Regulation of Genes Encoding the Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase and the Photosystem II Polypeptides D-1 and D-2 during the Cell Cycle of *Chlamydomonas reinhardtii*

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Abstract. Synthesis of the major chloroplast proteins is temporally regulated in light–dark-synchronized *Chlamydomonas* cells. We have used cloned chloroplast DNA probes, and in vitro and in vivo protein synthesis to examine the cell cycle regulation of photosystem II polypeptides D-1 and D-2, and the large subunit of ribulose-1,5-bisphosphate carboxylase (RuBPCase LS). Synthesis and accumulation of D-1 and D-2 mRNAs occurs during the first half of the light period (G1), correlating with increasing synthesis of the polypeptides. Rifampicin, added immediately before the light period, inhibited the normal increase in D-1, D-2 polypeptide synthesis. During the dark period D-1, D-2 mRNAs persist at high levels despite reduced rates of mRNA synthesis and translation during this period. Cell-free translation analyses indicate that the D-1 mRNA present during the dark period is efficient at directing synthesis of the D-1 precursor in vitro. We conclude that expression of the psbA (D-1) and psbD (D-2) genes are regulated primarily at the transcriptional level during the light-induction period but at the translational level for the remainder of the cell cycle.

Transcripts of the RuBPCase LS gene (rbcL) are also found at high levels during the light and dark periods but, unlike D-1 and D-2, LS mRNA levels do not increase until the last half of the light period and measurable synthesis and accumulation of this mRNA occurs during the dark. Furthermore, induction of LS polypeptide synthesis during the light period is insensitive to rifampicin. We conclude that LS production is regulated primarily at the translational level during the cell cycle.

THE growth and division of many unicellular algae can be easily synchronized by a repeating light–dark cycle. These organisms provide well-defined, naturally synchronous systems for investigating the regulation of biosynthetic processes associated with cell proliferation. Synchronously growing cultures of *Chlamydomonas reinhardtii* have been particularly useful, in part, because these cells can be grown autotrophically or heterotrophically and cell cycle mutants can be isolated (20, 26, 27, 55). In studies with *C. reinhardtii*, 12-h light/12-h dark cycles are typically used to synchronize the cells. With these conditions the cells divide during the middle of the dark period immediately after nuclear DNA replication. Total cellular protein and rRNAs accumulate during the light (i.e., G_1) period of the cell cycle (20). Stage-specific synthesis of a number of polypeptides and accumulation of some specific nuclear-derived mRNAs has also been described (1, 28).

Chloroplasts are prominent organelles in many algae and a large part of the work with synchronous cultures has been devoted to studying replication of the chloroplast (7). Chloroplast biogenesis in this system differs from the more well-studied "greening" phenomenon of higher plants, *Euglena* and the y-1 mutant of *Chlamydomonas* where rudimentary plastids develop into mature chloroplasts usually without cell division (25). In synchronous cultures of *Chlamydomonas*, chloroplast division occurs simultaneously with cytokinesis during the dark period, but chloroplast DNA replication occurs several hours earlier during the early to mid-light period (9). Several detailed studies have shown that synthesis of the major protein, pigment and lipid constituents of thylakoid membranes, and ribulose-1,5-bisphosphate carboxylase (RuBPCase)[1] occurs primarily during the light period of the cell cycle (4, 31–33). Further study of one of the light-harvesting chlorophyll-binding proteins of photosystem II (LHCII) has shown that synthesis of this protein correlates with the transient appearance of the mRNA during the light;

1. *Abbreviations used in this paper*: cpDNA, chloroplast DNA; LHCII, light-harvesting complex of photosystem II; LS, large subunit; PSII, photosystem II; RuBPCase, ribulose-1,5-bisphosphate carboxylase.

the mRNA disappears during the dark period even if the lights are left on (49). Together with previous work on tubulin and other cell cycle-regulated mRNAs (1) these data suggest that differential gene expression during the *Chlamydomonas* cell cycle results mainly from transcriptional control. These studies have used only nuclear-derived genes, however, as similar data are not available for specific chloroplast genes. The global analyses of Howell and Walker (29) and Matsuda and Surzycki (40) provide suggestive evidence of transcriptional and translational control in the chloroplast but cloned DNAs were not used to investigate specific genes.

We have investigated the expression of the chloroplast psbA, psbD, and rbcL genes during the light-dark cell cycle of *Chlamydomonas reinhardtii*. The psbA and psbD genes encode the 34-36-kD D-1 and 29-kD D-2 polypeptides, respectively, of photosystem II (PSII) (14, 17, 45). D-1 is believed to bind quinones and function as the secondary electron acceptor for PSII (35). D-1 has also been called Q_B and the herbicide-binding protein as it participates in binding of several herbicides which act on PSII (35). The function of D-2 is not known but it has been hypothesized to function in concert with D-1 (12). The psbA gene of *Chlamydomonas* contains four introns and is found within the inverted repeat region of the chloroplast genome, thus there are two copies per chloroplast DNA (cpDNA) molecule (17). The psbD gene, which is uninterrupted, is present as one copy per cpDNA molecule (45). The mRNAs for these hydrophobic membrane proteins are translated on thylakoid-bound ribosomes (21, 23). The rbcL gene, which encodes the large subunit (LS) of RuBPCase, is also present at one copy per cpDNA molecule and is uninterrupted (16). Our findings indicate that, in addition to transcriptional control, differential translation of stable mRNA plays a major role in expression of chloroplast-encoded genes during the cell cycle.

Materials and Methods

Growth and Labeling of Cells

The wild-type 137c mt+ strain of *Chlamydomonas reinhardtii* was grown photoautotrophically and synchronized with an alternating regimen of 12 h of light (2,000 lux) followed by 12 h of dark for at least three complete cycles (55). The light period begins at h 0, and the dark period at h 12 of the 24-h cycle; cell cycle time is also referred to as h of light or h of dark (e.g., L6 or D6). The specific growth characteristics of this wild-type strain under these conditions has been described (20). Briefly, the cells accumulate chlorophyll, protein, and thylakoid membranes only during the light period and divide in the middle of the dark resulting in a step-wise increase of two- to threefold in cell number. Experiments were routinely performed at a culture density of $\sim 1 \times 10^6$ cells/ml at the beginning of the light period.

