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EXAMINATION OF DIEL CHANGES IN GLOBAL TRANSCRIPT ACCUMULATION IN *SYNECHOCYSTIS* (CYANOBACTERIA)[1]

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Phytoplankton in nature must acclimate to a wide range of light conditions resulting from diel light cycles, ocean circulation and mixing, cloud cover, and the variable bio-optical characteristics of the water column. In this study, we used whole-genome cDNA microarrays to investigate the effects of a gradually fluctuating daily light cycle on gene expression in the cyanobacterium *Synechocystis* sp. strain PCC6803. From these data, we developed a conceptual framework depicting the diel regulation of metabolic pathways in the cell. The framework is focused on potential photoacclimation responses, including the regulation of the photosystems, cell division, and DNA replication. The mRNA abundance of genes involved in many metabolic pathways, and particularly those encoding proteins that function in photosynthesis and DNA replication, changed markedly over the course of the day. The levels of mRNA encoding polypeptides important for the formation of the light-harvesting apparatus, photosystems I and II, and cell division were found in high concentrations during the day. The transcript levels of many genes encoding enzymes involved in anabolic processes also increased considerably during the day. In contrast, transposon transcripts and mRNAs encoding proteins involved in DNA replication, cell wall synthesis, and respiratory activity were not found in high concentrations during the day. Although gradually varying light exposure induced significant changes in transcript accumulation within *Synechocystis*, the direction of these changes differed between our study and previous studies in which there was an abrupt transition between irradiances.

Key index words: **cyanobacteria; diel; light limitation; microarray; mRNA; *Synechocystis*; turbidostat**

Cyanobacteria are the oldest group of oxygenic organisms, making up the largest part of the autotrophic aquatic biomass. In addition to forming the base of the oceanic food web, cyanobacteria generate a substantial fraction of atmospheric oxygen and serve as a sink for a significant portion of atmospheric CO_2 (Schopf 1993, Summons et al. 1999, Blankenship 2002). Moreover, cyanobacteria inhabit a wide range of environments, from oceans to lakes, and from boiling hot springs to dry desert crusts. Many of these environments undergo large, rapid transitions in light, temperature, nutrient levels, and other properties. The cyanobacteria must have evolved mechanisms for acclimating in such diverse and dynamic habitats.

Light is among the most important and most variable environmental factors controlling the metabolism and growth of cyanobacteria (Tandeau de Marsac and Houmard 1993, Grossman et al. 1995), with short-term variation in irradiance resulting from variable cloud cover and mixing of ocean surface waters driven by wind and convection, superimposed on a more regular light cycle that varies over daily and seasonal timescales. To thrive under these dynamic light conditions, cyanobacteria must acclimate continuously throughout the day. All phytoplankton, including cyanobacteria, have evolved various mechanisms to optimize the use of light energy and to tailor the architecture of their photosynthetic apparatus to minimize the formation of damaging reactive oxygen species under high light conditions (Hihara et al. 1998, Huang et al. 2002, Muramatsu and Hihara 2003). During acclimation to changing light levels, cyanobacteria may alter both the level and characteristics of the light-harvesting phycobilisomes, protein

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complexes containing chl, or constituents of the photosynthetic reaction centers, photosystems I and II (PSI and PSII), to optimize the absorption of excitation energy and the efficiency of energy transfer (Tandeau de Marsac and Houmard 1993, Grossman et al. 1995, 2003). For example, soon after unicellular algae and cyanobacteria are shifted from low to higher light, photosynthesis saturates at the higher irradiances and both the absorbtion cross-section and the pigment content of the cell decrease (Escoubas et al. 1995, Grossman et al. 1995). When cells are exposed to high light for a significant amount of time, they dissipate excess absorbed light energy as heat in the short term (Grossman et al. 2001, Havaux et al. 2003), and adjust the stoichiometry of the photosynthetic complexes in the longer term (Fujita et al. 1987, Grossman et al. 1995). Many of these photoacclimation processes reflect functional changes in components of the photosynthetic apparatus that are controlled directly by light, or indirectly by the redox state of the cell, as set by the activity of the photosynthetic electron transport chain (Escoubas et al. 1995).

Recent batch culture studies have used whole-genome microarrays to examine the influence of changing light conditions on global transcript abundance in cyanobacteria. Although much of the phytoplankton population in oligotrophic marine environments is composed of the cyanobacteria genera *Prochlorococcus* and *Synechococcus*, many laboratory experiments use the freshwater cyanobacterium *Synechocystis* sp. strain PCC6803 (hereafter *Synechocystis*) because a complete genome sequence and whole-genome microarray, as well as a highly developed genetic system, have been available for this organism for a relatively long time. In addition, *Synechocystis* sp. PCC 6803 was first isolated from a dynamic light environment, a stratified lake, and various strains of *Synechocystis* spp. are environmentally numerous and important ecologically (Wood et al. 2002, Belykh and Sorokovikova 2003). Recently, microarray analyses were used to examine "light stress" responses in *Synechocystis* in order to examine how excess excitation modulates global, cyanobacterial gene expression and to identify possible regulatory systems (Hihara et al. 2001, 2003, Gill et al. 2002, Huang et al. 2002, Tu et al. 2004). However, the more "typical" responses of cyanobacteria to gradually fluctuating diel irradiances have yet to be examined extensively.

Studies in which *Synechocystis* was transferred from a continuous irradiance of 50–200 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ triggered a reduction in the level of transcripts encoding components of the photosynthetic apparatus, and an increase in the level of transcripts encoding enzymes required for CO_2 fixation, protection against photodamage, and protein synthesis (Hihara et al. 2001, Huang et al. 2002). In a similar experiment, Gill et al. (2002) used both whole- and partial-genome microarrays for *Synechocystis* to examine instantaneous transitions from darkness to bright light (250 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$). These authors found that approximately 35% of the genes examined responded to exposure to high light, particularly those genes encoding polypeptides associated with PSI and PSII, carbon fixation, and ATP synthase. Upon exposure of *Synechocystis* to pure UVB and pure white light, Huang et al. (2002) found that a 3-fold increase in UVB induced a 4- to 6-fold increase in the *psbA2* and *psbA3* transcripts (encoding the DI polypeptide of PSII), which they attributed to damage to PSII. They also found 5- to 31-fold increases in the abundance of transcripts encoding the family of Hli (high light inducible) proteins, which may serve as chl carriers, function in the dissipation of excess absorbed light energy within antennae complexes, or modulate the biosynthesis of chl (Havaux et al. 2003, Xu et al. 2004), and strong repression among many of the PSI and light-harvesting transcripts. Tu et al. (2004) also demonstrated marked changes in levels of transcripts encoding components of the photosynthetic apparatus when *Synechocystis* was transferred from low (30 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) to high (500 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) intensity illumination, and demonstrated that the *dspA* (*hik33*) mutant exhibited aberrant gene expression in both low and high light, suggesting that the mutant could not properly sense the light and/or intracellular redox conditions.

Although these studies demonstrate the utility of microarray analyses for monitoring cellular responses to changing environmental conditions, the light conditions used in these experiments differed markedly from a typical diel light cycle. The range of irradiances experienced by the cells was narrow and often high relative to the natural range, and measurements were made following abrupt transitions from low to high light, or after long continuous exposure to high light. Although these studies have provided significant insight into the responses of cyanobacteria to high light stress, specifically elucidating the short-term molecular basis for the frequently observed "shock" or "stress" responses, cyanobacteria more commonly undergo gradual changes in irradiance over the course of a day, and it is important to characterize their response to this light regime as well.

In the present study, we generated information on mRNA abundance in *Synechocystis* over a smoothly varying diel light cycle. *Synechocystis* was grown in a "cyclodyn" (a kind of turbidostat; see description in Methods section) under an illumination regimen that simulated diel changes in irradiance. Levels of mRNAs were monitored over the course of several days using whole-genome cDNA microarrays. This approach enabled us to determine how *Synechocystis* photoacclimates under gradually changing light conditions, eliminating some of the stress responses that may cloud interpretation of previous light acclimation studies as they apply to an understanding of the natural responses of the cyanobacteria.

METHODS

Inoculation and batch culture growth. Axenic, wild-type *Synechocystis* was grown in 50 mL of BG-11 medium (Stanier

et al. 1971) as batch cultures in continuous light of approximately 60 μmol photons $\cdot m^{-2} \cdot s^{-1}$ until the culture reached an OD_{750} density of ~ 1.0 (usually about 5 days). This culture was then used to inoculate ~ 1.5 L of BG-11 medium maintained under sterile conditions in a cyclodyn (Methods, *Cyclodyn apparatus*). The cyclodyn cultures were maintained under a diel light cycling schedule (Methods, *Light source*) at 27° C and continuously stirred and bubbled with 3% CO_2 in air. After approximately 1 week of growth under light cycling conditions, the density of the cyclodyn culture was stabilized at an OD_{750} of 0.6 (1.25×10^7 cells $\cdot mL^{-1}$). After approximately 3 days of additional growth at this density under light cycling conditions (approximately four generations), sampling was initiated and continued over a period of approximately 1 week.

