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18. Such manipulations change the value of the parameter f in the model. The steady-state amplitude is proportional to $1/(1 - f)r$ for large r (*7*). The factor f can be considered to be the fraction of releasable transmitter remaining after a spike, so $1 - f$ is the fraction of transmitter released. The steady-state synaptic strength is gA with g proportional to the amount of transmitter released, and thus to $1 - f$. As a result, the effective synaptic strength for sustained high rates is proportional to $(1 - f)/(1 - f)r = 1/r$ and is independent of f.
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Circadian Rhythms in Rapidly Dividing Cyanobacteria

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The long-standing supposition that the biological clock cannot function in cells that divide more rapidly than the circadian cycle was investigated. During exponential growth in which the generation time was 10 hours, the profile of bioluminescence from a reporter strain of the cyanobacterium *Synechococcus* (species PCC 7942) matched a model based on the assumption that cells proliferate exponentially and the bioluminescence of each cell oscillates in a cosine fashion. Some messenger RNAs showed a circadian rhythm in abundance during continuous exponential growth with a doubling time of 5 to 6 hours. Thus, the cyanobacterial circadian clock functions in cells that divide three or more times during one circadian cycle.

Circadian rhythms, oscillations of biological activities with a periodicity of approximately 24 hours in a constant environment, are observed in almost all organisms (*1*). The cellular components responsible for these rhythms and for the cell division cycle represent two major cellular oscillations that coexist in biological systems. The circadian clock is not a product of the cell division cycle because nondividing tissues such as the nervous tissue or mature leaves display robust circadian rhythms (*1*). However, it is unknown whether a circadian oscillation can exist when the cell division period is shorter than the circadian period. The circadian clock is thought to be dependent on state variables—that is, substances that reflect time, whose concentrations might be disrupted by the cell division process. Because several clock models implicate intracellular membrane structure directly or indirectly as a factor in circadian timing (*2*), it is important to investigate the relation between the cell cycle and circadian period. Autonomous models dependent on a clock gene, its mRNA, and its protein product have been proposed for the circadian clocks of *Drosophila* and *Neurospora* (*3*). These models assume that an important timing mechanism of a circadian feedback loop is the transport of a clock protein from the cytoplasm, where it is synthesized, to the nucleus, where it affects expression of its own gene. Cell division, which disrupts and divides the nuclear structure, might interfere with such a mechanism.

Stable circadian rhythms have been observed only in cells that divide more slowly than the circadian period. In *Tetrahymena* and *Euglena*, a circadian rhythm of cell division observed in slow-growing cultures does not persist when the generation time is less than a day (*4*). We used a transformed reporter strain (AMC149) of the cyanobacterium *Synechococcus* sp. PCC 7942 (*5*, *6*) to study circadian rhythms because it can divide much more rapidly than the circadian frequency and its circadian rhythm of bioluminescence can be monitored with high precision. A bacterial luciferase reporter gene inserted into the chromosome in AMC149 expresses a circadian bioluminescence rhythm. AMC149 displays a circadian rhythm that is physiologically equivalent to those of many eukaryotes (*7*). The generation time of *Synechococcus* can be as rapid as 5 to 6 hours and can be controlled easily by changing the light fluence or temperature. Here we demonstrate that cultures of cyanobacteria that divide twice or more per day display circadian rhythms of bioluminescence and levels of mRNA.

During an early stage of liquid culture, *Synechococcus* grew exponentially until the density reached 5×10^7 cells per milliliter (*8*). Because the optical density at 730 nm (OD_{730}) of the culture was below 0.05, the effective light intensity was not lowered by self-shading (*9*). Microscopic observation confirmed that *Synechococcus* also grew ex-

