CIRCADIAN PROGRAMS IN CYANOBACTERIA:
Adaptiveness and Mechanism

Carl Hirschie Johnson1 and Susan S. Golden2

1Department of Biology, Vanderbilt University, Nashville, Tennessee 37235; e-mail: carl.h.johnson@vanderbilt.edu; 2Department of Biology, Texas A&M University, College Station, Texas 77843; e-mail: sgolden@bio.tamu.edu

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Abstract At least one group of prokaryotes is known to have circadian regulation of cellular activities—the cyanobacteria. Their “biological clock” orchestrates cellular events to occur in an optimal temporal program, and it can keep track of circadian time even when the cells are dividing more rapidly than once per day. Growth competition experiments demonstrate that the fitness of cyanobacteria is enhanced when the circadian period matches the period of the environmental cycle. Three genes have been identified that specifically affect circadian phenotypes. These genes, kaiA, kaiB, and kaiC, are adjacent to each other on the chromosome, thus forming a clock gene cluster. The clock gene products appear to interact with each other and form an autoregulatory feedback loop.

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INTRODUCTION

What Is a Circadian Program?

Circadian rhythms are endogenous biological programs that time metabolic and/or behavioral events to occur at optimal phases of the daily cycle. They have three diagnostic characteristics. The first is that in constant conditions, the programs free-run with a period that is \( \sim 24 \) h in duration. The second is that, in an appropriate environmental cycle (usually a light-dark and/or temperature cycle), the rhythm will take on the period of the environmental cycle, that is, circadian rhythms will entrain to the environmental cycle. The final characteristic is that the period of the free-running rhythm is nearly the same at different constant ambient temperatures within the physiological range; that is, circadian rhythms are temperature compensated. It is these three characteristics that define circadian rhythms, not the details of their biochemical mechanisms. Indeed, questions of considerable interest are whether circadian mechanisms have evolved more than once and, if so, whether completely different biochemical processes have been harnessed to the task in different organisms. The fascination of circadian rhythms is how a biochemical mechanism can keep time so precisely over such a long time constant (\( \sim 24 \) h) at different ambient temperatures.

Before 1985, it was believed that circadian programs were exclusively a property of the eukaryotic domain (24). It was a reasonable assumption that rapidly growing prokaryotes would not have circadian organization, because it was thought that an endogenous timekeeper with a period close to 24 h would not be useful to organisms that divide more rapidly than once every 24 h, as do many prokaryotes. The assumption might be stated as, “Why have a timer for a cycle that is longer than your lifetime?” Although intuitive, this conclusion is flawed. It is based on the presumption that a bacterial cell is equivalent to a sexually reproducing multicellular organism, which it is not. A bacterial culture is more like a mass of protoplasm that grows larger and larger and incidentally subdivides. A mother cell does not die to make daughter cells; she is the daughter cells. From this perspective, it is reasonable that a 24-h temporal program could be adaptive to rapidly dividing protoplasm if the fitness of that protoplasm changes as a function of daily alterations in the environment (light intensity, temperature, etc).

Discovery of Circadian Programs in Prokaryotes

The proposal that prokaryotes might have circadian programs is not new. In the past 35 years, both *Escherichia coli* and *Klebsiella aerogenes* were proposed to exhibit circadian behavior (15, 54), but these studies were not persuasive (see below). That prokaryotic cells, either unicellular or multicellular, were too simple to express...
circular behavior became a dogma, despite the fact that there were almost no published reports of rigorous tests of the proposition (24).

Studies in the late 1980s on cyanobacteria began to change this mind-set. It was not researchers who were interested in the presence of circadian timekeepers in prokaryotes who initiated this research, but those who attempted to resolve how some unicellular (or non-heterocystous, filamentous) cyanobacteria could fix nitrogen. Why was this an apparent dilemma? Photosynthesis evolves oxygen, and oxygen strongly inhibits the nitrogenase enzyme. How then can a photosynthetic unicellular organism fix nitrogen? Nitrogen fixation appeared to be doomed by the essential process that provides cellular energy. An imaginative idea to reconcile these incompatible processes surfaced in the 1980s; photosynthesis and nitrogen fixation could be separated in time—photosynthesis in the day and nitrogen fixation at night [reviewed by Golden et al (12)]. The first data suggesting that this "temporal separation" could be a metabolic program controlled by a circadian clock were from the non-heterocystous, filamentous cyanobacterium *Oscillatoria* sp. (52). Those authors found a nocturnal rhythm of nitrogenase activity that persisted in continuous light (LL). Shortly thereafter, temporal separation of photosynthesis (in the day) and nitrogenase activity (in the night) was demonstrated in the marine unicellular cyanobacteria *Synechococcus* spp. Miami BG 43511 and 43522 (39). This pattern continued in LL for at least 3 days, but these authors preferred to interpret their data in terms of regulation by the cell division cycle rather than by a circadian clock (39).

