Regulation of the Nuclear-Coded Peptides of Yeast Cytochrome *c* Oxidase†

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ABSTRACT: We have analyzed the catabolite regulation of cytochrome oxidase by assaying changes in the synthesis of precursors of the nuclear-coded peptides (IV–VII) of cytochrome *c* oxidase in an in vitro reticulocyte cell-free system programmed with RNA isolated from cells grown in either glucose or raffinose. As a first step, we have characterized antibodies which bind to the precursors of subunits V and VI. Initial translation products for subunits IV and VII have also been tentatively identified by utilizing these antibodies. The messenger RNAs coding for the precursors of the nuclear-coded subunits fall in the expected size range of 8–15 S. Catabolite repression of the nuclear-coded oxidase peptides appears to be regulated by the abundance of their messenger RNAs. Translation of messenger RNA isolated from yeast cells grown on glucose indicates a coordinate and uniform increase in precursor synthesis during glucose derepression. In contrast, when RNA isolated from raffinose (derepressed) grown cells is used to direct cell-free translation, precursor abundance is high throughout growth, although the synthesis of some of the species changes in a complex pattern of ratio and abundance. These data indicate that the abundance of the messengers for the nuclear-coded precursors is regulated in a fashion dependent on the physiologic state of the cell.

The biogenesis of the mitochondrion is governed by interactions between the nuclear and mitochondrial genetic systems. In yeast, this interaction is influenced by a variety of physiologic states. Mitochondrial development can be repressed or derepressed, depending on the glucose concentration in the growth medium, on oxygen tension, and on the abundance of heme (Gollub et al., 1977; Linnane et al., 1972; Mahler et al., 1975b; Slonimski, 1953; Woods et al., 1975).

Yeast cytochrome *c* oxidase (cytochrome aa_3), the terminal component of the mitochondrial electron-transport chain, reflects the biosynthetic complexity of the mitochondrion as a whole. Cytochrome oxidase is an enzyme composed of seven peptides; three of these peptides (I–III), of molecular weights 40 000, 33 000, and 22 000, are specified by the mitochondrial genome, whereas the four subunits (IV–VII) of molecular weights 14 300, 13 200, 12 500, and 4500 are encoded by the nuclear genome (Mason et al., 1973; Mason & Schatz, 1973; Poyton & Schatz, 1975). The activity of the oxidase as well as the biosynthesis or accumulation of its subunit constituents appears to be regulated by physiologic states such as glucose repression (Perlman & Mahler, 1974), anaerobiosis (Mason et al., 1973; Slonimski, 1953), lipid depletion (Linnane & Crawford, 1975), and heme abundance (Gollub et at., 1977; Saltzberger-Muller & Schatz, 1978). The nature of this regulation is poorly understood at this time.

For investigation of the interactions between the nuclear and mitochondrial genomes which result in the synthesis and assembly of cytochrome oxidase, the nature of the primary transcripts for each of the cytoplasmic peptides of oxidase must be understood. Investigations from the laboratory of Poyton indicated the presence of a polyprotein precursor of all of the nuclear-coded peptides of oxidase (Poyton & McKemmie, 1979a,b). However, studies in the laboratories of Schatz (Lewin et al., 1980) and Blobel (Mihara & Blobel, 1980) as well as our own preliminary studies (Levens et al., 1980) have demonstrated the presence of individual precursors of the nuclear-coded peptides of cytochrome oxidase in the yeast cytoplasm and in the products of cell-free ribosomal translation systems directed by yeast mRNA. The nuclear-coded subunits of cytochrome bc_1 and oligomycin-sensitive ATPase are also present initially as discrete precursors (Cote et al., 1979; Maccecchini et al., 1979).

In this study, the oxidase precursors have been identified by means of immunologic techniques, and the approximate size of the transcripts coding for these products has been ascertained. The effect of catabolite regulation on the level of mRNA encoding the nuclear-coded precursors has been analyzed in an in vitro translation system. We have found that transcription was probably involved in the regulation of precursor synthesis during changes in the physiologic state of the yeast cell.

Materials and Methods

Conditions of Cell Growth. For most of our studies, cells from the *Saccharomyces cerevisiae* strains D273-10B or A364a were grown to stationary phase in 2% glucose, 1% bactopeptone, and 1% yeast extract followed by a 3-fold dilution in 0.3% glucose, 1% bactopeptone, and 1% yeast extract and growth for an additional 3 h. For studies on the regulation of the cytochrome oxidase precursors, cultures were grown to the cell densities noted in the text, in media containing either 1% glucose, 1% bactopeptone, and 1% yeast extract or 2% raffinose, 1% bactopeptone, and 1% yeast extract and fulfilling any auxotrophic requirements.

Isolation of Yeast RNA. Cells (30–40 g) grown under one of the conditions described above were collected by centrifugation and washed several times in cold distilled water. The cells were suspended in a buffer consisting of 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes)[1] (pH 7.6), 20 mM KCl, 3 mM $MgCl_2$, and 100 μg/mL heparin

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[1] Abbreviations: A_{260}, absorbance at 260 nm; DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; mRNA, messenger ribonucleic acid; TLCK, tosyllysyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; COAb, cytochrome oxidase antibody; CPAb, antibody to the cytoplasmic peptides.

(5–10 mL/g of cells) and broken manually with glass beads (8–15 g/g of cells) for 2 min at 0 °C (Lang et al., 1977). The lysate was centrifuged for 30 min at 10000 rpm in a Sorvall SS-34 rotor, adjusted to 0.1 M Tris (pH 9.0), 0.1 M NaCl, 1 mM NaEDTA, and 0.5% $NaDodSO_4$, and subjected to two consecutive phenol–chloroform–isoamyl alcohol (50:49:1) extractions. The final aqueous phase was adjusted to 0.3 M NaOAc (pH 5.8) and precipitated with 2–3 volumes of 95% ethanol. The precipitates were washed once with 2 M LiCl and once with 3 M NaOAc (pH 5.8) and 5 mM NaEDTA according to the technique of Shapiro et al. (1974). The final precipitate was washed with 95% ethanol, dried under nitrogen, and dissolved in water or 10 mM Tris-HCl (pH 7.4) at a concentration of 2–4 mg/mL.

