View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Cell, Vol. 101, 223–233, April 14, 2000, Copyright ©2000 by Cell Press

A KaiC-Interacting Sensory Histidine Kinase, SasA, Necessary to Sustain Robust Circadian Oscillation in Cyanobacteria

Hideo Iwasaki,* Stanly B. Williams,† Yohko Kitayama,* Masahiro Ishiura,*‡ Susan S. Golden,† and Takao Kondo*§
*Division of Biological Science
Graduate School of Science
Nagoya University
Nagoya 464-8602
Japan
†Department of Biology
Texas A&M University
College Station, Texas 77843

Summary

Both regulated expression of the clock genes *kaiA, kaiB,* and *kaiC* and interactions among the Kai proteins are proposed to be important for circadian function in the cyanobacterium *Synechococcus* sp. strain PCC 7942. We have identified the histidine kinase SasA as a KaiC-interacting protein. SasA contains a KaiB-like sensory domain, which appears sufficient for interaction with KaiC. Disruption of the *sasA* gene lowered *kaiBC* expression and dramatically reduced amplitude of the *kai* expression rhythms while shortening the period. Accordingly, *sasA* disruption attenuated circadian expression patterns of all tested genes, some of which became arrhythmic. Continuous *sasA* overexpression eliminated circadian rhythms, whereas temporal overexpression changed the phase of *kaiBC* expression rhythm. Thus, SasA is a close associate of the cyanobacterial clock that is necessary to sustain robust circadian rhythms.

Introduction

Circadian clocks are endogenous biological timing processes that have been observed ubiquitously in organisms from cyanobacteria to green plants and humans. Temporal regulation of various metabolic and behavioral activities by the clock (circadian rhythmicity) is thought to be adaptive for organisms to survive under daily alteration in environmental conditions, such as light, temperature, and humidity (Pittendrigh, 1993; Ouyang et al., 1998). To elucidate the molecular mechanism of the circadian oscillator, several clock genes and clock-related genes have been cloned and analyzed in various organisms (reviewed by Dunlap, 1999).

Cyanobacteria are the simplest organisms that exhibit circadian rhythms (reviewed by Golden et al., 1997). Using a bioluminescence reporter gene, we monitored circadian gene expression in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter referred to as *Synechococcus*) (Kondo et al., 1993) and isolated various rhythm mutants (Kondo et al., 1994). A locus composed of three genes, *kaiA*, *kaiB*, and *kaiC*, was cloned previously by genetic complementation (Ishiura et al., 1998). All three genes are essential for circadian rhythms of gene expression. Detailed genetic analyses suggested that negative feedback control of KaiC on *kaiBC* expression helps to generate circadian oscillation, and that KaiA sustains the oscillation by increasing the *kaiC* expression (Ishiura et al., 1998).

The Kai proteins interact in various combinations in yeast cells, in vitro, and in *Synechococcus* (Iwasaki et al., 1999). More recently, we found that KaiC has ATP-binding and autophosphorylating activities that are likely important for the circadian feedback processes (Nishiwaki et al., 2000). These observations provided some biochemical characteristics of the Kai-based oscillator. However, to elucidate a molecular mechanism by which Kai proteins contribute to generation of circadian rhythms, it is necessary to identify and characterize other clock-associated genes.

We used a yeast two-hybrid screen to search for genes that encode KaiC-associating proteins, and identified a histidine protein kinase gene, *sasA*. The functional relevance of SasA to the cyanobacterial circadian clock was analyzed by examining cyanobacterial transformants in which *sasA* was disrupted or overexpressed. The results indicated that SasA activates *kaiBC* expression and is important for maintaining robust rhythmicity in *Synechococcus*. Moreover, *sasA* contributes to adaptation of the cells to grow in light/dark cycles.

Results

Identification of SasA as a KaiC-Interacting Protein

To identify *Synechococcus* genomic DNA encoding potential KaiC-binding proteins, we used a LexA-KaiC hybrid protein as bait in a yeast two-hybrid screen (Fields and Song, 1989; Iwasaki et al., 1999). The *Synechococcus* genomic library, which was constructed in the two-hybrid vector pVP16 (Vojtek et al., 1993), was prepared from a *kaiABC*-deficient *Synechococcus* strain (Ishiura et al., 1998) to exclude the Kai proteins from the screening. Yeast transformants (5×10^6 independent clones) were screened, and 5 library clones were obtained for KaiC-dependent dual reporter gene activation (see Experimental Procedures). Four of these plasmids carried identical 0.3 kb DNA segments that encode the N-terminal portion of the two-component system histidine protein kinase, SasA (*Synechococcus* adaptive sensor) (Nagaya et al., 1993) (Figure 1A). The fifth plasmid was probably not significant as it contained *Synechococcus* DNA encoding only 6 amino acid residues.

Interestingly, the KaiC-interacting portion of SasA (amino acyl residues 1–97) is similar to the full-length KaiB protein (Figure 1A). Identity and similarity between the two polypeptides are approximately 26% and 60%, respectively. Indeed, we independently found SasA by its similarity to KaiB in a database (BLAST) (Altschul et al., 1990) search during the course of the two-hybrid screening.

§To whom correspondence should be addressed (e-mail: kondo@bio.nagoya-u.ac.jp).
‡Present address: Center for Gene Research, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan.

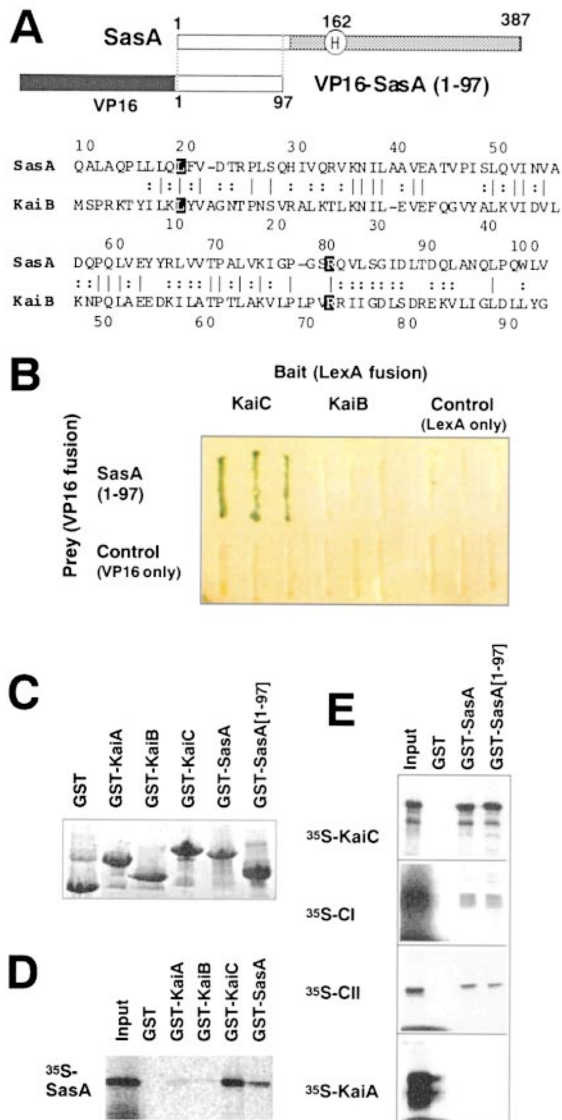

Figure 1. Identification of SasA as a KaiC-Interacting Factor

(A) Schematic representation of SasA (top) and the VP16:SasA-C-terminal domain fusion (middle). Putative histidyl residue for auto-phosphorylation (His 162) and conserved C-terminal kinase domain are indicated by shadowing on the SasA molecule. The amino acid sequence of the N-terminal SasA sequence was aligned with that of KaiB (bottom).

(B) Specific interaction of SasA(1–97) and KaiC in the two-hybrid system. Shown is the result of the filter assay for β-galactosidase reporter of yeast colonies cotransformed with the combination of bait and prey constructs indicated.

(C) Production of fusion proteins in *E. coli*. GST, GST fused to KaiA, KaiB, KaiC, SasA, or the N-terminal SasA fragment (residues 1–97) were produced in *E. coli* and purified by affinity chromatography with glutathione Sepharose 4B resin. The fusion proteins (about 5 μg each) were eluted from the resin and fractionated by SDS-PAGE on 10%–15% gradient gels, then stained with Coomassie brilliant blue (CBB).

(D) In vitro binding assay. GST, GST-KaiA, -KaiB, -KaiC, and -SasA (2 μg each) were immobilized on glutathione Sepharose 4B and then incubated with 2 μl of the reticulocyte reaction mixture containing ^{35}S-labeled SasA. Proteins associated with the resin were detected by SDS-PAGE on 12% gels followed by autoradiography.

