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Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria

(biological clocks/bioluminescence/*Synechococcus*)

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ABSTRACT **We have used a luciferase reporter gene and continuous automated monitoring of bioluminescence to demonstrate unequivocally that cyanobacteria exhibit circadian behaviors that are fundamentally the same as circadian rhythms of eukaryotes. We also show that these rhythms can be studied by molecular methods in *Synechococcus* sp. PCC7942, a strain for which genetic transformation is well established. A promoterless segment of the *Vibrio harveyi* luciferase structural genes (*luxAB*) was introduced downstream of the promoter for the *Synechococcus psbAI* gene, which encodes a photosystem II protein. This reporter construction was recombined into the *Synechococcus* chromosome, and bioluminescence was monitored under conditions of constant illumination following entrainment to light and dark cycles. The reporter strain, AMC149, expressed a rhythm of bioluminescence which satisfies the criteria of circadian rhythms: persistence in constant conditions, phase resetting by light/dark signals, and temperature compensation of the period. Rhythmic changes in levels of the native *psbAI* message following light/dark entrainment supported the reporter data. The behavior of this prokaryote disproves the dogma that circadian mechanisms must be based on eukaryotic cellular organization. Moreover, the cyanobacterial strain described here provides an efficient experimental system for molecular analysis of the circadian clock.**

Despite decades of study, the biochemical mechanism of circadian clocks remains a mystery. Circadian rhythms have been found in a wide spectrum of organisms (1) but, until recently, only in eukaryotes (2, 3). In the last few years circadian rhythms have been reported in the prokaryotic cyanobacteria (4–7). Unfortunately, these studies employed genetically intractable cyanobacterial strains and laborious assays to detect the rhythms. These difficulties have impeded the demonstration that the prokaryotic rhythms are equivalent to the circadian rhythms of eukaryotes.

Proof that prokaryotes have circadian pacemakers has threefold significance. (*i*) With regard to the evolutionary emergence of circadian behavior: Can the simpler organization of prokaryotes support a circadian mechanism? Is circadian behavior adaptive for prokaryotic niches as well as for eukaryotic niches? (*ii*) The previous failure of attempts to discover circadian clocks in prokaryotes has led to a "eukaryotes-only" dogma which limited the types of models that have been considered for the underlying clock mechanism (3). Now that prokaryotic cellular organization appears to be fully competent to generate circadian oscillations, a broader range of mechanisms can be seriously evaluated as candidates for the circadian pacemaker. (*iii*) The realization that prokaryotes express circadian behavior is significant from the perspective of designing an optimal strategy to discover the hitherto elusive secret of the circadian mechanism. That is, if prokaryotes display the phenomenon, then progress in elucidating its basis will probably be most rapid while using an appropriately chosen prokaryotic model; in prokaryotes, the mechanism itself may be simpler, and the average size of prokaryotic genomes, which is smaller than that of eukaryotic genomes, facilitates the goal of saturation mutagenesis for clock-related genes.

To reap the strategic benefits that prokaryotes offer, however, we must identify an organism which is amenable to molecular and genetic analysis and which exhibits circadian rhythms of a parameter that can be assayed continuously for many cycles by an automated system. We have developed such a strain by transforming a genetically tractable cyanobacterium, *Synechococcus* sp. strain PCC7942, with bacterial luciferase genes which function as a reporter of clock-controlled expression of the endogenous *psbAI* gene. The bioluminescence rhythm expressed by this reporter strain, called AMC149, is easily assayed by an automated monitoring system. The rhythm of AMC149 satisfies all three criteria of circadian rhythms (1, 2, 8, 9): persistence under constant conditions, entrainability by light/dark signals, and temperature compensation of the period.

MATERIALS AND METHODS

Strains and Growth Conditions. Wild-type *Synechococcus* sp. strain PCC7942 was grown in BG-11 medium (10) as modified by Bustos and Golden (11). Strain AMC149, which contains a modified Ω cassette (12), was cultured in the presence of spectinomycin sulfate (40 μg/ml). AMC149 was generated by transformation of wild-type *Synechococcus* with plasmid pAM977.

