J. Phycol. **37,** 933–942 (2001)

MINIREVIEW

ALGAE KNOW THE TIME OF DAY: CIRCADIAN AND PHOTOPERIODIC PROGRAMS[1]

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Eukaryotic algae have long served as model systems for analyses of circadian (daily) rhythms of many phenomena, including rhythmic gene expression, cell division timing, taxes, photosynthesis, and others. More recently, circadian clocks have been demonstrated in cyanobacteria, and rapid progress on the characteristics and mechanism of timekeeping in these prokaryotic algae ensued. These daily timekeepers enhance the fitness of algae and may contribute to seasonal responses.

Key index words: **circadian rhythms; *Chlamydomonas*; *kai* genes; *Lingulodinium*; photoperiodism; *Synechococcus***

Abbreviations: **CCTR, circadian-controlled translational regulator; LBP, luciferin-binding protein; PTM, photoperiodic time measurement**

Circadian (daily) rhythms are endogenous biological programs that time metabolic, physiological, and/or behavioral events to occur at optimal phases of the daily cycle. In addition to exhibiting a self-sustained oscillation in constant conditions that can be entrained to environmental cycles, circadian rhythms run at essentially the same speed at different ambient temperatures (i.e. they are "temperature compensated"). The overall organization of circadian systems is usually thought at the simplest level to include three major parts: (1) the input pathway (photoreceptors and thermoreceptors), (2) the central biochemical clockwork, and (3) output pathways that terminate on the observable rhythms (Johnson and Hastings 1986; a more detailed view is described in Johnson 2001). The first report of a circadian rhythm in a unicellular organism was that of phototaxis in the photosynthetic protist *Euglena* (Pohl 1948, Bruce and Pittendrigh 1956). Soon thereafter, reports appeared that demonstrated temperature-compensated daily rhythms in the algae *Lingulodinium polyedrum* (formerly called *Gonyaulax polyedra*; Hastings and Sweeney 1957, Sweeney and Hastings 1957), and *Acetabularia* (Sweeney and Haxo 1961).

From those roots, appreciation of the ubiquity of circadian rhythms among algae blossomed. A wide variety of rhythmic phenomena is exhibited by algae, including taxes (phototaxis and chemotaxis), the timing of cell division, photosynthetic capacity, bioluminescence, gene expression, sensitivity to UV light, and many others (Sweeney 1987, Edmunds 1988, Johnson and Golden 1999, Nikaido and Johnson 2000, Johnson and Kondo 2001). This review concentrates on studies of circadian clocks in two genetically tractable algae, the prokaryotic cyanobacterium *Synechococcus elongatus* PCC7942 and the eukaryotic alga *Chlamydomonas reinhardtii*. Other studies in other algae are mentioned as well. More complete descriptions of circadian phenomena in other algal species are published elsewhere (Sweeney 1987, Edmunds 1988, Johnson and Kondo 2001).

DO SINGLE ISOLATED CELLS HAVE CLOCKS AND IF SO, HOW MANY?

An important topic in "circadiana" for which algae have been important model systems is the issue of circadian organization at the population, cellular, and subcellular levels. Because unicellular algae express a variety of circadian rhythms, it might seem obvious that isolated single cells therefore can express circadian rhythms. But almost all studies on unicellular algae have been conducted on populations. Thus, it is conceivable that the circadian rhythms could be an emergent property of intercell interactions within the population. Perhaps cells within the population secrete a factor into the medium that accumulates to a threshold concentration that triggers the secretion of a compensatory second factor that degrades the first factor, and so on. This is not idle speculation: the intercellular signaling of *Dictyostelium* by secretion of cAMP is famous, and there is an example of a molecule made by an alga that affects the period of its circadian clock—the molecule is "gonyauline" from *Lingulodinium* (Roenneberg et al. 1991). Moreover, there is evidence in *Lingulodinium* that suggests a weak coupling of the circadian rhythms among cells in a population (Broda et al. 1985).

Although it is probably the case that the population rhythms of algae reflect circadian oscillations occurring independently within each cell, it may be unsettling to realize how few organisms have been shown to express a persisting circadian rhythm from a single isolated cell for more than one cycle. The most famous example is the alga *Acetabularia* (there is also evidence from *Lingulodinium*, but those measurements have been for one cycle or less). It was established long ago that single isolated cells of *Acetabularia* express circadian rhythms of photosynthetic oxygen evolution, even in cells without a nucleus (Sweeney and Haxo 1961, Karakashian and Schweiger 1976, Woolum 1991). More recently, other rhythms have been observed to persist for long free-runs in isolated cells of *Acetabularia* (Broda and Schweiger 1981). Finally, temperature compensa-

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tion has been shown using single isolated cells of *Acetabularia*, and free-running periods were measured at various temperatures in the same cell (Berger et al. 1992).

Can single cells have more than a single clockwork? Studies before 1992 in which two or more overt rhythms were monitored simultaneously from the same population of algae found that the periods and phase angles of various rhythms appeared to be rigidly constant. The best-documented example was that of *Lingulodinium*, where the phase relationships among several different rhythms did not waver during long free runs or after phase resetting (McMurry and Hastings 1972, Sweeney 1987). This and similar observations led circadian biologists to believe that each single cell houses only one circadian oscillator mechanism. More recent experiments in *Lingulodinium*, however, have demonstrated internal desynchronization of various rhythms under appropriate conditions of illumination and temperature (von der Heyde et al. 1992, Roenneberg and Morse 1993). In the decoupled state, the two oscillators exhibit phase responses to light:dark (L:D) signals that are partially independent (Morse et al. 1994). Although alternative explanations may exist, it now seems likely that even single cells can have multiple circadian pacemakers that are usually coupled but can decouple if the conditions are right.