(E) In vitro binding assay. GST, GST-SasA, and -SasA(1–97) (5 μg each) were immobilized on glutathione Sepharose 4B and then incubated with 3 μl of the reticulocyte reaction mixture containing ^{35}S-labeled intact KaiC, CI domain, CII domain, or KaiA. Proteins associated with the resin were detected by SDS-PAGE on 10% gels followed by autoradiography. Residues L11 and R76 in KaiB, whose mutations alter circadian periods, and the corresponding L19 and R80 in SasA, respectively, are reversed.

Although homotypic interaction of KaiB has been demonstrated using both yeast two-hybrid and in vitro binding assays (Iwasaki et al., 1999), and by bioluminescence resonance energy transfer (Xu et al., 1999), the hybrid protein VP16-SasA(1–97) (VP-16 fused to just the first 97 amino acyl residues of SasA) failed to interact with a hybrid LexA-KaiB protein in our yeast two-hybrid assay (Figure 1B).

We further established interaction between SasA and KaiC using an in vitro binding assay. Each protein, truncated SasA(1–97), full-length SasA(1–387), KaiA, KaiB, and KaiC, was individually expressed and purified with GST (glutathione-S-transferase) fused at the amino terminus (Figure 1C). The fusion proteins were immobilized on a glutathione sepharose affinity resin and incubated with in vitro–translated, ^{35}S-labeled SasA ([^{35}S]SasA). Resin-bound protein was eluted and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. As shown in Figure 1D, significant amounts of [^{35}S]SasA were retained by both immobilized GST-KaiC and GST-SasA fusions, but not by the GST, GST-KaiA, or GST-KaiB. In reciprocal assays, [^{35}S]KaiC interacted with both GST-SasA and GST-SasA(1–97), but not with the GST control (Figure 1E). [^{35}S]KaiA failed to interact with GST-SasA derivatives (Figure 1E).

The primary structure of KaiC contains a tandem repeat. The sequence of the first half, KaiC(1–260) called the CI domain, is easily aligned with and very similar to the sequence of the second half, KaiC(261–519), called the CII domain. Each half interacts separately with KaiA, KaiB, and KaiC in vitro (Iwasaki et al., 1999). Using the same procedure, we examined interaction between SasA and each half of KaiC, CI, and CII. Both [^{35}S]CI and [^{35}S]CII bound to both GST-SasA and GST-SasA(1–97) (Figure 1E). In summary, SasA interacted only with itself and KaiC. Either CI or CII was sufficient for the latter interaction. Interestingly, the first 97 amino acyl residues of SasA seem sufficient for all demonstrated interactions with KaiC and its derivative proteins.

Effects of *sasA* Disruption on Circadian Gene Expression

To test its function, we inactivated the *sasA* gene in a variety of reporter strains and monitored circadian gene expression patterns in continuous light (LL). The *sasA*-disrupted strains grew as well as their parental strains in LL (Nagaya et al., 1993) (for example, see Figure 6). Using promoter fusions to the *luxAB* genes encoding bacterial luciferase proteins, we determined patterns of gene expression from the *kaiA*, *kaiBC*, and *psbAI* promoters at two different light intensities after a single 12 hr dark period to synchronize the clock (Kondo et al., 1993; Ishiura et al., 1998).

At the higher light intensity (50 $\mu E\ m^{-2}\ s^{-1}$), amplitudes

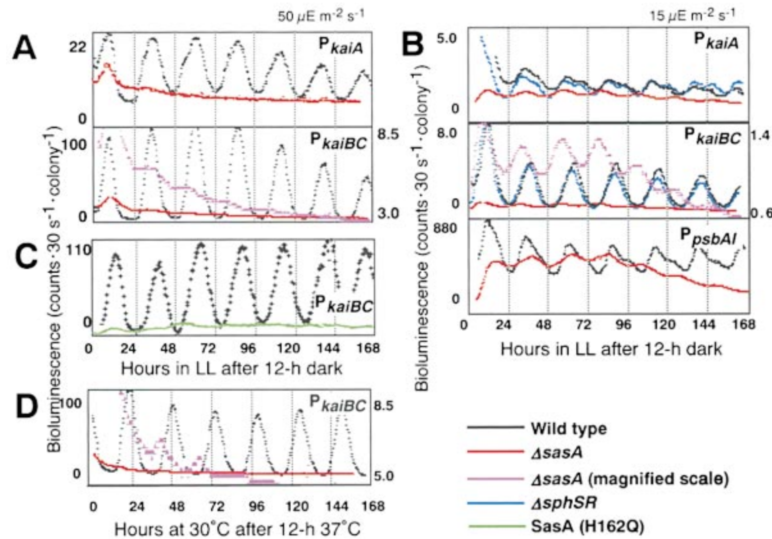

Figure 2. Disruption of *sasA* Attenuates Circadian *kai* Gene Expression

(A and B) Very low amplitude, short period bioluminescence rhythms in the absence of *sasA*. Wild type (black trace), *sasA*-disrupted (Δ*sasA*, red trace), and *sphSR*-disrupted (Δ*sphSR*, blue trace) cells that carry P_{kaiA}-, P_{kaiBC}-, or P_{psbAI}-reporter cassette were grown for 3–4 days on solid medium in LL at 50 μE m^{-2} s^{-1} (A) or 30 μE m^{-2} s^{-1} (B) to give 30 to 60 colonies (0.2 mm diameter). After a 12 hr dark treatment, cells were transferred to LL at 50 μE m^{-2} s^{-1} (A) or 15 μE m^{-2} s^{-1} (B), and the bioluminescence was measured by a photomultiplier tube. For clarity, bioluminescence profiles of the P_{kaiBC}-reporter strain lacking *sasA* are also shown in magnified scales (purple trace, maginified scale shown on the right).

(C) *kaiBC* expression profile in the H162Q mutant, in which a putative autophosphorylation residue was substituted with Gln. Bioluminescence from wild type (black) and the mutant (green) was monitored in LL at 50 μE m^{-2} s^{-1} as in (A).

(D) Nonphotic zeitgeber (temperature step-up) did not restore normal circadian phenotype in the *sasA*-disrupted strain. Cells were grown under LL (50 μE m^{-2} s^{-1}) at 30°C, treated with a temperature shift (37°C) for 12 hr, and then returned to 30°C. Other details are the same as described above.

of expression rhythms driven from both *kaiA* and *kaiBC* promoters (P_{kaiA} and P_{kaiBC}, respectively) were dramatically reduced and damped out to be arrhythmic within a few days (Figure 2A). Moreover, the magnitude of P_{kaiA} and P_{kaiBC} activity were lowered in the *sasA* disruptant cells to 40%–60% and 5%–10% of the peak levels, respectively.

At the lower light intensity (15 μE m^{-2} s^{-1}), the residual rhythmicity was more robust than at 50 μE m^{-2} s^{-1} (Figure 2B). Disruption of *sasA* shortened the period length of the P_{kaiBC} expression rhythm by 3 hr (23.0 ± 0.3 hr versus 25.8 ± 0.1 hr, n = 3). Both the magnitude of expression and the amplitude of the P_{kaiBC} rhythm were still diminished by inactivation of *sasA* (Figure 2B), but the magnitude of expression from both P_{kaiA} and P_{psbAI} was less affected.

In *Synechococcus*, expression of most or all genes is controlled by the circadian clock (Liu et al., 1995). Monitoring a larger set of reporter strains under low intensity LL revealed at least two classes of altered gene expression patterns in a *sasA* null background (Figure 3). In one class, which includes bioluminescence from *luxAB* gene reporter fusions to the *psbAII* (Golden et al., 1986), *cikA* (O. Schmitz, M. Katayama, and S. S. G., unpublished data) and *opcA* (Scanlan et al. 1992; H. Min and S. S. G., unpublished data) genes, inactivation of the *sasA* gene did not dramatically reduce bioluminescence levels but did result in arrhythmic, essentially constitutive, expression patterns (Figure 3A). In the other class, which includes *luxAB* fusions to the *rpoD2* and *ndhD* genes (Tsinoremas et al., 1996), *sasA* inactivation altered period, amplitude, or phase angle of the circadian expression rhythms (Figure 3B). Both phase angle and amplitude of the *rpoD2* expression rhythm were affected by inactivation of *sasA*, whereas the expression pattern from the *ndhD::luxAB* fusion retained little rhythmicity after *sasA* inactivation (Figure 3B).

Reduced amplitude of bioluminescence rhythms could be caused by availability of reduced flavin mononucleotide, a bacterial luciferase substrate. Thus, we also monitored expression patterns from a P_{kaiBC} gene fusion to the firefly luciferase gene, *luc*, which has different substrate requirements from the bacterial luciferase. Choice of reporter enzyme made no difference in the circadian phenotype of our wild-type or *sasA*-inactivated strains (data not shown).