Cyclodyn apparatus. The cyclodyn used for the continuous culture of *Synechocystis* was modeled after the cyclostat described by Bruyant et al. (2001). Our system was composed of a 1.5 L, 11.5 cm internal diameter Applikon (www.applikon.com) glass bioreactor with a sealed machined lid populated with ports that enable sparging, introduction of new medium, sample collection, and the measurement of temperature, pH, and culture density. The vessel was illuminated by a system of red-enriched fluorescent tubes (described below) and the entire apparatus was placed in a laminar flow hood to assist in maintaining sterility. Maintenance of the culture at an OD_{750} of 0.6 was accomplished using a Wedgewood Technology (San Carlos, CA, USA) Model BT65/E/A/3/D turbidity sensor (turbidometer) connected to a Wedgewood 652 cell growth monitor. Elevated turbidity was reported from the growth monitor to an Applikon ADI 1030 biocontroller, which activated a pump to drain medium from the bioreactor when the density exceeded the set point (OD_{750} of 0.6). The biocontroller also monitored the medium level in the vessel. When the medium level declined, as a consequence of out-pumping triggered by excess turbidity as above, a medium inflow pump was activated by the biocontroller to deliver fresh BG-11 medium to the vessel. When the reduction in culture density resulting from fresh medium influx reached the density set point ($OD_{750} = 0.6$), a signal from the turbidometer inactivated the outflow pump, and as the set medium level was attained, the inflow pump was inactivated. The outflow rate was set to be slower than the inflow rate; this prevented drainage of the system before the inflow pump could refill it. As a result of this balance, the dilution rate of the cyclodyn was approximately equivalent to the growth rate of the algae. We have used this system to maintain the density of a sterile culture at a desired set point for up to a month.

Light source. Synechocystis cultures were illuminated with white light from two U-shaped, cool fluorescent, dimmable light tubes (Sylvania Dulux DL, FT55DL, 841, 55W). The lights were positioned horizontally 4 in from the wall of the cyclodyn, and were maintained on a dimming schedule to simulate a diel cycle. No appreciable heat was given off by the lights, and a constant temperature was recorded in the cyclodyn throughout the day and night. Dimming of the lights was automated using a QBasic program in steps of 0–255 (255 = 5 V) according to the following equation:

$$L = 70 + (170 \sin(\pi \text{ seconds post 24 h}/86,400))$$
$$\text{If } L < ;0, \text{then Level} = 0, \text{ else Level} = L$$

The light reached a peak output of 400 μmol photons $\cdot m^{-2} \cdot s^{-1}$ measured with a dry/wet (photosynthetically active radiation) radiometer (Biospherical Instruments, San Diego, CA, USA. model QSL-2101) at the outside surface of the cyclodyn at approximately noon, after which time the irradiance gradually declined to near 0 just before 6:00 p.m. This range in irradiance is similar to what cyanobacteria, which are often found in stratified waters, would be exposed to at the surface. Because the fluorescent lamps would not dim to complete darkness, an electromechanical timer turned the lamp power off at 6:00 p.m. The lamp power was turned back on at 4:00 a.m. The spectral output of the Osram Sylvania fluorescent lights (measured using a spectroradiometer from Analytical Spectral Devices, Inc., Denver, CO, USA) and the measured irradiance at the surface of the cyclodyn over the diel cycle are shown in Fig. 1A. This spectrum was the closest to that of natural surface light that we could find in a commercially available dimmable fluorescent bulb. The spectrum did not change over the diel light cycle. The irradiance that penetrated to the center of the culture when the cultures were maintained at an OD_{750} of 0.6 was lower by a factor of over 10 (~ 17 μmol photons $\cdot m^{-2} \cdot s^{-1}$ at noon), resulting in an attenuation coefficient of 0.32 cm^{-1}. This attenuation is similar to what would be found in a large bloom of cyanobacteria in nature. The light measured throughout the interior and exterior of the apparatus is shown in Fig. 1B.

Sampling. Because of the amount of algal biomass required for analyses at each sampling point, only one or two time points were sampled over the course of each day for a period of 1 week, yielding a time series of at least one sample every 2 h over the 24 h diel cycle. At each sampling point, the cyclodyn tap was cleared by eliminating the first ~ 50 mL of culture that passed through it, after which ~ 150 mL of culture was collected in a flask that was cooled in liquid nitrogen. This sample (continuously cooled to close to 4° C) was sep-

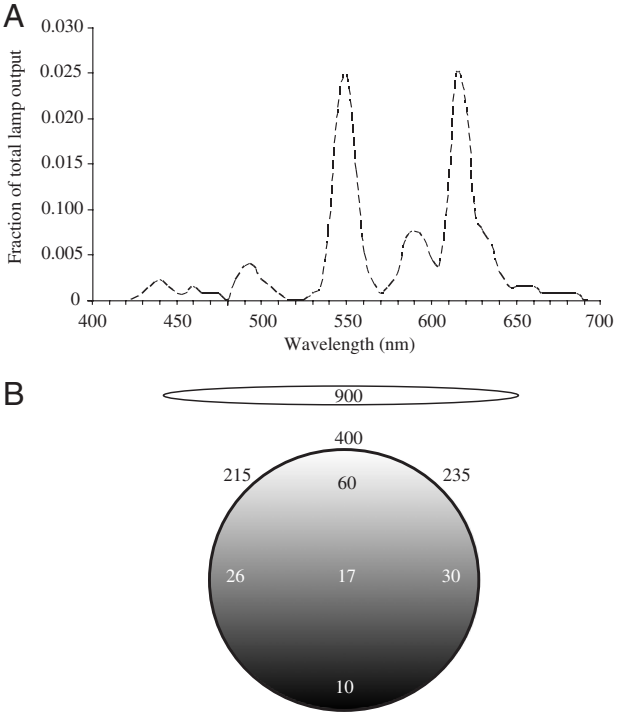

FIG. 1. (A) Output spectrum of the lamps used for growth in the cyclodyn as a fraction of total lamp output. (B) Light levels (integrated PAR) measured in the culture are depicted as shaded regions in this plan view of the cyclodyn. All measurements are as μmol photons $\cdot m^{-2} \cdot s^{-1}$. Note that there is an approximately 40-fold reduction in incident light (PAR) between the most proximal surface of the cyclodyn facing the lights and the distal surface. The maximal light level at the source was 900 μmol photons $\cdot m^{-2} \cdot s^{-1}$, while the maximum level at the proximal surface was 400 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and coincided with noon.

arated into four aliquots, two of which were poured into two 50 mL falcon tubes and immediately pelleted by centrifugation at 5000 rpm for 5 min at 4° C in an SA600 rotor. The supernatant was drained from the pelleted cells, the falcon tube was immersed in liquid N_2, and the tube with the frozen pellet was transferred to −80° C for storage. Duplicate 1 mL subset samples were used for measuring algal absorption *in vivo* from 300 to 800 nm (Beckman DU70 spectrophotometer, Fullerton, CA, USA), and another set of duplicate 1–2 mL subset samples were used for chl *a* and phaeopigment measurements. To measure the levels of chl *a* and phaeopigments per cell, samples were filtered onto Whatman GF/F 25 mm filters (Whatman, Maidstone, UK), extracted, and analyzed by previously established techniques (Holm-Hansen and Riemann 1978).

RNA isolation. Frozen pellets derived from the 50 mL aliquots of culture were resuspended in 500 µL of NAES extraction buffer (50 mM sodium acetate, pH 5.1, 10 mM EDTA, 1% sodium dodecylsulfate). An equal volume of acidic phenol (equilibrated with NAES) was added to the suspension (approximately 1.0 mL) along with 100 mg of glass beads (0.1 µm average diameter; Sigma, St. Louis, MO, USA) before agitation (two times for 30 s each) by vortexing. The beads and cell debris were pelleted by a brief centrifugation (5 s at full speed in an Eppendorf microfuge), and the supernatant was subjected to two phenol/chloroform (1:1) and one chloroform extraction. The aqueous phase was made 0.3 M NaOAc, and nucleic acid was precipitated by the addition of two volumes of ethanol, followed by a 2 h incubation at − 20° C. Precipitated nucleic acid was collected by centrifugation (10 min, full speed in an Eppendorf microfuge), washed with 70% ethanol, resuspended in Tris-EDTA, (ethylene diamine tetraacetic acid) and treated with 25 U of RNase-free DNase I (Amersham Biosciences, Piscataway, NY, USA) at room temperature for 30 min. The DNase I was denatured by two phenol/chloroform extractions and the aqueous phase from the second extraction was made 0.3 M NaOAc before precipitation of total RNA with two volumes of ethanol. A microarray reference sample was generated by mixing together an aliquot of each of the purified RNA samples, which contained approximately equal amounts of total RNA.

RNA blot hybridizations. Aliquots of purified RNA (10 µg, approximately 3 µL per sample) were added to 10 µL stock sample buffer [15% v/v H_2O%, 50% v/v deionized formamide, 10% v/v 10× MEN buffer (0.2 M MOPS buffer, 80 mM NaOAc and 10 mM EDTA, pH 8.0), 15% v/v 37% formaldehyde, 10% v/v 400 µL/mL ethidium bromide]. The samples were heated at 70° C for 5 min, placed immediately on ice, and then electrophoresed in a 1.3% agarose gel containing 2% formaldehyde. The gel was submerged in a running buffer consisting of 1× MEN containing 0.2% formaldehyde. The samples were electrophoresed at 100 V constant voltage until the lead dye (the lead dye was xylene cyanol and the lagging dye was bromophenol blue) had migrated $\frac{3}{4}$ of the way down the gel. The gel was photographed, washed in water for 15 min, and twice in 10× SSC for 5 min (with gentle agitation). The RNA in the gel was transferred to a Hybond™ Nylon membrane (Amersham Biosciences) as described by the manufacturer, and then cross-linked to the membrane using a Stratalinker 1800 UV cross linker (Stratagene, La Jolla, CA, USA).

