Studies on Chloroplast Development and Replication in Euglena

III. A STUDY OF THE SITE OF SYNTHESIS OF ALKALINE DEOXYRIBONUCLEASE INDUCED DURING CHLOROPLAST DEVELOPMENT IN EUGLENA GRACILIS

JAMES M. EGAN, JR. and EDGAR F. CARELL
Department of Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

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ABSTRACT

During chloroplast development in Euglena, the activity of a specific DNase, Euglena alkaline DNase, increases in a manner similar to that of chlorophyll synthesis, but without the lag customarily associated with the early hours of chlorophyll synthesis. The increase in Euglena alkaline DNase activity is not inhibited by chloramphenicol or by streptomycin, but is inhibited by cycloheximide. Euglena alkaline DNase activity is present in a group of aplastidic substrains which contain carotenoids. These results are interpreted to mean that this chloroplast-related DNase is synthesized in the cytoplasm, and that the genetic information for this enzyme is probably nuclear.

It is also shown that different bleached substrains exhibit substantial variation, both in total carotenoids and in Euglena alkaline DNase activity. These results are discussed in terms of the possibility that a cytoplasmic photoreceptor system is influencing the light-induced increase in Euglena alkaline DNase activity.

Extracts of Euglena gracilis contain an unusual DNase, with a pH optimum of 9.4 and an absolute requirement for Ca++. This enzyme, termed Euglena alkaline DNase, has a low specific activity in dark-grown cells and a much higher specific activity in light-grown Euglena. Exposure of dividing dark-grown Euglena to light induces an increase in the specific activity of this DNase. Such light-dependent properties have led to the hypothesis that Euglena alkaline DNase is a chloroplast-related enzyme (10).

This report provides further details of the relationship between the development of Euglena plastids and Euglena alkaline DNase. Using nondividing cells, we investigated the induction pattern of Euglena alkaline DNase during chloroplast development, as well as the effects of inhibitors of protein synthesis on this induction pattern. Finally, the levels of Euglena alkaline DNase activity and the levels of carotenoids were determined for a variety of aplastidic substrains derived from the Z and bacillaris strains of E. gracilis.

MATERIALS AND METHODS

Description of Mutants. Euglena gracilis, Klebs, var. bacillaris Pringsheim, and the bleached substrains, W, BUL, W, BHL, and W, BSmL, used in these experiments are described elsewhere (12, 30). Agar slants of the bacillaris strain and its three substrains were kindly provided by Dr. Jerome A. Schiff, Brandeis University, Waltham, Massachusetts.

E. gracilis, Klebs, Strain Z Pringsheim, from our stock culture was also employed (30). The bleached substrains Y, ZHL and W, ZHL were obtained by carrying light-grown strain Z through five rounds of culture on Hutter’s modified medium (29) at 32 C. The bleached substrains Y, ZSM and W, ZSM were produced by carrying light-grown cells through five rounds of culture on the same medium, containing 1 mg/ml of streptomycin at 25 C. In both treatments, the fifth culture was plated on agar. Yellow and white colonies appeared on the agar plates, and one colony of each color was isolated and designated as described elsewhere (33). The substrate Y, ZSL was obtained by plating light-grown cells of the Z strain and isolating a rare yellow colony from the plates. None of the Z strain mutants had detectable levels of chlorophyll, and no plastid remnants could be observed by fluorescence microscopy. No reversion to the wild type has been observed over a 2-year period; in addition, the white substrains did not revert to the yellow form during this time period.

Growth and Resting Conditions. After growing dark-adapted Euglena to a density of 5 × 10⁶ to 1 × 10⁷ cells/ml, the cells were aseptically transferred to Stern resting medium (37). The cells were kept in the dark for 72 hr and were then exposed to 150 ft-c of white fluorescent light for the specified times. At each time period, cells were harvested, rinsed, sampled for chlorophyll (9), and frozen. Further details of growth and resting conditions are presented in the figure legends.

