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Searching limiting steps in the expression of chloroplast-encoded proteins: relations between gene copy number, transcription, transcript abundance and translation rate in the chloroplast of *Chlamydomonas reinhardtii*

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Summary

We performed a systematic investigation of the quantitative relationship between genome copy number, transcription, transcript abundance and synthesis of photosynthetic proteins in the chloroplast of the green algae *Chlamydomonas reinhardtii* grown either in mixotrophic or phototrophic conditions. The chloroplast gene copy number is lower in the latter condition and the half-life and accumulation levels of most chloroplast transcripts are significantly reduced, although the relative rates of protein synthesis remain similar. Our study shows that, in most instances, chloroplast protein synthesis is poorly sensitive to changes in gene copy number or transcript abundance in the chloroplast. Treatment with 5-fluoro-2′-deoxyuridine, that inhibits chloroplast DNA replication and decreases extensively the number of copies of the chloroplast genome, had limited effects on the abundance of most chloroplast transcripts and little if any effect on the rates of protein synthesis. When using rifampicin, that selectively inhibits chloroplast transcription, we found no direct correlation between the level of transcripts remaining in the chloroplast and the rates of chloroplast protein synthesis. For two chloroplast genes, a 90% decrease in the amount of transcript did not cause a drop in the rate of synthesis of the corresponding protein product. Overall, our results demonstrate that there is no gene dosage effect in the chloroplast and that transcript abundance is not limiting in the expression of chloroplast-encoded protein.

Keywords: chloroplast, gene expression, rifampicin, FdUrd, *Chlamydomonas*.

Introduction

Transcriptional regulation of gene expression is widespread in the prokaryote kingdom, mainly because translation essentially occurs co-transcriptionally. As chloroplasts derive from ancestral cyanobacteria, but have evolved as endosymbiotic organelles, it is of interest to investigate the different regulatory mechanisms that underlie the expression of the chloroplast genome. In higher plants, it has been shown that regulation of chloroplast transcription is important with respect to the response of chloroplast gene expression to light changes and in the various developmental phases leading from proplastids to differentiated chloroplasts (Allison, 2000; Kuhlemeier, 1992; Mullet, 1993; Stern *et al.*, 1997). In *C. reinhardtii*, transcriptional activity of chloroplast genes is modulated by a circadian rhythm, and may to some extent be under nuclear control (Kawazoe *et al.*, 2000; Leu *et al.*, 1990). However, there is now overwhelming evidence for the predominance of post-transcriptional control in chloroplast gene expression that encompasses transcript maturation, stabilization and/or translational activation, these steps being controlled by general — as well as by target-specific — nucleus-encoded factors (for recent reviews see Barkan and Goldschmidt-Clermont, 2000; Hauser *et al.*, 1998; Rochaix, 2001; Stern and Drager, 1998; Wollman *et al.*, 1999; Zerges, 2000). Taken together, the studies on chloroplast gene expression have not yet defined the extent to which rates of transcription and transcript abundance actually control protein synthesis in the chloroplast. Furthermore, the possible influence of

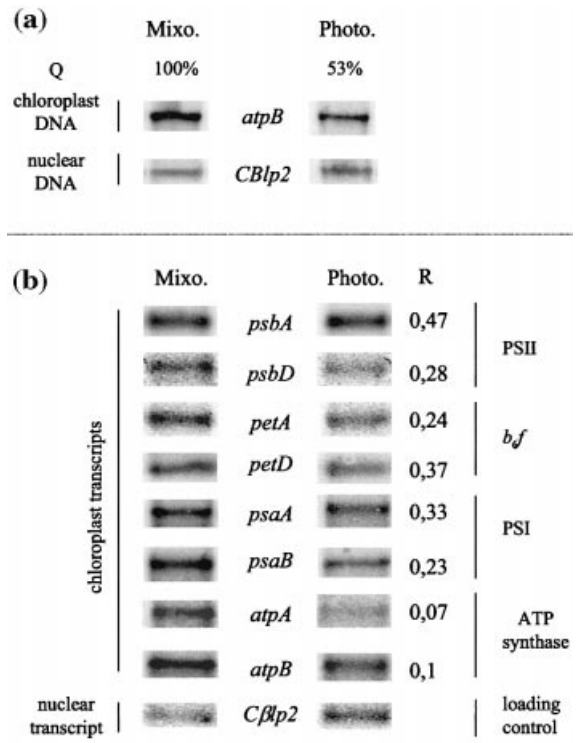

Figure 1. Panel a: chloroplast genome content (assayed by southern-blot experiments).
Q = relative chloroplast genome content in the indicated growth conditions. Signals were normalized to the signal obtained with the nuclear probe *Cβlp2*.
Panel b: overall mRNA accumulation levels (assayed by northern-blot experiments) in cells grown either in mixotrophic (left panel) or phototrophic (right panel) conditions.
R = ratio of the mRNA accumulation levels in phototrophic conditions to the levels obtained in mixotrophic conditions. Note that the lane corresponding to phototrophic conditions is overloaded as shown by the signal obtained for *Cβlp2*.

carbon metabolism on the expression of the chloroplast genome has not yet been systematically investigated.

The organelle genome is highly polyploid. When the unicellular green alga *C. reinhardtii* is grown heterotrophically, the unique chloroplast contains about 80 copies of a circular chromosome (Lau *et al.*, 2000; Rochaix, 1995), whereas in higher plants, a single plastid contains from 20 to 300 genome copies (Mullet, 1993; for a review see Sugiura, 1995). In addition, a single plant cell can accommodate up to 100 plastids. Thus, there is an important unbalance in gene copy number between the highly polyploid chloroplasts and the nucleus of a plant cell. This raises intriguing questions about gene dosage for protein expression in the chloroplast, given the composition of the major chloroplast protein complexes, whose nuclear and chloroplast-encoded subunits are present, in most cases, in a 1–1 stoichiometry. In this study we examined the relation between chloroplast genome copy number, transcription, transcript abundance and protein synthesis rates in the chloroplast of the green algae *C. reinhardtii.* To this end we combined approaches similar to those of Goodenough (1971) and Hosler *et al.* (1989) who attempted either to inhibit transcription or to modify chloroplast gene copy number. We show that despite contrasted effects of changes in gene copy number on the accumulation of some chloroplast transcripts, chloroplast protein synthesis is mostly insensitive to a decrease in gene copy number and that there is no close correlation between transcript abundance and translation rate. Based on recent studies (Heifetz *et al.*, 2000), we also wished to take into account a possible influence of carbon metabolism on the pattern of chloroplast gene expression. Therefore, both sets of experiments were undertaken with algae grown either in mixotrophic or in phototrophic conditions. These differential growth conditions reflect (i) ideal growth conditions commonly used for photosynthetic mutants, i.e. acetate containing medium and low-light (4 $\mu E\ m^{-2}\ sec^{-1}$) and (ii) truly phototrophic conditions for strains that are not impaired in photosynthesis, i.e. minimum medium with bubbling of 5% CO_2(g) under higher light intensity (20 $\mu E\ m^{-2}\ sec^{-1}$).

