Regulation of Mitochondrial Biogenesis: Enzymatic Changes in Cytochrome-deficient Yeast Mutants Requiring δ-Aminolevulenic Acid*

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Yeast cells almost completely deficient in all cytochromes were obtained by introducing two defective nuclear genes, cyd1 and cyc4, into the same haploid strain. The action of the two mutant genes is synergistic, since either gene acting singly results in only partial cytochrome deficiency. Normal synthesis of all cytochromes can be restored in the double mutant by adding δ-aminolevulenic acid to the growth medium. The optimum concentration of δ-aminolevulinate for restoration of cytochrome synthesis is about 40 μM; when higher concentrations are used, synthesis of cytochromes is partially suppressed, particularly that of cytochrome a₂.

Growth yield of the double mutant is stimulated by ergosterol and Tween 80, a source of unsaturated fatty acid. Methionine stimulates further. None of these nutrients is required for growth when sufficient δ-aminolevulenic acid is present in the growth medium. With respect to nutritional responses, the single-gene, cytochrome-deficient mutant, ole3, behaves like the double mutant. The frequency of the ρ⁻ mutation in the double mutant grown in the absence of ergosterol, Tween 80, and δ-aminolevulenic acid is at least 15%. The frequency can be reduced to less than 1% by either δ-aminolevulenic acid or Tween 80. Ergosterol alone does not decrease the ρ⁻ frequency. The ole3 mutant does not exhibit increased ρ⁻ frequency under similar conditions of unsaturated fatty acid deficiency.

The synergism between cyd1 and cyc4 does not result from mutations affecting two different δ-aminolevulinate synthetases. While δ-aminolevulenic acid synthetase activity in homogenates of cyd1 cells was very low, activity in homogenates of cyc4 cells was at least as high as in extracts of normal yeast. Therefore the cyd1 gene seems to control the structure or biosynthesis of δ-aminolevulenic acid synthetase (EC 2.3.1.37), while the cyc4 gene may regulate expression of synthetase activity or may control a subsequent enzyme in the porphyrin biosynthesis pathway. The cyd1 and ole3 mutant genes are functional alleles, and the latter mutation also results in greatly reduced δ-aminolevulenic acid synthetase activity in cell extracts.

Cytochromeless mitochondria isolated from cells of the double mutant retain significant ATPase activity which is inhibited by oligomycin and venturicidin. This result indicates that biosynthesis of the energy-conserving apparatus of the mitochondrion is not tightly linked to the biosynthesis of cytochromes. Cytochromeless mitochondria could provide a useful experimental system for studying the energy-transfer system in its native orientation without interference from the respiratory chain. Cytochromeless mitochondria contain succinic dehydrogenase; the specific activity of this enzyme is about 25% that observed in respiring mitochondria isolated from the double mutant containing cytochromes as a result of growth in medium containing 40 μM δ-aminolevulenic acid. Since the concentration of mitochondrial cytochromes can be controlled readily by regulating the exogenous supply of δ-aminolevulenic acid, the mutant provides a simple, versatile new system for investigating the progressive assembly of mitochondria.

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In a previous work, two mutations in *Saccharomyces cerevisiae*, *cyd1* and *cyc4*, were shown to cause partial cytochrome deficiencies which could be corrected by growing cells in medium supplemented with d-aminolevulinic acid (1). A mutant form lacking all cytochromes could be obtained from *cyd1* cells by destroying functional mitochondrial DNA with ethidium bromide to produce the p- state. Therefore, the *cyd1* defect, which is largely latent in the normal p- state, is unmasked by mutation to p-. Although the partial cytochrome deficiencies in *cyc4* and *cyd1* strains both respond to d-aminolevulinic acid (1), the *cyc4* gene is not allelic to *cyd1*, and the two genes segregate independently during meiosis. Among the meiotic segregants are found haploid strains containing both mutant genes simultaneously. These double mutants exhibit extreme deficiencies in cytochromes which can be restored to normal by adding exogenous d-aminolevulinic acid. To determine whether the apparent synergism between these two nuclear genes indicates the existence of two d-aminolevulinic acid synthetases, direct measurements of this enzymatic activity were made in homogenates of strains bearing one or the other mutant gene. The results show that only the *cyd1* mutation results in lowered d-aminolevulinic acid synthetase activity in vitro. In addition, some of the properties of cytochrome-deficient mitochondria isolated from the double mutant are described. In an independent study, Bard et al. (2) demonstrated that the fatty acid and ergosterol requirements of a yeast strain containing the *ole3* mutation (3) could be satisfied by adding d-aminolevulinic acid to the growth medium. Moreover, since deficiencies in respiration and cytochromes in the *ole3* strain were alleviated by added d-aminolevulinic acid, it was suggested that this mutation affected d-aminolevulinic acid synthetase directly. The present study demonstrates that *ole3* and *cyd1* affect the same genetic locus and that both mutations result in decreased d-aminolevulinic acid synthetase activity in vitro.

**MATERIALS AND METHODS**

**Mutant Strains and Genetic Methods**—The *Saccharomyces cerevisiae* strains containing the various mutations causing d-aminolevulinic acid-dependent cytochrome formation have been described previously. Strains containing *cyd1* and *cyd2* were isolated by Sanders et al. (1), the *cyd1* mutation was first described by Sherman (4), and the d-aminolevulinic acid-dependent cytochrome formation of *ole3* strains was described by Bard et al. (2). Strains containing these various genes together with appropriate auxotrophic markers were constructed by crossing and tetrad analysis, utilizing tester strains from The Johns Hopkins collection. Standard methods of mass mating, prototrophic selection, sporulation, ascus dissection, and tetrad analysis were used (5, 6). A Pepper Replicator-inoculator (Pentex, Inc., Kankakee, Ill.) was used for replica plating. Clotriplasmic petite (p-) formation was determined by tetrazolium staining of colonies formed on glyceral low glucose medium, as previously described (7).

