Light affects the structure of Chlamydomonas chloroplast chromosomes

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ABSTRACT

We have analyzed changes in the structure of chloroplast chromosomes in response to light in growing *Chlamydomonas* cells using a crosslinking assay based on the intercalation of HMT (4'-hydroxymethyl-4,5',8-trimethylpsoralen) into DNA. Our results show that the structure of chloroplast chromosomes in at least three widely separated regions is different in light-grown vs. dark-grown cells. Structural changes in chloroplast chromosomes occur within 3 hrs after exposure to light or darkness, respectively. The response to light is not inhibited by atrazine and can be elicited by dim blue light incapable of evolving O_2, indicating that it does not require photosynthesis. Inhibition of cytoplasmic protein synthesis with cycloheximide prevents this response to light, indicating that it depends, at least in part, on proteins imported from the cytoplasm.

INTRODUCTION

Light is a critical factor in development, maintenance and function of plant cells and their chloroplasts (for reviews see references 1–5). Here we have investigated whether the physiological responses of chloroplasts to light or darkness are accompanied by structural changes in chloroplast chromosomes. We have probed for such changes in chloroplast chromosome structure in living cells of the unicellular alga *C. reinhardtii*, using a DNA crosslinking assay that combines two methods described by Pettijohn and coworkers (6) and by Vos and Hanawalt (7). This assay (Fig. 1) is based on the different potential of chromosomes of different structure to intercalate HMT (4'-hydroxymethyl-4,5',8-trimethylpsoralen) (see below). Subsequent brief irradiation with near UV light (UVA) leads to crosslinking of chloroplast DNA as a function of the extent of intercalation (8).

Intercalation of psoralens increases with increasing torsional stress of underwound DNA (6,9). Additionally, DNA binding proteins can decrease psoralen binding to DNA, either because they relieve torsional stress of the DNA (10) or because they shield the DNA from the intercalator (11–13) or both. Light might potentially influence chromosome structure by altering torsional stress of the DNA and/or abundance or activity of certain DNA binding proteins.

Deproteinized chloroplast DNA is torsionally stressed because it is underwound (i.e. it is negatively supercoiled) (14). *In vivo*, chloroplast DNA is not bound to histones, but instead appears to be associated with small, basic proteins, some of which are antigenically related to eubacterial and cyanobacterial proteins HU (15,16). These and/or other proteins might constrain some of the torsional stress seen in deproteinized chloroplast DNA *in vitro* (10). Chloroplasts also contain DNA tracking proteins as well as relaxing (17,18) and supercoiling (17,19) topoisomerases. Partial inhibition of the latter enzyme with novobiocin rapidly reduces torsional stress of the chloroplast DNA (shown here) and alters synthesis of chloroplast transcripts *in vivo* (20). These considerations, together, suggest that chloroplast chromosomes, like bacterial chromosomes, maintain a certain homeostasis of torsional stress.

In contrast to higher plants, *C. reinhardtii* can develop fully functional chloroplasts in the dark (21). Under these conditions it can grow when supplied with an appropriate carbon source. Thus, by transferring dark-adapted algal cells into the light and vice versa, direct effects of light on potential HMT-dependent crosslinking of chloroplast DNA can be studied with few complications resulting from changes in chloroplast pigments, membranes, etc. We show here that exposure to light reduces the potential for HMT intercalation and DNA crosslinking in at least three widely separated regions of the chloroplast chromosome. This reduction does not depend on photosynthesis, but it does depend on cytoplasmic protein synthesis. We conclude that light affects the overall structure of the chloroplast chromosomes.

MATERIALS AND METHODS

Growth of the Algae

C. reinhardtii cw-15 (cell wall-) strain 278*gro*2 (a derivative of CC-278 described in reference 22) was used in all experiments. Medium and culture conditions for growing this alga were as previously described (22). Since potential HMT crosslinking of the chloroplast DNA changes as a function of the growth phase of the cells (see Fig. 3, below), it is important to compare light- and dark-adapted cells under otherwise similar physiological conditions. To obtain light-adapted cells, starter cultures were inoculated to 1×10^6 cells/ml and grown overnight in the light (for ca. 2 generations). These cultures were in turn diluted to 5×10^5 cells/ml and incubated an additional 18–24 hrs in the light. At this time, the light-adapted cells were in the mid-logarithmic growth phase ($2.5-3.5\times10^6$ cells/ml). To obtain dark-adapted cells, starter cultures were inoculated (with

stationary phase, light-grown cells) to an initial cell density of 4×10^5 cells/ml. They were incubated in the dark for two days (for ca. 3.0 generations). These cultures were in turn diluted to 8×10^5 cells/ml and incubated an additional 18–24 hrs in the dark. At this time, the dark-adapted cells were also in the mid-logarithmic growth phase ($2.5-3.5\times10^6$ cells/ml).

