labA: a novel gene required for negative feedback regulation of the cyanobacterial circadian clock protein KaiC

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In the cyanobacterium *Synechococcus elongatus* PCC 7942, circadian timing is transmitted from the KaiABC-based central oscillator to the transcription factor RpaA via the KaiC-interacting histidine kinase SasA to activate transcription, thereby generating rhythmic circadian gene expression. However, KaiC can also repress circadian gene expression, including its own. The mechanism and significance of this negative feedback regulation have been unclear. Here, we report a novel gene, *labA* (low-amplitude and bright), that is required for negative feedback regulation of KaiC. Disruption of *labA* abolished transcriptional repression caused by overexpression of KaiC and elevated the trough levels of circadian gene expression, resulting in a low-amplitude phenotype. In contrast, overexpression of *labA* significantly lowered circadian gene expression. Furthermore, genetic analysis indicated that *labA* and *sasA* function in parallel pathways to regulate *kaiBC* expression, whereas *rpaA* functions downstream from *labA* for *kaiBC* expression. These results suggest that temporal information from the KaiABC-based oscillator diverges into a LabA-dependent negative pathway and a SasA-dependent positive pathway, and then converges onto RpaA to generate robust circadian gene expression. It is likely that quantitative information of KaiC is transmitted to RpaA through LabA, whereas SasA mediates the state of the KaiABC-based oscillator.

[**Keywords:** Circadian clock; cyanobacteria; KaiC; *labA*; RpaA; SasA]

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The circadian clock is an endogenous timing system that controls various biological activities with a period of ~24 h. Most organisms use self-sustained oscillation to coordinate with and adapt to daily environmental changes. KaiA, KaiB, and KaiC have been identified as essential components for circadian oscillation in the cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus*). KaiC, an autokinase and autophosphatase, is the central component of the cyanobacterial circadian clock and interacts with KaiA and KaiB (Ishiura et al. 1998; Iwasaki et al. 1999; Nishiwaki et al. 2000; Taniguchi et al. 2001). KaiA enhances the autokinase activity of KaiC (Iwasaki et al. 2002; Williams et al. 2002; Uzunaki et al. 2004) and/or inhibits its autophosphatase activity (Kitayama et al. 2003; Xu et al. 2003), while KaiB attenuates the activity of KaiA (Williams et al. 2002; Kitayama

et al. 2003; Xu et al. 2003). KaiC phosphorylation oscillates with a period of ~24 h when the three recombinant Kai proteins are incubated in vitro in the presence of ATP (Nakajima et al. 2005). This oscillation period is refractory to changes in temperature, an important feature of circadian rhythms (Nakajima et al. 2005). Thus, Kai-based chemical oscillation is thought to be the basic circadian timing loop in *Synechococcus* (Nakajima et al. 2005; Tomita et al. 2005).

KaiC also interacts with a sensory histidine kinase, SasA (Iwasaki et al. 2000). Autophosphorylation of SasA is enhanced in response to KaiC binding (Smith and Williams 2006; Takai et al. 2006), and this phosphate group is then transferred to the putative DNA-binding protein RpaA (Takai et al. 2006). Inactivation of either *sasA* or *rpaA* dramatically reduces circadian gene expression (Iwasaki et al. 2000; Takai et al. 2006). Thus, the KaiC–SasA–RpaA pathway is likely to be a major positive pathway for circadian gene expression (Takai et al. 2006).

Notably, overexpression of KaiC strongly represses its own expression in a dose-dependent manner (negative

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feedback regulation) (Ishiura et al. 1998; Nishiwaki et al. 2004). In addition, most promoters in *Synechococcus* are clock-controlled (Liu et al. 1995) and are repressed to trough levels by KaiC overexpression (Nakahira et al. 2004). Transcriptional and translational oscillatory (TTO) processes involving negative feedback regulation of clock genes are thought to be essential for generating the basic circadian timing loop in most model organisms. In *Synechococcus*, negative feedback regulation of KaiC is important for circadian clock function, even though TTO processes are not necessary for circadian oscillation of KaiC phosphorylation (Nakajima et al. 2005; Tomita et al. 2005). It is thought that coupling of the KaiC phosphorylation cycle and the transcription–translation feedback process amplifies and stabilizes oscillation and mediates rhythmic physiological outputs (Tomita et al. 2005). However, the significance of negative feedback regulation of KaiC in the cyanobacterial circadian clock system has not been fully evaluated, because the mechanism of this feedback regulation is still unclear.

Here, we report a novel gene called *labA* (low-amplitude and bright) that is required for negative feedback regulation of KaiC. We demonstrate that *labA* and *sasA* cooperatively modulate *rpaA* function and cellular me-

tabolism. In addition, we discuss the physiological roles of feedback regulation of KaiC in the cyanobacterial circadian clock system.

Results

Identification of a novel gene required for negative feedback regulation of KaiC

We previously generated transgenic *Synechococcus* reporter strains in which the *Vibrio harveyi* luciferase gene was placed under the control of the *kaiBC* promoter ($P_{kaiBC}::luxAB$) and *kaiC* was placed under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter ($P_{trc}::kaiC$) (Ishiura et al. 1998; Iwasaki et al. 2002; Nakahira et al. 2004). In these strains, continuous IPTG-induced overexpression of *kaiC* results in complete repression of luciferase expression via negative feedback inhibition of the *kaiBC* promoter (Fig. 1A; Ishiura et al. 1998; Iwasaki et al. 2002; Nakahira et al. 2004).