The RNA bound to the nylon membrane was probed using the Gene Images CDP Star Hybridization Detection Bioluminescent Kit (Amersham Biosciences/General Electric, New Brunswick, NJ, USA). The membrane was moistened in 5 × SSC and then prehybridized with preheated (65° C) hybridization buffer (5%× SSC, 0.1% SDS, 5% dextran sulfate) for 30 min with constant agitation. The labeled DNA probes (polymerase chain reaction products synthesized using Taq polymerase from Fermentas Inc., Hanover, MD, USA) were prepared and labeled by incorporation of fluorescein (Fl)-labeled dUTP according to the CDP kit, boiled for 5 min, placed immediately on ice, centrifuged at full speed for 30 s in an Eppendorf microcentrifuge, and added to a glass tube containing the membrane with the bound RNA and hybridization buffer; the final probe concentration was ∼10 ng/mL. Hybridizations were performed overnight at 65° C. Following the hybridization, the membranes were washed in preheated 1 × SSC/0.1% SDS at 65° C for 15 min; ∼ 2 mL of the wash buffer was used per cm^2 of membrane. A second, more stringent 15 min wash was performed with 0.1%× SSC, 0.1% SDS, and the hybridized probe was detected using the CDP-Star Detection Module (Amersham Biosciences, RPN 3510), which relies on interaction of the Fluorescein dUTP incorporated into the probe with an anti-fluorescein alkaline phosphatase conjugate (AP). The AP is used to catalyze the decomposition of a stabilized dioxetane substrate, which is detected using an X-ray film. This assay was performed as described by the manufacturer.

Microarray hybridization and data analysis. Microarrays with array elements representing all genes on the *Synechocystis* genome were previously generated (Postier et al. 2003, Tu et al. 2004). The arrays were used to monitor changes in transcript levels over the diel cycle. Isolated total RNA (25 µg) from cells at different times of the diel cycle was incubated with a mixture of gene-specific reverse primers (a reverse primer was synthesized for each gene on the genome) and 30 U of Superscript II RNase H minus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for first-strand cDNA synthesis. This reaction was performed at 42° C for 1 h in a volume of 40 µL. Following first-strand synthesis, the RNA template was degraded with RNase H and second-strand synthesis was initiated with the Klenow fragment of DNA polymerase (USB, Cleveland, OH, USA), random primers, and Cy3- or Cy5-dUTP fluorescent nucleotides (Amersham Biosciences). For second-strand synthesis, we used 0.25 mM of each of dCTP, dATP, dGTP, 0.09 mM dTTP, 1 mM Cy3-dUTP or Cy5-dUTP, and 10 U per reaction of Klenow fragment of DNA polymerase. After incubating the reaction for 3 h at 37° C, the labeled cDNA fragments were purified using the QIAquick spin column (Qiagen, Valencia, CA, USA) and labeled cDNA representing 12.5 µg of input RNA was resuspended in hybridization buffer (3%× SSC, 0.3% SDS, 0.17 $\mu g\,\mu L^{-1}$ tRNA) to 30 µL, denatured at 95° C for 90 s, and applied to the array by capillary delivery under a glass lifter slip (Eric Scientific, Portsmouth, NH, USA). Multiple slides were sealed in custom-built hybridization chambers and the hybridization was performed at 65° C for 16 h. Approximately 1 mL of 3 × SSC was added to a well at the base of the incubation chamber to maintain humidity during hybridization. Following hybridization, arrays were washed for 2 min each at room temperature in low-stringency (2%× SSC, 0.03% SDS), moderate-stringency (1× SSC), and high-stringency (0.05× SSC) wash solutions, dried in a Savant Speedvac Plus (Savant, Holbrook, NY) by centrifugation for 2 min, and scanned using a GenePix Laser Scanner 4000 (Axon Instruments, Foster City, CA, USA). For each microarray, two hybridization reactions per time point were performed (dye swaps). For one analysis, equal amounts of Cy5-labeled cDNA from a test sample (RNA from each of the distinct time points of the diel cycle) and Cy3-labeled cDNA from a reference sample (pooled RNA from all samples collected over the course of the experiment) were mixed and hybridized to the array. A second set of hybridizations was performed in which test sample cDNA was labeled with Cy3-dUTP and reference sample cDNA was labeled with Cy5-dUTP. Each slide contained four copies of the array set representing the complete *Synechocystis* gene complement.

Data analysis. The images generated by the GenePix Laser Scanner 4000 were imported into GenePix Pro 3.0 (Axon Instruments) and spot intensities from scanned slides were quantified. Grids were predefined and manually adjusted to ensure good spot recognition. Spot signals that were distorted by dust or local high backgrounds were manually flagged and not included in subsequent analyses. After determining the relative ratios of the fluorescent signals from Channel 1 (Ch1; 532 nm) to Ch2 (635 nm), the data files (.gpr files) were imported into the Stanford Microarray Data base (genome-www5.stanford.edu and Gollub et al. 2003) and normalized using the standard SMD normalization procedure. The primary data sets can be viewed and downloaded at http://genome-www.stanford.edu/microarray/; the experiment ID numbers are: 40132, 44972, 44973, 37075, 40134, 44974, 40136, 44975, 44976, 55353, and 55354.

The microarrays used for these experiments contained four copies of array elements for the 3168 genes (total 12,672 array elements) that comprise the *Synechocystis* genome. In addition to removal of poor-quality spot images (e.g. with dust, as above), several layers of analytical filters were imposed in order to ensure high-quality data. Only the spot images with Ch1-normalized mean intensity/median background intensity greater than 2.5, Ch2-normalized mean intensity/median background intensity greater than 2.5, and either Ch1 net mean or Ch2-normalized mean intensities of greater than or equal to 250 were used in the analysis. A total of 13,356 spots passed these filters. We also compared data from time points collected during two separate runs to determine the reproducibility of the data. We found that for one time point at 12:00 (noon), the correlation was relatively good ($R^2 = 0.74$), while for the other time point (8:00 a.m.) the correlation was fair ($R^2 = 0.24$). This was not unexpected, given that the molecular responses change greatly over very short periods of time during the transitions from light to dark (or dark to light), and therefore the data collected during transitions would be highly variable, whereas data collected during the middle of the day outside of the transition period would be more predictable. In addition, we found that the concentrations of RNA were low in general during the early morning and late evening, decreasing the signal to noise significantly at these time points.

The signals from each of the four copies of a specific array element on a given slide, and across slides, were considered as separate observations. Two statistical tests, an analysis of variance (ANOVA) and correlation with light level, were performed on the resulting time series for each individual transcript. The ANOVA enabled us to decide for which genes there was a statistically significant change in the measure over time. For purposes of display and discussion, we used an F score cutoff of 5.0 to indicate significance ($P < 0.05$, df = 9). Correlation with light levels (time of day) for a given gene was carried out between the series of calculated light levels over time of day (L in the above equation, depicted in Fig. 2), and the mean of all the good measures at each time for that gene, that is, the mean of all data not missing or flagged bad, from both biological replicates and all four on-chip replicates.

For the purposes of discussion, we used a cutoff of a correlation coefficient greater than 0.6 (positive or negative, $P < 0.05$). We examined the effect of excluding outlier data points more than 2 SD from the mean of the value obtained for the transcript levels of each gene at each time point, but this did not significantly change the results; therefore, outlier values were retained.

A subset of metabolically significant transcripts for which there are meaningful annotations of the encoded proteins, with F scores greater than or equal to 5.0, only one missing value (time point) allowed per transcript, and abundances that exhibited the strongest correlations with light are considered the "cleanest" transcripts (these headings for these transcripts plus *psaA*, *psbA*, and *cpcB* are presented in Table 1, and the correlations with light are presented in Table 2). Appendix S1 (online) at: reports analyses for all meaningfully annotated transcripts with $F > 5.0$. The raw microarray images are available publicly through the Stanford Microarray Data base (http://genome-www.stanford.edu/microarray). Missing values were not replaced in order to avoid distorting the correlations, so the actual appropriate df and F score threshold for significance to $P < 0.05$ varies from one gene to the next, depending upon how many values were involved in the calculations. We used 5.0 (F) as a rough way of reducing the numbers of genes appearing in the in-text tables.

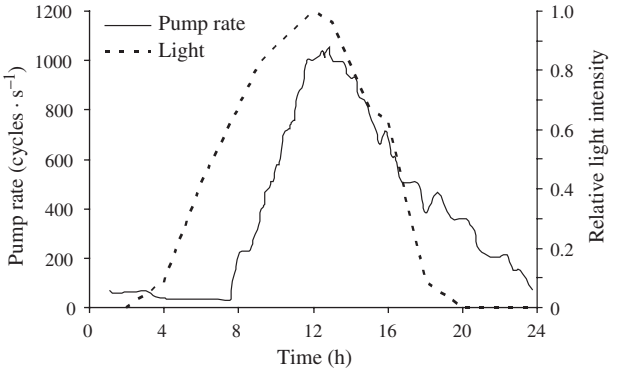

FIG. 2. Light level (dotted line) is overlain with the inflow of BG-11 enrichment medium (solid line). The light was activated at 4:00 a.m. and turned off at 6:00 p.m. The inflow of medium was measured as the hourly mean pump rate (pumps per sec) averaged over a 5 h window. The pump rate reflects the growth rate of the cells.

In interpreting the results of the present study, it is important to bear in mind the stringency that was used to determine whether or not a change in transcript abundance was strongly and significantly correlated with irradiance. The ANOVA criteria may exclude a transcript either because it does not change, or because the signal-to-noise ratio is too low on the microarray to detect a significant change. This difficulty occurs for many genes that are not expressed at high levels. A similar problem arises with the correlation analysis. Therefore, if a gene does not appear in these tables, this does not necessarily mean that it does not change over the course of the day because the effect may be masked by measurement noise.

