The Cyanobacterial Circadian System: From Biophysics to Bioevolution

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Abstract

Recent studies have unveiled the molecular machinery responsible for the biological clock in cyanobacteria and found that it exerts pervasive control over cellular processes including global gene expression. Indeed, the entire chromosome undergoes daily cycles of topology/compaction! The circadian system comprises both a posttranslational oscillator (PTO) and a transcriptional/translational feedback loop (TTFL). The PTO can be reconstituted in vitro with three purified proteins (KaiA, KaiB, and KaiC) and ATP. These are the only circadian proteins for which high-resolution structures are available. Phase in this nanoclockwork has been associated with key phosphorylations of KaiC. Structural considerations illuminate the mechanism by which the KaiABC oscillator ratchets unidirectionally. Models of the complete in vivo system have important implications for our understanding of circadian clocks in higher organisms, including mammals. The conjunction of structural, biophysical, and biochemical approaches to this system has brought our understanding of the molecular mechanisms of biological timekeeping to an unprecedented level.

Contents

BACTERIA HAVE CIRCADIAN RHYTHMS

Many biological oscillations have been analyzed biophysically, but most of those oscillators are of relatively high frequency (e.g., millisecond to second) and often involve ionic fluxes across membranes. Circadian rhythms, on the other hand, are ~24-h oscillations in biological processes that are controlled by an endogenous biochemical pacemaker. The processes for which activities are choreographed by these clocks range from gene expression, metabolism, and cell division, to development and behavior (12). Circadian rhythms are defined by three diagnostic properties: (*a*) persistence of the oscillations in constant conditions (usually constant darkness, DD, or constant light, LL, at constant temperature), (*b*) temperature compensation (the period length is only slightly affected by temperature changes, i.e., Q_{10} ~0.9–1.1), and (*c*) entrainment of the endogenous pacemaker to the environmental cycle of light and dark (12). Most difficult to explain from the biophysical perspective are the precision of this long time-constant oscillator (~24 h ± only a few minutes per day) and the temperature compensation property (which is true even for cells and tissues from endothermic animals) (12, 29, 95). However, from an evolutionary perspective, a temperature-dependent or imprecise clock is likely to be useless as an endogenous estimator of environmental time (12, 80). Therefore, a ~24-h clock with the conserved properties of temperature compensation, entrainment, and precision has been the product of natural selection in organisms from bacteria to human. What has not been conserved among cyanobacteria, fungi, plants, and animals are the sequences of the proteins, which are the gears and cogs of these clocks. This implies that circadian clocks have convergently evolved multiple times in response to the selective pressure of an environment with daily cycles (83).

The prokaryotic cyanobacterium *Synechococcus elongatus* PCC7942 has proven advantageous for circadian clock research (10, 43). Cyanobacteria have worldwide importance. The marine cyanobacterium *Prochlorococcus marinus* is possibly the most abundant photosynthetic organism on earth and certainly contributes a large proportion of total global photosynthetic activity (74). Moreover, cyanobacteria are being enlisted as platforms for production of biofuels. This includes *S. elongatus*, which is a unicellular bacterium that depends on photosynthesis autotrophically and therefore could

DD: constant darkness

LL: constant light

be used to produce biofuels by using sunlight as an inexpensive energy source (4, 5). Until the late 1980s/early 1990s, circadian biologists were reluctant to believe that organisms as simple as prokaryotes could have evolved an elaborate circadian timing mechanism (10, 34); they reasoned that a rapidly dividing bacterium whose lifetime was less than one day had no use for a timing mechanism that extended farther than its lifetime (80). However, *S. elongatus* can divide as rapidly as once every 5–6 h and can still show circadian rhythms without significant perturbation of its circadian pacemaker (32, 42, 55, 59). At this time, there is no question that the ~24-h rhythmic phenomena exhibited by *S. elongatus* are regulated by a bona fide circadian system (10, 43).

The circadian oscillator in *S. elongatus* has uniquely favorable characteristics for biophysical, biochemical, and genetic analyses (10). It is the only organism for which we have full structural information for the key clock proteins (in this case, KaiA, KaiB, and KaiC). *S. elongatus* has a genome size of 2.7 Mbp (smaller than that of *Escherichia coli*), and genetic tools abound (3, 21). Cyanobacteria are one of the few systems in which the adaptive significance of circadian programs has been rigorously tested (12, 31, 73, 103). Most significantly from a biophysical perspective, it is 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 (68). Although biophysical analyses of this oscillator have begun (6, 23, 63, 65, 81), we are at the watershed of understanding how this molecular oscillator really works (33, 53).

WHAT'S RHYTHMIC? OUTPUTS OF THE PACEMAKER IN CYANOBACTERIA

The first persuasive evidence for circadian rhythms in a prokaryote came from an investigation that studied the nitrogen-fixing cyanobacterium *Synechococcus* RF1 (10, 16). In this cyanobacterium, nitrogen fixation is regulated by the circadian clock such that it is maximal in the night phase. The enzyme that carries out the reduction of atmospheric nitrogen to ammonia is nitrogenase, an enzyme that is inhibited by oxygen. Because photosynthesis produces oxygen throughout the day and nitrogenase is sensitive to oxygen, turning on nitrogen fixation in the nocturnal phase allows the same cell to perform incompatible metabolic events: photosynthesis during the day and nitrogen fixation during the night (57). This is an example of how the evolutionary emergence of circadian systems may have enhanced fitness by optimizing temporal metabolic programs. In our search for a genetically malleable cyanobacterium, we and our collaborators settled on *S. elongatus* PCC 7942 (which incidentally does not fix nitrogen). The genetic properties of this organism facilitated our discovery of globally regulated gene expression by a circadian timekeeper. We use bacterial luciferase as a reporter of clock-regulated promoter activity; initially we studied the activity of the promoter for the *psbAI* gene (43), but we subsequently discovered that virtually all promoters in the *S. elongatus* genome are regulated by the circadian system (50). **Figure 1*a*** depicts rhythms based on a few selected promoter::reporter constructs, including the cyanobacterial promoters for the *psbAI*, *kaiA*, *kaiBC*, *purF*, and *ftsZ* genes. The majority of promoters are activated in the subjective day phase. However, the *purF* promoter is activated in the nocturnal phase (**Figure 1*a***), an interesting observation considering that its gene product is involved in an oxygen-sensitive pathway (*purF* encodes the enzyme catalyzing the initial step of de novo purine nucleotide biosynthesis; 49). Thus, the circadian regulation of the expression of this *S. elongatus* gene might constitute another example of temporal separation similar to that found for nitrogenase in *Synechococcus* RF1 (10, 16, 49, 50).

As expected from the global control of promoter activity, there is pervasive control by the circadian clock of mRNA abundances in cyanobacteria. In *S. elongatus* and other cyanobacterial species (*Synechocystis* sp. PCC

