Chloroplast Protein Synthesis in the Chromophytic Alga *Olisthodiscus luteus*\(^1,\) \(^2\)

**CELL CYCLE ANALYSIS**

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**ABSTRACT**

This study represents the first report on chloroplast protein synthesis during the synchronous cell growth of a chromophytic (chlorophyll \(a,c\)) plant. When the unicellular alga *Olisthodiscus luteus* is maintained on a 12-hour light:12-hour dark cycle, cell and chloroplast number double every 24 hours. A temporal separation between these two events occurs. Measurements of chloroplast and total cellular protein values suggest that polypeptide synthesis occurs mainly in the light portion of the cell cycle, and pulse chase studies demonstrate that chloroplast proteins made in the light are not degraded in the dark. Data support the following conclusions: (a) a similar complement of chloroplast DNA coded proteins is made at all phases of the light portion of the cell cycle, and (b) chloroplast protein synthesis is a light rather than a cell cycle mediated response.

A sophisticated program of genetic information is required to direct chloroplast construction and to determine final organelle complement within the replicating eukaryotic cell. This complex program consists of data originating from both nuclear and chloroplast genomes since the chloroplast DNA kinetic complexity is insufficient to code for all the polypeptides needed during plastid synthesis.

Though many aspects of the multifaceted problem of organelle biogenesis (*e.g.* growth, DNA synthesis, plastid division) have been individually approached using a number of experimental systems, few studies have occurred wherein the relationship among these control factors have been defined in a cell progressing through a normal cell cycle. In most organisms in which the cell cycle has been investigated, proteins are found to be differentially expressed. It would be of interest to know how the synthesis of chloroplast proteins is programmed. Does the plastid manufacture polypeptides continually (housekeeping proteins) or specifically (specialized proteins for chloroplast DNA replication, chloroplast division, etc.) during synchronous cell growth?

Higher plants contain multiple cell types within complex tissue structures, each tissue type displaying its own distinct cellular/organelar morphology and biochemical identity. These systems are not often amenable to cell cycle analysis. In contrast, many unicellular algae divide with a high degree of synchrony when induced by the application of an alternating light:dark cycle and therefore have been exploited in plant cell cycle research.

In this study, the alga *Olisthodiscus luteus*\(^4\) is used to analyze the synthesis of chloroplast DNA coded proteins throughout synchronous cell growth. The organism is ideally suited to this investigation for it has a simple morphology and cooperative physiology. The alga contains numerous, unbranched, peripherally located chloroplasts which are 3 to 4 \(\mu\)m in size, and the naturally wall-less *Olisthodiscus* cell is limited by only a plasma membrane which allows chloroplast number per cell to be easily monitored with statistical accuracy (4). Most significantly, a synchronous cell cycle can be maintained by subjecting cultures to a 12-h light:12-h dark regime. Under this growth condition, *Olisthodiscus* displays a temporal separation in cell and chloroplast division response (4). This chronological separation differs from that observed (3) in the well studied *Chlorella*, *Chlamydomonas* or *Euglena* systems where cell and chloroplast division occur almost simultaneously. Finally, a well defined molecular biology facilitates the analysis of cell cycle synthesis of *Olisthodiscus* ctdNA coded protein products. *Olisthodiscus* is one of the few unicellular algae for which both nuclear and chloroplast genome size, ploidy, and sequence organization are reliably known (1, 10, 11). Although ctdNA of *Olisthodiscus* is similar in size to that of green plants, the organelle DNA of this nongreen alga codes (25; Reith and Cattolico, unpublished data) for genes not usually found in the chloroplast (*e.g.*, RubPCase small subunit) and contains certain genes in more than one copy (large and small subunits of RuBPCase, the 32,000 \(Q_s\) protein, rRNA cistrons). Each cell maintains a homogeneous population of approximately 650 ctdNA molecules and thus is highly polyploid with respect to chloroplast DNA content (10). In contrast, the nuclear genome is haploid (11).