RESULTS

Synechocystis exhibited a diel cycle in growth rate that mirrored daily changes in light level. Specifically, growth was observed only during the daylight hours, with maximum growth coinciding with the highest irradiance, which was reached at noon (Fig. 2). The doubling time of the cells was approximately 19 h. Because of the cell density of the cultures, the mean light level within the cyclodyn at maximum light output was only 31 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (assuming rapid mixing; measured by integrating the irradiance at $\sim$ 20 places on the top surface of the cylinder and averaging over the surface area, 104 cm), which is well below the intensity that would saturate photosynthetic electron transport. As expected under such conditions, the culture accumulated high levels of chl *a* (mean $\pm$ SD = 2681 $\pm$ 207.9 μg/L, $n = 22$) and low levels of phaeopigments (46.16 $\pm$ 52.96 μg/L, $n = 22$), with a phaeopigment to chl *a* ratio of 0.01.

TABLE 1 Subset of transcripts strongly correlated with the diel cycles that have the most complete data (i.e. eight or nine out of nine timepoints represented), plus *cpcB*, *psaA*, and *psbA1* (italicized).

Gene	Subcategory	Transcript annotation
SLL1214	Adaptations and atypical conditions	Phytochrome-regulated gene (PNIL34 or AT103)
SLL1234	Amino acids and amines	S-adenosylhomocysteine hydrolase (ahcY)
SLR1390	**Cell division**	**Cell division protein FtsH (ftsH)**
SLR0009	CO_2 fixation	Ribulose bisphosphate carboxylase large subunit (rbcL)
SLL1525	CO_2 fixation	Phosphoribulokinase (prkor ptk)
SLR1777	**Cobalamin, heme, phycobilin, and porphyrin**	**Mg chelatase subunit ChlD (chlD)**
SLL1091	Cobalamin, heme, phycobilin, and porphyrin	391 aa (43 kD) bacteriochlorophyll synthase subunit
SLR1641	**Degradation of proteins, peptides, and glycopeptides**	**ClpB protein (clpB)**
SLL1427	**Degradation of proteins, peptides, and glycopeptides**	**Protease HhoB (hhoBor htrH)**
SLL1290	**Degradation of RNA**	**Ribonuclease II (rnb)**
SLR1543	DNA replication, restriction, modification, recombination, and repair	DNA-damage-inducible protein
SLR1350	Fatty acid, phospholipid, and sterol metabolism	Fatty acid desaturase (desA)
SLL0541	Fatty acid, phospholipid, and sterol metabolism	Acyl-CoA desaturase 1 (desC(des9))
SLR1756	Glutamate family/nitrogen assimilation	Glutamate–ammonia ligase (glnA)
SLL0018	Glycolysis	Fructose-16-bisphosphate aldolase (cbbA or cfxA)
SLR0394	Glycolysis	Phosphoglycerate kinase (pgk)
SLL1434	**Murein sacculus and peptidoglycan**	**Penicillin-binding protein 1B (mrcAor ponA)**
SLR0011	Other	Unknown function (presumed rbcX)
SLL0005	**Other**	**ABC1-like**
SLL1750	**Other**	**Urease α subunit (ureC)**
SLR2094	Other	GlpX protein (glpX)
SLL1479	**Pentose phosphate pathway**	**Glucose-6-P-dehydrogenase (devB)**
SLR1834	*Photosystem I*	*P700 apoprotein subunit Ia (psaA)*
SLR1181	*Photosystem II*	*Photosystem II D1 protein (psbA1)*
SLR0927	Photosystem II	photosystem II D2 protein (psbD2)
SLL0849	Photosystem II	photosystem II D2 protein (psbD)
SLL0427	Photosystem II	Photosystem II manganese-stabilizing polypeptide (psbO)
SLL1578	Phycobilisome	Phycocyanin a subunit (cpcA)
SLL1577	*Phycobilisome*	*Phycocyanin b subunit (cpcB)*
SLR0079	**Protein and peptide secretion**	**General secretion pathway protein E (gspE)**
SLL1814	Protein and peptide secretion	Preprotein translocase SecY subunit (secY)
SLR1226	**Purine ribonucleotide biosynthesis**	**Phosphoribosyl aminoidazole succinocarboxamide synthetase (purC)**
SLR1805	**Regulatory functions**	**Sensory transduction histidine kinase**
SLR0533	Regulatory functions	Sensory transduction histidine kinase
SLR2099	Regulatory functions	Receiver/transmitter
SLR1403	**Regulatory functions**	**Integrin α- and β4- subunit domain homologue**
SLR0322	Regulatory functions	CheA like protein
SLR1529	Regulatory functions	Nitrogen assimilation regulatory protein
SLL0779	Regulatory functions	PleD gene product homologue
SLR0473	**Regulatory functions**	**Phytochrome (phy)**
SLR0210	Regulatory functions	Sensory transduction histidine kinase
SLR1356	Ribosomal proteins: synthesis and modification	30S ribosomal protein S1 (rps1)
SLL0767	Ribosomal proteins: synthesis and modification	50S ribosomal protein L20 (rpl20)
SLR1643	Soluble electron carriers	Ferredoxin-NADP oxidoreductase (petH)
SLR2057	Transport and binding proteins	Water channel protein (apqZ)
SLL1982	**Transposon-related functions**	**Transposase (ISY352_c2)**

Negatively correlated with daytime are shown in bold, positively correlated are shown in plain text.

These values are indicative of healthy, actively growing cultures that are not experiencing light stress (Holm-Hansen and Riemann 1978, Parsons et al. 1984). In addition, analysis of the *in vivo* spectral absorption data revealed very little difference in peak ratios over the course of the experiment.

Relationship between light cycle and mRNA level. Although total transcript abundance was generally low at night, many individual transcripts showed dramatic diel variation. The transcripts from 1349 genes had *F* scores greater than 5.0, and 776 of these had meaningful Cyanobase annotations. The annotations were obtained from Cyanobase (www.kazusa.or.jp/cyano/cyano.html; last accessed July 1, 2003). A selected set of transcripts with meaningful annotations that are most strongly correlated with light level can be found in Table 1, while their abundances and the strength of their correlations with light can be found in Table 2; all significantly correlated transcripts (excluding unknown transcripts), their abundances at each light level, and the strength of their correlations with light can be found in Appendix S1. All transcripts referred to in the text can be found in Appendix S1.

Transcripts exhibiting positive correlations with light. In this group, transcripts encoding ATP synthase (Tables 1 and 2), carbon fixation enzymes (Tables 1 and 2, Fig. 3B), and light-harvesting and photosystem polypeptides (Tables 1 and 2, Fig. 3A) and specific

TABLE 2. Fold change difference between the sample and reference is shown under each time point (0:00 a.m.–8:00 p.m.) for genes described in Table 1.

Gene	0:00	6:00	8:00	10:00	12:00	14:00	16:00	18:00	20:00	R^2
SLL0005	**1.237**	**1.264**	**−1.003**	**1.131**	**−1.232**	...	**−1.454**	**1.168**	**1.333**	**0.321**
SLL0018	...	−5.108	2.041	−2.228	2.354	−1.896	−1.208	−5.334	−3.600	0.279
SLL0427	...	−3.956	1.253	−2.155	1.579	−1.165	1.480	−4.415	−4.330	0.398
SLL0541	...	−3.143	2.143	−2.202	1.766	−1.138	2.349	−4.748	−2.830	0.286
SLL0767	...	−2.226	1.630	−1.555	1.681	1.065	1.577	−2.460	−2.279	0.337
SLL0779	−1.061	−1.512	1.264	−1.019	1.187	1.159	1.311	−1.227	−1.432	0.384
SLL0849	...	−4.582	1.473	−2.580	1.987	−1.116	1.485	−7.221	−2.768	0.287
SLL1091	...	−3.882	1.107	−2.027	1.739	−1.460	−1.049	−3.141	−3.439	0.391
SLL1214	...	−3.584	1.639	−2.292	1.973	−1.483	1.469	−3.736	−3.232	0.305
SLL1234	−1.408	−3.025	1.397	−1.277	1.421	−1.116	1.187	−2.287	−2.929	0.542
SLL1290	**1.192**	**1.012**	**−1.174**	**−1.027**	**−1.346**	**−1.341**	**−2.047**	**1.021**	**1.268**	**0.293**
SLL1427	**1.251**	**1.044**	**−1.190**	**−1.097**	**−1.386**	**...**	**−1.825**	**−1.400**	**1.290**	**0.373**
SLL1434	**1.219**	**1.225**	**−1.030**	**1.083**	**−1.373**	...	**−1.359**	**1.004**	**1.137**	**0.308**
SLL1479	**1.215**	**−1.062**	**−1.091**	**−1.131**	**−1.326**	**...**	**−1.282**	**−1.009**	**1.205**	**0.403**
SLL1525	...	−3.215	1.357	−1.850	1.610	−1.457	1.374	−3.216	−3.579	0.419
SLL1577	...	...	*−1.086*	*−2.457*	*2.756*	*1.180*	*1.538*	...	*−5.093*	*0.364*
SLL1578	...	−11.970	1.007	−2.813	2.336	1.091	1.227	−12.464	−4.507	0.311
SLL1750	**1.129**	**1.278**	**−1.072**	**1.007**	**−1.031**	**...**	**−1.675**	**1.007**	**1.253**	**0.333**
SLL1814	...	−3.532	1.433	−1.871	1.805	−1.439	1.249	−3.342	−2.092	0.304
SLL1982	...	**1.590**	**1.254**	**1.111**	**−1.215**	**1.434**	**−1.368**	**1.963**	**2.150**	**0.367**
SLR0009	...	−6.254	1.119	−2.860	1.847	−1.969	1.193	−6.448	−3.921	0.322
SLR0011	...	−4.542	1.476	−2.874	2.471	−1.169	1.631	−5.278	−3.686	0.308
SLR0079	**1.035**	**1.338**	**−1.009**	**1.066**	**−1.047**	**...**	**−1.423**	**−1.016**	**1.351**	**0.302**
SLR0210	...	−1.828	1.140	−1.345	1.126	−1.115	1.194	−1.492	−1.986	0.404
SLR0322	−2.505	−1.833	1.491	−1.351	1.988	−1.143	1.373	−2.131	−1.574	0.374
SLR0394	−1.249	−3.962	−1.074	−1.397	1.697	−1.219	1.028	−2.910	−2.798	0.422
SLR0473	**−1.185**	**−1.095**	**−1.458**	**−1.474**	**−1.630**	**...**	**−1.095**	**−1.811**	**1.828**	**0.397**
SLR0533	...	−3.181	1.223	−1.777	2.625	−1.197	1.530	−3.563	−1.956	0.328
SLR0927	...	−3.725	1.540	−2.276	1.832	−1.099	1.596	−5.668	−2.449	0.280
SLR1181	*−1.397*	*−0.850*	*0.005*	*−0.734*	*0.760*	*−0.721*	*0.959*	*−1.123*	*−0.176*	*0.169*
SLR1226	**1.065**	**1.087**	**−1.095**	**1.005**	**−1.149**	**−1.379**	**−1.749**	**−1.146**	**1.274**	**0.319**
SLR1350	...	−1.889	1.337	−1.443	1.591	−1.327	1.096	−1.737	−1.706	0.277
SLR1356	...	−2.784	1.271	−2.354	1.509	−1.513	1.223	−3.294	−2.342	0.273
SLR1390	**1.097**	**1.162**	**−1.036**	**−1.037**	**−1.111**	**...**	**−1.292**	**−1.160**	**1.241**	**0.412**
SLR1403	**1.090**	**1.119**	**−1.244**	**−1.038**	**−1.262**	**−1.085**	**...**	**−1.038**	**1.148**	**0.355**
SLR1529	−2.663	−2.764	1.503	−1.803	1.878	−1.066	1.468	−3.436	−1.955	0.377
SLR1543	−1.456	−1.576	1.114	−1.212	1.273	−1.081	1.224	−1.765	−1.441	0.366
SLR1641	**1.089**	**1.527**	**1.066**	**1.179**	**1.022**	**−1.342**	**−1.532**	**1.065**	**1.687**	**0.333**
SLR1643	...	−4.150	1.208	−2.006	1.461	−1.580	−1.014	−3.201	−2.954	0.363
SLR1756	−4.630	−3.300	1.043	−2.686	1.650	−1.495	−1.654	−3.369	−2.963	0.346
SLR1777	**1.134**	**1.425**	**−1.081**	**1.185**	**−1.017**	**−1.303**	**−1.743**	**1.117**	**1.496**	**0.300**
SLR1805	**1.032**	**1.244**	**−1.061**	**1.099**	**−1.202**	**...**	**−2.034**	**1.073**	**1.565**	**0.294**
SLR1834	*−2.827*	*−1.766*	*−0.204*	*−2.028*	*1.316*	*−1.170*	*0.415*	*−2.961*	*0.062*	*0.048*
SLR2057	...	−3.826	−1.408	−2.151	1.967	−1.112	1.858	−3.765	−2.863	0.274
SLR2094	...	−5.138	1.550	−1.942	1.600	−1.166	1.517	−3.754	−3.826	0.484
SLR2099	...	−3.225	1.317	−1.744	1.551	1.082	1.756	−3.288	−2.078	0.349