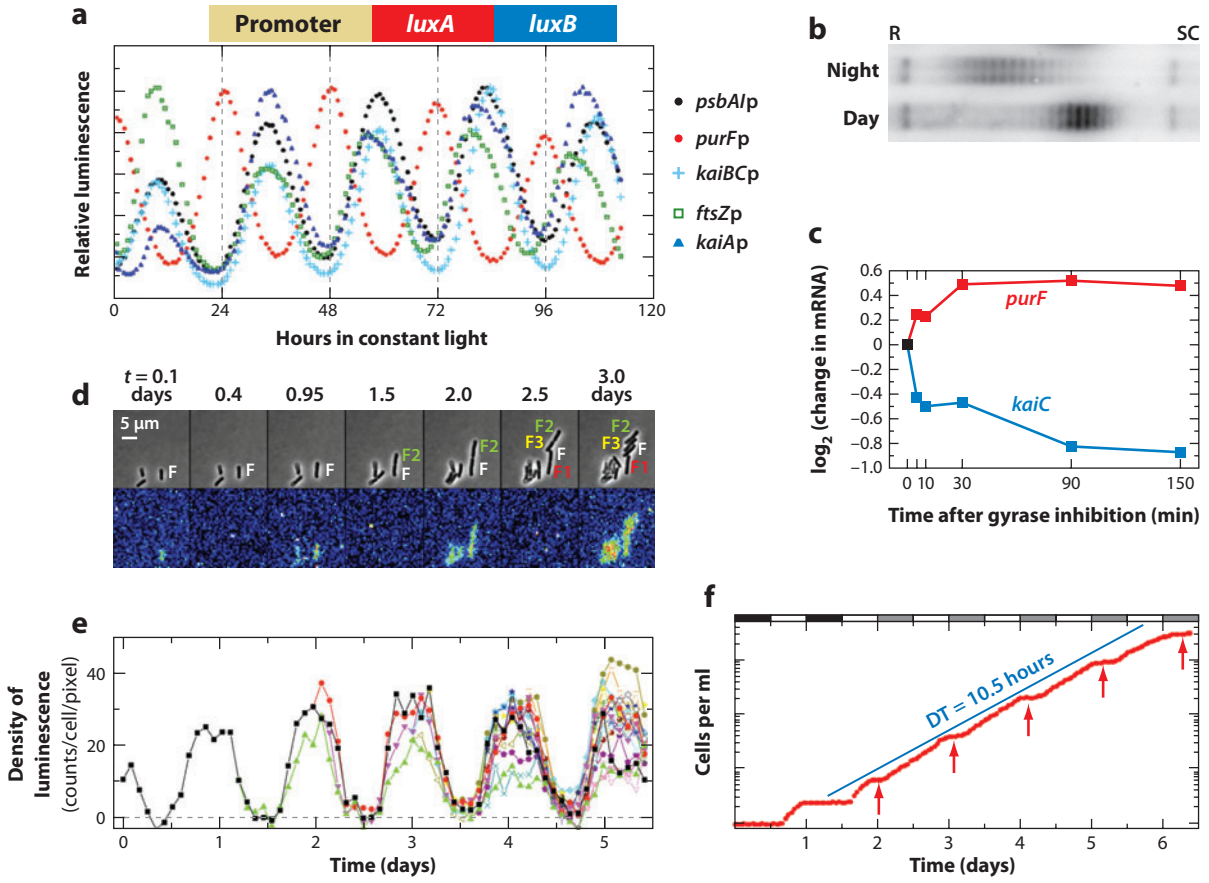


Figure 1

Circadian rhythms in *Synechococcus elongatus*. (*a*) Rhythms of luminescence emanating from cells transformed with bacterial luciferase (*luxAluxB*) fused to the promoters for the *psbAI*, *purF*, *kaiBC*, *ftsZ*, and *kaiA* genes. This plot illustrates circadian rhythms of gene expression. (*b*) Supercoiling of an endogenous plasmid indicates a circadian rhythm in chromosomal topology. In the subjective night topoisomers of the plasmid are more relaxed (R), whereas in the subjective day they are more supercoiled (SC) (104). (*c*) Gyrase inhibition results in an immediate change in gene expression due to drug-induced relaxation. Genes that have higher expression during relaxed circadian times immediately increase in gene expression (*purF*, *red*), whereas genes that have lower expression during relaxed circadian times immediately decrease in gene expression (*kaiC*, *blue*) (from Reference 100 with permission). (*d*) Micrographs of cyanobacterial cells at different times in constant light. Brightfield images (*upper panels*) show growth and cell division as a function of approximate circadian time. Observed luminescence (*lower panels*) reveals circadian rhythms in single cyanobacterial cells. The luminescence reporter was the *psbAI* promoter driving expression of bacterial luciferase. (*e*) Quantification of bioluminescence from a single cell as it divides in constant light. Cell division is indicated by differently colored traces for each daughter cell. (Panels *d* and *e* courtesy of Dr. Irina Mihalcescu from Reference 55). (*f*) Cell division in a population of *S. elongatus* cells is restricted by the circadian system. For the first 36 h the cells are in a light/dark (LD) cycle as indicated by the black and gray bars at the top of the panel. For the remaining time the cells are in constant light (LL). The cell count shows plateaus (*red arrows*) when the cells stop dividing. Plateaus occur during the night in LD cycles as well as the subjective night of LL. The average doubling time (DT) as indicated by the diagonal line was 10.5 h (59).

6803, *Crocosphaera watsonii*, and *Prochlorococcus* MED4), microarray analyses have confirmed that the transcripts of 10%–80% of genes in the genome exhibit circadian and/or daily oscillations of abundance (24, 44, 87, 100, 115). However, whereas promoter activity indicated essentially 100% of the genes are expressed rhythmically (50), microarray analysis in

S. elongatus found that only 30%–60% of mRNA abundances are rhythmic (24, 100). Presumably posttranscriptional regulation accounts for the differences in these two measurements. Transcripts that are either very unstable or very stable may be transcribed rhythmically, but the steady-state levels of the message may nevertheless be nearly constitutive. In initial studies of *S. elongatus* rhythms, the clarity of the *psbAI* promoter activity rhythm was much cleaner than that of *psbAI* mRNA abundance, implying posttranscriptional modulation of transcript abundances (43). These data suggest that in *S. elongatus* measurements of promoter activity are a more accurate indicator of circadian control over gene expression than of transcript abundance. An amusing aspect of studying gene expression in *S. elongatus* is that rhythmic gene expression does not always translate into rhythms of the implicated processes. For example, even though *psbAI* encodes a key photosynthesis protein (the three *psbA* genes encode the D1 protein of photosystem II), implying that photosynthesis is rhythmically regulated, experimental measurements of photosynthetic rate in *S. elongatus* have shown that there is a daily rhythm (in light/dark, LD) of photosynthetic capacity but not a circadian rhythm in LL (112).

How are the global rhythms of promoter activity mediated from the central clockwork? Two alternative scenarios have been proposed: a traditional transcription factor network and an oscillating chromosome model. The transcription factor model is based on studies implicating the putative transcription factor RpaA, which appears to be coupled to the cyanobacterial KaiABC oscillator by the histidine kinase SasA (28, 90). New data implicate other factors in this output pathway, including LabA and CikA (92). CikA may be an important component of both the input (86) and the output pathways. The other model for circadian regulation of global gene expression is the oscillating chromosome hypothesis (60, 104). One argument against mediation of global regulation solely by transcriptional factors that have coevolved with *cis* elements of *S. elongatus* promoters is that heterologous promoters from *E. coli* such as *conII*p and *trc*p exhibit circadian activity in *S. elongatus* (38, 67). Chromosomal topology/compaction provides a high-level way to influence promoter activity. In *S. elongatus* the circadian clock controls pervasive changes in the compaction and topology of the entire chromosome. Dramatic circadian changes are seen for both compaction/decompaction (visualized by DNA-binding dyes) (88) and DNA topology (indicated by plasmid supercoiling) (104) (**Figure 1*b***). DNA topology and torsion critically affect transcriptional rates, and it is therefore reasonable to hypothesize that such circadian changes in chromosomal topology could be partially responsible for daily modulation of promoter activity (56, 60, 88, 104). We have termed this hypothesis the oscillating nucleoid, or oscilloid model (60, 104).

LD: light/dark cycle

The role of chromosomal topology in regulating circadian gene expression has always been a "chicken or the egg" dilemma. In other words, is transcription rhythmic because the chromosome is being rhythmically supercoiled (i.e., the oscilloid hypothesis), or is the chromosome cyclically supercoiled because transcription is rhythmic? Recent results favor the oscilloid model (100). Gyrase is a key enzyme that regulates DNA superhelicity, and it is inhibited by the drug novobiocin, which thereby relaxes DNA. When novobiocin is added to *S. elongatus* cells at a phase in which the chromosome is normally negatively supercoiling, there is an immediate change in gene expression, and genes that are normally expressed in antiphase (*kaiBC* versus *purF*, **Figure 1*a***) respond in opposite directions (**Figure 1*c***). This result strongly supports the oscilloid hypothesis that *kaiBC* expression is turned on by negatively supercoiled DNA (and *purF* is turned off), and when the chromosome relaxes 12 h later, the relative expression levels of these two classes of genes flips (100). The two available scenarios for global expression patterns (oscilloid hypothesis versus transcriptional regulation by factors such as RpaA, SasA, LabA, and CikA) do not necessarily exclude each other. An analysis of stochastic gene

expression in cyanobacteria (8) provides support for the idea that circadian gene expression is regulated by multiple factors, e.g., changes in both DNA topology and transcriptional factor activity.

Rhythmicity has been recorded both for entire populations of cells (**Figure 1*a***) and for a single cyanobacterial cell, as demonstrated by luminescence rhythms from the luciferase reporter fused to the *psbAI* promoter (55) (**Figure 1*d***). Remarkably, this study also demonstrated that cell division does not perturb the circadian oscillator (**Figure 1*e***). The clock of a daughter cell ticks in phase with the clock of the mother cell. This 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 whether they do not divide at all) does not perturb the intrinsic ~24-h period of the circadian system (42, 59, 61). Moreover, the circadian oscillator specifies a checkpoint for division by regulating the timing at which cell division is permitted. It can be experimentally demonstrated that a population of cells rapidly dividing in LL (average doubling time of 10.5 h) will have cell division restricted by the circadian system (**Figure 1*f***). This circadian gating of cell division in *S. elongatus* has been studied recently by the Golden laboratory, and they reported that elevated ATPase activity of KaiC may provide the circadian checkpoint in cyanobacteria (11). Moreover, recent studies suggest that cell division in *S. elongatus* is influenced by the circadian-implicated genes *cikA* and *cdpA* (11, 51).

Pervasive changes in metabolism, cellular structure, and gene expression that accompany cell division do not perturb circadian timing. This imperturbability appears to be a general property of circadian pacemakers in cyanobacteria and in eukaryotes, and it might be argued that this constitutes evidence for a cyanobacteria-like pacemaking mechanism in eukaryotes (32).