Results

Chloroplast gene copy number and transcript accumulation levels for cells grown under mixotrophic or phototrophic conditions

To assess the contribution of gene copy number and transcript abundance to the expression level of chloroplast proteins, we first compared these figures in the two growth conditions widely used for *C. reinhardtii* cells, i.e. in strictly phototrophic and in mixotrophic conditions. DNA was extracted from the two types of cell cultures and analysed by DNA-filter hybridization experiments. The results obtained for the *atpB* gene, taken as a marker of the content in chloroplast DNA, were quantified and normalized to the signal obtained for the nuclear gene *Cβlp2* (Figure 1a). Cells grown under mixotrophic conditions displayed a chloroplast genome content twice as high as that of cells grown in phototrophic growth conditions, in agreement with the report of Lau *et al.* (2000). Transcript accumulation levels were monitored by RNA-filter hybridization experiments and are shown in Figure 1b. The signals obtained for various chloroplast transcripts were quantified and normalized to the signal for the nuclear *Cβlp2* transcript. For *psbD, petA, petD, psaA* and *psaB,* mRNA accumulation levels were reduced about three times in cells grown in phototrophic growth conditions when compared to cells grown in mixotrophic growth conditions. The *atpA* and *atpB* transcripts were more affected, their accumulation levels being about 10 times lower in phototrophic conditions. In contrast, *psbA*

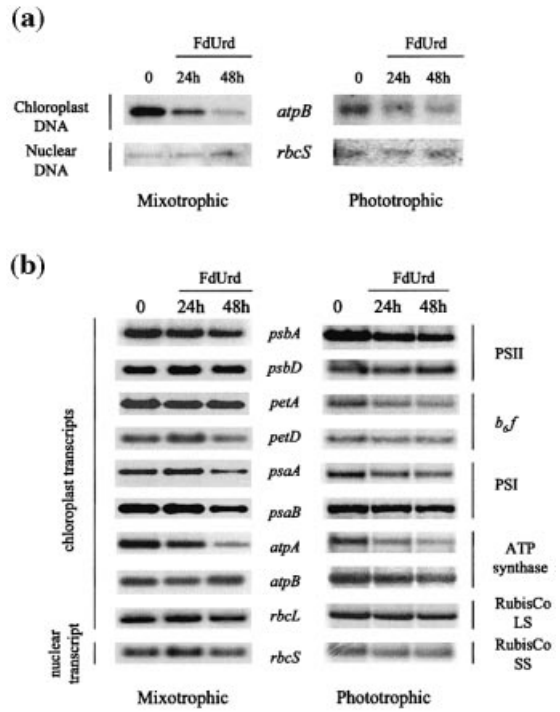

Figure 2. Panel a: Reduction in chloroplast genome copy number after FdUrd treatment for cells grown mixotrophically (left panel) or phototrophically (right panel). The DNA-filter hybridizations show the signals obtained for the chloroplast *atpB* and the nuclear *rbcS* genes, after 24 h or 48 h of treatment with 0.5 mM FdUrd.
Panel b: Transcript accumulation levels after FdUrd treatment. The RNA-filter hybridizations show the overall transcript accumulation levels after 24 h or 48 h of 0.5 mM FdUrd treatment for various transcripts, from cells grown either mixotrophically (left) or phototrophically (right). The nuclear transcript *rbcS* serves as a loading control.

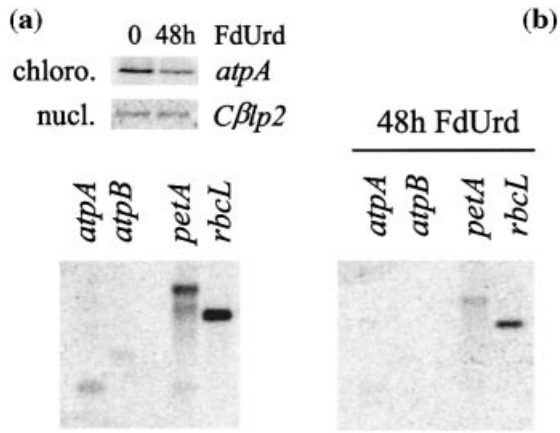

Figure 3. Panel a: Reduction of the chloroplast gene copy number upon FdUrd treatment assayed by DNA filter-hybridization. *atpA:* chloroplast gene; *Cβlp2:* nuclear gene. Reduction of the chloroplast genome copy number was close to three in this experiment.
Panel b: RNA-pulse-labelling experiment in FdUrd treated cells. Cells grown for 48 h in the presence (right) or absence (left) of 0.5 mM FdUrd were pulse-labelled using thaw/freeze permeabilized cells according to Gagne and Guertin (1992) with α-^{33}P-UTP for 15 min in order to detect transcription of chloroplast genes. The autoradiogram of labelled neotranscripts hybridized to DNA restriction fragments fixed on a nylon membrane is shown. An overall drop in transcription yield upon FdUrd treatment is observed. Similar results were obtained with another RNA pulse-labelling method using toluene-permeabilized cells as described in Guertin and Bellemare (1979) (not shown).

transcripts still accumulated in phototrophic conditions to about 50% of their level in mixotrophic conditions.