To identify novel factors involved in the negative feedback regulation of KaiC, we searched for mutants that were defective in their $P_{kaiBC}::luxAB$ reporter gene response to overexpression of KaiC. We randomly mutagenized the $P_{kaiBC}::luxAB$ reporter strain carrying the

Figure 1. Identification of *labA* as a gene required for negative feedback regulation of KaiC. (A) Bioluminescence traces of *labA* mutants in the *kaiC*-overexpressing $P_{kaiBC}::luxAB$ reporter strain. Cells were grown on agar plates, and their bioluminescence rhythms were measured under LL after entrainment with two LD cycles. IPTG (1 mM) or water was added at hour 24 in LL (LL 24). (Top panel) Bioluminescence rhythm of the parental reporter strain OX-*kaiC*(Ω)/NUC42. (Middle panel) Original *labA* mutant (Mutant 1) isolated from screen. (Bottom panel) *labA* mutant generated using pDlabA(Km^r) [OX-*kaiC*(Ω)/*labA*(Km^r)/NUC42]. (Red trace) Bioluminescence of cells treated with 1 mM IPTG; (black trace) bioluminescence of cells treated with water. The arrow indicates the timing of the addition of IPTG (or water). (B) Nucleotide sequence around the *labA* locus and deduced amino acid sequence of LabA. Transposon insertion sites of the three original mutants (Mutant 1–Mutant 3) are underlined (the transposon insertion created a 9-bp target site sequence duplication). The HpaI site used to generate pDlabA(Km^r) or pDlabA(Ω) is boxed (see Materials and Methods). The arrow indicates the start site of the downstream ORF, Synpcc7942_1892/syc2203_c. (C) Sequence comparison of LabA and LabA-related proteins from cyanobacterial species (PCC 7942, *S. elongatus* PCC 7942; PCC 7120, *Nostoc sp.* PCC 7120; PCC 7421, *Gloeobacter violaceus* PCC 7421; PCC 6803, *Synechocystis sp.* PCC 6803; BP-1, *Thermosynechococcus elongatus* BP-1).

$P_{trc}::kaiC$ transgene [OX-*kaiC*(Ω)/NUC42] by transposon insertion and monitored the bioluminescence of individual colonies of transformants in the presence of IPTG. We screened ~20,000 colonies and isolated three independent mutants that expressed the *luxAB* reporter gene even in the presence of IPTG. In these mutants, KaiC was overexpressed at high enough levels to disturb circadian rhythms, but the level of bioluminescence progressively increased and remained elevated, suggesting that feedback regulation of KaiC was abolished (Fig. 1A). In the absence of IPTG, mutants showed moderate, low-amplitude rhythms with higher trough levels of bioluminescence (Fig. 1A). Moreover, the average bioluminescence intensity of the mutant lines was 10–20 times higher than that of the parental reporter strain (“bright” phenotype) (Fig. 1A).

To determine what genes were altered in these mutants, we recovered kanamycin-resistant genomic fragments from mutant cells, and DNA regions flanking the transposons were sequenced. The three mutants carried independent transposon insertions in the same 561-base-pair (bp) putative ORF encoding a conserved hypothetical protein (locus tag; Synpcc7942_1891/Syc2204_c) with no known functional motifs or domains (Fig. 1B). We confirmed that the mutant phenotype could be reproduced by inserting a kanamycin resistance gene cassette (Km^r) into the *HpaI* site of the ORF [OX-*kaiC*(Ω)/*labA*[−](Km)/NUC42] (Fig. 1A,B). We named this gene *labA* after the phenotype of the mutants. LabA-related protein sequences are found not only in cyanobacteria (Fig. 1C), but also in various prokaryotic organisms, including some archaea (Supplementary Fig. S1A).

We next examined the levels of *labA* transcript in a wild-type $P_{kaiBC}::luxAB$ reporter strain (NUC42) by Northern blotting (Fig. 2). As a negative control, we con-

structed a *labA* mutant without the $P_{trc}::kaiC$ transgene [*labA*[−](Km)/NUC42] by transforming NUC42 with pD*labA*(Km^r). After release from a 12-h dark period, *labA* transcripts accumulated gradually under continuous light exposure (LL 0.1–18) and plateaued after LL 18, whereas no transcript was detected in the *labA* mutant (Fig. 2). These results suggest that the accumulation of *labA* transcripts showed little rhythmicity.

Characterization of *labA* mutant

To confirm that the “low-amplitude and bright” phenotype was not caused by the presence of the $P_{trc}::kaiC$ transgene, we monitored the bioluminescence rhythm of *labA*[−](Km)/NUC42 cells (Supplementary Fig. S2A). Disruption of *labA* increased the trough levels of bioluminescence, resulting in a low-amplitude rhythm with broad peaks (Supplementary Fig. S2A). In addition, the maximum bioluminescence intensity in the *labA* mutant was about sevenfold higher than that of the parental wild-type reporter strain (Supplementary Fig. S2A). These results demonstrate that the phenotype we observed in *labA* mutants was independent of the $P_{trc}::kaiC$ transgene.

To evaluate whether or not loss of *labA* affects expression at other promoters, we inactivated *labA* in the $P_{psbAI}::luxAB$ reporter strain AMC149 (Kondo et al. 1993). *psbAI* encodes a photosystem II protein, and its promoter drives expression with a robust circadian rhythm (Fig. 3A; Kondo et al. 1993). We found that *labA* mutants on an AMC149 background [*labA*[−](Km)/AMC149] exhibited a severe, low-amplitude rhythm with distorted waveforms, and the maximum bioluminescence intensity of the *labA* mutant was ~12-fold higher than that of the parental wild-type reporter strain (Fig. 3A), indicating that the *labA* mutation can affect expression at various promoters.