Attenuation of *kaiBC* Expression Rhythm by Mutation in the Putative Autophosphorylation Site of SasA

To examine whether histidine kinase function of SasA is involved in circadian *kaiBC* expression, we substituted the conserved histidine at residue 162 (H162) (Figure 1A), a putative autophosphorylation site expected to be essential for phosphoryl transfer activity, with glutamine (H162Q). As shown in Figure 2C, H162Q mutation dramatically reduced *kaiBC* promoter activity and lowered amplitude of the rhythm, as did disruption of *sasA*. Thus, this residue of SasA is indeed necessary for normal robust rhythmicity in *Synechococcus*, consistent with a requirement for autophosphorylation.

Both *sasA* and another *Synechococcus* histidine protein kinase gene, *sphS*, were originally cloned by heterologous complementation of sensor kinase function in *Escherichia coli* (Aiba et al., 1993; Nagaya et al., 1993). Because both SasA and SphS belong to the EnvZ-type histidine kinase subfamily, we measured bioluminescence from a P_{kaiBC} reporter strain that lacked both *sphS* and its cognate response regulator, *sphR* (Aiba et al.,1993). The *sphS/sphR* disruptant exhibited wild-type bioluminescence expression rhythms (Figure 2B, blue line). Therefore, although SasA and SphS behave similarly in a heterologous complementation assay that is fairly nonspecific for sensor kinase function, only SasA has a role in circadian control in *Synechococcus*.

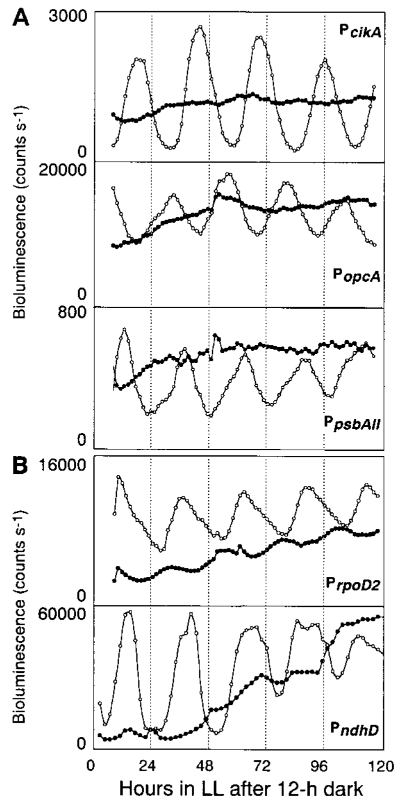

Figure 3. Two Phenotypic Classes Emerge after *sasA* Disruption

Bioluminescence rhythms comparing wild-type (open circles) to *sasA*-disrupted (filled circles) reporter strains. The promoter driving *luxAB* is indicated for each panel. Traces are from individual microtiter wells, but are representative of multiple independent experiments (see Experimental Procedures).

(A) Three reporter strains with arhythmic circadian phenotypes after *sasA* disruption are shown. Note that the P_{opcA} rhythm has a class 2 (180° offset) phase angle. (B) Two additional reporter strains that maintain detectable rhythmic expression patterns are shown.

Clock Resetting in the *sasA* Disruptant

Although unstable, the circadian clock in the *sasA*-disrupted strain is still entrainable by a 12 hr dark treatment (Figures 2A, 2B, and 3). To test whether a nonphotic zeitgeber (entrainment signal) is able to synchronize the circadian oscillator in the *sasA*-inactivated strain, we assayed P_{kaiBC} in wild-type and *sasA* backgrounds after a 12 hr exposure to high temperature. As shown in Figure 2D (red and purple lines), a 12 hr high temperature treatment (37°C) synchronized the *Synechococcus* clock to circadian time 12 (CT 12), which is subjective dusk, in both strains. Thus, *sasA* is not essential to reset the circadian clock by light or temperature. However, note that a 12 hr temperature step-up, concurrent with a 12 hr dark treatment still failed to recover robust rhythmicity in the *sasA*-disrupted strain (Figure 2D). Thus, attenuated circadian rhythmicity in this strain cannot be attributed to a defect in a light-specific signal transduction pathway to the clock.

Accumulation of *kai* Gene Products in the Absence of SasA

Inactivation of the *sasA* gene had dramatic effects on the expression of the *kai* genes without complete loss of the circadian timing mechanism (Figures 2 and 3). We examined the accumulation levels of *kai* mRNAs in an *sasA* strain by Northern (RNA) blot analysis. In wild-type cells, robust circadian rhythms of *kaiA* and *kaiBC* mRNA levels were observed under standard LL conditions (Ishiura et al., 1998) (Figure 4A). By contrast, no circadian fluctuations in these mRNA levels were evident in the *sasA* disruptant (Figure 4A). The levels of *kaiBC* and *kaiA* mRNAs were lowered in the mutant to 5%–15% and 40%–70%, respectively of those in wild-type cells at peak, correlating well with the bioluminescence profiles shown in Figure 2A.

Accumulation of the Kai proteins was also compared between wild-type and the *sasA* null strains. In wild-type cells, Y. Xu, T. Mori, and C. H. Johnson found KaiB and KaiC abundances exhibit circadian rhythms peaking at circadian time (CT) 15–16 (15–16 hr after lights-on) and troughing at CT 3–4, while the amplitude of KaiA accumulation rhythm is low (Figure 4B) (C. H. Johnson, personal communication; T. Nishiwaka, J. Tomita, and H. I., unpublished data). Accumulation of both KaiB and KaiC was dramatically reduced in the absence of SasA. KaiA accumulation was somewhat lower in the *sasA*-disruptant (∼70% of wild-type level), although to a lesser degree than that of KaiB and KaiC. These data are again consistent with our bioluminescence and Northern blot analyses.

Expression of *sasA* and Formation of SasA:KaiC Complex in *Synechococcus*

We monitored *sasA* expression patterns by inserting a promoterless *luxAB* gene set immediately downstream of the gene. As expected from evidence for global circadian control of gene expression (Liu et al., 1995), *sasA* promoter activity showed a circadian rhythm with period and phase angle similar to those of *kaiA* and *kaiBC*, while its amplitude was relatively low (Figure 4C). In correlation with the low amplitude expression rhythm, SasA protein appeared to accumulate constitutively throughout the day (Figure 4B and data not shown). As is the case for other clock-controlled genes in *Synechococcus*, the *kai* genes were required for rhythmic *sasA* gene expression (data not shown). However, disruption of the *kai* genes did not affect the magnitude of the *sasA* promoter activity (data not shown) nor SasA abundance (Figure 4B).

Because the KaiC-interacting portion of SasA is similar to KaiB, the two proteins may compete for binding to KaiC, and their relative ratios or affinities at different times of day could be important for their clock-related functions. To test whether KaiC and SasA/KaiB can be found in the same protein complex in vivo, we performed coimmunoprecipitation assays. *Synechococcus* lysates prepared from wild-type and *kaiABC*-deficient strains were separately immunoprecipitated with anti-KaiC antiserum, and the immunoprecipitated materials were blotted and probed with either anti-SasA or anti-KaiB antiserum. The results indicated that SasA and KaiC constitutively form a complex regardless of circadian time (Figure 4B and data not shown). In contrast, KaiC:KaiB complex is apparently more abundant at CT 16 than at CT 4 (Figure 4B).

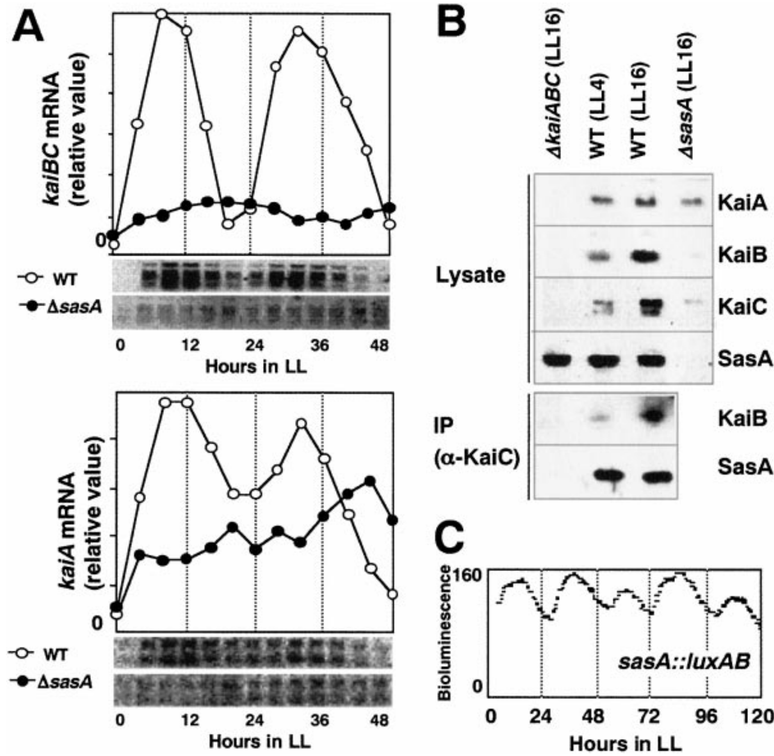

Figure 4. Expression of *kaiABC* and *sasA* Gene Products and SasA:KaiC Complex Formation

(A) Northern blot analysis. *kaiA* and *kaiBC* mRNA levels were examined in wild-type and *sasA*-disrupted strains at 4 hr intervals during hours 0–48 in LL (80 μE m^{-2} s^{-1}). Densitometric data of the blot are also shown. Multiple bands are due to both mRNA degradation and separation caused by the presence of rRNA. Abundance of *kai* mRNA was normalized for total RNA loading by using the signal of rRNA detected with ethidium bromide.