Poly(A+) RNA was isolated from total RNA by the technique of Aviv & Leder (1972). Poly(A+) RNA (1–2 A_{260} units) was incubated for 5 min at 68 °C in 200–400 μL of a buffer consisting of 10 mM Tris-HCl (ph 7.4), 1 mM NaEDTA, and 0.5% $NaDodSO_4$. The RNA was loaded onto a 12-mL, 5–20% linear sucrose gradient containing the same buffer. Gradients were centrifuged to an $\omega^2 t$ of 38000 in a Beckman SW41 rotor, and 0.6-mL fractions were collected by an ISCO gradient fractionator. Each fraction was adjusted to 0.3 M NaOAc (pH 5.8), ethanol precipitated, and dissolved in a minimal volume (50–100 μL) of 10 mM Tris-HCl (pH 7.4).

Conditions of the Reticulocyte Cell-Free Translation System. The New England Nuclear reticulocyte cell-free translation system was utilized with Mg^{2+} and K^+ optima of 0.6 and 80 mM, respectively. Either 3–6 μg of total RNA or 0.3–1.0 μg of poly(A+) RNA was used to direct the system. A 10-μCi sample of [^{35}S]methionine (800–1000 Ci/mmol; New England Nuclear) was used per 25 μL of reaction mixture. The incubation was carried out for 1 h at 37 °C or for 2 h at 26 °C.

When the translation assay was followed by an immunoassay, 200 μL of reaction mixture was diluted with 1 mL of immunobuffer [10 mM NaP_i (pH 7.4), 15 mM NaCl, 1% Triton X-100, and 0.2% $NaDodSO_4$] containing 0.5 mM TLCK and 10 mM methionine. The mixture was centrifuged for 10 min at 17000*g* in an Eppendorf minifuge, and the supernatant was used for the immunoassay.

^{35}S Labeling of Yeast Mitochondrial Protein. Mitochondrial lysates were labeled in vivo with [^{35}S]sulfuric acid (New England Nuclear) by the technique of Douglas & Butow (1976). Mitochondria were lysed for 5 min at room temperature in a small volume (100–200 μL) of 10 mM NaP_i (pH 7.4) and 2% $NaDodSO_4$ and diluted 10-fold with 10 mM NaP_i (pH 7.4), 1% Triton X-100, and 0.5 mM TLCK. The resulting supernatant was centrifuged for 10 min at 17000*g* in an Eppendorf minifuge for removal of unlysed mitochondria. Samples of the lysates were frozen at −20 °C prior to immunoassay.

Isolation, Fractionation, and Iodination of Cytochrome Oxidase. Yeast cells (Fleishman's) were broken by several passes through a Manton-Gaulin press, and the mitochondria were isolated by the technique of Schatz (Mason & Schatz, 1973). Cytochrome oxidase was isolated by the technique of Werner (1977), with suitable modifications in the cholate–KCl and ammonium sulfate fractionation steps. Submitochondrial particles were suspended in 10 mM Tris-HCl (pH 7.7) and 0.25 M sucrose at a concentration of 20 mg of protein/mL. The initial KCl–deoxycholate fractionation was performed as described, except that both the green pellet and the dense reddish brown layer were saved. The material was dissolved in 10 mM Tris-HCl (pH 7.4) at a concentration of 10 mg of protein/mL. Solid KCl was added to a final concentration of 1.2 M, and sodium deoxycholate (0.45 mg/mg of protein) was added. After the solution was mixed briefly, it was centrifuged at 20000 rpm for 30 min in a Sorvall SS-34 rotor, and a 0.5 volume of 2% sodium cholate was added to the supernatant. Ammonium sulfate fractionations were performed as described, except in the second fractionation where the cytochrome oxidase was precipitated in the 27–35% fraction. Total nuclear-coded subunits IV–VI were isolated by fractionation on 12% $NaDodSO_4$–polyacrylamide gels. Purified cytochrome oxidase was also kindly provided by G. Schatz.

Cytochrome oxidase was iodinated by a modification of Hunter's technique (Hunter, 1970). A 20-μg sample of cytochrome oxidase (3.5 mg/mL) was reacted with 400 μCi of $Na^{125}I$ (Amersham, 10 mCi/mL) in 100 μL of a buffer consisting of 0.12 M KP_i (pH 7.4) and 6 μg/mL chloramine-T for 5 min at 0 °C. Sodium metabisulfite (10 μg/mL) was added to terminate the reaction. The iodinated product was isolated in the void volume of a Sephadex G-50 (medium) column equilibrated in 10 mM NaP_i (pH 7.4) and 1% Triton X-100.

Production and Isolation of Antibodies. Antibodies directed against glyceraldehyde-3-phosphate dehydrogenase (Sigma), cytochrome oxidase, or isolated cytoplasmic peptides were raised in rabbits following multiple injections of 100–500 μg of protein. In the case of the isolated cytoplasmic peptides, the homogenized polyacrylamide gel was injected into the rabbits. γ-Globulin was isolated according to the technique of Palmiter et al. (1971). Antibodies prepared against subunit V (YR-9) and subunit VI (YR-10) were kindly provided by G. Schatz.

Conditions of Immunoassay. (*A*) *Indirect Immunoprecipitation.* Double antibody precipitation was performed by a modification of the technique of Shapiro et al. (1974). Either ^{35}S-labeled mitochondrial protein or ^{35}S-labeled cell-free lysate, at (0.5–1.5) × 10^6 cpm, or ^{125}I-labeled cytochrome oxidase (10000 cpm) in 200–500 μL of immunobuffer was reacted with 5 μg of nonimmune or specific γ-globulin for 16 h at 0 °C. Goat anti-rabbit antibody (50 μg) (Miles) was then added, and the mixture was incubated at 0 °C for 5 h. The suspension was loaded onto a step gradient consisting of 50 μL of 1 M sucrose in immunobuffer and 100 μL of 0.5 M sucrose in the same buffer and centrifuged for 10 min at 10000 rpm in a Sorvall HB-4 rotor. The pellet was suspended in 400 μL of immunobuffer, and the washing procedure was repeated twice.

