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Relationship between Gene Dosage and Gene Expression in the Chloroplast of *Chlamydomonas reinhardtii*[1]

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ABSTRACT

Expression of three chloroplast genes encoding proteins of different chloroplast complexes and the rRNA gene has been examined in cells having reduced numbers of chloroplast genomes as a result of growth in the presence of the thymidine analog 5-fluorodeoxyuridine. While accumulation of total mRNA for *rpl2* (ribosomal protein L-1), *rbcL* (ribulose bisphosphate carboxylase large subunit) and *atpA* (α-subunit of ATP synthase) declined with gene copy number, the levels of translatable mRNA and rates of synthesis of these three proteins were largely unaffected. Accumulation of rRNA declined less precipitously than mRNA levels for the three proteins in response to the reduction in chloroplast genome number. *Chlamydomonas* appears to compensate for reductions in the number of chloroplast genomes at several different levels. Populations of cells with only one-fourth the wild-type amount of chloroplast DNA per cell on average have half the normal level of chloroplast ribosomes and nearly normal rates of CO_2 fixation and levels of specific chloroplast encoded proteins. These results suggest that normal cells accumulate a large excess of transcripts for chloroplast genes and that levels of expression of these genes are regulated by posttranscriptional mechanisms.

A central question in plant development is how the synthesis and assembly of chloroplast protein complexes are coordinately regulated. Genes encoding the polypeptides making up these complexes are divided between the nuclear and chloroplast genomes and the proteins themselves are synthesized in two separate cellular compartments. The number of genomic copies present in the nucleus and the chloroplast is vastly different, with the ratio ranging from *ca.* 1:80 in the single chloroplast of the haploid green alga *Chlamydomonas reinhardtii* (8) to >1:10,000 in the case of higher plant cells which may have 100 or more chloroplasts (19). Nonetheless, nuclear and chloroplast encoded polypeptides making up the chloroplast complexes are assembled in defined stoichiometric ratios. Although nuclear genes encoding chloroplast polypeptides often occur as small gene families (27), there are too few gene copies in most families to alter significantly the highly biased genomic ratio between nucleus and chloroplast. Furthermore, the number of chloroplast genomes per organelle and, in higher plants but not *Chlamydomonas*, the number of chloroplasts per cell may increase or decrease during development or in response to environmental change (19), whereas the nuclear genome number remains constant.

C. reinhardtii provides a unique system in which to explore the effects of variation in the number of chloroplast genomes on chloroplast gene expression. First, nuclear gene families in this alga characterized to date are small (8). For example the nuclear *rbcS* gene encoding the small subunit of Rubisco[4] is present in only two copies (6). Second, each *Chlamydomonas* cell contains a single large chloroplast with an average complement of about 80 genomes (8). Third, the number of copies of the chloroplast genome can be reduced experimentally to about 10 to 20% of the normal level, without affecting the amount of nuclear DNA, by growing the alga in the presence of the thymidine analog FdUrd. (30). The mechanism by which FdUrd is thought to inhibit selectively chloroplast DNA replication, but not nuclear DNA replication, has been discussed previously (30). Fourth, pulse-labeling experiments to measure rates of synthesis of specific proteins in the cytoplasm or the chloroplast are simple to perform and appropriate antibodies for immunoprecipitation are available. Finally, specific DNA probes exist for measuring mRNA accumulation of individual genes.

This paper examines the effect of reducing the chloroplast genome number on the expression of four chloroplast genes, *rbcL* which encodes the Rubisco LS, *rpl2*, which encodes a ribosomal protein homologous to *Escherichia coli* L2 and designated in *C. reinhardtii* as L-1 (20, 23), *atpA* encoding the α- subunit of the ATP synthase, and the 16S rRNA gene. These results suggest that posttranscriptional regulation plays an important role in controlling the expression of these genes and that *Chlamydomonas* is able to compensate for reductions in the numbers of chloroplast genomes at several different levels.

MATERIALS AND METHODS

Growth Conditions

The wild-type *Chlamydomonas reinhardtii* strain CC-125 used in these experiments was obtained from the *Chlamydo-*

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[4] Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase; FdUrd, 5-fluorodeoxyuridine; LS, large subunit; SSPE, 0.18 M NaCl, 10 mM NaH_2PO_4 (pH 7.4), 1.0 mM EDTA; kb, kilobase pair.

monas Genetics Center, Department of Botany, Duke University, Durham NC 27706. Cells were grown phototrophically in 300 mL cultures of high salt medium (8) at 25°C under high intensity cool white fluorescent light (200 μmol photons m^{-2} s^{-1} PAR) and bubbled with 5% CO_2 in air. All cultures were inoculated at a density of 1 to 2 × 10^4 cells/mL from a pregrowth culture without FdUrd, and FdUrd (Sigma) was added to the appropriate concentration from a sterile stock solution. Cells were grown for *ca.* seven generations to early log phase (1 to 3 × 10^6 per ml) prior to harvest for molecular analysis and for 7.5 to 9 generations (3 to 8 × 10^6 per mL) for biochemical, physiological, and ultrastructural analysis.