**Cytochrome Determinations**—The cytochrome content of mutant and normal strains was estimated visually after freezing cell pastes, mixed with a few grains of sodium hydrosulphite, in liquid nitrogen. A Zeiss spectroscopy was used, as described previously (1, 4, 7). An arbitrary 10-point scale of relative concentration based on estimated density of absorption bands was used, as shown in the legend to Table V. Absolute spectra of cell suspensions were obtained using a split-beam spectrophotometer, with dilute whole milk placed in the reference cuvette as previously described (1). Cytochrome reduction was accomplished by adding glucose to the sample cuvette and bubbling the cell suspension with nitrogen.

**Media and Growth Conditions**—These were essentially as described previously (1). Growth media were as follows: (a) YPD: 1% yeast extract, 2% (w/v) peptone, 0.1% (w/v) dextrose, and 3% (v/v) glycerol. (c) SD (minimal medium): 0.61% yeast nitrogen base without amino acids (Difco), 2% dextrose. When strains auxotrophic for adenine were used, media were supplemented with 40 or 80 mg of adenine sulfate/liter. Cultures of strains requiring d-aminolevulinic acid were maintained on YPD medium supplemented with 0.5 mm d-aminolevulinic acid. For solid media, 1% Ionagar No. 2S (Wilson Diagnostics) was used. For genetic studies, SD medium was supplemented with adenine sulfate and various amino acids as previously described (7). In the study of cytochrome-linked nutritional requirements the following supplements were added to SD medium: Tween 80 (polyoxyethylene sorbitan mono-oleate), 1%; ergosterol, 10 μg/ml; methionine, 20 μg/ml. Stock solutions of Tween 80 and ergosterol dissolved in ethanol were used. Cultures were grown at 30°. A New Brunswick rotary shaker was used for growing liquid cultures. Dry weight was determined by collecting cells on Gelman Metriset filters, and drying them at 80° for at least 24 hours.

**Homogenization Procedures**—For making yeast homogenates, a Braun shaker operated at 4000 rpm for 60 s with constant cooling by CO2 was used. Acid-washed glass beads, 0.45 to 0.50 mm, were employed. Yeast cells grown on appropriate media were used. For mitochondrial studies growth was for 24 hours. For d-aminolevulinic acid phosphate, 0.07 μmol; coenzyme A, 0.52 μmol; NAD+, 0.6 μmol; potassium phosphate buffer, pH 7.2. Up to 9 g of cells could be homogenized, using proportionally higher quantities of glass beads and buffer. Cell debris and nuclei were removed by three successive centrifugations at 1000 × g for 5 min each and one for 10 min. Pellets were washed with aliquots of buffer equal in volume to the weight of cells used, and washed combined with the last supernatant fluid to produce the final homogenate. Protein was determined by the method of Lowry et al. (8).

To obtain submitochondrial particles for assay of ATPase and succinate-PMS reduction, a solution designated Medium A was used. It contained 0.4 m mannitol, 0.06 M Tris base (pH 8.2), and 0.001 M EDTA. Cells, glass beads, and Medium A were mixed in the proportions 3 g/5 g/6 ml. Up to 9 g of cells were employed. After homogenization, glass beads were removed by decantation and washed with a small volume of Medium A which was then mixed with the homogenate. Nuclei and cell debris were removed by two successive 5-min centrifugations at 1000 × g. The submitochondrial particle fraction was then collected by centrifuging at 25,000 × g for 20 min, and the supernatant fluid was discarded. A volume of Medium A, 1.5 ml/g of cells, was used to suspend the pellet. Protein was determined by the method of Murphy and Kies (9).

**d-Aminolevulinic Acid Assay**—d-Aminolevulinic acid synthetase was determined both colorimetrically and by an isotopic method. Since yeast cells may contain significant concentrations of free d-aminolevulinic acid under certain conditions (Labbe et al. (10, 11)), the colorimetric procedure required prior dialysis of homogenate. Homogenate (5 to 10 ml) was dialyzed at 4° overnight against 20 volumes of 0.1 M potassium phosphate, pH 7.2, containing 0.14 mM pyridoxal phosphate to stabilize the d-aminolevulinic acid synthetase (12).

For the colorimetric method, an incubation mixture similar to that described by Burnham and Lascelles (13) was employed, but instead of succinate, α-ketoglutarate and NAD+ were used, as described by Labbe (14). The mixture contained the following in a final volume of 1.5 ml: α-ketoglutarate, 100 μmol; glycine, 100 μmol; pyridoxal phosphate, 0.07 μmol; coenzyme A, 0.52 μmol; NAD+, 0.6 μmol; EDTA, 1.0 μmol; 2-mercaptoethanol, 10 μmol; MgCl2, 10 μmol; potassium phosphate (as buffer, pH 7.2), 70 μmol; homogenate protein, 15 to 30 mg. Incubation was carried out at 30° on a metabolic shaker for various time periods up to 60 min. Reaction was terminated by adding 0.25 ml of 30% trichloroacetic acid, followed by centrifugation. The d-aminolevulinic acid content of the supernatant fluid of each sample was determined by the method of Mauzerall and Granick (15) using acetylaceetone and modified Ehrlich's reagent.

