*cox5a*$^-$/*COX5a*$^+$ *reo1-1*/*reo1-4*	20	0	0
CT2 × DWS1-3B	*cox5a*$^-$/*COX5a*$^+$ *reo1-3*/*reo1-4*	15	0	0
CT3 × DWS1-3B	*cox5a*$^-$/*COX5a*$^+$ *reo1-3*/*reo1-4*	18	0	0
GD5a-4 × DWS1-3B	*cox5a*$^-$/*COX5a*$^+$ *REO1*/*reo1-4*	0	6	0

[a] Diploids were formed by crossing the indicated strains; see Table 1 for genotypes of haploid parent strains.

[b] "+" indicates wild-type growth on glycerol and ethanol; these strains are also Ura$^-$ indicating the presence of the *COX5a* gene rather than *cox5a*$_\Delta$::*URA3*. "S" indicates slow growth on glycerol and ethanol; these strains are Ura$^+$ and thus carry *cox5a*$_\Delta$::*URA3*. The slow growth phenotype indicates that they carry a *reo1* mutation. "N" indicates negligible growth on glycerol and ethanol; these strains are Ura$^+$ and therefore carry *cox5a*$_\Delta$::*URA3*. They carry the *REO1*$^+$ allele and hence do not produce enough Vb to grow significantly on nonfermentable carbon sources. See Figure 1 for further explanation of growth phenotypes.

that *reo1-4* is tightly linked to *reo1-1*, *reo1-2* and *reo1-3*. Based on phenotypic similarity, noncomplementation, and linkage of the *reo1* mutations, we conclude that *reo1-1*, *reo1-2*, *reo1-3* and *reo1-4* are mutant alleles in a single gene, *REO1*.

The *reo1* mutations affect a single gene that encodes a protein: Because the CT1, CT2, CT3 and CT4 mutants arose spontaneously at a frequency of roughly one in 10^6 cells, it is improbable that they carry more than one mutation that affects the growth phenotype on nonfermentable carbon sources. The best evidence that the *reo1* mutations affect a single genetic locus is the observation that the *reo1*$^-$ phenotype in *reo1*-4 strains is suppressed by amber suppressor tRNAs. CT1, CT2, CT3 and CT4 carry the amber mutations *his4-580* and *trp1-289*. Spontaneous His$^+$, Trp$^+$ revertants arise in these strains at a frequency of roughly 1 in 10^6 and most such His$^+$, Trp$^+$ revertants are expected to carry an amber suppressor tRNA gene. All of the six His$^+$ Trp$^+$ revertants of CT4 that were tested were unable to grow on nonfermentable carbon sources, whereas nearly all of the His$^+$ Trp$^+$ revertants of CT1, CT2 and CT3 were able to grow on nonfermentable carbon sources. From each of the six His$^+$, Trp$^+$ revertants of CT4, colonies that regain the ability to grow on nonfermentable carbon sources appeared spontaneously in cells patched to YPL or YPGE; each of these respiration-proficient (*reo1*$^-$) colonies was also His$^-$ and Trp$^-$. These observations that the *reo1*$^-$ phenotype reverts and re-reverts simultaneously with the His$^-$ and Trp$^-$ phenotypes in CT4 strongly suggests that the CT4 growth phenotype is due to an amber nonsense codon, that the mutated gene encodes a protein and, that the amber mutation results in a loss of function of the protein.

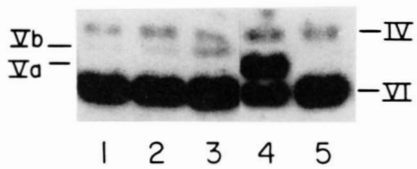

FIGURE 2.—Immunoblot analysis indicates that *reo1*$^-$ mutants have an increased amount of Vb. An aliquot of 25 μg of mitochondrial protein from strains CT1 (lane 1), CT4 (lane 2), CT1F (lane 3), JM43 (lane 4), and GD5a (lane 5) was electrophoresed on a 12.5% polyacrylamide gel containing 27% glycerol and 3.5 M urea. The gel was immunoblotted as described by VAESSEN, KREIKE and GROOT (1981), using antisera raised against cytochrome *c* oxidase subunits IV, V and VI from commercial Baker's yeast and ^{125}I-protein A. The positions of subunits IV, Va, Vb and VI are indicated. It is not possible to determine the amounts of Vb in the *reo1*$^-$ mutants relative to the amount of Va in JM43 because the antiserum was raised against commercial yeast subunits which contain predominantly Va rather than Vb, and the efficiency of cross-reactivity with Vb cannot be precisely quantitated.

