Properties of Cytoplasmic Mutants of *Saccharomyces cerevisiae* with Specific Lesions in Cytochrome Oxidase*

(mitochondrial genetics/mitochondrial biogenesis/biosynthesis of cytochrome c oxidase/cytochrome c oxidase mutants/genetics of cytochrome c oxidase)

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Communicated by Sarah Ratner, March 7, 1975

ABSTRACT Two mutants with specific defects in cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase; EC 1.9.3.1) have been isolated from cultures of *Saccharomyces cerevisiae* exposed to the mutagens ethylmethane sulfonate and Mn++. The mutations have been shown to be extranuclear by two criteria. The phenotype persists in diploids formed by a cross with a ρ0 strain of yeast of the opposite mating type. Tetrad analysis indicates a non-Mendelian segregation (4:0 and 0:4) of the mutations. Both mutants show a total absence of cytochrome oxidase activity and of spectral cytochromes a and a0. One of the mutants has been shown to be missing a polypeptide synthesized by mitochondria. The migration of this protein on polyacrylamide gels corresponds to the highest-molecular-weight subunit of cytochrome oxidase.

It has been established from recent studies with yeast (1, 2) and *Neurospora crassa* (3, 4) that the cytochrome oxidase complex of mitochondria contains both mitochondrially and cytoplasmically synthesized subunit proteins. In both organisms the enzyme has been shown to consist of seven distinct polypeptides, the three largest being mitochondrial translation products (1, 2, 4). Although it is generally assumed that the mitochondrial products are coded for by mitochondrial DNA, this has not been experimentally verified. A simple and direct demonstration of this relationship would be the isolation of cytoplasmic mutants with specific lesions in enzymes known to have mitochondrially synthesized proteins.

In this communication we report two mutants of *Saccharomyces cerevisiae* which show non-Mendelian segregation and whose primary defect is in the cytochrome oxidase complex. Biochemical analyses indicate that both mutants lack the visible absorption bands of cytochromes a and a0 and that one of the mutants is missing the largest subunit of the oxidase.

MATERIALS AND METHODS

The haploid prototrophic strain of α mating type, *S. cerevisiae* D273-10B, characterized by Sherman et al. (5) was used in this study.

Abbreviations: ρ, cytoplasmically inherited gene whose mutation leads to a pleiotropic loss of mitochondrial respiration, ATPase, and mitochondrial protein synthesis; YPD, yeast extract/peptone/dextrose; YEPG, yeast extract/peptone/ethanol/glycerol.

* This is paper XII in the series “Assembly of the Mitochondrial Membrane System.”

Isolation of Mutants. The details of the screening procedure used to isolate the mutants will be described elsewhere. Briefly, the selection method consisted of analyzing mitochondrial functions in a class of mutants that were unable to grow on glycerol but were still capable of mitochondrial translation.

Complementation Test. The tester strain used was *Saccharomyces carlsbergensis* CB11 (a *MAL6 ade*) which was subjected to two cycles of ethidium bromide treatment under conditions that should have deleted all of the mitochondrial DNA (6). The mutants were mixed with the tester strain on solid YPD medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). After overnight growth the mated cells were replicated to YEPG medium (1% yeast extract, 2% peptone, 2% ethanol, 3% glycerol (v/v), and 2% agar). Growth on this medium was checked after 1–2 days of incubation at 30°.

Tetrad Analysis. Approximately equal numbers of mutant cells and cells of *S. carlsbergensis* CB11 (a *MAL6 ade*) were mixed in liquid YPD medium. The suspensions were centrifuged and the pelleted cells were incubated at 30° for 5 hr. The cells were then washed with 5 ml of water and streaked on solid sporulation medium (2% potassium acetate, 0.1% glucose, and 2% agar). Tetrads could be observed after 2–3 days' incubation of the plates at room temperature. Spores were separated with a microneedle after digestion of the ascus wall with Glusulase (Endo Lab., Inc. N. J.) by standard procedures (7).