Construction of the Reporter Plasmid and Strain AMC149. Most restriction and modifying enzymes were purchased from Boehringer Mannheim and used as directed by the manufacturer. *Escherichia coli* strain DH5αMCR (Bethesda Research Laboratories) was the host for all plasmids. Transformants were propagated on LB (13) or Terrific Broth (14) medium in the presence of antibiotics at standard concentrations (13).

A 2.1-kb *Hin*dIII–*Sal* I fragment from pDH140 (15) containing upstream flanking sequences, the promoter (P_{psbAI}), and the amino-terminal coding region of *psbAI* (15) was ligated into *Hin*dIII/*Sal* I-cleaved pLAV1, which contains *luxAB*. Plasmid pLAV1 was constructed by L. Chlumsky as a derivative of pTB7 (16) (gift of T. Baldwin). The P_{psbAI}::*luxAB* fusion was excised from the resulting plasmid,

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Abbreviations: LL, constant illumination; LD, light/dark.

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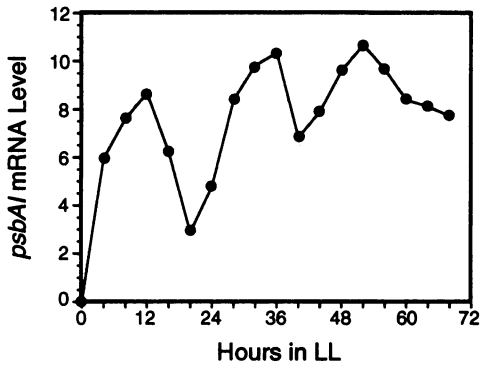

FIG. 1. Rhythmic abundance of *psbAI* message following entrainment to a LD cycle. Stationary-phase cells were entrained to a 12 hr/12 hr LD cycle as described in *Materials and Methods*. Aliquots were removed for RNA isolation at the given time points after release into LL. RNA samples (5 μg each) were subjected to electrophoresis, blotted onto a nylon membrane, and probed with a radiolabeled antisense RNA that recognizes the *psbAI* transcript. Band intensities from an autoradiogram were determined by a densitometer (Bio-Rad 620) and these values in arbitrary units were plotted versus time.

pAM976, as a 2.8-kb *Pvu* II fragment (having ≈150 bp upstream of P_{psbAI}); it was inserted into the *Synechococcus* neutral-site vector, pAM854 (11), at a *Sma* I site upstream of the Ω cassette, to produce pAM977.

Wild-type *Synechococcus* was transformed by pAM977, yielding spectinomycin-resistant colonies. These transformants carried the P_{psbAI}::*luxAB* fusion in the neutral site of the chromosome, as a result of homologous recombination, and lacked vector sequences. Southern analysis confirmed that the recombination event had occurred as predicted (data not shown).

Northern Blot Analysis of RNA from Light/Dark-Cycle-Entrained Cells. Wild-type *Synechococcus* cells were grown to stationary phase in modified BG-11 medium bubbled with 1% CO_2 in air under conditions of constant illumination (≈250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ outside of the carboy). The culture was transferred to a 30°C constant-temperature chamber, where it was stirred and provided with constant illumination for two more days (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ outside of the carboy). The light regime was then adjusted to 12 hr of light at 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ followed by 12 hr of darkness for five light/dark (LD) cycles. Samples were removed for RNA extraction at specific times. RNA was isolated and transferred to nylon membranes as described (17) and then probed with a gene-specific antisense RNA that recognizes the *psbAI* message (18).

