Light and Genetic Determinants in the Control of Specific Chloroplast Transcripts in *Chlamydomonas reinhardtii*¹

Received for publication January 20, 1984 and in revised form May 15, 1984

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**ABSTRACT**

We have looked for chloroplast genes whose expression is controlled by light by comparing the abundance of specific chloroplast transcripts in light-grown and dark-grown cells of *Chlamydomonas reinhardtii*. In addition, we have investigated whether genetic components influence expression of such genes.

Northern blot analyses of specific separated transcripts showed that in certain strains (e.g. CC-278), several chloroplast transcripts from the Hpa II 5 region of the chloroplast chromosome were more abundant in dark-grown cells than in light-grown cells. The increased abundance of these Hpa II 5-specific transcripts was dependent on the genetic background: we have isolated mutants and we have found distantly related wild type strains which do not overaccumulate these transcripts in the dark.

The strain which overaccumulated the Hpa II 5-specific transcripts, and only these strains, died after several cell divisions following transfer to the dark. Overaccumulation appears to be a necessary but not sufficient prerequisite for commitment to cell death in the dark.

By analogy to bacterial systems, we speculate that a critical event associated with accumulation of one of these specific transcripts involves control at the level of transcription initiation or termination-antitermination.

**MATERIALS AND METHODS**

**Algal Strains, Medium, and Culture Conditions.** *Chlamydomonas reinhardtii* cw-15 (cell wall minus) strains CC-278(−), CC-406(−), and CC-1311(−) were obtained from Dr. Elizabeth Harris, Chlamydomonas Genetics Center, Duke University, Durham, NC. Other strains mentioned in Table I were isolated in this laboratory by picking clones derived from single cells of strain CC-278.

A modified Bold's Basic Medium (4) was used in all experiments. Since the strains used in this study cannot use nitrate as the sole nitrogen source, sodium nitrate (250 mg/l) was replaced with ammonium nitrate (250 mg/l). In addition, sodium acetate (3 g/l), bactopeptone (4 g/l), and yeast extract (5 g/l) were added. All cultures were grown at room temperature (20–27°C) on a gyratory table (100 rpm). Cultures grown in the light received about 2000 lux of cool-white fluorescent light. Such cultures, at concentrations of 5 to 20 × 10⁶ cells/ml, were used to inoculate experimental cultures to an initial cell density of 1.5 × 10⁵ cells/ml. For subsequent growth in the dark, cultures were either placed in a light-proof box or were wrapped in a double layer of aluminum foil. Cell concentrations were determined with a hemacytometer.

Clones from single cells were obtained by plating cells onto sterile Whatman No. 1 filter paper which had been placed on the surface of 25 ml of solid medium (modified Bold's Medium containing 0.8% Difco agar) in 9 cm plastic (Falcon) Petri plates. The filter paper increased the plating efficiency.

**Test for Greening.** Various strains were grown in the light to saturation (1.5–3 × 10⁷ cells/ml). Ten μl of each culture were spotted onto filter paper on solid medium and the plates were incubated in the dark for at least 7 d.

**Preparation of Cellular RNA (7).** Total RNA was isolated from cells grown in the light or in the dark after about five or three to five cell doublings, respectively. Cells (2–5 × 10⁶) were sedimented, washed once with 0.1 M Tris, pH 9.5, and resuspended in 0.5 ml of this same buffer. Diethylpyrocarbonate (Sigma) was added, followed by 60 μl 10% SDS to lyse the cells. After about 20 sec, the lysate was extracted by adding 0.5 ml phenol/chloroform/isoamyl alcohol (12:12:1). This extraction was repeated once and was followed by one extraction with chloroform/isoamyl alcohol (24:1). The final aqueous phase was made 0.1 M in sodium acetate. DNA was precipitated by adding two volumes 95% ethanol. The tubes were gently inverted several times and the DNA was carefully spooned onto a glass rod. RNA was then precipitated at −20°C overnight. All manipulations prior to RNA precipitation were performed at room temperature. The precipitated RNA was washed once with 70% ethanol, dried, and dissolved in 0.1 M Tris, pH 9.5, and heated at 65°C for 5 min before spotting onto filter paper.