The increased bioluminescence in *labA* mutants suggested that *labA* might play a role in bacterial luciferase reactions. To test this possibility, we generated *labA* mutants in which the firefly luciferase (*luc*) gene was placed under the control of the P_{kaiBC} promoter (AMC541 strain). Interestingly, the average bioluminescence intensity per colony of *labA*[−](Km)/AMC541 was comparable to that of the parental AMC541 strain, in spite of the fact that disruption of *labA* caused low-amplitude rhythms with higher trough levels and broader peaks (Fig. 3B). In other words, the bright phenotype of *labA* was not observed in the AMC541 reporter strain. Similar results were obtained using a railroad-worm luciferase reporter strain, *labA*[−](Km)/P*kaiRE* (Supplementary Fig. S2B). The bacterial luciferase *luxAB* requires oxygen, reduced flavin mononucleotide (FMNH₂), and n-decanal as substrates to generate bioluminescence, whereas firefly and railroad-worm luciferase require ATP and luciferin instead of FMNH₂ and n-decanal. In our experiments, luciferin and n-decanal were provided exogenously, whereas ATP and FMNH₂ were derived from endogenous sources. Thus, a plausible explanation for the extreme bright phenotype when bacterial luciferase

Figure 2. Identification of *labA* transcripts. (A) NUC42 (wild-type) and *labA*-disrupted NUC42 [*labA*[−](Km)/NUC42] strains were cultured in BG-11M medium, and cells were collected every 6 h after entrainment with LD cycles. Total RNA (5 μ g) was separated by electrophoresis, and *labA* transcript was detected by Northern blotting using a DIG-labeled *labA*-specific probe. An image of ribosomal RNA (rRNA) stained with ethidium bromide is shown in parallel. (B) Densitometric data of the blot are also shown.

Figure 3. Characterization of *labA* mutants generated by pDlabA(Km^r). (A,B) Bioluminescence rhythms of *labA* mutants. *labA* was inactivated in *P_{psbAI}::luxAB* (A) or *P_{kaiBC}::luc* (B) reporter strains, and bioluminescence was monitored after entrainment with two LD cycles. (C) Fluctuation of *kaiBC* transcript in *labA* mutant [*labA⁻*(Km)/NUC42] cells detected by Northern blotting. (Panel *a*) Samples were prepared as described in Figure 2A except for the additional sampling at LL 9, and *kaiBC* transcript was detected by Northern blotting using a DIG-labeled *kaiBC*-specific probe. (Panel *b*) Densitometric data from three independent experiments. Data are expressed as mean $\pm$ SEM. (D) Accumulation profile of KaiC protein in the *labA* mutant [*labA⁻*(Km)/NUC42]. Cells were collected as described in Figure 2A, and total cellular proteins were prepared. Two micrograms of total cellular protein were loaded per lane, and KaiC was detected with anti-KaiC antiserum. The upper and lower bands correspond to phosphorylated KaiC (P-KaiC) and nonphosphorylated KaiC (NP-KaiC), respectively. (E) Slow-growth phenotype of the *labA* mutant. Colonies were grown in LL (40 μ E m⁻² sec⁻¹) at 30°C for 6 d.

was used as a reporter is that FMNH₂ may accumulate to high levels in the *labA* mutants, implying that *labA* may be involved in FMNH₂-related metabolism as well as in circadian gene expression. It was previously shown that disruption of *opcA*, which regulates the level of endogenous FMNH₂, affects the bioluminescence rhythm of *luxAB* reporter strains (Min and Golden 2000). However, since the bioluminescence waveform of *labA⁻*(Km)/NUC42 cells was similar to that of *labA⁻*(Km)/AMC541 cells (Fig. 3B; Supplementary Fig. S2A), whatever causes the bright phenotype does not affect the bioluminescence waveforms in *luxAB* reporter strains. The *labA* mutants also showed a slow-growth phenotype on agar plates (Fig. 3E), suggesting that *labA* is involved in cellular metabolism. It should be noted that the *labA* mutant did not exhibit slow growth in liquid culture (data not shown), indicating that this phenotype is dependent on culture conditions. The phenotypes in the mutants were not due to polar effects on the ORF downstream from the *labA* locus (locus tag; Synpcc7942_1892/syc2203_c) (see also Fig. 1B), because inactivation of Synpcc7942_1892/syc2203_c did not phenocopy our original mutants (data not shown). In addition, transgenic expression of *labA* in *labA* mutants was able to rescue the mutant phenotype (Supplementary Fig. S3).

Low-amplitude rhythms were also observed in the *labA* mutant by Northern blotting for *kaiBC* transcript (Fig. 3C). The average accumulation level of the *kaiBC* transcript in the *labA* mutant was also increased, probably due to the higher trough level of transcriptional oscillation (Fig. 3C). Western blotting confirmed that KaiC was expressed at higher levels in the *labA* mutant than in wild-type cells, although the circadian phosphorylation-dephosphorylation cycle of KaiC was still sustained in the mutant (Figs. 3D, 5E [below]). These results are consistent with the fact that negative feedback regulation of KaiC was abolished in *labA* mutants.

Effects of *labA* overexpression on circadian gene expression

To further analyze the function of *labA*, we examined the effect of *labA* overexpression on circadian gene expression in reporter strains carrying an IPTG-inducible *P_{trc}::labA* transgene. Overexpression of *labA* induced by 1 mM IPTG significantly reduced the amplitude of the bioluminescence waveforms in the *P_{kaiBC}::luxAB* reporter strain (OX-*labA*/NUC42) (Fig. 4A). This effect was dose-dependent and did not affect the period of the bioluminescence rhythm (Fig. 4C). Similar results were ob-

tained from experiments with the $P_{psbA1}::luxAB$ reporter strain (OX-*labA*/AMC149) (Fig. 4B,C).