The fold change is calculated from the log2 ratios as $2^{\log_2 \text{ ratio}}$, with the negative reciprocal taken of the values less than 1. Note that the table has been ordered alphanumerically by gene number.

chaperones (i.e. proteins responsible for preventing the formation of misfolded proteins or facilitating their renaturation) exhibited a dramatic transition from low or near-zero levels of mRNA when cultures were exposed to no or little light, to a near maximum by 8:00 a.m. (4 h after the activation of the light program). The temporal peak in transcript levels was often broad, extending from the early hours of the day until mid- or late afternoon. We did observe variability in the increase in transcript level over the course of the day (as seen in the dips and peaks in the graphs of Fig. 3), but the trends relative to the light cycle periods were robust.

Transcripts encoding the light harvesting (e.g. *cpcA, cpcB,* and *cpcC*, which encode for the α, β subunits of phycocyanin and a linker polypeptide of the phycobilisomes, respectively) and PSI and PSII polypeptides (e.g., *psaA*, *psbA1*, and *psbA2*) displayed a 10-fold or greater change in abundance over the course of the diel light cycle, as derived from the microarray data (Fig. 3A and Tables 1 and 2). It should be noted, however, that the three *psbA* genes of *Synechocystis* are highly conserved and the strong similarity among these genes at the nucleotide level precluded discrimination of the individual *psbA* genes using the array. In addition, no transcript from *psbA1* has been detected to date (Salih and Jansson 1997). Regardless, from analyses of the microarray data, we observed a strong positive correlation of *psbA* transcript levels with light, although it was also present to a significant extent

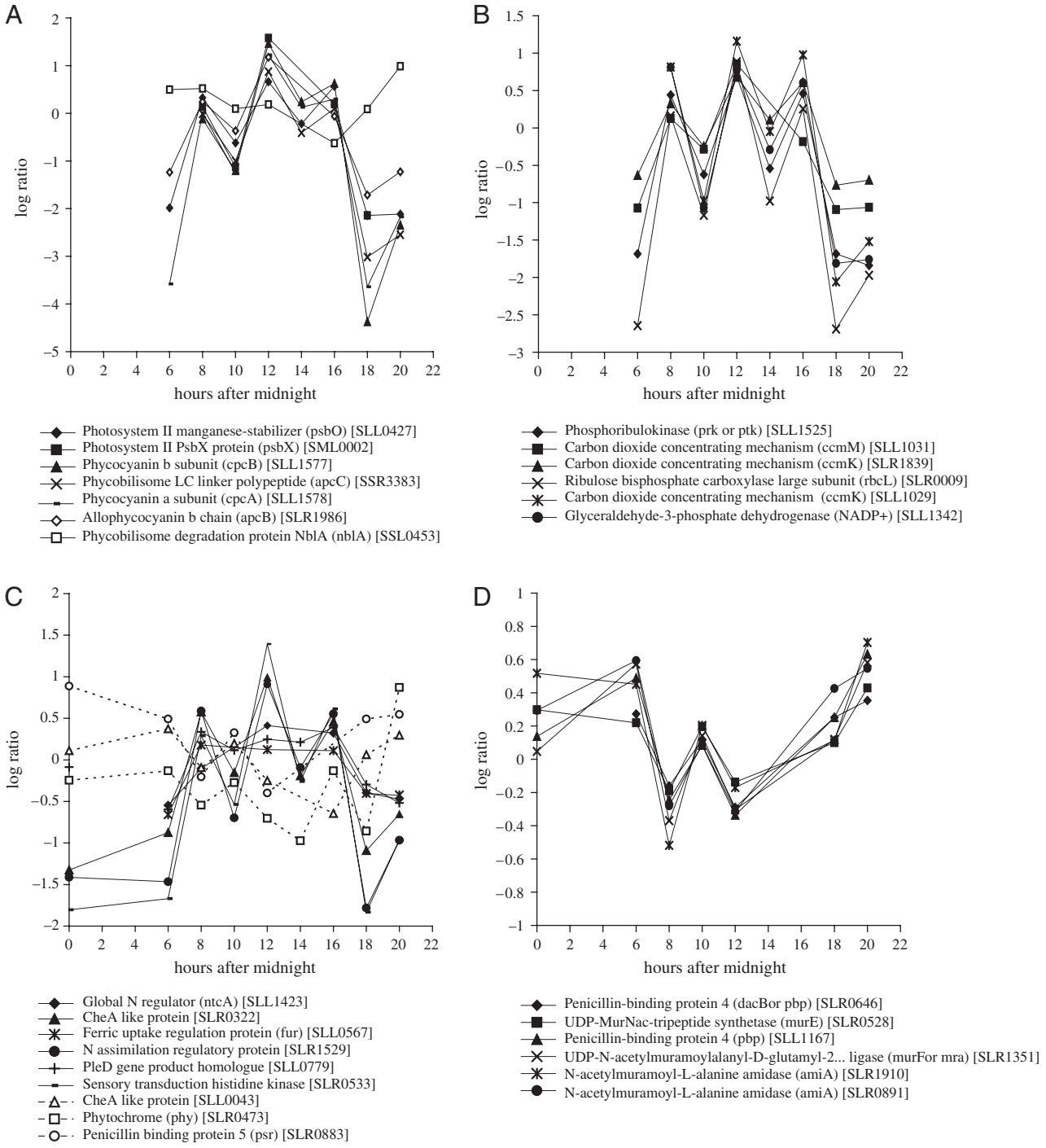

FIG. 3. Selected $\log_2$ ratio mRNA transcript abundance in four categories (according to Cyanobase categorization; see text for details). (A) Photosynthetic electron transport and light harvesting. (B) Carbon fixation. (C) Regulatory. (D) Cell wall associated.

in the dark, as demonstrated by both the microarray data (Tables 1 and 2) and RNA blot hybridization (Fig. 4).

