Chloroplast genes of the marine alga *Heterosigma carterae* are transcriptionally regulated during a light/dark cycle

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Abstract  
The transcription of four chloroplast-encoded genes (photosystem II gene psbA, the Calvin cycle genes rbcL and rbcS, and the chloroplast 23S rRNA) was analyzed in synchronous cultures of the marine chromophytic alga, *Heterosigma carterae*. A chloroplast run-on assay was developed for *H. carterae* to measure transcript initiation during a 12-hour light/12-hour dark cycle. Maximum expression of the psbA and rbcLS transcripts was shown to occur during the middle of the light portion of the cycle, with a 20- to 30-fold fluctuation between light (L6) and dark (D6), whereas rRNA levels remained essentially constant. Comparison of the run-on assays and Northern analysis indicated that control of transcript initiation is the primary mode of regulating steady-state levels of psbA and rbcLS mRNAs.

Introduction  
Photosynthesis is regulated at the cellular level by a variety of mechanisms, including control of gene transcription. In terrestrial plants, expression of both chloroplast and nuclear-encoded photosynthetic genes has been extensively studied in response to light cues. It is well documented that light affects gene expression in a cell-specific, organ-specific, and developmental stage–specific manner in both monocots and dicots (Kuhlmeier et al., 1987). The need to determine whether light-mediated changes in cellular RNA levels reflect an alteration in synthesis or turnover of a molecular species has resulted in the development of run-on transcription systems (Deng and Gruissem, 1987; Mullet and Klein, 1987). This technology allows the quantitative measurement of specific RNAs that have been newly initiated, and thus provides valuable insight into the RNA synthesis versus stability question. For example, during light-induced developmental changes in chlorophytes, nuclear genes (e.g., cab and rbcS) are transcriptionally controlled (Gilmartin et al., 1990), and chloroplast genes are predominantly, although not exclusively, posttranscriptionally regulated (Mullet and Klein, 1990; Gruissem, 1989).

Although a vast literature exists on the control of gene expression by environmental cues in terrestrial plants, essentially no information currently exists in the literature concerning control of chloroplast gene expression in marine plants. Recently, Valentin and colleagues (1992) examined the effect of light and sugar availability on messenger RNA (mRNA) levels of the chloroplast-encoded phycobilisome linker and a photosystem II gene. Steady-state levels of mRNA were higher in autotrophically growing cells exposed to light, but it was not determined whether this change in mRNA level was a transcriptionally or posttranscriptionally controlled response.

In this study, the unicellular alga *Heterosigma carterae* is used as a model chromophytic algal system. When maintained on a 12-hour light/12-hour dark (12L/12D) (diurnal) cycle, the cell cycle is synchronous, and logarithmically growing cells double once every 24 hours. Three photosynthetic genes are the focus of this study: the rbcL and rbcS genes encode the large and small subunits of ribulose 1,5 bis phosphate carboxylase (Rubisco),

*The taxonomic identity of this organism (previously known as Olisthodiscus luteus) has been recently revised (Taylor, 1992).*

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the first enzyme in the Calvin cycle; and the psbA gene encodes the 32-kd reaction center protein of photosystem II. Using a newly developed chloroplast run-on assay for H. carterae, we analyzed transcriptional regulation of these chloroplast-encoded genes, as well as the 23S ribosomal RNA (rRNA) during a 12L/12D diurnal cycle.

Results

Transcript abundance

Transcription of chloroplast-encoded photosynthetic genes psbA and the rbcL,S operon was analyzed during a 12L/12D cycle. In H. carterae, the rbcS gene is adjacent to the rbcL locus, and the genes are cotranscribed as a 2.6-kb mRNA (Reith and Cattolico, 1986; Hardison et al., 1992). The psbA transcript is 1.3 kb in length (Hardison, L.K. Personal communication). To determine the steady-state levels of these two transcripts, RNA was prepared from cells sampled during a 12L/12D cycle. The abundance of cellular rRNA species (including chloroplast, mitochondrial, and nuclear rRNAs) was found to be equivalent at all time points and was therefore used as a normalizing standard for this analysis (data not shown). Northern analysis was performed using H. carterae psbA and rbcS gene fragments as hybridization probes. Results indicated that there was a significant fluctuation in accumulation of the psbA and rbcL,S transcripts during the diel cycle, with a maximum at hour 6 in the light (L6) and a minimum at hour 6 in the dark (D6) (Figure 1). The quantitative difference between the maximum and minimum in two independent experiments averaged 33-fold for psbA and 21-fold for rbcL,S. A reproducible increase in mRNA levels for both transcripts occurred during the interval D9 to D12, although the magnitude of this increase was different in the two experiments. These data indicate that there is a major fluctuation in the abundance of psbA and rbcL,S transcripts in the plastid during a diurnal cycle.