**Northern Blot Analyses.** We have made a modified version of a Northern blotting procedure (20) to detect small amounts of RNA in samples. RNA was isolated from cultured cells grown in the light or in the dark, and the RNA was separated on formaldehyde-agarose gels (10). The DNA probe was recovered from the gel and used to detect specific chloroplast transcripts.

Structure and function of chloroplast DNA in various plant cells are rapidly being elucidated (for review, see 14). Little is known, however, about control of chloroplast genes. Since light is a critical environmental factor for plant cells, we have looked for differential expression of specific chloroplast genes in light-grown and dark-grown *Chlamydomonas reinhardtii* cells of different genetic backgrounds.

As a first step in studying light-dark control, we have measured the abundance of specific chloroplast transcripts under various conditions in various strains. This approach does not require that the affected transcripts are translated nor that the functions of the corresponding genes are known.

Chloroplast transcripts were analyzed by probing Northern blots of total cellular RNA with specific fragments of chloroplast DNA. This method can detect differences in specific transcripts with very high sensitivity. Most significantly, our results show that both light and genetic background control the abundance of certain chloroplast transcripts from the Hpa II 5 region of the chloroplast chromosome.

¹ Supported by National Cancer Institute Training Grant CA09385 and Cellular Molecular Biology Training Grant 2T32 07319 (R. J. T.), by National Institutes of Health Grant GM 13221-18 (G. M.), and by the Vanderbilt University Natural Science Committee Fund.

² Abbreviations: (−), mating type minus; kb, kilo base (RNA) or kilo base pair (DNA); LS, large subunit of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase; D2, a chloroplast encoded photosystem II associated protein.
dissolved in 50 μl water, and stored at −20°C. RNA prepared in this manner contained only trace amounts of DNA. The proportion of contaminating DNA varied between different RNA preparations.

**Northern Blot Analysis.** Total RNA was glyoxylated in DMSO (glyoxal and DMSO from Fisher), fractionated in 1% agarose gels, and blotted onto nitrocellulose (6, 13). For each blot of the different RNA samples, equal amounts of RNA, based on A260 were used. Blots were probed with specific DNA by one of two methods. In method A, prehybridization, hybridization (in the absence of dextran sulfate), and washing were as described in (13). In method B, prehybridization and hybridization employed 35% formamide and the final low-salt washes were at room temperature. After washing and drying the blots were exposed to Kodak XAR-5 x-ray film at room temperature or at −70°C in the presence of a DuPont Cronex Lightning Plus Screen. The autoradiograms were scanned with a Zeineh Soft Laser Scanning Densitometer.

DNA fragments to be used as probes came from three different sources. They were obtained by digesting total chloroplast DNA (prepared as in [2]) with Hpa II or BamH I, plasmid pCP43 DNA (see below) with EcoR I, or plasmid pRT1 DNA (see below) with BamH I and Pst I. Each total digest was 32P-labeled by the replacement synthesis method (6). The labeled DNA segments were separated in 0.7% agarose gels by electrophoresis in the presence of ethidium bromide. The desired separated DNA segments were cut out of the gel, boiled for 5 min, and added directly to 5 to 10 ml of hybridization mix (13) on ice.

**Plasmids and Bacterial Strains.** Plasmid DNA was prepared using the alkali lysis procedure described in (6). *Escherichia coli* strain C600 rII− m− containing plasmid pCP43 was obtained from Dr. J. D. Rocheix, University of Geneva, Switzerland. This plasmid contains chloroplast DNA restriction fragment EcoR I (5), containing the gene for LS (large subunit of RuBP carboxylase) (9), inserted into the EcoR I site of vector pBR1. Plasmid pRT1 was isolated in this laboratory by ligating chloroplast DNA restriction fragment Hpa II 5 into the Acc I site of vector pUC8 (8) and transforming *E. coli* strain JM103. Standard cloning procedures were used (6, 8). During the cloning, most of the Hpa II 5 insert was spontaneously deleted leaving behind a 0.8-kb fragment derived from one of its ends. (The reasons for this deletion are unknown.) Hpa II 5 was cloned intact (yielding plasmid pRT2) by ligation into the Cla I site of pBR322 and transformation of *E. coli* strain HB101.

