Eucaryotic cells contain at least two general classes of oxygen-regulated nuclear genes: aerobic genes and hypoxic genes. Hypoxic genes are induced upon exposure to anoxia while aerobic genes are down-regulated. Recently, it has been reported that induction of some hypoxic nuclear genes in mammals and yeast requires mitochondrial respiration and that cytochrome-c oxidase functions as an oxygen sensor during this process. In this study, we have examined the role of the mitochondrion and cytochrome-c oxidase in the expression of yeast aerobic nuclear COX genes. We have found that the down-regulation of these genes in anoxic cells is reflected in reduced levels of their subunit polypeptides and that cytochrome-c oxidase subunits I, II, III, Vb, VI, VII, and VIIA are present in promitochondria from anoxic cells. By using nuclear cox mutants and mitochondrial rho0 and mit mutants, we have found that neither respiration nor cytochrome-c oxidase is required for the down-regulation of these genes in cells exposed to anoxia but that a mitochondrial genome is required for their full expression under both normoxic and anoxic conditions. This requirement for a mitochondrial genome is unrelated to the presence or absence of a functional holocytochrome-c oxidase. We have also found that the down-regulation of these genes in cells exposed to anoxia and the down-regulation that results from the absence of a mitochondrial genome are independent of one another. These findings indicate that the mitochondrial genome, acting independently of respiration and oxidative phosphorylation, affects the expression of the aerobic nuclear COX genes and suggest the existence of a signaling pathway from the mitochondrial genome to the nucleus.

Both procaryotes and eucaryotes respond to intermittent and prolonged exposure to hypoxia (1). This response is crucial to survival in most organisms and can range from an immediate change in energy metabolism to the activation of gene expression pathways that help the organism cope with a low oxygen environment (1–3). Currently, the effect of oxygen concentration on gene expression is being studied intensively in a number of different organisms (c.f. Ref. 4). Studies with each of these experimental systems have made it clear that some genes (hypoxic genes) are up-regulated by exposure to anoxic or hypoxic conditions while other genes (aerobic genes) are down-regulated under these conditions. So far, most of the studies on the effects of hypoxia on gene expression have focused on the induction of hypoxic genes and have not addressed the down-regulation of aerobic genes. These studies on the induction of hypoxic genes have revealed that exposure to hypoxia can initiate a complex series of events, which begin with some sort of oxygen sensor and end with a signaling pathway that up-regulates hypoxic genes (1, 5–10).

Currently, the best understood oxygen sensors and signaling pathways are those in procaryotes (c.f. Ref. 1). Although progress has been made in understanding the transcriptional machinery involved in oxygen-regulated gene expression of eucaryotic genes, the underlying mechanisms of oxygen sensing and the signaling pathways that connect oxygen sensors to the transcriptional machinery in eucaryotes are poorly understood. However, there is growing evidence that the mitochondrial respiratory chain and cytochrome-c oxidase function in oxygen sensing and in the induction of some hypoxic genes in both yeast and mammalian cells (8, 9). Early evidence for the involvement of cytochrome-c oxidase in oxygen sensing in mammalian cells has come from spectral studies (11–13) that examined the influence of azide, cyanide, and carbon monoxide on chemosensory discharge, primarily in carotid body cells (14). More recently, it has been found that part of the hypoxic induction of some mammalian genes is sensitive to cyanide and that the mitochondrial respiratory chain is required for the hypoxic induction of a number of genes which are under the control of the HIF-1 transcription factor (10, 15).

Evidence for the involvement of the mitochondrial respiratory chain in the induction of some hypoxic genes has also been reported for the yeast Saccharomyces cerevisiae (16). Carbon monoxide affects the induction of a subset of hypoxic genes in this organism. It completely inhibits the induction of OLE1 (the gene for Δ-9 fatty acid desaturase) and CYC7 (the gene for iso-2 cytochrome c) and partially inhibits the induction of COX5b (the gene for cytochrome-c oxidase subunit Vb) but has no effect on the induction of seven other hypoxic genes (HEM13, HMG1, HMG2, ERG11, CPR1(NCP1), ANB1, and AAC3). This finding revealed two classes of yeast hypoxic genes: carbon monoxide-sensitive and carbon monoxide-insensitive. By using mutants deficient in the two major yeast carbon monoxide-binding hemeproteins (cytochrome-c oxidase and flavohemoglobin) it was found that cytochrome-c oxidase but not flavohemoglobin is required for the induction of the carbon monoxide-sensitive genes. These studies also revealed that OLE1 and CYC7 are not induced in rho0 strains (which lack a mitochondrial genome and respiration), in strains that are respiration deficient but
retain a mitochondrial genome, and in the presence of respiratory inhibitors. Together, these findings indicate that the mitochondrial respiratory chain is involved in the expression of some hypoxic yeast genes and that cytochrome-c oxidase is a likely oxygen sensor.

