Metabolic Compensation and Circadian Resilience in Prokaryotic Cyanobacteria

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Abstract

For a biological oscillator to function as a circadian pacemaker that confers a fitness advantage, its timing functions must be stable in response to environmental and metabolic fluctuations. One such stability enhancer, temperature compensation, has long been a defining characteristic of these timekeepers. However, an accurate biological timekeeper must also resist changes in metabolism, and this review suggests that temperature compensation is actually a subset of a larger phenomenon, namely metabolic compensation, which maintains the frequency of circadian oscillators in response to a host of factors that impinge on metabolism and would otherwise destabilize these clocks. The circadian system of prokaryotic cyanobacteria is an illustrative model because it is composed of transcriptional and nontranscriptional oscillators that are coupled to promote resilience. Moreover, the cyanobacterial circadian program regulates gene activity and metabolic pathways, and it can be manipulated to improve the expression of bioproducts that have practical value.

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INTRODUCTION

Circadian rhythms are circa-24-h oscillations in biological processes that are controlled by an endogenous biochemical pacemaker. This pacemaker conducts a symphony of processes ranging from gene expression, metabolism, and cell division to development and behavior (1). Three canonical properties define circadian rhythms: (*a*) persistence of the oscillations in constant conditions [usually constant dark (DD) or constant light (LL) at constant temperature], (*b*) temperature compensation (the period length is only slightly affected by different constant temperatures within the physiological range, namely a Q_{10} value of approximately 0.9–1.1), and (*c*) entrainment of the endogenous pacemaker to the environmental light/dark cycle (LD) (1). Two findings that are very difficult to explain from a biochemical perspective are the precision of these long time-constant oscillators (precision of plus or minus only a few minutes per day for a period close to 24 h) and the temperature compensation property, which applies even to cells and tissues from endothermic animals (1–5). One might argue that the realization of the temperature compensation property of circadian rhythms was the "Eureka moment" that first drew widespread attention to circadian clocks (6, 7). When first observed in the 1950s—the heyday of enzymological investigations—this phenomenon was first termed temperature independence (6, 8), and it seemed unbelievable that a complicated biological process could be so resilient to temperature, which was considered at that time to universally affect biochemical reactions.

However, from an evolutionary perspective, we should not have been surprised. A temperature-dependent or imprecise clock would poorly estimate environmental time (1, 7). Therefore, an ~24-h clock with the conserved properties of temperature compensation, entrainment, and precision has been the product of convergent evolution in highly divergent organisms: prokaryotic cyanobacteria and essentially all eukaryotes ranging from fungi to plants to animals. Moreover, a reliable timekeeper must be stable and resilient to noise that is generated both intracellularly (and intraorganismally for multicellular organisms) and environmentally. Examples of sources of intracellular noise include cell division;

coordination of metabolic pathways; and fluctuations in the number of messenger RNA (mRNA) and protein molecules due to rates of transcription, translation, and degradation (9, 10). Many environmental factors can create noise that would disrupt a nonresilient timer: temperature changes, light intensity changes, oxidation and redox status, nutrient availability, and so on, all of which can modulate metabolism. That light and temperature can significantly affect circadian period and phase has been known for more than 50 years (1, 11, 12). However, only recently have investigators learned the extent of the linkage between circadian clocks and metabolism (13–20). Moreover, most of the recent metabolism/clock investigations have studied how the clock regulates metabolism and the impact of metabolic feedback onto the clock. Lost in the flurry of findings that have reported only small effects of metabolic feedback onto circadian oscillators has been the realization of how profoundly these timekeepers are buffered against metabolic noise.

The thesis of this review is twofold. First, we describe how studies of circadian systems in prokaryotic cyanobacteria have altered our appreciation of the mechanisms by which stability of circadian oscillations can be accomplished, namely by coupling a biochemical oscillator to a transcription/translation oscillator. Second, we resurrect a forgotten, 40-year-old proposition that a canonical property of circadian clocks—temperature compensation—is actually a subset of a larger phenomenon, namely metabolic compensation, which creates a general homeostasis between the frequency of circadian oscillators and a host of factors that would otherwise destabilize these timekeepers' accuracy (21).

PERVASIVE CLOCK CONTROL OF GENE EXPRESSION IN PROKARYOTIC CYANOBACTERIA

Since the inception of the field of chronobiology, investigators have progressively discovered that all types of eukaryotes harbor circadian clocks. However, until 1986, prokaryotes were thought to be "too simple" to have evolved circadian systems (22). The first convincing evidence for circadian rhythms in prokaryotes came from an investigation of the nitrogen-fixing cyanobacterium *Synechococcus* sp. RF1 (23–25). In *Synechococcus* sp. RF1, nitrogen fixation is regulated by the circadian clock so that it occurs out of phase with photosynthesis. This finding is important metabolically because the primary nitrogen-fixing enzyme (nitrogenase) is inhibited by the oxygen that photosynthesis produces. Therefore, a biological clock mediates a temporal separation that allows the same cell to perform incompatible metabolic events: photosynthesis during the day and nitrogen fixation during the night (26). This observation exemplifies how circadian regulation of metabolism can benefit an organism. Inspired by the observations of rhythmicity in *Synechococcus* sp. RF1, we and our collaborators searched for a genetically tractable cyanobacterium and settled on *Synechococcus elongatus* PCC 7942, which incidentally does not fix nitrogen but does produce other oxygen-sensitive enzymes, such as PurF, during the night (**Figure 1**) (27). We developed a luciferase reporter system for *S. elongatus* that enabled the demonstration of the canonical properties of circadian rhythms in this prokaryote (28) and, subsequently, the first rigorous tests of the fitness advantage conferred by circadian organization in rhythmic environments (29, 30).

S. elongatus has uniquely favorable characteristics for biochemical, biophysical, and genetic analyses that have accelerated new discoveries into clock mechanisms (23). For example, *S. elongatus* is the only organism for which we have full structural information for the key clock proteins (in this case, KaiA, KaiB, and KaiC). Most significantly, from a biochemical perspective, cyanobacteria represent the only circadian system in which a molecular oscillator can be studied in vitro; persistence, precision, and temperature compensation can be reconstituted in vitro with three purified proteins (KaiA, KaiB, KaiC) and ATP (31, 31a). The genetic tools available for this organism facilitated our discovery of globally regulated gene expression by a circadian timekeeper. We

Circadian rhythm: a biological rhythm that persists under conditions of constant illumination, temperature, and other environmental factors with a period of ~24 h; its phase can be reset by an entraining agent, and its period length is relatively independent of various (constant) temperatures within the physiological range for that organism

Pacemaker: a biological oscillator that can both (*a*) sustain its own oscillation and (*b*) entrain/regulate other oscillators

DD: constant darkness

LL: constant light

LD: light/dark cycle

Entrainment: the process by which a daily environmental rhythm such as the LD regulates the period and phase of a self-sustained circadian pacemaker

Oscillator: a system that can oscillate, thereby producing a rhythm in some process

used bacterial luciferase as a reporter of clock-regulated promoter activity; initially we studied the activity of the promoter for the *psbAI* gene (*psbAI*p) (**Figure 1**) (28), but subsequently we discovered that virtually all promoters in the *S. elongatus* genome are regulated by the circadian system (32). **Figure 1*a*** depicts the luminescence rhythms of three selected promoter::reporter constructs: the original reporter for *psbAI*, the reporter for the clock gene *kaiBC*, and the reporter for the night-active *purF* gene. Global control of promoter activity predicts pervasive clock control of mRNA transcripts in cyanobacteria. Indeed, microarray studies of various cyanobacterial species (*S. elongatus* PCC 7942, *Synechocystis* sp. PCC 6803, *Crocosphaera watsonii*, and *Prochlorococcus* sp. MED4) confirmed that the transcripts of up to 80% of genes in the genome exhibit circadian and/or daily oscillations of abundance (33–38).