Antisera

Polyclonal antiserum against chloroplast ribosomal protein L-1 was prepared and characterized by Schmidt *et al.* (24). Polyclonal antiserum against Rubisco LS from garden pea was obtained from Dr. N.-H. Chua (Rockefeller University) and polyclonal antiserum against ATP-α of *C. reinhardtii* was from Dr. S. Merchant (University of California, Los Angeles).

Isolation and Quantification of RNA and DNA

Total cellular RNA from 2 × 10^8 cells harvested at a density of 1 × 10^6 cells/mL was extracted as described previously (25), except that successive LiCl precipitations were used (2). In a representative experiment we obtained 0.25 mg RNA/10^8 untreated cells and 0.28, 0.26, and 0.29 mg/10^8 cells grown in 0.1, 0.5, and 1.0 mM FdUrd, respectively. In higher plants, which have a far greater ratio of chloroplast to nuclear genomes than *C. reinhardtii* (>10,000:1 compared to 80:1), successive LiCl precipitations during RNA preparation reduce chloroplast DNA contamination to low or undetectable levels (1, 22). Northern blots of our RNA preparations probed with chloroplast genes such as *rpl2*, *atpA*, and *rbcL* routinely show hybridization to one or more bands of the predicted sizes for those genes, with no strong hybridization to either the high mol wt region where large fragments of the chloroplast genome would be expected to migrate or the low mol wt region that would contain a smear of RNA degradation products. Total cellular DNA from a similar number of cells was isolated as described previously (8) for analysis on dot blots. Slot blots of RNA and DNA were prepared by following Schleicher and Schuell Technical Literature No. 371, with each slot containing DNA or RNA (*ca.* 1 μg) extracted from an equal number of cells. As a control to ensure that the final autoradiographic signals of the experimental samples were within the linear response range of the film, a series of slots were loaded with various known amounts of DNA or RNA. DNA probes described below were nick-translated (specific activity from 1 to 3 × 10^8 cpm/μg) using enzymes from Bethesda Research Laboratories and [^{32}P]dATP from New England Nuclear (>3000 Ci/mmol). Unincorporated nucleotides from the nick-translation reactions were removed by chromatography with Elutip-d columns (Schleicher and Schuell). Blots were hybridized for 24 h at 42°C in roller bottles containing 5 mL of 4× SSPE (1× SSPE = 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.4], 1.0 mM EDTA), 50% formamide, 0.5% nonfat dry milk, 0.1% SDS, 50 μg/mL heparin, and 6 × 10^6 cpm of nick-translated probe. The blots were washed twice in 4× SSPE, 50% formamide, and 0.5% SDS at 45°C in the same bottles, transferred to pans, and washed twice with 0.1× SSPE, 0.2% SDS, and twice with 10 mM NaH_2PO_4 (pH 7.0), 0.2% SDS at 45°C before being dried and exposed to Kodak XAR-5 x-ray film.

Autoradiographic signals from slot blots were quantified using an LKB laser scanning densitometer and the signal peaks were integrated using an LKB Gel Scan program. The relative amounts of chloroplast and nuclear DNA were also measured in total cellular DNA extracts of 150 mL aliquots of mid-to-late log phase cells (3 to 8 × 10^6/mL) centrifuged to equilibrium in CsCl gradients in a Beckman model E ultracentrifuge (30). These gradients were quantified by recording the relative ultraviolet absorption of the DNA bands on Kodak commercial film No. 6127 and scanning the negatives with a Joyce-Loebl microdensitometer.

DNA Probes

The following probes for chloroplast genes were used: For r-protein L-1, a pUC18 plasmid containing a 230 base pair *Eco*RI/*Sau*3A fragment from the coding region of the *C. reinhardtii rpl2* gene with high homology to the *rpl2* genes from spinach and tobacco (26, 31). This *C. reinhardtii* gene was identified previously by heterologous hybridization (26) and further characterized by its ability to hybrid select mRNA for L-1 and by homology of its partial gene sequence to the comparable genes from higher plants (X-Q Liu, JP Hosler, JE Boynton, NW Gillham, unpublished results). For Rubisco LS, a pUC8 plasmid containing a 900 bp *Hin*dIII fragment completely internal to the *rbcL* coding sequence from *C. reinhardtii* (12). For 16S rRNA, a pUC8 plasmid containing the 0.9 kb *Eco*RI fragment from the 3′ end of the coding region of the 16S rRNA gene (9). For the ATP α-subunit, a pUC8 plasmid containing the 3.5 kb *Eco*RI 22 fragment which includes about 1.2 kb of the coding region of the *atpA* gene (29). Nuclear gene probes used included a pUC18 plasmid containing an 0.8 kb *Eco*RI cDNA fragment encoding the *rbcS* gene (6) and a pUC9 plasmid containing a 3.1 kb *Xho*I-*Sma*I fragment encoding the α_1-tubulin gene from *C. reinhardtii* (4). Entire plasmids, containing inserts, were used as probes since control experiments showed no signal using the vectors alone.