Changes in transcript abundance upon a FdUrd treatment

The thymidine analog 5-fluoro-2′-deoxyuridine (FdUrd) is known to inhibit specifically the chloroplast thymidilate synthase reducing further accumulation of thymidine in the chloroplast (Wurtz *et al.*, 1977). Incubation of *C. reinhardtii* cells with 1 mM FdUrd for a week or longer induces mutations in the chloroplast genome (Wurtz *et al.*, 1979). However, treatment of cells at lower FdUrd concentrations (0,5 mM) and for shorter time periods (48 h) has limited mutagenic effects while it still inhibits chloroplast DNA replication. Cells thus display a drop in the number of copies of the chloroplast chromosome after several rounds of mitotic divisions (Hosler *et al.*, 1989; Lau *et al.*, 2000; Matagne and Hermesse, 1981; Wurtz *et al.*, 1977). Here, we monitored the reduction in chloroplast genome copy number after 24 h and 48 h of FdUrd treatment by DNA-filter hybridization experiments (Figure 2a). Taking the *atpB* gene as a marker of the chloroplast genome, we observed the expected drop in genome copy number upon FdUrd treatment. As a control, we used a probe against the *rbcS* and/or *Cβlp2* (not shown) genes whose abundances are insensitive to FdUrd, because of their nuclear origin. We observed variations in the extent of reduction in chloroplast gene copy number in independent experiments relative to one growth condition, probably because of the non-irreversible but competitive mode of inhibition of FdUrd on chloroplast replication (Hosler *et al.*, 1989) and/or changes in the chloroplast content for thymidine, that should be affected by the metabolic state of the cell. Furthermore, reduction of chloroplast gene copy number appeared to be more pronounced when cells were grown mixotrophically. The decrease in chloroplast genome copy number for cells grown phototrophically showed an average reduction factor of 2 that is close to previous reports by Matagne and Hermesse (1981) and Hosler *et al.* (1989), whereas for cells grown mixotrophically, the decrease in genome copy number was consistantly larger, reaching as much as 20 in one experiment (close to the report by Lau *et al.*, 2000).

The rate of chloroplast transcription before and after a 48-h FdUrd treatment was then assayed by RNA-pulse labelling experiments. To this end we used either freeze-thaw-permeabilized (Gagne and Guertin, 1992) or toluene-permeabilized cells (Guertin and Bellemare, 1979). Both

Table 1. Chloroplast transcript accumulation after FdUrd or rifampicin treatment The table gives the mean value of the ratio of mRNA accumulation levels after 48 h of treatment with FdUr or 6 h of treatment with rifampicin to the accumulation levels in the untreated control. Northern blots were performed from one to five times, depending on the experiment. Signals obtained for the different chloroplast transcripts were normalized to the accumulation levels of the unaffected Cβlp2 and/or rbcS transcripts, of nuclear origin Standard deviations to the mean value of several experiments are indicated in brackets for the rifampicin experiment. As the ratio of reduction in chloroplast gene copy number fluctuated between independent experiments (see text), accumulation levels shown for the FdUrd treatment are relative to the described experiment only, and thus no standard deviation is indicated

	48h FdUrd		6h rifampicin	
Transcript	Mixo.	Photo.	Mixo.	Photo.
psbA	0.5	0.5	0.51(+/-0.01)	0.59(+/-0.08)
psbD	0.8	1.2	0.24(+/-0.13)	0.5(+/-0.13)
petA	0.9	0.4	0.30(+/-0.07)	0.02(+/-0.01)
petD	0.6	1	0.42(+/-0.06)	0.10(+/-0.01)
psaA	0.25	0.7	0.32(+/-0.03)	0.07(+/-0.01)
psaB	0.5	0.6	0.22(+/-0.01)	0.09(+/-0.01)
atpA	0.18	0.4	0.12(+/-0.08)	0.02(+/-0.01)
atpB	0.9	0.9	0.66(+/-0.14)	0.07(+/-0.02)
rbcL	1	0.9	0.38(+/-0.03)	0.24(+/-0.01)

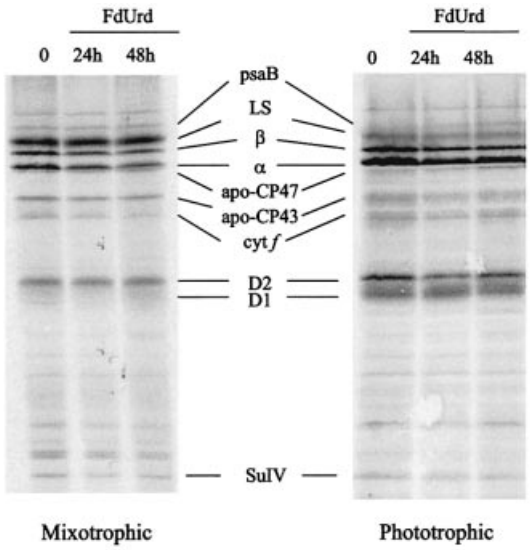

Figure 4. Chloroplast protein synthesis after FdUrd treatment.
Cells were pulse-labelled in presence of cycloheximide (inhibitor of cytoplasmic translation) with ^{14}C-acetate for 5 min after 24 h or 48 h of 0.5 mM FdUrd treatment for cells grown either mixotrophically (left panel) or phototrophically (right panel). An equal amount of cells was loaded onto each lane. Correspondence between genes and proteins are as follows: PSII complex: *psbC* (apo-CP43), *psbB* (apo-CP47), *psbD* (D2) and *psbA* (D1). The b_6f complex: *petA* (cyt *f*) and *petD* (SuIV). The PSI complex: *psaB* (psaB). The ATP synthase complex: *atpA* (α) and *atpB* (β). RubisCo: *rbcL* (LS, large subunit). The pulse-labelling experiments were carried out on the same samples that were used for RNA extraction and analysis.