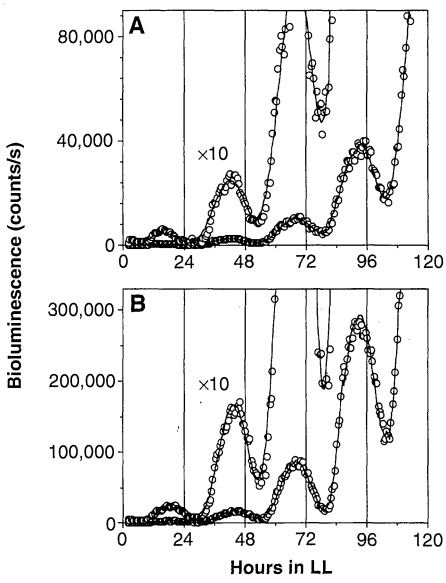

Fig. 1. Bioluminescence rhythm (open circles) and theoretical curve (solid lines) for exponentially growing *Synechococcus* in liquid culture and on an agar plate. (**A**) Measurements in liquid medium. AMC149 cells were cultured in modified BG11 medium (*13*) under LL to a density of 10^9 cells per milliliter. The culture was then diluted to 10^5 cells per milliliter. (**B**) Measurements on solid medium. Cells were spread on a 1-cm^2 area of an agar plate to form 1000 to 3000 microcolonies. (A and B) After 12 hours of darkness, a 1-ml aliquot of liquid culture or 1-cm^2 area of agar was transferred to a 20-ml vial, and 0.2 ml of 0.5% *n*-decanal solution (dissolved in oil) was placed in the vial separately. The bioluminescence from the vials was monitored continuously by the automated system with a photon-counting photomultiplier (*10*). The light fluence rate was 46 $\mu E\ m^{-2}\ s^{-1}$ during LL, and temperature was continuously maintained at 30°C. Bioluminescence from the vials is plotted against time in LL by open circles without a connecting line. The bioluminescence data were analyzed by Igor software (*11*) to find a best-fit curve to a model equation. Theoretical curves are superimposed in each plot. To visualize the profile of low-level bioluminescence during the early phase of each experiment, a 10-fold expansion of the bioluminescence is shown in each panel (×10 curve).

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ponentially on agar plates during microcolony formation. Cells continued to divide evenly and formed a round monolayer colony of 2000 to 3000 cells (*8*). Thereafter, cells formed multilayered colonies and the growth rate slowed, most probably as a result of limited resources (inorganic nutrients, carbon dioxide, and light) for the cells located in the interior of the colony. For both liquid and agar cultures, the generation time during exponential growth was 10 hours under 50 μE m^{-2} s^{-1} of continuous illumination (LL) (*8*).

A photon-counting method (*10*) was used to capture very weak bioluminescence signals from cultures of 10^4 to 10^5 cells. At the beginning of an experiment, the cell density of the liquid culture was about 10^5 cells per milliliter, and microcolonies on the agar plate were composed of about 10 cells. Thereafter, during assay periods of up to 110 hours, cells proliferated exponentially, with doubling times of approximately 10 hours, as reflected by an exponential increase of bioluminescence. Data points in Fig. 1 depict the bioluminescence profiles of exponential cultures of AMC149 in liquid medium and on an agar plate (the curves in Fig. 1 were generated independently as described below, rather than by connecting the data points). The bioluminescence profiles contained both exponential and circadian components.

To estimate exponential and rhythmic parameters quantitatively, the bioluminescence data shown in Fig. 1 were subjected to curve fitting by means of Igor software (*11*). The circadian bioluminescence rhythm expressed by the *psbAI* promoter in stationary phase cultures of *Synechococcus* can be approximately described as a cosine (or sine) function (*5*, *6*). Therefore, with the assumption that the cells grow as an exponential function and that the bioluminescence of each cell oscillates as a cosine function, we developed an equation that includes bioluminescence (B, counts per second) and time in LL (t, hours),

$$B = 2^{-(t/D)}\{A[\cos(2\pi t/T + 2\pi I/24) + 1] + C\}$$

where T is circadian period, D is generation time (hours), A is amplitude of the rhythmic component, C is a constitutive component, and I is the phase offset of the rhythm on a 24-hour scale (*12*). As shown by the curves superimposed in Fig. 1, the model equation predicts the real bioluminescence data. Parameters of the fitted equations, showing small standard deviations, are listed in Table 1. Several linear polynomial equations were also examined, but those models failed to fit the experimental data. Parameters that represent period (T) and

Table 1. Parameters of the fitted equation for the rhythm of AMC149. The parameters of the fitted equation and standard deviations are listed. Liquid and solid culture are for experiments shown in Fig. 1, A and B, respectively.