The isotopic method of Ebert et al. (16) was used with minor variations. The incubation mixture contained in a 2-ml volume: [2,3-14C]α-ketoglutarate, 1 μCi; glycine, 170 μmol; pyridoxal phosphate, 1.6 μmol; ATP, 1.5 μmol; coenzyme A, 0.32 μmol; EDTA, 17 μmol; MgCl2, 0.62 μmol; potassium phosphate buffer, pH 7.2, 80 μmol; dithiothreitol (or dithioerythritol), 0.20 μmol; Rhodopseudomonas spheroides succinyl-CoA synthetase enzyme. *The abbreviation used is:* PMS, phenazine ethosulfate.
tase preparation, 1 ml. Samples were incubated for 15 min at 30° in a metabolic shaker and the reaction stopped by adding 0.3 ml of 25% trichloroacetic acid. After centrifugation, supernatant layers were removed and transferred to 40-ml test tubes. Reaction vessels were rinsed with 5 ml of 15% trichloroacetic acid, and the rinse was used to wash the precipitated protein fractions. After a second centrifugation the supernatant fluid from a given sample (wash fluid) was combined with the corresponding fluid in the test tube. A 3-ml aliquot of 1 M sodium acetate buffer, pH 4.8, was added to the tube. The pH was adjusted to 7.4. The contents of the tube were then quantitatively transferred to a column (0.8 x 5 cm) of Dowex AG50 X-8 (100- to 200-mesh) previously equilibrated with 0.1 M sodium acetate buffer, pH 3.9. Radiactive succinate was eluted with 30 ml of this acetate buffer. The column was washed subsequently with 20 ml of methanol/acetate buffer (2:1 v/v), pH 3.9, then with 5 ml of 0.01 M HCl. The radiative δ-aminolevulinic acid was then eluted with 4 ml of 1 M NH₄OH. A 1.5-ml aliquot was then counted in 15 ml of the toluene-Triton X-100 counting mixture described by Patterson and Green (17). In some experiments 0.1 ml of 90% formic acid was added to scintillation vials to reduce background.

Succinate dehydrogenase was determined polariographically utilizing FMS as electron acceptor. A Clark oxygen electrode was utilized in a jacketed sample chamber of 2.5 ml capacity. Reaction temperature was 25°. The reaction mixture contained sodium succinate, 50 μmol, and antimycin A, 9 nmol, dissolved in 50 μM Tris-acetate buffer, pH 7.4. The electrode was attached to a strip-chart recorder which was calibrated to give full-scale deflection (100 graph units) after all the dissolved oxygen in the reaction mixture had been consumed. This was accomplished by injecting a few milligrams of actively respiring yeast cells into the sample chamber. After calibration, fresh reaction mixture was added to the sample chamber, 1 to 7 mg of submitochondrial particle protein were injected, followed by 50 μl of 100 mM PMS solution (freshly prepared and stored at 0° in the dark). Activity, in graph units/min, was determined from the slope of the linear trace obtained. Reaction was terminated by injecting 10 μl of 1 M sodium malate into the chamber.

ATPase activity was determined essentially as described by Goffeau et al. (18). The reaction mixture contained the following components: ATP, 3 μmol; MgCl₂, 6 μmol; Tris-HCl, 20 μmol; phosphoenolpyruvate, 2 μmol; pyruvate kinase, as anammonium sulfate suspension, 40 μg (14 to 20 μl); methanol, 15 μl; and in some samples, venturicidin, 0.3 μg, or oligomycin, 5 μg (added with methanol). The final pH of the reaction mixture was 8.6. The reaction was initiated with 20 to 100 μg of submitochondrial particle protein and allowed to proceed for 8 min at 30°. Reaction was terminated by adding 0.25 ml of 2.5% mercaptoacetic acid, and centrifuged supernatant fluids from each sample were analyzed for inorganic phosphate by the method of Gomori (19). Inorganic phosphate released was plotted as a function of submitochondrial particle protein added, and ATPase activity was determined from the slope of the resulting linear plot.

Chemicals and Enzymes—The following chemicals were purchased from Calbiochem: pyridoxal phosphate, α-ketoglutarate, NAD⁺, dibithreitol (Cleland's reagent), ergosterol, and glycine. The following were obtained from Sigma: δ-aminolevulinic acid, Tween 80, phenazine methosulphate, coenzyme A, antimycin A, pyruvate kinase (type II), crystalline, Tris base, and phosphoenolpyruvate, p-(Dimethylamino)benzaldehyde and 2-mercaptoethanol were obtained from J. T. Baker Chemical Co. Acetyl acetone was purchased from Aldrich, scintillation grade Triton X-100 from Amersham/Searle, suecinic acid from Fisher, [2,3-¹⁴C]suecinic acid from New England Nuclear (12.99 mCi/mmol), and antimycin A, 9 nmol, dissolved in 50 mM Tris-acetate buffer (10 ml/mM) and ATP from P-L Biochemicals. Venturicidin was a generous gift from Glaxo Laboratories, and was recrystallized from methanol before use. Oligomycin was a gift from the Upjohn Co. Dr. Diana Beattie and Dr. George Patton (the Mt. Sinai School of Medicine) kindly supplied succiny1-CoA synthetase which was prepared from a δ-aminolevulinic acid-requiring mutant of Rhodospseudomonas spheroides (20).