How are the global rhythms of promoter activity mediated from the central clockwork? (**Figure 2**) Two alternative—but not mutually exclusive—models have been proposed. The first model is a biochemical cascade pathway that impinges on globally acting transcription factors, RpaA and RpaB (39–42). RpaA appears to be coupled to the central KaiABC oscillator (see the next section) by the histidine kinase SasA through a classic two-component signaling pathway (39, 41–43). Also, there may be multiple parallel pathways, including LabA and CikA, all of which converge on the nodal factor RpaA (44, 44a). Interestingly, CikA may be an important component of both the input (45) and output pathways (39). The second model for circadian regulation of global gene expression is the oscillating chromosome/nucleoid hypothesis, the so-called Oscilloid Model (36, 46, 47). In *S. elongatus*, the circadian clock orchestrates dramatic circadian changes in DNA

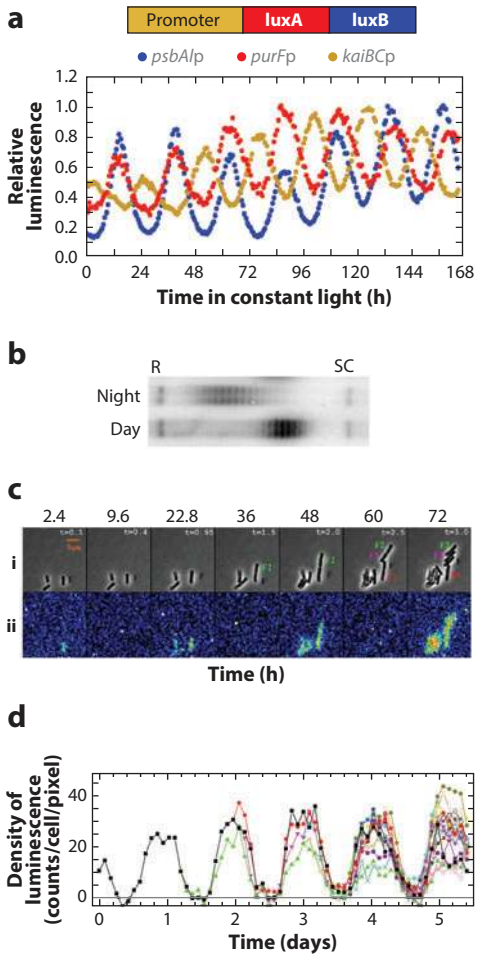


Figure 1

Circadian rhythms in cyanobacteria. (*a*) Circadian rhythms of gene expression monitored as rhythms of luminescence emanating from populations of cells transformed with bacterial luciferase (*luxAB*) fused to the promoters (p) for the *psbAI*, *kaiBC*, and *purF* genes. (*b*) Chromosomal topology shows a circadian rhythm as assayed by supercoiling of an endogenous plasmid. Topoisomers of the plasmid are more relaxed (R) in the subjective night and are more supercoiled (SC) in the subjective day (47). (*c*,*d*) Circadian rhythms of luminescence emitted by single cyanobacterial cells (10). (*c*) Micrographs of cyanobacterial cells at different times in constant light (LL). (*i*) Bright-field images showing growth and cell division as a function of hours in LL. (*ii*) Luminescence emanating from these cells (the luminescence reporter was *psbAI*p driving expression of *luxAB*). (*d*) Quantification of bioluminescence from a single cell as it divides into multiple cells as a function of time in LL. Starting at day 1.5, there are two differently colored traces as a result of cell division; the next division occurs at day 2.0; and so on. Panels *c* and *d* reproduced courtesy of I. Mihalcescu.

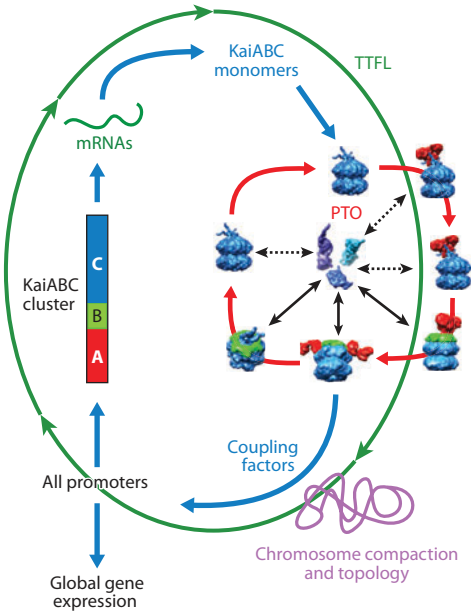


Figure 2

Organization of the circadian system in cyanobacteria. A posttranslational oscillator (PTO) is embedded within a transcription and translation feedback loop (TTFL). The KaiABC PTO (*cycle connected by red arrows*) is determined by phosphorylation of KaiC (*blue hexamers*) as regulated by interactions with KaiA (*red dimers*) and KaiB (*green monomers*). Robustness is maintained by synchronization of KaiC hexameric status via exchange of KaiC monomers, represented by the three molecular diagrams in the center of the PTO. The PTO regulates global transcription and chromosomal topology via coupling and transcriptional factors that include RpaA, RpaB, SasA, LabA, and CikA (39–44). The circadian transcription also drives new synthesis of KaiA, KaiB, and KaiC, which feeds new clock proteins into the PTO. Abbreviation: mRNA, messenger RNA.

topology (indicated by plasmid supercoiling) (**Figure 1*b***) (36, 47) and in chromosomal compaction and expansion (demonstrated by DNA-binding dyes) (48). DNA topology and torsion affect transcriptional rates; therefore, one may hypothesize that such circadian changes in chromosomal topology could be partly responsible for daily modulation of promoter activity (46–49). Supporting the Oscilloid Model is the fact that heterologous promoters from *Escherichia coli*, such as *conII*p and *trc*p, exhibit circadian activity in *S. elongatus* (37, 50, 51), a finding that is predicted to arise from an oscillating nucleoid. Moreover, the two models for global gene activity may be two sides of the same coin: The SasA/LabA/CikA/RpaA/RpaB pathway (44) may regulate gyrases and topoisomerases that mediate the oscillating nucleoid. Indeed, an analysis of stochastic gene expression in cyanobacteria (52) provided support for the idea that circadian gene expression is regulated by multiple factors, such as changes in both DNA topology and transcription factor activity.

The stability of the cyanobacterial circadian system is not disturbed by the pervasive changes in metabolism, cellular structure, DNA topology, and transcription that accompany cell division. In particular, the phase of the *psbAI*p activity rhythm is maintained in daughter cells when mother cells divide, as depicted in **Figure 1*c*** and ***d***, which illustrates technically difficult experiments on single cells (10). This result from single cells is consistent with the outcome of analyses of circadian timing in populations of dividing cells. Whether *S. elongatus* cells undergo rapid or slow division (or, alternatively, do not divide at all) does not perturb the intrinsic ~24-h period of the circadian system (46, 53–55). This oscillation's remarkable precision and stability are encoded in each separate cyanobacterial cell and are not stabilized by intercellular communication and coupling (10, 53). Moreover, the circadian oscillator specifies a checkpoint for division by regulating the timing with which cell division is permitted (46, 55, 56). In particular, elevated ATPase activity of KaiC may provide the circadian checkpoint for temporal "gating" of cell division in *S. elongatus* (57).

THE CYANOBACTERIAL CLOCK SYSTEM COUPLES A CORE BIOCHEMICAL POSTTRANSLATIONAL OSCILLATOR TO A TRANSCRIPTION AND TRANSLATION FEEDBACK LOOP

The core mechanism of the circadian clock in eukaryotic cells is widely held to be based on

TTFL: transcription and translation feedback loop

PTO: posttranslational oscillator

a transcription and translation feedback loop (TTFL) (58, 59), although there is evidence that this model may be incomplete or inaccurate (9, 60). The initial evidence in *S. elongatus* concerning the action of the KaiA, KaiB, and KaiC proteins also supported a TTFL model (61). However, our current understanding of the clock system in cyanobacteria is that a biochemical oscillator comprises the central "quartz crystal" of the clockwork (**Figure 2**). This core pacemaker operates within and regulates a larger TTFL that controls outputs and replenishes the oscillator's essential proteins (62–65). In the presence of ATP, the three–Kai protein core pacemaker can reconstitute an oscillator in vitro (31) and functions as a posttranslational oscillator (PTO) in vivo (**Figure 2**). On the basis of research from many labs, we know a great deal about this PTO's function and its relationship to the TTFL. These findings are briefly summarized here; see recent reviews for a fuller exposition (66, 67, 69; T. Kondo & S. Akiyama, manuscript in preparation).

During the in vitro cycle, the KaiABC system undergoes circadian changes in KaiC's phosporylation status that accompany the formation of complexes among the three proteins and entail ATP hydrolysis, ATP/ADP phosphotransfer, and conformational changes (the PTO panel of **Figure 2** depicts the cycle of KaiABC complexes). KaiC hydrolyzes ATP, a reaction that may be an intrinsically constant-rate timer (70) that drives KaiC autophosphorylation. The amount of net ATP hydrolysis produced by KaiC in 24 h is remarkably small [15 ATPs per day per KaiC monomer (70)], a finding that is probably important in the relative imperturbability of the cyanobacterial clockwork to metabolic fluctuations (see the end of the section titled Clock Control of Metabolism in Prokaryotic Cyanobacteria, below). The status of KaiC phosphorylation feeds back to regulate (*a*) the ATP hydrolytic rate (so that its intrinsic constant rate becomes rhythmic) and (*b*) switching of the KaiABC nanocomplex between the phosphorylating and dephosphorylating states. Therefore, the extent of KaiC phosphorylation is both a marker of circadian phase (71, 72) and a regulator of KaiC's manifold activities: ATPase, autokinase, autophosphatase, phosphotransferase, and nanocomplex formation (66, 73).

