THE SYNTHESIS OF CYTOPLASMIC DNA IN SYNCHRONIZED *EUGLENA*

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Chloroplastic DNA in *Euglena gracilis* is self-replicating (7), and presumably contains information directing reproduction and differentiation of the chloroplast. Mechanisms governing control of chloroplastic DNA replication in *Euglena* and the role of the nucleus in these events are unknown.

Knowledge of the time course of DNA synthesis in the chloroplasts of *Euglena*, when compared with that of the nucleus, could suggest whether or not common mechanisms control both events. A synchronous S period for chloroplasts and nucleus could be more economical to the cell in the production of enzymes necessary for DNA replication. However, ultraviolet inactivation of chloroplast development in synchronized *Euglena* suggested that this cytoplasmic DNA may be replicated early in the life of the cell, at a time different from that of nuclear DNA (3). Synthesis of total cellular DNA cannot be used to differentiate between these S periods, since the chloroplasts contain only about 3% of the total cellular DNA in *Euglena* (5), a difference not detectable by ordinary chemical techniques.

Study of the time course of chloroplastic DNA synthesis in *Euglena* has been pursued further by following the rate of cytoplasmic incorporation of a tritiated DNA precursor at different ages over the life cycle in synchronized populations. The results of these labeling experiments, described in this report, show that there are two separate peaks of activity in cytoplasmic incorporation: one of these is temporally correlated with nuclear DNA synthesis and chloroplast division; and the other is correlated with initiation of chloroplast development.

MATERIALS AND METHODS

Axenic populations of *Euglena gracilis* strain Z were grown in the salt medium of Cramer and Myers (4) at a temperature of 21.5°C. The synchronizing regime consisted of a repetitive cycle of 14 hr of light (1200 foot-candles) and given 10 hr of dark. A doublihg of cell number occurred in each dark period. Dilution of the culture with fresh sterile medium at appropriate times prevented the cells from entering the stationary phase. In the cycle used for the work reported here, the cells multiplied from 50,500 to 103,000 cells per milliliter during the dark period (Fig. 2), after a history of 20 previous light-dark cycles. This synchronous increase of 106% indicates that the cells were well behaved with respect to the synchronizing program.

Starting with the initiation of the light period (age 0), cells were collected at 2-hr intervals over the entire 24 hr cycle. These were packed by gentle centrifugation and suspended in 5 ml of the culture medium containing 1.0 mc of adenine-H^3 per ml (New England Nuclear Corp., Boston, Massachusetts, specific activity 0.5 mc/mM). The cells were incubated in this medium for 2 hr under the same light-dark conditions as the parent culture; afterwards, they were packed and fixed in ethanol–acetic acid (3:1) for 30 min, and given three 30-min washes in 70% ethanol. The cells were then passed through graded alcohols to water and digested overnight in buffered RNase (0.3 mg/ml, pH 7.0, 30°C). Subaliquots of the RNase-treated *Euglena* were also digested overnight in buffered DNase (0.5 mg/ml, pH 7.0, 3 mM $MgCl_2$, 30°C). After enzymatic digestion, the cells were extracted 2 times with ethanol–acetic acid and 3 times with 70% ethanol, and finally air-dried on slides. These preparations were dipped in liquid emulsion (Kodak NTB3, 45°C), exposed 3 days before developing, and stained through the emulsion with methyl green–pyronin. Since it was found that the cells became extremely distorted if allowed to dry at this point, the slides were kept in water until they could be examined. At this time, a drop of water was placed on the slide, a cover slip was added, and grains lying over the cytoplasm were scored under oil immersion.

Sagan (11) has demonstrated that while thymidine is not incorporated into DNA in *Euglena*, purines are. Adenine was selected for this study because of the high adenine-thymine content of chloroplastic DNA in *Euglena* (5). RNase removed most of the cytoplasmic incorporation of adenine-H^3 in 100 cells scored at random at each of the cell ages examined. Insignificant numbers of cytoplasmic grains were found over an equal number of cells of each age after DNase digestion. In this paper, therefore, incorporation remaining after RNase digestion, but removed by DNase digestion, is regarded as incorporation of adenine-H^3 into DNA.

Direct counts with the phase-contrast microscope determined the number of chloroplasts per cell. The plastids in *Euglena* are not easily visualized, because they are connected to one another by ribbonlike structures (6), and are, in addition, partially obscured by the numerous paramylum granules. It was found that brief treatment with ether of an aqueous suspension of *Euglena* caused the chloroplasts to assume a spherical shape, making them more conspicuous. The number of chloroplasts per cell was scored for 100 cells in aliquots collected at 2-hr intervals over the 24 hr light-dark cycle.