HMT Crosslinking of Chloroplast DNA

We have modified a procedure originally described by Vos and Hanawalt (7) to crosslink nuclear DNA in human VA2–6A3 cells (Fig. 1A). Approximately 3.5×10^8 algal cells were washed in 20 ml of minimal medium (i.e. the medium described in reference 22, but lacking yeast extract, bactopeptone and sodium acetate) and resuspended in 20 ml of this minimal medium (the complete medium strongly absorbed UVA light). The cells were chilled, HMT was added to yield a final concentration of 0.3–20 μg/ml (from a 0.25 mg/ml or 2.50 mg/ml stock in ethanol), and cells were chilled for 10 more min. The cells were then poured into 50 ml plastic petri dishes, placed on a sheet of aluminum foil on a rotary shaker and irradiated at room temperature for 1.5 min with two fluorescent black-light bulbs (Sylvania, F15T8-BLB, 15 W) while rotating at 90 rpm. The dose rate, measured with a calibrated silicon photo-diode (PIN IOUV) from United Detector Technology, was 3 W/m^2 at 350 nm. Following irradiation, the cells were pelleted, resuspended in 0.5 ml of Tris buffer, pH 9.5, and lysed by addition of 60 μl of 10% SDS. The lysate was extracted twice with phenol/chloroform/isoamyl alcohol (12:12:1) and then twice with chloroform/isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate and three volumes of 95% ethanol were added sequentially at room temperature. The precipitated DNA was collected by spooling onto a glass rod, rinsed twice with 70% ethanol and resuspended in 100 μl of TE buffer. Two μl of RNAase A (10 mg/ml) were added and the samples were incubated at 37°C for 30 min. The DNA was reprecipitated, washed as described above and finally resuspended in 50 μl of TE (10 mM Tris pH 8.0, 1 mM EDTA).

An aliquot of each DNA sample (1.5 μg) was restricted with *Eco*RI, *Hpa*II or *Pst*I (6 units overnight), 5 μg yeast tRNA were added as carrier and the nucleic acids were precipitated with ethanol at −70°C for 20 min. After centrifugation, the pellets were washed twice with 70% ethanol and resuspended in 9 μl of 4% sucrose, 0.25% bromophenol blue. The samples were alkali-denatured by adding 1 μl of 2 N NaOH at room temperature and were then immediately loaded onto 1% neutral agarose gels in Tris-acetate buffer (40 mM Tris-acetate pH 8.2, 2 mM EDTA) at 4°C. Following electrophoresis [for ca. 2 hrs at 100 V in an IBI mini-gel apparatus (Model QSH)], the DNA was blotted onto nitrocellulose (Schleicher and Schuell). The blots were probed with chloroplast DNA restriction fragments *Eco*RI 14, *Eco*RI 19 or *Hpa*II 5, or with a nuclear *Pst*I fragment containing the calmodulin gene. All probes were ^{32}P-labeled by the random primer method (23).

The HMT crosslinking assay is illustrated in Figure 1. Autoradiograms of the Southern blots were scanned with a Zeineh soft laser densitometer and the area under each peak was determined. The number of crosslinks in a restriction fragment should follow a Poisson distribution if crosslinking is random (7). Therefore, the average number of crosslinks in the DNA restriction fragment can be estimated using the equation: $P_0 = e^{-x}$, where P_0 is the percentage of the DNA fragment that was not crosslinked (i.e. that migrated as single-stranded DNA),