TABLE 6

Growth and respiration rates of a *reo1*$^-$ mutant and a faster-growing derivative of it

Strain[a]	Doubling time (hr)[b]	Percent of wild-type growth rate	Respiration rate[c]	Percent of wild-type respiration
JM43	2.9	100	94	100
CT4	21.5	14	9.3	10
CT4F	10.5	28	25.7	27

[a] See Table 1 for genotypes of strains.

[b] Cells were grown at 28° in YPL, which has the nonfermentable carbon source lactate. Doubling times were measured as described in MATERIALS AND METHODS.

[c] Cyanide-sensitive respiration rates are presented as pmol O_2 consumed per min per μg cell mass. Measurements and calculations are described in MATERIALS AND METHODS.

***reo1* mutations increase Vb production:** The observations that *reo1* mutations require a functional *COX5b* gene to exhibit a phenotype, and that poor *COX5b* expression is responsible for the low respiratory capacity of aerobically growth GD5a-4 (TRUEBLOOD and POYTON 1987) strongly suggest that the *REO1* gene product negatively affects the amount of Vb produced in aerobically grown wild-type cells. To determine whether the amount of Vb is increased in *reo1* mutants, immunoblot analysis was performed on each of the mutants and on faster-growing derivatives of each mutant (CT1F, CT2F, CT3F and CT4F, described in detail below). In Figure 2, we present an immunoblot of strains CT4, CT1, CT1F, JM43 and GD5a-4; Vb is not detectable in GD5a-4 (lane 4), but is observed in *reo1*$^-$ mutants (CT4 and CT1, lanes 1 and 2) and is more abundant in the faster-growing derivatives of *reo1*$^-$ mutants (*e.g.*, CT1F in lane 3). Results for the remaining mutants were similar (data not shown). The amounts of Vb polypeptide in these

strains parallels their ability to respire and to grow on lactate medium (Table 6). The *reo1*$^-$ mutants, CT4 and CT4F, have doubling times of 21 and 10 hr on lactate. These doubling times are significantly shorter than the doubling time, roughly 130 hr, observed for the *cox5a*$^-$, *REO1*$^+$ strain, GD5a-4, on lactate. The CT4 and CT4F growth rates are 14% and 28% of the growth rate of the isogenic wild-type strain, JM43 (doubling time 2.9 hr). Respiration rates of CT4 and CT4F during growth on lactate are approximately 10% and 27% of the wild-type rate in lactate, in good agreement with the growth rates in lactate, relative to JM43. Our interpretation of these results is that the *reo1* mutations lead to an increase in subunit Vb production, which increases the ability of the cell to respire and consequently the ability to grow on non-fermentable carbon sources, such as lactate.