To confirm that *labA* overexpression lowers the reporter gene expression, we examined the expression level of *luxAB* transcripts in OX-*labA*/NUC42 by Northern blotting. Cells were cultured in BG-11M liquid medium with or without 1 mM IPTG and sampled after entrainment by 12 h light/12 h dark (LD) cycles. The transcript of *luxAB* was subsequently detected by using a *luxAB*-specific probe. IPTG-induced overexpression of *labA* reduced expression of *luxAB* transcript, but the circadian oscillation of *luxAB* transcript was sustained (Fig. 4D). These results are consistent with those shown in Figure 4A, suggesting that *labA* represses circadian gene expression in a dose-dependent manner.

Cooperative modulation of circadian gene expression by *labA* and *sasA* through *rpaA*

The KaiC-interacting sensory histidine kinase SasA and its cognate response regulator RpaA are involved in the output pathways of the cyanobacterial circadian clock (Iwasaki et al. 2000; Takai et al. 2006). Inactivation of either *sasA* or *rpaA* dramatically decreases circadian gene expression (Fig. 5C,D; Iwasaki et al. 2000; Takai et al. 2006), indicating their positive regulatory roles in circadian gene expression. However, continuous overexpression of either *sasA* or *rpaA* also repressed *kaiBC* expression (Fig. 5A,B; Iwasaki et al. 2000). To assess whether mutations in *labA* affect transcriptional repression by *sasA* or *rpaA*, we generated *labA* mutants carrying the $P_{kaiBC}::luxAB$ reporter and either the $P_{trc}::sasA$ (OX-*sasA*/NUC42) or $P_{trc}::rpaA$ transgene (OX-*rpaA*/NUC42) and monitored their bioluminescence rhythms in the presence of 1 mM IPTG. As shown in Figure 5A, loss of *labA* function attenuated (not abolished) the negative effect of *sasA* overexpression, suggesting the additive effect of *labA* and *sasA*. However, this *labA* disruption did not substantially affect the negative effect of *rpaA* overexpression (Fig. 5B).

To further investigate the relationship between *LabA* and the SasA–RpaA pathway, we generated *labA*/*sasA* or *labA*/*rpaA* double mutants on a $P_{kaiBC}::luc$ (AMC541) background. The *sasA* single mutant [*sasA*[−](Km)/AMC541] exhibited a low-amplitude and short-period phenotype, as previously described (Fig. 5C; Table 1; Iwasaki et al. 2000). However, the level of the bioluminescence of the *labA*/*sasA* double mutant [*labA*[−](Ω)/*sasA*[−](Km)/AMC541] was significantly higher than that of *sasA* single mutants [*sasA*[−](Km)/AMC541] and lower than that of *labA* single mutants [*labA*[−](Ω)/AMC541], indicating that the *labA* mutation suppressed the low-amplitude phenotype of the *sasA* mutant (Fig. 5C; Table 1). The level of KaiC protein was also examined by Western blotting analysis (Fig. 5E). In the *sasA* mutant [*sasA*[−](Km)/AMC541], the level of KaiC was significantly reduced, as previously reported (Fig. 5E; Iwasaki et al. 2000; Takai et al. 2006). In the *labA*/*sasA* double mutant [*labA*[−](Ω)/*sasA*[−](Km)/AMC541], the level of KaiC was restored (Fig. 5E). The additive effect of compound

Figure 4. *labA* overexpression reduces circadian gene expression. (A–C) Effects of *labA* overexpression on bioluminescence rhythms of *luxAB* reporter strains. OX-*labA*/NUC42 (A), OX-*labA*/AMC149 (B), and parental reporter strains (Control) were exposed to 1 mM IPTG (red trace) or water (black trace). Arrows indicate the timing of the addition of IPTG (or water). (C) Dose-dependent effects on bioluminescence rhythms in OX-*labA*/NUC42 and OX-*labA*/AMC149 reporter strains. Parental strains NUC42 and AMC149 were assayed in parallel. Bioluminescence rhythm was monitored in LL after entrainment with two LD cycles, and various concentrations of IPTG were added to samples around LL 24. The bioluminescence intensity of the second peak (just following IPTG addition) was normalized with that of the first peak (before IPTG treatment), and the ratio value (relative second peak level) was plotted. (D) Expression of the *luxAB* reporter gene in OX-*labA*/NUC42. (Panel a) OX-*labA*/NUC42 and control NUC42 cells were cultured with or without 1 mM IPTG and sampled every 6 h. Five micrograms of total RNA were loaded per lane, and *luxAB* transcript was directly detected by Northern blotting using a DIG-labeled *luxAB*-specific probe. Ribosomal RNA (rRNA) stained with ethidium bromide is shown in parallel. (Panel b) Densitometric data of the blot are also shown.

Figure 5. Genetic interactions of *labA* with *sasA* and *rpaA*. (A,B) Effects of *labA* mutation on transcriptional repression caused by overexpression of *sasA* or *rpaA*. *OX-sasA/NUC42* and *OX-rpaA/NUC42* were transformed with *pDlabA(Δ)* to disrupt the *labA* gene. The resulting *labA* mutants and parental strains were exposed to 1 mM IPTG (red trace) or water (black trace), and bioluminescence rhythms were measured. Arrows indicate the timing of the addition of IPTG (or water). (C) Effects of *labA/sasA* double mutation on circadian gene expression. *AMC541, labA⁻(Δ)/AMC541, sasA⁻(Km)/AMC541*, and *labA⁻(Δ)/sasA⁻(Km)/AMC541* were grown on BG-11M agar plates under LL conditions. After entrainment with two LD cycles, bioluminescence rhythms of the cells were measured in the presence of 0.5 mM luciferin. (D) Effects of *labA/rpaA* double mutation on transcriptional oscillation. Bioluminescence rhythms of *AMC541, labA⁻(Δ)/AMC541, rpaA⁻(Km)/AMC541*, and *labA⁻(Δ)/rpaA⁻(Km)/AMC541* were monitored as described in C. (E) Accumulation levels of KaiC in the *labA/sasA* double mutant. KaiC detection was performed as described in Figure 3D. SasA was also detected in parallel using an anti-SasA antiserum.

mutations on circadian gene expression suggests that *labA* and *sasA* are involved in different output pathways.

