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Original Research Communication

Redox Modulation of Chloroplast DNA Replication in *Chlamydomonas reinhardtii*

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ABSTRACT

We constructed a plasmid probe containing DNA sequences unique for chloroplast (Cp) genome and nuclear genome of *Chlamydomonas reinhardtii*. Using this probe and quantitative Southern blot analyses, we determined the content ratio of Cp DNA/nuclear DNA in total DNA isolated from cells grown in different conditions. Algal cells grown photoheterotrophically with acetate as an added carbon source contain the highest amount of Cp DNA compared with cells grown in other conditions tested. We investigated the effect of nitrogen limitation, 5-fluorodeoxyuridine treatment, cadmium exposure, photoautotrophic growth, and heterotrophic growth in darkness. The change in the Cp/nuclear DNA ratio in cells shifted from one growth condition to another depended on cell division; Cp DNA content in undivided cells remained constant. Therefore, the reduction of Cp DNA content was attributed by under replication rather than selective degradation of Cp DNA. Cells with low Cp DNA content often contained less reduced glutathione, suggesting the possible effect of redox status. Low Cp DNA content was detected in cells treated with inhibitors that block electron flow of photosystems and in mutants with PS I defective phenotype. On the basis of these data, we propose that in *C. reinhardtii*, Cp DNA replication be modulated by redox status. Antiox. Redox Signal. 2, 529–535.

INTRODUCTION

In eukaryotic cells, energy metabolism takes place in mitochondria and chloroplasts (Cp). Both organelles contain their own genomes in multiple copy. The mitochondria genome has been studied extensively, as have the copy-number responses to developmental signals and functional needs; however, the factor(s) affecting copy number are not clear (Shadel and Clayton, 1997). Cp is the best-studied member of the plastid family, which consists of several related organelles unique for plant cells. Multiple copy genome was detected in many types of plastids, with the number varying from a few copies in a proplastid to a few hundred copies in a Cp. The dramatic increase in Cp number and Cp genome copy number that accompanies leaf development in higher plants has been documented (Bendich 1987), but the underlying mechanism as well as the necessity for the change remains to be investigated.

In this study, we analyzed the Cp DNA copy number change in the well-studied eukaryotic green alga *Chlamydomonas reinhardtii*. This unicellular alga is particularly suitable for this study for the following reasons. The cell contains a solitary Cp, so that the effect of Cp number on Cp DNA content can be eliminated. Treatment with 5-fluorodeoxyuridine (FdUrd)

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and mutant studies indicated that Cp DNA is present in excess of the minimal functional need (Harris, 1989). The alga uses acetate for heterotrophic growth and allows a wide range of growth condition manipulation.

MATERIALS AND METHODS

Algal strains and culture conditions

The selection of *C. reinhardtii* 950 from *C. reinhardtii* cc 178 (CW-15 mt^-) was reported in Tang *et al.* (1995). Other algal strains and mutants were obtained from the *Chlamydomonas* Genetics Center, Department of Bontany, Duke University (Durham, N.C.). The high salt (HS), nitrogen-free high salt (HS-N), high salt medium containing acetate (HSA), Tris-acetate-phosphate (TAP), culture media were prepared according to Harris (1989). TP was modified from TAP by using Tris HCl instead of acetic acid to adjust the medium pH. For cultures used in Cd treatment, glycerophosphate was used to provide phosphate source. $CdCl_2$ was added to the final concentration of 50 μM or 100 μM. The treatment time for Cd and other inhibitors was 48 hr. Algal cultures were kept at 21°C, on a shaker in a 12-hr light/12-hr dark cycle under 60 $\mu E/m^2$ sec light intensity in a culture chamber. Chemicals of reagent grade were purchased from Sigma (St. Louis, MO).

General molecular biology procedures

Quick isolation of total DNA from cells and the separation of Cp DNA from nuclear DNA were performed according to Tang *et al.* (1995). Construction of pBHg was described in Hsieh *et al.* (1991). Plasmid pCS2.1 was a generous gift from Dr Michel Goldschmidt-Clamont (Goldschmidt-Clamont, 1986). The 900-bp *Eco* RI fragment in pCS2.1 was purified (Sambrook *et al.*, 1989) and inserted into the *Eco* RI site of pBHg to generate pCN6. Plasmid DNA isolation, restriction enzyme digestion, and Southern blotting analysis were carried out according to Sambrook *et al.* (1989). Optimal range to measure hybridization bands was determined by loading different amounts of DNA in the gel and calibrating the band intensity with known standards. The relative intensity of hybridization bands was measured by using a PhosphoImager scanner (Molecular Dynamics).