Huang and coworkers were apparently the first to clearly recognize that cyanobacteria were exhibiting circadian rhythms, and, in a series of publications beginning in 1986, they demonstrated all three salient properties in the same organism, the unicellular freshwater *Synechococcus* sp. RF-1 (6, 7, 13, 16–20). These pioneers studied the rhythm of nitrogen fixation and of amino acid uptake in this cyanobacterium, and were also the first to report the isolation of mutants affecting these processes (20). Another ground-breaking study was that of Sweeney & Borgese (55), who were the first to demonstrate temperature compensation of a daily rhythm in the marine cyanobacterium *Synechococcus* sp. WH7803.

We now know of circadian programs expressed in a number of cyanobacterial species. In addition to those already mentioned, circadian rhythms have been demonstrated in *Synechococcus* sp. strain PCC 7942 (32 and below), and the genera *Synechocystis* (2, 3), *Anabaena* (T Kondo & M Ishiura, unpublished data; 35), *Cyanothece* (47, 48), *Trichodesmium* (5, 8, 42), and possibly *Prochlorococcus* (49). What about other prokaryotes? This issue is addressed later in this review.

**CIRCADIAN PROGRAMS IN CYANOBACTERIA**

**Nitrogen Fixation/Photosynthesis**

The yin/yang rhythms of nitrogen fixation and photosynthesis fit the expectation that a major role of circadian timers is to temporally program metabolic events to occur at optimal phases of the environmental cycle. In addition to the work
on *Oscillatoria* spp. and *Synechococcus* spp. Miami BG 43511/43522 and RF-1, another series of studies that supports the hypothesis of temporal separation has investigated the marine unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142 (9, 47, 48). A remarkable feature of this nitrogen-fixing cyanobacterium is the presence of large carbohydrate granules within the cells, which are easily visualized by electron microscopy. These granules accumulate progressively during daytime photosynthetic activity and dissipate during nocturnal nitrogen fixation (47).

There is, however, a counter-example to the temporal separation hypothesis among nitrogen-fixing cyanobacteria: the non-heterocystous, filamentous cyanobacterium *Trichodesmium* spp. fixes its nitrogen during the daytime, simultaneously with photosynthesis (5, 42). How do *Trichodesmium* spp. accomplish this feat? We don’t know, but it underscores the fact that cyanobacteria are capable of fixing nitrogen in the presence of oxygen and therefore that other mechanisms must exist for lowering oxygen in the vicinity of nitrogenase (11). For example, *Synechococcus* sp. RF-1 increases its aerobic respiration rate whenever nitrogen fixation is under way (14). Enhanced respiration may be an additional mechanism for depleting oxygen in the vicinity of nitrogenase, even in cyanobacteria that separate photosynthesis and nitrogen fixation in time.

How does the circadian clock regulate the nitrogenase output rhythm? In *Synechococcus* sp. RF-1, *Cyanothece* spp., and *Trichodesmium* spp., there are circadian rhythms of nitrogenase messenger RNA (mRNA) abundance. As expected from the phasing of nitrogen fixation, in *Synechococcus* sp. RF-1 and *Cyanothece* spp., the peak of nitrogenase mRNA abundance is nocturnal (9, 17), whereas in *Trichodesmium* spp., it is diurnal (8). These rhythms of mRNA abundance presumably drive circadian rhythms of nitrogenase abundance and activity.

**Luciferase Reporters Illuminate a Path for Genetic Analyses**

Our interest in cyanobacteria as a model system for studying circadian programs stemmed from the genetic advantages that some cyanobacteria offer (12, 24, 33). The rhythms of nitrogen fixation, amino acid uptake, and carbohydrate content are reproducible, but the labor-intensive nature of the assays would dismay any but the strong-hearted from using this type of rhythm for a mutant screen [although the Huang group was undaunted and succeeded in isolating some mutants (see 20)]. To reap the benefits that a genetically tractable prokaryote would offer, we searched for a cyanobacterium that is amenable to molecular/genetic analyses and exhibits circadian rhythms of a parameter that can be assayed continuously for many cycles by an automated system.

