# CIRCADIAN RHYTHMS IN *Synechococcus elongatus* PCC 7942: INSIGHTS INTO THE REGULATORY MECHANISMS OF THE CYANOBACTERIAL CLOCK SYSTEM

A Dissertation

by

SHANNON ROSE MACKEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Microbiology

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Approved by:

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# ABSTRACT

Circadian Rhythms in *Synechococcus elongatus* PCC 7942:
Insights into the Regulatory Mechanisms of the Cyanobacterial
Clock System. (August 2006)
Shannon Rose Mackey, B.A., The University of Texas at Austin
Chair of Advisory Committee: Dr. Susan S. Golden

Circadian rhythms of behavior have been well characterized in organisms including mammals, plants, insects, fungi, and photosynthetic bacteria. Cyanobacteria, such as the unicellular *Synechococcus elongatus* PCC 7942, display near 24-h circadian rhythms of gene expression. These rhythms persist in the absence of external cues, can be reset by the same stimuli to which they entrain, and are relatively insensitive to changes in ambient temperature within their physiological range. Key components have been identified as belonging to the central oscillator that comprises the timekeeping units, output pathways that relay temporal information to clock-controlled processes, and input pathways that synchronize the oscillator with local time. The emerging model of the cyanobacterial clock depicts the internal timekeeping elements KaiA, KaiB, and KaiC interacting with one another to form a large, multimeric complex that assembles and disassembles over the course of a day. Information is sent into and out of the oscillator via signal transduction pathways that include proteins involved in bacterial two-component systems. The research presented in this dissertation explores the regulatory mechanisms that exist at each level of the clock system. New components were

identified that interact with an important protein in the input pathway; these new players are involved in clock-associated phenomena, such as resetting the internal oscillation to external stimuli and maintaining proper circadian periodicity, as well as the process of cell division. The model formerly associated with the temporal, transcriptional regulation of the *kai* genes was redefined to reflect the unique properties of the prokaryotic oscillator. The differential output of the clock was examined by studying the circadian regulation of the *psbA* gene family. Overall, these data provide insight into the complex molecular events that occur to create a circadian timing circuit in *S. elongatus*.

# ACKNOWLEDGMENTS

I would like to thank my committee chair and friend, Dr. Susan Golden, for her endless support and guidance throughout this process. Her careful mentoring and undoubting faith provided the backdrop to my academic performance in both research and teaching.

In addition to my chair, the members of my committee, Drs. Deb Bell-Pedersen, Dave Earnest, and Ry Young, provided great interest and insights into my research and future scientific career. Their encouragement was, and will continue to be, greatly appreciated.

The many friends and colleagues I have made during my time at Texas A&M have made the experience enjoyable and unforgettable. I would especially like to thank Drs. Jayna Ditty, Eugenia Clerico, and Stan Williams for their infinite supply of humor and life lessons.

And most importantly, I thank Mom, Dad, Jenny, and Peter for their immeasurable patience with, and pride in, each hurdle I jump.

# NOMENCLATURE

| | |
|---|---|
| 6xHis | six adjacent histidine residues |
| 12:12 LD | 12 h light, 12 h dark |
| Ap | ampicillin |
| ATP | adenosine triphosphate |
| BG-11M | modified BG-11 medium |
| C-6xHis | six adjacent carboxy-terminal histidine residues |
| C-terminus | carboxy-terminus |
| CI | first of the tandem repeats of KaiC |
| CII | second of the tandem repeats of KaiC |
| CikA | circadian input kinase |
| CikA-I | CikA-interactor |
| CikA~P | phosphorylated CikA |
| Cm | chloramphenicol |
| CpmA | circadian phase modifier |
| cps | counts per second |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DBMIB | 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone |
| DNA | deoxyribonucleic acid |
| EMS | ethyl methane sulfate |
| FFT-NLLS | fast Fourier transform–nonlinear least square |
| FRP | free-running period |

| | |
|---|---|
| Gm | gentamycin |
| GTP | guanosine triphosphate |
| HPK | histidine protein kinase |
| Hyp-1 | hypothetical protein 1 |
| Hyp-2 | hypothetical protein 2 |
| I&A | Import and Analysis |
| IPTG | isopropyl-beta-D-thiogalactopyranoside |
| kb | kilobase |
| kDa | kiloDalton |
| Km | kanamycin |
| LD | light/dark |
| LdpA | light-dependent period |
| LL | constant light |
| mRNA | messenger ribonucleic acid |
| N-6xHis | six adjacent amino-terminal histidine residues |
| N-terminus | amino terminus |
| NHT-1 | aminotransferase |
| Ni-NTA | nickel-nitriloacetic acid |
| NS1 | neutral site I |
| NS2 | neutral site II |
| OD | optical density |
| ORF | open reading frame |

| | |
|---|---|
| Pex | period extender |
| PSII | photosystem II |
| PsR | *pseudo*-receiver |
| RR | response regulator |
| SasA | *Synechococcus* adaptive sensor |
| Sm | streptomycin |
| Sp | spectinomycin |
| Spk | serine-threonine protein kinase |
| WT | wild type |

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# CHAPTER I

# INTRODUCTION

The research presented in this dissertation identifies novel components of the *Synechococcus elongatus* PCC 7942 circadian clock system and helps to redefine the paradigm previously associated with the transcriptional control of the molecular timekeeping mechanism. Additionally, data are presented to demonstrate the differential circadian regulation of the *psbA* gene family.

## *The circadian clock*

Most organisms are subjected to daily fluctuations in light and temperature as a result of the full rotation of the Earth on its axis approximately every 24 h. Endogenous biological clocks that allow for anticipation of these daily variations have evolved within organisms and provide an advantage to those subjects (74, 95). These circadian clock systems are internal molecular mechanisms that control rhythmic gene expression which, in turn, regulates metabolic and/or behavioral processes (19, 20, 97).

Daily rhythmic behavior was recorded as early as 1729 by Jean Jacques deMairan who noticed that the opening and closing of heliotrope leaves persisted even after the plant had been moved to constant darkness (19). However, it was not until the 1970s that a molecular basis was ascribed to the control of these daily oscillations. The breakthrough came from *Drosophila melanogaster* and the work of Ronald Konopka and Seymour

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This dissertation follows the style of the Journal of Bacteriology.

Benzer (60). Flies that had been mutagenized with ethyl methane sulfonate (EMS) were examined for the persistence of two behaviors, pupal eclosion and locomotor activity, that were known to be controlled by the circadian clock. Flies displayed three categorical mutant phenotypes, those with activity rhythms much shorter than 24 h, or much longer than 24 h, or with no detectable rhythmicity at all. These phenotypes could each be complemented by just one locus, the *period* gene. Shortly after this discovery in fruit flies, the *frequency* gene was shown to be essential for rhythms in conidiation to persist in the filamentous fungus *Neurospora crassa* (23). In the 30 years to follow, the field of circadian biology accelerated at an amazing rate with the discovery and characterization of dozens of clock components in both prokaryotic and eukaryotic systems, including cyanobacteria, fungi, insects, mammals, and even humans (5).

Chronobiologists use three hallmark characteristics to define a process that is controlled by the circadian clock (Fig. 1.1) (19, 97). (1) The rhythm of the behavior must peak and trough with a periodicity near 24 h in the absence of an environmental cycle. The inability of a rhythm to continue under constant conditions implies that it is driven by an external stimulus, rather than being a product of an endogenous activity of the organism. The time needed for one oscillation to occur in constant conditions is known as the free-running period (FRP). The FRP of each model system is unique and is a result of the molecular happenings that occur in the absence of external stimuli. (2) The phasing of the peak or trough of the rhythm relative to a reference point (*e.g.*, lights on) can be changed by the environmental cues to which the clock entrains. Because FRPs are close

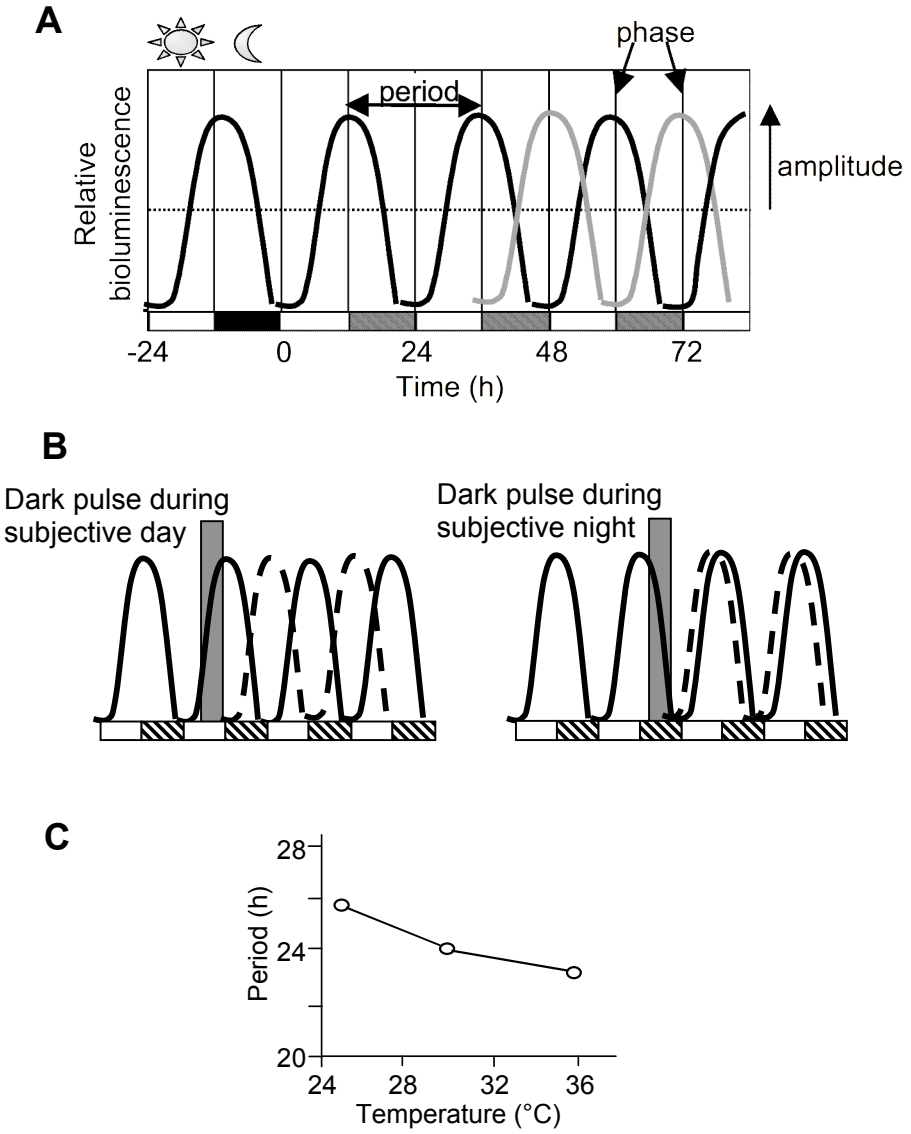


FIG. 1.1. Properties of a circadian rhythm. (A) Features of a circadian rhythm that are commonly measured are the period, phase, and amplitude. (B) The circadian rhythm can be reset when a dark pulse is administered during the time that corresponds to day when cells are kept in constant light (subjective day), but the same dark pulse will cause only a small shift when given during the subjective night. (C) The period of a circadian rhythm changes very little in response to changes in ambient temperature. The bars on the X-axis of (A) and (B) represent light (white), dark (black) and subjective dark (hatched). Negative time denotes light/dark cycles used to synchronize the cells' clocks. Figure adapted, with permission, from S. S. Golden and S. R. Canales, Nat. Rev. Microbiol. **1:**191-199, 2003.

to, but not exactly, 24 h the system must be able to reset its rhythm by environmental stimuli each day in order to avoid falling out of phase with its surroundings. (3) For a biological clock to be reliable in a natural setting, it must maintain a robust period of oscillation despite changes in ambient temperature. This property, termed temperature compensation, stems from the observation that the value of the FRP changes very little over different temperatures within the organism's physiological range. The effect of changes in temperature on the rate of most biological processes is measured by a $Q_{10}$ value, which is defined as the ratio of the rate of a given process at one temperature to the rate at a temperature 10ºC lower. The $Q_{10}$ value of the period of a circadian rhythm remains near 1, as opposed to other known biochemical reactions that have $Q_{10}$ values of 2 or 3.

Although these three cardinal rules are helpful to define circadian-controlled behaviors, they also oversimplify the complexity of the system. Although circadian rhythms persist in constant conditions, the FRP can vary slightly in response to changing light intensities. Diurnal organisms display a slightly shorter FRP (*i.e.*, faster clock) in higher light intensities than they do in low light; nocturnal organisms exhibit the inverse response with a longer FRP (*i.e.*, slower clock) in high light than in dim light. This phenomenon, originally described by Jürgen Aschoff, is known as "Aschoff's Rule" (4). Also, temperature compensation is not the same as temperature insensitivity. Temperature can be as strong a *zeitgeber* (literally, "time giver") in resetting the phase of the rhythm as the transition from dark to light (70). Individual reactions within the clock

are undoubtedly affected by changes in temperature, but the system as a whole is buffered such that the output of rhythmic behavior does not show large variations in its FRP.

A circadian clock can be generalized into three basic components. At the heart of the clock system is the central oscillator, whose proteins move at rates that are not on a 24-h time scale, but when acting together can integrate a series of biochemical events to create a near 24-h timing circuit. The temporal information produced by the oscillator is interpreted by components of the output pathways, which then play their own role, alone or in conjunction with other players, in controlling metabolic and behavioral processes. In order for the oscillator to maintain synchrony with the environment, input pathways interpret external stimuli as cues to advance or delay the phase of the endogenous oscillation. However, the more that is learned about the circadian clock, the more difficult it becomes to assign any one component to only one of the three divisions of the clock system.

***A cyanobacterial model system for circadian rhythms***

Until the 1980s, circadian clocks were believed to exist only in eukaryotes. Prokaryotes, with life spans much shorter than that of a full circadian cycle, were thought to have no need to maintain a 24-h timing mechanism. However, the existence of two incompatible processes – oxygenic photosynthesis and oxygen-sensitive nitrogen fixation – that occur in some unicellular cyanobacteria led researchers to investigate the mechanisms used to

separate these reactions. They discovered that in *Synechococcus* RF-1 a temporal separation occurred such that photosynthesis occurred during the day, and nitrogen fixation at night (79); the alternating rhythms display persistence in constant conditions, entrainability, phase resetting, and temperature compensation (34, 35, 42). Further studies showed that other cellular activities, such as amino acid uptake (9) and cell division (111), are also under control of an endogenous clock.

Despite the advances made in demonstrating that prokaryotes are capable of maintaining circadian rhythms of metabolic activity, the species used up to this point were not amenable to modern molecular biology techniques. In order to investigate the molecular mechanism that dictates timing within the cell, the obligately photoautotrophic cyanobacterium *S. elongatus* PCC 7942 became the prokaryotic model system for studying circadian behavior. This bacterium offers many technical advantages that the other unicellular species did not, including a small, fully-sequenced genome (2.8 Mb) and easy genetic manipulation; it is naturally transformable (33), conjugates with *Escherichia coli* (22), and has available vectors for cloning *S. elongatus* genes (32).

Because *S. elongatus* does not display an overt circadian behavior, like the leaf movement of plants or wheel running of mice, an observable "behavior" was created by fusing the promoter of the photosynthesis gene *psbAI* (P*psbAI*) to the *luxAB* bioluminescence genes from *Vibrio harveyi* (58, 69). This fusion creates a reporter gene whose activity can be monitored automatically in high-throughput assays (Fig. 1.2). The

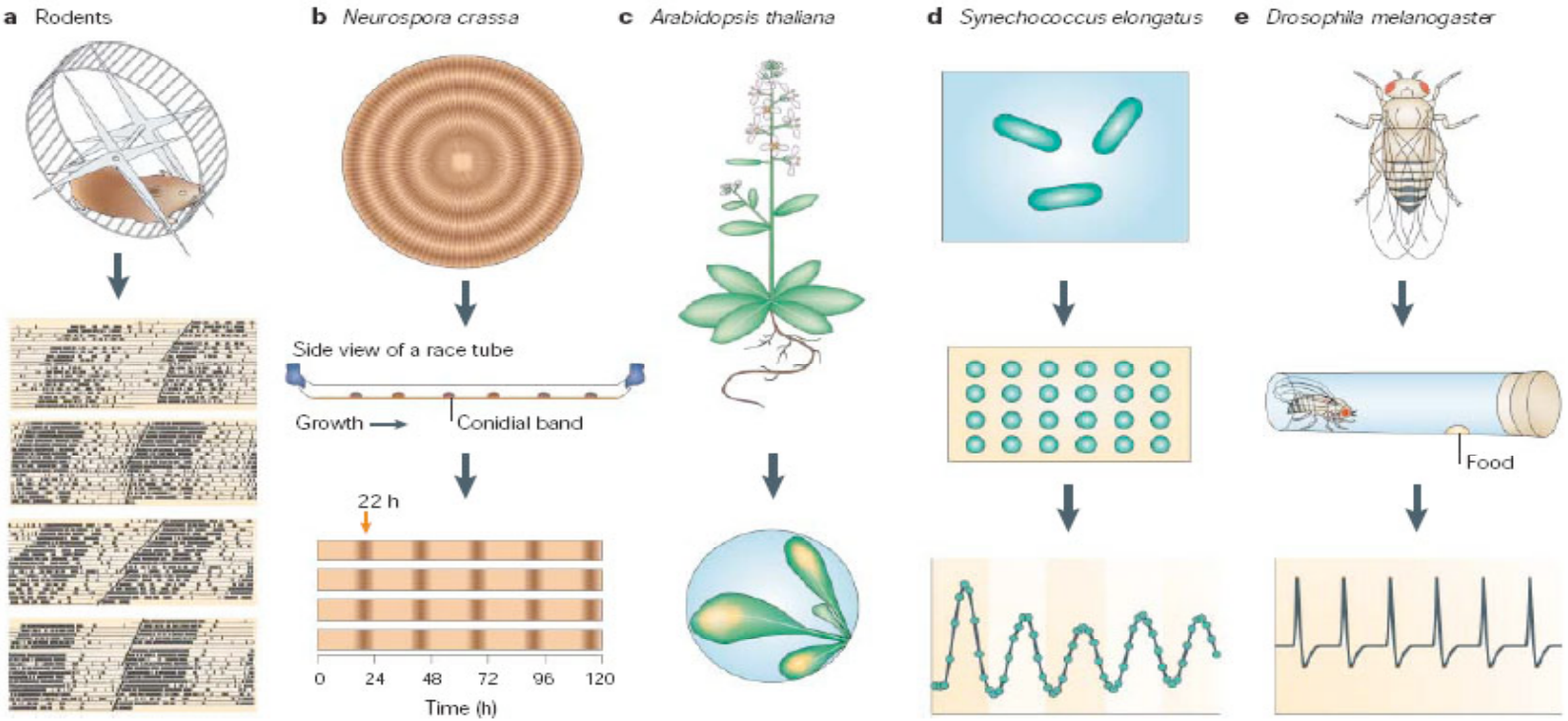


FIG. 1.2. Circadian rhythm model systems. Each model system for circadian research offers its own means of non-invasive or automatic means of recording daily rhythms. (A) Daily wheel-running activity of rodents occurs in constant darkness and is recorded in the form of an actogram. Bouts of activity are seen in black and provide information about the period and phase of the mammal's clock. (B) The fungus *N. crassa* produces asexual spores under the control of a biological clock. This process of conidiation can be measured using a specialized growth chamber called a race tube. (C) In *Arabidopsis thaliana*, rhythms of bioluminescence from luciferase fusions can be visualized for many days in constant light. (D) In the unicellular cyanobacterium, *S. elongatus*, luciferase fusions are used to monitor rhythms of promoter activity in high-throughput 96-well plates, allowing for saturation mutagenesis to determine components of the oscillator, as well as input and output pathways. (E) Daily flight movements of the model fly *D. melanogaster* break an infrared beam in a specialized tube. The number of breaks can be monitored electronically to detect the circadian pattern of locomotor activity. Figure and legend used, with permission, from S. S. Golden and S. R. Canales, Nat. Rev. Microbiol. **1:**191-199, 2003.

resulting rhythm of bioluminescence obeys all three rules that deem a process under circadian control. In fact, when random fragments from an *S. elongatus* genomic DNA library were fused upstream of the promoterless *luxAB* genes, all colonies that expressed bioluminescence were rhythmic in their expression, which suggests that there is global regulation of *S. elongatus* gene expression by the endogenous oscillator (72).

A noteworthy point to make is that the unicellular *S. elongatus* does not fix nitrogen (40), and without the need to separate photosynthesis and nitrogen fixation would not necessarily have a selective pressure to possess a robust circadian oscillator to regulate transcription. The rationale for maintaining this clock system became clear when cultures with different FRPs were mixed together in equal proportions and grown together in different light/dark (LD) cycles; the strain whose FRP most closely matches that of the given LD cycle prevails over its competitor (95). If cells are subjected to a 12 h light, 12 h dark (12:12 LD) cycle, a wild-type (WT) strain with FRP of 25 h outcompetes a 30-h mutant, yet this mutant grows better than WT if provided a 15:15 LD cycle. When either culture is grown independently in either constant light (LL) or LD cycles of various lengths, the growth rates are indistinguishable. Thus, the presence of a circadian oscillator that resonates with a period near that of the daily environmental cycles provides an adaptive advantage to that organism in a competitive environment.

***The Kai oscillator: keeping track of time***

The luciferase reporter system provided a practical method to screen tens of thousands of *S. elongatus* colonies to identify mutants defective in maintaining circadian time. Chemical mutagenesis with EMS generated a variety of circadian phenotypes that included arrhythmia and altered period lengths, which extended between the shortest at 16 h to the longest at 60 h (59). Complementation of the mutations with an *S. elongatus* genomic DNA library showed that all of the circadian defects could be rescued by a single locus that contained three adjacent genes (43). These genes were named *kaiA, kaiB,* and *kaiC* from the Japanese *kaiten*, which implies a cycle of events suggestive of the turning of the heavens. The *kai* genes are essential for circadian rhythms to persist in cyanobacteria. Deletion of any of these genes renders the clock arrhythmic, and their functions appear to relate only to the circadian system of the cells as they are not essential for cell viability and *kai* mutants do not display a growth deficiency when grown in pure culture (43).

The *kai* genes are expressed from two promoters: one promoter drives expression of *kaiA* (P*kaiA*), and a promoter upstream of *kaiB* (P*kaiBC*) expresses a *kaiBC* dicistronic message (43). Expressing the *luxAB* reporter genes from either of these promoters will produce rhythms in bioluminescence that peak at the light to dark transition in LD cycles, or about 12 h after release into LL and approximately every 24 h thereafter. Promoters with peak expression at the same time as the *kai* rhythms (including the aforementioned P*psbAI*) are termed “class 1” and make up the majority of promoters in

the *S. elongatus* genome; "class 2" promoters peak 12 h anti-phase to class 1 (72). The cyclic nature of *kai* transcription is mirrored in the accumulation of mRNA, with *kaiA* and *kaiBC* messages showing fluctuations in absolute levels throughout the day (43). However, only KaiB and KaiC protein levels display robust rhythmicity in either LD or LL conditions, with peak levels occurring 4 – 6 h after the peak in mRNA (125). KaiA accumulation is relatively constant throughout the circadian cycle and, if rhythms exist, the amplitude is much lower than that seen in KaiB or KaiC levels (125).

The *kai* genes and their protein products were originally thought to possess autoregulatory properties, like those of the eukaryotic circadian systems. The positive effector proteins CLOCK and CYCLE in *D. melanogaster*, and WHITE COLLAR-1 and WHITE COLLAR-2 in *N. crassa*, activate transcription of the negative effectors, PERIOD and TIMELESS in *D. melanogaster* (16), and FREQUENCY in *N. crassa* (10, 15). These clock proteins then translocate to the nucleus to inhibit their own transcription (3, 26). Much like these feedback loops, KaiA is a positive regulator of *kaiBC,* as overexpression of KaiA increases levels of bioluminescence from a P*kaiBC::luxAB* reporter (43). This activation is most likely indirect, as KaiA does not contain a predicted DNA-binding domain. KaiC will repress expression from the *kaiBC* promoter when produced in excess, suggesting a role in negative regulation of its own expression (43). However, some level of KaiC is necessary for full activity from the *kaiBC* promoter because levels of *kaiBC* expression are lower when *kaiC* is inactivated.

Together, these proteins were believed to form a negative transcription-translation feedback loop (Fig. 1.3).

Short-term overexpression of KaiC can shift the phase of peak expression in subsequent cycles (43). When the levels of *kaiBC* mRNA are on the rise during the subjective day, a pulse of elevated *kaiC* expression causes a phase advance. A short pulse of *kaiC* overexpression during the subjective night, when *kaiBC* levels are declining, causes a phase delay. The value of the phase shift is proportional to the phase difference between the time when the pulse was given and the time when *kaiBC* levels would normally be at their maximum.

Recent data suggest that the *kaiA* or *kaiBC* promoter regions do not contain specific *cis* elements needed to maintain circadian rhythms *in vivo.* Some mutations in clock-related genes disrupt the phase relationship between *kaiA* and *kaiBC* expression, yet self-sustained rhythms persist (52, 86, 119). Placing *kaiBC* under control of the heterologous P*trc* promoter from *E. coli* restores WT rhythms of bioluminescence and *kaiBC* mRNA accumulation in a reporter strain that lacks its endogenous *kaiBC* genes (126). Data presented in Chapter III of this dissertation show that driving either *kaiA* or *kaiBC* expression from a class 2 promoter, such that peak levels of *kai* expression are 12 h out of phase from that of their WT counterpart, can complement the respective *kai* null mutation. These experiments, published in *Microbiology*, were conducted in collaboration with Drs. Jayna L. Ditty and Stanly B. Williams.

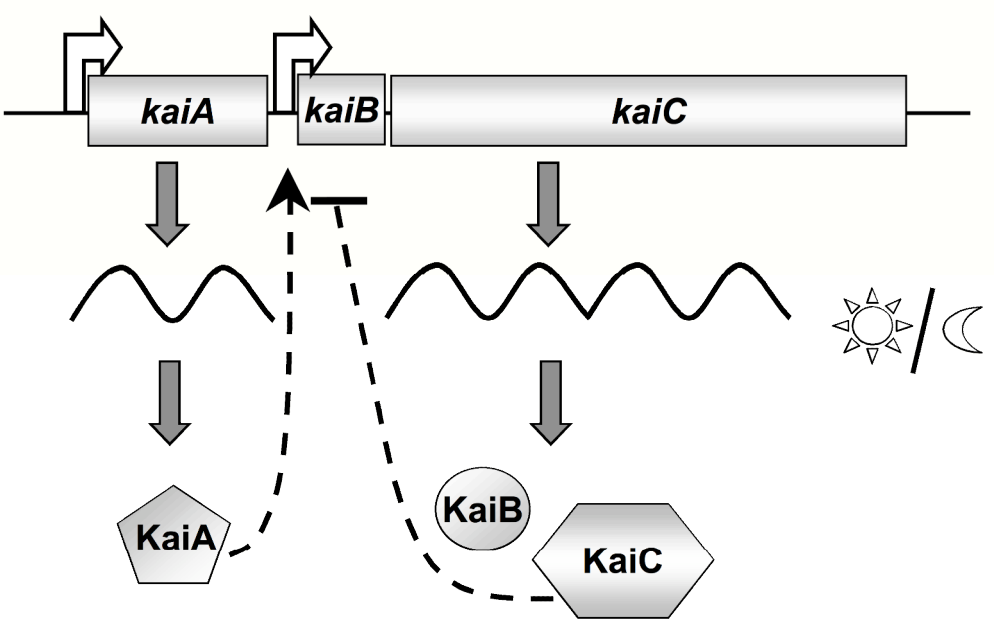


FIG. 1.3. Autoregulation of the *kai* locus. Transcription of the *kaiA* and *kaiBC* genes is under control of the circadian clock with peak expression levels occurring at subjective dusk in LL conditions. Overexpression of KaiA causes a decrease in the level of expression of *kaiBC,* while overexpression of KaiC inhibits its own expression. The resulting regulation creates a feedback loop with KaiA as the positive limb and KaiC and the negative regulator.

Bioinformatic analysis of the Kai primary sequences provided little information about function. Motif searches of the Kai proteins did not produce any significant matches for either KaiA or KaiB; the KaiC sequence, however, revealed domains that predicted testable functions (43). There is significant similarity between the first and second halves of the KaiC protein (CI and CII) (47), with each domain containing a P-loop ATP-binding motif (Walker's A motif), an imperfect Walker's B motif, and catalytic carboxylate glutamyl residues that are also present in other proteins known to bind ATP. The CI domain also contains two DXXG motifs (where X represents any amino acid residue) that are conserved among members of the GTPase family (43, 47).

These highly conserved domains of KaiC led investigators to test their predicted function. KaiC is able to bind ATP *in vitro*, and to a lesser extent GTP (92). Site-directed mutagenesis of the P-loop ATP-binding motif of CI disrupted the binding of ATP *in vitro*, and led to a failure to complement a *kaiC* null mutation *in vivo;* the corresponding mutation in CII had no effect on the ability of KaiC to bind ATP and was able to restore rhythmicity to the *kaiC* null, albeit with a 70 h period (92). KaiC protein autophosphorylates when incubated with radiolabeled ATP, and at least two phosphorylation sites have been shown to exist on adjacent serine and threonine residues (46, 96, 127). A third phosphorylation site is plausible due to its proximity to the other two residues, and it is thought that a phosphoryl group can shuttle between these sites (127). Amino acid substitutions at any of the three phosphorylation sites result in arrhythmicity from luciferase reporters *in vivo* (127).

Functions for KaiA and KaiB became apparent when they were incubated with KaiC in the presence of ATP. Although neither KaiA nor KaiB can undergo an autophosphorylation reaction, they can alter the rate of KaiC's autokinase activity (46, 54, 124). The addition of KaiA to KaiC *in vitro* increases the rate at which KaiC autophosphorylates by 2.5 fold. Addition of only KaiB to KaiC does not have any effect. Only when KaiB is added along with KaiA can KaiB's role be assigned: KaiB abrogates the positive activation of KaiA on KaiC, such that autophosphorylation by KaiC is reduced to about half of the KaiA/KaiC combination. The phosphorylation state of KaiC cycles throughout the day and is believed to be critical for circadian timing (46); mutants in the *kai* genes or other clock components that affect the ability of KaiC to phosphorylate in a rhythmic fashion alter the functionality of the clock (46, 126, 127).

As suggested by the biochemical relationship between the Kai proteins, KaiA, KaiB, and KaiC physically interact with one another both *in vitro* and *in vivo.* Initial yeast two-hybrid assays demonstrated both homotypic and heterotypic interactions among these clock components (47). KaiA and KaiB only weakly interact, but their interaction is strengthened in the presence of KaiC. Both the CI and CII domains of KaiC were shown to interact with the other two Kai proteins. The interaction between KaiA and KaiB was strengthened more by the CI domain than full-length KaiC; CII had the least positive effect on KaiA/KaiB binding and further demonstrates the differences in function of the two KaiC domains (47).

Early studies demonstrated a correlation between the binding strength of the proteins (as measured by colorimetric assays in yeast two-hybrid systems) and the period of the rhythm that resulted from those proteins *in vivo*. A mutation in *kaiA* that causes a 33-h FRP (*kaiA1*) enhances KaiA interaction with KaiB (47). Mutations in *kaiC* (*kaiC11* and *kaiC12)* that cause enhanced interactions between KaiA and KaiC lengthen the circadian period to 44 h and 60 h, respectively, whereas a KaiC mutant (*kaiC3*) that interacts weakly with KaiA results in a short 16-h period in LL (114). These data suggest that strong interactions between KaiA, KaiB, and KaiC can slow down the rate at which the clock proceeds.

The interactions between the Kai proteins have also been demonstrated *in vivo*. Affinity chromatography using a KaiC variant that carried an engineered tag of 6 adjacent histidine residues (6xHis) co-purified both the KaiA and KaiB proteins from *S. elongatus* protein extracts (47). Interestingly, the amounts of these proteins that were co-purified with KaiC were not constant throughout the circadian cycle. The greatest amounts of KaiA and KaiB proteins that interacted with KaiC occurred between 16 – 20 h after being released into LL (54), which suggests that the proteins form a large complex during the early subjective night.

The Kai proteins also form higher-order complexes through homotypic interactions. Sedimentation velocity and gel filtration chromatography showed that KaiC from either *S. elongatus* or its thermophilic cousin *Synechococcus lividis* P2 has a molecular mass

consistent with a hexamer when purified in the presence of ATP (38, 83). Electron microscopy and crystallographic structure analyses were able to elucidate this "double doughnut" shape, which consists of six KaiC monomers and 12 bound ATP molecules (83, 127). The CI and CII structural domains are connected by a thinner "waist" region and a channel exists in the center of the ring structures that narrows near the carboxy-terminal (C-terminal) regions of the KaiC monomers.

The X-ray crystal structure of KaiB reveals that it is comprised of four subunits that are organized into a dimer of two asymmetric dimers (49). Specific functions have not yet been attributed to KaiB based on its structure. The structural information obtained for KaiA, however, provided insightful hints at its function. KaiA forms a dimer in solution through interactions in its carboxy-terminus (C-terminus), which has a novel fold (122, 123). This C-terminal region is capable of binding a small peptide from the CII domain of KaiC in solution (123), and is alone responsible for the stimulation of KaiC autophosphorylation (124). The amino-terminal (N-terminal) domain of KaiA resembles that of a receiver domain of proteins typically involved in bacterial two-component signal transduction pathways (124). However, this domain does not contain the conserved aspartyl residue that normally accepts the phosphoryl group from a partner kinase and is, thus, termed a *pseudo*-receiver (PsR) domain. The atypical PsR of the AmiR protein in *Pseudomonas aeruginosa* acts through interactions with other protein products to elicit a response to external signals (93). Clock-associated proteins in *Arabidopsis thaliana* have been shown to interact with each other and with DNA-

binding transcription factors through their PsR domains (44). The hypothesized function of the N-terminus of KaiA is to act as a conduit to pass environmental information from the input pathways to the Kai oscillator by regulating the extent by which KaiA stimulates the autophosphorylation of KaiC (124).

Taken together, these data suggest that the strength and timing of the interactions among the Kai proteins are essential to the regulation of the phosphorylation state of KaiC, and, by consequence, the circadian cycle within the cyanobacterium. In fact, a temperature-compensated rhythm in the phosphorylation state of KaiC can be maintained for over three days *in vitro* when only KaiA, KaiB, KaiC, and ATP are present (89). However, the period (~22 h) of this oscillation in post-translational modifications is less than that of the *in vivo* oscillations of reporter bioluminescence, or mRNA and protein accumulation. Therefore, it is the work of the entire clock system, with its input and output pathways, that helps to coordinate this independent Kai oscillation with the environmental day/night cycle.

