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Circadian Rhythms in Cyanobacteria

Susan E. Cohen, Susan S. Golden

Center for Circadian Biology and Division of Biological Sciences, University of California, San Diego, La Jolla, California, USA

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SUMMARY

Life on earth is subject to daily and predictable fluctuations in light intensity, temperature, and humidity created by rotation of the earth. Circadian rhythms, generated by a circadian clock, control temporal programs of cellular physiology to facilitate adaptation to daily environmental changes. Circadian rhythms are nearly ubiquitous and are found in both prokaryotic and eukaryotic organisms. Here we introduce the molecular mechanism of the circadian clock in the model cyanobacterium *Synechococcus elongatus* PCC 7942. We review the current understanding of the cyanobacterial clock, emphasizing recent work that has generated a more comprehensive understanding of how the circadian oscillator becomes synchronized with the external environment and how information from the oscillator is transmitted to generate rhythms of biological activity. These results have changed how we think about the clock, shifting away from a linear model to one in which the clock is viewed as an interactive network of multifunctional components that are integrated into the context of the cell in order to pace and reset the oscillator. We conclude with a discussion of how this basic timekeeping mechanism differs in other cyanobacterial species and how information gleaned from work in cyanobacteria can be translated to understanding rhythmic phenomena in other prokaryotic systems.

INTRODUCTION

Circadian timekeeping was originally considered to be restricted to eukaryotic organisms, as bacteria were not considered complex enough to possess a circadian clock. Not only were bacteria thought to lack sufficient cellular complexity to support a circadian clock, but at the time it was believed that, in rapidly dividing cells (as would be the case for the many bacteria that can divide many times over a 24-h cycle), cellular functions would not be coupled to a circadian oscillator, a dogma also known as the "circadian-infradian" rule (1, 2). Although not initially associated with the circadian clock, rhythmic phenomena involving oscillations in photosynthesis (during the illuminated times of day) and nitrogen fixation (restricted to the dark portion of the day) were found in several diazotrophic strains of cyanobacteria (3–5). Oscillations in these activities that were found to persist under constant conditions and to be temperature compensated and/or to entrain to a light-dark (LD) cycle hinted at the existence of a circadian clock mechanism. However, at the time, those rhythms were attributed to other cellular processes and were not expected to be driven by a biological clock. It was not until 1986 that Huang and colleagues discovered a bona fide circadian rhythm of nitrogen fixation and amino acid uptake in *Synechococcus* sp. RF-1 that satisfied all three criteria of a true circadian oscillator: persistence, resetting, and temperature compensation (6–8).

We now know that circadian rhythms are not a property solely of eukaryotic cells. Currently, cyanobacteria are the simplest organisms and the only prokaryotes known to have a rigorously tested and robust circadian clock. The genetically tractable *Syn-*

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Address correspondence to Susan E. Cohen, susanc@ucsd.edu.

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echococcus elongatus PCC 7942 has emerged as a premier model organism for studying the molecular details and regulation of the clock. Pioneering work from the laboratories of Susan Golden, Carl Johnson, Masahiro Ishiura, and Takao Kondo established the use of luciferase as a reporter to monitor rhythms of gene expression enabling genetic investigations and the identification of the first clock mutants in cyanobacteria (9, 10).

OVERVIEW OF THE CLOCK AND RHYTHMIC PHENOMENA

The *S. elongatus* core oscillator, encoded by the *kaiA*, *kaiB*, and *kaiC* genes, regulates global patterns of gene expression (9, 11), the timing of cell division (12, 13), and compaction of the chromosome (14, 15). Environmental cues are transmitted to the oscillator via molecules that signal changes in cellular redox. Components such as CikA (circadian input kinase A) (16) and LdpA (light-dependent period A) (17) have been described as redox-sensitive proteins that are important for synchronizing the circadian oscillator with the external environment. Information from the oscillator is transmitted via an output pathway consisting of a two-component system, comprised of SasA (*Synechococcus* adaptive sensor A) (18) and RpaA (regulator of phycobilisome association A) (19), that is important for driving rhythms of biological activity, including gene expression and the timing of cell division (12, 20, 21).

Several lines of investigation, including random insertion of promoterless luciferase genes into the *S. elongatus* genome as well as more-recent transcriptomic analysis, have demonstrated that nearly all genes in the *S. elongatus* genome are expressed rhythmically (22–24). While gene expression profiles can be categorized into 5 or 6 different groups based on the phasing of peak expression time, the vast majority of genes fall into two classes. Class 1 genes show peak expression at dusk and are typified by the promoter of the *kaiBC* genes (P_{kaiBC}), and class 2 genes show peak expression at dawn and are typified by the *purF* promoter (P_{purF}). The circadian oscillator is not influenced by the periodicity of the cell cycle and maintains ~24-h periodicity independent of whether cells are dividing very rapidly or not at all (13, 25, 26). Although cell division is not synchronous among cells in the population or with the circadian cycle, the circadian clock regulates or "gates" the timing of cell division, defining a portion of the 24-h cycle in the early night in which cell division is disallowed (12, 13, 27). Additionally, the clock regulates the superhelical status and compaction of the chromosome (14, 15). Furthermore, the topological status of the chromosome is highly correlated with a distinct state in gene expression and has been proposed to be a key factor in imparting circadian gene expression patterns (28).

MECHANISM OF THE CIRCADIAN OSCILLATOR

In contrast to the transcription-translation feedback loop (TTFL) mechanism characteristic of eukaryotic circadian clocks, the cyanobacterial oscillator is a posttranslational oscillator (PTO) comprised of KaiA, KaiB, and KaiC proteins. KaiC is an autokinase, autophosphatase, and ATPase whose daily rhythms of phosphorylation at residues Ser431 and Thr432 and ATPase activity are key features of the timekeeping mechanism (Fig. 1) (29, 30). KaiC is a ring-shaped, bi-lobed homohexamer shaped like two stacked doughnuts. Each monomer is comprised of CI and CII domains that come together to form the linked CI and CII rings, as well as a C-terminal protruding tail referred to as the A-loop (Fig. 1) (31). While both CI and CII rings are able to bind and hydrolyze ATP,