PROKARYOTIC CLOCKS

After the prior work in eukaryotic algae, the discovery of circadian phenomena in cyanobacteria was a delightful surprise. Before the mid-1980s, it was believed that only eukaryotes had circadian systems (Johnson et al. 1996). The conclusion that only eukaryotes have circadian oscillators seemed reasonable, because it was assumed that an endogenous timekeeper with a period close to 24 h would not be useful to prokaryotic organisms that often divide more rapidly than once every 24 h (however, see below in the cell division section). In 1985 and 1986, several research groups discovered that diazotrophic cyanobacteria display daily rhythms of nitrogen fixation in both L:D cycles and in constant light. In particular, 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 demonstrated the salient characteristics of circadian rhythms in the unicellular freshwater cyanobacterium, *Synechococcus* sp. RF-1 (Grobbelaar et al. 1986, Huang and Grobbelaar 1995).

A crucial characteristic that needed to be demonstrated for cyanobacteria before they could be accepted into the circadian fold was that of temperature compensation. This criterion was first satisfied by studies of two species of *Synechococcus*: the marine *Synechococcus* WH7803 and the freshwater *Synechococcus* RF-1. Sweeney and Borgese (1989) showed that WH7803 displays temperature-compensated rhythms of cell division, whereas Chen et al. (1991) found temperature-compensated rhythms of amino acid uptake in the freshwater cyanobacterium isolated from rice fields, *Synechococcus* RF-1. *Synechococcus* RF-1 also expresses a nocturnal circadian rhythm of nitrogen fixation that has been well studied by the group in Taiwan, including the demonstration that a rhythm of nitrogenase mRNA abundance probably accounts for the nitrogen fixation rhythm (Huang and Chow 1990). These findings, along with those of others (including ours in non-nitrogen-fixing cyanobacteria), have displaced the "no circadian clocks in prokaryotes" dogma (Johnson et al. 1996). Despite the fact that this dogma is a relic of the past, however, cyanobacteria remain the only noneukaryotic system for which circadian organization is proven at this time.

CELL DIVISION VERSUS CIRCADIAN CYCLES: DO MULTIPLE TIMING CIRCUITS BECOME CONFUSED?

Circadian rhythms of the timing of cell division have been reported in many algal species. The marine dinoflagellate *Lingulodinium* was the first organism for which persuasive evidence of circadian cell division was demonstrated (Sweeney and Hastings 1958). *Lingulodinium* divides at the end of darkness when the cells are in an L:D cycle, and this periodicity of cell division continues for up to 14 days in constant dim light. Other eukaryotic unicellular organisms for which circadian gating of cell division has been demonstrated include *Chlamydomonas* (Goto and Johnson 1995), *Euglena* (Edmunds 1966, 1988), *Paramecium* (Volm 1964), and *Tetrahymena* (Wille and Ehret 1968). However, when populations of eukaryotic cells are grown in conditions that allow them to divide with a doubling time of less than 24 h, the daily periodicity of division disappears. From observations in eukaryotic microorganisms (especially *Lingulodinium*, *Euglena*, and *Tetrahymena*), an empirical rule called the "circadian-infradian rule" was promulgated, stating circadian rhythms are expressed only in cells that are dividing once per day or more slowly (Ehret and Wille 1970, Edmunds 1988). The rule implied that there is an interdependency between the cell division cycle (CDC) and circadian timing circuits such that when cell division is more rapid than once per day, the cells are unable to maintain an independent circadian oscillation (Mori and Johnson 2000).

In the cyanobacterium *Synechococcus elongatus*, cell division is regulated by the circadian clock (Mori et al. 1996). Unlike the case with eukaryotic algae, however, the circadian system in *S. elongatus* proceeds at its usual pace whether the cells are dividing rapidly (doubling time of 6–12 h) or slowly (doubling times of 50–100 h) (Mori et al. 1996). Overexpression of the *ftsZ* gene halted cell division but not growth, causing cells to grow as filaments without dividing (Fig. 1). The nondividing filamentous cells still exhibited robust circadian rhythms of gene expression (Mori and Johnson 2001a). These results indicate that in *S. elongatus*, the circadian timing system controls or "gates" rhythmic cell division but is otherwise independent of the cell division cycle. Therefore, *S. elongatus* does not obey the tenets of the circadian-infradian rule. Together with other results, these observations indicate that the cyanobacterial circa-