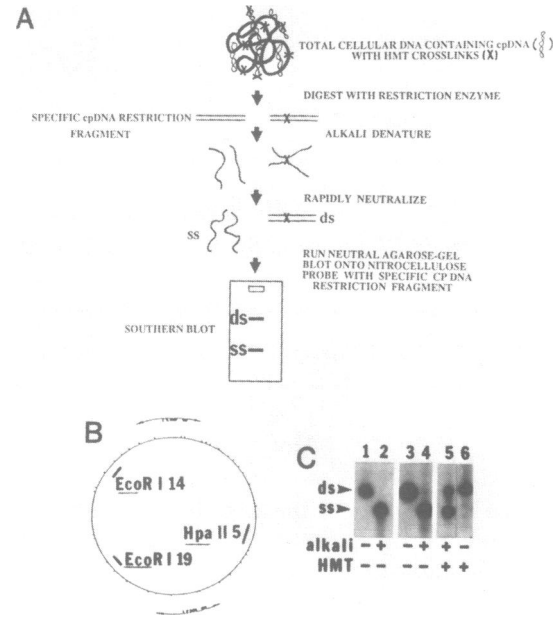

Figure 1. An assay for measuring the frequency of HMT crosslinking of specific regions of the chloroplast DNA. Panel A. Living algal cells were irradiated with UVA light in the presence of HMT to induce interstrand crosslinks in the chloroplast DNA (see Materials and Methods for details). After treatment, total cellular DNA was isolated and aliquots were restricted with *Eco*RI or with *Hpa*II. The DNA was then alkali denatured and separated in neutral agarose gels. The DNA was blotted to nitrocellulose and probed with labeled chloroplast restriction fragment *Eco*RI 14, *Eco*RI 19 or *Hpa*II 5 which encompasses promoter P_A (42). *Eco*RI 14 contains the *rbcL* gene, encoding the large subunit of ribulose bisphosphate carboxylase, the *psaB* gene, encoding a reaction center protein of photosystem I, and the 5′ end of the *atpA* gene, encoding the CF_1 alpha subunit of the chloroplast ATPase (43). *Eco*RI 19 encodes $tRNA^{tyr}$ (43). Cp, chloroplast DNA; ds, double-stranded DNA; ss, single-stranded DNA. Panel B. Map of *Chlamydomonas* chloroplast DNA showing the location of restriction fragments *Eco*RI 14, *Eco*RI 19 and *Hpa*II 5 used as probes in the crosslinking assay. Note that these fragments are well-separated. The nomenclature for the *Eco*RI fragments is that of Harris and coworkers (43). Other workers have named these fragments differently (44). The inverted repeats of the chloroplast DNA are indicated by arrows. Panel C. A representative Southern blot probed with chloroplast DNA restriction fragment *Eco*RI 14 is shown. Lane 1, non-denatured *Eco*RI 14 obtained from plasmid pCP43 (44); lane 2, denatured *Eco*RI 14; lane 3, non-denatured, *Eco*RI digested total cellular DNA (1.5 μg) from light-grown control cells (UVA-irradiated for 1.5 min in the absence of HMT); lane 4, same as lane 3 except that the DNA was denatured; lane 5, denatured, *Eco*RI digested DNA from light-grown cells irradiated with UVA light in the presence of HMT (0.5 μg/ml); lane 6, same as lane 5 except that the DNA was not denatured. The autoradiogram was purposely overexposed to show that there was no single-stranded algal DNA unless the DNA had been alkali-denatured, and that there was no double-stranded algal DNA after denaturation, unless the cells had been treated with HMT and UVA light.

and x is the average number of crosslinks in the DNA restriction fragment. For theoretical and technical reasons this estimate is most accurate when there are about equal proportions of single- and double-stranded DNA. To compare crosslinking in different restriction fragments, these data were expressed as crosslinks per 10 kbp.

We expected that crosslinking of chloroplast DNA from a given algal culture would increase with increasing HMT concentrations. Within the concentration range used in our experiments, the extent of HMT crosslinking was indeed proportional to the HMT concentration (Fig. 2). Crosslinking was also dependent on the UVA dose, as expected (data not shown).

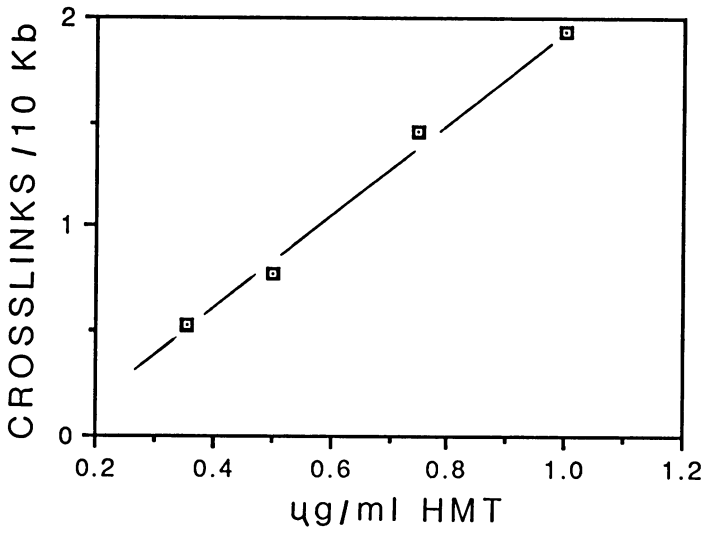

Figure 2. HMT crosslinking of chloroplast DNA as a function of HMT concentration. The data were obtained by crosslinking the *Hpa*II 5 region of the chloroplast chromosome from dark-adapted cells.

Enzymes and Chemicals

Restriction enzymes *Hpa*II, *Eco*RI and *Pst*I, and DNA polymerase I Klenow fragment were from Bethesda Research Laboratories. RNAase A and random primers were from Boehringer Mannheim. Cycloheximide, novobiocin and nalidixic acid were from Sigma. Atrazine was from Ciba-Geigy. HMT was from HRI Associates, Berkeley, CA. [α-^{32}P]dCTP (800 Ci/mol) was from Amersham.

RESULTS

HMT Crosslinking of Chloroplast DNA in vivo

HMT intercalates into DNA and forms covalent adducts with pyrimidines when exposed to UVA light (8). A monoadduct can subsequently crosslink with an appropriately positioned pyrimidine on the opposite DNA strand. After cutting with a restriction enzyme, alkali denaturation and neutralization, crosslinked restriction fragments will rapidly reanneal and migrate as double-stranded species, whereas the fragments containing no crosslinks will migrate as single-stranded species (7).