The increase in transcript abundance for most light-harvesting and photosystem polypeptides from the time when the lights were turned on (4:00 a.m.) until the time of maximum transcript accumulation was moderately steep, with the attainment of peak or near-peak levels of photosystem (e.g. *psaA* and *psbA2*) and light-harvesting (e.g. *cpcB and cpcC*) transcripts by 8:00 a.m. (Figs. 3A and 4). The levels of most of these transcripts from midnight to 4:00 a.m. (especially for photosystem and light-harvesting mRNAs) were so low that reliable transcript level data could not be obtained for these time points and are not shown in Figs. 3, A and B). The decrease from maximum to minimum

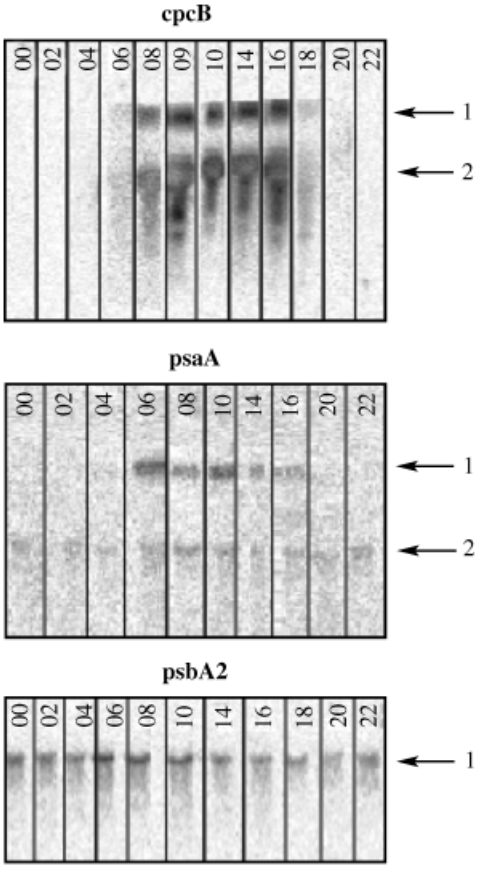

FIG. 4. RNA blot hybridizations for three photosynthetic transcripts, *cpcB* (top), *psaA* (middle). and *psbA2* (bottom). Each lane shown here represents an individual time point. Note the similar patterns between the RNA blot hybridization and microarrays, with dramatic increases in transcript abundances after 8:00 a.m. The RNA blot hybridizations have been reshuffled for display purposes—the original order was not in time sequence in order to avoid positional biases. For *cpcB*, arrows 1 and 2 designate higher and lower molecular weight polycistronic transcripts consisting of *cpcBAC2C1D* and *cpcBA*, respectively. For *psaA*, arrow 1 denotes the specifically hybridizing *psaA* transcript, while arrow 2 denotes a non-specific hybridizing transcript. For *psbA2*, arrow 1 represents the cumulative level of all *psbA* transcript because the probe would not specifically hybridize to *psbA2* mRNA.

mRNA levels as evening approached was generally fairly steep, with many attaining dark or near-dark levels by the time the light went off at 6:00 p.m. (Figs. 3 and 4). Interestingly, the transcript encoding NblA, a protein associated with degradation of the phycobilisome, showed a negative correlation with light (Fig. 3A, Tables 1 and 2).

There were also some transcripts encoding proteins that function in the maintenance and repair of DNA that were positively correlated with light, including those encoding two DnaK chaperones: a DNA ligase, and a DNA damage-inducible protein. These proteins may not be involved in replication per se, but may have a role in repairing damaged DNA during periods of rapid growth and exposure to excess excitation energy.

Many transcripts encoding non-photosynthetic proteins were also positively correlated with light. For example, mRNAs encoding enzymes involved in the assimilation of nutrients, including a transcript for nitrate transport protein NrtD, phosphate transporter polypeptides (PstB or PhoT) (Tables 1 and 2), the global nitrogen regulator (NtcA), and glutamate/ammonia ligase (GlnN) (Appendix S1), were highest during the day. The majority of the transcripts encoding proteins involved in cell division, including FtsZ, two out of four FtsH transcripts, and a cell division protein, were weakly positively correlated with light, with the FtsZ transcript peaking between 12:00 and 4:00 p.m. at a level >4 fold higher than at 12:00 p.m. Transcripts encoding proteins required for carbon dioxide fixation, including CcmK, CcmM, CcmL, RbcL, RbcS, and Prk/Ptk, were very strongly positively correlated with the light level (Fig. 3B).

Transcripts exhibiting negative correlations with light. Although the transcripts that were positively correlated with light showed much stronger diel changes in abundance than those that were negatively correlated, a number of transcripts were strongly negatively correlated with light level (Tables 1 and 2 and Appendix S1 in bold, Fig. 3, C and D). The levels of these transcripts were lowest at 10:00 a.m.–3:00 p.m. (peak light period) and highest between 10:00 p.m. and 6:00 a.m. (dark period and transition into light period). This category includes transcripts encoding proteins involved in DNA synthesis, maintenance, replication, and degradation, including the transcripts for DNA polymerase (DnaZ), a DNA primosomal protein (PriA), and a glucose-inhibited division protein (GidA) (Tables 1 and 2). In addition, an entire set of transcripts associated with the production of components of the cell wall (murein sacculus and peptidoglycan), including those encoding MurE, MurF, AmiA, RoDA, PonA, and PonB, was also strongly negatively correlated with light (Tables 1 and 2 and Fig. 3D). The majority of transcripts encoding the TCA cycle proteins, including succinate dehydrogenase, fumarase, and malate/lactate dehydrogenase, were also negatively correlated with light. Finally, while the transposons showed relatively weak correlations with time of day, of the few transposons with significant *F* scores, the majority were negatively correlated with the daylight period.

Behavior of transcripts with unknown function. Interestingly, the abundance of many mRNAs that encode polypeptides with no known function was significantly correlated (either negatively or positively) with time of day (the 20 with the highest correlation coefficients are shown in Table 3). These patterns may serve as a diagnostic that will help elucidate the physiological functions of the encoded polypeptides. We note that the levels of a number of these transcripts changed significantly just prior to the transition from light to dark and/or dark to light, indicating a possible circadian control (Table 3).

Confirmation by RNA blot hybridization. As noted earlier, some of the microarray results presented above

were confirmed by RNA blot hybridizations using total RNA derived from the same set of samples used for the microarray analyses. For this analysis, we selected three photosynthetic genes (*cpcB*, *psbA2*, and *psaA*) that were all positively correlated with light level. Application of RNA blot hybridizations to examine levels of the *cpcB*, *psbA2*, and *psaA* transcripts over the diel cycle yielded results comparable to those of the microarray analyses; a fairly abrupt change in mRNA levels took place during the morning hours, with a maximum at or near midday, decreasing to near zero after dark (Fig. 4). The multiple hybridizing transcripts observed on the RNA blot when the *cpcB* gene probe was used reflects the fact that *cpcB* is part of an operon that contains the *cpcB*, *cpcA*, *cpcC2*, *cpcC1*, and *cpcD* genes, with some transcripts resulting from transcription of the entire operon, which represents *cpcBAC2C1D* (band 1), and others resulting from transcription of *cpcA* and *cpcB* (band 2) (Plank and Anderson 1995). The pattern of abundance for both the long and short transcripts changed to a similar extent over the diel cycle (Fig. 4).

Timing of gene activation. We were able to classify groups of genes based on the specific times in the diel cycle during which they become most active (Fig. 5). Overall, many fewer transcript abundances were above the limit of detection during the night than during the middle of the day, and the low signals from the arrays that were hybridized to RNA isolated from cells during the evening hours made the analyses of these arrays more difficult. Transcripts categorized within the same metabolic pathway tended to peak within 2–4 h of each other during the diel cycle, with some notable exceptions.

A number of transcripts encoding polypeptides involved in DNA replication and protein degradation (including the DNA replication initiator DnaA, aminopeptidase P (PepP), and various proteases) showed peak levels at 6:00 a.m. Transcripts encoding proteins with similar functions (e.g. RecR, PriA) were high at other time points during the dark phase of growth. Other genes peaking just before daylight include some that encode polypeptides involved in transporter function (components of the ABC transporter family), peptidoglycan and cell wall biosynthesis (AmiA, and several transcripts involved in the synthesis of murein sacculus), cell wall-associated penicillin binding proteins (PonA, RodA, and spore maturation protein B), and an assortment of genes with regulatory and/or cofactor binding function.

The levels of some transcripts peaked between 8:00 and 10:00 a.m.; most of these are involved in photosynthetic function, but others are involved in the degradation (LysA, ATP-dependent Clp protease-regulatory subunit, ClpC, and ATP-dependent Clp protease proteolytic subunit, ClpP), modification (peptide chain release factor, PrfB, peptide methionine sulfoxide reductase, msrA or pms, and thiol:disulfide interchange protein, TrxA) and secretion of proteins (type 4 prepilin peptidase, HopD). In addition, peak nitrate transport (nitrate transporters NrtA and NrtC) coincided with the early morning.

Between 10:00 a.m. and 6:00 p.m., there was a peak in the levels of most transcripts encoding polypeptides involved in nutrient acquisition/assimilation, ribosomal protein synthesis and modification, and photosynthetic function. These included mRNAs encoding polypeptides of phycobilisomes (ApcA-E, CpcA-D and CpcG),

TABLE 3. Twenty unannotated (or "hypothetical") transcripts most strongly correlated with time of day.