Total cellular DNA was estimated spectrophotometrically according to the procedure of Schmidt and Thannhauser (12). For each determination, made in triplicate at each age examined, known numbers of cells (about 5×10^6) were used.

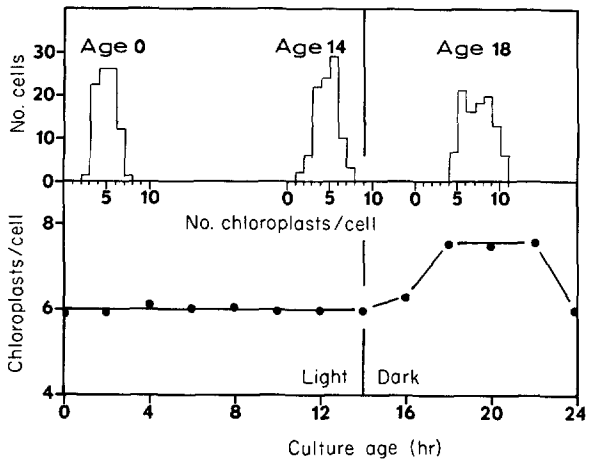

FIGURE 1 Lower curve: average number of chloroplasts in 100 *Euglena* collected at times indicated. Upper curve: representative histograms showing frequency distributions of chloroplasts in *Euglena* of three different ages. A bimodal distribution is seen during the period of cell division (age 18).

RESULTS

Fig. 1 is a plot of the average number of chloroplasts per *Euglena* in synchronized cultures, with frequency histograms for selected ages. The number of chloroplasts remains constant at about 6 per cell over the 14 hr light period, and increases to about 8 per cell during the period of cell division. These results are consistent with the view that *Euglena* chloroplasts divide just prior to cytokinesis. This view is supported by the histograms in Fig. 1, which show a normal distribution of chloroplast numbers in the light period, but a bimodal distribution of chloroplast numbers at age 18, i.e., in the middle of the dark period. In the

latter, the class containing the greater number of chloroplasts is presumably that of the cells which have not yet divided, but which have doubled the number of chloroplasts; the class containing the smaller number of chloroplasts could be made up of two groups of cells, those which have doubled the chloroplast number and completed cytokinesis, and undivided cells which have not yet doubled the chloroplastic number. It is concluded that the chloroplasts divide just before cytokinesis, and that chloroplast division is therefore synchronized by the same light-dark program which synchronizes cell division.

and less pronounced peak parallels the synthesis of total cellular DNA (as measured chemically).

DISCUSSION

DNA has been isolated from chloroplasts (5, 10) and mitochondria (1) of *Euglena gracilis*. In the studies reported here, cytoplasmic incorporation of adenine-H^3 doubtless reflects DNA synthesis in both chloroplasts and mitochondria. Generally, however, mitochondrial DNA comprises only about 0.5% of the total cellular DNA, while the amount of chloroplastic DNA in *Euglena* is about 6 times greater (5). It is assumed, therefore, that

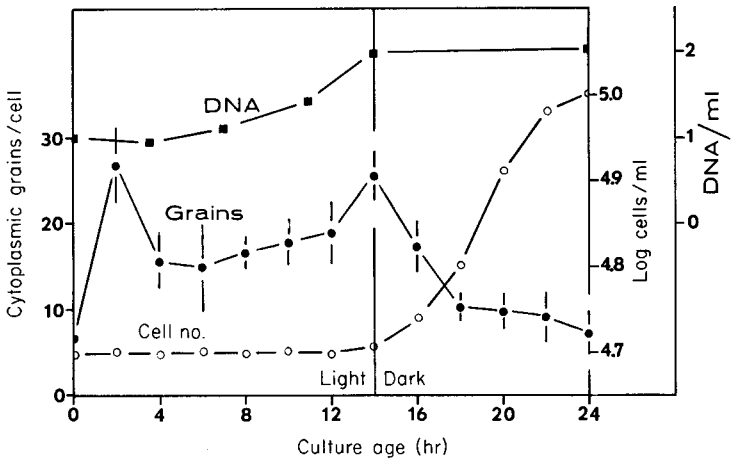

FIGURE 2 ●, number of grains found over the cytoplasm of *Euglena* following incubation with adenine-H^3 and subsequently digested with RNase. Cells were collected every 2 hr and incubated for 2 hr. ○, log number of cells/ml. ■, total DNA/ml of culture. Absolute amounts of DNA with an initial cell number of 50,000/ml can be obtained by multiplying the values by 0.108 μg. Total DNA is replicated in the latter half of the light period, but two peaks of cytoplasmic DNA synthesis are seen.