RESULTS

δ-Aminolevulinic Acid-dependent Cytochrome Formation—It has been demonstrated previously that two unlinked nuclear mutant genes, cydI and cyc4, cause yeast cells to be partially dependent upon exogenous δ-aminolevulinic acid (Alv) for maximum cytochrome biosynthesis (1). Fig. 1, panel A, illustrates the effect of δ-aminolevulinic acid on haploid strains containing one or the other of these two genes. The cytochrome deficiency is greater in the cydI mutant, GT6-1C, but cytochrome production in either strain is greatly enhanced by growing cells in δ-aminolevulinic acid-supplemented medium. In contrast, cells of heterozygous diploid strain GT20, produced by crossing the two haploid mutants, exhibit a normal cytochrome spectrum which is only slightly enhanced by growth in δ-aminolevulinic acid (Fig. 1, panel B). It may be concluded that cydI and cyc4 are both recessive, and probably nonallelic. That cydI and cyc4 represent mutations in different genes has been confirmed by demonstrating their independent meiotic segregation (1).

In the course of testing haploid meiotic progeny derived from strain GT20, it was found that about 20% of the δ-aminolevulinic acid-dependent haploid strains produced no detectable cytochromes when grown in the absence of exogenous δ-aminolevulinic acid. As Fig. 1 shows, neither the cydI gene nor the cyc4 gene, acting alone, produces such a complete deficiency. On the other hand, in cydI cells, inactivation of mitochondrial DNA by mutagen to the ρ⁻ state does result in a cytochromeless phenotype (1). When one of the cytochromeless haploid segregants derived from GT20 was treated with 40 μM δ-aminolevulinic acid, all cytochromes were produced in normal concentrations, as shown in Fig. 2. Clearly then, this strain contains normal mitochondrial DNA; that is, it is in the ρ⁺ state.

Genetic Studies—It seems likely, then, that this new cytochromeless phenotype requires the joint action of the two nuclear genes, cydI and cyc4. That both genes were present in strain GT20-12C could be deduced from the fact that this strain was derived from a tetrad containing only two δ-aminolevulinic acid-dependent segregants. The other two segregants in this tetrad were normal, indicating that they received neither the cydI nor the cyc4 gene. To confirm this deduction, complementation tests were performed by crossing strain GT20-12C with haploids containing singly either cydI or cyc4. Since both types of diploids resulting from these crosses exhibited considerable δ-aminolevulinic acid-dependent cytochrome formation, GT20-12C must contain both cydI and cyc4. Similar results were obtained with four other cytochromeless GT20 segregants. Finally, GT20-12C was crossed with a normal haploid strain, and tetrad analysis was performed on the resulting diploid. The data presented in Table I show, as expected, that no less than two mutant genes causing δ-aminolevulinic acid dependency must have been present in the parental diploid, GT40, since some tetrads contained three or four δ-aminolevulinic acid-dependent strains. Segregants with extreme cytochrome deficiencies were again observed, amounting to 25% of the δ-aminolevulinic acid-dependent strains. Although some abnormal tetrads were observed, the data indicate that when a tetrad contains only two δ-aminolevulinic acid-dependent segregants, both of these exhibit extreme cytochrome deficiencies, indicating the synergistic action of cydI and cyc4. These tetrads would therefore be parental ditype. In a nonparental ditype tetrad, two segregants would receive cydI and the other two cyc4, so that while all four segregants would show some degree of δ-aminolevulinic acid dependence, no extreme deficiencies would occur. Four such tetrads were observed. The predominant class of tetrads (tetratype) contained three δ-aminolevulinic acid-dependent segregants, one of which exhibited the extreme cytochrome deficiency. The symbol cydI will be used to indicate the cydI-2 mutation. The allele number will be indicated when cydI-1 was employed.
FIG. 1 (A, left and B, center). δ-Aminolevulinic acid (Alv)-dependent cytochrome synthesis in cyd1 and cyc4 haploids and in a heterozygous diploid. Cells were grown for 48 hours in YPD medium. Spectra were determined at 23°C with a split-beam spectrophotometer. Cell suspensions of 5% (wet w/v) were made in 50 mM phosphate buffer, pH 7.0, and an aqueous suspension of whole milk was used as a turbidity reference. Panel A, spectra of haploid parental strains GTG-1C (top) and B271 (bottom), containing, respectively, cyd1-2 and cyc4 mutations. Panel B, spectra of heterozygous diploid strain GT20, obtained by crossing GTG-1C and B271. When present, δ-aminolevulinic acid concentration was 40 μM. Alv, δ-aminolevulinic acid.

FIG. 2 (right). Effect of δ-aminolevulinic acid (Alv) concentration on cytochrome formation in a haploid cytochromeless mutant containing both cyd1 and cyc4. Cells were grown on YPD medium as indicated for Fig. 1. δ-Aminolevulinic acid was added to growth media at the indicated concentrations. Spectra were obtained as in Fig. 1. Haploid strain GT20-12C (double mutant) was obtained from strain GT20 by meiotic segregation. Alv, δ-aminolevulinic acid.

