

Three major output pathways from the KaiABC-based oscillator cooperate to generate robust circadian *kaiBC* expression in cyanobacteria

Yasuhito Taniguchi^{a,1,2}, Naoki Takai^{a,1,3}, Mitsunori Katayama^{a,4}, Takao Kondo^{a,b,5}, and Tokitaka Oyama^{a,b,5,6}

^aDivision of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan; and ^bCREST, Japan Science and Technology Corporation, Furo-cho, Chikusa, Nagoya 464-8602, Japan

Edited by Robert Haselkorn, University of Chicago, Chicago, IL, and approved December 31, 2009 (received for review August 30, 2009)

Circadian *kaiBC* expression in the cyanobacterium *Synechococcus elongatus* PCC 7942 is generated by temporal information transmission from the KaiABC-based circadian oscillator to RpaA, a putative transcriptional factor, via the SasA-dependent positive pathway and the LabA-dependent negative pathway which is responsible for feedback regulation of KaiC. However, the *labA/sasA* double mutant has a circadian *kaiBC* expression rhythm, suggesting that there is an additional circadian output pathway. Here we describe a third circadian output pathway, which is CikA-dependent. The *cikA* mutation attenuates KaiC overexpression-induced *kaiBC* repression and exacerbates the low-amplitude phenotype of the *labA* mutant, suggesting that *cikA* acts as a negative regulator of *kaiBC* expression independent of the LabA-dependent pathway. In the *labA/sasA/cikA* triple mutant, *kaiBC* promoter activity becomes almost arrhythmic, despite preservation of the circadian KaiC phosphorylation rhythm, suggesting that CikA largely accounts for the residual *kaiBC* expression rhythm observed in the *labA/sasA* double mutant. These results also strongly suggest that transcriptional regulation in the *labA/sasA/cikA* triple mutant is insulated from the circadian signals of the KaiABC-based oscillator. Based on these observations, we propose a model in which temporal information from the KaiABC-based circadian oscillator is transmitted to gene expression through three separate output pathways.

CikA | circadian clock | LabA | SasA | transcriptional feedback

Circadian clocks are endogenous self-sustained oscillators with a period of ≈ 24 h. A variety of organisms from bacteria to mammals use circadian clocks to adapt to daily environmental changes. Autotrophic cyanobacteria are the simplest organisms with a circadian clock, and one of the cyanobacterial species, *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus*), is a model organism for studying the circadian clock system (1). Conceptually, the circadian clock system can be divided into three components: the oscillator that generates the basic timing loop, the input pathway that transmits signals from the environment to entrain the oscillator, and the output pathway that transmits temporal information from the oscillator to regulate various cellular activities, such as transcription. Three proteins encoded by the *kaiABC* gene cluster, which is essential for the circadian rhythm, generate the basic timing loop of the circadian clock in *Synechococcus* (2, 3). A temperature-compensated, self-oscillatory circadian clock can be reconstituted by mixing the three Kai proteins, KaiA, KaiB, and KaiC, with ATP in a test tube (3). KaiC, the central component of the circadian clock, is an autokinase and autophosphatase, and its activity is modulated by KaiA and KaiB through dynamic interactions (4–6). These Kai protein reactions including KaiC phosphorylation oscillate both in an in vitro oscillatory system and in *Synechococcus* in a circadian fashion (3, 4, 6).

Temporal information generated by the KaiABC-oscillator in *Synechococcus* is transmitted to the transcriptional regulatory system. Circadian gene expression is widely observed in *Synechococcus*; *kaiBC* transcription shows a robust circadian rhythm

(2). The regulation of *kaiBC* expression forms a part of circadian transcriptional-translational feedback loop and thought to contribute the robustness of the entire circadian clock system in *Synechococcus* (7). The mechanism of circadian *kaiBC* expression has been gradually revealed, as well as the biochemical properties of the Kai proteins. A KaiC-interacting histidine kinase, SasA, and its cognate response regulator, RpaA, which has a putative DNA binding domain, are important output factors for transcription of clock-controlled genes including *kaiBC* (8). Disruption of either *sasA* or *rpaA* dramatically reduces circadian *kaiBC* expression and causes an almost arrhythmic phenotype, indicating that SasA and RpaA are circadian clock-controlled transcriptional activators (8, 9). This reduction of *kaiBC* expression results in reduction of KaiC accumulation in these mutants (8). SasA autophosphorylates in response to KaiC binding, and the phosphate is transferred to RpaA (8). It is likely that the KaiC-SasA-RpaA pathway activates *kaiBC* expression mainly during subjective day, depending on the status of the KaiABC-based oscillator (8).