Measurements of Translatable mRNA

Total cellular RNA was translated in rabbit reticulocyte lysates (Bethesda Research Laboratories) at a concentration of 167 or 333 μg/mL following the methods specified by the supplier. The large subunit of Rubisco, r-protein L-1, and ATP synthase α-subunit were immunoprecipitated from the reaction mixtures by the method described previously (25), with the exception that the IgG-antigen complexes were eluted from the protein A-Sepharose beads by boiling in SDS sample buffer. Following SDS-PAGE and fluorography (25), the signal associated with each protein on the fluorogram was quantified by scanning densitometry as described above. Control experiments were performed to establish that sufficient anti-

body was added to each *in vitro* translation reaction to precipitate quantitatively the L-1 and Rubisco LS proteins and that the concentration of RNA was within the linear response range of the *in vitro* translation system.

In Vivo Labeling

Cells (6×10^7) were harvested by centrifugation from cultures grown to a density of 1 to 3×10^6 cells/mL and resuspended in 1 ml of high salt medium and incubated under 200 μmol photons m^{-2} s^{-1} PAR with gentle stirring. After 10 min equilibration, $H_2[^{35}S]O_4$ (ICN, carrier free, 43 Ci/mg S) was added to a concentration of 700 μCi/mL. Labeling was terminated after 10 min by removal of 0.2 mL of cells to 1 mL of cold 100% acetone and the precipitated protein resuspended in 60 μL of denaturing solution (25). Immunoprecipitation of L-1, Rubisco LS, and ATP-α, electrophoresis, and fluorography were as described for the *in vitro* translations. To quantify the relative rate of protein synthesis, the protein bands were located on the gel by overlaying the fluorogram and then excised. Gel slices were dissolved in 30% H_2O_2 at 60°C for 24 h, mixed with 10 mL of liquid scintillation cocktail (NEN Biofluor) and counted. Alternatively, the appropriate bands on the fluorogram were quantified by densitometry as described above. Both techniques gave the same results.

Biochemical, Physiological, and Ultrastructural Measurements

Aliquots of cells at a density of 3 to 8×10^6/mL were harvested for assay of levels of whole cell protein and Chl, chloroplast ribosome composition, and ultrastructure as described previously (3). Whole cell photosynthetic rates were measured as follows: 10 mL aliquots of cells, diluted with high salt medium to a concentration of 2×10^6 cells/mL, were equilibrated for 15 min in 25 mL flasks at 25°C under saturating white light (300 μmol photons m^{-2} s^{-1} PAR) from a GE GF56/2MFL flood lamp. Then 1.5 ml of cells and 0.5 mL of a reaction mixture (0.2 M Tris-HCl [pH 8.0], 40 mM bicarbonate, 0.2 mCi/mmol $[^{14}C]O_2$ [NEN]) were mixed in a 25 mL flask and incubated in a water bath shaker at 25°C under the same light intensity for 15 min. The reactions were stopped and unfixed CO_2 driven off by addition of 2 mL of 1 N HCl followed by aeration for several hours. A 100 μL aliquot from each flask was added to a xylene-based scintillation fluid and counted.

RESULTS

Effect of FdUrd on Chloroplast DNA Levels

Growth of *C. reinhardtii* in medium containing 0.1 to 1.0 mM concentrations of the thymidine analog FdUrd was shown previously (30) to result in the specific loss of up to 80% of the chloroplast DNA, as measured by pulse labeling and equilibrium density gradient centrifugation. This corresponds to a decrease in the average number of chloroplast genomes per cell from approximately 80 to less than 20. The content of nuclear DNA per cell was unaffected by growth in FdUrd at this concentration. In this study, we have verified these findings both by density gradient centrifugation of total cell DNA (Fig. 1A) and by using cloned sequences from specific chloroplast and nuclear genes as hybridization probes for total cellular DNA bound to nitrocellulose (Fig. 1B). Low (0.1 mM) concentrations of FdUrd led to a reduction in the *rpl2* gene sequences to 50% of their level in the untreated control (Fig. 1) after seven generations of growth, whereas total chloroplast DNA was reduced to 60% of the untreated control in cells grown for 8.5 generations in FdUrd (Table I). However, the