Pulse-labeling of cells was performed by adding [^{3}H]arginine (10–20 Ci/mmol; New England Nuclear, Boston, MA) to a final concentration of 2 μCi/ml, and the cells were incubated for 1 h under ongoing growth conditions. Arginine was chosen as radioactive label because it is taken up and used for protein synthesis throughout the cell cycle (28). Care was taken to minimize exposure of the cells to light when labeling during the dark period and the cells were not centrifuged, starved, or disturbed in any way. Isotopic labeling was terminated by pouring the cells over crushed ice and all further manipulations were carried out at 0–4°C. The cells were harvested by centrifugation at 7,000 *g* for 10 min, washed, and resuspended in 0.25 M sucrose, 25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 25 mM KCl, 1 mM dithiothreitol (DTT). Incorporation of [^{3}H]arginine into total cellular protein increased linearly for the duration of the pulse (15 min–1 h), whether labeling in the light or dark periods. When cycloheximide was used, it was added 0.5 h before labeling to 10 μg/ml.

For labeling with [^{32}P]phosphate it was necessary to centrifuge the cells and resuspend them to $\sim 5 \times 10^7$ cells/ml in phosphate-free media containing 10 mM Tris-HCl, pH 7.5. [^{32}P]H_3PO_4 (carrier-free; ICN K&K Laboratories Inc., Plainview, NY) was added immediately to a final concentration of 600–700 μCi/ml and incubation continued for 1 h under ongoing growth conditions. [$^{32}PO_4$] incorporation into cold TCA-insoluble material (nucleic acids) was linear for the duration of the pulse; incorporation into hot TCA-insoluble material (protein) was negligible. Rifampicin, when used, was added to 250 μg/ml before labeling with [$^{32}PO_4$] (as described in the figure legends) and during labeling.

Preparation of Thylakoid Membranes

100–200-ml aliquots of pulse-labeled cells, harvested as described above, were broken by passage through a French press at 4,000 psi or by sonication with 3–4 bursts (15–30 s each) at 75% maximum setting on a Branson sonifier (Branson Sonic Power Co., Danbury, CT). Thylakoid membranes were isolated on discontinuous sucrose gradients as previously described (23). The protease inhibitors ε-amino-*n*-caproic acid, phenylmethylsulfonyl fluoride (PMSF) and benzamidine were included in the homogenization and isolation buffers. Thylakoid membranes isolated at different cell cycle stages showed identical Coomassie-stained polypeptide patterns and similar chlorophyll *a*/*b* ratios (2.0 ± 0.1) indicating that the same subcellular fraction was obtained in each isolation.

Isolation of Thylakoid Membranes with Bound Polysomes and In Vitro Protein Synthesis

The procedures used for isolation of thylakoids with bound polysomes and preparation of the *Escherichia coli* S-100 have been described (23). *E. coli* 29417 RNAse$^-$ (American Type Culture Collection, Rockville, MD) was used for the preparation of the S-100 extract. The cell-free protein synthesizing system contained the following: [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear) at 500 μCi/ml, 50 μM unlabeled amino acids, 70 mM KOAc, 7.5 mM $Mg(OAc)_2$, 42 mM NH_4Cl, 0.3 mM spermidine, 3 mM DTT, 55 mM Hepes-KOH, pH 7.8, 5 mM phosphoenolpyruvate, 30 μg/ml pyruvate kinase, 1 mM ATP, 0.2 mM GTP, 20% (vol/vol) *E. coli* S-100, and thylakoid membranes at 1 mg/ml chlorophyll. Protein synthesis was carried out at 24°C for 1 h. Rough thylakoids isolated during the light period routinely gave 10,000–15,000 cpm/μg chlorophyll with [^{35}S]methionine as label.

SDS Gel Electrophoresis and Fluorography

SDS PAGE was performed using the buffers described by Laemmli (36) and an acrylamide/bisacrylamide ratio of 37.5:1. The stacking gel contained 5% acrylamide and the resolving gels were continuous (14%) or linear gradients (7.5–15%). In some cases, a long resolving gel (30 cm) of 10–18% acrylamide with 2 M urea was used to analyze D-1 synthesis in vivo. Samples were prepared for electrophoresis by incubation at 100°C for 1–3 min in sample buffer (36) containing 2.5% (wt/vol) SDS and 50 mM DTT substituted for 2-mercaptoethanol. Electrophoresis was carried out at room temperature for 12–24 h and the gels were stained with 0.25% (wt/vol) Coomassie Blue R-250. M_r was estimated by co-electrophoresing polypeptides of known molecular mass, including β-galactosidase (110 kD), BSA (67 kD), ovalbumin (43 kD), α-chymotrypsinogen (25 kD), and myoglobin (17 kD).

For fluorography the gels were impregnated with EN^{3}HANCE (New England Nuclear) or sodium salicylate (8) and exposed to preflashed x-ray film at −70°C. The fluorographs were scanned with a Gelman automatic computing densitometer and the peaks of absorbance above the immediate background were integrated. The linearity of fluorography was checked by quantitating fluorographs of ^{3}H-labeled vesicular stomatitus virus proteins.

Isolation of RNA, Cell-free Translation, and Immunoprecipitation

Total cellular RNA was isolated as described previously (19). The RNA was translated in a nuclease-treated reticulocyte lysate system (New England Nuclear) at a concentration (400 μg/ml) within the linear response range of the lysate to total RNA. In addition, concentrations of RNA between 100 and 500 μg/ml did not show significantly different patterns of translation products when analyzed by SDS PAGE. Translations were performed for 1 h at 37°C in the presence of 1 mCi/ml [^{35}S]methionine (800–1,200 Ci/mmol) and routinely gave 50,000–60,000 TCA-precipitable cpm/μl. For immunoprecipitation, equal amounts of radioactive translation products (1.0×10^6 cpm) were denatured with 2% (wt/vol) SDS and incubation at

100°C for 1 min. Immunoprecipitation was performed as previously described (22) using excess antiserum and protein A-Sepharose.

Polyclonal antiserum to the major LHCII polypeptide of *Chlamydomonas* thylakoid membranes, polypeptide 11 in the nomenclature of Delepelaire and Chua (15), was generated in New Zealand white rabbits. The methods described previously for generating antiserum to polypeptides 4.1–4.2 were used (22), but with two modifications: chloroform-methanol-soluble thylakoid polypeptides (10) were used for preparative gel electrophoresis and gel slices containing the polypeptides were homogenized with Freund's adjuvant and administered directly until precipitating antibodies were obtained. Immunoprecipitation of acetone-extracted, detergent-solubilized thylakoid membranes with antiserum to polypeptide 11 gave two bands on an SDS gel, one co-migrating with polypeptide 11 and the other with the abundant LHCII polypeptide doublet 16–17 as expected (10).