It should be noted that the *labA* mutation also suppressed the short-period phenotype of the *sasA* mutant (Fig. 5C; Table 1). In the *sasA* mutant, phosphorylated KaiC was dominant at all the time points tested, as previously reported (Fig. 5E; Takai et al. 2006), whereas the circadian KaiC phosphorylation cycle was restored in the *labA/sasA* double mutant (Fig. 5E), probably due to restored *kaiBC* expression (Fig. 5C; Table 1). This restored KaiC phosphorylation cycle likely contributed to the suppression of the short-period phenotype of the *sasA* mutant.

We also examined the bioluminescence of *labA/rpaA* double mutants [*labA⁻(Δ)/rpaA⁻(Km)/AMC541*]. We found that the phenotype of the *labA/rpaA* double mutant was similar to that of the *rpaA* single mutant [*rpaA⁻(Km)/AMC541*], indicating that *rpaA* was epistatic to *labA* (Fig. 5D). These data are consistent with our earlier finding that the *labA* mutation could not suppress the *rpaA* overexpression phenotype (Fig. 5B). These

results suggest that *labA* and *rpaA* function in the same transcriptional regulation pathway.

Suppression of slow-growth phenotype of the *sasA* mutant by *labA* mutation

The *sasA* mutation causes a slow-growth phenotype under LD conditions (Iwasaki et al. 2000). We examined the effect of *labA* mutation on the slow-growth phenotype of the *sasA* mutant. On agar plates, the *sasA* mutant and the *labA/sasA* double mutant grew well under LL conditions (Fig. 6; Iwasaki et al. 2000). It should be noted that the slow-growth phenotype of *labA* mutants in this figure was obscured because the high-density cell cultures were plated and the growth was at saturation level in the conditions. Under LD conditions, the *sasA* mutant [*sasA⁻(Km)/AMC541*] showed a slow-growth phenotype (Fig. 6; Iwasaki et al. 2000), whereas *labA/sasA* double-mutant cells grew slower than wild-type cells (data not shown) but much faster than *sasA* mutant cells (Fig. 6). These results indicate that the *labA* mutation partially suppresses the slow-growth phenotype of *sasA* mutants under LD conditions.

Table 1. Phenotypic analysis of bioluminescence rhythm of *labA*[−]/*sasA*[−] double mutants

Genotype	Maximum bioluminescence intensity (counts/30 sec/colony)	Period length (h)
Wild type	30,547 ± 3994	25.52 ± 0.08
<i>labA</i> [−]	28,047 ± 2804	24.72 ± 0.24
<i>sasA</i> [−]	1751 ± 479	23.09 ± 0.30
<i>labA</i> [−] / <i>sasA</i> [−]	7320 ± 643	24.88 ± 0.30

Maximum intensity and period length of the bioluminescence rhythms of the wild-type strain [AMC541], *labA* mutant [*labA*[−](Ω)/AMC541], *sasA* mutant [*sasA*[−](Km)/AMC541], and *labA/sasA* double-mutant [*labA*[−](Ω)/*sasA*[−](Km)/AMC541] cells determined from their second peaks under LL conditions. Data are expressed as mean ± SEM from five independent experiments.

Discussion

In this study, we have identified *labA* as a novel cyanobacterial gene that modulates circadian gene expression. Disruption of *labA* increased the trough levels of circadian gene expression, resulting in a low-amplitude phenotype (Figs. 1A, 3A–C, 5A–D; Supplementary Fig. S2). In addition, overexpression of *labA* significantly reduced circadian gene expression (Fig. 4). Thus, we conclude that *labA* acts as an inhibitor of circadian gene expression. Moreover, mutations in *labA* abolished transcriptional repression by overexpression of KaiC (Fig. 1A), suggesting that negative feedback regulation of KaiC is mediated by LabA. Overexpression of a mutant version of KaiC that cannot be phosphorylated fails to repress *kaiBC* promoter activity (Nishiwaki et al. 2004), suggesting that phosphorylated KaiC is involved in feedback regulation. Therefore, LabA is likely to function downstream from phosphorylated KaiC as a negative output path of the Kai-based central oscillator.

The SasA–RpaA two-component system is also involved in the output pathway that causes global gene expression to reflect circadian oscillation (Takai et al. 2006). This phospho-relay can be modulated by the state of the Kai-based oscillator. Our epistasis analysis strongly suggested that *labA* and *sasA* are involved in different output pathways of the circadian clock, whereas *labA* and *rpaA* function in the same transcriptional regulation pathway (Fig. 5). The *labA* mutation did not substantially interfere with transcriptional repression produced by *rpaA* overexpression (Fig. 5B), suggesting that *rpaA* functions downstream from *labA*. The inhibitory role of *labA* in circadian gene expression suggests that LabA might function as a “brake” on RpaA, whereas SasA acts as an “accelerator” of RpaA (Takai et al. 2006). In other words, RpaA is likely to receive temporal information from the circadian clock through at least two pathways: the LabA-dependent negative pathway and the SasA-dependent positive pathway. Since the *labA/sasA* double mutant did show circadian gene expression (Fig. 5C), there are likely additional circadian pathways that regulate RpaA. Because LabA did not interact with any Kai proteins, SasA, or RpaA in our yeast

two-hybrid assays (Y. Taniguchi, unpubl.), LabA may interact indirectly with these clock-related proteins through yet unknown binding partners in cyanobacterial cells. The molecular partner of LabA remains to be elucidated.