(*B*) *Immunobinding.* A 1.5-g sample of protein A–Sepharose CL4B (Pharmacia) was equilibrated in 100 mM NaP_i (pH 7.4) and 150 mM NaCl and briefly centrifuged. The swollen resin (5 mL) was suspended in 5 mL of the same buffer. The γ-globulin fraction (200 μg) was incubated with 1 mL of the suspended resin overnight at 0 °C on an end-over-end rotator. The resin was briefly centrifuged, washed with immunobuffer, and centrifuged again. The final resin was suspended in an equal volume of immunobuffer. A 100-μL volume of the suspended resin was reacted with the cell-free product or mitochondrial lysate for 4 h at 0 °C. The resin was centrifuged for 2.5 min at 17000*g* in the Eppendorf minifuge and washed 3 times with 1 mL of immunobuffer.

Conditions of Electrophoresis. (*A*) *Sample Preparation.* Immunoprecipitates were dissociated in 100 μL of 50 mM Tris-HCl (pH 7.0), 6 M urea, 2% $NaDodSO_4$, and 5% β-mercaptoethanol and heated at 100 °C for 2 min prior to loading onto a slab gel. Immunobound products were dissociated from protein A–Sepharose CL4B in 100 μL of the same

buffer. After a brief centrifugation, the supernatant was heated for 2 min at 100 °C prior to loading onto the slab gel.

(*B*) *Slab Gel Preparation.* $NaDodSO_4$–polyacrylamide slab gels (12%) were cast, and electrophoresis was performed according to the method of Laemmli (1970). $NaDodSO_4$–polyacrylamide gels (15%) were prepared, and the protein was fractionated essentially according to the technique of Blatter et al. (1972). Fluorography was performed by the method of Laskey & Mills (1975).

Miscellaneous Analytical Techniques. Protein was estimated by the method of Lowry et al. (1951). Oxygen consumption was assayed with a Gilson oxygraph. Any dilutions that were required for an accurate assay were made in media isolated from the same stage of growth as that of the cells being assayed. Low-temperature spectra were obtained by the method of Cottrell et al. (1975).

Results

Characterization of Antibodies Used To Detect the Nuclear-Coded Precursors of Cytochrome Oxidase. Precursors of the nuclear-coded peptides of cytochrome oxidase have previously been identified by immunological and peptide digestion techniques. Immunoreaction using antibodies specific to individual or groups of cytoplasmic cytochrome oxidase peptides and immunocompetition with the purified peptides have led to the characterization of the initial translation products of subunits IV–VII (Lewin et al., 1980; Mihara & Blobel, 1980). The precursors for subunits V and VI have been further established by peptide mapping (Lewin et al., 1980). We have developed antibodies to holocytochrome oxidase and to the cytoplasmic peptides (specific to subunit V) and have compared their specificity with anti-subunit V and anti-subunit VI provided by Dr. G. Schatz. These antibodies have been utilized to examine the effect of catabolite regulation on precursor synthesis.

The antibodies were characterized by immunobinding with ^{35}S-labeled mitochondrial lysates (Figure 1A) and ^{125}I-labeled cytochrome oxidase (Figure 1B). Cytochrome oxidase antibody (COAb) reacts with each of the oxidase peptides. COAb binds subunits IV, V, and VII in ^{35}S-labeled mitochondrial lysates and subunit VI of ^{125}I-labeled cytochrome oxidase (Figure 1A, slot 2; Figure 1B, slot 2). The failure to detect subunit VI binding in ^{35}S-labeled lysates may be the consequence of the low methionine and cysteine content of this peptide (Poyton & Schatz, 1975). Only subunit VI of ^{125}I-labeled cytochrome oxidase was immunobound, probably because of the modification of other cytoplasmic peptides during the iodination procedure.

An antibody to the cytoplasmic peptides (CPAb) binds subunit V exclusively in ^{35}S-labeled lysates (Figure 1A, slot 3). The antibodies to subunits V and VI provided by G. Schatz and our antibodies (COAb, CPAb) reacted in a similar manner. Anti-subunit V only immunobound subunit V in ^{35}S-labeled lysates (Figure 1A, slot 4). Anti-subunit VI, on the other hand, reacted only with the ^{125}I-labeled subunit VI (Figure 1B, slot 5). In several antibody preparations, small amounts of higher molecular weight contaminants were also bound.

We used these antibodies to identify the primary translation products of the nuclear-coded cytochrome oxidase subunits synthesized in a translation system directed by total cytoplasmic RNA. COAb, CPAb, and anti-subunit V bind a peptide at 15 000 daltons (Figure 2A, slots 2–4; Figure 2B, slots 1 and 2) which has been shown to be a precursor (p5) of subunit V (13 200 daltons) (Lewin et al., 1980). Variable

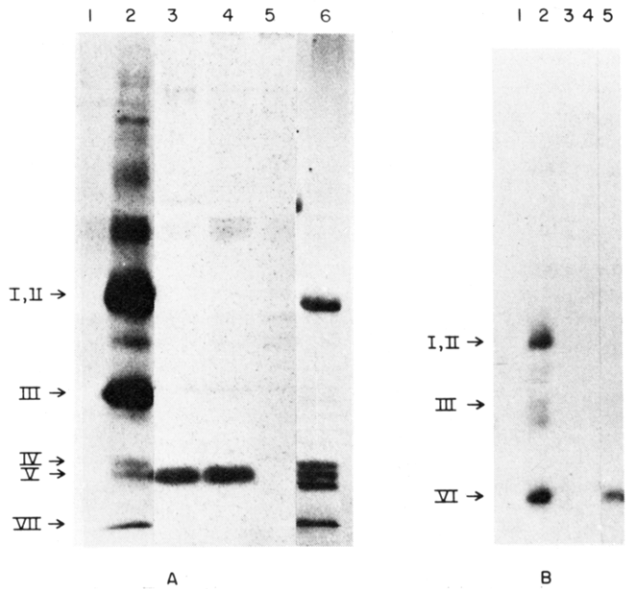

FIGURE 1: Specificity of cytochrome oxidase antibodies. Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of (A) ^{35}S-labeled mitochondrial lysates or (B) ^{125}I-labeled cytochrome oxidase, immunobound with (1) preimmune γ-globulin, (2) anti-cytochrome oxidase (COAb), (3) anti-cytoplasmic petites (CPAb), (4) anti-subunit V, and (5) anti-subunit VI. The cytochrome oxidase staining pattern (6) is shown in Figure 1A. ^{125}I-Labeled subunits IV, V, and VII react poorly with antibodies probably as a result of alterations during the iodination procedure. Arrows indicate the positions of the subunits of cytochrome oxidase (I–VII).