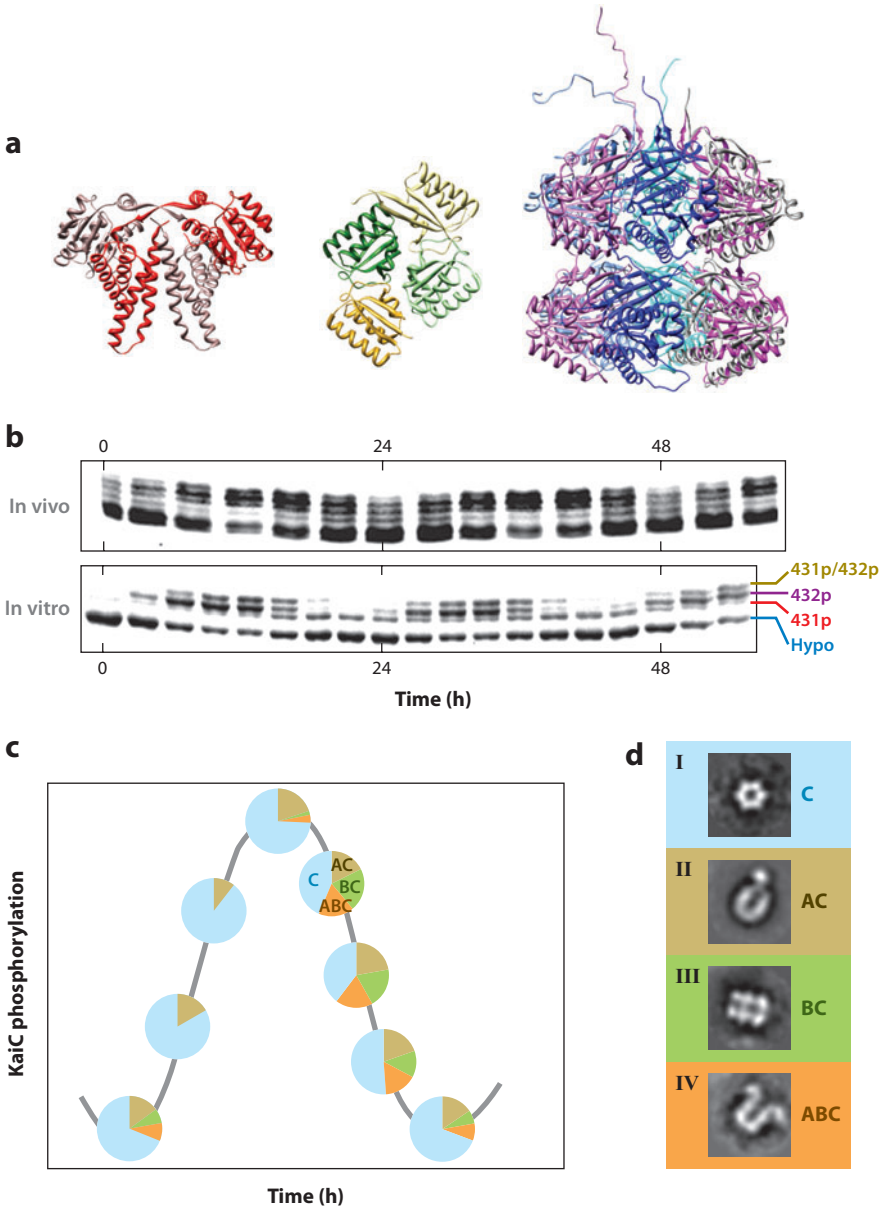
THE TIMEKEEPING MECHANISM: KaiA, KaiB, and KaiC ARE THE CLOCKWORKS' GEARS

The proteins KaiA, KaiB, and KaiC constitute the central components of the clockwork in *S. elongatus* (22). The *kaiA*, *kaiB*, and *kaiC* genes were first identified in 1998, and three-dimensional structures for the proteins they encode became available in 2004 (13, 15, 20, 76, 98, 111). They remain the only core circadian clock proteins for which full-length structures have been determined. KaiA is a domain-swapped dimer with an N-terminal bacterial receiver domain and a C-terminal α-helical

Figure 2

Structures, rhythmic phosphorylation, and associations of KaiA, KaiB, and KaiC. (*a*) Shown from left to right are the crystal structures of the *S. elongatus* KaiA dimer (111), the *Synechocystis* KaiB tetramer (20), and the *S. elongatus* KaiC hexamer (76, 78). Individual subunits of the multimeric proteins are represented in different colors. In the case of KaiC, the subunits are arranged around a central channel that runs vertically (behind the dark blue-colored subunit in this depiction (13). (*b*) Time courses of rhythmic KaiC phosphorylation in vivo and in vitro as assessed by SDS-PAGE (the lowest bands are hypophosphorylated KaiC and the upper bands are various forms of phosphorylated KaiC). Top: KaiC phosphorylation in vivo at different times in constant light (samples were collected every 4 h in constant light and immunoblotted). Bottom: KaiC phosphorylation in the in vitro reaction. Purified KaiA, KaiB, and KaiC were combined with ATP in vitro and samples were collected every 3 h and processed for SDS PAGE and staining. Four bands are obvious in these in vitro samples: hypophosphorylated KaiC and KaiC phosphorylated at the S431, T432, or S431/T432 residues (see labels on the right side of the panel). (*c*) Rhythms of KaiA·KaiB·KaiC complex formation during the in vitro cycling reaction. The color coding of the pie charts indicates the percentage of free KaiC hexamers (*blue*), KaiA·KaiC complexes (*brown*), KaiB·KaiC (*green*) complexes, and KaiA·KaiB·KaiC (*orange*) complexes (63). (*d*) Electron microscopy average images of free KaiC hexamers, KaiA·KaiC complexes, KaiB·KaiC complexes, and presumed KaiA·KaiB·KaiC complexes (63) (color coding is the same as in panel *c*).

bundle, and KaiB adopts a thioredoxin-like fold and forms dimers and tetramers. KaiC is by far the largest of the three and exists as a homohexamer with the appearance of a double-doughnut (**Figure 2*a***). The *kaiC* gene is the result of a gene duplication and KaiC displays similar N- and C-terminal domains at the monomeric level (the lobes are referred to as CI and CII, respectively). Six ATP molecules are bound between subunits in both the CI and the CII rings. The astonishing breakthrough from the Kondo laboratory in 2005 was the report that a molecular oscillator can be reconstituted when the three Kai proteins are combined together with ATP in a test tube (68). This in vitro oscillator ticks with a ~24-h period for at



EM: electron microscopy

SAXS: small angle X-ray scattering

least 10 days, with KaiC alternating between a hypophosphorylated and a hyperphosphorylated state (**Figure 2*b***) (23). Mass spectrometry and X-ray crystallography were used to establish that KaiC is phosphorylated at S431 and T432 in the CII lobe (72, 108). There appear to be no phosphorylation sites in the CI domain. Phosphorylation of T432 and S431 proceeds across the subunit-subunit interface (76, 108).

KaiC exhibits both autokinase and autophosphatase activities (27, 71, 85, 107), and it has been reported that 15 ATP molecules are rhythmically hydrolyzed per subunit during a complete in vitro 24-h cycle (93). KaiA serves as a promoter of the formation of the KaiC hyperphosphorylated state, and KaiB antagonizes KaiA's actions and promotes a return to the hypophosphorylated state. Structural and biophysical studies have provided insight into the KaiA·KaiC (78, 97) and KaiB·KaiC complexes (77). A range of techniques, including gel filtration chromatography, two-dimensional gel electrophoresis, negative-stain electron microscopy (EM), and small angle X-ray scattering (SAXS), have been employed to quantify the relative levels of KaiC versus KaiA·KaiC versus KaiB·KaiC versus KaiA·KaiB·KaiC complexes formed during the in vitro reaction cycle (**Figure 2*c*,*d***) (1, 37, 63). Another key aspect of the in vivo and in vitro KaiC phosphorylation cycle is the strict order of phosphorylation and dephosphorylation of the two P sites, which involves four steps: (*a*) T432 phosphorylation, (*b*) S431 phosphorylation, (*c*) T432 dephosphorylation, and (*d*) S431 dephosphorylation (**Figure 2*b***) (71, 85). The vital challenge is to understand the underlying molecular mechanisms of this clockwork.

GETTING TOGETHER: CYCLING INTERACTIONS AMONG THE Kai PROTEINS

KaiA enhances the autokinase activity of KaiC by binding to a C-terminal peptide from a subunit of the latter repeatedly and rapidly (**Figure 3**) (37, 81). A single KaiA dimer appears to be sufficient to upregulate phosphorylation of a KaiC hexamer to saturated levels (18), consistent with the higher abundance of KaiC hexamers in vivo relative to KaiA dimers (40). What is the mechanism underlying KaiA's function? NMR spectroscopy established that KaiA binds to the C-terminal tentacle peptides of KaiC (97). This interaction unravels an S-shaped loop within the contacted KaiC subunit as KaiA pulls the S-loop adjacent to the central channel of the KaiC hexamer (13). The KaiC crystal structure revealed that S-loop residues at amino acids 485–497 form hydrogen bonds across subunits at the periphery of the channel (76). Therefore, one can expect the disruption of the S-shaped loop of a single subunit to weaken the interface between adjacent CII lobes and to promote conformational changes within the CII ring conducive to phosphorylation at T432 and S431. A three-dimensional EM structure of the KaiA·KaiC complex revealed that KaiA assumes at least two orientations above the C-terminal dome of the KaiC hexamer. In one orientation, KaiA is tethered to KaiC via a flexible linker (78). In the second orientation, KaiA is engaged on the KaiC surface, which is suggestive of a transient interaction between an apical loop in the C-terminal domain of a KaiA monomer and the ATP-binding cleft on KaiC.