***The input pathways: keeping in sync with the environment***

Few components have been identified in *S. elongatus* that link diurnal variations, such as changes in light and temperature, to the circadian clock. These input pathway proteins recognize environmental signals and transmit the temporal cues to the central oscillator proteins, *i.e.*, the Kai complex. In many eukaryotic model systems, photoreceptors have been identified that directly connect the perception of light to the central oscillator to

control the phase of the endogenous rhythm (8, 25, 39). The molecular mechanisms for cyanobacterial synchronization are not as clearly understood.

The *period extender* (*pex*) gene has been tentatively placed in the input pathway to the central oscillator, although no specific role can be assigned. The *pex* gene was originally identified through what was originally thought to be its complementation of a 22-h short period mutant (65). Further analysis showed that this apparent WT rhythm was due to the presence of an ectopic copy of the *pex* gene, which resulted in a 2-h extension of the 22-h period. Overexpression of *pex* by an inducible promoter demonstrated a dose-dependent lengthening of the period of the circadian rhythm by Pex in both WT and circadian period mutant backgrounds. The Pex primary sequence does not provide any hint to its function because no recognizable domains can be assigned. Inactivation of *pex* causes a slightly shorter period, but more noticeably results in the increased expression of the *kaiA* gene (65), which suggests that the normal role of Pex protein is to repress *kaiA* expression, presumably in response to some environmental signal.

Two proteins, circadian input kinase (CikA) and light-dependent period (LdpA), have been shown to affect the ability of *S. elongatus* cells to respond to external stimuli. The CikA protein has been identified as a major component of an input pathway that is involved in nonparametric entrainment of the clock. Nonparametric entrainment resets the phase of the internal oscillation in response to the timing of an external stimulus, such as light, regardless of intensity (19). The *cikA* gene was originally identified in a

reporter strain that displayed slightly altered expression of the *psbAII* gene, which encodes the D1 protein of photosystem II. Additional analyses revealed a more striking phenotype with respect to the circadian clock. Cells that lack *cikA* reset very little (< 2 h) in response to pulses of darkness throughout the circadian cycle as compared to WT cells that can be reset by up to 8 h in response to the same stimulus (105).

Bioinformatic analysis of the CikA sequence provided some insight into its biochemical properties, but the mechanism of its function in the input pathway was not obvious. Although the N-terminal half of the protein contains a GAF domain, which typically binds a bilin chromophore in bacterial photoreceptors, the CikA GAF does not contain the conserved cysteine residue to which the bilin covalently attaches, and attempts to purify CikA with an attached bilin *in vivo* were unsuccessful (84). The central part of the protein consists of a histidine protein kinase (HPK) domain that undergoes autophosphorylation both *in vitro* and *in vivo*. This phosphorylation is essential for normal CikA function, as a histidine to alanine substitution at the phosphorylation site renders the protein inactive (84, 130). The C-terminal end of the protein contains a PsR domain, much like that of the N-terminus of KaiA. Experiments designed to visualize the transfer of a phosphoryl group from the HPK of CikA to its PsR *in vitro* did not show any phosphotransfer event occur (84). CikA localizes to the pole of the cyanobacterial cell and it is thought that this localization poises CikA in the proper position to relay its information to other components within the clock system. The PsR domain is necessary for this polarity of CikA (130).

Unlike the phase-resetting phenotype exhibited by a *cikA* mutant, a strain that lacks *ldpA* is affected in parametric entrainment. This entrainment is achieved by modulating the period of the internal rhythm in response to gradual changes in light intensity and/or quality (19). Like other diurnal organisms, *S. elongatus* follows Aschoff's rule, such that the circadian period increases as the light intensity decreases; however, mutants lacking *ldpA* maintain the shorter period associated with high light regardless of the actual intensity of light (51). Recent work showed that LdpA contains iron-sulfur clusters, which allow LdpA to sense the redox state of the cell. That information is then transduced to the Kai oscillator to acutely adjust period length (45).

Just as the Kai proteins of the central oscillator form a large, multimeric complex during the circadian cycle, the proteins of the input pathway are likely to form complexes, interactive with the oscillator components, that allow the clock to receive environmental information. Using a 6xHis-tagged LdpA variant, both the KaiA and CikA proteins were shown to interact with LdpA *in vivo* (45). The interaction between KaiA and CikA is likely indirect and possibly mediated by LdpA because the co-purification of LdpA and CikA was uninterrupted in a *kaiA* mutant, the interaction between LdpA and KaiA is maintained in a *cikA* mutant background (45). Additionally, neither CikA nor LdpA interacts with the Kai proteins directly in a yeast two-hybrid system (S. R. Mackey, J.-S. Choi, and S. S. Golden, unpublished data).

Proteins that have been identified through their interaction with CikA in a yeast two-hybrid system are critical for adjusting the period or phase of the *S. elongatus* circadian rhythm. This work, which was initiated by post-doctoral scientist Dr. Jong-Soon Choi, is presented in Chapter V and describes the effects of null and overexpression alleles of CikA-interacting proteins. Inactivation of a serine-threonine protein kinase alters the ability of the cyanobacterial clock to reset its phase in response to dark pulses. The overexpression of either an aminotransferase or a conserved hypothetical protein increases the period length of the endogenous rhythm. The effect on the circadian rhythm from overexpressing a second hypothetical protein has not yet been confirmed; however, this protein has been shown to play a role in the regulation of cell division.

The highly-conserved HPK domain and autokinase activity of CikA suggest that CikA is part of a bacterial two-component signal transduction system with a partner response regulator (RR) protein, named CikR. At this time, CikR has not been identified. Two independent projects were conducted to find a RR capable of accepting a phosphoryl group from CikA. Not the place to mention the undergrads. The first approach utilized a genetic screen in *E. coli* that had previously identified another clock-related HPK (85). The second approach took advantage of the finished genome sequence for *S. elongatus* and used bioinformatic analyses to select candidate RR proteins. The ability of CikA to *trans*-phosphorylate these proteins was tested directly *in vitro.* Chapter IV describes the experimental designs used and preliminary results. Although these projects proved unsuccessful in finding CikR, two clock-related proteins were discovered and many

techniques were developed that will provide useful tools for the characterization of other clock-related components in the future.

***The output pathways: regulating cellular processes***

The ability to maintain internal time is only beneficial to the organism if that information can be used to execute cellular processes at the time that provides the greatest benefit. The output pathways transduce the temporal information generated by the central oscillator to regulate metabolic and behavioral activities. One such activity is cell division. The circadian clock persists in cells with doubling times much shorter (as fast as 6 h) than a full circadian cycle (57, 81). DNA synthesis occurs at a constant rate, yet the number of cells in a continuously-diluted liquid culture displays a circadian rhythm in LL that matches the phase of its synchronizing LD cycle (81). In an arrhythmic *kaiC* mutant, the rhythm in cell division is driven by external LD cycles, but does not persist in LL (82). The circadian clock "gates" cytokinesis such that there are times throughout the circadian cycle where division is forbidden; for *S. elongatus*, this forbidden phase occurs in the late subjective day and early to mid-subjective night (81). The cell division cycle does not, however, regulate the clock. Cells maintain WT rhythms in bioluminescence from transcriptional fusion reporters regardless of doubling time (which varies with light availability) and in stationary phase when cells do not undergo cell division (82).

A number of proteins have been implicated in regulating the output from the circadian oscillator. The *Synechococcus* adaptive sensor (SasA) protein plays a critical role in maintaining circadian-controlled output. The circadian role of SasA was not recognized until years after its original identification as an HPK in a screen, performed in *E. coli*, to isolate genes involved in two-component signal transduction (85). The importance of SasA to the circadian timing mechanism was discovered simultaneously by two methods: it was recognized by bioinformatics to carry a domain that resembles KaiB, and found to interact directly with KaiC in a yeast two-hybrid system (48). Immunoprecipitation experiments further showed that either full-length or the N-terminus (residues 1 – 97) of SasA is capable of interaction with itself and with KaiC; either the CI or CII domain of KaiC was sufficient for this binding (48). This N-terminal domain of SasA has significant amino acid sequence similarity to the full-length KaiB protein, although their structures are different (49, 56). However, no interactions between SasA and KaiB or KaiA were detected *in vitro* or *in vivo* (48).

The use of a multitude of cyanobacterial reporter strains has shown that the inactivation of *sasA* decreases the amplitude of circadian gene expression, and rhythms damp to arrhythmia within four days; the clock is still running (albeit with a 2-h shortened period), but the transduction of the temporal information to the genome is blocked (48). Rhythmic accumulation of *kaiA* and *kaiBC* mRNA does not persist in the absence of *sasA*; KaiA protein levels are reduced to about 70% compared to those in WT, while KaiB and KaiC protein accumulation falls to nearly undetectable amounts (48).

Constitutive overexpression of SasA causes arrhythmia as measured by bioluminescence reporters, although short pulses of SasA overexpression can shift the phase of the rhythm with advances in the oscillation when *sasA* levels are decreasing and delays when *sasA* levels are on the rise (48). The direction of the phase shifts that result from short pulses of SasA overexpression differs from those seen when KaiC is overexpressed for short durations. This discrepancy further shows that SasA is important for the functionality of the clock system, but that its function is distinct and separate from that of the Kai complex.

The autophosphorylation activity of SasA is crucial to its function, as an amino acid substitution at the histidyl residue upon which autophosphorylation occurs results in a strain with a phenotype similar to the *sasA* null (48). Information has been shown to flow from the Kai oscillator to SasA: the addition of KaiC enhances the rate at which SasA autophosphorylates *in vitro* by 20 fold, though the reciprocal experiment does not show a SasA-dependent change in KaiC autokinase activity (109). Addition of the other Kai proteins lessens the stimulatory effect of KaiC on SasA (109). Thus, although SasA is not essential for the generation of circadian rhythms, it is in close physical association with the Kai complex and is crucial for the production of robust rhythms of circadian-controlled gene expression.

The pervasiveness of circadian control on gene expression in the *S. elongatus* genome hints at a global regulatory mechanism that exists in the bacteria to link the endogenous

rhythm to regulation of gene expression. One hypothesis for this global regulation is the rhythmic "breathing" of the cyanobacterial nucleoid over the circadian cycle, which would control access of transcriptional machinery to promoter elements. When *S. elongatus* cells are sampled over time and stained with DAPI (4', 6-diamidino-2-phenylindole) dye to visualize the nucleoid, a rhythm in the compaction/decompaction of the chromosome can be observed (109). This rhythm occurs on a 24-h time scale in 12:12 LD cycles, and persists with a period very near 24 h when cells are transferred to LL. The chromosome slowly condenses during the light (or subjective day) with full compaction occurring just before the anticipated light to dark transfer, and decompaction proceeds during the (subjective) night (109). The structure of KaiC resembles the hexameric proteins of the RecA/DnaB family, recombinase and helicase proteins that can bind DNA, and KaiC can interact with forked, double-stranded DNA molecules (83), which suggests its role in the changes in chromosome structure. In a *kaiC* null background, the rhythms in chromosome condensation are lost; however, the rhythm is not dependent solely upon the presence of KaiC, as the inactivation of *kaiA* produces a phenotype indistinguishable from the *kaiC* mutant (109). An inactivation of *sasA* still allows the chromosome to condense in a rhythmic fashion, but without SasA present the transfer of information is lost and the result is arrhythmic gene expression (109).

Other work has implicated the basic transcriptional machinery in contributing to circadian output pathways. In bacteria the RNA polymerase holoenzyme can include different sigma factors, the subunits that recognize promoter elements (36). The group 2

sigma factors in cyanobacteria are a family of proteins that have sequence similarity to the housekeeping sigma-70 RpoD1 protein, but they are not essential for viability of the cells (113). Inactivation of any of the group 2 sigma factor genes (*rpoD2, rpoD3 rpoD4,* or, *sigC*) either alone or in pairs alters the rhythm from a P*psbAI::luxAB* reporter by affecting the phase, period, or amplitude of expression (86, 119). The inactivation of *sigC* lengthens the period of expression from the *psbAI* promoter but has little effect on the expression patterns from either the *kaiBC* or *purF* promoters. The *kaiBC* promoter is only slightly perturbed as a result of the *rpoD2* single mutant, or the *rpoD3/rpoD4* and *rpoD2/rpoD3* double mutants, which suggests that there is greater buffering of that promoter compared to the others. The current model proposes that RNA polymerase forms holoenzymes with different group 2 sigma factors throughout the circadian cycle. Durations of activity for some sigma factors likely overlap; redundancy in their roles is indicated by discrepancies in their phenotypes. The connection between SasA and the regulation of the group 2 sigma factors is still unclear.

The *circadian phase modifier* (*cpmA*) gene has also been implicated as a component in output from the *S. elongatus* clock. Inactivation of the *cpmA* gene causes a severe change in the relative phase angle of a small subset of cyanobacterial reporters (52). In a *cpmA* background, rhythms from the *kaiBC* promoter are unaffected but the phase of expression from a P*kaiA::luxAB* reporter is shifted by 10 h, such that its expression peaks almost in anti-phase to that of *kaiBC* (52). Despite this discrepancy in timing from the *kai* promoters, robust rhythms of gene expression continue with WT period lengths.

This fact suggests that *cpmA* is not part of the oscillator, or a general output pathway, but rather specifically relays temporal information to a subset of genes.

Because *S. elongatus* relies on the sun as its sole energy source, the regulation of genes that produce proteins for the photosystem complexes is critical for the cells' survival. The three genes that encode the D1 protein of photosystem II (PSII) are known to be controlled at the transcriptional, post-transcriptional, translational, and post-translational levels (61, 63, 64, 67, 87, 102, 103, 108, 112, 115, 121). The *psbAI* gene encodes Form I of the D1 protein, and both the *psbAII* and *psbAIII* genes encode Form II. During acclimated growth conditions, Form I dominates the D1 pool within the thylakoid membrane. Under stress conditions, including high light or low temperature, the *psbAII* and *psbAIII* genes are induced and *psbAI* expression is down-regulated making Form II more abundant. The transcription of the *psbA* gene family is also controlled by the endogenous circadian rhythm of *S. elongatus*, as is illustrated in Chapter IV. During free-running conditions, expression from a *psbAII* reporter peaks 12 h out of phase with *psbAI* and *psbAIII*. When the *psbAI* gene is rendered inactive, the *psbAII* reporter adopts the phase of the rhythm of WT *psbAI* and *psbAIII* gene expression, which suggests a possible compensatory mechanism for the loss of *psbAI*.

***The periodosome***

The model that has emerged from the many years of work on the cyanobacterial clock system is the approximate 24-h assembly and disassembly of a large, heteromultimeric

complex, which influences the global expression of the *S. elongatus* genome. Size exclusion gel filtration chromatography and subsequent immunoblot experiments show that the members of the "periodosome" (KaiA, KaiB, KaiC, and SasA) can be co-purified in fractions of 400 – 600 kDa and are believed to be in physical association during the night (50). The abundance of the individual components, as well as the size of the complexes in which they are found, peaks at different times during a circadian cycle. These four proteins are the only four components that have been shown to make up this periodosome because analysis methods used antisera specifically against those proteins (50); it would be naive, however, to assume there are no other proteins in this complex that help to sustain rhythmicity in the cyanobacterial cell.

A pictorial representation of the cyanobacterial clock model is shown in Figure 1.4. Environmental information, such as light and temperature, is interpreted through the input pathway components CikA and LdpA. CikA likely propagates its signal through its predicted cognate RR, CikR. The input most likely leads to the N-terminal PsR domain of KaiA, which influences the C-terminal domain of KaiA to interact with KaiC. This interaction between KaiA and KaiC further stimulates the autokinase activity of KaiC and the formation of its hexameric structure. In the early night, SasA joins the KaiA/KaiC complex; KaiC stimulates the autophosphorylation of SasA, which then transfers its phosphoryl group to its predicted RR, SasR. SasR, along with other proteins in the output pathways, transduces the temporal information from the Kai oscillator throughout the cell. During the late day and early night, the KaiA and KaiC proteins,

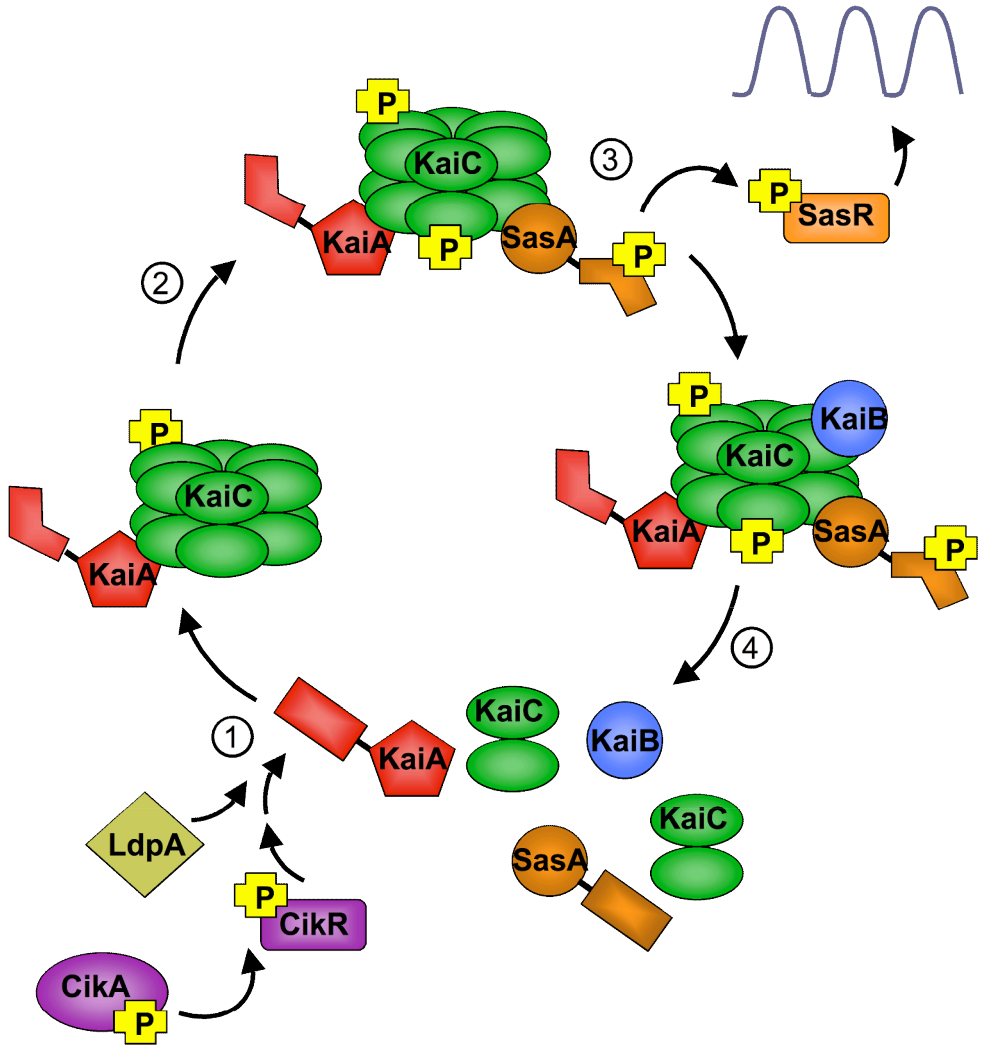


FIG. 1.4. A model for the cyanobacterial clock. (1) Environmental information is perceived by LdpA and CikA, which is predicted to pass its phosphoryl group to its unidentified CikR partner. The environmental cues are likely transmitted to the *pseudo*-receiver of KaiA. The interaction between KaiA and KaiC stimulates KaiC autokinase activity and the formation of a hexameric KaiC structure. (2) SasA joins the KaiA/KaiC complex where KaiC stimulates the autophosphorylation of SasA, and the phosphoryl group is predicted to be transferred from SasA to its response regulator SasR. (3) The Kai complex aids in the chromosome compaction, which with SasA/SasR regulates global rhythmic transcription of the *S. elongatus* genome. (4) The addition of KaiB to the complex abrogates the positive effect of KaiA on KaiC, which decreases the phosphorylation level of KaiC. By the morning, the complex has disassembled into its individual parts. Figure adapted, with permission, from S. S. Golden and S. R. Canales, Nat. Rev. Microbiol. **1:**191-199, 2003.

most likely in conjunction with other DNA-binding proteins, influence the degree of chromosomal condensation of the nucleoid, which modulates the level of gene transcription. In the late night, KaiB interacts with the KaiA/KaiC complex to abrogate the positive effect of KaiA on KaiC phosphorylation. By the morning, the complex has disassembled into its individual components, which may or may not maintain their homotypic interactions.

As with all of the model systems used to study circadian rhythms, the more that is learned about the cyanobacterial circadian clock system, the more complex and intricate it is found to be. The model described above paints a fairly complete picture of the means used to maintain timing in the oscillator and the way that information funnels into and out of that oscillator; yet, there are still gaps that need to be filled. The work presented in this dissertation has contributed greatly to the three major divisions of the cyanobacterial clock. This research has helped to reveal proteins involved in the input pathway of the clock system, describe clock-controlled regulation of genes involved in photosynthesis, and modify the paradigm associated with the autoregulation of components of a core circadian oscillator.

# CHAPTER II

# DETECTION OF RHYTHMIC BIOLUMINESCENCE FROM LUCIFERASE REPORTERS IN CYANOBACTERIA*

## *Introduction*

Cyanobacteria are among the growing group of organisms that have been shown to exhibit 24-h rhythms under the control of a central biological clock and *S. elongatus* PCC 7942 is the model organism for studying prokaryotic circadian rhythms. This unicellular photoautotroph does not display an overt circadian rhythm of behavior that can be easily monitored; however, it exhibits circadian control of expression of its genome (72). The organism has been genetically engineered to produce light using genes that encode a luciferase enzyme, as a readily measurable reporter of circadian gene expression. Luciferase is ideal for this purpose because it does not affect cellular growth and its short half-life accurately reflects real-time expression from promoter fusions because its protein product does not accumulate and obscure troughs of expression (58, 69). In addition, luciferase reporting provides an opportunity to measure the circadian rhythm of *S. elongatus* gene expression in a high-throughput and automated manner. We are able to utilize luciferases from both *V. harveyi* (58) and the firefly (105) to study circadian rhythmicity through mutant hunts and specific gene inactivations. This chapter describes the luciferase vectors and monitoring devices that are used to identify and characterize elements of the cyanobacterial circadian system, as well as the

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*Part of this chapter is reprinted with permission from *Methods in Molecular Biology*, by S. R. Mackey, J. L. Ditty, E. M. Clerico, and S. S. Golden, 2006, Humana Press, Totowa, NJ., in press.

transformation protocol used to introduce those vectors into the *S. elongatus* chromosome, and is intended to serve as a reference for future members of the Dr. Susan Golden laboratory.

### ***Description of* luxAB, luxCDE, *and* luc *vectors***

We have constructed a number of vectors that contain the promoterless *V. harveyi luxAB* genes, which encode the luciferase enzyme, and an antibiotic-resistance marker, flanked by the sequences from one of two identified "neutral sites" of the cyanobacterial genome. These neutral sites are regions of the *S. elongatus* chromosome that can be disrupted without any discernable phenotype (2) (neutral site I, NS1, GenBank accession number U30252; neutral site II, NS2, GenBank accession number U44761). Vectors for NS2 target two adjacent regions of the chromosome (NS2.1 and NS2.2) based on the location of the antibiotic-resistance cassette and cloning site for insertions within neutral site DNA on the plasmid. Because these vectors can replicate in *E. coli* but not in *S. elongatus*, a homologous recombination event (an apparent double cross-over in which the *trans*-gene and selective marker are inserted into the neutral site and the vector sequence is lost) must occur between the neutral site vector and the chromosome in order for genes to be expressed in the cyanobacterium (27). A number of unique and compatible restriction sites are available in each vector for ease of cloning *luxAB* transcriptional promoter fusions or translational fusions by standard recombinant DNA methods (100). Upstream of the multiple cloning site is a transcriptional terminator to

eliminate expression of *luxAB* from transcriptional elements other than the promoter being tested.

The product of a *luxAB* transcriptional or translational fusion is bioluminescent in the presence of its long-chain aldehyde substrate. A commercially available 10-carbon aldehyde (decanal) can be used to provide exogenous substrate to *S. elongatus luxAB* reporter strains for bioluminescence assays. It is difficult, however, to add the exogenous decanal to the high-throughput reporter system because the aldehyde is extremely volatile and, therefore, must be administered in the vapor phase. This vapor affects the ability of the clear, plastic Packard Topseal (Perkin-Elmer Life Sciences) to adhere to the 96-well microtiter plate and, as a result, the plate becomes stuck inside the machine. To avoid this ill-fated result, other neutral site vectors have been designed to express the *luxCDE* genes from *Photorhabdus luminescens* (ATCC 29999), which encode the enzymes required for synthesis of the long-chain aldehyde. We typically use the strong promoter of the *psbAI* gene to maintain a high level of expression from the *luxCDE* genes. Strains that express both *luxAB* and *luxCDE* are autonomously bioluminescent. The rhythm of light production reflects the transcriptional patterns of the promoter driving *luxAB*, even when levels of *luxAB* peak 12 h out of phase with the peak level of *luxCDE* expression, which suggests that the necessary reaction substrates for the luciferase reaction ($FMNH_2$, $O_2$, and long-chain aldehyde) are present in saturating levels throughout the circadian cycle (69).

In most of the autonomously-bioluminescent strains, both NS1 and NS2 are occupied by the *luxAB* and *luxCDE* constructs, respectively, making it difficult to introduce other *trans*-genes into the chromosome. We have developed two methods that leave one neutral site unoccupied for complementing a mutation or overexpressing a gene. One method introduces both P*psbAI::luxAB* and P*psbAI::luxCDE* into NS2.1, leaving NS1 (and NS2.2) available for genetic manipulation. We have also developed the use of an engineered allele of firefly luciferase, *luc*. Here, as in the *luxAB* system, a promoter of interest can be cloned upstream of the promoterless *luc* gene (Promega), sub-cloned to a neutral site vector, and crossed to the chromosome of the cyanobacterium. The resulting strain will produce light in the presence of exogenous 5 m*M* D-luciferin substrate, and has one neutral site available for introduction of other genes.

***Transformation, chromosomal segregation, and clonal propagation***

The following section describes the conditions typically used to transform *S. elongatus* with the bioluminescence vectors and the propagation of positive clones (adapted from Golden *et al.* [29]).

*S. elongatus* PCC 7942 is grown at 30ºC in 300 ml Erlenmeyer flasks that contain 100 ml modified BG-11 (BG-11M) liquid medium (6) [1.5 g/L $NaNO_3$, 0.039 g/L $K_2HPO_4$, 0.075 $MgSO_4 \bullet 7H_2O$, 0.02 g/L $Na_2CO_3$, 0.027 g/L $CaCl_2$, 0.001 g/L EDTA, 0.012 g/L $FeNH_4$ citrate, and 1 ml of the following microelement solution: 2.86 g/L $H_3BO_3$, 1.81 g/L $MnCl_2 \bullet 4H_2O$, 0.222 g/L $ZnSO_4 \bullet 7H_2O$, 0.391 g/L $Na_2MoO_4$, 0.079 g/L

$CuSO_4 \bullet 5H_2O$, and 0.0494 g/L $Co(NO_3)_2 \bullet 6H_2O$], shaking at 250 rpm with constant light of about 300 μE $m^{-2}$ $s^{-1}$ (1 Einstein = 1 mole of photons). Cells will reach an optical density at 750 nm ($OD_{750}$) of 0.7 in 4 – 7 days depending upon the inoculum used (typically 5 – 10 ml of a fully-grown culture into fresh medium). Cells used for transformation are typically used in log or early stationary phase. Cells are collected from 15 ml culture by centrifugation at 6000 × *g* for 10 min. The medium is discarded and the collected cell resuspended in 10 ml of 10 m*M* NaCl. Centrifugation is repeated at 6000 × *g* for 10 min. The pellet is resuspended in 0.3 ml of BG-11M liquid medium. Cells are concentrated to between $5x10^8$ to $1x10^9$ cells/ml because the transformation efficiency (per input DNA) increases linearly with increasing cell concentration (29). Plasmid DNA (50 ng to 2 μg) is added to concentrated cells. The efficiency of transformation depends on the properties of the donor DNA, including the conformation of the DNA (linear *vs.* circular). The efficiency of chromosomal recombination depends on the extent (number of uninterrupted base pairs) of identity between the *trans*-gene (or neutral site arms in the vector) and the chromosome, and the distance between the selectable marker and the end of the donor DNA fragment (31, 33). The tubes are then wrapped in aluminum foil to keep out light and incubated at 30ºC for 15 – 20 h with gentle agitation. The entire 0.3-ml cell suspension is plated on BG-11M agar that contains the appropriate selective antibiotic. BG-11M solid medium (29) contains equal volumes of twice-concentrated (2×) BG-11M liquid medium and Difco agar solution (3% in sterile water) that have been autoclaved individually and then mixed together with $Na_2S_2O_3$ added last (final concentration of 1 m*M*). The concentrations of antibiotics

used are as follows: 2 μg/ml gentamycin (Gm), 5 μg/ml kanamycin (Km), 2 μg/ml spectinomycin (Sp) + 2 μg/ml streptomycin (Sm), and 7.5 μg/ml chloramphenicol (Cm). The concentrations of individual antibiotics may need to be reduced when two or more are used in combination to select for multiple recombination events to the cyanobacterial chromosome. The usable ranges of antibiotics for transformed cells that carry resistance cassettes are: 5 – 20 μg/ml Km, 1 – 2 μg/ml Gm, 7.5 – 10 μg/ml Cm and 5 – 20 μg/ml Sp. To avoid spontaneous resistance to Sp, we use an equal amount of Sp and Sm, *e.g.* 2 μg/ml Sp + 2 μg/ml Sm; the omega cassette confers resistance to both (99). After 7 – 10 days of incubation under standard light conditions, transformed colonies will appear.

Single transformants are picked with a sterile toothpick and patched onto a fresh BG-11M agar plate that contains the selective antibiotic(s) to ensure that all chromosomes have incorporated the *trans*-gene. *S. elongatus* harbors 6 – 8 copies of its genome (81) and it is possible to obtain transformants in which the antibiotic-resistance cassette disrupted enough copies of the gene to allow the organism to live on selective medium even though the organism still harbors sufficient WT copies of the gene to carry out its function. Many single colonies can be streaked onto one plate if patches are kept to areas less than 1 $cm^2$. After 5 – 7 days of growth, the cyanobacteria can be used to inoculate a BG-11M liquid culture that contains the selective antibiotic(s), or used directly for the measurement of bioluminescence.

### *TopCount measurement*

The Packard TopCount Microplate Scintillation and Luminescence Counter (Perkin-Elmer Life Sciences) utilizes single photomultiplier tubes to measure the light produced from recombinant *luxAB* or *luc* reporter cyanobacteria in counts per second (cps). The advantage of using the TopCount to measure circadian bioluminescence is multi-fold: one can screen hundreds of strains at a time, and the automated counting protocol allows for rhythms to be monitored 24 h a day, for weeks at a time, with little attention from an otherwise sleep-deprived worker.

### Plate preparation

Black 96-well microtiter plates (ThermoLabsystems) and clear plastic lids are sterilized with 70% ethanol (EtOH), which can be evaporated in a laminar flow hood under UV light for at least 30 min before use. 50 ml of BG-11M-2× agar and 50 ml of BG-11M-2× salts are each placed in a 65°C water bath to bring to temperature. Antibiotics are added directly to the salts and $Na_2S_2O_3$ (final concentration 1 mM) is added directly to the melted agar. The agar and salts are mixed together in a laminar flow hood, and a multi-channel micropipette can be used to add 300 μl of agar medium to each well of a 96-well black plate. The plate is covered with the lid and allowed to solidify at room temperature for at least 30 min.

**Sample preparation**

Each well is inoculated with test strains. If using liquid culture, 20 – 40 μl of cell suspension (recommended $OD_{750}$ = 0.7) is administered to desired wells. Alternatively, cultures growing on solid medium may be streaked onto the agar pad of each well using a sterile toothpick. If using *luc* strains, inoculation of TopCount sample plates with liquid cultures provides superior traces to those inoculated from cultures grown on a solid medium. A flat toothpick may be placed on either side of the black plate immediately outside the outer wells to prevent the lid from directly contacting the samples. Sample plates can be incubated in constant light at 30°C overnight.

To synchronize the cells' clocks, a 12-h dark treatment is typically used. After dark treatment, in a laminar flow hood, the clear lid is replaced with a plastic Packard Topseal (Perkin-Elmer Life Sciences). When using *luc* reporter strains, 10 μl of a 5 m*M* D-luciferin solution must be added to each well before applying the Topseal. To allow gas exchange throughout the TopCount run, a 16-gauge sterile needle is used to poke a small hole in the plastic seal above each well that contains a sample, being careful not to touch the samples with the needle.

**TopCount protocol and interpretation of results**

*S. elongatus* is an obligate photoautotroph, for that reason LL conditions are used for circadian monitoring. A frame that surrounds the TopCount stackers was designed by D. Denke in the Department of Biology Instrumentation Shop to provide light to the

samples. We use a light source consisting of eight 40-W compact fluorescent bulbs (four bulbs on each side) that create a light intensity of about 1300 μE $m^{-2}$ $s^{-1}$ at the outer edge of the stacker and about 1000 μE $m^{-2}$ $s^{-1}$ in the middle of the stacker, which maintains a light gradient within the wells of the sample plate that ranges from about 230 μE $m^{-2}$ $s^{-1}$ in the outer wells that are closest to the light source to about 50 μE $m^{-2}$ $s^{-1}$ in the inner wells (86). Because the high intensity lamps cause an increase in temperature, we place a fan in front of the stackers, set at its lowest speed, to maintain a temperature of 30°C across the stackers (Fig. 2.1). The temperature within the measuring chamber is controlled automatically.