quantities of a peptide migrating near the position of subunit VI are also present and represent a contaminating species in the original antigen, or a degraded or incompletely translated product. COAb and anti-subunit VI react with a protein at 16 000 daltons (Figure 2A, slots 2 and 5) representing the precursor (p6) of subunit VI (12 500 daltons) (Lewin et al., 1980). In addition, COAb reacts with translation products at 18 500 and 4500 daltons (Figure 2A, slot 2). When the 4500-dalton product ("p7") is fractionated on a 20% $NaDodSO_4$–polyacrylamide gel, the peptide produced in vitro appears to have the same mobility as subunit VII (4500 daltons) (data not shown). Furthermore, addition of unlabeled subunit VII in the immunoassay results in the elimination of the binding of "p7" to COAb (Figure 2C, slots 6 and 7). This species appears to be analogous to the primary translation product of peptide VII observed by Mihara & Blobel (1980). Similarly, the peptide at 18 500 daltons appears to be analogous to that observed by Blobel as the precursor ("p4") of subunit IV (14 000 daltons) (Mihara & Blobel, 1980). We have designated the precursors of subunits IV and VII as "p4" and "p7" because of the lack of peptide mapping data and the absence of a direct comparison of the reactivity of our antibodies with those of Mihara & Blobel (1980).

Immunocompetition using highly purified cytochrome oxidase eliminates the binding of the presumptive precursors to COAb ("p4", p5, and p6) and anti-subunit VI (p6). It does not significantly affect the binding of higher molecular weight species to COAb. In this experiment, "p7" is obscured by a large amount of labeled material migrating near the front. Small quantities of higher molecular weight species (greater than 20 000 daltons) are also immunobound by CPAb and anti-subunit V. However, these antibodies, with identical subunit specificities, bind different higher molecular weight products.

Sizing of RNA Coding for Oxidase Precursors. Lewin et al. (1980) and Mihara & Blobel (1980) have shown that the

