Circadian orchestration of gene expression in cyanobacteria

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We wanted to identify genes that are controlled by the circadian clock in the prokaryotic cyanobacterium *Synechococcus* sp. strain PCC 7942. To use luciferase as a reporter to monitor gene expression, bacterial luciferase genes (*luxAB*) were inserted randomly into the *Synechococcus* genome by conjugation with *Escherichia coli* and subsequent homologous recombination. The resulting transformed clones were then screened for bioluminescence using a newly developed cooled-CCD camera system. We screened ~30,000 transformed *Synechococcus* colonies and recovered ~800 clones whose bioluminescence was bright enough to be easily monitored by the screening apparatus. Unexpectedly, the bioluminescence expression patterns of almost all of these 800 colonies clearly manifested circadian rhythmicity. These rhythms exhibited a range of waveforms and amplitudes, and they also showed a variety of phase relationships. We also found bioluminescence rhythms expressed by cyanobacterial colonies in which the luciferase gene set was coupled to the promoters of several known genes. Together, these results indicate that control of gene expression by circadian clocks may be more widespread than expected thus far. Moreover, our results show that screening organisms in which promoterless luciferase genes have been inserted randomly throughout the genome by homologous recombination provides an extremely sensitive method to explore differential gene expression.

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Circadian (daily) rhythms regulate an enormous variety of biological phenomena, including behavior, metabolism, and gene expression (Bünning 1973; Klopstech 1985; Dunlap 1993; Jacobshagen and Johnson 1994). These rhythms allow organisms to efficiently adapt to the day/night alterations of environmental illumination and temperature. As in development, where cell type-specific changes in gene expression allow different tissues to optimize their function, the circadian system programs a daily cycle of gene expression within each cell to optimize its function in a rhythmic environment. In the case of *Neurospora*, the comparison between developmental and circadian processes is not merely an analogy; the circadian clockwork controls the timing of a morphological differentiation, namely, the formation of aerial hyphae and conidiospores (Feldman and Dunlap 1983).

Another example of circadian programming of gene expression is the temporal separation of nitrogen fixation from photosynthesis in some cyanobacteria (Mitsui et al. 1986). These processes are mutually exclusive because the nitrogen-fixing enzyme nitrogenase is inhibited

by the oxygen produced by photosynthesis. Nitrogen-fixing photosynthetic organisms have solved this dilemma in different ways. One solution is the spatial differentiation of specialized nonphotosynthetic cells, called heterocysts, which perform the nitrogen-fixing duties (Tandeau de Marsac and Houmard 1993). The temporal programming strategy, on the other hand, exploits the natural environment's support of photosynthesis only in the daytime. The circadian clock of cyanobacteria that use this latter strategy balances the expression of photosynthesis versus nitrogen fixation genes so that photosynthesis occurs during the day to take advantage of the sunlight, and nitrogen fixation moonlights during the dark period when oxygen levels within the cells are low (Grobelaar et al. 1986; Mitsui et al. 1986; Schneegurt et al. 1994). This temporal programming is accomplished by rhythmic gene expression, because it has been shown that the abundance of nitrogenase mRNA is higher during the night phase in these cyanobacteria (Huang and Chow 1990).

We are interested in how the circadian clock controls gene expression patterns and in ascertaining how widespread this temporal control is. Additionally, we would like to know whether the circadian pacemaker can turn genes on and off during a variety of phases. Other organisms are known to express rhythms of mRNA abundance

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(Kloppstech 1985; Kay and Millar 1992; Jacobshagen and Johnson 1994). In some organisms, subtractive hybridization of mRNAs expressed at different circadian phases has been used to discover previously uncharacterized clock-controlled genes (ccgs). For example, 12 ccgs have been discovered in *Neurospora* by subtractive hybridization, 2 of which have been reported (Loros et al. 1989; J. Loros, pers. comm.). Both of these ccgs appear to be regulated by the circadian clock via transcriptional control (Loros and Dunlap 1991), and one has been identified as a gene involved in the differentiation of conidiospores (Bell-Pederson et al. 1992). In higher plants, subtractive hybridization has revealed two other rhythmically expressed genes, both of which encode RNA-binding proteins (Heintzen et al. 1994).