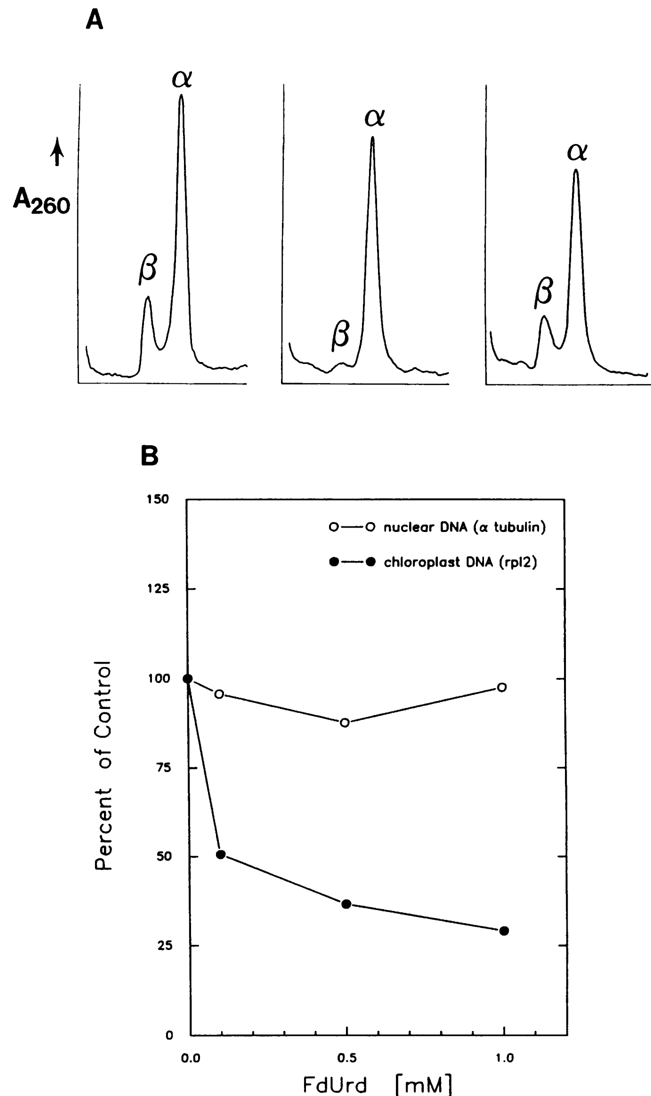


Figure 1. Thymidine analog FdUrd causes a selective reduction in levels of chloroplast DNA. A, UV absorbance profile of total cellular DNA isolated from control cells (*left panel*), cells grown for eight generations in 0.5 mM FdUrd (*center panel*) or in 0.5 mM FdUrd + 0.5 mM thymidine (*right panel*) and centrifuged to equilibrium in CsCl gradients. β = chloroplast DNA, 1.696 g/cm^3, α = nuclear DNA, 1.723 g/cm^3. B, Total cellular DNA isolated from cells grown in various concentrations of FdUrd for *ca.* seven generations was bound to nitrocellulose filters and hybridized to cloned probes for nuclear (α-tubulin) and chloroplast (*rpl2*) DNA. Each data point is the average of two representative hybridization experiments.

Table I. *Effect of Thymidine Analog FdUrd on Growth and Photosynthetic Capacity of C. reinhardtii*

Values for number of doublings, generation time, whole cell protein, Chl, and photosynthetic measurements are means from three separate experiments. Values for chloroplast DNA and ribosomes are from one of these experiments. In the same experiment, cell areas were estimated by tracing outlines of 10 cells from each FdUrd treatment in electron micrographs at 18,000× magnification selected without regard for normality of chloroplast ultrastructure. Standard errors for the cell size measurements ranged from 2.8 to 4.1 μm^2.

Parameter	FdUrd Concentration				
	Control	0.1 mM	0.5 mM	1.0 mM	0.5 mM + 0.5 mM thymidine
No. of cell doublings	9.1	8.5	7.8	7.5	8.0
Generation time (h)	5.6	6.0	6.5	6.8	6.4
Cell size (μm^2/median section)	32	39	44	47	39
Total cell protein (mg/10^9 cells)	21.9	18.8	28.8	34.1	26.4
Chloroplast DNA (% of total cell DNA)	18.4	11.1	4.5	3.5	19.8
Chloroplast ribosomes (% of total cell ribosomes)	35.1	25.8	16.9	17.5	35.8
Photosynthesis (μmol CO_2 fixed/h/10^9 cells)	104	105	80	59	107
Total Chl (mg/10^9 cells)	2.0	2.2	1.9	2.2	2.6

decline in chloroplast DNA levels occurred in a biphasic manner in response to increasing concentrations of the thymidine analog (Fig. 1B, Table I). A 10-fold increase in amount of FdUrd (from 0.1 to 1.0 mM) resulted in reduction of chloroplast DNA to only 20 or 30% of the control levels when measured on gradients or by hybridization, respectively. This biphasic curve does not result from incomplete dilution of preexisting chloroplast DNA at the higher FdUrd concentrations. An equilibrium would be approached after only three to four generations of growth when chloroplast DNA replication is inhibited by 50% (30). Addition of equimolar amounts of thymidine completely abolished the inhibitory effect of FdUrd on chloroplast DNA accumulation (Fig. 1; Table I). In contrast, the amount of nuclear DNA present in FdUrd grown cells, as measured using the cloned α-tubulin gene as a probe, was largely unaffected, indicating that nuclear DNA synthesis is not impaired by exogenous addition of the thymidine analog (Fig. 1B) (30). Similar results were obtained using the cloned *rbcL* and *rbcS* genes as probes to estimate chloroplast and nuclear gene copy number, respectively (data not shown).

Effect of FdUrd on Growth Rates, Chloroplast Ribosomes, Chloroplast Ultrastructure, and Photosynthesis

The reduction in chloroplast DNA per cell with increasing concentrations of FdUrd was accompanied by a modest increase in the generation time for photosynthetic growth (from 5.6 to 6.8 h) and an increase in both cell size and total cell protein (Table I). Rates of CO_2 fixation were not affected by reductions in chloroplast DNA of up to twofold (0.1 mM FdUrd), but were reduced by *ca.* 20% in cells with a fourfold reduction of chloroplast DNA (0.5 mM FdUrd). A 40% reduction in photosynthetic rates was seen in cells grown in 1.0 mM FdUrd, which had about 20% of the normal level of chloroplast DNA but normal Chl levels. In contrast, the levels of chloroplast ribosomes declined by 50% in cells grown in 0.5 mM FdUrd and were not further affected by doubling the concentration of the thymidine analog.