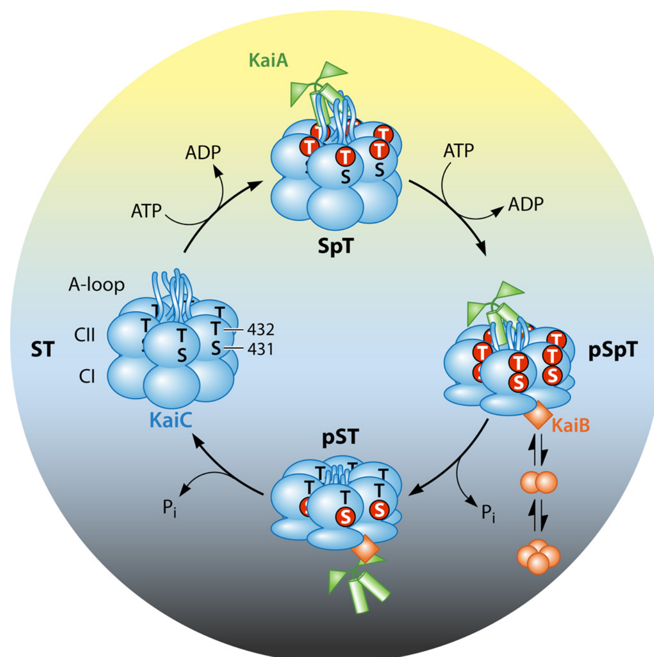

FIG 1 Mechanism of the cyanobacterial circadian oscillator. KaiC is a hexameric protein that comes together to form a "double doughnut" shape comprised of CI and CII rings with A-loop segments extending from the C terminus in the CII ring. KaiC in the nonphosphorylated state (ST) (far left) can interact with KaiA. KaiA, a dimer, binds to and stabilizes the A-loop segment in the exposed state, promoting KaiC autophosphorylation during the day. Red circles enclosing "S" and "T" represent phosphorylation of S431 and T432 residues, respectively, located in the CII ring. T432 becomes phosphorylated first (SpT), followed by S431, resulting in the doubly phosphorylated species (pSpT). Phosphorylation of S431 induces the stacking of the CII ring onto the CI ring. Ring-ring stacking exposes a binding site for KaiB (B-loop) on the CI ring. In order to for KaiB to interact with KaiC, both the B-loop must be exposed and KaiB must rearrange its tertiary fold from a ground-state fold found in tetramer and dimer species (orange circles) to a monomeric fold-switched form, fs-KaiB (orange diamond). Once both are achieved, fold-switched KaiB can bind to KaiC. fs-KaiB also interacts with KaiA, sequestering KaiA away from the A-loops on the CII domain. This action promotes burial of the A-loops into the hexamer and KaiC's autophosphatase activity during the nighttime portion of the cycle. Dephosphorylation occurs first on T432, resulting in pST species, followed by S431, resulting in a nonphosphorylated species (ST).

the phosphorylation events are limited to the CII ring (32). During the illuminated portion of the day, or of the subjective day, KaiA binds to the A-loop segment of KaiC, promoting KaiC autokinase activity (33). KaiB opposes KaiA's stimulatory activity by sequestering KaiA away from the A-loops, thereby promoting KaiC's intrinsic autophosphatase activity (33, 34).

KaiC phosphorylation occurs in a highly ordered, sequential manner where KaiC first phosphorylates Thr432 (SpT). Thereafter, Ser431 is phosphorylated, resulting in the doubly phosphorylated species (pSpT) (29, 35, 36). Dephosphorylation, occurring over the night/subjective night, also occurs in an ordered and opposite direction, with Thr432 becoming dephosphorylated first (pST) followed by Ser431, resulting in a nonphosphorylated species (ST) and completing one 24-h cycle (Fig. 1). More recently, highly sensitive nuclear magnetic resonance (NMR) techniques have highlighted the structural changes occurring in KaiC that drive the phosphorylation cycle in a clockwise direction. Specifically, the flexibility and stacking of the CI and CII rings relative to

each other change as a function of KaiC phosphorylation state and drive transitions from a KaiA-associated phosphorylation state to a KaiB-associated dephosphorylation state (34, 37). The phosphorylation of Ser431 results in tight stacking of the CI and CII rings, exposing a binding site for KaiB, referred to as the B-loop, located on the CI ring; exposure of the B-loops promotes the dephosphorylation phase of the cycle by allowing KaiB binding and the sequestration of KaiA away from the A-loops (Fig. 1) (34, 38). Consistent with the lack of motifs associated with typical protein phosphatases, KaiC autophosphatase activity occurs in an unusual manner. ATP is transiently formed via phosphotransfer from S431 and T432 to ADP, with the resulting ATP molecule hydrolyzed to ADP and P_i (39, 40). This unique mechanism is proposed to promote the ordered dephosphorylation events during the KaiC phosphorylation cycle.

KaiB FOLD SWITCHING

Major structural rearrangements in KaiB, specifically, rearrangement of the KaiB tertiary structure from a ground state (gs) to a fold-switched (fs) state, have recently come to light that play critical roles in driving the KaiC phosphorylation cycle in a clockwise direction, imparting time delay and linking the oscillator to output functions (41). X-ray crystallography has shown KaiB to exist as a homotetramer, organized as a dimer of dimers (42, 43). Despite the sequence similarity with the N terminus of output protein SasA, which exhibits a thioredoxin-like fold, the tetrameric form of KaiB adopts a rare fold not found in other proteins (20, 42, 44–46). While KaiB exists as a tetramer in solution, it was believed that KaiB bound to KaiC as a dimer (34, 47–49). However, recent studies have shown that KaiB binds to KaiC as a monomer (41, 47, 50). Surprisingly, this monomeric form of KaiB no longer exhibits the typical gs fold found in the dimeric and tetrameric forms but rather has completely rearranged, or fold-switched (fs), its tertiary structure to have a thioredoxin-like fold, similar to the N terminus of SasA (41). Mutations that stabilize the fold-switched version of KaiB (fs-KaiB) cause very rapid binding to KaiC: complete binding is observed in minutes, in contrast to wild-type (WT) or gs-KaiB, where hours transpire before an interaction is detected *in vitro* (41). Moreover, expression of fs-KaiB *in vivo* abolishes rhythms when expressed either as the only copy of *kaiB* or in addition to the endogenous copy. The fact that expression of fs-KaiB results in a dominant-negative phenotype supports the notion that fs-KaiB is the active form of KaiB (41). The transition from gs-KaiB to fs-KaiB is rare, and this low probability accounts for the slowness of the KaiB-KaiC complex association and for a significant element of the time delay necessary for 24-h timekeeping.

Thus, two conditions must be met in order for KaiB to associate with KaiC: KaiC must be in a state that is capable of interacting with KaiB, where the CII ring has stacked against the CI ring to expose the B-loop, and KaiB must also be in a fold-switched state. Once both of these events occur, KaiB can bind to KaiC and to KaiA. This trimolecular interaction sequesters KaiA away from the A-loop and promotes the dephosphorylation phase of the cycle. While there is some controversy regarding whether it is the CI or CII ring of KaiC that is bound by KaiB (51), new evidence clearly demonstrates physical binding of fs-KaiB to the CI ring (41); moreover, this binding is dependent on the presence of the B-loops, which are absent from CII (38, 41). These data suggest that, regardless of interactions KaiB may have with the CII domain, it is the interactions with the CI ring that are required to inhibit KaiA and promote the dephosphorylation phase of the cycle. Additionally, fs-KaiB plays a critical role in coordinating the activities of SasA and CikA, thus connecting oscillator timing to output activities (discussed below). Taken together, fold switching by KaiB, specifically, the rare transition to the fold-switched state, is a key factor that drives the clock in a clockwise direction, imparts a time delay, and links the oscillator to output function. KaiB is, so far, the only circadian clock protein described as a member of a class of proteins collectively known as metamorphic proteins that are capable of reversible fold switching (52).