KaiC's primary phosphorylation sites are T432 and S431 (74–77), and the rhythmic phosphorylation and dephosphorylation reactions follow a strict order (71, 72). The sequence of phosphorylation and dephosphorylation, TS → pTS → pTpS → TpS → TS (where T refers to T432, S to S431, and p to phosphate), can be explained as an unidirectional "ratchet" mechanism based on the structure of the KaiC molecule (67, 78). Concomitantly with the rhythms of phosphorylation and ATPase activity, KaiC cyclically forms nanocomplexes with KaiA and KaiB that have been extensively studied and that regulate both the nanoclock's precession (73, 79–86) and its outputs to clock-regulated processes such as gene expression (42, 83). In general, we have a better understanding of the KaiC-phosphorylating half-cycle and its stimulation by association with KaiA (66, 81, 87, 88) than we do of how KaiC loses its phosphates in the KaiC-dephosphorylating half-cycle. A recent surprise, however, is that dephosphorylation of T432 and S431 involves a phosphotransferase reaction that causes the regeneration of ATP from the T432 and S431 phosphates and the ADP bound between KaiC subunits (89, 90). This phosphotransferase activity implies that the 15 ATPs consumed per day per KaiC monomer represent only a net amount and that the total number of ATP molecules hydrolyzed during the daily clock cycle may be higher (70).

Therefore, the absolute numbers of ATP molecules that are hydrolyzed and then regenerated in a 24-h interval are not presently known. In this context, the observation that the KaiC S431A/T432A mutant protein (which cannot be phosphorylated) consumes 27 ATPs per subunit each day, as opposed to 15 ATPs by wild-type KaiC (KaiC-WT) (70), can be interpreted in two interesting ways. First, if phosphorylation on S431/T432 provides negative feedback to the ATPase activity, then the mutant KaiC S431A/T432A

protein may hydrolyze more ATP because the negative feedback is inactivated by the alanine mutations. Second, the difference of 12 ATPs between the two processes may be evidence that (*a*) in KaiC-WT 27 ATPs overall are hydrolyzed per day and 12 are regenerated by the ATP synthase (89) to yield a net consumption of 15 ATPs per day per KaiC molecule, but that (*b*) the ATP synthase/phosphotransferase activity is dead in the KaiC S431A/T432A mutant because there are no phosphates on the S431 and T432 residues to recycle back to the ADP to form ATP. Could this regeneration of ATP via the phosphotransfer activity have functional significance from a metabolic perspective? Indeed, this phosphotransferase activity is a potential mechanism to conserve ATP when its cellular concentration in cells declines during the night, given that *S. elongatus* is an obligate photoautotroph that is incapable of producing energy in the dark. Possibly significant is the interesting observation that the PTO (explicitly the KaiC phosphorylation rhythm) continues to run in the dark when other metabolic processes are shut down (91), and the phosphotransferase activity may be essential to this ability (89).

In the presence of ATP, the in vitro oscillator composed of KaiA, KaiB, and KaiC maintains a high-amplitude rhythm for at least 10 days (92). The PTO's resolute persistence is probably responsible for the aforementioned precision and stability in vivo in the absence of intercellular communication and coupling (10, 53). How is this resilience accomplished? From a biochemical perspective, it is inexplicable that the KaiABC oscillator can be (*a*) as slow as ~24 h, (*b*) surprisingly precise, and (*c*) reliably stable (93). Investigators have proposed at least two separate mechanisms that are not mutually exclusive to rationalize how the synchrony of KaiC phosphorylation and dephosphorylation (and, therefore, the high-amplitude rhythm of KaiC phosphorylation) is maintained in the PTO. First, during the dephosphorylating phase, KaiB binds to KaiC, allowing KaiC monomers to exchange among hexamers; this subunit exchange harmonizes the phosphorylation status among all the KaiC hexamers in the population of hexamers present in the in vitro reaction (and presumably also in vivo) (80, 92). Second, KaiA is sequestered and KaiA's activity is thereby progressively inactivated once the hyperphosphorylated state of KaiC is reached (72, 94–97). KaiA appears to be the limiting reactant in the in vitro reaction, and as KaiC becomes hyperphosphorylated, KaiB associates with it to form a KaiBC complex that then immobilizes KaiA into a stable ternary complex that quells KaiA's activity. Thus, KaiC's phosphorylation status provides negative feedback to regulate KaiA's activity such that the activities of the various species of Kai proteins are synchronized. We believe that both mechanisms (i.e., monomer exchange and KaiA sequestration) operate together to ensure stability within the PTO. Therefore, the timing and impact of the binding of KaiA and KaiB to KaiC—which mediate monomer exchange and KaiA sequestration—are critical for maintaining synchrony among KaiC hexamers, thereby promoting high-amplitude oscillations that persist for many cycles (72, 80, 92, 94–97).

THE COUPLED POSTTRANSLATIONAL OSCILLATOR/ TRANSCRIPTION AND TRANSLATION FEEDBACK LOOP SYSTEM PROMOTES STABILITY AND ROBUSTNESS

Beyond the maintenance of high-amplitude oscillations in the PTO, as analyzed with the in vitro KaiABC oscillator, is the greater issue of stability and robustness in the in vivo system, which introduces the relationship between the PTO and the TTFL in cyanobacteria. As mentioned in the Introduction, the first goal of this review is to use the cyanobacterial example to address mechanisms by which circadian oscillations can become robust. In the case of *S. elongatus*, coupling a biochemical oscillator (the PTO) to a transcription and translation oscillator (the TTFL) appears to achieve this end (62–65). As stated above, the cyanobacterial circadian program's exquisite precision and stability are encoded in each separate

Slave oscillator: an oscillator that is driven or entrained by a pacemaker

cyanobacterial cell by an intracellular biochemical network, whereas intercellular interactions and coupling appear to be negligible (10, 53).

Biochemical reactions that involve small numbers of molecules are intrinsically noisy because they can be dramatically altered by fluctuations in molecular concentrations (52, 63). In general, the number of transcription factor molecules in a prokaryotic cell is small; therefore, transcriptional activity can be particularly strongly affected by small changes in concentration, leading to high intrinsic noise (98). However, a PTO that is the emergent property of mass action among thousands of molecules is expected to be robust in the face of noise. In the case of KaiC, there are approximately 2,000 KaiC hexamers per cell (99). Various models of the PTO support the hypothesis that the KaiABC PTO is resilient to noise. For example, two studies based on experimental data from populations of cyanobacterial cells have modeled the hierarchical relationship between a coupled PTO/TTFL clock program either as a pacemaker/slave oscillator system (PTO → TTFL) (63) or as a hierarchically equivalent coupled oscillator system (PTO ↔ TTFL) (65). In both configurations, a complete timekeeping system built from the two tightly intertwined oscillators (the PTO and the TTFL) outperforms either oscillator individually in terms of robustness to noise generated either by temperature and metabolic fluctuations (63) or by cell division and growth (65). A subsequent analysis in which single cells in a small population were individually tracked concluded that although the PTO is sufficient to generate oscillations, the individual oscillators are less stable in the absence of the TTFL (64). Moreover, in that study, synchrony of circadian rhythms among cells in a population was not maintained as well in TTFL-less cells.

The foundations of the two oscillating systems are different: The PTO is a clock-specific nanomachine pacemaker based on thousands of redundant molecules that interact by mass-action biochemistry, whereas the TTFL involves other nanomachines that primarily mediate the key cellular processes of transcription, translation, and degradation. These different molecular foundations mean that the PTO may be affected by different types of perturbations than those affecting the TTFL and, thus, may compensate for each other to further enhance timekeeping resilience. Overall, these studies strongly support the idea that a mass-action biochemical oscillator (a PTO) embedded within a transcription and translation loop (a TTFL) stabilizes clocks against noise that can be generated by metabolic fluctuations, temperature, cell division, and transcription (52, 63–65).

CLOCK CONTROL OF METABOLISM IN PROKARYOTIC CYANOBACTERIA?

As summarized in the above section titled Pervasive Clock Control of Gene Expression in Prokaryotic Cyanobacteria, rhythmic gene expression is pervasive in cyanobacteria. What about metabolism? One might expect that global changes in transcription patterns over the circadian cycle would inextricably lead to circadian changes in metabolic pathways and metabolite concentrations. Indeed, in eukaryotes, a burgeoning literature suggests that there are synergistic nodes between the circadian transcriptome and specific metabolic pathways (13, 100). Because many species of cyanobacteria (including *S. elongatus*) are obligate photoautotrophs, the metabolism of these cells clearly is strongly manipulated by LDs (91, 101, 102). But what about in LL, when gene-expression patterns cycle comprehensively? We cannot assume that rhythmic gene expression necessarily leads to rhythmic metabolic patterns in LL. For example, the first report of circadian rhythms in *S. elongatus* showed that the gene encoding the key photosynthesis protein D1 (*psbAI*) exhibited persistent rhythms of the transcript (28), implying a circadian rhythm of photosynthetic capacity. However, a later study that measured photosynthetic oxygen evolution did *not* find a circadian rhythm of photosynthetic capacity in LL in the same species (103)! Therefore, we should be cautious about extrapolating from gene expression to activity of metabolic

pathways. Perhaps the function of rhythmic gene expression is to replace proteins rhythmically to preserve homeostasis. Regarding the example mentioned above, the D1 protein can become progressively inactivated by the irradiation of sunlight during the daily cycle; in this case, the role of rhythmic replacement of the protein during the day may be to preserve a constant photosynthetic capacity, given that the protein is rhythmically inactivated by LD.