Characterization of faster-growing derivatives of *reo1*$^-$ strains: Each *reo1*$^-$ mutant strain produces spontaneous derivatives (at a frequency of roughly 1 in 10^5) that grow more quickly on YPGE and YPL; see CT4F compared to CT4 in Figure 1. Both genomic DNA hybridization studies and genetic analysis indicate that these derivatives carry two copies of *COX5b*. Roughly twice as much genomic *COX5b* DNA is detected by a radioactively labeled *COX5b* DNA fragment in faster-growing derivatives compared to *reo1* mutants and JM43. In Figure 3A, we show a genomic DNA blot of CT4F and the *reo1* mutant from which it was derived, CT4. The amount of radioactive *COX5b* probe hybridized to CT4F DNA was calculated to be 2.2 times that hybridized to CT4, after normalization for the amount of DNA loaded in each lane. Qualitative DNA blots on the other mutants indicated that there is also more *COX5b* DNA in the haploid genomes of CT1F, CT2F and CT3F than in CT1, CT2 and CT3 (data not shown). Further evidence for duplication of *COX5b* comes from the observation of haploid strains that are Leu$^+$ (indicating the presence of *cox5b*::*LEU2*) and are able to grow slowly on YPGE (indicating the presence of *COX5b* and the *reo1* mutation), among the meiotic progeny of diploids formed from faster-growing derivatives and GD5b-101. DNA-DNA hybridization blot analysis confirmed that these haploid strains do carry both *cox5b*::*LEU2* and *COX5b* (Figure 3B). Haploid strains carrying both *cox5b*::*LEU2* and *COX5b* are expected from tetrad analysis only if the diploid strain carries more than one copy of the *COX5b* gene or more than one copy of the *cox5b*::*LEU2* gene. If a diploid strain received two physically unlinked copies of *COX5b* from a faster-growing derivative and one copy of *cox5B*::*LEU* from GD5b-101, between one-half and two-thirds of the Leu$^+$ (*cox5b*::*LEU2*) spores are expected to also carry *COX5b*, depending on the physical location of the extra *COX5b* gene. Of the Leu$^+$, Ura$^+$ spores one-quarter to one-third are expected to carry both *COX5b* and *reo1*, and therefore to be able to

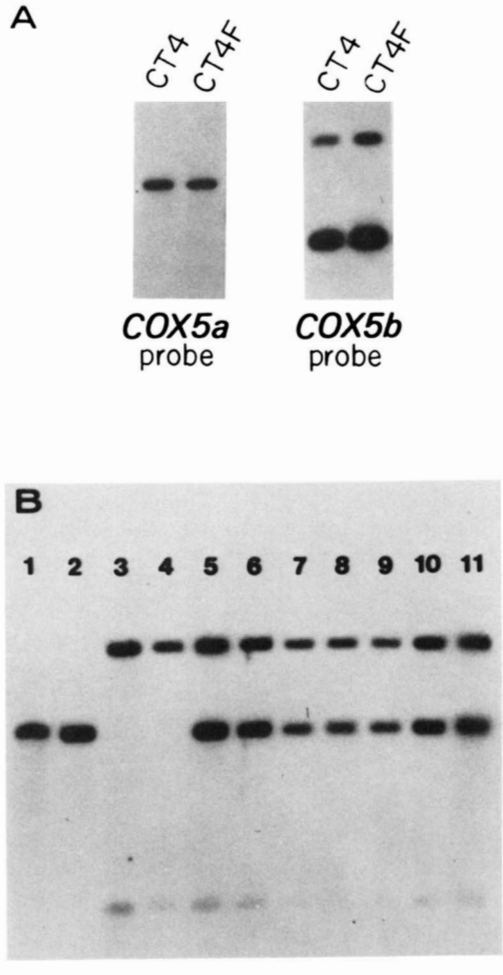

FIGURE 3.—Genomic DNA blot analysis indicates that faster-growing derivatives of *reo1*$^-$ mutants carry two copies of the *COX5b* gene. (A) Genomic DNA, isolated from strains CT4 and CT4F and digested with the restriction endonuclease *Hin*dIII, was electrophoresed on a 1% agarose gel, blotted to nitrocellulose and hybridized to either a *COX5a* or *COX5b* probe, as indicated (see MATERIALS AND METHODS for details). The *COX5a* probe hybridizes to a single *Hin*dIII restriction fragment whereas the *COX5b* probe hybridizes to two *Hin*dIII restriction fragments. The hybridized areas of the nitrocellulose were excised and the number of cpm of bound radioactive probe were determined in a scintillation counter; CT4 and CT4F bound 234 cpm and 179 cpm of the *COX5a* probe and bound 948 cpm and 1585 cpm of the *COX5b* probe, respectively. These data suggest that strain CT4F carries 2.2 times more *COX5b* DNA than strain CT4. (B) Genomic DNA was isolated from strains JM43, GD5a, GD5b and GD5ab (lanes 1, 2, 3 and 4, respectively) and from Leu$^+$ (*cox5b*::*LEU2*), Ura$^+$ (*cox5a*$_\Delta$::*URA3*) spores that are derived from the faster-growing derivatives of *reo1*$^-$ strains and are able to grow on YPGE (lanes 5–7, spores from CT2F; lanes 8 and 9, spores from CT3F; lane 10, spore from CT1F; lane 11, from CT4F). DNA was digested with *Cla*I, electrophoresed through a 1% agarose gel, blotted to nitrocellulose and hybridized to a ^{32}P-radiolabeled *COX5b* probe (see MATERIALS AND METHODS for details). The hybridization pattern in lanes 1 and 2 is that expected for *COX5b* and the pattern in lanes 3 and 4 is that expected for *cox5b*::*LEU*. The remaining lanes clearly exhibit both patterns, indicating the presence of both *COX5b* and *cox5b*::*LEU2* in these strains.