Determination of reduced glutathione content

The washed cells were resuspended in 10% sulfosalic acid and ground in liquid nitrogen. After removing debris by centrifugation, the clear supernatant was collected for protein and glutathione (GSH) measurements. Protein concentration was determined by using the commercial protein assay kit (Bio-Rad). Measurement of GSH was performed according to Rauser (1991) using a C18 reverse-phase column and Waters 600 high-performance liquid chromatography (HPLC) system.

RESULTS

Measurement of the Cp DNA/nuclear DNA content ratio by quantitative Southern blot analysis

Plasmid pCN6 contains 224-bp Cp-genome-specific and 900-bp nuclear-genome-specific sequence. In Figure 1A, the assignment of C and N1, N2 bands to Cp and nuclear genome, respectively, was based on its hybridization pattern to each component purified by CsCl gradient. The algal cell contains two copies of the *rbcS* gene (Goldschmidt-Clamont, 1986). The theoretical Cp DNA/nuclear DNA ratio for cells containing one copy of Cp DNA should be $224/900 \times 2 = 0.1244$. The experimental Cp DNA/nuclear DNA ratio was determined by C/N1 + N2. Figure 1B shows the statistical analysis of the Cp DNA/nuclear DNA ratio in cells grown in several conditions. The Cp DNA/nuclear DNA ratio showed that *C. reinhardtii* 950 in photoheterotrophic growth in HSA contained 98 ± 2 copies of Cp DNA per cell. Treatment with 1 m*M* FdUrd for 8 days reduced the Cp DNA content to 10 ± 2 copies per cell. The average Cp DNA copy number for cells grown in HS medium was 38 ± 4 per cell. After transferring cells to nitrogen-depleted HS (HS-N) for 24 hr, the Cp DNA content was further reduced to 17 ± 4 copy per cell. The effect of FdUrd treatment and nitrogen limitation to Cp DNA content was reported in cytological studies using DNA-specific fluorochrome, 4′,6-

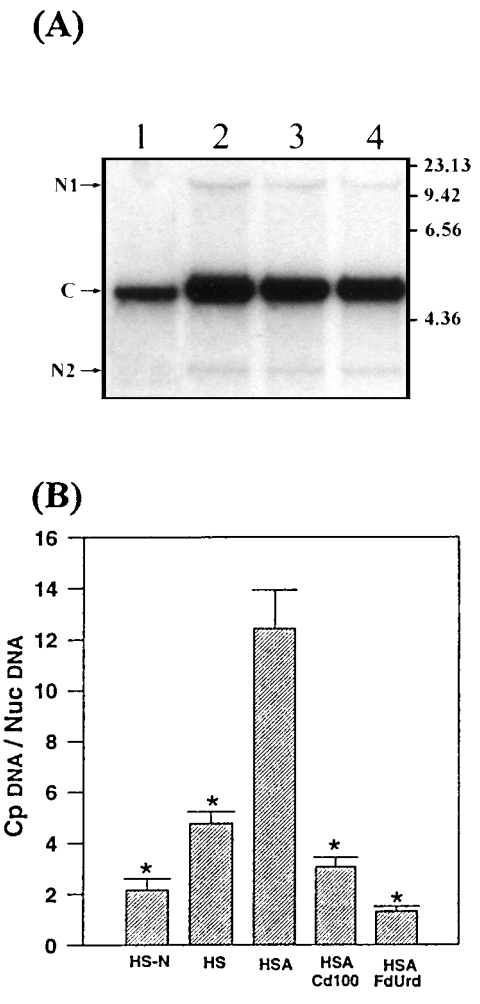

FIG. 1. (A) Southern blot used for quantitative analysis. N1, N2, and C mark the positions of two nuclear DNA and one Cp DNA hybridization bands, respectively. Positions of the size markers in kb are included. The *Eco* RI digest of purified Cp DNA was loaded in lane 1. Lanes 2, 3, and 4 contain the *Eco* RI digest of the total DNA isolated from strains 950, 124, and 125, respectively. All cells were grown photoheterotrophically in HSA. **(B)** The effect of different growth conditions on the Cp DNA/nuclear DNA ratio in *C. reinhardtii* 950. For each treatment, duplicate tests of at least five independently isolated DNA samples were carried out. Data are presented as the mean and standard deviation; all treatments show significant difference from that of cells grown in HSA (*$p < 0.05$).

diamidino-2-phenylindole (DAPI) (Matagne and Hermesse, 1981). Our results were in general agreement with theirs.