Ultrastructural studies revealed that about 40% of the cells in the cultures grown in 0.5 and 1.0 mM FdUrd (Fig. 2A) had defects in chloroplast organization of varying severity, reminiscent of those found in mutants defective in chloroplast ribosome assembly (3), whereas the remainder appeared to be indistinguishable from control cells (Fig. 2B). In contrast to the normal lamellar organization where the thylakoids are paired to form two- or three-disc grana, the chloroplast lamellae of the abnormal cells were either unpaired or abnormally stacked into giant grana. Cells grown on 0.1 mM FdUrd were all similar to control cells in terms of chloroplast structure. The presence of equimolar concentrations of thymidine in the FdUrd-grown culture normalized the effects of this analog on chloroplast ultrastructure. In summary, a greater than fivefold reduction in chloroplast DNA resulted in a twofold reduction in chloroplast ribosomes and a lesser reduction in CO_2 fixation, each of which depends on proteins encoded by the chloroplast genome.

Relationship of Chloroplast Gene Dosage to Chloroplast mRNA Levels

Nuclear and chloroplast gene probes were hybridized to slot blots of total cellular RNA isolated from cells grown in various concentrations of FdUrd. The accumulated levels of mRNA for r-protein L-1 and Rubisco LS declined in response to the drop in the copy number of the *rpl2* and *rbcL* genes (Fig. 3). This suggests that most copies of these chloroplast genes are actively transcribed and that the chloroplast does not compensate by greatly increasing transcription of the few remaining copies. Accumulation of 16S rRNA is much less affected by the decline in gene copy number (Fig. 4) than is the case for mRNA encoding the L-1 and Rubisco LS proteins. This might be due to stability of existing 16S rRNA

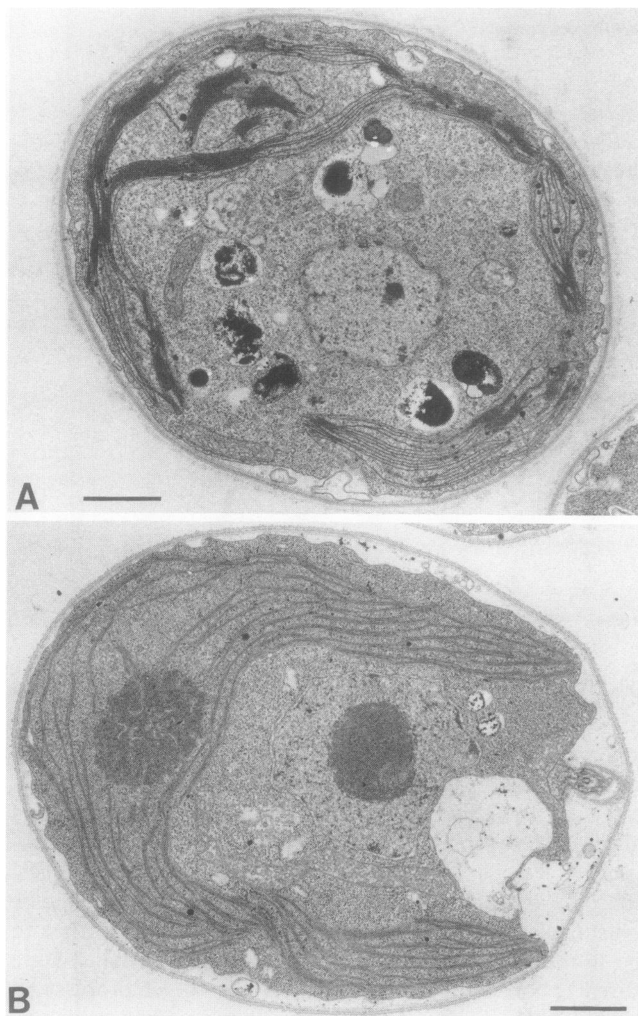


Figure 2. A, Median section through a cell of *C. reinnardtii* grown photosynthetically in 0.5 mM FdUrd for 8 to 9 generations. Chloroplast lamellae either remain unpaired or are stacked into abnormal giant grana, and the pyrenoid is absent. Bar = 1 μm. B, Median section through untreated control cell of *C. reinhardtii* grown photosynthetically. Single cup-shaped chloroplast with well-developed pyrenoid and lamellae paired to form two to three disc grana are evident. Bar = 1 μm.

molecules incorporated into chloroplast ribosomes, existence of two copies of the rRNA genes per chloroplast genome compared to the single copy protein genes, or increased transcription of the remaining rRNA genes.