TABLE 7

Faster-growing derivatives of *reo1*⁻ mutants carry two copies of *COX5b*

Cross[a]	Growth phenotypes[b] of *cox5aΔ::URA3*, *cox5b::LEU2* spores		Ratio YPGE⁺/YPGE⁻
	YPGE⁺	YPGE⁻	
CT11F × GD5b-101	2	6	0.25
CT12F × GD5b-101	5	8	0.385
CT13F × GD5b-101	4	6	0.40
CT14F × GD5b-101	3	8	0.27

[a] Diploids were formed by crossing the indicated haploid strains. See Table 1 for genotypes of parent strains.

[b] YPGE⁺ designates slow growth on YP glycerol + ethanol, indicating the presence of *COX5b* and the reo1 mutation. YPGE⁻ designates no growth on YP glycerol + ethanol indicating that the strain lacks either *COX5b* or the *reo1* mutation.

grow slowly on YPGE. Diploids formed by mating CT11F, CT12F, CT13F or CT14F to GD5b-101 produce Leu⁺ (*cox5b::LEU2*), Ura⁺ (*cox5a*$_{\Delta}$*::URA3*) spores that grow slowly on YPGE at frequencies in the predicted range (Table 7). As expected, diploids formed from slow-growing *reo1*⁻ mutants and GD5b-101 did not produce any Leu⁺, Ura⁺ spores that were able to grow on YPGE (Table 3).

In order to analyze how *COX5b* might have been duplicated, we examined the possibility that the chromosome on which *COX5b* resides has been duplicated. *COX5b* is located on chromosome *IX*, as indicated by orthogonal field alternating gel electrophoresis (R. WRIGHT, personal communication) and genetic analysis demonstrating linkage to *lys11* on the left arm of chromosome *IX* (L. K. DIRCKS and C. E. TRUEBLOOD, unpublished results). To test for chromosome *IX* disomy, we crossed CT1F, CT2F, CT3F, CT4F to strain K399-7D, which is marked by *lys1* on the right arm of chromosome *IX*. Tetrad analysis of these diploids (Table 8) produced a preponderance of 4 Lys⁺:0 Lys⁻ and 3 Lys⁺:1 Lys⁻ tetrads, indicating that the *LYS1* gene is duplicated in these strains. When the slow-growing *reo1* mutants (CT1, CT2, CT3 and CT4) were crossed to a *lys1* strain, tetrad analysis produced predominantly 2 Lys⁺:2 Lys⁻ tetrads, indicating that *LYS1* is not duplicated in these strains. These results strongly suggest that faster-growing derivatives of *reo1*⁻ mutants are disomic for chromosome *IX*. The increased ability of faster-growing derivatives to grow on nonfermentable carbon sources requires both the *reo1* mutation and the chromosome *IX* duplication; in a *REO1*⁺ strain, the chromosome *IX* duplication does not produce a detectable phenotype.