The HMT crosslinking assay is outlined in Figure 1A and a representative experiment is shown in Figure 1C. *Eco*RI-restricted total cellular DNA from HMT-treated cells was alkali-denatured and rapidly renatured. After electrophoretic separation, blotting, and probing of the Southern blot with *Eco*RI 14, only the two expected bands corresponding to double- and single-stranded *Eco*RI 14 hybridized with the labeled *Eco*RI probe (Fig. 1C, lane 5). As expected, non-denatured restriction fragments migrated as double-stranded DNA (Fig. 1C, lanes 1, 3 and 6), and alkali-denatured DNA which was not crosslinked migrated as single-stranded DNA (Fig. 1C, lanes 2 and 4).

For the reasons discussed in Materials and Methods, all subsequent comparisons were done at optimal HMT concentrations and UVA doses and we discuss our results in terms of potential HMT-dependent crosslinking of DNA.

Potential HMT Crosslinking of Chloroplast DNA Depends upon the Growth State

We suspected from preliminary experiments (not shown) that the potential for HMT crosslinking of chloroplast DNA is influenced by the physiological growth state of the cells. Further studies showed that HMT crosslinking of chloroplast DNA increased with increasing cell density (Fig. 3). Therefore, all subsequent experiments were conducted under controlled growth conditions (see Materials and Methods).

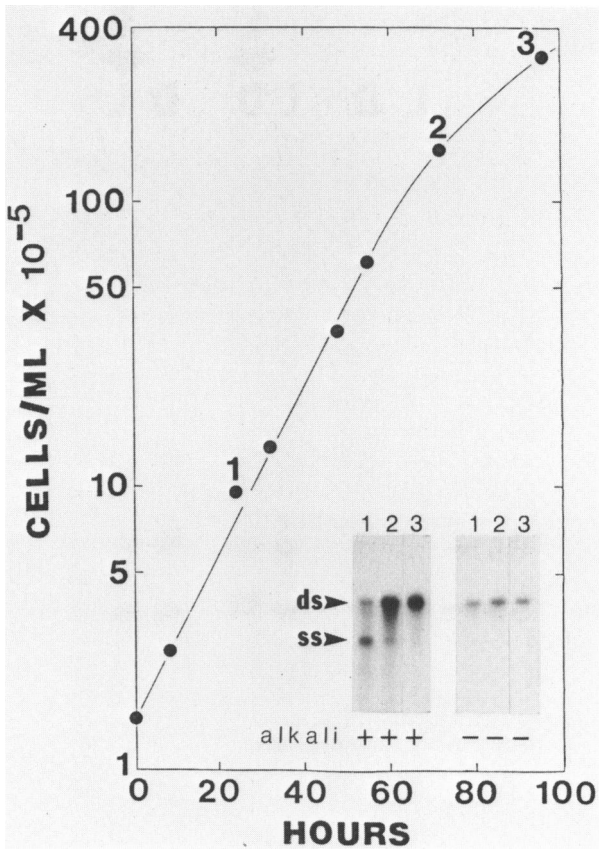

Figure 3. Potential HMT crosslinking of chloroplast DNA is dependent upon the growth state. Light-adapted cells were diluted to 2 × 10^5 cells/ml at 0 hrs and growth in the light was continued. Cell concentration was measured at the indicated times. At the indicated points (i.e. 1, 2 and 3), aliquots of the culture were used in the HMT crosslinking assay. The inset shows a Southern blot, analogous to the one shown in Figure 1C, probed with *Hpa*II 5. The HMT concentration was 1.0 μg/ml throughout.

White Light Reduces Potential HMT Crosslinking of Chloroplast DNA

Chloroplast DNA in light-adapted cells experienced less HMT crosslinking than the DNA in dark-adapted cells (Fig. 4, lanes 1 and 2 and Table I, section I). This result was found in three widely separated regions of the genome, i.e. in the *Eco*RI 19, *Hpa*II 5 and *Eco*RI 14 fragments (Fig. 1B and Table I, section I). Chloroplast DNA in light-adapted cells which were subsequently grown in the dark for 3 hrs showed an increase in potential HMT crosslinking in these same regions (Fig. 4, lanes 3 and 4 and Table I, section II). Conversely, chloroplast DNA in dark-adapted cells which were transferred into white light for 3 hrs showed a decrease in potential HMT crosslinking (Fig. 4, lanes 5 and 6 and Table I, section III). The crosslinking ratios of chloroplast DNA from light- versus dark-grown cells were similar (not statistically significantly different) in all three regions of the genome, regardless of the different conditions of the experiments shown in Tables 1 and 2 and in many other