methods gave the same qualitative information that is illustrated in Figure 3 in the case of thaw/freeze permeabilized cells. The extent of FdUrd-induced reduction in chloroplast gene copy number was approximately 3 in this particular experiment (Figure 3a). It was accompanied by a parallel decrease in the transcription rate of the four genes that we assayed, *atpA*, *atpB*, *petA* and *rbcL* (Figure 3b). This observation suggests that most if not all, copies of the chloroplast chromosome are transcriptionally active in *Chlamydomonas*. We then assayed the consequences of the decreased rate of chloroplast transcription, due to the reduction in genome copy number, on the abundance of various chloroplast transcripts (Figure 2b and Table 1). RNA-filter hybridization signals obtained for nine chloroplast transcripts, representative of the mRNAs for subunits of the five major photosynthetic complexes, PSII, PSI, Cytb_6f, ATP-synthase and RubisCo, were quantified and normalized to the signal obtained for the nuclear transcript *rbcS*. Given the fact that the extent of reduction in chloroplast genome content was different in independent experiments (see above), only one experiment by growth condition is shown, and therefore no standard deviations are indicated for the transcript accumulation levels. However, the same qualitative variations of the various transcript accumulation levels were reproducibly observed in independent experiments. Under mixotrophic growth conditions (Figure 2b, left panel), four transcripts, *psbD, petA*, *atpB* and *rbcL*, showed little if any changes after 48 h of FdUrd treatment. Three transcripts, *psaB*, *psbA* and *petD*, showed a significant but limited decrease reaching about 50% of their initial level after 48 h of treatment. Last, two transcripts, *atpA* and *psaA*, decreased extensively after 48 h of treatment, reaching about 20% of their initial accumulation level. Most of the chloroplast transcripts tested behaved similarly when the FdUrd treatment was applied to algae grown in phototrophic conditions. For instance the levels of *psbD*, *atpB* and *rbcL* transcripts

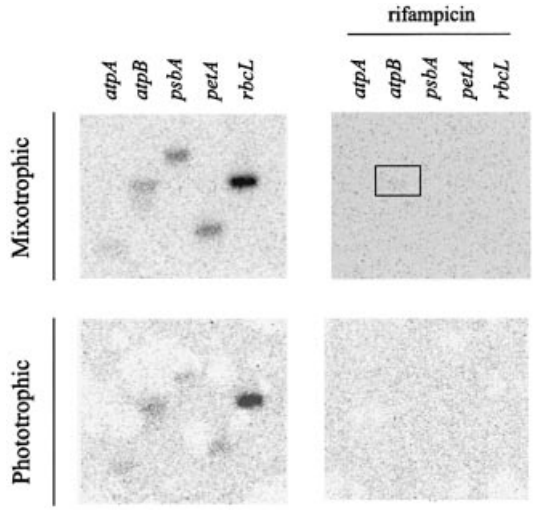

Figure 5. Inhibition of chloroplast transcription by rifampicin.
Rifampicin was added for 6 h at 350 μg ml^{-1} to cells grown either mixotrophically (upper panel) or phototrophically (lower panel), before the labelling of neosynthesized mRNAs with ^{32}P-α-UTP for 15 min using toluene-permeabilized cells. The autoradiogram of labelled neotranscripts hybridized to DNA restriction fragments fixed on a nylon membrane is shown.

remained poorly sensitive to the FdUrd treatment whereas the *atpA* transcript decreased most in both conditions (Figure 2b, right panel and Table 1). However, the *psaA* transcript became less sensitive to FdUrd in phototrophic conditions while the *petA* transcript showed an opposite behaviour.

Rates of chloroplast protein synthesis in FdUrd treated cells

The contrasted behaviour of distinct sets of chloroplast transcripts upon reduction of chloroplast genome copy number, raised the possibility that the pattern of protein expression in the chloroplast would be extensively modified after FdUrd treatment. In particular, the rates of synthesis of the *atpA* and *petA* products, the α subunit of CF1 of the ATP synthase complex and the cyt *f* of the b_6f complex, could be altered after FdUrd treatment in mixotrophic and phototrophic conditions, respectively. The ^{14}C-acetate-pulse labelling experiments of chloroplast translates, performed with FdUrd treated cells, are shown in Figure 4. In mixotrophic conditions (Figure 4, left panel), although some limited decrease in protein labelling occurred, we observed no major changes in the relative rates of chloroplast polypeptide synthesis. In particular, the moderate drop in labelling of the α subunit of the ATP synthase after 48 h of FdUrd treatment did not match the drastic drop in the accumulation of *atpA* transcripts. In phototrophic conditions (Figure 4, right panel), only did the large subunit of RubisCo (LS) show a significant decrease in synthesis rates after 24 h of FdUrd treatment.

The accumulation of chloroplast-encoded proteins was not altered by the FdUrd treatment in either growth condition, as revealed by gel electrophoresis and conventional immunodetection experiments using specific antibodies (not shown). Furthermore, the accumulation of the whole set of proteins in thylakoid membranes was not altered by the FdUrd treatment in either growth conditions as monitored by membrane purification and Coomassie or silver staining of thylakoid proteins separated by gel electrophoresis (not shown).

Changes in transcript abundance upon inhibition of chloroplast transcription by rifampicin

Rifampicin selectively and irreversibly binds to the β subunit of the *E. coli* RNA-polymerase, inhibiting further transcription initiation (Campbell *et al.*, 2001; McClure and Cech, 1978; for a review see Richardson and Greenblatt, 1996). The same mode of action of rifampicin has been described on the chloroplast-encoded, bacterial-like, RNA-polymerase (Surzycki, 1969). Full inhibition of chloroplast transcription can be obtained by treating cells for 1 h with 350 μg ml^{-1} rifampicin (Goodenough, 1971; Miller and McMahon, 1974; Surzycki and Rochaix, 1971; Surzycki, 1969; and Guertin and Bellemare, 1979). We thus chose to further investigate the transcript/translate relationship in the chloroplast, using cells grown for 3 and 6 h in the presence of 350 μg ml^{-1} rifampicin in either mixotrophic or phototrophic conditions. A longer treatment with rifampicin, up to 20 h, was lethal for the cells.