As of late 2013, metabolism as a function of circadian time in various species of *Synechococcus* has received very little attention. The concept of temporal separation of nitrogen fixation and photosynthesis discussed above was first suggested from research on marine synechococci (26), and its circadian underpinnings were revealed by extensive studies of the freshwater *Synechococcus* sp. RF1 (24, 25). Circadian expression of genes in *S. elongatus* (which does not fix nitrogen) encoding other oxygen-sensitive enzymes (e.g., *purF*) conformed to the nocturnally expressed, temporally separated pattern of nitrogenase activity in cyanobacterial species that can fix nitrogen (27). Photosynthesis is obviously rhythmic in LDs in all species, and in LL photosynthetic capacity is apparently rhythmic in some cyanobacterial species (e.g., *Synechocystis* sp. PCC 6803, *Synechococcus* sp. RF1, and *Cyanothece* sp. ATCC 51142) but not in other species (e.g., *S. elongatus* PCC 7942) (103, 104).

In contrast to that of *Synechococcus* species, the metabolism of *Cyanothece* sp. ATCC 51142, a unicellular, nitrogen-fixing cyanobacterium that temporally separates the processes of oxygen-producing photosynthesis and oxygen-sensitive nitrogen fixation, has been carefully examined under both diurnal (i.e., in LDs) and circadian (i.e., in LL) conditions. In this cyanobacterium, the metabolic processes that are under daily and/or circadian control include photosynthesis (O_2 evolution), carbohydrate (glycogen) granule accumulation, hydrogen gas (H_2) evolution, and nitrogen (N_2) fixation (104–106). A recent paper reported the fascinating observation that under some environmental conditions, *Cyanothece* sp. ATCC 51142 cells can switch between circadian and ultradian alternation of photosynthesis and nitrogen fixation in LL (107). This ultradian alternation can occur with a period as short as 10 h, and the ultradian periodicity is not temperature compensated. The ecological relevance of this ultradian mode is not yet clear. Obviously, we have much to learn about the temporal regulation of metabolism in cyanobacteria. Another example of a new insight into metabolism is that many cyanobacterial species have an unusual tricarboxylic acid (TCA) cycle that undergoes alternative enzymatic steps to several of the traditional TCA reactions; these alternative reactions may alter the ability of cyanobacteria to adapt to LD changes (108). It will be important to initiate studies of daily and circadian regulation of the cyanobacterial metabolome (i.e., metabolite composition) and to analyze the dynamic temporal changes in the metabolome by metabolic flux analyses (109, 110). This information can then be integrated with the known transcriptomic information (33, 36, 37) to assess the mechanism(s) by which the circadian system in cyanobacteria regulates metabolism.

Recent reports of non-TTFL rhythms in eukaryotes led to a flurry of excitement in chronobiological research (111–113). These studies show that the oxidation state of highly conserved peroxiredoxin proteins (PRXs) exhibit circadian oscillations in cells from humans to bacteria that persist in the absence of transcription or translation (111–113). PRXs are a family of antioxidant enzymes that scavenge reactive oxygen species (ROS), such as hydrogen peroxide, by catalyzing their own oxidation at a conserved cysteine group. Once oxidized, PRXs can be recycled by sulfiredoxin. In *S. elongatus*, one type of PRX (2-CysPRX) exhibits circadian cycles of oxidation (113) that may reflect an underlying metabolic rhythm of ROS generated by oxidative phosphorylation (111). A strain of *S. elongatus* in which the 2-CysPRX gene has been knocked out continues to express high-amplitude oscillations of gene expression and KaiC phosphorylation, suggesting that there is no input of the PRX oxidation rhythm (at least, of 2-CysPRX) into the KaiABC system (113).

ROS: reactive oxygen species

Given that daily and circadian PRX oxidation rhythms have now been reported in eukaryotes (e.g., humans, mice, *Drosophila*, *Neurospora*, *Arabidopsis*, and *Ostreococcus*) and prokaryotes (e.g., *S. elongatus* and *Halobacterium*), rhythmic PRX oxidation may be a conserved circadian marker across phylogenetic domains (113). Furthermore, the results obtained from PRX rhythms imply that the evolution of daily timekeeping mechanisms may be closely linked to metabolic responses to a rhythmic environment (16, 113, 114; see the below section titled Are Metabolic Changes Responsible for Entraining the Circadian System in Cyanobacteria?).

From an evolutionary perspective, obligate photoautotrophic cyanobacteria are intriguing organisms in which to study the relationship between metabolism and circadian timekeeping. In these species, transfer to darkness stops their ability to acquire new energy. Some cyanobacteria (e.g., *Cyanothece*) store photosynthetically derived energy in carbohydrate granules that can sustain metabolism in extended darkness (105). However, other cyanobacteria, such as the species that we study (*S. elongatus*), do not appear to store substantial energy reserves, so cells in darkness deplete cellular ATP levels (115). To conserve resources, *S. elongatus* cells "shut down," such that transcription and translation of all but the most essential genes are suspended (91, 102, 116). Consequently, transfer to dark has a major impact on the metabolism of these cyanobacteria, and yet cells can keep accurate circadian time for at least several days in darkness (91, 102, 117) despite large decreases in ATP concentration and energy charge (115). Even the in vitro oscillator is relatively imperturbable in the face of metabolic changes; despite its central characteristic—ATP hydrolysis—the period of the in vitro KaiABC rhythm lengthens by only 2 h when the percentage of ATP is reduced to only 50% of the optimal concentration (**Figure 3*a*,*b***) (115)! How does an energy-requiring timekeeping system cope with these dramatic changes in available energy and synthetic processes? First, KaiC uses only a tiny net amount of ATP in every cycle (70). Second, part of the answer may lie in the aforementioned phosphotransferase activity of KaiC, whereby the dephosphorylation reaction actually regenerates (at least in part) the ATP that was hydrolyzed to phosphorylate KaiC in the first place (89, 90). This unusual ability may help make the ATP/ADP cycle of the KaiABC clock less sensitive to cellular changes in the percentage of ATP during darkness (89). However, we do not presently have a quantitative estimate of the amount of ATP that is regenerated, so it is difficult to gauge how significantly this phosphotransferase activity buffers the clock against changes of [ATP] in the cell.

IS CIRCADIAN TEMPERATURE COMPENSATION A SUBSET OF A LARGER METABOLIC COMPENSATION?

How an energy-requiring timekeeping system can be buffered against dramatic changes in energy charge is perplexing (**Figure 3*a*,*b***) (115). However, this enigma reminds us of another long-unsolved problem in chronobiology, namely the phenomenon that put circadian rhythms "on the map"—temperature compensation. This term refers to circadian period. It means that the frequency of circadian rhythms is affected only slightly by different constant temperatures within the physiological range, specifically Q_{10} values of approximately 0.9–1.1 (**Figure 3*c*,*d***), but it does not mean that circadian clocks are totally insensitive to temperature. First, the Q_{10} for circadian periods is not exactly 1.0, indicating compensation rather than independence. Second, the phase of circadian rhythms can often be reset by acute temperature steps such that temperature cycles entrain the clocks of many organisms (1, 118). The phase sensitivity of circadian systems to temperature steps, in the face of the period being relatively insensitive to different constant temperatures, can be baffling for students of chronobiology, and it applies not only to organisms but also to the in vitro KaiABC system (31, 80, 119). What is the function of temperature compensation of the period if the phase