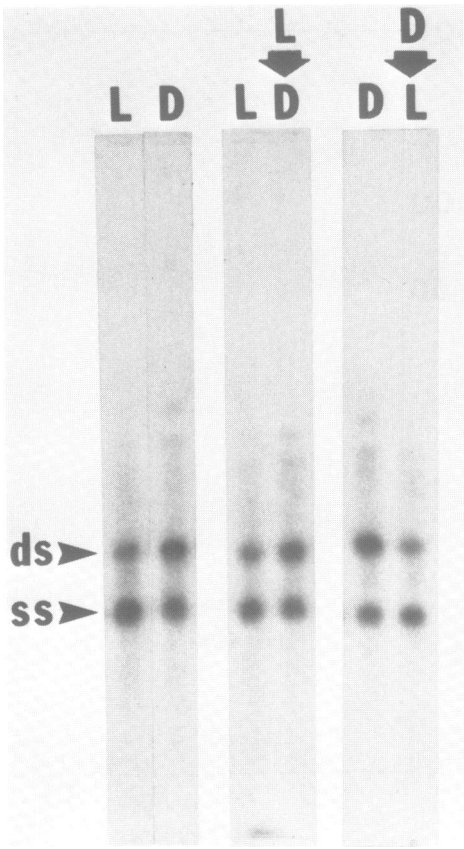

Figure 4. Light reduces potential HMT crosslinking of the chloroplast DNA. A representative Southern blot probed with chloroplast DNA restriction fragment *Eco*RI 19 is shown (ds, double-stranded *Eco*RI 19; ss, single-stranded *Eco*RI 19). In analogous experiments, HMT crosslinking in the *Hpa*II 5 and *Eco*RI 14 regions was analyzed (see Table I for quantitation). L, light-adapted cells (650 μW/cm^2 white light); D, dark-adapted cells; L→D, light-adapted cells shifted into darkness for 3 hrs; D→L, dark-adapted cells shifted into white light (650 μW/cm^2) for 3 hrs.

independent experiments (not shown). These ratios ranged from about 0.40 to about 0.75.

An HMT concentration of 1.0 μg/ml (Fig. 4 and Table I) allowed for the most accurate simultaneous determination of the crosslinking frequencies in the three regions under the three different light/dark treatments. Increasing or decreasing the HMT concentration in these and other experiments increased or decreased, respectively, the absolute frequency of crosslinking as expected. In spite of this, the effects of light on reducing the potential crosslinking were similar at different HMT concentrations (not shown). The much higher HMT concentrations required to crosslink the nuclear DNA (see below), crosslinked the chloroplast DNA to such an extent that quantitative comparisons could not be made at the same HMT concentration.

The light-induced decrease in potential HMT crosslinking was not due to increased shielding of chloroplast DNA from UVA light by pigments since we found no significant change in pigments during the 3 hr light or 3 hr dark treatments (data not shown). There was also no change in thylakoid membranes (electron micrographs not shown) which might sequester the hydrophobic HMT molecules. It should be recalled that *Chlamydomonas* chloroplasts develop normally and insert functional chlorophyll into thylakoid membranes in the dark (21).

Table 1. Light reduces potential HMT crosslinking of the chloroplast DNA.[a]

		EcoRI 19	*HpaII 5*	*EcoRI 14*
	dark (crosslinks/10 kb)	1.90	1.58	1.04
I	light (crosslinks/10 kb)	0.93	0.75	0.79
	ratio (light/dark)	**0.49**[1]	**0.47**[1]	**0.76**[1]
	light (crosslinks/10 kb)	0.88	1.11	0.74
II	3 hrs darkness (crosslinks/10 kb)	1.79	1.93	1.61
	ratio (light/dark)	**0.49**[2]	**0.57**[2]	**0.46**[2]
	dark (crosslinks/10 kb)	2.19	1.94	1.70
III	3 hrs light (crosslinks/10 kb)	1.07	0.89	0.93
	ratio (light/dark)	**0.49**[3]	**0.46**[3]	**0.55**[3]

[a]Autoradiograms like those shown in Figure 4 were scanned with a Zenith soft laser densitometer and the tracings were used to calculate the number of crosslinks/10 kb as described in Materials and Methods. Superscript numbers indicate ratios obtained from DNA of the same algal culture. The HMT concentration was 1.0 μg/ml in all cases. The cells were exposed to white light (650 μW/cm^2).