The unicellular freshwater cyanobacterium *Synechococcus* sp. strain PCC 7942 was a good candidate for this approach. Although PCC 7942 does not fix nitrogen, it has many advantages for genetic analyses: It is transformable by circular or linear DNA, recombines at homologous sites, can receive DNA by conjugation from *E. coli* at high efficiency, can express reporter genes, and has a genome that
is smaller than that of *E. coli* (12). Based on these characteristics, many genetic tools were developed (1).

We created a circadian reporter strain of *Synechococcus* sp. strain PCC 7942 by transforming it with a construct in which the *Vibrio harveyi* luciferase gene set *luxAB* is expressed under the control of the promoter for a *Synechococcus* photosystem II gene, *psbAI* (32). This reporter strain was named AMC149. The luminescence rhythm expressed by AMC149 in liquid cultures or from single colonies on agar medium is easily assayed by automated monitoring systems based on either photomultiplier tubes (1, 32) or CCD (charge coupled device) cameras (29, 33). The luminescence pattern conforms to all three salient properties of circadian rhythms: persistence in continuous conditions (LL) with a period close to 24 h, entrainability by light/dark (LD) signals, and temperature compensation (32). The luminescence rises during the day and falls during the night. We found that the luminescence rhythm is an accurate reporter of *psbAI* gene expression (36), confirming our expectation that this rhythm reflects circadian control over the promoter of the *psbAI* gene.

**The Cyanobacterial Clock Is Unperturbed by Rapid Cell Division**

Cultures of AMC149 that are growing with doubling times as rapidly as one division every 6–10 h continue to exhibit circadian rhythms of *psbAI* gene expression (31, 40). The amplitude of the luminescence patterns reflecting *psbAI* promoter activity is a function of the stage of the growth cycle for colonies on agar or for liquid cultures. For example, in early log phase, the luminescence patterns display a clear circadian rhythm that grows in amplitude exponentially (Figure 1). This

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**Figure 1** Rhythmic gene expression in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942. Generalized circadian patterns of *psbAI* promoter activity monitored by luminescence of the reporter strain AMC149 are shown in three different phases of the growth cycle: exponential, sustained, and senescent.
exponential-phase pattern can be precisely modeled by assuming only two essential components: (a) a circadian rhythm of psbAI promoter activity in every cell and (b) an exponential increase in the number of cells in the culture (31). After the growth rate of the culture slows, the luminescence pattern stabilizes into a circadian pattern of consistent amplitude (sustained phase). It is in this growth phase that the circadian oscillator can be most easily assayed. As the culture ages, the rhythm slowly damps (senescent phase). The damping observed in the senescent phase probably results from nutrient depletion (including what is very important to the photosynthetic cyanobacteria—diminution of light intensity by high cell densities).

Not only can the cyanobacterial clock keep track of circadian time in exponentially dividing cells, it can also gate cell division. In *Synechococcus* sp. strain PCC 7942 growing in LD cycles, division occurs only during the day. In LL, however, division is blocked by the circadian clock in the early subjective night phases and is allowed in the subjective day and late subjective night (40). Therefore, not only does the clock keep track of circadian time in cells that divide two or three times a day, it controls when that division is allowed (gate open) or forbidden (gate closed). Using flow cytometry, we determined that the DNA replication rate and the growth in size of each individual cell is not rhythmic over the circadian cycle, but it is the timing of division that is controlled by the circadian program (40). In the photosynthetic marine prokaryote genus *Prochlorococcus*, there also appears to be circadian control of division in rapidly growing cells (49). Together, these data highlight the fallacy of the bias described in the second paragraph of this review; cellular events can waltz to a circadian andante even when the cells are rapidly dividing in allegro.

**Global Orchestration of Gene Expression**

How many genes are controlled by the circadian clock in cyanobacteria? In *Synechococcus* sp. RF-1, Huang et al (16) conducted a study of protein synthetic patterns as a function of circadian time and found >10 polypeptides that exhibit circadian rhythms of translation and are expressed in a variety of phase relationships. We therefore wondered how extensive circadian control of gene expression is in *Synechococcus* sp. strain PCC 7942 and devised a strategy to globally search for rhythmic control over promoters (38).