Bioluminescence emitted by a luciferase reporter can be measured with exquisite sensitivity, providing an *in vivo* alternative to subtractive hybridization for estimating the extent of circadian control over gene expression. We have developed a method for random insertion of a luciferase reporter gene throughout the genome of the cyanobacterium *Synechococcus* sp. strain PCC 7942 with subsequent screening by a cooled-charge-coupled device (cooled-CCD) camera apparatus. Using the *Vibrio harveyi* luciferase genes (*luxAB*) as a reporter for the promoter activity of the *psbAI* gene, we have already shown that this cyanobacterium expresses circadian rhythms of gene expression (Kondo et al. 1993; Kondo and Ishiura 1994). The approach described herein allowed us to identify colonies of cyanobacteria in which the luciferase reporter had inserted into uncharacterized regions of the genome that induced expression of the luciferase and, subsequently, to ascertain whether the nearby promoter/enhancer regions were regulated by the circadian clockwork.

Bioluminescence patterns from clones that carried random luciferase gene insertions showed that circadian control over gene expression in this cyanobacterium is more extensive than we had imagined. More than 800 clones expressed clear rhythms of bioluminescence, and the phasing of their rhythms showed a wide range of phase relationships. That the circadian clock regulates many genes in this organism was also supported by experiments in which the luciferase genes were coupled to the promoters of known *Synechococcus* genes. Therefore, the circadian system in this cyanobacterium exerts widespread control over gene expression with a wide variety of temporal phasing patterns.

Results

Bioluminescence of colonies resulting from random insertion of luxAB

We created a library of *Synechococcus* genomic DNA fragments fused to a promoterless *luxAB* gene set that could be transferred to cyanobacteria by conjugation with *Escherichia coli*. This library was calculated to cover 99.9% of the *Synechococcus* genome (see Materials and methods). These sequences recombined into

Synechococcus by homologous recombination so that the region of DNA surrounding the insertion site is duplicated (Tsinoremas et al. 1994). Therefore, the insertion event should not disrupt the adjacent gene in most cases. Figure 1B shows one petri dish with colonies resulting from the conjugation procedure. Figure 1A is the same plate whose bioluminescence image in complete darkness has been captured with the cooled-CCD camera. The distribution of the bioluminescent colonies on the dish looks like a galaxy visualized through a powerful telescope. Approximately 10% of the clones resulting from conjugation expressed bioluminescence at a level that was detectable with our cooled-CCD camera apparatus (Fig. 1, cf. A and B).

Figure 1A also illustrates dramatically that the bioluminescence of some colonies is very bright, whereas that of other colonies is dim. This heterogeneity is not attributable to differences in colony size (Fig. 1B). Figure 2 shows the bioluminescence of 908 colonies that were recognized by our computerized colony recognition routine (Materials and methods). Most of the bioluminescence in the colonies is dim, with an approximately ex-

Figure 1. Bioluminescence of cyanobacterial clones resulting from conjugation. (A) A plate viewed in complete darkness with the cooled-CCD camera. Bright spots indicate the locations of bioluminescent colonies. (B) The same plate viewed under external illumination to show the location of every colony that has been transformed to a kanamycin-resistant phenotype.

Figure 2. Histogram of the bioluminescence intensity distribution of primary colonies after conjugation. In this experiment, 9600 kanamycin-resistant colonies were screened for bioluminescence, and 908 colonies were found to have bioluminescence that was detectable by the cooled-CCD camera in a 15-min exposure. The intensity of bioluminescence of each of these 908 colonies (1 mm diam.) is plotted here. The abscissa is the bioluminescence intensity in counts captured by the cooled-CCD camera after a 15-min exposure; the ordinate is the number of colonies expressing each intensity (organized into bins of 200 counts).

ponential decline in the number of colonies exhibiting increasingly brighter bioluminescence. This distribution of intensities suggests that the *luxAB* reporter gene set has randomly inserted into many areas throughout the genome near promoter regions, some of which are strongly active (the bright colonies) and others that are relatively weak (the dim colonies). Of ~30,000 colonies initially screened by the cooled-CCD apparatus, the time course of the bioluminescence of ~800 bright colonies and 40 dim colonies was analyzed in greater detail.

Bioluminescence profiles of bright clones

Almost every "bright" colony expressed a clear circadian rhythm of bioluminescence in constant light (LL), although the waveforms of those rhythms were quite variable among different colonies. We classified the waveforms into five separate categories, which are illustrated in Figures 3 and 4. Classes 1, 2, and 3 exhibit approximately a symmetrical sine curve. The distinction between these three classes is based on their average phase: the trough of class 1 rhythms is at approximately circadian time 21–24 (CT 21–24), those of class 2 at approximately CT 11–14, and those of class 3 at approximately CT 17–20 (Figs. 3 and 4). The rhythm of our previous *psbAI::luxAB* construct—named AMC149 (Kondo et al. 1993, 1994)—exhibits a phase relationship that falls into class 1. The waveforms of class 4 rhythms were significantly asymmetric, exhibiting a "sawtooth" pattern (Fig. 3). Finally, we grouped the remaining heterogeneous waveforms into class 5. Two common waveforms in

class 5 are those that are significantly bimodal and those that have "step-like" patterns (Fig. 3).