Preparation of Cells for Assays. Frozen cells were thawed and sonicated at the maximum setting on the Branson Biosonik sonicator. The sonication time was 30 sec in the experiment of Figure 3 and 2 min in all other experiments. While 30 sec of sonication was sufficient to release all enzyme activity, sonication periods of 2 min or longer did not appear to affect the specific activities (14). The sonicated suspension was then centrifuged at 30,000g for 30 min, and the supernatant was used as the source of enzyme.

Assay Methods. Two assay methods were used to measure the extent of hydrolysis of heat denatured DNA by Euglena alkaline DNase. In both methods, the incubation temperature was 37 C, CaCl₂, was present at a final concentration of 30 mm, and glycine-NaOH buffer pH 9.4, was added to a final concentration of 0.2 M.

The assay method used most frequently in this paper has been described (10). In this method, reaction mixtures received less than 0.1 mg of protein from the enzyme source. Protein was determined by the Lowry method (23), and the hydrolysis reaction was shown to be linear to at least 0.10 mg of protein. After enzyme addition, reaction mixtures received about 100
DNase Activities and Carotenoid Contents of Bleached Substrains. For the determination of DNase activity, substrains were grown to mid-logarithmic phase in Hutner's modified medium and were harvested, rinsed, and assayed as described above. For the determination of carotenoid contents, substrains in the mid-logarithmic phase were harvested, rinsed, sampled for cell counts, and extracted with 80% (v/v) acetone. The absorption spectrum of the extract was read with a Beckman DK scanning spectrophotometer with a recorder. The amount of carotenoid per cell was estimated from the \[ A_{475} \text{nm}, \] using an average extinction coefficient of \[ E_{475}^1 = 2,500 \] (37).

RESULTS AND DISCUSSION

Euglena Alkaline DNase Changes during Chloroplast Differentiation. Figure 1 shows that, when dark-adapted cells of the Z strain were exposed to continuous white light, the activity of *Euglena* alkaline DNase increased during the 72-hr period normally required for chloroplast differentiation in resting cells (37). The pattern of increase in DNase activity was similar to that of chlorophyll synthesis, except for the first 10 hr of chloroplast differentiation. During this early period, the rate of chlorophyll synthesis exhibited the customary lag reported for *Euglena* (37): *Euglena* alkaline DNase, however, began to increase almost immediately after the cells were exposed to light. Figure 1 also shows that when the cells were removed to darkness at either 15 or 45 hr of chloroplast development, the synthesis of both chlorophyll and *Euglena* alkaline DNase was halted.

During chloroplast development, the increase in DNase activity was the same, whether activity was expressed as specific activity (Fig. 1A) or as activity per cell (Fig. 1B). Thus, the light induction of *Euglena* alkaline DNase cannot be attributed to a decrease in total cell protein, or to a change in the quantity of protein extracted from the cells for assays. That the increase in DNase activity might be due to destruction of an enzyme inhibitor is also ruled out, since an extract of light-grown cells, when mixed with an equal quantity of protein from dark-grown cells, yielded 102% of the total activity expected. When the quantity of protein from dark-grown cells was doubled, 94% of the total activity was detected (14).

Many enzymes show an increase in activity during chloroplast development. Most often, these enzymes are involved in the photosynthetic functions of chloroplasts (15, 16), though recently it has been reported that two plastid related tRNA syntheses are induced (31). It has also been established that removal of light during chloroplast development halts synthesis of many chloroplast components in *Euglena* (25), including chlorophyll and the chloroplast enzyme, TPN triose phosphate dehydrogenase (5, 6). Finally, TPN triose phosphate dehydrogenase has been induced without the lag observed for chlorophyll synthesis (4, 32).

