Influence of growth conditions upon the number of chloroplast DNA molecules in *Euglena gracilis*  
(observed second-order rate constant)

JAMES R. Y. RAYSON AND CINDY BOERMA

Departments of Botany and Biochemistry, University of Georgia, Athens, Ga. 30602

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ABSTRACT The number of chloroplast DNA molecules in *Euglena gracilis* cells was measured by determining the shift in the observed second-order rate constant for the reassociation of 125I-labeled chloroplast DNA in the presence of unlabeled total cell DNA. Cells grown to stationary phase in the dark contained 217 molecules of chloroplast DNA. Cells grown to stationary phase in the light in either heterotrophic or autotrophic medium contained 590 and 1014 chloroplast DNA molecules, respectively. The observed second-order rate constant for the reassociation of 125I-labeled chloroplast DNA was not significantly altered in the presence of total cell DNA from a heat-bleached mutant, ZHB, which lacks chloroplast DNA. This evidence suggests that there is less than 0.3 of a chloroplast DNA molecule present in the nucleus of *Euglena*.

The fate of the genetic information in the chloroplast of *Euglena gracilis* during cell growth in the dark has provoked considerable discussion. Edelman et al. (1) demonstrated in dark-grown cells the presence of a satellite DNA that had a mean buoyant density similar to that of chloroplast DNA. Uzzo and Lyman (2) showed this satellite represented about 50% of that found in light-grown cells. A variety of different types of RNA-DNA hybridization experiments (3–5) indicate that chloroplast DNA transcripts do exist in dark-grown cells.

The precise number of chloroplast DNA molecules in both dark- and light-grown cells of *Euglena gracilis* has been difficult to determine. Previous measurements (1, 2) have depended solely upon resolving the relative amounts of chloroplast DNA appearing as a satellite in CsCl equilibrium density gradients. The small proportion of total cell DNA represented by chloroplast DNA and the poor resolution between chloroplast DNA and other DNA species (i.e., mitochondrial DNA) have contributed significantly to the uncertainty of these measurements.

We have measured the number of chloroplast DNA molecules in dark- and light-grown cells of *Euglena* by determining the shift in the observed second-order rate constant for the reassociation of radioactively labeled chloroplast DNA in the presence of unlabeled total cell DNA. Since the rate of DNA reassociation is directly proportional to the initial concentration of the reactants, the observed second-order rate constant of labeled chloroplast DNA in the presence of unlabeled total cell DNA will reflect the number of unlabeled sequences of chloroplast DNA in the reaction mixture (6).

We found that the number of chloroplast DNA molecules in *Euglena* cells is a function of the cell’s growth conditions. The number of chloroplast DNA molecules increases several-fold when the cells are grown in the light instead of the dark. Our data also indicate that in *Euglena* there are no sequences of chloroplast DNA complementary to nuclear DNA.

MATERIALS AND METHODS

Cell Growth. Axenic cultures of *Euglena gracilis*, var. Z, were grown to stationary phase in either an autotrophic medium (7) or a heterotrophic medium (8) containing glutamic acid and malic acid. A heat-bleached mutant of *E. gracilis*, var. ZHB (obtained from H. Lyman, SUNY, Stony Brook, N.Y.), which contains no chloroplast DNA, was grown to stationary phase in the light in the heterotrophic medium. All cells were harvested in a Sharples centrifuge and stored at −60°C.

Isolation of DNA. Chloroplast DNA was isolated from chloroplasts that had been purified on Renografin gradients (10). The DNA contained all those sequences found in a supercoiled, closed circular chloroplast DNA molecule (11). Total cell DNA was isolated as previously described (9) from cells grown either in the light or the dark. Salmon sperm DNA (Sigma) was dissolved in 0.15 M sodium chloride, 0.015 M sodium citrate, digested with pancreatic RNase ( Worthington), and then further purified by extensive deproteinization, as was total cell DNA. All DNA was centrifuged in preparative CsCl equilibrium density gradients to remove residual traces of protein, RNA, and polysaccharides. The DNA obtained from the CsCl gradients was dialyzed against sodium chloride, sodium citrate, concentrated by precipitation with ethanol, and stored in sodium chloride, sodium citrate over a drop of chloroform.

Labeling of Chloroplast DNA In Vitro. Purified chloroplast DNA with a single-stranded molecular weight of 120,000 (350 ± 50 nucleotides) was labeled in *vitro* with 125I-jodide (4, 12). The specific activity of the 125I-labeled chloroplast DNA, determined at optimal settings in a Packard Tricarb Liquid Scintillation Counter (model 3320), was 6.9 × 10^6 cpm/μg. Iodination of the DNA reduced its single-stranded molecular weight to 75,000 (230 nucleotides). Under these conditions for labeling the DNA and the efficiency of the scintillation counter, it was calculated that 1.5% of the deoxycytidine residues in the chloroplast DNA contain covalently bound iodine.

