Petite Mutation in Yeast

II. Isolation of Mutants Containing Mitochondrial Deoxyribonucleic Acid of Reduced Size

ELIZABETH S. GOLDRING, LAWRENCE I. GROSSMAN, AND JULIUS MARMUR
Department of Biochemistry and Department of Genetics (Division of Biological Sciences), Albert Einstein College of Medicine, Bronx, New York, 10461

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A series of petite mutants of Saccharomyces cerevisiae, generated after treatment for various times with ethidium bromide, was isolated, and the mitochondrial deoxyribonucleic acid size for each member was estimated. It was found that, as the treatment time with ethidium bromide was increased, the mitochondrial deoxyribonucleic acid isolated from the petite series was increasingly reduced in size.

Respiratory-deficient (petite) mutants of yeast are characterized by an inability to utilize non-fermentable substrates such as glycerol for growth. This effect is now well known to result from the presence of mitochondria which lack cytochromes (a + a₃) and b, as well as a number of respiratory enzymes, and are thus unable to engage in respiration (for reviews, see 9, 10).

Cytoplasmic petite mutations are irreversible; their deficiency is inherited in a non-Mendelian fashion (3) and usually associated with an altered buoyant density of mitochondrial deoxyribonucleic acid (DNA; see references 1, 2, 6). Their study may provide a useful approach to elucidating the function of mitochondrial DNA.

Respiratory-deficient mutants of the yeast Saccharomyces cerevisiae can be induced with close to 100% efficiency by the phenanthrene dye, ethidium bromide (EB; see reference 11). The dye acts by an unknown mechanism to cause the breakdown and eventual loss of mitochondrial DNA. After prolonged EB treatment, petites containing no detectable mitochondrial DNA were obtained (4; Goldring et al., Fed. Proc., 1970, p. 725).

The present work was undertaken to examine the degradation and eventual elimination of mitochondrial DNA at the individual cell level by isolating and culturing petite mutants at selected intervals during the EB treatment. A preliminary report of some of this work has appeared (Goldring et al., Fed. Proc., 1970, p. 725).

All of the petites used were isolated in a single experiment. The method of isolation of the mutants and the characterization of their DNA is shown diagrammatically in Fig. 1.

The amount of mitochondrial DNA present in each petite strain decreased as the treatment time with EB was increased. Approximately 10% of total cellular DNA is present in mitochondria of large strains. This proportion is somewhat low to allow accurate measurements of possible decreased mitochondrial DNA content in the petite strains. Thus, an amplification technique previously described (5) was used to increase the sensitivity of the measurements. This technique utilizes the fact that, in the presence of a cytoplasmic protein synthesis inhibitor such as cycloheximide (CH), about 85% of newly synthesized DNA is mitochondrial. When petite mutants were labeled in the presence of CH and the DNA was run in preparative CsCl gradients, the proportion of mitochondrial DNA synthesized relative to nuclear DNA could be measured.

The CsCl density gradient patterns of DNA extracted from this sequence of petites are shown in Fig. 2. The radioactivity in each nuclear DNA peak is 1825 ± 500 cpm. The DNA from strain KA shows a buoyant density pattern identical to its large parent when both are labeled in the presence of CH. Mutants isolated after more extensive treatment with EB show a decreased proportion of mitochondrial DNA. A mutant picked and cultured after being exposed to EB for 240 min (KO) contains mitochondrial DNA at the limit of detection by this method. Arrows (Fig. 2) bracket the region of mitochondrial DNA in each gradient.

The size of the mitochondrial DNA in the petite series was determined by pooling the mito-
chondrial region of each CsCl gradient shown in Fig. 2, dialyzing the preparation against SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7), and sedimenting it through a 5 to 20% sucrose gradient. Several of the sedimentation profiles are shown in Fig. 3. DNA from petite KA has a sedimentation pattern similar to that obtained from the grande, A664a/18A (4), although some more slowly sedimenting material is apparent, possibly indicating an early EB effect. Petites isolated after longer EB treatments show a marked decrease in the size of their mitochondrial DNA. The sedimentation data for these petities is summarized in Table 1, along with the EB treatment time used for their isolation and the nominal molecular weight calculated for each petite DNA from the sedimentation coefficient of the major peak.

Most petities are known to undergo a decrease in the buoyant density of their mitochondrial DNA from the value for grande cells (1.683 g/cc; references 1, 2, 6). The buoyant densities in CsCl of a number of the petities reported here were determined. Since most of these petity mutants contained reduced amounts of mitochondrial DNA, analytical buoyant density determinations were difficult to perform. Instead, 3H-labeled mitochondrial DNA from several of the EB-induced petities was centrifuged to equilibrium in preparative CsCl gradients containing 14C-labeled mitochondrial DNA from the grande strain. Figure 4 shows the results of such experiments. DNA from the petite KA is indistinguishable from the grande strain marker DNA, whereas other petities in the series gradually suffer a decrease in their mitochondrial DNA buoyant density.