We first assayed the efficiency of rifampicin in inhibiting chloroplast transcription by RNA-pulse-labelling experiments. *In vivo* transcription of five chloroplast genes was readily detected in the untreated controls using toluene permeabilized cells (Guertin and Bellemare, 1979), whereas no signal was obtained for *atpA, petA, psbA* and *rbcL* when cells were treated with rifampicin for 6 h (Figure 5). Therefore, chloroplast transcription of these genes was fully inhibited in our experimental conditions, which indicates that they are transcribed by a rifampicin-sensitive RNA-polymerase of bacterial origin (PEP, or *P*lastid *E*ncoded *P*olymerase). Only did the *atpB* gene show some residual transcription under mixotrophic growth conditions (Figure 5, upper panel): a faint band could still be observed for *atpB* after 6 h of rifampicin treatment (< 1% of the control). This could be indicative of the presence of a rifampicin-insensitive *n*uclear-*e*ncoded *p*olymerase (NEP) similar to the one which has been described in studies with higher plant chloroplasts (Hajdukiewicz *et al.*, 1997; Hedtke *et al.*, 1997). However, this signal was not detected when the RNA pulse-labelling experiment was performed on cells grown under photo-

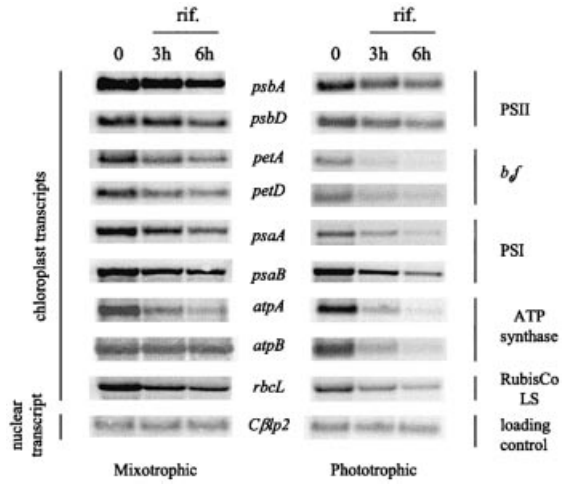

Figure 6. Transcript accumulation levels after rifampicin treatment.
Cells were grown either under mixotrophic (left) or phototrophic (right) conditions in the presence of 350 μg ml^{-1} of rifampicin for 3–6 h, and transcript accumulation analysed by RNA-filter hybridizations. The nuclear transcript *Cβ lp2* served as a loading control.

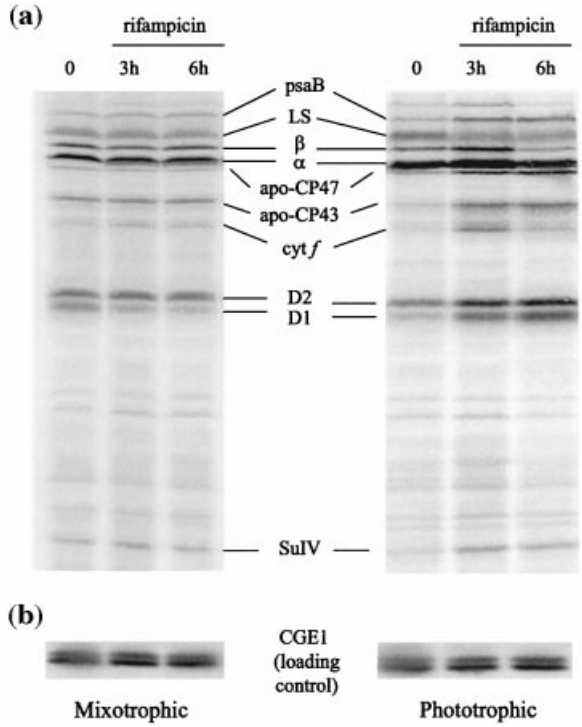

Figure 7. Chloroplast protein synthesis after rifampicin treatment.
Upon addition of 350 μg ml^{-1} rifampicin for 3–6 h, cells were subjected to a 5 min ^{14}C-acetate protein pulse-labelling experiment in the presence of cycloheximide. Labelled neosynthesized proteins were separated by gel electrophoresis and proteins transferred to a PVDF membrane. Radioactivity was revealed using a PhosphorImager (panel A). Loading was controlled by directly blotting the PVDF membrane with an antibody against CGE1, a nucleus-encoded protein (panel B). Correspondence between genes and proteins are as indicated for Figure 5. The pulse-labelling experiments were performed on the same samples that were used for RNA extraction and analysis.

trophic conditions (Figure 5, lower panel). The experimental procedure used to assay *in vivo* transcription of chloroplast genes has previously been assumed to allow detection of chloroplast, but not nuclear, transcription (Guertin and Bellemare, 1979; Kawazoe *et al.*, 2000). In agreement with this view, our attempts to detect transcription of the nuclear genes *rbcS* and *Cβlp2* did not give rise to any signal with this method (not shown).

We then addressed the stability of the transcripts in the chloroplast by performing RNA-filter hybridization experiments (Figure 6). Given that the generation time of *C. reinhardtii* in these asynchronous cultures is 5 h in phototrophic conditions and 12 h in mixotrophic conditions, cells should divide at most once or twice during the duration of the rifampicin treatment. Therefore, assuming that rifampicin completely blocks transcription soon after addition, the level of the most long-lived transcripts should decrease by a factor of 2 (for mixotrophic conditions) and 4 (for phototrophic conditions), due to their dilution during the experiment. Transcript accumulation levels of various chloroplast gene were analysed by RNA-filter hybridization experiments, quantified and normalized to the signal obtained for *Cβlp2* or *rbcS* (not shown), two transcripts that are unaffected by the rifampicin treatment because of their nuclear origin. One must note that, on Figure 6, accumulation levels can not be compared between the two growth conditions, because experiments were realised on different days using different transfers of RNA to nylon membranes and differently labelled probes. When grown in mixotrophic conditions (Figure 6, left panel) a majority of chloroplast transcripts from rifampicin-treated algae behaved in the same way. Their accumulation level was about 30% to 40% of that in untreated cells (Table 1). Two transcripts showed contrasting behaviours: the *atpA* transcript decreased more severely, reaching about 10% of its original level, whereas the *atpB* transcript was poorly sensitive to rifampicin, still showing 70% of its original accumulation level after 6 h of treatment (Table 1).

As a general rule, cells treated with rifampicin in phototrophic conditions displayed a more drastic decrease in their chloroplast mRNA content than their mixotrophic counterparts. The majority of the transcripts dropped below 10% of their original level after 6 h of rifampicin treatment, with *atpA* and *petA* being the most affected (Figure 6, right panel and Table 1). Noticeably, the *atpB* transcript, whose accumulation level was poorly sensitive to the rifampicin treatment in mixotrophic growth conditions, was highly affected in phototrophic conditions. In contrast, the *psbA, psbD* and *rbcL* transcripts showed a comparable behaviour in both growth conditions (Table 1).