To address the possibility that duplication of a chromosome *IX* gene other than *COX5b* is responsible for the faster-growth phenotype, we introduced a CEN plasmid carrying *COX5b* (YCp5b; see Table 2)

TABLE 8

Faster-growing derivatives of *reo1*⁻ mutants carry two copies of *LYS1*, a gene that resides on the right arm of chromosome *IX*

Diploid strain[a]	Haploid parent strains	Lysine phenotypes of tetrads			
		4⁺:0⁻	3⁺:1⁻	2⁺:2⁻	1⁺:3⁻
CT194	CT1F × K399-7D	4	1	0	0
CT195	CT2F × K399-7D	2	3	0	0
CT196	CT3F × K399-7D	4	0	0	0
CT193	CT4F × K399-7D	3	3	0	0
CT180	CT40-8B × K393-35C (*reo1-3*)	4	2	0	0
CT181	CT40-3C × K393-35C (*reo1-3*)	0	2	1	0
Total from crosses including fast-growing derivatives		17	11	1	0
CT190	CT1 × K399-7D	0	0	3	0
CT182	CT43-10C × K393-35C (*reo1-1*)	0	0	6	0
CT191	CT2 × K399-7D	0	1	4	0
CT192	CT3 × K399-7D	0	0	4	0
CT189	CT4 × K399-7D	0	0	4	1
Total from crosses including slow-growing strains		0	1	21	1

[a] Diploids were formed from the indicated haploid strains. See Table 1 for genotypes of haploid strains. K399-7D and K393-35C are lys1⁻ strains: *lys1* is a chromosome *IX* marker (STRATHERN, JONES AND BROACH, 1981).

or a CEN plasmid carrying the chimeric *COX5ba* gene (YCp5ba; see Table 2 and TRUEBLOOD and POYTON 1987) into a *reo1* strain that is disrupted for both *COX5a* and *COX5b* (CT39-10D). YCp5b and YCp5ba transformants of CT39-10D grow slowly on nonfermentable carbon sources. Since nondisjunction of CEN plasmids occurs more frequently than nondisjunction of chromosomes (KOSHLAND, KENT and HARTWELL 1985), we expected to observe a higher frequency of faster-growing derivatives in the population. Indeed, faster-growing derivatives arose quite frequently (roughly 1 in 100), leading to the conclusion that it is the duplication of *COX5b* itself that is responsible for the increased ability of faster-growing derivatives of *reo1*⁻ mutants to grow on nonfermentable carbon sources. Additional evidence supporting this point comes from the observation that a *reo1*⁻, *cox5a*⁻, *COX5b*⁺ strain (CT149-3D; see TRUEBLOOD, WRIGHT and POYTON 1988), which grows slowly on nonfermentable carbon sources, is directly converted to a faster-growing strain by transformation with CEN plasmid, YCp5ba (Table 2).

***REO1* is a negative regulator of *COX5b* in aerobically grown cells:** To test the effect of the *reo1* mutation on expression of the *COX5b* and *COX5a* genes, we analyzed the β-galactosidase activity in *reo1*⁻ and *REO1*⁺ strains transformed with plasmid pMC5bL, which carries the *COX5b-lacZ* gene or with

TABLE 9

***reo1* mutations increase *COX5b-lacZ* and *ANB1-lacZ* expression in aerobically grown, but not in anaerobicallly grown, cells.**

Strain[a]	*REO1* genotype	*lacZ* fusion gene[b]	β-galactosidase units[c] Aerobic	Anaerobic
CT149-3A	*reo1*⁻	*COX5b-lacZ*	21	22
CT149-3B	*REO1*⁺	*COX5b-lacZ*	2.1	16
CT149-3A	*reo1*⁻	*COX5a-lacz*	77	ND
CT149-3B	*REO1*⁺	*COX5a-lacz*	69	ND
CT39-10D	*reo1*⁻	*ANB1-lacZ*	25	56
CT44-6B	*REO1*⁺	*ANB1-lacZ*	0.6	35

[a] See Table 1 for complete geneotypes.