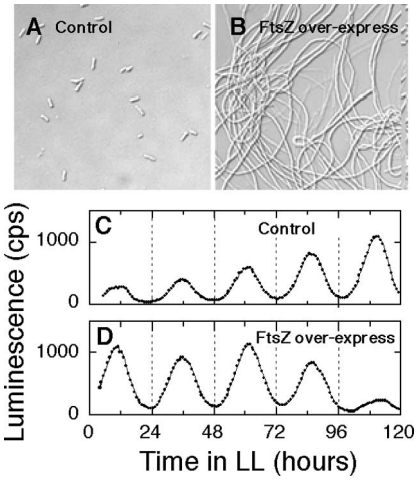

FIG. 1. Circadian rhythms of gene expression continue in dividing vs. nondividing cells of *Synechococcus elongatus*. Cell division of growing cyanobacteria is stopped by overexpression of the cell septum protein FtsZ. (A and B) Morphology of (A) control cells and (B) cells in which FtsZ is overexpressed. (C and D) Luminescence rhythms in (C) control cells and (D) cells in which division has been halted by FtsZ overexpression. Luminescence rhythms are monitored from reporters of luciferase-promoter fusions (Kondo et al. 1993). The reporter in C and D is the *kaiBC* promoter driving bacterial luciferase expression. LL, constant light. (Adapted from Figs. 4 and 5 in Mori and Johnson 2001a.)

dian system is stable and well sustained under a wide range of intracellular conditions.

CIRCADIAN GENE EXPRESSION

In cyanobacteria, circadian rhythms of gene expression are not only common, they are ubiquitous. A promoter trap strategy discovered that essentially every promoter in the chromosome of *S. elongatus* is regulated by the circadian clock (Liu et al. 1995), as are heterologous *Escherichia coli* promoters expressed in this species (Katayama et al. 1999). In the eukaryote *Chlamydomonas*, the extent of circadian gene expression has not been comprehensively measured, but it must be very significant. Relatively random "fishing expeditions" for rhythmically expressed genes in *Chlamydomonas* have already caught several clock-controlled genes (Hwang and Herrin 1994, Jacobshagen and Johnson 1994, Fujiwara et al. 1996, Hwang et al. 1996), and a systematic approach to gauging the number of clock-controlled genes would undoubtedly net a large "school" of such genes. Both nuclear-encoded (Hwang and Herrin 1994, Jacobshagen and Johnson 1994, Fujiwara et al. 1996) and chloroplast-encoded genes (Hwang et al. 1996) are controlled by the circadian pacemaker in *Chlamydomonas*.

Transcriptional control. How is circadian control over gene expression exerted? For most clock-controlled genes in photosynthetic organisms, mRNA abundances oscillate over the circadian cycle. Usually, these abundance rhythms have been found to be at least partially due to transcriptional control (McClung 2001). A curious observation of the studies of mRNA abundance rhythms is that in some cases they do not promote equivalently robust rhythms of the levels of their corresponding proteins (Beator and Kloppstech 1994, Heintzen et al. 1994). What is the function of the mRNA rhythms if there is no rhythm of the corresponding protein? For example, in *Chlamydomonas*, there are rhythms of the light-harvesting complex (*lhc*) protein synthetic rate even though the overall LHC protein abundance is essentially constant (Shan 1995). Apparently, these proteins turn over much more slowly than their mRNA counterparts. In such a case, one wonders about the adaptive significance of the mRNA abundance rhythms. One speculation is that the significance of the mRNA rhythm has to do with rhythmic *turnover* of the proteins, even though the total amount of protein is constant. For example, degradative rates of LHC proteins are likely to be higher during the day due to damage from sunlight; therefore, to maintain a constant level of intact LHCs, new synthesis of LHC protein is necessary every day.

Translational regulation. In contrast to the many studies implicating transcriptional control in controlling circadian gene expression, there are only a few examples of translational control of a circadian rhythm. The best characterized has been in the dinoflagellate *Lingulodinium*, which exhibits a precise circadian rhythm of spontaneous bioluminescent light emission that is one of the best characterized circadian outputs at the molecular level (Morse et al. 1990). The biochemical components of the bioluminescent reaction have been identified, so it has been possible to assess the mechanisms by which the circadian clock modulates each individual component so as to determine how the luminescence capacity is controlled rhythmically. The intracellular concentration of each of the three major components, the enzyme (luciferase), substrate (luciferin), and substrate-binding protein (luciferin-binding protein [LBP]), oscillates during the circadian cycle (Johnson et al. 1984, Morse et al. 1990). In fact, an entire organelle in *Lingulodinium*—the *scintillon*—undergoes circadian synthesis and degradation (Fritz et al. 1990). In another dinoflagellate, *Pyrocystis lunula*, the organization of rhythmic luminescence is different from that in *Lingulodinium*, but a common feature between the two species is rhythmic changes in the ultrastructural correlates of the luminescence system (Seo and Fritz 2000).

In *Lingulodinium*, the molecular control of the LBP has been extensively studied. Even though the abundance of LBP and the rate of LBP synthesis undergoes a large circadian change, the abundance of the mRNA that encodes LBP is *constant* over the daily cycle (Morse et al. 1990). The circadian clock controls when the translation of this mRNA is initiated. In subsequent studies of this apparent translational control, a protein that specifically binds to the 3′ untranslated region of the *lbp* mRNA was discovered and named circadian-controlled translational regulator (CCTR) (Mittag et al. 1994). The binding activity of this RNA-binding pro-

tein in extracts undergoes a circadian oscillation, with its lowest levels coinciding with the phases at which the *lbp* mRNA is translated. This result implicates the specific mRNA-binding protein as a translational repressor of LBP synthesis (Mittag et al. 1994).

