Title
LIGHT REGULATION OF THE CELL CYCLE AND GENE EXPRESSION IN EUGLEMA GRACILIS BACILLARIS

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(Ph.D. Thesis)

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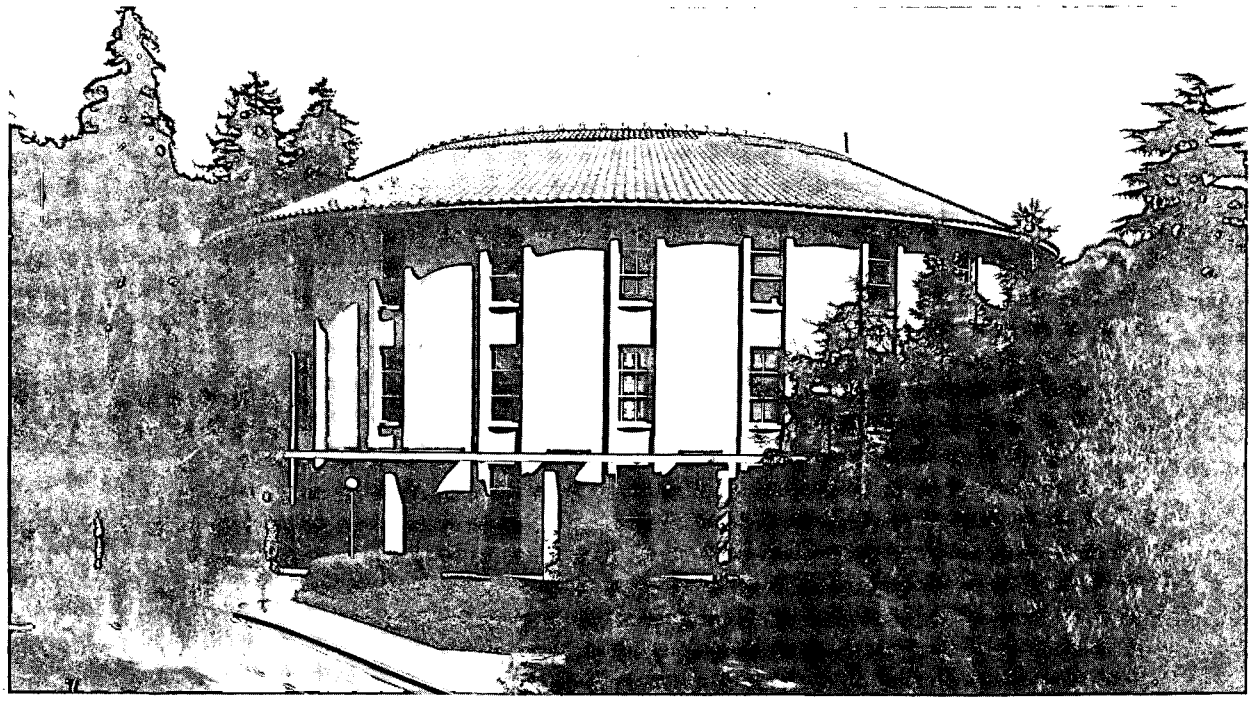

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Euglena gracilis bacillaris

by

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Light Regulation of the Cell Cycle and Gene Expression in *Euglena gracilis bacillaris*

Muh-ching Yee

Abstract

I have studied the light regulation of the cell division cycle in the photosynthetic unicellular alga *Euglena gracilis bacillaris*. *Euglena* are easily synchronized to a 12 hour light-12 hour dark regime. The cells carry out photosynthesis in the same manner as higher plants, with many chloroplasts per cell, and the cell size and genome size lend themselves to convenient analysis by flow cytometry.

By inoculating stationary phase, non-dividing cells into fresh media, and exposing the diluted cells to either light or darkness, I have determined that initiation of DNA synthesis for the cell division cycle is light dependent. By varying the amount of light to which synchronized cells are exposed, I have shown that commitment to the cell cycle requires exposure to more than six hours of light. I propose that this is to allow the accumulation, through photosynthetic electron transport, of an initiating factor that will enable DNA synthesis to begin. Incubation of synchronized cultures in brief periods of darkness during the light period has shown that the initiating factor has a half-life of 5 hours in the dark. Flow cytometry analysis also shows that once cells are committed to the cell cycle, they complete the cycle in the dark, so mitosis is a light independent step. Attempts to look for a link between photosynthetic activity and accumulation of the cell cycle initiating factor using the photosynthesis inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were complicated by the discovery that DCMU interferes with the cell cycle in *Euglena*. DCMU slows the rate of DNA synthesis and blocks mitosis in both phototrophic and heterotrophic wild type cells.

DCMU also blocks mitosis, but not DNA synthesis, in two chloroplastless mutants of *Euglena*, W_3BUL and W_{10}SmUL.

The levels of several photosynthetic messenger RNAs (mRNAs) have been studied in logarithmic phase and stationary phase cells under alternating light-dark conditions and continuous light conditions. RNA levels for *psbA*, which encodes the Photosystem II herbicide binding protein known as D1, display a strong circadian rhythm that persists for more than 3 days in continuous light. RNA levels for *rbcL*, which encodes the large subunit of ribulose 1,5-bisphosphate carboxylase, and for *rbcS*, which encodes the small subunit of that enzyme, have a shorter free-running circadian cycle. The chloroplast-encoded *rbcL* is more sensitive to cell cycle state because it does not accumulate in stationary phase cultures while the nuclear-encoded *rbcS* does accumulate.

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Chapter I

Introduction

The Cell Cycle

Normal, non-transformed cells are very sensitive to environmental factors. Lack of the essential amino acids, nutrients such as phosphate, glucose or lipids, or serum growth factors can stop all proliferation in both non-transformed animal cells and yeast (Pardee et al. (1978), Russell and Nurse (1986)). Such cells no longer carry out DNA synthesis but continue to have one genomic copy of DNA. RNA content and transcriptional activity in quiescent cells are decreased in comparison to cells going through the cell cycle (Darzynkiewicz et al. (1982), Rossini et al. (1976)), and the calmodulin level is increased (Chafouleas et al (1984)). Cells that are in a non-proliferating, or quiescent, state can be induced to re-enter the cell cycle by addition of the required nutrients. Under these conditions, synchronized passage through the cell cycle is observed, indicating that all the cells were arrested at the same point and that they are now all following a program of DNA replication and preparation for mitosis. Much effort has been focussed on elucidating the mechanism that brings quiescent cells into the cell cycle, and the program of activities that the cell follows in order to undergo mitosis.

The second part of the agenda has been more rapidly accomplished, partly with the help of advances in flow cytometry that have allowed direct observation of progress through the cell cycle (Shapiro (1981)). The cell cycle has been divided into 4 parts

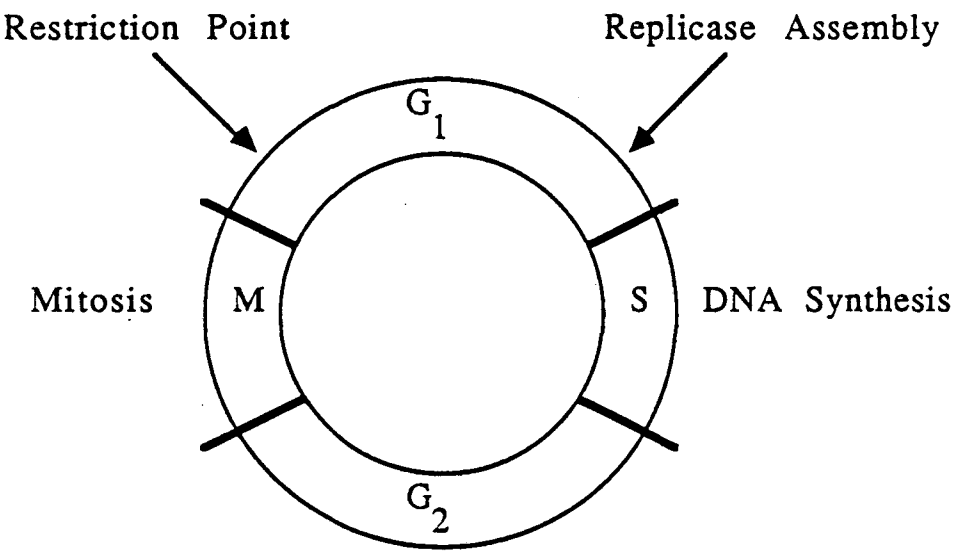
(Fig. 1-1): G_1, the period between mitosis and the start of DNA synthesis; S, the DNA synthesis period; G_2, the period between completion of DNA synthesis and mitosis, and M, mitosis.

G_1 is the period during which the cell prepares for DNA synthesis. It is the part of the cell cycle that is most variable in length, and is not even found in rapidly growing embryo cells (Gamow and Prescott (1970), Mukherjee (1976)). Comparison of cell size and length of time spent in G_1 by daughter cells after mitosis has shown that there is a correlation between mass increase and time spent in G_1 (Killander and Zetterberg (1965)). Cytokinesis frequently does not divide the cell into two equal halves and time lapse photography has shown that the larger daughter cell has a smaller mass increase than the smaller daughter cell during the G_1 phase following mitosis. Thus, the smaller a cell is the longer it will stay in G_1. This does not seem to be the case, however, for rapidly dividing embryo cells that carry out DNA synthesis without any increase in cell size (Prescott (1976)).

Cell Cycle Regulation

The major controlling point for entry into the cell cycle is at the G_1 phase. Cells that are subjected to conditions that are unfavorable to cell division (deprivation of serum growth factors, nutrients, etc.) revert to a G_1 state (Pardee, 1974). Virally transformed cells, or cells of tumor origin often exhibit loss of cell cycle control, so that cell proliferation continues during media depletion until the cells die for lack of nutrients (Medrano and Pardee (1980), Pardee and James (1975)). These cells have either lost or have a relaxed restriction point so that the cells are no longer sensitive to environmental conditions. Studies of cell cycle control will shed light on how cells regulate cell division and what causes the failure of such regulation in tumor cells. Complete understanding of this process will come with elucidation of how

Fig. 1-1. Division of the cell cycle into four phases. Functions that occur during each of the phases are indicated.

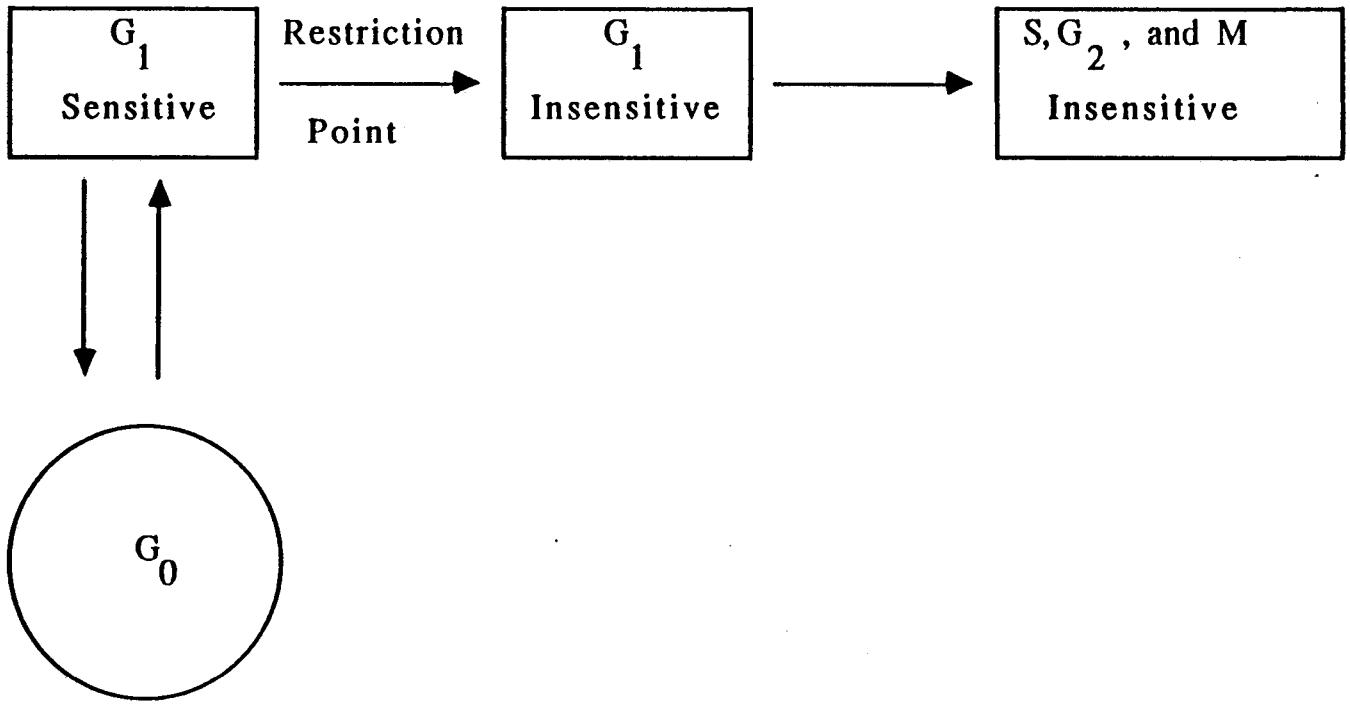

Restriction Point
Replicase Assembly
G1
Mitosis
M
S
DNA Synthesis
G2
Chromosome Condensation

environmental factors activate the complicated machinery needed for eukaryotic cell division.

The time at which the decision to go to the G_0 or quiescent state or to proceed with mitosis has been described as the "restriction point" (Pardee, 1974) (Fig. 1-2). Before this point, the cells are sensitive to environmental conditions that can induce them to enter quiescence or cell division. After the restriction point, the cells are no longer sensitive and will become committed to the cell cycle. Addition of cycloheximide in amounts that reduce protein synthesis to half the normal levels does not prevent Swiss 3T3 cells from completing the cell cycle once they are committed (Zetterberg and Larsson (1985)). Reduction of protein synthesis below that levels does stop DNA synthesis. The likelihood that cells will become committed to the cell cycle has been described as the "transition probability" (Smith and Martin (1973)). This probability is a function of environmental factors and cell size or cell metabolism. Favorable environmental factors, larger cell size and continuous protein synthesis give a higher transition probability of a cell becoming committed to the cell cycle (Brooks (1977), Killander and Zetterberg (1965)).

When growing mouse Swiss 3T3 cells are subjected to serum deprivation at different times after mitosis, it has been shown that cells 3 or fewer hours from mitosis are put into a quiescent state from which they will not emerge until placed in complete medium again for at least 8 hours (Zetterberg and Larsson (1985)). Cells that 4 or more hours away from mitosis are no longer dependent on serum and can proceed through the cell cycle despite serum deprivation for 8 hours. The authors describe the fourth hour from mitosis as the restriction point before which the cells are dependent on serum for continuation of a cellular program towards mitosis. After the restriction point, the cells are independent of serum and are committed to completion of the cell

Fig. 1-2. Model of cell cycle and controlling points. The G_1 phase is proposed to have periods sensitive and insensitive to environmental conditions. The steps after the restriction point are insensitive to environmental conditions.


G_1
Sensitive
Restriction
Point
G_1
Insensitive
S, G_2 , and M
Insensitive
G_0

cycle. The rate with which cells enter the cell cycle after the restriction point was found to be highly variable, and was explained as being caused by variation in cell size.

Ambiguities can arise from serum or nutrient deprivation experiments in animal and yeast cells due to incomplete removal of the nutrient and excessive handling of the cells. Cell surface contacts have been shown to cause stimulation of some cells into the cell cycle (Pardee 1975). Serum itself is an undefined mixture of proteins that presumably contain growth hormones. If very low levels of growth factors are required for giving a high transition probability to a cell, washing of a cell pellet in serum-free media might not be sufficient to remove all traces.

Algal Cell Cycle

Algal cells can be spontaneously synchronized in their cell cycle during growth in natural light. It was noted over 80 years ago that *Euglena* undergoes cell division in the dark (Dangeard, (1902)). The unicellular photosynthetic alga *Euglena gracilis* has a very simple, asexual life cycle (Leedale (1968)) so that the cell's only choice at each restriction point is quiescence or mitosis. Complicating factors in animal cells such as developmental pathways or meiosis do not interfere with cell cycle analysis in *Euglena*. The alga can be grown in completely defined media under phototrophic conditions (i.e., the cells must photosynthesize in order to survive) (Cramer and Myers (1952)). This means that light itself is an essential factor, and one that can be easily and quantitatively removed at will from a culture.

Previous studies have shown that the length of light exposure given to a phototrophic culture of *Euglena* will affect the increase in cell number. A natural light cycle of 12 hours light-12 hours dark gives less than a two-fold increase in cell number. Exposure to 16 hours of light followed by 8 hours of darkness is required for complete doubling in *Euglena gracilis bacillaris* (Cook and James (1960)). It has also been shown that DNA content increases during the second half of the light period, just before cell division. Reduction of the light period by varying amounts of time have shown that between 2 to 8 hours of light are necessary for the occurrence of events that lead to DNA synthesis (Edmunds (1964)).

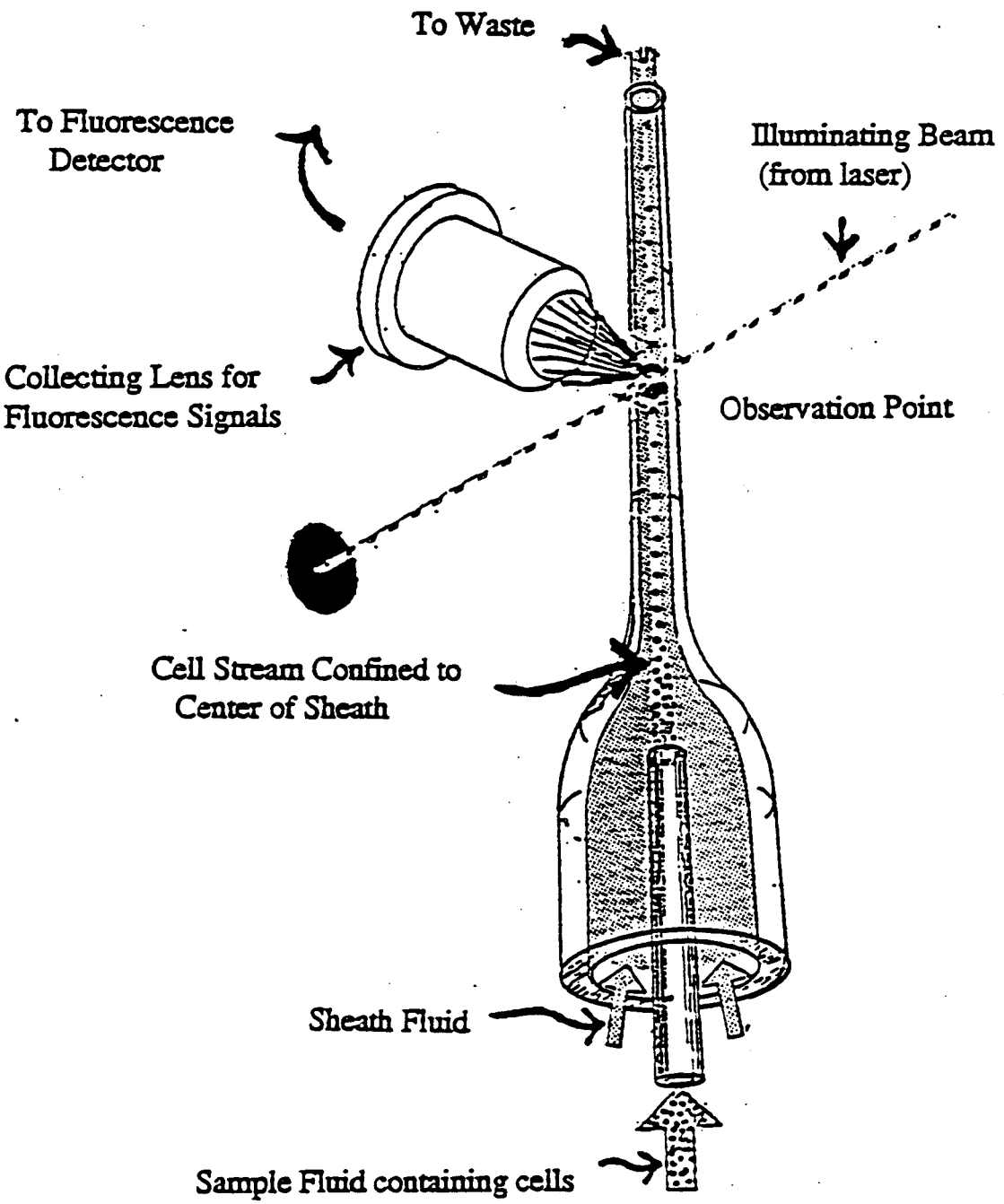
The mechanism of synchronization has been shown to result from a light dependent step during growth of cells in a light-dark regime and not due to entrainment of an endogenous cell clock. The unicellular photosynthetic alga *Chlamydomonas reinhardtii* has been studied using laser light scattering, which measures cell size and which has been shown to be correlated with passage through the cell cycle (Spudich

and Sager (1980)). Transfer of cells from light to dark during the early period of the light period causes cells to be arrested in the G_1 phase. Transfer of cells to darkness after the sixth hour of the light period does not interfere with completion of the cell cycle.

These results suggest that algal cells have controlling points in their cell cycle that are similar to those proposed in animal cells. If this is indeed the case, then algal cells offer an ideal system for studies of the light control of eukaryotic cells. To study the effect of light on entry of cells into the cell cycle, a sensitive method for measurement of DNA synthesis in synchronized cells is needed. Increase in cell size has been shown to occur in the G_1 phase (Prescott (1976)) but is not necessarily a good indicator of rates of entry into the S phase. Direct measurement of DNA allows the most detailed study of passage through the cell cycle, and this is a parameter whose measurement can be rapidly and quantitatively performed on the flow cytometer. Flow cytometry (FCM) is the passage of cells, one by one, through a laser beam and past a photo detector (Fig. 1-3). The detector measures a desired parameter at the rate of thousands of cells per second, which gives high statistical precision to the data collected (for reviews see Horan and Wheeless (1977), Shapiro (1985)). In the case of the experiments to be described, the increase in DNA content as cells carry on DNA replication is determined by measuring the fluorescence of a DNA binding dye in samples collected from synchronized populations of *Euglena*. Higher quantities of DNA will give higher fluorescence intensities (Fig. 1-4). Measurements of fluorescence intensities throughout a population will give the distribution of DNA content in that population. These measurements are usually presented as FCM histograms (Fig. 1-5).

Cells that contain one genome copy of DNA are said to be in the G_1 phase, cells

Fig. 1-3. Diagram of a generalized flow cytometer showing sample path, light source, and detector.



After a figure by Shapiro.

Fig. 1-4. Fluorescence signals obtained from cells in the G_1 or G_2 phase. Passage of a cell with a G_1 DNA content through the laser beam causes a pulse of light to be detected at the fluorescence detector, a photomultiplier tube. Passage of a cell with G_2 DNA content gives rise to a pulse with twice the fluorescence of a G_1 cell.