Table 2. Photosynthesis is not required for the decrease in potential HMT crosslinking of chloroplast DNA when cells were grown in the light.[a]

		Ratio of Crosslinks[b] *EcoRI 14*	*EcoRI 19*	*HpaII 5*
	light, no atrazine / dark, no atrazine	0.65	0.75	–
I	light, + atrazine / dark, no atrazine	0.51	0.62	–
	light, + atrazine / dark, + atrazine	–	–	0.50
II	dark, + atrazine / dark, no atrazine	1.04	1.02	–
III	blue light, no atrazine / dark, no atrazine	0.76	–	0.78

[a]Dark-adapted cells were exposed to white light (650 μW/cm^2) for 3 hrs in the absence or presence of 5 μM atrazine (the drug was added 30 min prior to exposure to light) (I); treated with atrazine for 3.5 hrs in the dark (II); or exposed to dim blue light (20 μW/cm^2) for 3 hrs (III). – : not determined.
[b]The number of crosslinks/10 kb for each sample was calculated as described in the legend to Table 1. These numbers were then used to calculate the indicated ratios. The HMT concentration was 0.5 μg/ml in all experiments. This concentration was used because it allowed for the most accurate quantitation of the crosslinking frequencies in DNA from dark-adapted cells.

As a control, we tested the effect of growth in the light on potential HMT crosslinking of nuclear DNA. Crosslinking was measured in a 3.2 kb *Pst*I genomic DNA fragment containing the calmodulin gene together with 5′ and 3′ flanking sequences (24). In contrast to chloroplast DNA, potential HMT crosslinking of this nuclear DNA fragment was similar under all growth conditions tested (i.e. continuous dark, dark with subsequent 3 hrs light, or continuous light) (Fig. 5, lanes 3–5). Like in chloroplast

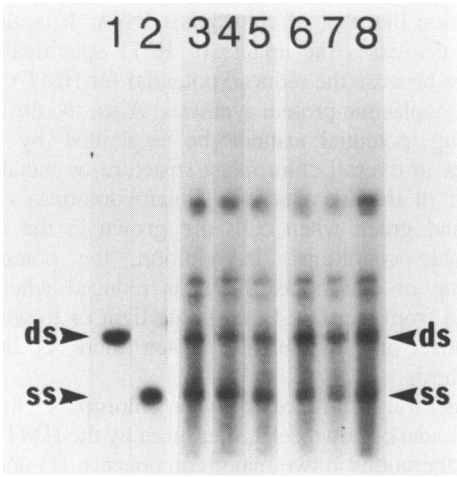

Figure 5. White light does not affect potential HMT crosslinking of nuclear DNA. Lanes 1−8 show a Southern blot probed with a 3.2 kb genomic *Pst*I fragment containing the calmodulin gene. Lanes 1 and 2, the cloned 3.2 kb fragment not denatured and alkali-denatured, respectively. Lanes 3−5, *Pst*I digested, alkali-denatured total cellular DNA from dark-adapted cells (lane 3), dark-adapted cells exposed to white light (650 $\mu W/cm^2$) for 3 hrs (lane 4) or light-adapted cells (lane 5). The HMT concentration used to crosslink the nuclear DNA *in vivo* was 9.5 $\mu g/ml$. Lanes 6−8, same as lanes 3−5, respectively, only the HMT concentration was 25 $\mu g/ml$.

DNA, increasing the HMT concentration increased crosslinking of nuclear DNA, as expected (Fig. 5, lanes 6−8). These results indicate that exposure to light does not interfere with HMT uptake or UVA transmission in the algal cells.

It should be noted that significantly greater amounts of HMT were required to crosslink the nuclear DNA as compared with the chloroplast DNA (compare Figs. 4 and 5). This result is consistent with the conclusion of other workers that eukaryotic nuclear DNA *in vivo*, in contrast to bacterial DNA and chloroplast DNA (see below), is under little or no torsional stress (6) and that binding of histones to the nuclear DNA reduces intercalation of HMT (11,13).

As a second control, we asked if light affects removal or repair, during sample preparation, of crosslinks that were formed in the chloroplast DNA in the cells. DNA of light-adapted cells was HMT crosslinked and then part of the culture was kept on ice and part was incubated at 25°C for 5 min to allow for repair. Both samples were then processed as usual at 0−4°C. This procedure takes about 10 min before the cells are lysed and DNA is extracted. HMT crosslinking of the chloroplast DNA was unaffected by the 5 min incubation at 25°C (not shown), indicating that there was little, if any, removal of HMT crosslinks during our normal assay conditions. We conclude that the difference in potential HMT crosslinking of chloroplast DNA that we found between dark-adapted cells and cells exposed to light is not due to a difference in the rate of removal of the crosslinks.

All these considerations together suggest that the light-dependent decrease in potential HMT crosslinking of the chloroplast DNA is due to a relatively rapid and stable change in the overall structure of chloroplast chromosomes.

Photosynthesis is not Required to Reduce Potential HMT Crosslinking of the Chloroplast DNA

Results of two experimental approaches indicate that photosynthesis is not required for the decrease in potential HMT crosslinking of the chloroplast DNA from light-grown cells. Firstly, we tested for effects of the photosynthesis inhibitor atrazine during growth in the light. As a second approach, we asked whether dim blue light of insufficient intensity to drive overall photosynthesis could reduce potential HMT crosslinking.