Phosphorylated KaiC is capable of repressing its own gene expression in a dose-dependent manner (i.e., negative feedback regulation of KaiC) (2, 10, 11). *labA* was identified as a gene required for this negative feedback regulation of KaiC (12). *kaiBC* promoter activity is maintained when KaiC is overexpressed in *labA* mutants, suggesting that the negative feedback regulation is abolished by the *labA* mutation. The *labA* mutation increases the troughs of circadian *kaiBC* expression, resulting in a low-amplitude rhythm, suggesting that *labA* represses *kaiBC* expression mainly during subjective night in wild-type *Synechococcus*. The lack of the negative feedback regulation in the *labA* mutant increases KaiC accumulation. More importantly, the *labA* mutation suppresses the phenotypes of the *sasA* mutant; circadian *kaiBC* expression and KaiC accumulation are restored in the *labA/sasA* double mutant (12). These results strongly suggest that *sasA* and *labA* govern *kaiBC* expression through

Author contributions: Y.T., M.K., T.K., and T.O. designed research; Y.T., N.T., and M.K. performed research; Y.T., N.T., M.K., and T.O. analyzed data; and Y.T. and T.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹Y.T. and N.T. contributed equally to this work.

²Present address: Global CMC Bios Japan, Pfizer Global Research and Development, Tokyo Laboratories, 3-22-7, Yoyogi, Shibuya-ku, Tokyo 151-8589, Japan.

³Present address: Laboratory of Animal Physiology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan.

⁴Present address: Department of Liberal Arts and Science, College of Industrial Technology, Nihon University, 2-11-1, Shinei, Narashino, Chiba, 275-8576, Japan.

⁵To whom correspondence should be addressed. E-mail: kondo@bio.nagoya-u.ac.jp or oyama@cosmos.bot.kyoto-u.ac.jp.

⁶Present address: Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan.

This article contains supporting information online at www.pnas.org/cgi/content/full/0909924107/DCSupplemental.

different pathways. In addition, bioluminescence from a $P_{kaiBC}::luc$ (firefly luciferase) reporter in the $labA/rpaA$ double mutant is similar to that in the $rpaA$ single mutant, indicating that $rpaA$ is epistatic to $labA$ in the bioluminescence assay (12). This suggests that $rpaA$ and $labA$ regulate $kaiBC$ expression through the same pathway. LabA is likely involved in the repression of circadian $kaiBC$ expression through RpaA, mainly during subjective early night when phosphorylated KaiC level is high. Thus, we propose a model in which the temporal information from the KaiABC-based oscillator diverges into two output pathways (the LabA-dependent negative pathway and the SasA-dependent positive pathway), which then converge onto RpaA to control the $kaiBC$ expression in a circadian fashion (12).

Although SasA and LabA are involved in the two major output pathways for circadian regulation of transcription, an additional output pathway has been postulated because the $labA/sasA$ double mutant in the $P_{kaiBC}::luc$ reporter strain background has a remarkable circadian bioluminescence rhythm (12). In this study, we report that CikA largely accounts for the circadian bioluminescence rhythm in the $labA/sasA$ double mutant. CikA was originally identified as a sensor histidine kinase involved in the input pathway of the cyanobacterial circadian clock (13). The $cikA$ mutation affects phosphorylation of KaiC and decreases phase shifts of the bioluminescence rhythm in response to 5-h dark pulses (13, 14). CikA is capable of binding to quinone molecules that sense the cellular redox state, and the stability of CikA protein *in vivo* is affected by the cellular redox state and environmental light intensity (14, 15). CikA is also likely to associate with the KaiABC-based protein complex (14, 15). Thus, it has been proposed that CikA senses changes in the cellular redox state, and through association with the KaiABC-based protein complex, CikA functions to reset the KaiABC-based circadian oscillator in harmony with the daily environmental cycle (14). However, it was unclear how the $cikA$ mutation causes a low-amplitude phenotype under constant light (LL) conditions (13). Here, we analyzed the molecular and genetic relationship between CikA and other clock-related components to demonstrate a role for CikA in the transcriptional regulation.