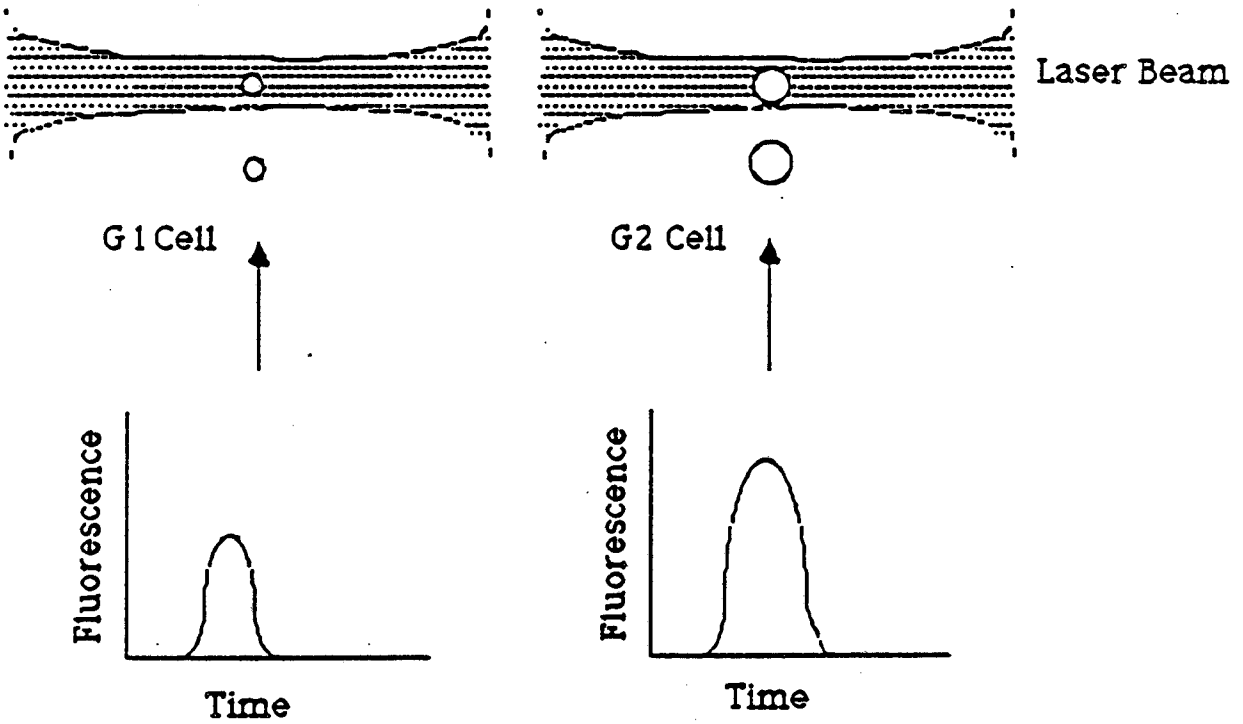
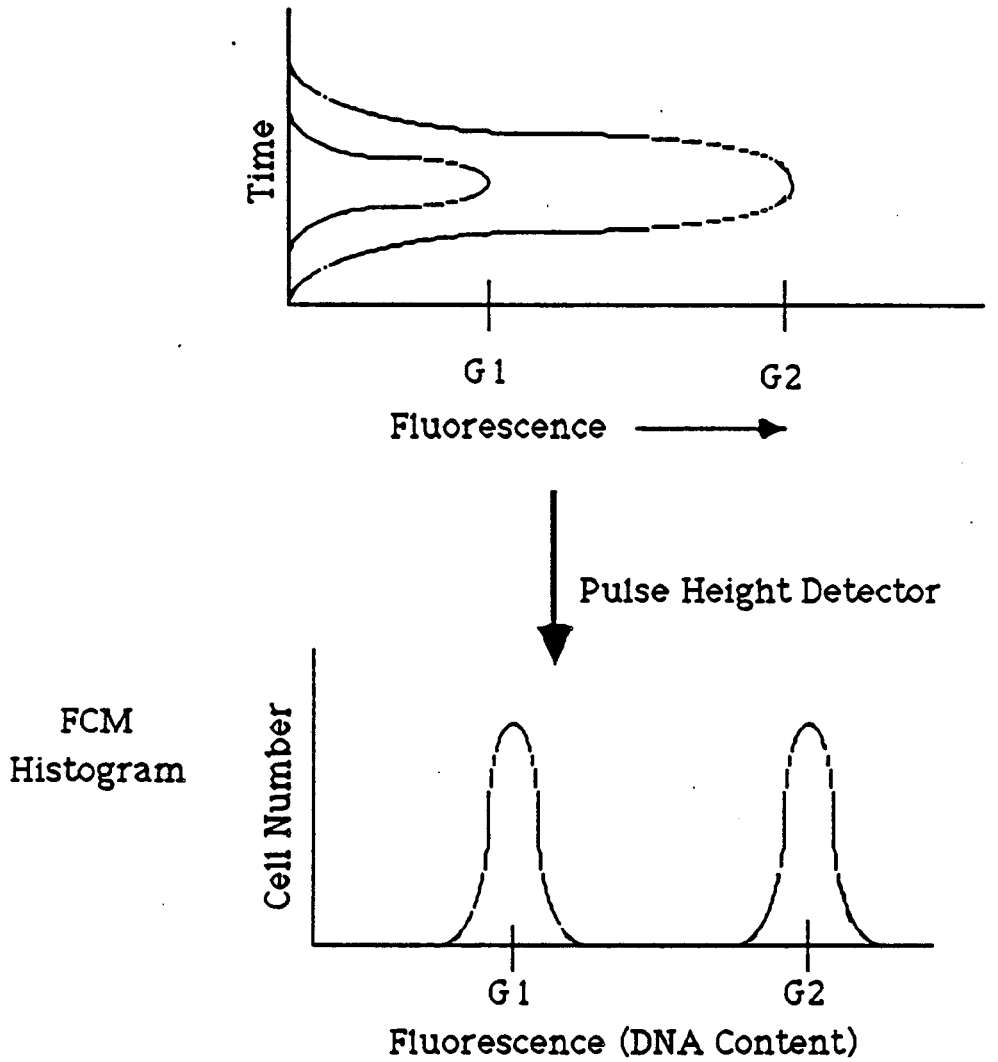

Laser Beam
G1 Cell
G2 Cell
Fluorescence
Time
Fluorescence
Time

Fig. 1-5. FCM histogram obtained by counting number of cells in the G_1 and G_2 phases of the cell cycle. A pulse height analyzer records the number of cells that have a particular fluorescent signal. Since fluorescence is proportional to DNA content, plotting fluorescence against cell number generates a histogram of the DNA distribution in the sample.

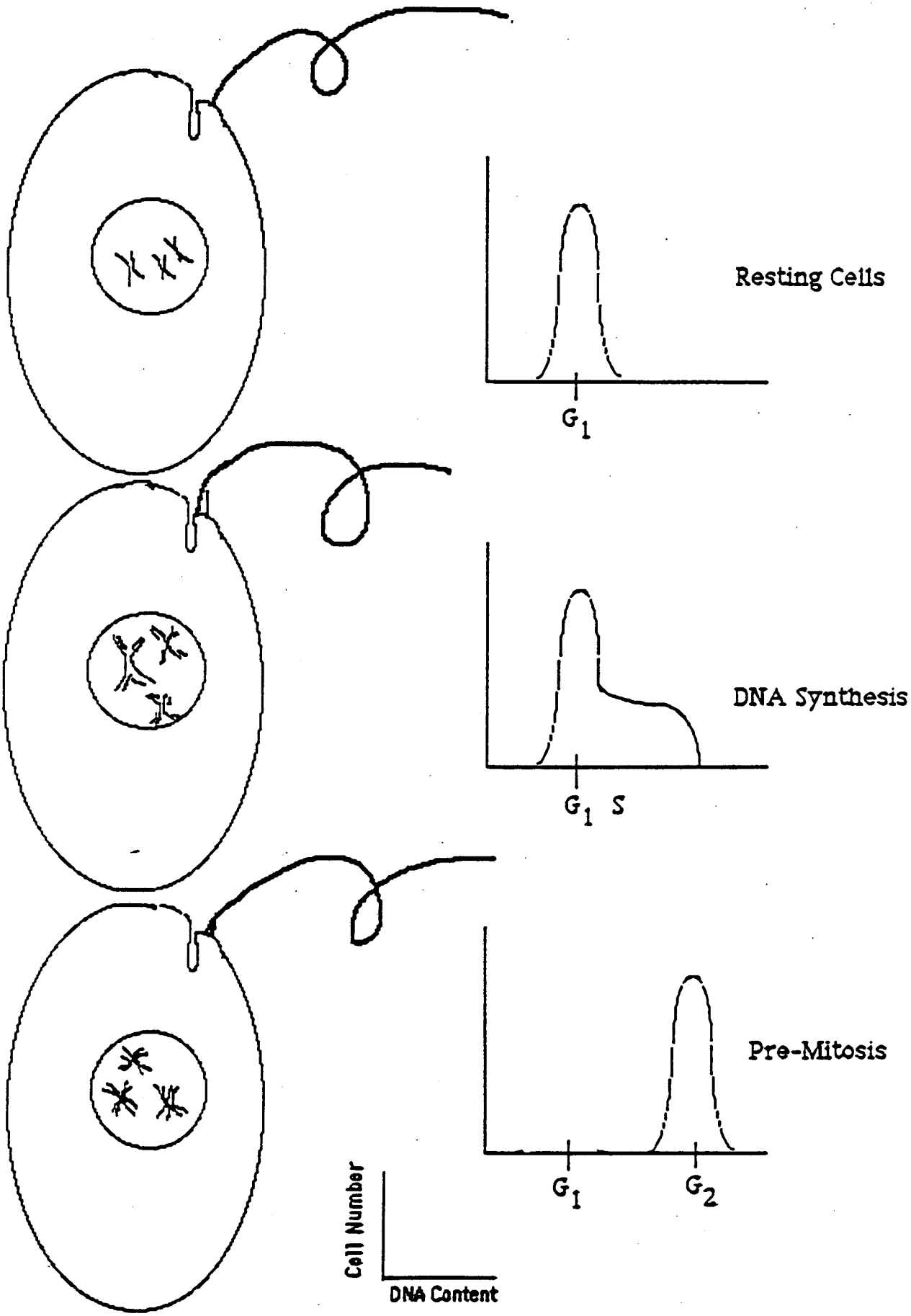


Frequency distribution of cells in G1 and G2.

actively synthesizing DNA are in the S phase, and cells that have completed DNA synthesis and have two genomic copies of DNA are in the G_2 phase. Plotting cell number against fluorescence (DNA content) shows the G_1 and G_2 phases as peaks and the S phase as the region between the peaks (Fig. 1-6).

Since intact particles, whether cells or organelles, are needed for data collection, very little manipulation can be performed on samples for FCM compared to that which is done on RNA or DNA for Northern and Southern blotting. By necessity this makes the procedure a rapid and direct one, and FCM is very convenient when used on intrinsic parameters that can be directly measured. In exchange for the limited sample manipulation that is done, FCM offers information on a cellular basis, so details that are usually lost upon sample homogenization or extraction are retained.

Fig. 1-6. Cartoon of the FCM histograms obtained from *Euglena* cells in the G_1, S or G_2 phases of the cell cycle.


Resting Cells
G_1
DNA Synthesis
G_1 S
Pre-Mitosis
G_1
G_2
Cell Number
DNA Content

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Chapter II

Light Regulation of the Cell Cycle in *Euglena gracilis bacillaris*

Introduction

Synchronous cell division is a well known and frequently studied phenomenon in many cells. Examination of a synchronized population allows transiently expressed messenger RNA's (mRNA's), gene products, or cell functions to be observed. Photosynthetic cells can be trained to follow a highly synchronous cell division cycle merely by growth in a light-dark regime, allowing the cellular functions to be easily tracked (Adams et al (1984)).

Photosynthetic algae naturally undergo synchronized cell division when grown in an alternating light-dark regime. Although the connection between the need for metabolites for cell division and the need for light to obtain photosynthates is an obvious one, the mechanism for how light acts upon the cells to regulate DNA synthesis is not so clear.

To study the molecular mechanism for the light inducible synchrony in photosynthetic algae, I have examined the cell cycle in *Euglena gracilis bacillaris* by flow

cytometry. *Euglena*, like higher plants, contain many chloroplasts per cell (Leedale (1982)). The cell size is approximately 50 by 10 by 10 microns, and the genome size is $3x10^6$ kilobases, so the algal cell size and genome size are similar to that of animal cells, while its photosynthetic apparatus is similar to that of plant cells (Cook (1968)). The alga offers a natural model of what occurs within different sectors of a leaf during growing or non-dividing phases. To obtain a similar sample from a higher plant would involve isolation of protoplasts and treatment with drugs such as hydroxyurea to synchronize cell division. Mechanical chopping of leaves releases nuclei that are representative of the cell cycle state of the tissue at the time of isolation, but the different developmental state of cells within each leaf will contribute to sample heterogeneity (Galbraith et al (1983)). Techniques have been established for studying *Euglena* by flow cytometry (Bonaly et al. (1987), this work), and exact distributions of the cells with respect to the cell cycle can be determined. In particular, the time of the start of DNA synthesis can be determined, so the effect of light on entry into the cell cycle can be studied.

It has been shown in the photosynthetic alga *Chlamydomonas reinhardtii* that completion of the cell cycle is dependent on photosynthetic electron transport (Spudich and Sager (1980)). If, prior to exposure to at least six hours of light, Chlamydomonas cells are put into the dark or are treated with the Photosystem II inhibitor dichlorophenyldimethylurea (DCMU), the cells are arrested in the early, G_1 phase of the cell cycle. When the cells are again placed in the light, they complete the cycle.

In this study, *Euglena* have been monitored through all phases of the cell cycle by flow cytometry. Exposure for different lengths of time in light has been used as a variable to look for changes in the passage of a culture through the cell cycle. The results show that *Euglena* have a light requirement for entry into the cell cycle; the

cells need to be exposed to light for at least 6 hours in the G_1 phase. Exposure to light for longer lengths of time causes more cells to enter the cell cycle. Once committed to the cell cycle, *Euglena* can complete their cell cycle in the dark. Cells containing two genomic copies of DNA go through mitosis in the dark, and the daughter cells return to the original, one genome copy state until the start of the next light period.

Materials and Methods

Growth of Cells

Euglena gracilis bacillaris was grown at 22°C in photoautotrophic medium containing essential salts and trace metals with 5% CO_2/95% air bubbled and stirred into the medium (Hallick et al (1982)). The cells were exposed to a 12 hour light-12 hour dark regime.

For synchrony experiments, *Euglena* were plated on *Euglena* Broth agar plates (Difco), and a single colony was picked and inoculated into phototrophic medium. The cells were allowed to grow to stationary phase, and cells from this stationary phase culture were inoculated at the indicated times into 1 liter of fresh medium to a final concentration of 2-5 x 10^4 cells/ml. Cells were collected with a 16 gauge cannula attached to a Gilson pump; the pump was controlled by a timer that allowed cells to be collected for 1-5 minutes every 2-3 hours. Cell samples of 20 ml were collected with an LKB 7000 fraction collector in an LKB refrigerated unit that was kept at 4°C. Cells kept at this temperature are halted in their progress through the cell cycle.

Flow Cytometry (FCM)

Euglena cells were fixed for analysis by flow cytometry by pelleting at 8000 rpm in an SM24 rotor (Sorvall) for 5 minutes, followed by two washes in 5 ml of 70% EtOH, then digestion with 0.1 mg/ml of heat-treated RNAse in 2 ml of Saline GM, 37° for 1 hr. (Saline GM is 0.27 M NaCl, 11 mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5). Cells were then re-pelleted (5 minutes on a table-top centrifuge, half-speed) and DNA was stained for at least 15 minutes with 2 ml of of propidium iodide (Calbiochem) (25-50 ug/ml) in Saline GM, before analysis by FCM. Samples were first examined under a fluorescent microscope to check that the

nuclei were well stained, and that the cytoplasm contributed no fluorescence. The propidium iodide was excited with 488 nm light, and fluorescence intensity per cell at greater than 620 nm was measured. Cells were analyzed in an FMF-77-100 flow cell (Research Developments, Los Alamos, NM) in a flow cytometer constructed as previously described (Hawkes and Bartholomew (1977)).

Results

Flow Cytometry Studies of Euglena

The use of flow cytometry on *Euglena* gives sharply defined and easily interpretable data sets for following the passage of cells through their cell cycle. Fixing cells in 70% EtOH removes the chlorophyll that could interfere with dye fluorescence. Staining of RNAse treated samples with propidium iodide results in cells whose fluorescence is due almost entirely to nuclear DNA.

Chloroplast DNA comprises about 15% of cellular DNA in fully green cells (Chelm et al). Since an overwhelming amount of the cellular DNA is nuclear, chloroplast and mitochondrial DNA do not significantly contribute to the DNA histograms.

The presence of chloroplast DNA does cause a broadening of all the peaks in a histogram due to the heterogeneity of chloroplast DNA copies per cell. The nuclear DNA content of a cell must exceed 2 standard deviations (σ) of the average value in order to be detectable as having exited from the G_1 phase. In the histograms examined here, 2 σ is about 28% of the average value. Even in the extreme case in which all chloroplast DNA replicated at the same time, such a change would not alter the percentage of cells calculated to be out of the G_1 phase.

When flow cytometry data are plotted as cell number vs. DNA content, the proportion of cells in a population that are to be found in each stage of the cell cycle is easily seen. The sensitivity of flow cytometry is good enough to distinguish between cells in G_1, which contain one genomic copy of DNA , cells in S phase, which are carrying out DNA synthesis, and cells in G_2, which have completed DNA synthesis and contain two genomic copies of their DNA. Even cells just starting DNA synthesis are easily discerned, so that times of entry into the S phase can be clearly identified.

Since all cells that are collected for flow cytometry are stored at 4°C before fixing, it was important to check that no DNA synthesis or passage through the cell cycle occurs at that temperature. Parallel samples were collected from a culture inoculated at L0, and one set was stored at 4°C for 12 hours before being fixed, while each sample in another set was fixed immediately after collection (Fig. 2-1).

The percentage of cells in the S and G_2 phases was calculated by peak integration, and the data are replotted as percentage of cells in the cell cycle versus time (Fig. 2-2). The 5th timepoint of the top set of histograms (Fig. 2-1a) was not included in the percentage calculation because that sample shows extreme DNA degradation. Fortunately, this kind of histogram was rarely seen in the experiments performed. Comparison of the flow cytometry profiles (FCM's) from the two sets of fixed cells show that very little cell cycling occurs during storage at 4°C.

The pulse height analyzer used for data collection consists of 256 channels that record the number of cells with fluorescence intensities that correspond to the value assigned to each channel. Plotting all 256 channels on the x-axis versus cell number on the y-axis generates an FCM histogram. When the instrument gain is kept constant, the position of the G_1 and G_2 peaks with respect to the x-axis in FCM histograms is proportional to the laser power used to excite the propidium iodide intercalated into the DNA. A small change in laser power can cause broadening of the peaks and a shift of the peaks to a higher x value (higher channel number). This is the case in the last histogram plotted in Fig. 2-1b. When a series of samples from the same culture are analyzed in a single FCM run, changes in peak position are due to changes in laser power. Changes in cellular DNA content are reflected in changes in relative peak heights.

Fig. 2-1 *Euglena* kept at 4°C do not progress through the cell cycle. Cells samples were collected at the same time and fixed either immediately (top, 1a) or fixed 12 hours after collection (bottom, 1b). Timepoints were collected at 2 hour intervals.

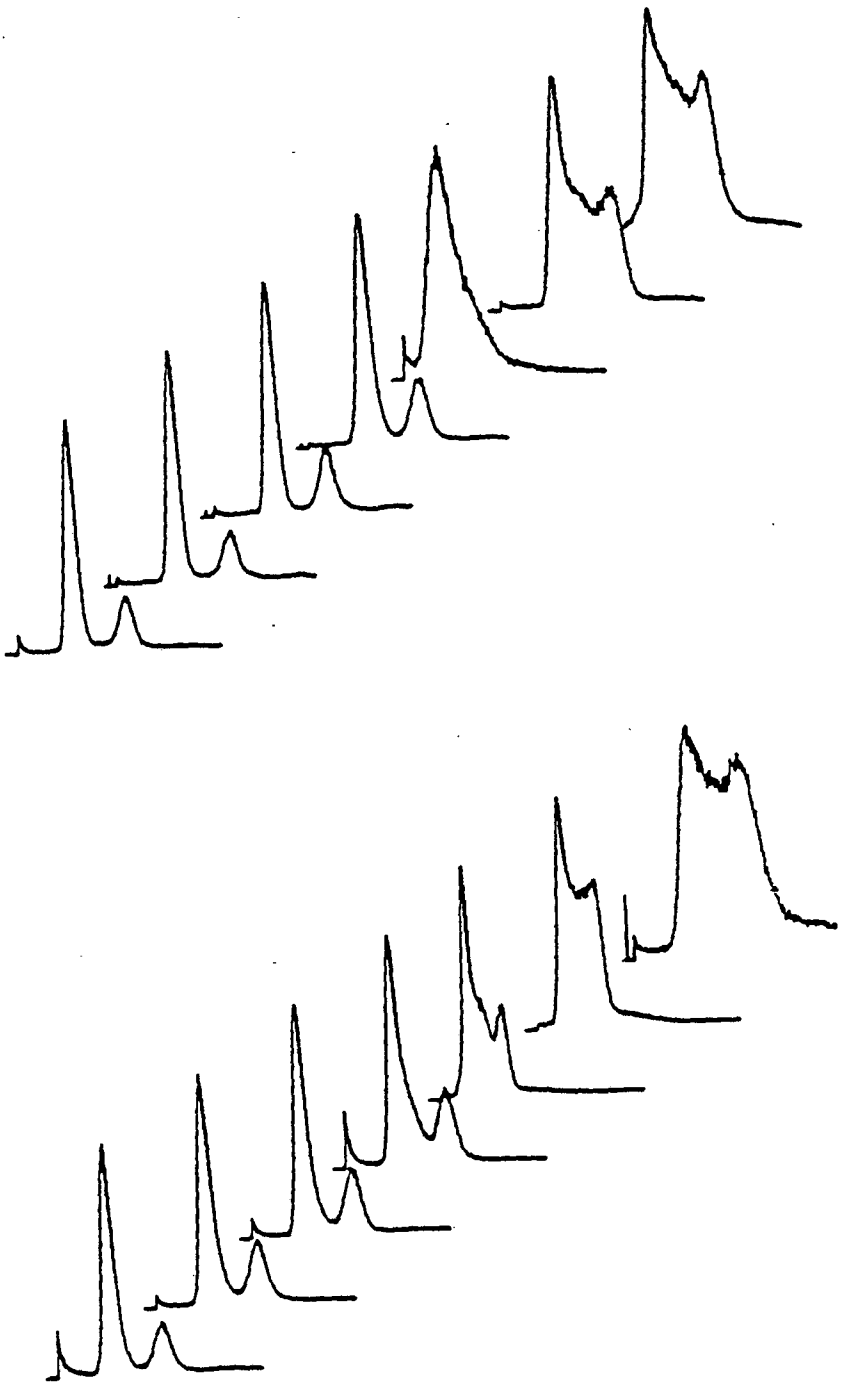
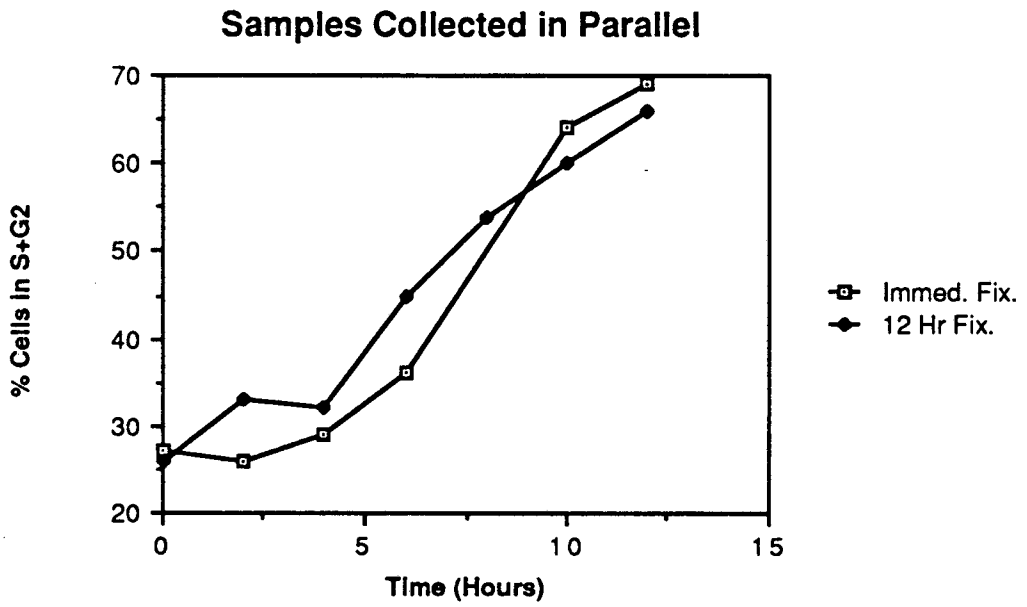


Fig. 2-2 Replotting of data from Fig. 2-1 as Percentage of Cells in S and G_2 phases versus Time. Rate of increase in percentage of cells entering the cell cycle is similar for cells fixed immediately or fixed 12 hours after collection.


Samples Collected in Parallel
% Cells in S+G2
70
60
50
40
30
20
0
5
10
15
Time (Hours)
Immed. Fix.
12 Hr Fix.

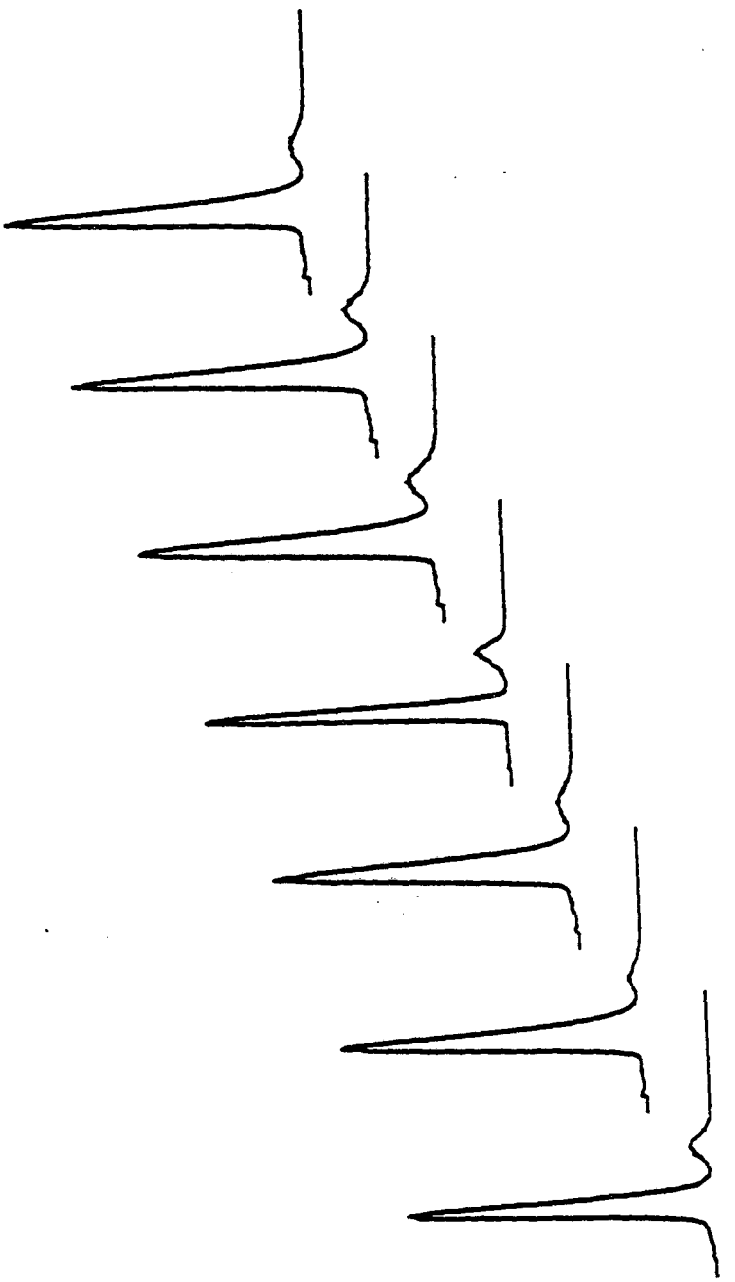
Light Influence on Cell Cycling

When cultures are grown to stationary phase, no change in cell number or passage through the cell cycle is seen (Fig. 2-3). The majority of the cells are in the G_1 (left side) peak, and stay there for all timepoints examined. A number of cells can be seen in a second peak on the right, which represents the G_2 phase. The G_2 peak of stationary phase FCM's represents a small proportion of the population, and these cells are either stopped in their G_2 phase or are continuing to carry on cell division while the rest of the population has stopped. Flow cytometry cannot distinguish between the two possibilities. However, if these cells are still cycling, they are doing so in a continuous fashion, because the size of the G_2 peak never changes. Because I am interested only in the synchronous movement through the cell cycle by the majority of the cell population when a stationary phase culture is used to inoculate fresh medium, the contribution from the much smaller number of cells in G_2 causes no interference.