Figure 4 summarizes the phase relationships of the rhythms in the various classes. Taken together, those categories expressing symmetrical sine curve waveforms (classes 1–3) show that gene expression in *Synechococcus* can display a variety of phases, with preferences for troughs occurring at about CT 11–14 (class 2), CT 17–20 (class 3), and CT 21–24 (class 1). Sawtooth waveforms (class 4, the largest class) primarily have their troughs at about CT 19–23. Class 5 waveforms bracket a range of phases from CT 18–3, but it should be remembered that phase determination for some class 5 rhythms (especially the step-like patterns) is not as accurate as for the other waveforms.

The bottom of Figure 4 summarizes all of the phase data for the bright colonies (total = 789 colonies). Clearly, rhythms can be expressed in a variety of phase relationships, with a strong preference for troughs at the end of the subjective night (CT 18–2) and a weaker preference for troughs in the early subjective night (about CT 10–15). These data demonstrate extensive temporal programming of gene expression in *Synechococcus*.

Figure 3. Representative traces of various classes of rhythmic waveforms expressed by bright colonies in LL. In comparison with Fig. 2, bright colonies are those expressing a level of bioluminescence of 2000 counts or more. In this experiment, the bright colonies were transferred from the primary plates and analyzed in a time-course experiment with the cooled-CCD apparatus. Bioluminescence intensity of a colony (3 mm diam.) is shown on the ordinate in counts of the cooled-CCD camera after a 3-min exposure. The distinctions between the five classes are explained in the text.

Figure 4. Distribution of phases for bioluminescence rhythms expressed by bright colonies in LL. The data are organized such that each panel depicts the phase distribution of one of the various classes of waveforms illustrated in Fig. 3. (Bottom panel) The composite of all the classes. The number of colonies in each class is indicated in each panel. (Abscissa) Phases of troughs of bioluminescence rhythms in CT, where CT 0 is the subjective dawn in LL, and CT 12 is the subjective dusk in LL (the white bar on the abscissa is the subjective day; the shaded bar is the subjective night). (Ordinate) Number of colonies (in bins of 0.5 hr of phase) whose bioluminescence trough is at the indicated phase.

Bioluminescence profiles of dim clones

The temporal pattern of bioluminescence was also checked for some colonies whose light emission was too low to be tracked efficiently in a time-course experiment with our cooled-CCD apparatus. We transferred 40 dim colonies to a photon-counting photomultiplier apparatus that was much more sensitive to low levels of bioluminescence (Materials and methods) and found that they also glowed rhythmically (Fig. 5). Although our analysis of these dim clones was much less extensive than for the bright clones, it appears that the phase of most of the dim clones is like that of the class 1 bright colonies (Fig. 5, A–C). However, one clone showed a rhythm whose phase appears to be class 2 (Fig. 5E), and two clones had a waveform similar to class 4 (one of which is depicted in Fig. 5D).

Confirmation of multiple-site insertions of the *luxAB* gene set

To confirm that the *luxAB* gene set had inserted into many sites throughout the *Synechococcus* genome, DNA was extracted from five class 1 clones. The DNA was digested with *Bam*HI and *Eco*RI, and a Southern blot experiment was performed using *lux* DNA as the probe (Fig. 6). The hybridization patterns were different among the transconjugant strains, indicating that the *luxAB* genes have been introduced into different regions of the genome even within clones of the same phase–relationship category. As the hybridization pattern of each clone is distinct, these data confirm the conclusion that our strategy resulted in the integration of the *luxAB* gene set into many sites throughout the *Synechococcus* genome. Sequence analysis indicated that the insertion site was also different for two randomly chosen class 2 clones (data not shown). However, Southern analysis of a sampling of class 2 clones indicated that one insertion site is

Figure 5. Representative traces of dim colonies. In comparison with Fig. 2, dim colonies are those expressing a level of bioluminescence of <2000 counts on the abscissa in Fig. 2. Bioluminescence was measured as described in Materials and methods. Units of bioluminescence on the ordinate are counts/sec of the photon counter and correspond to 1/30 to 1/100 of units on the ordinate in Fig. 3.