The bacterial luciferase gene set (*luxAB*) was inserted into the *Synechococcus* sp. strain PCC 7942 genome so as to achieve random insertions of *luxAB* throughout the chromosome (38). We screened the luminescence expression patterns from the ∼800 clones whose luminescence was bright enough to be easily monitored. Unexpectedly, the luminescence expression patterns of essentially all of these 800 colonies manifested clear circadian rhythmicity. These rhythmic colonies exhibited a range of waveforms and amplitudes, and they also showed at least two predominant phase relationships. We defined Class-1 genes as those whose expression peaks at the end of the day and Class-2 genes as those peaking at the end of the
Figure 2  Luminescence patterns of the promoter activity of Class-1 and Class-2 genes as assayed with the luxAB reporter. The upper trace is from the Class-1 gene psbAI, whereas the bottom trace is from the Class-2 gene purF. Data were recorded in constant light (LL), but the times of expected or subjective night are shown by the gray bars along the abscissa (from 37).

This promoter-trap experiment shows that circadian programming of gene expression is pervasive in cyanobacteria.

Our original luminescent strain, which was genetically engineered with the promoter of psbAI, is a Class-1 gene (peak at dusk; trough at dawn), as shown in Figure 2. The transcriptional activity of the reverse-phase Class-2 genes, however, is maximal at about dawn and minimal at about dusk (Figure 2). We have identified one of the Class-2 genes as purF, which encodes a key regulatory enzyme in the de novo purine synthetic pathway, glutamine PRPP amidotransferase (37, 38). It is intriguing that glutamine PRPP amidotransferase is sensitive to oxygen, as is nitrogenase in Synechococcus sp. RF-1, and that both of the genes encoding these enzymes are expressed in the night or Class-2 phase. Therefore, even though Synechococcus sp. strain PCC 7942 does not fix nitrogen, we hypothesize that the Class-2 expression pattern of the purF gene is related to the oxygen sensitivity of glutamine PRPP amidotransferase and that it is another example of the same "temporal-separation" program exhibited by the expression of nitrogenase in some of the nitrogen-fixing cyanobacteria (25, 37).

Based on our results with the random promoter-trap experiment, we formulated a model that incorporates both nonspecific circadian control and circadian
regulation by specific trans factors (38). In the simplest version of the model, there would be one (or a few) types of Class-1-specific cis elements turned on during the day by a Class-1-specific trans factor. A different set of Class-2-specific cis elements would be turned on at night by a Class-2-specific trans factor, and so on. 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 factor(s) could be involved as well. In support of our model for regulatory pathways that are specific for subsets of cyanobacterial genes, we discovered a gene whose altered expression significantly lowers the amplitude of the luminescence rhythm driven by some promoters (including that of \textit{psbAI}), but not of luminescence rhythms driven by other promoters (such as that of \textit{purF}). This gene encodes a sigma70-like transcription factor, \textit{rpoD2}, and is a member of a family of sigma factor genes in \textit{Synechococcus} sp. strain PCC 7942 (58). Apparently \textit{rpoD2} is a component of an output pathway of the circadian oscillator that affects the rhythmic expression of a subset of clock-controlled genes in \textit{Synechococcus} sp. strain PCC 7942.

\textbf{ARE THESE CLOCK PROGRAMS ADAPTIVE?}

\textbf{Competition as a Test of Fitness}

Although it is logical that circadian programming of the steps of gene expression, metabolic reactions, cell division, etc. could be adaptive, no rigorous test of the proposition had been performed in any organism, either eukaryotic or prokaryotic, prior to our recent work with cyanobacteria (41). We tested the value of circadian programming to reproductive fitness in cyanobacteria by using wild-type and mutant strains that exhibit different free-running periods (21, 33). The strains we used were C22a (period \(\sim 23\) h), wild-type (period \(\sim 25\) h), and C28a (period \(\sim 30\) h). Both C22a and C28a have point mutations in the \textit{kaiC} gene (see 21 and below). All three strains grew in pure culture at essentially the same rate in LL and in LD cycles, so there did not appear to be a significant advantage or disadvantage to having different circadian periods when the strains were grown in single-strain cultures (41).

However, when we mixed different strains together and grew them in competition with each other, a remarkable pattern emerged that depended on (a) the endogenous period of each strain and (b) the period of the LD cycle. We tested different LD regimens that had equal amounts of light and darkness but in which the frequency of the LD cycle differed: a 22-h cycle (LD 11:11), a 24-h cycle (LD 12:12), and a 30-h cycle (LD 15:15). In each case, the strain whose period most closely matched that of the LD cycle eliminated the competitor (41). Poor fitness was not necessarily associated with mutant phenotypes—in fact, the mutant strains easily defeated the wild-type if the mutant period was a better match to the LD cycle. These results were obtained for batch liquid cultures that were diluted every 8 days and for cultures maintained at constant cell density in a turbidostat (41).
Figure 3  Kinetics of competition between wild-type (period $\sim 25$ h) and the mutant strain C28a (period $\sim 30$ h). Raw data (open-symbol points and heavy lines) of competitions in LD 12:12 vs LD 15:15 are compared with the results of modeling (thin lines) using the equation shown in the panel. (In LL, both strains do equally well.) Terms: $w$, relative fitness ($w_{wt}$ for wild type, $w_{C28a}$ for C28a); $p$, fraction of a given strain in the mixed population ($p_t$ for generation $t$; $p_{t+1}$ for generation $t + 1$). Ordinate is the percentage of colonies in the population that are C28a; abscissa is the estimated number of generations (from 41).