Unlike KaiA, which binds as a dimer to the C-terminal tentacle portion of KaiC, KaiB does not exhibit any affinity to these tentacles. Moreover, whereas KaiA remains associated with KaiC during the entire phosphorylation cycle, KaiB displays a distinct preference for the phosphorylated form of the hexamer (37, 63, 71, 85). Hybrid structural biology approaches including cryo- and negative-stain EM and X-ray crystallography, along with native PAGE and fluorescence methods, revealed that KaiB dimers bind to the CII ring (77). Thus, EM images are consistent with KaiB dimers forming a third layer on top of CII without obscuring the central channel. This arrangement serves to prevent KaiA from approaching the ATP-binding clefts on KaiC, although the KaiA dimer is still tethered to the C-terminal CII peptide.

More recently, we discovered that although the initial interaction of KaiA with

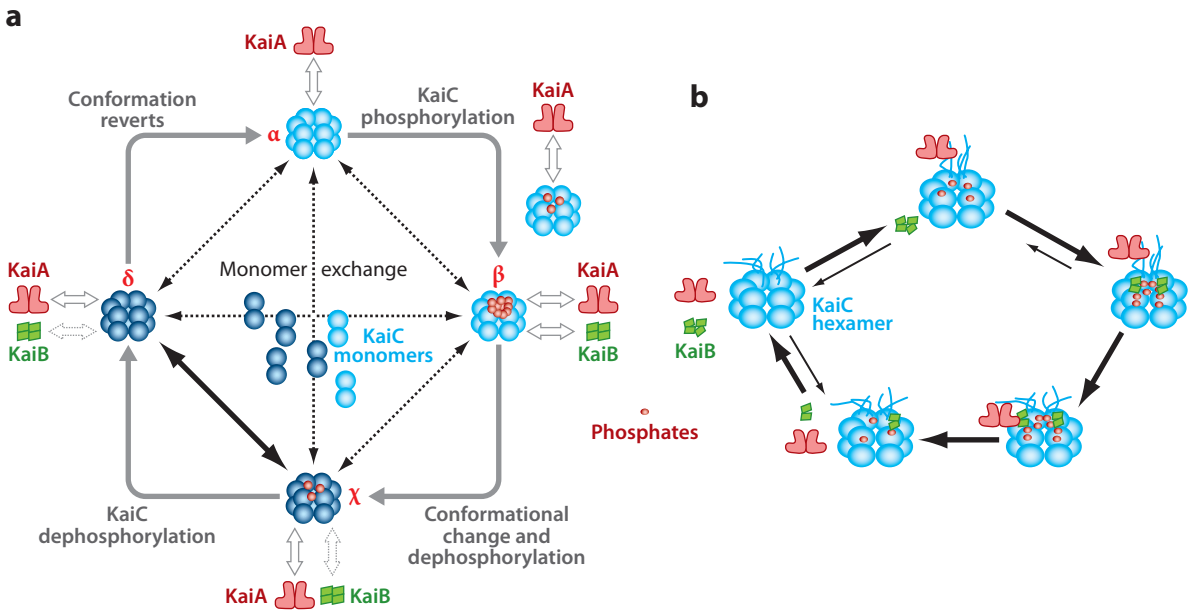


Figure 3

Models of the KaiABC oscillator. (*a*) Diagram representing the mathematical model for the KaiC phosphorylation cycle. The double circle dumbbell shapes in the center represent KaiC monomers. The KaiC hexamer can associate with and dissociate from KaiA and KaiB. KaiC hexamers are shown in light blue and dark blue, representing two conformational states (approximately equivalent to kinase versus phosphatase forms of KaiC). Red dots are phosphates at KaiC phosphorylation sites (residues S431 and T432). Monomer exchange between KaiC hexamers is depicted with the double-headed arrows in the center. The rates of monomer exchange vary among KaiC states, with a solid line indicating a high rate and a dashed line indicating a low rate (from Reference 63). (*b*) Diagram showing the formation of KaiA·KaiB·KaiC complexes. Starting from the leftmost molecular representation and proceeding clockwise: during the phosphorylation phase of the cycling reaction, KaiA (*red dimers*) repeatedly and rapidly interacts with KaiC's C-terminal tentacles. (KaiC molecules are the blue double-donut hexamers.) When KaiC becomes hyperphosphorylated (phosphates on T432 and S431 are depicted as red dots), it first binds KaiB (*green diamonds*) stably. Then, the KaiB·KaiC complex binds KaiA, sequestering it from further interaction with KaiC's tentacles. At that point, KaiC initiates dephosphorylation. When KaiC is hypophosphorylated, it releases KaiB and KaiA, thereby launching a new cycle (from Reference 81).

unphosphorylated KaiC is labile, once KaiC becomes hyperphosphorylated and binds KaiB, KaiA is incorporated into a stable A·B·C complex (81). Amazingly, this complex does not dissociate during a several-hour electrophoresis in native PAGE. Moreover, the formation of this stable complex is not dependent on KaiC's tentacles because KaiA and KaiB form the stable A·B·C complex with the hyperphosphorylated $KaiC^{489}$ mutant from which the C-terminal tentacles have been deleted (81). Our current hypothesis is that KaiA first repetitively interacts with the tentacles of hypophosphorylated KaiC to enhance KaiC's autokinase activity until KaiC is hyperphosphorylated, at which time the KaiC hexamer undergoes a conformational change that allows it to form a stable complex with KaiB (**Figure 3*b***) (27, 33, 37, 39, 63, 81, 97). This stable KaiB·KaiC complex exposes a novel binding site for KaiA, which sequesters KaiA in a stable KaiA·KaiB·KaiC complex. The sequestered KaiA is unable to further stimulate the autokinase activity of KaiC, and therefore the autophosphatase activity dominates such that KaiC dephosphorylates to its hypophosphorylated conformation from which KaiB and KaiA dissociate and the cycle begins anew (**Figure 3*b***) (81). Using electron spin resonance (ESR) spectroscopy, Mutoh et al. (65) found that spin-labeled Cys mutants

ESR: electron spin resonance

of KaiB from the thermophilic cyanobacterium *Thermosynechococcus elongatus* directly interacted with KaiA from the same species. Fluorescence and native PAGE assays of mixtures of *S. elongatus* KaiA and KaiB in the absence of KaiC did not reveal any interactions between the two proteins (77). Therefore, either the KaiA-KaiB interaction revealed by ESR is too transient to be captured by fluorescence or native gel analyses, or KaiB binding to KaiC creates a new interface for KaiA binding in *Synechococcus*.

KaiC subunits have two critical phosphorylation sites, S431 and T432. The role of a third potentially phosphorylated residue, T426, has been debated. In the crystal structure of the KaiC homohexamer from *S. elongatus*, all six T432 residues and four of the six S431 residues were phosphorylated (76, 108). Closer inspection of the surroundings of the two amino acids revealed that the protein chain curls between residues I425 and I430 so that residues D427, S428, and H429 form a loop in which T426 faces S431. In fact, the last two residues are closely spaced and the side chain of T426 forms a hydrogen bond with the phosphate group of S431-P (76, 108). In the absence of S431 phosphorylation, the two side chains are too far apart to interact. Single T432A, S431A, and T426A mutants are arrhythmic, and we had postulated that T426 might serve as a third phosphorylation site (108). Subsequently, we carried out more detailed biochemical and structural studies on in vitro and in vivo phosphorylation with T426 mutants (75, 109). A central question was whether the amino acid at position 426 must be phosphorylatable, or is it sufficient for it to form a hydrogen bond with S431-P? As a test, an asparagine that should be capable of hydrogen bonding to S431-P was substituted at position 426. This T426N mutant displayed the expected hydrogen bond between 426N and the phosphate on S431 in the crystal structure but was arrhythmic in vivo (75, 109). This observation and other data support the idea that position 426 must be phosphorylatable for the clock to function properly (75, 109). In vivo coexpression studies involving $KaiC^{WT}$ and mutants of KaiC with alternative residues at position 426 demonstrated dramatic effects on dominant/recessive relationships and also revealed that substitutions at T426 alter key properties, such as period, amplitude, robustness, and temperature compensation. Together with the finding that mutations of T426 critically affect the formation of complexes between KaiC and KaiA/KaiB, these observations confirmed that T426 is an important site that regulates the KaiC phosphorylation status in vivo and in vitro.

The association-dissociation process of Kai proteins in vivo and in vitro and the stoichiometry of their complexes in vitro have been analyzed by gel filtration chromatography, native gel assays, and negative-stain EM (36, 37, 63, 81). SAXS has also been used to study the dynamics of untagged Kai proteins as they assemble and disassemble over the in vitro oscillation (1). SAXS data provided support for the idea that the initial phase of the cyanobacterial circadian oscillator is determined largely by the assembly and disassembly of Kai proteins. Further, the period of the clock was resistant to intracellular noise, arising from collisions, crowding, and cytoplasmic viscosity.