The effect of acetate and cadmium on Cp DNA content

One unexpected finding was that cells grew photoautotrophically in HS medium that contained less Cp DNA. The result indicated that the increased demand for photosynthetic output did not increase the copy number and/or the presence of acetate increased the Cp DNA content. Figure 1B also shows the effect of cadmium (Cd) on Cp DNA content. The presence of 100 μM Cd reduced the cellular Cp DNA content from 98 ± 2 to 25 ± 3. This treatment condition did not inhibit growth, as determined by cell counting and chlorophyll measurement. To evaluate whether these effects were unique for the particular strain and culture medium, we studied the response in several strains in different culture media. Table 1 shows the results obtained from *C. reinhardtii* cc-125 mt^+. To test the effect of acetate, we grew cells in equivalent culture media with or without acetate, *e.g.*, HSA versus HS, TAP versus TP. In separate experiments, we observed a similar pattern and extent of changes in *C. reinhardtii* cc-124 mt^- and the cell wall-containing strains, *C. reinhardtii* 89 and *C. reinhardtii* 90 (data not shown). The results indicated that the effect of acetate or Cd was independent of strain, mating type, or culture media. In control condition of photoheterotrophic growth, the Cp DNA copy number of all tested strains showed no significant difference.

Under-replication of Cp DNA attributes to the reduction of Cp DNA content

The reduction of Cp DNA content could result from selective degradation and/or under replication of Cp DNA. To elucidate the possible cause, log-phase cells were transferred from one growth condition to another. Cell number

TABLE 1. THE EFFECT OF ACETATE AND CD ON CP DNA CONTENT OF *C. REINHARDTII*

Culture media	*Added ingredient*	*Cp DNA content (copies/cell)*
HS	None	42 ± 5
HSA	None	96 ± 3
HSA	50 μM $CdCl_2$	39 ± 4
HSA	100 μM $CdCl_2$	26 ± 3
TP	None	43 ± 6
TAP	None	97 ± 4
TAP	50 μM $CdCl_2$	46 ± 5
TAP	100 μM $CdCl_2$	28 ± 3

The algal strain used was *C. reinhardtii* (cc-125 mt^+).

Table 2. Changes of Cell Density and Cp DNA Content in Algal Culture Transferred from Photoheterotrophic Growth in HSA to Photoautotrophic Growth in HS

Days after transfer	*Cell density (10^6 cells/ml)*	*Cp DNA content (copies/cell)*
0	2.96 ± 0.12	98 ± 2
1	3.11 ± 0.09	96 ± 3
3	3.69 ± 0.13	89 ± 5
5	5.63 ± 0.21	56 ± 4
7	6.68 ± 0.27	47 ± 5

The algal strain used for this study was *C. reinhardtii* 950.

and Cp/nuclear DNA ratio was monitored periodically after the transfer. In cultures transferred from photoheterotrophic growth in HSA to photoautotrophic growth in HS, cell number did not increase significantly within 3 days, and the Cp DNA/nuclear DNA ratio remained constant. Within 5 days, cell number increased about two-fold and the Cp DNA content reduced from 98 ± 2 to 56 ± 4 copy per cell (Table 2). Similar types of results were obtained from culture transferred from photoheterotrophic growth in HSA to heterotrophic growth in HSA in darkness and from photoheterotrophic growth in HSA without Cd to HSA containing Cd (data not shown). The results suggested that the under-replication of Cp DNA was the major cause for the reduction of Cp DNA content. The constant Cp/nuclear DNA ratio in undivided cells indicated that Cp DNA was not selectively degraded under the conditions we investigated. When we reversed the direction of transfer, the increase of cellular Cp DNA content preceded the cell division (data not shown). The results again supported the contribution of Cp DNA replication to the changes of Cp DNA content.