[b] *COX5b-lacZ* and *COX5a-lacZ* have been described previously (TRUEBLOOD and POYTON 1987). *COX5b-lacZ* encodes a fusion protein consisting of the 17 amino acid leader peptide and 13 amino acids of mature Vb fused to amino acids 8 through 1024 of *E. coli* β-galactosidase. *COX5a-lacZ* encodes a fusion protein consisting of the 20 amino acid leader peptide and 13 amino acids of mature Va fused to amino acids 8 through 1024 of β-galactosidase. The *COX5a-lacZ* and *COX5b-lacZ* genes were introduced into yeast strains on plasmids pMC5bl and pCT5aL (see Table 2), derivatives of high copy number plasmid PSEY101 (DOUGLAS, GELLER and EMR 1984), which has *URA3* as a selectable marker. The *ANB1-lacZ* fusion gene was a generous gift of RICHARD ZITOMER (SUNY, Albany New York). The plasmid carrying the gene is YCpAZ6, a CEN plasmid with *TRP1* as a selectable marker.

[c] Strains transformed with plasmids were grown aerobically or anaerobically for 3–4 days at 28° as patches on synthetic dextrose plates containing necessary supplements at 40 μg/ml. A BBL anaerobic jar and Gas-Pak Plus were used to grow cells anaerobically. As soon as the anaerobic jar was opened cells were transferred into 1X Z buffer containing 100 μg/ml cycloheximide and assayed as described by MILLER (1972). Values are presented as MILLER units: $OD_{420}/OD_{600}/min \times 1000$. ND = not determined.

pCT5aL, which carries the *COX5a-lacZ* gene (see Table 2 and Table 9 footnotes). The *reo1-4* mutation results in a 10-fold increase in the level of expression of the *COX5b-lacZ* fusion gene in aerobically grown yeast cells and does not affect the expression of the *COX5a-lacZ* gene (Table 9). These results indicate that the *REO1* gene product affects production of Vb by influencing expression (transcription rate, transcript stability, or translational initiation frequency) and not by influencing post-translational steps (Vb polypeptide stability, targeting or assembly) since the latter steps should not affect β-galactosidase activity. The observation that the *reo1-4* allele is phenotypically suppressed by amber suppressor tRNAs (see above) provides strong evidence that the *reo1-4* mutation results in loss of function, rather than gain of function, of the *REO1* gene product. Therefore, we deduce that the normal role of the *REO1* gene product is to negatively influence *COX5b* expression.

To address the question of whether *COX5b* expression is modulated by the *REO1* gene product in response to oxygen concentration in the environment, we analyzed the effect of the *reo1-4* mutation on *COX5b-lacZ* expression in anaerobically grown cells (Table 9). The *reo1-4* mutation did *not* significantly increase β-galactosidase activity in anaerobically grown cells, which suggests that negative regulation of *COX5b* by *REO1* occurs only in aerobically-grown cells. Note that *COX5b-lacZ* expression is already elevated about eightfold in the *REO1*⁺ strain when it is grown anaerobically, rather than aerobically. This observation agrees with the data of M. HODGE and M. G. CUMSKY (personal communication) that *COX5b* transcripts are more abundant in anaerobically grown cells than in aerobically grown cells, and with our observation that *COX5b* transcripts are more abundant in heme-deficient cells than in heme-proficient cells (TRUEBLOOD, WRIGHT and POYTON 1988). Together, these findings implicate the *REO1* gene product as a negative regulator of *COX5b* in response to the environmental oxygen concentration.

To determine whether *REO1* is involved in regulation of other genes that are expressed at a higher level anaerobically than aerobically, we investigated the effect of the *reo1-4* mutation on expression of an *ANB1-lacZ* fusion gene. Although the function of the *ANB1* gene product is unknown, *ANB1* regulation has been investigated. *ANB1* transcripts are observed at much higher levels in anaerobically grown cells than in aerobically grown cells and transcription of the gene under anaerobic conditions is repressed by the addition of heme (LOWRY and LIEBER 1986), which indicates that aerobic repression is heme-mediated. The *reo1-4* mutation dramatically increases expression of *ANB1-lacZ* in aerobically grown cells, but has little effect on the already high level observed in anaerobically grown cells (Table 9). Thus, the *REO1* gene product is necessary for repression of *ANB1* expression in aerobically growing (heme-proficient) cells, but does not affect expression significantly in anaerobically growing (heme-deficient) cells.