WHY TIME IN THE IN VITRO OSCILLATOR IS UNIDIRECTIONAL

In the crystal structure of KaiC from *S. elongatus*, the T432 residues from all six subunits and the S431 residues from four subunits were phosphorylated (76, 108). T432's side chain oxygen atoms are closer on average to the ATP γ-phosphate (7.3 Å), compared with those from the S431 residues (8.4 Å). Once T432 residues are phosphorylated, new stabilizing interactions are formed across the CII subunits because the phosphate group of T432 becomes engaged in a salt bridge to R385 as shown in **Figure 4** (108). While it is reasonable to assume that local conformational fluctuations will be more limited after T432 is phosphorylated, there remains sufficient flexibility at the subunit

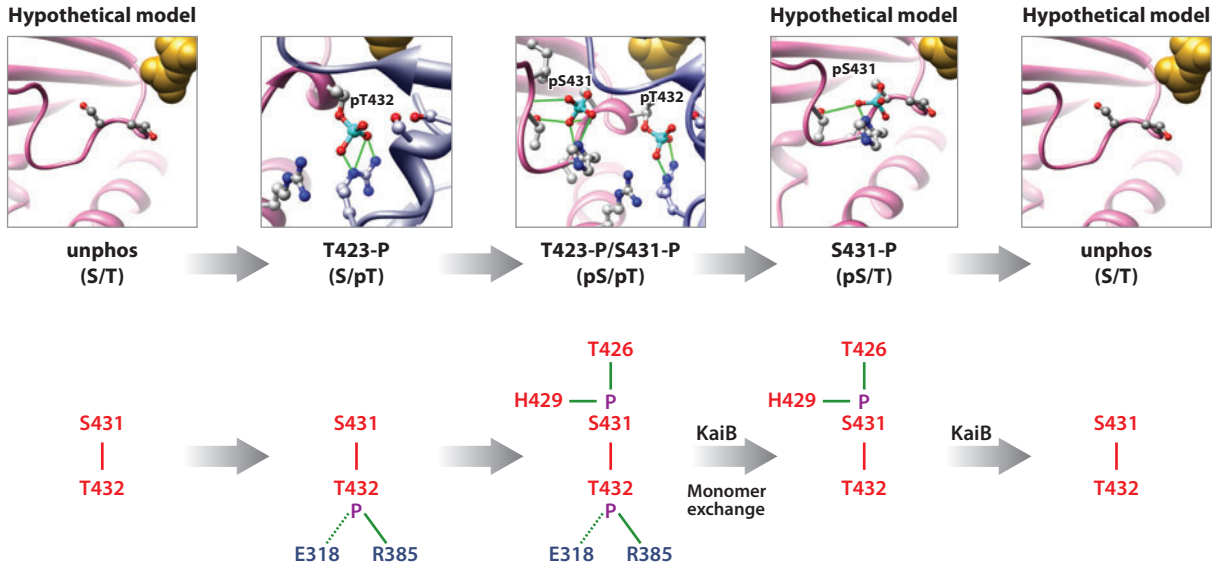


Figure 4

A ratcheting mechanism for unidirectional motion of the KaiABC oscillator. Starting with the unphosphorylated (unphos) form of KaiC (S/T), KaiC is first phosphorylated on T432 (S/pT), leading to the formation of a salt bridge (*solid green line*) to R385 on the adjacent KaiC subunit (*blue chain*; T432-P shows proximity to E318, as shown by the *dashed green line*). KaiC then autophosphorylates on S431, leading to the doubly phosphorylated form (pS/pT) that adds hydrogen bonds (*solid green lines*) to residues T426 and H429 on the same KaiC subunit (*pink chain*). The formation of these hydrogen bonds makes the reverse reactions unfavorable so that the KaiABC oscillator is unidirectional during the phosphorylation phase (33). The hyperphosphorylated KaiC (pS/pT) then interacts with KaiB and initiates monomer exchange and dephosphorylation, forming first pS/T and ultimately unphosphorylated KaiC (S/T) again. The S/T and pS/T forms of KaiC are inferred and labeled as hypothetical models on the figure because no crystal structures of these forms have been reported, whereas the S/pT and pS/pT forms have been successfully crystallized and reported (76, 75, 108).

interface to also allow transfer of a phosphate to S431 in the second step. Once phosphorylated, S431 can engage in additional hydrogen-bonding interactions with amino acids (T426 and H429) in the same subunit (**Figure 4**). These hydrogen-bonding interactions are probably crucial because mutation at T426 to alanine abolishes clock function (108, 109). In general, S431-P residues are more shielded inside a pocket formed by the phosphorylation loop compared with T432-P residues, which may explain the longer survival of the former during the dephosphorylation phase (**Figure 2*b***). Overall, the structural information on the phosphorylation events at the KaiCII subunit interfaces and the inter- and intrasubunit interactions formed by the phosphorylated residues indicates that the number of hydrogen bonds increases while first T432 and subsequently S431 are phosphorylated. This progressive increase in molecular interaction would make the reverse reactions unfavorable, causing a built-in ratcheting mechanism that drives the KaiC oscillator unidirectionally during the phosphorylation phase toward T432-P/S431-P (pS/pT) (33) (**Figure 4**).

We currently have no clear understanding of the mechanism of the autophosphatase activity of KaiC during the dephosphorylation phase. It is possible but not absolutely necessary that a conformational change occurs to drive KaiC forward to the phosphatase state and achieve dephosphorylation first of T432 and then of S431 in all six subunits. This order of phosphorylation and dephosphorylation events has been observed in biochemical assays as mentioned above (**Figure 2*b***) (71, 85). It appears

TTFL: transcription/translation feedback loop

that the interaction of KaiB with KaiC facilitates the formation of the phosphatase state. KaiB binds preferentially to a phosphorylated form of KaiC (specifically, the S431-P state; 71, 85). This behavior is different from that exhibited by KaiA, which can bind various forms of KaiC (37). Although the crystal structure of $KaiC^{WT}$ shows the homohexamer captured in the hyperphosphorylated state, with 10 out of 12 phosphate groups present at S431 and T432 sites (76), there are currently no hints from the structure regarding the dephosphorylation mechanism, although Mg^{2+} serves as a cofactor. In fact, to date no crystal structure of unphosphorylated KaiC has been reported.

A TICKING CLOCKWORK IN VITRO

The unanticipated finding that the three Kai proteins together with ATP generate stable oscillations within a ~24-hour period in a test tube (**Figure 2*b***) (68) provided a contradiction to the dogma that all circadian oscillators are dependent on a transcription/translation feedback loop (TTFL) (68, 82, 94, 107). Furthermore, the rates of KaiC phosphorylation, dephosphorylation, and ATP hydrolysis, as well as the in vitro rhythm, are all temperature compensated (64, 68, 93, 94). We take this as evidence that temperature compensation is built into the molecular characteristics of the three Kai proteins and the nature of their interactions. Temperature compensation remains an important unresolved question not just for the cyanobacterial system but for circadian clocks in general.

Terauchi et al. (93) have proposed that the rhythm of KaiC ATPase activity constitutes the most fundamental reaction underlying circadian periodicity in cyanobacteria. An alternative (and not exclusive) possibility is that ATP hydrolysis provides the energy needed for conformational changes in KaiC. Our results indicate that intermolecular dynamics of KaiA, KaiB, and KaiC determine the period and amplitude of this in vitro oscillator, leading to the hypothesis that (*a*) the basic timing loop of the KaiABC oscillator and (*b*) its outputs are mediated by conformational changes of KaiC in association with KaiA and KaiB. For example, mutations within KaiB that alter affinity to KaiC modulate the period of this clock in vivo and in vitro as predicted by mathematical modeling (81). Our interpretation is that the formation of Kai protein complexes is coupled with KaiC phosphorylation status; because different KaiB variants modulate the rate of KaiB·KaiC formation, they also affect the period of KaiC phosphorylation (81). At the very least, if the ATPase activity is the basic timing loop as suggested by Terauchi et al. (93), then the intermolecular associations with KaiB must regulate KaiC's ATPase activity in a deterministic way.

Now that an in vitro clock system has been identified, biophysical, biochemical, and structural tactics can be deployed to analyze the molecular nature of a circadian clockwork in a way that was previously impossible. The time-dependent formation of Kai protein complexes has been quantified with EM, gel filtration chromatography, SAXS, and native gel electrophoresis techniques (1, 9, 37, 63). Throughout the in vitro oscillation, KaiC exists in all possible combinations with KaiA and KaiB: free KaiC hexamers, binary KaiA·KaiC and KaiB·KaiC complexes, and ternary KaiA·KaiB·KaiC complexes (**Figures 2*c*,*d***). The proportions of these complexes vary in a phase-dependent manner, with free KaiC hexamers predominating at all phases. About 10% of KaiC hexamers are present as KaiA·KaiC complexes at all phases; by comparison, KaiB·KaiC and KaiA·KaiB·KaiC complexes are clearly rhythmic and are most common during the KaiC dephosphorylation phase (**Figure 2*c***) (37, 63). KaiC undergoes rhythmic changes in conformation, phosphorylation status, and interactions with KaiA and KaiB during the in vitro oscillation. Our working hypothesis is that the core of the oscillator is constituted by rhythmic changes in the conformation of KaiC that in turn modulate the interactions with KaiA and KaiB and the activity of transduction factors such as SasA and RpaA (37, 63, 90).