Culture	Period T (hours)	Phase I (CT)	Doubling time D (hours)	Rhythmic component A	Constitutive component C	A/C
Liquid	25.6 ± 0.3	10.9 ± 0.7	11.2 ± 0.3	66.9 ± 6.7	35.2 ± 4.5	1.90
Solid	24.6 ± 0.2	7.9 ± 0.6	10.6 ± 0.3	434.7 ± 50.2	214.1 ± 28.6	1.96

Table 2. Parameters of fitted equations for rhythms of period mutants. The parameters of the fitted equation of Fig. 3 are listed. Standard deviations of parameters were of similar magnitude to those in Table 1.

Mutant	Period T (hours)	Phase I (CT)	Doubling time D (hours)	Rhythmic component A	Constitutive component C	A/C
SP20	23.3	10.9	12.6	1.4	11.1	0.12
SP22	21.3	10.4	12.7	5.9	0.7	8.9
LP27	30.1	10.4	13.4	5.4	27.2	0.20
p30	27.7	11.9	13.8	17.5	25.4	0.69

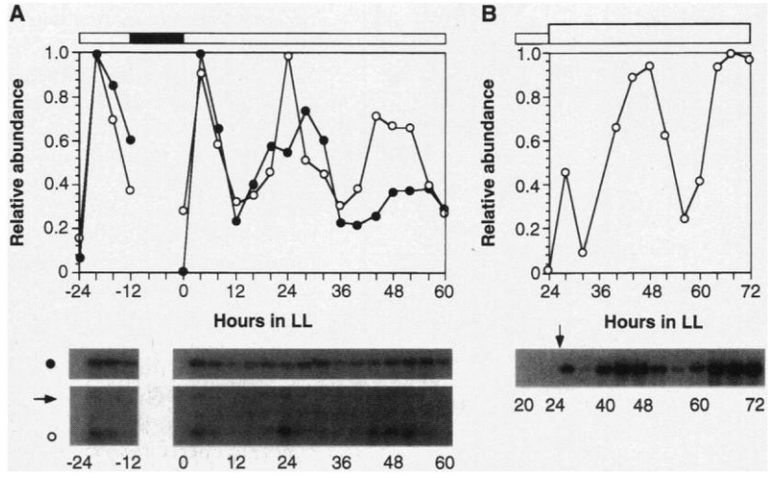

Fig. 2. Rhythms of mRNA abundance from *psbAI* and *psbAII* genes in wild-type *Synechococcus*. (**A** and **B**) Northern (RNA) blots of *psbAI* (solid circles) and *psbAII* (open circles) mRNA and densitometric data of the blots as a function of time in LL. To maintain exponential growth at higher cell density, wild-type *Synechococcus* was cultured by a turbidostat at 30°C in LL as described (*9*). The cell density of the culture was maintained by continuous dilution of the culture at an OD_{730} of 0.5 to 0.6 (about 10^9 cells per milliliter). The light fluence rate in LL (read spherically) at the times indicated by the white bar at the top of the graphs was 120 μE m^{-2} s^{-1}, which is similar to light fluence rates used in previous experiments (the generation time was 10 to 12 hours). (A) During the light portion of the entraining light (white bar above graph) and dark (black bar within white bar) cycle, and beginning with the initiation of LL conditions (0 hours), 40-ml samples were collected every 4 hours by centrifugation and stored at −70°C. After all samples had been collected, Northern blots of total RNA were prepared as described previously (*14*). Probes were antisense RNAs that hybridize to the unique 5′ untranslated leader of specific *psbA* messages, labeled with [α-^{32}P]uridine triphosphate with an in vitro transcription kit (Ambion). A Bio-Rad 620 densitometer was used to quantify the autoradiograms of the Northern blots. Even loading of the lanes was verified by densitometry of a negative image of ethidium bromide fluorescence from the 16*S* ribosomal RNA band. An open circle indicates the position on the Northern blot of a stable *psbAII* mRNA degradation product that was quantitated for the graph. An arrow indicates the position of the full-length *psbAII* message. (B) Northern analysis of full-length *psbAII* mRNA and densitometric data of the blot as a function of time in high-intensity LL. Before the onset of LL conditions (0 hours), the culture was entrained to a light and dark cycle as shown for (A) (*20*). Samples were taken every 4 hours beginning at 0 hours (except that the 36-hour sample was lost) and were processed as indicated for (A). RNA data are shown beginning with the 20-hour sample. A downward-pointing arrow indicates the time at which the light fluence rate, read spherically, was increased from 120 μE m^{-2} s^{-1} (generation time 10 to 12 hours; thin white bar above graph) to 400 μE m^{-2} s^{-1} (generation time 5 to 6 hours; thick white bar above graph), immediately after the 24-hour sample was taken.

phase (*I*) were very close to those obtained for fully grown colonies or for saturated liquid cultures. The parameter for generation time of the model (*D*) was also very close to the generation time estimated by real cell numbers of the culture (about 10 hours). Therefore, the model equation seems to accurately model parameters representing the circadian rhythm of *Synechococcus* in exponential growth.