Figure 6. Southern blot analysis to confirm multiple-site insertions of the *luxAB* genes. Genomic DNA from five independent clones belonging to class 1 was extracted and digested with *Bam*HI plus *Eco*RI. Southern blot analysis was performed as described in Materials and methods. These hybridization signals represent DNA from an *Eco*RI site near the start codon of *luxB* to the nearest *Eco*RI or *Bam*HI site upstream of *luxA* in the *Synechococcus* genome. Lane A is DNA from our previous *psbAI::luxAB* strain (AMC149; Kondo et al. 1993), Lanes B, C, D, and E are DNAs from four class 1 recombinant strains. Molecular sizes (in kb) are indicated for the migration of three relevant bands of a standard that was generated by digestion of bacteriophage λ DNA with *Bst*EII.

over-represented, and thus, class 2 clones may be rarer than Figure 4 suggests.

Confirmation of rhythmic gene expression by Northern analysis

We demonstrated previously that one class 1 gene, *psbAI*, shows a rhythm of promoter activity and a rhythm of mRNA abundance (Kondo et al. 1993; Liu et al. 1995), and that *luxAB* functions as an excellent reporter of class 1 promoter activity in *Synechococcus* (Liu et al. 1995). To confirm that *luxAB* functions as a reliable reporter of transcription for genes of other expression classes, we measured the mRNA abundance of a class 2 gene that we have identified (Y. Liu, unpubl.). This gene is *purF*, which encodes the enzyme catalyzing the initial step of the de novo purine nucleotide biosynthesis pathway, amidophosphoribosyltransferase (Markaroff et al. 1983; Ebbola and Zalkin 1987). Although the *purF* message is of low abundance and appears to be unstable, its level oscillates in antiphase to that of the class 1 *psbAI* mRNA (Fig. 7). These data indicate that *luxAB* accurately reports transcriptional activity of genes that are expressed at different phases of the circadian cycle.

Rhythmicity of bioluminescence from *luxAB* fusions to known genes

In addition to the random insertion strategy to identify clock-controlled genes, we also prepared fusions of a promoterless *luxAB* gene set to several promoters of *Synechococcus* genes that had already been identified. We chose three promoters that we expected to express different patterns of expression over the circadian cycle. The first was *psbAIII* (encoding the D1 protein of Photosystem II), whose response to high light is different from that of the class 1-type *psbAI* gene that we have used extensively (Kondo et al. 1993, 1994) and whose promoter has been well characterized (Li and Golden 1993). The second was *glnA* (glutamine synthetase), which is not light regulated (Bustos et al. 1990), and the third was the promoter for the rRNA gene (*rrnA*; Kuman et al. 1986), which we expected to be constitutive.

As shown in Figure 8, constructs of all three genes exhibited class 1 phase and waveform under our screening conditions. These data are consistent with those of the random insertion library; when measured by a sufficiently sensitive method, every promoter that we have tested shows at least a low amplitude rhythm. Additionally, these promoters are from well-characterized genes, so the data in Figure 8 confirm that rhythmic bioluminescence patterns are not a bizarre artifact of the random insertion protocol. Together, these data support the idea of extensive circadian control over gene expression.

Figure 7. Rhythms in LL of mRNA abundance of a class 1 gene (*psbAI*) vs. a class 2 gene (*purF*) in wild-type *Synechococcus*. (A) Northern blots of *psbAI* and *purF* mRNAs as a function of time in LL. (B) Densitometric data of the blots shown in A.

Figure 8. Traces of bioluminescence expressed from promoters of known genes. Bioluminescence was monitored from *Synechococcus* strains that carry *luxAB* gene fusions to the *psbAIII*, *glnA*, or *rrnA* promoters. The *psbAIII* and *glnA* reporter strains were measured as for the dim random library clones, whereas the *rrnA::luxAB* strain was measured by the cooled-CCD apparatus used for the bright clones. Ordinates of *psbAIII* and *glnA* are the same as those in Fig. 5, and *rrnA* ordinates are the same as those in Fig. 3.

Discussion

Extensive circadian modulation of gene expression

This investigation is the first overview of promoter activity in a genome over the circadian time scale. This feat was accomplished by virtue of the high efficiency of our gene transfer protocol via conjugation and by continuous monitoring of thousands of individual colonies by the cooled-CCD camera apparatus. The most striking result is that almost all of the clones that expressed bioluminescence showed a clear circadian rhythm. In addition, on the basis of the bright clones alone, the rhythms showed a variety of phase relationships. The data from the dim clones, although not as extensive as those of the bright clones, suggest that if a sufficiently large number of dim colonies were analyzed, the distribution of waveforms and phases of the dim colonies' rhythms may be similar to that of the bright colonies. These facts plus the results of our Southern blots (see Fig. 6) show that most of the colonies that we have screened are the result of linking the *luxAB* reporter construct to different promoters (see below).