**Enzymes and Reagents.** Restriction endonucleases were from Bethesda Research Laboratories or from Boehringer Mannheim (EcoR I). Digests were performed in TA buffer at 37°C for 1 to 2 h with 3 to 5 units of enzyme per μg of DNA. T4 DNA polymerase was purified by Dr. L. Rowen in our laboratory. 5′-[α-32P]dCTP (10 μCi/ml; ~800 Ci/m mol) was from Amersham and unlabeled dCTP, dATP, dGTP, and dTTP were from PL Biochemicals, Inc.

**RESULTS**

**Light-Dark Control of Hpa II 5 Transcripts.** Northern blots containing total RNA from light-grown (light RNA) and from dark-grown (dark RNA) CC-278 cells were probed with 32P-labeled Hpa II 5 DNA obtained from a digest of total chloroplast DNA. All transcripts originating from Hpa II 5 can be detected by this probe regardless of where they initiated or terminated. Figure 1A[a] shows that seven discrete major RNA species in the range of 3.1 to 1.4 kb were detected with this probe. We shall call the seven major species A through G and three additional minor RNA species H through J (Fig. 1A[b]) without attempting to distinguish between primary and processed or degraded transcripts. Species A (3.1 kb) dramatically overaccumulated in dark-grown cells as compared with light-grown cells (Fig. 1, A and B).

**Fig. 1.** Hybridization of Hpa II 5 to light RNA and dark RNA from strain CC-278. A, A Northern blot (7.5 μg cellular RNA per lane) was probed with 7.5 × 104 cpm of 32P-labeled Hpa II 5 obtained by digestion of total chloroplast DNA. Prehybridization, hybridization, and washing employed method A (see "Materials and Methods").Autoradiography was for 48 h (a) or for 2 weeks (b) at −70°C. Letters (A through J) refer to discrete transcripts. Transcript sizes were estimated from comigration with glyoxylated pBR322 restriction fragments of known sizes. B, Densitometer scans of the autoradiograms shown in A(a).

Transcripts B through E and G through I moderately overaccumulated whereas transcript F only slightly overaccumulated and transcript J was unaffected. These results indicate that in strain CC-278, light reduced the abundance of several transcripts originating from Hpa II 5. We do not yet know, however, whether in the light there was reduced synthesis, increased degradation, or a combination of the two.

The 8.0-kb Hpa II 5 fragment was mapped on the chloroplast chromosome by standard Southern blot and restriction endonuclease analyses (Fig. 2A). Using similar techniques, a cloned 0.8-kb subfragment of Hpa II 5 (from plasmid pRT1; see "Materials and Methods"), whose significance is discussed below, was mapped within Hpa II 5 (Fig. 2B).

We focused on the light-dark control of transcript A because it was clearly the most affected species and because it could be a precursor to some or all of the smaller species which overaccumulated in the dark (i.e. B–G). With the ultimate intent of identifying and characterizing the putative promoter region of transcript A, we wanted to first locate the general region of Hpa II 5 from which this species originated. Transcript A was also detected by probing Northern blots with the 0.8-kb subfragment of Hpa II 5 (see above) (Fig. 3, A and B). By using DNA probes labeled on one DNA strand only, we determined that transcript A was synthesized in the direction shown in Figure 2B. We therefore conclude that the putative promoter for transcript A is within Hpa II 5 and that it is in or near the 3.2-kb Hind III fragment of pRT2.