Estimation of the selective pressure by taking more time points suggested that the selective coefficient was surprisingly strong. As shown in Figure 3, a simple model that assumes (a) the selective pressure to be constant and (b) a lag in the initiation of selection suggested to a first approximation that the relative fitness of the less successful strain could be as low as 0.7–0.8 ($w_{wt} \sim 0.7$ in LD15:15, $w_{C28a} \sim 0.85$ in LD12:12). Because the growth rates of the strains in pure culture are not different by a factor of 20–30%, the modeling result depicted in Figure 3 indicates that we are observing a case of soft selection in which the poorer fitness of inferior genotypes is most obvious under competition. We cannot rule out that small, presently unmeasurable differences exist between the growth rates of these strains in pure culture, but soft selection seems to be the predominant mechanism responsible for the strong selection under competition. The results are unlikely to be caused by an unrelated mutation that is deleterious for growth because (a) mutant strains can outgrow wild types in the appropriate combination of biological and environmental periods and (b) a genetic test confirmed that only the differences between the $kaiC$ alleles of these strains are responsible for the competitive advantage/disadvantage (41).

How do the victorious strains win? Other than demonstrating that soft selection is operating, we do not yet know the mechanism of the selection. We do
know that the phasing of psbA\textit{I} gene expression is disrupted within strains in non-optimal LD cycles (41). This is consistent with the idea that the circadian program orders cellular processes to optimally match environmental cycles; when this order is disturbed, fitness is reduced. We conclude that the circadian pacemaker in cyanobacteria confers a significant competitive advantage when the period of the clock matches that of the environmental cycle, thus achieving optimal phasing of cellular events. This is the first rigorous demonstration in any organism of an advantage conferred by a circadian system to fitness.

**ROLES OF LIGHT AND DARK**

One of the big three properties of circadian clocks is their ability to be entrained to the environmental cycle. Entrainment means that the period of the biological clock becomes equal to that of the environmental cycle. Light and dark signals are usually considered to be the primary signals that set the phase of circadian clocks. In some organisms, the photopigments involved in circadian entrainment are known, such as phytochromes, rhodopsins, or cryptochromes (10, 23, 43, 51, 53, 56). In many cases, however, the identity of the relevant circadian photopigments remains a mystery (23, 43). The photopigments mediating the entrainment of cyanobacterial clocks fall into the latter group.

In \textit{Synechococcus} sp. strain PCC 7942, we have attempted to glean clues as to the identity of clock photopigments by action spectroscopy. Our preliminary data suggest that blue and red light are most effective in setting the phase of the cyanobacterial clock, whereas green and far-red light are ineffective (T Kondo & C Johnson, unpublished observations). The phasing effect of red light was not reversed by far-red light, nor was the effect of blue light reversed by red light [as reported for light-regulated gene expression by Tsinoremas et al (57)]. The spectrum does not coincide with that expected for photosynthesis in cyanobacteria, nor does it fit with the behavior of a classically acting phytochrome. In \textit{Synechococcus} sp. RF-1, red light of 680 nm was also found to be an effective phasing agent, and far-red light of 730 nm did not reverse red-light phasing (7). These data suggest that there are specific, unknown pigments that are the eyes of the clock of \textit{Synechococcus} spp. strains PCC 7942 and RF-1.