Primer extension experiments (data not shown) indicated that the same initiating nucleotide is used in the light and dark for both psbA (42 bases upstream of the initiation codon) and for rbcL,S (247 bases upstream of the initiation codon) and correspond to that previously reported (Hardison et al., 1992; Hardison, L.K. Personal communication). Thus, primary transcripts of these genes are qualitatively identical for cells sampled in either phase of the light/dark cycle.

Development of run-on assay

To determine whether transcription initiation or transcript stability establishes steady-state levels of mRNA as measured by Northern analysis, plastid run-on assays have been developed for several terrestrial plants (Deng and Gruissem, 1987; Mullet...
These assays monitor elongation of nascent transcripts in the presence of $^{32}$P-UTP. The chloroplast run-on assay we developed in *H. carterae* (described in Experimental Procedures) was characterized to demonstrate that elongation of preexisting transcripts rather than de novo transcription initiation was being measured. The kinetics of incorporation are rapid (Figure 2A), reaching a maximum by 10 minutes. Addition of an exogenous DNA template did not extend the incorporation time or increase total incorporation. These results support the assumption that reinitiation was not occurring (Figure 2B). Moreover, the addition of heparin, which binds free RNA polymerase and thereby prevents new initiations (Greenberg et al., 1984), does not decrease the level of incorporation observed. As noted by others (Mullet and Klein, 1987; Mulligan et al., 1991), a stimulation of incorporation occurred after addition of heparin.

Additional experiments demonstrate that the level of incorporation is dependent on the number of plastids added to the reaction (Figure 2C) and that linearity of the reaction is observed when as many as $6 \times 10^6$ plastids were added per reaction (data not shown). The run-on reaction is insensitive to rifampicin, as has been noted for terrestrial plant-lysed plastid assays and for in vitro transcription systems (Mullet and Klein, 1987; Orozco et al., 1985; Link, 1984). No incorporation occurs in the absence of unlabeled NTPs, indicating that incorporation does not represent an exchange reaction.

To identify genes being transcribed in these assays, the products of the plastid run-on reaction were used to probe filters containing bound chloroplast, nuclear, and mitochondrial fragments. Labeled RNA run-on products hybridized to *H. carterae* chloroplast genes (*rbcS*, *psbA*, and 23S rRNA) but not to a maize mitochondrial gene (*cox*).

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**Figure 2.** Parameters of the *H. carterae* run-on assay. (A) Kinetics of incorporation. Plastids were isolated from cells at L3. Aliquots were removed; the reaction was stopped after 2, 5, 10, 20, and 30 minutes; and the amount of incorporation was determined. (B) Incorporation is not due to reinitiation. Reactions using plastids isolated at L3 were carried out in the presence of 500 $\mu$g/mL heparin (■), 100 $\mu$g/mL rifampicin (□), or with addition of 2 $\mu$g of a plasmid containing the *H. carterae* psbA gene as an exogenous DNA template (▲). The control sample (no additions) is indicated by X. (C) Linearity of the reaction with respect to plastid number. Plastids were isolated from log phase cultures (L3) at a density of $4 \times 10^6$ cells/mL. Incorporation was measured 2 (■), 5 (□), and 10 (▲) minutes after the start of the reaction.
Table 1. Transcription of chloroplast ribosomal RNA. 

\(^{32}\)P-labeled RNA synthesized during run-on assays was hybridized to 23S rDNA bound to filters. Values indicate cpm hybridized. The cells used in preparing the cultures for each separate experiment (L6 and D6 time points) originated from the same experimental culture.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>L6</th>
<th>D6</th>
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<tbody>
<tr>
<td>1</td>
<td>685</td>
<td>810</td>
</tr>
<tr>
<td>2</td>
<td>286</td>
<td>245</td>
</tr>
<tr>
<td>3</td>
<td>124</td>
<td>248</td>
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<td>4</td>
<td>184</td>
<td>244</td>
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Although no nuclear genes have been isolated from *H. carterae*, several highly conserved genes, including histone H3 and H4 (from chicken) and tubulin (from the red alga *Porphyra umbilicalis*), were used for this analysis. These genes have been shown to hybridize under low stringency conditions with *H. carterae* nuclear DNA but did not hybridize at the same stringency conditions to the \(^{32}\)P RNA from the chloroplast run-on reactions (data not shown).