STAYING TOGETHER: MONOMER EXCHANGE AND THE MAINTENANCE OF SYNCHRONY AMONG HEXAMERS

Cyanobacterial cells in populations behave as autonomous oscillators that appear to be unable to communicate phase information intercellularly (2, 55). Once synchronized, however, cells in populations remain in sync for many cycles, implying a robust mechanism for maintaining a precise, high-amplitude rhythm inside each cell. Cellular events such as DNA synthesis, cell division, and metabolic changes generate "noise" or perturbations that pose a significant challenge for circadian oscillators (32, 55, 82). In the case of the in vitro oscillator, KaiC monomer exchange among different hexamers is a process that can potentially synchronize the phosphorylation status of individual hexamers within a population of hexamers, thereby sustaining a high-amplitude oscillation (**Figure 3*a***) (23, 37, 63). Monomer exchange was first observed using the technique of pull-down assays with FLAG-tagged KaiC proteins (37; the FLAG tag is an octapeptide protein tag with the following sequence: DYKDDDDK). Because the pull-down technique can suffer from aggregation/cross-reactivity problems, we investigated monomer exchange by fluorescence resonance energy transfer (FRET). Briefly, a population of KaiC labeled with IAEDANS [5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid] (EX 336/EM 470 nm) was mixed with a population of KaiC labeled with MTSF [2-((5-fluoresceinyl)aminocarbonyl)ethyl methanethiosulfonate-4-fluorescein] (EX 490/EM 515 nm). **Figure 5*a*** shows the time-dependent quenching of IAEDANS fluorescence (indicative of FRET) as a gauge of KaiC monomer exchange. Using this method, we confirmed that KaiC hexamers exchange their monomers, but we could not confirm the earlier report that KaiA inhibited KaiC monomer exchange (**Figure 5*b***) (37, 63). Subsequent experiments from the Kondo laboratory indicated that KaiC monomer exchange occurs primarily in the dephosphorylation phase of the KaiABC in vitro oscillation (23). Our model simulations show that phase-dependent monomer exchange

FRET: fluorescence resonance energy transfer

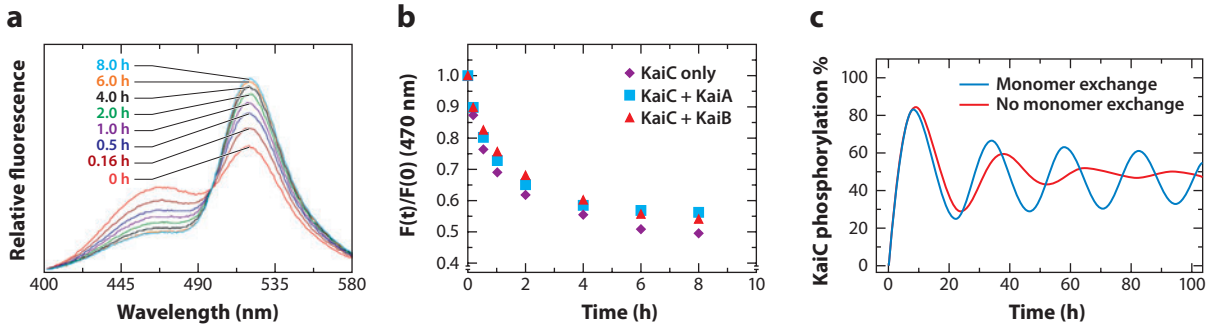


Figure 5

FRET analysis of KaiC monomer exchange. (*a*) A sample of KaiC labeled with IAEDANS (EX 336/EM 470 nm) was mixed with a sample of KaiC labeled with MTSF (EX 490/EM 515 nm). The emission spectrum of the mixture under excitation at 336 nm was recorded at the following times at 30°C: 0 h, 0.16 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, and 8 h. The decrease in fluorescence intensity at 470 nm of IAEDANS-labeled KaiC is indicative of energy transfer due to monomer exchange between the two labeled KaiC populations. (*b*) Effect of KaiA and KaiB on monomer exchange. Measurement of monomer exchange between IAEDANS-labeled and MTSF-labeled KaiC when KaiA (0.05 μg μl^{-1}) or KaiB (0.05 μg μl^{-1}) was added to the mixture of KaiC (0.2 μg μl^{-1} total concentration). The decrease in fluorescence intensity at 470 nm was plotted as a function of time. (*c*) Model prediction of the in vitro KaiABC oscillation in the presence (*blue line*) or absence (*red line*) of phase-dependent monomer exchange (from Reference 63). Abbreviations: FRET, fluorescence resonance energy transfer; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; MTSF, 2-((5-fluoresceinyl)aminocarbonyl)ethyl methanethiosulfonate-4-fluorescein.

PTO: posttranslational oscillator

allows the individual KaiC hexamers within the population of hexamers to maintain an equivalent level of average phosphorylation, such that the hexamers remain synchronized in terms of phospho-status (**Figure 5*c***) (63). In this fashion, dynamic rhythms of KaiC phosphorylation can be sustained for at least 10 cycles in vitro (23). Therefore, intracellular synchronicity is achieved by biochemical reactions occurring among thousands of Kai molecules per cell (40) that enable a posttranslational oscillator (PTO) of high precision and synchrony (23, 33, 63).

MODELING THE IN VITRO OSCILLATOR

Since the publication of the in vitro KaiABC rhythm (68), there have been many attempts to model this oscillator (7, 9, 14, 23, 37, 45, 47, 48, 54, 58, 63, 66, 82, 85, 91, 99, 102, 110, 113) and probably others of which we are unaware. As a representative example, we proposed in 2007 a model that stochastically simulates the kinetics of KaiC hexamers and the degree of phosphorylation of each monomer in every hexamer (**Figure 3*a***) (63). Beginning with a hypophosphorylated state of KaiC (state α), rapid and repeated association and disassociation of KaiA facilitate phosphorylation until the KaiC hexamer becomes hyperphosphorylated (state β) (27). Association of KaiB with KaiC then brings about a conformational change to a new state (KaiC*, state χ). Eventually, the KaiC* hexamer (state χ) undergoes dephosphorylation, reaches a relatively hypophosphorylated status (state δ), and relaxes to the original conformation (state α). Over the duration of the phosphorylation cycle of a hexamer, monomer exchange between any two hexamers in any of the states can occur. The rate of this subunit exchange reaches a maximum during the KaiC dephosphorylation phase, when KaiB is associated with KaiC (23). KaiB binds to the KaiC hexamer when the total degree of phosphorylation of the KaiC hexamer exceeds a threshold that places it in state β. Although we originally envisioned KaiA stochastically binding and unbinding rapidly from KaiC hexamers, we now know that when KaiB binds to hyperphosphorylated KaiC, KaiA is sequestrated to a novel site to form a stable KaiA·KaiB·KaiC complex (81). The model depicted in **Figure 3*a*** incorporates phase-dependent KaiC monomer exchange as a mechanism for keeping the phosphorylation state of hexamers synchronized in the population, and accurately predicts observed patterns of in vitro KaiC phosphorylation (63).

It is beyond the scope of this review to describe all the proposed models for the cyanobacterial clockwork (see Reference 7 for an evaluation of many of these models). However, one significant aspect in which the models differ is the mechanism of KaiC hexamer synchronization. In this respect, the various models fall mostly into two groups: synchronization by KaiA sequestration (6, 9, 85, 99) or synchronization by phase-dependent monomer exchange (23, 37, 63, 113). [Interestingly, monomer exchange was predicted by a modeling study before it was experimentally measured (14).] There is clear experimental evidence for phase-dependent monomer exchange (23, 63); however, several studies have also confirmed that KaiA is indeed sequestered into a stable A·B·C complex (6, 81, 85). These data suggest that KaiA sequestration may act in concert with monomer exchange to accomplish the synchrony of KaiC phosphorylation that enables the robust high-amplitude rhythms for many cycles in vitro (23). We have generated a combined model in which monomer exchange is a mechanism for maintaining phase synchrony among KaiC hexamers while KaiA sequestration is involved in the switch from autokinase to autophosphatase mode (81). Finally, modeling studies are beginning to address how the cyanobacterial pacemaker may regulate gene expression (82), metabolism (19), and cell division (110).

PTO AND TTFL: WHO IS DRIVING WHOM?