Results

Identification of $cikA$ as a Gene that Enhances Feedback Inhibition Induced by KaiC Overexpression. Previously, we identified $labA$ as a gene indispensable for negative feedback regulation of KaiC by a genetic screening (12). We also identified a $cikA$ mutant during the screen (Fig. 1). In this screen, we used a transgenic *Synechococcus* reporter strain [OX- $kaiC(\Omega)$ /NUC42] carrying additional loci in which the *Vibrio harveyi* luciferase gene ($luxAB$) 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 ($P_{IPTG}::kaiC$) (10). In the absence of IPTG, OX- $kaiC(\Omega)$ /NUC42 cells had a robust circadian bioluminescence rhythm that followed the circadian changes in $kaiBC$ promoter activity. IPTG treatment induced KaiC overexpression and dramatically decreased bioluminescence by repressing luciferase expression via negative feedback inhibition of the $kaiBC$ promoter (Fig. 1) (10). To identify mutants that lack this inhibition, we performed random transposon mutagenesis of OX- $kaiC(\Omega)$ /NUC42 and monitored the bioluminescence of individual colonies of the transformants in the presence of 1 mM IPTG. About 20,000 colonies were screened as described (12), and a $cikA$ mutant [OX- $kaiC(\Omega)$ / $cikA^-(Km)$ /NUC42] that expresses the $luxAB$ reporter gene even in the presence of IPTG was isolated (Fig. 1). Sequencing analysis of the genomic region flanking the transposon revealed that the transposon was inserted at nucleotide position 2218 (the first nucleotide of the translation initiation ATG was defined as "position 1"). In the absence of IPTG, OX- $kaiC(\Omega)$ / $cikA^-(Km)$ /NUC42 had a low-amplitude bioluminescence rhythm with higher

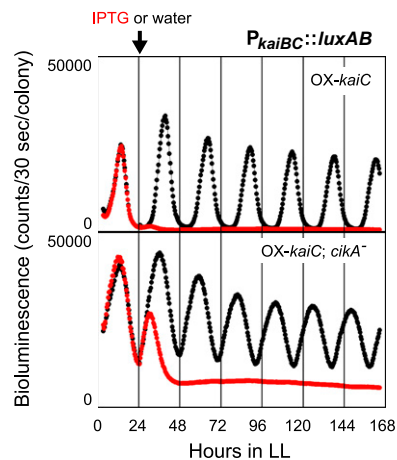


Fig. 1. Identification of $cikA$ as a gene that supports negative feedback regulation of KaiC. Bioluminescence traces of the $cikA$ mutant isolated from the screen [OX- $kaiC(\Omega)$ / $cikA^-(Km)$ /NUC42] is shown. Cells were grown on agar plates and their bioluminescence rhythms were measured under constant light (LL) conditions after entrainment with two 12-h light/12-h dark (LD) cycles. IPTG (1 mM) or water was added at hour 24 in LL (LL 24). (Upper) Bioluminescence rhythm of the parental reporter strain OX- $kaiC(\Omega)$ /NUC42; (Lower) bioluminescence rhythm of OX- $kaiC(\Omega)$ / $cikA^-(Km)$ /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 IPTG (or water) addition.

troughs and a short period (Fig. 1), as reported in a previous work (13). Addition of 1 mM IPTG rapidly decreased the bioluminescence intensity of OX- $kaiC(\Omega)$ / $cikA^-(Km)$ /NUC42, suggesting that negative feedback of KaiC occurs in this mutant. However, the feedback inhibition halted incompletely and bioluminescence remained at an intermediate level without rhythmicity, suggesting that the feedback regulation is attenuated in this strain (Fig. 1).

Genetic Interaction Between $cikA$ and $labA$. The phenotypes of the $cikA$ mutant shown in Fig. 1 (increased trough levels of circadian $kaiBC$ expression and attenuated negative feedback regulation of KaiC) strongly suggest that CikA negatively regulates $kaiBC$ expression. LabA is another negative regulator for $kaiBC$ expression (12). To clarify the roles of these two factors in the feedback regulation, we examined their genetic interaction. We generated a $labA/cikA$ double mutant [$labA^-(\Omega)$ / $cikA^-(Km)$ /AMC541] in a $P_{kaiBC}::luc$ reporter strain, AMC541, and evaluated the phenotype using a bioluminescence assay. We did not use $luxAB$ reporter strains in this experiment because the $labA$ mutation produces a "bright phenotype" in $luxAB$ reporter strains (12). As reported (12, 13), the $labA$ single mutant [$labA^-(\Omega)$ /AMC541] had a low-amplitude rhythm with high trough levels, whereas the $cikA$ single mutant [$cikA^-(Km)$ /AMC541] had a short period and low-amplitude rhythm with high trough levels (Fig. 2A). The $labA/cikA$ double mutant had a much more severe phenotype: a very low-amplitude rhythm with a higher overall level (Fig. 2A; see also Fig. 3B), indicating an additive effect of these two mutations on the amplitude of the output oscillation.