Analysis of mRNA Levels by Blot Hybridization

Electrophoresis and northern blot hybridization of total RNA was performed as described (19). In some cases, RNA blots were hybridized sequentially to different DNA probes after removal of the previously hybridized DNA by incubation of the nitrocellulose in boiling H_2O for 3 min. Relative mRNA levels were also measured by applying the RNA samples (up to 5 μg) to nitrocellulose using a dot-blot manifold (Bethesda Research Laboratories, Gaithersburg, MD). The RNA was denatured with formaldehyde/formamide as for the northern blots and then brought to 3.0 M NaCl, 0.2 M NaH_2PO_4, pH 7.4, 10 mM EDTA (20× SSPE) before binding to nitrocellulose. After hybridization and washing, the nitrocellulose dots were cut and counted by liquid scintillation spectrometry.

Cloned DNA Probes

Plasmid pEC23 contains the 5.6-kb R14 fragment of *Chlamydomonas* chloroplast DNA in the vector pBR325 (21). Fragment R14 contains 4 of the 5 exons of the polypeptide D-1 gene (psbA) and hybridizes predominately to the 1.2-kb D-1 mRNA (17, 21). Cloned DNA probes for RuBPCase LS and D-2 were obtained from J.-D. Rochaix (University of Geneva). Plasmid R15.4 contains a 760-base pair (bp) HindIII internal fragment of the *Chlamydomonas* LS gene (rbcL) in the plasmid vector pBR322 and hybridizes exclusively to the 1.6-kb LS mRNA (16). Plasmid pCP55 contains the 2.6-kb R3 fragment of *Chlamydomonas* chloroplast DNA and most of the D-2 structural gene (45). It hybridizes exclusively to the 1.1–1.2-kb D-2 mRNA.

Cloned plasmid DNAs were maintained in cultures of *E. coli* C600 and HB101 which were grown in Luria-Bertani broth in the presence of 25 μg/ml kanamycin for pCP55 and 50–100 μg/ml ampicillin for pEC23 and R15.4. Plasmid DNA was isolated by CsCl–ethidium bromide centrifugation using a rapid boiling procedure (24). DNA probes were radioactively labeled by nick-translation to a specific activity of 1–3 × 10^8 cpm/μg (39).

Analysis of mRNA Synthesis

^{32}P-RNA was purified from cells that were pulse-labeled with [^{32}P]PO_4 as described above, and hybridized to excess immobilized DNA. For the ^{32}P-RNA isolation, cells were harvested by centrifugation, resuspended to ~1 × 10^8 cells/ml in 50 mM Hepes-KOH, pH 7.5, 10 mM EDTA, and then lysed with SDS (2% [wt/vol]) and *N*-lauroyl sarcosinate (2% [wt/vol]) in the presence of 1% (vol/vol) diethylpyrocarbonate. NaCl was added to 0.1 M and the mixture was extracted twice with phenol/chloroform/isoamyl alcohol (24:23:1) and once with chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated with 2.5 vol of ethanol and, after resuspension of the pellet in 50 mM Hepes-KOH, pH 7.8, 5 mM $Mg(OAc)_2$, 50 mM NH_4Cl, the DNA was removed by digestion with DNAse I (Sigma Chemical Co.; EP) for 15 min at 37°C followed by extraction with phenol and precipitation with ethanol as before. The RNA precipitate was collected by centrifugation, resuspended in H_2O, and reprecipitated with 2.5 M LiCl, 1 mM $MgCl_2$ and incubation for 6–12 h at 0°C; this step was necessary to separate the RNA from radioactive material that bound nonspecifically to nitrocellulose in the subsequent hybridizations. Final RNA pellets were resuspended in H_2O and stored at −70°C. RNA obtained by this procedure was judged to be intact and not contaminated with DNA when analyzed by denaturing agarose gel electrophoresis.

Radioactivity in specific mRNA molecules was determined by hybridization to DNA which had been bound to nitrocellulose by the procedure of Kafatos et al. (34). Nitrocellulose filters, containing 5–10 μg DNA/dot, were prehybridized overnight at 42°C in 50% (vol/vol) formamide, 5× SSPE, 0.5% (wt/vol) SDS, 50 μg/ml poly A, 200 μg/ml denatured sperm DNA, 500 μg/ml *E. coli* tRNA; and hybridization was then performed with a fresh aliquot of the same solution containing 20 μg ^{32}P-RNA for 72 h at 42°C. The DNA dot-blots were washed at 50°C in 0.1× SSPE, 0.1% (wt/vol) SDS and then exposed to x-ray film for a visual record before cutting out the dots and counting them by liquid scintillation spectrometry. Hybridization was judged to be essentially complete since the addition of fresh filters containing recombinant plasmid DNA and subsequent hybridization did not yield detectable signals above that obtained with the control plasmid. The vector plasmids pBR325, pBR322, and pCR1 did not hybridize significantly to *Chlamydomonas* ^{32}P-RNA preparations; therefore, pBR325 was routinely used to measure nonspecific binding of radioactivity.

Miscellaneous Measurements

Protein was measured using the procedure of Lowry et al. (38) in the presence of 0.1% (wt/vol) SDS with BSA as standard. Samples for protein determination were obtained by extracting whole cells or thylakoids with 90% (vol/vol) acetone and resuspending the protein pellets in 0.5 M NaOH/1% (wt/vol) SDS with heating. Radioactivity in protein was determined as described previously (23). RNA was quantitated by UV spectrophotometry (20 A_{260} = 1 mg/ml). Radioactivity in RNA or DNA was determined by precipitating samples in cold 10% TCA, 1% sodium pyrophosphate, 100 μg/ml single-stranded carrier DNA, and collecting the precipitates on GF/C filters (Whatman Inc., Clifton, NJ). Cell number was determined with a hemacytometer.