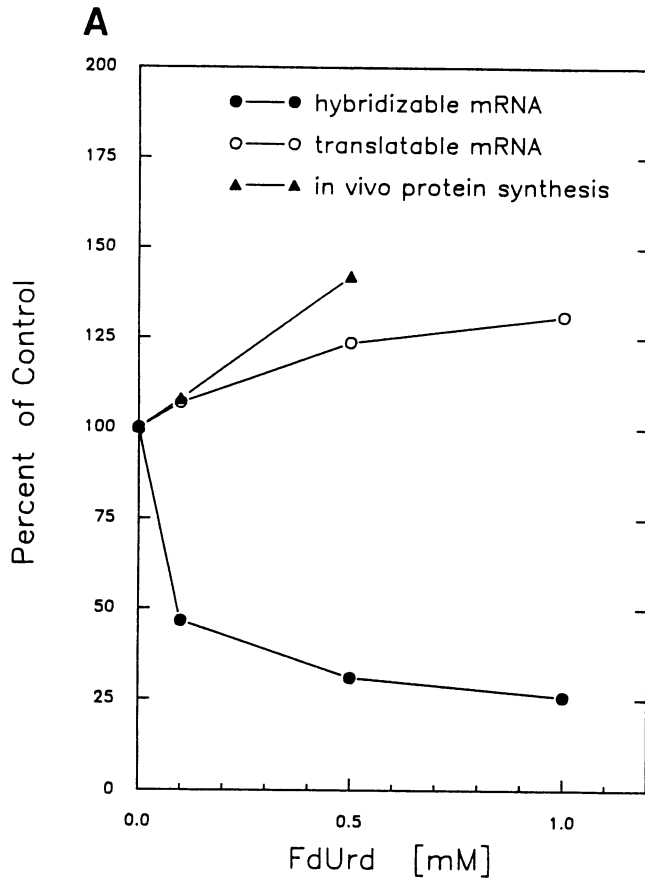
Relationship of Plastid Gene Dosage to Chloroplast Protein Synthesis

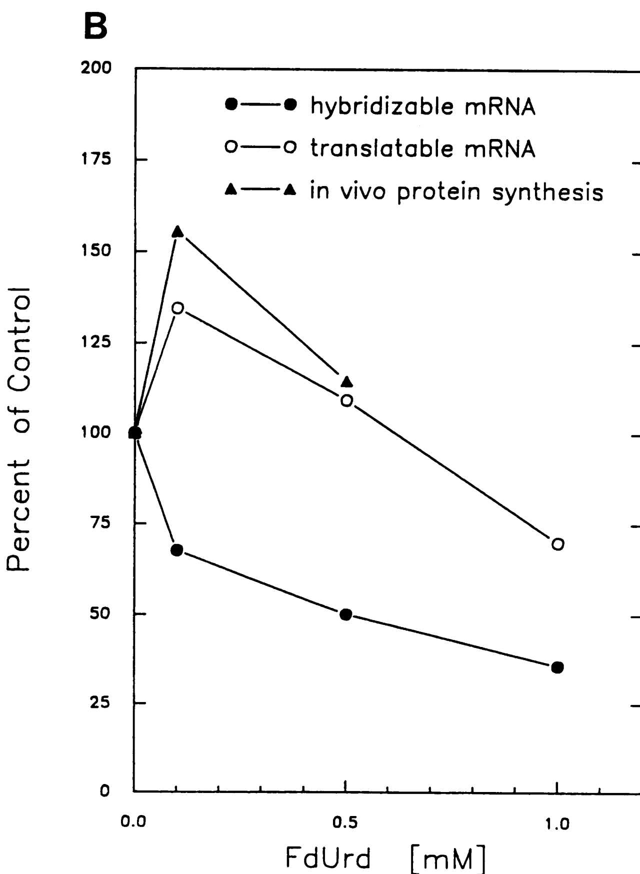
The relative rates of L-1 and Rubisco LS synthesis were determined by a 10 min pulse of cells grown in 0.1 and 0.5 mM FdUrd with $[^{35}S]O_4$, followed by immunoprecipitation, electrophoresis and fluorography of the proteins. Both proteins were synthesized at higher rates in cells grown in 0.1 and 0.5 mM FdUrd than in control cells (Figs. 3 and 5). This might result from increased energy reserves available for protein synthesis in the FdUrd-grown cells due to their greatly reduced synthesis of chloroplast DNA and RNA. The high level of synthesis of L-1 and Rubisco LS, under conditions where the mRNA levels for these proteins is reduced by 50 to 70%, clearly shows that the chloroplast of exponentially growing cells accumulates severalfold greater levels of mRNA than are required to maintain the observed level of synthesis of these two proteins. Similar experiments were not performed with cells grown in 1.0 mM FdUrd because their reduced photosynthetic rate could complicate interpretation of the results.