REGULATION OF THE Kai-BASED OSCILLATOR

Perhaps one of the most remarkable features of the Kai-based oscillator is its unique ability to be reconstituted *in vitro* (53). By combining purified KaiA, KaiB, and KaiC in a test tube with ATP, oscillations of KaiC phosphorylation can be observed for days and even weeks. *In vitro*, the Kai oscillator has a periodicity of 24 h that is temperature compensated, solidifying the notion that the phosphorylation of KaiC is the main time-keeping mechanism in cyanobacteria (53). This *in vitro* preparation has proven to be a powerful tool for investigating the mechanism of clock action, and how the oscillator senses environmental inputs is described below.

While oscillations in KaiC phosphorylation are a major component of the timekeeping mechanism, the *kai* genes are subject to additional layers of regulation *in vivo*. Maintaining the appropriate stoichiometries among the Kai proteins is critically important for sustained rhythmicity, both *in vivo* and *in vitro* (54). The *kaiABC* locus is expressed rhythmically, showing peak expression at dusk, with *kaiA* driven from its own promoter (P_{kaiA}) and *kaiB* and *kaiC* expressed as an operon under the control of the P_{kaiBC} promoter (9). Inactivation or overexpression of any of the *kai* genes stops the clock (9, 55). In addition to oscillations in transcription, KaiB and KaiC exhibit robust oscillations in total protein levels, peaking early in the subjective night (circadian time 15 to 16) (from 25,000 to 10,000 molecules/cell for KaiB and from 15,000 to 5,000 molecules per cell for KaiC) (56), whereas KaiA protein levels remain constant or vary with a low amplitude at around 500 molecules/cell (Fig. 2) (56, 57).

In addition to the PTO, a TTFL mechanism similar to that of eukaryotic systems is also at play in the cyanobacterial circadian system. KaiA is a positive regulator, promoting expression of P_{kaiBC}, whereas KaiC is a negative regulator of expression from P_{kaiBC} (9). However, unlike eukaryotic core clock components, none of the Kai proteins are transcription factors, and Kai autoregulatory functions are indirect. Interactions of kinases (CikA and SasA) with the KaiABC complex influence the phosphorylation state of the master regulator of circadian gene expression, RpaA. RpaA is a transcription factor, discussed in more detail below, that binds directly to and regulates expression from P_{kaiBC}, another mechanism by which feedback is achieved to drive rhythmic gene expression (58). However, this TTFL mechanism is not as important for driving circadian rhythmicity as the PTO because KaiC phosphorylation cycles persist when cells are maintained in the dark, where transcription and translation are severely attenuated, or in the presence of transcription/translation inhibitors (59). More recently, it has been determined that the TTFL can drive rhythmicity under certain circumstances. For example, when mild KaiA overexpression locks KaiC into a hyperphosphorylated state to eliminate the PTO, rhythms of gene expression

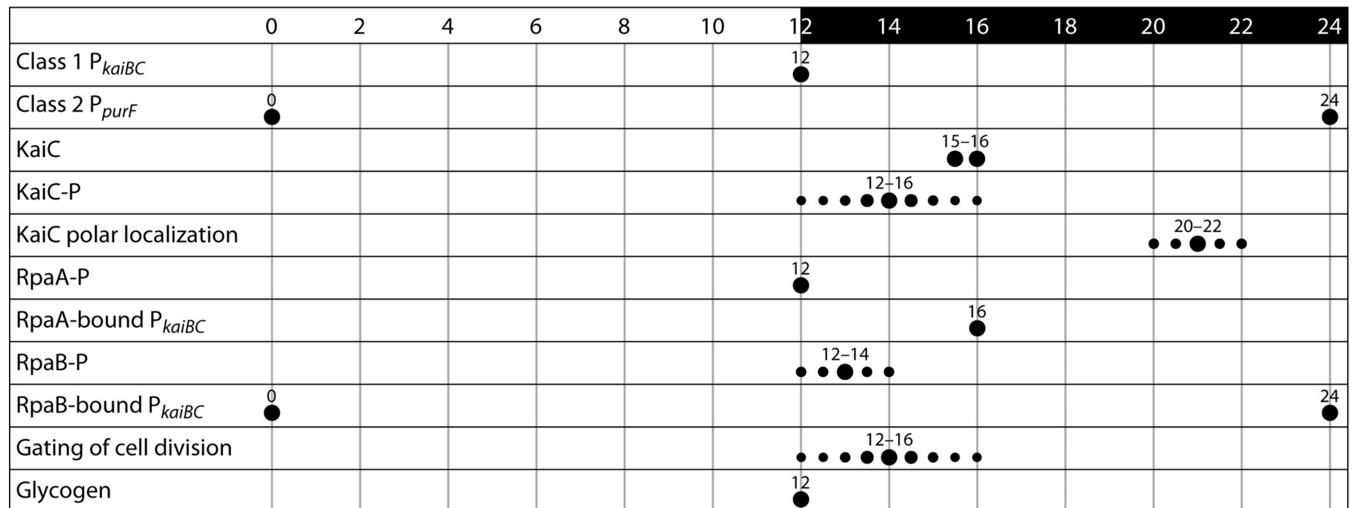

FIG 2 Timeline of circadian events. Peak timing for various events that occur over the circadian cycle is depicted both numerically and graphically (dots). All data except for RpaB phosphorylation status were collected from experiments performed in constant light.

(P_{kaiBC}) are still observed (60). Moreover, experiments in which *kaiBC* was placed under the control of an exogenous promoter in order to eliminate the TTFL showed that, under conditions that favor rapid growth, enhanced variations in phase are observed among individual cells within the population (61). These data, coupled with mathematical modeling, suggest that the TTFL is required for the generation of robust circadian oscillations specifically under conditions of rapid growth (61, 62). In contrast to what is observed in eukaryotic systems, the phasing of the cyanobacterial clock is inherited from mother cell to daughter cell with negligible intercellular coupling (63, 64). Combined, these results would suggest that the fidelity of cellular phase is compromised in individual cells without the TTFL.

SIGNALING STATES OF THE OSCILLATOR

Of the four KaiC phosphorylation states that occur over a 24-h cycle, how does each state contribute to generating rhythms of gene expression and how is information from the oscillator interpreted to produce rhythms of different phases? In order to address these questions, phosphomimetic variants of the 4 possible KaiC phosphorylation states were used to examine their influence on the rhythms of gene expression. In the absence of *kaiC*, class 1 (dusk-peaking) genes are constitutively expressed near the peak of normal oscillations whereas class 2 (dawn-peaking) genes are constitutively expressed near the trough (65). Surprisingly, the expression of most KaiC phosphomimetic variants had no effect on gene expression in a *kaiC* null strain, which behaved the same as a *kaiC* null strain for both class 1 (P_{kaiBC}) and class 2 (P_{purF}) reporters. The only exception was the expression of the KaiC-ET variant, a phosphomimetic of the pST state (Fig. 1), which was able to reverse the pattern of gene expression when expressed as the only copy of *kaiC* (65). In a KaiC-ET-expressing background, class 1 genes show expression near the trough, whereas class 2 genes have high expression near the peak. This effect is observed only when *kaiA* and *kaiB* are also present, suggesting that the entire oscillator is required to signal output from the clock (65). From these data, Kai-complex output activity (KOA) was measured and was found to peak near dawn. Taken together, these data suggest that the pST state of KaiC, occurring three-quarters of the way through the cycle, is responsible for signaling output that results in rhythms of gene expression. Additionally, the pST state of KaiC is capable of exerting opposite effects on class 1 (inhibition) and class 2 (activation) genes (65).