Black sample plates are necessary so that luminescence from neighboring wells does not interfere with measurements. We place 6 – 8 sample plates in the TopCount stackers and separate each black sample plate with three clear 96-well plates to allow sufficient light to reach the cells **(**Fig. 2.1**)**. Each plate is read every 1.5 – 2 h depending on the number of plates used. The plates enter the machine and are kept in darkness for 3 min at 30°C to allow fluorescence from the photosynthetic apparatus to dissipate before measuring bioluminescence. It takes approximately 10 min for the TopCount to count and record bioluminescence from each 96-well plate, thus placing the cells in the dark for a total of 13 min every 2 h, if using 8 sample plates. This brief introduction to darkness does not have an effect on the rhythms of gene expression (as far as synchronization, entrainment, or resetting of the cultures) as displayed by the bioluminescent reporters. Very clear

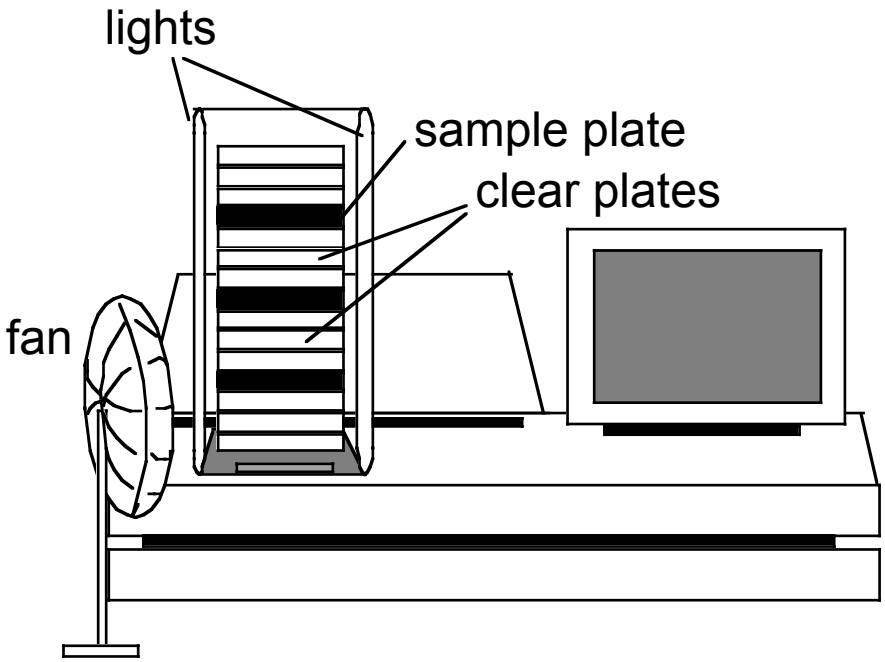


FIG. 2.1. Schematic representation of the Packard (Perkin-Elmer) TopCount Microplate Scintillation and Luminescence Counter**.** A set of four compact fluorescent light bulbs lines each side of the TopCount stackers, which hold black sample plates separated by three clear plates to allow sufficient light penetration of the sample wells. To reduce the heat supplied by the lights, a fan is placed in front of the stacker to maintain a temperature of 30ºC.

rhythms have been recorded from both *lux* and *luc* reporters by the TopCount for over two weeks with no detectable change in rhythm characteristics (30).

Measurements recorded by the TopCount can be downloaded and interpreted using the Import & Analysis (I&A) program (98) designed by the S. A. Kay laboratory (available at http://www.scripps.edu/cb/kay/shareware/). The I&A program creates a Microsoft Excel worksheet that displays the bioluminescence cps for each timepoint collected for each sample in the 96-well plate. From this worksheet, the bioluminescence emitted from the culture in each microtiter well can be graphed. Each graph displays circadian properties that can be analyzed to determine if a particular mutation or environmental cue has caused an alteration of the rhythm (Fig. 2.2). The period of the circadian rhythm is defined as the amount of time between two adjacent peaks or troughs; the WT period for *S. elongatus* is between 23.5 h and 25 h depending on light intensity. This difference in periodicity occurs because the cells obey Aschoff's Rule, a phenomenon of the circadian clock wherein the period of the circadian rhythm of diurnal organisms shortens with increasing light intensity (4, 51). Following Aschoff's Rule, the cyanobacterial cultures in the outer wells of the 96-well plate, that are closer to the light source, show consistently shorter periods than cultures in the inner wells (Fig. 2.3). Because light intensity has a minor effect on circadian period, and because absolute expression levels (but not circadian parameters) vary with cell number (18, 51), all mutant and control analyses should be calculated from samples carefully paired on the monitoring device for equivalent illumination from samples grown in parallel under identical conditions.

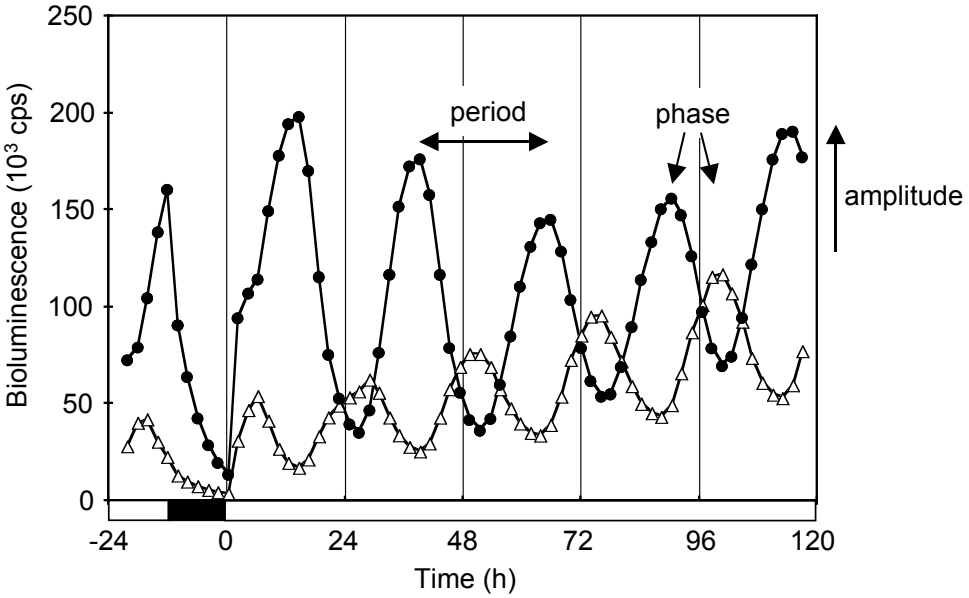


FIG. 2.2. Characterisitcs of the *S. elongatus* circadian rhythm. Negative time represents time during LD cycles to synchronize the cells' clocks. The period length (time between the occurrence of peaks or troughs) for the P*kaiBC::luxAB* (closed circles) and P*purF::luxAB* (open triangles) are nearly identical, but their phase of peak expression with respect to time entering LL are 12 h apart. P*kaiBC::luxAB* peaks at subjective dusk, while P*purF::luxAB* peaks at subjective dawn in constant conditions. Another measured property of circadian curves is the amplitude, which measures the expression level from the midline of the curve to either the trough or peak.

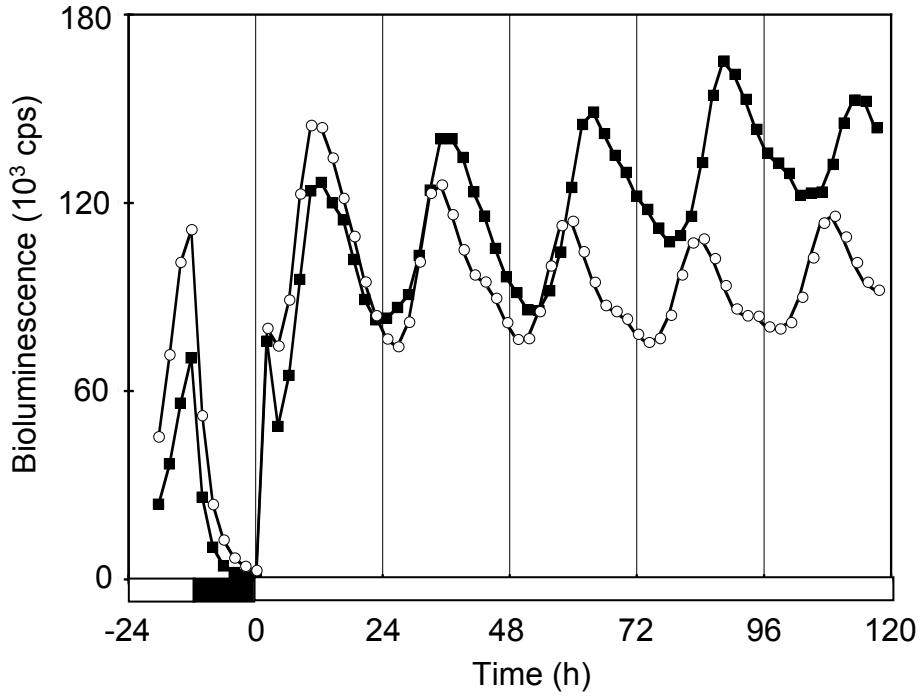


FIG. 2.3. *S. elongatus* circadian behavior displays Aschoff's Rule. Bioluminescence traces from a P*psbAI::luxAB* reporter strain exhibits a shorter circadian period at high light conditions (open circles; FRP = 24.07 h) than at low light (closed squares; FRP = 25.46 h).

Another noticeable element of the rhythm is relative phase, which is the positioning of the rhythm peak with respect to a reference point, such as when the culture was placed into constant light. A third characteristic is amplitude, the distance from the mid-line of the curve to either the peak or the trough of the rhythm. This property, though important, is the most variable of the three even among WT samples. These characteristics can be analyzed using the I&A computational interface FFT-NLLS, which provides statistical period, phase, and amplitude information.

# CHAPTER III

# STABILITY OF THE CIRCADIAN CLOCK UNDER DIRECTED ANTI-PHASE EXPRESSION OF THE *kai* GENES*

## *Introduction*

In *S. elongatus*, the circadian pacemaker is comprised of the products of at least three genes, *kaiA*, *kaiB*, and *kaiC*. The *kai* locus is expressed from two promoters ── one upstream of *kaiA* (P*kaiA*, monocistronic message) and one upstream of *kaiB* (P*kaiBC*, dicistronic *kaiBC* message) ── which drive transcription in the same circadian phase in WT cells, with peak expression at the time that corresponds to dusk when cells are kept in LL (subjective dusk) (43). Central to models for the mechanism of eukaryotic circadian clocks are delayed transcriptional-translational feedback loops that regulate timing of clock gene expression. These feedback loops involve positive effector proteins that stimulate core clock gene expression. The core clock components then negatively regulate their own expression, to generate an oscillation of gene expression and clock protein production that results in a 24-h regulatory mechanism (19, 37, 129).

Some aspects of the regulation of clock genes in *S. elongatus* are reminiscent of the autoregulatory feedback models. Previous work has shown that KaiA is required for expression from P*kaiBC*, and that overexpression of *kaiA* enhances expression from P*kaiBC*, suggesting a role in positive activation (43). KaiC is required for normal levels

*Reprinted with permission from "Stability of the *Synechococcus elongatus* PCC 7942 circadian clock under directed anti-phase expression of the *kai* genes" by J. L. Ditty, S. R. Canales, B. E. Anderson, S. B. Williams, and S. S. Golden, 2005. Microbiology **151:**2605-13.

of expression from its own promoter; however, overexpression of *kaiC* blocks expression from P*kaiBC*, suggesting a role in negative autoregulation (43). Hence, these data were interpreted to be consistent with eukaryotic circadian timing models. More recent data have shown the mechanism to be more complex (46, 117, 126).

There has been increasing evidence that the feedback loops that are central to eukaryotic circadian clock models are not necessary for rhythms to persist in the cyanobacterial clock. Expression of *kaiC* from an *E. coli* consensus promoter was shown to complement a *kaiC* null strain, which demonstrates that *cis* elements in the *kaiBC* promoter sequence are not specifically required to maintain rhythmicity in the cells (126). Some mutants of *S. elongatus* affect the phase relationship between P*kaiA* and P*kaiBC* expression, but do not disrupt the self-sustained rhythmicity of the circadian clock itself. For example, the *cpmA* mutation results in expression of P*kaiA* 10 h out of phase from P*kaiBC* (52). Similarly, null mutations in any of the known group 2 sigma factors, which are important for promoter recognition and subsequent transcription in *S. elongatus,* also show differential effects on circadian expression (86, 119). In particular, *sigC* inactivation lengthens the period of expression from P*kaiA*, but not from P*kaiBC*, by 2 h; thus, the peak expression from P*kaiA* with respect to P*kaiBC* falls out of phase by up to 8 h (86). This finding suggests that the relative timing of transcriptional activity from at least the *kaiA* promoter is not important for generating circadian rhythms.

To test directly whether the coordinated timing of transcription of the *kai* promoters is

important for circadian timekeeping in *S. elongatus*, the native transcriptional regulation of *kai* genes was bypassed to force expression in an unusual phase. Expressing the *kaiA* gene or *kaiBC* dicistron from the *purF* promoter (78), such that expression was delayed by 12 h with respect to WT expression timing, forced a peak at subjective dawn (class 2) rather than the normal subjective dusk (class 1). This work shows that individual *kai* loci, expressed independently from a class 2 promoter, function normally in the core circadian timing mechanism and support downstream circadian rhythms in *S. elongatus*. Therefore, coincident transcriptional patterns of expression of the *kai* genes are not necessary for precise and persistent circadian rhythms in the cyanobacterium.

## *Methods*

### Bacterial strains and plasmids

The strains and plasmids used in this study are described in Table 3.1. Cyanobacterial reporter strains were created in *S. elongatus* PCC 7942. All luciferase (*luc*) reporter fusions are integrated at NS2 and recombinant complementation constructs at NS1 of the *S. elongatus* chromosome. These neutral sites are regions of the *S. elongatus* chromosome that can be disrupted without any discernable circadian or growth phenotype (2).

### Media and growth conditions

All cyanobacterial strains were grown and maintained as described in Chapter II. Upon reaching an $OD_{750}$ of 0.2, cultures were synchronized by placement in a cycle of 12:12

TABLE 3.1. Bacterial strains and plasmids used to examine *kai* regulation

| ***S. elongatus* strain** | **Genetic background** | **Ectopic *kai* plasmid*** | ***kai* plasmid characteristics** | **Reporter plasmid†** | **Reporter characteristics‡** | **Source or reference** |
|---|---|---|---|---|---|---|
| AMC541 | WT | none | none | AM2105 | P*kaiBC::luc*; $Cm^R$ | 18 |
| AMC601 | WT | none | none | AM2016 | P*purF::luc*; $Km^R$ | 105 |
| AMC603 | WT | none | none | AM2019 | P*psbAI::luc*; $Km^R$ | Lab collection |
| AMC702 | *kaiA* deletion | none | none | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC703 | *kaiB* deletion | none | none | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC704 | *kaiC* deletion | none | none | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC705 | *kaiBC* deletion | none | none | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1099 | WT | AM2246 | P*kaiA::kaiA* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1101 | WT | AM2482 | P*purF::kaiA* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1161 | *kaiA* insertion | none | none | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1233 | *kaiA* insertion | AM2246 | P*kaiA::kaiA* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1234 | *kaiA* insertion | AM2482 | P*purF::kaiA* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1271 | WT | AM2245 | P*kaiBC::kaiB* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1272 | *kaiB* deletion | AM2245 | P*kaiBC::kaiB* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1273 | WT | AM2302 | P*kaiBC::kaiC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1274 | *kaiC* deletion | AM2302 | P*kaiBC::kaiC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1276 | WT | AM3109 | P*kaiBC::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1277 | WT | AM3139 | P*purF::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1278 | *kaiB* deletion | AM3109 | P*kaiBC::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1279 | *kaiB* deletion | AM3139 | P*purF::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1280 | *kaiC* deletion | AM3109 | P*kaiBC::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1281 | *kaiC* deletion | AM3139 | P*purF::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1282 | *kaiBC* deletion | AM3109 | P*kaiBC::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| AMC1283 | *kaiBC* deletion | AM3139 | P*purF::kaiBC* | AM2105 | P*kaiBC::luc*; $Cm^R$ | This study |
| STC100 | *kaiBC* deletion | AM3139 | P*purF::kaiBC* | AM2016 | P*purF::luc*; $Km^R$ | This study |
| STC101 | *kaiBC* deletion | AM3139 | P*purF::kaiBC* | AM2019 | P*psbAI::luc*; $Km^R$ | This study |

TABLE 3.1. *continued*

| Plasmid | Characteristics | Antibiotic resistance‡ | Source or reference |
|---|---|---|---|
| pHP45Ω-Km | source of $Km^R$ Ω-cassette | Ap; Km | 24 |
| pRL278 | *sacB* vector for hit and run allele exchange | Km | 2 |
| pUCBM20 | *E. coli* cloning vector | Ap | Boehringer Mannheim |
| pAM1303 | NS1 cloning vector | Sp/Sm | 2 |
| pAM1725 | source of *kai* DNA | Ap | Lab collection |
| pAM1978 | *kaiA* in-frame deletion plasmid | Km | This study |
| pAM1979 | *kaiB* in-frame deletion plasmid | Km | This study |
| pAM1980 | *kaiC* in-frame deletion plasmid | Km | This study |
| pAM2314 | pAM1303 + additional engineered restriction sites | Sp/Sm | Lab collection |
| pAM2380 | P*purF* in pUCBM20 | Ap | This study |
| pAM2383 | P*purF::kaiA* in pUCBM20 | Ap | This study |
| pAM2969 | $Km^R$-Ω cassette in the *Bam*HI site of *kaiA* in pAM1725 | Ap; Km | This study |
| pAM3110 | Gateway neutral site I cloning vector | Sp/Sm | This study |
| pAM3135 | P*purF::kaiBC* in pUCBM20 | Ap | This study |

* All ectopic *kai* alleles are integrated at neutral site I (NS1), following homologous recombination with the indicated plasmid, which does not replicate or persist in the cyanobacterial cells.
† All reporter constructs are integrated at NS2, following homologous recombination with the indicated plasmid, which does not replicate or persist in the cyanobacterial cells.
‡ Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp/Sm, spectinomycin/streptomycin.

LD for two days and then returned to LL conditions for sampling. Cultures of at least an $OD_{750}$ of 0.2 were used for measurement of bioluminescence via the Packard TopCount luminometer (Perkin-Elmer Life Sciences), as previously described in Chapter II.

**DNA manipulations and sequencing**

Plasmid DNA to be used for sequencing and subcloning was prepared by the QIAprep miniprep kit (QIAGEN). Sequencing was performed with BigDye terminator mix (Applied Biosystems) per manufacturer's instructions. PCR primers were generated, and sequencing reactions were run, at the Gene Technology Laboratory (Institute of Developmental and Molecular Biology, Texas A&M University, College Station, TX). Restriction endonuclease digestions were performed according to manufacturer's instructions (New England Biolabs). DNA fragments for subcloning were purified from agarose gel slices by CONCERT Rapid Gel Extraction System (Invitrogen).

**Construction of *kai* mutant strains**

An in-frame deletion of each *kai* gene was generated by removing codons for 254, 79, and 409 amino acids of *kaiA*, *kaiB*, and *kaiC* products, respectively (see Fig. 3.1 for further details of the deletions). Each in-frame deletion construct was cloned into pRL278, a *sacB* hit-and-run negative selection vector (2), used to transform AMC541 (29), and crossed into the chromosome via homologous recombination to replace each WT *kai* gene with the deleted allele (27). Chromosomal DNA from each deletion strain was prepared (29) and regions surrounding each deletion were amplified by PCR.

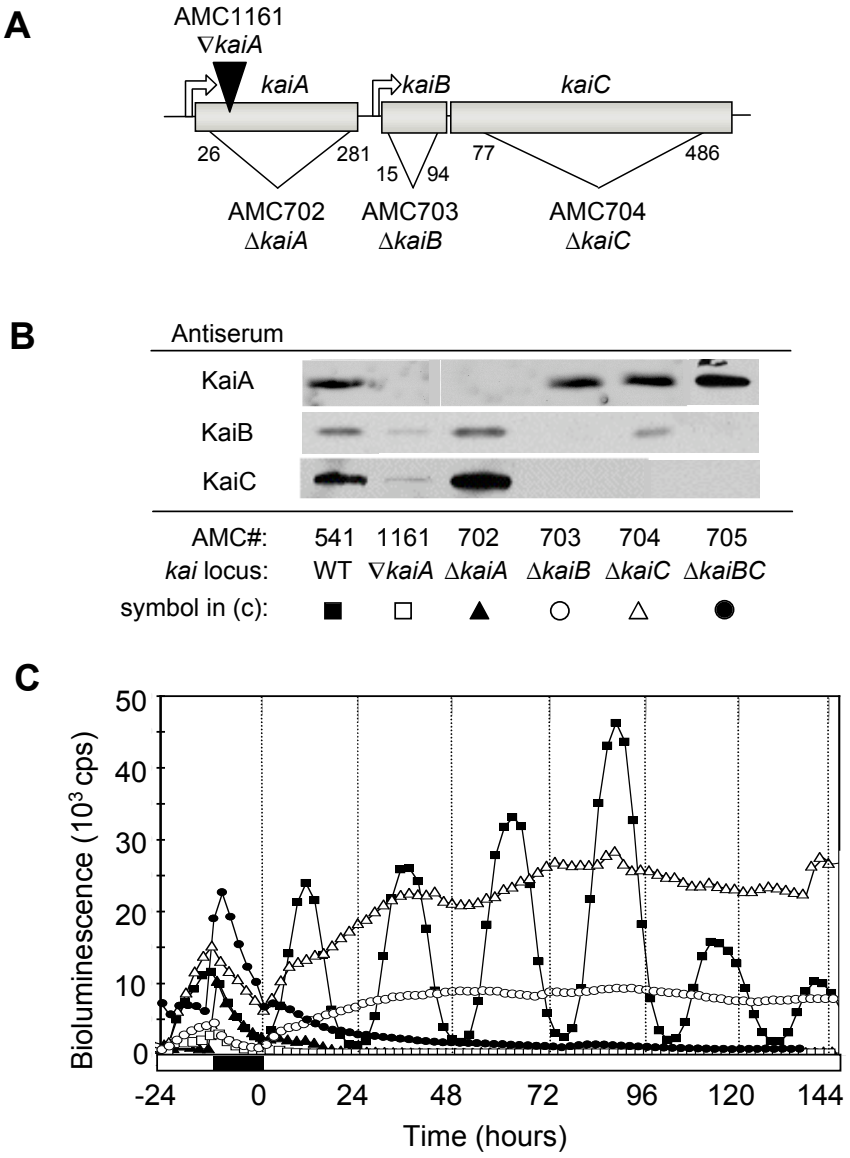


FIG. 3.1. Construction and characterization of *kai* mutant strains. (A) Representation of the *kai* locus. The numbers under each gene represent the amino acid residues for which codons were removed. The inverted triangle represents the $Km^R$ Ω-cassette that was inserted in *kaiA*. (B) Immunoblot of 20 µg total soluble protein showing relative protein levels and that the Kai proteins are not made in the respective null strain. (C) Bioluminescence in counts per second (cps) from a P*kaiBC::luc* reporter are arrhythmic in each *kai* null background. On the X-axis, negative time denotes time during LD cycles to synchronize the cells' clocks.

Amplification products were de-salted using CONCERT Rapid PCR Purification System (Invitrogen) and sequenced to verify the in-frame deletion of each *kai* gene.

Insertion of a Km-resistance Ω-cassette from pHP45Ω-Km into the *Bam*HI site of *kaiA* (in pAM1725), which is 309 bp downstream of the *kaiA* GTG start codon, created pAM2969. This construct was used to transform AMC541 to create AMC1161 (∇*kaiA*).

**Construction of a neutral site I Gateway vector**

A Gateway technology–compatible (Invitrogen) NS1 vector, pAM3110, was designed by amplifying the recombination cassette from pDONR221 (Invitrogen), which includes the sequences for *att*P1, the *ccdB* gene, a Cm-resistance gene, and *att*P2. The primers anneal 25 bp upstream of the M13 Forward sequence and 15 bp downstream of the M13 Reverse sequence, and incorporate *Mlu*I and *Xho*I restriction sites, respectively. The PCR product was cloned into *Mlu*I/*Xho*I-digested pAM2314 to create pAM3110. Exogenous DNA to be recombined at NS1 of the *S. elongatus* chromosome can be amplified with primers containing the *att*B1 and *att*B2 sequences, and cloned into pAM3110 using a BP Clonase reaction; the recombinant construct is inserted adjacent to an Sp/Sm-resistance Ω-cassette, both of which are flanked by *S. elongatus* NS1 DNA.

**Construction of ectopic *kai* alleles**

The *kaiA* gene and its native promoter region (P*kaiA::kaiA*) (43) were amplified using PCR and cloned into the NS1 cloning vector pAM1303 that was digested with *Bam*HI

and *Not*I. The *kaiA* segment in this plasmid (pAM2246) extends from 312 bp upstream of the *kaiA* GTG start codon to 80 bp downstream of the *kaiA* TGA stop codon, and was generated using primers that incorporate *Bgl*II and *Not*I restriction sites, respectively. P*purF::kaiA* was constructed as follows: an 88 bp P*purF* promoter fragment (78) was amplified by PCR using primers to incorporate an upstream restriction site (*Bam*HI) and a downstream restriction site (*Mlu*I). P*purF* was cloned into pUCBM20 as a *Bam*HI/*Mlu*I fragment creating pAM2380. A promoterless *kaiA* gene was amplified using PCR with primers flanking the *kaiA* gene from 84 bp upstream of the *kaiA* GTG start codon to 80 bp downstream of the *kaiA* TGA stop codon, incorporating *Mlu*I and *Not*I restriction sites, respectively, and subsequently cloned into the *Mlu*I/*Not*I sites of pAM2380 to create P*purF::kaiA* (pAM2383). This construct was cloned as an *Eco*RI/*Not*I fragment into the NS1 cloning vector pAM1303 to create pAM2482.

The *kaiBC* native promoter (43) was amplified along with either the *kaiB* gene alone (P*kaiBC::kaiB*) or *kaiBC* (P*kaiBC::kaiBC*). In both cases the upstream primer annealed 494 bp upstream of the *kaiB* ATG start and incorporated a *Bam*HI site. The downstream primer had an endpoint 30 bp downstream of the *kaiB* TAA stop codon for P*kaiBC::kaiB* and 146 bp downstream of the *kaiC* TAG stop codon for P*kaiBC::kaiBC*; both incorporated a *Not*I restriction site. Insertion of each fragment into *Bam*HI- and *Not*I-digested pAM1303 generated pAM2245 (P*kaiBC::kaiB*) and pAM3109 (P*kaiBC::kaiBC*). The P*kaiBC::kaiC* allele was generated as for P*kaiBC::kaiBC* except

that pAM1979 (the *kaiB* deletion construct) served as template and the downstream primer had an endpoint 46 bp downstream of the *kaiC* TAG stop codon.

The P*purF::kaiBC* construct used the same class 2 promoter segment as described for the generation of P*purF::kaiA*. A promoterless *kaiBC* operon was amplified by PCR using primers to flank *kaiBC* from 68 bp upstream of the *kaiB* ATG start codon to 146 bp downstream of the *kaiC* TAG stop codon. Amplification incorporated an upstream (*Mlu*I) and downstream restriction site (*Not*I). The resultant PCR product was cloned downstream of the *purF* promoter in pAM2380 to create pAM3135. The entire P*purF::kaiBC* construct was amplified from pAM3135 using primers that contain *att*B1 and *att*B2 sequences, respectively. The purified PCR product was then used in a BP Clonase recombination reaction with pAM3110 to create pAM3139.

**Measurement and analysis of *in vivo* bioluminescence**

Automated measurement of bioluminescence from the various *S. elongatus luc* reporter strains was performed as described in Chapter II. Data were recorded from each inoculated 96-well microtiter plate for two LD cycles (for synchronization) and 5 – 7 days in LL. All data acquired by the TopCount were graphed and analyzed by the I&A Excel interface (98) as described in Chapter II. The circadian period and standard deviations of all reporter fusions were calculated from at least five cycles of data obtained in LL by FFT-NLLS, a Fast Fourier statistical package associated with I&A (98). Each strain was measured in at least three independent experiments. The circadian

periods of the total number of individual wells of each strain from all experiments were used to calculate the average and standard deviation values displayed in Tables 3.2 and 3.3. Circadian phase of each trace is determined by the time of peak expression of bioluminescence with respect to the time the cells were placed in LL after the synchronizing LD cycles. Reporters termed class 1 peak at subjective dusk, with their first peak at 12 h after their entry into LL and peaking again approximately every 24 h. Class 2 reporters peak at subjective dawn; after an initial acute increase, peak expression occurs 24 h after entering LL.

**Whole-cell extract preparation and immunoblot analyses**

Whole-cell extracts of *kai* deletion strain cultures were prepared from 10 ml of an $OD_{750}$ of 0.3 (or higher) culture grown under constant conditions, harvested by centrifugation at 6,000 × *g* for 15 min, resuspended in 100 µl of BG-11M medium, and frozen at –80 °C. Cell suspensions were thawed on ice, and mixed with an equal volume of 106 µm and finer glass beads (Sigma Chemical) in a 1.5 ml centrifuge tube. The slurry was shaken vigorously with a vortex at high speed for 20 cycles of 30 s shaking, 30 s on ice. The samples were harvested by centrifugation (1,500 × *g*, 1 min) and the supernatant fraction collected. The remaining bead slurry was washed with 50 µl BG-11M, shaken briefly with the vortex, and the supernatant fraction was collected after centrifugation (1,500 × *g*, 1 min). The two supernatant fractions were combined and any remaining whole cells were discarded after subsequent centrifugation at 16,000 × *g* for 10 min and collection of

TABLE 3.2. Circadian periods of *kaiBC*-complemented strains

| **Strain** | **Background** | ***kaiBC* construct** | **Period (h)** | **n** |
|---|---|---|---|---|
| **P*kaiBC::luc* reporter** | | | | |
| AMC541 | WT | none | 24.8+/-0.44 | 80 |
| AMC703 | *kaiB* deletion | none | AR | 12 |
| AMC704 | *kaiC* deletion | none | AR | 13 |
| AMC705 | *kaiBC* deletion | none | AR | 15 |
| AMC1271 | WT | P*kaiBC::kaiB* | 23.8+/-0.64 | 14 |
| AMC1272 | *kaiB* deletion | P*kaiBC::kaiB* | AR | 22 |
| AMC1273 | WT | P*kaiBC::kaiC* | 25.8+/-0.42 | 29 |
| AMC1274 | *kaiC* deletion | P*kaiBC::kaiC* | 26.4+/-0.33 | 20 |
| AMC1276 | WT | P*kaiBC::kaiBC* | 25.2+/-0.51 | 14 |
| AMC1277 | WT | P*purF::kaiBC* | 25.3+/-0.52 | 27 |
| AMC1278 | *kaiB* deletion | P*kaiBC::kaiBC* | 25.5+/-0.62 | 18 |
| AMC1279 | *kaiB* deletion | P*purF::kaiBC* | 25.8+/-0.70 | 42 |
| AMC1280 | *kaiC* deletion | P*kaiBC::kaiBC* | 24.9+/-0.35 | 22 |
| AMC1281 | *kaiC* deletion | P*purF::kaiBC* | 25.9+/-0.69 | 27 |
| AMC1282 | *kaiBC* deletion | P*kaiBC::kaiBC* | 25.5+/-0.52 | 26 |
| AMC1283 | *kaiBC* deletion | P*purF::kaiBC* | 26.6+/-0.64 | 24 |
| **P*purF::luc* reporter** | | | | |
| STC100 | *kaiBC* deletion | P*purF::kaiBC* | 26.3+/-1.04 | 12 |
| **P*psbAI::luc* reporter** | | | | |
| STC101 | *kaiBC* deletion | P*purF::kaiBC* | 24.96+/-0.89 | 19 |

TABLE 3.3. Circadian periods of *kaiA*-complemented strains

| **Strain** | **Background** | ***kaiA* construct** | **Period (h)** | **n** |
|---|---|---|---|---|
| **P*kaiBC::luc* reporter** | | | | |
| AMC541 | WT | none | 24.8+/-0.44 | 80 |
| AMC702 | *kaiA* deletion | none | AR | 73 |
| AMC1099 | WT | P*kaiA::kaiA* | 23.3+/-0.63 | 15 |
| AMC1101 | WT | P*purF::kaiA* | 23.7+/-0.45 | 30 |
| AMC1161 | *kaiA* insertion | none | AR | 36 |
| AMC1233 | *kaiA* insertion | P*kaiA::kaiA* | 25.9+/-0.73 | 30 |
| AMC1234 | *kaiA* insertion | P*purF::kaiA* | 25.5+/-0.66 | 35 |

the soluble protein fraction. Soluble protein concentrations were determined by the method of Lowry *et al*. (73).

Equal amounts of total protein (10 – 20 μg) from each whole cell extract sample were separated by SDS-PAGE (12.5%) for immunoblot analyses. Protein was transferred to 0.2 μm PROTRAN nitrocellulose membranes (Schleicher & Schuell) by capillary transfer for KaiA and KaiB detection as previously described (75), or to 0.45 μm PROTRAN nitrocellulose membranes (Schleicher & Schuell) by semi-dry blot transfer per manufacturer's instructions (Bio-Rad Laboratories) for KaiC. Polyclonal rabbit antiserum to KaiA and KaiB were used at dilutions of 1:2,000 and 1:1,000 respectively, and were detected using peroxidase-conjugated goat anti-rabbit IgG (Calbiochem). Polyclonal chicken antiserum to KaiC (Aves Labs) was used at a dilution of 1:2,000, and detected with peroxidase-conjugated goat anti-chicken IgY (Aves Labs). Detection of Kai antibody binding was visualized with the SuperSignal West Pico Chemiluminescent Substrate detection system (Pierce) per manufacturer's instructions. Under the stated conditions, the phosphorylated and unphosphorylated KaiC proteins are not resolved. Therefore, KaiC immunoblots detect the total level of KaiC present in the cell.

## *Results*

### Construction and characterization of *kai* gene mutant strains

A series of *S. elongatus* strains was created that harbors in-frame deletions of *kaiA*, *kaiB*, and *kaiC* (Fig. 3.1A) to serve as clean genetic backgrounds for ectopically-expressed *kai*

alleles. Expression of a P*kaiBC::luc* reporter in these strains (AMC702, AMC703, AMC704, and AMC705), while light-responsive under LD cycles, is arrhythmic under LL conditions (Fig. 3.1C). The in-frame deletion strains have no discernable growth phenotype compared to the WT strain, AMC541 (data not shown). Immunoblot analysis of each deletion strain showed that the respective protein is absent (Fig. 3.1B); both KaiB and KaiC are absent in the Δ*kaiBC* strain (AMC705). Although it was designed to avoid polar inactivation of *kaiC,* the Δ*kaiB* strain does not produce KaiC (Fig. 3.1B), and expression of *kaiB* alone is not sufficient to complement the Δ*kaiB* strain (Table 3.2). The P*kaiBC::kaiC* construct was generated using the Δ*kaiB* allele; and KaiC is expressed from this construct in NS1 of AMC1273 and AMC1274, producing active KaiC protein as shown by immunoblot (data not shown) and its ability to complement the Δ*kaiC* strain AMC704 (Table 3.2). It is possible that during the hit-and-run allele replacement procedure a secondary mutation occurred in the cyanobacterium that caused KaiC to no longer be produced.

Previous experiments have shown that *kaiA* expression has a positive effect on P*kaiBC* because in the absence of KaiA, the amount of expression from a P*kaiBC* reporter decreases (43). Therefore, it was expected that an in-frame deletion of *kaiA* would cause a decrease in the amount of KaiB and KaiC proteins expressed in this mutant background. This expected phenotype was reported by the P*kaiBC::luc* promoter fusion in the Δ*kaiA* strain (AMC702); levels of bioluminescence from the reporter are depressed to below background levels (Fig. 3.1C). However, noticeably elevated levels

of KaiB and KaiC protein were detected in the Δ*kaiA* strain (Fig. 3.1B). The data are consistent with the hypothesis that a negative element was removed from the P*kaiBC* promoter region in the process of deleting the *kaiA* gene, as the stop codon of the *kaiA* gene is only 90 bp upstream of the start codon for *kaiB* (43). Others have observed evidence for the presence of a negative regulatory element in this region (66). The elevated level of KaiB and KaiC protein production was not detected at the transcriptional level by the P*kaiBC::luc* promoter fusion because the reporter includes this putative negative element.