Transcript initiation

The *H. carterae* plastid run-on assay was utilized to differentiate between RNA abundance and transcript initiation capacity for the specific chloroplast genes psbA, rbcL, and rbcS throughout the diel cycle (12L/12D). Ribosomal RNA transcription was used as an internal standard to normalize the values of the run-on transcription experiments. The choice of rRNA as a standard is supported by the 4 separate experiments shown in Table 1. These results show that the amount of rRNA incorporation at L6 and D6 (times of maximum and minimum psbA and rbc mRNA levels) varied 2-fold or less. The variation seen from one experiment to the next in absolute levels of incorporation into rRNA (e.g., experiments 1 and 2) is probably due to slight differences in the culture conditions and histories.

The transcripts measured by hybridization were shown to represent the sense transcript for psbA, rbcL,S, and 23S rDNA. As seen in Figure 3, the run-on products hybridized at least 50 times more strongly to antisense DNA or RNA than to sense strand for each gene tested. The small amount of hybridization to the sense RNA or DNA is not sufficiently to affect our quantitation.

When the level of transcription of psbA and rbcL,S was expressed relative to rRNA transcription at a chosen time in the cell cycle, the data in Figure 4 was obtained. High levels of transcription of both psbA and rbcL,S genes occurred during the light portion of the cycle, with a maximum at L6. Transcription was much reduced during the dark portion of the cycle, with an average difference

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**Figure 3.** Hybridization to antisense and sense probes. \(^{32}\)P-labeled run-on transcripts from L3 were hybridized to filters containing bound antisense (AS, top) and sense (S, bottom) DNAs or RNAs for each of the genes analyzed. (A) rbcS antisense and sense RNAs (B) psbA antisense and sense DNAs (C) rRNA antisense and sense RNAs.

**Figure 4.** Transcription run-on assays. (A) Filters were hybridized with RNA made from plastids isolated at the times indicated. (B) The amount of transcription for the psbA and rbcL,S genes is based on densitometric scanning of the hybridization data and expressed relative to that of the rRNA genes, with the maximum transcription corresponding to a value of 1. ■ = rbcS; ▲ = psbA.
between maxima and minima of 24-fold for psbA and 22-fold for rbcL,S using data from 4 independent experiments. The timing and magnitude of the fluctuation in initiation is very similar to that seen for mRNA accumulation.

Two explanations for these observations are (1) the primary mechanism for controlling transcript levels during a diel cycle is regulation of transcription initiation; or (2) a rapid degradation of newly made transcripts occurs in the dark. Several experiments were performed to differentiate between these alternatives. First, the kinetics of incorporation in the dark (at D6) are similar to those seen in the light (L3), increasing rapidly in the first 10 minutes to a maximum level, which is maintained up to 30 minutes (data not shown). Equal levels of incorporation have been observed for L6 and D6 run-ons as well. This result indicates that general degradation of transcripts does not occur in the dark. Second, to determine whether the specific psbA and rbcL,S transcripts are selectively degraded in the dark, a pulse-chase experiment was performed using plastids isolated at D6. Aliquots of the chased and unchased reactions were hybridized to filters containing DNA fragments of the psbA, rbcS, and 23S genes. The amount of hybridization that resulted was the same for the chased and unchased samples for the 10' interval, although there was a slight decrease in hybridization to rbcDNA with an additional 5 minutes of chase (Figure 5A). Therefore, during the 10-minute interval used for all run-on experiments, no significant degradation of either psbA or rbcL,S transcripts took place during the dark. As a final control for degradation,

\[ ^{32}P \text{-labeled run-on transcripts made from plastids isolated at L3 were added to a nonradioactive run-on transcription reaction containing plastids isolated at D6, and the fate of the L3 RNA was followed by hybridization analysis. Again, no degradation of L3-made RNA occurred in the presence of lysed D6 plastids (Figure 5B). These results lead us to conclude that transcript initiation is the regulating step in determining transcript abundance of the H. carterae chloroplast genes psbA and rbcL,S.}]