Table I
Segregation of δ-aminolevulinic acid-dependent cytochrome deficiencies in strain GT40

Strain GT40 was prepared by crossing strain GT20-12C (cyd1-2 cyc4) with normal tester strain GT7-6B. Cytochrome concentrations were estimated visually in a spectroscope after freezing dithionite-treated cells in liquid nitrogen. Each segregant was grown on YPD medium with and without 0.5 mM δ-aminolevulinic acid (Alv) supplement. PD, NPD and T refer to parental dittype, nonparental dittype, and tetraspl type tetrads, respectively. Four abnormal tetrads were found: two contained three δ-aminolevulinic acid-dependent segregants, but one of these gave a very weak response to δ-aminolevulinic acid. The other two tetrads contained one extremely deficient segregant and one with a very weak response to δ-aminolevulinic acid.

<table>
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<th>Tetrad type</th>
<th>No. of Alv-dependent segregants</th>
<th>No. of segregants with extreme deficiencies</th>
<th>No. of tetrads</th>
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<tr>
<td>PD</td>
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<td>2</td>
<td>4</td>
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<tr>
<td>NPD</td>
<td>4</td>
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<td>T</td>
<td>3</td>
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deficiency characteristic of the synergistic action of the two mutant genes. This indicates that the one normal segregant received neither mutant gene, while one of the other two δ-aminolevulinic acid-dependent strains received cyd1 and the other cyc4. It is not clear why four tetrads did not conform to this pattern. The presence of a modifier gene or gene-conversion could be considered.

Changes in δ-Aminolevulinic Acid Synthetase Activities—A possible explanation for the apparent synergism between cyd1 and cyc4 is that each of these genes controls the structure of a different δ-aminolevulinic acid synthetase. To test this possibility, direct assays for δ-aminolevulinic acid synthetase were performed on haploid strains containing each gene singly, and on haploid strain GT20-12C, which contains both mutations. The results presented in Table II show that homogenates prepared from cells containing the cyd1 mutation exhibit markedly less δ-aminolevulinic acid synthetase activity than normal cell extracts. In contrast, δ-aminolevulinic acid synthetase activity in homogenates of cyc4 cells is equal to or greater than normal. The enzymatic activities in extracts of the double mutant were below the level of detection by either assay employed. From these results it appears very unlikely that the cyc4 gene is a structural gene for a separate δ-aminolevulinic acid synthetase, but the cyd1 mutation may well represent such a structural gene alteration. Since cyd1 cells do produce significant amounts of cytochrome (Fig. 1), the very low δ-aminolevulinic acid synthetase activity in homogenates of cyd1 cells suggests that the enzyme may be quite labile in vitro.

Effects of Cytochrome Deficiencies on Lipid Biosynthesis and Fidelity of Replication of Mitochondrial DNA—Bard et al. (2), in a study of mutants with nutritional requirements for unsaturated fatty acid, recently demonstrated that these mutants were also blocked in ergosterol biosynthesis. One of these mutants, bearing only the ole3 mutation, was found to lack all detectable cytochromes. Supplementation of growth medium with δ-aminolevulinic acid restored cytochrome biosynthesis and permitted mutant growth in the absence of unsaturated fatty acid (Tween 80) and ergosterol. A comparison between an ole3 strain (W1) and the double mutant GT20-12C is given in Table III.
The addition of a low concentration of δ-aminolevulinic acid (10 μM) which is sufficient for only very limited synthesis of mitochondrial cytochromes in GT20-12C (Fig. 2), replaces the lipids in stimulating growth. Apparently, the ole3 mutant is less efficient in utilizing δ-aminolevulinic acid than strain GT20-12C, since at 10 μM no cytochrome bands were evident in ole3 cells, and growth stimulation was slightly less than with lipids.

Another important difference between the two mutants is the effect of cytochrome deficiency on the spontaneous frequency of the mutation ρ+ to ρ− (Table III), which involves an inactivation of functional mitochondrial DNA. Cells of GT20-12C grown without Tween 80 or δ-aminolevulinic acid have a high frequency of this mutation which is not observed in the ole3 mutant. In some experiments more than 50% of GT20-12C cells became ρ−. When the double mutant is grown on medium containing δ-aminolevulinic acid, or Tween 80 plus ergosterol, ρ− frequency drops to the normal range (0.5 to 3%) found in various wild type strains. Apparently the critical component in maintaining mitochondrial DNA in the normal ρ+ condition during cell replication is unsaturated fatty acid rather than cytochromes. Other experiments in which ergosterol was the sole lipid supplement (data not shown) indicate that ρ− frequency remains high. Since mitochondrial DNA may be closely associated with the mitochondrial inner membrane, these data suggest that the unsaturated fatty acid content of mitochondrial membrane phospholipid may be of considerable importance in maintaining the fidelity of replication (or repair) of the organelle DNA. The basis for this phenotypic difference between ole3 and cycl 1 cycl 4 has not been determined, but a difference in a mitochondrial membrane component, possibly related to cycl 4, may be considered.

An alternative explanation for the high frequency of ρ− is merely that the selective growth advantage that a ρ− cell normally has is largely lost in medium lacking both δ-aminolevulinic acid and lipids.

As noted above, the concentration of δ-aminolevulinic acid was chosen so that it would allow only limited synthesis of mitochondrial cytochromes. Since addition of lipids together with this concentration of δ-aminolevulinic acid increases cell yield of strain GT20-12C above that obtained with either lipids or limited δ-aminolevulinic acid alone, it appears that at 10 μM δ-aminolevulinic acid concentration may be limiting for microsomal lipid synthesis as well as for mitochondrial cytochrome synthesis. Nevertheless, it appears that with 10 μM δ-aminolevulinic acid sufficient unsaturated fatty acid is produced to suppress ρ− mutation frequency to a low level, or to reestablish a selective growth advantage for ρ+ cells.