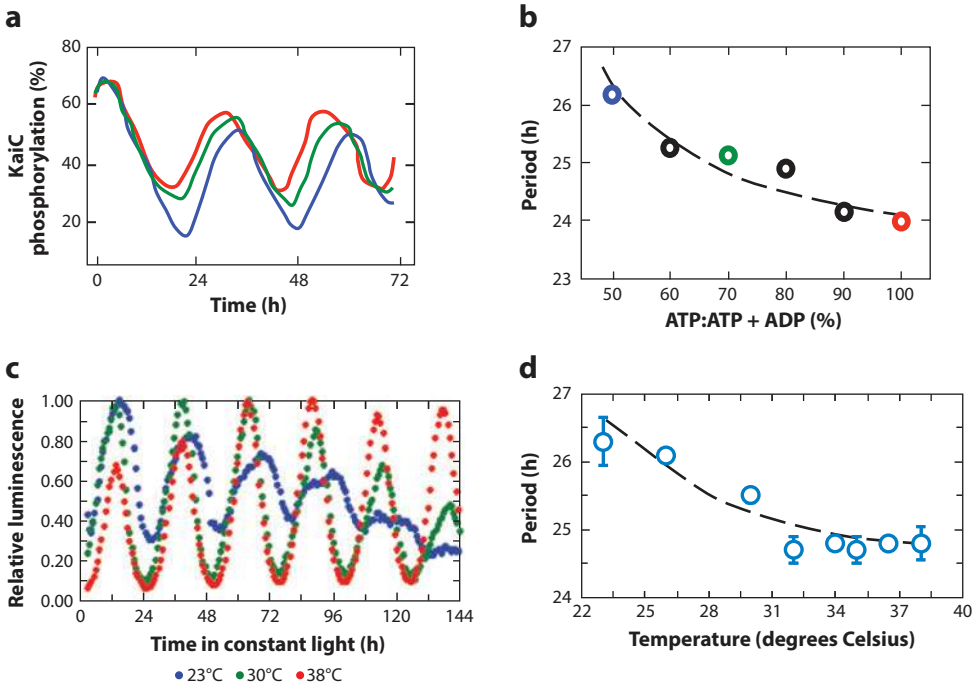


Figure 3

Comparison between circadian period modulation by ATP:ADP (in vitro rhythm) and temperature (in vivo rhythm). (*a*,*b*) Rhythmic KaiABC in vitro reactions in buffers with various ATP:ADP ratios. (*a*) In vitro rhythms of KaiC phosphorylation at 100% ATP (*red*), 70% ATP (*green*), and 50% ATP (*blue*). (*b*) Summary data for the period of the in vitro oscillation as a function of the ATP:ADP ratio. (*c*,*d*) In vivo bioluminescence rhythms with the *kaiBC* promoter::*luxAB* reporter at different temperatures. (*c*) In vivo rhythms at 23°C, 30°C, and 38°C. (*d*) Summary data for the period of the in vivo rhythm as a function of temperature over the range from 23°C to 38°C. Panels *a* and *b* modified from Reference 115, and panels *c* and *d* modified from Reference 124.

of the clock can be modified by temperature transitions? The answer probably lies in buffering the biochemistry of the clock against running faster or slower when the temperature changes so that an appropriate phase relationship to the solar day is conserved in the presence of day-to-day temperature variations (1, 118). Circadian rhythms are phased to the solar day by entrainment to daily cues that consist of changes in light and temperature. Therefore, circadian programming aims to conserve the daily phase of clock-controlled processes, and temperature compensation of the angular velocity (i.e., the period) of the clock is a means to this end.

As mentioned in the Introduction, the second goal of this review is to revisit a forgotten, 40-year old proposition that a universal property of circadian clocks—temperature compensation—may actually be a subset of a larger phenomenon, namely metabolic compensation, which creates a general homeostasis of the frequency of circadian oscillators. In 1973, Pittendrigh & Caldarola (21, p. 2697) wrote: "Given the fact of temperature compensation . . . we should long ago have turned to a more general proposition: if circadian oscillations are to function as a reliable framework for a temporal organization . . . , their frequency (or period) must be essentially invariant in the

face of all variations they encounter in the cellular milieu." Unfortunately, at the time of this proposition, there was little evidence beyond the effects of temperature to support the concept of metabolic compensation.

Changes in metabolism can enhance or repress biochemical reaction rates such as transcription and translation, and intracellular metabolite concentrations can be highly sensitive to limiting nutrients (120, 121). Therefore, it is surprising that recent interest in the link between clocks and metabolism (13–19, 113) has not independently stumbled upon the same insight that Pittendrigh & Caldarola (21) proposed. Because the rates at which many biochemical nanomachines operate are dramatically affected by metabolite concentrations, an accurate timekeeping nanomachine must either (*a*) operate in an invariant cellular milieu or (*b*) be capable of compensating for the metabolic fluctuations it encounters. Perhaps some multicellular organisms can maintain an essentially invariant milieu, but cyanobacterial cells cannot control their extracellular environment to any appreciable extent and therefore must adapt to, rather than control, metabolic fluctuations. If so, it is almost certainly the case that cyanobacteria are not alone in this capability. Therefore, this review posits that metabolic compensation is a primary mechanism by which circadian clocks keep accurate timing. The idea that temperature compensation of circadian systems in eukaryotes is intertwined with metabolism has not been totally neglected and was occasionally suggested in several different contexts (16, 122). Moreover, a recent paper on the eukaryotic fungus *Neurospora* introduced the concept of clock compensation against intracellular changes of metabolic status due to glucose-dependent modulation of biochemical rates (123). We propose that this metabolic compensation of circadian rhythms has profound, far-reaching implications and is related to temperature compensation.

Figure 3 depicts a critical correlation that supports the comparison between metabolic compensation and temperature compensation in the in vitro KaiABC oscillator. When the [ATP]:[ADP] ratio is experimentally modulated, the period changes only slightly (**Figure 3*a***) (115). Even [ATP]:[ADP] ratios as low as 50% ATP cause only an ~2-h lengthening of period (**Figure 3*b***). These results are relevant to metabolism in vivo because *S. elongatus* is an obligate photoautotroph that derives its energy exclusively from photosynthesis and photophosphorylation, which can occur only in the light. Consequently, when *S. elongatus* cells are transferred to darkness, ATP levels decline significantly, reaching levels as low as 50% ATP ([ATP]/[ATP]+[ADP]) after only 8 h (115). Nevertheless, the rhythm of KaiC phosphorylation continues to operate in darkness for at least 2 to 3 days (91, 117). Obviously, both in vitro and in vivo, the ATP-hydrolyzing cyanobacterial clock (70) can compensate for major metabolic changes that deplete ATP levels.

The above-mentioned impact of changing ATP levels is very similar to the well-known effect of temperature on cyanobacterial clocks in vivo (28, 124) and in vitro (31), as illustrated for the in vivo rhythm in **Figure 3*c***. Even a decrease in temperature of 15°C lengthens the in vivo period by <2 h (**Figure 3*d***). Obviously, a temperature change of this magnitude will have a major effect on the metabolic rates of an organism such as *S. elongatus*, which cannot regulate its temperature. Indeed, we know that the growth rate of these cells is optimal at 30°C and that it decreases significantly either above or below this optimal temperature (124). Therefore, not only does temperature affect the activity of circadian clock proteins, it also affects metabolism globally by changing the biochemical rates of the enzymes of metabolic pathways.

The correlation between the compensated effects of constant temperature and constant percentage of ATP on circadian period (**Figure 3**) is remarkable because circadian phase is not compensated for, but rather reset by, acute changes in either temperature or the percentage of ATP. Numerous papers address the phase-resetting effects of temperature pulses, steps, or cycles on a wide variety of

organisms (1, 118, 125) and, more recently, the in vitro KaiABC oscillator (80, 119). Moreover, the KaiABC oscillator is reset by pulses of low [ATP] (115). For reactions to changes in both acute temperature and the percentage of ATP, these responses provide entrainment cues (1, 115, 118). Consequently, the effects of temperature and metabolism on period versus phase are, again, strikingly similar. Does a common response mechanism underlie these similarities? Specifically, is temperature compensation a subset of a larger, metabolic compensation? And if so, will the realization that circadian oscillators are buffered against metabolic changes in general aid our efforts to understand circadian compensation—efforts that have not made much progress because they have focused on temperature compensation?

These observations in *S. elongatus* (**Figure 3**) are relevant to other circadian systems, including eukaryotes. Even endotherms such as mammals experience temperature compensation of circadian rhythms in cells and tissues isolated and assayed in vitro (2–5). Temperature compensation could be considered unnecessary in endotherms because their body temperature is relatively constant and, therefore, this property may have been lost during evolution. Yet when cells and tissues of endotherms are isolated in vitro, temperature compensation is preserved. Why? Some research supports the hypothesis that the low-amplitude body-temperature cycle in mammals (~1–2°C) functions as a systemic cue to entrain individual oscillators in peripheral tissues so that optimal phasing of all tissues in an endotherm is maintained (126). If the intraorganismal environment of endotherms includes a temperature cycle, then is a temperature compensation and entrainment mechanism still relevant? The metabolic compensation thesis of this review, however, suggests that general compensation and homeostasis against metabolic perturbation may be adaptive for higher organisms and evolutionarily conserved. If temperature compensation is a subset of a more general metabolic compensation, this property may accompany metabolic homeostasis by natural selection as a "fellow traveler." Other evidence of metabolic compensation in higher organisms includes the observation that transcription of mammalian cells can be pharmacologically inhibited by up to 50% and yet the period of circadian rhythms changes only by ~2 h (60); in fact, the change in period counterintuitively *shortens* by ~2 h, whereas if transcriptional rate were integral to the clock mechanism, one would expect the period to lengthen. This finding suggests, again, a robust compensatory response to a treatment that significantly inhibits metabolism. Additional evidence is the aforementioned analysis of compensated responses to sugar-dependent manipulation of metabolism in the eukaryotic fungus *Neurospora* (123). Finally, the pervasive changes in metabolism, cellular structure, and gene expression that accompany cell division have relatively minor effects on the circadian period. This imperturbability to cell division appears to be a general property of circadian pacemakers in cyanobacteria and in eukaryotes, and one may argue that it constitutes further evidence of a metabolic compensation mechanism (9, 10, 54, 55, 57, 127, 128). In fact, metabolic compensation may be more generally relevant to circadian precision in endotherms than is the property of temperature compensation.