Non-transformed animal cells will stop cell cycling at high cell density due to limitation of factors in the medium. Photosynthetic cells in stationary phase might be stopped in their cell cycle for several reasons: lack of light, lack of space, or accumulation of inhibitory waste products, all caused by the high culture density. Dilution of such cells into fresh medium alleviates all problems, and the following experiments were performed to determine which effect, available light or space or inoculation into fresh medium, is dominant in committing *Euglena* to the cell cycle.

Two cultures inoculated at the same time into fresh medium were exposed to either 12 hours of light, or 12 hours of darkness. For the light exposed cells, DNA synthesis began 4-6 hours after inoculation (Fig. 2- 4a). At 6 hours after inoculation, the G_2 peak on the left can be seen to be growing as the DNA content per cell increases. Inoculation of cells into fresh medium followed by a 12 hours' exposure

Fig. 2-3 FCM histograms collected at 4 hour intervals from a culture grown to stationary phase in alternating light and dark. Cells in stationary phase do not progress through the cell cycle.



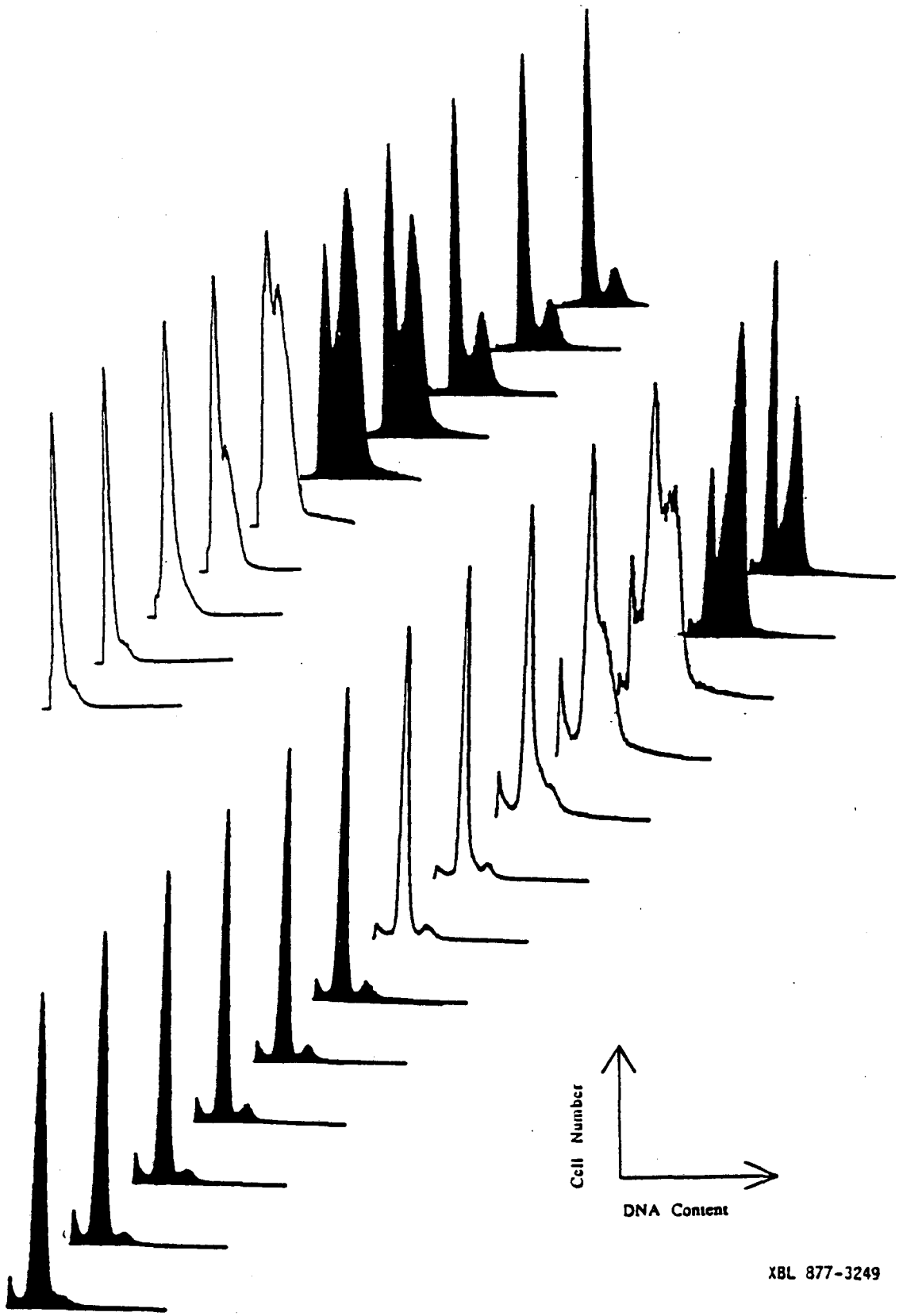
to darkness shows almost no movement through the cell cycle for the first 18 hours (Fig. 2-4b). Not until 6 hours into the next light period do the majority of the cells enter the S phase (Fig. 2-4b, frame 9).

Calculation of the percentage of cells in each histogram that were in the S and G_2 phases was done by peak integration. When the histogram data are replotted as the percentage of cells in the S and G_2 phases vs. time, one can clearly see that for both cultures an increase in the number of cells in the S and G_2 phases occurs only after exposure to 6 hours of light (Fig. 2-5).

If the start of DNA synthesis in *Euglena* were caused merely by dilution of cells into fresh medium with a lower concentration of waste products, or to an internal, light independent cell clock, one would expect the same time of initiation of DNA synthesis in cultures inoculated in the light or the dark. No cell cycle activity is seen in cells inoculated in the dark during the 12 hour dark period, and entry into the S phase is seen only after exposure to at least 6 hours of light. Cells inoculated into fresh media and kept in darkness for 18 hours show no cell cycling activity until exposure to 6 hours of light (Fig. 2-6a). Finally, cells exposed to 12 hours of light followed by 36 hours of darkness go through only one round of replication during the first 18 hours, then stay in G_1 for the rest of the dark incubation (Fig. 2-6b). These experiments give a clear indication that light, and not available space or an internal 24 hour clock, is the determining factor for entry into the cell cycle.

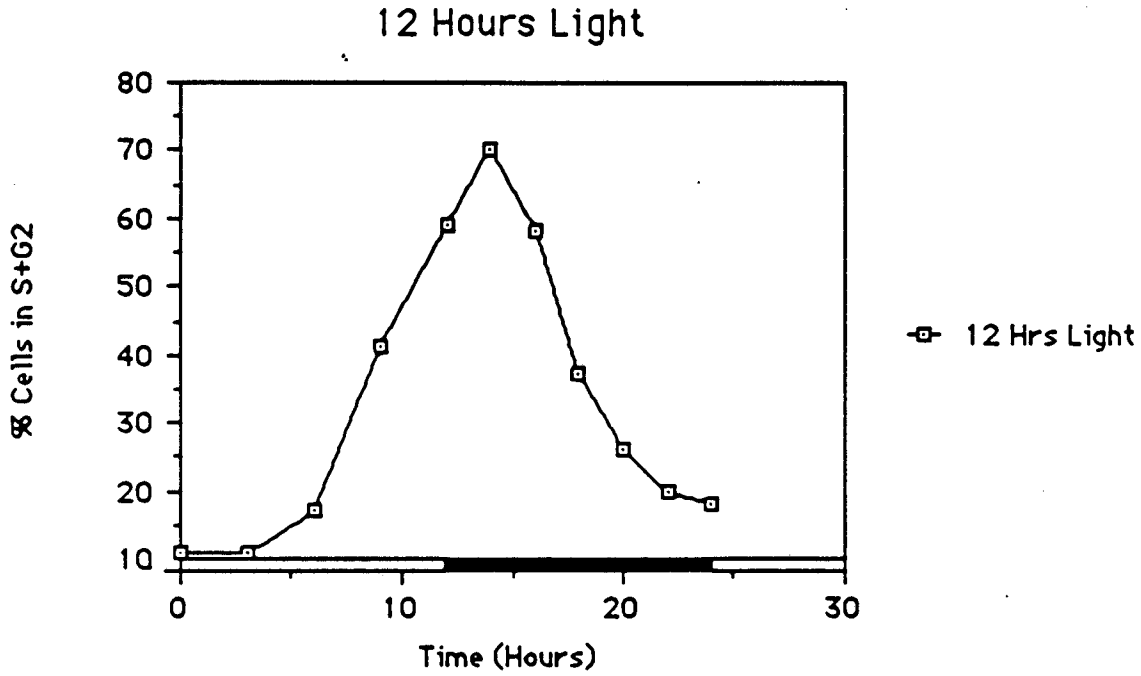
The six hour delay in the light period before the start of DNA synthesis indicates that there is a minimum amount of light required for cell cycling. Newly diluted cells were exposed to varying lengths of light exposure to determine whether the light requirement is in the nature of a simple switch that starts all the cells in a population into the cell cycle after enough exposure to light has occurred, or more like a dose

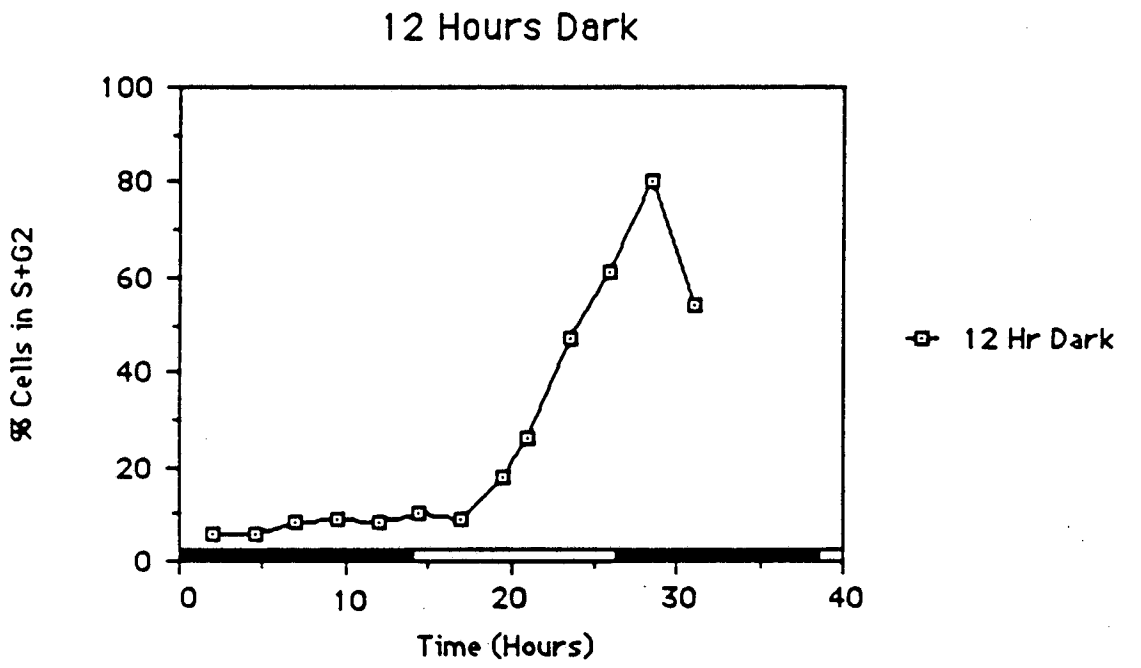
Fig. 2-4. Cultures diluted at the same time into fresh medium and exposed to 12 hours of light (Fig. 2-4a) or 12 hours of darkness (Fig. 2-4b). Cells were collected at 3 hour timepoints (Fig. 2-4a) or at 2.5 hour timepoints (Fig. 2-4b). Filled-in FCM profiles represent samples collected during the dark period.



XBL 877-3249

Fig. 2-5. Replotting the data from Fig. 2-4 as percentage of cells in S and G_1 against Time. The area outside the G_1 peak in each histogram was calculated by computer integration and is plotted as percentage of total cells.

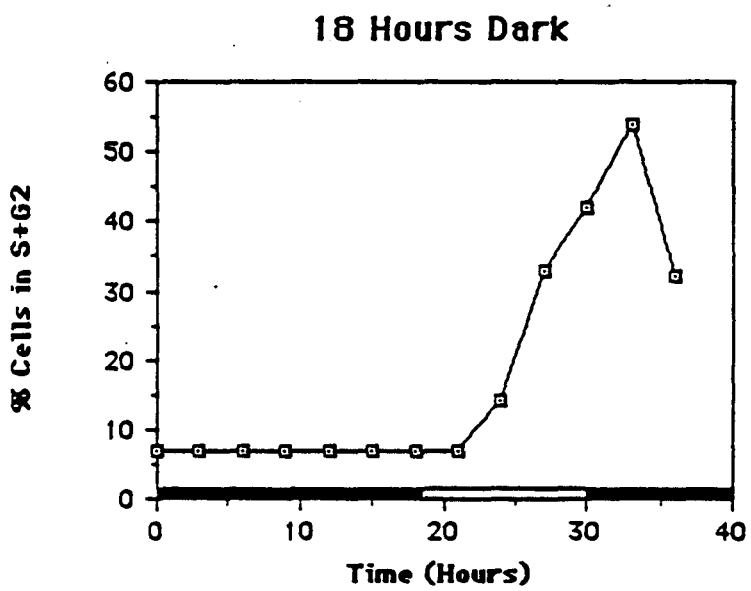

12 Hours Light
% Cells in S+G2
80
70
60
50
40
30
20
10
0
10
20
30
Time (Hours)
12 Hrs Light

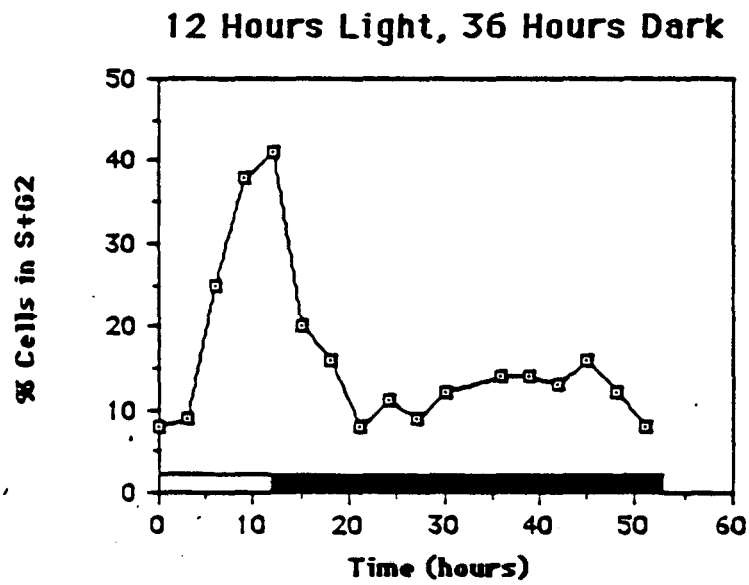

12 Hours Dark
% Cells in S+G2
100
80
60
40
20
0
0
10
20
30
40
Time (Hours)
12 Hr Dark

XBL 877-3251

Fig. 2-6a Percentage of cells in S and G_2 from histograms of cells exposed to 18 hours darkness followed by 12 hours light. Cells do not enter the S phse until 6 hours after the start of the light period.

Fig. 2-6b Percentage of cells in S and G_2 from histograms of cells exposed to 12 hours light followed by 36 hours darkness. Cells go through one round of replication during the first 18 hours, then stay in G_2 for th remainder of the dark period.


18 Hours Dark
% Cells in S+G2
60
50
40
30
20
10
0
0
10
20
30
40
Time (Hours)


12 Hours Light, 36 Hours Dark
% Cells in S+G2
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (hours)

response, so that longer light exposure causes more cells to enter the cell cycle.

Cultures exposed to 6, 9, or 12 hours of light all enter the cell cycle within the first light period (Fig. 2-7a). Cells exposed to fewer than 6 hours of light stay mostly in the G_1 phase until the next light period, when the cells begin DNA synthesis after exposure to 6 hours of light (Fig. 2-7b). Cells exposed to 3 hours of light do show a small number of cells entering the S phase after the first light period, suggesting that some cells in the population can begin DNA synthesis with less than 6 hours of light, but that the majority of the population is dependent on an exposure of 6 hours of light.

Exposure to more than 6 hours of light causes progressively more cells to enter the S phase. Cultures exposed to 12 hours of light have a greater maximum percentage of cells in the S and G_2 phases than cells exposed to 9 hours of light, which have more than cells exposed to 6 hours of light.

Because *Euglena* are capable of non-photosynthetic growth on heterotrophic media, cells grown in the absence of light in heterotrophic media must have different metabolic requirements for cell cycling. FCM profiles of wild-type cells grown in the dark for many generations show that, when inoculated into fresh heterotrophic medium, the cells still show a 6 hour lag period before the start of DNA synthesis (Fig. 2-8).

Fig. 2-7 Determining minimum light exposure required for cell cycling.

Fig. 2-7a Cells exposed to 6, 9, or 12 hours of light all enter the S phase at the same time.

Fig. 2-7b Cells exposed to 0 or 3 hours of light don't enter the S phase until the middle of the next light period.

Fig. 2-7a

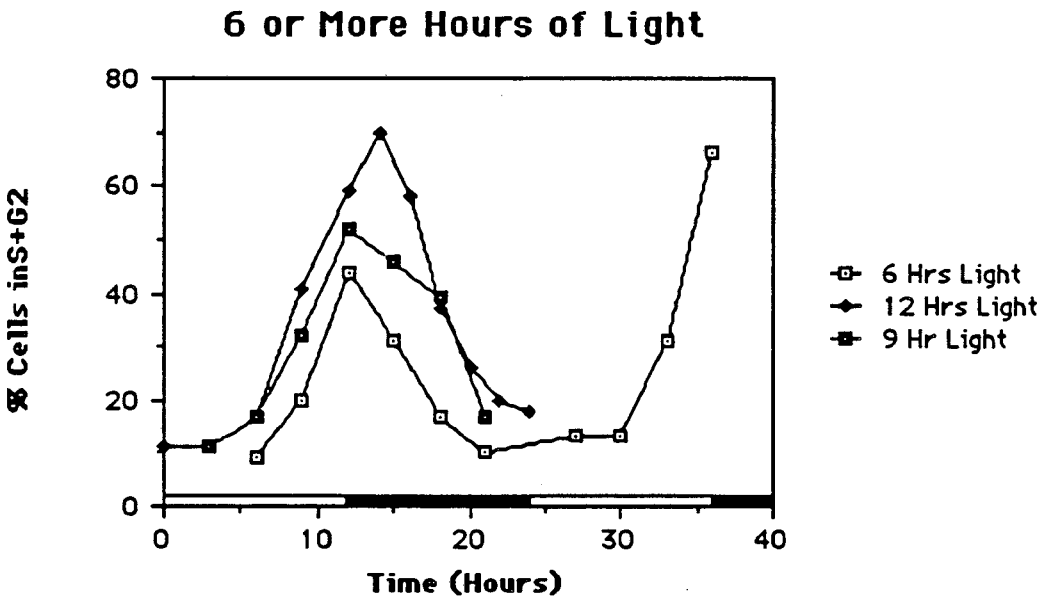


Fig. 2-7b

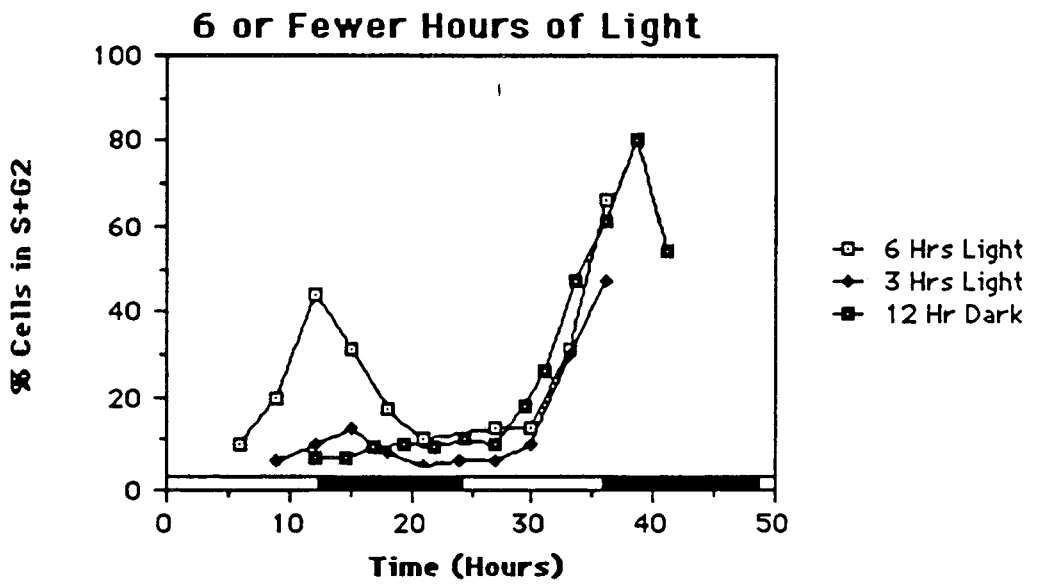
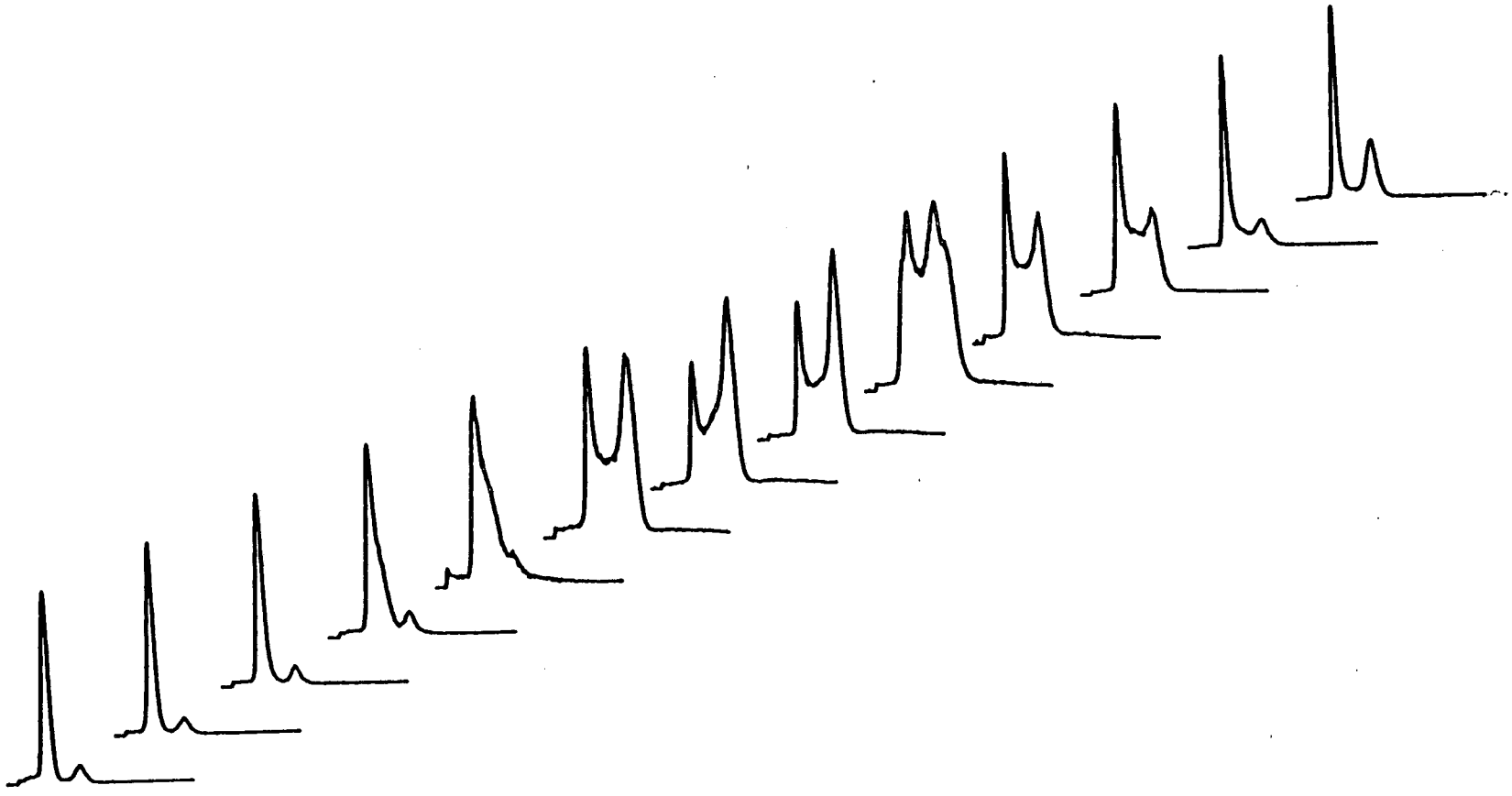


Fig. 2-8 Dark grown cells are also synchronized upon inoculation into fresh media.