The above observations have increased interest in assessing how cytochrome-c oxidase functions in oxygen-regulated gene expression (c.f. Refs. 9 and 10). At present, little is known about the oxygen-sensing function of this enzyme. However, it is clear that oxygen concentration has a profound affect on both its biosynthesis and catalytic functions (c.f. Ref. 17 and 18). This oligomeric protein of the inner mitochondrial membrane contains subunit polypeptides encoded by both nuclear and mitochondrial genomes. The three largest subunits (I, II, and III) are encoded by mitochondrial genes (COX1, COX2, and COX3); they form the catalytic core of the enzyme (17, 19). Oxygen affects the expression of two of these genes post-transcriptionally. The other polypeptide subunits are encoded by nuclear genes; some of them modulate catalysis, whereas others function in the assembly or stability of the holoenzyme. Abnormal preparations of yeast cytochrome-c oxidase contain at least six subunit polypeptides (IV, Va or Vb, VI, VII, VIIa, and VIII) encoded by nuclear COX genes (COX4, COX5a or COX5b, COX6, COX7, COX9, and COX8, respectively) (20). COX5a and COX5b encode interchangeable isoforms, Va and Vb, of subunit V (21). The other nuclear-encoded subunits are encoded by single-copy genes. Oxygen affects the levels of expression of all of these genes at the level of transcription. All of these genes except COX5b are aerobic genes; COX5b is a hypoxic gene. The inverse regulation of COX5a and COX5b by oxygen is especially interesting because the isoforms encoded by these genes have differential effects on holocytochrome-c oxidase activity. By altering an internal step in electron transport between heme a and the binuclear reaction center the "hypoxic" isoform, Vb, enhances the catalytic constant (TN) of the enzyme 3–4-fold (22, 23). Hence, these isoforms allow cells to assemble functionally different types of holoenzyme in response to oxygen concentration.

We are using S. cerevisiae as a model system to further explore the role of the mitochondrial and cytochrome-c oxidase in adaptation to hypoxia. In this study, we focus on the role of anoxia and the mitochondrial on the expression of aerobic COX genes. Our findings provide support for the existence of a signaling pathway, which acts independently of respiration, from the mitochondrial genome to the nucleus.

**EXPERIMENTAL PROCEDURES**

**Strains**—The S. cerevisiae strains used in this study are described in Table 1. Two respiratory-competent parental strains were used: D273-10B and JM43. Strain JM43 was constructed by crossing D273-10B with strain AB35-13D (MATa leu2-2, 112, ura3-52, his4-580, ade2), as described (24) and then extensively backcrossed with D273-10B. These two strains are isogenic except for the auxotrophic markers that were introduced into JM43. Strain JM43pΔ and D273-10BpΔ were derived from their respective parent strains by treatment with acriflavine, for 18 h, and then selecting for rho- colonies, as described (25). The absence of DNA was initially assessed by crossing to JM8, a rho- tester strain and then verified by cesium chloride centrifugation in the presence of 4.6-diamidino-2-phenylindole (26). Strain JM43GD9 was produced as described previously (27). Strain JM43GD6 was constructed as follows. The COX6 gene in JM43 was disrupted by use of the plasmid pVirSt3 (28), which is derived from pUC19. The URA3 gene was inserted into the HindIII site that is within the COX6 coding region on pVirSt3 and the resulting plasmid was used to transform JM43 by the lithium acetate procedure (29). Confirmation that the COX6 gene was disrupted was achieved by Southern blot analysis, Northern blot analysis, and Western immunoblotting of mitochondrial proteins. Strains aM10–150-4D, M9-3, DS-80, and aM17–162-4D were derived from strain D273-10B; they were kindly provided by Dr. Alexander Tzagoloff. The mitochondrial DNA in aM10–150-4D carries a 7.5-kilobase deletion that spans the region from 47.5 to 60 map units and that completely removes the coxl genetic locus (30, 31). Strain aM17–162-4D carries a mit- mutation in COB2 region of the the cytb gene (32). Strain DS-80 is a rho- strain that has a mitochondrial genome consisting of a tandemly repeated segment of mitochondrial DNA that is 2.6 kilobase pairs in length. This 2.6-kilobase pair repeat spans the paromomycin-resistant locus and encodes the 15-s RNA gene. Strain M9-3 carries a mit- mutation in the cox3 gene (33). Strain VC36 carries a cox2 promoter mutation which prevents transcription of cox2 (34). Genetic complementation tests were routinely performed to be sure that the neither the nuclear cox mutants nor the mit- mutants used in this study were rho-. The nuclear cox mutants, JM43GD6 and JM43GD9, were checked for their ability to complement the respiratory deficiency in JM6 and the mit- mutants, aM10–150-4D, VC36, and aM17–162-4D, were checked for their ability to complement the respiratory deficiency in M9-3. Yeast were propagated in Escherichia coli HB101 or DH5α and purified by alkaline hydrolysis (35).

**Growth Conditions**—Yeast cells were grown in either YPGal (1% Bacto-yeast extract, 2% Bacto-peptone, 1% galactose) or a semi-synthetic galactose medium containing Tween 80, ergosterol, and silicon antifoam (SSG-TEA; per liter: 10 g of galactose, 3 g of Bacto-yeast extract, 1 g of KH2PO4, 0.8 g of (NH4)2SO4, 0.7 g of MgSO4·7H2O, 0.5 g of NaCl, 0.4 g of CaCl2, 5 µg of FeCl3, 0.1% Tween 80 (v/v), 20 µg of ergosterol, and 350 ppm Dow Corning FG-10 silicon antifoam). To obtain a more uniform dispersion, the Tween 80, ergosterol, and silicon antifoam were sonicated in solution prior to autoclaving. Amino acids and nucleotides were added, as appropriate, at a concentration of 40 µg/liter. Cells were grown at 28–30 °C either in YPGal in Delong flasks on a shaker (200 rpm) or in semi-synthetic galactose medium in a New Brunswick Bioflo IIc fermentor, equipped with a gas-flow train that allows cells to be cultured at any desired oxygen concentration and to be shifted between oxygen concentrations (36). The fermentor was inoculated with precultures that were grown aerobically on a shaker at 28–30 °C to mid-exponential phase. The temperature, pH, and sparge rate were maintained as described (16). The dissolved oxygen concentration in the fermentor was monitored continuously and the oxygen concentration (µM) in the vessel was calculated from the measured dissolved oxygen level and based upon the oxygen solubility in the growth media at 28 °C, the ambient barometric pressure, and the pressure with the vessel (cf. Ref. 36). Anoxic and low-oxygen cultures (0–5 µM O2) were grown in the dark to prevent photoinhibition of growth (37). Nominal oxygen-free (anoxic) cultures were grown in O2-free N2 containing 2.5% CO2. To prevent trace O2 from entering the fermentor,
the gas mixture was passed through an Oxyclear O2 absorber (Lab-Clear, Oakland, CA). Cells were harvested at mid-exponential phase. During harvest cells were quick-chilled to −4 °C, washed twice with ice cold diethylpyrocarbonate-treated distilled water, and either processed for RNA immediately or frozen in liquid N2. Cycloheximide was added to cultures at a final concentration of 25 μg/ml prior to harvesting.