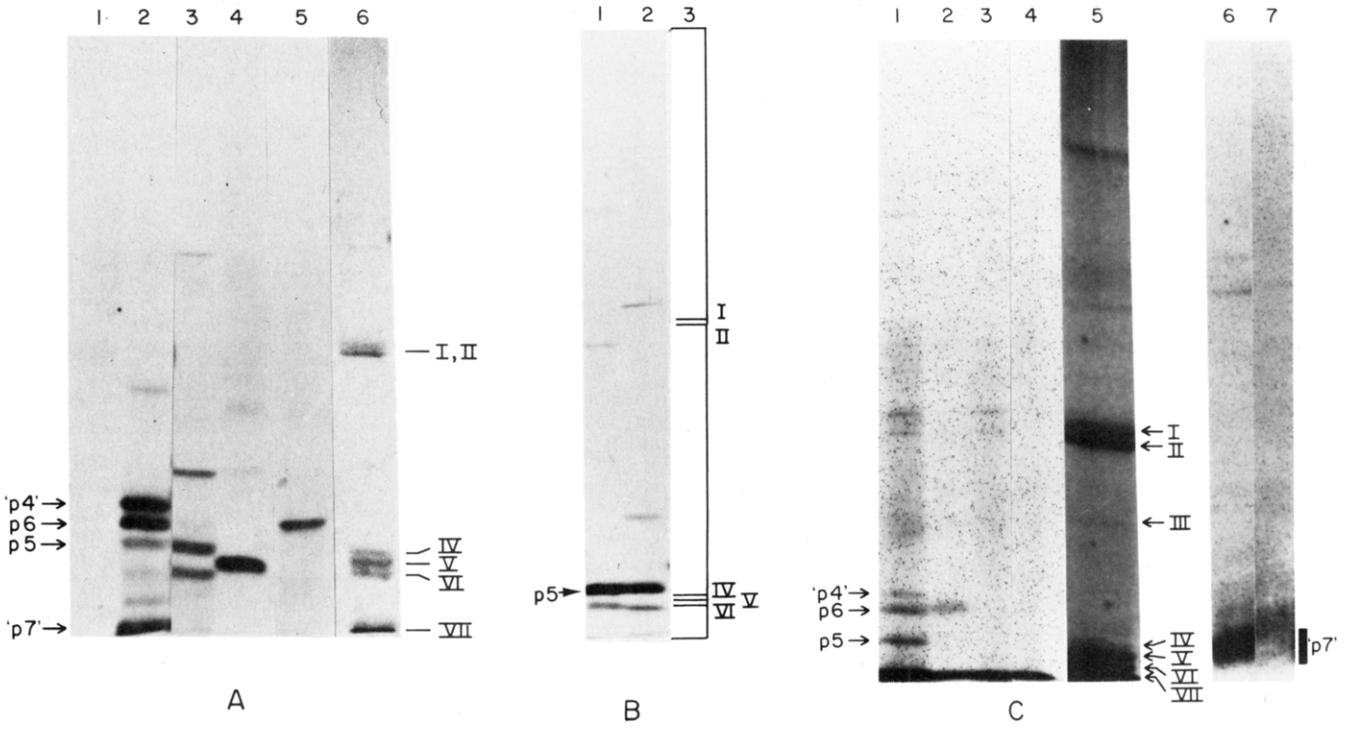

FIGURE 2: Identification of cytochrome oxidase precursors in vitro. (A) Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of [^{35}S]-methionine-labeled cell-free translation products immunobound with (1) preimmune γ-globulin, (2) anti-cytochrome oxidase (COAb), (3) anti-subunit V, and (5) anti-subunit VI and ^{35}S-labeled mitochondrial lysates immunobound with (4) anti-subunit V; (6) staining pattern of cytochrome oxidase. (B) Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of [^{35}S]methionine-labeled cell-free translation products immunobound with (1) anti-cytoplasmic peptides (CPAb) and (2) anti-subunit V; (3) representation of cytochrome oxidase staining pattern. (C, 1–5) Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of ^{35}S-labeled cell-free translation products immunobound with anti-cytochrome oxidase (COAb) (1, 3) or anti-subunits VI (2, 4) in the absence (1, 2) or presence (3, 4) of highly purified cytochrome oxidase (10–20 μg); (5) staining pattern of cytochrome oxidase. Arrows indicate the positions of the precursors. (C, 6 and 7) Fluorogram of $NaDodSO_4$–polyacrylamide gel (20%) of ^{35}S-labeled cell-free translation products directed by 8S RNA immunobound in the absence (6) or presence (7) of 0.45 μg of subunit VII purified by 20% $NaDodSO_4$–polyacrylamide gel electrophoresis. The shaded region on the right indicates the position of "p7".

oxidase precursors label with ^{35}S-labeled formylmethionine, indicating that they represent primary translation products. We have further confirmed this point by determining the size of transcripts coding for the low molecular weight species. Cytoplasmic poly(A+) RNA was fractionated on 5–20% linear sucrose gradients. Nine fractions were collected, and the RNA isolated from each fraction was used to direct the translation system (Figure 3). The ability of the gradient to discriminate broadly between RNA species has been ascertained from the molecular weight range of the translation products directed by the various fractions (data not shown) and by the fractionation of the transcripts coding for the abundant protein, glyceraldehyde-3-phosphate dehydrogenase (Figure 4, DH).

The results (Figure 4) clearly show that low molecular weight RNA (fractions 1–3; 8–15 S) codes for the precursors. Fraction 1 (8S) RNA directs the synthesis of only "p7"; fractions 2 and 3 (12–15S) RNA code for all of the precursors. Cytochrome oxidase antibody binds the 18 500- ("p4"), 16 000- (p6), and 4500-dalton ("p7") species, whereas CPAb binds the 15 000-dalton (p5) peptide. In this experiment, COAb bound poorly to p5. RNA of size greater than 15 S does not code for significant quantities of the low molecular weight products (fractions 4–9). Thus, the low molecular weight RNA codes for the short immunoreactive forms ("p4", p5, p6, "p7"). These proteins, therefore, are likely to represent primary translation products and not degradation products of a larger protein.

Catabolite Regulation of Cytochrome Oxidase Precursors. Cytochrome oxidase and a variety of other mitochondrial respiratory proteins (e.g., cytochromes *b* and *c*) are capable of undergoing repression and derepression, depending on the

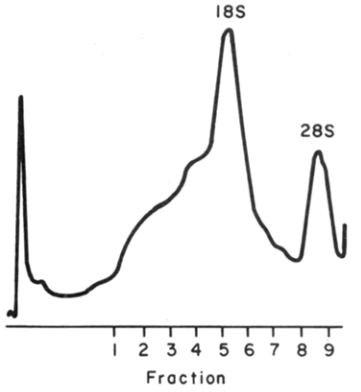

FIGURE 3: Sucrose-gradient fractionation of RNA. A_{260} profile of poly(A+) RNA fractionated on a 5–20% linear sucrose gradient. The fraction numbers are indicated at the bottom of the figure. The positions of 18S and 28S ribosomal RNA are marked.

conditions of growth (Ephrussi et al., 1956; Slonimski, 1953; Linnane et al., 1973; Perlman & Mahler, 1974). To investigate the involvement of nuclear transcription of cytochrome oxidase precursors in this process, we utilized cell-free translation and assayed changes in precursor synthesis as a function of the physiologic state. Since equal amounts of ^{35}S-labeled product are used in each immunoassay, a change in the abundance of a particular translated protein most likely reflects an alteration in the abundance of a specific transcript, although the possibility of changes in the "translatability" of the mRNA cannot be eliminated.

RNA was isolated from cells grown under various conditions. Cells were grown to midlogarithmic phase on either 5%