**Discussion**

Development of a chloroplast run-on assay for the chromophyte alga *H. carterae* has allowed us to determine that strong diurnal fluctuations occur in transcription initiation of the chloroplast genes psbA and rbcL,S. Analogous experiments that distinguish between transcriptional and posttranscriptional control of chlorophytic chloroplast gene expression during light/dark cycles have not been reported. However, diurnal fluctuations for the nuclear encoded *cab* genes of terrestrial plants have recently been reported to be due to transcriptional control (Taylor, 1989; Millar and Kay, 1991). Studies of chloroplast gene regulation in terrestrial plants (chlorophytes) have focused on developmental expression and the response to light cues. Although a general increase in chloroplast gene transcription has been shown to occur during plastid biogenesis in spinach and sorghum (Deng and Gruissem, 1987; Schrubar et al., 1990), differential modulations in steady-state mRNA levels for specific genes have been attributed either exclusively to changes in message stability (in spinach; Deng and Gruissem, 1987) or to a combination of transcriptional and posttranscriptional control (in barley; Mullet and Klein, 1987; Klein and Mullet, 1990; Sexton et al., 1990).

Our observation that control of psbA and rbcL,S transcript levels is transcriptional rather than posttranscriptional raises the question of whether control of steady-state levels of messages is different when chromophytes and chlorophytes are compared. To determine whether transcriptional control is a mechanism that is used extensively or exclusively in regulating gene expression in *H. carterae*, expression patterns of additional chloroplast-encoded photosynthetic genes as well as those whose products are not directly involved in the photosynthetic response are currently under investigation. Other differences in transcription between

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**Figure 5.** Controls for nuclease activity at D6. (A) Pulse chase experiment. Samples were removed for hybridization at the times indicated. Lanes 1–3: unchased samples removed after 5, 10, and 15 minutes. Lanes 4–5: chased samples removed at 10 minutes (5-minute pulse + 5-minute chase) and 15 minutes (5-minute pulse + 10-minute chase). (B) RNA was hybridized to filters with *rbcS*, psbA, 23S rRNA fragments bound. Probes were RNA from run-on reactions including (1) *^{32}P*-labeled L3 RNA and D6 plastids and (2) *^{32}P*-labeled L3 RNA without D6 plastids.
chlorophylls and the chlorophyte *H. carterae* are worth noting. Multiple promoters and 5' and 3'-processed transcripts are produced for several chloroplast genes of terrestrial plants (Grussem, 1989), whereas we have observed a single transcript and start site for the two messages analyzed to date in *H. carterae* (Hardison et al., 1992; Hardison, H.K. Personal communication). Variations in transcription properties between chlorophytic and chlorophytoid chloroplasts, such as those detailed herein, could reflect a different chloroplast evolutionary history. Distinctions between chloroplast genomes of these two major plant phyla have already been noted. Some nuclear encoded genes (e.g., *rbcS*, *tufA*, *atpG,D*) of terrestrial plants are plastid encoded in chlorophytes (Valentin et al., 1993). Moreover, phylogenies constructed using the *rbcL* gene place terrestrial plants and chlorophytes within a 5- and *γ*-purple bacterial cluster, respectively (Delaney et al., 1993). These observations have prompted speculation that the primary endosymbiotic event in chloroplast acquisition may have involved the engulfment of different ancestral photosynthetic prokaryotes (Valentin et al., 1993; Delaney et al., 1993). This evolutionary scenario could explain the genetic/functional differences observed that have been documented to occur among chloroplasts of extant plant lineages.

Three alternative hypotheses can be proposed to account for the changes in transcription that occur during a light/dark cycle in *H. carterae*. Cells may be responding directly to a light cue, with a signal transmitted to the chloroplast causing an increase in transcription. However, because a reproducible increase in the amount of transcript has been shown to occur before the light portion of the cycle begins, it seems more likely that the transcriptional response is based on an endogenous cue. Because cells grown on a 12L/12D cycle are synchronized by this light regime, the fluctuation in RNA levels may be a consequence of the temporal position of cells in the cell cycle. A final hypothesis is that the fluctuation is a circadian-based phenomenon in which the light-entrained endogenous rhythm directs the transcriptional activity. In each case, light serves as the inductive cue, either directly or indirectly.

In summary, we adapted the run-on transcription assay for use in a chlorophytic alga to analyze chloroplast gene transcription. This assay is an essential tool in the comparative assessment of transcriptional versus posttranscriptional control. Using this method, the effects of other environmental factors (e.g., macronutrient or micronutrient availability) on transcription of specific chloroplast genes can be examined.

**Experimental Procedures**

**Cell growth and media**

*H. carterae* was cultured axenically in O-3 medium (McIntosh and Cattolico, 1978) on a 12L/12D light regime using cool-white fluorescent illumination of 50 to 75 µmol/sec/m².