XBL 8710-4111

Discussion

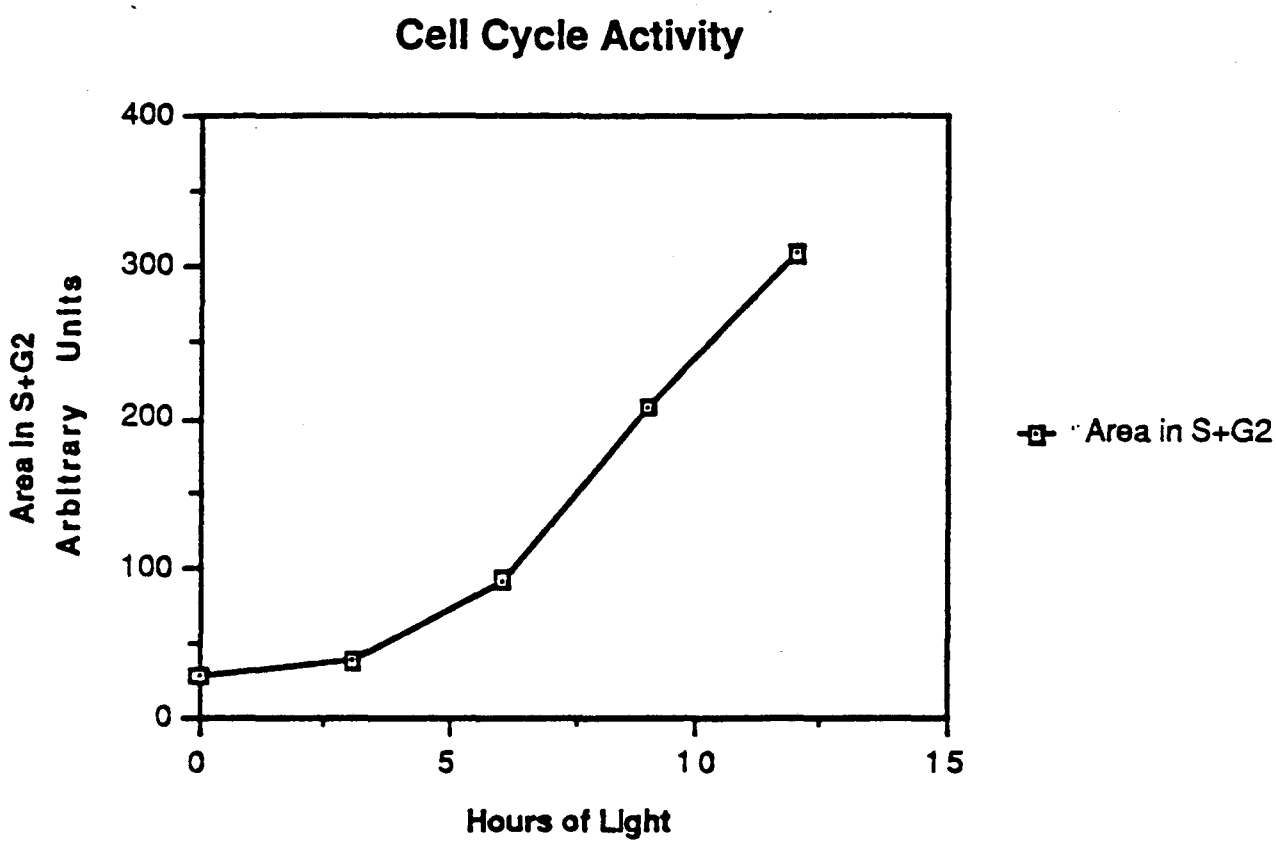
Light Requirement

These experiments have shown that *Euglena* can be easily followed through the cell cycle by the use of flow cytometry. The degree of synchrony is very high in these cells, and all phases of the cell cycle are clearly defined by their FCM profiles. Entry into the cell cycle is a light dependent phenomenon for phototrophically growing cells. Inoculation of stationary phase cells in the dark does not show any change in the FCM profile of the diluted culture.

By varying the length of light exposure after inoculation, it has been shown that at least six hours of light are required for cells to be committed to the cell cycle. In this respect, light induction is like a switch that gives an on/off response. When the area under the curves for the first 24 hours in Figs 6a and 6b are plotted versus hours of light exposure (Fig. 2- 9), it can be seen that exposure to less than six hours of light causes almost no entry into the cell cycle, while exposure to more than six hours of light causes entry of 50-80% of a population into the cell cycle. When cultures are exposed to 9 or 12 hours of light, more cells enter the cell cycle than in cultures exposed to 6 hours of light; while 6 hours of light is the minimum light exposure needed to initiate the cell cycle, light exposure after that point causes a dose response, so that more light allows more cells to enter the S phase.

Entry of all cells into the cell cycle is never seen in these experiments; whether the cells left in G_1 during the first round of cell division can enter the cell cycle during the next round, or whether these are dead cells, can't be determined by FCM in this case. Incorporation of bromodeoxyuridine (BrdUrd) into nuclear DNA followed by immunological detection with a monoclonal antibody against BrdUrd has been used to study DNA replication in animal cells. However, *Euglena* lack cytoplasmic thymidine

Fig. 2-9. Area under curves in Figures 6a and 6b during the first 24 hours plotted versus hours of light exposure received by each culture. Cultures exposed to fewer than 6 hours of light show very few cells in the S and G_2 phases. Cells exposed to more than 6 hours of light show a linear response to light.



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kinase (Sagan (1965)) and do not incorporate [^{3}H]-thymidine into nuclear DNA, so total DNA per cell, but not DNA replication can be measured.

In the case of cells exposed to only 3 hours of light, a small amount of cell cycling is seen at the beginning of the dark period. If the light requirement were a dose response, cells exposed to 3 hours of light ought to have half as many cells entering the S phase as cells exposed to 6 hours of light. Instead, the population of cells entering the S phase in the 3 hour light exposed cells is much smaller than that seen in the 6 hour light exposed culture. The low percentage of cells entering the S phase is not due to large number of dead cells either, since the majority of the population enters the cell cycle in the next light period. These cells that require only 3 hours of light for entry into the S phase probably represent the Gaussian variance that is to be expected as a culture goes through many cell divisions.

Examination of the FCM's presented here shows that all cells that have initiated DNA replication complete their cell cycle in the dark. While six hours of light is required for entry into the S phase, completion of DNA synthesis and mitosis occurs in the dark, so passage through these phases is clearly independent of light. If completion of the cell cycle were also light dependent, one would expect to see cells maintain the same FCM profile all through the dark period and only move on to the next phase of the cell cycle when the light came on again. Instead, once cells have started into the S phase, they can progress through the rest of the cell cycle in the dark until they have returned to the G_1 phase, where they remain until exposed to at least another six hours of light.

Threshold Light Response

The six hour light requirement for entry into the cell cycle, and the repression of cell cycling in *Chlamydomonas* upon addition of the photosynthesis inhibitor DCMU

(Spudich and Sager (1980)) indicate accumulation of a factor that needs a photosynthetic byproduct for initiating cell cycling. In the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Saccharomyces pombe*, a cell cycle control gene, *CDC28* and *cdc2* respectively, has been found (Piggott et al. (1982), Russell and Nurse (1986)). The gene product functions in late G_1 to commit cells to a new round of DNA replication, and in late G_2 to initiate mitosis (Reed et al. (1985), Simanis and Nurse (1986)). From sequence analysis of the cloned gene and functional analysis of the proteins, both *CDC28* and *cdc2* has been shown to be phosphoproteins with protein kinase activity. Expression of a human cDNA library in *S. pombe* has been used to identify a human gene that can complement the *cdc2* mutation (Lee and Nurse (1987)). An analogous system might be present in *Euglena*, and light-induced entry into the S phase of the cell cycle in the alga might be linked to accumulation of enough energy by photophosphorylation to activate a cell cycle control protein.

Linear Light Response

A chloroplast synthesized protein has also been implicated as the cause of the light inducible synchrony seen in photosynthetic algae (Blamire et al (1977)). Treatment of cells with low levels of inhibitors of chloroplast protein synthesis causes inhibition of nuclear DNA replication, while chloroplast DNA replication continues. After the first 6 hours of light has been received, the linear effect of light on the number of cells entering the S phase could be due to the activity of this chloroplast factor. Several chloroplast transcripts have been shown to be stable only in the light and not in the dark (Deng and Gruissem (1987)) which would account for why cells past the restriction point commit to the cell cycle only during the light period.

Conclusions

These experiments have shown that phototrophically grown *Euglena* require ex-

posure to 6 hours of light before initiation of the cell cycle. We propose that this light requirement is the time needed to allow activation of a "start" factor analogous to the *cdc2* and *CDC28* gene products in yeast. Once exposure to the minimum amount of light has been achieved, further exposure to light allows more cells to enter the cell cycle. We propose that light induces factor that increases the transition probability of entry into the cell cycle. This factor is short-lived, with a half-life of 6 hours or less. The longer the light exposure, the more factor is available, the higher the transition probability, and the more cells enter the cell cycle. If the light exposure is shorter than 6 hours, the factor degrades without stimulating significant entry into the cell cycle.

In the case of heterotrophic dark grown cells, a 6 hour lag is still observed, so a cell cycle control protein in *Euglena* should be activated by the energy pool available in the cell, and not directly by photosynthesis. Dark grown cells contain immature chloroplasts known as proplastids, which do not have a functional photosynthetic apparatus. They do however contain approximately 1400 copies of the chloroplast genome per cell (Chelm et al. (1977)) and the genomes are transcriptionally active even in the dark (Keller et al. (1982)), so activation of the cell division cycle by a chloroplast encoded protein is not ruled out even in dark grown cells.

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Chapter III

The Effects of Darkness and DCMU on the Cell Cycle in *Euglena*

Introduction

Photosynthetic algae have long been studied as a model system for cell division because of the ease with which the cells can be synchronized to a light-dark program (Edmunds (1964)). The basis of the light dependence of the cell cycle in algae is attributable to the reliance of the cells on photosynthesis as their sole nutritional source. However, the rapidity of change in the rate of entry into the cell cycle in response to transfer from light to darkness is difficult to assign directly to a decrease in photosynthetically-derived nutrients. Previous experiments performed by our group have shown that there is a basal light requirement in *Euglena* that must be fulfilled before cell cycling can begin, followed by a light dose effect that determines how many cells in a population will actually enter the cell cycle (Yee and Bartholomew (1988)). We have designated the light dose dependent activity that causes commitment to the cell cycle the initiating factor. We have investigated the light dose effect to see if it is directly dependent on the photosynthetic activity of the cells, and to determine the lifetime in the dark of the factor that causes commitment to the cell cycle. We wanted to compare the effects of inhibition of photosynthesis and the effects of darkness to see if they gave similar results in synchronized cells. We assumed that the inhibitor used affects only Photosystem II electron transport, and any differences observed

between the inhibitor and dark treatments would be due to a non-photosynthetic light dependence.

From our previous results, it is clear that *Euglena* loses the cell cycle initiating factor after a 12 hour dark incubation. If the cell cycle initiating factor were stable in the dark, one would expect a culture that had undergone one round of cell division to begin a new round of cell division immediately after the start of the next light period. Instead, cells that have gone through one round of cell division under a 12 hour light-12 hour dark regime require another 6 hours of light exposure before initiating a second round of DNA synthesis. Other groups that have grown *Euglena* in a 14 hour light-10 hour dark regime have also noted that cells grown under those conditions undergo synchronized cell division at the beginning of the dark period, with no further increase in cell number until the same timepoint 24 hours later. (Edmunds, 1964). This demonstrates that 10 hours of darkness is enough to "reset" *Euglena* to a state that is uncommitted to the cell cycle

The results presented below show that the cell cycle initiator has a half-life of 5 hours in the dark. Presumably, the levels of the initiator fall below a threshold level after 10 hours of darkness so that a 6 hour light exposure is needed to allow cells to enter the next round of replication.

We wanted to see if the cell cycle initiator required light alone or photosynthetic activity in order to accumulate. Cells were treated with the Photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the expectation that it would stop photosynthesis but allow cell cycling to proceed. A mutant strain of Euglena has been isolated that is resistant to the effects of DCMU on its photosynthesis apparatus (Laval-Martin et al. (1979)), so it was reasonable to assume that DCMU affects wild type photosynthetic electron transport but not cell cycle activities. However, DCMU affects

the *Euglena* cell cycle in ways other than those due to interruption of photosynthesis. DCMU causes a decrease in the rate of DNA synthesis in wild type cells grown under dark, heterotrophic conditions, when the cells do not carry out photosynthesis. DCMU also causes cells to become blocked or greatly retarded in their ability to carry out mitosis.

The effects of DCMU are not limited to wild type cells, but are also found in chloroplastless mutants of *Euglena*. DCMU-treated mutant cells exhibit a G_2 blockage, but DNA synthesis is not affected as in wild-type cells.

Methods

Euglena gracilis bacillaris was grown phototrophically or heterotrophically as described previously (Yee and Bartholomew (1988)). Samples were prepared and analyzed on the flow cytometer as previously described. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was recrystallized from methanol and water and used as a 2 mM stock dissolved in ethanol. DCMU was used at a final concentration of 10uM.

Two chloroplastless strains of *Euglena gracilis bacillaris*, W_3BUL and W_{10}SmUL were obtained from Prof. Jerome Schiff.

Results

Lifetime of the Cell Cycle Initiator

Our previous experiments showed that phototrophically-grown *Euglena* has a cell cycle initiation factor that becomes active after 6 hours of light exposure. Cells cease to enter the cell cycle within 3 hours of transfer into darkness, and incubation in 12 hours of darkness causes all cells to return to G_1 until another 6 hours of light re-activates the cell cycle initiation.

The lifetime of the cell cycle initiator in the dark was investigated by monitoring the rate of DNA synthesis in cultures exposed to dark periods of varying lengths in the middle of a 12 hour light period. The rate of increase in the percentage of cells in S and G_2 after the brief dark exposure was used as a measure of the amount of active initiator still available to the cells.

3 Hours Darkness Causes Slight Delay in Cell Cycle Entry

Wild type, phototrophically-grown *Euglena* was diluted into fresh, phototrophic medium and exposed to 9 hours light to allow DNA synthesis to begin. The cells were then placed in darkness for 3 hours followed by light for 3 hours, and finally, into darkness for the rest of the experiment. The percentage of cells in the S and G_2 phases was monitored by FCM and plotted in Fig. 3-1a.

6 hours after inoculation, the percentage of cells entering the S and G_2 phases can be seen to rise sharply. This rate continues even during the 3 hour dark incubation. Cells enter S and G_2 at only a slightly slower rate during the next 3 hour light incubation. In the last dark incubation, the cells enter G_2 and undergo mitosis.

Fig. 3-1a Synchronized cultures of *Euglena* were subjected to brief periods of darkness in the middle of a light period to study the lifetime of the cell cycle initiating factor. Phototrophically-grown cells were inoculated into fresh phototrophic medium and exposed to 9 hours light, 3 hours darkness, 3 hours light, followed by darkness. The rate of entry into the cell cycle was monitored by flow cytometry (FCM) and the percentage of cells in S and G_2 plotted against time.

Fig. 3-1b. Phototrophically-grown cells were inoculated into fresh medium and exposed to 6 hours light, 6 hours dark, 6 hours light.

Fig. 3-1a

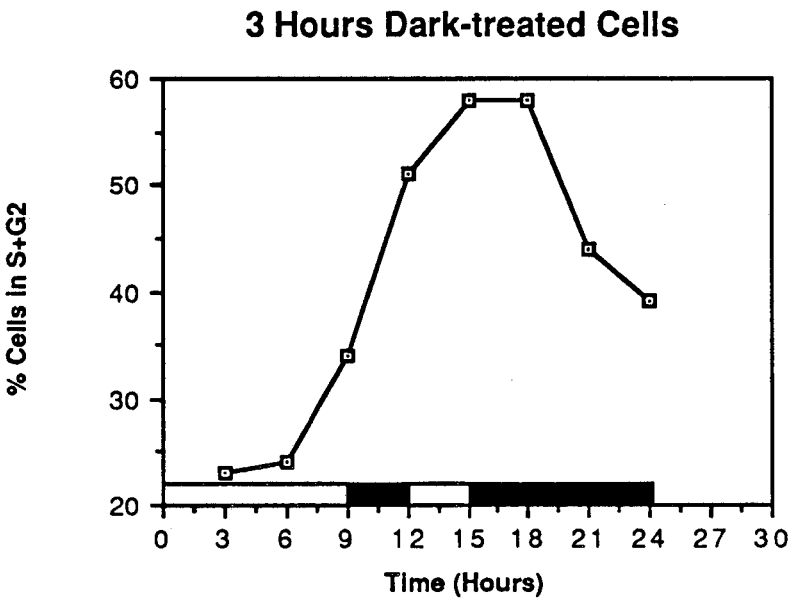
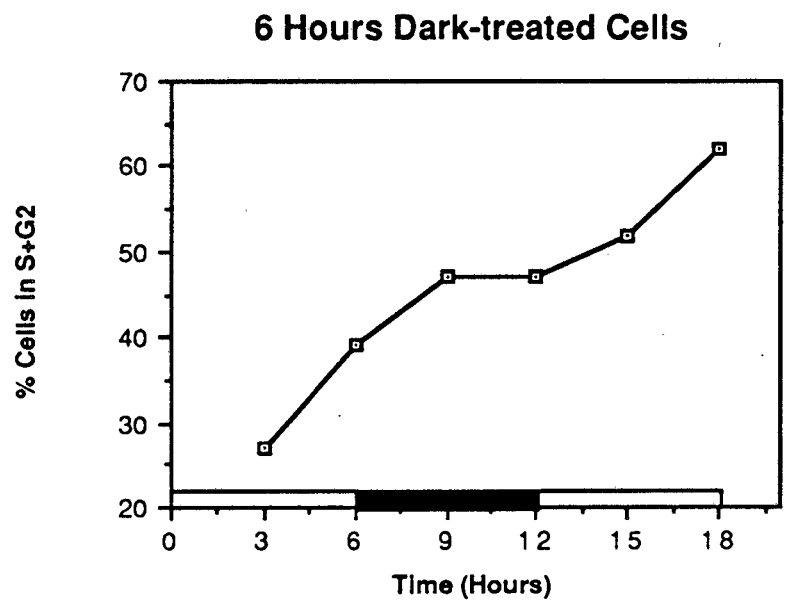


Fig. 3-1b



Cells Still Enter the Cycle after 6 Hours of Darkness

Another culture of phototrophically-grown cells was diluted into fresh medium and exposed to 6 hours light, 6 hours darkness, then 6 hours light (Fig. 3-1b). The cells cease entering the cell cycle after the 3rd hour in darkness, but immediately begin entering the S phase again, though at a diminished rate, when the culture is returned to the light.

These results show that the cell cycle initiator is stable for only a brief period in darkness. The rate of entry into S and G_2 after 3 hours of darkness is 2.3% of the population per hour compared to 3.3% per hour just before the dark treatment. The rate of entry after 6 hours of darkness is 1.6% per hour compared to 4% per hour just before the dark treatment. From this, we calculate that the cell cycle initiation factor has a half-life of 5 hours.

The Effects of DCMU Differ from those of Darkness

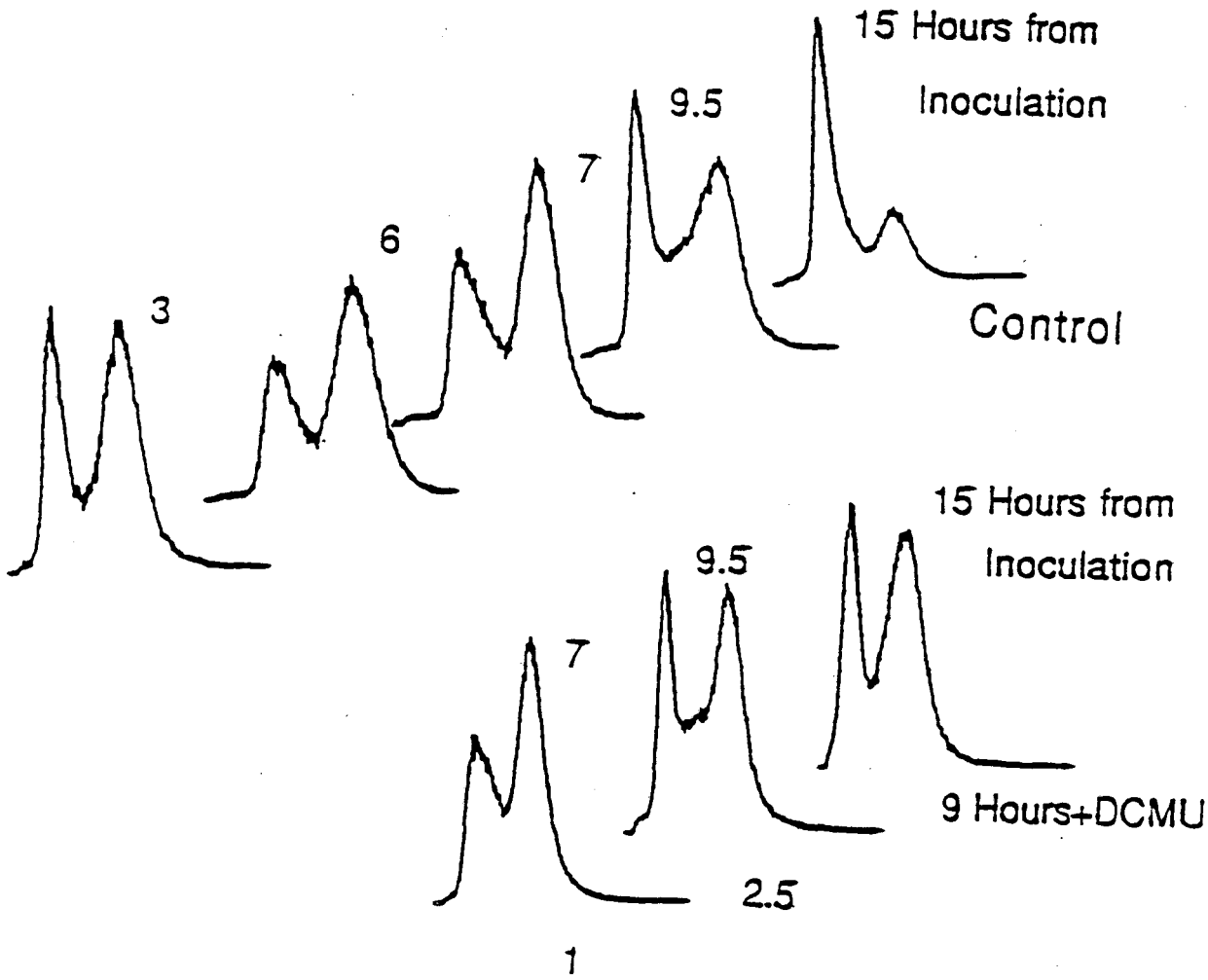
Flow cytometric analysis of *Euglena* cultures subjected to either DCMU or darkness was performed to see if the two treatments give the same effect with respect to DNA synthesis. Wild type *Euglena gracilis bacillaris* was inoculated into duplicate flasks containing phototrophic medium and allowed to grow in the light for 6 hours to enable DNA synthesis to begin. DCMU was then added to one flask and samples were taken to monitor the progress of the culture through the cell cycle. Examination of the DNA histograms show that the effect of DCMU is quite different from that of darkness. Untreated control cells stop entering the S phase once the culture is put in the dark. Cells that were in S phase complete DNA synthesis and the culture undergoes synchronized mitosis after 3-6 hours in the dark (Fig. 3-2a).

DCMU-treated cells do not behave like cells put in darkness. Addition of DCMU

Fig. 3-2a. Phototrophic cells were inoculated into duplicate flasks of phototrophic medium. Control cells were exposed to 12 hours light and 12 hours darkness. Numbers above histograms indicate the hour after inoculation at which sample was taken.

Fig. 3-2b. DCMU (10 uM) was added to second culture 6 hours after inoculation. Numbers below histograms indicate the hour after addition of DCMU at which sample was taken.

Phototrophic Cells, DCMU-treated in Light



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causes an immediate decrease in the rate of DNA synthesis, so that the time spent in the S phase is prolonged by at least 6 hours (Fig. 3-2b). Cells also accumulate in the G_2 phase, so mitosis is also blocked. Replotting of the data as percentage of cells in S and G_2 shows that no further increase in the number of cells entering the cell cycle occurs after addition of DCMU (Fig. 3-3).

Both completion of the S phase and mitosis normally occur in the dark, in light-independent steps. These results indicate that DCMU affects the cell cycle in ways other than by inhibition of Photosystem II.

DCMU Blocks Non-photosynthesizing Cells

To see if DCMU has the same inhibitory effects on *Euglena* grown under non-photosynthetic conditions, light-grown wild type cells were inoculated into duplicate flasks of heterotrophic medium in the dark, DCMU was added 6 hours after inoculation, and samples were collected for FCM (Fig. 3-4). The data are replotted as percentage of cells in the S and G_2 phases versus time (Fig. 3-5). Comparison of DCMU-treated and control cultures show that DNA synthesis is again retarded, though cells continue to enter the cell cycle, and that the cells are still blocked in late S. Thus, DCMU affects DNA synthesis and mitosis even in non-photosynthesizing wild type cells.

To see if these functions are sensitive to DCMU in cells that do not contain Photosystem II, experiments were performed on several cell types that lack active chloroplasts. Two mutant strains of *Euglena*, W_3BUL and W_{10}SmUL, were examined to see if DCMU affects chloroplastless cells. Mutant cells from near stationary phase cultures of either strain were inoculated into duplicate flasks. The cultures were allowed to grow for 2 hours to enable DNA synthesis to begin, then DCMU was added to one flask. Samples for flow cytometry were taken at 2 or 3 hour intervals. Comparison of the FCM histograms and plot from control and DCMU-treated cells

Fig. 3-3. Plotting of the data in Fig. 3-2 as percentage of cells in S and G_2 against time. Entry of cells into the cell cycle in the DCMU-treated culture is blocked.