An insertional mutant of *kaiA* (∇*kaiA*, AMC1161) was generated to inactivate the gene without affecting potential regulatory elements of *kaiBC* (Fig. 3.1A). As in the Δ*kaiA* mutant background, rhythms of bioluminescence from the P*kaiBC::luc* reporter are arrhythmic in the ∇*kaiA* strain (Fig. 3.1C). Conversely, protein levels of KaiB and KaiC are decreased relative to WT in ∇*kaiA* (Fig. 3.1B). Therefore, this *kaiA* insertion mutant strain was used for further complementation studies.

KaiC has been shown to act as a negative regulator of the *kaiBC* promoter; when KaiC is overexpressed, expression from a P*kaiBC* reporter decreases significantly (43). The in-frame *kaiC* deletion strain (AMC704) shows that expression of the P*kaiBC::luc* reporter increases over time when *kaiC* is not present (Fig. 3.1C), supporting the role of KaiC as a negative regulator. However, regulation of the *kaiBC* promoter is more complicated in that KaiC is also required for normal levels of *kaiBC* expression. This necessity is

demonstrated in the slightly lower levels of KaiB protein in the Δ*kaiC* mutant background (Fig. 3.1B).

**Complementation of ∇*kaiA* with class 2 P*purF::kaiA* restores WT circadian rhythms**

Because mutants of *S. elongatus* have been identified that uncouple the transcriptional phasing of the two circadian clock *kai* operons (52, 86), the effect of directly changing the transcriptional phasing of *kaiA* (to 12 h out of phase with that of *kaiBC*) was examined. Expression of an ectopic copy of *kaiA* from either its native promoter or the heterologous *purF* promoter in an otherwise WT strain shortened the circadian period by about an hour (Table 3.3). Each *kaiA* construct was also tested for its ability to complement the ∇*kaiA* strain (AMC1161). Expression of either P*kaiA::kaiA* or P*purF::kaiA* in the ∇*kaiA* strain (AMC1233 and AMC1234, respectively) restored circadian rhythmicity with periodicities very near that of the P*kaiBC::luc* reporter in a WT background (Table 3.3). Also, the phase of the rhythm was not altered when *kaiA* was produced 12 h out of phase from its native timing (Fig. 3.2B). Levels of Kai proteins in these strains are comparable to those in the WT (Fig. 3.2A). Therefore, the peak of the oscillation in transcription of *kaiA* does not have a substantial effect on the properties of the cyanobacterial circadian rhythm.

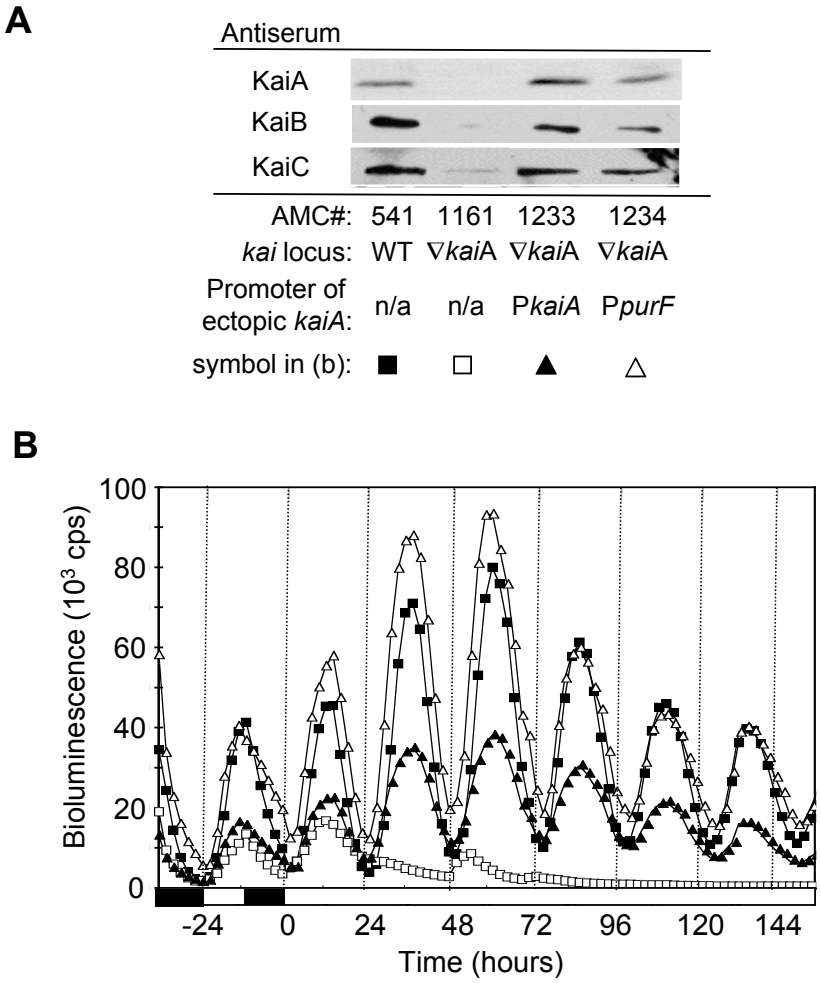


FIG. 3.2. Complementation of the ∇*kaiA* strain by P*purF::kaiA*. (A) Immunoblot of 20 μg total soluble protein showing that WT protein levels of KaiA are produced when *kaiA* is expressed from P*kaiA* and P*purF*. (B) Bioluminescence traces from a P*kaiBC::luc* reporter. Axes labeled as for Fig. 3.1(C).

**P*purF::kaiBC* can restore WT rhythms to a Δ*kaiBC* strain**

Throughout the circadian cycle, the overall level of KaiA protein remains constant, or fluctuates with low amplitude over a 24-h time scale (125). Therefore, altering the expression of the *kaiA* gene may not result in a change in the amount of KaiA protein over time and, thus, would not alter the phasing of the clock itself. The KaiB and KaiC proteins, however, accumulate in a circadian fashion with peak protein levels occurring CT 16 – 20 (a 4-h lag from peak mRNA levels) and these oscillations, along with changes in phosphorylation levels, have been proposed to be required for rhythmicity (43, 117, 126). Therefore, the necessity for peak transcriptional activity of *kaiB* and *kaiC* to occur at subjective dusk was examined.

An ectopic copy of the entire *kaiBC* dicistron expressed from either its native promoter or the *purF* promoter was tested for its ability to complement a strain that lacks both *kaiB* and *kaiC.* In a WT background a second copy of *kaiBC* from either promoter had little effect on the circadian rhythm of the cells (Table 3.2, AMC1276 and AMC1277). In the Δ*kaiBC* strain expression of P*kaiBC::kaiBC* from NS1 (AMC1282) drives P*kaiBC::luc* expression in class 1 phase, peaking at subjective dusk (Fig. 3.3B) with a period very close to that of WT (Table 3.2). Moreover, expressing *kaiBC* from the heterologous *purF* promoter in the Δ*kaiBC* strain (AMC1283), such that transcription would peak 12 h out of phase from its WT pattern and from expression of *kaiA*, did not alter the period of luciferase expression from a P*kaiBC::luc* reporter (Table 3.2). In addition, the phase of peak bioluminescence from P*kaiBC::luc* was not altered, despite

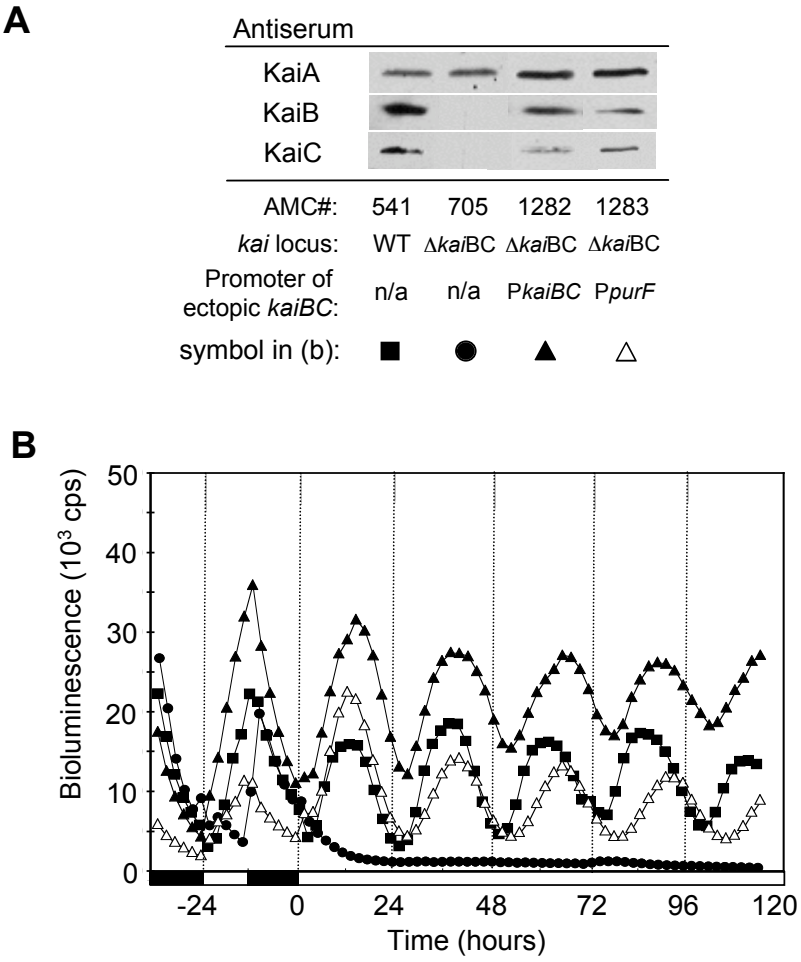


FIG. 3.3. Complementation of the Δ*kaiBC* strain by P*purF::kaiBC.* (A) Immunoblot of 20 μg total soluble protein showing that WT levels of KaiB and KaiC proteins are produced in the complemented strains. (B) Bioluminescence traces from a P*kaiBC::luc* reporter. Axes labeled as for Fig. 3.1(C).

the fact that these core clock genes are being transcriptionally activated 12 h out of phase from their normal time (Fig. 3.3B). Levels of Kai proteins in these complemented strains are similar to those seen in WT (Fig. 3.3A).

**Pervasive effects of P*purF::kaiBC* expression on both class 1 and class 2 reporters**

To determine the effect of mis-timed *kaiBC* expression on promoters whose activities are not central to the clock, well-characterized class 1 (P*psbAI::luc*) and class 2 (P*purF::luc*) reporters were measured for rhythms in bioluminescence in the P*purF::kaiBC*-complemented strain. Consistent with the results for the P*kaiBC::luc* reporter, both P*psbAI::luc* (STC101) and P*purF::luc* (STC100) reporters displayed rhythms with WT period and phasing (Table 3.2 and Fig. 3.4). In LL, the peak of expression from the *purF* reporter is 12 h out of phase from P*psbAI::luc.* However, in the LD cycles prior to release into LL, P*purF::luc*, and all known class 2 reporters (S. R. Mackey and S. S. Golden, unpublished data), peak only 4 h before class 1 rhythms, as has been previously described (71). The class 2 rhythms exhibited by P*purF::luc* in the P*purF::kaiBC*-complemented strain demonstrates that the *purF* promoter is still recognized as class 2 and is driving *kaiB* and *kaiC* in that phase from NS1. A P*kaiA::luc* reporter also maintained its WT circadian properties in this complemented background (data not shown).

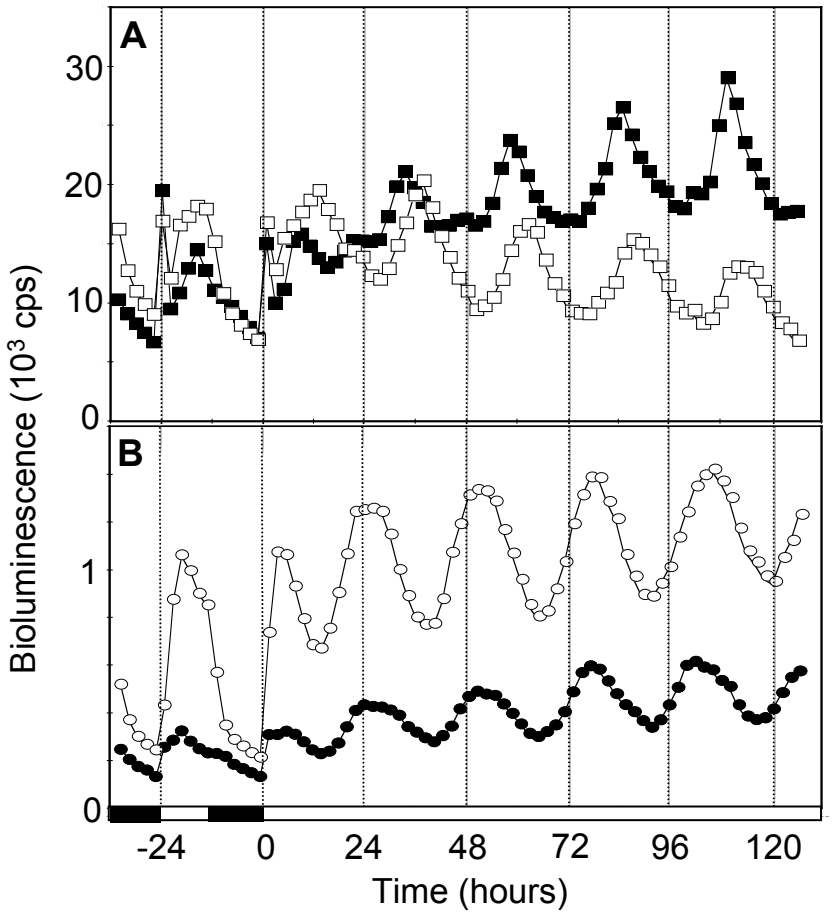


FIG. 3.4. A P*purF::kaiBC*-complemented clock can differentiate between class 1 and class 2 promoters to produce a WT period and relative phase. (A) Bioluminescence traces from a P*psbAI::luc* reporter. Closed squares, AMC603; open squares, STC101. (B) Bioluminescence traces from a P*purF::luc* reporter. Closed circles, AMC601; open circles, STC100. Axes labeled as for Fig. 3.1(C).

***Discussion***

We demonstrated that the promoter elements of the *kai* locus are not specifically needed to support circadian control of gene expression; in fact, the synchrony of expression of *kai* loci is not needed to maintain rhythms. Either *kaiA* or *kaiBC* can be expressed from the class 2 *purF* promoter, 12 h out of phase from the other *kai* locus, and preserve the persistence and precision of the circadian mechanism. However, *cis* elements do exist that regulate the level of expression of these genes: a *kaiC* null causes an increase in the level of expression from its promoter (Fig. 3.1C), while overexpression causes a decline (43). The bioluminescence rhythm from a P*kaiBC* reporter has a high amplitude as compared to other reporters, such as P*psbAI* (compare Fig. 3.1C and Fig. 3.4A) and declines to near background levels at its trough. The negative regulatory element may assist in the robust, high-amplitude expression from P*kaiBC* because removal of a portion of the promoter region results in increased levels of KaiB and KaiC proteins in the Δ*kaiA* strain. When this element is missing, as in the Δ*kaiA* strain, the trough levels may be increased such that there is a higher basal level of *kaiBC* expression, which would account for the observed increase in protein level.

The amounts of each Kai protein affect the period length of the rhythm. Expression of a second copy of *kaiA* in a WT background shortened the circadian rhythm by about an hour (Table 3.3). Previous experiments have demonstrated that KaiA increases the rate in which KaiC is autophosphorylated by about 2.5 fold *in vitro* (124). Having two copies of *kaiA*, as seen in AMC1099 and AMC1101, may lead to an increase in the

phosphorylation state of KaiC protein, and increase the rate of progression through the circadian cycle, which may account for the shortened period in these strains. The regulation is more complicated, though, as expression of an ectopic copy of *kaiB* in a WT strain (AMC1271) also shortens the period of circadian rhythmicity in LL (Table 3.2). KaiB abrogates the stimulatory effect of KaiA on the autophosphorylation of KaiC; a second copy of *kaiB* would not be expected to cause the same effect on the period length as the ectopic copy of *kaiA*. The presence of an additional copy of *kaiC* in the WT strain (AMC1273) causes a slight lengthening of the period of the rhythm (Table 3.2). The stability of KaiC and its complexes has been shown to be a determining factor of the period length in the cell (50, 117, 126). The extra copy of *kaiC* and the resulting increased level of KaiC protein present in the cell may provide more periodosome scaffolds to form, assembly and disassembly of which could lengthen the cycle.

These data and those from other labs provide increasing evidence that the mechanisms for maintaining the clock in cyanobacteria are not dependent on the negative and positive limbs of a transcriptional-translational feedback loop. In addition to the ability to sustain circadian rhythms when *kai* genes are expressed from heterologous promoters (126), *S. elongatus* continues to tell time in the dark, when *de novo* expression of the *kai* genes, and thus protein synthesis, is not detectable (117, 126). Under these conditions, which would disrupt a transcriptional-translational feedback loop, circadian rhythms in the phosphorylation state of KaiC continue (117). Moreover, the Kai proteins can establish a temperature-compensated circadian rhythm of KaiC phosphorylation *in vitro*,

directly demonstrating that there is a post-translational clock mechanism in the cyanobacterium (89). Thus, understanding the post-translational dynamics of core clock proteins will be needed to elucidate the stabilizing and time-keeping properties of the Kai-based clock.

# CHAPTER IV

# DIFFERENTIAL CIRCADIAN REGULATION OF *psbA* GENES IN *Synechococcus elongatus* PCC 7942

## *Introduction*

The cyanobacteria are a group of prokaryotes that are distinct in their ability to carry out oxygenic photosynthesis like that of higher plants. Their photosynthetic apparatus contains two photosystems, which are large protein complexes that reside in the thylakoid membrane. Photosystem II (PSII) is the site of water splitting and oxygen evolution. The reaction center core of PSII contains two structurally-similar proteins, D1 and D2, which house the photoreactive chlorophyll and other cofactors involved in electron transport. The genes that encode the D1 (*psbA*) and D2 (*psbD*) proteins occur in the cyanobacteria as gene families, in contrast to plants where the conserved genes are present in single copies. The regulation of the *psbA* genes has been extensively studied in the unicellular, photoautotrophic *S. elongatus* PCC 7942. *S. elongatus* harbors three *psbA* genes that encode two different forms of the D1 protein (28). Form I, encoded by *psbAI*, is the predominant form of the D1 protein at low light intensities (13, 61). In response to stress-induced conditions, such as high light, the *psbAII* and *psbAIII* genes are upregulated to result in an increase of Form II of the D1 protein (64); Form II differs from Form I by only 25 amino acids, 12 of which are located in the first 16 N-terminal residues (28).

The two forms of D1 protein suggest that they have distinct functions to provide a selective advantage for maintaining them. The induction of the genes that encode Form II under high light intensity hints that this Form serves to protect the photosynthetic apparatus (64). When light intensity exceeds the rate of photosynthesis electron transport, the cells enter a state known as photoinhibition. The source of photoinhibition is thought to be the D1 protein, which is damaged during the reactions that occur during photosynthesis, cleaved, and then replaced in order to maintain electron transport (11). When the rate of degradation of D1 is greater than the rate of synthesis and thylakoid incorporation, photoinhibition occurs. In cells transferred from low light to high light, Form I is quickly replaced with Form II in the thylakoid membrane (13, 62). Those cells that incorporate Form II exhibit a more effective conversion of energy in photosynthesis reactions (12). Cells capable of producing only Form I cannot acclimate to high light intensity and do not recover sufficiently after being exposed to high light (13).

The regulation of the *psbA* gene family occurs at multiple levels. In low light, the majority of *psbA* transcripts are from *psbAI* and only Form I can be detected in the thylakoid membrane (13, 61). Immediately after introduction to photoinhibitory light intensities, the *psbAII* and *psbAIII* transcripts are induced. The rise in overall levels of these transcripts results from increased rates of transcription, not an increased stability of the transcripts (64, 102). The *psbAI* and *psbAIII* transcripts are actively degraded at high light by an unknown protein factor (64). The level of *psbAI* transcript will eventually rebound from its initial decline after a longer exposure (24 h) to high light; despite these

higher levels of detectable *psbAI*, Form II is the predominant form of D1 protein incorporated in the thylakoid when the cells are exposed to higher light intensities (61).

Factors other than light intensity can influence the level of *psbA* gene transcription and stability of the resulting transcripts. Low temperature, exposure to UV-B light, and reduced redox state of the cell increase the levels of *psbAII* and *psbAIII* mRNA accumulation (7, 101, 116, 120). Some of these same stimuli also influence the parametric and nonparametric entrainment properties of the circadian rhythm in *S. elongatus* (4, 45, 51). Although the circadian clock has been shown to regulate the rhythmic expression from all *S. elongatus* promoters (72), the extent to which the clock controls the timing of *psbA* expression has not been extensively studied. The differential regulation of these genes in response to environmental changes as well as the distinct functions of the two D1 protein products suggest that there may also be an adaptive advantage through differential regulation of these genes by the endogenous circadian rhythm. In particular, it is not evident why the strain carries both *psbAII* and *psbAIII* genes, whose environmental responses and products are the same; partitioning of their expression into different circadian phases might explain this conundrum. Therefore, we monitored the patterns of bioluminescence from luciferase reporters driven by the promoters of the *psbA* gene family (P*psbA::luxAB*) in LL. The rhythms from P*psbAII* and P*psbAIII*, which encode Form II of the D1 protein, were also examined in the absence of the endogenous *psbAI* gene. The presence of a circadian element within the promoter regions of each gene was also tested by removal of previously-described *cis*

elements. The results show that a *psbAII* reporter displays a class 2 rhythm with expression patterns 12 h anti-phase to those of the class 1 *psbAI* or *psbAIII*. However, in the absence of the *psbAI* gene, *psbAII* adopts a class 1 rhythm as a possible compensatory mechanism for the loss of the predominant form of the essential D1 protein.

## *Methods*

### Bacterial strains and growth conditions

The strains used in this study are described in Table 4.1. Cyanobacterial reporter strains were created in *S. elongatus* PCC 7942. All cyanobacterial strains were grown and maintained as described in Chapter II. Cultures of at least an $OD_{750}$ of 0.3 were used for measurement of bioluminescence on the Packard TopCount luminometer (Perkin-Elmer Life Sciences), as described in Chapter II.

### Measurement and analysis of *in vivo* bioluminescence

Automated measurement of bioluminescence from *psbA* and *purF* reporter strains and subsequent analysis was performed as described in Chapter II. Cells' clocks were synchronized with two 12:12 LD cycles and then monitored in LL for at least five days. For LD sampling, cells were inoculated onto micotiter plates, their clocks were synchronized by 12 h dark exposure, and bioluminescence was monitored for at least four consecutive 12:12 LD cycles using protocols described in Chapter II.

TABLE 4.1. Cyanobacterial strains used to study *psbA* regulation

| **Strain*** | **Promoter driving *luxAB*** | **Promoter endpoints†** | **Antibiotic resistance‡** | **Source or reference** |
|---|---|---|---|---|
| AMC408 | *purF* | -500 to -422 | Cm; Sp/Sm | 51 |
| AMC537 | *psbAIII* | -38 to +39 | Cm; Sp/Sm | 77 |
| AMC543 | *psbAI* | -40 to +230 | Cm; Km | 52 |
| AMC776 | *psbAI* | -115 to +43 | Cm; Sp/Sm | 87 |
| AMC777 | *psbAI* | -54 to +43 | Cm; Sp/Sm | 87 |
| AMC781 | *psbAI* | -115 to +1 | Cm; Sp/Sm | 87 |
| AMC1264 | *psbAII* | -870 to +134 | Cm; Sp/Sm | 115 |
| AMC1293 | *psbAII* | -870 to +134 | Cm; Km; Sp/Sm | This study |
| AMC1294 | *psbAIII* | -38 to +39 | Cm; Km; Sp/Sm | This study |
| AMC1295 | *purF* | -500 to -422 | Cm; Km; Sp/Sm | This study |
| AMC1510 | *psbAIII* | -38 to +1 | Cm; Sp/Sm | This study |

* All reporter strains also contain P*psbAI::luxCDE*. AMC543 contains P*psbAI::luxAB* in NS2.1 and P*psbAI::luxCDE* in NS2.2. The other reporter strains contain P*psbAI::luxCDE* in the NS (either 1 or 2) not occupied by the *luxAB* reporter.
† Nucleotide numbers are relative to the ATG start codon at +1 for the *purF* promoter, and relative to the transcriptional start site at +1 for each of the *psbA* promoters.
‡ Cm, chloramphenicol; Km, kanamycin; Sp/Sm, spectinomycin/streptomycin.

***Results***

To determine the regulation of *psbA* expression by the circadian system, each *psbA* promoter (P*psbA*) was engineered to drive expression of the bioluminescence reporter genes *luxAB.* The reporter strains were exposed to two LD cycles to synchronize the cells' clocks and the levels of bioluminescence from each reporter fusion were monitored in LL. All three strains exhibited rhythmic expression with periodicities very near 24 h (Fig. 4.1). The *psbAI* promoter expressed the highest levels of bioluminescence, consistent with previous measurements of RNA, protein, and *lacZ* reporter data (28, 61, 102), followed by *psbAII*, and the *psbAIII* reporter displayed the lowest level of expression. The P*psbAI::luxAB* and P*psbAIII::luxAB* reporters exhibited class 1 rhythms with the phase of peak expression occurring 12 h after the release from LL and approximately every 24 h thereafter (Fig. 4.1). However, the P*psbAII::luxAB* reporter displayed a class 2 rhythm with peak expression occurring 12 h out of phase from either *psbAI* or *psbAIII,* like that of the well-characterized P*purF::luxAB* reporter (Fig. 4.1) (78).

Because cyanobacteria are rarely in an environment where they are exposed to continuous light, the different *psbA* reporters were maintained in multiple, successive LD cycles to determine their temporal patterns in a more natural setting. The highest point of expression for the class 1 reporters, P*psbAI* and P*psbAIII*, occurred at the transition from light to dark (Fig. 4.2). The class 2 P*psbAII* reporter consistently peaked 4 h prior to the light to dark transition and the class 1 genes (Fig. 4.2); this phase lead is

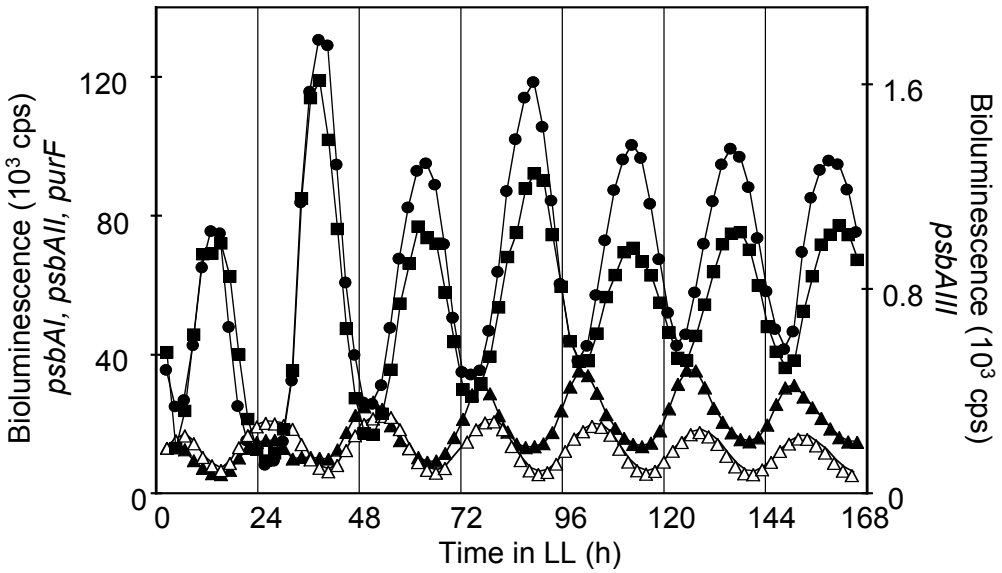


FIG. 4.1. Expression from P*psbAII::luxAB* peaks anti-phase to that of P*psbAI* or P*psbAIII.* Bioluminescence in counts per second (cps) from a P*psbAI::luxAB* (closed circles) or P*psbAIII::luxAB* (closed squares) reporter peaks at subjective dusk. The P*psbAII::luxAB* reporter (closed triangles) peaks 12 h out of phase from the other two *psbA* promoters, like that of the class 2 P*purF::luxAB* reporter.

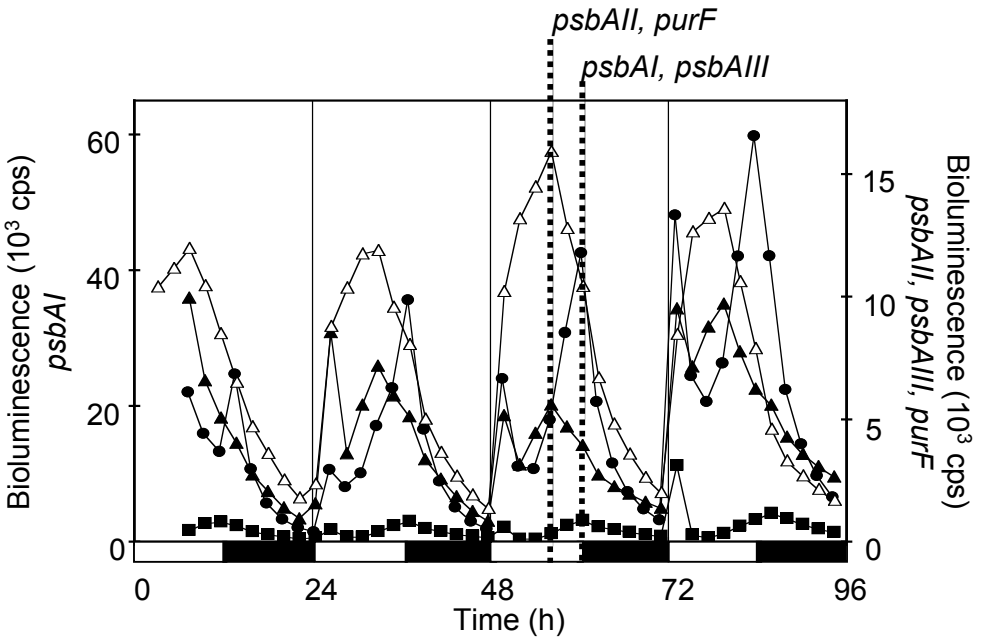


FIG. 4.2. Expression of *psbA::luxAB* reporters in LD cycles. The peak of expression from P*psbAI* and P*psbAIII,* which are both class 1 genes in LL, occurs at the transition from light to dark. The class 2 reporters, P*psbAII* and P*purF*, consistently peak 4 h earlier than the light to dark transition in each LD cycle. On the X-axis, white and black bars indicate light and dark, respectively.

also seen from other class 2 promoters, such as P*purF* (Fig. 4.2) and P*opcA* (data not shown). Taken together with the results from the LL experiments, the biological clock in cyanobacteria creates an additional level of differential transcriptional regulation of the *psbA* gene family.

The protein product of the *psbAI* gene (Form I) is the major form of the protein at normal physiological conditions. However, at high light the *psbAII* and *psbAIII* genes are upregulated and *psbAI* transcript is actively degraded (64) to result in a net increase in the levels of Form II (61). In the absence of *psbAI*, overall levels of Form II protein are markedly increased even in low light conditions (13, 62, 103). To determine whether the circadian timing of transcription from P*psbAII* or P*psbAIII* was dependent upon the presence of the *psbAI* gene, the effect of inactivating the *psbAI* gene on relative circadian phase in LL was tested in strains that harbor either P*psbAII::luxAB* or P*psbAIII::luxAB* reporters. In the absence of *psbAI,* the class 1 P*psbAIII::luxAB* continued to exhibit rhythms in bioluminescence that closely matched its WT counterpart (Fig. 4.3A). In contrast, the phase of P*psbAII::luxAB* expression was changed by 12 h when *psbAI* was not present in the cell (Fig. 4.3B). This change in phase was not exhibited by all class 2 promoters because the inactivation of *psbAI* did not have an effect on the circadian rhythm from P*purF::luxAB* reporter (Fig. 4.3C). Thus, when *psbAI* is not present, the expression from P*psbAII* adopts the timing of expression of the absent, and normally predominant, *psbAI* gene.

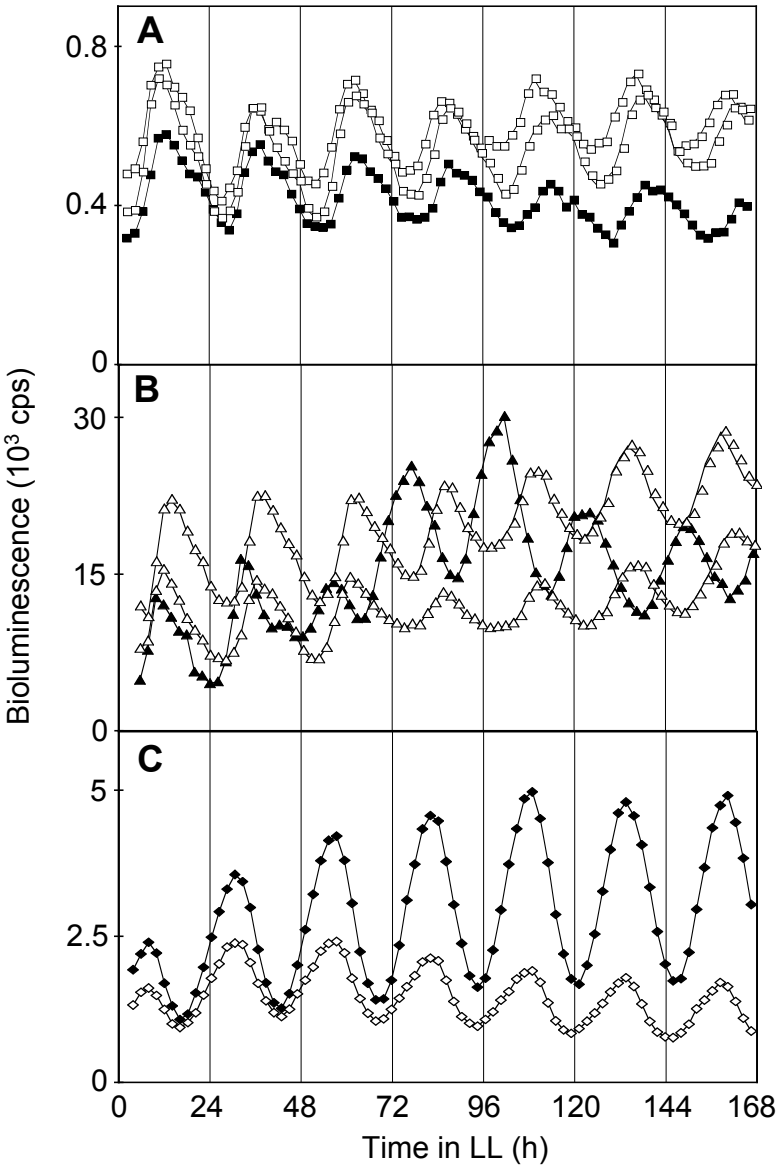


FIG. 4.3. The phase of expression from P*psbAII* is affected by the absence of the *psbAI* gene. The endogenous *psbAI* gene was interrupted by a Km-resistance cassette in the P*psbAII,* P*psbAIII*, and P*purF* reporter strains. Each strain was synchronized by two days of 12:12 LD and bioluminescence was measured from each strain in LL. Closed symbols represent (A) P*psbAIII::luxAB,* (B) P*psbAII::luxAB*, and (C) P*purF::luxAB* reporters in otherwise WT cells, open symbols represent each reporter in the *psbAI* inactivation strain. Two traces of the *psbAI:Km* strains are shown for the P*psbAII* and P*psbAIII* reporters.