In comparison with the results of searches in other organisms for clock-controlled genes, an unexpected result of our study is that almost every bioluminescent colony expressed a clear rhythmic pattern, and none was definitively arrhythmic. Thus, circadian gene expression in *Synechococcus* is pervasive. The circadian clock in cyanobacteria does not merely control a specialized set of genes, but it dominates the entire metabolism of the cell.

Why do our results differ from those of clock researchers working with eukaryotic organisms? Of course, it is

possible that circadian modulation of gene expression in the prokaryotic cyanobacteria is more extensive than in eukaryotes. On the other hand, perhaps when enough colonies (or individuals) can be screened by a method of high sensitivity in intact cells of other organisms, circadian clock control will be found to be widespread. Consistent with this idea is the observation that some genes in eukaryotic organisms are constitutively expressed under some conditions but rhythmically expressed under other conditions, for example, the cytochrome *c* gene in *Chlamydomonas* (Jacobshagen and Johnson 1994). Another possible explanation is that our screen was for rhythmic promoter activity, not rhythmic mRNA abundance; perhaps clock control of transcription is widespread, but post-transcriptional effects sometimes mask rhythmic promoter activation. A support for this interpretation comes from experiments with one of the *cab* genes of *Arabidopsis*, which shows no rhythm of mRNA abundance, but transcriptional fusions of its promoter to a reporter gene indicate that the promoter is rhythmically activated by the circadian clock (Millar and Kay 1991).

Although it is clear that we have tagged many different genes in our screening, it is difficult to make an exact estimate of the number of genes that have been assayed. The library of *Synechococcus* DNA fragments generated by *Sau3AI* cleavage was calculated to cover 99.9% of the genome (Materials and methods). The conjugation method that we used is very efficient (Tsinoremas et al. 1994), and the genome of *Synechococcus* sp. PCC 7942 is small (2.7 Mb; M. Sugiura, pers. comm.), so the coverage of the genome theoretically should be excellent. Our Southern blot data suggest that many of the insertions of the *luxAB* gene set are unique, which supports the conclusion that most of the rhythmically bioluminescent colonies are the result of insertions into different genomic loci.

There are several reasons why our coverage of the genome is not likely to equal the theoretical 99.9%, however. For example, some *Synechococcus* DNA segments could carry a coding sequence for a protein that interferes with *E. coli* metabolism, so that such segments might have been lost during propagation in *E. coli*. Also, insertion of the antibiotic-resistance sequence, a random fragment of *Synechococcus* DNA, and the *luxAB* gene set may disrupt gene expression in some locations in the *Synechococcus* genome. Nevertheless, our calculations plus (1) the heterogeneity of Southern blot patterns of both the *E. coli* library cells and the target *Synechococcus* clones (Fig. 6), and (2) the large range of bioluminescence patterns expressed by the bright colonies indicates that a large enough proportion of the genes in *Synechococcus* has been tagged to make a statistically valid statement about the extensive influence of the circadian clock over gene expression in this cyanobacterium.

Phased gene expression

Class 1 colonies express a rhythm whose phase is very close to that of our previously constructed *psbAI::luxAB*

reporter strain (named AMC149), in which the bioluminescence increases progressively during the subjective day and peaks near subjective dusk, with a corresponding trough near subjective dawn (Kondo et al. 1993). The phasing displayed by classes 1, 3, 4, and 5 genes covers a broad but similar range (although assigning a definite phase to the variable waveforms of some of the class 5 rhythms is difficult). Therefore, up to 85% of the bright colonies oscillated with this phase relationship, as did most of the dim clones we tested. Because many clones oscillated with this approximate phasing, perhaps this range is a kind of “default” phase of rhythmic gene expression. If this phase is maintained in a light/dark cycle, then these genes would be expected to be most active in the daytime. Under environmental light/dark cycle conditions, the energy metabolism of these cells should be most active during the day in this photoautotrophic organism, so it is not surprising that many rhythmic genes should exhibit this phase relationship. Additional support for a default phase of gene expression is provided by studies in which we fused promoters with no additional *cis*-acting elements to the *luxAB* gene set and observed circadian rhythms with class 1 patterns (data not shown).

Because the daily cycle of sunlight is crucially important to cyanobacteria, we would predict that some genes might be controlled by the circadian clock to anticipate dawn, thereby maximizing the harvesting of light energy. Class 2 clones (15% of the total screened) apparently reflect genes of precisely this type: Their rhythms of bioluminescence peak in early subjective day (trough in early subjective night). The bioluminescence of class 3 rhythms peaks in the mid-subjective day (trough in mid-

subjective night). Class 3 colonies numbered 6% of the total colonies screened. Our data support the hypothesis that efficient temporal organization in cyanobacteria can be achieved by programming some genes to be expressed at a variety of phases ranging from late subjective night throughout the subjective day under constant conditions.