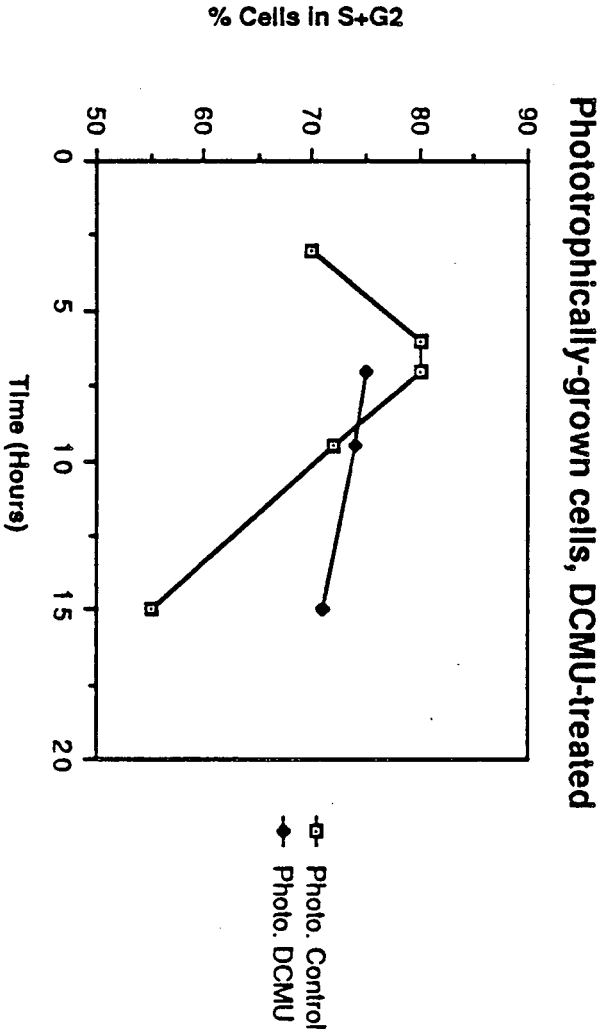
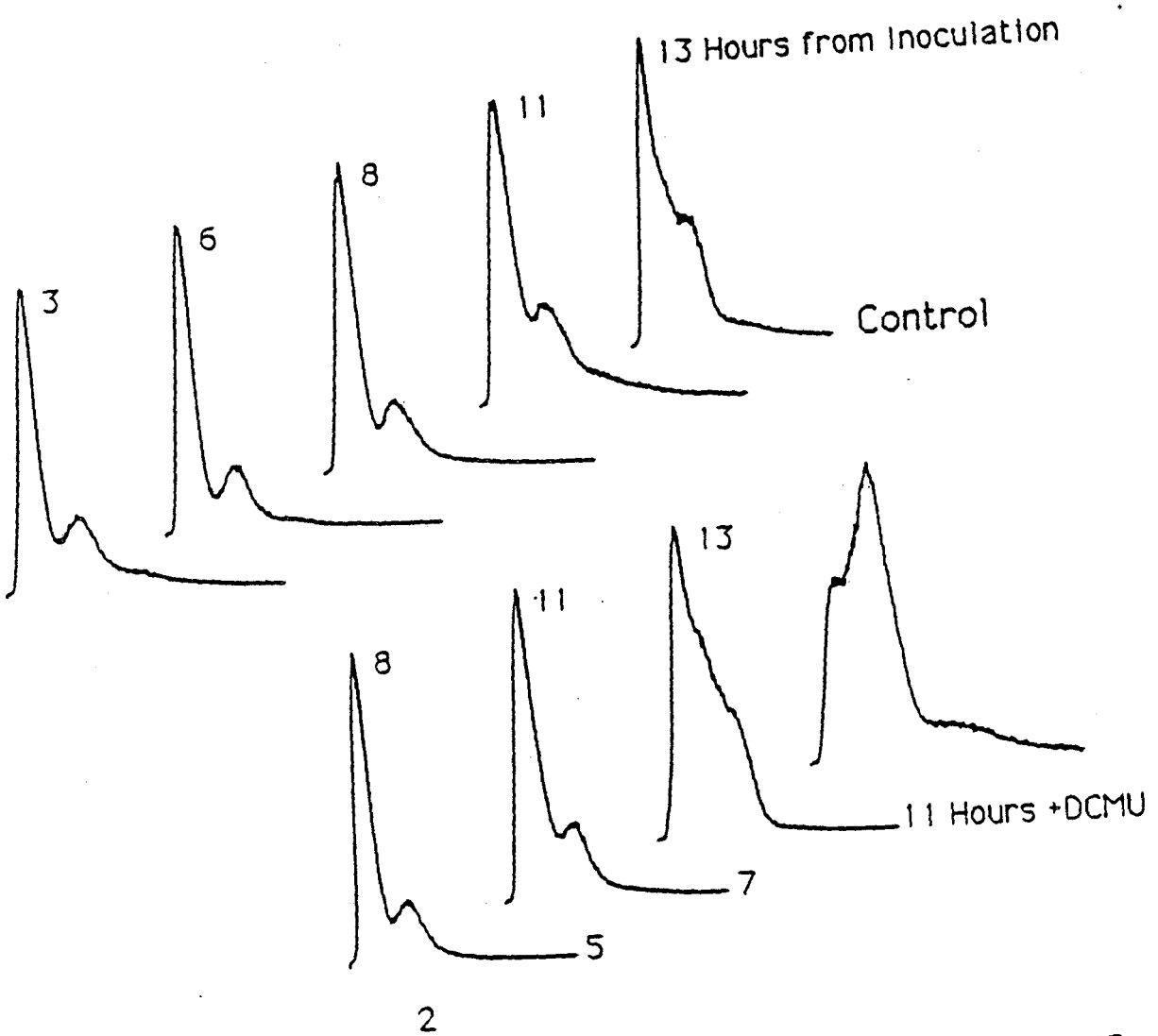

Phototrophically-grown cells, DCMU-treated
% Cells in S+G2
50
60
70
80
90
0
5
10
15
20
Time (Hours)
Photo. Control
Photo. DCMU

Fig. 3-4. Cells from a phototrophic culture were inoculated into duplicate foil-wrapped flasks containing heterotrophic medium to block photosynthesis while allowing the cells to grow. DCMU (10 uM) was added to one flask 6 hours after inoculation.

Fig. 3-5. A plot of the percentage of cells in S and G_2 from Fig. 3-4 against time shows that the rates of DNA synthesis and mitosis are decreased in the DCMU-treated culture.

Light-grown cells, DCMU- and Dark-treated



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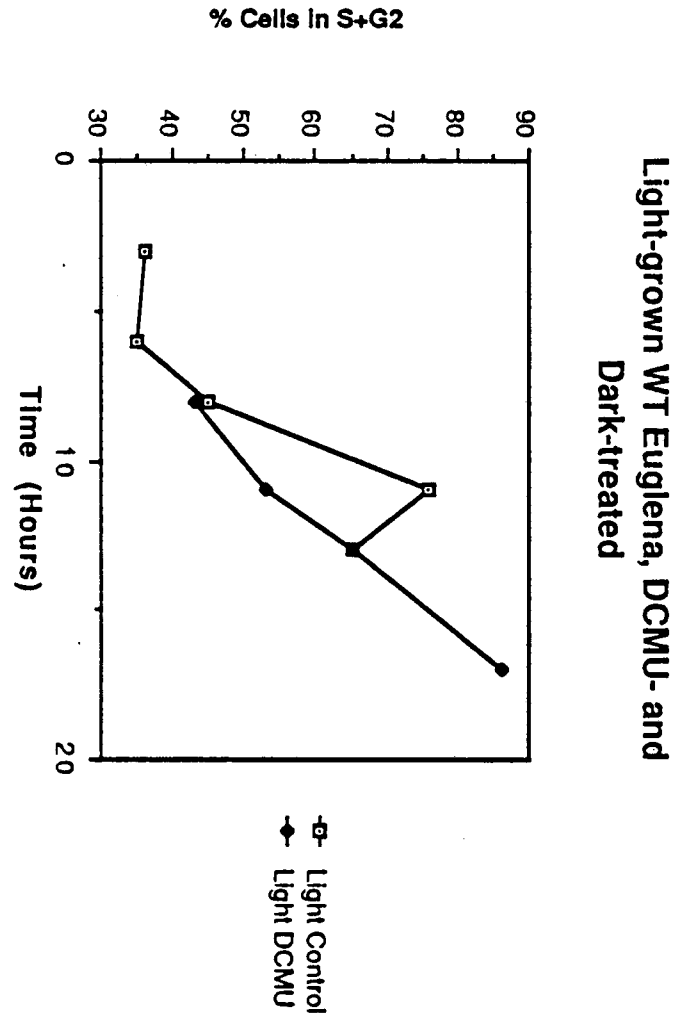

Light-grown WT Euglena, DCMU- and Dark-treated
% Cells in S+G2
30
40
50
60
70
80
90
0
10
20
Time (Hours)
Light Control
Light DCMU

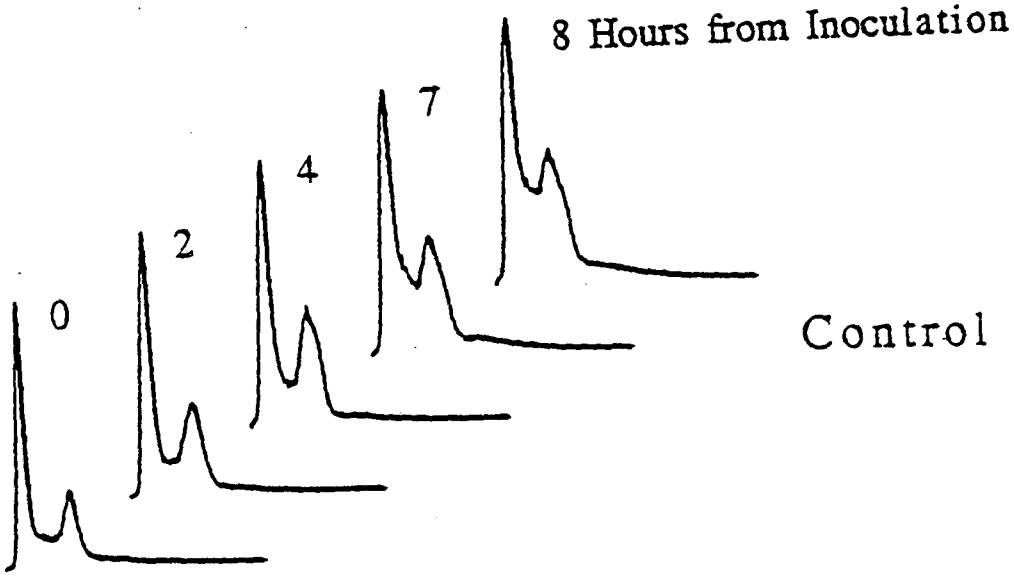
for W_{10}SmUL show that DCMU has no effect on the rate of DNA synthesis in this chloroplastless strain (Fig. 3-6, 3-7) . However, the DCMU-treated cells are found to accumulate in the G_2 phase instead of proceeding with mitosis. W_3BUL cells also show no inhibition of DNA synthesis at all by DCMU (Fig. 3-8), and accumulate in G_2. The G_2 accumulation is more easily seen in the FCM histogram (Fig. 3-9). The percentage of cells in the S and G_2 phases in the final timepoints is the same in control and DCMU-treated cells because the mutant cells immediately re-enter the cell cycle after completing one round of replication. The two samples differ because the control cells have more cells in S and the DCMU-treated cells have more cells in G_2.

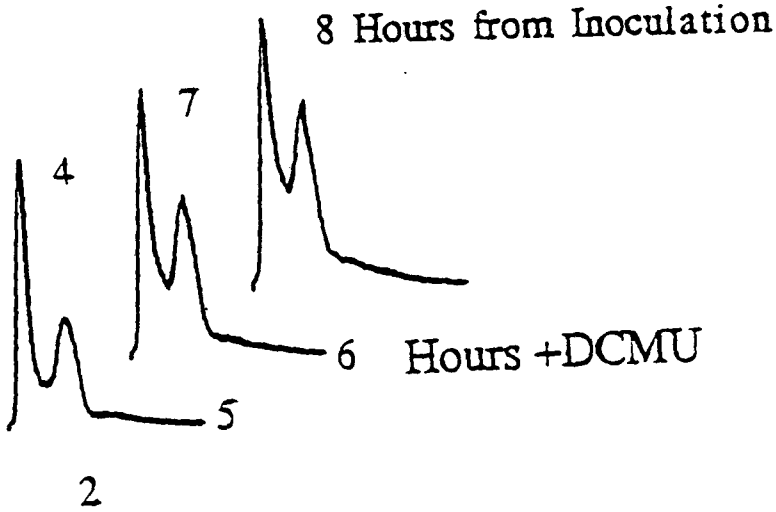
When grown in the dark under heterotrophic conditions, wild type *Euglena* rapidly loses chlorophyll and becomes non-photosynthetic. Such cells still contain chloroplast DNA, but no longer have mature chloroplasts. Dark-grown, non-green wild type cells were inoculated into duplicate flasks and allowed to grow for 6 hours to begin DNA synthesis. Then DCMU was added to one flask and samples were collected for FCM (Fig. 3-10). Comparison of the percentage of cells in S and G_2 in the control and DCMU-treated cultures shows that DNA synthesis is slower in the DCMU-treated culture, and the cells are again blocked in G_2 (Fig. 3-11). The block is not complete, since cells are seen to begin exiting G_2 by the last timepoint.

Fig. 3-6. Cells of the chloroplastless *Euglena* mutant W_{10}SmUL were inoculated into duplicate flasks containing heterotrophic medium. DCMU was added to one flask 2 hours after inoculation.

Fig. 3-7. A plot of the percentage of cells in S and G_2 in Fig. 3-6 are plotted against time. No change in the rate of DNA synthesis is seen for the DCMU-treated culture, but the cells are blocked in G_2.

W_{10}SmUL treated with DCMU





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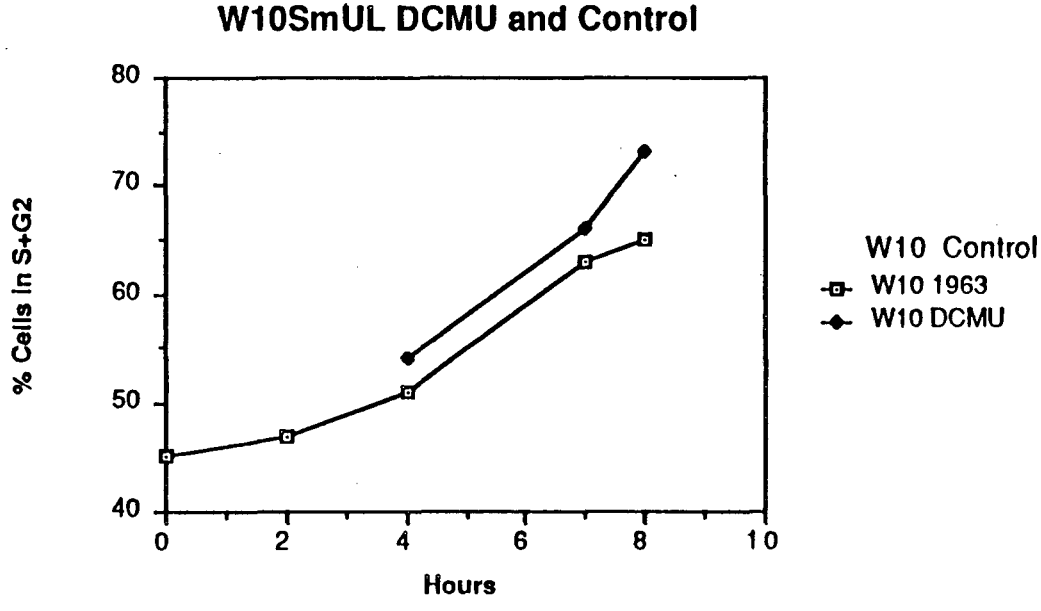
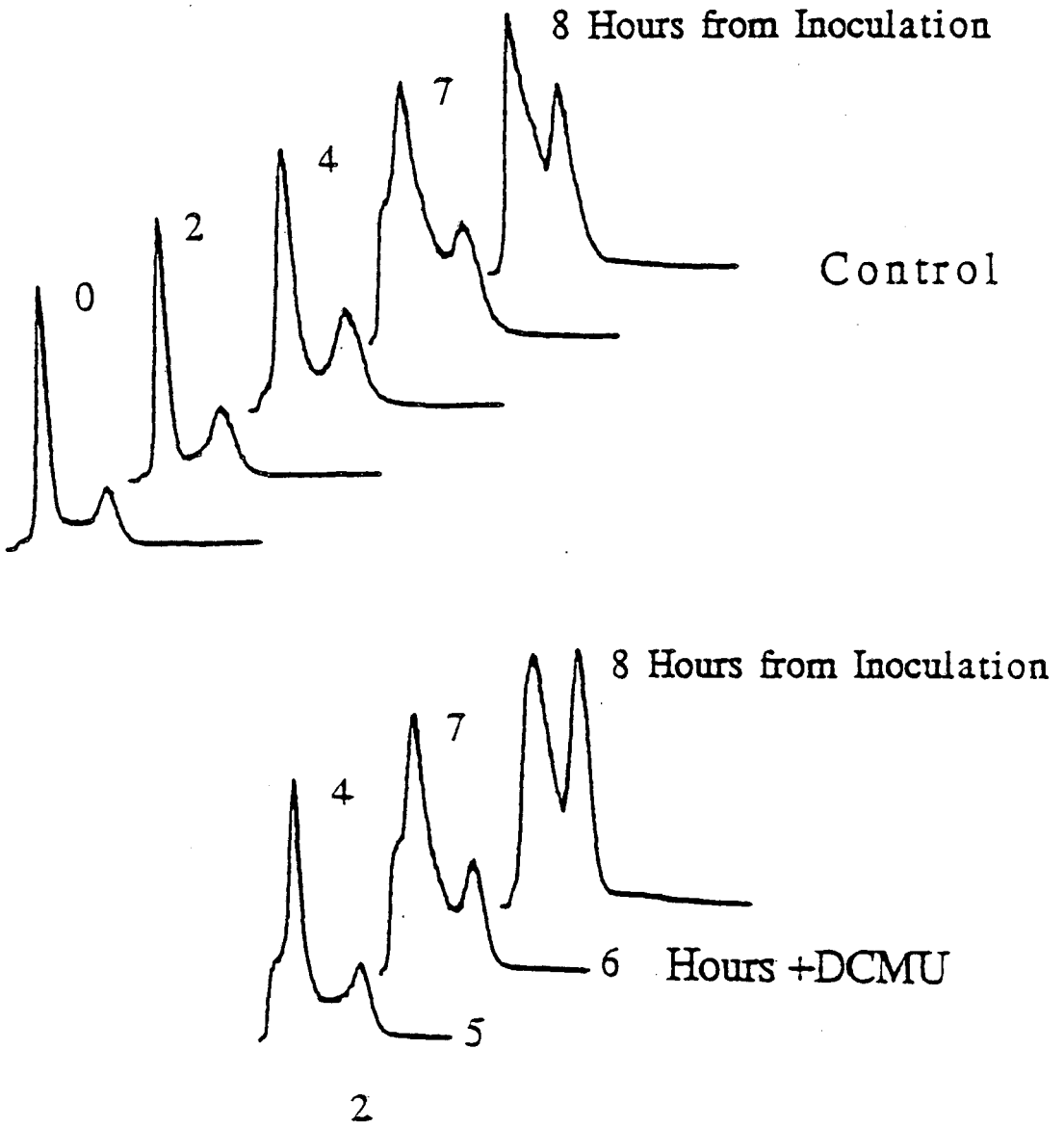

W10SmUL DCMU and Control
% Cells In S+G2
80
70
60
50
40
0
2
4
6
8
10
Hours
W10 Control
W10 1963
W10 DCMU

Fig. 3-8. Cells of the chloroplastless *Euglena* mutant W_3BUL were inoculated into duplicate flasks containing heterotrophic medium. DCMU (10uM) was added to one flask 2 hours after inoculation. No change in the rate of DNA synthesis is seen for the DCMU-treated culture.

Fig. 3-9. DNA histograms of the W_3BUL cultures show that the DCMU-treated culture is also blocked in the G_2 phase in comparison to the untreated culture.

W_3BuL treated with DCMU



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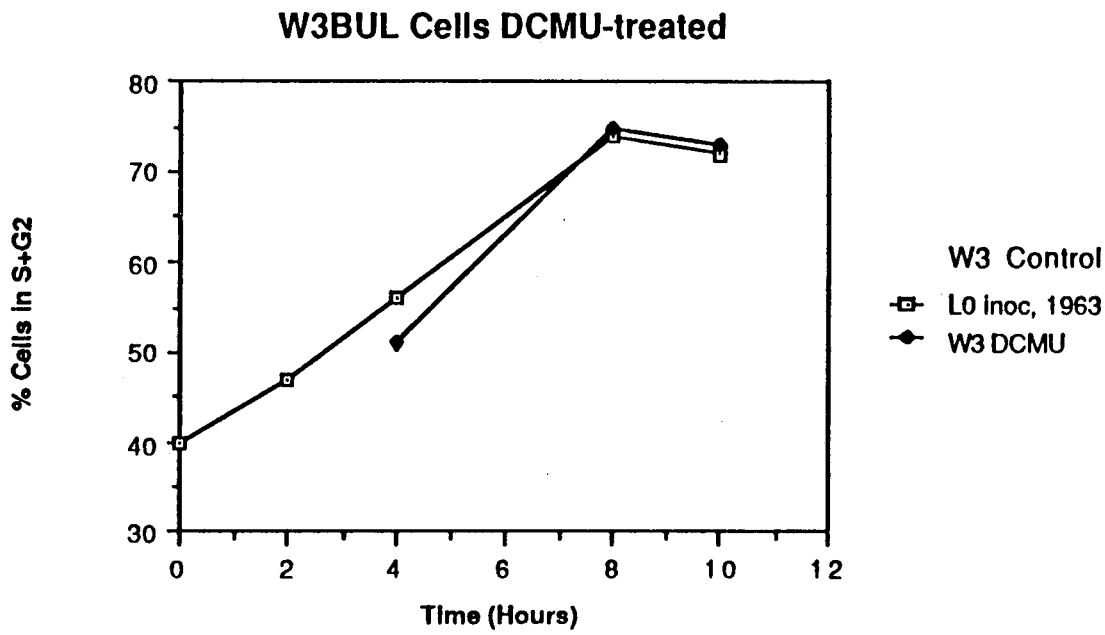
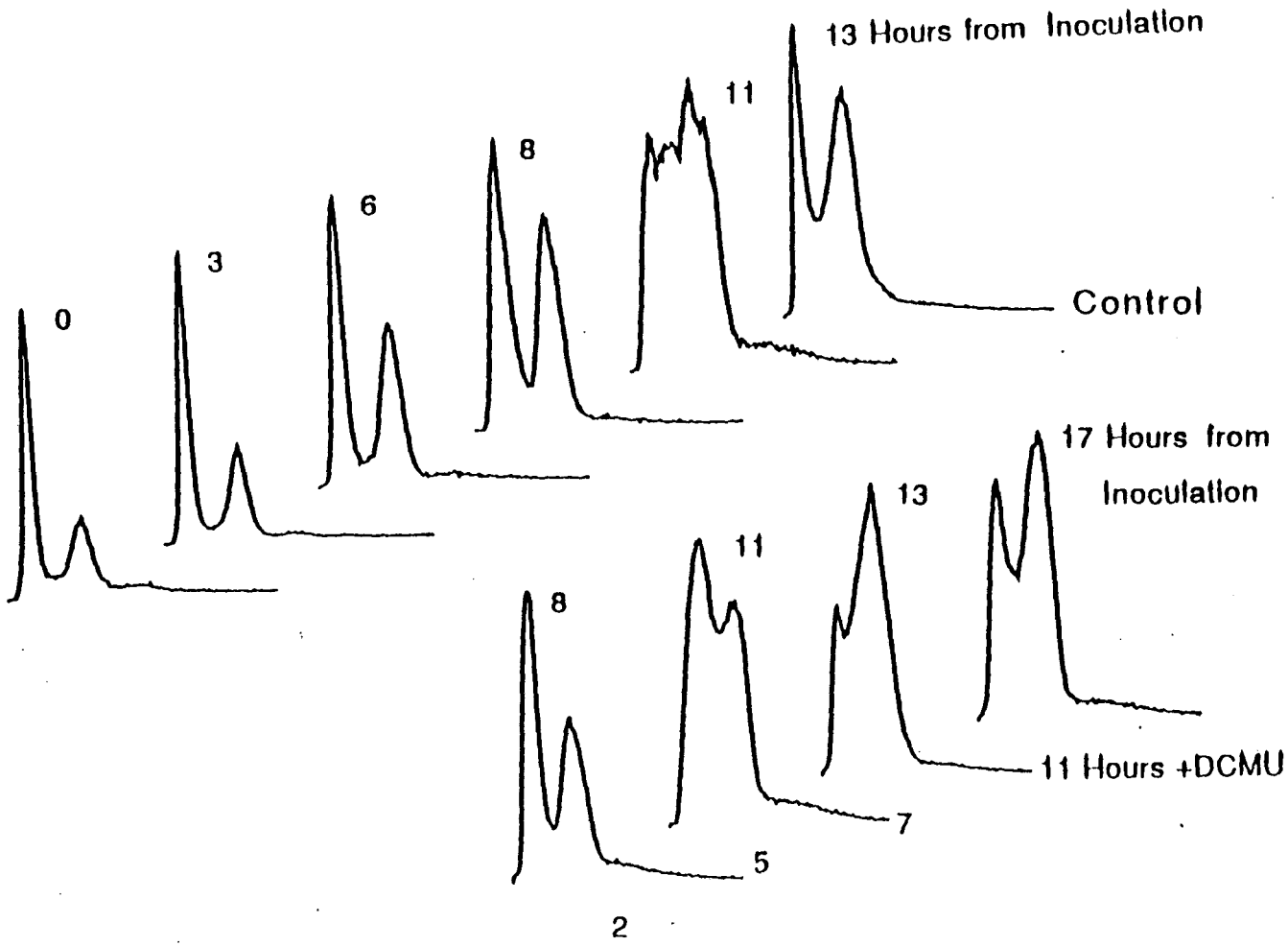

W3BUL Cells DCMU-treated
% Cells in S+G2
80
70
60
50
40
30
0
2
4
6
8
10
12
Time (Hours)
W3 Control
L0 inoc, 1963
W3 DCMU

Fig. 3-10. Dark-grown, non-green wild type *Euglena* was inoculated into duplicate foil-wrapped flasks containing heterotrophic medium. DCMU was added to one flask 6 hours after inoculation.

Fig. 3-11. The percentage of cells in S and G_2 in Fig. 3-10 are plotted against time. The rate of DNA synthesis is lower in the DCMU-treated culture, and the cells are partially blocked in G_2, though some are escaping the block by the last time point.