Assay of the Bioluminescence Rhythm. The transformed strain AMC149 was grown in a 12 hr/12 hr LD cycle with continuous shaking (100 rpm). The light fluence rate was 46 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during illuminated periods and temperature was maintained at 30°C. When the culture reached stationary phase (≈10^9 cells per ml), 5 ml of cell suspension was transferred to a 20-ml vial. An open, sterile microcentrifuge tube containing *n*-decanal dissolved in dimethyl sulfoxide was placed upright in the vial. The vial was sealed to allow the volatile *n*-decanal to escape into the gas phase at a constant limited rate; then it was placed in front of a photomultiplier tube (Hamamatsu 931B) without shaking. The sample and photomultiplier detector were enclosed in a light-tight box. A microcomputer controlled the box to open for 25 min to illuminate the culture at 46 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and then to close for 5 min. Bioluminescence from the cells was measured during the 5-min dark period (2 min of darkness to allow chlorophyll fluorescence to decay, then two 1.5-min measurements of bioluminescence). Usually measurements were started 12 hr after the transfer of culture to the vial, to avoid a period of transient changes in physiological state. Measurements were repeated every 30 min for 1 week under continuous illumination (LL) with 5-min dark pulses as described above. Brief, repetitive dark pulses can have minor effects on circadian clocks but do not cause major interference (19). Output of the photomultiplier tube was amplified electronically and read by a microcomputer through an analog-to-digital converter. The reading was averaged over the measurement period and plotted on the ordinate in millivolts as "Bioluminescence." One unit (1 mV) roughly corresponds to a light emission of 10^5 photons per second from the vial. For the experiment shown in Fig. 4 we used a multichannel measuring assembly in which a housing that contained the photomultiplier tube moved along a guide rail to measure bioluminescence sequentially from in-line-placed vials (19). The measuring protocol was the same as for the single-channel system. Both measuring assemblies were placed in a temperature-controlled chamber to maintain constant temperature of the vials.

RESULTS AND DISCUSSION

A previous study suggested that photosynthetic activity is rhythmic in cyanobacteria (5). We reasoned that photosynthesis genes might be regulated by a circadian clock to achieve efficient photosynthesis. *Synechococcus* cells were grown to stationary phase, entrained to a LD cycle, released into constant light (LL), and sampled for RNA extraction. Northern (RNA) blot experiments indicated that the *Synechococcus psbAI* message exhibits rhythmic changes in abundance in constant conditions (Fig. 1). This message encodes D1, a major protein component of the photosystem II reaction center.

We capitalized upon the observation by fusing this gene's promoter to a reporter gene encoding bacterial luciferase. We chose this reporter because its activity (bioluminescence) can be assayed repeatedly without perturbing the cells, and its hydrophobic and volatile substrate, *n*-decanal, easily diffuses into *Synechococcus* cells at a constant rate when cultures are exposed to *n*-decanal vapor. Bioluminescence is a convenient parameter to monitor as an overt rhythm for circadian oscillators, as has been demonstrated in the naturally bioluminescent dinoflagellate *Gonyaulax* (8). We fused a promoterless segment of the wild-type *Vibrio harveyi* luciferase

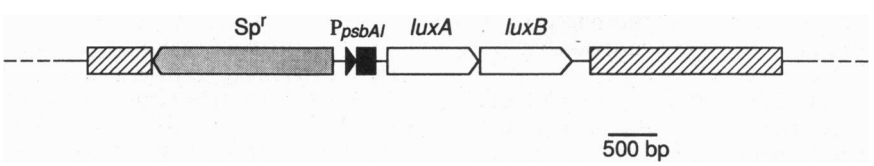

FIG. 2. Schematic representation of reporter vector pAM977, which carries a P_{psbAI}::*luxAB* transcriptional fusion. Arrows represent the coding regions of the spectinomycin/streptomycin-resistance gene (Sp^r) and the *luxAB* genes and indicate the direction of transcription for each. The *psbAI* promoter is designated as P_{psbAI} (filled triangle), and it is followed by a segment of *psbAI* amino-terminal coding region (filled rectangle) which is fused out-of-frame with *luxA*. Hatched boxes represent *Synechococcus* DNA sequences in the vector that target insertion of the cloned sequences to a locus termed a neutral site on the cyanobacterial chromosome (12). The pBR328 base of the vector is not shown, because it is not transferred to the *Synechococcus* chromosome during transformation.