Recent studies (25) in our laboratory have shown that by labeling *Olisthodiscus* cells in the presence of cycloheximide or anisomycin at L4 in the synchronous cell cycle, approximately 50 labeled proteins (35 soluble and 15 membrane) can be detected by two-dimensional gel electrophoresis. The synthesized proteins range in size from 12,000 to greater than 100,000 \(D\) in mol wt and nearly all electrofocus in the range of \(pH\) 5 to 8. They include a small number of basic proteins and a number of proteins which have hydroporphic characteristics.

We have now extended these studies to investigate the expression of ctdNA coded proteins during the cell cycle. It should be noted that *Olisthodiscus* is a chromophytic (Chl \(a,c\)) plant. To date information on the regulation of chloroplast protein synthe-

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\(^{4}\) The taxonomic affinity of *O. luteus* is disputed. The organism has been described as a xanthophyte, a chrysophyte, and a chloromonad.

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sis has only been available for chlorophytic (Chl a,b) plant types. This study represents the first attempt to analyze cell cycle events associated with ctDNA expression in a chromophytic plant species.

**MATERIALS AND METHODS**

**Cell Growth.** *Olisthodiscus lutes* Carter was grown on an artificial seawater medium (25) and synchronized by a 12-h light:12-h dark cycle. All cultures were stringently monitored for bacterial and fungal contamination (25). Cell counts were made using a model ZBI Coulter cell counter. Chloroplasts were counted according to Cattolico et al. (4). Cells late in the exponential phase of growth (1-5 × 10^6 cells/ml) were used in these experiments. In the nomenclature, L0, L4, etc. and D0, D4, etc. refer to the length of time (h) that cells were in the light (L) or dark (D) phase of a synchronous 24-h cycle.

**Determination of Cell and Chloroplast Protein during the Cell Cycle.** To determine the amounts of cellular and chloroplast protein present throughout the cell cycle, two 800-ml cultures were grown under identical growth conditions except that one culture was maintained under reversed light regime. At each time point, a 120-ml sample was axenically withdrawn from each culture. From each sample, a 10-ml aliquot was taken and cell density of this aliquot was determined using a model ZBI Coulter counter. A second 10-ml aliquot was centrifuged at 2500g for 2 min, resuspended in 50 μl of distilled H_2O, and used to determine total cell protein (25). Chloroplasts were then isolated (26) from the remaining 100 ml of sample as follows. Cells were collected by centrifugation (165g, 10 min) through a 90% Percoll pad and further concentrated by recentrifugation (3000g, 5 min). The loose pellet was resuspended in 0.33 M sorbitol, 50 mM Hepes, 1 mM MgCl_2, 6 mM H_2O and 2% BSA (pH 7.6) and the cell suspension passed through a French press at 450 p.s.i. Chloroplasts were collected by differential centrifugation (2600g, 15 s; 5900g, immediate braking) and resuspended in 0.33 M sorbitol, 50 Hepes (pH 7.6). Aliquots of the isolated chloroplasts were counted using a Levy-Hauser counting chamber. The isolated chloroplasts were then pelleted at 7000g for 1 min, resuspended in 50 μl of distilled H_2O, and used to determine chloroplast protein.

**Cell Labeling and Protein Electrophoresis.** Cells were labeled (25) at times indicated by incubating approximately 100 ml of culture with 25 μCi of NaH^14CO_3 for 30 min. CHI^3 (1 μg/ml) was added 5 min prior to the addition of label. Controls (25) demonstrate that this level of antibiotic allows only chloroplast protein synthesis in the *Olisthodiscus* cell. Following incubation, labeled cells were collected by centrifugation at 1470g for 5 min. The cells were then separated into membrane and soluble fractions by resuspending the pellet in 100 μl of solution A (0.1 M Hepes, 0.1 M DTT [pH 7.6] with KOH [5]), and homogenizing 5 to 6 times using a Dounce homogenizer. The samples were centrifuged 5 min at 15,000g and the supernatant containing the soluble proteins was removed. The membrane-containing pellet was washed with 1 ml of 50 mM Tris (pH 7.6) and resuspended in a small volume of solution A. Incorporation of radioactivity was assayed as previously described (25, 26). Three volumes of sample were mixed with two volumes of solution B (5% SDS, 30% sucrose, 0.1% bromphenol blue [5]), heated in a boiling water bath for 30 s, and cooled on ice. Samples were electrophoresed on 10 to 20% linear gradient polyacrylamide gels as previously described (26).