Influence of redox status to Cp DNA content

Using this measurement method, we also observed that cells survived in extreme conditions contained less Cp DNA. We searched for common factor(s) among these conditions. Upon exposure to Cd, algal cells synthesized phytochelatins to sequester this heavy metal (Howe and Merchant, 1992). Concomitant with the synthesis of phytochelatins that use GSH as substrate, a decrease in the cellular GSH pool was predicted. We actually detected the reduction of cellular GSH content from 3.17 ± 0.34 to 2.08 ± 0.21 nmol/mg protein upon treatment with 100 μM Cd for 48 hr (Table 3). After measuring the GSH content in cells subjected to other treatments that affected the Cp DNA content, we detected a general correlation between low GSH level and low Cp DNA content. GSH is a major reducing cofactor for oxygen detoxification and contributes significantly for the maintenance of cellular redox homeostasis. In this connection, we carried out the following experiments.

Effect of photosynthetic inhibitors on Cp DNA content

In photosynthetic cells, photosystem (PS) I is the main source of the reducing equivalents. Paraquat blocks PS I electron flow and can provide information on the possible involvement of PS I (Hess, 1980). We tested the effect of Paraquat and other photosynthesis inhibitors on Cp DNA content (Table 4). When cells were grown photoheterotrophically in HSA, presence of 0.3 μM Paraquat in the culture medium for 48 hr did not affect cell division but reduced the Cp DNA content from 98 ± 2 to 61 ± 8 copies per cell. Paraquat could form superox-

Table 3. Cp DNA and GSH Content in *C. reinhardtii* Grown in Different Culture Conditions

Culture condition	*GSH content (nmol/mg protein)*	*Cp DNA content (copies/cell)*
Control	3.17 ± 0.34	98 ± 2
Treated with 100 μM Cd	2.08 ± 0.21	25 ± 3
Deplete of nitrogen	2.33 ± 0.08	28 ± 5
Treated with methylamine	3.26 ± 0.21	118 ± 15

The strain used for this study was *C. reinhardtii* 950. Control was photoheterotrophic growth in HSA. The treatment time was 48 hr.

Table 4. The Effects of Photosynthesis Inhibitors on Cp DNA Content in *C. reinhardtii*

Inhibitor	*Function and target site*	*Cp DNA content (copies/cell)*
Control	None	98 ± 2
Paraquat	Blocks the electron flow of PS I	61 ± 8
DCMU	Electron flow between PS II and PS I	78 ± 5
Methylamine	Uncoupler of photophosphorylation	118 ± 15
Hydroxylamine	Inhibits water molecule splitting	116 ± 13

The algal strain used for this study was *C. reinhardtii* 950.

ide that damages the lipid components. To determine whether Paraquat reduced Cp DNA content through its inhibition of photosynthetic electron transport or its effect on the lipid component, we tested the effect of 3-(3′,4′-dichlorophenyl)-1, 1-dimethylurea (DCMU). DCMU blocks electron transport between the primary acceptor of PS II and plastoquinone. Noncyclic photosynthetic electron transport generates ATP and NADPH by operating PSI and PSII in series; it is affected by DCMU. The treatment with DCMU at 10^{-6} *M* did not affect cell division but reduced the Cp DNA content to 78 ± 5 copies per cell. We also tested the effect of the uncoupler of photophosphorylation, methylamine, and the inhibitor of water molecule splitting, hydroxylamine. Both inhibitors did not reduce Cp DNA content over the tested concentration range of 10^{-8} *M* to 10^{-3} *M* for 48 hr. These results suggested that Cp DNA replication is more sensitive to the redox status than the energy status.

Mutants with defective PS I contain less Cp DNA

To assay the *in vivo* effect of PS I, we measured the Cp DNA content in PS I-defective mutants with different lesions. *C. reinhardtii* cc-742 contains mutation in the Cp genome. The two nuclear mutants tested were *C. reinhardtii* cc-1062 F23 mt^+ that was mapped at the AC 212 locus and *C. reinhardtii* cc-1042 which lacks P700, Cp1, and polypeptide 2 (Harris, 1989). We detected low Cp DNA and GSH content in all mutants. The results are summarized in Table 5. Therefore, the limited supply of reducing equivalents within the cell is correlated with the low Cp DNA content.