EB treatment of respiratory-sufficient cells was previously shown to cause degradation and eventual elimination of mitochondrial DNA without demonstrably affecting the extent of synthesis of nuclear DNA; in addition, mitochondrial DNA synthesized in the presence of EB was reduced in size and amount (4; Goldring et al., Fed. Proc., 1970, p. 725). Some of these results were recently confirmed (7). In the present work, we examined the properties of mitochondrial DNA in petite strains generated by treatment with EB; these strains originated as single colonies and were analyzed after many generations of growth in the absence of the dye.

The size to which preexisting labeled mitochondrial DNA is degraded after a given length of EB treatment (4) correlates strikingly with the size of mitochondrial DNA found in these stable strains which were isolated after treatment with dye for similar time periods. Thus, one may infer that removal of the dye at any time during the gradual degradation of mitochondrial DNA stops further breakdown and allows the cells thereafter to remain and replicate the remaining

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Fig. 1. Plan of the experiment. EB (10 μg/ml) was added to a culture of log-phase cells. Samples were removed at various times and diluted with SP (0.15 M NaCl, 0.02 M potassium phosphate, pH 7.4); a sample was plated and scored for the proportion of petities, as previously described (4). The kinetics of petite induction (reproduced as described in reference 4) are shown. Another sample was similarly plated to isolate the mutants. After 2 to 3 days of growth at 30°C, petite colonies were picked and cultured in the absence of EB. Their DNA was then radioactively labeled and centrifuged to equilibrium in CsCl; the mitochondrial region of each gradient was pooled, dialyzed, and used for size determination by sucrose gradient centrifugation.

Fig. 3. Sucrose gradient sedimentation of petite mitochondrial DNA. (a) Grande DNA (reproduced from reference 4); (b) KA DNA; (c) KH DNA; (d) KO DNA. Fractions enclosed by arrows in Fig. 2 were pooled, dialyzed against SSC, and sedimented through a 5 to 20% sucrose gradient. Running and processing of gradients was previously described (4). Each sample contains 14C-DNA from phage T7 as a sedimentation marker. The sedimentation coefficients of major peaks, measured relative to T7 DNA (taken as 32S), are also indicated. The direction of sedimentation is to the left.
Fig. 2. Preparative CsCl gradient patterns of several petites. Log-phase cultures (15 ml) were labeled for 5 hr with 3H-adenine (15 μCi/ml) in the presence of CH (5). Cells were converted to spheroplasts (4) and lysed, and the DNA was centrifuged to equilibrium in an angle rotor (5) and collected; a sample of each fraction was processed (5). The mitochondrial DNA region is bracketed by arrows. Buoyant density increases to the left in each gradient. The nuclear DNA peak positions represent a density of 1.699 g/cc. The time in each panel refers to the length of treatment of a culture with EB prior to isolating each strain.
TABLE 1. Origin and properties of the petite strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment time (min)</th>
<th>Petites in population (%)</th>
<th>$S$</th>
<th>Mol wt $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A664a/18A</td>
<td>30</td>
<td>21$^*$</td>
<td></td>
<td></td>
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<tr>
<td>KA</td>
<td>5</td>
<td>4</td>
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<td>21</td>
</tr>
<tr>
<td>KC</td>
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<td>3.5</td>
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<td></td>
</tr>
<tr>
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<td>17</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
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<td>0.6</td>
</tr>
<tr>
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<td>98.9</td>
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<td></td>
</tr>
<tr>
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<td>120</td>
<td>99.5</td>
<td>11</td>
<td>1.1</td>
</tr>
<tr>
<td>KO</td>
<td>240</td>
<td>99.9</td>
<td>7</td>
<td>0.31</td>
</tr>
</tbody>
</table>

$^a$ EB was used at 10 $\mu$g/ml. Samples were removed at the indicated times, diluted with SP (0.15 m NaCl, 0.02 m potassium phosphate, pH 7.4), and distributed on yeast-peptone plates (4). After incubation at 30 C for 2 to 3 days, the proportion of petites was determined by a tetrazolium overlay method (8).
$^b$ After the treatment times indicated in column 2.
$^c$ Shown for the major sedimenting species.
$^d$ Calculated from the sedimentation values of column 4 using the equation of Studier (11). Figures shown to be multiplied times 10$^4$.
$^e$ This value is reproducibly obtained from the grande strain but probably does not represent intact molecules.
$^f$ Not determined.

mitochondrial DNA. Although the size of the mitochondrial DNA remaining after removal of EB appeared to be stable during the course of these experiments, we have no data on its persistence for longer periods. Treatment with EB until degradation is complete produces strains devoid of detectable mitochondrial DNA (4, 7; Goldring et al., Fed. Proc., 1970, p. 725).

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LITERATURE CITED

**FIG. 4.** Buoyant densities of several mitochondrial DNA samples. The mitochondrial region of CsCl preparative density gradients analogous to those shown in Fig. 2 were isolated and rerun. Petite DNA samples were labeled with $^3$H-adenine; grande mitochondrial DNA, present in each gradient as a density marker, was labeled with $^{14}$C-adenine. Running and processing of gradients was previously described (5). Density increases to the left: grande DNA has a buoyant density of 1.683 g/cc. $^3$H petite mitochondrial DNA (●). $^{14}$C grande mitochondrial DNA (○).