**RNA Isolation and Hybridization—**Total RNA was isolated from washed cells as described previously (38). RNA samples were denatured and separated on 1.8% agarose gels containing MOPS/formaldehyde buffer (20 mM MOPS, 40 mM sodium acetate, 8 mM EDTA, and 220 mM formaldehyde; cf. Ref. 39). The RNA samples were transferred to nylon membranes (PerkinElmer Life Sciences) and hybridized as described previously (40). Approximately 30 μg of RNA was loaded per lane; loading was adjusted to give equal signals for hybridization to the ACT1 gene. DNA probes were prepared by random-primer labeling of double-stranded DNA fragments using [α-32P]dCTP or [DuPont NEN; cf. Ref. 35] “All-In-One” random prime mixture (Sigma). Either plasmid-based probes or PCR fragments were used. The plasmid based probes were as follows: the probe for COX4 was an 800-bp XbaI fragment, derived from pUC-4SH (41); the probe for COX5α was a 500-bp PstI fragment from plasmid pSap5000; the probe for COX6 was a 500-bp Stul/BglII fragment from pVIST3 (28); the probe for COX6 was a 570-bp XhoI-Styl fragment from the probe for COX6; the probe for COX5b was a 240-bp ClaI/EcoRI fragment derived from pVIIas3 (27); and the probe for ACT1 was a 520-bp Styl fragment from plB155 (43). A PCR fragment of each gene was obtained by amplifying genomic DNA. The sequence of the PCR primer pairs and their coordinates are as follows: the COX4 fragment corresponded to bp 2 to 461 of the gene and was amplified with the primer pair 5′-GGATCTGGTTGCTGTTGTTGTT-3′ and 5′-ACCCGGCCAATGCTTCC-3′; the COX5α fragment corresponded to bp 10 to 454 of the gene and was amplified with the primer pair 5′-AACACCTTTACTAGAAGGTGGAAC-3′ and 5′-ATGAGCCTGAGATAAACC-3′; the COX6 fragment corresponded to bp 4 to 844 of the gene and was amplified with the primer pair 5′-CAAGGCGCATTACGAGAATTCC-3′ and 5′-AGAAGCTTGGGATAGCCTC-3′; the COX5b fragment corresponded to bp 10 to 251 of the gene and was amplified with the primer pair 5′-ATGTTGTGCCAACAGATGAT-3′ and 5′-CAAGGGCCATATTCAGAGA-3′; the COX9α fragment corresponded to bp 11 to 165 and was amplified with the primer pair 5′-CCTCAATTCGGAGTCGATC-3′ and 5′-TTTCTCCTCAGCTGAC-3′; and the ACT1 fragment corresponded to bp 3 to 546 of the gene and was amplified with the primer pair 5′-ATGTTGTGCCAACAGATGAT-3′ and 5′-AGAAGCTTGGGATAGCCTC-3′. After amplification, PCR fragments were gel-purified on a 1% TAE-agarose gel (35). Stringency washes were performed as described previously (21). Signal intensity was measured with either an AMBIS Radiolabel Imaging System or a Molecular Dynamics Storm 860 PhosphorImager.

To quantify the transcripts, the relative signal strength was normalized to the level of ACT1 mRNA. To determine whether levels of actin mRNA are affected by mitochondrial genotype, we prepared total nuclear acids from JMG3 and JM43/3 cells grown to mid-exponential phase in YPGal, chemically quantitated the amounts of RNA and DNA, and probed a Northern blot of the RNA with the [32P]-labeled ACT1 probe. When normalized to cellular DNA the amount of actin mRNA did not vary by more than 5%, indicating that the expression of ACT1 is unaffected by mitochondrial genotype.

**Cell Fractionation—**Whole cell extracts were prepared by shaking with glass beads (44). For preparation of mitochondrial and cytosolic fractions, cells were grown to mid-exponential phase, harvested, and spheroplasted as described (23), except that cycloheximide was added to a concentration of 25 μg/ml to all buffers used prior to the production of spheroplasts from anoxic cells. All steps after spheroplasting were performed at 4 °C. Spheroplasts were harvested by centrifugation (5 min at 3,000 × g), washed gently in post-spheroplast buffer (1.5 M sorbitol, 1 mM Na2EDTA, 0.1% bovine serum albumin, pH 7.0), and sedimented at 3,000 × g for 5 min. Washed spheroplasts were resuspended in lysis buffer (0.6 M mannitol, 2 mM Na2EDTA, 0.1% bovine serum albumin, pH 7.4), lysed in a Sorvall Omnimixer at low speed for 3 s at 4 °C, centrifuged for 5 min at 30,000 × g, and the pellet was dissected into fractions, cells, nuclei, and debris. The resulting supernatant was decanted and centrifuged for 10 min at 12,100 × g to pellet mitochon-

dria. The mitochondrial pellet was washed by resuspension in mitochondrial lys buffer minus bovine serum albumin (pH 7.0), homogenized with a glass-Teflon homogenizer, and centrifuged at 1,651 × g for 5 min. The resulting supernatant was decanted and centrifuged at 25,500 × g for 10 min to pellet the mitochondria. The post-mitochondrial supernatant, collected after the 12,100 × g centrifugation, was used as the cytosol.