Because almost all tests for circadian rhythmicity in cyanobacteria have used LL as the constant condition, it is appropriate to ask whether this circadian clock requires the presence of light to run. Most cyanobacteria are obligate photoautotrophs, including \textit{Synechococcus} sp. strain PCC 7942. In constant darkness (DD), all luciferase reporter strains derived from PCC 7942 show rapidly damped oscillations in DD. Is this because the clock has stopped, or is it possible that the central timekeeper is still running and the outputs are turned off as an energy-saving device? One line of inquiry to answer this question in \textit{Synechococcus} sp. strain PCC 7942 used light pulses that can reset the phase of the clock as probes of the pacemaker’s phase in DD. The data suggested that the clock continued to run in this photoautotroph, even in DD (30).
Is the presence of light a necessity for the unimpeded precession of the clock, or is it merely that a certain metabolic rate must be maintained for the clock to express itself? This question has been addressed in cyanobacterial species that can grow heterotrophically. After a period of adaptation, *Synechocystis* sp. strain PCC 6803 and *Cyanothece* spp. can grow heterotrophically: on glucose for *Synechocystis* and on glycerol for *Cyanothece*. Using the *dnaK*:luxAB reporter strain of *Synechocystis* sp. growing on glucose, Aoki & coworkers were able to show that the luminescence rhythm persisted for many cycles in DD (3). Similarly, the rhythms of nitrogenase activity and carbohydrate content persisted robustly for >4 days in *Cyanothece* spp. grown on glycerol (48). Therefore, in these species of cyanobacteria it is clear that the clockwork can run in DD if metabolism is maintained and that the clock is not directly light dependent. It seems likely that, in the absence of other energy sources, DD is perceived as a signal to shut down unnecessary processes. But the central clock of photoautotrophic cyanobacteria appears to have favored status and continues to run in DD unlinked to its energy-guzzling outputs—rather like an automobile engine idling in neutral gear.

**GENETIC DISSECTION OF THIS “CLOCKWORK GREEN”**

**The kai Clock Gene Cluster**

Using the PpsbAI::luxAB reporter strain (AMC149) described above, we screened >500,000 clones of *Synechococcus* sp. strain PCC 7942 that had been treated with the mutagen ethylmethanesulfonate. Over 100 mutants exhibiting various circadian phenotypes, including arhythmia, altered waveforms, and atypical periods (ranging between 14- and 60 h) were isolated (33). Most of these mutants grow apparently as well as the wild type and exhibit no other obvious phenotype besides circadian anomalies.

Efficient rescue of mutant phenotypes is possible in *Synechococcus* spp. by the introduction of libraries of wild-type *Synechococcus* DNA, and we succeeded in rescuing >30 mutants of various phenotypes. DNA fragments from several rescued mutants complemented other mutant phenotypes, including short-period, long-period, and arhythmic phenotypes. These rescue experiments allowed us to pinpoint a cluster of three adjacent genes, named *kaiA*, *kaiB*, and *kaiC* (21; *kai* means “rotation” or “cycle” in Japanese). All of the mutants that have been complemented so far can be rescued by a plasmid carrying the entire *kaiABC* cluster, and 19 mutations were mapped by DNA sequencing to the three *kai* genes. All are missense mutations resulting from single nucleotide exchanges. Most of the mutations are recessive (rescue by wild-type DNA is complete), but a few are semidominant, such as that of the 60-h-period mutant C60a. Each of the three genes has at least two clock mutations mapped to it, and the largest gene, *kaiC*, has many mutations that include all the possible clock phenotypes: short period, long period, low amplitude, and arhythmia. No significant similarity was found among the *kai* genes and any other previously reported genes in prokaryotes or eukaryotes, except that there is a possible homolog of the *kaiC* gene among unidentified open
reading frames in the genomic sequences of archaebacteria (see below). Moreover, there are two P-loop motifs in the kaiC gene. This motif, [G or A]XXXXGK[T or S], is a GTP/ATP nucleotide-binding region (46). The kaiABC cluster appears to be a clock-specific region of the chromosome in cyanobacteria, because deletion of the entire cluster or of any one of the kai genes separately does not affect viability (in single-strain cultures), but does cause arhythmicity (21).

Figure 4 summarizes much of our knowledge about the relationships among these components (21). Promoter activities were found in the upstream regions of both the kaiA and kaiB genes. The kaiA promoter gives rise to a monocistronic
kaiA mRNA, whereas the kaiB promoter produces a dicistronic kaiBC mRNA. Both kaiA and kaiBC transcripts are rhythmically abundant. Inactivation of any single kai gene abolished these rhythms and lowered kaiBC promoter activity. Continuous overexpression of kaiC repressed the kaiBC promoter (negative feedback), whereas kaiA overexpression enhanced it (positive feedback). Overexpression of the kaiC gene for a few hours reset the phase of the rhythms. Consequently, the level of KaiC expression is directly linked to the phase of the oscillation.