INPUT MECHANISMS

Although the circadian clock is built upon a self-sustained biological oscillator, it is highly sensitive to its environment and can be reset by environmental stimuli. In cyanobacteria, changes in the day-night cycle are not sensed directly via photoreceptors as in eukaryotic systems; rather, cells monitor changes in their environment indirectly by sensing the redox status of the cell, which varies as a function of photosynthesis status. Several redox-sensitive input components have been identified, including CikA and LdpA (57, 66). In addition, the core oscillator components KaiA and KaiC are also known to directly detect metabolites of photosynthesis in order to synchronize the clock with the external environment (67, 68). While the primary components that make up the input and output pathways are known, we highlight recent work that has shed light onto how these components interact with each other as well as with the oscillator to synchronize the clock with the environment and drive rhythmic outputs.

REDOX SENSING BY THE CLOCK

CikA was the first input component identified (16). Mutation of *cikA* results in a variety of phenotypes, including a short circadian period, low-amplitude oscillations, elongated cells, and the inability to reset oscillations of gene expression after receiving a 5-h dark pulse (16, 69). The fact that *cikA* mutants do not reset the phase of the oscillation after receiving a resetting dark pulse solidified the notion of CikA as a member of an input pathway responsible for relaying information from the environment to the oscillator, although we now know that CikA is additionally responsible for modulating output activities (70). This environmental sensing is achieved by interactions between CikA, as well as central oscillator component KaiA, and the cellular quinone pool.

The abundance and redox status of many cellular metabolites

change as a function of photosynthetic activity in cyanobacteria, including the redox state of the plastoquinone pool. Specifically, the quinone pool becomes rapidly oxidized upon the onset of darkness (71). CikA and KaiA, through their respective pseudoreceiver (PsR) domains, can bind to the oxidized form, but not the reduced form, of quinone (66, 68). Remarkably, the addition of oxidized quinone (Q_0), proposed to serve as a proxy for darkness, applied during the subjective day is sufficient to induce phase shifts both *in vivo* and *in vitro* that mimic the effects of a true dark pulse *in vivo* (71). Both CikA and KaiA are peripheral membrane proteins that localize to the poles of cells, which may facilitate interactions with quinones in the thylakoid membranes (72, 73). Moreover, CikA protein levels are subject to both circadian regulation and environmental changes, where CikA abundance is inversely proportional to light intensity and exhibits the highest levels in the dark (66). The addition of oxidized quinone results in the aggregation of CikA and KaiA *in vitro* and their rapid disappearance, as detected by immunoblot, *in vivo*, suggesting that aggregation followed by degradation of these components is a potential mechanism by which the clock becomes synchronized with the day-night cycle (66, 68).

In addition to CikA/KaiA interactions with quinones, changes in cellular redox are relayed to the clock via the input component LdpA, which is an iron-sulfur center-binding redox-active protein (17, 57). *ldpA* mutants exhibit 1-h period shortening and a lack of sensitivity to the light gradients that normally modulate the circadian period (17). LdpA is responsible for the fine-tuning of the period of circadian rhythms as a consequence of sensing changes in electron transport that are dependent on light intensity and is also required for light-dependent changes in CikA abundance, suggesting cooperativity between CikA and LdpA in relaying environmental signals to the oscillator.

METABOLIC INPUTS TO THE CLOCK

In addition to cellular redox, the circadian clock receives inputs from other metabolites of photosynthesis that change over the course of the day. Rust et al. reported that KaiC can directly sense changes in the ATP/ADP [specifically, ATP/(ATP+ADP)] ratio in the cell over the course of the day (67). Irrespective of when a dark pulse is administered, cells experience a large decrease in the ATP/ADP ratio gradually over the duration of the pulse. When similar transient changes to the ATP/ADP ratio are mimicked *in vitro*, large phase shifts in the phosphorylation rhythm of KaiC are observed (67). Although the cellular response to darkness was the same at all circadian times, the oscillator displayed differential levels of sensitivity to the cue at different times in the cycle, as is characteristic of circadian responses. Specifically, the oscillator was most sensitive to changes in ATP/ADP during the middle of subjective day, corresponding to a "daytime" point when KaiC phosphorylation was increasing. Taken together, these data suggest that the redox status of the quinone pool and the ATP/ADP ratio act in concert to signal both the onset and duration of darkness to the oscillator (71). Both cues contribute to the ability of a circadian clock to encode a photoperiod.

Other connections between cellular metabolism and the clock have been uncovered recently. Specifically, the circadian clock regulates rhythms of glycogen abundance under conditions of continuous illumination (74, 75), where photosynthetic carbon assimilation results in glycogen accumulation during the subjective day. Analysis of mutants that display various abilities to shift circadian phase after receiving a dark pulse revealed that the amount of glycogen present in the cell is inversely proportional to the ability to phase shift (75). *cikA* mutant strains, which cannot reset the phase in response to a 5-h dark pulse, accumulate larger amounts of glycogen and higher levels of ATP/ADP in the dark and have an exaggerated dusk-like transcriptional state compared to WT strains (75). In contrast, *glgC* mutants, which are not capable of accumulating glycogen, exhibit stronger phase shifts when a dark pulse is administered during the day. When *cikA*-like changes in ATP/ADP were mimicked *in vitro*, a defect in the ability to shift the phase of KaiC phosphorylation was detected, suggesting that the inability of *cikA* mutants to reset is in part due to changes in cellular energy charge (75). Taken together, these results suggest that the clock regulates metabolic rhythms that can feed back to the oscillator and allow the cell to anticipate nightfall.

OUTPUT MECHANISMS

The mechanisms that relay the temporal information from the clock to generate circadian rhythms of cellular physiology rest centrally on a two-component signaling pathway comprised of the histidine kinase SasA and cognate response regulator RpaA (20, 21). The SasA/RpaA output pathway is required to drive global rhythms of gene expression as well as gating of cell division (12). However, rhythms of chromosome compaction are independent of SasA (14), suggesting the existence of other output pathways. The following section highlights recent advances that have shed light on how RpaA exerts its influence to generate rhythmic outputs and have identified novel players that modulate output activities.