Isolation of Mitochondria. Mutant and wild-type cells were grown aerobically at 30° in 2% galactose medium containing 0.3% yeast extract and the basic salts of Ephrussi and Slonimski (8). The cells were harvested in early stationary phase, washed, and suspended at a concentration of 5–10 g of wet weight per 30 ml of a solution containing 0.25 M mannitol, 0.02 M Tris-acetate, pH 7.5, and 1 mM EDTA. The cells were broken in a Bronwill homogenizer and mitochondria were isolated as described previously (9). Submitochondrial particles were prepared by sonic irradiation of mitochondria with a Raytheon 10 kHz-Sonic Oscillator (9).

Biochemical Analysis of Mutants. The submitochondrial particles were assayed for rutamycin-sensitive ATPase (ATP

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phosphohydrolase; EC 3.6.1.3), cytochrome oxidase (ferro-cytochrome c oxidoreductase; EC 1.9.3.1), and NADH-cytochrome c reductase as described previously (10). Spectra were recorded at room temperature with a Cary 14 spectrophotometer. Proteins were determined by the method of Lowry et al. (11).

**Labeling and Detection of Mitochondrial Products.** Mitochondrial products were labeled in vivo under the following conditions. The cells were grown in 2% glucose medium containing 0.3% yeast extract, and the basic salts of Ehrusni and Slonimski (8). After overnight aeration at 30°C (early stationary culture) the cells were harvested and transferred to fresh medium supplemented with 2 mg/ml of chloramphenicol and further aerated for 3 hr. The chloramphenicol-treated cells were washed with water and inoculated into a medium containing 2% glucose, salts, 50 μM cycloheximide, and 20 μCi/ml of [3H]leucine. After 2 hr of labeling in this medium, the cells were harvested and washed with the mannitol/Tris/EDTA solution, and submitochondrial particles were prepared as described above. The mitochondrial products labeled during the cycloheximide incubation were analyzed in whole submitochondrial particles by gel electrophoresis.

**Gel Electrophoresis.** Submitochondrial particles were dissociated in a solvent containing 2% sodium dodecyl sulfate, 0.1 M Tris-acetate, pH 8.0, and 1% mercaptoethanol (v/v). The depolymerized proteins were separated on 15% polyacrylamide gels (0.6 x 10 cm) in the presence of 0.5% sodium dodecyl sulfate and 0.1 M Tris-acetate, pH 8.0 (3). The gels were frozen, sliced into 1 mm sections, dissolved in H₂O₂, and analyzed for radioactivity as described previously (2).

### RESULTS

#### Enzymatic properties of mutants

Three mutagens were used to obtain mutants of *S. cerevisiae* with defects in mitochondrial functions—N-acetyl-l-amino-2-nitro-N-nitrosoguanidine, ethylmethane sulfonate, and Mn++. Mn++ has been recently shown to increase the frequency of cytolytic drug-resistant mutants in yeast (12). Out of approximately 4000 mutants examined that showed lack of growth on glycerol, two strains had specific defects in cytochrome oxidase and exhibited non-Mendelian segregation of the phenotype. One mutant, E3-19, was isolated from a stock mutated with ethylmethane sulfonate and the other, M3-9, was derived by mutagenesis with Mn++.

**TABLE 1. Mitochondrial respiratory and ATPase activities of S. cerevisiae**

<table>
<thead>
<tr>
<th>Strain</th>
<th>NADH-cyt. c reductase</th>
<th>Cytochrome oxidase</th>
<th>ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>D273-10B</td>
<td>0.35</td>
<td>0.50</td>
<td>5.0</td>
</tr>
<tr>
<td>E3-19</td>
<td>0.18</td>
<td>&lt;0.01</td>
<td>2.8</td>
</tr>
<tr>
<td>M3-9</td>
<td>0.4</td>
<td>&lt;0.01</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Submitochondrial particles were prepared from cells grown to early stationary phase on 2% galactose medium as described under Methods. The specific activities are based on particle protein. ATPase was assayed at 37°C and cytochrome oxidase at 23°C.