The role of *REO1* as a negative regulator of *COX5b* and *ANB1* in response to oxygen and heme raises the possibility that *reo1* mutations are allelic to the recessive *rox1* or *rox3* mutations. *rox1* mutations were originally isolated by their ability to increase expression of an *ANB1-CYC1* fusion gene, in aerobically grown cells (LOWRY and ZITOMER 1984) and have been shown to increase *ANB1* transcript levels (LOWRY and LIEBER 1986). *rox3* mutations and additional *rox1* alleles were isolated by their ability to increase expression of a *CYC7-galK* fusion gene, in aerobically grown cells; alleles of *rox1* and *rox3* isolated in this latter screen (including *rox1-210* and *rox3-202*) also increase expression of the *ANB1* gene (R. ZITOMER, personal communication). The *rox1-210* and *rox3-202* mutations each increase expression of *COX5b* slightly; the increase in *COX5b* expression in *rox1* and *rox3* strains enables a *cox5a*⁻, *COX5b*⁺ strain to grow slowly on

TABLE 10

reo1 mutations define a complementation group distinct from the rox1 or rox3 complementation groups

Diploid Strain[a]	Parental Strains	β-galactosidase activity[b] Aerobic	Anaerobic
CT166-AZ	αLR1 (*ROX1*$^+$ *ROX3*$^+$) × CT48-6B(*reo1-4*)	0.5	28.7
CT167-AZ	αLR1-210 × CT48-6B(*reo1-4*)	0.6	33.7
CT168-AZ	αLR1-202 × CT48-6B(*reo1-4*)	0.8	35.2

[a] Diploid strains were selected on SD + trp after indicated parent strains were mated. See Table 1 for genotypes of haploid strains. Diploid strains CT166, CT167 and CT168 were transformed with plasmid YCpAZ6, a CEN plasmid that carries the *TRP1* and *ANB1-lacZ* fusion genes (see Table 2).

[b] Measured and calculated as described by MILLER (1972) (OD_{420}/OD_{600}/min)×1000; "aerobic" indicates that the yeast strains were grown aerobically in SD liquid medium and "anaerobic" indicates that the yeast strains were grown anaerobically on SD solid medium.

nonfermentable carbon sources when three copies of *COX5b* are present, but not when one or two copies are present. To perform complementation tests between *reo1* and *rox1* and between *reo1* and *rox3*, diploid strains CT167 and CT168 were constructed by crossing a *reo1*$^-$ strain (CT48-6B) to a *rox1*$^-$ *strain (αLR1-210) and a rox3*$^-$ strain (αLR1-202), respectively. As a control, αLR1, the ROX$^+$ strain from which *αLR1-210* and *αLR1-202* were isolated, was also mated to the same *reo1*$^-$ strain, to produce strain CT166. These strains were transformed with a plasmid carrying the *ANB1-lacZ* fusion gene, yielding strains CT166-AZ, CT167-AZ and CT168-AZ. If the *reo1* mutation were in this same gene as either the *rox1* or the *rox3* mutations, CT167-AZ or CT168-AZ would be expected to exhibit high β-galactosidase activity when grown aerobically, since no intact *ROX1* or *ROX3* gene product would be present to repress *ANB1-lacZ* expression. As shown in Table 10, CT166-AZ, CT167-AZ and CT168-AZ do *not* express β-galactosidase when grown aerobically, indicating that complementation *has occurred* between the *reo1* and *rox1* alleles, and between the *reo1* and *rox3* alleles (*i.e.*, these diploid strains must be producing functional *ROX1*, *ROX3* and *REO1* gene products, since expression is effectively repressed aerobically). When grown anaerobically, all three strains exhibit high levels of β-galactosidase activity, confirming the presence of an intact anaerobically expressible *ANB1-lacZ* fusion gene in each strain. These β-galactosidase results strongly suggest that *reo1* mutations are not in the *ROX1* or the *ROX3* gene, but we have not ruled out the unlikely possibility that intragenic complementation has occurred in one of these diploids.