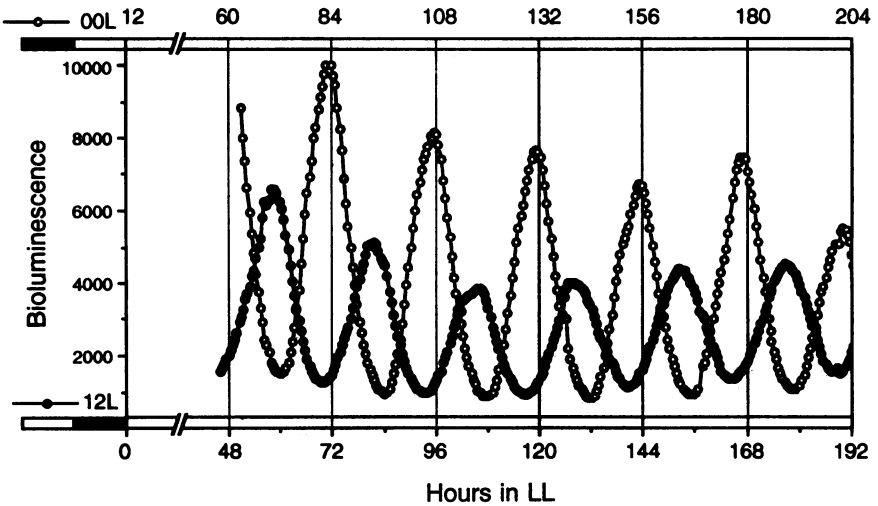

FIG. 3. Circadian rhythm of bioluminescence in continuous light conditions. The transformed strain AMC149 was cultured at 30°C under a 12 hr/12 hr LD cycle (46 μmol·m^{-2}·s^{-1}) with continuous shaking (100 rpm) and then transferred to vials for measurement of bioluminescence. The two traces are from cultures that were previously entrained to LD cycles which were 12 hr out of phase. The last LD cycles preceding LL are illustrated on the abscissa (open bar, light period; filled bar, dark period).

operon (*luxAB*) to the promoter of the *Synechococcus psbAI* gene (Fig. 2). The reporter construct was introduced into *Synechococcus* on a vector that targets cloned DNA to a nonessential region (termed a neutral site) on the chromosome (12). The resulting transformed strain was designated AMC149.

Fig. 3 shows the time course of bioluminescence in LL measured concurrently from two AMC149 cultures which had been entrained previously to LD cycles that were 12 hr out of phase. Under constant conditions, the bioluminescence from both cultures oscillated with a period of ≈24 hr, but with opposite phases. These results indicate that the bioluminescence rhythm is an expression of an endogenous clock within the cells and that the phase of the rhythm is determined by the LD cycle which precedes the continuous conditions. The peak of bioluminescence occurred 12 hr after the onset of the LL and then at 24 hr intervals. Addition of a photosystem II inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (1 μM), or an inhibitor of bacterial translation, chloramphenicol (10 μM), abolished the rhythm. However, the eukaryotic translation inhibitor cycloheximide (35 μM) had no effect. This indicates that the rhythm originated with the cyanobacterium and was not contributed by a signal from a contaminating eukaryotic organism.

Single light or dark signals can reset the phase of circadian rhythms to a new phase, and the direction of the phase shift depends upon the phase at which the signal is administered (1, 2). This characteristic is crucial for the circadian clock to entrain its cellular activities to the solar LD cycle (9). Fig. 4 depicts the resetting of the bioluminescence rhythm in AMC149 by a single dark pulse: 4-hr dark pulses administered to cultures maintained otherwise in LL shifted the phases of the rhythms. The direction and magnitude of the resulting phase shift were a function of the phase of the rhythm at which the dark pulse was given; variation between the phases of control cultures was <1 hr.