**RESULTS**

**Protein Accumulation during Synchronous Growth.** A synchronous cell cycle (4) was induced in our experimental system by maintaining *Olisthodiscus* cultures on a 12-h light:12-h dark photoperiod. On this regime (Fig. 1A) cell division initiates at approximately D4 and continues to L3. Under optimal conditions, logarithmically growing cultures will double in cell number during this 10- to 12-h division period. Chloroplast replication within the culture begins at L10 and continues to D5. Approximately 30 chloroplasts are present in these logarithmically growing *Olisthodiscus* cells.

When levels of protein are examined in this synchronously growing culture, the data of Figure 1B are obtained. Total cellular protein begins to increase in amount when the light is turned on (L0) and this accumulation terminates at the onset of the dark period (L12/D0). A similar pattern of protein biosynthesis is observed to occur when chloroplasts which have been isolated at different phases of the cell cycle are analyzed for protein content (Fig. 1B). A linear increase in chloroplast protein occurs only during the light portion of the cell cycle and as in whole cells, protein accumulation in the dark phase is undetectable. Throughout the cell cycle, chloroplast protein represents a constant 25 to 35% of the total cellular protein.

These data indicate that light availability has a significant effect on the quantitative production of chloroplast polypeptides. Moreover, light must differentially influence the processes of chloroplast growth and chloroplast replication for the plastids of *Olisthodiscus* predominantly divide in the dark (Fig. 1A) whereas chloroplasts increase in volume during the light phase of the cell cycle (R. A. Cattolico, unpublished data).

**Synthesis of ctDNA Coded Proteins during Cell Cycle Light Phase.** The synthesis of ctDNA coded proteins in the light phase of the cell cycle has been investigated by labeling cells with NaH^14CO_3. The use of NaH^14CO_3, a nonspecific label, and the fact that cell density had to be kept low (2 × 10^6 cells/ml) to
ensure cell cycle maintenance, resulted in a low specific activity in the recovered proteins and restricted polypeptide analysis to a one dimensional gel system.

Choosing NaH\(^{14}\)CO\(_3\) as the labeled precursor for these studies was dictated by the fact that neither radioactive leucine, methionine, a 20-amino acid mixture, sulfate, or \(^{3}\)H\(_2\)O were incorporated into \textit{Olisthodiscus} cells at significant levels. Technical difficulties in obtaining highly labeled macromolecules for cell cycle analysis occurs frequently when dealing with algal systems. As seen above, many precursor molecules simply are not incorporated, and changes in precursor pool sizes or transport may occur during synchronous growth. Although transient nutrient deprivation is often successful in obtaining a highly labeled product, this approach has been shown (17) to alter the cell cycle itself.

At L1 of the cell cycle approximately 27 proteins (12 soluble, 10 membrane) can be detected by our analytical method (Fig. 2). Four proteins identified in previous studies (25), include the large (a) and small (b) subunits of RuBPCase, the 32,000-D Q\(_a\) protein (c) and the apoprotein of CP1 (d). It should be noted that all other samples (L4, L8, L11) are identical in protein profile and individual polypeptide labeling intensity. These data strongly support the conclusion that the pattern of proteins synthesized does not change either qualitatively or quantitatively during the light phase of synchronous cell growth.

**Synthesis of ctDNA Coded Proteins during Cell Cycle Dark Phase.** The negligible accumulation of protein observed in chloroplasts during the dark phase of the cell cycle might be the result of two possibilities: (a) protein synthesis shuts down completely in the dark or (b) protein synthesis continues in the dark but at a rate equal to the rate of protein degradation. The first alternative implies that in the dark phase of cell growth the proteins synthesized in the light portion of the cell cycle turn over very slowly or not at all whereas the second alternative suggests that a very rapid rate of protein turnover occurs. The test for \textit{de novo} protein synthesis during the dark was complicated by the fact that NaH\(^{14}\)CO\(_3\) was the only precursor which would incorporate into the \textit{Olisthodiscus} cell, and that the utilization of this particular precursor is light dependent.