When *Lingulodinium lbp* mRNA was tested with extracts from *Chlamydomonas*, an analogous circadian binding activity was discovered in that alga, implying a conservation of translational regulatory mechanisms in diverse algae (Mittag 1996). This analogous protein was called "Chlamy 1," and both it and CCTR bind specifically to UG repeats in the *lbp* mRNA sequence. The binding activity of Chlamy 1 is also controlled by the circadian clock, but in contrast with CCTR, which binds maximally in the day, Chlamy 1 binds maximally during the night (Mittag 1996, 1998). UG-repeat sequences in untranslated regions and coding regions of mRNA are common throughout the eukaryotes, so this might be a common but unappreciated method of regulation (Mittag 1996, 1998).

WHY DO ALGAE HAVE ENDOGENOUS TEMPORAL PROGRAMS?

Although circadian and photoperiodic timers are found in photosynthetic and nonphotosynthetic organisms, the L:D cycle is absolutely crucial to photosynthetic organisms because light is their source of energy and life. Therefore, it is probably no accident that the unicells in which circadian rhythms were first discovered were photosynthetic: for eukaryotes, *Euglena*, and for prokaryotes, the cyanobacterium *Synechococcus*.

One hypothesis concerning the advantage of having a daily biological clock is that it organizes a temporal *program* that cues processes to occur at specific phases throughout the daily environmental cycle. This programming can enable the organism to *anticipate* changes in the environment that are important and enable it to respond appropriately. For example, a circadian alarm clock that heralds dawn can allow an organism to prepare its photosynthetic apparatus so as to capture the first photons of dawn. Temporal programming also permits the phasing of incompatible processes to occur at different times, as in the case of photosynthesis and nitrogen fixation in unicellular cyanobacteria (Mitsui et al. 1986). The nitrogen-fixing enzyme nitrogenase is inactive in the presence of oxygen, which creates a dilemma for organisms whose photosynthesis releases oxygen. As one of several tactics to allow these mutually incompatible tasks to proceed, some cyanobacteria have separated photosynthesis and nitrogen fixation *temporally*: photosynthesis in the daytime and nitrogen fixation at night. Another example of temporal separation of processes in algae is the separation of motility taxes in the alga *Chlamydomonas*: *photo*taxis in the daytime and *chemo*taxis (to ammonium) at night (Byrne et al. 1992).

Evolution of circadian timers. The question of why organisms have endogenous temporal programs is closely linked with identifying the selective forces that encouraged the original evolution of these timers. The presence of circadian oscillators in cyanobacteria, whose ancestors appear in the fossil record at least 3.5 billion years ago, implies that circadian clocks might have been an ancient invention of evolution (although it remains possible that circadian timers may have been absent in ancient cyanobacteria and evolved relatively recently). It is possible that a selective pressure for elaborating an internal timekeeper could have been the advantage of anticipation and/or temporal separation of metabolic activities suggested above.

Perhaps an initial driving force for the early evolution of circadian clocks could have been the advantage inherent in phasing cellular events that are inhibited by sunlight to occur in the night. This idea has been called the "escape from light" hypothesis (Pittendrigh 1993). That speculation seems plausible when one considers the numerous examples of microorganisms with 24-h cell division cycles in which DNA replication and cell division occur during the night (Edmunds 1988). Some of the events of the cell division cycle in these microorganisms might be sensitive to sunlight (e.g. S phase).

If this hypothesis about the early evolution of circadian programs is correct, then we would predict that present-day organisms might still exhibit temporal regulation of light-sensitive processes to the night. Because the most generally deleterious wavelengths of sunlight are in the UV range, we tested whether there is a daily rhythm of sensitivity to UV light in *Chlamy-*

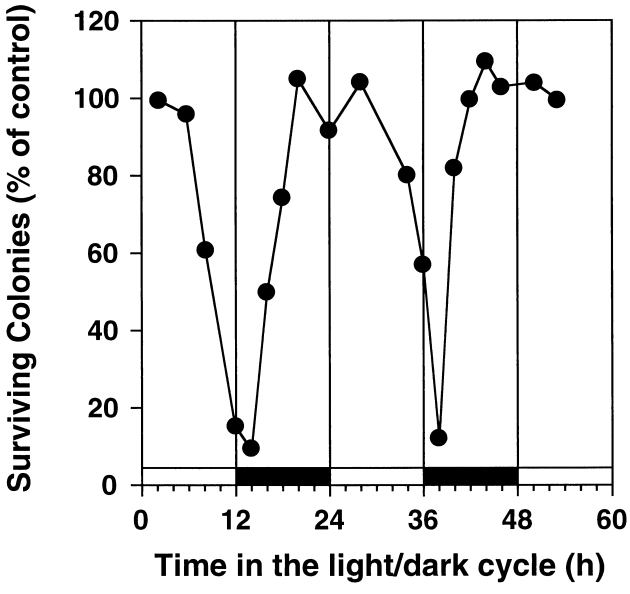

FIG. 2. Survival of *Chlamydomonas* cells after irradiation by UV light as a function of the time in an L:D cycle. *Chlamydomonas* cultures were plated onto agar medium and treated with equal amounts of UV light at different phases of a L:D 12:12 cycle. Survival was measured as the colony-forming ability of cells after treatment as compared with cells that were not irradiated with UV light. (Adapted from Fig. 3 in Nikaido and Johnson 2000.)

domonas (Nikaido and Johnson 2000). As shown in Figure 2, these algae are more sensitive to UV light near sunset and into the early night. The rhythmic sensitivity persists in constant conditions (Nikaido and Johnson 2000). These data are consistent with the hypothesis that the circadian system in *Chlamydomonas* has programmed UV-sensitive processes to occur at phases of the daily cycle when UV levels will be low or absent (Nikaido and Johnson 2000).