**Western Immunoblotting—**Whole cells extracts were subjected to SDS-polyacrylamide gel electrophoresis in gels containing 16% acrylamide, 10% glycerol, and 4.8 M urea (20). Western immunoblotting was done as described (45), except that proteins were blotted onto Immobilon PVDF (Millipore) or Hybond ECL (Amersham Pharmacia Biotech) nitrocellulose membranes, according to the manufacturer’s instructions. Anti-cytochrome-c oxidase subunit-specific sera were raised in rabbits to high performance liquid chromatography purified yeast cytochrome-c oxidase subunits (20). Immuneactivity was detected either by 125I-protein A followed by autoradiography, or horseradish peroxidase-linked secondary antibodies followed by chemiluminescence using a luminol detection kit, as indicated in the figure legends. Immunoblots were quantitated digitally using Kodak ID software.

**Measurement of Mitochondrial Cytochromes and Respiratory Chain Activities—**Absorption spectra of cytochromes were measured in whole cells by low temperature difference spectroscopy at room temperature, with an Amino DW-2000 double beam dual wavelength spectrophotometer, as follows. Cells were grown in YPGal to midexponential phase and harvested at mid-exponential phase, washed cold diethylpyrocarbonate-treated distilled water, and either processed for preparation of mitochondrial and cytosolic fractions, cells, nuclei, and debris. The resulting supernatant was decanted and centrifuged for 10 min at 12,100 × g to pellet mitochon-
dria. The mitochondrial pellet was washed by resuspension in mitochondrial lys buffer minus bovine serum albumin (pH 7.0), homogenized with a glass-Teflon homogenizer, and centrifuged at 1,651 × g for 5 min. The resulting supernatant was decanted and centrifuged at 25,500 × g for 10 min to pellet the mitochondria. The post-mitochondrial supernatant, collected after the 12,100 × g centrifugation, was used as the cytosol.

**RESULTS**

**Down-regulation of Aerobic Nuclear COX Genes and Their Protein Products in Anoxic Cells—**Previously, we have found that mRNA levels from COX4, COX5α, COX6, COX7, COX8, and COX9 are all reduced in hypoxic and anoxic cells and that the level of expression of these genes is determined by oxygen concentration per se (40). Each gene is down-regulated at reduced oxygen concentrations, with the largest decrease in expression of these genes occurring at oxygen concentrations below 1 μM O2. The effects of oxygen concentration on the expression of these genes is conveniently assayed by Northern blot hybridization using gels on which the transcript load for ACT1, a gene whose expression is unaffected by oxygen concentration (47), is held constant. Comparison of transcript levels in normoxic and anoxic cells is shown in Fig. 1A. Transcript levels for these genes in anoxic cells have been estimated previously at 17 (for COX4), 23 (for COX5α), 31 (for COX6), 26 (for COX7), 25 (for COX8), and 39% (for COX9) (40). It is surprising that these genes are transcribed at all in anoxic cells, given that they encode subunits of an enzyme, cytochrome-c oxidase, a protein that uses oxygen as a substrate, and given that anoxic cells lack cytochrome-c oxidase activity and cytochromes aa3 (48). Insofar as the precise function of many of these nuclear-encoded subunits in holocytochrome-c oxidase is not yet established it is conceivable that they have functions in anoxic cells that are unrelated to their function as subunits of holocytochrome-c

---

2 The abbreviations used are: MOPS, 4-morpholino propane sulfonic acid; PCR, polymerase chain reaction; bp, base pair(s).
3 C. Dagsgaard, and R. O. Poyton, unpublished data.
oxidase. Of course, this assumes that the transcripts for these subunits are translated in anoxic cells and that the subunits themselves are stable under anoxic conditions. To determine whether this is the case we examined promitochondrial and cytosolic fractions from anoxic cells, using cytochrome-c oxidase subunit-specific antibodies. From Fig. 1B it is clear that subunits VI, VII, and VIIa are present in promitochondria and that subunits IV and VIII are essentially absent. As expected, the subunit V isoform present in aerobic mitochondria is Va and the predominant subunit V isoform present in promitochondria is Vb. To determine the relative levels of these subunits in promitochondria we quantitated immunoblots produced from two independent preparations of mitochondria and promitochondria. Their average level in promitochondria is 0.2 (subunit IV), 9.5 (subunit Va), 52 (subunit VI), 23 (subunit VII), 51 (subunit VIIa), and 0% (subunit VIII) of their level in mitochondria.

Mitochondrial Respiration Is Not Required for the Down-regulation of Aerobic COX Genes during a Shift to Anoxia—The above results confirm that the aerobic nuclear COX genes are down-regulated in response to reduced oxygen concentration. The kinetics of this down-regulation for COX4 and COX5a, after shifting cells from normoxic to anoxic conditions, is seen in Fig. 3A, lanes 2–6. It is obvious that transcript levels for each gene decline rapidly and that both reach their anoxic levels by 2 h after the shift (compare Fig. 3A, lanes 6 and 7). Thus, transcript levels from these genes change quickly in response to a shift from normoxia to anoxia. The down-regulation of these aerobic genes during a shift from normoxia to anoxia is interesting in light of the recent finding that the mitochondrial respiratory chain is required for the induction of some hypoxic nuclear genes in yeast and mammalian cells (15, 16) during this sort of shift. Indeed, it raises the question of whether the respiratory chain contributes to the down-regulation of these aerobic genes during a shift from normoxia to anoxia. To address this we examined the down-regulation of these same two genes in the rho0 strain, JM43p0. This strain lacks a mitochondrial genome and is respiration-deficient (Table II). From Fig. 3B it is clear that transcript levels from both COX4 and COX5a are reduced in JM43p0. It is also clear that they decline rapidly after the shift with decay kinetics that are similar, if not identical, to those observed for JM43 (Fig. 3A). These results indicate that neither mitochondrial respiration nor cytochrome-c oxidase is required for the reduction in COX4 and COX5a transcript levels as cells adapt to anoxic conditions.