![Absorption spectra of mitochondrial extracts from strains D273-10B (Wild type, WT), E3-19, and M3-9. Submitochondrial particles from cells grown on 2% galactose were extracted with 1% deoxycholate, 1 M KCl, and 50 mM Tris-HCl, pH 8.0, at a protein concentration of 8 mg/ml. The suspensions were centrifuged at 165,000 Xg for 20 min and the clear supernatants were used for the difference spectra. The extract in the reference cuvette was oxidized with a small amount of potassium ferricyanide and that in the sample cuvette was reduced with a few grains of sodium dithionite. The difference spectra were obtained at room temperature with a Cary 14 spectrophotometer.](image)

The three mitochondrial enzyme activities assayed, only cytochrome oxidase was totally absent in both mutants. NADH-cytochrome c reductase was somewhat lower in E3-19 but normal in M3-9. The rutamycin-sensitive ATPase in both mutants was approximately 60% of wild type (Table 1). Difference spectra of reduced versus oxidized extracts of submitochondrial particles are shown in Fig. 1. These spectra correlate with the enzymatic properties. Both E3-19 and M3-9 showed the presence of b- and c-type cytochromes and a total absence of cytochromes a and a₂.

**Genetic properties of mutants**

Two genetic tests indicated that the mutations in E3-19 and M3-9 are cytolytically inherited. (1) When crossed to a ρ+ strain of opposite mating type, neither mutant produced diploids capable of growth on glycerol. The mutations, therefore, do not appear to be complemented by a cell with a normal complement of nuclear genetic information for cytochrome oxidase. (2) When the mutants were crossed to a ρ+ haploid strain, the segregation of the phenotype in tetrad analyses was non-Mendelian. E3-19 and M3-9 were crossed to a respiratory competent haploid strain, *S. carlsbergensis* CB11 (a MAL6 ade1), and the diploids were sporulated on potassium acetate medium. The spores derived from the crosses were scored for growth on glycerol. The results of the tetrad analyses (Table 2) show a 4:0 and 0:4 segregation pattern of growth on glycerol for both mutants. It is interesting, however, that in one tetrad derived from M3-9 the spores segregated as 1:3. We do not know whether this result was due to a false tetrad or whether it has a real genetic basis.

The enzymatic phenotype of the meiotic segregants showing inability to grow on glycerol was examined in more detail. The four progeny cells from a single (0:4) tetrad obtained by
TABLE 2.  Tetrad analysis of E3-19 and M3-9

<table>
<thead>
<tr>
<th>Ascus number</th>
<th>E3-19</th>
<th>M3-9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

E3-19 and M3-9 were crossed to *S. carlsbergensis* CB11 (a *MAL6 ade1*). The diplods were sporulated on solid potassium acetate medium. Tetrad were separated and the spores were grown on YPD medium for 2-3 days. They were then patched onto YEPG medium and growth was checked after 2 days. The plus signs indicate growth on glycerol, zeros indicate lack of growth on glycerol. The following spore viabilities were found: E3-19—7 tetrads with 4 viable spores, 4 tetrads with 3 viable spores, 3 tetrads with 2 viable spores, and 2 tetrads with 1 viable spore. M3-9—7 tetrads with 4 viable spores, 3 tetrads with 3 viable spores, 4 tetrads with 2 viable spores and 2 tetrads with 2 viable spores. Only the results from tetrads in which all four spores germinated are reported.

A cros of E3-19 and M3-9 to *S. carlsbergensis* CB11 (a *MAL6 ade1*) were grown in 2% galactose and the mitochondria were assayed for NADH-cytochrome c reductase, cytochrome oxidase, and rutamycin-sensitive ATPase. The results of this experiment (Table 3) indicate that the four segregants from each of the two crosses preserve the enzymatic phenotype of the parental mutant. It is also of interest that both E3-19 and M3-9 complemented nuclear mutants with specific lesions in cytochrome oxidase of which 20 were tested.