Results

Previous work from this laboratory (20) and others (4) has shown that synthesis of the abundant thylakoid polypeptides is restricted to the light phase in light–dark-synchronized *C. reinhardtii*. In addition, we have shown that the membrane-bound synthesis of D-2 is restricted to the light peaking near the middle of the light period (23). The cell cycle synthesis of D-1, however, was not clear from these studies. Therefore, we have investigated D-1 synthesis during the cell cycle by in vivo pulse-labeling and in vitro synthesis with thylakoid-bound polysomes. Thylakoid membranes were purified from pulse-labeled cells and analyzed by SDS PAGE under conditions that separate D-1 from polypeptides 9 and 10 (Fig. 1 *A*). Polypeptide 10 is apparently a chlorophyll *a*/*b*-binding polypeptide (15); the function of polypeptide 9 is unknown. The fluorograph shows that synthesis of D-1 occurs throughout the light period but is not detectable during the dark period. Densitometric scanning of the fluorograph and correction for differences in specific radioactivity of the labeled thylakoids indicates that synthesis of D-1 is ~3–4 times greater at the mid-light period than during the first hour of the light. Although equal radioactivity was applied to each lane of the gel shown in Fig. 1 *A*, the specific radioactivity of thylakoids from dark-labeled cells was quite low and there is little to no radioactivity in identifiable thylakoid proteins. The radioactivity that did co-purify with thylakoids labeled during this period was concentrated in high molecular mass proteins, most of which barely entered the 10–18% polyacrylamide gel. The nature of these low mobility proteins is unknown but they are apparently not thylakoid protein aggregates since the Coomassie stain profile of all samples was indistinguishable (not shown) indicating equal solubilization of thylakoids from light- and dark-labeled cells.

To verify that synthesis of D-1 is restricted to the light phase, the protein synthetic capacity of thylakoid-bound polysomes was examined. Although these polysomes synthesize a number of thylakoid membrane proteins (21–23), D-1 is the major product when [^{35}S]methionine is used as label (21). D-1 was abundantly synthesized by polysomes isolated during the light but was not detected as a product of dark-

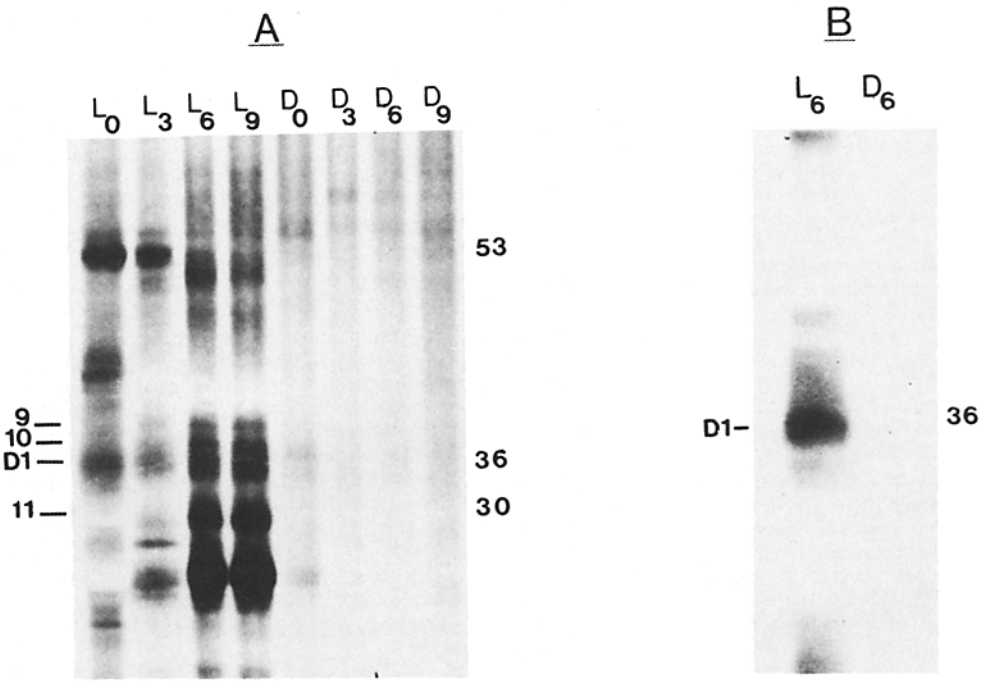

Figure 1. Synthesis of D-1 during the cell cycle of *C. reinhardtii*. (*A*) Aliquots of synchronous cells were removed at the indicated times, pulse-labeled for 1 h with [^{3}H]arginine and thylakoid membranes purified on discontinuous sucrose gradients. Approximately equal amounts of radioactive thylakoid membranes (60,000 cpm) were electrophoresed on a 10–18% polyacrylamide gel which was fluorographed. Relevent polypeptides are labeled to the left according to the nomenclature of Chua and Gillham (11) and only a portion of the fluorograph is shown. (*B*) Thylakoid-bound chloroplast polysomes were isolated at the indicated times of the light–dark cycle and used for protein synthesis (at equal chlorophyll concentration) with [^{35}S]methionine as radioactive label. Electrophoresis was performed on a 14% acrylamide gel. The position of D-1 is indicated to the left in *A* and *B*, and M_r ($\times 10^{-3}$) is indicated to the right.

period thylakoid polysomes (Fig. 1 *B*). Thus the cell cycle synthesis of D-1 is similar to that previously described for D-2 (23) except for a more noticeable synthesis of D-1 during the first hour of the light period (Fig. 1 *A*).

D-1, D-2, and RuBPCase LS mRNA Abundance during the Cell Cycle

A number of genes encoding photosynthetic proteins have been localized on the physical map of *Chlamydomonas* cpDNA, including those for D-1 (psbA), D-2 (psbD) and the LS of RuBPCase (rbcL) (44). These genes have been obtained as cloned DNA and used to probe the cell cycle for the complementary mRNA (Figs. 2 and 3). D-1 mRNA, as determined by northern (Fig. 2) and dot-blot (Fig. 3) hybridization is present throughout the cell cycle showing a 2.5-fold increase during the first half of the light period and declining slightly (25%) in the dark. The cell-cycle pattern of D-2 mRNA abundance, measured by northern (Fig. 2) and dot-blot (Fig. 3) hybridization, is similar to D-1. D-2 mRNA levels increase during the light period and decrease in the dark returning almost to the levels observed at the onset of the light period. The difference between the maximum (at L6) and minimum (at L1) levels is about threefold, a slightly greater variation than the D-1 mRNA.