On the basis of the metabolic compensation hypothesis, we predict that mutations in clock proteins that suppress temperature compensation may render the circadian system more sensitive to other metabolic perturbations. Preliminary observations of mutations in the *kaiABC* genes suggest that this prediction is upheld for circadian rhythmicity in *S. elongatus* (Y. Xu & C.H. Johnson, unpublished observations). Also, might the PRX oxidation rhythm be related to metabolic compensation, in that it might be a mechanism to respond to (and compensate for) varying levels of ROS that are generated by fluctuations of metabolic rate that tend to vary over the daily cycle (111–113)?. Are circadian clockwork mechanisms equipped with a metabolic sensor (perhaps of ATP levels) that adjusts circadian rate constants to changes in intracellular metabolic rates?

***kaiABC* gene cluster:** the adjacent genes in the *S. elongatus* PCC 7942 genome that encode the central cyanobacterial clock components KaiA, KaiB, and KaiC

ARE METABOLIC CHANGES RESPONSIBLE FOR ENTRAINING THE CIRCADIAN SYSTEM IN CYANOBACTERIA?

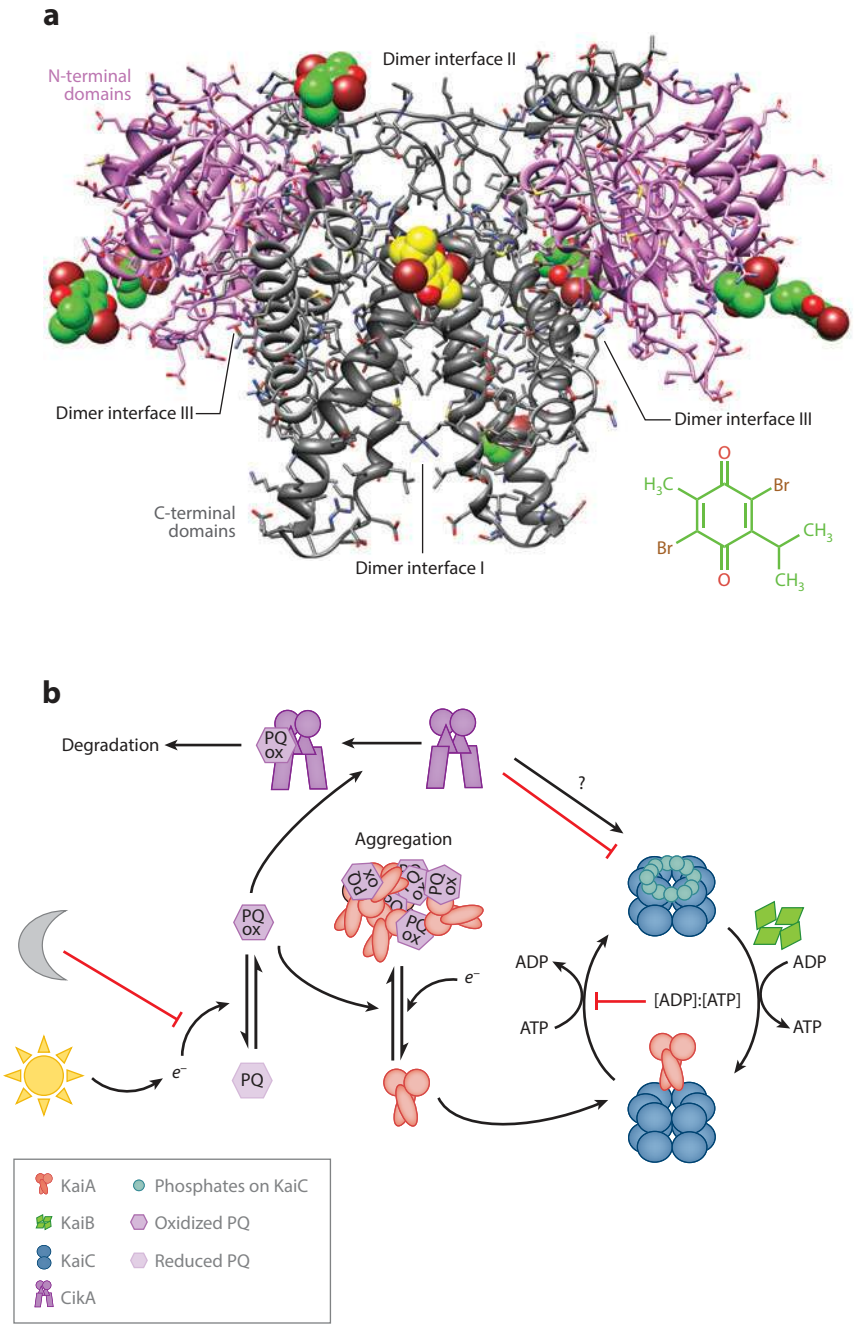
Daily LD and temperature changes are the primary signals by which circadian systems entrain to the environmental 24-h cycle; LD cues usually trump temperature cues when the timing of both daily signals conflicts (1). In most eukaryotes, specific transduction pathways are dedicated to the task of circadian photoentrainment, and these pathways usually involve a specific photoreceptor molecule that harbors a chromophore (e.g., melanopsin, cryptochrome, phytochrome, White Collar). In contrast, current evidence for circadian phototransduction in cyanobacteria does not implicate a specialized pathway. For example, although there appear to be cryptochrome-like and phytochrome-like homologs in the *S. elongatus* genome, knockouts of those genes have no discernible impact on circadian photoentrainment. Instead, broad changes in metabolism and redox appear to be the functional signals that entrain the cyanobacterial clock. Genetic analyses from the Golden group (45) identified the bacteriophytochrome-like protein CikA, which affects the efficacy of light-induced phase resetting. Furthermore, a series of biochemical investigations from the same group suggested that CikA transduces environmental signals by binding a quinone and using cellular redox state as an entraining cue in *S. elongatus*, given that darkness quickly oxidizes the intracellular plastoquinone pool. Intriguingly, the central clock protein KaiA also binds the oxidized quinone analog DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), causing aggregation of KaiA that blocks KaiA stimulation of KaiC phosphorylation (**Figure 4**) (129). We confirmed the precise binding sites of DBMIB to KaiA by analyzing the structure of *S. elongatus* KaiA crystals soaked in the plastoquinone analog (**Figure 4*a***) (130). These binding modes are consistent with aggregation of KaiA in the presence of oxidized DBMIB, a finding established both by native PAGE (polyacrylamide gel electrophoresis) and light scattering and by a potential interference with binding of a C-terminal KaiC peptide by KaiA (129, 130). These data are consistent with the idea that KaiA and/or an input pathway involving CikA senses day and night signals as changes in redox state, thereby entraining the central clock (129).

However, more recent studies have implicated even more fundamental metabolic factors in the entrainment process. As mentioned above, because *S. elongatus* is an obligate photoautotroph, when cells are placed into darkness and photosynthesis (and ATP photophosphorylation) shuts off, ATP levels decline (115). The reduction in the [ATP]:[ADP] ratio causes a partial shutdown of many cellular processes, and an almost total cessation of transcription and translation (91, 116). Treatment of the in vitro KaiABC oscillator with reductions in the [ATP]:[ADP] ratio that mimic the changes occurring in vivo cause in vitro phase resetting that appears to be very similar to what occurs in living cells in response to dark-pulse phase-shifting (115).

Figure 4

(*a*) Interactions between KaiA dimer and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) (bottom right; oxidized form). The N- and C-terminal domains of KaiA are colored pink and gray, respectively. DBMIB molecules are in space-filling mode with carbon atoms colored green. A single DBMIB molecule exclusively bound to the C-terminal domain is colored yellow. (*b*) Putative mechanism of KaiABC posttranslational oscillator (PTO) entrainment by the redox state of plastoquinone (PQ) and the [ATP]/[ADP] ratio. Oxidation and reduction of PQ are controlled by photosynthetic electron transport as a function of light availability and by respiratory electrons in the dark. Oxidized quinone is bound by the pseudoreceiver domain of CikA and KaiA, leading to KaiA aggregation, which abrogates the KaiA-mediated stimulation of KaiC phosphorylation. The mechanism by which CikA influences KaiC (indicated by a question mark) is not known. Modified from Reference 131.

Therefore, it appears that photosynthetic activity, which has major effects on ATP levels and intracellular redox, entrains the circadian clock system in *S. elongatus*. KaiC phosphorylation status is directly influenced by the [ATP]:[ADP] ratio (115), and KaiA stimulation of KaiC phosphorylation is blocked by oxidized quinones in vitro and in vivo (131). Therefore, these two fundamental metabolic consequences of photosynthetic activity may act together to



inform the cyanobacterial clock of dark signals as entraining cues (**Figure 4**) (131).