The stability of E3-19 and M3-9 was checked both with respect to reversion to wild type and conversion to cyto-

TABLE 3.  Enzyme activities of meiotic segregants of E3-19 and M3-9

<table>
<thead>
<tr>
<th>Spore</th>
<th>Specific activities (umol/min per mg)</th>
<th>NADH-cytochrome c reductase</th>
<th>ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rut.</td>
<td>+rut.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E3-19</td>
<td>M3-9</td>
</tr>
<tr>
<td>A</td>
<td>&lt;0.01</td>
<td>0.15</td>
<td>2.72</td>
</tr>
<tr>
<td>B</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>2.95</td>
</tr>
<tr>
<td>C</td>
<td>&lt;0.01</td>
<td>0.13</td>
<td>2.73</td>
</tr>
<tr>
<td>D</td>
<td>&lt;0.01</td>
<td>0.20</td>
<td>2.50</td>
</tr>
</tbody>
</table>

E3-19 and M3-9 were crossed to *S. carlsbergensis* CB11 (a *MAL6 ade1*) and sporulated as described in the legend to Table 2. A single tetrad from each cross in which the four segregants did not grow on glycerol was selected for the enzymatic analyses. The cells were grown on 2% galactose medium to stationary phase, and mitochondria were isolated and assayed as described under Materials and Methods.

plasmic petites. The frequency of spontaneous revertants in E3-19 was approximately 1 per 10⁶ cells. No spontaneous revertants were found in M3-9 when 10⁶ cells were plated on YEPG. The conversion to cytoplasmic petites could not be studied by the standard mating procedure, which involves crossing to a ρ₀ tester strain and scoring for growth of the diploids on glycerol, since the mutants themselves do not complement the ρ₀ tester. In our studies of nuclear mutants of cytochrome oxidase, however, we have noted that the cytoplasmic petites produced from such mutants grow more slowly on YPD plates and therefore appear as smaller colonies. We have found that this circumstance allows cytoplasmic petites to be accurately estimated on the basis of colony morphology alone. When this test was used, the conversion to cytoplasmic petites was found to be 20% for E3-19 and less than 2% for M3-9. Two presumptive cytoplasmic petites produced by E3-19 were picked and examined for their ability to carry out mitochondrial protein synthesis. Neither was capable of synthesizing any of the mitochondrial protein products seen in wild-type cells.

Mitochondrial Products in E3-19 and M3-9. The products of mitochondrial protein synthesis in E3-19 and M3-9 were compared to those of wild-type cells by in vivo labeling with [³H]leucine in the presence of cycloheximide. The gel patterns of the total mitochondrial products present in submitochondrial particles are shown in Fig. 2. Although there is some reduction in the radioactive protein bands of E3-19, the overall pattern of proteins made is similar to that seen in wild type. In M3-9, however, the second largest protein band is
almost totally absent. This protein migrates with the same mobility as the highest-molecular-weight subunit of cytochrome oxidase.

**DISCUSSION**

Several types of mitochondrial mutants have been described in which it has been shown that the genetic defect resides in mitochondrial DNA. Among these mutants are those resistant to drugs that affect mitochondrial translation, such as erythromycin (13) and chloramphenicol (14), mutants that are resistant to inhibitors of oxidative phosphorylation and the ATPase complex (oligomycin, tributyltin) (15), and most recently pleiotropic mutants which are still capable of mitochondrial protein synthesis (16). With the exception of the drug-resistant mutants all cytoplasmically inherited mutations in *S. cerevisiae* have been found to be pleiotropic with a general loss of mitochondrial respiratory activities and of oligomycin-sensitive ATPase. The two cytoplasmic mutants isolated in this study are novel in that the mutations seem to be restricted to cytochrome oxidase, as determined by enzyme activity and the spectral properties of the mitochondria. Analysis of the mitochondrial products formed in the mutants indicates an almost complete absence in M3-9 of a protein that corresponds in migration to the largest subunit of cytochrome oxidase. Since M3-9 does not revert it is possible that the mutation involves a deletion of a segment of mitochondrial DNA that codes for one of the subunits of the oxidase. It cannot be excluded, however, that the mutation is in a gene that controls the translation of a product of mitochondria that is involved in the assembly of the cytochrome oxidase enzyme.

The mutants should be valuable in studies on the biosynthesis of cytochrome oxidase and of the role of mitochondrial gene products in this process. Secondly, the mutants may also be of value from the standpoint of mitochondrial genetics. Since the genes affected appear to be concerned with the development of functional cytochrome oxidase, it should be of interest to map them relative to some of the cytoplasmic drug-resistant mutants presently available.

This research was supported by Grants no. 1RO1-HE 13003 and GM 18888 from the National Institutes of Health, U.S. Public Health Service.