SasA was identified simultaneously in two laboratories as a KaiC-interacting protein and as exhibiting sequence similarity to KaiB (20). It is comprised of an N-terminal KaiB-like domain and a canonical histidine kinase domain. Loss of *sasA* results in a short circadian period and low-amplitude rhythms of gene expression. SasA interacts with KaiC via its N-terminal KaiB-like domain, which stimulates SasA autophosphorylation and phosphotransfer to response regulator RpaA (Fig. 3) (20, 21). Early structural analysis raised questions of the functional significance regarding the sequence similarity between KaiB and the N terminus of SasA because the differences that occurred created critical differences in secondary and tertiary structures (46). This conundrum was solved by the discovery of fs-KaiB, which shares the tertiary structure of the N terminus of SasA (41). Once KaiB has attained the fs-KaiB conformation, these two proteins compete for binding to overlapping binding sites on KaiC (38). fs-KaiB plays a critical role in regulating the timing of output functions and has been shown to be able to displace SasA from KaiC (41). Moreover, fs-KaiB can interact with the pseudoreceiver (PsR) domain of CikA (41) and association with the KaiBC complex stimulates the phosphatase activity of CikA toward RpaA (41, 70). Additionally, KaiA promotes the displacement of SasA from KaiC by KaiB, implicating KaiA in regulation of clock output functions as well (38).

While the loss of *sasA* results in altered rhythmicity (20), loss of *rpaA* completely abolishes rhythms of gene expression, highlighting RpaA as a key factor for conveying circadian outputs (21). These data also suggest the existence of auxiliary pathways that converge on RpaA. Although the SasA/RpaA pathway was first

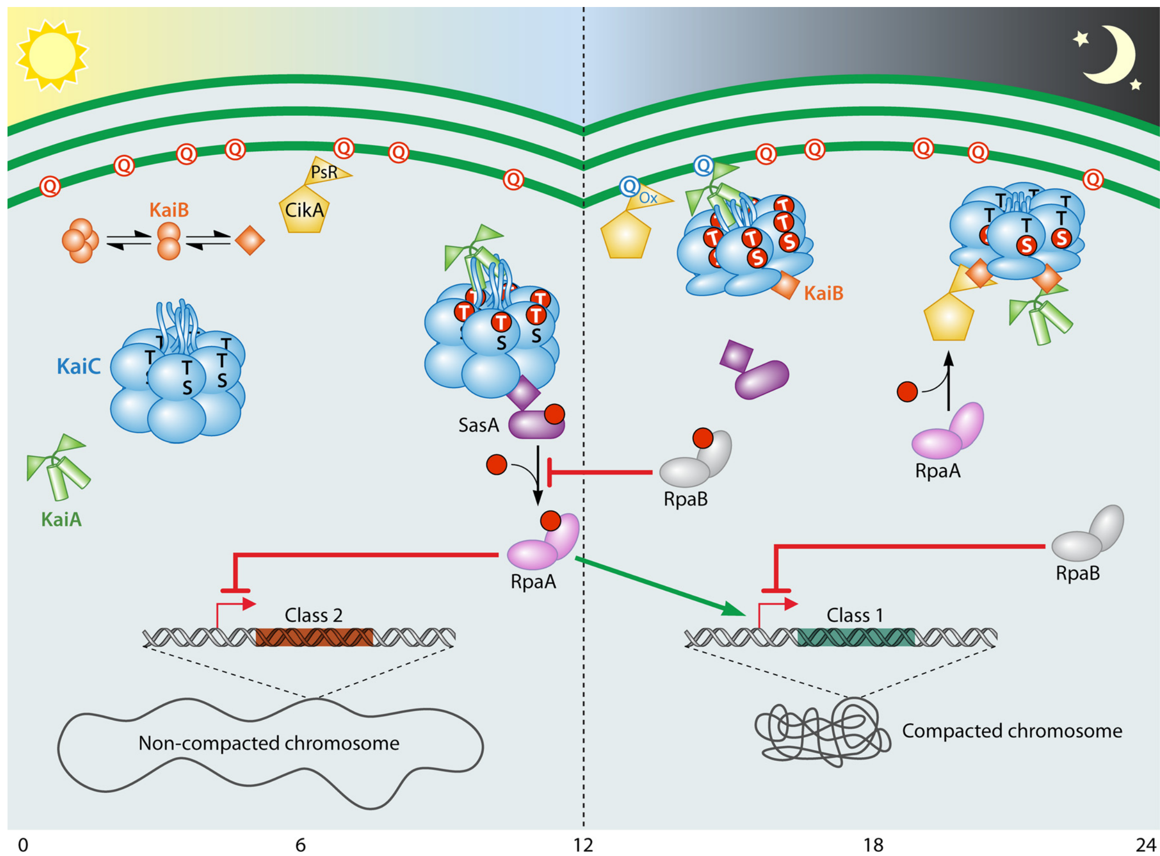

FIG 3 Model for how the circadian network functions over time and within a cell. Two cells are displayed side by side showing the progression of the oscillator over time (indicated by shading from sun to moon at the top and numerically at the bottom). During the day (left panel), KaiA and KaiC are found primarily in the cytosol. KaiA interacts with KaiC, promoting KaiC phosphorylation during the illuminated portion of the cycle. Phosphorylation events are indicated by a red dot. CikA is localized to the poles of cells. Quinones mobile in the thylakoid membranes are reduced during the day (red "Q"). SasA is capable of interacting with KaiC, promoting autophosphorylation of SasA and phosphotransfer to RpaA. RpaA-P accumulates over the course of the day, peaking at the day-night transition or ~12 h after lights on. RpaA-P represses the expression of class 2 (dawn-peaking) genes and activates class 1 (dusk-peaking) genes. Environmentally controlled RpaB can inhibit the phosphorylation of RpaA. Chromosomes (gray) are in a noncompacted state. At night or during the subjective night (right panel), the quinone pool becomes transiently oxidized. KaiA, KaiC, and CikA are all colocalized to the cell poles where CikA and KaiA can interact with oxidized quinones (blue "Q"). Fold-switched KaiB competes with SasA for binding to KaiC and sequesters KaiA away from the A-loop, promoting KaiC dephosphorylation through the night. CikA competes with KaiA for binding to fold-switched KaiB. The KaiBC complex promotes CikA's phosphatase activity toward RpaA. At night, chromosomes (gray) are in a compacted state.

identified in the early 2000s, it was not until recently that we obtained a more comprehensive understanding of how RpaA, a DNA-binding response regulator, exerts its influence to generate robust oscillations of biological activity. O'Shea and colleagues demonstrated that RpaA is active as a DNA-binding transcription factor in its phosphorylated state and that phosphorylated RpaA (RpaA-P) binds directly to ~100 targets in the *S. elongatus* genome (58). RpaA-P promotes expression from the *kaiBC* promoter (P_{kaiBC}) by binding to a site at positions −29 to −51 relative to the transcription start site (58), a region that had been shown previously to be an important regulatory region for expression of P_{kaiBC} (76). The binding of RpaA to these targets occurs in a circadian manner, with similar phases at all 110 sites in the genome (Fig. 2). The RpaA regulon consists of 134 transcripts, including 170 genes and 41 noncoding RNAs. Eight of the genes expressed as part of the RpaA regulon are transcription factors, including 4 sigma factors (58). These targets are likely to be important for the more pervasive rhythmicity that occurs throughout the genome, including genes that are not directly under RpaA control.