An interesting result was that no or very few colonies expressed a rhythm with a minimum phase in the first three-quarters of the subjective day (CT 3–9), which would correspond to peak activity in the first three-quarters of the subjective night (CT 12–21). For an autotrophic photosynthetic organism, it is possibly significant that gene expression is relatively quiescent during the phase of the daily cycle in which light energy is unavailable. Because we screened a large number of clones, the absence of rhythms with this phase probably reflects a true “silent” phase and is not an artifact of having missed such colonies.

Control of gene expression by the circadian clock

Because we observed rhythms of gene expression that show a variety of phase relationships, some of these genes are likely to be directly regulated by the clock via *cis*-acting elements and *trans*-acting factors that coordinately regulate the expression of single genes or groups of genes (Fig. 9). In the simplest version of this scenario, there would be one (or a few) types of class 1-specific *cis*-acting elements turned on by a class 1-specific *trans*-acting factor expressed by the clock during the day. A different set of class 2-specific *cis*-acting elements would be turned on by a class 2-specific *trans*-acting factor that the circadian oscillator induces at night, and so forth.

Figure 9. Model for circadian control of gene expression in *Synechococcus*. Coding regions of genes are depicted as rectangles; adjacent promoter regions are depicted as boxes with arrowheads. Sine curves illustrate the phasing of various overt rhythms (far right) and rhythms of *trans*-acting factor/nonspecific factor activity (center). The circle (left) is the central circadian clockwork. Some ensembles of genes may be controlled by rhythmic activity of specific *trans*-acting factors that coordinately regulate groups of genes (top two diagrams). These *trans*-acting factors could be activated at different specific phases (note the difference in phase between activity rhythms of *trans*-acting factors A and B). Promoters responding to specific *trans*-acting factors may be regulated coordinately, e.g., open-box promoters respond to *trans*-acting factor A, closed-box promoters respond to *trans*-acting factor B. Other genes, especially those in the default phase, may be regulated by global, but nonspecific factors that oscillate, as illustrated in the bottom diagram. In the latter case, promoter regions of the regulated genes may vary but still be rhythmically activated by nonspecific global factors.

Because of the large number of genes that are apparently influenced by the clock in cyanobacteria, however, it seems unlikely that each of them is controlled by a specific regulatory factor. Some global factors could be involved as well. For example, because the energy status of these cells is dependent on photosynthesis, energy charge will certainly oscillate during the daily cycle, and this could influence the transcriptional rate of many genes, perhaps resulting in a waveform with the default phase as depicted in Figure 9. Another candidate for non-specific control could be the circadian control of a global event, such as DNA replication. If the rate of DNA replication undergoes circadian modulation, then overall transcription rates could also be affected (Theisen et al. 1993). Another global but nonspecific factor might be the status of the supercoiling of the chromosome; in *Chlamydomonas*, the supercoiling of the chloroplast's chromosome is influenced by light/dark conditions (Thompson and Mosig 1990). On the other hand, bioluminescence rhythms that are the result of such a non-specific factor would not be expected to display a variety of phase relationships and waveforms. Therefore, the data are best explained by a model that incorporates both nonspecific circadian control and circadian regulation by specific *trans*-acting factors.

Further analyses of rhythmic colonies in *Synechococcus*

An important question about the organization of circadian systems is the elucidation of the mechanism by which the clock controls rhythmic gene expression. The different phasic classes of rhythmic gene expression revealed by our screening indicates that there are at least several independent regulatory signals issued by the clock. Presently, we do not know the identities of most of the genes that are linked to the clock.

Reporter constructs provide a powerful tool to answer this question. Because the insertion sites are tagged with the *luxAB*, kanamycin-resistance, and pBR322 sequences, the adjacent regions of *Synechococcus* DNA can be recovered in *E. coli* as a kanamycin-resistance plasmid and sequenced (Tsinoremas et al. 1994). This procedure can identify the relevant gene and promoter. As mentioned above (and see Fig. 7), we have identified one of the class 2 genes by this technique and found it to be the *purF* gene encoding the rate-limiting enzyme of the purine nucleotide biosynthetic pathway, amidophosphoribosyltransferase (Markaroff et al. 1983; Ebbole and Zalkin 1987). We plan to carry out this rescue procedure for other representative clones of each class to better understand circadian orchestration of gene expression.

Random insertion of promoterless luciferase genes, followed by screening of bioluminescence patterns, is an elegant and sensitive method of screening for differential gene expression in general. This method is particularly applicable to studies of development and responses to environmental stress (Wolk et al. 1991). By using a sensitive cooled-CCD camera, both temporal and spatial patterns of gene expression can be detected.