Chloroplast protein synthesis in cells treated with rifampicin

Under mixotrophic conditions, the rates of synthesis of most chloroplast-encoded proteins did not decrease even after 6 h of treatment with rifampicin, despite the significant changes in transcript abundance that we observed in several instances (Figure 7a, left panel). Here again, the rate of synthesis of the α subunit of the chloroplast ATPsynthase showed no significant changes whereas the corresponding *atpA* transcript dropped dramatically after rifampicin treatment. The synthesis of cytochrome *f* even seemed to increase slightly, whereas the accumulation level of the corresponding *petA* transcript was much reduced after 6 h of rifampicin treatment. We noted that the synthesis of the PSII subunit D1, but not of D2 (another subunit of PSII) decreased whereas the transcripts levels of the two subunits were similarly affected. For cells grown under phototrophic conditions, the dramatic drop in the level of most chloroplast transcripts was not accompanied by reduced rates of synthesis for most of the chloroplast proteins. However, the pattern of protein labelling varied markedly with the time of rifampicin treatment (Figure 7a, right panel). LS showed a continuous and marked decrease in its rate of synthesis after rifampicin treatment. The synthesis of many polypeptides, among which the ATP-synthase subunits α and β, cyt *f* and sulV from the cytochrome b_6f complex, appeared to be transiently up-regulated (compare the 0 and 3 h lanes on the right panel of Figure 7), then returning to either the initial level as for the α subunit and cytochrome *f*, or even severely decreasing as for the β subunit. Figure 7(b) shows direct immunoblotting of the membrane with an antibody against the nucleus-encoded *C*hloroplast *G*rp*E* homologue *1* (CGE1) protein (Schroda *et al*., 2001) that was used as a loading control. The result shows that the 3 and 6 h lanes are slightly overloaded, when compared to the untreated control, but this can not on its own account for the up-regulation of synthesis of most chloroplast translates at the 3 h point. Furthermore, the up-regulation of synthesis after 3 h of rifampicin treatment in phototrophic conditions was reproducibly observed in three independent experiments. The major PSII subunits, apoCP47/apoCP43/D2 and particularly D1 showed a marked and continuous increase in synthesis after 6 h of rifampicin treatment.

These changes in the rates of synthesis of the chloroplast–encoded products were not accompanied by any significant changes in their accumulation levels, as checked by immunoblotting with specific antibodies (not shown). Also, accumulation of thylakoid membrane proteins was not altered by rifampicin treatment in either growth conditions as monitored by membrane purification and Coomassie or silver staining of thylakoid proteins separated by gel electrophoresis (not shown).

Discussion

A common feature of bacteria is that transcription rate and mRNA abundance most often control the rates of protein synthesis. Since chloroplasts derive from ancestral cyanobacteria, our aim was to provide a better view of the pre-translational contributions to the expression of chloroplast-encoded proteins. The use of FdUrd and rifampicin in two distinct growth conditions allowed us to demonstrate that extensive changes in genome copy number, in transcription rates and in the steady-state level of chloroplast transcripts are not directly correlated to changes in the rate of synthesis of most chloroplast-encoded proteins.

Transcription of chloroplast genes

Using FdUrd as an inhibitor of chloroplast DNA replication, we observed a parallel decrease in genome copy number and transcription rates in the chloroplast. This supports the view that most copies of the chloroplast chromosome are transcriptionally active and that the RNA polymerases are not present in limiting concentration. In most instances however, the decreased rates of transcription due to lower chloroplast ploidy, were not accompanied by significant changes in the abundance of the vast majority of the chloroplast transcripts tested. Among the exceptions were the decreased accumulation of *psaA* transcripts for algae grown in mixotrophic conditions, of *petA* transcripts for algae grown phototrophically and *atpA* transcripts in either growth condition. The poor sensitivity of the post-transcriptional step to FdUrd treatment can be readily explained by the presence of limiting amounts of nucleus-encoded factors that select a subset of neosynthesized transcripts in the chloroplast, protecting them from nucleolytic degradation after transcription. Therefore, the concentration of these factors (and neither the genome copy number nor the actual transcription rate) would determine the abundance of transcripts in the chloroplast. Nuclear factors that protect chloroplast mRNAs from nucleolytic degradation by acting on their 5′UTR have been described for most of the chloroplast transcripts that we tested but not for *atpA (*see Barkan and Goldschmidt-Clermont, 2000; Nickelsen, 1998; Stern and Drager, 1998; Wollman *et al*., 1999; Monde *et al*., 2000). If such a factor is missing for *atpA,* its accumulation would be predicted to follow the fraction of transcriptionally active chloroplast chromosomes contrarily to the other transcripts that can accumulate in the chloroplast in proportion to the concentration of their nuclear-encoded stabilizing factors. An additional prediction of this proposal is that the *atpA* mRNAs should decline more severely than all other chloroplast transcripts when transcription is blocked, a behaviour that we indeed observed after treatment of the algae with rifampicin.

Relation between transcript accumulation levels and rates of translation

A correlation between transcript accumulation levels and protein synthesis rates has been established in some instances in higher plants (Mullet, 1993; Pfannschmidt *et al.*, 1999; Rapp *et al.*, 1992). However, other developmental studies have shown that transcriptional activity is not necessarily tightly linked to rates of chloroplast protein synthesis, pointing to an extensive post-transcriptional control of chloroplast gene expression (Mullet, 1988). The analysis of several translation-defective strains of *C. reinhardtii* has shown that the level of the non-translated transcript either increased, as for *atpA* (Drapier *et al.*, 1992), or decreased, as for *psbA* (Girard-Bascou *et al.*, 1992) or *petA* (Wostrikoff *et al.*, 2001). Thus, as discussed by Nickelsen (1998), there is no general rule linking translation and mRNA stability in the chloroplast of *C. reinhardtii*.