Though the light induction of *Euglena* alkaline DNase appears similar to that of other chloroplast enzymes, this enzyme cannot be assigned a role in either photosynthesis or in translational mechanisms associated with chloroplasts. Nonetheless, the results of Figure 1 and of other work strongly indicate that *Euglena* alkaline DNase is involved in chloroplast metabolic activities. For example, in preliminary studies, we have found that *Euglena* alkaline DNase increases substantially as light-grown *Euglena* become depleted of vitamin B₁₂ (unpublished). During vitamin B₁₂ depletion, the chloroplasts continue to divide while cell division slows to a halt (9). Also, in subcellular localization experiments, we found that a portion of *Euglena* alkaline DNase activity could not be removed from highly purified chloroplast fractions, even after repeated washings of these fractions (14).

**Fig. 1.** *Euglena* alkaline DNase and chlorophyll appearance during light-induced chloroplast development. Dark-adapted cells of strain Z were grown in Hutner's modified medium (29) and were rested in Stern, pH 5.5, resting medium (37). The cells were then exposed to light at 0 time, and aliquots were taken for measurements at the times indicated. Cells were returned to the dark by covering individual flasks with black cloth.

The concentration of ³²P-DNA was determined as described by Lehman (22, see also 14). The volumes were adjusted to 0.30 ml with double distilled water. After an incubation period not exceeding 4 hr (linearity was maintained for at least 5 hr), the reactions were halted by adding 0.2 ml of 2.5 mg/ml calf thymus DNA and 0.5 ml of 3.5% perchloric acid. Following centrifugation to remove the precipitate, 0.20 ml of the supernatant was plated, dried, and counted (14). Enzyme activity was expressed as nmoles of PCA solubile ³²P-DNA products per hour (22).

In the second assay method used to obtain the data of Figure 3, reaction mixtures received up to 0.30 mg of protein of the enzyme source (the reaction was linear up to 0.34 mg of protein). The reaction mixtures then received 0.24 mg of calf thymus DNA (Sigma Chemical Co.), and the volumes were adjusted to 0.60 ml. Following an incubation period not exceeding 3 hr, the reactions were halted by adding 1.4 ml of 3.5% PCA; the reaction in this method was linear for at least 4 hr. The \[ A_{475} \text{nm} \] of the PCA-soluble supernatant was then determined by using a Gilford spectrophotometer. After subtracting the appropriate blanks, the final \[ A_{475} \text{nm} \] was converted to nmoles of PCA solubile-DNA products by assuming a millimolar absorptivity of 10 (27).

**Specific Activity and Activity per Cell.** Specific activity in each assay was defined as nmoles of PCA-soluble products per mg of protein per hour. Comparable values of specific activity were obtained with both assay methods.

Activity per cell was defined as nmoles of PCA-soluble products per cell per hour. Cell counts were performed manually, using the Sedgwick-Rafter counting method (9).

Abbreviation: PCA: perchloric acid.
Effects of Inhibitors of Protein Synthesis on the Induction of Euglena Alkaline DNase. The induction of Euglena alkaline DNase might involve new synthesis of this enzyme. New synthesis must occur in either the chloroplasts or the cytoplasm of Euglena, since both compartments contain the machinery essential for protein synthesis (32). We therefore sought to test the hypothesis of new synthesis by treating resting cells with either chloramphenicol, streptomycin, or cycloheximide. Chloramphenicol and streptomycin do not inhibit cell division in Euglena (1, 34), but do inhibit protein synthesis by isolated chloroplasts (8, 28), and both antibiotics have been reported to inhibit the in vitro activity of 70S chloroplast ribosomes (7, 8). Cycloheximide does not inhibit protein synthesis in isolated chloroplasts (36) and is generally assumed to inhibit protein synthesis directed by the 87 to 89S ribosomes in the cytoplasm of Euglena (36), since it completely inhibits cell division (3).

Figure 2 summarizes the results of one of many experiments in which cells of the Z strain were treated with chloramphenicol. It is evident that chloramphenicol did not inhibit Euglena alkaline DNase activity; indeed, the levels per mg of protein and per cell were higher in the chloramphenicol-treated cells than in the control samples. The levels of chlorophyll per cell were 50 to 60% lower in the chloramphenicol-treated cells, indicating that chloroplast development was substantially inhibited by this antibiotic.

