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DNA-DEPENDENT RNA SYNTHESIS IN CHLOROPLASTS OF *EUGLENA GRACILIS*

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Isolated chloroplasts of *Euglena gracilis* have been shown to be capable of incorporating amino acids into protein (2). This incorporation is inhibited by actinomycin D, a fact which suggests that the chloroplasts might be capable of DNA-dependent RNA synthesis. Such RNA synthesis is also indicated in a recent report (6) showing inhibition of chlorophyll synthesis by actinomycin. In conjunction with our studies of chloroplast synthesis and replication in *Euglena*, we have investigated this phenomenon and present here evidence for DNA-dependent RNA synthesis in isolated chloroplasts of this organism. Chlorophyll formation in colorless *Euglena* cells is also re-investigated with regard to the synthesis of RNA and DNA. Much evidence has been accumulating which indicates that the DNA of *Euglena* chloroplasts may have a genetic role in the inheritance of chloroplasts (5, 7). The ability of this DNA to direct RNA synthesis might add support for this contention.

Euglena gracilis strain Z was grown heterotropically on defined medium in light or dark at room temperature (25°C) with constant agitation at moderate speed, as described previously (5).

Effects of Actinomycin D and Mitomycin C on the Synthesis of Chlorophyll

Dark-grown colorless cells in the log phase of growth were collected and transferred into fresh neutral medium, pH 6.8–7.2, containing actinomycin D (20 μg/ml) or mitomycin C (30 μg/ml) and allowed to become green under normal illumination. Aliquots of cells were taken at intervals of several hours. The number of cells was determined by haemocytometer and the chlorophyll was determined by measuring the optical density at a wavelength of 665 mμ. The results are shown in Table I.

Although mitomycin C–treated cells do not undergo any further division, there is no inhibition of chlorophyll formation. In fact, for a period of 38 hr they make almost twice the amount of chlorophyll per cell as compared with nontreated controls. Actinomycin D, on the other hand, inhibited synthesis of chlorophyll soon after its introduction into the medium. After 38 hr in actinomycin D, the cells appear to have less than 17% of the amount of chlorophyll found in control cultures.

RNA Synthesis in Isolated Chloroplasts

Since actinomycin D was inhibiting chlorophyll synthesis, studies were undertaken to test for the degree of in vitro inhibition of DNA-dependent RNA synthesis in isolated chloroplasts.

Pure chloroplasts were isolated in 75% sucrose at 23,000 × *g* for 30 min in a Spinco centrifuge, according to the method of Eisenstadt and Brawerman (1). That the fraction contained only chloroplasts was checked by microscope observation of the plastids and by staining a few test slides with the Feulgen method. RNA synthesis was studied by incubating the chloroplasts at 30°C for 20 min in a medium containing 50 μmoles of Tris (pH 8.0); 8 μmoles of $MgSO_4$; 4 μmoles of cysteine HCl; 50 mμmoles of CTP, GTP, UTP; and 10 mμmoles of 8-C^{14}-ATP (8.9 mc/mmole) in a total volume of 0.8 to 0.9 ml, as described by Kirk (4). The reaction was stopped by the addition of 0.2 N cold perchloric acid (PCA), and the chloroplasts were washed twice

with this reagent for a period of 1 hr. The chloroplasts were then rinsed in water and their chlorophyll was removed with two changes of absolute ethanol. The residue was digested in 3 ml of hydroxide of hymine 10-x (Packard Instrument Co., La Grange, Illinois) at 60°C for 15 min. The solution was cooled, and 12.5 ml of scintillation solution was added and counted in an automatic counter, model 720 (Nuclear-Chicago Corporation, Des Plains, Illinois). The results are shown in Table II.

The results shown in Table II indicate that there is considerable incorporation of ATP-C^{14} into the residue. This activity was almost completely inhibited by the addition of actinomycin D or DNase or RNase prior to incubation. The system was also sensitive to the lack of one or more nucleoside triphosphates in the medium. Heat-treated chloroplasts also failed to incorporate ATP-C^{14}. The activity appeared to be in RNA of chloroplasts since most of it was removable by RNase digestion. The intensity and consistency of incorporation and its resistance to repeated washes following incubation make it highly unlikely that the incorporation in chloroplast RNA could be due to contaminated adsorbed DNA from nuclei.

The effects of actinomycin D and mitomycin C suggest that the light-induced development of chloroplasts and synthesis of chlorophyll require synthesis of new RNA but not necessarily synthesis of new DNA. These results are in accord with the observations of Smillie (8) who reported that 5-fluorouracil (FU) inhibited chlorophyll synthesis while 5-fluorodeoxyuridine (FUDR) was found to be ineffective. This is significant in view of the fact that the loss of chlorophyll-forming ability of the bleached cells is due not to the loss of plastids but to the loss of proplastids to differentiate into chloroplasts (3). Thus, mitomycin C and 5-fluorodeoxyuridine (FUDR), which interfere with DNA synthesis, do not inhibit chlorophyll synthesis or chloroplast development, whereas actinomycin D, by preventing synthesis of messenger RNA, does so.

The effect of actinomycin D on chlorophyll synthesis in intact cells suggests an interruption of DNA-dependent RNA synthesis. However, it does not indicate whether this RNA was dependent on extranuclear DNA. The in vitro studies of RNA synthesis in isolated chloroplasts show that this RNA synthesis is inhibited by actinomycin D, DNase, and RNase. These results support the notion that isolated chloroplasts are capable of carrying out DNA-dependent RNA synthesis.

TABLE I

Effects of Mitomycin C and Actinomycin D on the Synthesis of Chlorophyll in Chloroplasts in Cells

Cells	Chlorophyll (μg) for eight million cells		
	12 to 15 hr* after incubation	24 to 28 hr after incubation	36 to 38 hr after incubation
Control	14.01	41.24	76.86
Mitomycin C–treated	19.31	84.43	144.61
Actinomycin D–treated	12.08	13.11	12.78

* The hours are expressed as hours of incubation in light.

TABLE II

Incorporation of ATP-C^{14} into Isolated Chloroplasts of Euglena

Experimental conditions	CPM	% of complete system
1. Complete system*	4873	100
2. Complete (boiled chloroplasts; 10 min, 100°C)	268	5.5
3. Complete minus GTP, CTP, UTP	341	7.0
4. Complete minus CTP, UTP	436	9.0
5. Complete minus UTP	452	9.2
6. Complete plus 20 μg of actinomycin-D	259	3.3
7. Complete plus 50 μg of DNase	237	4.8
8. Complete plus 50 μg of RNase‡	263	5.0
9. RNase extract of complete system§	4508	92.5

* The complete system consisted of the reaction mixture (see text) plus chloroplasts which contained 0.225 mg of chlorphyll, 12.7 mg of protein, 72 μg of DNA, and 440 μg of RNA.

‡ Actinomycin, DNase, and RNase were added to the reaction mixture 10 min prior to incubation. The RNase (Worthington Corporation, Freehold, New Jersey) was heated at 80°C for 10 min to remove any contaminating DNase.

§ The extraction of RNA from the reaction mixture by RNase (1 mg/ml) was done at 37°C for 8 hr, at pH 6.8, in McIlvain's buffer.

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