Atrazine, at a concentration of 5 μM for 30 min prior to and during growth in the light, clearly did not prevent the light-induced decrease in potential HMT crosslinking of the chloroplast DNA (Table 2, parts I and II). Under our conditions, atrazine strongly inhibited phototrophic growth in liquid medium at a concentration as low as 0.1 μM but had little, if any, effect on heterotrophic growth in the dark even at 10 μM. We therefore believe that the 5 μM concentration of atrazine used in our crosslinking experiments was sufficient to inhibit photosynthesis.

Growth in dim blue light (20 $\mu W/cm^2$), incapable of causing measurable O_2 evolution (25), also clearly reduced potential HMT crosslinking of the chloroplast DNA (Table 2, part III). These results do not support nor contradict the idea that a blue light receptor is involved in this response. Other wavelengths of light are currently being tested.

Together, these results indicate that growth in the light reduces potential HMT crosslinking of chloroplast DNA under conditions where photosynthesis is strongly inhibited. We conclude that photosynthesis plays little, if any, role in this light response. This conclusion, in turn, makes it unlikely that our results are due to light-induced changes in overall chloroplast metabolism.

Table 3. Cycloheximide inhibits the light-dependent reduction of potential HMT crosslinking of chloroplast DNA.[a]

		Ratio of Crosslinks[b] *EcoRI 14*	*HpaII 5*
I	light, no cycloheximide / dark, no cycloheximide	0.58	0.66
	light, + cycloheximide / dark, no cycloheximide	1.05	0.97
II	dark, + cycloheximide / dark, no cycloheximide	–	0.88

[a]Dark-adapted cells were exposed to white light for 3 hrs in the absence or presence of 10 $\mu g/ml$ cycloheximide (the drug was added 30 min prior to exposure to light) (I); or treated with cycloheximide at the same concentration for 3.5 hrs in the dark (II).
[b]These ratios were determined as in the experiments summarized in Table 1. The HMT concentration was 0.5 $\mu g/ml$ throughout.

Cytoplasmic Protein Synthesis is Required for Light to Reduce Potential HMT Crosslinking of the Chloroplast DNA

Cycloheximide (an inhibitor of cytoplasmic ribosomes) at a concentration of 10 $\mu g/ml$ (26) clearly blocked the light-induced reduction of potential HMT crosslinking of the chloroplast DNA (Table 3, part I). This concentration of cycloheximide, which was more than 25 times the concentration required to arrest growth, had little, if any, effect on potential HMT crosslinking of chloroplast DNA in the dark (Table 3, part II and data not shown). We conclude that at least one of the components required for the light response is synthesized on cytoplasmic ribosomes. This requirement for cytoplasmic protein synthesis also supports the conclusion that light does not reduce potential HMT crosslinking of the chloroplast DNA by simply inhibiting uptake of HMT into the chloroplast.

Table 4. HMT crosslinking of chloroplast DNA in novobiocin-treated, nalidixic acid-treated and gamma-ray-irradiated cells.

	Ratio of Crosslinks[a] (treated/control)	
	EcoRI 14	*HpaII 5*
novobiocin[b]	0.32	0.22
nalidixic acid[c]	0.28	–
gamma-irradiation[d]	0.71	–

[a]These ratios were determined as in the experiments summarized in Table 1. The HMT concentration was 2.0 μg/ml. This higher HMT concentration was used to insure that measurable proportions of crosslinked chloroplast DNA fragments were present after the novobiocin and nalidixic acid treatments, both of which greatly reduced potential HMT crosslinking. (Also note that in these experiments we used light-adapted cells and not dark-adapted cells as in the experiments summarized in Tables 2 and 3.)
[b]Light-adapted cells were treated with 200 μg/ml novobiocin for 2 hrs.
[c]Light-adapted cells were treated with 100 μg/ml nalidixic acid for 1 hr.
[d]Light-adapted cells were irradiated with 12.5 krad of gamma-rays from a cobalt source over a 15 min period. The cobalt source available to us had considerably lower intensity than the source used by Pettijohn and coworkers (6). The longer exposure time that we had to use also increased the chance for repair of the radiation-induced nicks.

Novobiocin, Nalidixic Acid and Gamma-Ray Irradiation, All of Which Reduce Torsional Stress, Reduce Potential HMT Crosslinking of the Chloroplast DNA

In bacteria, the inactivation of DNA gyrase results in an increase in the average linking number (27) and a decrease in the average torsional stress of the DNA as measured by psoralen binding (6). As expected, *in vivo* inhibition of the chloroplast supercoiling topoisomerase (17,19) with sublethal concentrations of novobiocin (200 μg/ml) greatly reduced the number of HMT crosslinks both in the *Hpa*II 5 and in the *Eco*RI 14 region within 2 hrs after addition of the drug (Table 4, line 1). Higher novobiocin concentrations had an even greater effect (data not shown). Similar effects were seen after inhibition of this chloroplast topoisomerase with nalidixic acid (100 μg/ml) (17,19) within 1 hr after addition of the drug (Table 4, line 2).