Resetting of the circadian rhythm is dysfunctional in $cikA$ mutants. Thus, desynchronization of circadian rhythms among cyanobacterial cells may produce normalized output signals with a severely low-amplitude rhythm in the $labA/cikA$ double mutant. To assess this possibility, we examined KaiC accumulation and phosphorylation using immunoblotting. In the $labA$ mutant, total KaiC was increased by ≈ 2 - to 6-fold, and the circadian rhythm of KaiC phosphorylation was robustly sustained (Fig. 2B), as

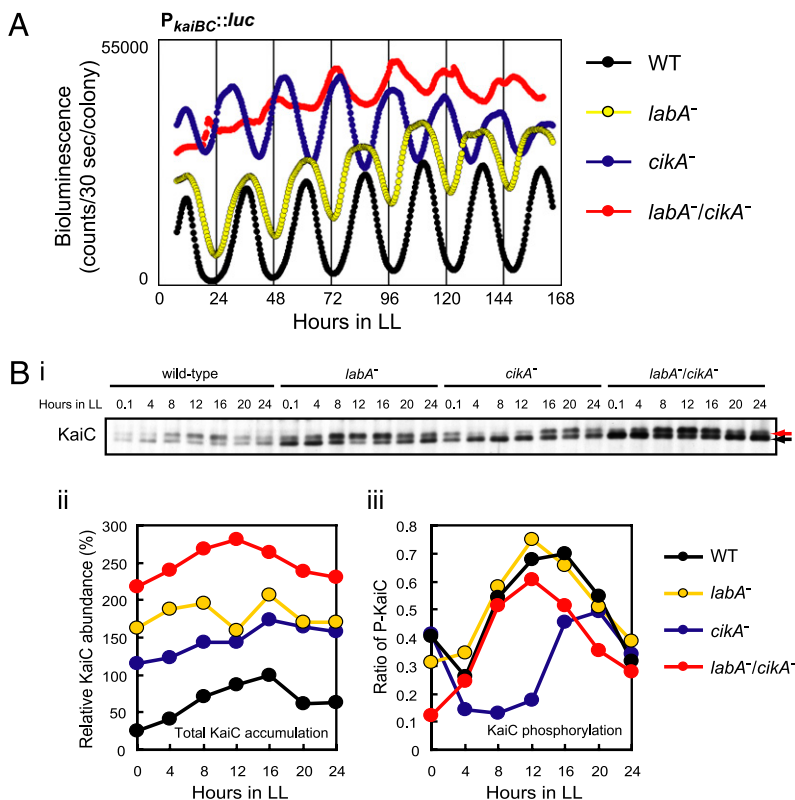


Fig. 2. Circadian phenotypes of the *labA/cikA* double mutant. (A) Bioluminescence rhythm of the *labA/cikA* double mutant. Wild-type AMC541 (WT), $labA^-(\Omega)$ /AMC541 ($labA^-$), $cikA^-(Km)$ /AMC541 ($cikA^-$), and $labA^-(\Omega)/cikA^-(Km)$ /AMC541 ($labA^-/cikA^-$) were grown on BG-11M agar 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. (B) Total abundance and phosphorylation profile of KaiC in the *labA/cikA* double mutant. AMC541, $labA^-(\Omega)$ /AMC541, $cikA^-(Gm)$ /AMC541, and $labA^-(\Omega)/cikA^-(Gm)$ /AMC541 were cultured in BG-11M medium, and cells were collected every 4 h after entrainment with LD cycles. Total cellular proteins (2 μ g per lane) were analyzed by immunoblotting using anti-KaiC antiserum. (B) One of blots from two independent experiments is shown. The upper and lower bands correspond to phosphorylated KaiC (P-KaiC; red arrow) and nonphosphorylated KaiC (NP-KaiC; black arrow), respectively. (Bii and Biii) Densitometric analysis of the blot shown in B; (Bii) total KaiC abundance; (Biii) KaiC phosphorylation rhythm.

reported (12). The KaiC phosphorylation profile in the *labA* mutant was similar to that in the wild-type strain (Fig. 2Biii). Total KaiC level was also increased in the *cikA* mutant, similar to the *labA* mutant (Fig. 2Bi and Fig. 2Bii). As Ivleva et al. reported (14), the *cikA* mutation altered the phase of the phosphorylation rhythm after dark treatment (Fig. 2Bi and Fig. 2Biii). KaiC level in the *labA/cikA* double mutant was higher than that in *labA* or *cikA* mutant (Fig. 2Bi and Fig. 2Bii), suggesting that *labA* and *cikA* mutations have an additive effect on KaiC level. More importantly, the KaiC phosphorylation ratio in the *labA/cikA* double mutant fluctuated similar to the wild-type strain (Fig. 2Bi and Fig. 2Biii), suggesting that circadian oscillations of KaiC phosphorylation are synchronized among the *labA/cikA* double-mutant cells. Therefore, the severely low-amplitude rhythm of the bioluminescence reporter in the *labA/cikA* double mutant is likely caused by abnormalities in the cellular output from the oscillator, rather than desynchronization of individual oscillatory cells. These results suggest that *cikA* negatively regulates *kaiBC* expression independent of *labA*, and then it supports feedback regulation of KaiC.