To differentiate between the two possibilities cited above and to circumvent precursor assimilation problems, cells were labeled with NaH\(^{14}\)CO\(_3\) in the presence of CHI during the final 30 min of the light period (L11.5–D0). Following the labeling period, CAP was added to a concentration of 100 \(\mu\)g/ml and the culture was sampled at D0, D4, D8, and D11.5. The presence of both inhibitors blocks any possible resynthesis of protein (26) during the dark period and thus this procedure represents a classical pulse-chase experiment.

As seen in Table I, the amount of radioactivity in the cells remains constant throughout the dark period. Furthermore, when these samples are run on gels (Fig. 3), the pattern of labeled proteins is also shown to be constant and identical to the spectrum of proteins which is observed in light. These data demonstrate that the ctDNA coded proteins which are labeled during the final 30 min of the light period of the cell cycle remain labeled throughout the dark period and that turnover and new synthesis of plastid proteins is minimal during the dark.

**Potential for Chloroplast Protein Synthesis during the Dark.** Since chloroplast protein synthesis appears to be low or nonexistent in the dark, it was of interest to investigate the condition of the chloroplast protein-synthesizing apparatus during this period. First, it may be that light is an absolute requirement for chloro-

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**FIG. 2.** Autoradiographs of proteins synthesized by \textit{Olisthodiscus} in the presence of CHI at various points in the light phase of the cell cycle. Cells were labeled for 30 min at the time indicated and separated into membrane and soluble fractions. The samples were run on SDS gels and fluorographed. Previously identified proteins are: (a) large subunit of RuBPCase; (b) small subunit of RuBPCase; (c) apoprotein of CP1; (d) the 32,000 dQ\(_a\) protein weight markers are BSA (68,000 D), ovalbumin (45,000 D), carbonic anhydrase (29,000 D), and myoglobin (17,000 D).
Table I. Maintenance of Radioactive Protein during the Dark Period of the Cell Cycle

Cells were labeled during the final 30 min of the light period with NaH\(^4\)CO\(_3\) in the presence of CHI. Following addition of CAP to 100 \(\mu\)g/ml at D0, samples were collected at the times indicated and incorporation and protein amounts determined as described in the "Materials and Methods" section.

<table>
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<th>Sample</th>
<th>cpm (\times 10^{-5})</th>
<th>Total Protein</th>
<th>Specific Activity</th>
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<td></td>
<td>Membrane</td>
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</tr>
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<td>D11</td>
<td>2.55</td>
<td>1.16</td>
<td>3.37</td>
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</tbody>
</table>

Table II. Labeling of Light-Growing or Dark-Growing Cells in the Presence of Light

Cells were sampled at the time indicated and labeled with NaH\(^4\)CO\(_3\) in the presence of CHI under standard light conditions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>cpm (\times 10^{-5})</th>
<th>Total Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
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</table>

plast protein synthesis and its absence is the only factor preventing the synthesis of chloroplast proteins. Alternatively, during the dark portion of the cell cycle, the chloroplasts may be programmed to halt protein synthesis and only upon reaching a specific time in the cell cycle (i.e. L0) would the chloroplast regain synthetic competency. To differentiate between these possibilities, cells in the dark phase of the cycle (D4 and D8) were preincubated with CHI for 5 min. These cells were then transferred to the light and labeled with NaH\(^4\)CO\(_3\) for 30 min. Incorporation of label by these cells was comparable to that of cells labeled during the light period of the cycle (Table II). The pattern of proteins synthesized (Fig. 4) is identical to that found during the light phase of the cycle. It should be noted that identical polypeptide patterns were obtained from cells removed at all points in the dark phase of the cell cycle (results not shown). Thus, the synthesis of cDNA coded proteins requires the presence of light.

**DISCUSSION**

*Olisthodiscus luteus* is an obligate photoautotroph (4). Recent studies (R. A. Cattolico, unpublished data) demonstrate that light plays an indispensable role in the maintenance of chloroplast complement within this organism. Significant coupling between
cell and organelle division rates results in a predictable and remarkably regulated program of organelle biogenesis, so that cells maintained under a given light regime have a specific chloroplast number.