Gamma-ray irradiation which nicks and thereby partially relaxes the chloroplast DNA also decreased potential HMT crosslinking *in vivo* (Table 4, line 3).

These results show directly that the HMT crosslinking assay can readily detect a reduction of average torsional stress in the chloroplast DNA.

DISCUSSION

We have shown that the potential for HMT crosslinking of chloroplast DNA in three widely separated regions is higher in dark-grown than in light-grown Chlamydomonas cells. The higher or lower potential is established within less than three hours after growing cells are transferred to the dark or to the light, respectively. Based on these results, we conclude that growth of cells in the light alters the overall structure of the chloroplast chromosome. Trivial explanations for our results can be excluded from results of several control experiments.

The different potential for HMT crosslinking of chloroplast DNA cannot be explained by different uptake of HMT into cells under these conditions, because there is no difference in HMT crosslinking of nuclear DNA from light-vs. dark-grown cells even though the actual extent of crosslinking depends on the HMT concentration like that of chloroplast DNA. It is also unlikely that light decreases the uptake of HMT specifically into the chloroplast because the reduced potential for HMT crosslinking requires cytoplasmic protein synthesis. Also, the different HMT crosslinking potential cannot be explained by significant differences in overall chloroplast structure or metabolism. As mentioned in the Introduction, Chlamydomonas chloroplasts develop and green when cells are grown in the dark under heterotrophic conditions. In addition, the potential HMT crosslinking of chloroplast DNA is reduced when cells are transferred from the dark to dim blue light or to white light in the presence of atrazine, i.e. when there is little or no photosynthesis.

The structural differences between chloroplast chromosomes of light- vs. dark-grown cells, measured by the HMT assay, can be due to alterations in two major components: (1) unconstrained torsional stress of the DNA and (2) DNA binding proteins which relieve average torsional stress and/or shield the DNA.

Within the framework of current models on the maintenance of torsional stress in DNA, especially the twin supercoiling domain model (28) and the homeostatic balance model (29,30), light might alter torsional stress of the chloroplast DNA in several ways: e.g. by affecting concentrations or activities of one or more chloroplast topoisomerases, by altering concentrations or activities of certain DNA binding proteins which constrain torsional stress, and/or by affecting transcription or other DNA-protein interactions which involve proteins tracking along the DNA.

In bacteria, certain environmental signals activate signal-transduction pathways leading to changes in superhelicity and, by implication, in torsional stress of DNA (31–36). *Chlamydomonas* cells might contain an analogous transduction pathway that is light-activated, ultimately leading to a partial relaxation of torsional stress of the chloroplast DNA. Results of Kochel and Sinden (37) show that a two-fold reduction in the superhelical density of bacterial plasmid DNA results in an approximately two-fold reduction in the frequency of HMT crosslinking of this DNA. Growth in light (vs. dark) results in approximately two-fold reduction in HMT crosslinking potential. This is less than the reduction induced by chloroplast DNA gyrase inhibitors and indicates that even if light were exclusively to reduce unconstrained torsional stress, it would relax only a fraction of it.

The extreme view of a possible alternative explanation is that light induces a change in the amount and/or activity of chloroplast chromosomal proteins without causing a significant change in the torsional stress of the chloroplast DNA. Such proteins could theoretically 'coat' a significant fraction of the chloroplast DNA and/or promote a condensation or compaction of the chloroplast chromosomes. In either case, these proteins could reduce the potential HMT crosslinking of chloroplast DNA by shielding the DNA from HMT or UVA light required for crosslinking, or both. This extreme explanation is probably not a true alternative, because many DNA binding proteins, including histones, alter the structure of DNA to which they bind (reviewed in references 38 and 39).

A light-induced change in binding of DNA sequence-specific repressor and/or activator proteins or proteins that bind to alternative DNA structures in chloroplast DNA cannot be responsible for the difference in HMT crosslinking. Such proteins would not be expected to affect the HMT reactivity of the entire chloroplast chromosome, especially at the low levels of HMT crosslinking employed in our experiments (i.e. 1–2 crosslinks/10 kb).

Our results indicate that changes in HMT crosslinking of different regions of the chloroplast DNA are not strictly correlated with changes in transcription from the same regions (data not shown).

The physiological significance of the alterations of chloroplast chromosome structure in response to light is a matter of speculation. As mentioned above, all processes that involve unwinding of the DNA potentially affect and are affected by changes in torsional stress and by binding of proteins to DNA (40). Thus, changes in chromosome structure may affect replication, transcription, recombination and/or repair of chloroplast DNA. In this respect, it is interesting to note that in phototrophically grown, synchronized Chlamydomonas cells, chloroplast DNA replication starts approximately 3 hrs after transfer of cells from the dark to the light (41). This time frame is similar to that when the light-induced change in chromosome structure becomes apparent. Investigating this possible correlation is of great future interest.

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