Dark-grown Cells DCMU- and Dark-treated



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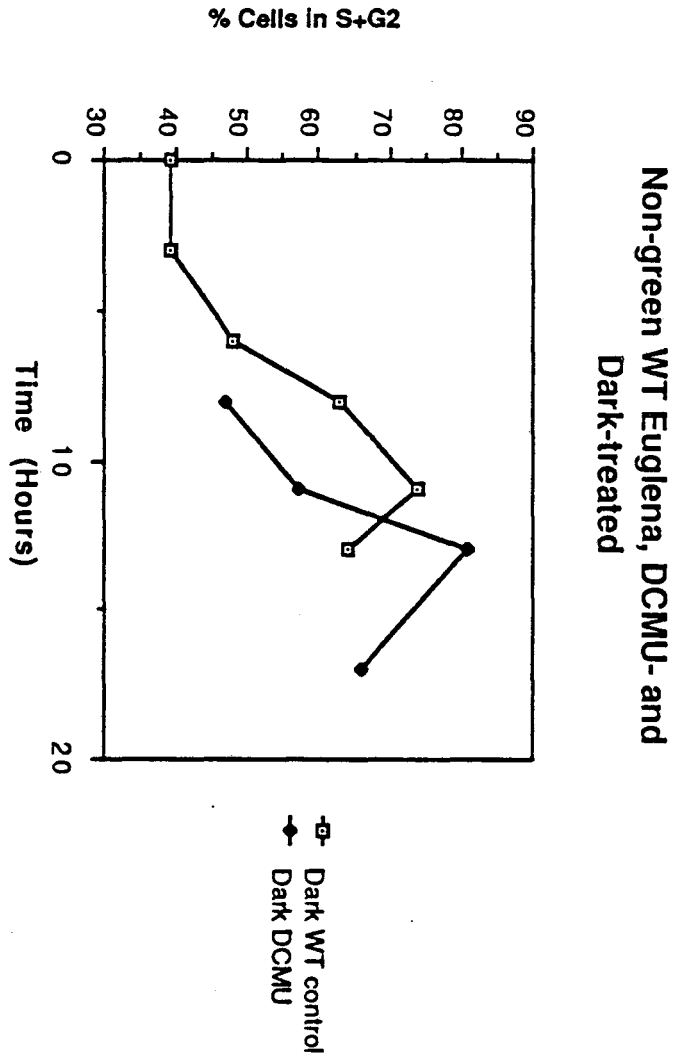
Non-green WT Euglena, DCMU- and Dark-treated
% Cells in S+G2
90
80
70
60
50
40
30
0
10
20
Time (Hours)
Dark WT control
Dark DCMU

Discussion

The Lifetime of the Cell Cycle Initiator

The brief dark incubations show that the cell cycle initiator has a half-life of 5 hours in darkness. If the factor continues to decay at that rate, after 12 hours of darkness, about 15% of the maximum level of initiator should still be in the cells. However, cells exposed to 12 hours of darkness need 6 hours of light exposure before beginning DNA synthesis again. This indicates that the amount of initiator left isn't enough, and that a higher minimal level is needed before the cells will enter the next round of replication.

The Effects of DCMU

Attempts to separate the effects of photosynthesis from the effects of light on the cell cycle initiator were blocked by the surprising effects of DCMU on the cell cycle in *Euglena.* The G_2 blockage seen in chloroplastless mutants and in dark-grown, non-photosynthesizing wild type cells shows that DCMU affects cytoplasmic and/or nuclear functions as well as photosynthesis in green cells.

DCMU has been used by other workers to identify photosynthesis-linked effects in *Euglena.* Monroy and Schwartzbach (1983) showed that addition of DCMU to *Euglena* exposed to light inhibited the accumulation of several polypeptides found in control cells. Addition of DCMU to dark-grown cultures caused no change in the polypeptide levels. This indicates that the cell cycle effects of DCMU observed in dark-grown *Euglena* are not due to inhibition of protein synthesis.

The presence of a small percentage of cells entering the cell cycle in both green and non-green wild type cells grown in dark heterotrophic conditions after DCMU treatment, in contrast to the stop in cell cycle entry in phototrophically-grown cells,

can be attributed to the availability of nutrients from the heterotrophic medium. This indicates that photosynthesis is needed to a small extent for commitment to DNA synthesis in phototrophic cultures. However, the strong retardation of DNA synthesis in heterotrophically-grown wild type cells is puzzling to see since there should be no need for chloroplast function under dark conditions.

Mutant cells are less sensitive than wild type cells to DCMU during DNA synthesis. This is not due to slower penetration of DCMU into mutant cells compared to wild type cells. An accumulation of cells in G_2 can been seen in W_{10} cells within two hours of DCMU treatment so the cells are rapidly affected. The major difference between wild type cells and the W_{10}SmUL and W_3BUL strains is the lack of chloroplasts or wild type proplastids in the mutant cells. Phototrophically-grown cells remain green throughout the first day of growth in dark, heterotrophic medium, so those cells still contain an active chloroplast. Dark-grown wild type cells contain a proplastid that begins maturation into a chloroplast within 30 minutes of illumination (Osafune et al. (1980)). W_{10}SmUL lacks any proplastid structures that are detectable by electron microscopy (Osafune and Schiff (1983)). W_3BUL contains a proplastid that undergoes very limited development in light (Osafune and Schiff (1980)). DCMU may be affecting a chloroplast or plastid function that is related to nuclear DNA synthesis.

Possible Targets of DCMU The existence of a DCMU effect on the cell cycle in *Euglena* suggests that two mutations may be required in order to give rise to a DCMU-resistant strain. A chloroplast mutation i the *psbA* coding region would be needed to alter the DCMU binding site in the Photosystem II reaction center, and a second muttion in an unknown locus would be needed to allow the cells to continue DNA synthesis and mitosis in the presence of DCMU. Since mutants lacking both a chloroplast and chloroplast DNA in *Euglena* are shown to be sensitive to DCMU, it

is likelythat the second mutation is located in the nuclear genome.

Conclusion

Euglena has been shown to possess a cell cycle initiating factor that accumulates in the light period during phototrophic growth. The stability of the initiating factor is 5 hours in the dark. Cellular levels of the factor are high enough to allow entry into the cell cycle after up to 6 hours of dark incubation. However, after 10-12 hours of darkness, levels of the factor are too low to enable DNA synthesis to begin and another incubation in light is needed before *Euglena* will undergo another round of replication.

Addition of DCMU causes a cessation in the increase of cells entering the S and G_2 phases and blocks mitosis in phototrophically-grown cells. In heterotrophically-grown cells, DCMU causes a retardation in the rate of cells entering S and G_2 and blocks mitosis, while in chloroplastless mutants DCMU leaves DNA synthesis unaffected while still blocking mitosis.

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Chapter IV

Initiation of the Cell Cycle in Wild Type and Mutant *Euglena*

Introduction

Cell treatments that block passage through the cell cycle, such as low pH, low serum, and high cell density, all yield cultures whose populations are predominantly in the G_1 peak of an FCM histogram. Such quiescent cultures are thought to present a model for non-dividing cells such as those found in differentiated and aging tissues. Much effort has been devoted to studying quiescent cells in order to see what physical differences they have to prevent them from entering the cell cycle.

One school of thought is that there is no difference internally between quiescent and proliferating cells. Quiescent cells merely lack external factors, such as growth hormones or nutrients, and are blocked from the cell cycle due only to lack of those ingredients. Many workers have shown that quiescent cells do carry on cell division, but at a much reduced rate compared to proliferating cells. When serum starved human fibroblast cells are incubated [^{3}H]-thymidine, flow sorting of the cells in the G_2 peak of the FCM histogram show that 5% of the cells have incorporated the label into their nuclear DNA (Dell'Orco et al). This shows that serum-starved cells still traverse the

cell cycle, but at a much reduced rate compared to proliferating cells. Smith and Martin have proposed a transition probability model to explain such results. Cells after mitosis have a probability of entering S that is environmentally dependent. Good growth conditions give a high probability, and poor growth conditions give a low probability (Fig. 4-1). Such a model does not require a G_0 state for quiescent cells. Instead, quiescent cells can be considered to be in prolonged G_1 state, in which all cells have the capacity for re-entering the cell cycle, but do not for lack of environmental factors. Support for this comes from 3T3, 3T6 and SV3T3 cells which have no kinetic lags that would indicate a traverse from the G_0 to G_1 states (Shields and Martin).

Other workers have shown that quiescent cells have a lowered RNA content (Darzynkiewicz et al.), reduced transcriptional activity (Rossini et al.), and increased calmodulin levels (Chafouleas et al). WI-38 human fibroblast cells become more difficult to revive when kept for prolonged periods in a quiescent state (Rossini et al). These results suggest that, at least in some cells, a separate G_0 phase exists and cells must exit that phase in order to re-enter the cell cycle (Fig. 4-2).

Zetterberg has proposed that the G_1 phase detectable by FCM be divided into three temporal regions: G_0, the quiescent state, G_1 pre-mitosis (G_{1pm}), which is the period before the restriction point when the cell decides to commit to the next round of cell division during which the cells are dependent on environmental factors such as serum, and G_1 pre-synthesis (G_{1ps}), which is the period after the restriction point (Zetterberg and Larsson). During the G_{1pm} period, the cells are sensitive to both external factors, such as serum, and internal activities, such as protein synthesis. Removal of an essential factor at this time prevents commitment to the cell cycle (Brooks, Zetterberg and Larsson). After the G_{1pm} period, cells are relatively insensitive to their environment and will proceed through the cell cycle in the absence of serum or under

Fig. 4-1. Transition probability model of entry into the cell cycle. Cells in a post-mitosis state (G_{1pm}) have a probability of entering the cell cycle that is dependent on environmental conditions such as nutrients levels. A low nutrient level will cause a low transition probability, so the number of cells going into the pre-DNA synthesis state (G_{1ps}) and entering the cell cycle is low. Conversely, a high nutrient level will give rise to a high transition probability.

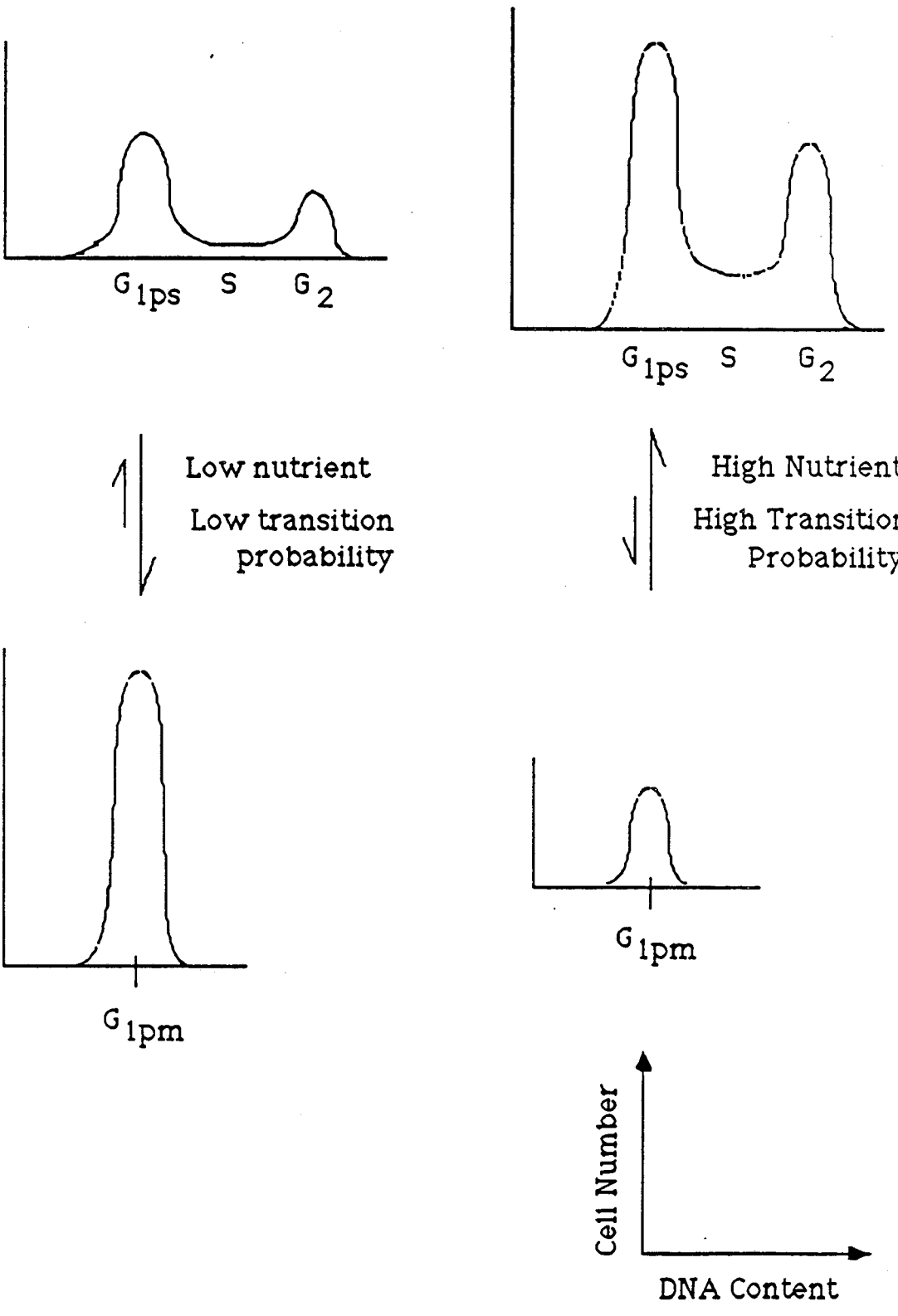
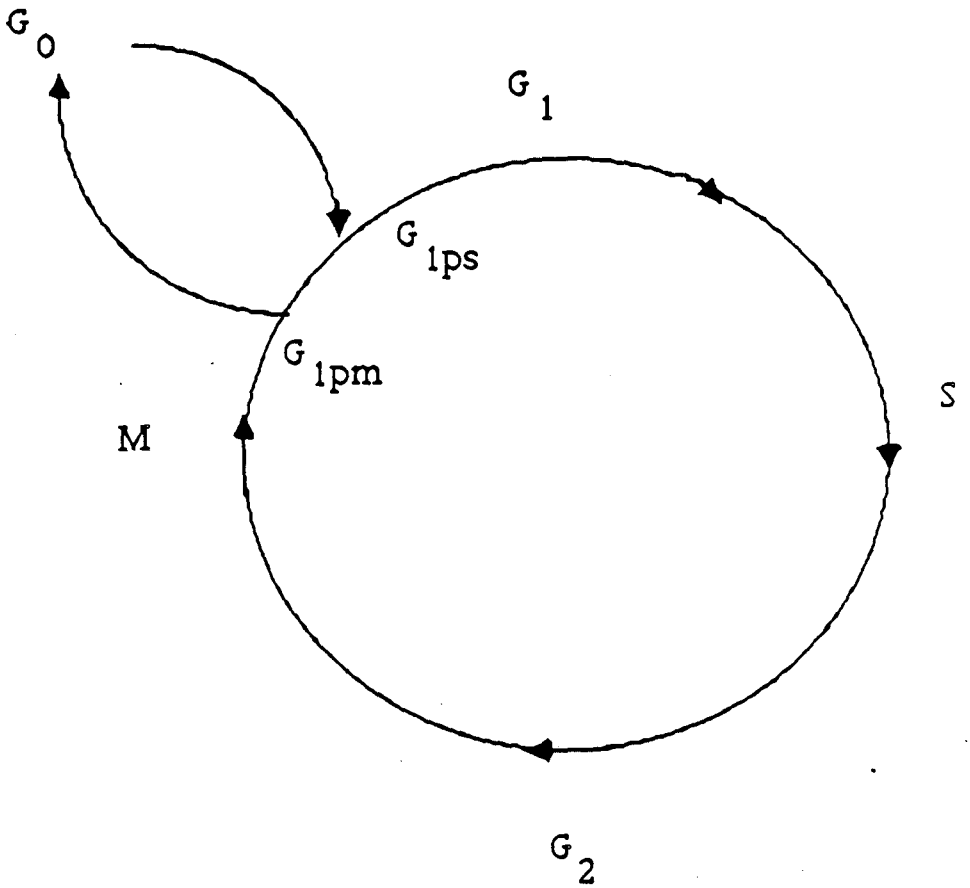

G1ps S G2
G1ps S G2
Low nutrient
Low transition probability
High Nutrient
High Transition Probability
G1pm
G1pm
Cell Number
DNA Content

Fig. 4-2. G_0 model of entry into the cell cycle. Quiescent cells are in a separate state called G_0. Treatment of quiescent cells with a cell cycle inducer will cause cells to traverse from G_0 to G_1 before entering the cell cycle. Quiescent cells in a G_0 state should take more time to begin the S phase than proliferative cells that have just finished mitosis.


G 0
G 1
G 1ps
G 1pm
S
M
G 2

partial inhibition of protein synthesis.

After cells have committed to entering the cell cycle, or, in Pardee's terminology, passed the restriction point (Pardee, 1974), the rapidity with which they begin the S phase is described as their transition probability (Smith and Martin). Under optimal growth conditions, the transition probability for an entire culture is high, while under starvation conditions it is low. Variation in the transition probability is seen within each culture. In phototrophic *Euglena*, this is exemplified by some cells which require only 6 hours of light to enter the S phase while other cells require 12 hours of light. Such variation arises due to differences in cell size or chloroplast number. Phototrophic growth represents the extreme of environmental dependence, since the cells absolutely require light for commitment to the cell cycle. The controlling points and kinetics uncovered under such circumstances represent the most sensitive response available from the cell. In contrast, heterotrophic growth allows continuous availability of all nutrients, so that the most rapid growth is obtained from the cell.

The regulation of the cell cycle in phototrophically grown *Euglena* presented in the previous chapter can be explained by light-dependent initiation of entry into the cell cycle. However, *Euglena* also grows under non-photosynthetic conditions in the dark, and non-photosynthetic, chloroplastless mutants of *Euglena* exist. Therefore, another mechanism for cell cycling must exist in these cultures. Comparison of cells grown under phototrophic and heterotrophic conditions allows determination of whether cell cycle controlling points are common to cells grown in both conditions, or whether certain phases or controls can be eliminated under optimal growth conditions.

Materials and Methods

Wild type *Euglena gracilis bacillaris* was grown in both phototrophic and heterotrophic conditions as described in the previous chapter. Mutant strains W_3BUL and W_{10}SmUL were grown aseptically in 125 ml flasks containing 50 ml of Euglena Broth (Difco). Cells were grown both in light and in foil-wrapped flasks. Sample collection, cell fixing and staining, and flow cytometry were performed as previously described.

Results and Discussion

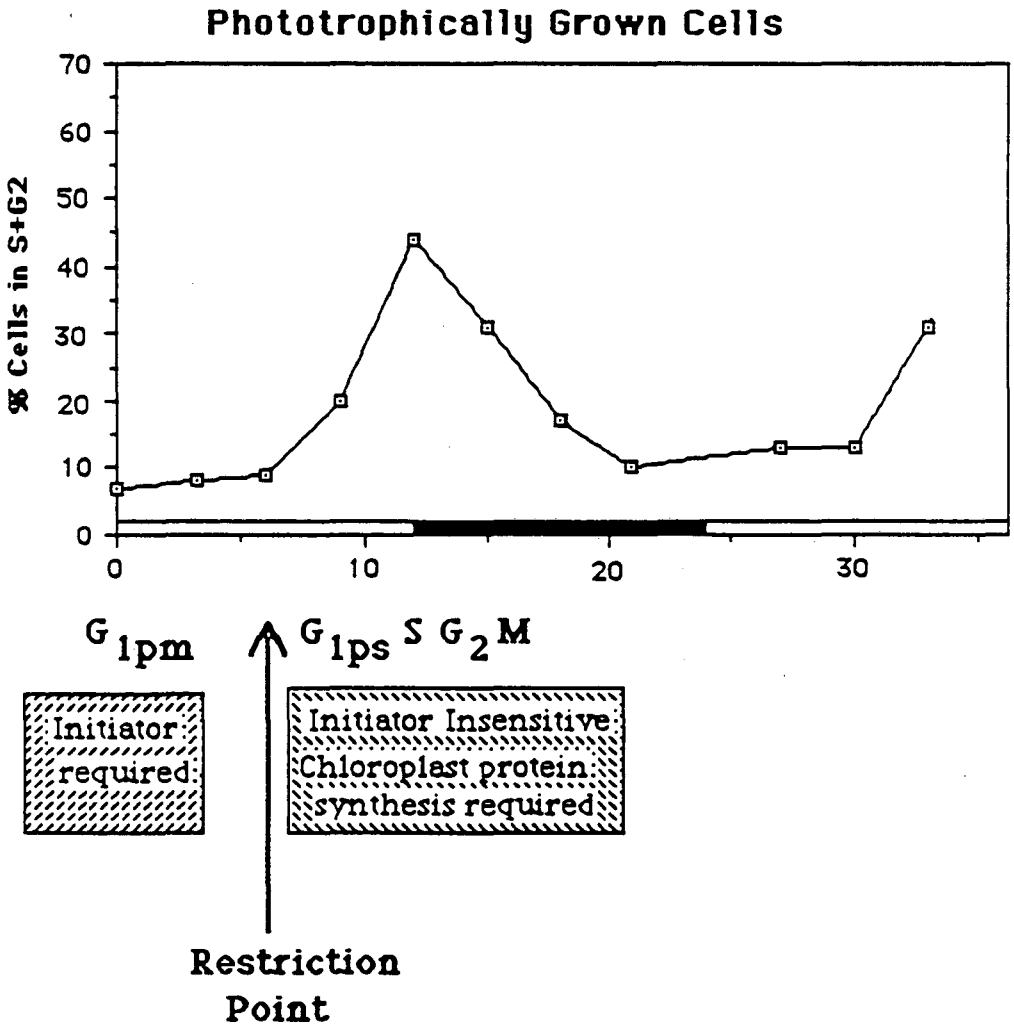
Does Euglena *have a G_0 phase?*

The percentage of cells in the S and G_2 phases from phototrophically grown cells can be compared against a cell cycle "time line" (Fig. 4-3). The first 6 hours of light exposure after inoculation show no cell cycle activity and continued light exposure is needed for entry into the cell cycle, so this period corresponds to the G_{1pm} phase in Zetterberg's terminology. After the sixth hour, the transition probability of cells entering the S phase increases with increasing light exposure, so this is the G_{1ps} and S periods. The rest of the cell cycle, the remainder of S, G_2 and M, is completed in the dark. No movement outside of G_1 is seen until the next light period.

On the second day after inoculation, a 6 hour lag period is again seen before entry of cells into the S phase. This shows that the cells are going through a G_{1pm} phase again before commitment to another round of cell division. The length of this period is the same as that of the first day. No extra lag for a G_0 to G_1 transition is detected. The time required for wild type *Euglena* to go from stationary phase into active cell cycling is the same as for active cells to commit to another round of cell cycling, i.e., the cells are constantly in a state of readiness for entry into the cell cycle whenever environmental conditions allow. Such an ability to stay poised at the G_{1pm} phase accounts for the rapid growth in nature seen in algal blooms in ponds and streams when phosphate levels are suddenly increased.

Heterotrophic media supplies more nutrients than phototrophic media, so a high level of nutrients is always available. FCM histograms of heterotrophically dark-grown wild type cells show a synchronized wave of cells that enter the S phase 6 hours after inoculation (Fig. 4-4). Cells continue to enter the S and G_2 phases for several hours longer than seen in phototrophic cultures exposed to 12 hours of light.

Fig. 4-3. Plot of percentage of phototrophically grown cells in the S and G_2 phases versus time. The parts of the cell cycle and the controlling points are plotted along the time axis.


Phototrophically Grown Cells
% Cells in S+G2
70
60
50
40
30
20
10
0
0
10
20
30
G1pm
G1ps S G2 M
Initiator required
Initiator Insensitive Chloroplast protein synthesis required
Restriction Point

This is expected, since it has been shown that *Euglena gracilis bacillaris* require 16 to 18 hours of light exposure for doubling of the cell number in a phototrophic culture (Cook and James).

The 6 hour lag seen after inoculation in heterotrophically grown wild type cultures indicates that a G_{1pm} period still needs to be traversed when stationary phase cells are newly diluted. The length of the lag is the same as that seen in phototrophically grown cells, so dark-grown wild type cells are also poised at the G_{1pm} phase and not in a G_0 phase. This indicates that the controlling element at this point is a cytoplasmic or nuclear activity, and not a photosynthetic activity.

Mutant cells may not be able to sustain themselves in a G_{1pm} state for very long. We have never observed a short, 6 hour lag followed by entry into the cell cycle in newly inoculated cultures of W_3BUL or W_{10}SmUL cells. These cells display a very long, greater than 24 hour, lag period before entry into the cell cycle (Fig. 4-5). The very long lag period may indicate a true G_0 phase in mutant cells.

Is a chloroplast needed for G_{1pm}?

Both light- and dark-grown *Euglena* can enter the cell cycle after a 6 hour lag from the time of inoculation, while mutant cells show a much longer delay. An immediate question is whether the chloroplast is involved in the ability of wild type cells to be prepared to enter the cell cycle more readily than chloroplast-less mutants.

A strong argument for the case that there is no chloroplast involvement in passage through the cell cycle during dark growth comes from study of tobacco suspension cells (Heinhorst et al). Such cells, grown in the dark in Murashige-Skoog medium, can carry out nuclear DNA synthesis independently of chloroplast activity. Addition of chloramphenicol, an inhibitor of chloroplast protein synthesis has no effect on the

Fig. 4-4. Plot of the percentage of heterotrophic, dark-grown, wild type cells in the S and G_2 phases versus time.