Do daily clocks enhance fitness? The rhythm of UV sensitivity described above implies that the temporal program regulated by the circadian clock is adaptive. However, there had been no direct definitive tests for whether circadian clocks enhance fitness. We performed the first rigorous test of the adaptive significance of circadian programs by using competition experiments between different strains of *S. elongatus* (Ouyang et al. 1998). These cyanobacteria do not conjugate under laboratory conditions, and many mutant strains have been isolated that exhibit different free-running periods (Kondo et al. 1994). For asexual microbes, differential growth of one strain under competition with other strains is a good measure of reproductive fitness. The circadian phenotypes of the strains used had free-running periods of about 22, 25 (wild-type), and 30 h. In pure culture, the strains grew at about the same rate in constant light and in L:D cycles, so there did not appear to be a significant advantage or disadvantage to having different circadian periods when the strains were grown individually. The fitness test was to mix different strains together and to grow them in competition to determine if the composition of the population changes as a function of time. The cultures were diluted at intervals to allow growth to continue (Fig. 3A).

The results of this competition test were satisfying (Ouyang et al. 1998). When each strain was mixed with another strain and grown together in competition, a pattern emerged that depended on the frequency of the L:D cycle and the circadian period. When grown on a 22-h cycle (11 h light, 11 h dark), the 22-h-period mutant became the dominant cell type in the mixed cultures. On a 30-h cycle (15 h light, 15 h dark), the 30-h-period mutant ("C28a") could defeat either wild-type or the 22-h-period mutant. On a "normal" 24-h cycle (12 h light, 12 h dark), the wild-type strain could overgrow either mutant. Figure 3B shows results from the competition between wild-type and the 30-h-period mutant C28a. Clearly, the strain whose period most closely matched that of the L:D cycle eliminated the competitor. Under a nonselective condition (in this case, constant light), each strain was able to maintain itself in the mixed cultures. Because the mutant strains could defeat the wild-type strain in L:D cycles in which the periods are similar to their endogenous periods, the differential effects that were observed are likely to result from the differences in the circadian clock. A genetic test was also performed to demonstrate that the clock gene mutation was specifically responsible for the dif-