Relationship of Plastid Gene Dosage to Translatable mRNA Levels

Accumulation of mRNA in excess of amounts necessary to make normal levels of L-1 and Rubisco LS raises the question of whether all mRNA for these two proteins is synthesized in a translatable form, or whether a large pool of non-translatable mRNA is synthesized and subsequently processed to yield mRNA capable of being translated on chloroplast ribosomes. To differentiate between these two possibilities, total cellular RNA from cells grown in different concentrations of FdUrd was translated in rabbit reticulocyte lysates and the labeled L-1 and Rubisco LS polypeptides synthesized were immunoprecipitated and separated on acrylamide gels, and fluorographs of the gels were quantified by densitometry. If a single pool of translatable mRNA for each protein were present within the chloroplast, the levels of translatable mRNA should decline in the same manner as the total mRNA levels in cells having reduced amounts of chloroplast DNA. This should be true even if specific factors modulate translation of these mRNAs within the chloroplast, since the rabbit reticulocyte translation system would be highly unlikely to contain such factors. However the data (Figs. 3 and 5) show that the levels of translatable mRNA for L-1 and Rubisco LS do not decline in parallel with the levels of total mRNA in response to the FdUrd-induced reduction of chloroplast DNA. Rather, the levels of translatable mRNA correlate well with the observed rates of synthesis of the L-1 and Rubisco LS proteins (Figs. 3 and 5). This suggests that two pools of mRNA exist in the chloroplast and that the size of the smaller translatable pool is maintained even as the larger pool of nontranslatable mRNA decreases by 50 to 70%. Differences seen in amounts of translatable RNA and rates of *in vivo* synthesis between Rubisco LS and r-protein L-1 at higher FdUrd concentrations possibly reflect differences in posttranscriptional regulation of these two genes (11).

All of the experiments presented above for expression of the *rbcL* and *rpl2* genes were also performed for the *atpA* gene encoding the α-subunit of the chloroplast ATP synthase. Results for the α-subunit of the ATP synthase (data not shown) were similar to those obtained for r-protein L-1 in terms of hybridizable mRNA levels and to Rubisco LS in terms of translatable RNA levels and protein synthesis. Thus our observations indicate that only a small fraction of the normal number of gene copies and corresponding mRNAs encoding proteins of three separate chloroplast complexes are necessary for synthesis of normal amounts of these chloroplast components.





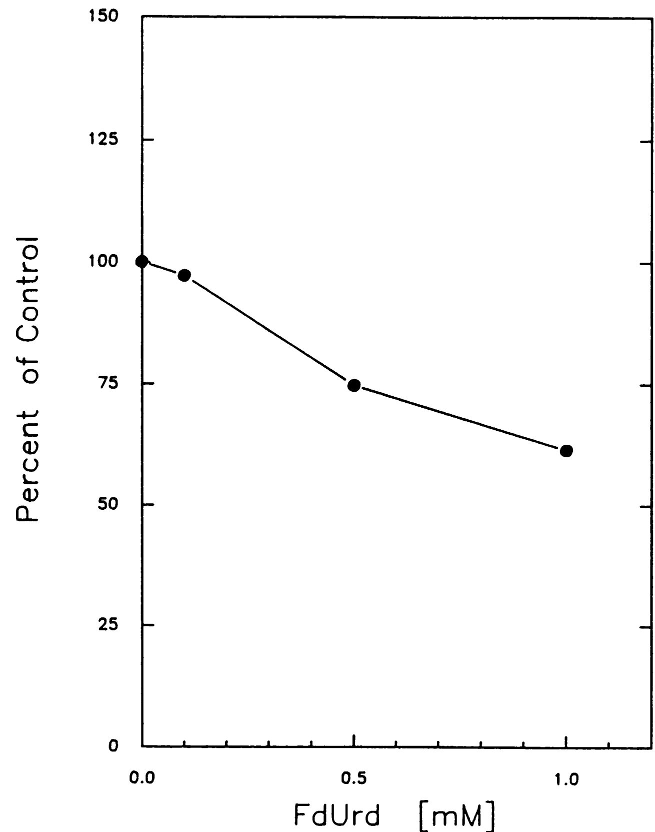


Figure 4. Accumulation of chloroplast 16S rRNA in cells grown in various concentrations of FdUrd. Each data point is an average of three experiments and has a standard error equal to or less than 10%.

DISCUSSION

Our results lead to four main conclusions regarding the expression of chloroplast genes in *C. reinhardtii*. First, they confirm those published earlier (30) that growth of this alga in low concentrations of the thymidine analog FdUrd leads to a reduction in chloroplast DNA copy number, whereas nuclear DNA levels and growth rate are largely unaffected. The reduction in the amount of chloroplast DNA per cell seen in a population of FdUrd grown cells likely occurs as a distribution around the mean value, since individual FdUrd-grown cells stained with 4′6-diamidino-2-phenylindole show considerable variation in the number and size of chloroplast nucleoids (13) and cells grown at 0.5 and 1 mM FdUrd show varying degrees of abnormalities in chloroplast ultrastructure. Second, transcription of the chloroplast genes encoding r-protein L-1, Rubisco LS and ATP-α appears to be constitutive, in that reductions in template levels are paralleled by a

Figure 3. Accumulation of hybridizable and translatable mRNA and rates of synthesis of the chloroplast ribosomal protein L-1 (A) and the large subunit of Rubisco (B) in cells grown in various concentrations of FdUrd for 7 generations. Data points for hybridizable mRNA accumulation are averages of three determinations for equal numbers of cells, with each point having a standard error equal to or less than 10%. Representative experiments are presented for the levels of translatable mRNA, loaded on an equal A_{260} basis, and for the relative rates of protein synthesis for equal numbers of cells.