Materials and methods

Strain and growth conditions

Wild-type *Synechococcus* sp. strain PCC 7942 was cultured in modified (Bustos and Golden 1991) BG-11 medium (Allen 1968) under constant illumination [50–70 $\mu\text{E}/\text{m}^2$ per sec (E is the energy of 1 mole of photons)] at 30°C. Agar plates (1.5%) with modified BG-11 medium plus 5% Luria broth (vol/vol) were used for conjugation (Tsinoremas et al. 1994).

Construction of the DNA library and transfer to *Synechococcus*

A 2.8-kb *Sma*I fragment of plasmid pAM1040 (C.A. Strayer and S.S. Golden, unpubl., based on pLAV1; Chlumsky 1991), which carries the promoterless carboxy-terminal coding region of *luxD* and the intact *luxA* and *luxB* genes, was isolated and inserted into the *Eco*RV site of the mobilizable vector pAM1153 (a modified pBR322 plasmid that encodes kanamycin resistance; Tsinoremas et al. 1994). The resulting plasmid, pAM1224, in which the *luxAB* and kanamycin-resistance genes have the same orientation, was linearized upstream of *luxA* by digestion with *Bgl*II and dephosphorylated with calf intestinal phosphatase (Promega). Genomic DNA from *Synechococcus* sp. strain PCC 7942 was isolated as described by Bustos et al. (1990), partially digested with *Sau*3AI, and separated by electrophoresis on 1% agarose gels (Sambrook et al. 1989). Genomic DNA fragments of 1.5–4 kb were isolated from the gels and purified. Approximately 100 ng each of the pooled DNA fragments and the *Bgl*II-digested/dephosphorylated pAM1224 was mixed and treated with T4 DNA ligase. An aliquot of the ligation mixture was introduced into *E. coli* DH10B cells by electroporation (Cell Porator, BRL) and kanamycin-resistant *E. coli* colonies were selected on agar plates. DNA was prepared from >50 randomly chosen *E. coli* transformants. Digestion with *Eco*RI confirmed that ~85–90% of the transformants contained plasmids with random restriction patterns (data not shown). These data suggest that the inserts were random samples of the *Synechococcus* genome.

The remainder of the ligation mixture was introduced into *E. coli* strain AM179, which contains the conjugation helper plasmid pRL528 and is chloramphenicol resistant (Elhai and Wolk 1988). More than 10,000 transformants resistant to kanamycin (50 $\mu\text{g}/\text{ml}$) and chloramphenicol (170 $\mu\text{g}/\text{ml}$) were collected by washing plates with 4 ml of antibiotic-containing LB medium to form a chromosomal DNA:*luxAB* library. The library was stored in aliquots in the presence of 15% glycerol at –70°C until needed for conjugation. The genome size of *Synechococcus* is ~2.7 Mb, and the average size of the inserts was ~2.7 kb; statistically, therefore, this library should contain >99.9% of the *Synechococcus* genome.

Transfer of this library into *Synechococcus* cells was achieved by triparental conjugal mating (Elhai and Wolk 1988; Tsinoremas et al. 1994). The cells were spread on agar plates containing BG-11 plus 5% LB (vol/vol). After incubating overnight under LL (~60–70 $\mu\text{E}/\text{m}^2$ per sec) at 30°C, the plates were underlaid with kanamycin (10 $\mu\text{g}/\text{ml}$, final concentration). After ~2 weeks, >20,000 kanamycin-resistant colonies were collected by washing the plates with BG-11 medium containing kanamycin. The cells were replated on BG-11 plates, allowed to develop kanamycin-resistant colonies, and screened for bioluminescence as described below. This replating procedure excluded transconjugants whose growth was slowed by the insertion. Because the average size of the insert is ~2.7 kb and the insertion results in a duplication after a single homologous recombina-

tion (Fig. 10), in most cases the endogenous genes should not be interrupted by the insertion of the DNA::luxAB constructs.

Screening method

Transformed cells (~2000 cells) were spread on 9-cm plates of BG-11 agar medium. After incubation in LL at 26°C, the bioluminescence of plates was captured by a cooled-CCD detector (TE/CCD512BKS, Princeton Instruments) with an exposure of 15 or 20 min. Selected bright bioluminescent colonies were transferred as single colonies to new plates with sterile toothpicks. After incubation for 4–5 days in LL and a 12-hr dark exposure to synchronize the circadian clocks in the various colonies, bioluminescence of colonies was monitored continuously by an automated monitoring system (Kondo et al. 1994) in LL for 4–5 days. The position of the trough of the rhythm was computed by a regression procedure similar to that used for the rhythm of *Chlamydomonas* (Kondo et al. 1991). The positions of the first troughs were estimated by extrapolation of times of troughs in LL and expressed in CT scale. CT 0 is the subjective dawn in LL, and CT 12 is the subjective dusk in LL.