Extensive studies on the promoters of the *psbA* genes have provided insight into the function of the different regulatory elements. It is possible that there is a circadian element within these *cis* sequences that provides temporal information to determine the phase of expression. To test this hypothesis directly, the rhythms of bioluminescence from luciferase reporters driven by promoters that lack one or more of the functional *cis* elements for the *psbA* genes were examined in LL. The full-length promoter region for *psbAI* extends from –115 to +43, relative to the transcriptional start site at +1 (Fig. 4.4A). The basal promoter includes bases –54 to +1 and a positive element exists just upstream of the basal promoter (–115 to –54) that increases the level of expression from the promoter by ~ 6 fold (87). The sequence downstream of the basal promoter (+1 to +43) also increases the strength of the *psbAI* promoter (87). Removal of either of these positive regulatory elements did not change the phase of peak expression of bioluminescence (Fig. 4.4B). However, the shapes of the waveforms are altered such that the troughs of expression are delayed by about 8 h when the downstream stimulatory element was removed, and nearly 10 h in the absence of the upstream positive element (Fig. 4.4B). As expected, the latter strain (AMC777) displayed severely decreased overall levels of expression.

The *psbAIII* promoter contains a basal promoter from bases –38 to +1 relative to the transcriptional start site at +1 (Fig. 4.5A) with an upstream negative element (–75 to +38) (67). Downstream of the basal promoter lies a true enhancer (+1 to +39) that increases the level of gene expression in a position- and orientation-independent manner

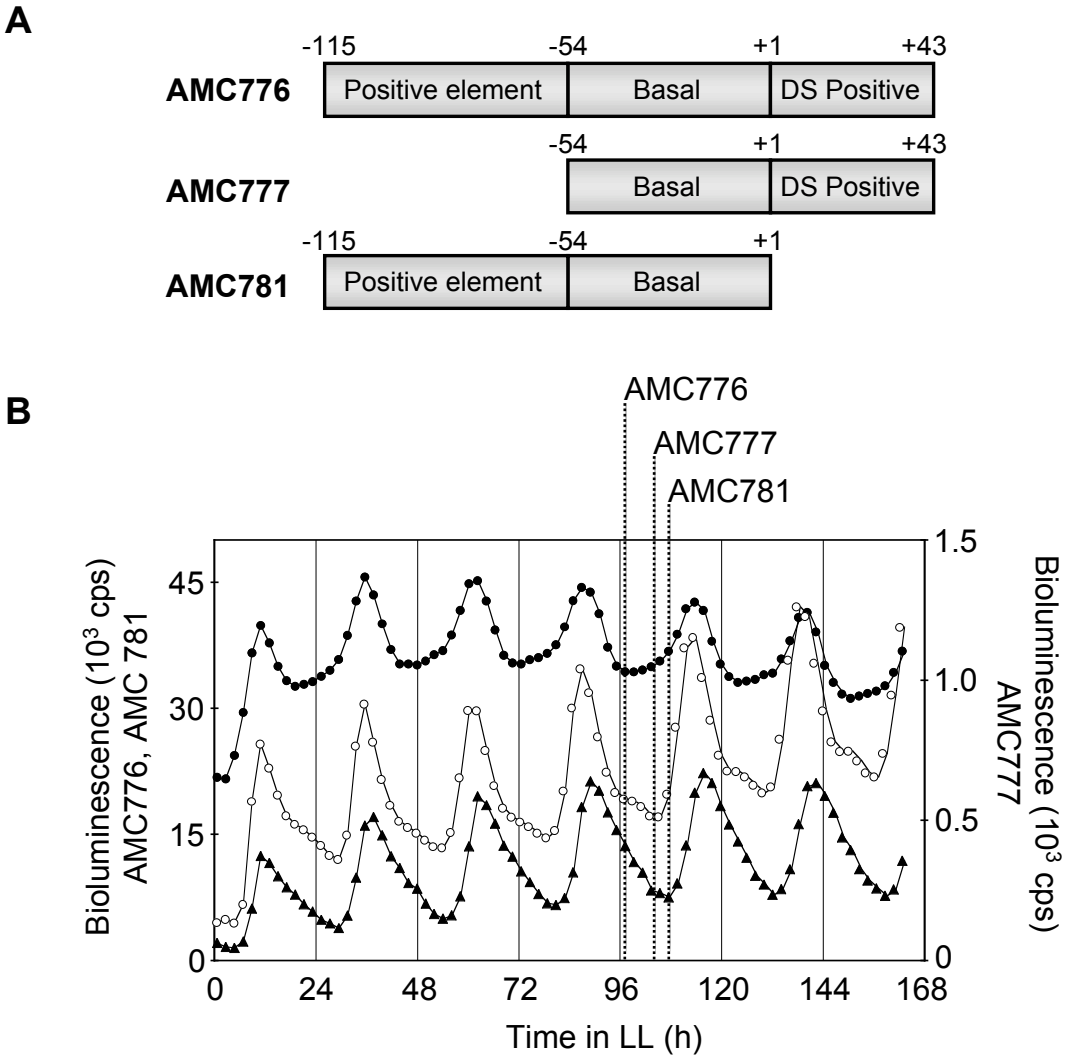


FIG. 4.4. Removal of promoter elements does not change the circadian properties of expression from a *psbAI* promoter. (A) Schematic representation of the *psbAI* promoter elements in each reporter strain. (B) Bioluminescence from P*psbAI::luxAB* derivatives peak in the same phase and maintain WT period length. AMC776 (closed circles) contains the full *psbAI* promoter from bases -115 to +43, relative to the transcriptional state site at +1. AMC777 (closed triangles) does not contain the positive element (bases -115 to -54). AMC781 (open circles) lacks the untranslated downstream (DS) positive element from +1 to +43. Dotted lines show the relative trough in expression from each of the reporters.

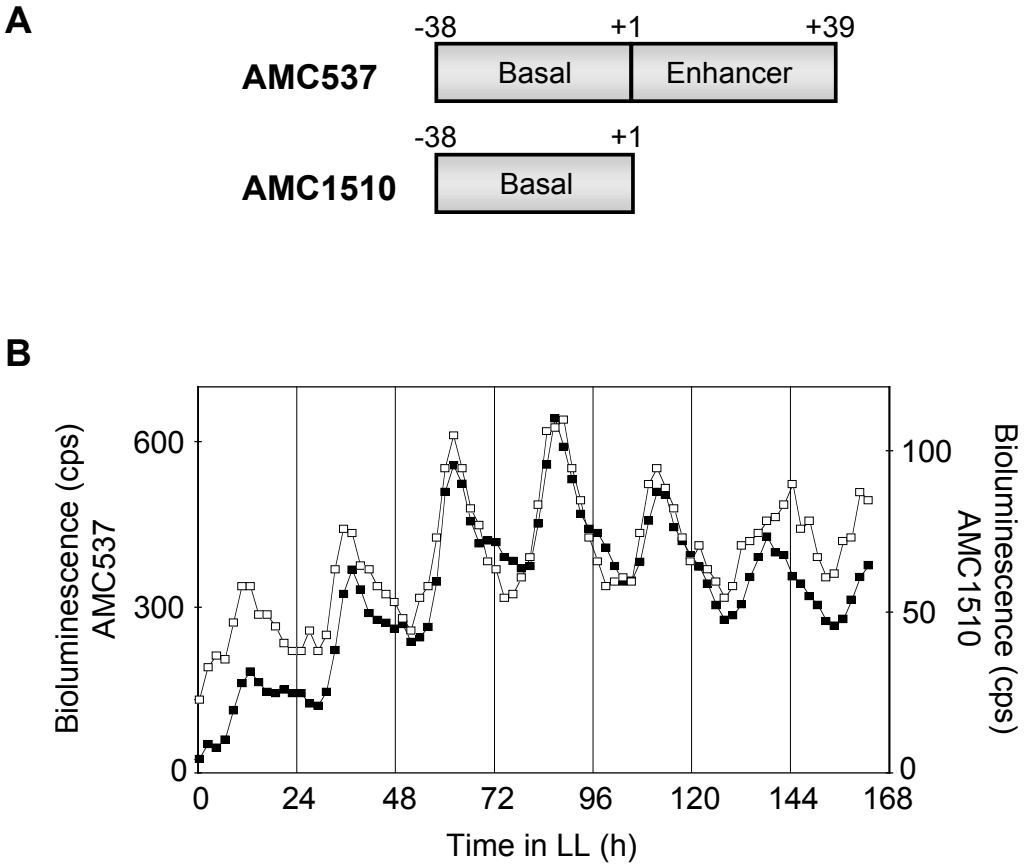


FIG. 4.5. Removal of promoter elements does not change the circadian properties of expression from a *psbIII* promoter. (A) Schematic representation of the *psbAIII* promoter elements in each reporter strain. (B) Bioluminescence from P*psbAIII::luxAB* derivatives peak in the same phase and maintain WT period length. AMC537 (closed squares) contains the basal promoter and enhancer element of the *psbAIII* promoter from bases -38 to +39, relative to the transcriptional start site at +1. AMC1510 (open squares) does not contain the enhancer element.

(67). Due to the very low level of expression exhibited by the basal promoter (Fig. 4.5B), a reporter also harboring the negative element, which would decrease the level of expression even further, was not tested. Removal of the enhancer element decreased the level of bioluminescence but did not alter the period or phase of the rhythm of bioluminescence (Fig. 4.5B).

Based on these data, the promoter elements tested for the class 1 *psbAI* and *psbAIII* genes do not contain a specific circadian element to designate their phase of peak expression. However, over 90% of promoters in the *S. elongatus* genome (72), as well as a heterologous *E. coli* promoter (88), display class 1 rhythms in LL suggesting that the default state of a promoter is to peak at the dusk to dawn transition. If a circadian element exists that changes the peak phase of expression to something other than class 1, it would most likely be present in the promoters of class 2 genes. Previous work using the *purF* promoter was unable to define a sequence that specifically conferred class 2 gene expression (78); nonetheless, the different promoter elements of the *psbAII* gene will be tested for their effect on phase. Each of these constructs will be monitored in the presence and absence of *psbAI* in order to ascertain that gene's effect on transcriptional regulation of *psbAII*.

***Discussion***

The experiments completed thus far in this study have addressed the circadian regulation of the *S. elongatus psbA* gene family. The findings add yet another level of complexity

to the coordinated regulation of the *psbA* genes. In addition to the previously-described transcriptional control of these genes in response to light intensities, the circadian rhythm also differentiates the timing of gene expression throughout the daily cycle.

Not surprisingly, the transcription of each member of the *psbA* gene family, like that of the entire *S. elongatus* genome, is under clock control with a periodicity of promoter activity that peaks once a day (Fig. 4.1). The relative strengths of the promoters as determined by levels of bioluminescence are mirrored in their relative transcript abundance described previously (61). The main difference that exists in their regulation by the biological clock system is the phase of peak expression from the *psbAII* promoter, which occurs 12 h out of phase from either *psbAI* or *psbAIII* (Fig. 4.1). The other class 2 promoters that have been characterized previously (P*purF* and P*opcA*) were both proposed to confer an advantage in that their maximal expression occurs during subjective night, when oxygen-producing photosynthesis is declining, because their protein products are oxygen-sensitive (71, 77). This argument does not hold true for expression of the *psbAII* gene, whose protein would be directly involved in photosynthesis. The distinction between the timing of class 1 and class 2 genes in consecutive LD cycles shows that their expression peaks are not completely out of phase with one another but instead are separated by only 4 h (Fig 4.2).

Within the *psbA* gene family, two genes (*psbAII* and *psbAIII*) encode the same Form II of D1. This sequence deviates from Form I by only 25 amino acids and is involved in the

protection of the photosynthesis apparatus in response to harsh conditions. Both genes are upregulated in response to high light, low temperature, and low $CO_2$ levels (101, 102, 112). Although their regulation is similar on many levels, one major difference has been shown to exist post-transcriptionally. The *psbAIII* transcript is actively degraded at high light intensities, like that of *psbAI,* whereas the *psbAII* transcript remains stable (64). The alternative phases of gene expression as controlled by the circadian rhythm now demonstrate an additional discrepancy between their regulations and perhaps provide insight into the selective advantage of maintaining two copies of the gene.

When the *psbAI* gene is missing from the cyanobacterial cell, the overall levels of *psbAII* and *psbAIII* transcript, as well as Form II of D1, are dramatically increased at low light conditions as an apparent compensatory mechanism for the loss of Form I, the predominant D1 protein in standard conditions (62). It is only in the absence of Form I that detectable levels of Form II protein are present in the thylakoid membrane under low light (13, 61, 62). Presented here are data that show the clock-controlled expression of *psbAII* also exhibits compensatory regulation when the *psbAI* gene is missing from the system; the timing of highest expression is altered to better mimic the pattern of expression normally exhibited by the class 1 *psbAI* promoter. Because the *cis* regulatory elements of the *psbAI* gene are still present, it is the absence of activity of the gene (and perhaps its protein product) that results in altered expression pattern exhibited by the *psbAII* reporter.

The relationship between the biological clock and transcriptional regulation of the *psbA* genes may involve a family of transcriptional regulators belonging to the LysR family. The CmpR protein is a member of the CbbR subfamily of LysR transcription factors. A strain that lacks *cmpR* displays a 3-h delay in the phase of expression from the class 1 P*kaiBC::luxAB* reporter (S. Kutsuna, personal communication). The CmpR protein has also been shown to bind to the palindromic, consensus sequences (typical of binding sites for the CbbR subfamily) in the enhancer elements of both the *psbAII* and *psbAIII* promoter regions (112). In the absence of *cmpR*, the levels of *psbAII* and *psbAIII* transcripts are lower than those of WT cells; however, CmpR is not necessary for the induction of expression by exposure to high light (112).

The *psbAI* untranslated downstream positive element also contains a CbbR consensus binding site, but CmpR is most likely not the protein that binds to that element because there is no discernable difference between levels of *psbAI* transcript with or without CmpR in standard or high light conditions (112). Removal of this stimulatory element showed an 8-h shift in the time of lowest expression of bioluminescence as compared to the full-length promoter (Fig. 4.4). Previous competition experiments demonstrated that protein from cyanobacterial soluble extracts that is capable of binding to the *psbAI* consensus site *in vitro* is also able to bind to the true enhancer element of the *psbAII* promoter (87). Thus, this family of transcription factors may influence the circadian timing of expression through their interactions with elements in the promoters of the *psbA* gene family.

A large chasm exists in understanding how the inactivation of a gene whose product is involved in photosynthesis can lead to the altered timing and overall levels of transcription of other specific genes. Connecting the dots of current research suggests that there may be an internal metabolic feedback from the cell upon the clock and the processes that it controls. It has been shown that the LdpA protein is able to detect changes in the redox state of the cell (*i.e.*, light intensity) to fine-tune the period of the internal oscillation (45). The periodicity of the clock's oscillation controls the rhythmic breathing of the cyanobacterial chromosome; the nucleoid of the cell is diffuse during the day and then tightly compacts in anticipation of the (subjective) light to (subjective) dark transition (109). It is thought that the change in chromosome condensation and the resulting alterations in DNA topology and supercoiling affect the ability of the transcriptional machinery to access the promoter regions of certain genes. Dissection of the *purF* promoter failed to provide evidence of any specific *cis* regulatory elements that confer class 2 phase to the expression pattern of bioluminescence reporters (78). The current model depicts the class 2 genes under control of promoters that are accessible only when the nucleoid is in the more compacted, negatively-supercoiled state. The binding of transcription factors, like those of the CbbR family, could also lead to subtle changes in supercoiling that could affect patterns of gene expression in coordination with clock-controlled topology alterations. The fact that the inactivation of the *psbAI* gene can alter the phase of *psbAII* expression suggests that within the cell a complex metabolic connection exists that links the photosynthetic apparatus, via the clock, to the

adjustment of chromosome condensation that would result in a 12 h change in phase of peak expression from at least the class 2 *psbAII* promoter.

Overall, the clock-controlled transcription of the *psbA* family offers another opportunity for the cyanobacterium to adjust the ratios of D1 proteins in the cell. The ability to utilize numerous inputs to regulate a family of genes provides greater flexibility for individual gene control and a greater overall stability to the system.

# CHAPTER V

# CikA-INTERACTING PROTEINS INFLUENCE THE PERIOD AND PHASE OF THE CYANOBACTERIAL CIRCADIAN RHYTHM

## *Introduction*

At the heart of the *S. elongatus* circadian system are the *kaiA, kaiB,* and *kaiC* genes, whose protein products comprise the central oscillator that generates the endogenous circadian rhythm (43). The Kai proteins, together with other clock components, form a large heteromultimeric complex – termed a periodosome – during the circadian cycle that is essential for oscillations to be maintained (50). Deletion of any of the *kai* genes results in arrhythmicity (43); missense mutations in the Kai proteins can cause various phenotypes that include arrhythmia, short period, long period, and altered phase of peak gene expression (43, 91). The temporal information of the periodosome proceeds through output pathways to control genome-wide transcription (72). The known components of the output pathway include the group 2 sigma factors (86) and an HPK, SasA (48), which physically associates with the periodosome at specific times throughout the circadian cycle (50).

The internal oscillation produced by the Kai proteins is reset daily by LD cycles to produce an exact 24-h rhythm and maintain synchrony with the environment. The input pathway of the circadian system receives these external signals and transmits the information to the oscillator. Photoreceptors that directly connect the perception of light to the phase of the rhythm have been identified in many eukaryotic model systems. In *N.*

*crassa* the blue light photoreceptor, WHITE COLLAR-1, forms a complex with the WHITE COLLAR-2 protein; this heterodimer can then bind to the light-responsive elements in the promoter of the central oscillator gene, *frq*, to induce high levels of expression and reset the clock (25, 39). The *D. melanogaster* photoreceptor CRYPTOCHROME undergoes a conformational change in response to light, which allows it to bind to the clock protein TIMELESS and trigger the degradation of TIMELESS (8). This sudden decrease in TIMELESS protein will cause either phase advances or delays depending on the time within the circadian cycle that the organism is exposed to light.

The means by which the circadian rhythm of *S. elongatus* is synchronized with environmental signals is not well understood. The free-running circadian period can adjust acutely in response to changes in light intensity through parametric entrainment (51). At low light intensity (25 μE $m^{-2}$ $s^{-1}$), the FRP of WT *S. elongatus* is nearly 26 h. When the intensity of light increases to 250 μE $m^{-2}$ $s^{-1}$, the period length decreases (reflective of a faster clock) to approximately 24.5 h. Strains that lack the *ldpA* gene no longer recognize the differences in light intensity as a signal to alter the circadian period in the cell. The *ldpA* mutant maintains the short period associated with higher light intensities regardless of the amount of light it receives (51). The LdpA protein contains iron-sulfur clusters that allow LdpA to sense the redox state of the cell (45), which in cyanobacteria reflects the amount of light acquired and used in PSII, and pass that temporal information to the central clock components by an unknown mechanism.

Nonparametric entrainment (*i.e.*, phase resetting) of the clock has been shown to occur in response to dark pulses (105), pulses of KaiC overexpression (43), and pulses of SasA overexpression (48). The CikA protein is a major component of this nonparametric pathway. Cells that lack *cikA* do not reset the phase of peak expression from a bioluminescence reporter in response to dark pulses that, at specific times during the circadian cycle, can reset the phase of WT cells by up to 8 h (105).

The CikA protein contains three recognizable functional domains. The N-terminal region contains a GAF domain that lacks the conserved cysteine residue typically involved in bilin binding of phytochrome-type photoreceptors (105). Attempts to purify CikA with a covalently-bound bilin *in vivo* failed (84), and current models hypothesize that CikA does not act as a photoreceptor. The central HPK domain is capable of binding and hydrolyzing ATP to autophosphorylate at a conserved histidine residue (84). HPK proteins are usually part of two-component bacterial signal transduction systems. The HPK protein autophosphorylates in response to a signal and then transfers that phosphoryl group to a partner RR protein, which in turn brings forth an appropriate reaction within the cell to properly respond to the stimulus (110). At present, the RR for CikA has not been identified (discussed further in Chapter VI). At the C-terminus of CikA is a PsR domain that is hypothesized to be involved in protein-protein interactions (130). It is the PsR domain that is responsible for the *in vivo* localization of CikA to the poles of the cell; when the PsR domain is removed, a fluorescent variant of CikAΔPsR can be visualized as a diffusible signal throughout the cytoplasm (130).

The prediction that the PsR domain interacts with other proteins that are likely involved in the circadian clock system has led to multiple sets of experiments in an attempt to identify these components. CikA is part of a complex that contains at least the LdpA and KaiA proteins (45). The interaction between CikA and KaiA is likely indirect (possibly via LdpA) because the copurification of LdpA and CikA is maintained in the absence of *kaiA*, and the interaction between KaiA and LdpA is not interrupted in a *cikA* mutant strain (45). Additionally, yeast two-hybrid systems failed to detect an interaction between CikA and any of the three Kai proteins (S. R. Mackey, J.-S. Choi, and S. S. Golden, unpublished data).

This accumulation of anecdotal evidence for CikA's involvement in protein-protein interactions suggests that there are previously-unidentified proteins that are critical for adjusting the period or phase of the rhythm to match that of the environmental day. To find these clock components, we used a yeast two-hybrid system to identify proteins that directly interact with CikA. Five proteins were discovered that consistently passed the stringent selective requirements of the system by their interaction with CikA in yeast. The role of each of these proteins in the circadian system was assayed using both null and overexpression alleles. The data in this chapter show that, of the five candidates, one CikA-interacting protein affects the resetting ability of the clock and two others help to maintain correct periodicity of the *S. elongatus* circadian rhythm.

## *Methods*

### Yeast two-hybrid assay

Expression vectors for CikA variants fused to the GAL4 DNA-binding domain were created by amplifying the open reading frame (ORF) of *cikA* using PCR with primers that incorporate *Nde*I and *Sal*I sites and ligating the digested PCR product to *Nde*I/*Sal*I-digested pGBKT7 (Clontech). The full-length *cikA* sequence encodes 754 amino acids, CikA-GHR bait encodes residues 87 – 754, CikA-HR encodes amino acids 322 – 754, and CikA-R encodes residues 611 – 754. A prey library was obtained from H. Iwasaki (Nagoya University) that contains 1 – 3 kb fragments of *Sau*3AI-digested *S. elongatus* genomic DNA ligated to the *Bam*HI site of the prey vector, pGADT7 (Clontech).

Bait plasmids were introduced to yeast strain AH109 using the small-scale transformation procedure described by Clontech. Yeast strains that contained the CikA bait were then transformed with the prey library using the large-scale transformation protocol. Doubly-transformed cells were plated on Minimal Synthetic Dropout medium (SD) that lacks tryptophan, leucine, adenine, and histidine at 30ºC for 5 – 7 days. For the β-galactosidase colony lift filter assay, colonies were patched onto selective plates and allowed to grow at 30ºC for 2 – 3 days. Yeast cells were transferred to 75 mm diameter filters (VWR) that had been soaked in Z buffer/X-gal solution [60 mM $Na_2HPO_4$, 40 mM $NH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, and 250 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)], immersed in liquid nitrogen for 10 s, allowed to thaw at room temperature, and then incubated at 30ºC. The positive controls provided with the

Clontech system encode fusions of the GAL4 DNA-binding domain to murine p53 (P53) and the GAL4 activation domain to the SV40 large T-antigen (T), which strongly interact with one another. The negative control uses a human lamin C bait (Lam) that does not interact with the T-antigen prey (Clontech).

Prey plasmids were extracted from yeast strains and used to transform DH10B *E. coli* cells. These clones were extracted from *E. coli* using the QIAprep miniprep kit (Qiagen), sequenced, and used in BLAST (1) analysis to provide a prediction of their function based on their similarity to other sequences in the database.

**Bacterial strains and plasmids**

The plasmids and strains used in this study are described in Tables 5.1 and 5.2, respectively. All plasmids were maintained in DH10B *E. coli* cells. Cyanobacterial reporter strains were created in *S. elongatus* PCC 7942. Recombinant overexpression constructs were integrated at NS1 of the *S. elongatus* chromosome. AMC462 (P*kaiBC::luxAB*) and its derivatives integrate the luciferase reporter at NS1, and the genes required for synthesis of the long-chain aldehyde substrate, driven by the *psbAI* promoter (P*psbAI::luxCDE*), are integrated at NS2. AMC669 (P*psbAI::luxAB;* P*psbAI::luxCDE*) and its derivatives carry both the luciferase reporter and necessary enzymes at NS2.1. AMC1004 and its derivatives harbor the P*kaiBC::luxAB* reporter at NS2.1 and P*psbAI::luxCDE* at the adjacent NS2.2. Plasmids that contain genes interrupted by antibiotic-resistance cassettes were used to transform cyanobacterial

TABLE 5.1. Plasmids used in the study of *cikA-I* genes

| Plasmid | Characteristics | Antibiotic resistance‡ | Source or reference |
|---|---|---|---|
| 1A8-L4 | Source of *Mu* insertion in *nht-1* | Ap; Cm; Km | Lab collection |
| pBlueScript | *E. coli* cloning vector | Ap | Strategene |
| pGADT7 | Prey vector for yeast two-hybrid | Ap | Clontech |
| pGBKT7 | Bait vector for yeast two-hybrid | Km | Clontech |
| pHP45Ω-Km | Source of $Km^R$ Ω-cassette | Ap; Km | 24 |
| pAM2152 | $Gm^R$ cassette in *cikA* | Ap; Gm | 105 |
| pAM2956 | $Km^R$ Ω-cassette from pHP45Ω-Km in pBlueScript at *Eco*RI site | Ap; Km | Lab collection |
| pAM2957 | $Km^R$ Ω-cassette from pHP45Ω-Km in pBlueScript at *Bam*HI site | Ap; Km | Lab collection |
| pAM2991 | NS1 cloning vector; IPTG-inducible promoter | Sp/Sm | 45 |
| pAM3032 | pGADT7-*nht-1* | Ap | This study |
| pAM3034 | pGADT7-*spk* | Ap | This study |
| pAM3035 | pGADT7-*gaf-1* | Ap | This study |
| pAM3036 | pGADT7-*hyp-1* | Ap | This study |
| pAM3037 | pGADT7-*hyp-2* | Ap | This study |
| pAM3038 | pGBKT7-*cikA-GHR* | Km | This study |
| pAM3040 | pGBKT7-*cikA* | Km | This study |
| pAM3041 | pGBKT7-*cikA-R* | Km | This study |
| pAM3044 | $Km^R$-Ω cassette in the *Hind*III site of *hyp-1* in pAM3036 | Ap; Km | This study |
| pAM3045 | $Km^R$-Ω cassette in the *Bam*HI site of *gaf-1* subcloned in pBlueScript | Ap; Km | This study |
| pAM3055 | $Km^R$-Ω cassette in the *Apa*I/*Xba*I sites of *hyp-2* in pAM3037 | Ap; Km | This study |
| pAM3291 | *gaf-1* overexpression construct in pAM2991 | Sp/Sm | This study |
| pAM3292 | *spk* overexpression construct in pAM2991 | Sp/Sm | This study |
| pAM3307 | *nht-1* overexpression construct in pAM2991 | Sp/Sm | This study |
| pAM3330 | $Km^R$-Ω cassette in the *Hind*III/*Xba*I sites of *spk* subcloned in pBlueScript | Ap; Km | This study |

TABLE 5.1 *continued*

| Plasmid | Characteristics | Antibiotic resistance‡ | Source or reference |
|---|---|---|---|
| pAM3627 | *hyp-1* overexpression construct in pAM2991 | Sp/Sm | This study |
| pAM3697 | *hyp-2* overexpression construct in pAM2991 | Sp/Sm | This study |
| pAM3749 | pGBKT7-*cikA-HR* | Km | This study |

‡ Ap, ampicillin; Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin; Sp/Sm, spectinomycin/streptomycin.

TABLE 5.2. Cyanobacterial reporter strains used to study the effects of CikA-I proteins on circadian rhythmicity

| ***S. elongatus* strain** | **Genetic background** | ***cikA-I* plasmid*** | **Reporter†** | **Antibiotic resistance‡** | **Source or reference** |
|---|---|---|---|---|---|
| AMC462 | WT | none | P*kaiBC::luxAB;* NS1 | Sp/Sm; Cm | 86 |
| AMC564 | *cikA* | none | P*kaiBC::luxAB;* NS1 | Sp/Sm; Cm; Gm | 105 |
| AMC669 | WT | none | P*psbAI::luxAB*; NS2.1 | Cm | 86 |
| AMC1004 | WT | none | P*kaiBC::luxAB*; NS2.1 | Cm; Km | 130 |
| AMC1005 | *cikA* | none | P*kaiBC::luxAB*; NS2.1 | Cm; Km; Gm | 130 |
| AMC1245 | WT | pAM3045 | P*kaiBC::luxAB;* NS1 | Sp/Sm; Cm; Km | This study |
| AMC1300 | WT | none | P*kaiBC::luxAB;* NS1 | Sp/Sm; Km | Lab collection |
| AMC1343 | WT | pAM3330 | P*kaiBC::luxAB;* NS1 | Sp/Sm; Cm; Km | This study |
| AMC1434 | WT | pAM3627 | P*psbAI::luxAB*; NS2.1 | Sp/Sm; Cm | This study |
| AMC1459 | WT | pAM3291 | P*psbAI::luxAB*; NS2.1 | Sp/Sm; Cm | This study |
| AMC1460 | WT | pAM3292 | P*psbAI::luxAB*; NS2.1 | Sp/Sm; Cm | This study |
| AMC1465 | WT | pAM3307 | P*kaiBC::luxAB*; NS2.1 | Sp/Sm; Cm; Km | This study |
| AMC1472 | *cikA* | pAM3307 | P*kaiBC::luxAB*; NS2.1 | Sp/Sm; Cm; Km; Gm | This study |
| AMC1473 | *cikA* | pAM3330 | P*kaiBC::luxAB;* NS1 | Sp/Sm; Cm; Km; Gm | This study |
| AMC1475 | *cikA* | none | P*psbAI::luxAB*; NS2.1 | Cm; Gm | Lab collection |
| AMC1476 | WT | 1A8-L4 | P*kaiBC::luxAB*; NS1 | Sp/Sm; Cm; Km | This study |
| AMC1478 | WT | pAM3697 | P*psbAI::luxAB*; NS2.1 | Sp/Sm; Cm | This study |
| AMC1481 | *cikA* | pAM3292 | P*psbAI::luxAB*; NS2.1 | Sp/Sm; Cm; Gm | This study |
| AMC1482 | *cikA* | pAM3697 | P*psbAI::luxAB*; NS2.1 | Sp/Sm; Cm | This study |

* All overexpression constructs are integrated at neutral site I (NS1), following homologous recombination with the indicated plasmid, which does not replicate or persist in the cyanobacterial cells.

† Reporter constructs are integrated at NS1 or NS2 following homologous recombination. All reporter strains also contain P*psbAI::luxCDE* in the NS (either 1 or 2) not occupied by the *luxAB* reporter. AMC669 and its derivatives contain both P*psbAI::luxAB* and P*psbI::luxCDE* in NS2.1.

‡ Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin; Sp/Sm, spectinomycin/streptomycin

reporter strains. Following homologous recombination, mutant allele replacement at the native locus was verified by PCR and compared to the WT allele (14, 27).

**Media and growth conditions**

All cyanobacterial strains were grown and maintained as described in Chapter II. Cultures were grown at 30ºC with continuous illumination of 200 μE $m^{-1}$ $s^{-2}$. Cultures of at least an $OD_{750}$ of 0.3 were used to monitor the levels of bioluminescence using the Packard TopCount luminometer(Perkin-Elmer), as described in Chapter II.

**DNA manipulations and sequencing**

Plasmid DNA for sequencing and subcloning was purified using QIAprep miniprep kit (Qiagen). Sequencing was performed using the BigDye terminator mix (Applied Biosystems) according to the manufacturer's instructions. PCR primers were generated by Integrated DNA Technologies, Inc., and sequencing reactions were run at the Gene Technology Laboratory (Institute of Developmental and Molecular Biology, Texas A&M University). Restriction endonuclease digestions were performed using the manufacturer's instructions (New England Biolabs) and DNA fragments for subcloning were purified from agarose gels using the Wizard SV Gel and PCR Clean-up System (Promega).

**Construction of *cikA-interactor* (*cikA-I)* null alleles**

Independent null alleles of each *cikA-I* gene were created by insertion of an antibiotic-resistance cassette in the coding region of each gene. A Cm-resistance *Mu* transposon inserted in the *nht-1* gene gave rise to the 1A8-L4 clone. The Km-resistance Ω-cassette ($Km^R$-Ω) from pHP45Ω-Km (24) was used to inactivate the other four genes. The full-length sequence of *spk* was cloned into pBluescript (Stratagene) at *Hin*dIII and *Xba*I sites. This clone was digested with *Sty*I and *Sfo*I, which removed almost 800 bp of the ORF, and ligated to the $Km^R$-Ω from pAM2957 that had been digested with *Xba*I and *Eco*RV to create pAM3330. In constructing *gaf-1ΩKm*, the full-length *gaf-1* sequence was cloned in pBluescript. Inverse PCR was used to create two *Bam*HI sites 400 bp apart within the *gaf-1* ORF. The resulting inverse PCR product and pHP45Ω-Km were digested with *Bam*HI and ligated to create pAM3045. The *hyp-1ΩKm* construct (pAM3044) was created by inserting a *Hin*dIII-digested $Km^R$-Ω into the *Hin*dIII site of pAM3036. To create *hyp-2ΩKm* (pAM3055), $Km^R$-Ω was first subcloned into the *Eco*RI site of pBluescript to create pAM2956. pAM3037 and pAM2956 were each digested with *Apa*I and *Xba*I and $Km^R$-Ω from pAM2956 was ligated to pAM3037.