In addition to the two PSII proteins D-1 and D-2, cell-cycle steady-state levels of RuBPCase LS mRNA were measured. Like D-1 and D-2, the synthesis of LS polypeptide is confined to the light period (20, 28, 31). LS mRNA, as determined by northern (Fig. 2) and dot-blot (Fig. 3) hybridization is also present at high levels throughout the cell cycle,

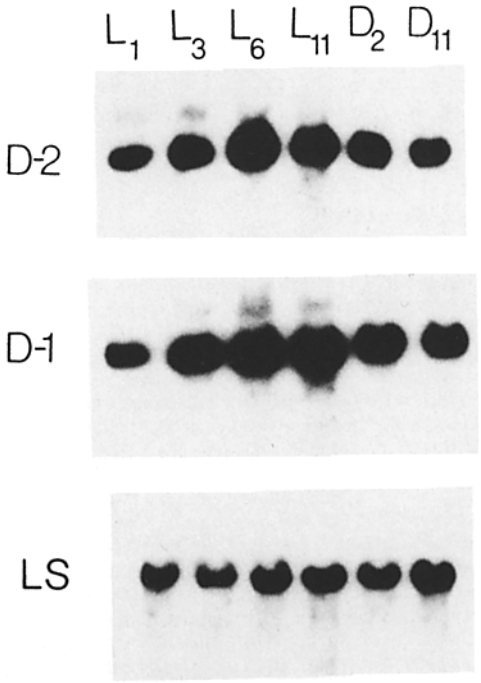

Figure 2. Relative mRNA levels for D-1, D-2, and RuBPCase LS determined by northern blot hybridization. Total RNA (8 μg), isolated at the indicated times of the cell cycle, was electrophoresed on 1.5% agarose/6% formaldehyde gels and blotted onto nitrocellulose. The blots were probed with ^{32}P-labeled plasmids R15.4 (*LS*), pEC23 (*D-1*), and pCp55 (*D-2*). The blots are representative of three hybridizations for each probe.

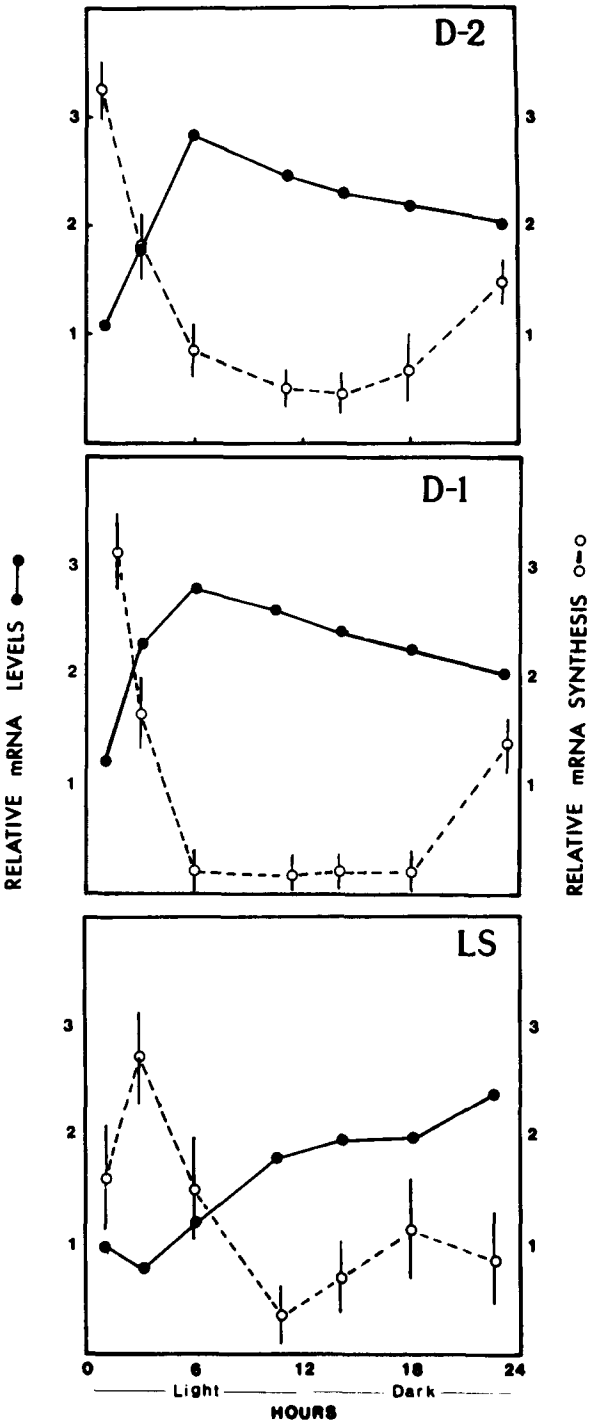

Figure 3. Synthesis and accumulation of RuBPCase LS, D-1, and D-2 mRNAs during the cell cycle. LS, D-1, and D-2 steady-state mRNA levels were measured by RNA dot-blot hybridization of the ^{32}P-labeled plasmids to 5 μg total RNA from each cell cycle time point (●). The same pattern was obtained with 2.5 μg RNA/dot. Relative mRNA synthesis was measured by hybridization of ^{32}P-RNA to DNA dot-blots of the same plasmids used in Fig. 2 (o). Hybridization was quantitated by liquid scintillation counting of the nitrocellulose dots and the cpm obtained with filter-bound pBR325 was subtracted from each. The data was normalized by dividing the cpm obtained throughout the cell cycle for each DNA probe by an arbitrarily chosen number so the values could be plotted on the same numerical scale and the degree of change more easily evaluated. The relative mRNA synthesis values are averages ± SEM of three hybridizations for LS and D-2, and two for D-1.

but unlike D-1 and D-2, accumulates during the last half of the light period and during the dark resulting in a final increase of 2.5-fold.

D-1 mRNA is very abundant and the D-1 polypeptide precursor is a major translation product of *Chlamydomonas* RNA in the reticulocyte lysate translation system (19, 21). Consequently, it was possible to determine, for at least D-1, if the mRNA present during the dark period is functional as a template for protein synthesis. Cell-free translation of total RNA from different points in the cell cycle reveals little change in translatable mRNA coding for the 36-kD precursor to D-1 (pD-1) (Fig. 4 *A*) compared to mRNA encoding the major LHCII protein precursor (pLHCII) (Fig. 4 *B*). An increase in translatable D-1 mRNA is apparent during the first half of the light period which is followed by a slight but noticeable decline in the dark. Thus, the cell-cycle pattern of D-1 mRNA abundance assayed by cell-free translation is similar to that observed with a cloned hybridization probe (Figs. 2 and 3). This indicates that the in vitro translatability of D-1 mRNA is similar throughout the cell cycle. The assay for translatable LHCII mRNA was performed to show that, under these conditions, the abundance of a major cytoplasmic mRNA varies dramatically during the cell-cycle as previously reported (49).