Selected dim bioluminescent colonies were transferred to 1-cm² patches on new plates. After incubation for 4–5 days in LL and a 12-hr dark exposure, each patch was transferred to a 20-ml scintillation vial. The bioluminescence from the vials was measured by an automated photomultiplier tube apparatus described previously (Kondo et al. 1993), except that the sensitivity was increased by routing the output to a photon counter. A low-noise photomultiplier (Hamamatsu R2693P) was used at 1000 V, and this signal was processed by a photon-counting unit (Hamamatsu C3866) that removes thermal noise by discrimination of peak heights (signal at peak heights of 0.2 V or above). Signal pulses from the C3866 were detected by an electronic counter (Iwatsu SC-7201). Because of (1) the high gain of the R2693P photomultiplier tube, and (2) the discrimination between thermal noise and signal afforded by the photon counter, the effective sensitivity of light detection was 30–100 times greater than the previous analog system reported by Kondo et al. (1993).

Figure 10. Integration of the random *luxAB* gene library into the *Synechococcus* chromosome. The chromosomal DNA::*luxAB* library was introduced into *Synechococcus* by conjugation. After a single homologous recombination, the DNA inserts in the library are duplicated as shown and the *luxAB* genes are inserted into the genome. The 1.5- to 4.0-kb fragments of the *Synechococcus* chromosome are indicated by the solid bars. The hatched bar denotes the homologous fragment in the wild-type *Synechococcus* chromosome. (Km^r) The kanamycin-resistance gene; (P) The promoter of a hypothetical operon; (a,b,c,d) different regions of that operon.

Southern blotting analysis of DNA from different *Synechococcus* clones

Minipreps of total DNA (Bustos et al. 1990) were prepared from randomly chosen bioluminescent clones from class 1. Each sample (~250 ng) was digested with *Bam*HI plus *Eco*RI and subjected to electrophoresis on 1% agarose gels. DNA was transferred onto nylon membranes (Hybond-N, Amersham; Sambrook et al. 1989). An *Eco*RI fragment of pAM1040 that includes all of *luxA* and 32 bp of *luxB* was labeled with [α -³²P]dCTP by the random primer method (DECAprime DNA labeling kit from Ambion, Inc.) and used as a probe.

Northern blotting analysis

For the experiment depicted in Figure 7, wild-type cyanobacteria were grown at 30°C in LL to an approximate cell density of OD₇₅₀ = 0.5 and given a 12-hr exposure to darkness to synchronize the cells. Beginning 6 hr after the reinitiation of LL conditions, 40-ml samples were collected every 6 hr for 54 hr by centrifugation, frozen quickly in liquid nitrogen, and stored at -70°C. After all samples had been collected, Northern blots of total RNA were prepared as described previously (Liu et al. 1995). The membrane was first hybridized with a radioactive probe for *psbAI* mRNA (Liu et al. 1995), and the blot was then stripped of *psbAI* probe and hybridized with a radioactive probe for *purF* mRNA. The *purF* probe was an antisense RNA that specifically recognizes the coding region of the *purF* gene, and it was labeled with [α -³²P]UTP by using an in vitro transcription kit (Ambion, Inc.). A computerized densitometer (Technology Resources MC1000) was used to quantify the autoradiographs of the Northern blots.

Fusion of *luxAB* to promoters of known genes

A promoter assay vector (pAM1293) was constructed in which promoter-bearing fragments can be inserted upstream of the promoterless *luxAB* gene set and targeted to a neutral site of the *Synechococcus* genome (Bustos and Golden 1992). A 2.8-kb *Sma*I-*Pvu*II fragment containing promoterless *luxAB* (from pAM1040, see above) was inserted into the *Sma*I site of pAM854 (Bustos and Golden 1991). Promoter fragments were inserted into a unique *Sma*I site upstream of the *luxA* gene. The *psbAIII* promoter/enhancer fragment (-38 to +39; Li et al. 1993) was generated by PCR, as was a 195-bp fragment containing the *rnaA* promoter (-142 to +53; Kumano et al. 1986). The *glnA* promoter was excised from pSG2.4 [a gift from S.J. Robinson, University of Oregon, Eugene], as a 2.4-kb *Hind*III-*Eco*RI fragment, which has its downstream end at +541. The promoter::*luxAB* plasmids were used to transform *Synechococcus* to spectinomycin resistance, transferring the reporter genes to the neutral site (Bustos and Golden 1991).

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