(B) Western blot analysis and coimmunoprecipitation assay. Accumulation levels of KaiA, KaiB, KaiC, and SasA were examined. Cells were collected at hours 4 and 16 in LL. Proteins (10 μg) were prepared from cells of wild-type, *kaiABC*-deficient, and *sasA*-disrupted strains, subjected to SDS-PAGE on 10% gels, and then analyzed by immunoblotting using each anti-Kai antiserum (upper four panels). The protein extracts were also used for immunoprecipitation (IP) with anti-KaiC IgG as a primary antiserum. Immune complexes were resolved and analyzed by Western blotting with anti-KaiB or anti-SasA antiserum.

(C) Low amplitude rhythm of *sasA* gene expression in LL, monitored by a bioluminescence reporter inserted just downstream of the *sasA* gene in the chromosome. Bioluminescence was monitored as described in Figure 2.

Effects of *sasA* Overexpression on *kai* Gene Expression

We also determined the effects of both constitutive and temporal overexpression of the *sasA* gene by placing its transcription under control of the IPTG (isopropyl β-thiogalactoside)–inducible *trc* promoter. The effects of overexpression were assayed in a P_{kaiBC} reporter strain (Figure 5A). Sustained overexpression of *sasA* was induced at hour 24 in LL (see Experimental Procedures). After one cycle, *kaiBC* expression became arrythmic with low bioluminescence levels (Figure 5A). Note that the subsequent circadian cycle after IPTG treatment was somewhat phase advanced relative to the uninduced control cycle. Loss of P_{kaiBC} activity after the subsequent cycle suggests that SasA has some role in negative regulation of *kai* gene expression as well as positive control of it (see Discussion).

In addition, we examined the effect of temporal overexpression of *sasA* on the phase angle of the expression rhythm from P_{kaiBC} by 6 hr administration of IPTG (Figures 5B and 5C). Transient elevation of *sasA* expression delayed oscillation when native *kaiBC* mRNA levels were increasing (from midsubjective day to midsubjective night, hours 24 to 30 and 30 to 36 in LL) and advanced the oscillation when these levels were decreasing (from midsubjective night to midsubjective day, hours 36 to 42). Based on Western blot analysis, our 6 hr administration of IPTG during hours 30 to 46 increased SasA protein levels 4- to 5-fold over endogenous levels (data not shown). This significant phase shifting by increased levels of SasA further suggests a close association of this protein with normal circadian clock function (see Discussion).

Growth of *sasA*-Disrupted Cells under Light/Dark Cycles

Inactivation of *sasA* did not affect the growth rate of *Synechococcus* under various LL conditions (light fluences of 6, 30, and 60 μE m^{-2} s^{-1} on agar plates) (Nagaya et al., 1993) (Figure 6A). However, we found that a *sasA*-disrupted strain grows much slower than the wild-type or a *kaiABC*-deficient strain under LD cycles (periodic alteration of 12 hr light and 12 hr darkness, 12L12D) of various light fluences (6, 30, or 60 μE m^{-2} s^{-1}). In addition, the *sasA*-disrupted strain in 12L12D (60 μE m^{-2} s^{-1}) appears to form colonies slower than it does in dim LL (6 μE m^{-2} s^{-1}), whereas wild-type cells grew faster in 12L12D (60 μE m^{-2} s^{-1}) than in dim LL. Thus, slower growth under LD cycles in the absence of SasA reflects a defect in adapting to light/dark transitions rather than by a simple difference in the quantity of light irradiation each day. Cells lacking both the *sphS* and *sphR* genes grew at similar rates to the wild-type strain in both LL and LD (Figure 6B). In addition, the *sasA*-deficient cells grew normally in LL at 6 or 60 μE m^{-2} s^{-1} under a temperature cycling regime (12 hr at 37°C and 12 hr at 30°C or 12 hr at 30°C and 12 hr at 25°C) (Figure 6B and data not shown). These results suggest a role for SasA in adapting the cell's metabolism specifically to natural light and dark transitions rather than in setting the circadian clock.

It should be noted that the abnormal circadian phenotypes observed in *sasA*-inactivated strains (Figures 2–4) cannot be attributed simply to a growth abnormality. This follows from two observations. First, the slow growth phenotype was not observed in the *sasA* null strains under LL conditions after the single 12 hr dark

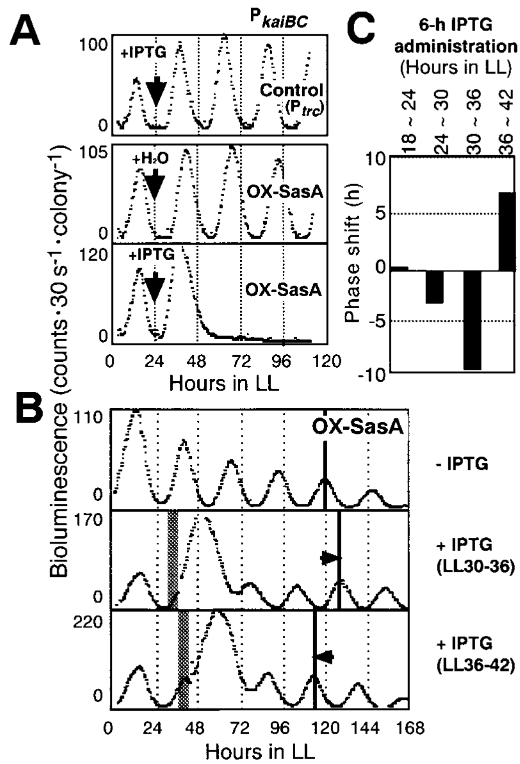

Figure 5. Overexpression of *sasA* Affects Circadian Rhythms

(A) Continuous overexpression of *sasA* disrupts circadian rhythms. Bioluminescence from a P_{kaiBC} reporter strain carrying a P_{trc} vector only or a P_{trc}::*sasA* construct (for overexpression of SasA) was monitored. Cells were grown on agar plates in LL, exposed to darkness for 12 hr to synchronize their clocks, returned to LL (50 μE m^{-2} s^{-1}) and treated with 2 mM IPTG or water at hour 24 in LL after the dark exposure (the arrows indicate the timing of addition of IPTG or water). Monitoring of bioluminescence and representation of data were the same as described in Figure 2.

(B and C) Transient (6 hr) SasA induction changes the phase of the circadian clock in a phase-dependent manner. Bioluminescence from P_{kaiBC}-reporter strains carrying the P_{trc}::*sasA* construct was monitored. Cells were grown and treated with IPTG within indicated duration (gray zone). Phase responses to *sasA* overexpression administrated at four distinct circadian times are indicated.

period used to entrain the clock (data not shown). In addition, expression levels from P_{kaiBC} were greatly reduced in the *sasA* inactivated strains even in the absence of any light to dark cues (Figure 6B).

Discussion

We have identified and characterized the *sasA* gene as encoding a salient function within the *Synechococcus* circadian timing mechanism. Although *sasA* was originally isolated from *Synechococcus* via heterologous complementation of two-component-type histidine protein kinase function in *E. coli* (using *envZ* or *phoR/creC* backgrounds, see Nagaya et al., 1993), its physiological functions were unknown. We have revealed functions of SasA which include: (1) physical interaction with the circadian clock protein KaiC through a KaiB-like region, (2) maintenance of robust circadian oscillation of gene expression under continuous light conditions, (3) enhancement of *kaiBC* expression, and (4) adaptation of growth to LD cycles. This is the first report of a two-component system signaling molecule that is intimately involved in circadian timing.

SasA as a Circadian Amplifier for Robust Rhythmicity

We conclude that SasA is necessary to maintain robust circadian oscillations in *Synechococcus*. This may arise through enhancement of transcription of the *kaiBC* genes, suggested to be a "state variable" of the cyanobacterial oscillator (Ishiura et al., 1998). The following data from our *sasA* null strains support this initial conclusion: (1) both promoter activities and *kai* mRNA levels, especially those from the *kaiBC* operon, were greatly reduced; (2) the average levels of expression of genes other than *kaiBC* were much less affected; (3) accumulation levels of the KaiB and KaiC proteins were reduced, whereas that of KaiA was less affected; (4) the amplitude and period of the circadian expression of all clock-controlled genes tested were evidently altered—in some cases, expression patterns were completely arhythmic.