In this study, the synthesis of chloroplast coded proteins has been investigated throughout the synchronous cell cycle of *Olis-thodiscus luteus*. This cell cycle has been induced by the application of a specific light regime. The underlying mechanism of protein production in *Olis-thodiscus* seems quite simplistic. Data demonstrate that during the entire light phase of the cell cycle, the pattern of polypeptides which are synthesized is both qualitatively and quantitatively constant, but in the dark, chloroplast protein synthesis is significantly reduced and may be completely absent. However, cells which are in the dark phase regain the capacity to produce polypeptides if they are transferred from the dark to the light, and the profile of the proteins made after this transition is identical to that observed during the normal phase of the cell cycle growth. These results are consistent with earlier observations which have been made on protein synthesis in other synchronized algal systems. Net whole cell protein accumulation occurs only in the light period of the *Chlamydomonas* (15) and *Euglena* (8) cell cycle. Although only small amounts of information are available on specifics of chloroplast protein synthesis during synchronous growth in other plant systems, it is known that both the holoenzyme and the large subunit of RuBPCase are synthesized only during the light phases of the *Euglena* (30) and *Chlamydomonas* (15) cell cycles. In fact, Howell et al. (12) have shown that the synthesis of the *Chlamydomonas* RuBPCase large subunit is strictly light dependent, for a dark to light cell transfer initiates the synthesis of this enzyme. This data, plus the fact that the synthesis of ctDNA coded membrane proteins of *Chlamydomonas* also occurs only in the light portion of the cell cycle (20) suggests that as in *Olis-thodiscus*, the *Chlamydomonas* system also has a light dependent production of chloroplast proteins.

The regulation of protein synthesis by light could occur either at the level of transcription or translation. Variations in transcription of ctDNA have been observed to occur (21, 24) in differentiating plastids. However, data from observation (14) of the synchronous *Chlamydomonas* system indicate that light primarily influences translation during normal cell cycle growth. Throughout the *Chlamydomonas* system, the levels of certain mRNAs remain constant (13). The fact that most of the *Chlamydomonas* ctDNA EcoRI restriction fragments synthesize mRNA during the dark phase of the cell cycle (19) provides additional support to the hypothesis that light minimally affects the transcription of this chloroplast genome during synchronous cell growth.

Three lines of evidence further support the suggestion that light may have a significant effect at the translational level during synchronous cell cycle. First, a rapid recruitment of chloroplast ribosomes into polysomes at the beginning of the light period of the *Chlamydomonas* cell cycle has been observed (2) to occur. Ribosome recruitment into polysomes has often been used as a measure of protein synthesis (22, 29). Second, the attachment of chloroplast ribosomes to thylakoid membranes has been shown to be a necessary step in the synthesis of some chloroplast proteins (6, 18). Chua et al. (6) have found that, in *Chlamydomonas*, this attachment of ribosomes to thylakoid membranes occurs only in the presence of light. Finally, light appears to be the primary energy source for protein synthesis. Protein synthesis in chloroplasts isolated from *Euglena* and a variety of higher plants (see 9 for review) has been found to be light dependent. In fact, Ramirez et al. (23) have shown that, in isolated pea plastids, protein synthesis is dependent on ATP produced by cyclic photophosphorylation.

Are these data inconsistent with the fact that dark maintained etioplast or proplastids synthesize proteins (see 16 for review) though frequently at a repressed level (7)? It has been observed that greening in both *Euglena* (28) and higher plants (31) is blocked by the addition of inhibitors of respiration and oxidative phosphorylation. Furthermore, Siddell and Ellis (27) have shown that pea etioplasts, isolated during a 4-d greening period, became progressively less capable of using ATP as an energy source for protein synthesis and progressively more capable of using light. These studies suggest that dark-grown plastids are able to utilize energy provided by the breakdown of storage products in the mitochondria. Upon exposure to light, this ability is lost and light becomes the primary source for chloroplast protein synthesis. We would speculate that since *Olis-thodiscus* is an obligate photoautotroph, its chloroplasts would be more like that of a fully differentiated higher plant plastid—that is, its chloroplasts would not have the ability to utilize energy from the mitochondria.

In summary, further studies of chloroplast protein synthesis during synchronous cell growth will provide information on the regulatory mechanism(s) which function *in vivo* during normal chloroplast biogenesis and thus give new insight on the change which must occur at the translational and/or transcriptional level to permit plastid differentiation.

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