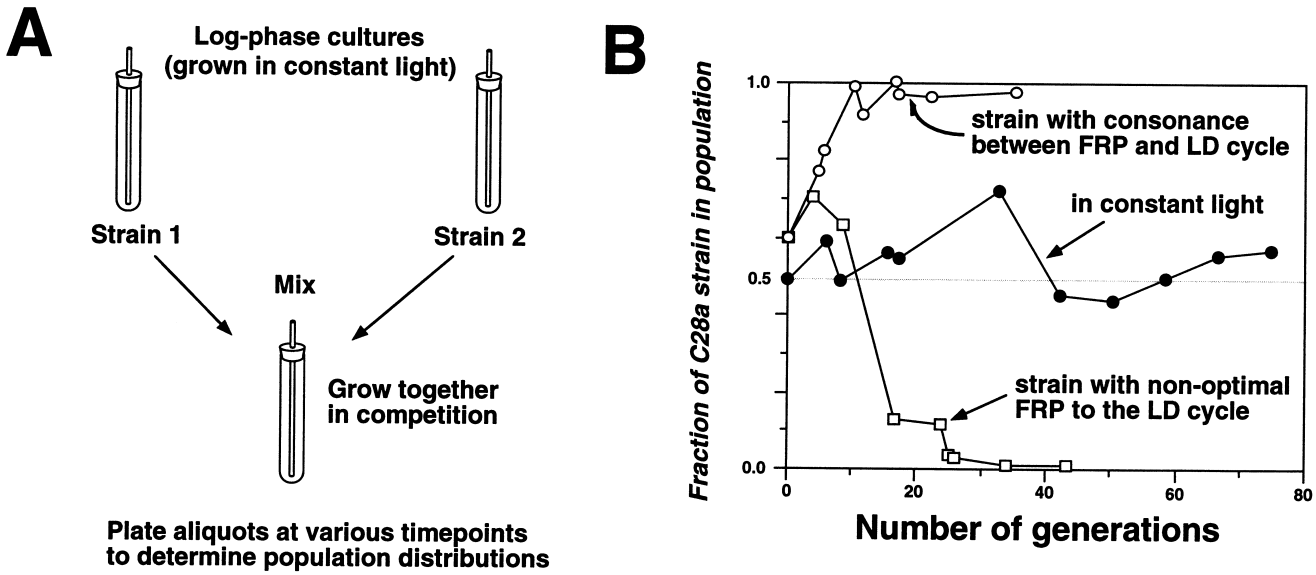

FIG. 3. Competition of circadian strains in different light/dark cycles. (A) Different strains of cyanobacteria were mixed together in batch cultures and grown in competition under different light/dark cycles. Every 8 days, the cultures were diluted with fresh medium. At various times during the competition, aliquots were plated as single colonies and their luminescence rhythms were monitored to determine the frequency distribution of the different circadian phenotypes. (B) The strain whose endogenous free-running period most closely matched that of the environmental light/dark cycle was able to out-compete strains with a nonoptimal period. In constant light (nonselective conditions), all the strains were able to maintain their initial fraction in the population. FRP, free-running period; LD, light/dark cycle. These data show the competition between a 30-h mutant (C28a) and wild-type (FRP = 24 h). The upper trace is on a 30-h light/dark cycle, the middle trace is from constant light, and the lower trace is on a 24-h light/dark cycle. Ordinate, fraction of C28a mutant in the mixed population. (Adapted from Fig. 3 in Ouyang et al. 1998.)

ferential effects in the competition experiment (Ouyang et al. 1998). This was the first rigorous demonstration in any organism of a fitness advantage conferred by a circadian system.

PHOTOPERIODISM AND SEASONAL RESPONSES

One clock-involved process for which the adaptive significance is clear is that of photoperiodic time measurement (PTM). PTM is the ability of plants and animals to sense the season of the year by measuring the duration of the day and/or night in the natural environment and respond appropriately so as to adapt to seasonal changes in their environment (Thomas and Vince-Prue 1997). This phenomenon is of fundamental importance to the biological adaptations of organisms to their environment, especially in the cases of reproduction and development. The first demonstration that organisms sense the changing season by measuring the duration of the day or night was in plants, where the seasonal flowering of plants was shown to be controlled by photoperiod in 1920 (Garner and Allard 1920). The model for PTM that has become generally accepted is that a circadian (daily) clock is the timer that somehow measures the length of the night and triggers the developmental events controlled by photoperiodism, as first proposed by Erwin Bünning in 1936 (Bünning 1936) and now supported by multiple lines of evidence (Thomas and Vince-Prue 1997, Johnson 2001).

To date, PTM is well documented in multicellular eukaryotes, but it seems logical that unicellular organisms might also benefit from being able to anticipate and respond to seasonal changes in their environment. From the perspective of the evolution of PTM and responses to season, the question of whether unicellular organisms can accomplish photoperiodic timing is important. Nevertheless, there has been only one prior report of a photoperiodic response in unicells, namely in the dinoflagellate alga *Lingulodinium* (Balzer and Hardeland 1991). The authors of that paper reported that *Lingulodinium* cells formed "temporary cysts" in response to cool temperatures and short photoperiods, and they hypothesized that these cysts were the first step toward differentiation of a permanent dormant cyst for overwintering. This was an attractive hypothesis because an annual cycle of *Lingulodinium* "permanent cysts" in ocean sediments had been reported by a different group (Anderson and Keafer 1987). However, Balzer and Hardeland (1991) did not demonstrate that the temporary cysts could develop into permanent cysts, so the ecological relevance of their observations is unclear.

We recently completed a study of PTM in the green alga *Chlamydomonas reinhardtii* (Suzuki and Johnson unpublished data). In the life cycle of *Chlamydomonas*, nonoptimal conditions (e.g. nitrogen deprivation) provoke the differentiation of haploid vegetative cells into gametes, which mate and form diploid zygospores. Zygospores are dormant cells that cannot divide until after they undergo meiosis and germinate into four haploid vegetative cells, and they resist stressful environmental conditions, such as freezing, darkness, desiccation, and starvation, better than vegetative or gametic cells (Harris 1989, Suzuki and Johnson unpublished data). As shown in Figure 4, we found that the germination efficiency of *Chlamydomonas* zygospores is enhanced (~90% germination) in long-day L:D cycles (i.e. photoperiods longer than 10 h) but is suppressed (<25% germination) in short-day L:D cycles (i.e. photoperiods of 8–9 h) (Suzuki and Johnson unpublished data). Zygospores that differentiate in short-day conditions can be germinated after a prolonged treatment with darkness, so these short-day zygospores are not a terminal stage. Comparisons of photoperiod