It is important to note that the *sasA*-disrupted strain did not completely lose rhythmicity, whereas rhythmicity is completely abolished by disruption of any *kai* gene (Ishiura et al., 1998). However, the rhythms in the *sasA* null strain appear too weak to function as a global, temporal coordinator of cellular metabolism. Also, in spite of severe reduction of *kaiBC* expression (less than 10% of wild-type levels) by *sasA* disruption, the period of the residual rhythm was shortened but surprisingly still in a circadian range. Therefore, SasA is not an essential component of the basic timing loop, but evidently a close associate of the oscillator to amplify it to be sufficient as a robust temporal coordinator.

Continuous overexpression of *sasA* completely suppressed the *kaiBC* promoter (Figure 5A), and temporary elevation of SasA advanced or delayed the phase of the *kaiBC* expression rhythm in a phase-dependent manner (Figure 5B). Interestingly, the overexpression of *sasA* allowed one additional, robust *kaiBC* expression cycle before strong suppression of P_{kaiBC} activity. In contrast, *kaiC* overexpression immediately represses *kaiBC* expression (Ishiura et al., 1998). Phase shifting patterns after pulsed *sasA* overexpression were also different from those created by *kaiC* temporal overexpression. These results not only support a close association between SasA function and the central oscillatory mechanism, but also provide both functional differences between SasA and KaiC (Figure 7A and see below).

KaiC Interacts with the Sensor Domain of SasA

The sensory histidine kinase and the response regulator are two families of phosphorylation-based signal transduction proteins that constitute the two-component regulatory system (Stock et al., 1989; Parkinson, 1993). In sensory kinases, the amino-terminal portion of the protein is thought to be a signal input domain. As demonstrated above (Figure 1), only the amino-terminal, 97 amino acyl residues of SasA are necessary for its interactions with KaiC. Moreover, we confirmed that disruption of His 162 of SasA, presumably an essential residue for histidine kinase function, abolished SasA's function in circadian gene regulation (Figure 2C). Thus, it is plausible that binding to KaiC changes enzymatic activity of

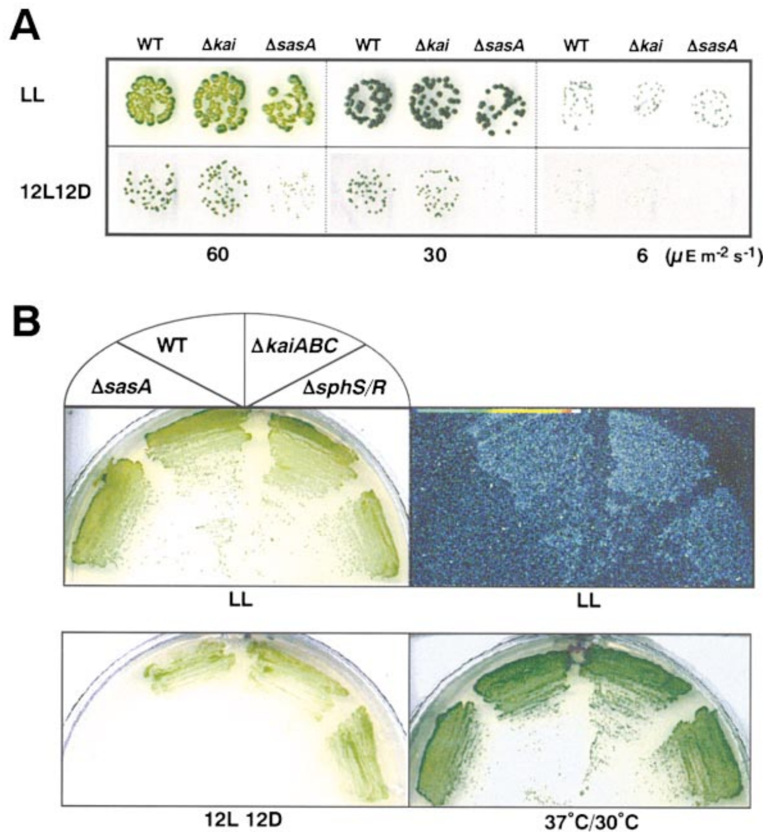

Figure 6. Slow Growth Phenotype in the Absence of *sasA* in LD but Not in LL or Temperature Cycle Conditions

(A) Colonies of wild-type, *kaiABC*-deficient or *sasA*-disrupted strains were allowed to form on solid media for 10 days under continuous illumination (LL) or 12 hr light/12 hr dark (12L 12D) conditions at the indicated light intensity.

(B) Growth phenotypes of wild type and strains lacking *sasA*, *kaiABC*, or *sphSR* on agar plates grown under LL (50 μE m^{-2} s^{-1}) and 12L (50 μE m^{-2} s^{-1}) 12D at 30°C, or LL (50 μE m^{-2} s^{-1}) with 12 hr 30°C/12 hr 37°C cycles. These strains carried a P_{kaiBC}*::luxAB* reporter. Bioluminescence image of the cells grown in LL was captured by a cooled CCD camera (upper right panel).

SasA and thereby mediates a clock-controlled, phosphoryl relay system (see below).

Regardless of the evolutionary relationships among the circadian timing mechanisms in prokaryotes and eukaryotes, it seems evident that "clocks" have recruited similar regulatory mechanisms to exhibit their control of cellular activities (Dunlap, 1999). Note that an open reading frame (ORF sll0750) that encodes a protein similar to SasA and structural homologs of *kaiABC* genes are present in the genome of *Synechocystis* sp. strain PCC 6803 (Mizuno et al., 1996), in which we have found circadian rhythms (Aoki et al., 1995). These cyanobacterial circadian-timing systems will provide an instructive evolutionary contrast to the fungal, insect, and mammalian systems (reviewed by Dunlap, 1999). It is also possible that identification of SasA will uncover phylogenic "missing-links" of circadian systems between prokaryotes and eukaryotes. Recent studies have revealed many proteins belonging to the two-component regulatory families in diverse eukaryotic species including yeast, *Neurospora*, *Dictyostelium*, and higher plants (reviewed by Wurgler-Murphy and Saito, 1997).

Implications of Similarity between SasA and KaiB

It is intriguing that the presumed input domain of SasA and KaiB share similar amino acid sequence. Moreover, SasA and KaiB exhibit similar protein-binding profiles in yeast or in vitro: (1) binding to KaiC, (2) lesser interaction with KaiA, and (3) homotypic interaction (Figure 1) (Iwasaki et al., 1999). Surprisingly, KaiB and SasA do not bind to each other in our assays.

Previous studies identified two mutations in the *kaiB* gene that cause a period shortening of approximately 3 hr in the expression rhythm from a *kaiBC* promoter (Ishiura et al., 1998). Recall that inactivation of *sasA* also reduces the period of this rhythm to a similar extent (Figure 2). Interestingly, both *kaiB* mutations result in amino acid changes (L11F, a leucine to phenylalanine change at residue 11, and R76W, an arginine to tryptophan substitution at residue 76) at positions that are identical in our sequence alignment of these proteins (L19 and R80, respectively, in SasA) (Figure 1A). These similarities suggest commensurate or overlapping function for SasA and KaiB in the circadian clock mechanism. It is likely that their mutual interactions with KaiC results in this functional overlap. KaiC and SasA form a complex constitutively in vivo, whereas KaiC-KaiB complex is more prevalent at CT 16 than at CT 4. KaiB and SasA may have similar affinities for KaiC, hence, the ratio of KaiB-KaiC complex and SasA-KaiC complex may simply reflect the relative abundance of KaiB and SasA (Figure 4B). It is possible that KaiB additionally binds to a KaiC molecule in the KaiC-SasA complex to form a larger multimer when KaiB levels are high at CT 16 (Figure 7A, right).

Function of SasA:KaiC Complex

How does SasA modulate *kaiBC* gene expression? In general, sensory kinases function by autophosphorylation and phospho-transfer activities at the beginning of a phosphoryl-relay system that terminates by modifying