Synthesis of D-1, D-2, and LS mRNAs during the Cell Cycle

Because of the unexpected finding that LS, D-1, and D-2 mRNAs are present at high levels during the light and dark periods, it was of interest to characterize further the synthesis and accumulation of these mRNAs by pulse-labeling cells with [^{32}P]PO_4 for 1 h at selected times of the cell cycle. Although shorter pulse-labeling periods may have given a more accurate estimate of mRNA synthesis, a 1-h labeling period was required to obtain high specific activity RNA from cells grown under these conditions and not starved for PO_4. After pulse-labeling, ^{32}P-RNA was isolated and radioactivity in specific mRNA species measured by hybridization to cloned DNA probes (Figs. 3 and 5). D-1 and D-2 mRNAs showed similar patterns of synthesis during the cell cycle; peak levels of pulse-labeled RNA were observed at L1–L2, which then declined rapidly and reached low levels by L6–L7. The low level of D-1 and D-2 mRNA synthesis continued until the end of the dark period when pulse-labeled D-1 and D-2 mRNA begins to accumulate again. These results would indicate that the accumulation of these mRNAs during the period L0-L6 is due to high rates of mRNA synthesis during this period (Fig. 3). The diminished synthesis of D-1 and D-2 mRNA in the dark is accompanied by a 25% decline in D-1 and 35–40% decline in D-2 mRNA levels by the end of the dark period.

RuBPCase LS mRNA showed a different pattern of synthesis (and accumulation) during the cell cycle compared to the D-1 and D-2 mRNAs (Figs. 3 and 5). The peak rate of LS mRNA synthesis occurred at L3–L4, which was immediately followed by increased accumulation of LS mRNA during the mid to late light period (L3–L10). LS mRNA synthesis is noticeable during the dark and is apparently responsible for the continued accumulation of this mRNA during the dark period.

Rifampicin has been shown to inhibit chloroplast transcription and rRNA synthesis in *Chlamydomonas* (48).

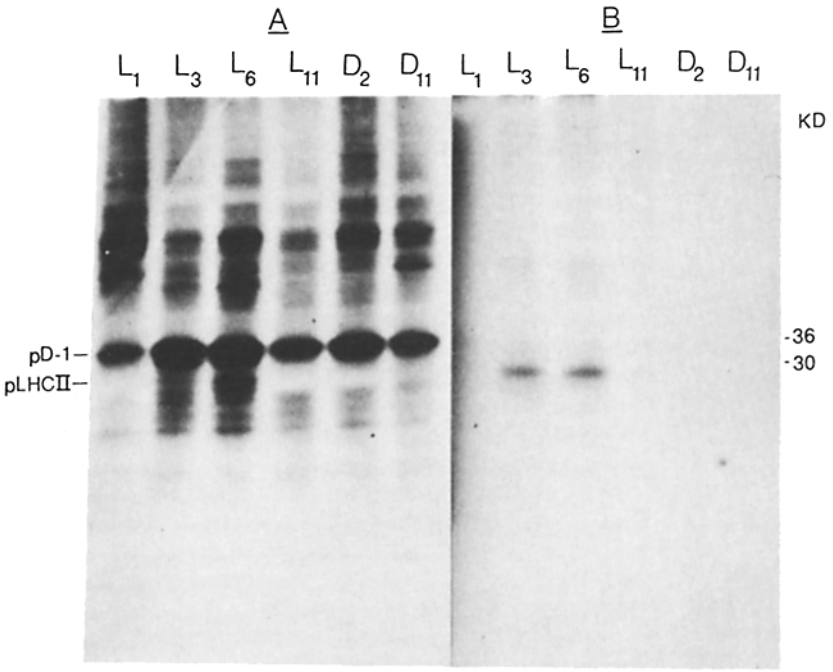

Figure 4. Translatable mRNA for D-1 and the major LHCII precursor during the cell cycle. Equal amounts of total RNA, isolated at the indicated times of the cell cycle, were translated in the reticulocyte lysate with [^{35}S]methionine; 5 μl of each translation assay, containing 2–3 × 10^5 cpm, were applied to the gel (*A*). For *B*, ~1 × 10^6 cpm of radioactive protein from each of the translations was immunoprecipitated with antiserum to the major LHCII protein and one half of each immunoprecipitate was electrophoresed on the same gel. The immunoprecipitation is representative of two experiments while total translation products were analyzed a number of times. SDS PAGE was performed on a 14% polyacrylamide gel. The precursors to D-1 (*pD-1*) and the major LHCII protein (*pLHCII*) can be identified among total translation products.