**Northern analysis**

Total RNA was isolated from *H. carterae* using a guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987). For each time point, 5 µg RNA was electrophoresed on a 0.007% formaldehyde, MPS, 1% agarose gel (Ausubel et al., 1987). RNA was transferred to nylon (Nytran) filters and crosslinked by ultraviolet irradiation. Filters were hybridized at 65° for 20 hours according to Church and Gilbert (1984) using internal fragments of the *H. carterae rbcS* and *psbA* genes as probes. The *rbcS* probe was generated from plasmid pOC560,8, which contains a 0.8-kb SphI-EcoRI *rbcS* fragment cloned into *puc118*. The 1.1-kb internal *psbA* fragment was generated from pOCP8.5 by digestion of this plasmid with HindIII and NsiI. To confirm that equivalent amounts of RNA were transferred, the blot was hybridized with a 23S rRNA probe, and appropriate concentration corrections were made. A 3.0-kb PvuII fragment, which includes the 23S rRNA gene from the chloroplast genome of *H. carterae*, was generated from plasmid pOCXba25.

**Synthesis of sense and antisense DNA and RNA**

To obtain plasmids from which sense and antisense RNA of the *rbcS* and 23S chloroplast rRNA genes could be synthesized, a 0.8-kb HindIII-EcoRI *rbcS* fragment was cloned into the vector *pGEM3*, and the 2.16-kb Hpal fragment from the 23S rRNA gene was ligated to the vector Bluescript II KS⁺ (Stratagene), forming plasmids *pSSRBC1* and *pSSRRN1*, respectively. Sense and antisense RNAs were generated according to manufacturers' specifications by in vitro transcription using SP6, T7, and T3 polymerase. Plasmids *pSSPSB4* and *pSSPSB0.0*, which contain the 1.1-kb HindIII-NsiI internal *psbA* fragment cloned into the vector *pGEM7*⁺ and *pGEM7*⁺ (Promega), respectively, were used to generate single-stranded DNA, representing the sense and antisense DNA strands of the *psbA* gene.
Chloroplast run-on assay

Plastids were recovered from logarithmically growing \textit{H. carterae} cells (density of 2–4 × 10^6 cells/mL) as described previously (Reith and Cattolico, 1985), except that concentrated cells (2 × 10^7 cells/mL) were broken by gentle Dounce homogenization. Intact chloroplasts, which were refractile under phase contrast microscopy, were collected by centrifugation at 1200 g for 5 minutes at 5°C and resuspended in 0.33 mol/L sorbitol, 0.05 mol/L Heps, 1 mmol/L MgCl_2, and 0.1% b-mercaptoethanol. The plastids (4–6 × 10^8 per reaction) were treated with Triton X-100 (0.15%) for 2 minutes at 25°C to lyse the organelles. The run-on assay was initiated by adding the lysed plastids to a 50-μL reaction containing 500 μg/mL heparin; 25 mmol/L potassium acetate; 50 mmol/L Heps (pH 7.9); 10 mmol/L MgCl_2; 10 mmol/L DTT; 125 μmol/L ATP, CTP, and GTP; 12.5 μmol/L UTP; and 100 μCi 32P-UTP (3,000 Ci/mmol) at 25°C. The reaction was terminated after 10 minutes by the addition of 10 μg proteinase K, SDS (0.2%) and 10 μg tRNA at 65°C. The amount of 32P-UTP incorporated was determined by measuring the trichloroacetic acid insoluble radioactivity. For analysis of specific gene transcription, 140 ng of the 0.8-kb rbcS fragment, 140 ng of the 1.1-kb psbA fragment, and 80 ng of the 3.0-kb 23S rRNA fragment were bound to Nytran filters using a Schilderich and Schuell slot-blotting apparatus and crosslinked by UV irradiation. Filters were hybridized with RNA made from plastids isolated at specified times during the diurnal cycle. Hybridization was carried out at 65°C for 40 hours in 3 mL hybridization solution (Church and Gilbert, 1984). For lower stringency hybridizations involving heterologous mitochondrial or nuclear DNA, the temperature at which the hybridization and washes were done was lowered to 55°C.

The pulse chase experiment was performed as follows. The standard reaction mixture containing 12.5 μmol/L 32P UTP was allowed to proceed for 5 minutes; the reaction was then divided, and an excess of unlabeled UTP (1mmol/L) was added to one subsample to initiate a chase experiment.

Acknowledgments

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