It is generally assumed that the mechanism of circadian clocks in eukaryotes is dependent on

autoregulatory TTFLs (12, 17). Indeed, the discovery of the KaiA, KaiB, and KaiC proteins as key clock components in *S. elongatus* (22) did not initially raise any doubts about the importance of a TTFL at the core of this prokaryotic clockwork. This interpretation was based on the same kind of evidence that currently supports the existence of TTFL oscillators in eukaryotes, namely (*a*) rhythms of abundance for mRNAs and proteins encoded by clock genes, (*b*) feedback of clock proteins on their gene's transcription, and (*c*) phase setting by experimental expression of clock proteins (12, 22, 33). However, a number of more recent observations seemed to be inconsistent with a core TTFL oscillator in cyanobacteria. For example, the circadian rhythm of KaiC phosphorylation appeared unaffected by global inhibition of transcription and translation (94). Moreover, replacement of the promoters driving *kaiBC* gene expression with nonspecific heterologous promoters did not disturb the circadian rhythm (67, 107). When cyanobacterial cells were treated with the protein synthesis inhibitor chloramphenicol for extended periods of time, no phase changes were observed in the circadian system after return to normal conditions (35, 106). The discovery of the in vitro KaiABC oscillator in 2005 proved that a TTFL was not necessary for circadian oscillations in cyanobacteria (68), and that this KaiABC system was likely to act as a PTO in vivo.

The experimental observations that the *kaiABC* gene cluster is essential for rhythms in vivo and that rhythmic KaiC phosphorylation runs without a TTFL in vitro and in vivo (35, 68, 94, 106) implied that the KaiABC PTO was the self-sustained core pacemaker. Therefore, transcription and translation were posited to be involved only in output (68, 94). Subsequently, Kitayama et al. (41) suggested that oscillations in KaiC abundance based on transcription and translation are also important for generating the in vivo circadian rhythm. In particular, those authors reported that constitutive hyperphosphorylation of KaiC (either by overexpression of KaiA or by using a mutant of KaiC that mimicked constitutive hyperphosphorylation) allowed rhythmicity to proceed in vivo. Their interpretation was motivated by the observation that cyanobacterial cells apparently exhibited oscillations when the KaiABC oscillator was inactivated by stalling the phosphorylation status of KaiC. In other words, the KaiABC oscillator (PTO) did not seem to be an obligatory core oscillator in cyanobacteria because transcription and translation oscillated even in the absence of the KaiC phosphorylation cycle and the oscillation persisted regardless of the phosphorylation state and kinase activity of KaiC (41). Another group modeled these results and found that a hypothetical pacemaker composed of tightly intertwined PTO and TTFL cycles can generate robust circadian rhythms over a broad range of growth conditions (116).

We decided to extend the experiments by Kitayama et al. and have recently arrived at a different conclusion regarding the hierarchy between the PTO and the TTFL. Our current working model is that the PTO is the core pacemaker and the TTFL is a damped slave oscillator (82) (**Figure 6**). We base this interpretation on our finding that the rhythms generated by cells expressing hyperphosphorylated KaiC have a long period and are clearly damped. Moreover, these damped rhythms are not compensated for by changes in metabolic activity and therefore cannot be considered a bona fide circadian phenomenon. Modeling studies showed that the experimental data were compatible with a core PTO driving the TTFL and that the combined PTO/TTFL system is resilient to noise. Modeling also suggested that de novo synthesis of clock proteins coupled with KaiC monomer exchange results in phase shifts or entrainment of the core PTO pacemaker (**Figure 6**). Our new model of the cyanobacterial oscillator explains how the core pacemaker can be a PTO while receiving input from a TTFL (82). This interpretation has exciting implications for eukaryotic clock systems and suggests that the existence of a common mechanism at the heart of circadian oscillations in all biological systems merits a re-evaluation (32, 82, 83).

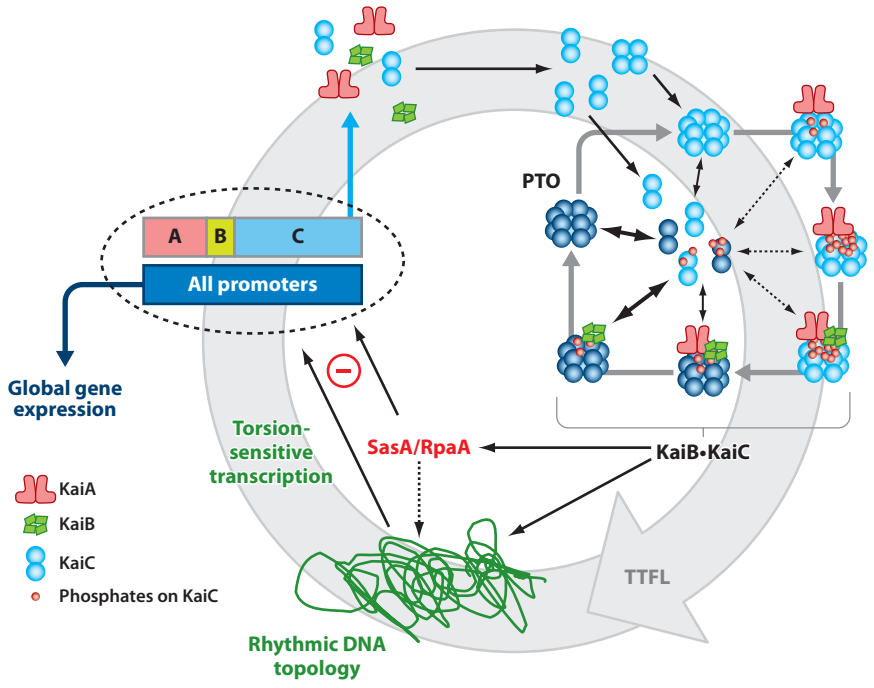


Figure 6

The core posttranslational oscillator (PTO) is embedded in a larger transcription/translation feedback loop (TTFL). The PTO is linked to the damped TTFL by transcription and translation of the *kaiABC* cluster. Global gene expression is mediated by rhythmic modulation of the activity of all promoters, including those driving the expression of the central clock gene cluster, *kaiABC* (ABC in figure). Rhythmic DNA torsion and/or transcriptional factor activity (e.g., SasA/RpaA) modulates global promoter activities. Cyclic changes in the phosphorylation status of KaiC regulate DNA topology/transcriptional factors. The PTO is determined by KaiC phosphorylation as regulated by interactions with KaiA and KaiB (compare with **Figure 3*a***). Robustness is maintained by synchronization of KaiC hexameric status via monomer exchange (depicted by dumbbell-shaped KaiC monomers exchanging with KaiC hexamers in the middle of the PTO cycle). The shade of KaiC hexamers (*dark versus light blue*) denotes conformational changes that roughly equate to kinase versus phosphatase forms. New synthesis of KaiC feeds into the KaiABC oscillator as nonphosphorylated hexamers or as monomers that exchange into pre-existing hexamers. If the new synthesis of KaiC occurs at a phase when hexamers are predominantly hypophosphorylated, the oscillation of KaiC phosphorylation is reinforced (enhanced amplitude). If new synthesis of unphosphorylated KaiC happens at a phase when hexamers are predominantly hyperphosphorylated, this leads to an overall decrease in the KaiC phosphorylation status, thereby altering the phase of the KaiABC oscillator (phase shift), reducing its amplitude, or both. Phase shifts accomplished by this mechanism could be partially or totally responsible for entrainment in vivo (from Reference 82).

ENTRAINING THE ENDOGENOUS CLOCK TO ENVIRONMENTAL TIME

One of the three salient properties of circadian rhythms is their ability to be entrained by the daily cycle in the environment so that their endogenous ~24-h period takes on a period of exactly 24 h with the appropriate phase relationship (12). By virtue of its day-to-day consistency, the LD cycle is the most important environmental signal for circadian entrainment in most organisms. In cyanobacteria, mutagenesis screens designed to find factors involved in phase resetting elicited by pulses of darkness were successful in identifying CikA, a histidine kinase and pseudobacteriophytochrome

(86). Because of its similarity to bacteriophytochromes, CikA initially seemed to be a light-absorbing photoreceptor. However, unlike a classical photoreceptor, CikA does not absorb light, but rather it appears to sense the redox state of the intracellular plastoquinone pool (25, 26). Light drives large changes in redox potential via photosynthesis, and this could be an entraining cue for the cyanobacterial clock (52). Quinone not only directly binds to CikA, it also affects the stability of CikA (26). More recently, redox effects were further implicated in clock entrainment by the observation that KaiA can bind quinone and this binding destabilizes the KaiA protein, thereby reducing its ability to stimulate KaiC autophosphorylation (105). These data suggest that KaiA senses environmental signals as changes in redox state and modulates the circadian clock. Other proteins implicated in light resetting of the cyanobacterial clock include Pex and LdpA, in addition to four new proteins that interact with CikA (NhtA, PrkE, IrcA, and CdpA) (25, 51, 89). These proteins may contribute to the input pathway (51).

However, a new paper has proposed a dramatically different explanation for LD entrainment that is based on the observation that the ratio of ATP to ADP declines in the dark in *S. elongatus* cells, which are obligately photosynthetic (84). When ATP:ADP ratio changes that are similar to the in vivo measurements in darkness were simulated in the in vitro oscillator, they caused phase shifts similar to those observed with dark pulses in vivo. These data were interpreted to mean that exposure of cells to darkness changes the ATP/ADP ratio, and this change directly resets the phase of the PTO in vivo. A mathematical model based on these data effectively modeled entrainment by dark pulses (84). Although this new study has the potential to explain entrainment of the cyanobacterial circadian clock, it is presently unclear how this interpretation will fit together with the other data implicating CikA (and other proteins) in the entrainment pathway.