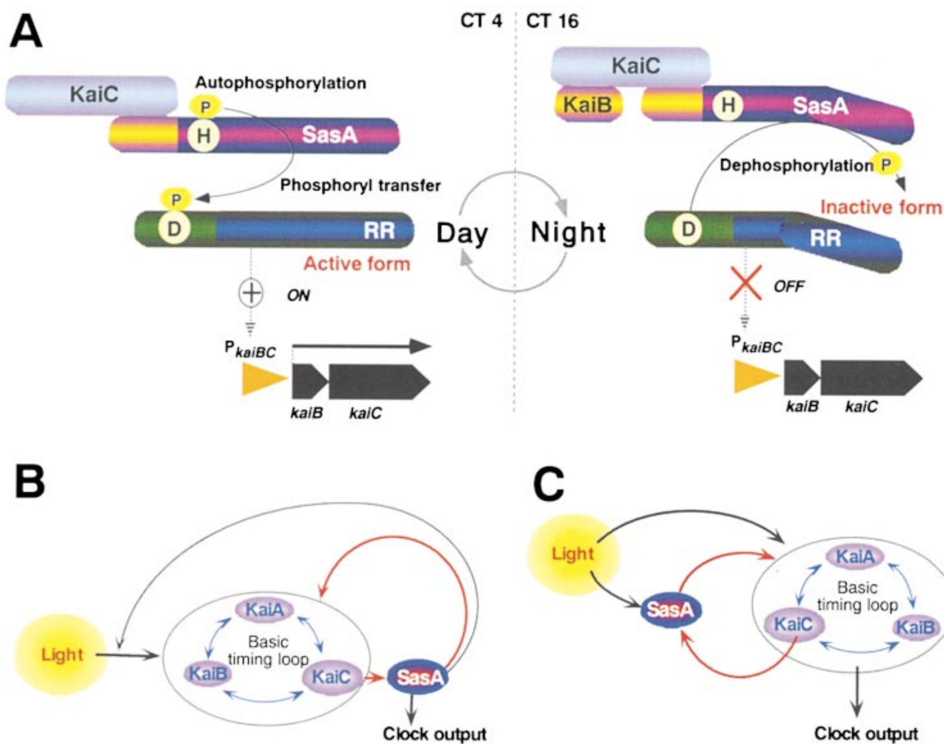

Figure 7. Possible Models for SasA Function in the Circadian System

(A) A model for function of the SasA:KaiC complex in *kaiBC* expression. In the early morning, when total KaiC abundance is low, SasA may bind with KaiC, stimulating autophosphorylation to activate its cognate response regulator (RR) through phosphoryl transfer from His (H) to Asp (D). Activated RR directly or indirectly activates expression of *kaiBC* genes from P_{kaiBC} (left). As the KaiBC concentration gets higher in the evening, KaiC:SasA complex is additionally bound with KaiC or KaiB to form a larger complex(es), which reduces SasA autophosphorylation. Hence, SasA may then act as a phosphoryl protein phosphatase, inhibiting RR-mediated stimulation of P_{kaiBC} activity (right).

(B and C) SasA may function as the first output of the clock to regulate downstream clock-controlled processes (B) or as an element of redundant input pathways (C). In both models, SasA and clock protein KaiC form an outer feedback loop (red arrows) to amplify the KaiABC-based basic timing process (gray circle) by modulating the *kai* gene expression as depicted in (A). In model (B), KaiC:SasA complex controls its cognate response regulator and thereby directly controls downstream gene expression. In model (C), SasA controls robustness of the circadian oscillation in a light-dependent manner, although SasA is not essential to entrain the clock. SasA may sense light signals directly or indirectly, and binding of SasA to KaiC would alter SasA's enzymatic activity. See Discussion for more detail.

function of a DNA-binding, transcription-regulating, response regulator. Thus, SasA-mediated phosphoryl relay would, by necessity, include at least one additional component—the SasA cognate response regulator. Considering the negative effects of *sasA* gene inactivation on expression levels from the *kaiBC* promoter (Figures 2 and 4), this response regulator may function to activate *kaiBC* gene transcription. The role for this postulated response regulator in the *Synechococcus* circadian oscillator would thus be comparable to those described for PAS-containing transcription factors, the dClock/Cycle (dBmal1) and CLOCK /BMAL1 proteins in *Drosophila* and mammals, and the WC-1 and WC-2 proteins in *Neurospora* (reviewed in Dunlap, 1999). Most histidine protein kinases like SasA have both kinase and phosphoryl-protein phosphatase activities with respect to their cognate regulatory proteins (Pratt and Silhavy, 1995). In the absence of the phosphatase activity, such as in a *sasA* null strain, low levels of phosphorylated (activated) response regulator can accumulate via nonspecific kinase activities (Kim et al., 1996). This nonspecific response regulator activation could account for the residual rhythmicity seen in *sasA* null strains (Figures 2 and 3).

Based on these considerations and data described in this paper, we propose a model for SasA-mediated modulation of the *kaiBC* expression (Figure 7A). In this model, both SasA and KaiC can be positive and negative elements in feedback control of *kai* gene expression. During subjective day, total KaiC level is relatively low, but considerable amounts of KaiC:SasA complex exist in the cell (Figure 4B). This complex functions as a positive element, possibly through stimulating SasA autophosphorylation, and thereby activating a SasA-coupled response regulator that directly or indirectly enhances *kaiBC* expression (Figure 7A, left). A positive function of KaiC for *kaiBC* expression has been suggested by our previous observation that P_{kaiBC} activity is somewhat downregulated in an arhythmic mutant mapped on *kaiC* (Ishiura et al., 1998).

By contrast, SasA may inactivate its cognate response regulator with its phosphoryl-protein phosphatase activity when SasA does not bind to KaiC or when KaiB (or/and KaiC itself) additionally binds the SasA:KaiC complex. During subjective night such KaiB:KaiC:SasA (Figure 7A, right) or/and multimerized KaiC:SasA complexes can be formed to negatively regulate *kaiBC* expression. It is still possible that KaiB could compete with SasA

for binding KaiC to release free SasA molecules functioning as negative elements, although we failed to detect reduction in the SasA:KaiC complex level at CT 4 in our experimental condition (Figure 4B).

Our model accounts for the additional expression cycle followed by loss of *kaiBC* expression when *sasA* is continuously overexpressed. Within several hours after IPTG administration at CT 0, when the KaiC level is not very high, the SasA level gradually increases and forms a complex with KaiC to enhance *kaiBC* expression. About 10 hr after induction, in turn, abnormally highly accumulating KaiC-uncoupled SasA gradually inactivates *kaiBC* expression to close one cycle and further abolishes recovery of its activation.

SasA Function in Cyanobacterial Circadian Organization

In theory, circadian organization can be divided into three major constituents: input pathways, the central oscillator, and output pathways. The oscillator generates fundamental circadian timing. Input pathways mediate environmental stimuli to synchronize the oscillator with environmental cycles. The oscillator is coupled to clock-controlled processes through output pathways. Based on our observations presented here, two alternative possibilities for the function of SasA in the cyanobacterial circadian organization can be assigned (Figures 7B and 7C).

The first possibility is that SasA functions as the first output from the circadian timing mechanism, and thus transmits timing signals to all clock-controlled processes (Figure 7B). This regulation includes feedback upon *kai* gene expression, upon light sensitivity of the oscillator, and also, necessarily, upon gene expression responsible for metabolic adaptation to natural growth conditions, which include light to dark transitions (see below).

An alternative possibility is that SasA modulates a photic input pathway(s) into the oscillator to act as a circadian amplifier (Figure 7C). Because the attenuated clock in the *sasA*-inactivated strain is still entrainable to both 12 hr darkness and 12 hr temperature shifts (Figure 2), SasA is not essential to entrain the clock. Nonetheless, SasA may function in one of several potential photic-input pathways because the amplitude of the circadian oscillation in a *sasA* mutant is more sensitive to light fluence than a wild-type strain (Figures 2A and 2B). This model postulates that SasA senses light signals either directly or indirectly, and that binding of SasA to KaiC would alter its sensitivity to further regulate SasA's enzymatic activity. Both models reflect both light-regulated *kaiBC* expression and a feedback control of the *kai*-based oscillator to its input. More importantly, as depicted in Figures 7B and 7C, SasA-KaiC interaction amplifies a KaiABC-based fundamental timing process (gray circle) by forming an outer feedback loop that modulates *kai* gene expression (red arrows and Figure 7A).

SasA as an Adaptive Sensory Kinase for Light/Dark Cycles

Growth is severely suppressed in a *sasA*-disrupted strain only under LD cycles (Figure 6). Microscopic observation did not reveal any morphological difference in cell shape and cell size between the *sasA* null and wild-type strains grown in LD conditions (data not shown). These results suggest that *sasA* is important for normal growth under natural diurnal conditions. The cause of slower LD growth in the absence of SasA could be perturbation of general metabolism in the dark. Alternatively, *sasA* mutant cells may be very sensitive to stress caused by abrupt light/dark transitions. Because *kaiABC*-deficient cells grow as well as wild-type cells in 12L12D, the effect of *sasA* disruption on circadian function alone is not likely to explain the maladaption to diurnal growth. However, it is still possible that the function of SasA in adaptation to LD transitions is regulated by the circadian clock through the SasA–KaiC interaction. In either case, SasA may allow *Synechococcus* to adapt to day–night alternation by maintenance of robust circadian rhythms and by directly adjusting metabolism to LD transitions.

Experimental Procedures

Bacterial and Yeast Strains

Saccharomyces cerevisiae strain L40 was used for the two-hybrid analysis (Vojtek et al., 1993). As hosts for transgenic analyses of *sasA*, we used wild-type *Synechococcus* reporter strains CR1 (P_{psbAI}::*luxAB* strain) (Kutsuna et al., 1998), NUC35, and NUC39 (P_{kaiA}::*luxAB* and P_{kaiBC}::*luxAB* strains carrying a chloramphenicol-resistance gene as a selective marker gene at a specific targeting site called NSI). NUC38 (Iwasaki et al., 1999) was used as a *kaiABC*-deficient P_{kaiBC} reporter strain. The *sphS/sphR*-deficient reporter strain was generated by transformation of NUC39 with pHAI140 (Aiba et al., 1993). *Synechococcus* was cultured in modified BG-11 medium (BG-11M) (Bustos and Golden, 1991).