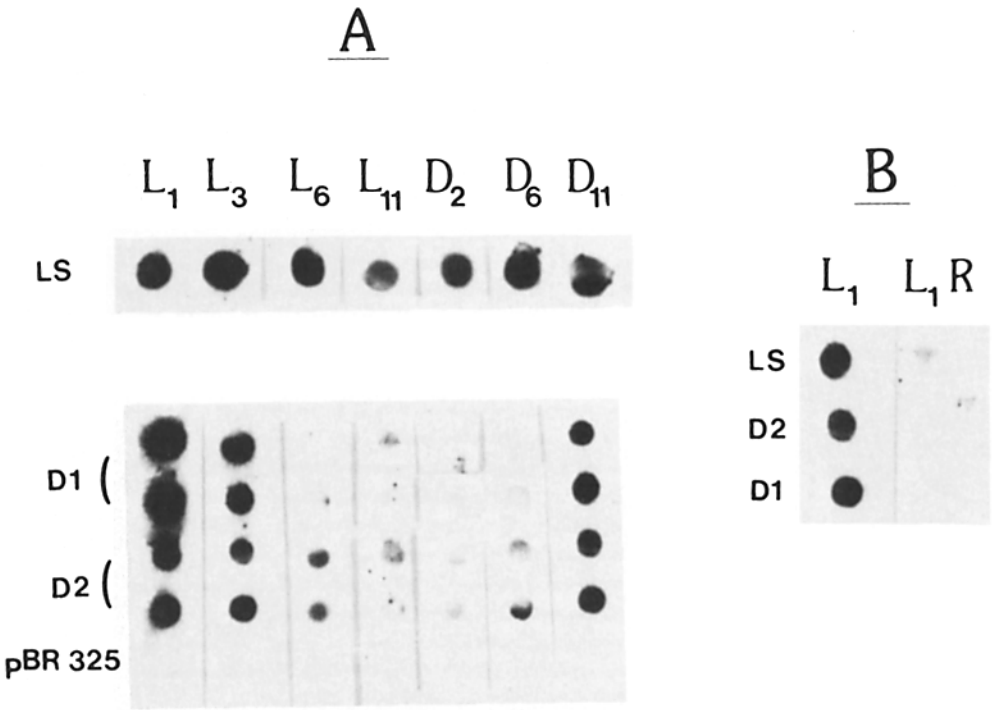

Figure 5. Synthesis of RuBPCase LS, D-1, and D-2 mRNAs during the cell cycle and inhibition by rifampicin. 10-μg aliquots of plasmids R15.4 (*LS*), pCP55 (*D-2*), pEC23 (*D-1*), and pBR325 (as a control) were bound to nitrocellulose and then 20 μg of RNA, extracted from cells pulse-labeled for 1 h with [^{32}P]PO_4 at the indicated times of the cell cycle, was hybridized to the filter-bound DNA for 72 h (*A*). In *B*, hybridization was carried out with RNA extracted from cells labeled with [^{32}P]PO_4 at L1, with (L_1R) and without (L_1) rifampicin present since L0. In *A*, plasmids pCP55 and pEC23 were spotted in duplicate while pBR325 and R15.4 were single determinations.

Labeling of synchronous cells with [^{32}P]PO_4 in the presence of rifampicin (250 μg/ml), and hybridization of isolated ^{32}P-RNA to cloned cpDNAs shows that this drug also inhibits transcription of chloroplast structural genes (Fig. 5 *B*). Labeling of D-1, D-2, and RuBPCase LS mRNAs was completely blocked by rifampicin while labeling of total high molecular weight RNA was inhibited only 50%. The absence of ^{32}P-RNA in the rifampicin-treated cells complementary to the cpDNA probes also confirms the specificity of the hybridization conditions for detecting pulse-labeled chloroplast mRNAs.

Effects of Rifampicin on D-1, D-2, and RuBPCase LS Synthesis

The correlation between high rates of D-1 and D-2 mRNA synthesis, increasing D-1 and D-2 mRNA levels (Fig. 3), and increasing synthesis of D-1 (Fig. 1) and D-2 polypeptides (23) during the first 6 h of the light period suggests that transcription may ultimately regulate the synthesis of these polypeptides during this period. This hypothesis was tested by inhibiting transcription with rifampicin during the first 5 h of the light period and the effect on D-1 and D-2 protein synthe-

sis examined by labeling cells with [^{3}H]arginine during the sixth hour of the light. Labeling with [^{3}H]arginine was carried out in the presence of cycloheximide so that synthesis of D-1 and D-2 could be visualized without interference from co-migrating cytoplasmically synthesized proteins. SDS PAGE analysis shows that rifampicin, added at L0, resulted in a substantial, but not complete, inhibition of the synthesis of polypeptides D-1 and D-2 during the period L5–L6 (Fig. 6). This result supports the observed correlations between mRNA and protein synthesis, and provides further evidence that the increasing rate of D-1 and D-2 polypeptide synthesis during the light depends to a large extent on transcription of these genes during the early light period.

In contrast to D-1 and D-2, RuBPCase LS polypeptide synthesis increases and peaks during the light period (20) before substantial increases in LS mRNA are observed (Fig. 3). Fig. 6 shows that inhibition of LS gene transcription during the period of L0–L5 with rifampicin resulted in no detectable inhibition of RuBPCase LS synthesis at L5–L6. We conclude that synthesis of RuBPCase LS polypeptide during the light period does not require de novo mRNA synthesis during the light. The ineffectiveness of rifampicin toward LS synthesis also argues against the possibility that secondary effects of the drug are responsible for the inhibition of D-1 and D-2 synthesis.

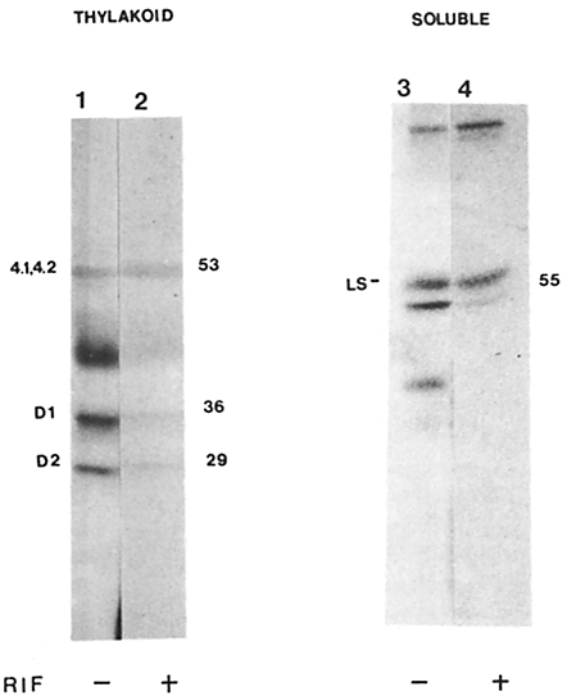

Figure 6. Rifampicin treatment during the light period inhibits synthesis of D-1 and D-2 but not RuBPCase LS. Rifampicin (250 μg/ml) was added to cells at L0, then at L5 the cells were pulse-labeled with [^{3}H]arginine for 1 h in the presence of cycloheximide. Soluble protein and thylakoid membranes were prepared, electrophoresed on 7.5–15% polyacrylamide gels, and fluorographed. Lanes *1* and *2* contained thylakoid membranes (equal chlorophyll) and lanes *3* and *4* contained equal amounts of soluble protein. Polypeptides D-1, D-2, and RuBPCase LS are identified to the left of the lanes, and M_r ($\times 10^{-3}$) to the right.