**Methionine Requirements of Cytochrome-deficient Yeast**—
A methionine requirement in ole3 and other lipid-requiring, cytochrome-deficient yeast mutants has previously been reported (2, 23). Table IV shows that the double mutant responds like the ole3 mutant to this amino acid. In completely defined medium (SD medium), lipids alone support little or no growth, methionine alone causes a significant weight change, and lipids plus methionine stimulate growth even more. The methionine requirement appears to be a consequence of the δ-aminolevulinic acid (or cytochrome) deficiency, since maximal growth is obtained when a nonlimiting concentration
of δ-aminolevulinic acid (0.2 mM) is added, and methionine causes no significant additional growth stimulation.

Functional Allelism of cydl and ole3—The biochemical similarities between ole3 strains and the double mutant suggested the possibility that ole3 might be allelic to either cydl or cyc4. To test this possibility, genetic complementation tests were performed as described previously (1). Table V shows that diploids constructed by crossing ole3 haploids with cydl haploids (GT42, GT43, GT46, and GT47) exhibited marked cytochrome deficiencies which could be overcome by growing cells in medium supplemented with δ-aminolevulinic acid. In contrast, diploids produced by crossing ole3 haploids with a cyc4 haploid (GT44 and GT48) behaved like controls in which ole3 strains were crossed with normal strain D311-3A (GT41 and GT45). None of these latter four diploids exhibited significant cytochrome deficiency or δ-aminolevulinic acid dependence. Since both cydl and ole3 are recessive, it is highly probable that the δ-aminolevulinic acid dependence of the cydl x ole3 diploids indicates functional allelism. That is, ole3-cydl-1, and cydl-2 represent separate mutations in the same nuclear gene. The ole3 mutation differs from the two cydl mutations in causing an almost complete δ-aminolevulinic acid dependence. Apparently, the abnormal gene products produced by the cydl strains retain considerable activity in vivo, while ole3 strains produce a gene product with a very low activity in vivo. The low level of residual cytochrome production in the cydl x ole3 diploids would accordingly reflect mainly the residual activity of the cydl gene product.

To confirm that ole3 and cydl are mutations at the same locus, tetrad analyses were performed using strains GT42 and GT43. Fifteen tetrads of each were analyzed and the δ-aminolevulinic acid-dependence of each segregant was determined (data not shown). Since all of the 120 strains tested exhibited δ-aminolevulinic acid dependence, it is clear that recombination between ole3 and cydl must be relatively rare. Consequently we conclude that ole3 and cydl probably represent mutations in the same gene.

Activity of δ-Aminolevulinic Acid Synthetase in Homogenates of an ole3 Strain—In order to test the prediction that the ole3 mutation alters δ-aminolevulinic acid synthetase, assays of this enzymatic activity were carried out in homogenates of ole3, cydl, and normal strains. As the data in Table VI show, δ-aminolevulinic acid synthetase activity of the homogenate derived from the ole3 strain GT39-6B was less than 2% of that observed in a comparable homogenate of normal strain Q1. As in the previous experiments (Table II), in vitro activity of δ-aminolevulinic acid synthetase in the cydl-2 strain Q2 was much less than normal. However, it is slightly greater than that found with the ole3 preparation. Therefore, the probable molecular basis for the functional allelism of cydl and ole3 is established: both mutations result in defective δ-aminolevulinic acid synthetase activities, which, in turn, lead to the cytochrome deficiencies observed in vivo.

Energy Transfer and Electron Transport Activities in Mitochondria Isolated from Cytochrome-deficient Yeast—Since the concentration of cytochromes produced by the double mutant, GT20-12C, can be manipulated over a very wide range simply by regulating the exogenous δ-aminolevulinic acid supply in the growth medium, this mutant has potential value in the
study of mitochondrial assembly and its regulation. In the previous study (1) it was shown that sedimentation behavior of mitochondria isolated from a cydl mutant differed depending on the presence or absence of 8-aminolevulinic acid in cell growth medium. It was suggested that significant compositional differences in mitochondria were responsible. This approach has been extended to an examination of the effects of 8-aminolevulinic acid-dependent cytochrome concentration on specific enzymatic activities associated with mitochondrial energy transfer and the segment of the electron transport chain which precedes the cytochrome sequence. The double mutant, rather than a cydl strain, was used in order to examine the full range of cytochrome concentrations.

Table VII compares mitochondrial ATPase activity in membrane fractions isolated from 8-aminolevulinic acid-supplemented and unsupplemented cells of the double mutant, GT20-12C. One expression of the energy conservation apparatus of the mitochondrion is the membrane-bound ATPase which is sensitive to oligomycin and related antibiotics. Venturicidin was used in these experiments because it is much more potent than oligomycin in inhibiting ATPase and oxidative phosphorylation in yeast mitochondria (24). Oligomycin gave similar results.

Submitochondrial particles from unsupplemented cells exhibited significant venturicidin-sensitive ATPase activity, about 25% as much as a comparable fraction prepared from 8-aminolevulinic acid-supplemented cells. Venturicidin insensitive ATPase activities were very similar in the two preparations. The presence of significant venturicidin-sensitive ATPase activity in cytochromeless mitochondria demonstrates that while heme and cytochrome formation may have a substantial effect on the expression of this component of the energy conservation mechanism, it does not exert stringent control on this aspect of mitochondrial assembly.