Metabolism as reflected by the [ATP]:[ADP] ratio and oxidized quinone pools may not fully explain entrainment, however. Several studies suggested that changes in the relative synthesis and abundance of the Kai proteins can cause clock resetting. For example, we described a mechanism by which the TTFL can feed into the PTO such that new synthesis of clock proteins can phase-shift or entrain the core PTO pacemaker. This synthesis of new KaiC—which must, by definition, be unphosphorylated—can perturb the proportion of phosphorylated KaiC in the total KaiC pool. We experimentally tested and confirmed predictions based on this model by entraining the in vivo circadian system with cycles of new clock protein synthesis that modulate the phosphorylation status of the clock proteins in the PTO (63). Consequently, we concluded that the TTFL can provide entraining input into the PTO. Nakajima et al. (132) suggested another approach that was based on the observation that the relative [KaiA]:[KaiB] and [KaiA]:[KaiC] ratios oscillate in light but are constant in darkness. This variation in Kai protein ratios should cause changes in the angular velocity of the PTO in the light but not in the dark, allowing parametric entrainment. Hosokawa et al. (133) also found that the TTFL is important for the circadian entrainment in *Synechococcus*. These authors determined that the amplitude of the KaiC phosphorylation cycle (indicative of the PTO) changed according to the [KaiA]:[KaiC] ratio, which was cyclic under LL. When *Synechococcus* cells were transferred from LL to DD at subjective dawn, the amplitude of the KaiC phosphorylation cycle was attenuated and exhibited a higher [KaiA]:[KaiC] ratio with a high overall KaiC phosphorylation level, compared with cells that were transferred to DD at subjective dusk. On the basis of these results, Hosokawa et al. (133) proposed that the TTFL enhances resetting of the Kai-based PTO in cyanobacteria. Although these three studies (63, 132, 133) do not rule out a role for metabolic changes in the entrainment mechanism, they imply that a full understanding of circadian entrainment in cyanobacteria may require an approach that integrates more factors than have been considered previously.

APPLICATIONS: MANIPULATING THE CLOCK TO ENHANCE EXPRESSION OF USEFUL BIOPRODUCTS

Because they grow by capturing solar energy and because they are genetically malleable, photoautotrophic cyanobacteria are appealing cell reactors for the production of useful bioproducts and biofuels (134–136). However, despite the potential of engineering direct photosynthetic routes to useful bioproducts in cyanobacterial hosts, the productivity of heterologous proteins is currently too low for industrial application. Furthermore, few tools are available that specifically address the challenges of redirecting or enhancing flux in photosynthetic microbes along metabolic pathways toward synthesis of useful bioproducts or their precursors (136, 137). This lack of tools limits researchers' ability to translate exciting laboratory findings into practical industrial platforms for photosynthetic bioproduction. Despite these limitations, *S. elongatus* and related genetically malleable cyanobacteria have become preferred subjects for development of this biotechnology, including production of aldehydes, alcohols, and sugars (138–140).

As discussed above, the circadian system in *S. elongatus* globally controls virtually all gene expression (32, 33, 36, 47). Therefore, we reasoned that the clock system could be manipulated to increase the expression of genes that encode useful bioproducts (37). This task could be accomplished in two ways. First, the expression of many genes is turned on during the daytime and peaks at the subjective dusk phase (Class I genes; expression of most of the genes in *S. elongatus* peaks at this phase), whereas others are maximally expressed at subjective dawn (Class II genes; expression of

fewer genes in *S. elongatus* peaks at this phase) (**Figure 5*a***). Therefore, if the clock could be locked into a phase at which the expression of a heterologous gene peaks, then the expression of that gene could be maximized. Second, because biotechnological production would be potentiated by a constant 24/7 schedule (24 h per day, 7 days per week), placing cyanobacteria in LL and inactivating rhythmic expression would enhance the total amount of bioproduct produced (**Figure 5*b***). The available neutral sites from which heterologous genes are typically expressed in *S. elongatus* are usually regulated in the subjective dusk phase pattern (Class I genes) (37). We found that overexpression of KaiA would constitutively hyperphosphorylate the KaiC protein (37, 63, 141, 142), which manipulates KaiABC-regulated gene expression to provide the maximum transcriptional activity of genes that are expressed in the predominant subjective dusk phase (Class I genes), including heterologous genes inserted into neutral sites of the chromosome (**Figure 5*a***) (37). Moreover, because constitutive overexpression of KaiA latches clock-regulated gene expression in an arrhythmic, maximal mode (for Class I genes), relatively constant synthesis of the desired product could be achieved if the cyanobacteria were maintained in LL under a constant production schedule. By contrast, if rhythmic expression were allowed, much less bioproduct would ultimately be generated (**Figure 5*b***) (37).

We applied this strategy to the expression of heterologous proteins with potentially valuable products, namely human proinsulin, foreign luciferase, and exogenous hydrogenase (37), but this tactic could be used to increase the expression of any industrially important protein or pathway. Moreover, the overall principle of inactivating the circadian system so that it latches at the peak expression is not restricted to cyanobacteria but may be useful for ongoing 24/7 industrial applications using any organism harboring a circadian clock that regulates gene-expression patterns (e.g., transgenic expression of bioproducts using plants as bioreactors).

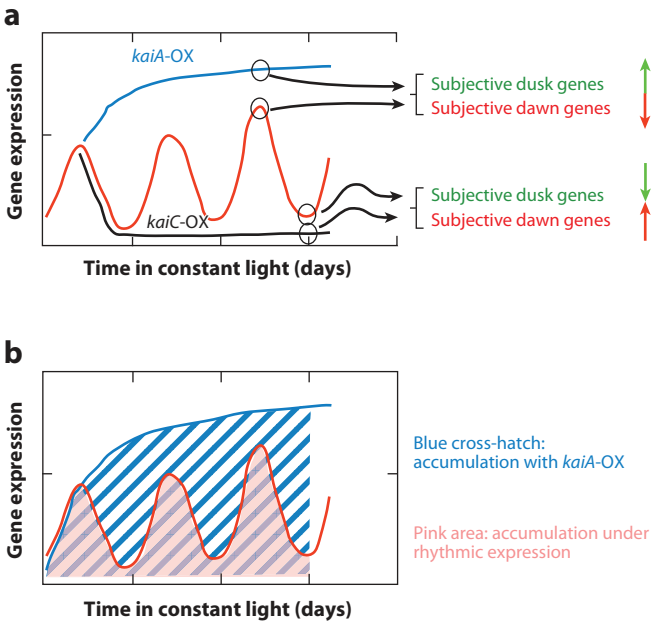


Figure 5

Manipulation of the circadian system to enhance the expression of useful bioproducts. (*a*) The red curve depicts the normally rhythmic expression pattern of subjective dusk genes. By constantly overexpressing *kaiA* (*kaiA*-OX), subjective dusk (Class I) genes are upregulated (*green arrow*), subjective dawn (Class II) genes are downregulated (*red arrow*), and the rhythmicity of gene expression is lost (*blue*). Constant overexpression of *kaiC* (*kaiC*-OX) has the opposite effect on Class I versus Class II gene expression (*green and red arrows*) and represses activity of subjective dusk genes (*black*). (*b*) Latching the complementary gene-expression patterns by *kaiA*-OX enhances the expression of dusk genes—including heterologous genes inserted into the NSI or NSII sites—and leads to a greater accumulation of gene products in constant light (*blue*) than would be possible if the same gene were expressed under control of the native rhythmic system (*pink*). Modified from Reference 37.

EVOLUTION OF CIRCADIAN SYSTEMS: DID METABOLIC CYCLES PLAY A ROLE?

Cyanobacteria are an ancient group. They are probably one of the earliest life forms, dating back 3.5 billion years (or more). Therefore, over the span of their evolution, they were subjected to the Earth's original anoxic atmosphere, and they are considered the primary organism that transformed Earth's atmosphere by photosynthetic oxygen emission into its current oxygenated state—a process that is thought to have begun approximately

2.5 billion years ago. Some researchers have proposed that high-frequency metabolic feedback loops—under selective pressure from the daily environment—were recruited to coalescence of an approximately daily period (16). Another model for the evolution of circadian timing—the so-called escape-from-light hypothesis (7, 143, 144)—posits that light can damage cellular metabolism (e.g., DNA damage caused by UV light), which could have been a selective force for the evolution of circadian pacemakers. Briefly, the escape-from-light hypothesis proposes that light during the sunlit portion of the daily cycle had profound and mostly deleterious effects on early life; therefore, phasing cellular processes that are hypersensitive to light so that they occur in the dark nighttime may have driven the early evolution of circadian timers.