DISCUSSION

In *C. reinhardtii*, the change in the DAPI staining pattern of Cp nucleoids has been studied during the mating process (Kuroiwa *et al.*, 1982) and other developmental stages (Coleman, 1984). Due to the dynamic morphological changes of Cp nucleoids (Ehara *et al.*, 1990), and the strong interference of nuclear DNA, quantitative measurement of Cp DNA content by DAPI staining is difficult. Separation of Cp DNA from nuclear DNA and mitochondria DNA for the purification of each component has been successful, but this method is again difficult for quantitative detection of each component. The current method is more suitable for quantitative estimate of Cp DNA content, particularly for cells grown in a wide range of stress conditions.

We demonstrated that in *C. reinhardtii* Cp

Table 5. Cp DNA and GSH Content in Different Mutant Strains of *C. reinhardtii*

Strains	*PS I phenotype*	*Location of mutation*	*Cp DNA content (copies/cell)*	*GSH content (nmol/mg protein)*
950	Wild type	None	98 ± 2	3.17 ± 0.34
cc-106	Defective	Nuclear	35 ± 8	2.28 ± 0.17
cc-1042	Defective	Nuclear	32 ± 6	2.35 ± 0.23
cc-742	Defective	Cp	68 ± 13	2.66 ± 0.15

DNA replication is more sensitive than nuclear DNA replication to changing redox status. In all experiments, we tried to collect data from synchronous culture by inoculating the testing culture with synchronous cells and monitoring the synchrony by periodic cell counting. We detected in some treatment conditions cells that did not divide synchronously, particularly in heterotrophic growth. Therefore, the measurement is not the absolute value. Our data showed that cells with limited supply of reducing equivalents support a lower level of Cp DNA replication and produced progeny cells with reduced Cp DNA content. The Cp DNA replication system appears to be responsive to the availability of reducing equivalents that is the difference between production and demand. The cells can generate reducing equivalents from either biological oxidation of organic carbon source(s) and/or photosynthetic electron transports. The demand for reducing equivalents involves various assimilation pathways and stress responses. This hypothesis is consistent with the following observations; the reduction of Cp DNA content in Cd-treated cells and the enhancing effect of acetate to Cp DNA content for many strains under photoheterotrophic growth. It also explains the reduction of Cp DNA content in cells subjected to nitrogen limitation. When carbon is available, nitrogen limitation leads to increased content of fatty acids and carbohydrates (Behren *et al.,* 1994) by triggering biosynthetic pathways that demand additional reducing equivalents.

The redox status affect signal transduction (Staal *et al.,* 1994), apoptosis (Sato *et al.,* 1995), transcription (Storz *et al.,* 1990; Ziegelhoffer and Kiley, 1995), translation (Danon and Mayfield, 1994), post-translational regulation (Malter and Hong, 1991), and protein activity (Fontecave *et al.,* 1989; Rainwater *et al.,* 1995; Ross *et al.,* 1995). A few examples are cited here. This is the first report to implicate redox modulation of DNA replication. Further studies are required to determine whether this is a unique feature for Cp DNA of this alga or a common phenomenon for the replication of other DNA. Redox regulation is usually mediated by thiol-disulfide balance to modulate protein conformation change and/or protein affinity with DNA or RNA. The regulatory role of iron–sulfur (Fe-S) proteins in redox sensing has been reported. The redox state of the Fe-S center of the *E. coli* global transcription factor, FNR, affects DNA-binding activity and regulates gene expression in response to oxygen deprivation (Khoroshilova *et al.,* 1995). In a superoxide stress pathway, the Fe-S center of the SoxR protein is involved in sensing the redox state by oxidation-reduction (Hidalgo *et al.,* 1998). Previously, we have reported the sequence-specific interaction of a Fe-S protein with the cloned Cp DNA replication origin *in vitro* and *in vivo* (Hsieh *et al.,* 1991; Wu *et al.,* 1989, 1993). The role of this protein–DNA interaction in the redox modulation of Cp DNA replication requires further investigation.

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ABBREVIATIONS

Cd, cadmium; Cp, Chloroplast; DAPI, 4′,6-diamidino-2-phenylindole; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FdUrd, 5-fluorodeoxyuridine; Fe-S, iron-sulfur; GSH, glutathione; HAS, high salt medium-containing acetate; HPLC, high-performance liquid chromatography; HS, high salt; HS-N, nitrogen-free high salt; N_2, nitrogen; PS, photosystem; TAP, tris acetate phosphate.

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