Results of a similar experiment relating 8-aminolevulinic acid-limited cytochrome formation to mitochondrial succinate-PMS reductase is presented in Table VIII. As in the case of mitochondrial ATPase, a membrane fraction (submitochondrial particles) from cytochrome-deficient GT20-12C cells exhibited a significant, but greatly diminished level of this mitochondrial activity relative to a comparable submitochondrial particle fraction prepared from 8-aminolevulinic acid-supplemented cells. It may be concluded, therefore, that assembly of that segment of the respiratory chain preceding the cytochromes is strongly influenced by, but not tightly linked to, the formation of 8-aminolevulinic acid or heme.

**DISCUSSION**

The observations reported here appear to rule out the existence of two 8-aminolevulinic acid synthetases as the basis for the synergism between the cydl and cyc4 mutations. Rather, the evidence supports the view that Saccharomyces cerevisiae contains only a single, major 8-aminolevulinic acid synthetase. While 8-aminolevulinic acid synthetase activity in homogenates of cydl cells is very low, the comparable activity in cydl preparations is not decreased relative to that of control extracts.

It was previously predicted (1) that if either cydl or cyc4 represented a modification of a unique structural gene for 8-aminolevulinic acid synthetase, it should be possible to produce mutations at this same locus which would result in complete loss of 8-aminolevulinic acid and cytochrome formation, even in p+ cells. The demonstration that both the lipid requirements and respiration deficiency of the cytochromeless ole3 mutant could be alleviated by 8-aminolevulinic acid (2) suggested that the ole3 mutation might represent just such a structural gene alteration. Since the data given here show that diploids formed by crosses of the type (cydl x ole3) exhibit 8-aminolevulinic acid-dependent cytochrome synthesis, we may conclude that such diploids are homozygous for a gene which controls 8-aminolevulinic acid formation. The observation that homogenates of ole3 cells contained barely detectable 8-aminolevulinic acid synthetase activity and that cydl extracts had very low activity confirms that the two mutations affect the same enzymatic reaction, the synthesis of 8-aminolevulinic acid from succinyl-CoA and glycine. The similar 8-aminolevulinic acid dependency of cytochrome production, the lack of genetic complementation and recombination, and the similar decreases in enzymatic activity in the two mutants are clearly consistent with the hypothesis that both cydl and ole3 represent mutations in a structural gene for 8-aminolevulinic acid synthetase. The low, but significant activity of this enzyme in extracts of cydl-2 cells suggests that cydl-2 is a missense mutation. Alternatively, the low 8-aminolevulinic acid synthetase activities in cydl and in ole3 could conceivably reflect defects in a regulatory gene which specifically controls transcription of the 8-aminolevulinic acid synthetic machinery.
synthetase structural gene. The present data do not distinguish between these two alternatives.

Although cytochrome production in ole3 strains is much less than in cycl1 strains, it is still sufficient to permit extremely slow growth in defined medium lacking lipids and methionine. Moreover, very faint traces of cytochrome bands have been detected in ole3 cells grown without added \( \delta \)-aminolevulinic acid. The low, but significant, residual \( \delta \)-aminolevulinic acid synthetase activity observed in extracts of ole3 cells suggests several possibilities: (a) The ole3 mutation may be a missense mutation in the \( \delta \)-aminolevulinic acid synthetase structural gene. (b) The ole3 mutation may cause a more serious block in specific transcription than cycl1 does. (c) \( S. \) cerevisiae may contain two enzymes with \( \delta \)-aminolevulinic acid synthetase activity, a major one controlled by the cycl1-ole3 locus, and a minor one accounting for about 3–4% of the total specific activity. The existence of two \( \delta \)-aminolevulinic acid synthetases in yeast has been suggested by Mahler and Lin (25). (d) The residual activity in ole3 extracts may represent a “proenzym” with very low catalytic activity. In this case the ole3 and cycl1 mutations would most probably cause defects in processing of such a proenzyme to produce a more active form (1). It was surprising to find that the \( \textit{in vitro} \) \( \delta \)-aminolevulinic acid synthetase activity of the cycl1 strain was almost as low as that found in ole3 extracts. The relative \( \textit{in vivo} \) activity of the enzyme, as indicated by cytochrome formation in the absence of added \( \delta \)-aminolevulinic acid, is apparently much higher in a cycl1 strain than in a strain containing ole3. Either the cycl1 enzyme is very labile \( \textit{in vitro} \), or other genetic factors, differing in the two strains, are responsible for the \( \textit{in vivo} \) differences in cytochrome formation. Additional studies will be required to distinguish between these two possibilities. Notwithstanding the lack of quantitative correlation between relative activities \( \textit{in vivo} \) and \( \textit{in vitro} \), the \( \textit{in vitro} \) data clearly demonstrate that both the cycl1 and ole3 mutations result in greatly diminished \( \delta \)-aminolevulinic acid synthetase activity relative to that found in normal yeast.

Since the cycl4 mutation by itself does not result in diminished \( \delta \)-aminolevulinic acid synthetase activity \( \textit{in vitro} \), the normal allele, CYC4, cannot be a gene which regulates the transcription of the \( \delta \)-aminolevulinic acid synthetase structural gene. Instead, it may be concerned either with the expression of \( \delta \)-aminolevulinic acid synthetase activity \( \textit{in vivo} \) or with the efficiency of utilization of \( \delta \)-aminolevulinic acid in cytochrome formation. It has previously been noted that because of its response to added \( \delta \)-aminolevulinic acid, the cycl4 mutant might be classified as porphyrin-deficient rather than as cytochrome-deficient (1). Sugimura and co-workers (26) have described a mutant which accumulates porphyrins, which are probably formed by spontaneous oxidation of accumulated porphyrinogens. Cells of a strain containing this mutation exhibit porphyrin absorption bands at 538 and 575 nm. In contrast, cycl4 strains do not exhibit detectable absorption at these wavelengths. Therefore, if the partial requirement for \( \delta \)-aminolevulinic acid exhibited by cycl4 reflects a partially defective step in porphyrin biosynthesis, this step probably involves the initial formation of tetrapyrrole or an earlier precursor in the biosynthetic sequence.