Genetic Analyses of the *sasA/cikA* Double Mutant and the *labA/sasA/cikA* Triple Mutant. Next, we evaluated genetic interaction between *cikA* and *sasA* using a *sasA/cikA* double mutant. We generated a *sasA/cikA* double mutant strain in the AMC541 background [$sasA^-(Km)/cikA^-(Gm)$ /AMC541] and assayed its bioluminescence (Fig. 3A). The *sasA* single mutant in the AMC541 background [$sasA^-(Km)$ /AMC541] had a low-amplitude rhythm and dramatically reduced bioluminescence level (Fig. 3A), as described (12). The bioluminescence trace of the *sasA/cikA* double mutant was arrhythmic or almost arrhythmic, with an extremely low-amplitude rhythm that was more severe than that of the *sasA* mutant (Fig. 3A). These results suggest that *cikA* may generate the residual rhythmicity of the *sasA* mutant by regulating circadian gene expression independent of *sasA*.

We also evaluated the bioluminescence of the *labA/sasA/cikA* triple mutant in the AMC541 background [$labA^-(\Omega)/sasA^-(Km)/cikA^-(Gm)$ /AMC541] (Fig. 3B). The *labA/sasA* double mutant in the AMC541 background [$labA^-(\Omega)/sasA^-(Km)$ /AMC541] had a low-amplitude, but remarkably robust, bioluminescence rhythm (Fig. 3B), as described (12). However, bioluminescence trace of the *labA/sasA/cikA* triple mutant was almost arrhythmic or had a severely low-amplitude rhythm (Fig. 3B), suggesting that *cikA* largely accounts for the bioluminescence rhythm in the *labA/sasA* double mutant. The bioluminescence level of the triple mutant was consistently higher than that of the *sasA/cikA* double mutant, indicating that the *labA* mutation has a positive effect on bioluminescence level irrespective of *sasA* and *cikA*. These results are consistent with those for the *labA/sasA* and the *labA/cikA* double mutants (Figs. 2A and 3B) (12). Bioluminescence level of the *labA/sasA/cikA* triple mutant was greater than that of the *rpaA* single mutant, *rpaA^-(Km)*/AMC541 (Fig. 3B). These results suggest that RpaA can be activated by factors other than LabA, SasA, and CikA. Note that our attempts to isolate a *cikA/rpaA* double mutant for further genetic analyses resulted in failure, probably because of a synthetic lethal interaction between the two (Fig S1).

KaiC levels in these multiple mutants were examined by immunoblotting (Fig. 3C). KaiC decreased to undetectable levels in the *sasA* single mutant in this experiment (Fig. 3C). KaiC could be detected in the *sasA/cikA* double mutant, but the level was much lower than that in the wild-type strain (Fig. 3Ci and Fig. 3Cii). KaiC phosphorylation was persistently elevated in the *sasA/cikA* double mutant and its rhythmicity was obscure, probably because of low KaiC level (Fig. 3Ci and Fig. 3Ciii). Total KaiC level in the *labA/sasA/cikA* triple mutant was greater than that in the *sasA/cikA* double mutant, and the phosphorylation rhythm of KaiC was restored, likely a result of restoration of KaiC accumulation (Fig. 3C). In other words, like the in vitro KaiC phosphorylation rhythm, the cellular Kai-based oscillator is capable of working synchronously regardless of LabA, SasA, and CikA levels, if Kai accumulation in

mutant analysis of *cikA* and *sasA* suggests that CikA also regulates *kaiBC* transcription independent of SasA (Fig. 3). Thus, the CikA-dependent transcriptional regulatory mechanism appears to be independent of LabA and SasA. Because LabA and SasA function independently to regulate *kaiBC* transcription (12), the regulatory system appears to consist of at least three separate pathways. It is interesting to note that the low-amplitude phenotype in the *sasA* single mutant becomes more severe under strong light conditions (8, 9). Because accumulation of CikA protein is inversely related to light intensity (15), it is possible that the decreased CikA level in *sasA* mutant cells under high-light conditions exacerbates the low-amplitude phenotype, similar to effect observed in the *sasA/cikA* double mutant.

The *labA/cikA* double mutant and the *labA/sasA/cikA* triple mutant clearly demonstrate the separation between transcriptional regulation and the circadian rhythm of the KaiABC-based oscillator in vivo (Figs. 2 and 3), strongly suggesting that the regulatory machinery for circadian transcription is mainly composed of three output pathways. In addition, *kaiBC* transcription is evident in strains that lack LabA, SasA, and CikA, although it is almost arrhythmic. These data suggest that RpaA can be activated by other factors through signaling pathways independent of the circadian clock. This finding seems to be related to the phenotype of the Δ *kaiABC* mutant: *kaiBC* promoter activity was evident in the Δ *kaiABC* mutant and similar to that in the triple mutant in bioluminescence assays (Fig. 3B) (4). The nullification of the oscillator appears to be equivalent to that of these three output pathways in terms of transcriptional regulation of *kaiBC*, implying that the regulatory machinery for basic *kaiBC* transcription is independent of the circadian system.