In the present study we found only in one instance, *atpB*, some correlation between transcript abundance and translation rates. In the experiments where the accumulation of *atpB* transcripts was mostly unaffected (namely after FdUrd treatment performed in either growth condition or rifampicin treatment of algae grown in mixotrophic conditions) the rates of synthesis of the β subunit was not affected. However, in the single experiment where the accumulation level of the *atpB* transcript was dramatically reduced (rifampicin treatment of algae grown in phototrophic conditions), the rate of synthesis of the β subunit displayed a significant decrease. In all other cases, our study provides extensive experimental support to the lack of a direct relationship between translation rates and transcript abundance in *C. reinhardtii* chloroplasts. The *atpA* and *petA* transcripts that decreased severely after FdUrd or rifampicin treatment still allowed rates of protein synthesis that were comparable to those observed in the untreated control. For instance, in rifampicin-treated cells grown phototrophically, as little as 2% of *atpA* and *petA* transcripts were sufficient to provide near wild-type synthesis of the corresponding proteins. The fact that severely reduced amounts of those transcripts still permits near wild-type synthesis of the corresponding proteins may be explained by either of two ways: (i) a smaller amount of transcripts is more intensively translated or (ii) in the regular growth conditions with no drug added, only a subset of those transcripts is actually loaded onto polysomes for translation. That the concentration of chloroplast transcripts is not rate-limiting for translation is consistent with a previous report by Hosler *et al.* (1989) that a reduction in chloroplast genome copy number in *C. reinhardtii* cells grown phototrophically produced a decrease in the accumulation levels of the chloroplast *atpA, rpl2* and *rbcL* transcripts, whereas synthesis of the corresponding proteins, the α subunit of ATP-synthase, the r-protein L1 and LS remained largely unaffected (however, the behaviour of LS reported by Hosler *et al.* (1989) is in contrast with our data: we observed a reduction in synthesis rate of LS for the FdUrd treatment performed in phototrophic conditions). Overall, it appears that translation must be controlled by rate-limiting nucleus-encoded factors imported in the chloroplast (for reviews see Hauser *et al.*, 1998 and Zerges, 2000), as has been documented for pet494p (Steele *et al.*, 1996) or pet111p (Green-Willms *et al.*, 2001), which are nuclear-encoded translational activators of the *COX3* and *COX2* mRNA in yeast mitochondria, respectively. Thus, as pointed out by Hosler *et al.* (1989), there may be two distinct pools of mRNA in the chloroplast, a pool of non-translatable mRNAs and a pool of mRNAs that are activated for translation by nucleus-encoded factors.

A remarkable illustration of the prominent role of translational regulation can be found in our study of the expression of the *psbA* and *rbcL* genes, which encode, respectively, the PSII subunit D1 and LS: a similar decrease in *psbA* transcripts in mixotrophically grown and phototrophically grown algae (for both the FdUrd and rifampicin experiments) was accompanied in one case by a down regulation in the synthesis of the D1 protein, whereas it was up-regulated in the other case. This behaviour reflects the complex translational regulation for this PSII subunit that encompasses ribosome pausing (Kim *et al.*, 1991; Zhang *et al.*, 2000) and is the most sensitive to photoinduced damage (Danon and Mayfield, 1994a, 1994b; Kim and Mayfield, 1997; Trebitsch *et al.*, 2000). Up-regulation of the synthesis of the D1 subunit in high light without significant changes in *psbA* transcript accumulation levels has also been reported by Shapira *et al.* (1997). For *rbcL*, comparable accumulation levels of the transcript in cells treated with FdUrd in either growth conditions, led to a decrease in the synthesis rate of the corresponding LS protein for cells grown phototrophically, whereas it remained constant for cells grown mixotrophically.

Effect of growth conditions

Under standard conditions, our results are in agreement with those published by Lau *et al.* (2000), i.e. that *C. reinhardtii* cells grown mixotrophically contain about twice as much chloroplast DNA as cells grown phototrophically. A likely explanation, given the shorter generation time of cells grown in phototrophic conditions (5 h) than in mixotrophic growth conditions (12 h), is that higher number of mitotic cell divisions per time unit lead to an increased partitioning of non-replicated chloroplast genomes, as is the case for bacteria. The lower decrease in chloroplast genome copy number upon FdUrd treatment

of cells grown phototrophically may also originate from their lower polyploidy, if one assumes that there is a lower limit for the number of chloroplast chromosomes required for cell viability.

Photosynthetic activity and light quality are also known to participate in the regulation of chloroplast genome expression. At the transcriptional level, it has been shown that *psbD*, *psbC* and *petG* genes are under the control of a blue-light activated promoter in higher plant chloroplasts (Christopher *et al.*, 1992; for a review see Stern *et al.*, 1997). In *C. reinhardtii*, variations in chloroplast transcription rates were observed in a circadian way, with a peak of transcription at the onset of the light period (Leu *et al.*, 1990). These results have been extended to a general light-dependent variation in chloroplast mRNA levels, that are due to both transcriptional and post-transcriptional regulations, the degradation of chloroplast transcripts being up to five times higher in the light period when compared to the dark period of synchronously grown cultures (Salvador *et al.*, 1993). We observed a similar change in chloroplast mRNA stability when comparing the rifampicin-treated algae grown under mixotrophic or phototrophic conditions. Only did the *rbcL* transcripts show limited destabilization in phototrophic conditions and the two PSII transcripts tested (*psbA* and *psbD*) remained as stable as in mixotrophic conditions. All the other mRNAs tested displayed a severe drop in transcript stability, their degradation being four to five times higher in phototrophic conditions. The higher stability of the *psbA* transcript correlates well with the limited changes in its abundance when changing growth conditions (Figure 1b). The comparable stability of the *rbcL* transcript in both conditions is in good agreement with a report by Shiina *et al.* (1998) showing that in tobacco, accumulation levels of *rbcL* transcripts are independent of light, although in their case modification of transcription rate and mRNA stability appeared to play a role. On the other hand, this significantly lower life-time of most chloroplast transcripts for cells grown phototrophically fully accounts for their lower steady-state mRNA levels, when compared to cells grown mixotrophically, non-regarding the limited difference in gene copy number (by only a factor of 2). Particularly, the very short half-life of *atpA* and *atpB* transcripts in phototrophic growth conditions (Figure 6, right panel) may explain on its own the 10 times drop in their steady state accumulation levels when compared to cells grown mixotrophically (Figure 1b).

The decreased half-life of *atpB* and *psaB* in phototrophic conditions may be due to the same degradation process that is triggered by DTT for these two transcripts (Salvador and Klein, 1999), suggesting a role of thioredoxin mediated regulations of chloroplast transcript stability. Further support for a regulatory role of thioredoxins in phototrophic conditions comes from the examination of the pattern of synthesis of chloroplast-encoded proteins in phototrophically grown algae treated with rifampicin for 6 h (Figure 7, right panel): it became enriched in its PSII components. Several reports suggest that translation of the *psbA* transcript is strongly enhanced under phototrophic growth conditions due to a translational activation complex that preferentially binds the *psbA* messenger in the light through a thioredoxin-controlled mechanism (Danon and Mayfield, 1994a, >1994b; Fong *et al.*, 2000; Trebitsch *et al.*, 2000). It thus appears that photosynthetic activity has a major impact on chloroplast gene expression, most likely through the action of redox activated regulatory factors.