A novel recent explanation for the evolution of circadian systems beginning with cyanobacteria is based on a genomic analysis that suggests that the *kaiABC* genes coevolved with *Prx* genes. Specifically, when the evolution of the sequence for *kaiC* (the oldest member of the *kai* clock gene triumvirate) was compared with that of other conserved gene families, the three highest correlations were for the other two *kai* clock genes (*kaiA* and *kaiB*) and for *Prx* genes (113). These evolutionary relationships extend beyond those that would be expected simply on the basis of time elapsed since a common ancestor. The authors of this paper suggested that most organisms that survived the transition to an aerobic environment 2.5 billion years ago were those that respired and/or evolved oxygen. Electron-transport chains involving oxygen inevitably produce ROS by-products, and due to daily cycles of photosynthesis and metabolism driven by daily LD and temperature changes, these ROS were produced rhythmically over the span of the daily cycle (113, 144). Therefore, a system to anticipate and manage the concomitant daily generation of intracellular ROS was adaptive. These findings led the authors to propose that in cyanobacteria an intimate coevolution of cellular timekeeping with redox homeostatic mechanisms occurred, beginning at least 2.5 billion years ago (113).

Other genomic sequence analyses have studied the evolution of circadian timekeeping among cyanobacteria. These analyses have been applied to the circadian or circadian-related genes *kaiABC*, *cikA*, *sasA*, *ldpA*, *pex*, *labA*, *rpaA*, and *cpmA*, but only the conclusions derived from the study of the core *kaiABC* cluster are discussed here (145). The *kaiC* gene and its homologs occur pervasively throughout eubacteria and Archaea. In many of these bacterial and archaebacterial groups, *kaiC* occurs as a single-domain gene that is most closely homologous to the CI domain of the double-domain *kaiC* gene of *S. elongatus*. As mentioned above, *kaiC* is probably the oldest of all circadian genes, and it belongs to the RecA (DNA recombinase)/DnaB (replicative helicase) superfamily of genes (146). This relationship implies that the function of *kaiC*'s ancestors was involved in DNA damage, DNA replication, or RNA metabolism (RNA helicases have been implicated in transcription, translation, and many other processes). The similarity between KaiC and helicases is intriguing. Our attempts to identify a DNA or RNA helicase activity by KaiC have been unsuccessful, but negative "wet-lab" results for helicase activity are not unusual for genes that are predicted by sequence or structural analyses to have a helicase function. The helicase similarity is particularly fascinating because clock protein complexes in eukaryotes include essential RNA helicases (114, 147). Is there a conserved and essential function of helicase-like proteins in clock mechanisms from bacteria to mammals?

The fact that single-domain *kaiC* homologs, among other bacterial species, are more similar to the CI domain of *S. elongatus*'s KaiC suggests that the CII domain (including the C-terminal tentacles that interact with KaiA) diverged more recently toward its current circadian function (145, 148). The *kaiB* gene is also widespread among eubacteria and Archaea and is often found in an operon with *kaiC*. In some species, there is a double-tandem *kaiB* gene in association with *kaiC*. However,

in other species, *kaiC* is found alone without *kaiB*. In contrast to the widespread occurrence of *kaiB* and *kaiC*, *kaiA* appears only among cyanobacteria (148; T. Mori & C.H. Johnson, unpublished data). Does this mean that a circadian clock system based on *kai* genes exists only in cyanobacteria? What does KaiC do when KaiA and KaiB are not present? Does it have a different function, possibly related to DNA damage, DNA replication, or RNA functions?

The case of cyanobacterial *Prochlorococcus* is particularly interesting. *Prochlorococcus marinus* is possibly the most abundant photosynthetic organism on Earth (149), and the various *Prochlorococcus* species show a very interesting phenomenon; some have lost all of the *kaiA* gene and others have lost most of the *kaiA* gene and retain only a truncated sequence that is unlikely to be functional (150; T. Mori & C.H. Johnson, unpublished data). Has the clock "de-evolved" in *Prochlorococcus*? Concomitantly with the loss of *kaiA*, laboratory experiments have found daily rhythms with *Prochlorococcus* in LD (38, 150) but not yet a sustained rhythm in constant conditions that would be diagnostic of a bona fide circadian system. In vitro assays using purified $KaiC^{Pro}$ and $KaiB^{Pro}$ have confirmed that $KaiC^{Pro}$ can autophosphorylate without KaiA but that $KaiB^{Pro}$ does not appear to promote dephosphorylation of $KaiC^{Pro}$ in vitro (151). Therefore, *Prochlorococcus* has lost the *kaiA* gene, with the result that it either may have a residual, highly damped circadian oscillator or has lost sustained circadian capabilities altogether and now has a simpler, hourglass timing mechanism.

Several studies have speculated that *Prochlorococcus* species are quickly evolving into new ecological niches and are under selective pressure to reduce the sizes of their genomes, a consequence of which is the loss of *kaiA* (150–152). These authors argue that in the relatively constant environment of the ocean, a circadian mechanism is not necessary and an hourglass timer that is reset every day by LD may satisfy the need to temporally gate cellular metabolism and cell division to a consistent LD (150–152). This hypothesis is imaginative; nonetheless, we note that the oceanic environment is not entirely consistent, especially with regard to temperature and the duration of photoperiod. *Prochlorococcus* is virtually ubiquitous in the oceans within the latitudinal band extending from 40°N to 40°S; at increasing distances from this band, *Prochlorococcus* is still found, but its concentrations decline fairly rapidly (149). Within this distribution range, thermal clines and upwelling can change the temperature. Moreover, photoperiod can change dramatically between 0° and 40°N or 40°S, and ocean currents can move *Prochlorococcus* around within this wide range of photoperiods. An hourglass timekeeper that gates cellular processes to dawn or dusk could choose a different phase to gate cellular processes when the cells are exposed to a long photoperiod versus a short photoperiod, and this phase variability might be maladaptive. Therefore, elucidation of the timing process in *Prochlorococcus* and how it interacts with the environment may yield new clues about the selective pressures involved in circadian systems in general.

SUMMARY POINTS

1. The cyanobacterial clock system couples a core biochemical oscillator (a PTO) to a TTFL.
2. This coupled PTO/TTFL organization promotes stability and robustness.
3. The circadian system pervasively controls gene-expression patterns in cyanobacteria, and this clock control may be manipulated for practical goals to enhance the expression of useful bioproducts.

4. Although gene expression is globally regulated by the clock, we do not yet know whether metabolism is similarly regulated. Nevertheless, acute changes in metabolism can apparently entrain the circadian clock of cyanobacteria.
5. A property that has long been a defining characteristic of circadian clocks—temperature compensation—may actually be a subset of a larger phenomenon, namely metabolic compensation, which creates a general homeostasis between the frequency of circadian oscillators and a host of factors that would otherwise destabilize these timekeepers' accuracy.
6. The *kaiC* gene is widespread among prokaryotes (eubacteria and Archaea), but it may perform a nonclock function in prokaryotic species outside the cyanobacteria. The elucidation of this nonclock function could provide fascinating clues about the selective pressure(s) that led to the evolution of circadian clocks and about whether metabolic cycles played a role.

FUTURE ISSUES

1. We cannot explain how a biochemical oscillator can have such a long time constant (~24 h) and remain so precise. Nothing about phosphorylation reactions, ATP hydrolysis, protein–protein interactions, or other known properties of proteins in general or of the Kai proteins in particular can explain the long time constant (93).
2. We do not have a mechanistic explanation for how the KaiABC system is compensated either for temperature or for changes in the ATP:ADP ratio.
3. Optimal expression of the in vitro reaction requires significantly more KaiA in the in vitro reaction than is present in vivo (compare Reference 31 with Reference 99). Moreover, although early reports suggested that the period and amplitude of the in vitro rhythm are relatively invariant within an allowed range of Kai protein concentrations (153), recent data indicate that there are significant effects on the period of the in vitro oscillator when the ratio of [KaiA] is varied relative to the concentration of [KaiB]+[KaiC] (132). Perhaps a major function of the TTFL is to maintain Kai protein concentrations within a range that maintains a dependable timekeeper?
4. We have crystal structures of the three individual Kai proteins, but not of all the Kai proteins together in a complex or while interacting with SasA, CikA, and so on. Moreover, we should pursue structural analysis methods (e.g., small-angle X-ray scattering, NMR, electron microscopy) that are compatible with structural determinations under dynamic conditions.
5. Regarding input, how do light and other cues entrain the clock? Despite evidence of metabolic inputs to the central clock (**Figure 4**), current explanations involving redox versus the ATP:ADP ratio versus unknown pathways seem to be incomplete.
6. Regarding output, how are the Kai proteins coupled to the downstream control of gene expression? One study proposed that KaiC's ATP hydrolytic activity is the key output of the KaiABC oscillator (57). Moreover, we previously proposed that the rhythm of chromosomal topology is a global regulator of rhythmic gene expression (46, 47, 49).

Presently, the dominant output model evokes a biochemical cascade and two-component signaling pathway involving SasA, RpaA, RpaB, LabA, and CikA (39–44), but further research on this topic may yield surprises.

7. What is the mechanism for the competition and selection phenomena that illustrate the adaptive significance of the *S. elongatus* clock in rhythmic environments (29, 30)?
8. Will the circadian clockwork in *S. elongatus* ultimately prove to be completely distinct from that of eukaryotes? Or will the insights gleaned from cyanobacteria induce a reassessment of clocks in higher organisms (9, 63, 78)?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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