Library Construction

Synechococcus genomic DNA was prepared from NUC39, partially digested with DNaseI (50 mM $MnCl_2$), and the resulting DNA fragments were repaired with T4 DNA polymerase and Klenow polymerase, and size-fractionated. Five-tenths to three kb fragments were ligated with NotI linkers and cloned into the NotI site of pVP16 (Vojtek et al., 1993). The resulting ligation products were transformed by electroporation into *E. coli* DH10B. Plasmid DNA was prepared from 1×10^6 transformant colonies. Approximately 70% of the clones were estimated to be recombinant, and inserts ranged from 0.3 to 2 kb.

Two-Hybrid Screening

The yeast strain L40 expressing LexA-KaiC (Iwasaki et al., 1999) was transformed with the *Synechococcus* genomic DNA library as described (Gietz et al., 1992). Approximately 5×10^6 transformants were screened for activation of the *HIS3* reporter gene (30°C for 3 days). Five of twenty-two of His^+ clones exhibited strong β-galactosidase activity. Library plasmids were then recovered from these clones and reintroduced into appropriate yeast reporter strains to verify a plasmid-linked, KaiC-dependent, His^+ $LacZ^+$ phenotype.

In Vitro Binding Assay

GST fused to either full-length SasA or N-terminal SasA fragments at the N terminus was produced by induction of derivatives of the pGEX-3X vector (Pharmacia) with 1 mM IPTG in an *E. coli* DH5α background. The resulting inclusion bodies were purified as described (Aiba et al., 1989), lysed with 1× PBS containing 6 M urea, and collected by centrifugation. The supernatant was dialyzed with 1× PBS containing 2 M urea and then with 1× PBS for 20 hr each. Then, fusion proteins were purified with and immobilized on glutathione Sepharose 4B (Pharmacia) as described (Iwasaki et al., 1999). ^{35}S-labeled KaiA, KaiC, CI, or CII was synthesized in vitro and interaction assays were performed as described previously (Iwasaki et al., 1999).

Disruption of the *sasA* Gene

One plasmid for disruption of *sasA*, pDsasA, carries three DNA segments in succession: a 2.1 kb DNA fragment carrying the upstream region of *sasA* (nucleotides [nt] 2 to 2099 relative to the left BamHI site of the 4.8 kb BamHI-KpnI segment from plasmid pCS33 [Nagaya et al., 1993] [A gift from Dr. H. Aiba]), a 1.5 kb HpaI fragment from plasmid pCKm carrying a kanamycin resistance gene (M. I., unpublished data); and the 1.8 kb SmaI-KpnI segment from pCS33. This 5.4 kb fragment was cloned into pBluescript KSII (Stratagene) to complete pDsasA. An additional disruption plasmid, pAM2076, was constructed by cloning the 4.8 kb BamHI-KpnI fragment from plasmid pCS33 into plasmid pUC19 (Promega) and then inserting a 1.4 kb XmnI-SmaI fragment from pDAH346 (provided by Dr. D. Hodgson) into the SmaI site within *sasA*. The pDAH346 fragment encodes a gentamycin resistance gene derived from pXS1 (Hirsch et al., 1986). *Synechococcus* strains NUC35 and NUC39 were transformed with pDsasA. Strains AMC520 (*psbAII::luxAB*), AMC538 (*rpoD2::luxAB*), AMC551 (*ndhD::luxAB*), AMC589 (*cikA::luxAB*), and AMC617 (*opcA::luxAB*) were transformed with pAM2076. The clones resistant to kanamycin sulfate (33 μg/ml) or gentamycin (2 μg/ml) were checked by Southern blot and PCR to confirm disruption of the *sasA* gene.

H162Q Mutant Strain

A 3.6 kb SacII-KpnI fragment of pCS33 (nt 1284 to 4850) was inserted into pBluescript KSII to construct pBSSA2. Next, the Ω fragment carrying a spectinomycin resistance gene (Prentki and Krisch, 1984) was inserted into the BstBI site of pBSSA2 (corresponding to nt 3491 of pCS33) to create pCsasΩ. Site-directed mutagenesis of pCsasΩ was performed as described (Horton, 1993) and designated pCsasΩHQ. NUC39 was transformed with pCsasΩHQ and selected with spectinomycin (40 μg/ml). Transformant colonies on agar plates were subjected to bioluminescence assay (Kondo and Ishiura, 1994) to screen for reduced bioluminescence clones. Seven low bioluminescent clones were identified from ~2000 transformants, and their *sasA* loci were confirmed for the H162Q mutation.

Northern Hybridization

Cells were grown with aeration in LL of 80 μE s^{-1} m^{-2} at 30°C to maintain an optical density of 0.3 at 730 nm (OD_{730}) using a continuous culturing system. The culture was exposed to 12 hr of darkness to synchronize the circadian clock, and then returned to LL. At 4 hr intervals in LL, cells were harvested, immediately frozen, and stored at −80°C. RNA extraction and Northern hybridization were performed using digoxigenin-labeled *kaiA* and *kaiC* probes as described (Ishiura et al., 1998).

Monitoring *sasA* Gene Expression as Bioluminescence from a Luciferase Reporter

The coding region of *luxAB* gene set from *Vibrio harveyi* (Baldwin et al., 1984) was inserted into the XbaI site of pCsasΩ (corresponding to nt 3398 of pCS33, ~50 bp downstream of the stop codon of *sasA*) to construct pCsas-lux. Then, *Synechococcus* was transformed with pCsas-lux to target *luxAB* just downstream of the *sasA* ORF of the chromosome. Transformant clones were selected with spectinomycin, grown under LL on BG-11M agar plates until colonies were 0.2 mm in diameter, and then assayed for bioluminescence rhythms.

Preparation of Anti-SasA Antiserum

Purified GST-SasA(1–97) was used to immunize rabbits. Antigen specificity of antisera was confirmed by Western blotting using cell extract prepared from NUC39 and pDsasA-transformed NUC39 as described previously (Iwasaki et al., 1999) except anti-SasA antiserum at 1000-fold dilution as a primary probe. The antiserum specifically detects SasA protein with molecular mass of ~45 kDa (Figure 4B).

Immunoprecipitation

Cells were grown with aeration in LL of 30 μE s^{-1} m^{-2} at 30°C to maintain the optical density at 730 nm of 0.2 as described above. After treatment with 12 hr of darkness, cells were returned to LL and harvested. Cyanobacterial cell extracts were prepared as described (Iwasaki et al., 1999). Protein content of each extract was adjusted to 200 μg in 400 μl of extraction buffer and incubated with 25 μl bed volume of AffiGel-Hz beads (BioRad) coupled to purified rabbit anti-KaiC IgG at 4°C for 12 hr. After six washes with 1 ml of extraction buffer, the beads were resuspended in 80 μl of SDS-sample buffer without reducing agent. Proteins were eluted by vortexing gently for 10 min at room temperature. After centrifugation, the supernatant was collected, supplemented with 0.1% 2-mercaptoethanol and 0.1% bromophenol blue, and subjected to SDS-PAGE and immunoblotting. KaiB, KaiC, and SasA were reacted with specific antisera at 1:1000, 1:2000, and 1:1000 dilution, respectively, and detected with the enhanced chemiluminescence (ECL) method (Pharmacia), according to the manufacturer's protocol.

Overexpression of the *sasA* Gene

Nco-BamHI segments carrying a sequence encoding SasA were ligated with NcoI- and BamHI-digested p322P*trc* (Kutsuna et al., 1998) to obtain $p322P_{trc}$::*sasA*. The smaller BglII fragment from $p322P_{trc}$::*sasA* was inserted into the BamHI site of the pTS2KC (Kutsuna et al., 1998) to obtain pTS2C-P_{trc}::*sasA*. NUC35 and NUC39 were transformed with each plasmid as described previously (Kutsuna et al., 1998) to harbor the expression construct at a specific genomic targeting site of the chromosome (NSII) (Andersson et al., 2000). Conditions for overexpression were the same as described previously (Ishiura et al., 1998), except that 2 mM IPTG was used for induction.

Bioluminescence Assays

Most bioluminescence rhythms were monitored and processed as described previously (Ishiura et al., 1998), except that we used a higher sensitivity photomultiplier tube (Hamamatsu R329P). For Figure 6B, bioluminescence from streaks of *Synechococcus* on agar plates was analyzed by a cooled CCD camera system as described previously (Kondo and Ishiura, 1994). For the data shown in Figure 3, cultures were grown for 3 to 4 days under standard LL conditions in liquid BG-11M containing the appropriate antibiotic before being transferred to 96-well microtiter plates containing BG-11M agar medium. Eighty wells, 40 for wild type and 40 for *sasA*-disrupted reporter strains, were used on each plate. The cultures were incubated in situ in the microtiter wells for 24 hr in LL before being subjected to a 12 hr dark interval to synchronize the circadian clock. Bioluminescence rhythms were subsequently measured and recorded in LL using a Packard TopCount luminometer as described elsewhere (Katayama et al., 1999; Andersson et al., 2000).