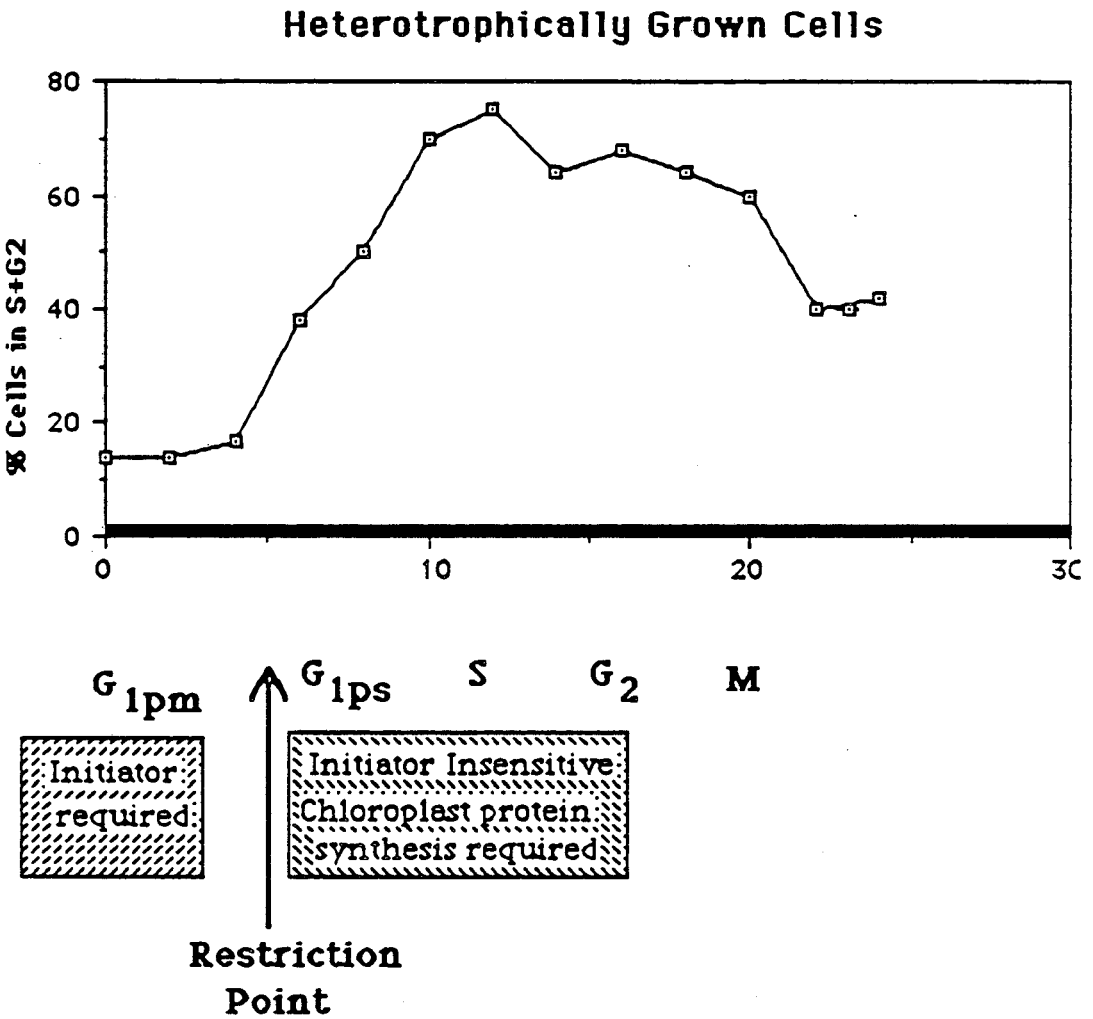
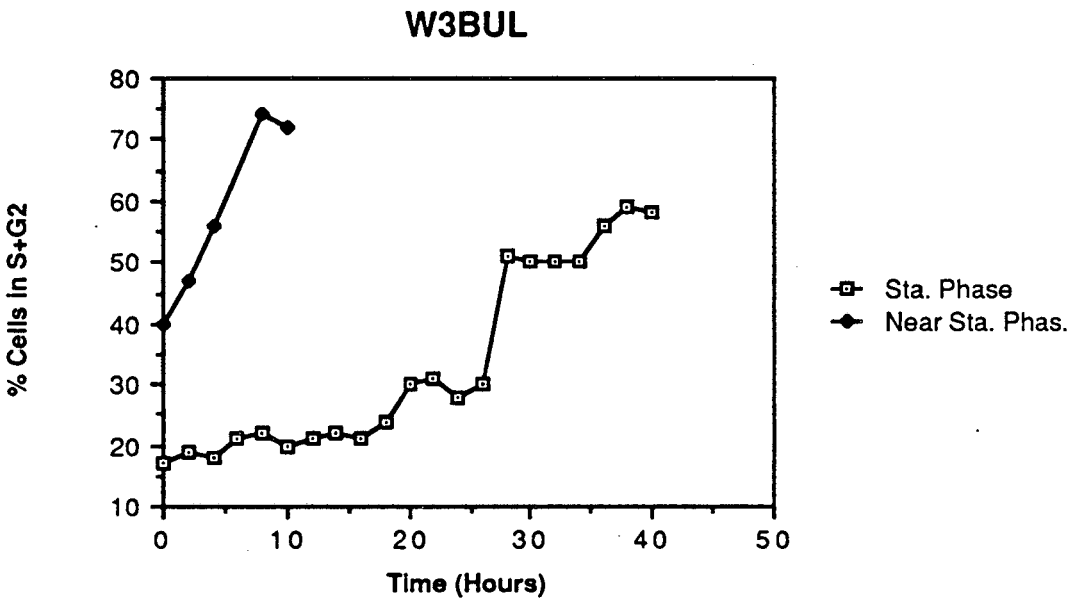
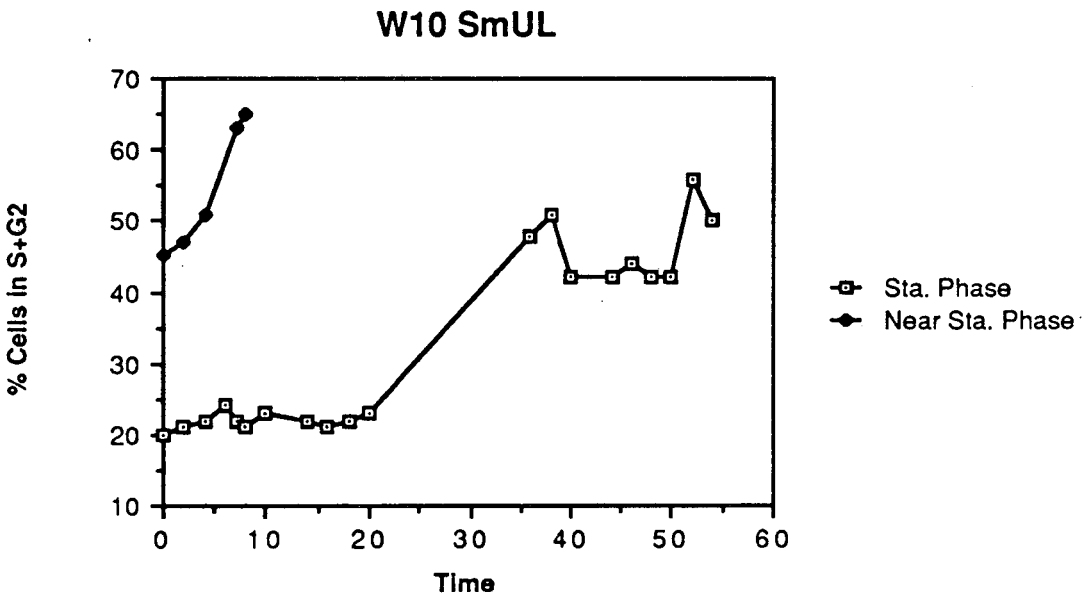

Heterotrophically Grown Cells
% Cells in S+G2
80
60
40
20
0
0
10
20
30
G1pm
G1ps
S
G2
M
Initiator required
Initiator Insensitive
Chloroplast protein synthesis required
Restriction Point

Fig. 4-5a. Plot of the percentage of heterotrophically grown mutant W_3BUL cells in the S and G_2 phases versus time.

Fig. 4-5b. Plot of the percentage of heterotrophically grown mutant W_{10}SmUL cells in the S and G_2 phases versus time. Timepoints from 24th to 36th hour were not collected.


W3BUL
% Cells in S+G2
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Time (Hours)
Sta. Phase
Near Sta. Phas.


W10 SmUL
% Cells in S+G2
70
60
50
40
30
20
10
0
10
20
30
40
50
60
Time
Sta. Phase
Near Sta. Phase

rate of incorporation of [^{3}H]-thymidine into nuclear DNA. Addition of cycloheximide, an inhibitor of nuclear protein synthesis, causes immediate repression of nuclear DNA synthesis followed by a delayed repression of chloroplast DNA synthesis. This shows that a functional chloroplast is not essential for entry into the S phase during heterotrophic growth.

The *Euglena* mutant W_3BUL (Osafune and Schiff, 1980a) was generated by uv irradiation of wild type cells and selection of bleached colonies, so pleiotrophic effects in nuclear genes are not unexpected. The mutant W_{10}SmUL (Osafune and Schiff, 1980b) was generated by growing wild type cells in streptomycin, which should inhibit only chloroplast protein synthesis and eventually give rise to daughter cells that are lacking a chloroplast. However, loss of protective pigments such as chlorophyll and carotenoids followed by growth in the light can cause damage to nuclear DNA as well. Such damage might be the cause of the long delay before entry of newly diluted W_3BUL and W_{10}SmUL cells into the cell cycle.

Is there a G_2 Controlling Point?

Studies of cell cycle mutants in the fission yeast *Schizosaccharomyces pombe* have shown that there are two cell cycle controlling points, one at the G_1 to S transition, and one at the G_2 to M transition (Simanis and Nurse). The same protein kinase, *cdc2* has been found to initiate both transitions. Since *Euglena* clearly has a G_1 controlling point for commitment to the cell cycle, it would be interesting to see if *Euglena* also has a G_2 controlling point.

Nuclear DNA synthesis under photoautotrophic conditions is vitamin B_{12} dependent (Bré et al., Lefort-Tran et al). Cells starved of vitamin B_{12} have a varying DNA content, showing repression of DNA synthesis during the S phase; starved cells that have completed DNA replication are then blocked in mitosis. No G_1 cells are

observed during vitamin B_{12} starvation, however, so initiation of DNA synthesis still occurs. Cells deprived of vitamin B_{12} and grown in continuous light undergo cell division inhibition after 6 generations, when intracellular vitamin sources are depleted. When vitamin B_{12} deprived cells are grown in an alternating light-dark cycle, cell division inhibition occurs less rapidly (after about nine generations). Growth in darkness with lactate as a carbon source shows no inhibition. The authors have concluded that in vitamin B_{12} starved cells, photosynthetic activity inhibits cell division. While a chloroplast factor may be needed for initiation of the cell cycle (Blamire), a vitamin B_{12} mediated activity is needed for completion of the cell cycle. Presumably, photosynthesis also needs vitamin B_{12} and competes against cell division under conditions of vitamin B_{12} starvation. Since vitamin B_{12} starved cells are found to be blocked all through the S phase, a vitamin B_{12}-dependent step is probably not the G_2 controlling point.

During all of our experiments with varying the light exposure, no cells were found to be arrested in G_2. The results presented in the previous chapter show that all cells that initiate the cell cycle complete it, and completion can be performed in the dark. If there is a G_2 controlling point in *Euglena* it will probably not be found without the aid of cell cycle mutants since the G_2 to M transition is light independent.

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Chapter V

Circadian Cycle and Cell Cycle Regulation of RNA Accumulation in *Euglena gracilis*

Introduction

A circadian behavior is one that can be entrained by a light-dark program and that can persist in continuous darkness or continuous light with a free-running period that is close to 24 hours. Circadian rhythms in activity and temperature change have long been described in many eukaryotic systems ranging from humans (Minors and Waterhouse (986)) to the unicellular photosynthetic alga *Chlamydomonas reinhardtii* (Bruce (970)). The imposition of periodicity by light has been shown to be the regulator of circadian cycles. Transfer of an entrained organism to a new light-dark regime causes the circadian rhythms to be reset (for review see Takahashi and Zatz (1982)). The photoreceptor responsible for this entrainment has been shown to be located in the retina in mammals.

The molecular basis for these rhythms that are coupled to the day-night cycle have only recently begun to be addressed (for review see Feldman (1982)). Genetic analysis of the *per* locus in *Drosophila* and the *frq* locus in *Neurospora* have shown that these genes control circadian behaviors, though they may not be the pacemakers

of the cycle. Mapping of circadian behaviors to specific genes implies that the cyclic activity is a function of gene expression, either at the RNA or the protein level.

The evident light dependence of a circadian rhythm naturally makes it of interest for plant biologists to investigate the role such a cycle may play in the molecular response of photosynthetic organisms to light. Many groups have examined the changes in RNA levels as dark-grown seedlings or etiolated plants are exposed to light (Link (982), Thompson et al. (1983), Tobin (1981)). A program of RNA accumulation stimulated by phytochrome has been suggested as the mechanism for driving these changes. However, these are not the normal conditions under which photosynthetic organisms grow. Several workers have recently shown that plants grown in a light-dark regime display a circadian cycle of RNA accumulation (Kloppstech (1985), Piechulla and Gruissem (1987)). The onset of increase in RNA levels has been shown to occur before a phytochrome-elicited response would be expected, suggesting that other light or metabolic receptors may be involved in the circadian response.

Klein and Mullet (1987) have shown that RNA levels of several chloroplast genes decrease with increasing plant age in barley seedlings. This correlates with a decrease in the growth rate of chloroplast number and chloroplast size as the number of chloroplasts per cell and chloroplast volume approach that found in mature leaves. Their results suggest that RNA accumulation may also be related to cell cycle activity in the organelle.

We have investigated circadian accumulation of a nuclear-encoded and two chloroplast-encoded photosynthetic genes inactively dividing (log phase) and non-dividing (stationary phase) cultures of *Euglena gracilis*. Because *Euglena* is a unicellular photosynthetic organism, we can follow RNA accumulation without interference from a developmental program. We can monitor the cultures to determine whether the cells

are passing through the cell cycle or not. In this way, it is possible to distinguish a circadian regulated program of RNA accumulation from a cell cycle regulated program.

Materials and Methods

Growth of Cells

Euglena gracilis bacillaris was grown at 23°C in photoautotrophic medium (Hallick et al.) with 5% CO_2 bubbled and stirred into the medium. Cells were grown either in an alternating 12 hour light-12 hour dark cycle or under continuous light. Cells were also maintained in the dark in foil-wrapped flasks containing Euglena Broth (Difco), which allows the cells to carry out non-photosynthetic growth. Continuous light grown cells were transferred from a light-dark grown culture and maintained for 3 days in continuous light, then transferred to fresh medium before RNA samples were collected.

Synchrony of cells was determined by flow cytometry as previously described (Yee and Bartholomew (1988)).

RNA Isolation

All plasticware and glassware used in RNA steps was first treated with diethylpyrocarbonate (DEP) to inactivate RNAses. All solutions were autoclaved 60-90 minutes before use. RNA isolation was by the method described in*Current Protocols in Molecular Biology* (Ausubel et al. (1987). Approximately 10^8 cells were spun in the SA600 rotor, 10,000 rpm, 5 minutes. Cells were resuspended in 0.5 ml RNA extraction buffer (TLE) for each 40 ml of original volume. TLE buffer is Tris (pH 8.2) (0.2M), LiCL (0.1 M), EDTA (5 mM). 50 ul 20% SDS were added and the cells vortexed vigorously. The lysate was extracted with TLE equilibrated phenol:chloroform:isoamyl alcohol (25:24:1) 3 times until no interface was seen. The aqueous layer was then extracted once with $CHCL_3$:isoamyl alcohol, equal volume of 4M LiCl was added, and the RNA was precipitated overnight at 4°C. A soft, white RNA pellet was obtained

after spinning in an Eppendorf centrifuge at 4°C. The pellet was resuspended in 200 ul DEP-treated water, 20 ul NaOAc (3 M) was added and the RNA reprecipitated in 2.5 volumes EtOH for 1 hour at -20°C. The RNA was pelleted and resuspended again in 200 ul water for measurement of absorbance at 260 and 280 nm.

RNA gels and Northern blots were performed according to the methods of Schloss et al. Unless otherwise indicated, 5 ug of RNA was loaded into each lane. For staining and photography, gels were washed for 5 min. each in 6 changes of distilled water. Gels were then stained for 20-30 min. in 5 ug/ml ethidium bromide, 0.5 M NH_4OAc, and destained overnight in distilled water.

Cloned Probes

Two plasmids containing cloned genes were used as the chloroplast probes. pEZC.514 contains the Euglena *psbA* gene which codes for D1, the Photosystem II reaction center herbicide-binding protein, and also codes for $tRNA^{leu}$ (Keller and Stutz, 1984). pCR34.1 contains the 3' region of the *Chlamydomonas* ribulose 1,5-bisphosphate carboxylase large subunit (*rbcL*) (Stiegler et al.(1982)) Both plasmids were obtained from the lab of Prof. Richard Hallick. A plasmid containing the gene for β-tubulin β37 from *Chlamydomonas reinhardtii* was used as a non-photosynthetic negative control for light regulated expression (Schloss et al (1984)). The plasmid contains the 3' end of the β-tubulin gene and was obtained from the lab of Dr. Joel Rosenbaum.

Plasmid DNA was isolated by the method of Maniatis et al. except that 3% Triton-X100 was used instead of 10% SDS to lyse cells. Just before phenol:chloroform extraction, cell lysates were treated with 0.1 mg/ml RNAse A, 15 min, room temperature, then with 0.1 mg/ml pronase, 30 min, 37°C.

Synthesis of Oligonucleotides

Oligonucleotides for use as probes were made on the Applied Biosystems DNA Synthesizer 381A. DNA was extracted from the cyanomethylphosphoramadite column by washing with 1.5 mls NH_4OH. The DNA in NH_4OH were incubated in a glass vial with Teflon cap at 55°C for 8-12 hours. The samples were divided into 3 Eppendorf vials each, dried down, DNA pellets rinsed briefly with 70% EtOH (no vortexing), dried again, and resuspended in 1-3 mls 0.1 M triethylammonium bicarbonate (TEAB). The DNA was then diluted 1:200 in TE buffer and absorption spectra taken.

Short oligonucleotides resulting from premature termination were removed from full length oligos on a preparative 20% polyacrylamide gel, 40 watts, 3 hours. Oligos were visualized by ultraviolet shadowing and the full length product cut out from the gel. Oligos were extracted from the gel by overnight incubation in 0.5 M NaCl at 37°C. The extracted oligo was further purified by passage through a Sep-pak column (Millipore).

Preparation of Radioactive Probes

DNA from the plasmids coding for chloroplast genes, pEZC.514 and pCR34, were labelled by nick translation with α-[^{32}P]-dATP (400 Ci/mmol) as described by Maniatis. Probes were labelled to 3-5 x 10^7 cpm/ug.

The β-tubulin cDNA clone β37 was removed from its pBR322 vector by restriction digest with Pst I and purified by isotachoelectrophoresis as described by Ofverstedt et al. (1984). The β-tubulin fragment was labelled by using the large fragment of DNA polymerase with the Amersham random priming kit and α[^{32}P]-dCTP (3000 Ci/mmol) following the instructions of the manufacturer. Probes were labelled to 5-10 x 10^8 cpm/ug.

The RuBisco small subunit 35-mer was labeled using polynucleotide kinase and γ-[^{32}P]-ATP (5000 Ci/mmol) as described by Maniatis. Preparative acrylamide gel

electrophoresis followed by Cerenkov counting of the isolated kinased oligo showed that the DNA was labelled to 1-5 x 10^8 cpm/ug.

Filter Hybridization

For hybridization with cloned probes, Northern blots were prehybridized in 0.1 ml/cm^2 of 50% formamide, 5XSSC, 10X Denhardt's, 50 mM $NaPO_4$ (pH 6.8), and 100 ug/ml salmon sperm DNA at 42°C for at least two hours. SSC is 0.15 M NaCl, 0.015 M trisodium citrate. 100X Denhardt's is 2% bovine serum albumin (Sigma, Fraction V), 2% Ficoll, 2% polyvinylpyrrolidone.

Double stranded probes were denatured with 1/10 volume 3 N NaOH for 5 minutes, neutralized with 1/5 volume 1 M Tris (pH 7.0) and 1/10 volume 3 N HCl, and added to prehybridized filters at 2-5 x 10^5 cpm/ml of hybridization solution. Filters were allowed to hybridize at 42°C overnight. Filters were washed in 2XSSC, 0.1% SDS at 42°C, 10 minutes twice. If high background was detected by Geiger Counter, filters were washed further in 0.2XSSC, 0.1% SDS at 42°C for 10 minutes and at 55°C for 10 minutes.

For hybridization with the oligonucleotide probe, Northern blots were prehybridized in 0.1 ml/cm^2 6XSSPE, 10X Denhardt's, 1 mM ATP, and 100 ug/ml salmon sperm DNA at 42°C for at least 2 hours. 10 SSPE is 1.8 M NaCl, 100 mM $NaPO_4$ (pH 7.7), 10 mM EDTA. Kinased oligonucleotide probe was added at 10 pm/ml and allowed to hybridized at 42°C for at least 6 hours. Filters were washed in 6XSSPE, 0.1% SDS at room temperature for 10 minutes, then at 42°C for 10 minutes and finally at 55°C for 10 minutes. All filters were exposed to X-ray film with intensifying screens (Dupont) at -70°C overnight.

Densitometry

Due to the range of hybridization intensities obtained from log phase, stationary phase and continuous light Northerns, several exposures of each Northern were made. First, exposures of each Northern within the linear response range of the X-ray film were made for densitometry traces. Traces were performed on a Hoefer (San Francisco) GS300 Transmittance/Reflectance Scanning Densitometer. Next, exposures of all Northerns hybridized to the same probe were made onto the same film, and relative intensities were determined. The densitometry readings of each Northern were scaled by factors to reflect the relative intensities of one Northern versus another.

Results

Oligonucleotide Probe for the Small Subunit

The *Euglena* small subunit polypeptide of ribulose 1,5-bisphosphate carboxylase has been sequenced (Sailland et al (1986)). Several regions of homology exist between the *Euglena* protein and those of higher plants and cyanobacteria. The region from amino acid 78 to 83 was chosen as a promising site of highly conserved sequence. Comparison of the DNA sequence of this region from *Lemna* (Stiekema, et al. (1983)), pea (Bedbrook et al. (1980), Coruzzi et al. (1983)), soybean (Berry-Lowe et al. (1982)), tobacco, (Pinck et al. (1983)), and petunia (Dunsmuir et al. (1983)) showed a high level of similarity, with little variation in codon usage (Fig. 5-1a).

A 35 base oligonucleotide (SSu 35) containing a deduced coding sequence from amino acids 74 to 85 was made (Fig. 5-1b). Three-way degeneracies in the higher plant sequences were replaced by inosine in the oligo sequence. Two-way degeneracies were retained in the oligo sequence so that a mix of 16 sequences was generated. The oligo is specific for the small subunit gene. Test hybridization of SSu35 to dot blots of a LHCP clone and a pea small subunit cDNA clone showed specific hybridization to the pea small subunit plasmid (data not shown).

During light synchronized growth, the levels of rRNA species in photosynthetic algae increase linearly during the light period (Wilson and Chiang (1977)). Previous experiments and these Northerns have demonstrated that all the rRNA species increase at the same rate, so that the ratio of each rRNA to total RNA stays constant (Fig. 5-2a). This increase of rRNA is presumably the result of preparation for cell division. Since rRNAs comprise 95% of the total RNA in the cell, the Northern data shown here represent essentially equal quantities of rRNA in each lane, and the changes in specific mRNA levels represent changes relative to rRNA.

Fig. 5-1a Alignment of cloned sequences of the small subunit of ribulose 1,6-bisphosphate carboxylase (*rbcS*) with the amino acid sequence from *Euglena*. The region is highly conserved, with only one amino acid difference between the *Euglena* and higher plant sequences.

Fig. 5-1b. Sequence of the 35-base oligonucleotide (SSu 35) used as a probe for *Euglena rbcS* mRNA. The 4 degeneracies and 2 inosines in the sequence were used at positions that are not conserved in the plant sequences.

RuBisco Small Subunit Sequences

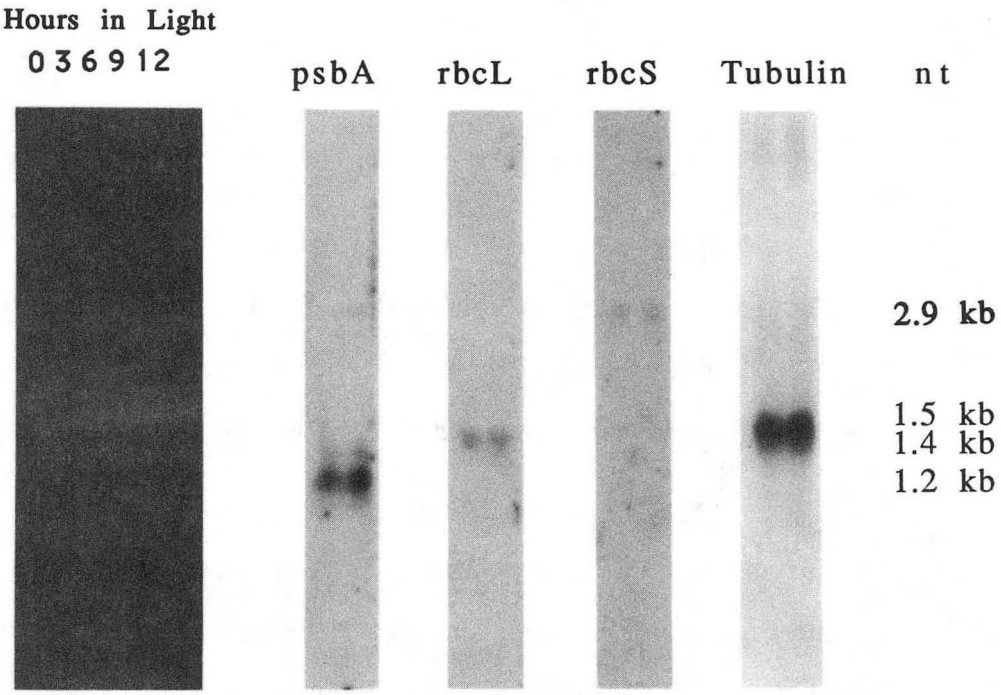
Euglena Amino Acid		**Tyr**	**Tyr**	**Asp**	**Asn**	**Arg**	**Tyr**	**Trp**	**Thr**	**Met**	**Trp**	**Lys**	**Leu**	
Petunia DNA	5'	TAC	TAT	GAT	GGA	AGA	TAC	TGG	ACA C	ATG	TGG	AAG	CTT G	3'
Lemna DNA	5'	TAC	TAC	GAC	GGG	AGG	TAC	TGG	ACG	ATG	TGG	AAG	CTG	3'
Pea DNA	5'	TAC	TAT	GAT	GGA	AGA	TAC	TGG	ACA	ATG	TGG	AAG	CTT	3'
Soybean DNA	5'	TAC	TAT	GAT	GGA	CGC	TAC	TGG	ACA C	ATG	TGG	AAG	CTT G	3'
Tobacco DNA	5'	TAC	TAT	GAT	GGC	AGA	TAC	TGG	ACC	ATG	TGG	AAG	CTA	3'
SSu Oligo Probe	3'	ATG	ATG A	CTG A	TTG A	GTI	ATG	ACC	TGE	TAC	ACC	TTC	GA	5'

Fig. 5-2A. Ethidium bromide-stained gel of total DNA from 5 timepoints throughout a light period. The fluorescence intensity of the rRNA bands stay the same for all timepoints, showing that the rRNA species change at the same relative rates during the light period.