Mitochondrial Involvement in the Expression of Aerobic Nuclear COX Genes—To further explore a possible involvement of the mitochondrion in the expression of the aerobic nuclear COX genes we next compared the levels of the mRNAs encoded by COX4, COX5a, COX6, COX7, COX8, COX9, and ACT1, as described under “Experimental Procedures.” The ACT1 transcript was used as an internal control for loading. Lane 1, blot of RNA from normoxic cells. Lane 2, blot from anoxic cells. Panel B, Western blots of mitochondria and promitochondria with subunit-specific antisera to cytochrome-c oxidase subunits IV–VIII. Subunit-specific antisera to subunits IV, VI, VII, VIIa, and VIII were made from high performance liquid chromatography purified subunits. The antiserum to subunit V was made to a synthetic peptide which duplicates the 20-amino acid sequence common to both Va and Vb at their carboxyl terminus. Proteins were separated on a 16% SDS-polyacrylamide gel containing 10% glycerol and 4.8 M urea and transferred to nitrocellulose. Cytochrome-c oxidase subunits were detected with a subunit-specific primary antibody followed by horseradish peroxidase-linked secondary antibodies. Immunoreactivity was detected by chemiluminescence using a chemiluminescence detection kit (PerkinElmer Life Sciences). 10 µg of mitochondrial protein was loaded in each lane. Lane 1, aerobic mitochondria. Lane 2, anaerobic promitochondria.
mRNA from all five COX genes are reduced in the rho<sup>o</sup> strain (Fig. 4A). To quantitate the down-regulation of these genes in rho<sup>o</sup> strains, we used Northern blots representing three separate cultures of JM43 and JM43<sup>ρ<sup>o</sup></sup>. In addition, we compared the level of expression of these genes in three different cultures of D273-10B<sup>o</sup>, a respira
tion-competent strain, D273-10B<sup>ρ<sup>o</sup></sup>, a respiration-deficient rho<sup>o</sup> derivative of D273-10B, and DS-80, a rho<sup>−</sup> derivative of D273-10B. Northern blots were quantitated by counting the radioactivity associated with each band, and normalizing to mRNA from ACT1, a gene whose expression is not affected by the presence or absence of a mitochondrial genome (see “Experimental Procedures,” and Ref. 49). From
Northern blots were quantitated with an Ambis radiolabelled imager or a Molecular Dynamics Storm PhosphorImager and normalized to the level of ACT1 mRNA. To facilitate comparison between strains, transcript levels are expressed as a decimal percentage relative to their levels in their respective respiratory-proficient parent strains (D273-10B for strains D273-10B and DS80, and JM43 for JM43). Values and standard deviations are given for three independent determinations for each strain and their respective parents.

| Strain        | COX4       | COX5a      | COX6      | COX8      | COX9      |
|---------------|------------|------------|-----------|-----------|-----------|
| JM43\(^{r0}\) | 0.39 ± 0.04| 0.29 ± 0.02| 0.29 ± 0.07| 0.39 ± 0.04| 0.33 ± 0.09|
| D273–10B      | 0.31 ± 0.03| 0.33 ± 0.07| 0.35 ± 0.05| 0.33 ± 0.02| 0.42 ± 0.05|
| DS80          | 0.28 ± 0.06| 0.30 ± 0.07| 0.30 ± 0.04| 0.29 ± 0.02| 0.38 ± 0.08|

Table III it is clear that the levels of the nuclear aerobic COX transcripts are reduced by 2.5–3.5-fold in the both of the rho\(^{0}\) strains and in the rho\(^{-}\) strain, relative to their respective parental strains. Further evidence for the down-regulation of COX4, COX5a, COX6, COX8, and COX9 in a rho\(^{0}\) mutant strain comes from comparing the levels of their protein products in JM43 and JM43\(^{r0}\). The Western immunoblot in Fig. 4B demonstrates that the levels of subunits IV and VI are reduced while subunits Va, VIIa, and VIII are absent in JM43\(^{r0}\). Subunits IV and VI are present in JM43\(^{r0}\) at 20 and 16%, respectively, of their level in JM43.

Rho\(^{0}\) cells differ from rho\(^{-}\) cells both genotypically and phenotypically. They differ genotypically in lacking a mitochondrial genome and they differ phenotypically in lacking mitochondrial respiration. To determine whether it is the absence of respiration that leads to the down-regulation of the nuclear COX genes we first analyzed the levels of their mRNAs in two nuclear mutants, JM43GD6 and JM43GD9 (Fig. 5). These strains are isogenic with JM43 except that they carry null mutations in the nuclear COX genes, COX6 and COX9, respectively. Moreover, they are rho\(^{-}\) and carry a fully functional mitochondrial genome, as judged by their ability to complement a rho\(^{0}\) strain. Both JM43GD6 and JM43GD9 are respiration-deficient (Table II). From Fig. 5 and Table IV it is clear that the levels of transcripts from COX4, COX5a, COX6, COX8, and COX9 in either JM43GD6 or JM43GD9 are comparable to their levels in their respiratory-competent parent strain, JM43, and higher than levels in JM43\(^{r0}\). It should be noted that both JM43GD6 and JM43GD9 are null mutants in an essential nuclear-encoded subunit of cytochrome-c oxidase. They are devoid of cytochrome-c oxidase activity and spectrally detectable cytochromes aa\(_3\) (Fig. 6) but retain normal levels of the other respiratory chain complexes (Ref. 27, and data not shown). In short, they lack an assembled active holocytocrome-c oxidase but have an otherwise normal respiratory chain. The finding that these two strains express the nuclear aerobic COX genes at the normal levels found in their respiration-competent parent indicates that the presence or absence of a functional holocytochrome-c oxidase does not affect the expression of these genes.