Peak RpaA-P occupancy occurs early in the subjective night (58) (Fig. 2), which correlates well with previous reports that Kai-complex output activity (KOA) peaks near dawn (65). Loss of *rpaA* results in global loss of rhythmic gene expression, arresting cells in a dawn-like state, with class 2 genes showing high expression and class 1 genes showing low expression (58). Expression of a phosphomimetic variant of the active-state RpaA (D53E) arrests gene expression in a dusk-like state and inhibits cell division, resulting in elongated cells, suggesting that phosphorylated RpaA is the active state both for driving rhythms of gene expression and for gating of cell division (58). When expression of *kaiBC* is placed under the control of a promoter that lacks an RpaA-binding site (P_{trc}), rhythms of KaiC phosphorylation persist in the absence of *rpaA*, although gene expression is arrhythmic (58). This finding suggests that the loss of gene expression rhythms in an *rpaA* null strain is not due to an effect on the oscillator itself, further solidifying the notion of RpaA as a downstream component of the clock mechanism.

ROLES FOR CikA IN THE OUTPUT

CikA is a histidine kinase, capable of autophosphorylation on a conserved His residue (77); however, the identity of a response regulator partner involved in this pathway long remained a mystery. Phosphoprofiling experiments identified RpaA as the only response regulator (of 24 tested) capable of phosphotransfer from CikA *in vitro* (70). Histidine kinases can act either as a kinase or phosphatase toward their cognate response regulator (78, 79). Indeed, while CikA is capable of transferring a phosphoryl group to RpaA *in vitro*, it appears that the main role for CikA *in vivo* is as a phosphatase, involved in removing phosphoryl groups from RpaA that are added by SasA (70). Earlier genetic studies had also implicated CikA in roles in modulating circadian outputs via negative regulation of RpaA, although at the time the mechanism was not understood (80). The activities of SasA and CikA toward RpaA are timed by the oscillator. The fully phosphorylated state of KaiC (pSpT) is required for maximal stimulation of SasA's kinase activity (21, 70), while the presence of both KaiB and KaiC is needed to stimulate the phosphatase activity of CikA (70). The current model explains this requirement, because it is fs-KaiB, stabilized by binding to KaiC, that binds and stimulates CikA. Taking the data together, SasA and CikA antagonize each other to create robust rhythms of RpaA phosphorylation, suggesting that RpaA is a key node for integrating circadian inputs and generating rhythmic outputs.

NOVEL COMPONENTS OF THE OUTPUT PATHWAY

crm (circadian rhythm modulator) and *rpaB* (regulator of phycobilisome association B) have recently been identified as loci that play roles in the circadian clock system. How these components were identified to regulate the output pathway and how they exert their influence on the clock are discussed in the following section.

crm is a small, previously unannotated gene that potentially encodes a 62-amino-acid peptide with no functional domains; it was found upstream of *rpaA* by a transposon insertion mutation that was originally thought to affect RpaA expression. A transposon insertion allele in the *crm* gene (*crm1*), similarly to the disruption of *rpaA*, results in arrhythmic expression of luciferase reporters (81). Prior to this work, the only mutations known to cause complete arrhythmia were changes in the *kai* genes or *rpaA*. However, RpaA steady-state levels are not altered in a *crm1* mutant and *crm1* phenotypes are distinct from those of an *rpaA* null strain, suggesting that the arrhythmia is not due to a polar effect on *rpaA*. Although expression of an intact *crm* in *trans* is sufficient to complement the arrhythmicity of a *crm1* strain, an in-frame deletion of *crm* has no discernible phenotype with respect to rhythms of gene expression, suggesting that the effect on the circadian clock is allele specific (81). While the identity of the mechanism of action remains unresolved, a Crm peptide is proposed to function as an allosteric modulator of RpaA, consistent with the requirement for a translation-initiating codon in *crm* to complement a *crm1* mutant. Perhaps the shortened or altered peptide produced by the transposon insertion is capable of exerting adverse effects on the clock in a recessive manner.

The RpaB response regulator has been implicated in circadian transcriptional regulation. RpaB (19) is an essential, highly conserved response regulator that, in conjunction with cognate histidine kinase NblS, regulates a large number of genes in response to stress conditions, including high light, cold, and osmotic and oxidative stress (82–84). In a phosphorylated state, RpaB can bind to an element called HLR1 (high light regulatory 1) to regulate expression (82). RpaB has been reported to function as an activator of gene expression but primarily as a repressor, inhibiting expression from downstream promoters (82, 85). A HLR1 element present in the P_{kaiBC} promoter (positions −51 to −44 relative to the transcription start site, overlapping with the reported RpaA-binding site) is bound by RpaB *in vivo* and *in vitro*, with peak binding observed at subjective dawn under conditions of constant light (86), ~12 h offset from peak RpaA binding to P_{kaiBC} (Fig. 2) (58). Taken together, these reports suggest that RpaA and RpaB act cooperatively to regulate expression of circadian targets, perhaps by RpaA displacement of RpaB. Genes included as part of the RpaA regulon, including *kaiBC* (class 1) and *purF* (class 2), have also been shown to be negatively regulated by RpaB (87).

While the phosphorylation of RpaA oscillates in a circadian manner, oscillations in RpaB phosphorylation are observed only when cells are grown under diel conditions (12 h of light followed by 12 h of dark) and are not regulated by the clock (87). Moreover, overexpression of the RpaB receiver domain resulted in decreased RpaA phosphorylation levels, suggesting not only that RpaA and RpaB are competing for binding to targets but also that RpaB can act as a modulator of RpaA activity via regulation of RpaA phosphorylation status (87). These new findings highlight how cross talk occurs between a circadian (SasA-RpaA) pathway and an environmentally controlled pathway (NblS-RpaB) and how the pathways cooperate to generate rhythms of gene expression.

INTEGRATED CIRCADIAN NETWORK

Once thought of as a linear pathway consisting of input → oscillator → output, the cyanobacterial clock can be more accurately described as an integrated network, with components making multiple connections and serving multiple roles in order to generate robust circadian oscillations (Fig. 3). Roles for KaiA in both the central timekeeping mechanism and input signaling (68), as well as roles for CikA in input redox sensing and modulation of output activity, had been previously appreciated (66, 70). Recently, mutations that map to *sasA* were found to suppress *cikA*-associated phenotypes (88). Suppressor mutations of *sasA* (*sasAY290H* and *sasAP263L*) can restore WT patterns of gene expression rhythms, both period and amplitude, when expressed in strains lacking *cikA*. Remarkably, these mutations also restore the ability to reset the phase of the rhythms after receiving a dark pulse to *cikA* null cells (88). *sasA* suppressor mutations continue to support phase resetting in a *cikA* mutant background when *kaiBC* is driven from the P_{trc} promoter and rhythmic expression of this locus is no longer dependent on direct RpaA binding; this finding suggests that these mutations are exerting their influence not indirectly through RpaA feedback but perhaps through direct modification of the oscillator (88). Such a mechanism is consistent with the knowledge that SasA physically binds to KaiC and competes for binding with KaiB (41). These data suggest that input and output, as well as the fundamental oscillation, result from a highly connected network of clock components.