Gene	0:00 a.m.	6:00 a.m.	8:00 a.m.	10:00 a.m.	12:00 p.m.	2:00 p.m.	4:00 p.m.	6:00 p.m.	8:00 p.m.	r^2
Positively correlated with daytime										
SLL1665	−1.198	−2.111	−1.304	−1.241	1.009	−1.532	1.053	−2.593	−1.821	0.173
SLR0551	−2.388	−2.020	1.404	−2.336	1.318	−2.299	1.114	−2.328	−1.447	0.248
SLR0848	−1.088	−1.241	1.020	1.092	1.176	−1.271	−1.227	−1.326	−1.145	0.117
SLR1187	−1.047	−1.409	1.208	−1.145	1.148	−1.361	1.066	−1.702	−1.301	0.205
SLR1506	−1.209	−1.242	1.105	1.063	1.115	−1.141	−1.050	−1.119	−1.222	0.244
SLR1816	−1.351	1.023	1.144	1.196	−1.046	−1.172	1.187	1.075	−1.049	0.152
SLR1855	−1.716	−1.545	1.018	−1.279	1.144	−2.003	−1.254	−1.998	−1.640	0.228
SLR2087	−1.547	−1.174	1.082	−1.070	1.161	1.066	−1.073	1.050	−1.369	0.471
SSR0536	−1.247	−1.328	1.205	−1.053	1.178	1.100	1.048	−1.335	−1.281	0.322
SSR2848	−1.377	−1.246	1.038	−1.187	1.497	−1.217	−1.218	−1.540	−1.094	0.119
Negatively correlated with daytime										
SLL0148	1.389	1.201	−1.031	1.084	−1.183	−1.266	−1.437	1.033	1.201	0.330
SLL0264	1.244	1.354	−1.195	1.198	−1.221	−1.284	−1.507	1.232	1.403	0.326
SLL0355	1.122	1.246	−1.291	1.003	−1.336	−1.607	−1.851	−1.072	1.239	0.485
SLL0471	1.523	1.356	−1.150	1.080	−1.387	1.021	−1.523	1.137	1.348	0.501
SLL1233	1.281	1.461	1.191	1.084	−1.233	1.401	−1.076	1.247	1.359	0.357
SLR0617	1.596	1.240	−1.206	1.194	−1.352	−1.347	−2.948	1.094	1.500	0.482
SLR0689	1.230	1.321	1.092	1.284	1.010	−1.320	−1.372	1.056	1.495	0.328
SLR1462	1.182	1.062	−1.439	1.147	−1.457	−1.353	−1.952	1.092	1.244	0.364
SLR1603	1.044	1.357	−1.090	1.178	−1.333	1.214	−2.198	−1.013	1.616	0.363
SSR0756	1.217	1.092	−1.309	−1.018	−1.292	−1.511	−1.388	−1.072	−1.007	0.342

Data presented here include the gene number, fold change values at each time point (calculated as in Table 2), and the results for the correlation (r^2) between transcript response and time of day. Only genes for which all time points contained all values are included.

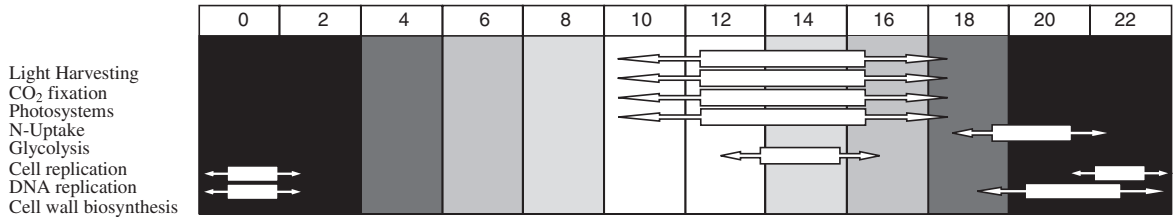

FIG. 5. Qualitative summary depicting the peak transcript abundances over the diel cycle for selected Cyanobase categories, including CO_2 fixation, cell division, DNA replication, light harvesting, photosystems, nitrogen uptake, transposons, and glycolysis, based on transcripts with at least four good time points. The length of the arrow and box represents the approximate span of time over which the abundance of different transcripts in a category peaks.

PSI (PsaA-B, PsaE-F, and PsaL), PSII (PsbA2, PsbA3, PsbJ, PsbT-V, PsbO, and PsbX), carbon fixation and concentrating mechanisms (CcmK through CcmM, RbcL, RbcS, Tpi, and Ptk/Prk), ATP synthesis (AtpA-I), chaperone function (GroES, GroEL, and DnaK), cell division (one out of two significantly correlated FtsH transcripts and, FtsZ, the "cell division cycle protein," maf protein, and the septum site-determining protein, MinD), NADH dehydrogenase (NdhA, NdhB, NdhD, NdhF, NdhG, NdhH, NdhI, and NdhK-L), nitrogen assimilation (GltB, GlnA, GlnN, NirA, and CynS), various regulatory and transport processes (particularly, sensory histidine kinases, and nitrogen and phosphate transporter components), as well as many ribosomal polypeptides. Other transcripts encoding polypeptides involved in photosynthesis and inorganic carbon fixation and metabolism peaked by the late afternoon (4:00 p.m.), including ApcF, PsaD, PsaK, PsbA2, PsbH, PsbL, CcmA, NdhC, and NdhJ. This group also contained genes encoding chaperone polypeptides (GrpE) and proteins associated with the uptake of sulfate, phosphate, iron, and other metal ions.

Many transcripts encoding polypeptides involved in DNA replication and repair and transposition peaked between 6:00 and 12:00 p.m. (including PriA, glucose-inhibited division proteins, GidA and GidB, RecJ, RecG, RecN, DnaG, DnaX, LigA, and various helicases), as did some genes encoding polypeptides associated with glycolysis (pgi, Gap1, PykF, and PfkA), transposons, and protein degradation. Peak abundances for mRNAs encoding enzymes of the structural components of the cell wall (PonB, AmiA, and MurC through MurF) and some transporter function (Na/H antiporters, BraE, ABC transporter, and iron transporters) also peaked between 10:00 p.m. and midnight.

It should be noted that some transcripts associated with particular metabolic pathways did not follow the same pattern as the majority of the other transcripts. For example, under the DNA replication and repair Cyanobase categories, the majority of the transcripts encoding the chaperones DnaJ and DnaK peaked during the day, unlike the majority of the other DNA replication, transcription, and degradation transcripts. In addition, under the light-harvesting and photosystem categories, the transcript encoding the PSII PsbN protein (*psbN*) and the phycobilisome degradation protein NblA (*nblA*) peaked during the night, instead of during the day when the rest of the light-harvesting transcripts reached their maximum level.

DISCUSSION

Photosynthetic organisms such as cyanobacteria must balance available light energy over the diel cycle with their basal energy needs, their reproductive demands, and the repair of systems that are damaged as a consequence of metabolic activity (e.g. photosynthesis, which generates reactive oxygen species). Therefore, these organisms are constantly monitoring their light environment and adjusting gene expression and metabolic processes to light conditions or according to the daily cycle. In this study, we found that over the diel cycle, the growth of cells in the cyclodyn occurred exclusively during the day and was most rapid during the afternoon when photosynthesis was at its maximum. However, many transcript abundances increased much earlier than the peak in irradiance or growth, consistent with a response to redox state (Li and Sherman 2000, Van Waasbergen et al. 2002), circadian (Liu et al. 1995, Kondo et al. 1997, Golden and Canales 2003), or photoreceptor control (Franklin and Whitelam 2004). Gene expression that anticipates the change in light conditions may be regulated by a circadian clock, and this pattern is witnessed in the levels of several transcripts, such as *psbA*.

Transcript abundance during the day. Many transcripts involved in biosynthetic and metabolic processes are abundant during the day, when anabolism—the building up of complex molecules—appears to be most active. The transcripts that peak during the day include those encoding components that are part of the light-harvesting apparatus and the photosystems, and that function in cell division, inorganic carbon fixation, nitrogen uptake and utilization, and aspects of gene regulation (based on increased levels of mRNAs for regulatory elements).

Transcript encoding components of the light-harvesting/photosynthetic apparatus remained high throughout the daylight period, a phenomenon not observed in previous studies that assessed photoacclimation under light-stress conditions (Muramatsu and Hihara 2003, Hsiao et al. 2004). Many mRNAs encoding polypeptides of the photosynthetic apparatus were positively correlated with light, with the strongest

encoding PsaA, CpcB, and CpcA; the first polypeptide is a subunit of the reaction center of PSI while the latter two are components of the light harvesting phycobilisomes. The *psaA* and *cpcB* transcripts were nearly undetectable during the dark phase of the diel cycle. These results suggest that the cells increase their potential to synthesize the light-harvesting/photosynthetic apparatus throughout the day and that more transcripts are required for increased translation of polypeptides of the photosynthetic apparatus during periods of active growth.

Several transcripts encoding polypeptides responsible for cell division were most abundant during the day, including those encoding FtsZ. This agrees with the work of Holtzendorff et al. (2001) on the marine cyanobacterium *Prochlorococcus marinus* 9511. In *Prochlorococcus marinus*, the mRNA encoding the cell division protein FtsZ was positively correlated with light, showing a maximum transcript level ~ 10 h after the onset of light. The coordination of increased growth with the timing of maximum light level (typically noon) has been observed in many bacteria, including cyanobacteria, and in both freshwater and marine species of cyanobacteria (Holtzendorff et al. 2001, 2002, Jacquet et al. 2001). The timing of cell division may be either circadian or light quality and/or irradiance controlled, although the former appears to be responsible for the timing of cell division in phytoplankton such as *Prochlorococcus* spp. (Shalapyonok et al. 1998). It should be noted that one transcript encoding the cell division protein FtsH is most abundant at night, but other FtsH transcripts are most abundant during the day.

The maximum transcript abundances for the carbon and nitrogen assimilation pathways coincided with the maximum light level, and the transcripts were maintained at a high level into the evening hours. This suggests that light harvesting and energy transfer are in synchrony with the accumulation of fixed carbon and the uptake of nutrients into the cell. Specifically, the transcript encoding RUBISCO (RbcS and RbcL), which is known to be the rate-defining protein for carbon fixation and accumulation in the cell, is highest during the day, as are transcripts associated with the carbon-concentrating mechanism. In addition, transcripts encoding proteins for glutamate-ammonia ligase and ferrodoxin-nitrite reductase, together with nitrogen assimilation regulatory proteins and regulatory elements involved in nutrient acquisition and assimilation, follow a pattern similar to, albeit weaker than, that of the light-harvesting and photosystem transcripts. This pattern of transcript accumulation makes sense given the need for cellular materials during the time when cell division is taking place and energy is available to reduce carbon and nitrogen. Interestingly, many transcripts encoding polypeptides involved in nutrient assimilation exhibited neither a night-time increase nor a constant level during the day. Instead, these transcripts peaked during mid-day, similar to the light-harvesting transcripts, suggesting that nutrient assimilation may indeed be more prevalent during the day, when energy generated by photosynthetic electron transport can be used for assimilatory processes. Diel cycles in nutrient uptake similar to those that we have observed at the transcript level have been found during studies of the open ocean and lakes, as well as in laboratory cultures (Litchman et al. 2004). It should also be noted that during rapid growth, the transcripts needed for basic cell function such as the nutrient assimilation and light-harvesting transcripts would most likely be replicated to avoid dilution during the growth and division of the cell.