In a second experiment, we analyzed the levels of mRNA from the aerobic nuclear COX genes in mutants that carry missense mutations in two mitochondrial COX genes (cox1 and cox2) and in the cytochrome b gene, cyt6. These mutants are isochromosomal with the respiratory-proficient strain, D273-10B, and like the rho\(^{0}\) strain, D273–10B\(^{r0}\), derived from D272-10B, they are respiration-deficient (Table II). Aside from carrying mit\(^{-}\) mutations in cox1, cox2, or cyt6 these strains carry a rho\(^{0}\) mitochondrial genome, as judged from their ability to complement one another and produce respiration-proficient diploid strains when mated. From Fig. 7 and Table IV it is clear that the levels of transcripts from COX4, COX5a, COX6, COX8, and COX9 in the aM17–162–4D, aM10–150–4D, and VC36 are equivalent to those levels observed in the respiratory competent parent strain, D273-10B. Together, these data with JM43GD6, JM43GD9, aM17–162–4D, aM10–150–4D, and VC36 demonstrate that a deficiency in respiration per se cannot account for the decrease in COX gene mRNA levels observed in rho\(^{0}\) cells.

The Mitochondrial Genome Is Essential for Optimal Expression of Aerobic Genes under Anoxic Conditions—Because the three subunit polypeptides (Atp6, Atp8, and Atp9) that make up the proton membrane channel sector of the yeast ATP synthase are encoded by mitochondrial genes (50) rho\(^{0}\) cells lack the ability to perform oxidative phosphorylation. It is difficult to determine whether oxidative phosphorylation is responsible for the down-regulation of the nuclear COX genes observed in rho\(^{0}\) cells by using ATP synthase mutants because yeast strains that carry mutations which affect the function or assembly of the ATP synthase have highly unstable mitochondrial genomes (51). In addition, uncouplers, which can be used to inhibit oxidative phosphorylation in isolated mitochondria, are problematic for intact yeast cells because they affect its plasma membrane H\(^{+}\)-ATPase (52). Fortunately, it is possible to assess whether the lack of oxidative phosphorylation leads to a down-regulation of nuclear COX genes directly in rho\(^{0}\) cells by comparing their level of expression in anoxic rho\(^{0}\) and rho\(^{-}\) cells. Promitochondria from anoxic rho\(^{0}\) cells lack mitochondrial respiration and oxidative phosphorylation but contain a mitochondrial genome, while promitochondria from rho\(^{-}\) cells lack respiration, oxidative phosphorylation, and a mitochondrial genome. If a defect in oxidative phosphorylation is responsible for the down-regulation of nuclear COX genes in rho\(^{0}\) cells...
one would expect the level of expression of these genes to be the same in anoxic rho^0 and rho^1 cells. Conversely, if it is the lack of a mitochondrial genome per se that is responsible then the level of expression of the nuclear COX genes should be higher in anoxic rho^1 cells than in anoxic rho^0 cells. To test which of these possibilities is correct, strains JM43 and JM43GD6 were cultured in 2.5% CO2 in oxygen-free N2 and mRNA levels from COX4, COX5a, COX6, COX8, and COX9 were measured. From Fig. 8 it is clear that the levels of expression of these genes is reduced in JM43^r0 relative to JM43. Their expression in anoxic JM43^r0 cells was between 16 and 36% of their levels in anoxic JM43 cells.

These findings imply that the down-regulation of nuclear COX genes in rho^0 cells is independent of both respiration and oxidative phosphorylation, and that the mitochondrial genome is essential for optimal expression of these genes even in anoxic cells. This suggests that it is the absence of one or more mitochondrial genes, or their ability to be expressed, that leads to the down-regulation of aerobic COX genes in rho^0 cells. Although anoxia and the lack of a mitochondrial genome both result in the down-regulation of aerobic nuclear COX genes these results also indicate that these two effectors of nuclear COX gene expression work independently of one another.

**DISCUSSION**

The results of this study provide interesting new insight concerning the regulation of aerobic COX genes in yeast. First, they demonstrate that both oxygen and the mitochondrial genome exert a positive effect on the expression of these genes but that mitochondrial respiration per se has no effect. Second, they show that a mitochondrial genome is required for optimal expression of these genes under both normoxic and anoxic conditions. Third, they show that the down-regulation brought about by reduced oxygen concentration and the down-regulation brought about by the absence of a mitochondrial genome are independent of one another, ruling out the possibility that the down-regulation of these genes in hypoxic or anoxic cells is mediated by the mitochondrion. Together, these findings imply that at least one mitochondrial gene is involved in a signaling pathway to the nucleus, that this pathway is operative under normoxic and anoxic conditions, and that this pathway does not involve respiration.

**Table IV**

Relative transcript levels in respiration-deficient strains

| Strain   | Gene | COX4 | COX5a | COX6 | COX8 | COX9 |
|----------|------|------|-------|------|------|------|
| JM43     | 1.00 | 1.00 | 1.00  | 1.00 | 1.00 | 1.00 |
| JM43GD6  | 0.87 | 1.15 | 0.94  | 1.02 | 0.88 |
| JM43GD9  | 0.84 | 1.02 | 0.95  | 1.00 | 0.87 |
| D273-10B | 1.00 | 1.00 | 1.15  | 1.23 | 1.13 |
| aM10–150-4D | 0.90 | 1.05 | 0.98  | 0.98 | 0.96 |
| VC36     | 1.12 | 1.30 | 0.98  | 0.98 | 0.96 |
| aM17–162-4D | 0.93 | 1.04 | 0.98  | 0.98 | 0.96 |

* Genes and their transcripts deleted from this strain.