Discussion

Previous studies of the cell cycle expression of LHCII, tubulin, and other nuclear-encoded genes of *Chlamydomonas* have demonstrated control of mRNA levels (1). Accumulation of these mRNAs may be determined by transcriptional and/or posttranscriptional controls in the nucleus or post-transcriptional control over mRNA stability in the cytoplasm (3, 13). In contrast, the present analysis of three major chloroplast-encoded genes (rbcL, psbA, and psbD) shows that these mRNAs are abundant throughout the cell cycle. In addition, we have recently found that mRNAs encoding the 51- and 47-kD polypeptides of the PSII reaction center (the psbB and psbC genes, respectively) are also abundant throughout the light–dark cycle (our unpublished results). Although direct measurements of mRNA half-lives have not been made, comparison of the mRNA synthesis and mRNA accumulation data (Fig. 3) indicate that D-1, D-2, and LS mRNAs are relatively stable; synthesis of these mRNAs is periodic during the cell cycle and immediately succeeded by, or coincident with, mRNA accumulation. Also, under these same conditions, cytoplasmic mRNA for the major LHCII precursor disappears within a few hours of peak accumulation (Fig. 4 *B*). We conclude that differential synthesis of chloroplast-encoded proteins during the cell cycle involves strong control over mRNA translation. Moreover, these data indicate that the constitutive presence of these mRNAs is due in large part to stable molecules rather than continuous mRNA synthesis. Finally, this type of gene control, i.e., differential translation of stable mRNA, is relatively unique among proliferating cells (53), nor is it characteristic of gene expression in procaryotes or mitochondria, organisms and organelles whose translation systems have much in common with that of chloroplasts (25).

Thylakoid polypeptides D-1 and D-2 are partially homologous PSII components (45). It has also been suggested that they interact physically within the PSII reaction center core (12). Therefore, it is not surprising that the cell cycle programs for expression of these polypeptides are similar. Increased synthesis and accumulation of D-1 and D-2 mRNAs occurs during the first half of the light period coincident with increasing synthesis of the polypeptides. Further evidence of transcriptional control during the light was obtained with rifampicin which inhibited the increase in D-1, D-2 polypeptide synthesis. The inhibition of D-1, D-2 synthesis by rifampicin is consistent with the observations of Armstrong et al. (2) who showed that this drug inhibits the increase in photosynthetic oxygen evolution which normally occurs during the light period. The light-induction period of D-1, D-2 gene expression occupies only 25% of the cell cycle, however. For the remainder of the cell cycle D-1, D-2 mRNA levels decrease only 25–35% and for much of this time in the virtual absence of mRNA synthesis or translation. Thus, synthesis of D-1 and D-2 are regulated primarily at the transcriptional level early in the light period but at the translational level for most of the cell cycle. The similar cell cycle patterns of D-1, D-2 mRNA synthesis suggest that transcription of these genes is coordinatedly regulated. How the coordinate transcription of these genes could be achieved is not obvious, however. The single copy of the psbD gene is not closely linked to the two copies of the psbA gene on the chloroplast genome (17, 45). Also, sequence homology in the 5′ control regions of these genes was not indicated (45).

The enhanced transcription and translation of the psbA and psbD genes during the early light period may have a physiological as well as a developmental role in cell growth. Spudich and Sager (52) have shown that cell division during the dark period depends on photosynthetic electron transport through PSII during the first half of the light period. Based on the known physiological properties of D-1 (35), and the probable similarity of D-2 (45), it seems likely that the high rates of D-1, D-2 protein synthesis seen during the first half of the light period are necessary to sustain electron transport through PSII during this period.

Like D-1 and D-2, RuBPCase LS synthesis during the *Chlamydomonas* cell cycle is initiated by light and then increases and peaks during the light period; synthesis is low to undetectable during the dark period (20, 31). RuBPCase LS mRNA is also present at high levels throughout the cell cycle but, unlike D-1 and D-2, a significant increase in the steady-state mRNA level was not apparent until the last half of the light period (Fig. 3). Furthermore, blocking transcription of the rbcL gene with rifampicin did not inhibit the light-induced increase in LS polypeptide synthesis (Fig. 6). The lack of inhibition of LS synthesis by rifampicin is also consistent with the observations of Armstrong et al. (2) that rifampicin does not inhibit the increase in RuBPCase enzymic activity which normally occurs during the light period. We conclude that RuBPCase LS production is controlled primarily at the translational level and that the bulk of LS synthesis within a given cell cycle occurs on mRNA accumulated during previous growth cycles. It should be mentioned that we have not excluded the possibility of posttranslational mechanisms preventing accumulation of LS during the dark period.

Regulation of the psbD gene has not been studied previously, but expression of the psbA and rbcL genes has been investigated during chloroplast development in *Euglena* and higher plants. These studies have shown that levels of psbA and rbcL mRNAs increase during development indicative of transcriptional control (5, 37, 42, 43, 47, 50, 51). Recently, however, evidence has been presented that the increased levels of rbcL mRNA during greening are not equivalent with the degree of LS protein accumulation in peas (30), nor are they commensurate with the increased in organello rates of protein synthesis by plastids isolated during development in *Euglena* (41). These investigators have suggested the possibility of translational control for the rbcL gene during greening. In addition, Berry et al. (6) found that in dark-grown amaranth cotyledons, LS mRNA accumulated transiently and at a lower level than light-grown plants, but the mRNA persisted for 2 d after synthesis of the protein could not be detected by in vivo labeling. Thus, these studies suggest that translational regulation of rbcL expression is widespread, occurring in systems with light-dependent chloroplast biogenesis as well as during replication of the chloroplast in *Chlamydomonas*.

Evidence for translational regulation of the psbA gene in mature chloroplasts of the higher plant *Spirodela* has recently been presented (18). Light-grown plants, shifted into darkness, showed a substantial decline in D-1 synthesis as measured by in vivo labeling, but mRNA levels were relatively unaffected. This situation could be considered analogous to the light-dark transition in the *Chlamydomonas* cell cycle. However, in addition to in vivo labeling we show that there is no synthesis of D-1 in the dark by thylakoid polysomes, thus ruling out any possibility of synthesis and rapid degradation occurring in the dark. Furthermore, we propose that persistence of psbA mRNA in dark-shifted *Spirodela*, and rbcL mRNA in dark-grown amaranth (6), is due to stable mRNA molecules.

There are two important questions concerning translational regulation in the chloroplast of *Chlamydomonas*. What is the physical state and/or location of the untranslated mRNAs, and why are they not translated in the dark period of the cell cycle? Our preliminary results indicate that the psbA and psbD mRNAs remain bound to thylakoid membranes in the dark period, but whether they are sequestered in ribonucleoprotein particles or in some other form has not been determined. The absence of translation of these mRNAs during the dark period is apparently not a cell-cycle requirement (46), but may result from decreased ATP levels in the dark (54). These questions are currently under study.

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