Physiological roles of LabA in the circadian clock system

Previous work suggested a model for the cyanobacterial circadian clock system in which the KaiC phosphorylation cycle is coupled to a transcriptional–translational feedback loop (Tomita et al. 2005). Thus, LabA is likely to affect the entire circadian clock system through feedback regulation of KaiC (Fig. 1A).

What are the physiological roles of LabA in this system? Our data show that LabA increases the peak/trough ratio of circadian gene expression via transcriptional repression. Earlier work suggested that the SasA–RpaA pathway is likely to be activated mainly in the subjective-day phase (Takai et al. 2006). Because the peak of SasA–RpaA phosphorylation in vitro occurs 4–8 h prior to that of phosphorylated KaiC, changes in its amount itself may not be the primary factor for the circadian regulation of this pathway (Takai et al. 2006). Consistent with this SasA–RpaA phosphorylation, *kaiBC* transcript fluctuates in vivo with a peak at circadian time (CT) 9–12 (subjective dusk) and a trough at CT 0 (subjective dawn) (Figs. 3C, 7A; Ishiura et al. 1998; Iwasaki et al. 2000; Imai et al. 2004; Takai et al. 2006). The fluctuation in KaiC protein and KaiC phosphorylation is shifted later in phase relative to that of *kaiBC* transcript. On the other hand, LabA is a key component for the feedback

Figure 6. The *labA* mutation suppressed the slow-growth phenotype of the *sasA* mutant under LD conditions. Cultures of wild-type (AMC541), *labA* mutant [*labA*[−](Ω)/AMC541], *sasA* mutant [*sasA*[−](Km)/AMC541], and *labA/sasA* double mutant [*labA*[−](Ω)/*sasA*[−](Km)/AMC541] were streaked onto BG-11M agar plates and incubated under LL or LD cycle conditions at 30°C for 5 d (for LL) or 15 d (for LD). Note that the slow-growth phenotype of the *labA* mutant was obscured in these experiments because the growth was at saturation level.

Figure 7. Models for LabA function in the transcriptional-translational feedback loop of the cyanobacterial circadian clock system. (A) Fluctuation of LabA activity relative to changes in the level of KaiC (probably phosphorylated). Profiles of *kaiBC* mRNA (black trace) and KaiC protein (red trace) abundance in cyanobacterial cells are depicted. In the *labA* mutant, *kaiBC* expression exhibited a low-amplitude rhythm due to lack of KaiC–LabA-dependent transcriptional repression during the subjective night phase (blue trace). (B) A possible model for LabA functions in the cyanobacterial circadian clock system. Details are described in the text.

regulation responding to the amounts of (probably phosphorylated) KaiC (Fig. 1A).

Thus, we propose that transcriptional inhibition caused by LabA oscillates according to changes in the amount of phosphorylated KaiC. Total levels of KaiC and KaiC phosphorylation oscillate with a peak at CT 15–16 (subjective night) and a trough at CT 4 (subjective morning) (Figs. 3D, 7A; Xu et al. 2000; Iwasaki et al. 2002; Kitayama et al. 2003). Accordingly, KaiC–LabA is likely to strongly repress RpaA activity during subjective night, when KaiC is abundant, and to lower the trough level of transcriptional oscillation (Fig. 7A). During the subjective day, when KaiC levels are low, KaiC–LabA repressive activity is likely to be lower (Fig. 7A). The low-amplitude phenotype of *labA* mutants is consistent with this model (Fig. 7A). The LabA-dependent negative pathway, which appears to be regulated by the amount of clock components, presumably functions in harmony with the SasA-dependent positive pathway, which is regulated by the post-translational state of the Kai-based

chemical oscillator. In our model, this system produces a phase difference between LabA and SasA activity that amplifies circadian oscillation (Fig. 7A). In addition, our data suggest that the level of LabA can determine at least in part the expression level of clock-controlled genes (Fig. 4C), presumably by modulating feedback regulation of KaiC. The balance between the positive activity of SasA and the negative activity of LabA is likely to be important for determining the amplitude of the circadian oscillation of clock-controlled genes.

LabA and the cellular metabolic/redox system

LabA also appears to be involved in the cellular redox/metabolic system, since *labA* mutants showed slower colony growth on agar plates (Fig. 3E). The extreme bright phenotype of *labA* mutant in the *luxAB* reporter strains implies excessive accumulation of FMNH₂, an endogenous substrate for bacterial luciferase (Figs. 1A, 3A, 5A,B; Supplementary Fig. S2A). The circadian clock is able to affect cellular metabolic/redox states via transcriptional regulation, but since changes in *kai* gene expression did not substantially affect cellular growth rate on agar plates (Ishiura et al. 1998; Iwasaki et al. 2000; Nakahira et al. 2004), changes in clock gene expression in *labA* mutants may not explain the slow-growth phenotype in these cells. Through its regulation of both transcription and cellular metabolism, LabA may play a role in adaptation to light/dark alternation, since the *labA* mutation suppressed the slow-growth phenotype of the *sasA* mutant under LD conditions (Fig. 6). LabA and SasA might cooperatively modulate the cellular redox/metabolic system to adapt the daily light/dark alternation.

A model for cyanobacterial circadian gene expression system

We propose a model for LabA function in the cyanobacterial circadian clock system (Fig. 7B). First, Kai proteins generate the basic timing loop by regulating Kai-based chemical oscillation (Nakajima et al. 2005). Following its interaction with KaiC (Iwasaki et al. 2000), SasA is autophosphorylated and activates the putative transcription factor RpaA (Takai et al. 2006). It is presumed that RpaA controls circadian gene expression globally through the basic transcriptional machinery (Takai et al. 2006). The SasA–RpaA pathway appears to be activated by Kai proteins mainly during the subjective day phase (Takai et al. 2006), and the rhythmic activation of RpaA through KaiC–SasA generates the minimal transcriptional rhythm. Concurrently, KaiC represses RpaA function through the LabA-dependent pathway, mainly during the subjective night phase. LabA and SasA also cooperatively modulate the cellular redox/metabolic system. This KaiC–LabA–RpaA pathway model is largely based on our genetic analysis, and the molecular mechanism of this signal transduction pathway remains to be solved.