**Fig. 7.** Northern blot analysis of nuclear COX gene mRNAs in strains D273-10B, D273-10B^r0, aM10–150-4D, VC36, and aM17–162-4D. Poly(A') RNA was prepared, electrophoresed, blotted, and hybridized with gene specific probes for ACT1, COX6, COX4, COX9, COX5a, and COX8. Northern blot of mRNA from D273-10B (lane 1); D273-10B^r0 (lane 2); aM10–150-4D (lane 3); VC36 (lane 4); and aM17–162-4D (lane 5).
All three mitochondrially encoded subunits of cytochrome oxidase, including the two subunits (I and II) that make up its catalytic core, are expressed under conditions where its substrate (i.e. oxygen) is unavailable is intriguing. One is led to wonder if they coassemble with one another into a protein complex and what their function, if any, might be in the promitochondria of aerobic cells.

Mitochondrial Effects on Expression of Aerobic Nuclear COX Genes—The results presented here, together with those of previous studies with yeast and mammals, demonstrate that the mitochondrion can affect the expression of nuclear genes in two fundamentally different ways. In the first, mitochondrial respiratory function is essential for the induction of some hypoxic genes as cells are shifted from normoxia to hypoxia or anoxia (15, 16). In the second, the mitochondrial genome, acting independently of its respiratory function, is essential for optimal expression of aerobic nuclear genes under both normoxic and anoxic conditions.

Recent studies with mammalian cells in culture have suggested that the mitochondrial respiratory chain participates in the regulation of hypoxic genes via the production of reactive oxygen species (15, 54, 55). It is not yet clear if reactive oxygen species are also involved in mitochondrial control of nuclear gene expression in yeast. So far, the only mitochondrially initiated pathway known to affect nuclear gene expression in this organism is retrograde regulation (49, 56, 57). This pathway is used, by yeast cells, to sense the energy state of their mitochondria. It functions to up-regulate some aerobic genes in response to the lack of mitochondrial respiration. Components of this pathway include: two subunits (Rtg1p and Rtg3p) of a heterodimeric transcription factor, Rtg2p, a cytoplasmic protein that contains an hsp-like ATP-binding site (58), and the Tup1-Cyc8 protein complex, which interacts with the Rtg1-Rtg3 heterodimer and which can either activate or repress transcription (59). It is unlikely that this pathway is involved in the expression of nuclear COX genes because cells carrying null alleles of RTG1 and RTG2 are capable of respiration-dependent growth (57) and because nuclear COX genes lack the R-box binding site for the Rtg1-Rtg3 heterodimer.

We refer to the type of mitochondrial-nuclear cross-talk uncovered by the studies presented here as intergenomic signaling. It is distinguishable from retrograde regulation in three ways. First, mitochondrial respiration is important for retrograde regulation but not for intergenomic signaling. Second, those genes that are subject to retrograde regulation are up-regulated in the absence of mitochondrial respiration while those genes that are subject to intergenomic signaling are down-regulated in the absence of a mitochondrial genome. Third, intergenomic signaling affects expression of respiratory protein genes while retrograde regulation probably does not.

The finding that the transcription of aerobic nuclear COX genes is reduced in cells that lack a mitochondrial genome but not in cells that lack respiration suggests that it is the absence of one or more mitochondrial genes, or their ability to be expressed, that is involved. This is surprising because all known mitochondrial gene products (proteins and RNA) are assumed to participate either in the function or biogenesis of the mitochondrial respiratory chain. This raises the possibility that an as yet unidentified mitochondrial gene is involved. Until recently, our understanding of the yeast mitochondrial genome sequence was based of a conglomerate sequence derived from several polymorphic strains (60, 61). This sequence was incomplete and contained multiple errors (62). Recently, the entire mitochondrial genome sequence was determined for a single yeast strain, FY1679 (62). The overall organization of the mitochondrial genome of FY1679 is similar to the “short mitochondrial genomes” of the two respiration-proficient strains, D273-10B and JM43, used here except for two deletions and some differences in the flanking regions of some protein-coding genes. Surprisingly, the sequence of the FY1679 mitochondrial genome revealed seven new small open reading frames. The function of these putative protein-coding genes is not yet known so it is possible that one or more of them is involved in intergenomic signaling. Alternatively, it is possible that a mitochondrial gene product involved in respiration is multifunctional and participates in both respiration and intergenomic signaling. Precedent for the multifunctionality of mitochondrial proteins comes from the finding that cytochrome c can function both as an electron carrier in the mitochondrial respiratory chain and in the activation of cytosolic caspases during apoptosis (63), and from the finding that Atp6, which is a mitochondrial gene product that functions as a subunit of ATP synthase, is important for the stability of the mitochondrial genome (51). Finally, it is possible that the physical presence of the mitochondrial genome, or a complex of which it is a part, is required.

The identification of which mitochondrial gene(s) is (are) involved in intergenomic signaling should help in understanding how the mitochondrial genome sends “signals” to the nucleus. It will require an exhaustive analysis of mit− mutants in individual mitochondrial genes and rho− mutants that retain different parts of the mitochondrial genome. The limited set of mitochondrial mutants (e.g. am17–162-4D, aM10–150-4D,
VC36, and DS-80) used in this study indicate that the ctb, cox1, cox2, and 15S rRNA genes, by themselves, are not involved.