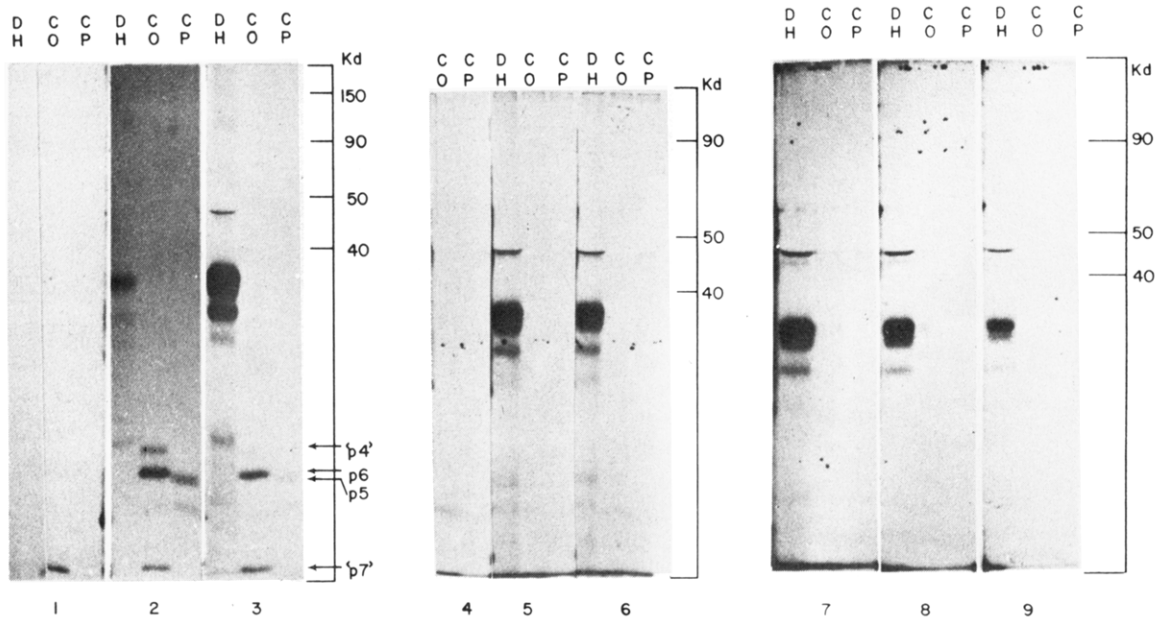

FIGURE 4: Translation and immunobinding of products directed by fractionated RNA. Fluorogram of $NaDodSO_4$–polyacrylamide gel (12%) of [^{35}S]methionine-labeled cell-free translation products directed by RNA fractionated on the 5–20% linear sucrose gradient (fractions 1–9) and immunobound with anti-glyceraldehyde-3-phosphate dehydrogenase (DH), anti-cytochrome oxidase (CO), and anti-cytoplasmic peptides (CP). The immunobound products of fractions 1–3, 4–6, and 7–9 were subjected to electrophoresis on individual gels. The amounts of ^{35}S-labeled products utilized for each immunobinding were identical except in the case of fraction 1, in which one-third of the radioactivity was used as a result of the low abundance of this RNA fraction. The arrows indicate the positions of each of the precursors.

glucose (repressive) or 2% raffinose (derepressive), or during a high (2%) to low (0.3%) glucose shift (derepressive). The reproducible pattern of immunobound peptides allowed us to identify the precursors (p5, p6) and the possible precursors ("p4", "p7"). Synthesis of all the precursors, but not total translatable mRNA activity, was significantly repressed when glucose-grown RNA was used to direct the translation system. The RNAs isolated from derepressive carbon sources directed the synthesis of greater amounts of the precursors, particularly in the case of the high- to low-glucose shift (Figure 5). Precursor synthesis in vitro, therefore, is capable of responding to the physiologic state of the yeast cell.

To establish the temporal relationship between the appearance of the various precursors and their release from catabolite repression, we grew cells to different densities on 1% glucose, and the isolated RNAs were used to direct translation in vitro. The results clearly demonstrate that the increase in precursor synthesis during logarithmic and early stationary phases correlates with the onset of enzymatic induction as assayed by cytochrome content and oxygen consumption (Figure 6).

The synthesis of the precursors increased during the logarithmic phase at a fairly uniform rate and appeared to reach plateau values at similar stages of cellular growth. This suggests a similar mode of regulation for the transcripts coding for each of the precursors. These results, obtained at glucose concentrations up to 5%, were identical in the two strains studied (data not shown).

Cells grown on trisaccharide raffinose to a midlogarithmic phase exhibited significantly higher cytochrome content than did the glucose-grown cells as the same stage (compare right side of Figure 6 with Figure 7). However, raffinose-grown cells also exhibited a derepressive phenomenon late in the logarithmic phase, resulting in a 3–4-fold induction of all of the cytochromes.

To determine whether the precursors of cytochrome oxidase respond differently during raffinose and glucose derepression, we isolated RNA from cells grown to several densities, using

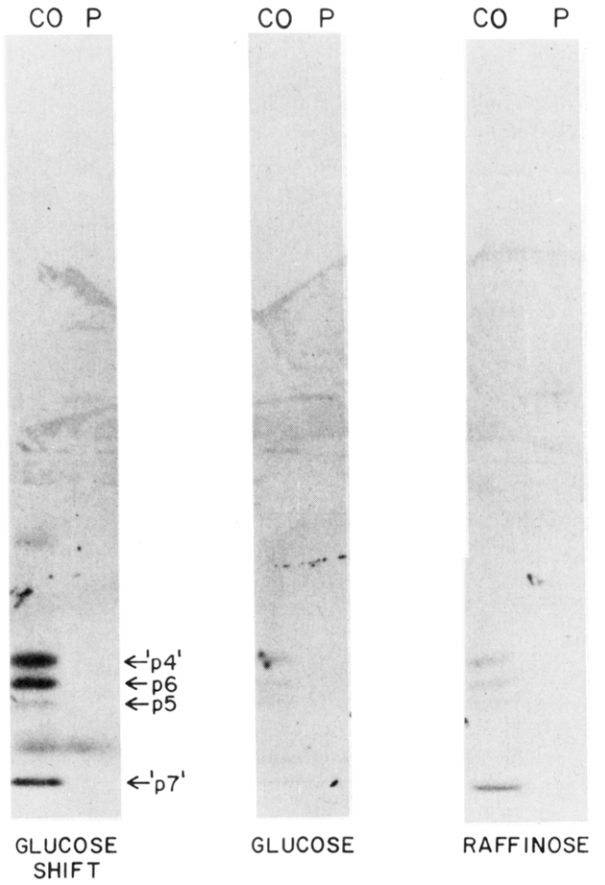

FIGURE 5: Physiologic regulation of cytochrome oxidase precursors (p4–p7). Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of ^{35}S-labeled translation products immunobound with anti-cytochrome oxidase (CO) and preimmune γ-globulin (P). Identical counts per minute of ^{35}S-labeled products were used for each immunoassay. The RNA used was isolated from cells grown to midlogarithmic phase on glucose or raffinose, or during a high- to low-glucose shift. The arrows indicate the position of each of the precursors.