Overall, the fact that chloroplast protein synthesis rates are mostly insensitive to mRNA accumulation levels allows the algae to respond to physiological changes in chloroplast genome copy number and transcript accumulation levels that naturally take place, when the growth conditions vary with naturally occurring changes in availability in carbon sources. Post-transcriptional regulatory mechanisms therefore offer a good preservation of photosynthetic competence in changing environmental conditions, although gene copy number and mRNA levels in the chloroplast would be affected.

The lack of correlation between gene copy number, transcript abundance and protein translation in the chloroplast illustrate the deep evolution in gene expression that has developed with the engulfment of a prokaryote in a host cell. The control of chloroplast protein expression by nuclear-encoded factors provides the framework for our current understanding of these specific traits of organellar gene expression. How these nuclear-encoded factors are delivered to their targets in the organelle therefore stands as a key issue for further studies of chloroplast biogenesis.

Experimental procedures

Strains and growth conditions

The *C. reinhardtii* wild-type (A1, *mt*-) strain, a derivative of the 137c strain presented in Harris (1989) was used for all the described experiments. For mixotrophic growth conditions, cells were grown in a liquid Tris-Acetate-Phosphate (TAP) medium (pH 7.2), as described in Harris (1989), under continuous low light (4 μE m^{-2} s^{-1}) at 25°C and agitation. For phototrophic growth conditions, cells were grown in a minimum medium (pH 7.2) (comparable to the HS medium described in Harris (1989)) under an illumination of 20 μE m^{-2} s^{-1} and with the addition of 5% bubbled CO_2(g) at 25°C and agitation.

Treatment with drugs: FdUrd treatment

Cells were incubated for up to 48 h with 0.5 mM of 5-fluoro-2′-deoxyuridine (Sigma), a treatment known to reduce chloroplast genome copy number (Wurtz *et al.*, 1977).

Table 2. Intragenic DNA fragments from chloroplast or nucleus origin, used for hybridization of Northern or Southern blots.

Gene	Origin	Size	Reference
psbA	entire R14 fragment of the chloroplast DNA	2.3 kB	Rochaix (1978)
psbD	entire R3 fragment of the chloroplast DNA	2.7 kB	Rochaix (1978)
petA	*Hin*dIII fragment of the chloroplast R26 fragment	3.5 kB	Büschlen *et al.* (1991)
petD	*Hin*dIII fragment of the chloroplast R26 fragment	1.1 kB	Büschlen *et al.* (1991)
psaA	*Hin*dIII-*Eco*RI fragment of the chloroplast R11 fragment	2.2 kB	Choquet *et al.* (1988)
psaB	*Bam*01 fragment of the chloroplast DNA	580 bp	Rochaix (1978)
atpA	*Eco*RI-*Pst*I fragment of the chloroplast R7 fragment	947 bp	Rochaix (1978)
atpB	*Eco*RI-*Kpn*I fragment of the chloroplast Ba5 fragment	2.9 kB	Rochaix (1978)
rbcL	*Hin*dIII fragment of the chloroplast R15 fragment	890 bp	Rochaix (1978)
rbcS	*Alw*NI-*Sst*II fragment of the coding region of rbcS2	370 bp	Schroda *et al.* (1999)
Cβlp2	entire cDNA of the nuclear Cβlp2 gene	1 kB	Schroda *et al.* (2001)

Rifampicin treatment

Cells were incubated for up to 6 h with 350 μg ml^{-1} of rifampicin (Sigma) to inhibit transcription of chloroplast but not nuclear genes (Surzycki, 1969).

RNA analysis

Total RNA was extracted and analysed by Northern blots as in Drapier *et al.* (1998). The bands shown in Figures 1,2 and 6 correspond only to the mature mRNAs of the corresponding genes (in some cases, maturation intermediaries were observed, but were discarded for quantifications and not included in the figures). Radiolabelled gene-specific probes were derived from intragenic DNA fragments of nuclear or chloroplast origin (Table 2). Labelling was performed for each probe using the Nonaprimer Labelling Kit (Quantum, Appligene) with 5 ng of DNA and 25 μCi of α-^{33}P- dATP.

In vivo *RNA-pulse labelling*

Cells were assayed for *de novo* chloroplast RNA synthesis using toluene permeabilized cells as described in Guertin and Bellemare (1979) with the following modifications: cells were solubilized with 1% toluene and pulse-radiolabelled with 165 μCi ml^{-1} of α-^{32}P-UTP (400 Ci $mmol^{-1}$, Amersham) for 15 min RNA, extracted as described in Drapier *et al.* (1998), was then hybridized with unlabelled DNA restriction fragments of chloroplast or nuclear origin separated by gel electrophoresis and transferred to a nylon membrane. RNA pulse-labelling experiments were also performed using thaw/freeze permeabilized cells according to Gagne and Guertin (1992) with the modifications described in Sakamoto *et al.* (1993).

DNA-filter hybridizations

Total DNA was prepared as described in Rochaix (1980), separated by gel electrophoresis, transferred to nylon membranes and probed with radiolabelled intragenic DNA fragments from chloroplast or nuclear origin.

Protein analysis

Pulse-labelling experiments were carried out as described in Drapier *et al.* (1992) with 5 μCi ml^{-1} of ^{14}C-acetate (50 mCi $mmol^{-1}$, Amersham) in the presence of an inhibitor of cytoplasmic translation (6,6 μg ml^{-1} cycloheximide, Sigma). Proteins of solubilized cells were separated in urea/SDS-polyacrylamide gels as described in Piccioni *et al.* (1981) and transferred to a PVDF membrane which was then exposed in a PhosphorImager to detect radiolabelled proteins. Loading of the gels was controlled by immunoblotting of the PVDF membrane with antibodies directed against nucleus-encoded proteins (CGE1 (Schroda *et al.*, 2001), OEE2 and/or OEE3).

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