One clue to the mechanism of this clockwork is that the Kai proteins appear to interact. Yeast two-hybrid and in vitro binding assays indicate that the KaiA, KaiB, and KaiC proteins interact both homotypically and heterotypically (22). One long period mutant exhibits an altered heterotypic interaction between KaiA and KaiB; this result suggests that inter-Kai contact is important to the clock mechanism (22). Using a novel method based on resonance energy transfer to assay protein-protein interactions, we have confirmed that KaiB polypeptides interact (59). Taken together, these results suggest that there is negative feedback control of kaiC expression by the KaiC protein to generate a circadian oscillation in cyanobacteria involving protein-protein interactions and that KaiA sustains the oscillation by enhancing kaiC expression.

The model is still very preliminary, however. It is likely that this feedback model—which borrows many of its features from clock models of eukaryotes—is oversimplified. For example, the model implies that the Kai protein levels are rhythmic and that changes in Kai protein levels within the physiological range will elicit phase resetting. Mutations of the genes encoding Kai proteins that affect protein-protein interactions should alter circadian properties. Does KaiC bind ATP and/or GTP, and, if so, does modification of the P-loop motif disrupt normal time-keeping? Are there other molecular players, such as Kai-interacting transcriptional factors? These and other predictions demand extensive testing.

The kai genes apparently encode products that are crucial for the activity of a clockwork in cyanobacteria. But is this the only clock mechanism in cyanobacterial cells? This question cannot be dismissed lightly, because multiple clock mechanisms have been discovered in the unicellular eukaryotic alga Gonyaulax spp. (44). So far, no concrete evidence indicates the presence of more than one clock mechanism in Synechococcus spp. For example, most mutations that affect the period of one reporter strain similarly affect the period of other reporter strains; for example, the periods of the PpsbA1::luxAB, PpurF::luxAB, and PkaiBC::luxAB reporter strains respond similarly to mutations in kaiABC (21; C Johnson & S Golden, unpublished data). Nevertheless, we must remain alert to the possibility that new data may indicate that even in simple prokaryotic unicellular organisms, multiple circadian oscillators could coexist.

**Other Clock Genes?**

One of the questions posed in the previous section was whether there are more genes involved in the circadian mechanism of Synechococcus sp. strain PCC 7942. Although we have achieved saturation mutagenesis with ethylmethanesulfonate,
we do not have enough pieces to fit the puzzle together yet. For example, if feedback regulation of the kai cluster is crucial, then what are the transcription factors? None of the Kai proteins has a DNA-binding motif. It is possible that a Kai protein complex interacts with a general transcription factor. We might not have discovered this factor in our screens to date if it is an essential transcription factor that confers a lethal phenotype when mutated. To identify other components in the circadian clockwork, imaginative new screens (and possibly new mutagens) need to be tested. For example, transposon mutagenesis has identified a histidine protein kinase gene whose inactivation causes a short-period phenotype (O Schmitz & SS Golden, unpublished observations). Another tactic is to search for Kai-interacting proteins by a yeast two-hybrid (or other) screen.

Yet another strategy to uncover new genes that encode components of the clockwork is to isolate extragenic suppressors. One suppressor that emerged from the initial attempts to rescue clock mutations is the period-extender gene, pex (34). When the copy number of the pex gene is increased, pex lengthens the period of wild-type and other strains by 2 h. Disruption of pex shortens the period by 1 h, and overexpression lengthens the period by up to 3 h. No meaningful homologs to pex were detected in DNA or protein databases (34). Screens that have been specifically designed to find other extragenic suppressors (e.g. by mutation of clock mutants to find altered phenotypes) have not yielded non-kai candidates, but the hunt continues.

**EVOLUTIONARY ASPECTS**

**Evolution of the kaiC Gene**

We have found strong candidates for homologs to the kaiC gene in the genomic sequences of the archaea *Methanobacterium thermoautotrophicum* (50), *Methanococcus jannaschii* (4), *Pyrococcus horikoshii* (27), and *Archaeoglobus fulgidus* (28). As shown in Figure 5 in which the archaeabacterial sequences are aligned roughly end to end with those of the kaiC gene from the cyanobacteria *Synechoccus* sp. (21) and *Synechocystis* sp. (26), the amino acid sequence identities between the cyanobacteria and these archaeabacteria range between 25.7% and 34.2% over the entire length of the proteins. More important, there is conservation of the P-loop motif GXGGXXGKT or S], which is a GTP/ATP nucleotide-binding region (46) and is indicated on Figure 5 by the double underlines. This motif appears to be highly conserved among the kaiC genes of the cyanobacterial species and the putative kaiC homologs of the archaea. The kaiC homolog of *Methanococcus* sp. is shorter than the others, and the P-loop motif appears only once in the *Methanococcus* sequence, whereas it appears twice in the sequences of all the other genes.