In addition to causing daily oscillations in mRNA and protein abundance, the circadian system also undergoes a circadian orchestration of subcellular organization, suggesting that clock proteins localize to the appropriate position within the cell in order to execute their functions. The input/output protein CikA had been previously demonstrated to localize as a discrete focus at one pole of the cell in a manner that requires the quinone-binding PsR

domain (73). More recently, we demonstrated by using fully functional fusions to fluorescent proteins that the oscillator components KaiA and KaiC similarly localize to a single pole of the cell (72, 89). Whereas CikA is localized to the cell pole throughout the course of the day, KaiC exhibits spatiotemporal changes in subcellular localization, such that enhanced polar localization is observed at night in a clock-dependent fashion (72). Oscillations in KaiC localization that range from diffuse throughout the cell during the day to highly localized to the poles at night are observed under both diel and constant conditions and are dependent on the presence of a functional clock, demonstrating that regulation of these spatiotemporal changes is circadian in nature (72). CikA and KaiC are capable of independently localizing to the poles in *Escherichia coli* as well, suggesting that the mechanism of localization is not dependent on a cyanobacterial, clock, or photosynthetic factor. In contrast, the polar localization observed for KaiA is entirely dependent on the presence of KaiC, specifically, of the A-loop segment of KaiC to which KaiA is known to bind (72). Consistent with the dependence of KaiA localization on the presence of KaiC, KaiA and KaiC colocalize with each other as well as with CikA (72), suggesting that the spatial distribution of the clock components and the colocalization of factors that differ in space and time contribute to the complexity of the circadian system.

Although the mechanism that targets KaiC to the poles remains mysterious, localization patterns due to the formation of inclusion bodies or regulated proteolysis by the ClpXP protease at the poles have been ruled out. However, one clue to the biological relevance underlying polar localization comes from the use of *cikA* mutant variants. CikA variants that lack the PsR domain or the phosphoralatable histidine in the kinase domain are able neither to complement rhythms of gene expression nor to support polar CikA localization (72, 73). Expression of these *cikA* variants results in striking delocalization of KaiC, suggesting that polar localization of clock components may contribute to mechanisms of entrainment (72).

Expression of a *cikA* variant lacking the GAF domain, which is not able to restore normal periodicity (73), displayed an intriguing localization pattern in which CikA was able to localize to the poles only at night (72). The GAF domain of CikA is similar to that of phytochrome photoreceptors that typically bind bilin cofactors; however, the GAF of CikA lacks a conserved cysteine or histidine to serve as a bilin-binding site (16). While CikA is capable of binding to a bilin cofactor *in vitro*, the complex is not photoactive and CikA does not copurify with a bilin cofactor, suggesting that CikA itself does not function as a photoreceptor (77). Rather, it is the PsR domain of CikA that has been deemed important for CikA's role as a redox sensor and for relaying environmental signals via interactions with oxidized quinone (66). However, the localization patterns exhibited by the CikA (ΔGAF) variant, distributed throughout the cell during the day and localized to the poles at night, in contrast to being constitutively localized, suggest that the GAF domain of CikA is important for differentiation of day from night. Whether this ability to alter subcellular localization patterns comes from previously undetected direct photoreception activity or from some other mechanism remains undetermined. Taking the data together, the subcellular localization of clock components that vary as a function of space and time contributes to the complexity of the circadian network in cyanobacteria and may function to facilitate interactions with metabolites of photosynthesis and protein complex assembly, thereby contributing to the synchronization and robustness of the circadian clock.

CIRCADIAN SYSTEMS IN OTHER CYANOBACTERIA

While the *S. elongatus* clock system is by far the best-understood cyanobacterial circadian clock, natural variations of the basic *kaiABC* system exist. For example, marine *Prochlorococcus* spp., important primary producers, carry *kaiB* and *kaiC* genes but lack a (full-length) *kaiA* gene (90). While *Prochlorococcus marinus* PCC 9511 has been shown to exhibit robust diel oscillations of gene expression and cell cycle progression, these oscillations rapidly damp under constant conditions (91), leading to the notion that the *Prochlorococcus* clock mechanism is not a self-sustained oscillator but rather functions more like an hourglass, requiring daily environmental input to reset and drive the clock. Intriguingly, diel oscillations of gene expression in marine cyanobacteria, including *Prochlorococcus* spp., have also been observed in the ocean with peak expression similar to that observed in monocultures grown in the laboratory for many of the genes tested (92). Unexpectedly, this same sampling study conducted by DeLong and colleagues found diel oscillations in gene expression of heterotrophic bacterial populations as well. They have proposed metabolic coupling between primary producers (e.g., cyanobacteria) and consumers (e.g., heterotrophic bacteria) to be the underlying mechanism by which oscillations occur among the heterotrophic bacterioplankton groups (92). These studies suggest that the cyanobacterial circadian clock can more globally drive rhythmic phenomena in nature, including among organisms that do not themselves keep time.

While some cyanobacterial species, including *Prochlorococcus*, have lost *kaiA* from the system, other species, including *Synechocystis* sp. PCC 6803, encode additional KaiB and KaiC paralogs. In addition to the standard *kaiAB1C1* cluster, *kaiC2 kaiB2*, *kaiB3*, and *kaiC3* genes are found at 4 genomic loci (90). How the Kai proteins encoded by these multiple genes might interact and whether they all function in timekeeping mechanisms is not well understood. However, it has been proposed that the *kaiA kaiB1 kaiC1* locus of *Synechocystis* sp. 6803 plays a critical role in establishing circadian rhythmicity, as has been demonstrated in *S. elongatus* PCC 7942, and that *kaiB3* and *kaiC3* may provide fine-tuning (93, 94).

Kai PROTEINS OUTSIDE CYANOBACTERIA

While *kaiA* appears to be specific to cyanobacterial genomes, potential homologs of *kaiB* and *kaiC* are widespread in species of *Archaea* and, to a lesser extent, in genomes of *Proteobacteria*, *Chloroflexi*, and *Bacteroides* (90, 95). Both *kaiB* and *kaiC* genes are found in some genomes, while only orthologs of *kaiC* can be identified in others. Interestingly, most of the *kaiC* homologs found in species of *Archaea* are single-domain versions of *kaiC* whereas those found in *Proteobacteria* mostly encode both CI and CII domains (90). Our current model of the circadian mechanism of *S. elongatus* implies that two domains are needed to support timekeeping.

What are these *kai* homologs doing? Are they participating in timekeeping activities, or are they adapted for participation in other cellular processes? Both may be true. In the purple photosynthetic protoebacterium *Rhodobacter sphaeroides*, which carries both *kaiB* and *kaiC* homologs, rhythms of gene expression on either circadian or ultradian time scales could be detected using a

luciferase reporter depending on the presence or absence of oxygen (96). These rhythms exhibited a 20.5-h circadian period under aerobic conditions, whereas an ultradian 10.6-to-12.7-h period was observed under anaerobic conditions (96). These rhythms could be entrained to light or temperature cycles and persisted under continuous conditions. Although it was not demonstrated that *kaiB* and *kaiC* play a role in this timekeeping mechanism, the work does suggest that *kai* genes may also be important for biological timekeeping in organisms other than cyanobacteria.