**Construction of *cikA-I* overexpression constructs**

The ORF of each *cikA-I* gene was amplified from *S. elongatus* genomic DNA by PCR with primers that incorporate the codons to encode six adjacent amino- or carboxy-terminal histidine residues (N-6xHis or C-6xHis, respectively) for immunoblot detection and restriction enzyme sites that were compatible for cloning into the NS1 vector

pAM2991. The primers for *nht-1, hyp-1,* and *hyp-2* incorporated 5' *Eco*RI and 3' *Bam*HI sites and encoded an N-6xHis tag. The primers for *gaf-1* each included *Bam*HI sites and encoded a C-6xHis tag. The *spk* primers each included *Eco*RI primers and encoded a C-6xHis tag. Each PCR product was purified with the Wizard SV Gel and PCR Clean-up System (Promega), digested with *Eco*RI and/or *Bam*HI, and ligated to digested pAM2991.

**Whole-cell extract preparation and immunoblot analyses**

Whole-cell protein extracts from *cikA-I* overexpression strains were prepared as described elsewhere (17) from 50 ml of an *S. elongatus* culture with an $OD_{750}$ of 0.4 (or higher) grown in LL. Equal amounts of soluble protein (10 – 20 μg) were separated on 12.5% SDS-PAGE and transferred to 0.45 μm Protran nitrocellulose membrane by semi-dry blot transfer per manufacturer's instructions (Bio-Rad). His-tagged proteins were detected using a Penta-His antibody (Qiagen) according to the manufacturer's directions and using a peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). The signal was visualized with the SuperSignal West Pico chemiluminescent substrate detection system (Pierce) and exposed to X-ray film. Overexpression of NHT-1, Spk, and Hyp-2 proteins were verified. Prior to induction, the His-tagged Spk and Hyp-2 proteins could not be detected and NHT-1 produced only a very low signal with the Anti-His immunoblot. After induction, protein levels increased substantially – approximately 30 fold – although this value is unreliable as the uninduced signal was so low. His-tagged Hyp-1 was not detected, most likely because the placement of the 6xHis

tag resulted in its being cleaved. Hyp-1 overexpression was verified in *E. coli* by visualizing protein levels with Coomassie Brilliant Blue strain. GAF-1 protein could not be detected in either cyanobacterial or *E. coli* cell extracts despite using multiple constructs and experimental conditions.

**Measurement and analysis of *in vivo* bioluminescence**

Automated measurement of bioluminescence from *S. elongatus* reporter strains was performed as described in Chapter II. Data were recorded from inoculated 96-well plates for two synchronizing LD cycles and 5 – 7 days in LL. For overexpression experiments, 1 mM final concentration isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to WT and *cikA* mutant strains that do not harbor the overexpression constructs as negative controls; the addition of IPTG to these strains did not change the period or phase of the rhythm of bioluminescence. Period values for each strain, with or without the overexpression construct, were determined using at least two cycles before the addition of IPTG (pre-IPTG) and at least three cycles after the addition of IPTG (post-IPTG).

All data obtained from the TopCount were graphed using the I&A Excel interface (98), as described in Chapter II. The circadian period and standard deviation were calculated using at least three cycles of data obtained in LL by the Biological Rhythms Analysis Software System (available from A. Millar and P. Brown, University of Edinburgh, Edinburgh, UK).

Phase resetting was assayed by removing an inoculated 96-well plate from the stacker of the TopCount machine and placing it in the dark for 5 h at 30ºC; an empty, black plate was put in the stacker in its place. Plates were removed during the first cycle in LL at 3, 5, 8, or 10 h after the last synchronizing LD cycle. Phase shifts were determined by comparing the phase of peak expression of bioluminescence from samples that had been subjected to the dark pulse to an identical plate that remained in LL.

**Light microscopy**

Bright-field images were captured through an Olympus IX-70 microscope with a Hamamatsu ORCA-ER camera using the 100X immersion oil objective lens. Images were modified with Adobe Photoshop 7.0 to increase resolution and improve contrast.

***Results***

**Identification of CikA-I proteins**

The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used to identify proteins from *S. elongatus* that interact with CikA. The HPK- and PsR-encoding regions of the *cikA* gene were cloned into pGBKT7 to be expressed as a protein fusion to the GAL4 DNA-binding domain as "bait" (CikA-HR, Fig. 5.1A). A "prey" library from H. Iwasaki (Nagoya University) was obtained that contains 1 – 3 kb fragments of *S. elongatus* genomic DNA in pGADT7 which are expressed as fusion proteins to the GAL4 activation domain. Interaction between CikA bait and a prey protein activated

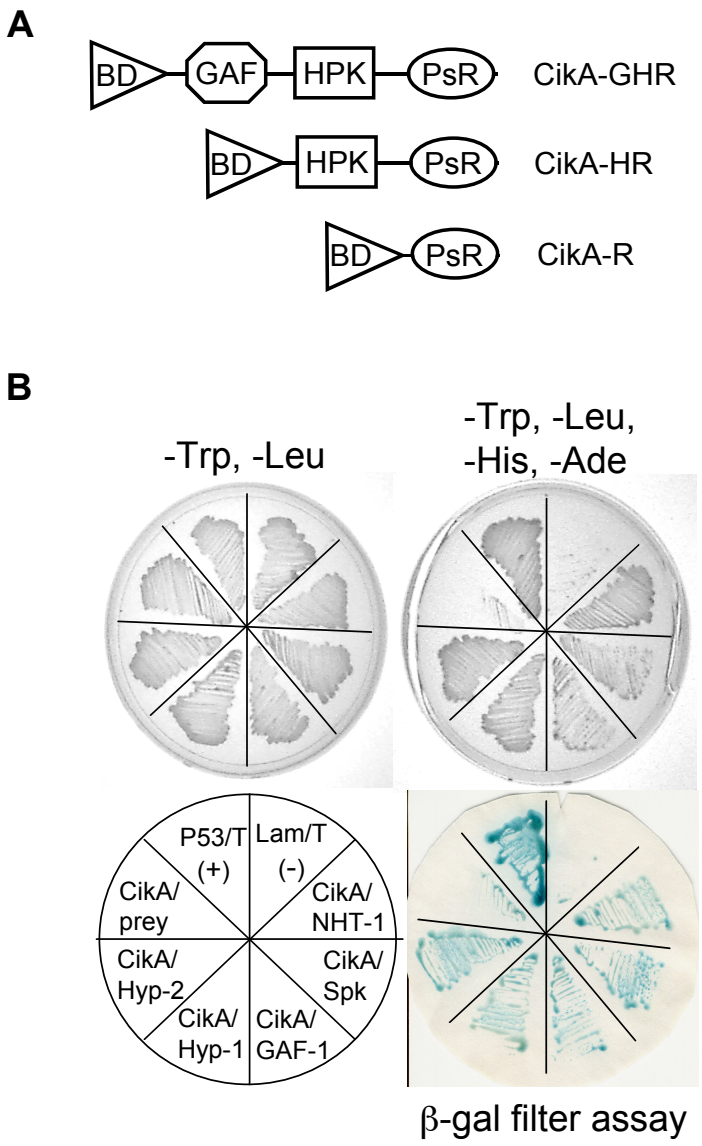


FIG. 5.1. Identification of CikA-interacting proteins. (A) Schematic representation of the CikA bait constructs. Each bait contains the GAL4 DNA-binding domain (BD) fused to the GAF, HPK, or PsR domains of CikA. (B) One colony of doubly-transformed yeast clones that contain one prey and one bait were streaked onto agar media deficient in tryptophan and leucine (top left) to select for the plasmids or deficient in tryptophan, leucine, histidine, and adenine (top right) to select for interaction between the prey and bait proteins. β-galactosidase activity was monitored by a filter assay in the presence of X-gal (bottom right). A plate map (bottom left) shows the bait and prey constructs used.

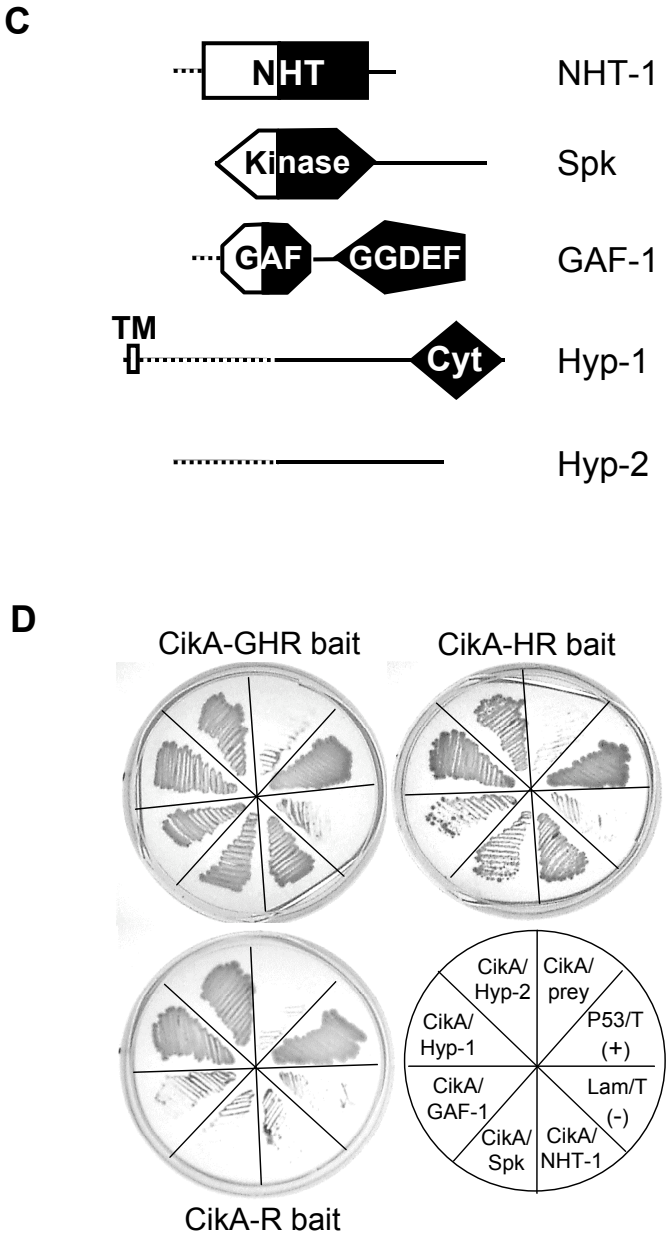


FIG. 5.1. *continued*. (C) Schematic representation of the functional domains of each CikA-interacting protein. Solid lines and black shapes with white print depict the portion of the protein fused to the GAL4 activation domain in the prey. Dotted lines and white shapes with black writing depict the remainder of the full-length protein. TM, transmembrane domain; Cyt, cytochrome c-like domain. (D) Domain mapping of interactions between variant CikA baits and CikA-I preys. CikA-I preys were doubly-transformed with CikA-GHR bait (top left), CikA-HR bait (top right), or CikA-R bait and plated on medium that is deficient in tryptophan, leucine, histidine, and adenine to select for interaction. A plate map (bottom right) depicts the order of plating the cells.

transcription of four reporter genes, two of which encode enzymes necessary to complement nutritional deficiencies in the adenine and histidine biosynthetic pathways.

Seven clones, representing five genes, expressed proteins that consistently supported growth in the presence of CikA-HR in yeast on selective medium (Fig. 5.1B), and produced a dark blue color in colony lift filter β-galactosidase assays (Fig. 5.1B). Sequence similarity suggested homology to proteins that contain the following functional domains: a class V aminotransferase (NHT-1), a serine/threonine protein kinase (Spk), a GAF-GGDEF (GAF-1), and two different conserved hypothetical proteins (Hyp-1 and Hyp-2). Schematic representations of the predicted domains are shown in Figure 5.1C. Spk and Hyp-1 were each identified from two independent prey clones. The full-length sequences of the two hypothetical proteins are conserved among the completed cyanobacterial genomes that are available with the exception of *Gloeobacter violaceus* PCC 7421, which interestingly also does not contain *kai* genes and is the only cyanobacterium known that does not possess thylakoid membranes (90). The N-terminal region of Hyp-1 contains a predicted signal peptide sequence and a single transmembrane domain; the C-terminus has a domain similar to the active domain of cytochrome *c*, which may be involved in sensing the redox state of the cell. No predicted domains can be assigned to the primary sequence of Hyp-2.

To further test the interaction between CikA and the identified proteins, bait constructs were used that encode different domains of the CikA protein. All five preys were able to

interact with either CikA-GHR (GAF, HPK, and PsR) or CikA-HR bait (as was used in the original screen; Fig. 5.1D); only the two conserved hypothetical proteins interacted with bait that contained only the PsR domain (Fig. 5.1D).

**Inactivation of *spk* alters phase resetting**

In addition to the inability to reset their clocks in response to darkness, strains that lack *cikA* have a shortened FRP of approximately 22 h in LL conditions (105), compared with approximately 25 h for WT strains. To determine the role that each *cikA-I* plays in the circadian rhythm of *S. elongatus*, null alleles were created by interrupting each ORF with an antibiotic-resistance cassette (Table 5.1; see Methods for details). The resulting insertional mutations were used to transform cyanobacterial P*kaiBC::luxAB* luciferase reporter strains, and rhythms in bioluminescence were monitored in LL. No difference in period, phase, or amplitude could be detected between WT and each mutant reporter strain for any of the loci (data not shown). A possible explanation for this result is that the targeted genes are essential for viability, and the strains selected are merodiploids; *S. elongatus* harbors 6 – 8 copies of its genome (81) and it is possible to obtain transformants in which the antibiotic-resistance cassette disrupted enough copies of the gene to allow the organism to live on selective medium even though the organism still harbors sufficient WT copies of the gene to carry out its function. PCR analysis of genomic DNA from putative null mutant strains showed that the inactivated alleles for *hyp-1* and *hyp-2* were not segregated: both inactivated and WT copies of the gene were present (data not shown). The other three null strains (*nht-1, spk,* and *gaf-1*) were fully

segregated but showed no mutant circadian phenotype in standard free-running LL conditions.

Because each of these five CikA-I proteins interacts with CikA in the yeast system, it was predicted that they are involved in an input pathway to the clock. A *cikA* null strain is defective in its ability to reset the phase of its rhythm in response to 5-h dark pulses (105); therefore, each mutant strain was tested for the ability to recognize an exposure to darkness and reset its rhythm as a result. After two synchronizing LD cycles, the three fully-segregated mutant strains (*nht-1, gaf-1,* and *spk*) were subjected to dark pulses at four different points (LL3, LL5, LL8, and LL10) during the first cycle of LL to assess their roles in phase resetting. The response of the WT cells was the largest and most stable at LL8, a point in mid-subjective day when the cells would normally be in the light; therefore, we focused on the phase shift that occurred at that time point. When WT cells were subjected to 5 h of dark beginning at LL8, the bioluminescence from the P*kaiBC::luxAB* reporter consistently advanced the phase of its peak expression, such that its peak occurs about 6 h earlier than cells maintained in LL (Fig. 5.2A). Both the *nht-1* and *gaf-1* strains showed WT resetting (data not shown); the *spk* null, however, showed a different magnitude of phase shift as compared to WT. A reporter strain lacking the *spk* gene is not blind to the pulse of darkness; as shown in Figure 5.2B, the *spk* mutant resets its rhythm unpredictably, even among independent samples of the same clone, with phase advances ranging from 6 h to 11 h. With each experimental trial, the *spk* mutant consistently shifted differently than WT, although the magnitude and direction of

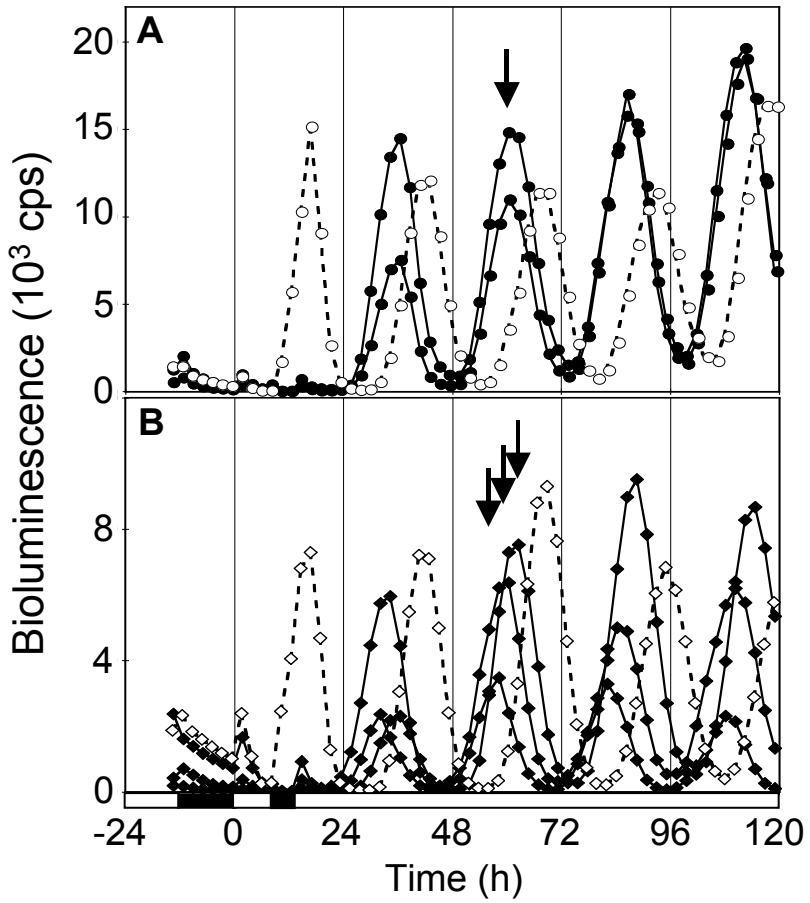


FIG. 5.2. Proper phase resetting requires the *spk* gene. Bioluminescence from (A) a WT P*kaiBC::luxAB* reporter and (B) an *spk* inactivation mutant. Open symbols, LL with no dark pulse; closed symbols, 5-h dark pulse at LL8. Two independent traces of WT demonstrate the reproducible phase resetting. Three independent traces of the *spk* mutant are shown to demonstrate the unpredictable magnitude of the phase shift. Arrows represent the phases of peak expression after the dark pulse.

the phase shift varied between trials; at times, the same liquid culture of an *spk* mutant strain would exhibit both advances and delays even when all other experimental parameters were identical (Fig. 5.3). In contrast, all samples of a WT clone respond identically, and the direction and magnitude of resetting in the WT is predictable and reproducible.

Because CikA is necessary for the circadian system to reset to environmental cues, the ability to respond to dark pulses was also tested in a strain that lacks both *spk* and *cikA*. Consistent with previously published data, the *cikA* null strain was blind to the dark pulse at LL8 and did not change its pattern of rhythmicity (Fig. 5.4A). The double mutant resembled that of the *cikA* strain with no noticeable phase resetting (Fig. 5.4B). Neither the *cikA* nor the *spk/cikA* mutant was capable of recognizing the external signal at other times throughout the circadian cycle (data not shown). Thus, the altered resetting exhibited by the absence of the Spk protein requires CikA, and without CikA present the cells are unable to respond to environmental cues and adjust their circadian timing accordingly.

**Overexpression of NHT-1 or Hyp-1 protein causes a long circadian period**

Overexpression of many cyanobacterial clock proteins, including KaiA, KaiC, and CikA, will cause arrhythmia (43, 130), presumably by titrating away interacting clock components and disrupting the flow of communication. Overexpression of other clock-related proteins alters circadian period but the rhythm remains robust (86). We tested

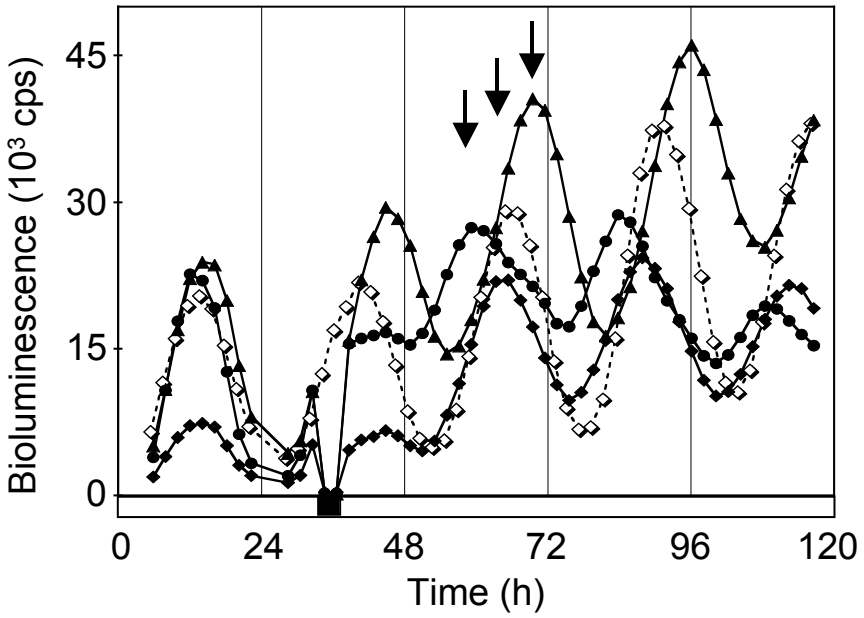


FIG. 5.3. An *spk* mutant resets its phase in an unpredictable manner. Bioluminescence from a P*kaiBC::luxAB* reporter in an *spk* null background. Open symbols, LL with no dark pulse; closed symbols, 5-h dark pulse. Three independent traces represent the erratic direction and magnitude of phase shifts that occur in the absence of *spk*. Arrows represent the phases of peak expression after the dark pulse.

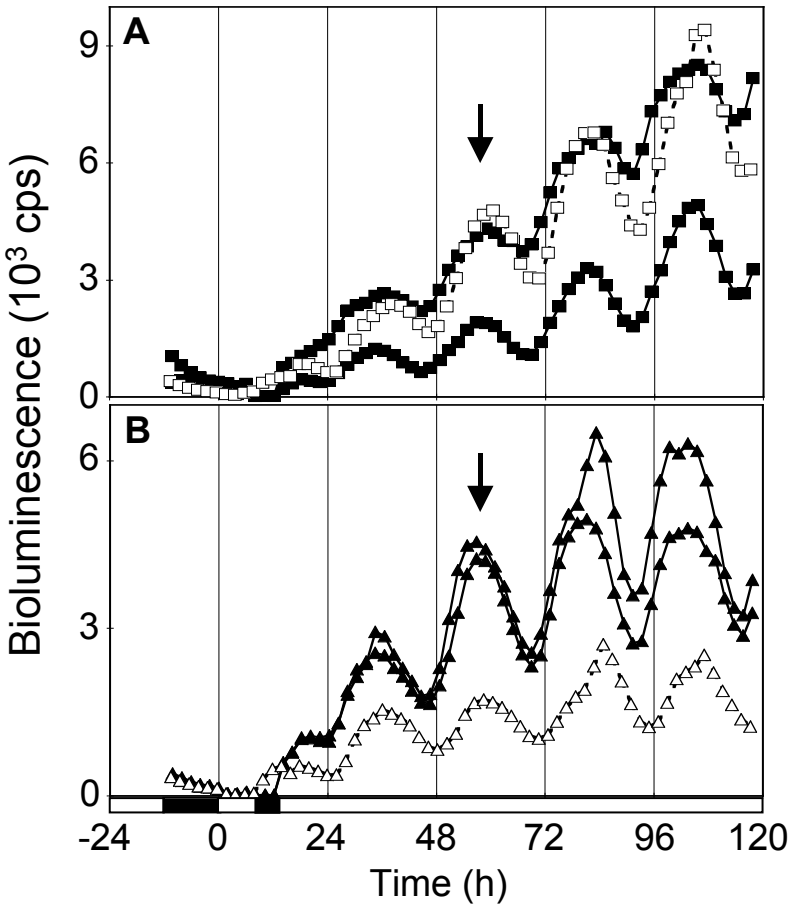


FIG. 5.4. Erratic resetting of the *spk* mutant requires *cikA*. Bioluminescence from a P*kaiBC::luxAB* reporter in a (A) *cikA* null and (B) *cikA/spk* double mutant. Open symbols, LL with no dark pulse; closed symbols, 5-h dark pulse at LL8. Two independent traces demonstrate the reproducible lack of phase resetting when *cikA* is not present in the cell. Arrows represent the phase of peak expression after the dark pulse.

whether overexpression of each of the putative CikA interactors would help to elucidate their roles in the clock where the null alleles may have failed, either because the mutant alleles were unable to segregate completely or because of functional redundancy with other proteins in the cell. Each ORF was cloned downstream of an IPTG-inducible promoter and integrated at NS1 of the *S. elongatus* chromosome of reporter strains. For each of the overexpression experiments, IPTG was added to the WT and *cikA* mutant strains that do not carry the overexpression construct; IPTG did not have an effect on the properties of the rhythm of bioluminescence in those strains. The overexpression of Spk, confirmed by immunoblot analysis, did not cause any significant change to the period or phase of bioluminescence in LL (data not shown). Despite numerous efforts to construct a clone that would overexpress the GAF-1 protein, we were unable to induce expression of this protein in either cyanobacteria or *E. coli*.

The *nht-1* and *hyp-1* overexpression constructs each caused a lengthening of the circadian period upon induction by IPTG (Figs. 5.5 and 5.6). Induction of excess NHT-1 protein caused the period of oscillation to increase by approximately 1 h (Fig. 5.5A and C). To determine whether this long period phenotype is dependent on the presence of the CikA protein, NHT-1 was overexpressed in a *cikA* null strain. A *cikA* mutant displayed the characteristic short period as compared to WT in LL (105) (Fig. 5.4B and C). When NHT-1 was overexpressed in the absence of *cikA,* the period was lengthened by almost 2 h, such that the period matched that of the WT strain with excess NHT-1 protein (Fig. 5.5A and C). Thus, NHT-1 is epistatic to the *cikA* null mutation. These results suggest

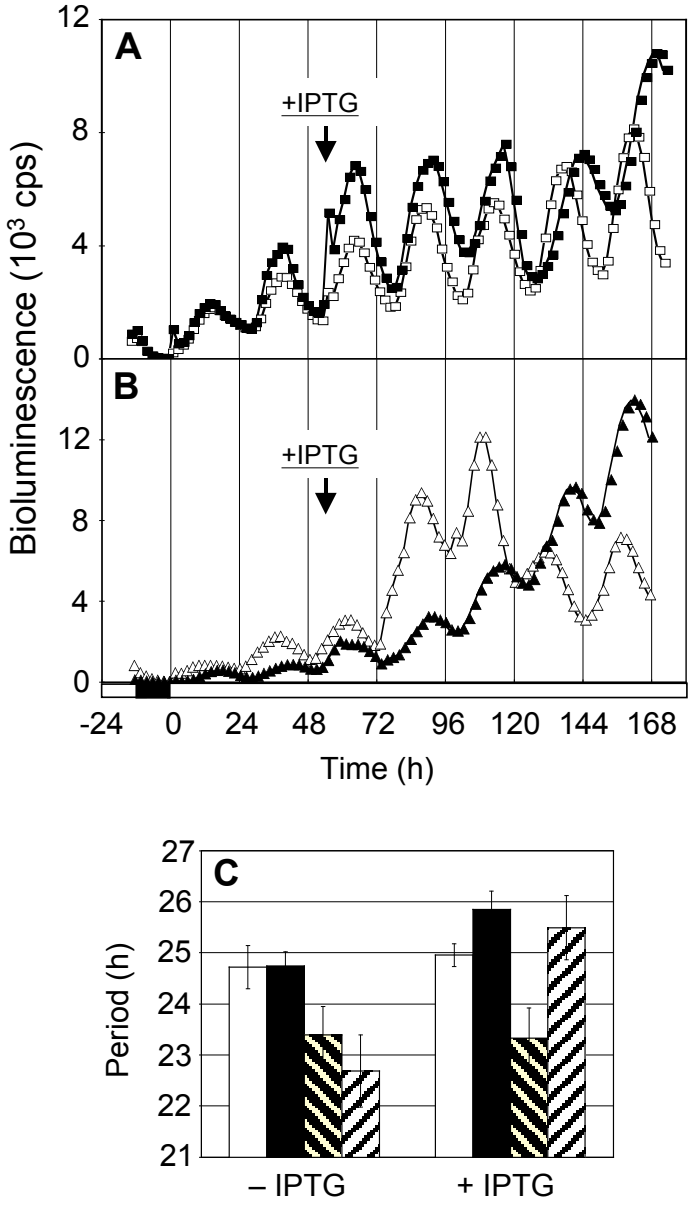


FIG. 5.5. Overexpression of NHT-1 lengthens circadian period independent of *cikA*. (A) Bioluminescence from a P*kaiBC::luxAB* reporter. Open squares, WT; closed squares, NHT-1 overexpression. (B) Bioluminescence from a P*kaiBC::luxAB* reporter in a *cikA* mutant background. Open triangles, *cikA* null; closed triangles, *cikA* null with NHT-1 overexpression. Arrows represent at what point in time 1 mM IPTG was added. (C) Average period length before and after the addition of IPTG. White bars, WT; black bars, NHT-1 overexpression; thick hatching, *cikA* null; thin hatching, *cikA* null with NHT-1 overexpression.

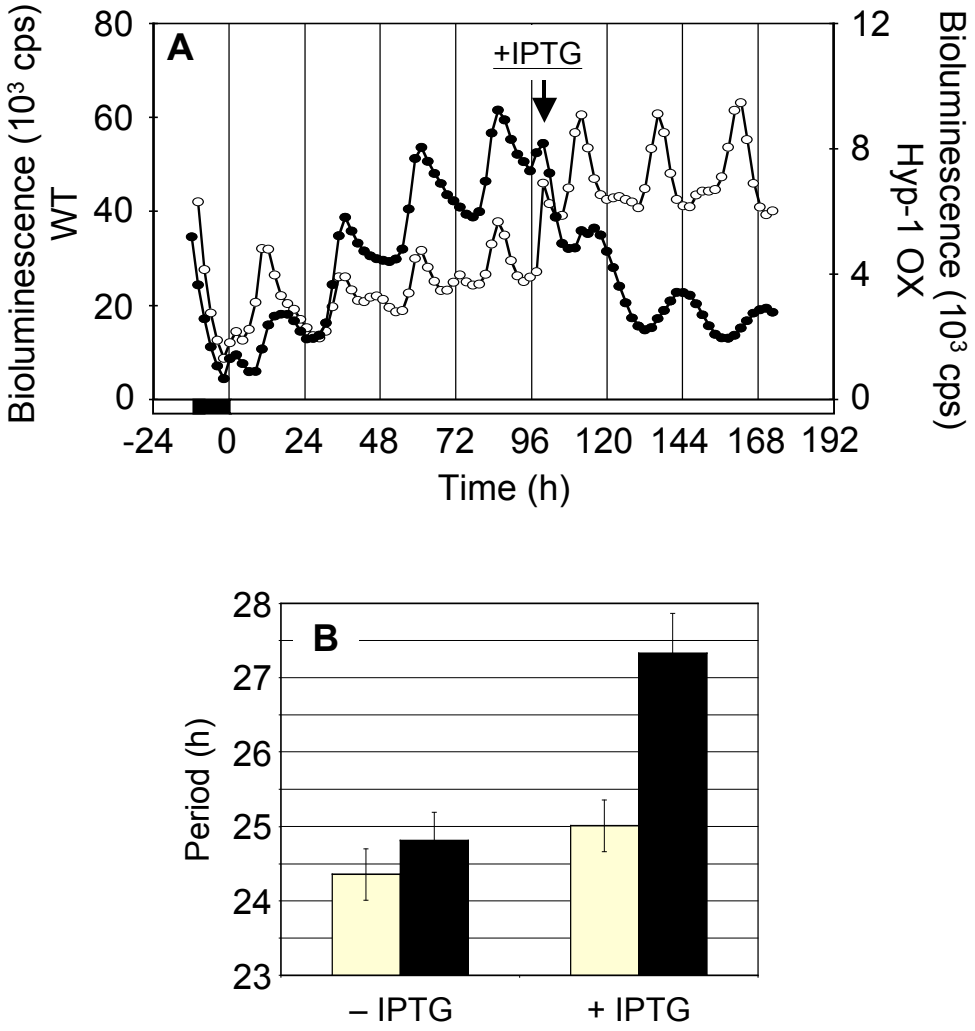


FIG. 5.6. Overexpression of Hyp-1 lengthens circadian period. (A) Bioluminescence from a P*psbAI::luxAB* reporter. Open circles, WT; closed circles, Hyp-1 overexpression. Arrows represent at what point in time 1 mM IPTG was added. (B) Average period length before and after the addition of IPTG. White bars, WT; black bars, Hyp-1 overexpression.

that the increased period length of the rhythm when NHT-1 is supplied in excess does not require CikA and that NHT-1 exerts its effect downstream of CikA in the pathway to the central oscillator.

Overexpression of Hyp-1 also affected the *S. elongatus* circadian rhythm. Hyp-1 production was induced in another standard class 1 reporter P*psbAI::luxAB*, which peaks at subjective dusk like that of the P*kaiBC* reporter. This increased amount of Hyp-1 protein caused the period to lengthen to almost 27 h in LL, 2.5 h longer than that of the WT strain (Fig. 5.6) but the phase of the rhythm did not change.

**Overexpression of Hyp-2 suppresses the cell division defect of a *cikA* mutant**

The Hyp-2 protein has no recognizable motifs in its primary amino acid sequence, and attempts to inactivate the gene *in vivo* were unsuccessful, which suggests that the gene is essential. To ascertain the role that Hyp-2 plays in the circadian system, the protein was overexpressed in both WT and *cikA* null reporter strains. Preliminary data showed that after the addition of IPTG in either a WT or *cikA* mutant background that harbors the Hyp-2 overexpression construct, the majority of wells in a microtiter plate showed a sharp decrease in overall bioluminescence and no discernable rhythm (data not shown). To determine if the induced expression of *hyp-2* was toxic to the cells, Hyp-2 was overexpressed in cyanobacteria and the morphology of the cells was examined 24 h after induction and compared to that in the uninduced samples. The addition of IPTG to the WT strain did not have a noticeable effect on the size or shape of the cells (Fig. 5.7A).