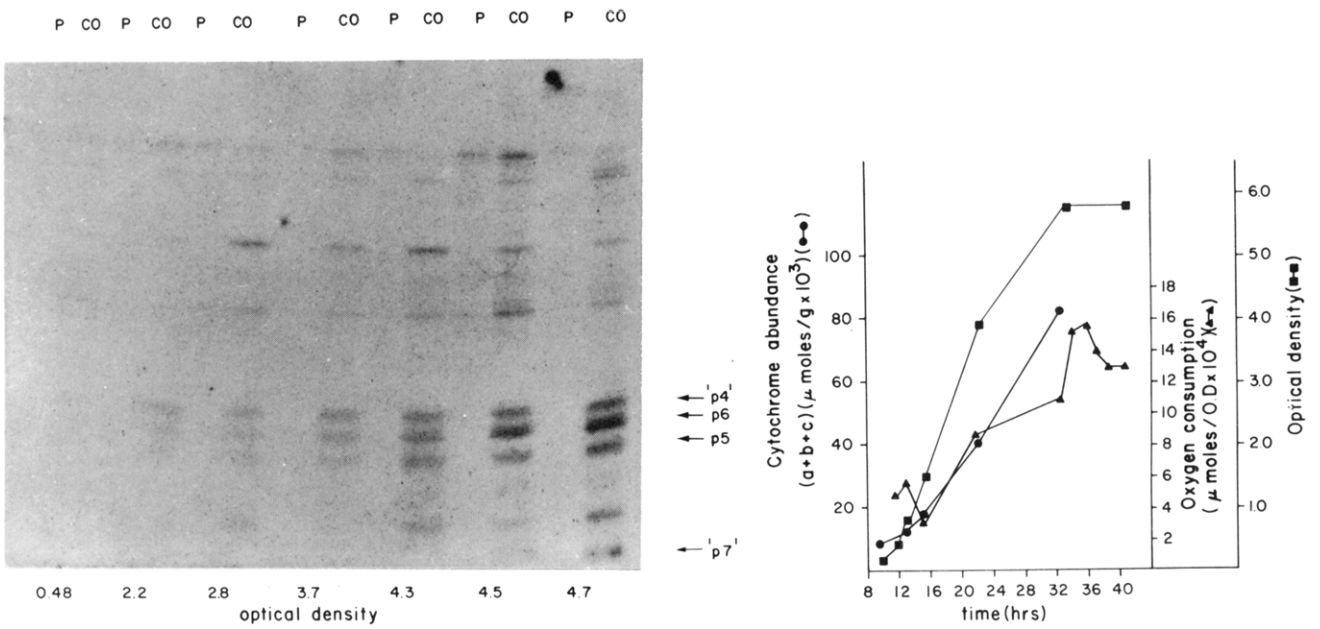

FIGURE 6: Physiologic regulation of oxidase precursors during glucose growth. (Left panel) Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of immunobound translation products in vitro directed by RNA isolated from A364a cells which have been grown to various stages on 1% glucose. Preimmune (P) and anti-cytochrome oxidase (CO) γ-globulin (Ab) were utilized for immunobinding. Identical amounts of ^{35}S-labeled cell-free products were utilized for each immunobinding. The arrows on the right side indicate the position of each of the precursors. (Right panel) Total cytochrome abundance (●), oxygen consumption (▲), and optical density (■), measured as a function of time during growth of A364a cells on 1% glucose.

2% raffinose as the carbon source (Figure 8). All of the precursors were synthesized at high levels (Figure 8, left and right panels). However, the absolute amounts and ratio between "p4", p5, and "p7" varied throughout growth. In particular, "p7" reached peak synthesis during midlogarithmic growth (Figure 8, right panel). These data are not the result of the variability of cell-free analysis of immunobinding since only slight variations in the ratio of these peptides were observed among independent cell-free translation experiments directed by a given RNA source. Furthermore, no significant variations in the ratio and abundance of precursors were found when different RNA concentrations were used in the translation system (data not shown). Thus, our data reveal a pattern of precursor synthesis during raffinose growth distinct from that observed during glucose derepression.

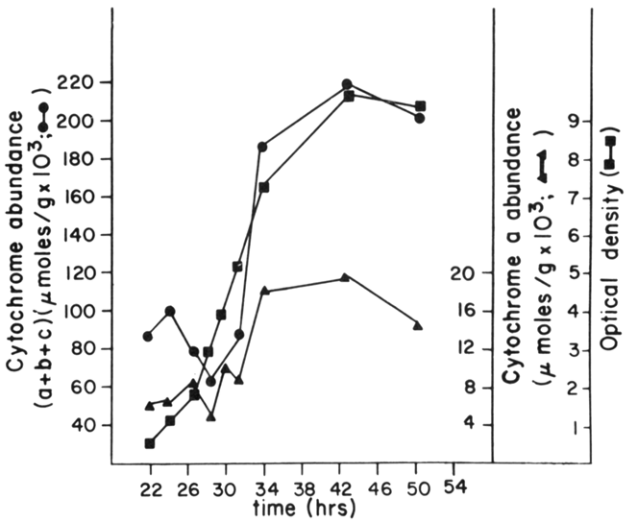

FIGURE 7: Total cytochrome abundance (●), cytochrome *a* abundance (▲), and optical density (■), measured as a function of time during growth of D273-10B cells on 2% raffinose.

Discussion

Mitochondrial catabolite repression in yeast is expressed through the inhibition of activities related to respiration, such as citric acid cycle enzymes and the cytochromes. The abundance of these enzymes in cells growing on a moderate concentration of glucose increases in the late-logarithmic and stationary phases of growth. That the rate of growth is not an essential factor is shown by the fact that release from the repression has been observed under nongrowing conditions (Mahler et al., 1975a).

Although the phenomenon of catabolite repression has been well documented, its regulation is still poorly understood. By utilizing respiratory-deficient petite mutants and selective inhibitors of mitochondrial protein synthesis and transcription, Perlman & Mahler (1974) and Kovac (1972) have shown that the mitochondrial genome is not essential for derepression. However, the involvement of the mitochondrial genome in this process has been suggested by the isolation of a mitochondrial temperature-sensitive mutant that affects the derepression of some proteins involved in respiration (Bandlow, 1977). Thus, the mitochondrial genome may have a regulatory effect on derepression. Nuclear mutants have also been isolated which eliminate the derepression of some, but not all, proteins induced during derepression (von Ciriacy, 1977), indicating the presence of more than one regulatory factor. Cyclic AMP, one of the factors involved in bacterial derepression (Zubay et al., 1970), has been proposed by Mahler and co-workers as a mediator of derepression at the level of nuclear and/or mitochondrial RNA polymerase (Mahler & Lin, 1978).

The isolation of the isocytochrome *c*-1 gene by Montgomery et al. (1978) has allowed a careful analysis of the transcriptional regulation of cytochrome *c* derepression. By analyzing the hybridization of transcripts to isolated gene sequences, Zitomer et al. (1979) and Boss et al. (1980) have found that the repression of cytochrome *c* is directly related to a reduced abundance of the cytochrome *c* mRNA, indicating a transcriptional mode of catabolite regulation.