Acknowledgments—We thank Drs. John Trawick and Norbert Kraut for early contributions to this study and Dr. Alexander Tzagoloff for strains.

REFERENCES

1. Bunn, H. F., and Poyton, R. O. (1996) Physiol. Rev. 76, 839–885
2. Hochachka, P. W., Buck, L. T., Dull, C., and Land, S. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9493–9498
3. Hochachka, P. W. (1998) J. Exp. Biol. 201, 1243–1254
4. Sehmer, G. L. (2000) Adv. Exp. Med. Biol. 475, 303–310
5. Acker, H. (1994) Annu. Rev. Biochem. 71, 3–10
6. Huang, L. E., Ho, V., Arany, Z., Kmysia, E., Gasson, D., Tendler, D., Livingston, D. M., and Bunn, H. F. (1997) Kidney Int. 51, 548–552
7. Batcliffe, P. J., Maxwell, P. H., and Pugh, C. W. (1997) Nucleol. Biol. Transplant. 12, 1842–1848
8. Semenza, G. L. (1999) Cell 96, 281–284
9. Poyton, R. O. (1999) Respir. Physiol. 115, 119–133
10. Chandel, N. S., and Schumacker, P. T. (2000) J. Exp. Biol. 203, 1880–1889
11. Wilson, D. F., Mokashi, A., Chugh, D., Vinogradov, S., Osanai, S., and Lahiri, S. (1994) FEBS Lett. 151, 370–374
12. Wilson, D. F., Mokashi, A., Lahiri, S., and Vinogradov, S. A. (2000) Adv. Exp. Med. Biol. 475, 259–264
13. Lahiri, S., Buerk, D. G., Chugh, D., Osanai, S., and Mokashi, S. (1995) Brain Res. 684, 194–200
14. Acker, H., and Xu, D. (1995) New Physiol. Sci. 10, 211–216
15. Chandel, N. S., Maltepe, Goldwasser, E., Chugh, D., Mokashi, S. C., and Schumacker, P. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11715–11720
16. Kow, T. E., Burke, P. V., Staalsh, B., and Poyton, R. O. (1996) Proc. Natl. Acad. Sci. U. S. A. 96, 5446–5451
17. Poyton, R. O., and McEwen, J. E. (1996) Biochem. J. 319, 18672–18677
18. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-itoh, K., Nakashima, R., Yasuo, R., and Yoshikawa, S. (1996) Biochem. Biophys. Res. Commun. 223, 325–331
19. TerLinde, J. J. M., Liang, H., Davis, R. W., Steensma, H. Y., van Dijken, J. P., and Pronk, J. T. (1999) J. Biol. Chem. 274, 14705–14712
20. Butow, R. A. (1988) Mol. Biol. Cell. 19, 110–118
21. Cumskey, M. G., Ko, K., Trueblood, C. E., and Poyton, R. O. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 2235–2239
22. Mattick, J. S., and Nagley, P. (1977) Mol. Gen. Genet. 152, 267–276
23. Williams, D. H., and Penneu, D. J. (1975) Methods Cell Biol. 12, 335–351
24. Wright, R. M., Dirks, L. K., and Poyton, R. O. (1986) J. Biol. Chem. 261, 17183–17191
25. Williamson, D. H., and Fennell, D. J. (1975) J. Biol. Chem. 250, 1136–1144
26. Maarse, A. C., VanLoon, A. P. G. M., Riezman, H., Gregor, I., Schatz, G., and Grivell, L. A. (1984) EMBO J. 3, 2831–2837
27. Patterson, T. E., and Poyton, R. O. (1986) J. Biol. Chem. 261, 17192–17197
28. Patterson, T. E., and Poyton, R. O. (1986) J. Biol. Chem. 261, 17192–17197
29. McEwen, J. E., Ko, C., Kloeckener-Gruissem, B., and Poyton, R. O. (1986) J. Biol. Chem. 261, 11872–11879
30. Tschau, H., Staezel, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
31. Tzagoloff, A., Akai, A., and Needleman, R. J. (1975) Biol. Chem. 250, 223–235
32. TerLinde, J. M., Liang, H., Davis, R. W., Steensma, H. Y., van Dijken, J. P., and Pronk, J. T. (1999) J. Bacteriol. 181, 7409–7413
33. Rogers, P. J., and Stewart, P. R. (1973) J. Bacteriol. 115, 88–97
34. Lia, X., Small, V. L., Stere, P. A., and Butow, B. A. (1991) Mol. Cell. Biol. 11, 38–46
35. Petersen, P. L., and Amzel, L. M. (1993) J. Biol. Chem. 268, 9937–9940
36. Butow, R. A. (1988) Philos. Trans. R. Soc. Lond. B Biol. Sci. 319, 127–133
37. Lia, X., and Butow, R. A. (1993) Cell 72, 61–71
38. Rothermer, B. A., Shyjan, A. W., Etheridge, J. L., and Butow, R. A. (1995) J. Biol. Chem. 270, 29476–29482
39. Conlan, R. S., Goulardali, N., Hatzi, P., and Tzamarias, D. (1999) J. Biol. Chem. 274, 205–210
40. de Zamarro, C., and Bernardi, G. (1996) Gene (Amst.) 41, 155–177
41. Tzagoloff, A., and Myers, A. M. (1986) Annu. Rev. Biochem. 55, 249–285
42. Fournier, F., Roganti, T., Leclerc, N., and Purnelle, B. (1998) FEBS Lett. 440, 325–331
43. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
44. Li, M., Tzagoloff, A., Underbrink-Lyon, K., and Martin, N. (1982) J. Biol. Chem. 257, 5921–5928