We also found that circadian rhythms of mRNA abundance were evident in continuous cultures maintained in exponential phase at a higher cell density by growth in a turbidostat (*13*). Figure 2A shows the changes in *psbAI* and *psbAII* transcripts in LL when the culture was grown with a generation time of about 10 hours. At this light intensity, the *psbAII* gene is poorly expressed, but a stable degradation product recognized by the gene-specific probe was evident after entrainment by a light and dark cycle. The abundance of the *psbAI* mRNA and the full-length and degraded *psbAII* mRNAs oscillated with a 24-hour period and phase similar to the bioluminescence rhythm (*14*). At a higher light intensity, the *psbAII* full-length message was abundant and showed circadian cycling. An acute response to the shift to high light intensity was also evident (Fig. 2B). Under these conditions, the doubling time was once per 5 to 6 hours. Therefore, we conclude that the circadian clock in *Synechococcus* maintains a 24-hour period regardless of cell division rate or cell density.

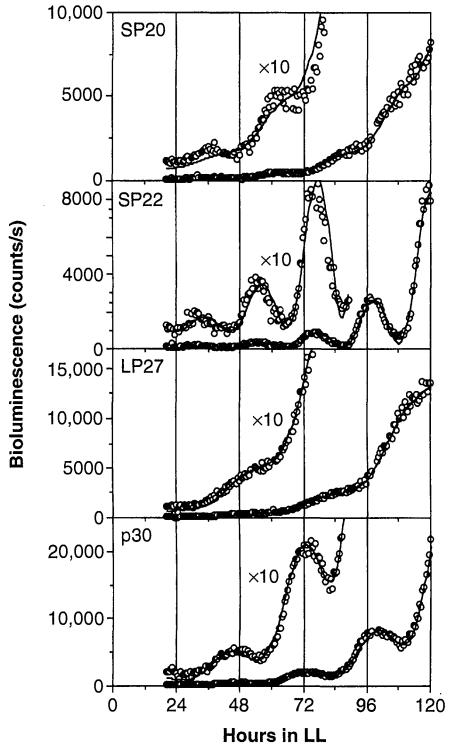

Fig. 3. Bioluminescence rhythm (open circles) and theoretical curves (solid lines) of exponentially growing *Synechococcus* clock mutants. The bioluminescence of clock mutants (SP20, SP22, LP27, and p30) was analyzed as in Fig 1B. To visualize profiles of low-level bioluminescence during the early phase of each experiment, a 10-fold expansion of the bioluminescence is shown in each panel (×10 curve).

We examined the circadian rhythm of several period mutants (*15*) in exponential phase (Fig. 3 and Table 2). Although the bioluminescence rhythm of the wild-type strain (AMC149) was not influenced by growth conditions (Fig. 1 and Table 1), the amplitudes of rhythms in SP20, LP27, and p30 (*15, 16*) were weak in exponential culture (the A/C ratio was less than 1), whereas fully formed colonies of these mutants displayed well-sustained rhythms (*15*). Calculated periods of exponential cultures of these mutants were shorter or longer than those obtained for mature colonies. However, the amplitude of the SP22 rhythm was larger than that of the wild type and its period was not altered. These observations imply that some mutations altered the clock system not only for period but also for stability of the oscillation, a phenotype that was not evident in the stationary culture.

In a concurrent study, Mori *et al.* performed flow cytometry of *Synechococcus* and found that cell division is prohibited for several hours in the circadian cycle, but cell length continues to increase while cell division is arrested (*17*). Thus, the total cell mass in the batch culture should continue to increase exponentially. The gating of cell division they observed is not evident in our data, but our result is expected if bioluminescence correlates not with cell number but with total cell mass.