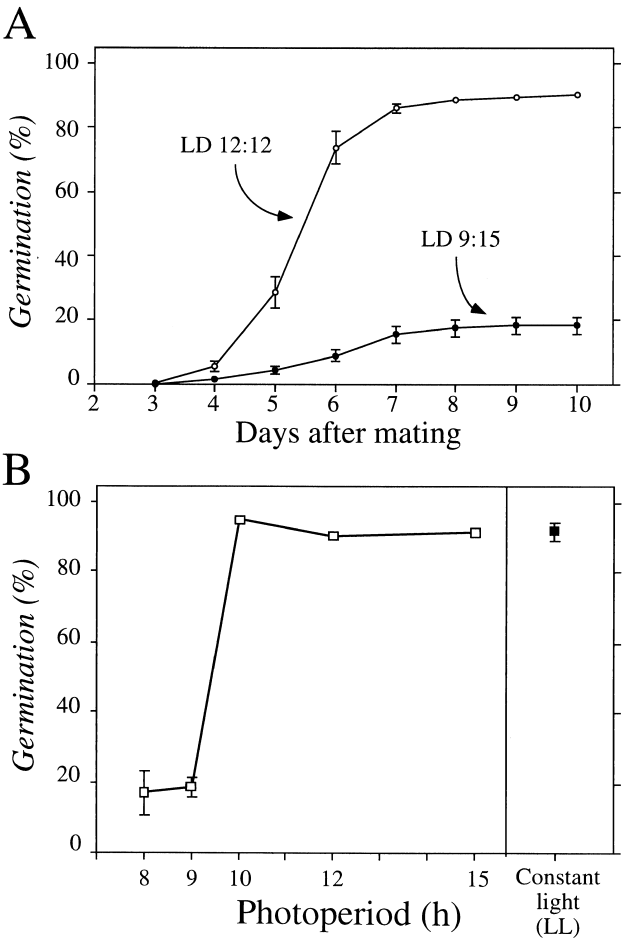

FIG. 4. Germination in different photoperiods in *Chlamydomonas*. (A) Germination kinetics. Germination of zygospores of strain 137c (mt+/mt−) was monitored in long-day conditions (light/dark, 12:12, open circles) and in short-day conditions (light/dark, 9:15, closed circles). LD, light/dark. (B) Photoperiodic response curve. The germination efficiencies 10 days after mating are shown as a function of the light duration in light/dark cycles of 24 h total duration (open squares). Constant light (LL, closed square) represents cells continuously exposed to light for 24 h a day. (A and B) Germinated cells were counted at 10 days after mating, and all data are shown with SEM. (Adapted from Fig. 1 in Suzuki and Johnson an unpublished manuscript.)

versus fluence demonstrated that *Chlamydomonas* germination was a *bona fide* photoperiodic response and not merely a response to fluence (Suzuki and Johnson unpublished data). Thus, it is likely that the suppression of germination by short days is an adaptive response for the overwintering of *Chlamydomonas*.

A "CLOCKWORK GREEN"

Without a doubt, the most successful strategy for unveiling circadian components has been screening for mutants with altered phenotypes, cloning the genes that have been mutated, and characterizing the expression patterns and products of those genes. The genetic approach to analyzing circadian rhythms has been applied to two algae, the eukaryote *Chlamydomonas* and the prokaryotic cyanobacterium *Synechococcus*.

Chlamydomonas. At about the same time that circadian period mutants were first reported in *Neurospora* (Feldmann and Waser 1971) and *Drosophila* (Konopka and Benzer 1971), the first mutants affecting circadian rhythms in *Chlamydomonas* were isolated (Bruce 1972, 1974, Bruce and Bruce 1978). Bruce used the phototaxis rhythm to screen for mutations induced by nitrosoguanidine. He found four long-period mutants that were named *per* for "period" but were not homologs of the *Drosophila* or mammalian *per* genes. At 22° C, the mutants had the following periods: *per-1* (27 h), *per-2* (26.5 h), *per-3* (26.5 h), and *per-4* (28 h). Unlike the first clock genes identified in *Neurospora* and *Drosophila*, these mutants appeared to be at different loci (Bruce 1974). In addition to the Bruce mutants, Mergenhagen identified a spontaneous mutant with an 18-h period (Mergenhagen 1984). Mergenhagen and Mergenhagen (1987) blasted this short-period mutant and wild-type *Chlamydomonas* into outer space and obtained clear data showing that circadian rhythms of phototaxis persist robustly away from earth. Unfortunately, neither the Bruce mutants nor the Mergenhagen mutant have been further characterized to any significant degree nor is there other information on how the *Chlamydomonas* clock ticks. We hope the sequencing of the *Chlamydomonas* genome that is underway (see www.biology.duke.edu/chlamy_genome) will initiate a new phase of scrutiny into clock mutants and the clockwork of *Chlamydomonas*.

Cyanobacteria. On the other hand, there has been significant progress in the analysis of the cyanobacterial clockwork. More than 100 mutants exhibiting various circadian phenotypes, including arhythmia, altered waveforms, and atypical periods (ranging between 14 and 60 h), have been isolated from *S. elongatus* (Kondo et al. 1994). Over 30 of these mutants have been rescued by the introduction of libraries of wild-type *S. elongatus* DNA. DNA fragments from several rescued mutants complemented other mutant phenotypes, including short-period, long-period, and arhythmic phenotypes.

These rescue experiments pinpointed a cluster of three adjacent genes, named *kaiA*, *kaiB*, and *kaiC* (Ishiura et al. 1998, "kai" is Japanese for "rotation" or "cycle number"). Nineteen mutants were rescued by transformation with a plasmid carrying the entire *kaiABC* cluster, and the relevant mutations were mapped to the three *kai* genes by DNA sequencing. All are missense mutants resulting from single nucleotide exchanges. Most mutations are recessive (rescue by wild-type DNA is complete), but a few are semidominant. Each of the three genes has at least two clock mutations mapped to it, and the largest gene, *kaiC*, has multiple mutations that include many clock phenotypes: short period, long period, low amplitude, and arhythmia. 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 individually does not affect viability (in single-strain cultures) but causes arhythmicity. None of the Kai proteins has an obvious DNA-binding motif.