Acknowledgments

We thank C. H. Johnson (Vanderbilt University) for sharing unpublished results, H. Aiba and T. Mizuno (Nagoya University) for the kind gift of pCS33 and pHAI140, H. Min and O. Schmitz for use of the *opcA* and *cikA* reporter strains, respectively, and D. Hodgson (University of Warwick) for pDAH346. We are grateful to members of the Kondo lab for fruitful discussion and advice, especially T. Nishiwaki and J. Tomita for Western blotting, C. Inouye for the continuous culturing system, S. Kutsuna for RNA techniques, and Y. Taniguchi for the two-hybrid screen.

This research was supported by grants from the Japanese Ministry of Education, Science and Culture (11233203, 11440234, 11151213, 11558089, and 11694199), Research for the Future Program of the Japan Society for the Promotion of Science (JSPS, JSPS-RFTF96L00601) and the Mitsubishi Foundation to T. K., by NSF grant MCB9513367 to S. S. G., and by Human Frontier Science Program grants (to both T. K. and S. S. G.). H. I. was supported by JSPS Fellowship 09001527, and S. B. W. by NRSA Fellowship F32 GM19644 from the National Institutes of Health.

Received November 11, 1999; revised March 15, 2000.

References

Aiba, H., Mizuno, T., and Mizushima, S. (1989). Transfer of phosphoryl group between two regulatory proteins involved in osmoregulatory expression of the *ompF* and *ompC* genes in *Escherichia coli.* J. Biol. Chem. *264* 8563–8567.

Aiba, H., Nagaya, M. and Mizuno, T. (1993). Sensor and regulator proteins from the cyanobacterim *Synechococcus* species PCC7942 that belong to the bacterial signal-transduction protein families: implication in the adaptive response to phosphate limitation. Mol. Microbiol. *8*, 81–91.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Andersson, C.R., Tsinoremas, N.F., Shelton, J., Lebedeva, N.V., Yarrow, J., Min, H. and Golden, S.S. (2000). Application of bioluminescence to the study of circadian rhythms in cyanobacteria. Methods Enzymol., in press.

Aoki, S., Kondo, T., and Ishiura, M. (1995). Circadian expression of the *dnaK* gene in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J. Bacteriol. *177*, 5606–5611.

Baldwin, T.O., Berends, T., Bunch, T.A., Holzman, T.F., Rausch, S.K. Shamansky, L., Treat, M.L., and Ziegler, M.M. (1984). Cloning of the luciferase structural genes from *Vibrio harveyi* and expression of bioluminescence in Escherichia coli. Biochemistry *23*, 3663–3667.

Bustos, S.A., and Golden, S.S. (1991). Expression of the *psbDII* gene in *Synechococcus* sp. strain PCC 7942 requires sequences downstream of the transcription start site. J. Bacteriol. *173*, 7525–7533.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. Cell *96*, 271–290.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature *340*, 245–246.

Gietz, D., St. Jean, A., Woods, R.A., and Schiestl, R.H. (1992). Improved methods for high efficiency transformation of intact yeast cells. Nucleic Acid Res. *20*, 1425.

Golden, S.S., Brusslan, J., and Haselkorn, R. (1986). Expression of a family of *psbA* genes encoding a photosystem II polypeptide in the cyanobacterium *Anacystis nidulans* R2. EMBO J. *5*, 2789–2798.

Golden, S.S., Ishiura, M., Johnson, C.H., and Kondo, T. (1997). Cyanobacterial circadian rhythms. Annu. Rev. Plant Physiol. Mol. Biol. *48*, 327–354.

Hirsch, P.R., Wang, C.L. and Woodward, M.J. (1986). Construction of a Tn5 derivative determining resistance to gentamicin and spectinomycin using a fragment cloned from R1033. Gene *48*, 203–209.

Ishiura, M., Kutsuna, K., Aoki, S., Iwasaki, H., Andersson, C.R., Tanabe, A., Golden, S.S., Johnson, C.H., and Kondo, T. (1998). Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. Science *281*, 1519–1523.

Iwasaki, H., Taniguchi, Y., Ishiura, M., and Kondo, T. (1999). Physical interactions among circadian clock proteins, KaiA, KaiB and KaiC, in cyanobacteria. EMBO J. *18*, 1137–1145.

Katayama, M., Tsinoremas, N.F., Kondo, T., and Golden, S.S. (1999). *cpmA*, a gene involved in an output pathway of the cyanobacterial circadian system. J. Bacteriol. *181*, 3616–3624.

Kim, S., Wilmes-Riesenberg, M.R., and Wanner, B.L. (1996). Involvement of the sensor kinase EnvZ in the in vivo activation of the responsse-regulator PhoB by acetyl phosphate. Mol. Microbiol. *22*, 135–147.

Kondo, T., and Ishiura, M. (1994). Circadian rhythms of cyanobacteria: monitoring the biological clocks of individual colonies by bioluminescence. J. Bacteriol. *176*, 1881–1885.

Kondo, T., Strayer, C.A., Kulkarni, R.D., Taylor, W., Ishiura, M., Golden, S.S., and Johnson, C.H. (1993). Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc. Natl. Acad. Sci. USA *90*, 5672–5676.

Kondo,T., Tsinoremas, N.F., Golden, S.S., Johnson, C.H., Kutsuna, S., and Ishiura, M. (1994). Circadian clock mutants of cyanobacteria. Science *266*, 1233–1236.

Kutsuna, S., Kondo, T., Aoki, S., and Ishiura, M. (1998). A period-extender gene, *pex*, that extends the period of the circadian clock in the cyanobacterium *Synechococcus* sp. PCC 7942. J. Bacteriol. *180*, 2167–2174.

Liu, Y., Tsinoremas, N.F., Johnson, C.H., Lebedeva, N.V., Golden, S.S., Ishiura, M., and Kondo, T. (1995). Circadian orchestration of gene expression in cyanobacteria. Genes Dev. *9*, 1469–1478.

Mizuno, T., Kaneko, T., and Tabata, S. (1996). Compilation of all genes encoding bacterial two-component signal transducers in the genome of the cyanobacterium, *Synechocystis* sp. strain PCC6803. DNA Res. *3*, 407–414.

Nagaya, M., Aiba, H., and Mizuno, T. (1993). Cloning of a sensory-kinase-encoding gene that belongs to the two-component regulatory family from the cyanobacterium *Synechococcus* sp. strain PCC7942. Gene *131*, 119–124.

Nishiwaki, T., Iwasaki, H., Ishiura, M. and Kondo, T. (2000). Nucleotide binding and autophosphorylation of clock protein KaiC as a circadian timing process of cyanobacteria. Proc. Natl. Acad. Sci. USA *97*, 495–499.

Ouyang, Y., Andersson, C.R., Kondo, T., Golden, S.S., and Johnson, C.H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. Proc. Natl. Acad. Sci. USA *95*, 8660–8664.

Parkinson, J.S. (1993). Signal transduction schemes of bacteria. Cell *73*, 857–871.

Pratt, L.A. and Silhavy, T.J. (1995). Porin regulon of *Escherichia coli*. In Two-Component Signal Reduction, J. Hoch and T.J. Silhavy, eds. (Washington, DC: ASM Press), pp. 105–127.

Prentki, P., and Krisch, H.M. (1984). In vitro insertional mutagenesis with a selectable DNA fragment. Gene *29*, 303–313.

Scanlan, D.J., Newman, J., Sebaihia, M., Mann, N.H., and Carr, N.G. (1992). Cloning and sequence analysis of the glucose-6-phosphate dehydrogenase gene from the cyanobacterium *Synechococcus* PCC 7942. Plant Mol. Biol. *19*, 877–880.

Stock, J.B., Ninfa, A.D., and Stock, A.M. (1989). Protein phosphorylation and regulation of adaptive response in bacteria. Microbiol. Rev. *53*, 450–490.

Tsinoremas, N.F., Ishiura, M., Kondo, T., Tanaka, K., Takahashi, H., Johnson, C.H., and Golden, S.S. (1996). A sigma factor that modifies the circadian expression of a subset of genes in cyanobacteria. EMBO J. *15*, 2488–2495.

Vojtek, A.B., Hollenberg, S.M., and Cooper, J. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell *74*, 205–214.

Wurgler-Murphy, S.M. and Saito, H. (1997). Two-component signal transducers and MAPK cascades. Trends Biol. Sci. *22*, 172–176.

Xu, Y., Piston, D.W., and Johnson, C.H. (1999). A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc. Natl. Acad. Sci. USA *96*, 151–156.