Another criterion of circadian rhythms is that their period length is approximately constant at different ambient temperatures, a phenomenon known as temperature compensation of the period (1, 2). Fig. 5 shows that the bioluminescence rhythm of AMC149 meets this criterion: period length was 25.5 hr at 25°C, 24 hr at 30°C, and 23 hr at 36°C. The calculated Q_{10} value for frequency (1/period) of this rhythm is 1.1. This value lies within the range for the temperature dependence of circadian rhythms in eukaryotes and is far from that of most biological processes, such as growth or respiration, for which the Q_{10} values are usually between 2 and 3. Temperature compensation of the amino acid uptake rhythm has been reported for another cyanobacterium, *Synechococcus* RF-1 (7). Therefore, the rhythms of *Synechococcus* satisfy the three fundamental criteria for circadian rhythms.

Does the rhythm of bioluminescence in AMC149 prove that this promoter is under circadian control? Not necessarily. Because bacterial luciferase catalyzes the following reaction,

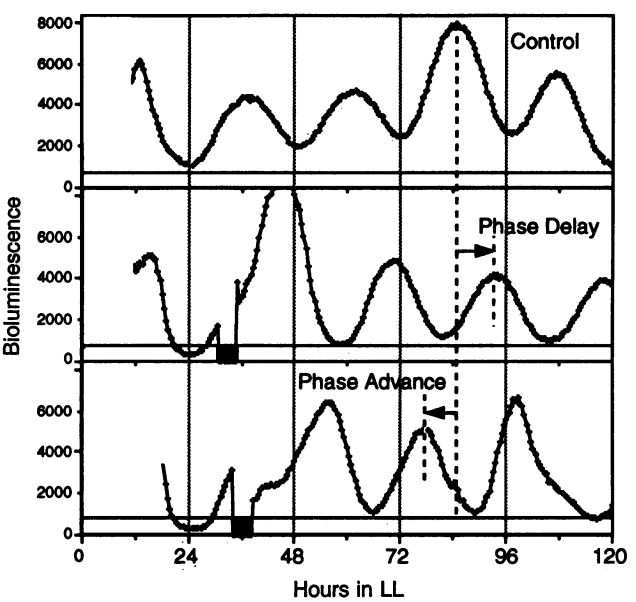

FIG. 4. Resetting of the bioluminescence rhythm by dark pulses. Three cultures were entrained to 12 hr/12 hr LD cycles. At time 0, each culture was released into LL (open bar). A single dark pulse of 4 hr (black bar) was given to the second and third cultures at the times indicated (for the middle panel, beginning 30 hr after the onset of LL; for the bottom panel, beginning 34 hr after LL onset). After the pulse, the cells were returned to LL for the duration of the experiment. The dark pulses "delayed" the phase of the rhythm by 8 hr (middle) or "advanced" it by 6 hr (bottom). Assignment of phase shifts as "advances" or "delays" is a convenient designation, although it is arbitrary in most cases (9). Experimental procedures and presentation of results are the same as in Fig. 3.