It is interesting to note that the key transcriptional factors crucial for circadian rhythm are likely to be regulated both positively and negatively by the circadian clock in other organisms. For instance, in *Neurospora*, the activity of the key circadian transcriptional activator White Collar Complex (WCC) is suppressed by the clock protein FRQ in the nucleus during morning/midday phase, whereas WCC accumulation is supported by cytosolic FRQ during evening phase (Brunner and Schafmeier 2006; Schafmeier et al. 2006). In mammals, CLOCK/BMAL1 heterodimers cooperate with coactivators such as p300/CREB-binding protein to activate transcription (Etchegaray et al. 2003), and with CRYPTOCHROME to repress transcription (Konratov et al. 2006). These regulatory functions likely converge on the C terminus of BMAL1 to produce the appropriate balance of transcriptional activation and suppression (Kiyohara et al. 2006). Thus, the basic framework of transcriptional control by the circadian clock may be conserved and may contribute to the robustness of circadian clock systems.

LabA and the DUF88/COG1432 domain family

At present, no known domain/motif has been found in LabA. According to the Conserved Domain Database of the National Center for Biotechnology Information (Marchler-Bauer et al. 2003), the entire coding sequence of LabA belongs to the uncharacterized DUF88/COG1432 domain family (Supplementary Fig. S1A). Although many genes belonging to this family have been found in the genome of various prokaryotic species, their function and biochemical properties are unknown. To our knowledge, this is the first study that describes the functional characterization of a member of this family. Interestingly, a BLAST search (Altschul et al. 1990) revealed that a protein encoded by a putative ORF, MCA1614 of *Methylococcus capsulatus*, possesses a DUF88/COG1432 domain at the N-terminal region and a putative DNA/RNA-binding domain at the C-terminal region (Supplementary Fig. S1B). This implies that the DUF88/COG1432 domain has regulatory role(s) for protein functions. Biochemical characterization of LabA will be crucial for elucidating the mechanism by which LabA functions in the cyanobacterial circadian clock system.

Materials and methods

Bacterial strains and media

Synechococcus elongatus PCC 7942 was used as the background strain for the cyanobacterial reporter strains in this study. The NUC42 ($P_{kaiBC}::luxAB$) (Nishimura et al. 2002), AMC149 ($P_{psbAI}::luxAB$) (Kondo et al. 1993), and PkaiRE ($P_{kaiBC}::P_{xhRE}$) (Kitayama et al. 2004) reporter strains were constructed in previous studies. The firefly luciferase reporter strain, AMC541 [$P_{kaiBC}::luc$ reporter inserted into NSII (Cm^R)], was a kind gift from Dr. S.S. Golden (Texas A&M University, College Station, TX) (Ditty et al. 2003). The $P_{kaiBC}::luxAB$

reporter strain (NUC42) carrying the $P_{trc}::kaiC$ transgene (OX-*kaiC*/NUC42) was described previously (Iwasaki et al. 2002). The $P_{kaiBC}::luxAB$ reporter strain (NUC42) carrying the $P_{trc}::sasA$ transgene (OX-*sasA*/NUC42) was provided by H. Iwasaki (Waseda University, Tokyo, Japan) (Iwasaki et al. 2000). The other cyanobacterial strains described were constructed in this study. Generation of the $P_{kaiBC}::luxAB$ reporter strain carrying the $P_{trc}::rpaA$ transgene (OX-*rpaA*/NUC42) is described in the Supplemental Material.

Cyanobacterial cells were grown in modified BG-11 medium (BG-11M) (Bustos and Golden 1991) or on BG-11M plates containing 1.5% Bacto Agar (Difco) at 30°C under continuous illumination (LL) of 40 $\mu E m^{-2} sec^{-1}$ from white fluorescent lamps. Transformation of *Synechococcus* was described previously (Porter 1988; Kutsuna et al. 1998). For construction and propagation of plasmids, the *Escherichia coli* strains DH5 α or TOP10 (Invitrogen) were used. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plate containing appropriate antibiotics.

Screening using transposon mutagenesis

To permit screening for a transposon carrying the kanamycin-resistance gene, the Km^r gene of OX-*kaiC*/NUC42 was disrupted by an Ω cassette. A plasmid carrying the Km^r gene (pACYC177) was digested with HindIII and PstI, and the Ω cassette was inserted. OX-*kaiC*/NUC42 was transformed with the resulting plasmid, and eight spectinomycin-resistant clones were selected. Among them, a kanamycin-sensitive clone [OX-*kaiC*(Ω)/NUC42] was used for further experiments. Sau3AI-digested genomic DNA from *Synechococcus elongatus* PCC 7942 was subcloned into the BamHI site of pBR322-inv-omega (the average insert size was ~6.5 kb). This genomic library was mutagenized with the EZ::TN <KAN-2> Insertion Kit (Epicentre) according to the manufacturer's protocol. OX-*kaiC*(Ω)/NUC42 cells (2.5 OD₇₃₀) were transformed with 0.5 μg of the mutagenized genomic library, and plated onto 20 BG-11M agar plates containing 5 $\mu g/mL$ kanamycin. The bioluminescence rhythm of kanamycin-resistant clones (~20,000 colonies) was monitored by a CCD camera apparatus (Kondo et al. 1994) in the presence of 1 mM IPTG. Among these, we picked 29 luminous colonies and confirmed their phenotypes again (IPTG was added at the third day of the assay). Finally, 10 confirmed clones were selected for further analysis. Genomic DNA purified from the clones was digested with EcoRI and subcloned into pBluescript II. DH5 α was transformed with the ligation mixture, and kanamycin-resistant clones were selected. Plasmid was prepared from them, and regions flanking the transposons were sequenced with KAN-2 FP-1 primer and KAN-2 RP-1 primer (included in the EZ::TN <KAN-2> Insertion Kit). The sequence of *labA* has been deposited in the DDBJ/GenBank/EMBL databases (accession no. AB281186).