Although LD cycles are usually the most important entraining agent, cyclic exposure to temperature changes can often entrain circadian clocks (30). There has not yet been a report of temperature cycle entrainment for the cyanobacterial system in vivo (except for a brief mention in Reference 114), but there have been two reports of temperature resetting of the in vitro oscillator (63, 114). In 2007, we reported the first phase response curves for the in vitro system, generated by 6-h pulses of either 16°C or 37°C away from the free-running temperature of 30°C (63). This analysis demonstrated that the in vitro oscillation shares characteristic phase-dependent responses to perturbation with most circadian systems (12, 30). A more extensive analysis of temperature-induced phase resetting that used steps between 30°C and 45°C also found that the temperature stimuli shifted the phase of the in vitro rhythm in a phase-dependent manner suggestive of a nonparametric entrainment mechanism (12, 114).

Nakajima et al. (69) have suggested that daily changes in the intracellular concentrations of KaiB and KaiC could modulate the angular velocity of the pacemaker in vivo and provide an entraining stimulus. KaiB and KaiC abundance levels oscillate in LL, whereas KaiA levels remain rather constant (27, 40, 94, 106). Although some studies have reported that the abundance of Kai proteins is cyclic in LD cycles as well (36), we find considerable variability of Kai cycling in LD; in some experiments, Kai protein levels seem not to oscillate in LD when the KaiC phosphorylation rhythm is robust (82). Therefore, the hypothesis that entrainment in vivo is based on changes of Kai protein abundances warrants more experimental testing (69). Our studies of the relationship between the PTO and TTFL have led us to propose that new synthesis of Kai proteins in the daytime alters and adjusts KaiC phospho-status as needed for proper entrainment to the environment (**Figure 6**) (82).

EVOLUTION OF CIRCADIAN TIMING

Cyanobacteria are an ancient group. They were likely one of the earliest life forms, dating back

at least 3.5 billion years. Therefore, over the span of their evolution they have been subjected to the original earth's anoxic atmosphere, which was also characterized by high levels of ultraviolet (UV) radiation. Indeed, cyanobacteria are thought to be the primary organism that transformed earth's atmosphere by photosynthetic oxygen emission into its current oxygenated state with the concomitant creation of the ozone layer, which helps to shield earth's inhabitants from the deleterious effects of UV radiation. Currently, the most plausible model for the evolution of circadian timing—the escape from light hypothesis (32, 70, 79, 80)—strongly implicates daily gating of cell division and DNA damage as the selective forces that resulted in the evolution of circadian pacemakers. In a nutshell, the escape from light hypothesis proposes that the illuminated portion of the light/dark cycle had profound and mostly deleterious effects on early life; therefore, a strong initial driving force for the early evolution of a circadian timer could have been the advantage of phasing to the nighttime those cellular processes that are vulnerable to light. For example, UV light produces thymidine dimers in DNA, which if not repaired accurately mutate the DNA. Moreover, even visible light can modulate metabolism and other processes because it is absorbed by omnipresent pigments in cells such as cytochromes. Consequently, cellular events that are hypersensitive to light (such as DNA damage and electron transport) would be best performed at night in organisms that cannot shield themselves from irradiation. The escape from light hypothesis predicts that the vestiges of nocturnal programming of light-sensitive processes might have been retained in present-day organisms. This prediction was tested in the eukaryotic unicell *Chlamydomonas*, where it was found that the cells were indeed most sensitive to UV light in the early night (70).

FUTURE DIRECTIONS FOR CYANOBACTERIAL CLOCKS

As Jacques Monod said in his Nobel Prize acceptance speech in 1965, "The ambition of molecular biology is to interpret the essential properties of organisms in terms of molecular structures. This objective has already been achieved for DNA, and it is in sight for RNA, but it still seems very remote for the proteins." Although we may be on the verge of realizing Monod's ambition with the KaiABC system, many outstanding and unresolved issues remain:

- A perplexing concern about the in vitro oscillator when seen in the context of its in vivo operation relates to the stoichiometry among the KaiA, KaiB, and KaiC proteins for optimal operation. The original publication (68) used concentrations of the Kai proteins that were supposedly based on estimates of in vivo concentrations (40), but in fact significantly more KaiA is needed in the in vitro reaction than appears to be present in vivo. In addition, while early reports suggested that the period and amplitude of the in vitro rhythm were relatively invariant within an allowed range of Kai protein concentrations (37), recent data indicate significant effects on the period of the in vitro oscillator when the ratio of [KaiA] was varied relative to the concentration of [KaiB]+[KaiC] (69). Modeling indicates that the PTO is robust and resilient within a limited scope of Kai protein concentration fluctuations (82), but these recent results (69) raise the concern that the in vitro oscillator might not be a reliable pacemaker in vivo. Perhaps a function of the TTFL is to maintain Kai protein concentrations within a range that maintains a dependable timekeeper.
- What is the configuration of KaiC in the unphosphorylated state? At the present time, no three-dimensional structure of unphosphorylated KaiC has been reported.
- We have crystal structures of the three Kai proteins individually, but not of all three Kai proteins in a complex or interacting with SasA, CikA, etc. Moreover, methods of structural analysis that allow

dynamic measurements of structure (i.e., SAXS, NMR, and EM) should be pursued further because crystal structures are snapshots of proteins whose structures are almost certainly dynamic, and these dynamic changes will be important for fully understanding the mechanism of the KaiABC oscillator.

- Despite progress, a complete mechanistic understanding of phosphorylation (autokinase), dephosphorylation (autophosphatase), and ATPase activity remains obscure.
- Is the KaiC hexamer a propeller? Do all the monomers in the KaiC hexamer act in concert, or is there spatial distribution of activities? Considering that KaiC and F1-ATPase share structural similarities, perhaps there is a rotary phosphorylation/activity within KaiC as in F1-ATPase (96, 101).
- How are the phosphorylation and dephosphorylation rates of KaiC compensated for temperature (68, 94)? The mechanism of temperature compensation, which is a key property of all circadian systems, remains a key mystery.
- How are the Kai proteins coupled to the downstream control of gene expression? One study has proposed that the ATP hydrolytic activity of KaiC is the key output of the KaiABC oscillator (11). Alternatively, an output pathway for KaiC that is based on two-component signaling was proposed whereby KaiC influences the phosphorylation status of the histidine kinase SasA, which in turn regulates the activity of a response regulator, RpaA, that may act as a transcriptional factor (90). Other factors have also become implicated in this output pathway (92). The sequence and structural similarity of KaiC to RecA and DnaB has suggested another possibility, namely that the Kai nanomachine may have a helicase activity (46, 62). This possibility—which does not exclude the participation of a SasA/RpaA or ATPase pathway—is particularly intriguing given the global rhythms of chromosomal topology (60, 100, 104). If KaiC can act on DNA, perhaps it directly mediates the pervasive supercoiling. However, although we found that KaiC has weak binding affinity for forked DNA substrates (62), hitherto attempts to measure helicase activity from KaiC have been unsuccessful.
- Is an oscillating chromosomal topology the basis for rhythmic global gene expression (60, 100, 104)? Systems biology approaches may yield answers.
- What is the mechanism for the competition/selection phenomena that illustrate the adaptive significance of the *S. elongatus* clock in rhythmic environments (19, 31, 73, 103)?
- Will the clockwork in *S. elongatus* ultimately prove to be completely distinct from the clocks of eukaryotes? Or will the insights gleaned from cyanobacteria induce a reassessment of clocks in higher organisms (32, 33, 82, 83)?

SUMMARY POINTS

1. Biological clocks have played an important role in bioevolution as evidenced by their existence in organisms that initially evolved as far back as 3.5 billion years ago.
2. Prokaryotic cyanobacteria have a circadian timekeeping system that enhances fitness.
3. These cells exhibit pervasive circadian regulation of gene expression, possibly by regulation of chromosomal topology.

4. A circadian rhythm of the phosphorylation of the central clock protein KaiC can be reconstituted in vitro with three proteins derived from the cyanobacterium *S. elongatus* (KaiA, KaiB, and KaiC) and ATP.
5. KaiA, KaiB, and KaiC are the only circadian proteins for which the three-dimensional structure of full-length proteins is known.
6. Structural, biochemical, and biophysical methods have been used to study the mechanism by which KaiC is rhythmically phosphorylated and dephosphorylated. Dephosphorylation is temporally coordinated with monomer exchange, which may also function to maintain synchrony of individual Kai molecules in the population of molecules.
7. Modeling has been applied to the in vitro and in vivo systems. Models of the complete in vivo system have indicated the existence of a core biochemical oscillator that controls a larger transcription/translation feedback loop.
8. The *kaiC* gene is widespread among prokaryotes (Eubacteria and Archaea), but it may be performing a nonclock function in prokaryotic species outside of the cyanobacteria. The elucidation of this nonclock function could lead to fascinating clues about the selective pressure(s) that led to the evolution of circadian clocks.

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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