Fig. 5-2B. Total RNA isolated from one timepoint from logarithmic phase cells was run on a gel (5 ug/lane) and blotted to nitrocellulose. Two lanes each were probed with clones for *psbA, rbcL* or β-tubulin, or with the SSu 35 oligonucleotide. Hybridization conditions are as described in *Material and Methods*.

A. Ethidium bromide stained gel

B. Test filter hybridizations



XBB 881-192

Test Hybridizations

RNA extracted from a phototrophically-grown log phase culture was run in duplicate lanes on a 1% formaldehyde gel. After transfer to nitrocellulose, each set of duplicate lanes was allowed to hybridize with one of the four probes — pEZC.514, pCR.34, β-37 or SSu 35 —in order to determine the optimal hybridization and wash conditions (Fig. 5-2b).

Accumulation of Chloroplast Encoded Photosynthetic RNAs

Two chloroplast encoded photosynthetic genes were used to study message accumulation in cycling (logarithmic phase) and non-cycling (stationary phase) cells. pEZC.514 contains an Eco RI fragment of *Euglena* chloroplast DNA that encodes the *psbA* gene (Reardon and Price (1984), Keller and Stutz(1984)). In light-dark synchronized, logarithmically growing (log phase) cells, the mRNA for *psbA* is seen to accumulate to a maximum in the first half of the light period (Fig. 5-3). Message levels in the mid-light period are 50% higher than at the beginning or the end of the light period (Fig. 5-4). Light-dark synchronized stationary phase cells show a much smaller accumulation of *psbA* mRNA, with a maximum increase of 10% in the middle of the light period. The message level in stationary phase cells is approximately half that of the logarithmic phase cells. Cells grown in continuous light also show an increase in *psbA* message, though the levels are not as high as those seen in log phase cells.

A plasmid containing the 3' end of the *rbcL* gene from *Chlamydomonas* was used as a second chloroplast probe (Fig. 5-5). Gingrich and Hallick (1985) have shown that there is about 80% homology between the *Chlamydomonas* and *Euglena* genes. The *Chlamydomonas* gene has been used to identify the *Euglena* gene (Stiegler et al (1982)). Densitometry scans of probed Northerns again show an increase in message

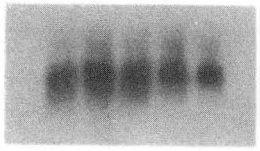
Fig. 5-3. *psbA* mRNA steady state levels determined by hybridization. Total RNA (5 ug/lane) from light/dark synchronized logarithmic phase and stationary phase cells, and from continuous light-grown cells was probed with the plasmid pEZC.514.

Fig. 5-4. Comparison of changes in *psbA* levels among the three cultures based on densitometry scanning of the Northerns probed with pEZC.514. Each point is the average of three hybridizations. Relative levels were determined by intensity of the signal on the exposed X-ray film, units are arbitrary.

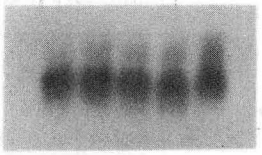
Photosystem II Reaction Center Hybridization

Hours in Light
0 3 6 9 12

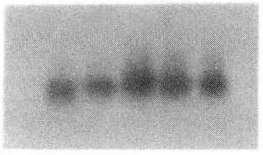
Logarithmic Phase



Stationary Phase



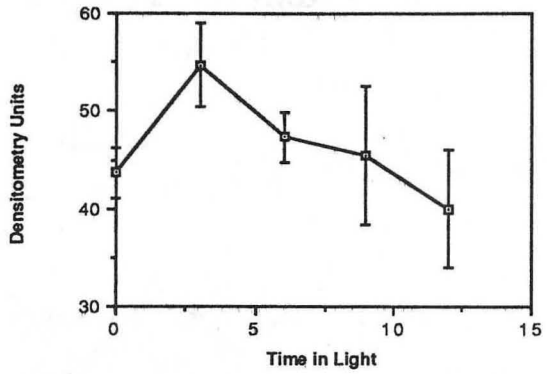
Continuous Light-grown



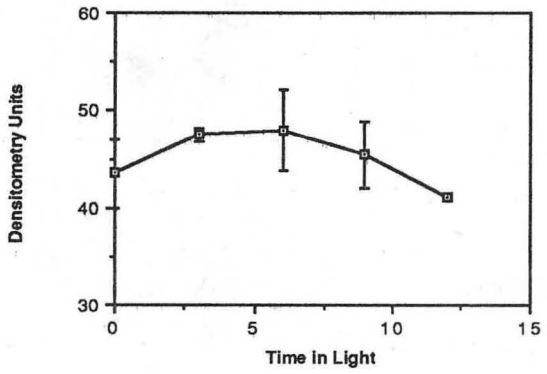
XBB 881-198

psbA Hybridization

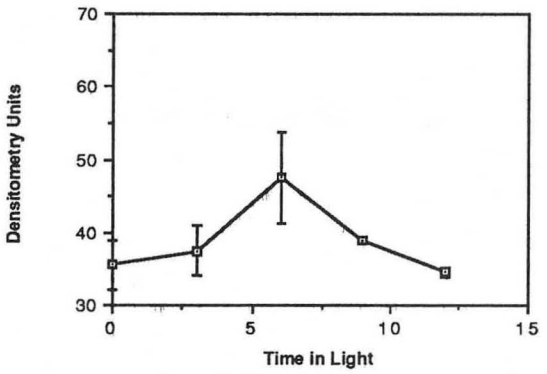
Logarithmic Phase Cells Hybridization



Stationary Phase Cells Hybridization



Continuous Light-grown Cells Hybridization



levels in log phase cells in the first half of the light period (Fig. 5-6). The maximum message level is 100% higher than at the beginning or the end of the light period, and the message level drops off much more sharply than that of *psbA*. The peak of accumulation in stationary phase cells occurs three hours later than that of the logarithmic phase cells, and the maximum accumulation is much less than that of the logarithmic phase cells. Cells grown in continuous light show very little fluctuation in the level of *rbcL* mRNA.

Accumulation of a Nuclear-encoded Photosynthetic Gene

The SSu 35 probe gave strong hybridization to log phase and stationary phase Northerns (Fig. 5-7). Densitometry scans show that *rbcS* message levels have only small increases during the light period, with log phase cultures again increasing to a maximum before stationary phase cultures (Fig. 5-8). Message levels for both log phase and stationary phase cultures increase about 30%, and the levels drop off slowly throughout the course of the light period in contrast to the sharp decrease seen for the chloroplast-encoded photosynthetic genes.

Comparison with a non-photosynthetic gene

Levels of mRNA for β-tubulin were examined in the three culture conditions described above to see how messages for a nuclear-encoded, non-photosynthetic gene varied in comparison with the chloroplast-encoded genes. Tubulin mRNA has been shown to be regulated by the cell cycle in the photosynthetic alga *Chlamydomonas reinhardtii* (Ares and Howell (1982)).

Probing of Northern blots with β-tubulin shows that tubulin mRNA accumulates to a maximum in the early light period of logarithmically growing *Euglena* (Fig. 5-9). Continuous light-grown cells also display an increase in the mid-light period. This

Fig. 5-5. *rbcL* mRNA steady state levels determined by hybridization. Total RNA (5 ug/lane) from light/dark synchronized logarithmic phase and stationary phase cells, and from continuous light-grown cells was probed with the plasmid pCR34.1.

Fig. 5-6. Comparison of changes in *rbcL* levels among the three cultures based on densitometry scanning of the Northerns probed with pCR34.1. Each point is the average of three hybridizations.

RuBisco Large Subunit Hybridization

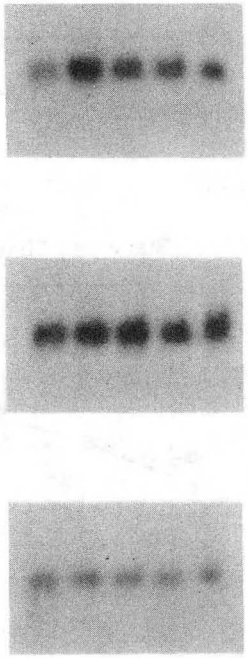
Hours in Light

0 3 6 9 12

Logarithmic Phase

Stationary Phase

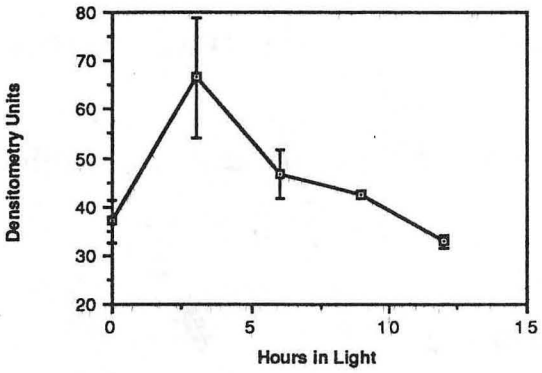
Continuous Light-grown



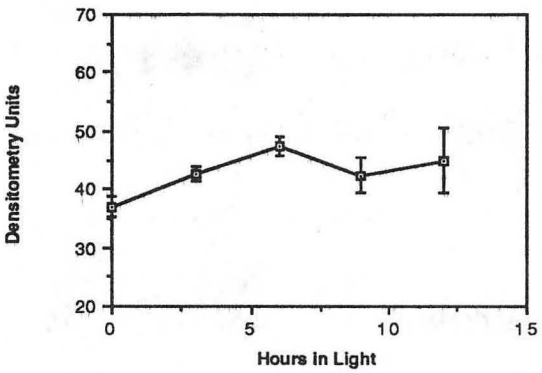
XBB 881-197

rbcL Hybridization

Logarithmic Phase Cells Hybridization



Stationary Phase Cells Hybridization



Continuous Light-grown Cells Hybridization

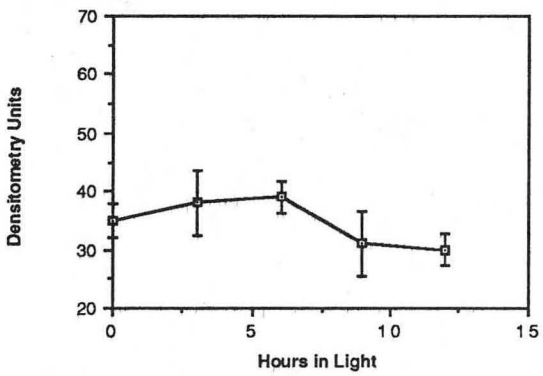
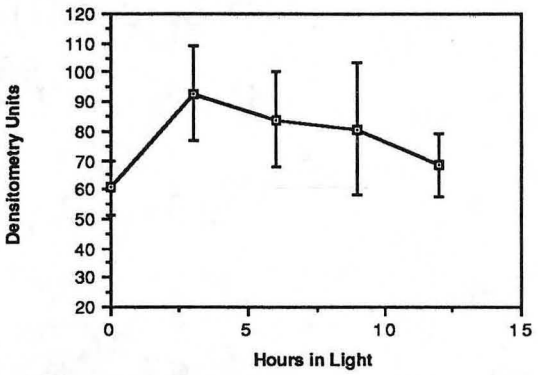


Fig. 5-7. *rbcS* mRNA steady state levels determined by hybridization. Total RNA (5 ug/lane) from light/dark synchronized logarithmic phase and stationary phase cells, and from continuous light-grown cells was probed with the SSu 35 oligonucleotide. **Fig. 5-8.** Comparison of changes in *rbcS* levels among the three cultures based on densitometry scanning of the Northerns probed with SSu 35. Each point is the average of three hybridizations.

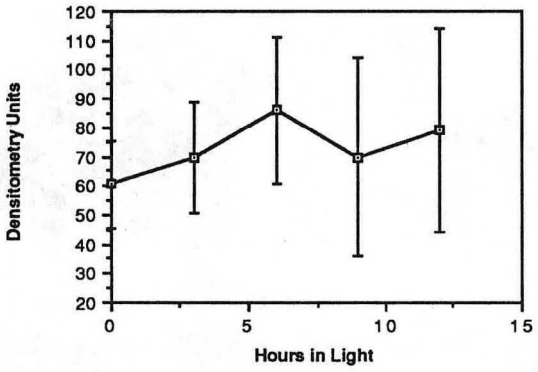
indicates that despite growth for several days in continuous light, *Euglena* still retains some synchronous cell cycle behavior. Densitometry scans show that tubulin message levels are relatively constant in the stationary phase culture (Fig. 5-10).

rbcS Hybridization

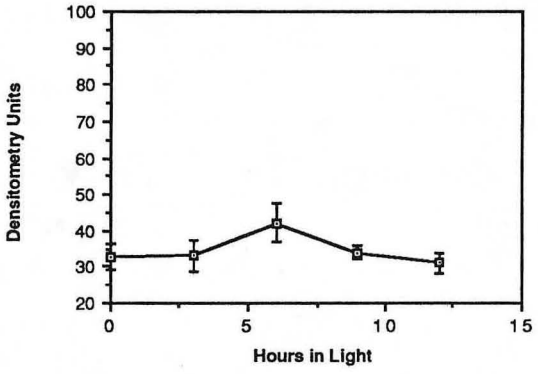
Logarithmic Phase Cells Hybridization



Stationary Phase Cells Hybridization



Continuous Light-grown Cells Hybridization

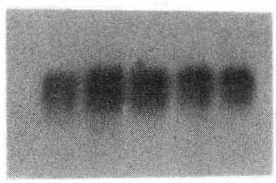


RuBisco Small Subunit Hybridization

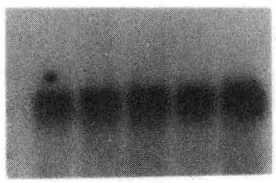
Hours in Light

0 3 6 9 12

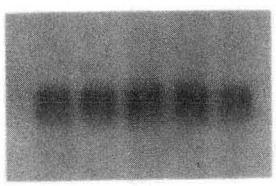
Logarithmic Phase



Stationary Phase



Continuous Light-grown



XBB 881-193

Fig. 5-9. β-tubulin mRNA steady state levels determined by hybridization. Total RNA (5 ug/lane) from light/dark synchronized logarithmic phase and stationary phase cells, and from continuous light-grown cells was probed with the β-37 plasmid.

Fig. 5-10. Comparison of changes in β-tubulin levels among the three cultures based on densitometry scanning of the Northerns probed with β-37. Each point is the average of three hybridizations.

Tubulin Hybridization

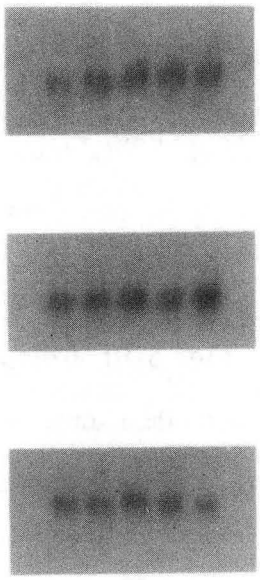
Hours in Light

0 3 6 9 12

Logarithmic Phase

Stationary Phase

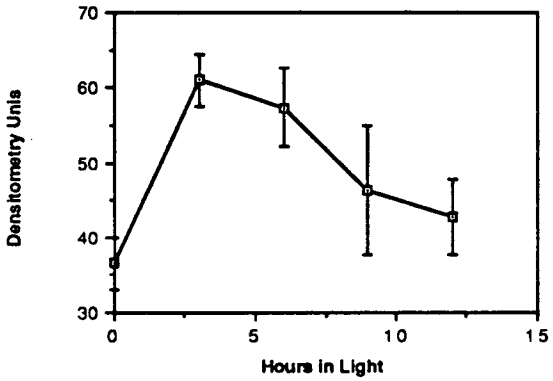
Continuous Light-grown



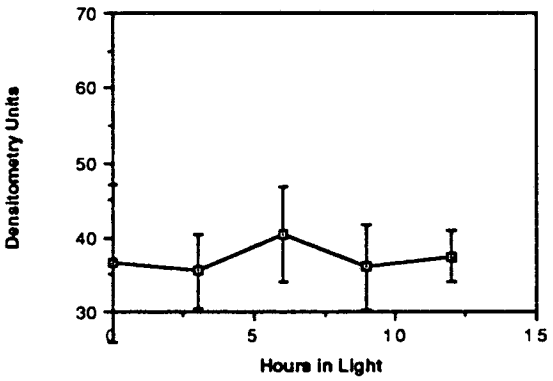
XBB 881-196

Tubulin Hybridization

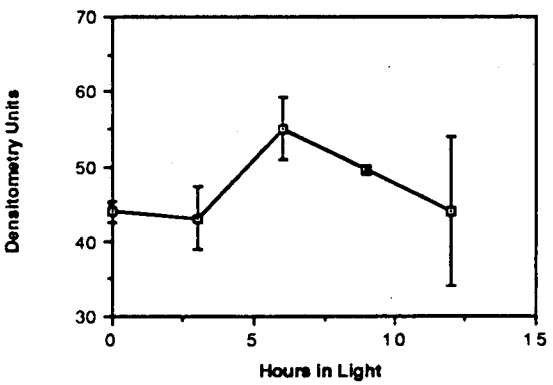
Logarithmic Phase Cells Hybridization



Stationary Phase Cells Hybridization



Continuous Light-grown Cells Hybridization



Discussion

β-Tubulin RNA in *Chlamydomonas* has been shown to be accumulated in a cell cycle dependent fashion (Ares and Howell (1982). These results show that in *Euglena* β-tubulin RNA is accumulated in log phase cells and shows a free-running rhythm of accumulation in continuous light-grown cells. As expected, it does not display significant changes in steady state RNA levels in stationary phase cells. The small amount of change seen may be due to a low number of cells in the culture that are still going through the cell cycle. We have taken the amount of variation seen in stationary phase Northerns probed with β-tubulin as a baseline in the analysis of RNA accumulation of other genes.

Comparison of RNA accumulation levels for the two chloroplast-encoded photosynthetic genes show that steady state levels of both RNA species are increased in the first half of the light period in log phase cells over stationary phase cells. However, the free-running rhythm of accumulation for *psbA* is much longer than for*rbcL*. A strong circadian cycle of *psbA* RNA accumulation is still evident after 3days of growth in continuous light while *rbcL* accumulation is dampened and shows less variation than stationary phase cells. This shows that two different programs for RNA regulation exist in the chloroplast so that *rbcL* accumulation is more sensitive to the cell cycle state of the organism than *psbA* accumulation.

rbcS RNA accumulation is seen to have a diurnal variation in both log phase and stationary phase cells. The free-running circadian cycle is as short as that of *rbcL* RNA accumulation. However, the nuclear-encoded subunit is not as sensitive to the cell cycle state as the chloroplast-encoded subunit since *rbcS* RNA accumulation is still seen in stationary phase cells.

These results indicate that there is a circadian pacemaker in *Euglena* that controls

RNA accumulation. Steady state levels of RNAs display a diurnal pattern during log phase growth under light-dark conditions. The free-running circadian behavior varies from gene to gene and seem uncorrelated to the genome in which it is encoded. Both nuclear- and chloroplast-encoded genes can display a long or short free-running rhythm.

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Chapter VII

Conclusion

Cell Cycle Response to Light

Earlier experiments in synchronization of algae had shown that phototrophic growth causes a coordinated entry of a population into the cell cycle (Edmunds (1964), Leedale (1959)), and that such synchronization was dependent on light alone, and not on an endogenous cell clock (Spudich and Sager (1980)). However, the mechanism by which light triggers entry into the cell cycle, and possible similarities between light-induced algal proliferation and serum- or amino acid-induced growth in animal cells were not known.

Flow cytometry has enabled us to elucidate the kinetic mechanism of light regulation of the cell cycle. The results presented in the previous chapters have shown that phototrophically grown *Euglena* has a two-part dependence on light for entry into the cell cycle. The first is a threshold light requirement for initiation of the cell cycle which is similar to the restriction point found in animal cells (Pardee (1974)). The requirement is light-dependent in phototrophically grown cells, but light is not directly linked to the response. Dark grown cells diluted into rich nutrient medium also exhibit

the same response. Thus, the threshold response is due to accumulation of a cellular component to a critical level before initiation of DNA synthesis.

After the threshold restriction point is passed, a linear light response is seen. This may be dependent on photosynthetic capacity or on chloroplast number per cell. Zetterberg's proposal that larger cells enter the S phase faster than smaller cells (Zetterberg and Larsson (1985)) could be analogous to *Euglena* cells with more chloroplasts having a shorter G_1 phase than *Euglena* cells with fewer chloroplasts. Since exit from G_1 is dependent on light exposure in algae, the determination of chloroplast parameters that affect entry into S can be definitively performed. If variation in chloroplast number per cell is what gives rise to the heterogeneity in entry of cells into the S phase, one would expect a more homogeneous response in *Chlamydomonas reinhardtii*, a unicellular photosynthetic alga that contains only one chloroplast per cell.

Determination of the Lifetime of the Cell Cycle Initiator

We have designated the linear light response that causes commitment to the cell cycle the initiating factor. Treatment of cells with brief periods of darkness in the middle of the light period S phase has allowed us to determine that the half-life of the cell cycle initiating factor in darkness is 5 hours. After 12 hours of darkness, the level of the initiating factor is too low to allow cells to enter the cell cycle until the culture has been exposed to 6 more hours of light.

The Effect of DCMU on the Cell Cycle

During attempts to separate the effects of light from the effects of photosynthesis on accumulation of the cell cycle initiating factor, we found that the photosynthesis inhibitor DCMU strongly affects the cell cycle in *Euglena*. DCMU greatly slows the rate of DNA synthesis in wild type cells grown under photosynthetic or non-photosynthetic

conditions. DCMU also inhibits or greatly slows mitosis in all strains of *Euglena*, both wild type and chloroplastless mutants, under all growth conditions. Since DCMU has been shown to have no effect on protein synthesis in nonphotosynthetic wild type cells, its effect on the *Euglena* cell cycle must be due to a direct effect on S and G_2 activities.

Attempts to further examine the accumulation of the cell cycle initiating factor may be done with red and blue light treatments of phototrophically growing cells instead of inhibitors. Red light has been shown to stimulate chloroplast activity and blue light has been shown to stimulate nuclear and chloroplast activity in dark-grown *Euglena* transferred to light conditions (see Schiff and Schwartzbach for review).

Attempts to Isolate the Chloroplast from Nuclear DNA Replication

Newly inoculated dark-grown wild type *Euglena* were shown to have a 6 hour lag period similar to newly inoculated phototrophically grown cells. Since such cells still contain large quantities of chloroplast transcripts, chloroplast participation in the nuclear cell cycle could not be ruled out. Experiments with synchronization of chloroplastless mutants did not provide a definitive answer as to whether a chloroplast is needed for efficient entry into the cell cycle. Mutant cells diluted from near stationary phase cultures immediately entered the S phase. Mutant cells diluted from stationary phase cultures displayed a lag time about three times longer than the wild type after inoculation before entry into the cell cycle. The length of the lag was so great that when cells did enter the cell cycle they were longer synchronized. If a chloroplast were not necessary for the cell cycle, one would expect mutant cells to have a 6 hour lag period followed by synchronized entry into the cell cycle. If the lack of a chloroplast were the only deterrent to efficient entry, one would expect a delay of greater than 6 hours followed by synchronized entry at a later time. The lack of any synchronized

behavior indicates that some mutant cells are more viable than others, and that more than one change has occurred in these cells. Conclusions about chloroplast effects alone can not be drawn from these cells.

Circadian and Cell Cycle Regulation of RNA Accumulation

Analysis of steady state RNA levels in log phase and stationary phase cultures of *Euglena* grown under a light-dark cycle and then shifted to continuous light have shown several different patterns of RNA accumulation. One chloroplast-encoded gene, that for *psbA*, displays a diurnal accumulation that has a circadian free-running time of greater than 3 days in continuous light. A second chloroplast-encoded gene, that for *rbcL*, has a diurnal accumulation that is lost after 3 days in continuous light. The nuclear-encoded *rbcS* gene also has an RNA accumulation diurnal pattern that is rapidly lost after 3 days growth in continuous light. These results indicate that there is a circadian pacemaker in *Euglena* that affects RNA accumulation during light-dark entrainment. However, the free-running time displayed by each RNA can vary and is not dependent on its genome.

Future Applications of Flow Cytometry in Algae

The application of flow cytometry to the study of algae has allowed elucidation of circadian and cell cycle interactions. The use of flow cytometry to determine the proliferative state of cells has enabled us to separate the effects of light-dark entrainment from cell cycle initiation on mRNA accumulation. Another fundamental question in chloroplast molecular biology is whether gene expression is proportional to genome number. Application of new *in situ* RNA hybridization techniques will allow exact determination of RNA levels in individual cells. The averaging effect due to RNA extraction and the need to compare all values to rRNA then will be eliminated.

Flow cytometry is already being used to measure protein levels in cells by binding of primary antibodies to the protein of interest followed by binding of a secondary antibody conjugated to a fluorescent marker (for review see Jacobberger et al. (1986)). In order to correlate mRNA levels with protein levels in photosynthetic cells, it would be very important to examine the levels of both an mRNA and its protein on a cell by cell basis in order to see if the diurnal RNA effects described here have any influence on protein levels.

The advantages for study of light control of photosynthetic gene expression by flow cytometry in algae include the ease with which the cells can be synchronized to light, the ability of the algae to survive heterotrophically, and the growth of algae as unicellular organisms, which obviates the need to disrupt tissue to obtain experimental material and allows examination of chloroplast DNA, RNA and protein on a cell by cell basis.

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