Disruption of labA

A fragment containing the *labA* ORF was amplified by PCR using two primers: 5'-GACAACCCAGTTGGTAATTGCG-3' and 5'-GGGCAAAGGCCAATCGGTGAC-3'. The PCR product was subcloned into pGEM-T (Promega), and a Km^r or Ω cassette was inserted into the HpaI site of the *labA* gene to construct pDlabA(Km^r) or pDlabA(Ω), respectively (Fig. 1B). AMC149, NUC42, AMC541, PkaiRE, OX-*kaiC*(Ω)/NUC42, OX-*sasA*/NUC42, and OX-*rpaA*/NUC42 were transformed with pDlabA(Km^r) or pDlabA(Ω), and the transformants were selected in 25 $\mu g/mL$ kanamycin or 40 $\mu g/mL$ spectinomycin, respectively. The cells were checked by PCR to confirm *labA* disruption (data not shown). We monitored the biolumines-

cence rhythm of the cells essentially as described previously (Iwasaki et al. 1999). For entrainment of the circadian rhythm, cells were exposed to two LD cycles. For insect luciferase reporter strains, luciferin instead of n-decanal was added to a final concentration of 0.5 mM immediately before the assay. Addition of IPTG (for Figs. 1A, 5A,B) was performed as described previously (Ishiura et al. 1998).

Disruption of *sasA* and *rpA*

pDsasA, a construct to disrupt the *sasA* gene, and an inactivation cassette to disrupt the *rpA* gene were described previously (Iwasaki et al. 2000; Takai et al. 2006). AMC541 and *labA*[−](Ω)/AMC541 were transformed with either construct, and mutants were selected in 25 μ g/mL kanamycin. The bioluminescence assay was performed as described above.

Assaying the growth phenotype

For Figure 3E, cells were cultured in BG-11M medium under LL conditions (40 μ E m^{−2} sec^{−1}) with shaking for 6 d. The cultures were diluted with BG-11M medium, and 10 μ L of each culture were spotted onto a BG-11M agar plate. After growing under the LL conditions at 30°C for 6 d, colonies were photographed. For Figure 6, the cyanobacterial liquid cultures grown under the LL conditions for several days were diluted to OD₇₃₀ = 0.2, and aliquots (10 μ L) were streaked onto BG-11M agar plates and incubated at 30°C under LL or LD conditions for 5 d or 15 d, respectively.

Overexpression of *labA*

To construct an IPTG-inducible *labA* vector, we performed PCR using two primers: 5'-CAAGACTCCATGGCATTTCGCCTAGTCG-3' (the NcoI site is underlined) and 5'-GTTGATGGGATCCAGGCTCATGCCGAGC-3' (the BamHI site is underlined). The PCR product was inserted into the NcoI-BamHI site of p322P_{trc} (Kutsuna et al. 1998) to obtain p322P_{trc}::*labA*. p322P_{trc}::*labA* was digested with BglII, and the shorter fragment carrying *lacI*^q-P_{trc}::*labA* was subcloned into the BamHI site of the pTS2KC targeting vector (Kutsuna et al. 1998) to obtain pOX-*labA*. NUC42 and AMC149 were transformed with pOX-*labA*, and kanamycin-resistant clones were obtained (the resulting strains were OX-*labA*/NUC42 and OX-*labA*/AMC149, respectively). For the complementation experiment in Supplementary Figure S3, *labA*-disrupted NUC42 [NUC42 transformed with pD*labA*(Ω)] was transformed with pOX-*labA*. The bioluminescence assay and the addition of IPTG were performed as described previously (Ishiura et al. 1998). IPTG-dependent overexpression of *labA* was confirmed by Northern blotting (data not shown).

Northern and Western blotting analyses

Cells were precultured in BG-11M medium with aeration for four LD cycles and then transferred to a continuous culture system to maintain an OD₇₃₀ of 0.2. The culture was additionally exposed to a 12-h dark pulse, and then returned to LL (40 μ E m^{−2} sec^{−1} at 30°C with aeration). For *labA* overexpression, NUC42 and OX-*labA*/NUC42 were cultured with or without 1 mM IPTG in a continuous culture system as described above. Sampling was performed under the LL conditions every 3 or 6 h, and harvested cells were immediately frozen at −80°C. RNA preparation and Northern blotting analysis were performed as described previously (Ishiura et al. 1998; Kutsuna et al. 1998). Five micrograms of total RNA were loaded per lane. Di-

goxygenin (DIG)-labeled probes were prepared using the DIG DNA Labeling Mix (Roche). To prepare a probe for *labA*, two primers—5'-CAAGACTCCATGGCATTTCGCCCTAGTCG-3' and 5'-GTTGATGGGATCCAGGCTCATGCCGAGC-3'—were used. For the *luxAB* probe, 5'-GGGGACTGCTTTTTGTACAACTTGATGAAATTTGGAACTTCCTTCTCAC-3' and 5'-GGGGACAACCTTTGTATAGAAAAGTTGTACGAGTGGTATTTGACGATG-3' were used. Preparation of *kaiBC* probe was described previously (Ishiura et al. 1998). Western blotting analysis (2 μ g of total protein per lane) was performed as described previously (Nishiwaki et al. 2004). All experiments were performed two or three times.

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***labA*: a novel gene required for negative feedback regulation of the cyanobacterial circadian clock protein KaiC**

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