We have begun a study of the nuclear control of cytochrome

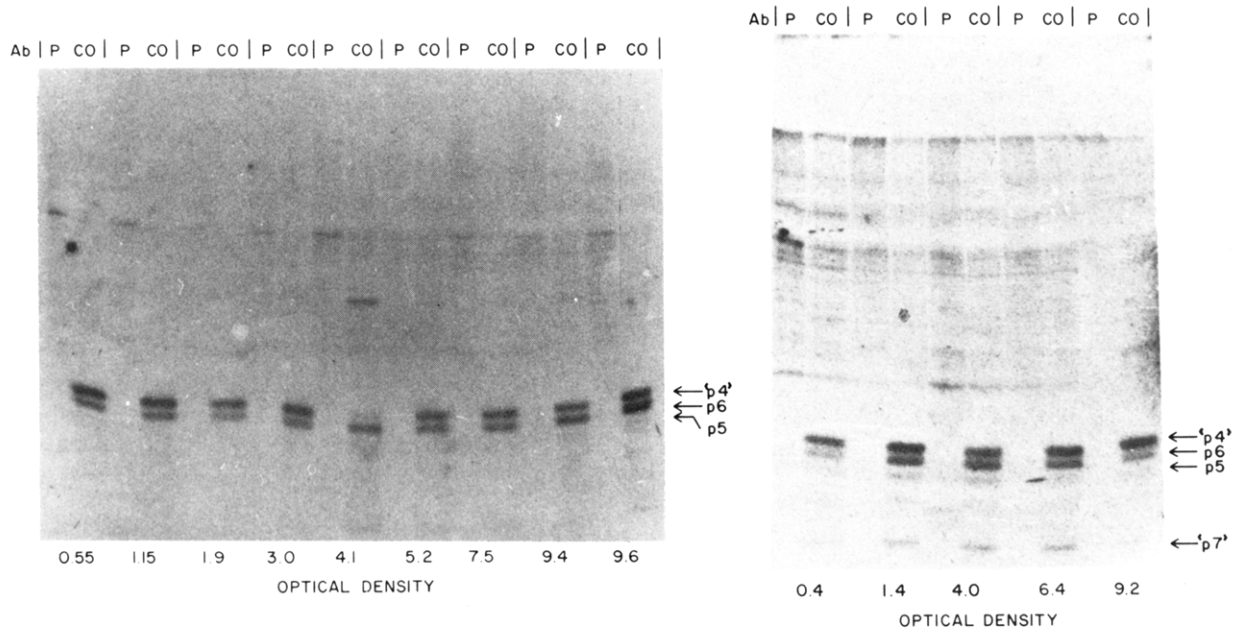

FIGURE 8: Physiologic regulation of oxidase precursors during raffinose growth. (Left panel) Fluorogram of $NaDodSO_4$–polyacrylamide gel (15%) of immunobound translation products in vitro, directed by RNA isolated from D273-10B cells grown to various stages on 2% raffinose. Preimmune (P) and anti-cytochrome oxidase (CO) γ-globulin (Ab) were utilized for immunobinding. Equal counts per minute of ^{35}S-labeled products were used in each immunoassay. Due to the low reactivity with COAb, subunit V is present only in low quantities on this gel. The arrows on the right indicate the position of each of the precursors. The right panel is the same as the left panel except that an independent set of RNAs isolated from D273-10B cells grown to different stages on 2% raffinose was utilized for cell-free translation. In addition, the products were subjected to electrophoresis for a shorter period of time so that "p7" could be resolved, and they were overexposed so that p5 could be visualized.

oxidase catabolite repression by analyzing the regulation of the nuclear-coded cytochrome oxidase peptides. Individual precursors for the cytoplasmic subunits similar to those observed by Mihara & Blobel (1980) and Lewin et al. (1980) have been detected in an in vitro translation system. These peptides of molecular weight 18 500, 16 000, 15 000, and 4500 appear to be the progenitors of subunits IV, VI, V, and VII, respectively. The detection of these precursors has allowed an analysis of message size as well as the physiologic control of message abundance.

We have found that RNAs of small size (8–15 S) encode the oxidase precursors. This observation indicates that each precursor is synthesized from independent RNAs as a discrete product. The precursors, therefore, are not degradation products of a large protein such as the polyprotein described by Poyton (Poyton & McKemmie, 1979a,b). These data are in agreement with the assignment of individual precursors on the basis of formylmethionine incorportation as described by Lewin et al. (1980) and Mihara & Blobel (1980). The higher molecular weight immunobound forms detected in the cell-free translation system are likely to result from contaminants within the immunogen and may be the basis of ambiguities concerning the nature of the precursors. This is indicated by the finding that two different antibodies of identical subunit specificity bind different high molecular weight products. The demonstration that highly purified cytochrome oxidase competes with the immunobinding of only the lower molecular weight species supports this view.

The uniform increase in the abundance of the different precursors detected by cell-free translation in vitro during growth on glucose correlates with the onset of enzymatic derepression. Thus, the mRNAs for these precursors are likely to be present in higher amounts during the process of derepression than in the repressed state. Our data may indicate a transcriptional response of oxidase genes to a single factor during the process of glucose derepression. This conclusion depends on the validity of the cell-free system as a tool in assaying mRNA abundance. A good correlation between the abundance of specific transcripts and cell-free translation has been observed in several cases including cytochrome *c* (Zitomer et al., 1979), ovalbumin (McKnight et al., 1975), and vitellogen (Mullinix et al., 1979). However, the alternative possibility that the massages may be more "translatable" as a result of a specific modification of a certain class of messages or through differential degradation of particular messages cannot be eliminated.

In contrast to the behavior of precursors on glucose, most of the precursors are present in high abundance throughout raffinose growth as expected under conditions of derepression. We have further observed a series of changes in relative ratio and abundance of "p4", "p7", and p6. If "p4" and "p7" do indeed represent precursors, then these data suggest that each gene may be capable of independent regulation. However, none of the changes observed in precursor abundance correlates with the induction of cytochromes late in raffinose growth, suggesting that this process involves a complex mode of regulation possibly with controlling elements at levels other than transcription. The isolation of cloned genes for the nuclear-coded oxidase precursors will aid in the further elucidation of the role that transcription plays in these processes.

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