DISCUSSION

The low level of expression of *COX5b* in aerobically grown cells has provided both the means and the motivation for selecting mutants that exhibit increased *COX5b* expression. Since strains deleted for *COX5a* do not produce enough cytochrome *c* oxidase subunit V to allow significant growth on nonfermentable carbon sources, mutations that increase *COX5b* expression can be directly selected. Selection for growth on nonfermentable carbon sources has the potential of identifying both *cis*-regulatory sites upstream of *COX5b* and *trans*-acting genes involved in *COX5b* regulation. We isolated four independent spontaneous mutants of the *cox5a* deletion strain, GD5a-4, that grow slowly on lactate (and other nonfermentable carbon sources), produce more Vb than the parent strain, and exhibit an increased respiration rate. Each mutant carries a recessive mutation that is responsible for these phenotypes and the four mutations define a single complementation and linkage group: the *REO1* gene. Derivatives of each *reo1*$^-$ mutant that are able to grow faster on lactate arise spontaneously at a frequency of roughly 1 in 10^5. These derivatives are likely to be the product of nondisjunction events that result in chromosome *IX* disomy, since they have a duplication of at least two chromosomal *IX* genes: *COX5b* on the left arm and *LYS1* on the right arm. The duplication of *COX5b* on chromosome *IX*, or on a CEN plasmid, increases the ability of cells to grow on nonfermentable carbon sources in *reo1*$^-$, *cox5a*$^-$ mutants but not in *REO1*$^+$ or *COX5a*$^+$ strains.

The effects of the *reo1* mutations on expression of the *COX5b* and *ANB1* genes are similar; the *reo1-4* mutation dramatically increases expression of both genes in aerobically grown (heme-containing) cells but alters expression very little in anaerobically grown (heme-deficient) cells, where expression of *COX5b* and *ANB1* is already elevated. Since the *reo1-4* mutation appears to create an amber nonsense codon, we propose that *REO1* encodes a protein that plays a role in aerobic expression of the *COX5b* and *ANB1* genes and that the *reo1-4* mutation, and the other *reo1* mutations, lead to loss of function of the *REO1* protein. The available data do not identify a specific regulatory role for the *REO1* protein. However, since heme repression of *COX5b* and *ANB1* affects transcript abundance (TRUEBLOOD, WRIGHT and POYTON 1988; LOWRY and LIEBER 1986), we speculate that *REO1* is involved in transcriptional regulation, possibly as a repressor of transcription or as a transcriptional activator required for expression of a repressor (or repressors) of *COX5b* and *ANB1*. The possibility that *reo1* mutations influence expression of *COX5b* and *ANB1* by affecting heme biosynthesis, has not been ruled out. However, the following observations indi-

cate that *reo1* mutations (including the presumed null allele, *reo1-4*) do not abolish or severely limit heme biosynthesis: (1) *reo1*$^-$ strains respire at a significant rate (Table 6) and have normal levels of cytochromes *b*, *c* and c_1 (R. O. POYTON, unpublished observations); and (2) the *COX5a* gene, which is positively regulated by heme, is unaffected by the *reo1* mutation. Based on these observations and our observations on *COX5a* and *COX5b* expression in *hem1*$^-$, *reo1*$^-$ double mutants (TRUEBLOOD, WRIGHT and POYTON 1988), we argue against involvement of the *REO1* protein in heme biosynthesis.

Two genes that are involved in negative regulation of *ANB1* have been described previously; the *rox1* and *rox3* mutations increase *ANB1* expression in aerobically grown cells (LOWRY and ZITOMER 1984; LOWRY and LIEBER 1986). These mutations have a small effect on *COX5b* expression, and, as shown in Table 10, are not in the same complementation group as the *reo1* mutations. It seems likely that there are at least three genes (*ROX1*, *ROX3* and *REO1*) that are involved in aerobic repression of *ANB1*. *REO1* is clearly involved in aerobic regulation of *COX5b*; the importance of *ROX1* and *ROX3* in *COX5b* regulation remains ambiguous. The roles of the *ROX1*, *ROX3* and *REO1* gene products have not been defined nor have any interactions between them been examined. Further genetic and biochemical investigation of the regulatory control pathways of *COX5b* and *ANB1* should answer the questions raised here and thereby increase understanding of heme-mediated anaerobic/aerobic regulation in yeast.

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