Interestingly, all the other major transcripts which hybridized to Hpa II 5 (i.e. B–G) were also detected after probing with the 0.8-kb subfragment of Hpa II 5 (Fig. 3, A and B). Only transcripts H through J failed to hybridize to the 0.8-kb probe (compare Figs. 1 and 3). The estimated sizes of transcripts A through G (Fig. 1), their hybridization with the 0.8-kb subfragment, and the fact that they are all probably synthesized in the same direction (determined as for transcript A) indicated that some or all of these transcripts overlap.

In other cases where light has been shown to affect the abundance of specific plant transcripts, phytochrome was involved
and short periods of light were sufficient to elicit the response (5, 11, 12). Since phytochrome activity depends on red light ($\lambda_{max}$ of 660 nm) we asked whether red light of that wavelength (4 min light per 12 h darkness) would inhibit transcript A in strain CC-278. We found that such red light did not reduce the abundance of transcript A (data not shown); thus, it is unlikely that phytochrome mediates the regulation of transcript A that we have described above. We do not know the nature of the receptor(s) that is (are) involved in this regulation.

**Effects of Light Versus Darkness on Other Chloroplast Transcripts.** Light RNA and dark RNA from CC-278 cells were probed with chloroplast DNA restriction fragments BamH I 11/12 (rRNA genes) (3) and EcoR I 15 (LS gene). In contrast to the transcripts detected with Hpa II 5, the major and most of the minor transcripts detected with the BamH I 11/12 probe appeared in very similar proportions in light RNA and dark RNA (Fig. 4, A and B). One exception was a relatively large transcript (arrow in Fig. 4A[b]) which appeared in reduced proportions in light RNA. Two transcripts that hybridized to EcoR I 15 (1 and 2 in Fig. 5, A and B) appeared in similar proportions in light RNA and dark RNA and one (transcript 3) appeared in slightly reduced proportions in light RNA. Quantitatively, all differences between light RNA and dark RNA detected with these probes were minor in comparison with the large difference observed with the 3.1-kb transcript A from Hpa II 5.

**Genetic Background Influences Hpa II 5 Transcript A.** To investigate whether genetic background affected the abundance of transcript A, Northern blots of light RNA and dark RNA from various strains were probed with the 0.8-kb Hpa II 5 subfragment. Representative results obtained with RNA from strain 278gro1 are shown in Figure 6 and the results of using all strains are summarized in Table I. Strains 278gro1 and 278gro2 were selected for improved growth in the dark (see below) from strain CC-278 while strain 278gro1 was selected because it greened in the dark (see below). The results clearly show that overaccumulation of transcript A in the dark depended on the genetic background: it did not occur in the two gro mutants nor in two other wild type strains obtained directly from the CGC (i.e. CC-406 and CC-1311).

**Growth and Greening of Different Strains.** Strains CC-278, CC-406, and CC-1311 all have the cell wall minus phenotype and require reduced nitrogen for growth. CC-278 and CC-406 are otherwise considered to be wild type strains while CC-1311 contains the str1 (streptomycin resistance) and pfl2 (paralyzed flagella) mutations. In liquid medium, strains CC-406 and CC-1311 grew logarithmically in the light and in the dark (Fig. 7 and data not shown). In contrast, strain CC-278 grew logarithmically in the light but grew poorly (Fig. 7) and eventually died in the dark (Table I). After about three cell doublings in the dark, many cells were enlarged and their content appeared granular in the light microscope. Eventually, these cells disintegrated, such that most cultures contained few if any viable cells after about 5 d. However, spontaneous mutants (see below) which survived in the dark appeared in some cultures. Obviously these mutants had selective advantage in the dark. Such mutants were isolated from strain CC-278 as rapidly growing colonies on agar plates incubated in the dark (Table I). These mutants were purified by two cycles of single colony isolation and have remained stable thereafter. Representative examples are the mutants 278gro1 (Fig. 7; Table I) and 278gro2 (Table I).

The results summarized in Table I show that overaccumulation of transcript A in the dark occurred only in cells which were committed to death in the absence of light. During the course of this study a second culture of strain CC-278 was obtained from the CGC. These cells also exhibited dark-lethality and overaccumulated transcript A in the dark (data not shown).