Previously, we proposed a model for feedback regulation of *kaiBC* in which the LabA-dependent negative pathway and the SasA-dependent positive pathway regulate the putative master transcriptional regulator, RpaA, to generate circadian gene expression (12). Based on our current results, we have added a third CikA-dependent circadian clock output pathway to our model. CikA is a negative factor and thus, CikA-dependent negative pathway could not function if the target promoters are not active. Because RpaA appears essential for activation of *kaiBC* expression, we speculate that the CikA-dependent negative output pathway requires the transcriptional activator RpaA as a downstream factor. Although biochemical interaction between SasA and RpaA (8) and genetic relationship between *labA* and *rpaA* (12) were demonstrated in previous studies, so far we have not established the role of RpaA in the CikA-dependent pathway under the KaiABC-based oscillator (Fig. S1). Further studies should be required for the elucidation of the CikA-dependent transcriptional regulatory network.

The bioluminescence trace of the *labA/sasA/cikA* triple mutant was not completely arrhythmic. Thus, there may be additional output pathways. Alternatively, the bioluminescence of the *labA/sasA/cikA* triple mutant may oscillate because of cellular activity, such as metabolism, translation, and protein degradation, which fluctuates in a circadian fashion under direct control of the KaiABC-based circadian oscillator.

When does CikA function during the circadian cycle? Under LL conditions, CikA accumulation increases and decreases in subjective night and day, respectively (14). CikA protein seems to fluctuate with a peak at CT 20 to 24 (subjective late night to dawn) (14). Thus, it is plausible that CikA represses *kaiBC* expression mainly during the subjective late night phase under constant conditions. The low-amplitude phenotype of *cikA* mutants suggests that CikA deepens the trough levels of circadian *kaiBC* expression (Figs. 1, 2, and 3A) (13). Furthermore, CikA proteins are unstable in the light (15), suggesting that CikA functions mainly during the dark phase under natural cyclic conditions. Combined regulation by the circadian clock and light must amplify the fluctuation in CikA level under natural con-

ditions. CikA is also involved in phase-setting in response to a dark pulse (13), and the circadian profile of CikA level is reasonable for resetting the circadian clock in response to the dark-light transition at dawn under natural conditions.

Considering all of the results, we propose a revised model for regulation of circadian *kaiBC* expression in *Synechococcus* (Fig. 4). The KaiABC-based complex is thought to serve as the basis for cyanobacterial circadian rhythm generation. KaiC has two phosphorylation sites and they are circadianly phosphorylated and dephosphorylated in sequence (16). KaiC phosphorylation oscillates with a peak around CT 16 (4). In vitro, SasA-RpaA phosphorylation occurs 4 to 8 h before the peak of KaiC phosphorylation (8). Therefore, we can extrapolate that SasA phosphorylation fluctuates with a peak around CT 8 to 12 (subjective day to dusk) in vivo and activation of the SasA-dependent pathway up-regulates *kaiBC* expression via RpaA mainly during the subjective day phase (8). Because phosphorylation of KaiC is indispensable for negative feedback regulation of KaiC (11), LabA appears to function downstream of phosphorylated KaiC. Both accumulation and phosphorylation of KaiC oscillate with a peak around CT 16 (subjective night) (4, 5). Thus, repression of *kaiBC* expression by LabA is strongest around CT 16 (12), when doubly phosphorylated KaiC is most prevalent (16). The LabA-dependent negative pathway appears to function by repressing RpaA activity. CikA abundance fluctuates with a peak around CT 20 to 24 (14). Thus, CikA may repress *kaiBC* expression during the subjective late night, possibly by associating with the KaiABC-based protein complex. Because CikA is involved in both input and output pathways of the circadian clock, the interaction of CikA with the KaiABC-based oscillator should function not only in the modulation of the phasing of the KaiABC-based oscillator but also in the activation of a negative circadian output pathway. We speculate that the CikA-dependent pathway also functions through RpaA to regulate transcription. It is likely that RpaA is also regulated by noncircadian mechanisms.