Each cell in the culture that doubles every 12 hours should divide more than once per day even though cell division is gated by the circadian clock because division is suspended for only a few hours in the circadian cycle. Detailed imaging that shows individual cells of a microcolony forming on an agar plate revealed that no cell remained undivided (*8*). Similar conclusions can be drawn from curve fitting to a model equation, which included a term that represents the bioluminescence of nondividing cells. This term was always negligible (less than 0.1% of total bioluminescence). Therefore, observations reported here clearly demonstrate circadian rhythmicity in cells that are dividing faster than circadian frequency; that is, cell division cycling does not interfere with the circadian clock of *Synechococcus*.

Because three features of circadian rhythms are common to all organisms, a universal molecular mechanism for the circadian clock might be predicted. However, current models of the clock in *Drosophila* and *Neurospora* (*3*), in which the putative protein state variables Period, Timeless, and Frequency have been localized during the circadian cycle, are tied strongly to compartmentalization events involving export and import across the nuclear membrane. If the clock mechanism is similar among prokaryotes and eukaryotes, cell division and replication events are less disruptive of the circadian information than is intuitively obvious, and an alternative compartment must serve the sequestration function in prokaryotes. Alternatively, adaptation of the clock to eukaryotic intracellular compartmentation may have occurred after the circadian clock was introduced to ancestral eukaryotes by an endosymbiotic prokaryote (*18*); the eukaryotic clock may have evolved to function only in cells that divide slowly. Thus, intraorganellar events may have been incorporated that have no parallel in cyanobacteria.

The prokaryotic clock may be less affected by replication than is that of eukaryotes. The results of Mori *et al.* (*17*) suggest that DNA synthesis is continuous in the cyanobacterial population. This implies that replication does not interfere with circadian timekeeping in cyanobacteria. This might be made possible by more rapid completion of replication in bacteria or an absence of eukaryotic chromosome structure, or both.

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7. A transformed strain of the cyanobacterium *Synechococcus* sp. PCC 7942, AMC149, carries the promoter of the *psbAI* gene, which encodes the D1 protein of photosystem II, fused to the *Vibrio harveyi* luciferase gene set (*luxAB*) at a nonessential region of the genome (*5*). Both liquid cultures (*5*) and colonies on agar plates (*6*) of AMC149 display a robust rhythm of bioluminescence that satisfies the three salient features of circadian rhythms: persistence in constant conditions, resetability of phase by a light signal, and temperature compensation of period length.
8. AMC149 was used for most experiments in this study. For liquid cultures of low density, an aliquot of

the culture was spread on an agar plate at various times and colonies that formed later were counted. For early stages of colony formation on agar plates, one microcolony was repeatedly photographed at various times in LL under phase-contrast microscopy, without the use of a cover slip for repetitive observation. The cell number in the colony was directly counted. The generation time of the cells was calculated from the slope of the growth curve obtained by linear regression analysis.

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10. The bioluminescence from the vials was measured every 30 min by an automated photomultiplier tube apparatus as described (*5*). A low-noise photomultiplier tube (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 (selecting those of 0.2 V or above). Signal pulses from the C3866 were integrated in an electronic counter (Iwatsu SC-7201). As a result of high gain of the R2693P photomultiplier tube and the rejection of thermal noise by the photon counter, the effective sensitivity of light detection was 30 to 100 times greater than that of the previous analog system. The counter was read by computers (Sharp PCE-600 and Macintosh Plus) and stored as a computer file for curve fitting by the Igor program. At each measurement, the dark count of the photomultiplier was subtracted automatically from the signal count.
11. Igor (version 1.28; WaveMetrics, Lake Oswego, OR) was used for curve fitting by the Levenberg-Marquardt method [W. H. Press *et al.*, *Numerical Recipes in C* (Cambridge Univ. Press, Cambridge, 1988)].
12. Because the term $\cos(2\pi t/T + 2\pi I/24) + 1$ in the model equation oscillates between 0 and 2, coefficient A of this term could be considered as an amplitude of rhythm, whereas parameter C represents a constitutive component of bioluminescence of the cell—that is, bioluminescence level at the trough of the rhythm. Because both parameters are a function of absolute bioluminescence level, the A/C ratio can be used to evaluate the rhythmicity. Parameter I defines an initial phase of cosine oscillation. If $I = 12$, the oscillation starts from the trough.
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