In a previous report (1) it was demonstrated that the cycl1 defect could be unmasked by converting a strain containing this mutation from the \( \rho^+ \) to the \( \rho^- \) state. This observation revealed a close relationship between one or more genes in mitochondrial DNA and a gene in the nucleus, CYD1, which controls a key reaction in mitochondrial biogenesis. Since the present data strongly suggest that CYD1 controls the primary structure of \( \delta \)-aminolevulinic acid synthetase, an intramitochondrial enzyme (27), the unmasking of the cycl1 defect may well result from the inability of a partially defective \( \delta \)-aminolevulinic acid synthetase to withstand the abnormal environmental stress encountered when mitochondria are rendered defective by the \( \rho^- \) mutation. The nature of this presumed stress is not known: it might stem from changes in intramitochondrial binding sites for \( \delta \)-aminolevulinic acid synthetase or from failure of \( \rho^- \) organelles to incorporate the partially defective enzyme. Following the same line of thought, the present demonstration that the cycl1 defect can also be fully unmasked by the nuclear mutation, cycl4, suggests that the cycl4 mutation might also cause an abnormality in the incorporation or binding of \( \delta \)-aminolevulinic acid synthetase within mitochondria. Since succinyl-CoA, one of the substrates for the enzyme, is produced and utilized inside the mitochondria, any unincorporated, extramitochondrial \( \delta \)-aminolevulinic acid synthetase probably could not function for lack of substrate.

The close similarity in nutritional responses given by the cytochromeless cycl1 cycl4 strain and an ole3 strain lend additional support to the hypothesis advanced by Bard et al., (2) that primary defects in porphyrin biosynthesis, by causing deficiencies in hemoproteins such as cytochrome \( \beta \) and cytochrome P450, result in defective lipid biosynthesis. The observed difference in susceptibility of the two types of strain to the \( \rho^- \) mutation may provide a clue to the role of the normal CYC4 gene-product. However, one or more genes derived from The Johns Hopkins genetic stocks which differ from their allelic counterparts in the Sheffield stocks may govern this difference. Therefore, appropriate breeding experiments must be performed before implicating cycl4 as the determinant responsible for the difference in \( \rho^- \) frequency. Regardless of the genetic basis for the higher susceptibility of the double mutant, it is clear that it requires a deficiency in unsaturated fatty acid for expression. It seems likely, then, that a component of the mitochondrial membrane, closely associated with lipid, is somehow involved in maintaining the integrity of mitochondrial DNA. The existence of this postulated component is revealed by the differences in the two mutant types, cycl1 cycl4 and ole3. As noted under “Results,” an alternative explanation for the effects of unsaturated fatty acid on \( \rho^- \) frequency is that unsaturated fatty acid controls the selective growth advantage of \( \rho^+ \) cells relative to \( \rho^- \) cells. Further study is therefore needed to define the role of cycl4.

The nutritional requirement for methionine observed when ole3 strains are grown on minimal medium may well be the result of defective sulfur metabolism. Since inorganic sulfate is the sole source of sulfur in this medium, methionine and cysteine biosynthesis are dependent upon the metabolic pathway which reduces sulfate to sulfide. One of the enzymes of this pathway, sulfite reductase, probably contains a porphyrin-related prosthetic group. Murphy et al. (28) have shown that the sulfite reductases (EC 1.8.1.2) of \( \textit{Escherichia coli} \) and other bacteria contain siroheme, an iron-tetrahydroporphyrin with 8 carboxylate side chains. Since the spectra and other properties of the purified yeast enzyme are very similar to those of the \( \textit{Escherichia coli} \) enzyme (29), it is quite likely that yeast sulfite reductase also contains siroheme. Consequently, defective porphyrin
biosynthesis would be expected to result in defective conversion of inorganic sulfate to amino acid sulfur and in the methionine requirement observed.

Since the δ-aminolevulinic acid synthetase-deficient mutants characterized in this work produce almost no detectable cytochromes, yet retain functional mitochondrial DNA, they have important potential as experimental systems for studying mitochondrial assembly. With these strains it is possible to manipulate mitochondrial development over a very wide range simply by varying the δ-aminolevulinic acid content of the growth medium. This system therefore provides a valuable new companion to two other widely used systems in investigating the organelle assembly process in yeast: aerobic adaptation of anaerobically-grown cells and derepression of glucose-repressed yeast. A particularly attractive prospect for this new system is that it can be used to manipulate mitochondrial development under highly aerobic conditions in the absence of glucose repression.

Another potential application of these mutants is in studying mechanisms of mitochondrial energy-transfer. Since mitochondria lacking cytochromes contain significant venturicidin-sensitive ATPase, it should be possible to use these organelles to investigate the energy-transfer system in its native orientation without interference from the respiratory chain. Cytochrome-less mitochondria may also provide useful starting material for purifying components of the energy-transfer system.

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