The three major output pathways likely regulate *kaiBC* promoter activity according to different temporal information sources that are generated by a single circadian clock. SasA activates *kaiBC* promoter activity according to the reaction states of KaiC,

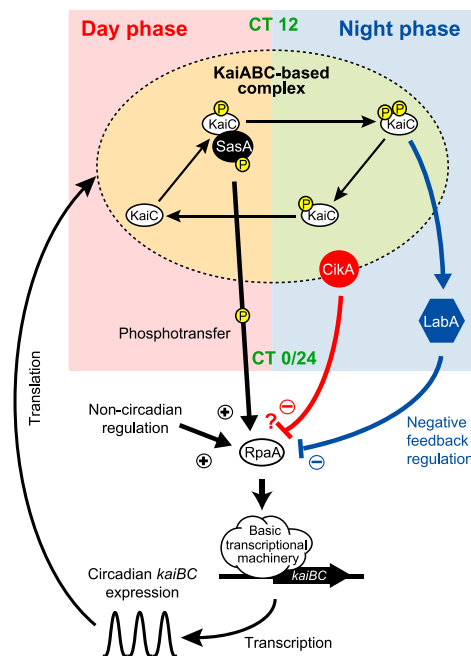


Fig. 4. A possible model for the transcriptional-translational feedback loop of the cyanobacterial circadian clock. Details are described in the text.

whereas LabA represses *kaiBC* promoter activity in response to accumulation of phosphorylated KaiC. The function of CikA in transcriptional regulation likely depends on CikA level, which is under the control of the clock, as well as binding to the KaiABC-based complex. This scenario explains the phase of the *kaiBC* mRNA expression rhythm: peak at subjective dusk (CT 12) and trough at subjective dawn (CT 0/24) (12). Around subjective dawn, the SasA-dependent positive pathway is likely inactive and noncircadian regulatory mechanisms for RpaA activation may be diminished by the CikA-dependent negative pathway. After subjective dawn, the negative effect of the CikA-dependent pathway gradually decreases and the noncircadian regulatory mechanisms and the SasA-dependent positive pathway can activate RpaA. The positive effect of the SasA-dependent pathway peaks around subjective dusk. Later, the positive effect of the SasA-dependent pathway decreases as KaiC is dephosphorylated. The negative effect of the LabA-dependent pathway peaks in the early- to midsubjective night, and then the positive effects of noncircadian regulatory mechanisms and residual SasA-dependent activity are suppressed. The phase differences among these pathways should increase the amplitude of circadian *kaiBC* expression. Regulations by three pathways likely ensure robust circadian *kaiBC* expression against perturbations in cellular systems and the clock system. The robustness appears to be achieved by the mechanism of the Kai protein-based oscillation and oscillatory *kaiBC* expression, which can be uncoupled from the phosphorylation rhythm of the protein oscillator (7). The output pathways for *kaiBC* expression are likely required for the machinery to coordinate these rhythmic molecular events. Furthermore, in *Synechococcus*, the circadian clock regulates many promoters; they exhibit a range of waveforms, amplitudes, and phase angles (17, 18). The combination of three output pathways and noncircadian regulation may underlie the generation of a variety of circadian promoter activities.

In summary, we have highlighted the contribution of CikA on circadian *kaiBC* expression. Although this and previous studies revealed that CikA plays important roles in both phasing of the KaiABC-based oscillator and circadian gene expression, underlying mechanisms of these roles are still unclear. Further analysis is needed to reveal the functional relationship between CikA and the KaiABC-based oscillator in the cyanobacterial circadian-clock system.

Materials and Methods

Bacterial Strains and Media. All cyanobacterial reporter strains used in this study were derivatives of *Synechococcus elongatus* PCC 7942. The $P_{kaiBC}::luxAB$ reporter strain NUC42 (19) and the $P_{kaiBC}::luc$ reporter strain AMC541 (1) were generated previously. OX-*kaiC*(Ω)/NUC42, *labA*⁻(Ω)/AMC541, *sasA*⁻(Km)/AMC541, *labA*⁻(Ω)/*sasA*⁻(Km)/AMC541, and *rpaA*⁻(Km)/AMC541 have been described (12). The other cyanobacterial strains were constructed in this study. Transformation of *Synechococcus* was performed as described (20). AMC541 and *labA*⁻(Ω)/AMC541 were transformed with pDcikA(Km) (described later) to generate *cikA*⁻(Km)/AMC541 and *labA*⁻(Ω)/*cikA*⁻(Km)/AMC541, respectively. AMC541, *labA*⁻(Ω)/AMC541, *sasA*⁻(Km)/AMC541, and *labA*⁻(Ω)/*sasA*⁻(Km)/AMC541 were transformed with pAM2152 (21) to generate *cikA*⁻(Gm)/AMC541, *labA*⁻(Ω)/*cikA*⁻(Gm)/AMC541, *sasA*⁻(Km)/*cikA*⁻(Gm)/AMC541, and *labA*⁻(Ω)/*sasA*⁻(Km)/*cikA*⁻(Gm)/AMC541, respectively. *Synechococcus* cells and *Escherichia coli* strains were cultured and maintained as described (12).