It is also clear that transcripts for a number of regulatory elements showed a sustained increase during the daylight hours. Some of these encode sensory histidine kinases and others encode response regulators. Our results suggest that many regulatory factors are associated with growth and/or with light responses. For example, the response regulator encoded by slr0081, a member of the *ompR* family of osmotic response regulators (Kenny 2002), is positively correlated with light. While OmpR is involved in osmotic stress responses, other members of this gene family in *Synechocystis* (slr0115 and slr0947, or *rpaA* and *rpaB*) regulate the coupling of phycobilisomes to photosynthetic reaction centers (Ashby and Mullineaux 1999). The polypeptide encoded by slr0081 is very similar to the RpaA and RpaB polypeptides. In addition, Li and Sherman (2000) found that sll0797 or *rppA*, encoding another polypeptide similar to OmpR, was part of a two-subunit response regulator controlling the synthesis of PSII through redox sensing. The mRNA abundance pattern of sll0797 was similar to that of slr0081, although the significance level was lower. This aggregate information raises the possibility that the slr0081 polypeptide plays a role in controlling a specific light response. The transcripts encoding other regulatory factors such as CheA (also called TaxA), which play important roles in light-responsive motility (Bhaya et al. 2001), are also responsive to light levels.

It is important to keep in mind that increases in transcript abundance are not necessarily related to the metabolic rate; increases in some transcripts in the light period appear to reflect changes in the rates of degradation of proteins, and in some instances, an increase in the level of mRNA may not lead to a change in the steady state level of the encoded protein. For example, under oxidative stress, the *psbA* transcripts, which encode the light responsive reaction center polypeptides of PSII, increase significantly (Clarke et al. 1993, Kulkarni and Golden 1994). This is primarily because the D1 protein, which is encoded by *psbA*, is susceptible to oxidative damage within the reaction center and is rapidly degraded in high light. Therefore, more transcripts are needed for translation to replace the rapidly degrading D1 polypeptide. In this study, because the average peak irradiance was significantly below that saturating photosynthesis, the light-harvesting requirements of the cells were always high. Furthermore, even though the phycocyanin mRNA level may have increased by 10-fold during the day, the

in vivo spectral absorption data showed that the level of the phycocyanin polypeptide in the cell did not dramatically increase. These results suggest that an increase in transcripts encoding phycobilisome polypeptides serves in the synthesis of new phycobilisomes during rapid growth in the light period.

Transcript abundance at night. The set of metabolic processes that are thought to be more active in the evening include DNA replication, cell wall biosynthesis, aspects of energy metabolism, and potential transposon activity. Replication of DNA and cell division were previously observed to occur during different phases of the cell cycle (Binder and Chisholm 1990, Holtzendorff et al. 2001). This is consistent with the changes in the mRNA content of the cell that we noted in this study. Cell division was observed at mid-day and coincided with peak abundance of mRNAs that encode FtsZ and other cell division polypeptides. Meanwhile, a number of transcripts associated with DNA replication were more abundant at night (e.g. GidA, PriA, DnaZ, DnaJ). Interestingly, we found that transcripts encoding polypeptides for cell wall biosynthesis were more abundant at night and in the early morning than they were during mid-day. A regulatory element (slr0883 in Fig. 3D) controlling the synthesis of certain polypeptides with cell wall function also peaked during the evening. These data suggest that the building blocks for cell wall biosynthesis are generated during the night or in the early morning, and that these building blocks are then used for rapid assembly of cell wall components during mid-day cell division.

Our observation that some transcripts encoding enzymes involved in glycolysis and the oxidative pentose phosphate pathway (e.g. Gap1, DevB) peaked in the evening is consistent with existing physiological data (Stal and Moezelaar 1997, Knowles and Plaxton 2003). For example, although knowledge of dark carbon energy metabolism is as yet incomplete, some studies have shown that glycogen accumulated during the day is catabolized by glycolysis and the oxidative pentose phosphate pathway during the night, producing ATP and anabolic precursors (Stal and Moezelaar 1997). Our data further show that the maximum transcript levels encoding these two enzymatic pathways lag the peak abundance of carbon fixation transcripts, which are highest during the day. These findings suggest that the cells accumulate fixed carbon during the day when excitation energy can be harvested, while the net organic carbon levels decrease in the evening when the cells are undergoing catabolic processes required for cell maintenance in the absence of photosynthesis.

Curiously, we found that transcription associated with many of the *Synechocystis* transposons (whose presence indicates genetic mobility) (DuFresne et al. 2003) appears to have occurred in the evening when the cells are not growing. While it is difficult to interpret the significance of this result, we suggest that such activity might be beneficial for the generation of bacterial variants when the cells are maintained under conditions that would prevent their growth. Such variants may find new ways to utilize environmental resources, possibly giving them a selective advantage over their competitors.

Overall changes in gene expression. The present study provides us with a global view of the metabolic changes that occur in cells in response to diel changes in irradiance**.** While our data suggest that broad categories of activities in *Synechocystis* vary during diel cycling, these results must be substantiated at the protein and/or enzyme activity levels. However, analyses of the levels of the transcripts within the broad temporal divisions used here (day vs. night) under conditions of naturally varying light levels demonstrate that increased transcript levels are generally sustained over an entire phase of the light cycle. Often, genes that become active in the morning hours show sustained activity throughout the day.

Even under diel light cycling where differences in perceived irradiance between night and day are relatively small (dark vs. $\sim 31\ \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), we found that changes in the abundance of transcripts encoding proteins in the photosynthetic apparatus or other metabolic pathways were significant. These results have implications concerning the way in which the input light is sensed. Specifically, although light may be sensed through the activity of photosynthetic electron transport and the redox state of the cell (Escoubas et al. 1995, Li and Sherman 2000, Im and Grossman 2002, Van Waasbergen et al. 2002), transcripts for a number of genes monitored in this study become abundant in the early morning hours when light levels are very low; such conditions would not elicit marked changes in either photosynthetic activity or cellular redox potential. Rather, the transcript levels and gene activities may be controlled by specific photoreceptors that either work independently of, or in conjunction with, the circadian clock. If this is correct, it will be critical to identify those molecules that act as photoreceptors and the wavelengths of light that are being used as signals, as light quality as well as irradiance affects the potential for these phytoplankton to photoacclimate successfully in nature (Grossman et al. 2001).

Our study of the molecular responses of *Synechocystis* to gradual changes in the diel light level differ markedly from studies where cyanobacteria underwent light stress during transitions from low light (or darkness) to high light, or where they were maintained in sustained high light. In our study, many of the photosystem transcripts, including *psaA*, increased in abundance at the beginning of the light period and remained high throughout the day, with some transcripts maintained at moderate levels after the lights were turned off. Under sustained high light conditions, many transcripts encoding proteins of PSI have been shown to decline upon exposure of cells to high light. In the work of Hihara et al. (2001), the transcript for *psaA* declined by 3-fold when *Synechocystis* cells were abruptly transferred from low to high light. Under the latter condi-

tions, photosynthetic electron transport was saturated and the cells were undergoing high light stress. After the cells adjusted to the high light conditions, the *psaA* transcript level returned to 50–100% of that observed under low light conditions.

A similar situation occurred for a number of transcripts encoding proteins of the light-harvesting apparatus. For example, in our study, a number of the *cpc* transcripts strongly increased in abundance during the daytime, but in the study conducted by Hihara et al. (2001), the *cpc* transcripts declined by approximately 95% immediately following exposure of the cells to high light. It is notable that after 15 h in the high light environment of the Hihara et al. (2001) study, the transcript levels increased to approximately 50% of the initial low light level. The differences in transcript levels observed after an abrupt change in the intensity of light to which the cells are exposed may reflect an extreme stress response, which is alleviated to some extent as the cells photoacclimate and establish a new cellular homeostasis.

Our data show that naturally varying light exposure induced significant changes in mRNA levels within *Synechocystis*, but that the sign of the changes often differed between our study and those studies in which there was a sudden and dramatic increase in light levels (Hihara et al. 2001), conditions that can elicit a stress response in the cells. However, in determining the absolute difference between a stress response and the gradual diel response, the timing of sampling of the culture is critical; cells adjust their photosynthetic state on the scale of hours, so a sampling gap longer than two hours between large shifts in irradiance would not capture the initial response of the cells. In addition, our global examination of mRNA abundances revealed that many pathways in the cell appear to be timed with diel changes in light. In addition to the photosynthetic pathway, fluctuations in transcript levels for mRNAs encoding proteins involved in DNA replication and repair, cell division, the synthesis of cell wall material, and nutrient assimilation pathways were strongly correlated to the light cycle. Further studies will reveal how well the metabolic state of the cell is reflected in mRNA transcript abundance, and whether the correlations discovered in this study are valid for most other phytoplankton.

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Supplementary Material

The following supplementary material is available for this article online:

Appendix S1. Examination of diel changes in global transcript accumulation in *Synechocystis* sp. strain PCC6803.