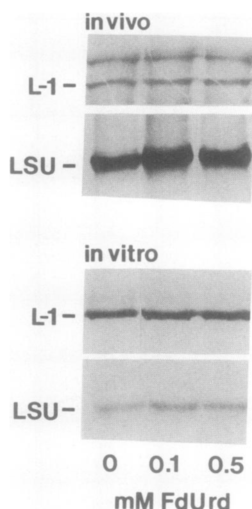


Figure 5. Effect of growth of cells in FdUrd on synthesis of chloroplast r-protein L-1 and Rubisco large subunit (LSU) *in vivo* and *in vitro*. Rates of synthesis *in vivo* were measured in cells pulse-labeled with $[^{35}S]O_4$ for 10 min. Immunoprecipitated protein from equal numbers of cells was loaded in each lane (see "Materials and Methods"). Relative levels of translatable mRNA *in vitro* were determined in a rabbit reticulocyte lysate system (see "Materials and Methods") using 333 μg/mL total RNA for each L-1 lane and 167 μg/mL for each LSU lane.

similar lowering of transcript levels. Third, the normal 80-fold copy number of these three genes is not required to maintain high levels of synthesis of proteins encoded by the chloroplast genome. Moreover, since the photosynthetic capacity of the cells is not greatly reduced by a fourfold reduction in the number of chloroplast genomes seen in cells grown in 0.5 mM FdUrd, one can predict that the high copy number of other chloroplast genes encoding proteins involved in photosynthesis or protein synthesis may not be required for normal rates of photosynthetic growth. Fourth, the chloroplast apparently accumulates excess mRNA for r-protein L-1, Rubisco LS and ATP-α, and only a fraction of the mRNA present for these proteins is translated. If this were not the case, one would expect the decline in hybridizable mRNA levels observed with increasing FdUrd concentrations to be paralleled by a similar decline in the amounts of translatable mRNA measured *in vitro*. Instead, much smaller changes are seen in the levels of mRNA translatable *in vitro*.

The observation that accumulation of the *rpl2*, *rbcL*, and *atpA* transcripts appears to be controlled largely by chloroplast gene dosage in *C. reinhardtii* is similar to findings made in several studies with chloroplasts of higher plants (1, 10, 14, 21). Our finding that the level of 16S rRNA accumulation is affected somewhat less than levels of *rbcL* and *rpl2* mRNA by a reduction in the copy number of these chloroplast genes may be attributable to differences in the relative stability of these RNAs. The 16S rRNA is very likely protected by its association with the proteins of the chloroplast ribosome (see 14), and in fact, the level of 16S rRNA accumulation (Fig. 4) correlates quite well with measurements of chloroplast ribosomes in FdUrd grown cells (Table I).

Numerous studies have shown the existence of post-transcriptional control mechanisms for synthesis of various proteins within chloroplasts (see 7, 15 for general reviews; 11). Many of these reports demonstrate translational control of protein synthesis during greening, *i.e.*, translatable mRNA for a particular protein is present, but is not translated *in vivo*. In our experiments with exponentially growing phototrophic cells of *C. reinhardtii*, protein synthesis rates are always high and the cells appear to control synthesis of the L-1, Rubisco LS and ATP-α polypeptides posttranscriptionally, possibly by conversion of nontranslatable mRNA to a translatable form. Under normal growth conditions, transcripts of these three genes accumulate to levels beyond those required for normal rates of protein synthesis. Hence the alga may have evolved posttranscriptional mechanisms to prevent the synthesis of excess proteins.

Several chloroplast mRNAs have been shown to undergo posttranscriptional processing, but less is known about the extent to which chloroplast mRNA processing limits the rate of protein synthesis. In spinach, pea, and maize chloroplasts, *rbcL* transcripts are processed by removal of 115 to 235 bases from their 5′ ends (16). In chloroplasts from pea leaves (16) and greening maize leaves (5), the smaller *rbcL* transcript is less abundant. Similar processing events in *C. reinhardtii* could account for the existence of translatable and nontranslatable mRNA pools. In *Petunia*, *rbcL* transcripts from a green cell suspension culture are not translated by rabbit reticulocyte lysates while *rbcL* transcripts isolated from chloroplasts of mature leaves are translatable by this *in vitro* system (28).

Transcription of all chloroplast rRNA gene copies in a cell at maximal levels is clearly not essential for synthesis of normal amounts of chloroplast rRNA. This is supported by our observation that chloroplast rRNA declines less precipitously than chloroplast DNA in FdUrd grown cells and the previous finding that a chloroplast deletion mutant of *C. reinhardtii* with only one set of rRNA genes per genome makes normal levels of chloroplast ribosomes (17). Furthermore, peas and certain related legume species which have only one set of rRNA genes per genome (reviewed in 18) instead of the usual two, have normal photosynthetic rates and presumably have normal levels of chloroplast ribosomes. Nonetheless, the amount of chloroplast rRNA made may limit the production of chloroplast ribosomes since both 16S rRNA accumulation and the number of chloroplast ribosomes decline in cells with reduced numbers of chloroplast genomes, while the rate of synthesis of at least one r-protein remains high.

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