Genetic Screening Using Transposon Mutagenesis. Genetic screening using transposon mutagenesis to identify the *cikA* mutant shown in Fig. 1 [OX-*kaiC*(Ω)/*cikA*⁻(Km)/NUC42] has been described (12). The genomic DNA of OX-*kaiC*(Ω)/*cikA*⁻(Km)/NUC42 was digested with EcoRI, subcloned into pBluescript II, introduced into DH5 α , and kanamycin-resistant clones were selected. The plasmid obtained from the clone was pDcikA(Km). pDcikA(Km) was sequenced with KAN-2 FP-1 primer and KAN-2 RP-1 primer to determine the transposon insertion site.

Bioluminescence Assay. Bioluminescence assays were performed as described (12). The results shown in the figures are representative of two or more independent experiments.

Immunoblotting. Cell culture, sampling, KaiC immunoblotting, and densitometric analysis were performed as described (11). The results shown in the figures are representative of two independent experiments.

ACKNOWLEDGMENTS. We thank R. Ito for collaborating in the screening, and H. Kondo and T. Nishikawa for technical support. We also thank Dr. S. S. Golden (University of California, San Diego) for providing pAMC2152 and pAM2477. This research was supported in part by Grants-in-Aid Grant 15G50308 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. K. and T.O.), and the Japan Society for the Promotion of Science Grant 17370088 (to T.O.). Analysis of DNA sequences was conducted in conjunction with the Life Research Support Center at Akita Prefectural University.

- Ditty JL, Williams SB, Golden SS (2003) A cyanobacterial circadian timing mechanism. *Annu Rev Genet* 37:513–543.
- Ishiyama M, et al. (1998) Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. *Science* 281:1519–1523.
- Nakajima M, et al. (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* 308:414–415.
- Iwasaki H, Nishiwaki T, Kitayama Y, Nakajima M, Kondo T (2002) KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. *Proc Natl Acad Sci USA* 99:15788–15793.
- Kitayama Y, Iwasaki H, Nishiwaki T, Kondo T (2003) KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. *EMBO J* 22:2127–2134.
- Kageyama H, et al. (2006) Cyanobacterial circadian pacemaker: Kai protein complex dynamics in the KaiC phosphorylation cycle in vitro. *Mol Cell* 23:161–171.
- Kitayama Y, Nishiwaki T, Terauchi K, Kondo T (2008) Dual KaiC-based oscillations constitute the circadian system of cyanobacteria. *Genes Dev* 22:1513–1521.
- Takai N, et al. (2006) A KaiC-associating SasA-RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria. *Proc Natl Acad Sci USA* 103:12109–12114.
- Iwasaki H, et al. (2000) A kaiC-interacting sensory histidine kinase, SasA, necessary to sustain robust circadian oscillation in cyanobacteria. *Cell* 101:223–233.
- Nakahira Y, et al. (2004) Global gene repression by KaiC as a master process of prokaryotic circadian system. *Proc Natl Acad Sci USA* 101:881–885.
- Nishiwaki T, et al. (2004) Role of KaiC phosphorylation in the circadian clock system of *Synechococcus elongatus* PCC 7942. *Proc Natl Acad Sci USA* 101:13927–13932.
- Taniguchi Y, et al. (2007) *labA*: a novel gene required for negative feedback regulation of the cyanobacterial circadian clock protein KaiC. *Genes Dev* 21:60–70.
- Schmitz O, Katayama M, Williams SB, Kondo T, Golden SS (2000) CikA, a bacteriophytochrome that resets the cyanobacterial circadian clock. *Science* 289:765–768.
- Ivleva NB, Gao T, LiWang AC, Golden SS (2006) Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock. *Proc Natl Acad Sci USA* 103:17468–17473.
- Ivleva NB, Bramlett MR, Lindahl PA, Golden SS (2005) LdpA: a component of the circadian clock senses redox state of the cell. *EMBO J* 24:1202–1210.
- Nishiwaki T, et al. (2007) A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J* 26:4029–4037.
- Liu Y, et al. (1995) Circadian orchestration of gene expression in cyanobacteria. *Genes Dev* 9:1469–1478.
- Ito H, et al. (2009) Cyanobacterial daily life with Kai-based circadian and diurnal genome-wide transcriptional control in *Synechococcus elongatus*. *Proc Natl Acad Sci USA* 106:14168–14173.
- Nishimura H, et al. (2002) Mutations in KaiA, a clock protein, extend the period of circadian rhythm in the cyanobacterium *Synechococcus elongatus* PCC 7942. *Microbiology* 148:2903–2909.
- Porter RD (1988) DNA transformation. *Methods Enzymol* 167:703–712.
- Zhang X, Dong G, Golden SS (2006) The pseudo-receiver domain of CikA regulates the cyanobacterial circadian input pathway. *Mol Microbiol* 60:658–668.