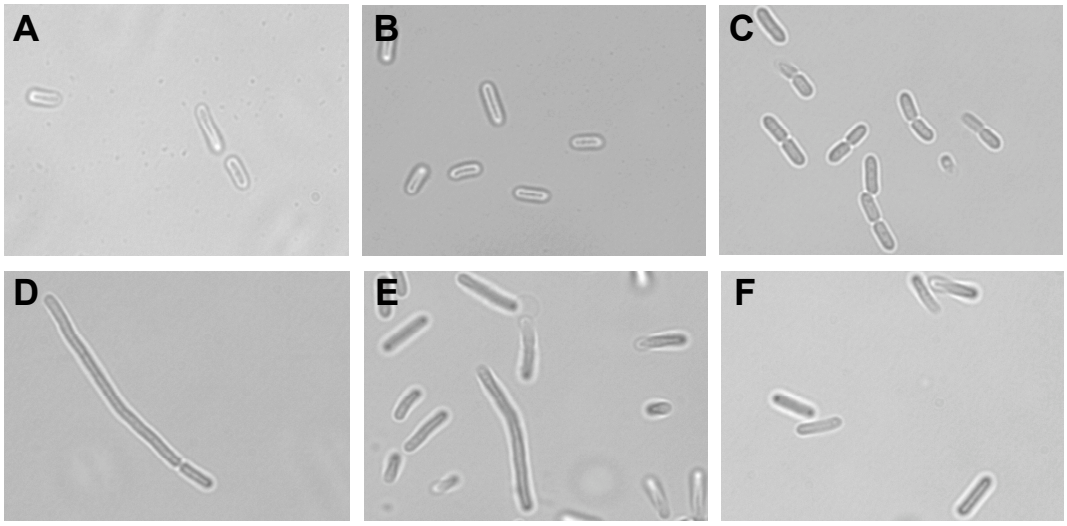


FIG. 5.7. Overexpression of Hyp-2 suppresses the cell division defect of a *cikA* mutant. A second copy of *hyp-2* under the control of an IPTG-inducible promoter was introduced into a WT or *cikA* null cyanobacterial strain and its effect on cell morphology was examined after 20-h induction using 0.5mM IPTG. Addition of IPTG did not alter the size of (A) WT or (D) *cikA* mutant cells. (B) Uninduced cells that harbor the *hyp-2* overexpression construct display a WT cell morphology. (C) Overexpression of *hyp-2* in an otherwise WT background stimulates cell division. (E) In the absence of IPTG, cells that lack *cikA* and carry the *hyp-2* overexpression construct have the characteristic snake-like cell length of a *cikA* mutant. (F) The overexpression of *hyp-2* in a *cikA* background is able to suppress the cell division defect of the *cikA* mutant and the cell size resembles that of WT cells.

Cells that harbor the overexpression construct displayed a WT phenotype in the absence of inducer (Fig. 5.7B). Upon addition of IPTG, the high levels of Hyp-2 protein stimulated cell division such that 20-h after induction nearly every cell visualized was in the process of cell division (Fig. 5.7C).

Cells that lack *cikA* display a defect in cell division such that their length is over twice that of the WT cell (80). In the absence of inducer, cells that lack *cikA* and carry the inducible *hyp-2* construct displayed the characteristic elongated *cikA* cell phenotype (Fig. 5.7D and E). However, when Hyp-2 was expressed at high levels in the absence of *cikA*, the cell division defect was suppressed and cells regained their WT length (Fig. 5.7F), presumably through newly-initiated cell division events. Therefore, the Hyp-2 protein appears to be involved in the regulation of cell division and when produced at high levels can suppress the negative effects exerted by the absence of CikA. The role that Hyp-2 plays in the cyanobacterial circadian rhythm requires more detailed studies.

***Discussion***

Five proteins have been identified through their interaction with the clock input pathway component CikA, three of which have been shown to affect the properties of the circadian rhythm, and one that affects cell division. However, the predicted CikR protein did not come out of this screen. Although some cognate HPK-RR pairs are able to be detected using the yeast two-hybrid system (53, 94), many interactions are brief and

would not provide sufficient interaction to overcome the nutritional selection of the system.

Two of the CikA-interacting proteins, NHT-1 and Hyp-1, substantially changed the period of gene expression when they were produced in excess. Overexpression of NHT-1 increased the period to the same length in either a WT or *cikA* mutant background (Fig. 5.5), which is consistent with a role in the circadian system, specifically downstream of the CikA protein. The NHT-1 sequence is highly conserved among the available cyanobacterial sequences and has significant similarity to the family of class V aminotransferases. The NifS protein also belongs to this family and is involved in the synthesis of iron-sulfur clusters. The presence of such clusters is important to the function of the LdpA protein in sensing changes in redox state (*i.e.*, light intensity) to finely adjust the period length of the *S. elongatus* circadian rhythm. The exact role for NHT-1 will be difficult to determine as there is no general assay that can be used to glean information about its mechanism. However, the rate of CikA autophosphorylation *in vitro* can be examined in the presence of NHT-1 to determine if the interaction between these proteins can enhance or repress the kinase activity of CikA.

The overexpression of Hyp-1 also increased the period length of clock-controlled expression in LL (Fig. 5.6). The predicted domains of Hyp-1 do not suggest any testable enzymatic assays to elucidate its function in the cyanobacterial biological clock, but bioinformatic searches revealed a signal peptide and transmembrane domain at the N-

terminus of the protein. In the yeast two-hybrid system, the C-terminal half of Hyp-1 interacts specifically with the PsR domain of CikA, which is known to be the region that is essential for CikA's localization to the cell poles (130). The working model shows Hyp-1 as the membrane-spanning protein to which CikA binds to localize at the poles; this localization is critical for proper function of CikA because variants that do not localize (those lacking PsR) are not capable of complementing a *cikA* null strain (130). Additionally, the PsR domain has been implicated in repressing the kinase activity of CikA (84) and binding of the PsR domain to Hyp-1 is predicted to remove the repression of the PsR domain to allow phosphotransfer to occur to the predicted CikR protein. A direct test of the role of Hyp-1 in localizing CikA at the pole is complicated by the fact that Hyp-1 appears to be essential for viability. It may be possible to conditionally deplete Hyp-1 by using an antisense RNA strategy (41) to determine whether CikA localization is affected.

The effect of Hyp-2 protein overexpression has not yet been clearly shown to influence the circadian rhythm of the clock, but Hyp-2 does play a role in another cellular process – cell division – in which CikA is involved. A link between cell division and the clock has previously been demonstrated. The biological clock gates cytokinesis such that there are times within the circadian cycle that division is forbidden; the forbidden period in *S. elongatus* extends from late day into early night (81). One possible reason for prohibiting cytokinesis during certain stages of the circadian cycle is that the periodosome complex that contains at least the core components of the central oscillator

may be vulnerable during that time. During the same time period as the phase that cell division is forbidden, the Kai proteins and SasA are beginning their formation of a large multimeric complex that assembles and disassembles over the course of a day; the temporal information that this complex maintains in the mother cell is passed on to the daughter cells with a heritable and precise period and phase (76). Attempting to split up this complex during the forbidden phase of the circadian cycle, when the complex is vulnerable to perturbations, might lead to a disruption of the timing mechanism within the cell.

The functions for CikA and Hyp-2 in the gating process are not yet understood. The use of flow cytometry on strains that express these null and overexpression alleles throughout the course of a day may provide hints at the involvement of these proteins that could more closely link components of the clock system to the regulation of cell division. Time-lapse microscopy after the induction of Hyp-2 production may also offer clues to its function by showing how long after overexpression is initiated the first cell division takes place. The initial thought that increased Hyp-2 protein was lethal to the cells does not appear to be true because growth of those same cells was not hindered when they were examined for cell morphology. On the contrary, the *hyp-2* overexpressing cells appear to divide more than their WT counterparts. The preliminary arrhythmic expression of bioluminescence after the induction of *hyp-2* expression may be the result of a disrupted periodosome that comes about by a forced cell division event.

The unprecedented, erratic phase resetting of the *spk* null mutation places the Spk protein in the input pathway of the cyanobacterial clock. The disrupted resetting caused by the *spk* null requires CikA and, when CikA is absent, the cells cannot respond to light/dark signals to adjust the phase of their rhythm to match their environment. Even among samples that came from the same original liquid culture, the individual wells of microtiter plates exhibit unpredictable resetting in response to a 5-h dark pulse (Fig. 5.2). Individual cells monitored for circadian period and phase in microcolonies inherit the circadian properties through cell divisions without apparent coupling between cells (76). Thus, it is unclear how the entire population of *spk* mutant cells within a well can reset in unison, yet the resetting from clonal siblings on the same plate can be quite different.

By sequence similarity, the function predicted for Spk is to phosphorylate other proteins at serine and/or threonine residues. Among the many possible substrates of Spk is KaiC, which has been shown to autophosphorylate on two residues *in vitro* (92). When either of these phosphorylation sites is eliminated, the rhythms of bioluminescence are lost *in vivo* (127). Although this phosphorylation is due to autokinase activity, it is possible that *in vivo* the autophosphorylation event is required before additional phosphorylation of KaiC by other proteins can occur. A mutation in *kaiC* exists (*pr1*) that abolishes the ability of the strain to reset to dark pulses (55). This mutation also severely decreases the rhythm in the levels of phosphorylated KaiC over the circadian cycle (55). In the *pr1* mutant, KaiC exists in both the phosphorylated and unphosphorylated forms in approximately equal amounts in LL for at least two days. Thus, varying levels of the

post-translational modifications to KaiC are critical for its complete function in the oscillator of the cyanobacterial clock. In the absence of Spk, the phosphorylation status of KaiC may be compromised, which could lead to an inability of the oscillator to accurately reflect changes in the environment.

# CHAPTER VI

# THE HUNT FOR THE PREDICTED RESPONSE REGULATOR PARTNER OF CikA

## *Introduction*

Two-component signal transduction systems are adaptable regulatory systems that have been found to exist in prokaryotes, archaea, and some eukaryotes. These systems act to sense and respond to a myriad of environmental conditions, including such diverse processes as osmoregulation and chemotaxis in *E. coli,* sporulation in *Bacillus subtilis,* and virulence systems that encode vancomycin resistance in *Enterobacter faecalis.*

As the name implies, the original identification of these signal transduction pathways included two parts: an HPK that contains a conserved kinase domain, and an RR that includes a receiver domain (reviewed in reference 110). The HPK senses environmental changes, which trigger the protein to autophosphorylate at a conserved histidine residue using the γ-phosphoryl group from ATP as its phosphodonor (Fig. 6.1). The phosphorylated HPK transfers this phosphoryl group to the RR on a conserved aspartic acid residue in the receiver/regulatory domain. The *trans*-phosphorylation event typically causes a conformational change in the RR receiver domain that results in the activation of an associated effector domain to bring forth an appropriate response to the external stimulation.

More elaborate histidine-to-aspartate phosphotransfer systems exist that utilize the basic signaling elements of the two-component system and incorporate them into more

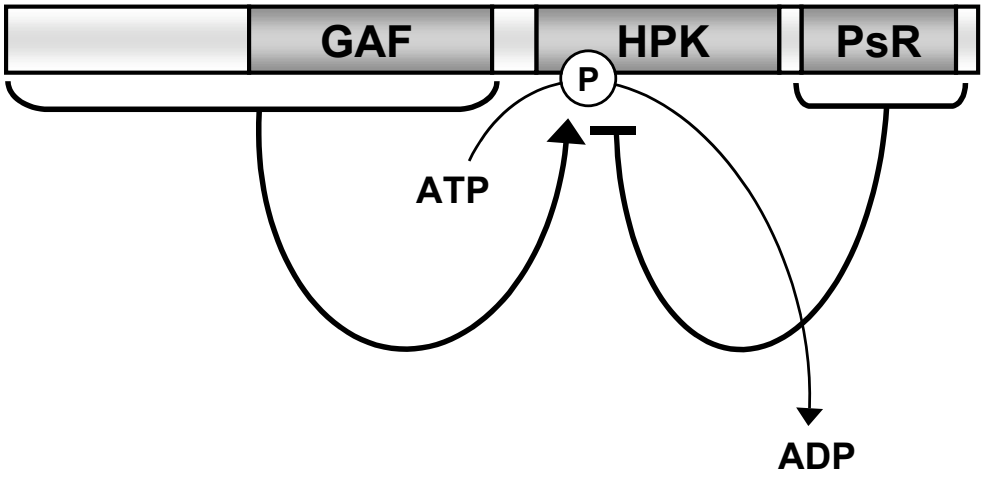


FIG. 6.1. Autoregulation of CikA kinase activity *in vitro*. Schematic representation of the functional domains of the CikA protein. Removal of the GAF-like domain (GAF) decreases the rate of autophosphorylation at the histidine protein kinase (HPK) domain, suggestive of a role in activation. The *pseudo*-receiver (PsR) domain acts to negate the positive role of the GAF by repressing kinase activity; removal of the PsR shows a ten-fold increase in autophosphorylation. Figure adapted from Mutsuda, M. *et al.* J. Biol. Chem. **278:**19102-19110, 2003.

sophisticated phosphotransfer and phosphorelay pathways (110). Many HPK proteins are able to propagate their signal by phosphorylating more than one RR, each of which can then trigger the downstream events that are needed to aid in the cell's adaptation to its environment. Other systems have multiple HPK proteins to regulate only one RR; the effect brought about by that RR is suitable to respond to more than one environmental condition. Hybrid HPKs exist that contain two HPK domains (HPK1 and HPK2) that lie upstream and downstream of a receiver domain, respectively. The phosphoryl group is transferred from HPK1 to the flanked receiver and then again to HPK2; a separate RR protein is able to accept a phosphoryl group from either HPK domain depending upon the cellular conditions. The combination of different domains in each protein, as well as the diversity of the proteins themselves, has resulted in the optimization of responses to a variety of signals.

In *S. elongatus*, at least two HPK proteins – CikA and SasA – are part of the biological clock system. SasA plays a critical part in the output pathway(s) of the clock that transduces temporal information from the Kai oscillator to regulate global gene expression (48). CikA is essential for the ability to reset the endogenous timekeeper such that the circadian rhythm matches that of the external day/night cycle (105). The phenotypes associated with the null and overexpression alleles of these kinase proteins demonstrate their importance in providing information to and from the central oscillator. The ability to autophosphorylate is essential for the functions of each of these proteins; amino acid substitutions at the conserved histidine residues mimic the null phenotypes

(48, 84). Surprisingly, research conducted thus far has failed to identify the cognate RR(s) for these predicted two-component systems.

The phosphorylation state of CikA is regulated by the other regions of the CikA protein. The HPK domain of CikA is flanked by an N-terminal atypical GAF domain and a C-terminal PsR domain, which act opposingly to adjust the level of CikA autophosphorylation (84). Removal of the GAF domain *in vitro* decreases the level of CikA autokinase activity, whereas a CikA variant that lacks the PsR domain has 10-fold higher kinase activity than the WT protein. It is therefore proposed that these domains act as positive and negative elements, respectively, such that the PsR counteracts the enhancement from the GAF domain to repress the level of autophosphorylation *in vivo* (Fig. 6.1).

The CikA PsR domain does not contain the conserved aspartic acid residue that typically accepts the phosphoryl group from an HPK (105); nonetheless the possibility existed that CikA functions as a hybrid HPK/RR protein and is capable of phosphotransfer to its own PsR domain at a different, novel amino acid. The PsR was ultimately eliminated as the true RR through experiments involving two CikA variants (84). A *cikA* mutant lacking the PsR domain (ΔPsR) was allowed to autophosphorylate with radiolabeled [γ-$^{32}$P]ATP and was then combined with a *cikA* mutant that cannot autophosphorylate (H393A) but contains a functional PsR. After a 2-h incubation, no phosphotransfer from ΔPsR to H393A could be detected. It is thus presumed that CikA instead transfers its

phosphoryl group to a true RR that plays a critical role in the input pathway of the cyanobacterial biological clock.

The connection between CikA and the Kai-based oscillator is still unclear. The functional HPK domain of CikA and the failure to transfer a signal via phosphorylation of its own PsR domain both suggest that there is a partner RR for CikA. Additional evidence arose when cyanobacterial soluble protein extracts from a *cikA* null strain were radiolabeled with [$\gamma$-$^{32}$P]ATP in the presence or absence of recombinant full-length CikA protein. Radioactive bands representing proteins of approximately 30 kDa and 45 kDa appeared only when CikA was added (Fig. 6.2; M. Mutsuda and S. S. Golden, unpublished data), suggestive of at least one RR that can accept the phosphoryl group, although the possibility that those bands were degradation products of CikA has not been fully tested.

The work described in this chapter used three different methods in attempt to identify and characterize the RR of CikA, named CikR. The projects include a genetic screen in *E. coli* that was previously used to identify SasA, *in vitro* affinity chromatography of five candidate receiver proteins and subsequent phosphotransfer experiments, and introduction of null alleles of candidate *cikR* genes in cyanobacterial reporter strains to assess their role in the circadian rhythm of *S. elongatus*. The experiments were conducted by three undergraduate students under my direct supervision. Lynette

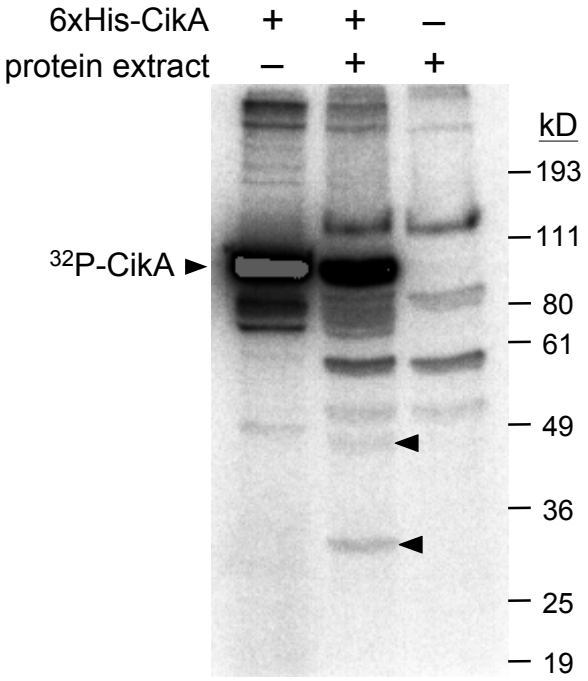


FIG. 6.2. Phosphorylation of two bands in cyanobacteria requires CikA. Soluble protein extracts from a *cikA* null strain were radiolabeled with [γ-$^{32}$P]ATP in the presence or absence of recombinant 6xHis-tagged CikA. Two bands of about 30 kD and 45kD, marked by arrows, are visible in the extract that contains CikA but not in its absence.

Albrecht used a genetic screen in *E. coli*, and Erica Schoeller and Hannah Malcolm used a biochemical approach to attempt to identify CikR.

## *Methods*

### Bacterial strains and plasmids

All plasmids were maintained in DH10B *E. coli* cells. Cyanobacterial reporter strains were created in *S. elongatus* PCC 7942. AMC669 (P*psbAI::luxAB;* P*psbAI::luxCDE*) and its derivatives carry both the luciferase reporter and necessary enzymes at NS2.1. AMC1004 and its derivatives harbor the P*kaiBC::luxAB* reporter at NS2.1 and P*psbAI::luxCDE* at the adjacent NS2.2. Plasmids that contain genes interrupted by antibiotic-resistance cassettes were used to transform cyanobacterial reporter strains. Following homologous recombination, mutant allele replacement at the native locus was verified by PCR and compared to the WT allele (14, 27).

### Media and growth conditions

The *E. coli* cultures were maintained in LB broth or on LB agar plates with antibiotics in the following concentrations: Cm, 17 μg/mL; Ap, 100 μg/mL; and Km, 50 μg/mL. All cyanobacterial strains were grown and maintained as described in Chapter II. Cultures were grown at 30ºC with continuous illumination of at least 200 $\mu E\ m^{-1}\ s^{-2}$. Cultures of at least an $OD_{750}$ of 0.3 were used to monitor the levels of bioluminescence using the TopCount luminometer, as described in Chapter II.

**Construction of *cikA* expression plasmids**

A 3.5 kb DNA fragment from cosmid 7E6 that contains the full-length ORF of *cikA* and its native promoter region was digested with *Nhe*I and *Hind*III. This fragment was cloned into the *Xba*I and *Hind*III sites of pBGS19 and pACYC184 to create pAM3101 and pAM3143, respectively. The making of expression vector pQE-80/*cikA* (pAM2478) is described elsewhere (84).

**Construction of *cikR* overexpression plasmids**

The potential *cikR* genes were amplified using PCR. The forward primer for each gene contained a 5' CACC sequence for directional cloning into the pENTR/D-TOPO vector (Invitrogen); the resulting plasmid was then used to transform DH10B *E. coli* cells. The plasmid DNA was sequenced using the BigDye terminator mix (Applied Biosystems) as per manufacturer's instructions. From the pENTR/D-TOPO plasmid, each *cikR* gene was then cloned into the pDEST17 (Invitrogen) vector using an LR recombinase (Invitrogen) reaction and used to transform DH10B cells. The resulting plasmids were isolated from *E. coli* using a QIAprep kit (Qiagen) and the genes were sequenced to assure no mutations occurred during the recombinase reaction. The pDEST17 derivatives were used to transform BL21(DE3)pLysE ($Cm^R$) *E. coli* cells (Novagen), which harbor an IPTG-inducible T7 polymerase because the pDEST17 vector uses a T7 promoter to drive expression of the *cikR* candidate gene. The coding sequence for six histidine residues lies just upstream of the potential CikR ORF and serves as an affinity tag for purification of the protein of interest.

The *134RR2* gene was also cloned into the pET15b vector (Novagen) by Carl Carruthers in Dr. Andy LiWang's laboratory in the Department of Biochemistry at Texas A&M University. This vector includes a T7 promoter and codons that encode an N-6xHis tag. This plasmid was also used to transform BL21(DE3)pLysE cells for overexpression and purification of 134RR2.

**Protein overexpression**

The pDEST17/*cikR* and pET15b/*134RR2* plasmids in the BL21(DE3)pLysE *E. coli* strain were used to overexpress each CikR candidate protein. CikA was overexpressed from pAM2478 ($Ap^R$), which is a pQE80 (Qiagen) derivative that drives expression of *cikA* from a T7 promoter and produces an N-6xHis-tagged CikA protein. A 5 mL overnight culture was used to inoculate 500 mL LB with appropriate antibiotics, and the culture was grown at 30ºC shaking at 250 rpm until it reached an $OD_{600}$ of 0.5. The culture was then induced with IPTG (1 mM final concentration). 124RR1, 132RR2, and 134RR2 were induced for approximately 4 h at 30ºC before the entire culture was collected by centrifugation at 5000 rpm (JA-10 rotor) for 10 min at 4ºC. CikA, 135RR1, and 135RR4 were induced overnight at room temperature before being collected by centrifugation at 5000 rpm (JA-10 rotor) for 10 min at 4ºC. Cell pellets were frozen at –80ºC until ready for use.

**Purification of CikA and CikR candidates**

The frozen *E. coli* pellets were thawed on ice for 15 min before being resuspended in 3 mL lysis buffer per gram pellet (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole). Once the cells were resuspended, lysozyme was added (1 mg/ml final concentration) and the cells were incubated on ice for 30 min. The cells were broken open by sonication for 5 min (30 s on, 30 s off), RNase (10 µg/mL) and DNase (10 µg/mL) were added, and the lysates were incubated on ice for 15 min. Lysates were then fractionated by centrifugation at 10,000 rpm (JA-20 rotor) for 30 min at 4ºC to separate soluble protein from particulate material. Soluble protein was then transferred to a 15 mL conical tube.

A nickel-nitriloacetic acid (Ni-NTA) slurry (Qiagen, 2 mL) was added to a flow column and allowed to settle before draining the buffer. The column was washed with 5 mL lysis buffer. The soluble protein containing each overexpressed CikR candidate protein was added to the equilibrated column, allowed to flow through the column, and collected. The collected soluble protein was then added to the column again to assure that the majority of 6xHis-tagged CikA or CikR protein bound to the column. The column was washed with 1 mL wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole) 8 times. The protein was eluted using 3 volumes of 1 ml elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole) each. Each eluate was collected and stored at 4ºC until ready to use. Purified CheY protein was provided by Run-Zhi Lai in the laboratory of Dr. Michael Manson in the Department of Biology at Texas A&M University.

**Phosphotransfer experiments**

The purified CikR candidates and CikA were dialyzed against the storage buffer (50 mM Tris•HCl, pH 7.5, 4ºC, 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) overnight, changing the buffer after the first 4 h. The dialyzed protein was then concentrated using an Amicon Ultra centrifugal filter device (Millipore). Purified CikA protein (10 µg/µL) was allowed to autophosphorylate (CikA~P) for 60 min in kinase buffer (50 mM Tris•HCl pH 8, 50 mM KCl, 10 mM $Mg_2Cl$, 0.1 µCi [$\gamma$-$^{32}$P] ATP). The radiolabeled CikA~P was then added to purified CikR candidate protein [10 µg/µL] or CheY protein [13.5 µg/µL] and excess cold ATP (0.5 mM) for 10 s, 30 s, 1 min, or 2 min. Reactions were stopped using equal volume 2X sample buffer (120 mM Tris•HCl, pH 6.8, 20% glycerol, 4% SDS, 10% mercaptoethanol (added just before use), 0.1% bromophenol blue). Samples were separated by electrophoresis on 16% polyacrylamide gels. The gels were dried and radiation was detected using a phosphoimager after either a 20-min or overnight exposure. All experiments were completed at room temperature.

**Construction of *cikR* null alleles**

Independent null alleles of each *cikR* candidate gene were created by insertion of an antibiotic-resistance cassette in the coding region of each gene. A Cm-resistance *Mu* transposon was inserted in the *124RR1* gene to give rise to the 7G3-W6 clone, and into the *135RR4* gene to create 4G8-AAA4. The Sp/Sm-resistance Ω-cassette ($Sp^R$/$Sm^R$-Ω) from pHP45Ω-Sp (24) was used to inactivate the *134RR2* gene. The full-length sequence of *134RR2* was cloned into pBluescript (Stratagene) at *Eco*RI and *Xho*I sites. The

$Sp^R/Sm^R$-Ω cassette was digested with *Eco*RI and ligated at the *Mfe*I site of the subcloned *134RR2* gene to create pAM3337. Plasmids that contain genes interrupted by antibiotic-resistance cassettes were used to transform cyanobacterial reporter strains (14, 27). Following homologous recombination, mutant allele replacement at the native locus was verified by PCR as compared to the WT allele.

**Measurement and analysis of *in vivo* bioluminescence**

Reporter strain AMC1300 (P*kaiBC::luxAB*) and its derivatives integrate the luciferase reporter at neutral site I (NS1) and the proteins required for synthesis of the long-chain aldehyde substrate, driven by the *psbAI* promoter (P*psbAI::luxCDE*), are integrated at neutral site II (NS2). AMC1004 (P*kaiBC::*luxAB) carries the luciferase reporter at NS2.1 and P*psbAI::luxCDE* at the adjacent NS2.2. AMC669 (P*psbAI::luxAB;* P*psbAI::luxCDE*) and its derivatives carry both the luciferase reporter and necessary enzymes at NS2.1. Automated measurement of bioluminescence from the *S. elongatus* reporter strains was performed as described in Chapter II; data acquisition and analysis were performed as described in Chapter III.

***Results***

**Use of a genetic screen in *E. coli***

The original identification of the protein SasA came through a genetic screen in *E. coli* that was used to search for cyanobacterial HPK proteins (85). At the time, members of the RR family had been discovered in the cyanobacteria (68, 104) but no published

reports existed for sensor kinases. The screen takes advantage of an *E. coli envZ* mutant strain (DZ225) and the ability of some HPK proteins to "cross-talk," *i.e.*, transfer a phosphoryl group to a RR protein other than their partner RR when the partner RR is not present. The *envZ* gene encodes an inner membrane protein. Its N-terminal periplasmic sensory domain detects changes in external concentration of ionic salts, which triggers an autophosphorylation reaction to occur at its C-terminal kinase domain (for a review of the EnvZ/OmpR system see reference 21). The phosphorylated form of EnvZ transfers its phosphoryl group to the transcription factor, OmpR. OmpR directly activates expression of *ompC* and represses *ompF* expression to influence the pore size of the structures in the outer membrane. The OmpC and OmpF porins have diameters of 1.08 nm and 1.16 nm, respectively, such that when there is greater incorporation of OmpC in the membrane, solutes can diffuse less readily into the cell.

In the DZ225 strain, EnvZ is not functional and a *lacZ* reporter gene is driven by the *ompC* promoter (P*ompC::lacZ*) (85). Without any perturbations or additions to the DZ225 strain, the cells cannot utilize lactose. In an attempt to isolate proteins that are capable of phosphorylating OmpR in the absence of its cognate HPK EnvZ, a plasmid library that contained *Sau*3AI-digested *S. elongatus* genomic DNA was used to transform DZ225 cells and then screened for the ability to ferment lactose by plating on MacConkey/lactose plates. The cells that expressed a protein capable of activating expression of the P*ompC::lacZ* reporter, presumably by stimulation of OmpR, could ferment lactose to cause the decrease in pH that creates the characteristic magenta

colony color on MacConkey/lactose agar. The protein found to fulfill this criterion was SasA.

An HPK that functions in the input pathway of the cyanobacterial system (CikA) has been identified and characterized, but the RR with which it pairs has evaded detection. Because the *E. coli* screen proved fruitful in the discovery of SasA, we used the same system to look not for another HPK, but instead for its RR. We proposed that if CikA is able to cross-talk with the *E. coli* RR OmpR to produce the initial *lac*+ phenotype on MacConkey/lactose agar (magenta colony color) by activating the P*ompC::lacZ* reporter, expression of the real CikR would compete with OmpR for access to the phosphoryl group of CikA to ultimately produce a *lac*- phenotype visible by its white colony color on MacConkey/lactose (Fig. 6.3).

The full-length *cikA* gene was first cloned into the plasmid pQE-80 (Qiagen), which produces high levels of the protein product due to its high copy number (the plasmid contains a ColE1 replication origin that produces an estimated 150 copies in the cell) and strong T5 promoter that drives expression of the gene of interest. This plasmid was used to determine if the screen in the DZ225 strain would be practical to use in searching for CikR candidates. The possibility existed that CikA would not be able to cross-talk in *E. coli* and OmpR would not accept the phosphoryl group from CikA. If that occurred, all of the transformed colonies would be white in color (*i.e.*, *lac*- phenotype) on MacConkey/lactose. If CikA was capable of phosphorylating OmpR, active OmpR

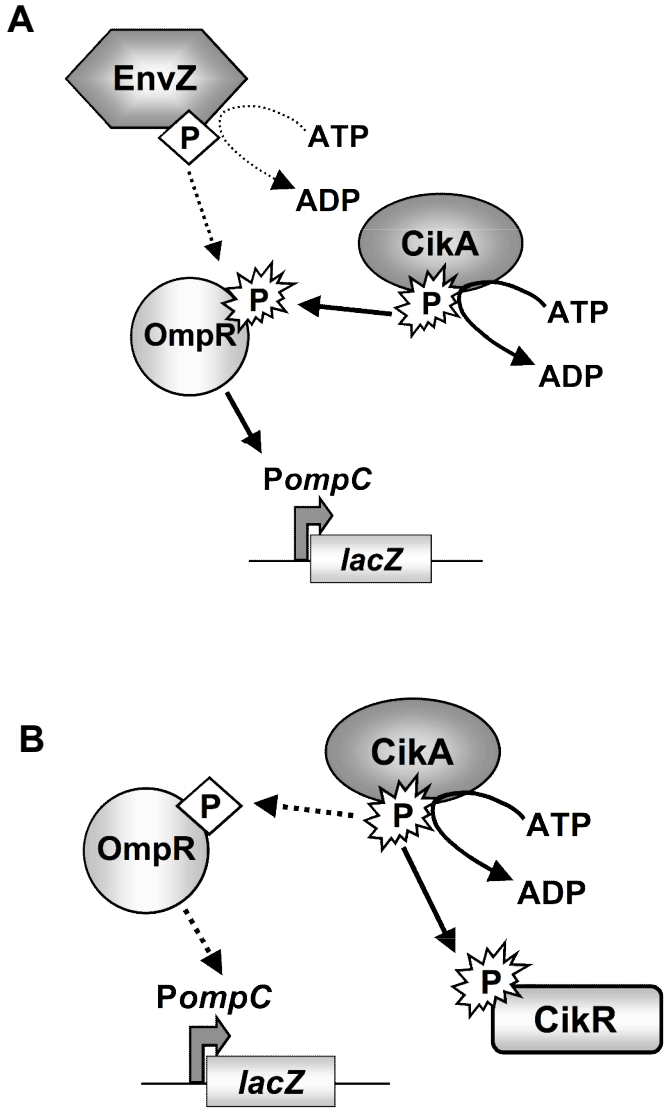


FIG. 6.3. Proposed research plan to find CikR using a genetic screen. The DZ225 *E. coli* strain lacks EnvZ, which normally phosphorylates its cognate response regulator OmpR. The phosphorylated form of OmpR activates transcription of the reporter fusion, P*ompC::lacZ*. (A) Transformation of DZ225 with a plasmid that expresses *cikA* may compensate for the loss of EnvZ because CikA can act as an alternative phosphodonor to OmpR to confer a *lac*+ phenotype. (B) Transformation of DZ225+*cikA* with an *S. elongatus* genomic library may identify RR(s) of CikA because CikA preferentially phosphorylates its cognate RR rather than OmpR to confer a *lac*- phenotype.

protein would activate transcription of the *lacZ* reporter gene and result in magenta colonies (*i.e.*, *lac*+ phenotype). When the pQE-80/*cikA* plasmid was used to transform the DZ225 strain, the cells had a mucoid appearance on LB agar plates and were unable to grow on MacConkey agar. The control strains that harbored either an empty pQE-80 vector or a *sasA*-expressing plasmid both displayed circular colonies on LB and a *lac*- phenotype or *lac*+ phenotype, respectively, on MacConkey/lactose (Fig. 6.4).