There are putative homologs of KaiC in other prokaryotes, especially Archaebacteria, but no homologs to any of the Kai proteins have yet been discovered in eukaryotes (Johnson and Golden 1999). A recent genomic survey claims that KaiC is a member of the bacterial RecA/DnaB family (Leipe et al. 2000). RecA is an ATP-dependent DNA recombinase, and DnaB is the replication fork helicase in bacteria. The *kaiC* gene appears to be an internally duplicated version of a RecA/DnaB-like gene: it has two parts that are very similar to each other (Iwasaki et al. 1999). In each half of the *kaiC* gene, there is a Walker A or "P-loop" motif. This motif, [G or A]XXXXGK[T or S], is often an ATP/GTP nucleotide binding region. Indeed, KaiC binds ATP (and GTP with a lower affinity), and when the Walker A motifs are mutated, the nucleotide binding is abolished and rhythmicity is severely disrupted or abolished (Nishiwaki et al. 2000, Mori and Johnson 2001b). Therefore, this motif is essential in both parts of the KaiC molecule for rhythmicity.

The rhythmic expression patterns of the *kai* genes are reminiscent of eukaryotic clock genes from *Drosophila* and *Neurospora*. 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 *kaiBC* promoter produces a dicistronic *kaiBC* mRNA (Ishiura et al. 1998). Both *kaiA* and *kaiBC* transcripts are rhythmically abundant (Ishiura et al. 1998, Iwasaki et al. 2000). Continuous overexpression of *kaiC* repressed the *kaiBC* promoter (negative feedback), whereas *kaiA* overexpression enhanced it (positive feedback). The abundances of the KaiB and KaiC proteins undergo circadian oscillations in constant light, whereas the pattern of KaiA abundance exhibits little, if any, circadian variation (Xu et al. 2000). Pulsatile expression of the *kaiC* gene from an inducible promoter to physiological levels resets the phase of the rhythms (Ishiura et al. 1998, Xu et al. 2000). Therefore, it appears that the level of KaiC expression (or some form of KaiC) is directly linked to the phase of the oscillation.

A feature of the Kai proteins that is reminiscent of clock proteins in eukaryotes is that Kai proteins appear to interact. Two-hybrid, *in vitro* binding, and resonance energy transfer assays indicate that the KaiA,

KaiB, and KaiC proteins interact both homotypically and heterotypically (Iwasaki et al. 1999, Xu et al. 1999). One long-period mutant exhibits an altered heterotypic interaction between KaiA and KaiB, suggesting that inter-Kai contact is important to the clock mechanism (Iwasaki et al. 1999). Many of the period-altering mutations in *kaiC* have been mapped to two KaiA-binding domains in KaiC (Taniguchi et al. 2001). Another KaiC-interacting protein is a histidine protein kinase, SasA (Iwasaki et al. 2000). The N-terminal sequence of SasA is similar to the sequence of KaiB. Disruption of *sasA* lowers *kaiBC* expression and results in arhythmicity of the expression of most genes in moderate to high light intensities. Clearly, SasA is necessary for robust expression of the cyanobacterial clock (Iwasaki et al. 2000). Interestingly, low amplitude rhythms were observed in dim constant light, implying that the role of SasA has something to do with light perception or light intensity compensation. Another gene that appears to be involved in light perception by the cyanobacterial clock is the phytochrome-like gene, *cikA* (Schmitz et al. 2000).

Despite these advances, the mechanism of the cyanobacterial clockwork is still a tantalizing figure obscured by shimmering veils of disparate observations. Several working hypotheses have been proposed for 1) the central pacemaker invoking rhythms of transcription/translation (Ishiura et al. 1998) and 2) the global regulation of gene expression invoking regulation of transcriptional factors (Liu et al. 1995, Tsinoremas et al. 1996). We recently proposed a new model for the fundamental mechanism of the clockwork in cyanobacteria (Mori and Johnson 2001b) that was inspired by a prior publication demonstrating daily rhythms of chromosomal topology in the chloroplast chromosome of *Chlamydomonas* (Salvador et al. 1998). Because the key clock protein KaiC is related to bacterial recombinases and helicases, KaiC might act directly on DNA. These observations tempt us to consider that KaiC might mediate both its own negative feedback regulation (Ishiura et al. 1998) and global regulation of the cyanobacterial genome (Liu et al. 1995) by orchestrating oscillations in the condensation and/or supercoiling status of the entire cyanobacterial chromosome by analogy to the observations in the *Chlamydomonas* chloroplast chromosome (Salvador et al. 1998). We therefore propose that a fundamental component of the cyanobacterial clockwork is that the condensation/supercoiling status of the chromosome rhythmically changes such that it becomes an oscillating nucleoid, or "oscilloid" (Mori and Johnson 2001b). Time will tell us whether this or another hypothesis about the cyanobacterial clockwork is correct.

Research on circadian programming in algae has advanced the circadian field, from the pioneering work on cell division, physiology, and biochemistry—most notably in *Lingulodinium* and *Acetabularia*—to genetics in *Chlamydomonas* and *Synechococcus*. Circadian control permeates all levels of biological organization. Algae have been, and will undoubtedly remain, important model systems for revealing the secrets of circadian programs at all levels of biological complexity.

C. H. J. thanks Drs. Takao Kondo and Susan Golden for an exciting and productive collaboration on circadian rhythms in algae (in the case of Dr. Kondo, extending back to 1985 and concerning work on both *Chlamydomonas* and cyanobacteria). We are grateful for support from the National Institute of Mental Health, the National Science Foundation, and the Human Frontier Science Program.

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