Investigations of the *kaiB* and *kaiC* homologs in the human pathogen *Legionella pneumophila*, on the other hand, have suggested that the *kai* genes may participate in other biological processes. In *L. pneumophila*, *kaiB* and *kaiC* are organized in an operon that is under the control of the stress sigma factor RpoS and that has a growth-phase-dependent expression profile (97). While *L. pneumophila* KaiC is capable of autophosphorylation, no interaction between KaiB and KaiC was detected using two-hybrid approaches (97). Mutation of the *kaiC* operon resulted in strains with enhanced sensitivity to oxidative and salt stress, suggesting that *kaiBC* may play a role in cellular stress responses (97). Interestingly, *kaiC2* from *Synechocystis* sp. PCC 6803, proposed to not have a role in clock function, is most similar to *kaiC* homologs found in genera of *Archaea* and other *Proteobacteria*, including *Legionella*. Although rigorous investigations into roles for the *Legionella kaiB* and *kaiC* homologs in circadian timekeeping have not been undertaken, the results of these studies suggest that *kaiBC* homologs found outside cyanobacteria may participate in other biological processes. Interestingly, the crystal structure of *L. pneumophila* KaiB revealed that it assumes the fold-switched form (97). Remarkably, the amino acid substitutions that were introduced to stabilize the fold-switched (KaiC-interacting [41]) state in *S. elongatus* are the same residues that are found naturally in those positions in *L. pneumophila* as well as in other organisms. It is postulated that the ancestral KaiB protein had a thioredoxin-like, fold-switched structure and that evolution of the ground-state KaiB fold enabled the time delay that contributed to the circadian clock (41).

ARCHAEAL RHYTHMS

While prokaryotic rhythms have been attributed to cyanobacteria, preliminary evidence suggests that *Archaea* also display rhythmic activities reminiscent of those driven by a circadian clock. Specifically, the expression of ~12% of genes from the halophilic archaeon *Halobacterium salinarum* NRC-1 has been found to oscillate with either ultradian (13-h) or circadian (22-h) periodicity under constant conditions after 3 days of entrainment in an LD cycle (98). The halophilic archaeon *Haloferax volcanii* harbors 4 cryptic *kaiC*-like genes, *cirA*, *cirB*, *cirC*, and *cirD*, that share 28% to 33% identity with and 48% to 55% similarity to KaiC from *S. elongatus* and possess predicted Walker A and B motifs (99). Expression of the *cir* genes was found to be rhythmic under diel conditions, and these oscillations are lost in *cir* mutant backgrounds lacking *cirB*, *cirC*, or *cirD*, whereas *cirA* could not be eliminated (99). While more-detailed studies will be required to determine if these oscillations represent a bona fide circadian rhythm, these results pose the very interesting hypothesis that circadian rhythms may also be a property of some *Archaea*.

PEROXIREDOXINS AS A MARKER OF CIRCADIAN RHYTHMS

Peroxiredoxins (Prx) are a family of antioxidant proteins that help control intracellular peroxide levels (100). Peroxiredoxin oxidation rhythms have been described as a conserved marker for circadian rhythms (101–103). Using an antibody against the oxidized active state, 24-h redox cycles that are entrainable and temperature compensated and persist under continuous conditions are observed. Remarkably, in eukaryotic cells, peroxiredoxin oxidation rhythms have been demonstrated to be independent of transcription-translation feedback loops (102, 103). Rhythms of peroxiredoxin oxidation have been observed for many species, including mice, files, and *Neurospora*, as well as in prokaryotes, including *S. elongatus* and the archaeon *Halobacterium salinarum* sp. NRC-1 (101). These data add to the mounting evidence regarding archaeal circadian rhythms. Interestingly, in *S. elongatus*, peroxiredoxin oxidation rhythms persisted in a *kaiA* mutant background, suggesting that the observed rhythms are not driven by the canonical clock mechanisms and that another oscillator may be present (101). This yet-to-be-identified oscillator may represent a more ancient clock, present in all domains of life, where it has been proposed that oxidative cycles could have driven the evolution of circadian rhythms.

PERSPECTIVES

In diverse organisms, circadian rhythms are vitally important for controlling temporal programs of cellular physiology that facilitate adaptation to daily environmental changes (104). While cyanobacteria are the only prokaryotes currently known to possess a bona fide circadian clock, rhythmic activities, reminiscent of circadian rhythms, have been observed in other prokaryotic organisms. The model organism *Synechococcus elongatus* PCC 7942, a cyanobacterium for which genetic manipulation is simple, circadian rhythms of gene expression are readily measured, and extensive genetic tools are available, has not only led to our understanding of how the cyanobacterial circadian clock works but also has provided fundamental insights that extend beyond circadian biology. Although the cyanobacterial clock represents a more simplified system with a relatively small number of players, many unknowns still exist. More-recent studies have increased our understanding of how the core oscillator is integrated with other cellular processes and how temporal information from the oscillator is transmitted to result in oscillations of biological activity. What was once considered to be a linear model, consisting of input, oscillator, and output, has now morphed into a more integrated network model. Many components of the clock network have been shown to be multifunctional, with roles in timekeeping, input, and/or output mechanisms. The fact that single compensatory point mutations that suppress the loss of *cikA* can be isolated in *sasA* suggests the presence of a highly connected circadian network. Although the KaiABC oscillator can run in isolation in a test tube, the Kai-based oscillator must make many connections with this clock network as well as with other components of the cell to affect subcellular localization to the poles of cells, interactions with the thylakoid membranes, and other cellular functions. These past few years have seen a profound increase in our knowledge of the cyanobacterial circadian clock, and the future promises to reveal further insights into the mechanisms of this amazing system.

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Susan E. Cohen obtained a B.S. in microbiology (2003) from the University of California, Santa Barbara, and a Ph.D. in molecular biology (2009) from the Massachusetts Institute of Technology, where she worked on mechanisms of DNA repair and damage tolerance in *Escherichia coli* in the laboratory of Graham C. Walker. She is an American Cancer Society and University of California President's Postdoctoral Fellow in the laboratory of Susan S. Golden at the University of California, San Diego, where she studies the cell biology of the cyanobacterial circadian clock.

Susan S. Golden obtained a B.A. in biology (1978) from the Mississippi University for Women and a Ph.D. in genetics (1983) from the University of Missouri. After postdoctoral research at the University of Chicago, she began her independent career on the faculty of biology at Texas A&M University. In 2008 she moved her laboratory to the University of California, San Diego, where she is currently a Distinguished Professor in the Division of Biological Sciences and the director for the Center for Circadian Biology. Her earlier interests centered on investigating light-regulated gene expression in *Synechococcus elongatus*. Research in her laboratory is now focused on elucidating the mechanism of the cyanobacterial circadian clock and developing cyanobacteria as platforms for industrial production of valuable products.