The inability of the DZ225 cells that express *cikA* to grow on MacConkey agar was unexpected. One visible difference seen in the colonies that express *cikA*, but not in either the positive or negative control, was the abnormal, mucoid colony morphology on LB agar when CikA is produced, which suggests that the integrity of the cell membrane is disrupted in the presence of high levels of CikA protein. In the DZ225 strain, the phosphorylated form of OmpR activates transcription from the *ompC* promoter, which would express not only the *lacZ* reporter but also the endogenous *ompC* gene. If CikA is able to phosphorylate OmpR to activate *ompC* expression, the OmpC porin protein would be produced and incorporated into the cell membrane. In the presence of EnvZ, the transcription of *ompC* is down-regulated by the phosphatase activity of EnvZ on phosphorylated OmpR once the cell has adjusted its outer membrane composition to accommodate the changes in external osmotic pressure. In the absence of EnvZ, but in the presence of a kinase, *e.g.*, CikA, that can only phosphorylate OmpR and not remove the phosphoryl group, OmpR would be active for a longer duration than when EnvZ is present. This high level of active OmpR protein may lead to an increased amount of the

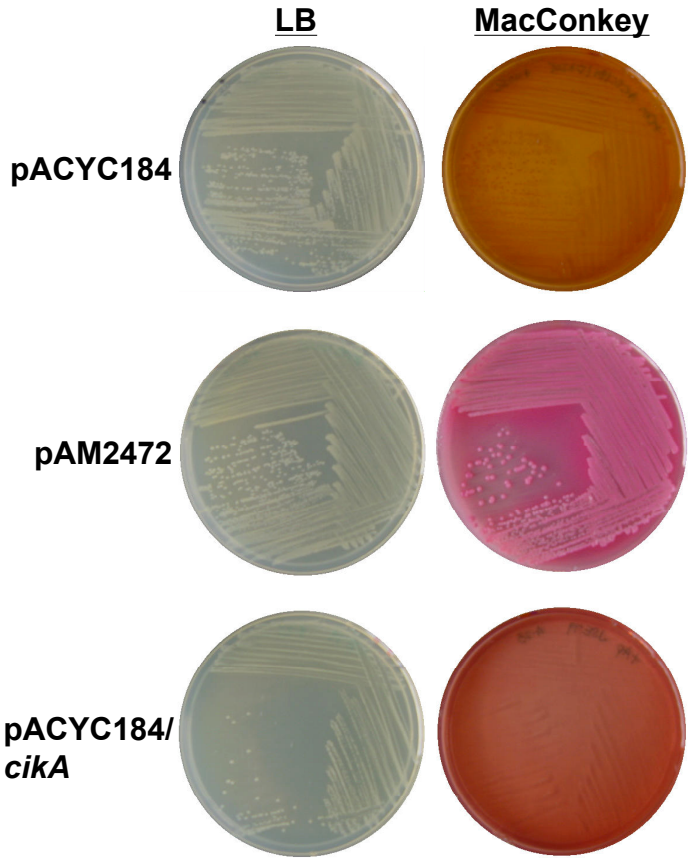


FIG. 6.4. Phenotypes of DZ225 strains that produce SasA or CikA proteins. DZ225 transformed with pACYC184 vector alone (negative control) exhibits a *lac*- phenotype on MacConkey agar. MacConkey agar is differential for bacteria that can ferment lactose, *i.e.,* produce *lacZ.* Cells expressing *lacZ* will be pink in color and those not expressing *lacZ* will be colorless/white on the medium. Transformation with the positive control pAM2472, a *sasA*-expressing vector, shows the *lac*+ phenotype. Introduction of CikA into strain DZ225 produces mucoid colonies on LB agar and cells cannot grow on MacConkey agar.

OmpC porin in the outer membrane, which may permit more salts to pass through the membrane. In the presence of the harsh bile salts in MacConkey agar, the cells are not able to survive.

In order to decrease the amount of CikA in the cell and attempt to lower the amount of active OmpR protein, *cikA* was cloned under control of its native promoter into pBGS19 (pUC19 derivative; MB1 origin; 50 – 100 copies per cell) or pACYC184 (p15A origin; 20 – 30 copies per cell). Despite the attempts to lower the amount of CikA protein, introduction of either of these plasmids into DZ225 caused the same mucoid cell morphology on LB, and no colonies were able to grow on MacConkey/lactose agar.

The strong effect that production of CikA had on the DZ225 cells predicted that the system could be used as a selection, rather than a colorimetric screen. Because DZ225 cells that harbor a plasmid that encodes CikA are still viable on LB agar, LB broth was inoculated with DZ225 cells that carried the pBGS19/*cikA* plasmids and those cells were used to introduce a pSE380 library that carried fragments of *S. elongatus* genomic DNA. The transformed cells were plated on LB agar selective for both the *cikA* plasmid and library plasmid. The colonies that appeared were then washed from the surface of the agar and plated on MacConkey/lactose agar with selective antibiotics. We predicted that if the true RR for CikA was produced in the doubly-transformed cells, CikA would preferentially phosphorylate its cognate RR instead of OmpR, which would lead to a decrease in the activation of transcription from the *ompC::lacZ* reporter and result in

cells that could grow on MacConkey agar and display a *lac*- phenotype. Those colonies that did not produce a protein to titrate the transfer of phosphoryl groups away from OmpR would be unable to grow, like those cells that express *cikA* alone. The majority of the resuspended cells did not grow on MacConkey/lactose but eighteen *lac*- colonies emerged. The total plasmid DNA that was expected to contain both the library plasmid and the *cikA* plasmid was extracted from these colonies and used to transform DH10B *E. coli* cells. Half of each transformation reaction was plated on LB+Ap, to select for the library plasmid; the other half of the reaction was plated on LB+Km to select for the *cikA* plasmid. For each of the eighteen DH10B transformations, no colonies grew on the plates containing Km. These results imply that, during the library transformation procedure, a small number of DZ225 cells had lost the pBGS19/*cikA* plasmid, which allowed for growth on MacConkey, but also picked up a secondary mutation that allowed for their growth in the presence of Km. Therefore, even as a selection, the use of the DZ225 system to identify the CikR protein proved unsuccessful.

**Use of bioinformatics, protein purification, and *in vitro* phosphorylation**

The full *S. elongatus* genome was sequenced and partially annotated by the Joint Genome Institute in collaboration with Dr. Susan Golden. This new information about the contents of the cyanobacterial genome allowed for proteins predicted to encode receiver domains to be identified by using bioinformatic methods. Once the genes that encode such proteins were discovered, the overall goal of this project was to clone and overexpress the genes in order to partially purify the proteins, and ultimately visualize

the transfer of information from CikA to one (or more) of the CikR candidates via phosphotransfer of radiolabeled ATP.

The genome database was searched for predicted ORFs that would encode an amino acid sequence similar to the consensus sequence of receiver domain(s) with the conserved aspartic acid residue that accepts the phosphoryl group from an HPK in two-component systems. The *S. elongatus* genome encodes nineteen proteins that have significant similarity to that of a true receiver domain to produce an E-value of 1e-05 or less. The sequence encoding the receiver domains for each of those nineteen candidates was then used to search the genome sequence database until no new loci were found.

Of the nineteen genes predicted to encode receiver domains, thirteen were removed from further consideration. Elimination of some sequences occurred because the function of the encoded protein was already known to be unrelated to the clock, or the gene was located directly downstream of the ORF for another HPK; it is predicted that those HPK and RR proteins are corresponding partners. Other genes were removed from the list because they are not predicted to encode the conserved aspartic acid residue that has been shown to receive the phosphoryl group from HPK proteins. Additionally, CikR is predicted to be a single domain protein because we do not expect it to bind DNA but rather to interact with other proteins in the input pathway; thus, those gene sequences that encode multi-domain proteins were not initially considered as likely *cikR* candidates. Based on those criteria, five potential *cikR* genes remained and were named

*124RR1*, *132RR2*, *134RR2*, *135RR1*, and *135RR4* based on their assignment within the genome-sequencing project.

The five genes to be investigated were cloned using Gateway Technology (Invitrogen) because it allows for the rapid insertion of genes into vectors without the need for restriction enzyme digestion and ligation reactions. This project was the first in Dr. Golden's laboratory to use Gateway Technology for protein production in *E. coli* and served to determine the success of the system. Briefly, the commercial entry vector (pENTR/D-TOPO) has been linearized by the topoisomerase I enzyme from *Vaccinia* virus, which binds to double-stranded DNA, cuts the backbone, and forms a covalent bond with the DNA (106). The 5' hydroxyl group of the PCR product of the gene of interest can reverse this reaction to allow the PCR product to anneal to the linearized pENTR/D-TOPO vector (107). The gene of interest is then transferred into the destination vector (pDEST17) through a modified, bacteriophage lambda LR recombination reaction, which can be completed at room temperature. The pDEST17 vector was used for this project because it contains the strong T7 promoter upstream of the cloning site, codons that encode an N-6xHis tag for protein purification, and an ampicillin-resistance cassette for clone selection.

To determine if any of the five candidate proteins is capable of accepting the phosphoryl group from CikA, 6xHis-tagged CikA and each 6xHis-tagged CikR candidate protein were overexpressed in *E. coli*. Cell pellets were broken open using sonication and the

soluble protein extract was used to partially purify each protein by affinity chromatography with a Ni-NTA column.

The first step of the phosphotransfer experiment was to establish conditions that allowed CikA to autophosphorylate. Conditions had been previously determined that would allow for the autophosphorylation and phosphotransfer of almost all of the known HPK/RR systems from *E. coli* (128). The great success of these experiments encouraged the use of their protocols in the hunt for CikR. In the presence of [$\gamma$-$^{32}$P] ATP, CikA could autophosphorylate in a time-dependent manner (Fig. 6.5). For the remainder of the phosphotransfer experiments, CikA that had been incubated with radioactive ATP for either 40 or 60 min was used.

The difficulty in trying to create an environment that supports the transfer of a radiolabeled phosphoryl group from CikA to the CikR candidates is that there are numerous variables that need to be ideal in order for the reaction to occur. To help to narrow the range of variables that could cause problems in the reaction and/or detection, conditions were first determined that were favorable for CikA to transfer to a known RR protein, CheY. CheY is a single receiver domain involved in chemotaxis in *E. coli*. CheY is a "promiscuous" acceptor of information from HPK proteins *in vitro*, as was shown by its ability to receive phosphoryl groups from a number of different HPK proteins when its cognate HPK protein is not present (128). The phosphorylation of CheY by its partner HPK (CheA) was shown to occur within 10 s, with no detectable

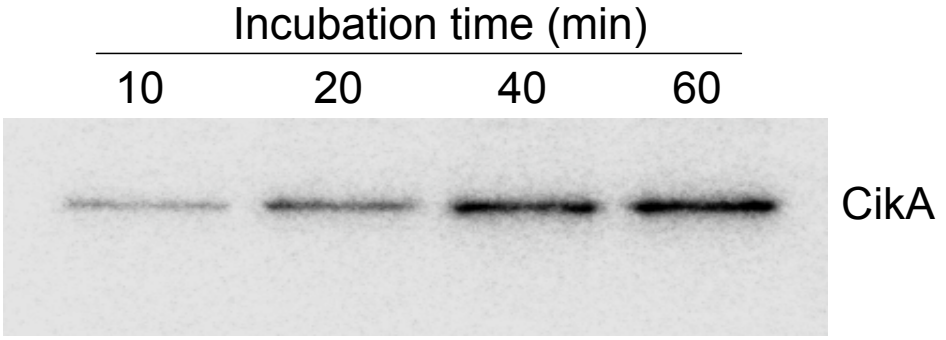


FIG. 6.5. Autophosphorylation of CikA *in vitro*. Purified CikA was combined with 0.1 μCi [γ-$^{32}$P] ATP for 10, 20, 40, or 60 min. Samples were stopped with 2X-stop buffer and separated on 16% SDS-PAGE. The gels were dried and radioactivity was detected using a phosphoimager.

radioactive CheY remaining after only 2 min of incubation with a phosphorylated HPK protein (128). Thus, short time courses were used to detect phosphorylation events that may occur even when the lifetime of the phospho-protein is short. After CikA was allowed to autophosphorylate, purified CheY protein was added. Figure 6.6 shows that the conditions used permitted the phosphotransfer from CikA to CheY within 10 s and the decay of the signal followed a pattern similar to that seen previously in the CheA/CheY reaction (128). The same experimental conditions that were used for the phosphotransfer reaction from CikA to CheY were applied to demonstrate transfer to each of the five CikR candidate proteins. Despite numerous attempts, no phosphotransfer could be detected (Fig. 6.7; see Discussion).

**Use of reverse genetics**

The predicted function of CikR is to act in the input pathway of the cyanobacterial biological clock to connect (probably indirectly) CikA to the Kai proteins of the central oscillator. The absence of this protein would likely cause an abnormal circadian phase-resetting response to a 5-h dark pulse and may also affect the circadian timekeeping in LL if it is tightly linked to the core clock components. To assess the circadian function of the *cikR* candidate genes *in vivo*, three of the five ORFs were interrupted with an antibiotic-resistance cassette in cyanobacterial luciferase reporter strains and the resulting rhythm of bioluminescence examined.

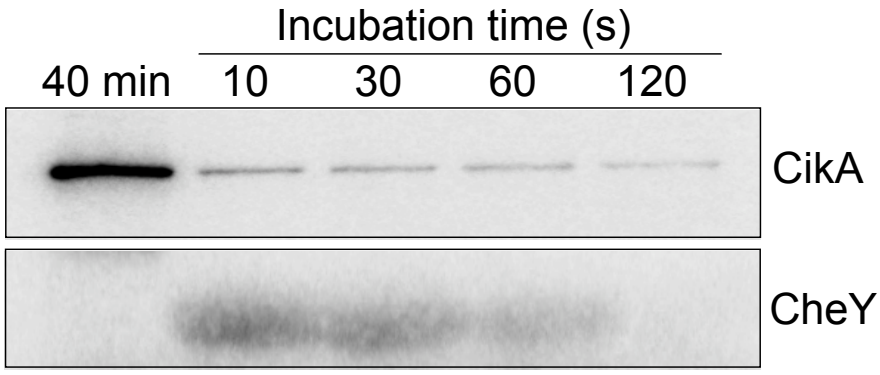


FIG. 6.6. Phosphotransfer from CikA to CheY *in vitro*. Purified CikA was combined with 0.1 μCi [γ-$^{32}$P] ATP for 40 min. Purified CheY protein was added to the radiolabeled CikA and samples were allowed to incubate for up to 2 min. Reactions were stopped with 2X-stop buffer and proteins were separated on 16% SDS-PAGE. The gels were dried and radioactivity was detected using a phosphoimager.

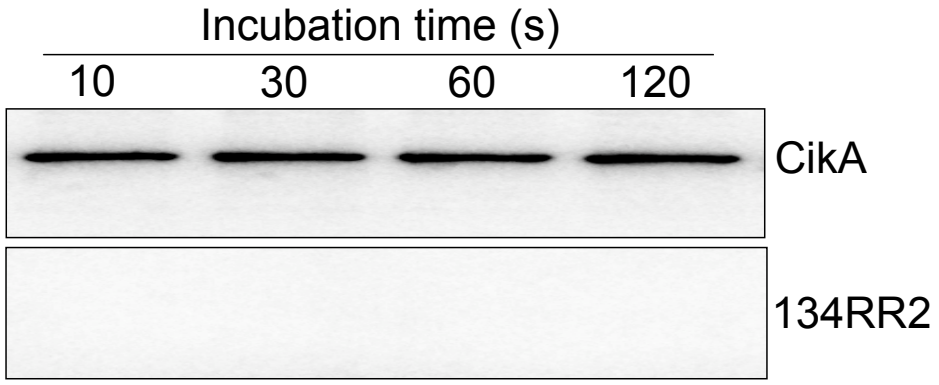


FIG. 6.7. Attempted phosphotransfer from CikA to CikR candidate 134RR2 *in vitro*. Purified CikA was combined with 0.1 μCi [γ-$^{32}$P] ATP for 40 min. Purified 134RR2 protein was added to the radiolabeled CikA and samples were allowed to incubate for up to 2 min. Reactions were stopped with 2X-stop buffer and proteins were separated on 16% SDS-PAGE. The gels were dried and radioactivity was detected using a phosphoimager. Similar results were achieved with each of the five CikR candidates.

Preliminary analysis of the *cikR* null alleles showed that, under LL conditions, the disruption of *135RR4* did not have a noticeable effect on the circadian properties of luciferase expression from a *kaiBC* promoter (Fig. 6.8A). Inactivation of *124RR1* resulted in a circadian period similar to that of the WT strain, but the phase of peak expression consistently occurred about 4 h before that of the WT reporter (Fig. 6.8B). Complete replacement of each WT allele with the interrupted allele was determined by PCR analysis of genomic DNA (data not shown).

The *134RR2* gene appears to be essential for cell viability because attempts to replace the WT copy of the gene with the interrupted allele resulted in merodiploids, as was determined by PCR analysis of genomic DNA from both WT and transformed cyanobacterial cells (data not shown). *S. elongatus* harbors 6 – 8 copies of its genome (81) and it is possible that the antibiotic-resistance cassette disrupted enough copies of the gene to survive on selective medium, but some WT alleles remain functional. In a P*kaiBC::luxAB* reporter, the partial loss of function caused both the period and phase of the rhythm to be different from those of the WT strain (Fig. 6.8C). To verify that this phenotype was due to a disruption of the clock and not specific altered regulation of the *kaiBC* promoter, a P*psbAI::luxAB* reporter strain was also transformed with the *134RR2* inactivation allele. The merodiploid state of *134RR2* in the *psbAI* reporter was verified by PCR (data not shown) and this strain also displayed altered phasing and a slightly shorter period than WT (Fig. 6.8D). Thus, two of the five CikR candidates have a marked effect on the biological timing system of the cyanobacterial cell.

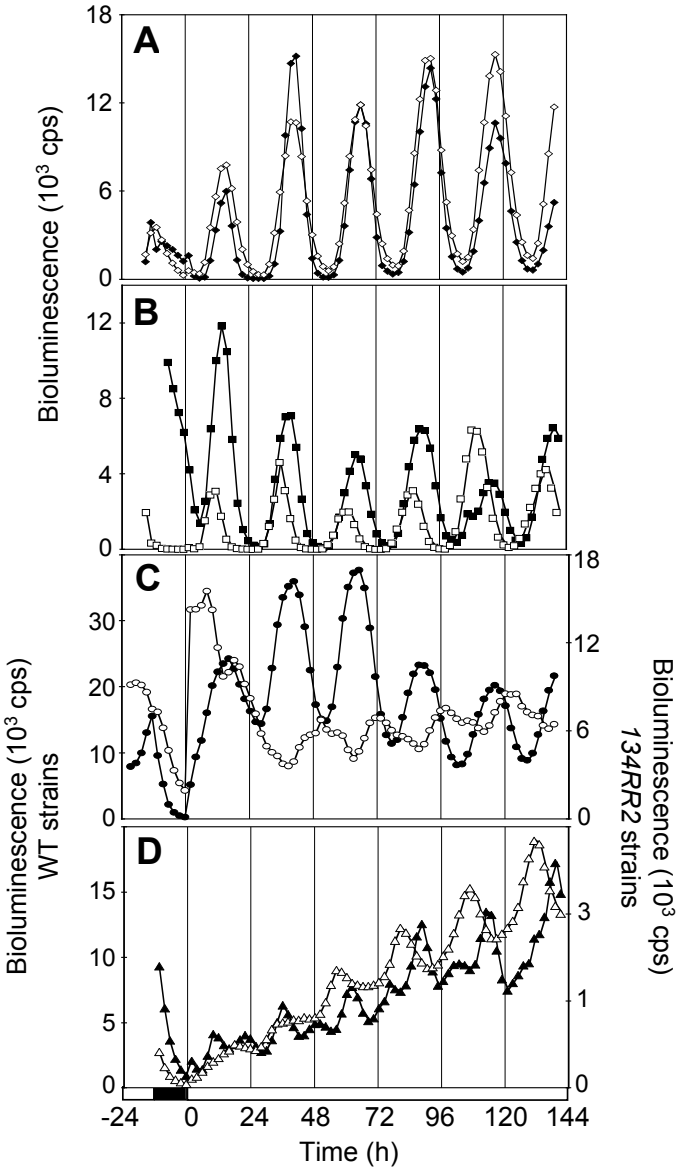


FIG. 6.8. Circadian phenotypes of *cikR* inactivation alleles in LL. (A) *135RR4* interrupted by a $Cm^R$ *Mu* transposon insertion (open diamonds) does not display a difference in period or phase from the WT P*kaiBC::luxAB* reporter strain (closed diamonds). (B) A $Cm^R$ *Mu* transposon insertion in *124RR1* (open squares) causes the peak of expression to occur 4 h before the WT P*kaiBC::luxAB* reporter (closed squares). *134RR2* was disrupted by a $Sp^R/Sm^R$-Ω cassette. The mutation causes altered periodicity and phasing in (C) a P*kaiBC::luxAB* reporter (WT, closed circles; *134RR2* mutant, open circles) or (D) a P*psbAI::luxAB* reporter (WT, closed triangles; *134RR2* mutant, open triangles).

***Discussion***

Despite the many different experiments designed to elucidate the nature of the CikR protein, its identity remains a mystery. Why have the methods used to identify CikR failed to do so? One possibility is that a "helper" protein is required *in vivo* for the interaction between and phosphotransfer from CikA to CikR. This other protein (or set of proteins) would not have been present in either the *E. coli*, *in vitro*, or yeast two-hybrid system (discussed in Chapter V). Evidence that strongly supports the need for protein-protein interactions in the function of CikA stems from research conducted with variants of the CikA protein. As mentioned earlier, the other domains of CikA influence its rate of autophosphorylation with the most outstanding change (a 10-fold increase) resulting from the removal of the PsR domain (84). The PsR is also the domain that appears to be involved in protein-protein interactions that tether CikA to the pole of the cell (130). Full-length CikA that is fused to a fluorescent protein is found predominantly at the cell poles, whereas a fluorescent CikAΔPsR variant has lost this polar positioning and can be seen throughout the entire cell. The current working model for CikA function is that the PsR domain binds to an unknown protein at the cell pole. This binding causes two things to happen: (1) repression of the PsR on CikA's autokinase activity is removed, which allows CikA to autophosphorylate at a much higher rate, and (2) the complex of proteins (including CikR) with which CikA associates locates together at the pole to allow the complex to fulfill its duty in the input pathway of the clock system. Two candidate helper proteins (Hyp-1 and Hyp-2) have been identified through the yeast two-hybrid experiments described in Chapter V that used CikA as bait.

Another possible explanation for the inability to identify CikR is that the five candidate proteins found in the bioinformatic search and used in subsequent biochemical experiments are not CikR. It is predicted that CikR is a small, diffusible factor that contains a conserved receiver domain. This prediction stems from preliminary experiments that showed the presence of smaller protein bands from cyanobacterial extracts becoming radioactive only when radiolabeled, phosphorylated CikA protein is added to the soluble protein extracts from *cikA* null strains (Fig. 6.2**)**. However, this prediction could be incorrect.

One candidate protein (133RR3) remains to be tested. This protein satisfied the criteria for being designated a CikR candidate in the initial bioinformatic search except that it is very large – 929 amino acid residues – and contains seven predicted functional domains, one of which is a receiver domain with the conserved aspartic acid residue for accepting the phosphoryl group. None of the domains is predicted to be a kinase domain, and the ORF of *133RR3* does not lie immediately downstream of another HPK protein. This gene is currently being cloned in order to conduct the same types of experiments with its protein product that were done with the first five candidates.

In addition to testing 133RR3, further phenotypic tests need to be conducted with the original five *cikR* candidates. Preliminary *in vivo* analysis of inactivation alleles showed that 124RR1 and 134RR2 are needed to maintain proper phasing and periodicity of circadian rhythms. Interestingly, the 134RR2 protein appears to be essential not only for

proper circadian timing, but also essential for cell viability because the WT copies of the gene could not be completely replaced by the inactivated allele. The presence of a functional clock is not necessary for proper cellular processes, as *kai* deletions that render the clock arrhythmic do not display a discernable growth phenotype. The function of 134RR2 is likely not limited to the clock and may contribute to cellular housekeeping activities. Another reason for further dissection of the phenotype and molecular mechanisms of *134RR2* is that its protein product was independently identified through a yeast two-hybrid system that used the N-terminal PsR domain of KaiA as bait (S. R. Mackey and S. S. Golden, unpublished data). 134RR2 could be a one-step connection between receiving environmental information from CikA and transferring that signal directly to the Kai complex via interactions with KaiA.

Data still need to be collected to ascertain the effect that null alleles of *132RR2* and *135RR1* have on the biological clock. If any of the candidate proteins is the true CikR, it would be expected that a null allele would also exhibit a difference in response to LD cues as is typical of input pathway components. Thus, these mutant strains will be tested for their ability to "sense" differences in light intensities and to reset to dark pulses. Overexpression of proteins involved in the clock often results in period changes or arrhythmia (43, 48, 130). Because functional redundancy often occurs within a cell and obscures the effect of inactivating genes, each CikR protein should be overexpressed and its effect on the clock determined by the pattern of bioluminescence from reporter constructs.

From the outside looking in, it may appear that the described experiments have failed because they did not answer the one big question: What is the identity of CikR? However, the combined experimental procedures have identified two new players involved in the cyanobacterial clock system that are necessary for proper phase and period of the rhythm. The tools used and developed during these projects will allow for the roles of these two components in the biological rhythm of *S. elongatus* to be assessed and fully appreciated. Most importantly, the true success of these projects stems from the invaluable educational and research experiences that three undergraduates received.

# CHAPTER VII

# CONCLUSIONS

A circadian clock system has long been described in presentations as three separate entities – input, oscillator, output – in a linear pathway connected by unidirectional arrows. The dissection of pieces that comprise each division has only recently provided a bit-by-bit explanation for the resulting rhythmic behaviors. The discovery of a biological clock in the prokaryote *S. elongatus* allowed for the unique opportunity to explore the complicated web of proteins involved in making a single cell tick.

Central to the clock systems of eukaryotic organisms are complex and interweaving transcription-translation feedback loops where overall levels of opposing factors peak out of phase from one another (5). Despite the novelty of a prokaryotic circadian rhythm, the preconceived notions remained that the *S. elongatus* oscillator proteins acted in a similar eukaryotic-biased feedback system. However, the *kai* promoters do not contain specific *cis* information required to preserve circadian control of gene expression; furthermore, the synchrony of expression of the *kai* loci need not be maintained to support cyanobacterial circadian rhythms (Chapter III). The current data now depict the cyanobacterial oscillator as the product of post-translational modifications to core oscillator proteins. The working model does not discount the fact that overall levels of Kai proteins cause subtle changes in the period of the rhythm (Chapter III), or that the overexpression of KaiA increases transcription from the *kaiBC* promoter while increased amounts of KaiC repress its own expression (43), as well as that of all other tested

promoters (88). The converging hypotheses suggest that although the clock tightly regulates the rhythm of transcription of the entire *S. elongatus* genome, the converse is not true; rhythmic gene expression is not necessary for the endogenous oscillator to function. Studies conducted on *S. elongatus* cells that have been maintained in the dark, where *de novo* transcription and translation do not occur, demonstrate that rhythms of KaiC phosphorylation persist with a near WT period (117). Furthermore, in the presence of ATP the Kai proteins are capable of establishing a temperature-compensated circadian rhythm in KaiC phosphorylation *in vitro* (89).

One of the caveats of the test-tube clock is that the resulting *in vitro* rhythm is 2 – 3 h shorter than the rhythms of bioluminescence exhibited by luciferase reporters and of mRNA and protein accumulation *in vivo*. Because the endogenous rhythm generated by the Kai proteins alone is slightly shorter than what is seen in intact cells, a mechanism must exist to slow down the clock in order to match the environmental day. The role of interpreting external information to fine-tune the period of the endogenous oscillator to mirror the environmental cycle belongs to the input pathway of the clock system. Interestingly, null mutations of any of the known input pathway components – CikA, LdpA, and Pex – display rhythms with periods that closely match that of the *in vitro* rhythm of KaiC phosphorylation (51, 65, 105). These data suggest that when the input pathways to the oscillator are disrupted, the oscillator cannot receive the proper information from the environment that is needed to modulate the endogenous 22-h rhythm in the phosphorylation state of KaiC to reflect the daily cycles of light and dark.

Although there is a correlation between the period lengths of input pathway mutants and the *in vitro* oscillating system, a clear connection that links those proteins together has not yet been firmly established. In multiple experiments that were designed with the intent of leading us to the identity of the much-anticipated CikR partner protein, we instead discovered five new components (NHT-1, Spk, Hyp-1, 124RR1, and 134RR2) that play necessary roles in the cyanobacterial biological clock (Chapters V and VI). Further analyses of these proteins will help to fill in the gaps that exist in the original input → oscillator → output scheme long held as axiomatic by researchers in circadian rhythm studies.

***CikA research in a new light***

A major focus of other laboratories currently conducting research on the daily rhythms of *S. elongatus* is the careful examination of the core oscillator Kai proteins. Although these proteins are remarkable in their properties, their function appears to be specific to the clock; null mutations of any of the *kai* genes do not cause a discernible defect in cell growth (43). CikA, however, appears to be involved in many different realms of cellular activity, only one of which is the short-period, defective phase-resetting phenotype that affects the circadian rhythm (105). Cells that lack *cikA* also have altered responses in gene expression to changes in light intensity (105) and are defective in cell division (80). This protein appears to be the first (of many) proteins that intertwine temporal and cellular/metabolic processes.

One of the original goals of the research presented here was to identify and characterize CikR, the predicted cognate response regulator for CikA. However, CikR remains unknown and there is a real possibility that it does not exist. If this is true, CikA function might instead depend upon the modulation of its kinase activity through interactions with other protein components or ligands. The GAF domain activates the autokinase activity of CikA, as is demonstrated by the decreased levels of phosphorylation when the GAF domain is removed (84). Although the GAF does not contain the cysteine residue typical for photoreceptors, it likely binds a different cofactor to bring forth a response to external conditions. The PsR domain represses CikA autophosphorylation and its removal greatly increases the rate of kinase activity (84). Structural modeling with the NMR structure of the PsR domain and a modeled structure of the CikA HPK show possible electrostatic interactions between these two domains that conceals the histidine phosphoacceptor (T. Gao, X. Zhang, S. S. Golden, and A. LiWang, unpublished data). The regions of the PsR that are not likely to interact with the HPK domain are believed to form protein-protein interactions with other components. These intermolecular contacts could serve two functions: localize CikA to the cell pole, and relieve the repression of the PsR on the autokinase activity of CikA. It is entirely possible that the phosphoryl group of CikA does not become transferred to another protein but instead facilitates the formation of complexes at the pole of the cell. CikA has been shown to interact (indirectly) with KaiA, LdpA, SasA and KaiC *in vivo* (45) and with five other proteins through the yeast two-hybrid system (Chapter V).

The polar focus of CikA poises the protein to accept the external signals needed to reset the rhythm of the internal oscillation. In eukaryotic systems, there are photoreceptors dedicated to receiving light information, which facilitates their function of manipulating the abundance of core clock components to result in a change in the phase of the rhythm. In cyanobacteria, there does not appear to be a photoreceptor needed to sense light quality or quantity, but instead at least two proteins that are capable of sensing the redox state of the cell, which is representative of light intensity. The spectrum of light that is capable of resetting the phase of the internal oscillation in *S. elongatus* is indistinguishable from that needed for photosynthesis (T. Kondo, personal communication). Additionally, inactivation of either of the two proteins in the *S. elongatus* genome that contain GAF domains with the conserved cysteine residue that would be predicted to be involved in red/far red photoreception does not have an effect on the properties of circadian rhythmicity in LL or in response to dark pulses (S. R. Mackey, X. Zhang, and S. S. Golden, unpublished data). Therefore, it seems unlikely that there is a red light photoreceptor dedicated to the resetting of the clock. Instead, the properties of the photosynthetic apparatus that provide information about the intensity of light in the form of redox changes in the thylakoid membrane are likely to be the necessary components for transmitting external information to the proteins of the input pathway.

Both LdpA and CikA are sensitive to the effects of the electron transport inhibitor 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) (45). DBMIB blocks electron

transport from plastoquinone to the cytochrome $b_6f$ complex within the photosynthetic apparatus to create an overall reduced state of the plastoquinone pool (118). By sensing the redox state of the cell, LdpA and CikA are able to interpret this signal as a representative of light intensity, which would fluctuate over the course of the day and provide environmental information to the cell. The PsR domain is directly sensitive to DBMIB and may itself bind a quinone (N. B. Ivleva, T. Gao, A. LiWang, and S. S. Golden, unpublished data). The close association of CikA to the photosynthetic apparatus, through its polar localization, aids in the measurement of the redox state of the plastoquinone pool as an indirect measurement of light intensity. Under these circumstances, a true photoreceptor would not be necessary for the synchronization of the internal oscillation with the outside world.

The protein sequence of CikA fails to identify a full-length homolog in other cyanobacterial genomes. Specifically, the N-terminal 183 residues of CikA do not have similarity to any known functional domain; in fact, using that 183 amino acid stretch to find similar sequences in the BLAST database results in no hits with an E-value of less than 1e-04. A CikAΔ183N variant complements the period of a *cikA* null strain in LL and its overexpression causes arrhythmia like that of its WT counterpart (130). However, this sequence is not entirely dispensable. CikA protein that lacks these N-terminal residues does not have WT levels of autophosphorylation *in vitro* (84), nor does it respond like the WT protein to the electron transport inhibitor DBMIB (N. B. Ivleva and S. S. Golden, unpublished data). Determining the structure of this mysterious sequence

by either NMR or crystallography may provide insight into its function, as these techniques have done for the PsR domains of both CikA and KaiA.

***The black box of clock output***

Because the study of circadian rhythms in *S. elongatus* began decades after the study of other systems, researchers launched into studying the detailed mechanisms of how the clock ticks without knowing what it controls. In work published June 2006, the core oscillator has been shown to control condensation of the chromosome, which likely explains the fundamentally rhythmic transcription of the genome (109). Two distinct classes of expression have been documented that peak in opposite phases from one another in constant conditions. The majority of genes peak during the time when the nucleoid is decondensed and the cell's genome is accessible to transcriptional machinery. A small minority of genes peak during maximal condensation and are proposed to require tightly-coiled topology in their promoter regions to be transcribed (72, 78). The clock system also gates the time at which cell division can occur, such that there is a forbidden time when the cells cannot undergo cytokinesis (81). One reason for this gating may be that the periodosome complex is sensitive to perturbations during this time period. Another possibility may be that the tightly compacted chromosome that exists at the light to dark transition, the same window of time in which cell division is forbidden, cannot be disrupted without harming the integrity of the nucleoid. For these reasons and perhaps many others, the clock confers an advantage to those cells whose internal periodicity closely matches that of the world around it (95).

***Closing comments***

The cyanobacterial clock system has been shown to be tightly linked with many of the major processes of the cell: photosynthesis, transcription regulation, post-translational modifications, nucleoid condensation, and cell division. With each new discovery, it becomes increasingly difficult to separate these processes into the different divisions of the clock system and to separate clock-controlled processes from those of housekeeping and metabolic processes.

Because these distinctions are no longer clear, most, if not all, of the non-essential genes involved in the clock have been isolated using the various methods of saturating mutagenesis. With the functional genomics project currently being done under the direction of Dr. Susan Golden, each predicted ORF in the genome is being inactivated and tested for its effect on the circadian rhythm (41). Through this project, numerous genes that appear to be essential for both viability and for the maintenance of internal rhythms have been discovered. By using anti-sense RNA to knock down the activity of essential genes in cyanobacterial cells, their effect on the clock can be assessed in an otherwise viable genetic background (41).

Two recent additions to the arsenal of techniques used to unravel the tick-tockings of the clock system are structural determination of proteins and subcellular localization of fluorescent protein fusions. Many of the structures already completed have provided insight into the function of the respective protein that was not obvious from its primary

structure alone. The initial use of subcellular localization assays revealed which domain of CikA was required to confine the protein to the cell poles (130). The combined use of genetics, molecular biology techniques, protein localization, and protein structure provides the tools needed to attack the unknown elements of the *S. elongatus* clock system. With these tools at our disposal, *S. elongatus* will probably be the first organism for which a comprehensive understanding of circadian rhythmicity will be achieved.

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