Renaturation of DNA. The observed second-order rate constant for the reassociation of 125I-labeled chloroplast DNA was determined by measuring the relative amounts of single-stranded to double-stranded DNA in a reaction mixture on hydroxyapatite columns (9, 13). Salmon sperm DNA and the different total cell DNA samples were sonicated (9) to lower the molecular weights of the DNA to 120,000. Conditions for DNA renaturation and hydroxyapatite chromatography have been previously described (9). The single-stranded and double-stranded fractions eluted from hydroxyapatite were adjusted to 10% trichloroacetic acid and collected on Millipore filters. The filters were dried and placed in toluene-Omnifluor (New England Nuclear) scintillation fluid. Radioactivity was determined as described above.

The extent of duplex formation was monitored as a function
FIG. 1. Reassociation kinetics of $^{125}$I-labeled chloroplast DNA alone and in the presence of either total cell DNA from the ZHB mutant of salmon sperm DNA. $^{125}$I-Labeled chloroplast DNA (0.0694 μg/ml) was reassociated alone (○) and in the presence of either 500 μg/ml of total cell DNA from the ZHB mutant (●) or 500 μg/ml of salmon sperm DNA (●). Samples of 50 μl of the various mixtures were prepared in 0.48 M sodium phosphate (pH 6.8), boiled for 5 min, and incubated for varying periods of time at 62°. The fraction of $^{125}$I-labeled chloroplast DNA reassociated was measured by hydroxyapatite chromatography.

The observed second-order rate constants for the reassociation of $^{125}$I-labeled chloroplast DNA in the presence of various DNA samples were calculated by fitting the experimental points to a single-component, second-order rate reaction. The data were analyzed by the nonlinear least squares computer program in the Biomedical Computer Program Library developed at UCLA (15).

RESULTS

Fig. 1 shows the reassociation kinetics of $^{125}$I-labeled chloroplast DNA from Euglena. Chloroplast DNA was renatured alone and in the presence of either salmon sperm DNA or ZHB total cell DNA. The observed second-order rate constants for each reaction were essentially the same in all three cases. Table 1 shows the observed second-order rate constant and the standard deviation for each reaction. The difference in the observed k values for each reaction is within the calculated standard deviation and is similar to that expected for a molecule of this complexity (16).

The identity of the observed k values for the renaturation of $^{125}$I-labeled chloroplast DNA alone and in the presence of either salmon sperm DNA or ZHB total cell DNA indicated two important features of this reaction. One, the viscosity of the DNA at concentrations similar to those used for the following experiments does not measurably slow down the renaturation reaction. Two, the ZHB mutant has few, if any, sequences complementary to the chloroplast DNA. The latter point is important in arguing that only unlabeled chloroplast DNA in total cell DNA preparations will alter the observed k for the reassociation of $^{125}$I-labeled chloroplast DNA.

<table>
<thead>
<tr>
<th>Origin of DNA driver</th>
<th>$^{125}$I-labeled chloroplast DNA/*</th>
<th>Observed second-order rate constant† of $^{125}$I-labeled chloroplast DNA</th>
<th>Factor of increased rate‡</th>
<th>Chloroplast DNA molecules per Euglena cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast DNA</td>
<td>—</td>
<td>3.63 ± 0.42</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+ ZHB</td>
<td>1.388 x 10⁻⁴</td>
<td>3.46 ± 0.36</td>
<td>0.85</td>
<td>0 ± 0.3</td>
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<tr>
<td>+ Salmon sperm</td>
<td>1.388 x 10⁻⁴</td>
<td>4.12 ± 0.40</td>
<td>1.13</td>
<td>—</td>
</tr>
<tr>
<td>+ Heterotrophic-dark</td>
<td>1.446 x 10⁻⁴</td>
<td>275.8 ± 28.6</td>
<td>79.9</td>
<td>217 ± 23</td>
</tr>
<tr>
<td>+ Heterotrophic-light</td>
<td>1.581 x 10⁻³</td>
<td>64.5 ± 9.48</td>
<td>17.8</td>
<td>530 ± 83</td>
</tr>
<tr>
<td>+ Heterotrophic-light</td>
<td>2.979 x 10⁻⁴</td>
<td>338.9 ± 51.2</td>
<td>93.4</td>
<td>550 ± 84</td>
</tr>
<tr>
<td>+ Heterotrophic-light</td>
<td>1.385 x 10⁻⁴</td>
<td>772.7 ± 108.0</td>
<td>212.9</td>
<td>590 ± 80</td>
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<tr>
<td>+ Heterotrophic-light</td>
<td>4.094 x 10⁻⁴</td>
<td>2597.7 ± 430.3</td>
<td>715.8</td>
<td>585 ± 97</td>
</tr>
<tr>
<td>+ Autotrophic-light</td>
<td>1.280 x 10⁻⁴</td>
<td>1440.5 ± 207.4</td>
<td>396.8</td>
<td>1014 ± 146</td>
</tr>
</tbody>
</table>