Figure 3 summarizes the results of another experiment in which cells of the bacillaris strain were treated with streptomycin or cycloheximide. It should be noted that we have not observed any obvious differences in the alkaline DNase of the bacillaris and the Z strains of Euglena. For example, both strains had virtually identical Ca⁺⁺ requirements (14), and, as shown in Table I, both had substantial light-dark differences in dividing cells.

The results of Figure 3 indicate that streptomycin did not inhibit the light induction of Euglena alkaline DNase; indeed, as with chloramphenicol, an increase over the control values was observed. In contrast, cycloheximide completely prevented the induction of Euglena alkaline DNase. Both antibiotics prevented light-induced chlorophyll synthesis, as reported elsewhere (21, 32).

Since streptomycin and chloramphenicol did not inhibit the induction of Euglena alkaline DNase while cycloheximide did, we infer that the site of synthesis for Euglena alkaline DNase is probably cytoplasmic. In this regard, our enzyme appears to be similar to another chloroplast enzyme, TPN triose phosphate dehydrogenase, the induction of which also was not inhibited by chloramphenicol and streptomycin (4). However, the levels of TPN triose phosphate dehydrogenase were about the same or slightly lower in the streptomycin-treated cells as in the control cells (32). The reasons both of our chloroplast specific antibiotics gave increased Euglena alkaline DNase activities may be related to one possible plastid function of this hydrolytic enzyme. It is interesting to note that we have also found that nalidixic acid gave increased specific activities when compared to controls (14). In dividing Euglena, streptomycin and nalidixic acid are potent bleaching agents (2, 24). Chloramphenicol usually does not cause bleaching, but up to 25% bleaching has been reported (26). Perhaps high levels of Euglena alkaline DNase are induced to degrade chloroplast DNA, which presumably becomes incapable of duplicating itself when cells are exposed to bleaching conditions.

Euglena Alkaline DNase Activity in Bleached (Aplastidic) Substrains. Since the site of synthesis for Euglena alkaline DNase was cytoplasmic, we reason that the genome controlling this enzyme may not be in the chloroplasts; thus, aplastidic substrains should contain low but detectible levels of Euglena.

![Figure 2](image2.png)  
*Fig. 2. Euglena alkaline DNase and chlorophyll appearance during light-induced chloroplast development in the presence and absence of 1 mg/ml chloramphenicol. Conditions were as in Figure 1, except that chloramphenicol was added just prior to the exposure of the resting cells to light.*

![Figure 3](image3.png)  
*Fig. 3. Euglena alkaline DNase and chlorophyll content during light-induced chloroplast development in: (a) untreated cells; (b) cells treated with 15 ug/ml cycloheximide; (c) cells treated with 0.05% streptomycin sulfate. Dark-adapted cells of the bacillaris strain were grown in Hutner's pH 8 medium (18), and were rested in Stern, resting medium (37) adjusted to pH 6.8 with K2HPO4. Cycloheximide was added just prior to exposure of the cells to light. Streptomycin was added 8 hr before exposure of the cells to light.*
Table I. *Euglena* Alkaline DNase and Total Carotenoids in Several Light-grown and Dark-adapted Bleached Substrains Derived from Strains \( Z \) and bacillaris