Since it has been reported that y-1 mutants, which do not green in the dark, die in the dark (15), we tested if dark-lethality was correlated with an inability to green in the dark. Various strains (Table I) were pregrown in the light, spotted onto solid medium, and subsequently grown in the dark. The results (Table I) clearly show that dark-lethality and overaccumulation of transcript A were unrelated to the potential to accumulate Chl in the dark.

**DISCUSSION**

We have found that certain *C. reinhardtii* strains, when grown in the dark, overaccumulate a family of overlapping chloroplast transcripts (A through G in Fig. 3) as compared with light-grown cells. Red light which activates phytochrome does not affect this overaccumulation, suggesting that the process is not regulated by phytochrome. These overaccumulated transcripts hybridize to an 0.8-kb piece of chloroplast DNA derived from one end of the 8.0-kb Hpa II 5 fragment (Figs. 1–3). Consistent with reports of
the gene products, (see "Materials and Methods") present in plasmid pRT1. Prehybridization (etc.) employed method B (see "Materials and Methods"). Autoradiography was for 18 h at -70°C.

others (7), in light-grown cells this region of the chloroplast chromosome shows low transcriptional activity when compared with other regions, e.g. restriction fragments BamH I 11/12 (rRNA genes) (Figs. 3 and 4), EcoR I 15 (LS gene) (Figs. 3 and 5), and EcoR I 3 (D2 gene) (data not shown).

The largest transcript of this family (A of 3.1 kb) became most abundant (Fig. 3). Significantly, when transcript A was overaccumulated so were the smaller transcripts, while conditions that reduced abundance of transcript A also reduced abundance of the smaller transcripts. These results show that failure to process transcript A to one or more of the smaller species cannot solely be responsible for overabundance of transcript A under these conditions. We propose that light, together with several specific gene products, control initiation and/or termination (for review of both, see 10) of transcript A. Our mutants that are altered in this control (e.g. 278groI in Table I) should be useful in dissecting the mechanism of this control. Hopefully, the DNA sequence of the putative promoter for transcript A will also be useful in this regard. We are sequencing the region of Hpa II 5 which we suspect contains this promoter (Fig. 2B).

We do not know the origin of the smaller transcripts which overlap with transcript A. They may be the products of degradation, of alternative splicing, of premature termination, or of one or more promoters distinct from the putative transcript A promoter. We stress that although these transcripts and their

Fig. 3. Hybridization of a cloned 0.8-kb subfragment of Hpa II 5 to light RNA and dark RNA from strain CC-278. A, A Northern blot (10 μg cellular RNA per lane; different RNA preparations than in Fig. 1) was probed with 3.0 × 10⁶ cpm of the 0.8-kb chloroplast DNA insert present in plasmid pRT1. Prehybridization (etc.) employed method B (see "Materials and Methods"). Autoradiography was for 18 h at -70°C.

Fig. 4. Hybridization of BamH I 11/12 (rRNA probe) to light RNA and dark RNA from strain CC-278. A, A Northern blot (10 μg cellular RNA per lane; same RNA preparations as in Fig. 3) was probed with 6.5 × 10⁶ cpm of BamH I 11/12 obtained by digestion of total chloroplast DNA. Prehybridization (etc.) employed method B (see "Materials and Methods"). Autoradiography was for 20 h at room temperature (a) or for 12 h at -70°C (b). B, Densitometers scans of the autoradiograms shown in A(a).

Fig. 5. Hybridization of EcoR I 15 (LS probe) to light RNA and dark RNA from strain CC-278. Details are as in the legend to Figure 4 except that the probe (2 × 10⁶ cpm) was the 5.7-kb chloroplast DNA insert present in plasmid pCP43. Transcript 3 is the mature LS mRNA (1).
commitment to lethality in the dark that we have described here.

Acknowledgments—We thank E. Harris for sending us several algal strains and J. D. Rochaix for plasmids pCP43 and pCP55. G. M. thanks F. Konig (University of Frankfurt) for many stimulating discussions and the Alexander von Humboldt foundation for a fellowship that initiated these discussions.

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