Fig. 2 is a plot of the average number of cytoplasmic grains found over synchronized *Euglena* after RNase digestion as a function of cell age. One standard deviation from the mean is indicated. Incorporation was found at all ages examined. However, the rate of incorporation varied considerably. Entrance into the light period results in a fourfold increase. The rate of incorporation then falls to about half this level in the middle of the light period, after which a gradual increase occurs. At the end of the light period, the rate is about equal to that found in the early hours of the light period. The number of grains found over cytoplasm decreases to minimal levels during the dark period. These data are interpreted as showing two distinct peaks of activity in the incorporation of adenine-H^3 into cytoplasmic DNA. The second

the significant features of cytoplasmic DNA synthesis described here are essentially due to chloroplast activity.

Relatively long term radioautographic studies of the type reported here, which span an entire life cycle, require considerable caution in interpretation. The apparent demonstration of two periods of increased rates in synthesis of cytoplasmic DNA, for example, assumes invariant precursor levels. Our own preliminary results suggest that this is almost certainly not the case; while actual levels of nucleotide pools have not yet been determined in synchronized *Euglena*, we have found that measurable deoxyadenosine monophosphate (dAMP) kinase activities are transient and confined to a brief period shortly before initiation of DNA synthesis (J. R. Cook and M. Carver, un-

published). Such transient activity suggests that significant levels of dATP will be available to the cell only in the latter half of the light period, during nuclear DNA synthesis. Increased deoxyadenosine triphosphate (dATP) levels at this time would tend to dilute the labeled precursor. Since the rate of cytoplasmic incorporation of the label increases in the latter half of the light period, in spite of a presumed increase in dATP levels, it is concluded that the observed increase in the rate of cytoplasmic DNA synthesis is real.

Interpretation of the initial burst of cytoplasmic DNA synthesis rests on more uncertain ground. However, synthesis at this time agrees well with the ultraviolet inactivation studies (3). It may be pointed out that this burst of synthesis is completed by the 4th hr of the light period, well before nuclear DNA synthesis begins. Presumably, deoxynucleotide levels would be low at this time, resulting in an increase in concentration of the labeled precursor. The relatively low rate of incorporation at the 4th hr suggests that the first peak of cytoplasmic DNA synthesis is real.

These results imply the presence of two distinct S periods of cytoplasmic DNA synthesis, presumably chloroplastic, in synchronized *Euglena*. One S period occurs just after chloroplast division, the other just before. It may be asked if the S period in chloroplastic replication is interrupted? Or do these data indicate isolated S periods for two separate species of chloroplastic DNA?

Ray has shown that two distinct species of DNA are present in *Euglena* chloroplasts (10). One of these can be found in a bleached mutant of *Euglena* (10), which contains self-replicating proplastids, but is incapable of synthesizing chlorophyll (8). Gibor and Granick (8) have proposed that the DNA of such mutants is responsible for replication of the proplastids but contains no information for chlorophyll synthesis. Presumably, such information would reside in the other species of DNA found in *Euglena* chloroplasts, although it is absent in the bleached mutants. Taken together, these data suggest that there are two species of chloroplastic DNA: one directing chloroplastic morphogenesis (including chlorophyll synthesis), and the other directing chloroplast (or proplastid) replication.

The present data support such a concept. The later peak of cytoplasmic DNA synthesis comes just before initiation of chloroplast division and is temporally correlated with the nuclear S period. This correlation suggests that this species of chloroplastic DNA is associated with replication of the plastid. The earlier peak of cytoplasmic DNA synthesis occurs just after the light period begins, and is therefore associated with chlorophyll synthesis, which is initiated immediately with the onset of the light period in synchronized *Euglena* (2).

It is of interest to consider possible control mechanisms governing chloroplastic synthesis of DNA in synchronized *Euglena*. Since the second peak of DNA synthesis corresponds to the nuclear S period, and chloroplastic division occurs synchronously with cell division, it seems likely that this species of chloroplastic DNA and the nuclear DNA are both subject to the same control mechanisms, and could utilize common nucleotide pools and enzymes of DNA synthesis. However, the first peak of cytoplasmic incorporation is apparently unrelated to the nuclear S period. In fact, this first burst of incorporation is so much sharper than any of the other events in synchronized *Euglena*, including cell division and nuclear DNA synthesis, that it appears to be largely unrelated to any aspect of cell growth or division. The fact that this burst of incorporation occurs immediately after the onset of the light period strongly suggests that light may act as a stimulus for synthesis of this chloroplastic DNA. Iwamura (9) has shown that light increases the "turnover" of chloroplastic DNA in *Chlorella*. If this species of DNA were informational for the synthesis of RNA controlling growth and development of the chloroplasts in synchronized *Euglena*, replication early in the light period would provide two functional templates over most of the light period.

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