<table>
<thead>
<tr>
<th>Strain or Substrain</th>
<th><em>Euglena</em> Alkaline DNase Specific Activity</th>
<th>Total Carotenoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light-grown</td>
<td>Dark-adapted</td>
</tr>
<tr>
<td></td>
<td>pg/mg protein</td>
<td>pg/mg protein</td>
</tr>
<tr>
<td>Strain Z</td>
<td>0.126 ± 0.110</td>
<td>0.295 ± 0.256</td>
</tr>
<tr>
<td>Bacillaris</td>
<td>0.126 ± 0.110</td>
<td>0.295 ± 0.256</td>
</tr>
<tr>
<td>W4BUL</td>
<td>0.126 ± 0.110</td>
<td>0.295 ± 0.256</td>
</tr>
<tr>
<td>W3BHL</td>
<td>0.126 ± 0.110</td>
<td>0.295 ± 0.256</td>
</tr>
<tr>
<td>Y7ZHL</td>
<td>0.080 ± 0.013</td>
<td>0.055 ± 0.013</td>
</tr>
<tr>
<td>Y2ZSmL</td>
<td>0.070 ± 0.012</td>
<td>0.055 ± 0.013</td>
</tr>
<tr>
<td>W4BSmL</td>
<td>0.070 ± 0.012</td>
<td>0.055 ± 0.013</td>
</tr>
<tr>
<td>W4ZHL</td>
<td>0.070 ± 0.012</td>
<td>0.055 ± 0.013</td>
</tr>
<tr>
<td>W4ZSmL</td>
<td>0.070 ± 0.012</td>
<td>0.055 ± 0.013</td>
</tr>
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1 95% confidence interval of the reported mean.

Euglena alkaline DNase in these substrains led us to consider the possibility that carotenoid production and Euglena alkaline DNAase synthesis was somehow linked in the bleached substrains. A re-examination of the carotenoid-containing substrains in Table I indicates a relationship between specific activity and total carotenoids. For example, light-grown W4BUL had 80 to 100% higher levels of both *Euglena* alkaline DNAase and total carotenoids than did dark-adapted W3BHL. Also, light-grown W4BHL had 30 to 40% higher levels of both DNAase activity and carotenoids than did dark-adapted W3BHL. Finally, Y7ZHL and Y2ZSmL had the same specific activity in light- or dark-adapted cells; similarly, the carotenoids of light-grown Y7ZHL, Y2ZSmL, and Y2ZSL suggest a strain difference among our selection of substrains. In this regard, it should be noted that dark-adapted strain Z had less than half the carotenoids of the dark-adapted bacillaris strain (Table I).

Another interesting correlation of our results can be made with the results obtained from a study of patterns of RNA labeling following the exposure to light of resting W4BUL, W4BHL, and W4BSmL (38). A substantial increase in RNA labeling for W4BUL was found; less of an increase was reported for W4BHL, and only a small increase was observed for W4BSmL. These results appeared qualitatively similar to our results for Euglena alkaline DNAase and carotenoids of the same substrains. Thus, bleaching treatments seem variably to affect a cytoplasmic regulatory system involved in light-induced RNA synthesis, light-induced synthesis of enzymes required for production of carotenoids, and light-induced synthesis of Euglena alkaline DNAase. This points to an effect of the bleaching agent on a cytoplasmic photoreceptor system. Such a photoreceptor has been indicated from other work, not only with bleached substrains (11, 38), but also with bacillaris (20). It has been suggested that this photoreceptor system might be a blue-far red-absorbing pigment, which, in the wild type cells, functions to assist in the mobilization of the cytoplasm during the early hours of chloroplast differentiation (32, 35).

CONCLUSIONS

The essential results of this work are interpreted to mean that, although *Euglena* alkaline DNAase functions in metabolic activities associated with chloroplasts, its site of synthesis is in the cytoplasm, and the genetic information for...
this enzyme is probably localized in the nucleus. A mito-
chondrial site of synthesis and of genetic information is also possible; however, the existence of mitochondrial ribosomes in Euglena has not been conclusively demonstrated, and the mitochondrial genome is extremely small (13).

In terms of a model proposed for chloroplast development, enzymes such as Euglena alkaline DNase and TPN triose phosphate dehydrogenase should be under partial control of a cytoplasmic photopigment system which, during plastid development, operates synergistically with the well-known chlo-
roplast-localized protochlorophyllide-chlorophyllide transformation (32, 35). If we are correct in suspecting that the physiologial variation among amastidic substrains is a result of differential effects of bleaching agents upon this cytoplasmic photoreceptor system, then studies of action spectra taken from carefully chosen substrains might allow a precise identi-
fication of the chemical nature of this photoreceptor.

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