Prompted by considerations relating to the endosymbiotic theory of the origin of eukaryotic organelles, we searched the databases for possible kai homologs in eukaryotes. At present, no encouraging candidates have appeared. In particular, there are no apparent homologs in the chloroplast genomes of tobacco or other
higher plants, nor is there any significant hybridization between kai DNA and the chloroplast genome of the eukaryotic alga *Chlamydomonas* (Y Xu, S Surzycki & CH Johnson, unpublished observations). Therefore, there are no data that yet encourage the view that the clockwork that evolved in cyanobacteria was transferred to eukaryotes by endosymbiosis or another mechanism of gene transfer.

**Are There Circadian Timepieces in Other Prokaryotes?**

As discussed in the first section of this review, it used to be thought that prokaryotic organization and lifestyle were incompatible with circadian clocks. As addressed herein, we now realize that circadian programming is an integral part of cyanobacterial organization and that this temporal program has adaptive value. But what about non-photosynthetic prokaryotes? This is a challenging question, with momentous implications for understanding the early evolution of circadian rhythmicity. The previous suggestion that *E. coli* might have a circadian clock (15) was based on an old study (45) in which *E. coli* cells were grown in rich nutrient medium in an apparatus whose temperature control was almost certainly poor. Therefore, the possible daily trends noted by Halberg & Conner (15) are likely to be merely a result of a diurnal cycle of temperature, to which the growth rate of *E. coli* would be exquisitely sensitive. (Incidentally, there are no kai homologs in the genome of *E. coli*.) A later study that was more careful to control temperature purported to discover circadian rhythms of growth rate in *Klebsiella* spp. (54), but a circadian trend is not obvious from the raw data and requires extensive statistical analyses to uncover. Moreover, the only circadian property addressed in that study was an ∼24-h oscillation; the other salient properties—temperature compensation and entrainment—were neglected. At this writing, there is no persuasive evidence that circadian clocks reside in prokaryotes other than cyanobacteria.

Nonetheless, we think that there is a good chance that circadian clocks will be found in other eubacteria for which a daily timekeeper will enhance fitness. The keys are to find the proper conditions and to choose an appropriate parameter to measure. What about the third great domain of biology, the *Archaea*? Despite the fact that the ecological niche occupied by some archaeabacteria, e.g. the halobacteria, is not very different from that occupied by cyanobacteria [i.e. both live in an aqueous habitat and derive their energy from the daily sunlight cycle (in the case of halobacteria, under anaerobic conditions)], there is no direct evidence that archaeabacteria have invented an endogenous daily clock. In fact, it is not clear that anyone has yet looked seriously for circadian clocks in archaeabacteria. The existence of putative *kaiC* homologs in *Archaea* encourages a concerted hunt for clocks, but we must also caution that the *Archaea* in which a *kaiC* homolog has been found are methanogens and/or extremophiles that inhabit environments for which an endogenous 24-h clock would not appear to be adaptive (e.g. deep-sea methane vents, hot springs, etc). Discovering circadian clocks in archaeabacteria could help us to understand much about the evolution of circadian rhythms.
Figure 5 Deduced amino acid sequences of the products of kaiC gene from *Synechococcus* sp. strain PCC 7942 and its putative homologs from the cyanobacterium *Synechocystis* sp. strain PCC 6803 and the archaea *Archaeoglobus fulgidus, Pyrococcus horikoshii, Methanobacterium thermoautotrophicum*, and *Methanococcus jannaschii* (data from References 4, 21, 26–28, 50). An asterisk underlines residues that are identical in the majority of these proteins. Putative ATP/GTP-binding site motifs (P-loop, GXXXXGKT/S) are indicated by a double underline.
Figure 5  (continued)
CONCLUDING REMARKS

Just as fruitfly pupae metamorphose and eclose as winged adults, so has the circadian clock field undergone a dramatic transformation in the past 10 years. Much of that transformation is caused by the rapid progress on identifying clock components in \textit{Drosophila melanogaster}, \textit{Neurospora crassa}, plants, and mammals. More subtle transformations, but no less profound, include the dislodging of several entrenched dogmas, including the idée fixe that prokaryotes were too simple to have complex timepieces and that rapidly growing cells cannot keep track of circadian time. Studies of cyanobacteria have single-handedly disposed of these dogmas and have also provided the first rigorous demonstration of the fitness value of circadian programming. We expect that, with the shattering of the prokaryotic barrier, new insights into the distribution, mechanism, adaptiveness, and evolution of circadian clocks will be forthcoming.

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