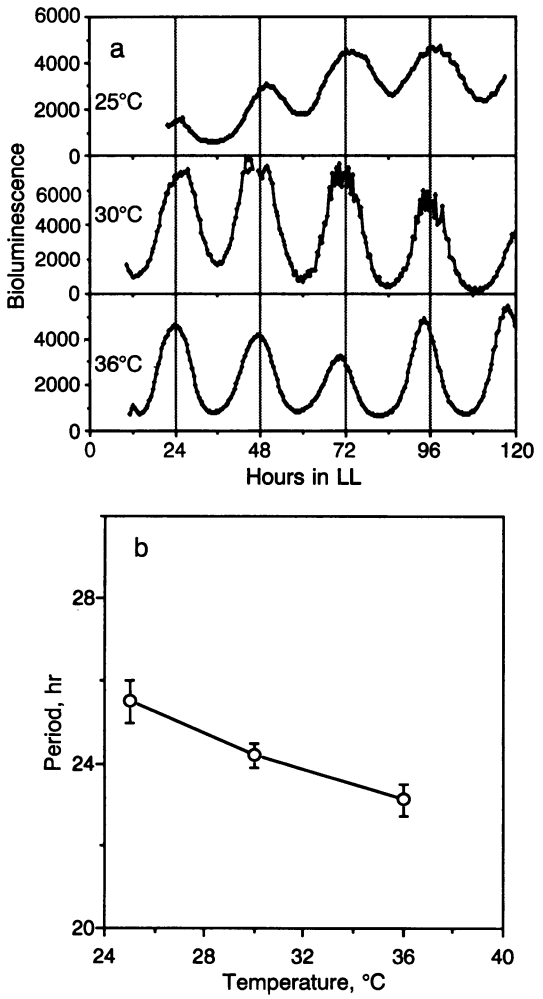

FIG. 5. Temperature compensation of the period. Experimental procedures are the same as in Fig. 3, except that the bioluminescence monitored at three temperatures is shown. (*a*) Rhythms of bioluminescence. (*b*) Periods of the rhythm vs. temperature (bars indicate standard deviation of three or four replicates).

$FMNH_2$ + *n*-decanal + $O_2 \rightarrow$ FMN + *n*-decanoic acid + H_2O + light, bioluminescence can be changed not only by changes in the intracellular level of luciferase but also by the levels of substrates. Since *n*-decanal was supplied exogenously, its effective level should be constant, but the levels of $FMNH_2$ and O_2 are unknown. To address this question, we extracted luciferase from cells cultured in LL and measured the activity *in vitro*. The activity of luciferase at the phase of maximum bioluminescence (hour 60 of LL) was 2.5 times higher than that at the minimum phase (hour 48 of LL). Note that *psbAI* mRNA levels in nontransformed cells also changed rhythmically in LL (Fig. 1). The peaks of the rhythm in mRNA level occurred at approximately the same times in LL as in the bioluminescence rhythms, that is, at hour 12, 36, and so on. Therefore, although levels of $FMNH_2$ and O_2 could be changed by photosynthesis or respiration, it is most likely that the bioluminescence rhythm exhibited by AMC149 is due to rhythmic changes in the intracellular level of luciferase which is caused by a circadian regulation of the *psbAI* promoter. Since the D1 protein plays a critical role in electron transfer in photosystem II, circadian regulation of the *psbAI* gene may be important in the genetic regulation of photosynthesis.

We report here that *Synechococcus* has a circadian clock whose fundamental characteristics are the same as those exhibited by eukaryotes. The presence of a circadian clock in cyanobacteria suggests that circadian mechanisms evolved much earlier than has been previously assumed: the first circadian system may have evolved before the branching of eubacteria and eukaryotes in the universal phylogenetic tree (20).

The molecular mechanism of circadian behavior can now be dissected in a prokaryote by using luciferase as a reporter by which to continuously monitor the rhythm. A recent report demonstrates that firefly luciferase can be used to assay circadian rhythmicity in *Arabidopsis* (21). The advantage of using bacterial luciferase in *Synechococcus* is that cells can be continuously exposed to decanal vapor without any perturbation, whereas firefly luciferase requires periodic administration with fresh luciferin substrate. Therefore, using a bacterial luciferase reporter allows us to automatically assay the bioluminescence rhythm of an undisturbed culture at a high enough frequency to gauge the waveform of a circadian output with high precision. Furthermore, because *Synechococcus* is a unicellular prokaryote with well-developed gene transfer techniques (22), its clockwork should be simpler to analyze than that of a multicellular eukaryote. Automated monitoring makes AMC149 an ideal strain for identifying and analyzing mutants affected in the function of the circadian clock.

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