* The concentration of $^{125}$I-labeled chloroplast DNA in all cases was 0.0694 μg/ml.
† The observed k was calculated with the computer program described in Materials and Methods.
‡ Factor of increased rate = (observed k of $^{125}$I-labeled chloroplast DNA + total cell DNA/k of $^{125}$I-labeled chloroplast DNA).
Number of chloroplast DNA molecules per *Euglena* cell

The number of chloroplast DNA molecules in total cell DNA can be calculated from the ratio of the observed $k$ of $^{125}$I-labeled chloroplast DNA in the presence of a known amount of unlabeled total cell DNA to the observed $k$ of $^{125}$I-labeled chloroplast DNA alone. Fig. 3 shows the kinetics of reassociation of a constant amount of $^{125}$I-labeled chloroplast DNA in the presence of similar amounts of total cell DNA obtained from cells grown under different conditions. The calculations for determining the number of chloroplast DNA molecules per cell were identical to those of Gelb et al. (6). The observed $k$ values for the reassociation of the labeled chloroplast DNA in different total cell DNA preparations were calculated as described in Materials and Methods and are listed in Table 1.

A model calculation to determine the number of chloroplast DNA molecules per cell is shown in Table 2. The number of chloroplast DNA molecules in *Euglena* cells grown under different conditions is listed in Table 1. Cells grown to stationary phase in the dark in heterotrophic medium contain 217 chloroplast DNA molecules per cell. Dark-grown cells transferred to the light and grown to stationary phase in the same heterotrophic medium show a 2.5-fold increase in the number of chloroplast DNA molecules per cell. Cells grown to stationary phase in autotrophic medium show a 4.7-fold increase in chloroplast DNA molecules per cell over dark-grown cells.

**DISCUSSION**

The number of chloroplast DNA molecules in *Euglena* cells has been shown to be dependent upon growth conditions. In cells grown in the dark to stationary phase, 1.08 ± 0.12% of the total cell DNA is of chloroplast origin. When the cells are grown to stationary phase in the light in the same heterotrophic medium, 2.95 ± 0.43% of the total cell DNA is chloroplast DNA. Total cell DNA from cells grown in the light to stationary phase in an autotrophic medium contain 5.07 ± 0.72% chloroplast DNA. Since the number of proplastids in dark-grown *Euglena* cells (18) is equivalent to the number of chloroplasts in light-grown
Table 2. Calculation of the number of chloroplast DNA molecules per cell

| Molecular weight of chloroplast DNA = 9 x 10^7 * |
| Molecular weight of Euglena DNA per cell = 1.8 x 10^12 † |
| Molecular weight of Euglena DNA/molecular weight of chloroplast DNA = 2 x 10^4 |

125I-Labeled chloroplast DNA molecules/Euglena cell = (μg of 125I-labeled chloroplast DNA/μg of total cell DNA) x 2 x 10^4

Factor of increased rate = (observed k of 125I-labeled chloroplast DNA plus total cell DNA/k of 125I-labeled chloroplast DNA)

Total concentration of chloroplast DNA = (125I-labeled chloroplast DNA molecules/Euglena cell) x factor of increased rate

Chloroplast DNA molecules per cell = total concentration of chloroplast DNA - (125I-labeled chloroplast DNA molecules/Euglena cell)

* Manning et al. (16).
† Parenti et al. (17).

cells, this increase in the number of chloroplast DNA molecules per cell must be an actual increase in the DNA content per chloroplast. Electron microscopy of serial sections of proplastids and chloroplasts from several higher plants, also, indicates a similar dependency upon chloroplast development and the number of chloroplast DNA molecules per organelle (19).

The reassociation kinetics of 125I-labeled chloroplast DNA in the presence of ZHB total cell DNA was identical to that of 125I-labeled chloroplast DNA alone. The standard deviation of the observed k of the labeled chloroplast DNA in the presence of 500 μg/ml of ZHB total cell DNA represents slightly less than 0.3 chloroplast DNA molecule per mutant cell. These measurements discount any significant sequence similarity between nuclear and chloroplast DNAs in Euglena. This conclusion is in contrast to Richards’ (20), who showed that 2% of the nuclear DNA was complementary to chloroplast DNA.

The variance in the number of chloroplast DNA molecules in Euglena as a result of the growth conditions requires that there be a method for regulating the relative rates of chloroplast and nuclear DNA synthesis. Although it may be fortuitous, the observation by Manning and Richards (21) that in exponentially growing cells chloroplast DNA replication proceeds faster than nuclear DNA replication suggests that this type of mechanism does exist in Euglena. The effect of growth conditions upon the number of chloroplast DNA molecules per chloroplast may be one possible means of regulating the expression of the chloroplast DNA sequences necessary for chloroplast development by gene dosage.

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